LECTINS AND THEIR LIGANDS IN SHAPING IMMUNE RESPONSES

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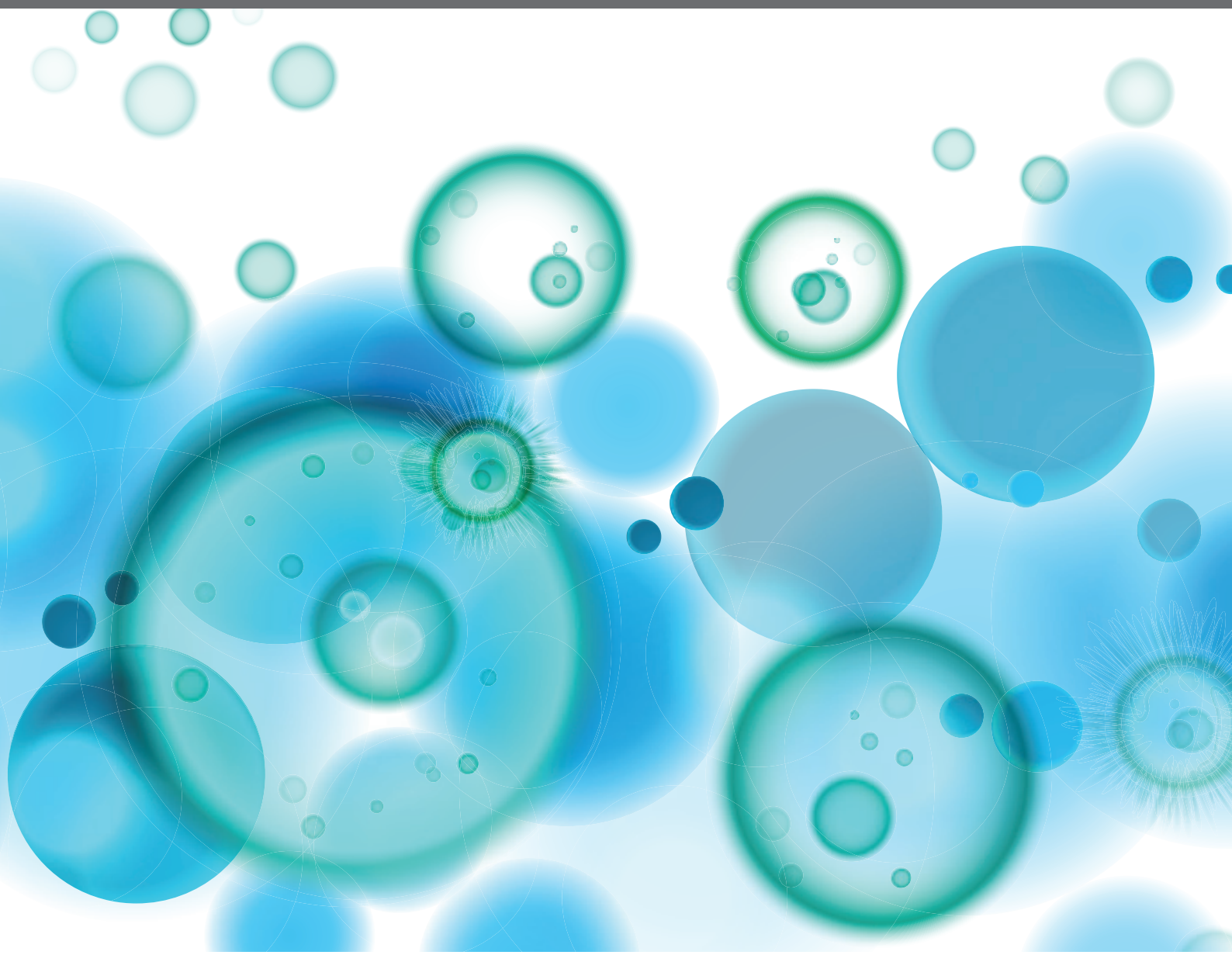

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LECTINS AND THEIR LIGANDS IN SHAPING IMMUNE RESPONSES

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EDITORIAL
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Editorial: Lectins and Their Ligands in Shaping Immune Responses

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Editorial on the Research Topic:

Lectins and Their Ligands in Shaping Immune Responses

Lectins are glycan-binding proteins that are involved in numerous biological processes including cell development, cell–cell interactions, signaling pathways, and the immune response. In innate immunity, lectins often act as pattern recognition receptors (PRRs) and recognize pathogen-associated molecular patterns (PAMPs), but also damage-associated molecular patterns (DAMPs). Thus, lectins may contribute to a protective immune response, for instance during infections, but they may also be involved in immune pathology, for instance during sterile inflammation. Main classes of lectins in innate immunity include C-type lectin receptors (CLRs), siglecs, and galectins. Due to the manifold functions of these different classes of lectins in anti-microbial defense as well as immune homeostasis, lectin targeting is a promising strategy to shape immune responses in the context of infections, autoimmunity, cancer, or vaccination.

This Research Topic provides original research and discussions focusing on aspects such as ligand recognition by lectin receptors, induced signaling pathways, and lectin-mediated effector functions during infections and inflammatory processes. It also addresses the identification and characterization of novel lectin ligands and discusses how lectin targeting can be exploited to stimulate or modulate immune responses. The contributions to this Research Topic provide in-depth insights into current research on the impact of lectins/lectin ligands on immune responses. The collection of 18 articles published in this Research Topic comprises original Research Articles, Methods and Opinion Articles, as well as Comprehensive and Mini-Reviews.

NOVEL LINK BETWEEN DECTIN-2 AND LC3-ASSCOCIATED PHAGOCYTOSIS OF MYCOBIOTA

It is well-known that the CLR Dectin-2 contributes to fungal recognition. Previous studies have shown an involvement of the microtubule-associated protein light-chain 3 (LC3)-associated phagocytosis (LAP) in host/fungi interactions. The study by Lamprinaki et al. describes a novel link between Dectin-2 and LAP in the host response to commensal fungi. By using defined knockout systems, the authors show a connection between Dectin-2 binding of commensal yeasts, internalization, and signaling via association with LAP.

CLRS AS NOVEL MARKERS OF DC SUBSETS

Conventional dendritic cells (cDCs) in humans can be subdivided into two subsets, $CD141^+$ DCs (cDC1) and $CD1c^+$ DCs (cDC2). Heger et al. identify the CLR CLEC10A (also known

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as Macrophage galactose-type C-type lectin) as a specific marker for human cDC2. CLEC10A targeting using a CLEC10A-specific monoclonal antibody as well as a bivalent ligand resulted in a rapid internalization into cDC2 and increased cytokine responses upon TLR7/8 stimulation. This study highlights CLEC10A as a candidate receptor for *in vivo* antigen-targeting approaches.

CLR TARGETING FOR ANTIGEN DELIVERY AND IMMUNE STIMULATION

Targeting CLRs expressed by antigen-presenting cells is a promising strategy to enhance immune responses and to increase the efficacy of vaccines. Numerous studies have shown that the CLR DC-SIGN serves as an attractive target for the delivery of antigens into DCs; however, the mechanism of how DC-SIGN affects cross-presentation of antigens has not yet been fully elucidated. The study by Horrevorts et al. addresses the dynamics of DC-SIGN-mediated internalization, antigen processing, and cross-presentation and analyzes the impact of Toll-like receptor-4 (TLR4) on these processes. The authors conclude that the combined use of DC-SIGN and TLR4 ligands may serve as an antigen-targeting platform to boost antigen cross-presentation to $CD8^+$ T cells.

Velasquez et al. use an antibody-mediated DC-SIGN targeting approach to enhance $CD4^+$ T cell responses against mycobacterial antigens. Using the hSIGN mouse expressing human DC-SIGN under control of the murine CD11c promotor, they observed increased frequencies of antigen-specific IFN-γ^+IL-2^+TNF-α^+ polyfunctional $CD4^+$ T cells upon antigen targeting to DC-SIGN. Using a similar targeting approach for CD209a/SIGNR5 (mouse DC-SIGN), Schetters et al. show that ovalbumin (OVA)-conjugated anti-SIGNR5 antibody elicited robust antigen-specific $CD4^+$ and $CD8^+$ T cell responses and enhanced OVA-specific antibody responses *in vivo*. These findings highlight the utility of this CLR as a target for antigen delivery and immune stimulation.

IDENTIFYING NOVEL CLR/BACTERIA INTERACTIONS

In order to identify novel interactions of lectin receptors with pathogens, appropriate research tools are needed. In their Methods article, Mayer et al. describe applications of CLR-Fc fusion proteins to screen for yet unknown CLR/bacteria interactions. Using *Campylobacter jejuni* as an example, they identify Dectin-1 as a candidate CLR in *C. jejuni* recognition whose functional role can now be investigated in further studies.

ASSOCIATION OF C-TYPE LECTIN WITH PULMONARY TUBERCULOSIS

CLRs have been implicated in the recognition of mycobacteria and the induction of anti-mycobacterial immunity (see the review article by Wagener et al. in this Research Topic). Klassert et al. used an AmpliSeq-based approach to screen main CLR gene clusters and CLR pathway-related genes for single nucleotide polymorphisms (SNPs) associated with pulmonary tuberculosis in an Indian population. One SNP in the gene encoding for the Mannan-binding lectin serine protease 1 (MASP1) was found to be significantly associated with pulmonary tuberculosis in this population, thus suggesting an involvement of the lectin pathway of the complement system in tuberculosis pathogenesis.

ROLE OF SIGLECS AND GALECTINS IN IMMUNE MODULATION

While most of the articles included in this Research Topic deal with CLRs, the two other main classes of lectins in innate immunity, siglecs, and galectins, must not be underestimated. Siglecs represent a lectin superfamily that is widely expressed by immune cell subsets and characterized by binding to sialic acid residues. The study by Nagala et al. investigates a potential role of Siglec-E as a negative regulator of TLR4-mediated endocytosis and signaling as proposed in previous studies. However, while Siglec-E induction by bacterial lipopolysaccharide (LPS) modulated the phenotype of macrophages, their study does not support a significant role for Siglec-E in TLR4-mediated endocytosis and signaling functions.

Galectins share a common structural fold and exhibit a preference for *N*-acetyllactosamine-containing glycoconjugates. In their Perspective article, Sundblad et al. discuss findings on galectin functions in intestinal inflammation. Their data indicate that galectins represent active players in the intestinal mucosa to preserve immune and epithelial homeostasis. Thus, galectins may serve as promising biomarkers and therapeutic targets during severe mucosal inflammation.

CLRS IN INFECTION, INFLAMMATION, AND AUTOIMMUNITY

The Research Topic is complemented by comprehensive review and opinion articles focusing on the functions of CLRs in infection, inflammation and autoimmunity. In their review article, Goyal et al. describe CLR interaction with human pathogenic fungi. The authors summarize known functions of CLRs, such as Dectin-1, Dectin-2, Mincle, Mannose receptor (MR), and DC-SIGN, in fungal recognition and highlight their relevance in orchestrating antifungal responses. Besides their role in antifungal immune responses, CLRs are also involved in viral recognition. How CLRs contribute to antiviral immunity on the one hand, but may also be exploited by viruses to escape immune responses on the other hand, is the focus of the review article by Bermejo-Jambrina et al. The authors provide an overview of known interactions between viruses and CLRs, including the impact of CLR engagement on virus internalization, transmission, and cross-presentation of viral antigens. In addition, CLRs contribute to innate and adaptive immune responses during viral infection, such as type-I interferon responses or T helper cell polarization. Cross-talk mechanisms between CLRs and complement receptors in opsonization

and virus internalization are discussed in this review as well. Wagener et al. review the relevance of the Dectin-1/Syk/CARD9 signaling axis in anti-mycobacterial immunity. The authors specify Dectin-1 functions in recognition of mycobacteria and discuss recent findings on the crucial role of Syk signaling and the adaptor protein CARD9 in anti-mycobacterial immunity. While a distinct mycobacterial PAMP recognized by Dectin-1 still remains to be identified, this review highlights the relevance of the Dectin-1 pathway in mounting an immune response to *Mycobacterium tuberculosis*, which may lead to potential applications in tuberculosis vaccine adjuvant development.

CLRs not only play an important role in the recognition of pathogens, but may also sense DAMPs and contribute to sterile inflammation and autoimmunity. Chiffoleau reviews this essential role of CLRs as driving players of sterile inflammation and highlights their relevance for autoimmune diseases, allergy, or cancer. Specific aspects of how myeloid CLRs sense their cognate ligands and crosstalk with heterologous receptors are detailed by del Fresno et al. Their review article deals with the diversity of signaling modules in CLRs. Signaling may require CLR multimerization; the relative affinity or avidity of CLR ligands may determine whether an activatory or inhibitory signal is finally conveyed into the CLR-expressing cell. In conclusion, further research is needed to understand how different signaling pathways triggered by CLRs and heterologous receptors act in concert.

Two articles in this Research Topic focus on CLRs in autoimmune diseases. While Hadebe et al. review current knowledge on the role of CLRs recognizing fungus-derived and other allergens in asthma, the opinion article contributed by te Velde discusses clues for a role of the CLR Mincle in Crohn's disease. The Research Topic is rounded by two review articles on structural aspects of ligand recognition by the CLR Mincle and the identification and characterization of novel Mincle ligands. Williams discusses the structural basis of Mincle recognition of lipidic ligands including sterols, glucose- and glycerol-based glycolipids, and glycosyl diglycerides. This review also details how variations of Mincle ligands have provided insights into structure-activity relationships and may help to identify novel Mincle ligands. Braganza et al. focus on the chemical nature of known Mincle ligands and the rational synthesis of analogs to screen for potent Mincle agonists. Interestingly, subtle changes to functional groups in the lipid backbone of Mincle ligands were found to markedly affect their immune stimulatory properties. In conclusion, Mincle agonists display promising potential as vaccine adjuvants and immunotherapeutics.

CONCLUSIONS

Mounting evidence has demonstrated that lectin receptors play crucial roles in innate immunity. This Research Topic provides numerous examples of how lectins are involved in immune responses during infections or inflammatory processes. It also highlights the utility of lectin targeting for antigen delivery to enhance vaccine efficacy. Future studies will unravel the potential of lectins as therapeutic targets during infections, autoimmune diseases, or cancer.

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All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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OPINION
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The C-Type Lectin Mincle: Clues for a Role in Crohn's Disease Adjuvant Reaction

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The term adjuvant is predominantly used when discussing vaccines, but only mimics how normally infections activate the immune system to secure that an innate immune reaction induces dendritic cells (DCs) to become optimally stimulatory for T cells. The interaction with the pathogen determines the different signals that are needed for a DC to become fully operated and give the proper polarizing factors to the differentiating T cell. Optimal co-stimulation requires a signal that is provided by upregulated receptors (CD80 and CD86) on DCs, and the T cell polarizing signal is mediated by various soluble or membrane-bound factors, like IL-12 for Th1 cell polarization. These signals are provided by ligation of pattern recognition receptors (PRRs), such as toll-like receptors (TLRs) and C-type lectins that can sense infection through recognition of pathogen-associated molecular patterns (PAMPs) or various inflammatory tissue factors (1).

Resting macrophages have low major histocompatibility complex class II and co-stimulatory molecules expressed on their surface. Macrophages can take up microorganisms *via* receptors such as scavenger receptors, complement receptors, and C-type lectins for degradation in phagosomes resulting in peptides for presentation. Macrophages also continuously scavenge dead or dying cells. These are a rich source of self-antigens, so it is very important that they do not activate naïve T cells when there is no ongoing microbial infection.

In Crohn's disease (CD), a chronic inflammatory bowel disease (IBD), multiple factors have been described that contribute to disease pathogenesis (2). The exact etiology of IBDs still remains unknown, although it is thought that the diseases result from an excessive immune response directed against microbial or environmentally derived antigens that can be triggered by the disruption of the intestinal epithelial barrier integrity. The resulting inflammation is a very general reaction; a specific antigen mediating the inflammation has never been identified. The response is induced by the luminal microbiota, where microbial antigens as adjuvants stimulate the immune reaction. This results in activated innate (macrophages and neutrophils) and adaptive (Th1/Th17 and B lymphocytes) responses (3). In this respect, CD includes many characteristics of an immunologic adjuvant reaction.

Most proteins are poor immunogens when injected alone. Various substances that induce co-stimulatory, adjuvant, activity have been added in vaccines for a long time to induce appropriate antibody responses. Vaccines containing bacterial products, necessary for T cell responses, are very potent and therefore use in humans is limited. In recent years, the development of adjuvants that induce a strong cellular response has shifted from an empirical to a rational process based on knowledge of molecular mechanisms. A major breakthrough was the identification of the C-type lectin Mincle (macrophage-inducible C-type lectin) as one of the main receptors involved (4–6).

In this opinion article, I provide clues that the cellular adjuvant reaction that characterizes the pathophysiology of CD might be mediated by signaling *via* Mincle.

MINCLE

Mincle (also called clec4e or clecsf9) was first described as a downstream target of NF-IL6 (also named C/EBP-β), a transcription factor, in macrophages (7). They demonstrate that Mincle mRNA was strongly induced in response to several inflammatory stimuli, such as LPS, TNF-α, IL-6, and IFN-γ in murine macrophages. A few years later, Mincle was grouped together with macrophage C-type lectin (MCL), DC immunoreceptor, and Dectin-2 (DC-associated lectin-2) as type II-related C-type lectins (8). These genes were mapped in an arthritis susceptibility locus in a rat model and the first indication for an immune activating function of Mincle was proposed (9). Mincle serves as a receptor for various bacteria, fungi, and other molecules (listed in **Figure 1A**). Mincle signals *via* association with the FcRγ chain that contains an activating receptor coupled with an immunoreceptor tyrosine-based activation motif, ultimately resulting in activation of NF-κB (10).

The first suggestion that Mincle is a receptor for a cell wall component of *Mycobacterium tuberculosis*, the glycolipid trehalose-6,6′-dimycolate (TDM, also named cord factor), was made when it appeared to be involved in a characteristic process of mycobacterial infection: the formation of granulomas (4). Mycobacteria can persist in normal tissues (39); recruitment of Mincle by TDM coupled to immunoglobulin G-opsonized beads interferes with phagosome maturation (40). The activity of Mincle is mediated by a ligand binding site that is conserved in a wide range of mammalian species (41). The Th1/Th17 adjuvanticity of TDM and its synthetic analog trehalose-6,6′-dibehenate (TDB) including its molecular mechanism *via* Syk and Card9 was confirmed in several studies (5, 6, 42).

Mincle protein is barely detectable on resting cells (4, 42). In experiments in various tissues in rhesus macaques, the frequencies of $CD14^+$ gated cells that express Mincle in colon and ileum were low compared with bone marrow, liver, spleen, and lymph nodes (43). Induction of Mincle expression was shown to be induced by several pathogenic and non-pathogenic stimuli. Mincle was shown to be induced by TDM in the absence of Mincle protein expression *via* MCL (also called dectin-3) that was constitutively expressed in myeloid cells (44–46) through protein–protein interaction *via* its stalk region (47). C/EBP-β is the central hub in Mincle expression and connects TLR4 signals to TDB/TDM responsiveness through MyD88-dependent upregulation of Mincle (32, 48).

MINCLE AND CD

In literature, there is no direct link that connects Mincle to CD. There is, however, already a lot of information that links Mincle to other diseases. Most of these are also inflammatory-mediated diseases, such as rheumatoid arthritis (49, 50), allergic skin inflammation (28) and post-ischemic inflammation (51, 52), and other experimental inflammatory models (53–58).

Mincle has been shown to regulate numerous cellular responses including phagocytosis, endocytosis, respiratory burst, Nlrp3 inflammasome activation, NET formation, pro-inflammatory cytokine, and chemokine production and promotes Th1/Th17 responses [recently reviewed in Ref. (59, 60)]. These are all inflammatory reactions that have been described to play a role in CD. In **Figure 1A**, the different factors that are involved in Mincle signaling and also associated with CD are highlighted and discussed beneath.

As indicated, Mincle can act as a receptor for several different pathogens. The question is if these microorganisms have also been associated with CD. For *Mycobacterium avium* subspecies paratuberculosis, this is well known, it can be isolated from intestinal tissues and blood samples from CD patients at higher frequency than healthy persons (61). Treatment with antimycobacterial regimens in clinical trials achieved reversal of CD symptoms (62, 63). Also other bacteria linked to Mincle have been associated with CD: *Listeria monocytogenes*, *Klebsiella pneumonia*, *Streptococcus pneumonia*, *Pneumocystis pneumonia*, and *Escherichia coli* (64–70). A dysfunction in both a specialized form of autophagy, xenophagy, and HIF-1α was demonstrated to be involved in adherent invasive *E. coli* infections in CD (71). HIF-1α-induced inducible nitric oxide synthase produces nitric oxide (NO) that was shown to be upregulated in the inflamed mucosa in response to pro-inflammatory cytokines (72, 73).

In around 50% of CD patients' granulomas can be detected (74). Granulomas are linked to mycobacterium, and Mincle has been shown to be important (75). Several cytokines, including IL-1 and TNF-α, have been shown to promote the formation of granulomas (76). These cytokines can be secreted upon stimulation *via* Mincle after stimulation with TDM-mediated granuloma formation (4).

Toward fungal glycans, it has been demonstrated that human peripheral blood mononuclear cells (PBMCs) from CD patients show a hyperresponsiveness with a central role for Syk and Src signaling (77). PBMCs from patients with CD produce more IFN-γ and IL-17 upon exposure to *Candida* (78).

A well-known complication of CD is intestinal fibrosis. Recently, it was demonstrated that this was mediated *via* a PKCδ-mediated redox-dependent signaling process by accumulated advanced oxidation protein products (79).

CARD9 has been an autoimmune disease-associated gene, and differential expression of this gene might be a functional mechanism underlying observed GWAS signals (80). It coordinates Th17- and IL-22-producing cells in intestinal immune responses after epithelial injury in mice (81). Aberrant regulation of *CARD9*, either through genetic mutation (e.g., polymorphism) or activation by environmental triggers *via* Mincle, could contribute to pathological immune activation.

Crohn's disease patients treated with TNF blockers demonstrated an increased risk of opportunistic infections such as mycosis, aspergillosis, pneumocystosis, or cryptococcosis (82) and also Pityrosporum (Malassezia) folliculitis (83), and cutaneous lesions of Leishmaniasis (84).

About 50% of the world's population carry the *Helicobacter pylori* bacterium. In a meta-analysis, a negative association was found between *H. pylori* infection and CD. They conclude that *H. pylori* could exert an immunomodulatory effect in IBD (85) maybe by Mincle-mediated anti-inflammatory signaling (18).

Finally, danger/damage-associated molecular pattern (DAMP)-derived triggers from dead cells may contribute *via*

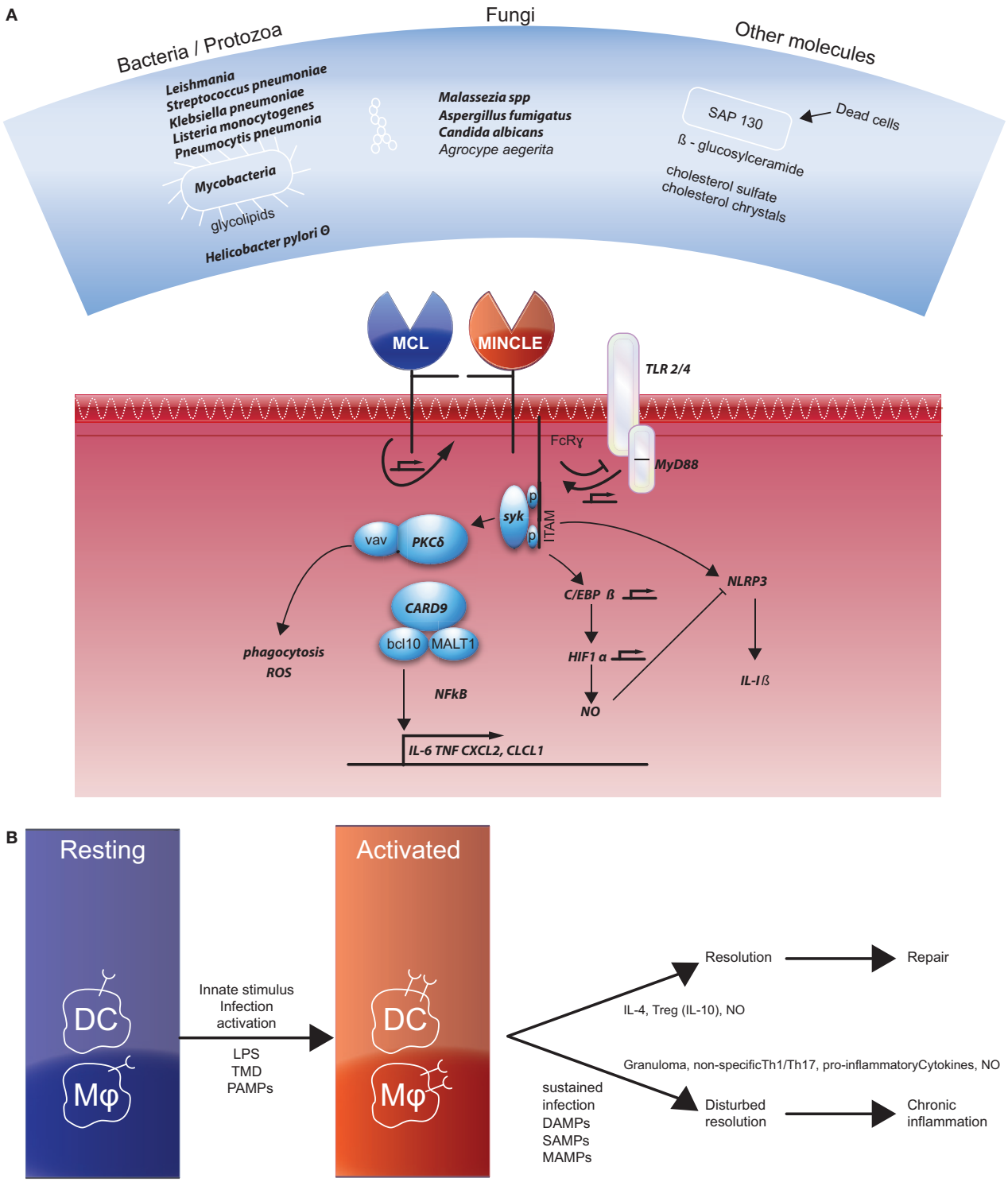

FIGURE 1 | **(A)** Overview of the ligands of Mincle (top panel) and the signaling pathways described in the current literature. Factors that were also documented in literature related to Crohn's disease are depicted in bold. References of the different ligands not mentioned in the text are for bacterial pathogen species (11–13), *Leishmania* (14), mycobacteria, and corynebacteria (15, 16), *Streptococcus pneumonia* (17), *Helicobacter pylori* (18), *Malassezia* (19, 20), *Aspergillus fumigatus* (21), *Candida albicans* (22, 23), SAP130 (10, 24, 25), β-glucosylceramide (26), cholesterol crystals (27), and cholesterol sulfate (28). References for the various signaling molecules not mentioned in the text are for TLR4/MyD88 (29), PKC (30), vav proteins (31), HIF1α (32) and Nlrp3 (33, 34). **(B)** Resting dendritic cells (DCs) and macrophages also do not express Mincle, but they do express macrophage C-type lectin (MCL). Different activating signals induce Mincle expression. Two different pathways induce repair or chronic inflammation as indicated. References for activation and chronic inflammation are given in the text, for the resolution: Th2 skewing *via* IL-4 (35, 36), IL-10 (37), and NO (38).

Mincle to excessive and sustained inflammation in CD patients with active disease (86).

Taken together, numerous Mincle-related ligands and signaling molecules can be linked to CD.

HOW COULD MINCLE MEDIATE CD INFLAMMATION?

The efficacy by which Mincle handles microbes, microbial products, and damaged cells directs whether the outcome will be with suppression or with excess inflammation (see **Figure 1B**). These pathways can be polarized, but since CD is a chronic relapsing disease the activating signals and wound healing processes might also be present more or less in parallel. Mincle functions as a receptor for different bacteria and fungi, leading to proper immune responses that functions to eradicate pathogens (38, 82). Early response mediated *via* TLRs and MCL expressed on macrophages by a primary infectious stimulus (PAMP) results in the upregulation of Mincle expression. This leads to a sustained signaling process *via* the activating motif of the FcRγ chain and the production of pro-inflammatory cytokines and finally a non-specific activation of Th1/Th17 response. In an appropriate immune response, the end product is the eradication of the infectious agent and resolution of the inflammation. Mincle stimulation can help by inducing anti-inflammatory genes and genes involved in wound healing. In case of a sustained infection when pathogenic ligands are still present or because of tissue damage resulting in the presence of DAMPs or self-associated molecular patterns (SAMPs) continuous Mincle signaling remains. TLR- and Mincle co-dependent genes are enriched among genes required to handle persisting D/M/SAMP signals (38).

There are several pathways that counter-regulate Mincle on macrophages and DCs. Among them is the observed effect of IL-4 on Mincle expression of monocyte-derived DCs (87). This is, however, an artificial system, because these cells co-express surface markers (CD83 and DC-SIGN) that are not found to on the same cells in the *in vivo* situation (88).

In CD, there is evidence that a dysregulated macrophage function and a consecutive defective acute inflammatory response result in the impaired clearance of commensal bacteria. The persistence of the bacteria leads to a chronic granulomatous inflammation. Pathogenic infections may act as triggers or contributing factors for the chronic inflammation (2, 89) that is mediated by other stimuli of various nature, involving microbial-associated molecular patterns, DAMPs, or SAMPs, all described to be ligands of Mincle.

It will be of potential interest to study the direct role of Mincle as a predominant activating C-type lectin receptor *via* a Syk/Card9-dependent signaling mechanism in CD. Genetic susceptibility, barrier defects, or bacterial handling, dysbiosis or infection, sustained innate immunity, and defective regulation are all layers of a multi-hit model of intestinal inflammation (2). They are combined with different homeostatic modules such as autophagy, ER stress, antimicrobial proteins, the microbiota, PRRs, cytokine modules, and regulatory T cells. Defective modules may predispose people to the development of chronic intestinal inflammation. Determination of the role of Mincle in these layers and modules will reveal if Mincle is an important receptor of mediator of the chronic nature of CD, which could be relevant for therapeutic intervention. Targeting Syk has been suggested as a treatment for allergic and autoimmune disorders (90). In rheumatoid arthritis, inhibition of Syk has been studied as a treatment option (91). Although there is no direct evidence on the role of Mincle in CD, the data on the expression and role of Mincle in health and disease reveal numerous potential starting points. The synergy and antagonisms of the various PRRs, whether these are C-type lectins or TLRs, and their differential regulation on cells of the innate immune system, macrophages, and DCs is an important topic to understand the endogenous adjuvant reaction that they might induce. There are probably multiple mechanisms and interactions that result in the observed pathogenic immune reaction that is the fundament of CD. Here, the surprising overlap between features of CD and the roles that Mincle plays in a (chronic) immune reaction might indicate that CD could be an adjuvant reaction induced by Mincle triggering.

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The author confirms being the sole contributor of this work and approved it for publication.

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LC3-Associated Phagocytosis Is Required for Dendritic Cell Inflammatory Cytokine Response to Gut Commensal Yeast *Saccharomyces cerevisiae*

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The human fungal microbiota known as mycobiota is increasingly recognized as a critical factor in human gut health and disease. Non-pathogenic commensal yeasts such as *Saccharomyces cerevisiae* promote homeostasis in the gut, whereas dysbiosis of the gut mycobiota is associated with inflammation. Glycan-binding receptors (lectins) are key host factors in host–mycobiota interaction in the gut. They are expressed on immune cells such as dendritic cells (DCs) and recognize fungal polysaccharides. This interaction is imperative to mount appropriate immune responses for immune homeostasis in the gut as well as clearance of fungal pathogens. Recent studies demonstrate that microtubule-associated protein light-chain 3 (LC3)-associated phagocytosis (LAP) is involved in lectin–fungi interactions. Yet, the biological impact of LAP on the lectin function remains largely elusive. In this report, we demonstrate that in mouse LAP is linked to dendritic cell-associated lectin 2 (Dectin-2), a C-type lectin specific to fungal α-mannan polysaccharide. We found that mouse Dectin-2 recognizes commensal yeast *S. cerevisiae* and *Kazachstania unispora*. Mouse bone marrow-derived DCs (BMDCs) produced inflammatory cytokines TNFα and IL-1β in response to the yeasts in a Dectin-2 and spleen tyrosine kinase (Syk)-dependent manner. We found that *S. cerevisiae* and *K. unispora* induced LAP in mouse BMDCs upon internalization. Furthermore, LC3 was activated by stimulation of BMDCs with the yeasts in a Dectin-2 and Syk-dependent manner. To address the biological impact of LAP on Dectin-2 yeast interaction, we established a knock-in mouse strain (Atg16L1^{E230}, thereafter called E230), which BMDCs exhibit autophagy-active and LAP-negative phenotypes. When stimulated with yeasts, E230 BMDCs produced significantly less amounts of TNFα and IL-1β. Taken together, we revealed a novel link between Dectin-2 and LAP that enables host immune cells to respond to mycobiota.

Keywords: LC3-associated phagocytosis, dendritic cell-associated lectin 2, fungi, dendritic cell, autophagy

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INTRODUCTION

The human gut contains various fungal species, which form together the fungal microbiota or mycobiota. The number of intestinal commensal fungi represents relatively small fraction of human microbiota when compared to bacteria; however, they play critical roles in gut health and disease (1). Mycobiota dysbiosis is associated with exacerbated gut inflammation in mouse and humans (2, 3). The most dominant fungal species are *Candida albicans* and *Candida tropicalis* in humans and mice, respectively (1, 4, 5). Both species affect the severity of inflammatory bowel diseases. In several human randomized clinical trials, once taken orally, a number of commercially available strains of *Saccharomyces cerevisiae* known as *S. boulardii* has been shown to prevent *Clostridium difficile*-associated diarrhea (6). However, molecular and cellular mechanisms underpinning the impact of mycobiota remain largely unknown.

One of the key host factors includes cell surface lectin receptors expressed on immune cells. Such receptors recognize fungal polysaccharides and induce immune cell activation. Fungal β-glucan is recognized by dendritic cell-associated lectin-1 (Dectin-1) (7), chitin by macrophage mannose receptor (MR) (8), β-mannan by Galectin-3 (9), and α-mannan by dendritic cell-associated lectin 2 (Dectin-2) (10). Dectin-2 possess exclusive specificity to mannose, as all the identified glycan ligands for Dectin-2 contain mannose (10–12). Dectin-2 binding to fungi activates the spleen tyrosine kinase (Syk) signaling pathway and induces immunomodulatory function of DCs including downstream cytokine production (13). This, in turn, induces a Th17 response to remove invasive fungi such as *C. albicans* (14, 15). Another key function of Dectin-2 is internalization of the ligand into the cell. An earlier study shows that anti-Dectin-2 antibody (Ab) binding induces rapid receptor internalization (16), and recently it is shown that Dectin-2 directs intracellular cargo to lysosomes (15). While these reports demonstrate that Dectin-2 is an endocytic/phagocytic receptor, how this pathway impacts on the immunomodulatory function of Dectin-2 remains unknown.

Another key host factor involved in host–fungi interactions is microtubule-associated protein light-chain 3 (LC3)-associated phagocytosis (LAP). LC3 is originally characterized as an autophagy-associated protein that initiates the formation of the autophagosome (17). Cellular stresses such as nutritional change, infection, and oxidative damage recruit LC3 to newly synthesized membrane components that encapsulate damaged organelles and intracellular pathogens for degradation (17). Recent reports indicate that LC3 is also involved in phagocytosis, to fuse phagosomes with lysosomes, thereby facilitating degradation of exogenous components such as invasive fungi. In an *Aspergillus fumigatus* infection model in mouse, LAP is shown to be critical for fungal killing (18). LAP-deficient and autophagy-active bone marrow-derived macrophages (BMMs) exhibit reduced fungal killing (18). LC3β KO bone marrow-derived DCs (BMDCs), which lack both autophagy and LAP, were less efficient in presenting fungal antigens to MHC class II pathway (19). Furthermore, LC3β KO BMMs show enhanced inflammatory cytokine production in response to *C. albicans* (20). Since LC3β KO cells are both autophagy and LAP-negative, these findings need to be confirmed in an autophagy-active and LAP-negative system to assess the role of LAP in immune cell response to fungi.

In this report, we employed a new genetic tool to assess the link between LAP and Dectin-2 in DC–mycobiota interaction. We found that mouse Dectin-2 recognizes two human commensal yeasts *S. cerevisiae* and *Kazachstania unispora*. These yeasts induced the cytokines TNFα and IL-1β from BMDCs in a Dectin-2 and Syk-dependent manner. Using a transgenic mouse strain with a mutation in a key autophagy gene, *Atg16L1*, which is autophagy-active and LAP-deficient ($Atg16L1^{E230}$, thereafter called E230), we found that LAP was required for the inflammatory cytokine production from BMDCs in response to the commensal yeasts. The commensal yeasts induced LAP, shown by the recruitment of LC3 to the internalized yeasts in WT BMDCs, but not in E230 BMDCs. Consistently, we found these yeasts induced LC3 activation and LAP in a Dectin-2-dependent manner. Taken together, our data demonstrate for the first time that interaction between Dectin-2 and commensal non-pathogenic yeast induces LAP, which is required for immunomodulatory function of Dectin-2 in DCs.

MATERIALS AND METHODS

Mice

C57BL/6J WT, $Atg16L1^{E226}$, and $Atg16L1^{E230}$ mice (thereafter called E226 and E230 mice, respectively), which are defective in autophagy and/or LAP, respectively, and Dectin-2 KO mice were maintained in the specific pathogen free animal facility at the University of East Anglia (Norwich, UK). Generation and characterization of E226 and E230 mice will be described elsewhere. The inducible $Syk^{flox/flox}$/rosa26CreERT2 mouse strain was maintained at Toronto University (Toronto, ON, Canada) (21). The tamoxifen induction protocol and characterization of the Syk knockout phenotype have been described (22, 23). Control littermates were treated with 10% v/v ethanol in sunflower oil, the diluent for tamoxifen. Bone marrow cells were harvested and frozen. Animal use in this study was conducted under the project license (70/8177 and 70/8332) authorized by the UK home office, and University of Toronto Faculty Advisory Committee on Animal Services and Toronto General Research Institute Advisory Committee on Animal Services, and conducted in accordance with the guidelines of the Canadian Council on Animal Care.

Reagents

Chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise stated. *N*-succinimidyl ester conjugated (NHS)-Alexa647 and Cell trace violet (CTV) were from ThermoFisher Scientific (Waltham, MA, USA). α-Mannan from *Malassezia furfur* was purchased from InvivoGen (San Diego, CA, USA). Scleroglucan (β-glucan) was obtained from Elicityl (France). Lipopolysaccharide (LPS) from *Klebsiella pneumoniae* O1 was obtained from Dr. Chris Whitfield (University of Guelph, Canada). Alexa647-labeled anti-mouse Dectin-1 Ab (clone 2A11, rat IgG2b) was purchased from Bio-Rad (Hercules,

CA, USA). Alexa647-labeled anti-Dectin-2 Ab (clone 2B4, rat IgG2a) was generated as described previously (11). Biotinylated anti-mouse Dectin-2 Ab (clone 2B4) was also generated using NHS-LC-biotin (ThermoFisher Scientific). Alexa647-labeled isotype-control Abs, mouse Fc receptor blocking Ab (clone 93, BioLegend), and *R*-phycoerythrin (PE)-labeled streptavidin were purchased from BioLegend (San Diego, CA, USA). ELISA kits for mouse TNFα and IL-1β were from BioLegend and R&D systems (Minneapolis, MN, USA), respectively. Anti-LC3 A/B (#4108S), β-actin (#4970S), and anti-rabbit IgG conjugated with horseradish peroxidase (HRP) (#7074P2) were from Cell Signaling Technologies (Danvers, MA, USA).

Cell Lines

RAW macrophage cell line expressing mouse Dectin-2 was given from Dr. Kiyoshi Ariizumi (UT Southwestern, Dallas, TX, USA) and was cultured in RPMI1640 (Lonza, Walkersville, MD, USA) supplemented with 25 mM HEPES, 2 mM L-glutamine, 100 U/mL penicillin/streptomycin, 10% FBS (ThermoFisher Scientific), and 55 μM mercaptoethanol (R10). BWZ.36 cells expressing wild-type mouse Dectin-2 (Dectin-2^{WT}), carbohydrate-binding incompetent mouse Dectin-2 (Dectin-2^{QPD}), and the mock transfectant were maintained as described previously (11).

Yeasts

Saccharomyces cerevisiae isolated from human feces (#2966) and *C. albicans* (#3779) was from National Collection of Yeast Culture (Norwich, UK). *K. unispora* isolated from human feces (#CBS 3004) was obtained from CBS-KNAW Fungal Biodiversity Centre (Netherlands). Yeasts were initially cultured in yeast media medium at 25°C for 3 days. On the day of experiment, fungi culture was diluted at 1:20 and grown at 37 and 30°C for *S. cerevisiae* and *K. unispora*, respectively. Yeast culture was in exponential growth phase (OD = 0.7) when harvested for subsequent experiments.

Reporter Assay

Reporter assay was performed as previously described (11). Briefly, 1×10^5 of BWZ.36 cells expressing mouse Dectin-2 were incubated with living yeasts at the indicated multiplicity of infection (MOI). Cells were lysed, and β-galactosidase activity was monitored by a colorimetric assay (11).

Flow Cytometry

For the binding assay of yeasts to Dectin-2-RAW cells, yeasts were suspended in PBS at 1×10^7 cells/ml and fluorescently labeled with 10 μg/ml NHS-Alexa647 for 1 h at 25°C. Mouse Dectin-2-RAW cells (1×10^7 cells/ml) were labeled with 0.3 μM CTV in PBS for 10 min at 25°C. After labeling, 2.0×10^5 of Dectin-2-RAW cells were incubated with the fluorescent yeasts (MOI = 5) for 1 h at 37°C in FACS Buffer (HBSS containing 25 mM HEPES and 0.1% BSA) supplemented with 2 mM $CaCl_2$ or 10 mM EGTA. Cells were washed and analyzed by Fortessa cell analyzer. To analyze lectin expression on mouse BMDCs, cells were incubated with Fc receptor blocking Ab for 10 min at 4°C. The cells were stained with Alexa647-labeled anti-mouse Dectin-1 and Dectin-2 Abs, or the isotype-matched control Abs for 30 min at 4°C. Biotinylated anti-Dectin-2 and the isotype-control Abs were also used in combination with PE-labeled streptavidin. The stained cells were washed with FACS buffer and incubated with 0.33 μg/ml propidium iodide before analysis by Fortessa. All data were processed in FlowJo (TreeStar, USA).

Analysis of Cytokine Production from BMDCs

Mouse BMDCs used in this study were generated as described before (11). To analyze cytokine production, 1×10^5 of BMDCs were cultured in a 96-well round-bottom plate in the presence of *S. cerevisiae*, *K. unispora*, and *C. albicans* at a MOI of 5, 100 μg/ml α-mannan, or 100 ng/ml LPS, and 1 mg/ml curdlan (Wako Chemicals, Tokyo, Japan), a β-glucan, at final concentration for 16 h. The amount of TNFα and IL-1β in the culture supernatant was measured by ELISA.

Western Blot Analysis of LC3 Lipidation

Bone marrow-derived DCs were generated in a 24-well plate (1.9×10^5 cells/well). On day 6, *S. cerevisiae* and *K. unispora* were added to the well (MOI = 5 and 10) and incubated for 60 min at 37°C. After incubation, culture supernatant was removed and 80 μl of lysis buffer (20 mM Tris–HCl, 150 mM NaCl, 1 mM EDTA, 1% Triton-X 100, 2 mM sodium orthovanadate, 10 mM sodium fluoride, and one complete ULTRA tablet, Mini) was added to the well and incubated for 5 min at 4°C. After the cell lysis, cell debris was removed by centrifugation at $21.1 \times g$ for 10 min at 4°C. The supernatant was collected, mixed with 4× of laemmli buffer (Bio-Rad) containing 1.43 M β-mercaptoethanol, and heated for 15 min at 75°C. Twenty microliters of cell lysate were loaded onto a 4–15% gradient TGX mini gel (Bio-Rad) and ran for 30 min at 200 V. Proteins were transferred to PVDF membrane (Thermo Scientific, Waltham, MA, USA) for 1 h at 100 V. Membrane was blocked with 5% non-fat milk (Sigma-Aldrich) in PBS containing 0.05% Tween-20 (PBS-T) for 1 h at 25°C. Membrane was washed 4 times for 5 min with PBS-T. The membrane was incubated with primary Abs anti-LC3 A/B (1:1,000), β-actin (1:5,000) in PBS containing 1% BSA for 16 h at 4°C. Membrane was washed in PBS-T as above and incubated with anti-rabbit IgG conjugated with HRP (1:3,000) in 5% non-fat milk in PBS-T for 1 h at 25°C. The membrane was washed in PBS-T as above and then incubated with ECL detection reagent (GE Healthcare Life Sciences, Marlborough, MA, USA). Image was obtained and the band intensity was quantified using Fluorochem E (ProteinSimple, San Jose, CA, USA).

Fluorescent Microscopy

Mouse BM cells were cultured on coverslips (VWR, Radnor, PA, USA) placed in 24-well plate in the culture medium for BMDC generation (11). To test phenotype of E226 and E230 cells, on day 6 BMDCs were either left in the culture medium, incubated in HBSS for 2 h at 37°C to induce starvation-driven autophagy, or incubated with polystyrene beads (Polysciences, Warrington, PA, USA) for 1 h at 37°C and then Monensin (Sigma-Aldrich)

for 1 h at 37°C to induce LAP. The cells were washed with PBS three times and fixed with ice-cold 100% methanol for 10 min at −20°C. The cells were washed and incubated with PBS containing 5% goat serum (Gibco, Waltham, MA, USA), 0.3% Triton-X for 30 min at 25°C. The fixed cells were incubated with anti-LC3 Ab (1:500, Cell Signaling, Danvers, MA, USA) in PBS containing 1% BSA, 0.3% Triton-X for 16 h at 4°C on a rocker (20 rpm). The cells were washed with PBS three times and incubated with Alexa488 goat anti-rabbit IgG (1:1,000, Life Technologies, Carlsbad, CA, USA) in PBS containing 1% BSA, 0.3% Triton-X for 2 h at 25°C. The cells were washed with PBS three times and stained with DAPI (1.0 µg/ml, Thermo Scientific) in PBS for 5 min at 25°C on a rocker (20 rpm). The cells were washed twice with PBS, the coverslip was mounted in fluoromount (eBioscience) and kept at 4°C in the dark until being imaged with Zeiss using a software Axio Vision fluorescence imager. Images were taken using 63× objective setting with an immersion oil Type LDF (Cargill, Wayzata, MN, USA). To monitor yeast-induced LAP in BMDCs, yeasts were washed twice with PBS and incubated with 4% paraformaldehyde (PFA) in PBS for 1 h at 25°C. The PFA-fixed yeasts were washed with PBS and suspended at 1.0×10^7 cells/ml in BMDC culture medium. The yeasts were further incubated with 10 µg/ml NHS-Alexa555 (ThermoFisher Scientific) for 1 h at 25°C in the dark and washed with the medium. On day 6, BMDCs were incubated with Alexa555-labeled yeast for 2 h at 37°C at a MOI of 10. Around 100 cells for WT, E226, and E230, BMDCs were analyzed to count the number of starvation-induced LC3 puncta in the cell. The puncta size was defined by a diameter of 1.67 µm, any LC3-punta smaller or larger were not included.

Statistical Analysis

One-way ANOVA followed by Tukey's test were used for statistical analysis on Prism software (GraphPad). $p < 0.05$ was considered as statistically significant.

RESULTS

Mouse Dectin-2 Recognizes Commensal Yeasts

We tested Dectin-2 binding to the commensal yeast *S. cerevisiae* and *K. unispora* isolated from human feces. To check the binding of *S. cerevisiae* and *K. unispora* to Dectin-2, we employed the mouse Dectin-2-expressing BWZ.36 reporter cell assay in which we measured β-galactosidase activity as a readout for Dectin-2 interaction with yeasts (11). Importantly, human and mouse Dectin-2 has conserved carbohydrate-binding specificity (10, 24). We found that the commensal yeasts bound to mouse Dectin-2 in the reporter assay (**Figure 1A**). The binding was mediated *via* the carbohydrate-recognition domain of Dectin-2, as the carbohydrate-binding incompetent mutant of Dectin-2 (Dectin-2^{QPD}) failed to bind to the yeasts (**Figure 1A**) (11). Dectin-2 interaction with the yeasts was further confirmed using RAW macrophage expressing mouse Dectin-2 by flow cytometry. We found that after 1 h incubation with yeasts, Dectin-2-RAW cells bound to both *S. cerevisiae* and *K. unispora* (**Figure 1B**). Of importance,

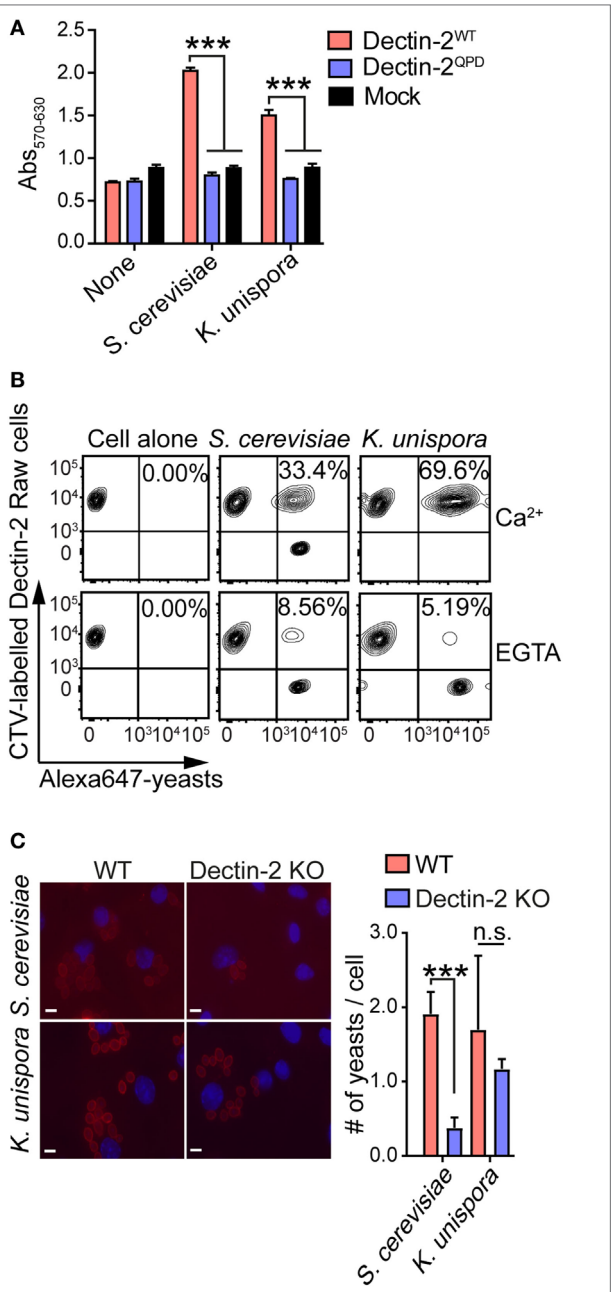

FIGURE 1 | Mouse Dectin-2 binds to gut commensal yeasts. **(A)** BWZ reporter cells expressing mouse Dectin-2^{WT}, Dectin-2^{QPD}, and mock transfectant were incubated with the indicated yeasts at a MOI of 5. After 1 day incubation, β-galactosidase activity in the reporter cells was monitored by a colorimetric assay. **(B)** Mouse Dectin-2-expressing RAW macrophages were incubated with Alexa647-labeled yeasts or left alone. The binding was analyzed by flow cytometry. **(C)** Mouse BMDCs were incubated with the Alexa555-labeled and paraformaldehyde-fixed yeasts (red) at a MOI of 10. After 2 h incubation, images of BMDCs were taken with the DAPI staining (blue). White bar indicates 5 µm. The number of yeasts associated with BMDCs was quantified. Data shown are the mean of triplicates ± SD from one representative experiment and reproducible in three independent experiments. Statistical analyses were performed by one-way ANOVA followed by Tukey's test. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; n.s., not statistically significant.

the binding was diminished when the calcium ion chelator EGTA was added (**Figure 1C**), indicating Ca^{2+}-dependent interaction of Dectin-2 with yeasts. We also tested mouse BMDC interaction with the yeasts. To this end, we incubated mouse BMDCs with Alexa555-labeled yeasts and counted the number of fluorescent yeasts associated with the cell. We found that Dectin-2 KO BMDCs showed fewer number of yeasts associated with the cell when compared to WT BMDCs (**Figure 1C**). Taken together, these data demonstrate that Dectin-2 recognizes commensal yeasts including *S. cerevisiae* and *K. unispora* through Ca^{2+}-dependent glycan recognition.

Gut Commensal Yeasts Stimulate Mouse BMDCs in Dectin-2 and Syk-Dependent Manner

Commensal yeasts and their components are increasingly recognized to stimulate host immune cells, which is important for gut health and disease (2, 4, 5). Therefore, we assessed the contribution of mouse Dectin-2 and its signaling pathway to the immune response to commensal yeasts. We found that *S. cerevisiae* and *K. unispora* stimulated mouse BMDCs, as shown by production of the cytokines, TNFα and IL-1β, after incubation at a MOI of 5 (**Figure 2**). The response was partially mediated by Dectin-2, as Dectin-2 KO BMDCs showed more than 50% reduction in cytokine production in response to the yeasts (**Figure 2A**). The residual cytokine production is likely mediated by other fungal recognition receptors including Dectin-1, MR, and TLRs, as shown previously (5, 25). This reduced cytokine production was only seen when Dectin-2 KO cells were stimulated with Dectin-2 ligands, as Dectin-2 KO BMDCs show normal response to ligands for TLR4 and Dectin-1 for instance (Figure S1 in Supplementary Material) (11, 14).

Since Syk is required for intracellular signaling of the C-type lectins including Dectin-2 (13), we sought to assess the contribution of Syk in BMDC response to commensal yeasts. Of note, *K. unispora* failed to stimulate Syk KO BMDCs, suggesting a dominant role of Syk-coupled fungal recognition receptors such as Dectin-1 and Dectin-2 (**Figure 2B**). Syk KO BMDCs were still able to respond to *S. cerevisiae* but to a lesser extent than WT BMDCs, implying contribution of non-Syk-coupled activation receptors such as TLRs (**Figure 2B**) (5). We confirmed that Syk KO BMDCs express Dectin-2 at a level indistinguishable with that of WT cells (**Figure 2C**). Taken together, our data indicate that gut commensal yeasts induce TNFα and IL-1β production from BMDCs in Dectin-2 and Syk-dependent fashion.

Gut Commensal Yeasts Induce LAP and LC3 Lipidation in BMDCs in a Dectin-2-Dependent Manner

Since previous studies suggest that pathogenic fungi *C. albicans* and *A. fumigatus* induce LAP (18, 20), we sought to test whether gut commensal yeasts also do so. To this end, we generated BMDCs from mice carrying mutations in autophagy protein Atg16L1 that affect autophagy and LAP. The E230 mouse strain carries a stop codon at the end of the coiled coil domain that

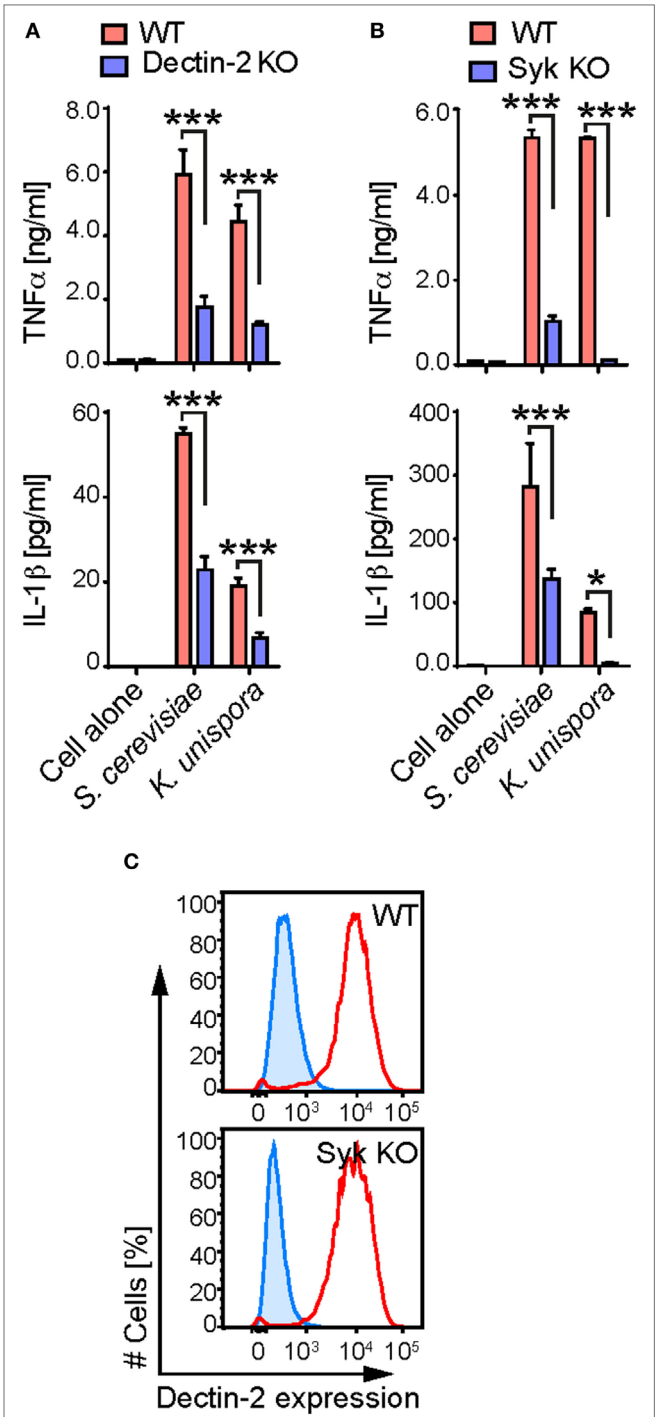

FIGURE 2 | Dectin-2 and Syk-dependent cytokine production by gut commensal yeasts. **(A,B)** WT, Dectin-2, and Syk KO BMDCs were incubated with the indicated yeasts at a multiplicity of infection of 5. After 16 h incubation, the supernatant was harvested, and the amount of TNFα and IL-1β was measured by ELISA. **(C)** WT and Syk KO BMDCs were stained with anti-Dectin-2 Ab (red) or the isotype-control Ab (blue). The stained cells were analyzed by flow cytometry. Data shown are the mean of triplicates ± SD from one representative experiment and reproducible in three independent experiments. Statistical analyses were performed by one-way ANOVA followed by Tukey's test.

contains glutamate residues E226 and E230 required for autophagy but lacks the linker region and the WD repeat domains required for LAP (26). The E226 mouse strain contains glutamate 226 and two additional amino acids (alanine and glycine) followed by a stop codon, which prevents expression of glutamate at position 230 and is therefore deficient in both autophagy and LAP (26). The autophagy and LAP phenotype of E226 and E230 BMDCs, respectively, were confirmed *in vitro*. E226 BMDCs exhibited the absence of both starvation-induced autophagy and polystyrene bead-induced LAP, whereas E230 BMDCs showed active autophagy to a lesser extent to that of WT and complete absence of LAP (**Figures 3A,B**). We found that E226 BMDCs produced significantly higher amount of IL-1β in response to LPS (**Figure 3C**), which was consistent with the previous reports demonstrating the inhibitory role of autophagy in inflammasome activation (17). On the other hand, IL-1β production from E230 BMDCs was almost indistinguishable with that of WT BMDCs, suggesting LAP is not involved in LPS-induced IL-1β production in BMDCs (**Figure 3C**).

When WT BMDCs were incubated with PFA-fixed and Alexa555-labeled yeasts, we observed LAP formation in the cell. In WT BMDCs, internalized *S. cerevisiae* was surrounded by LC3, indicating LAP formation (**Figure 4A**). This was completely abolished in both E226 and E230 BMDCs (**Figure 4A**). Likewise, Alexa555-labeled and PFA-fixed *K. unispora*-induced LAP in WT BMDCs, but not in E226 nor E230 BMDCs (Figure S2 in Supplementary Material). LC3 is known to be covalently ligated to phosphatidylethanolamine upon autophagy and LAP formation (17). Indeed, we observed lipidation of LC3 in BMDCs upon stimulation with the yeasts, occurring after 1 h incubation (LC3-II in **Figure 4B**). Of importance, this yeast-induced LC3 lipidation was Dectin-2 dependent, as it was partially reduced in Dectin-2 KO BMDCs (**Figure 4B**). Furthermore, LC3 lipidation occurred in a Syk-dependent manner (**Figure 4B**), which was consistent with previous study (20). We also observed LC3 lipidation in response to α-mannan (**Figure 4B**), suggesting that Dectin-2 ligation is sufficient to induce LC3 lipidation. The α-mannan used in this study bound to Dectin-2, but not Dectin-1 in a reporter assay (Figure S3 in Supplementary Material). Next, we sought to test whether Dectin-2 is involved in LAP formation when cells were stimulated with the commensal yeasts. Indeed, LAP formation in Dectin-2 KO BMDCs was significantly reduced when incubated with *S. cerevisiae* and *K. unispora* (**Figures 5A,B**). Taken together, these data demonstrate commensal yeasts induce LC3 lipidation and LAP in a Dectin-2-dependent manner.

LAP Is Required for Inflammatory Cytokine Production from BMDCs in Response to Gut Commensal Yeasts

To assess the involvement of LAP in immunomodulatory function of DCs, we compared yeast-induced cytokine production from WT, E226, and E230 BMDCs. When these BMDCs were stimulated with living yeasts, E230 BMDCs produced less amount of TNFα and IL-1β, suggesting that LAP is required for cytokine production in BMDCs in response to the commensal yeasts (**Figure 6A**). E226 BMDCs also showed modest reduction in both TNFα and IL-1β production in response to *S. cerevisiae*. In case of *K. unispora*-induced cytokine production in E226 BMDCs, reduction in TNFα was statistically significant, but IL-1β not. We observed the reduced TNFα production in E226 and E230 KO BMDCs in response to the PFA-fixed yeasts (Figure S4 in Supplementary Material). While the PFA-fixation of yeasts abrogated the IL-1β response (Figure S4 in Supplementary Material), these data suggest that the reduced cytokine production in E226 and E230 BMDCs was not due to the overgrowth of yeasts. We confirmed that WT, E226, and E230 BMDCs express fungal polysaccharide receptors, Dectin-1 and Dectin-2, at an indistinguishable level (**Figure 6B**). These data demonstrate that LAP is required for cytokine production in BMDCs in response to commensal yeasts.

DISCUSSION

The mycobiota is increasingly recognized as a key component of our mucosal surface that affects both bacterial microbiota and host physiology (1). The host immune system is a critical factor that interacts with the mycobiota both directly and indirectly through fungal metabolites and outer membrane vesicles (OMVs) that contain enzymes, nucleic acids, and polysaccharides (1, 27). While recent studies have identified host immune cell receptors responsible for fungal polysaccharide recognition (5), we still do not fully understand the molecular mechanisms underpinning their cell signaling. In this report, we have shown that LAP is involved in Dectin-2 cell signaling induced by gut commensal yeasts.

Commensal non-pathogenic yeasts such as *S. cerevisiae* are habitants in the human gut and provide beneficial effects to both the bacterial microbiota (28) and the host (1). Our results suggest that the commensal yeasts *S. cerevisiae* and *K. unispora* may induce immunomodulatory functions *via* Dectin-2 in the gut. In this regard, our preliminary data indicate that Dectin-2 is expressed in human and mouse myeloid cells in the lamina propria of the small intestine (Wittmann et al., unpublished data). Future studies must include *in vivo* animal models to test whether these commensal yeasts regulate gut immunity upon colonization, and whether Dectin-2 plays any roles in the immune regulation *in vivo*.

The most outer polysaccharide on the fungal cell surface is α-linked mannan, which can be recognized by mannose-specific C-type lectins including MR, DC-SIGN, and Dectin-2 (29). Interestingly, DC-SIGN has been shown to recognize *C. albicans N*-linked α-mannan but not those expressed in *S. cerevisiae* (30), suggesting that fungal surface α-mannan structure can vary among commensal yeasts. On the other hand, Dectin-2 recognizes both *C. albicans* and *S. cerevisiae*, as shown in this study and many others (10, 14). This difference might be explained by the recent structural study on α-mannan recognition by human Dectin-2. Feinberg et al. have suggested that Dectin-2 could recognize internal α-linked mannose residue in α-mannan polysaccharide, while DC-SIGN would only recognize the terminal mannose disaccharide structure because the binding requires 2- and 3-hydroxyl groups of non-reducing terminal mannose, which is not available in the internal mannose residues (24). It is of great interest to determine precise α-mannan structure in *C. albicans*

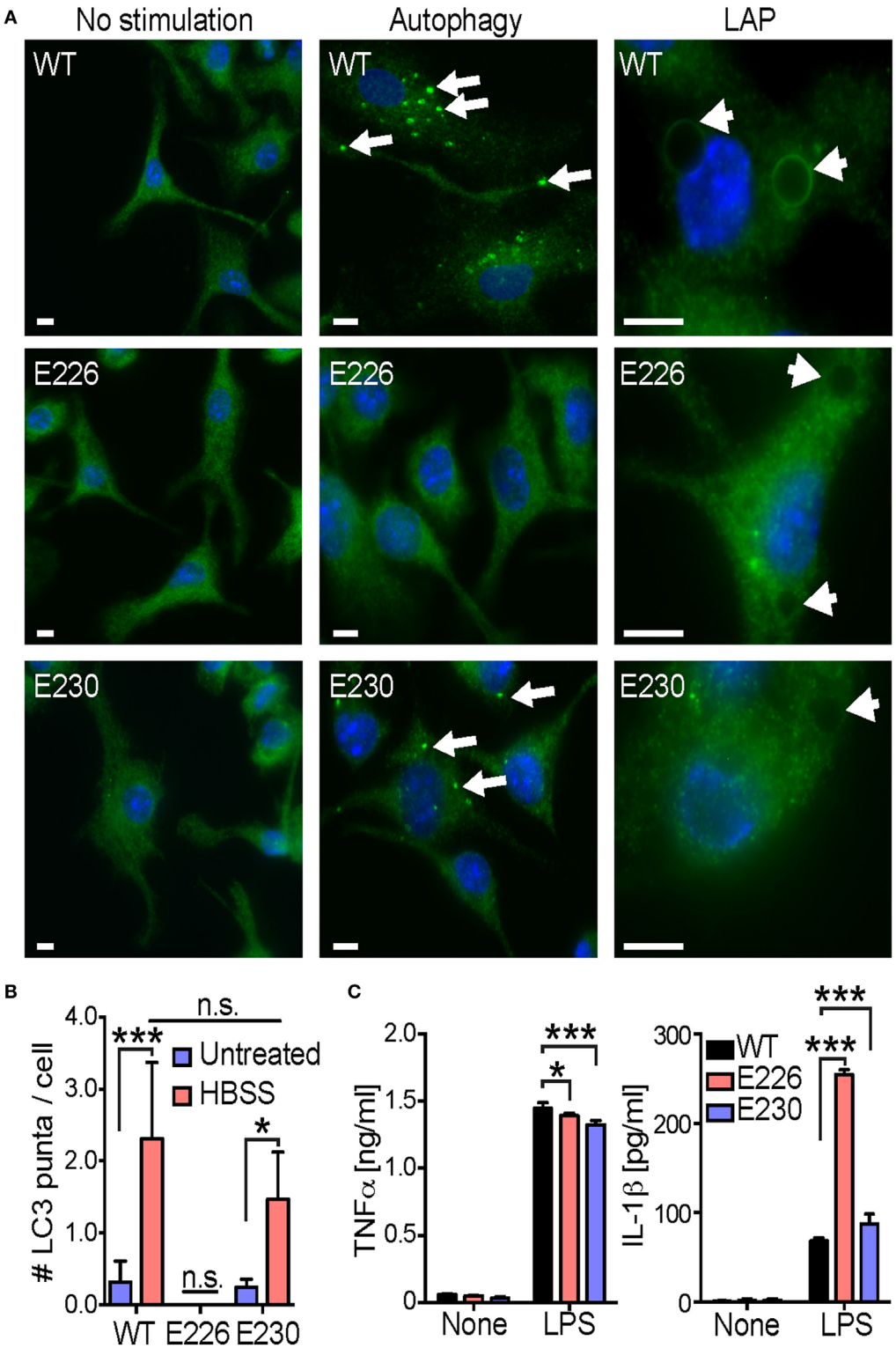

FIGURE 3 | Analysis of autophagy and LAP in E226 and E230 BMDCs. **(A)** The autophagy and LAP phenotype in WT, E226, and E230 BMDCs were analyzed by staining LC3, when cells were starved (autophagy) and polystyrene beads were taken up (LAP), respectively. White bar indicates 5 μm. Arrows and arrow heads show LC3 puncta and internalized beads, respectively. **(B)** The number of starvation-induced LC3 puncta in the cell was counted around 100 cells per each genotype. **(C)** WT, E226, and E230 BMDCs were stimulated with 100 ng/ml of lipopolysaccharide. After 16 h incubation, the supernatant was harvested, and the amount of TNFα and IL-1β was measured by an ELISA. Data shown are the mean of triplicates ± SD from one representative experiment and reproducible in three independent experiments. Statistical analyses were performed by one-way ANOVA followed by Tukey's test.

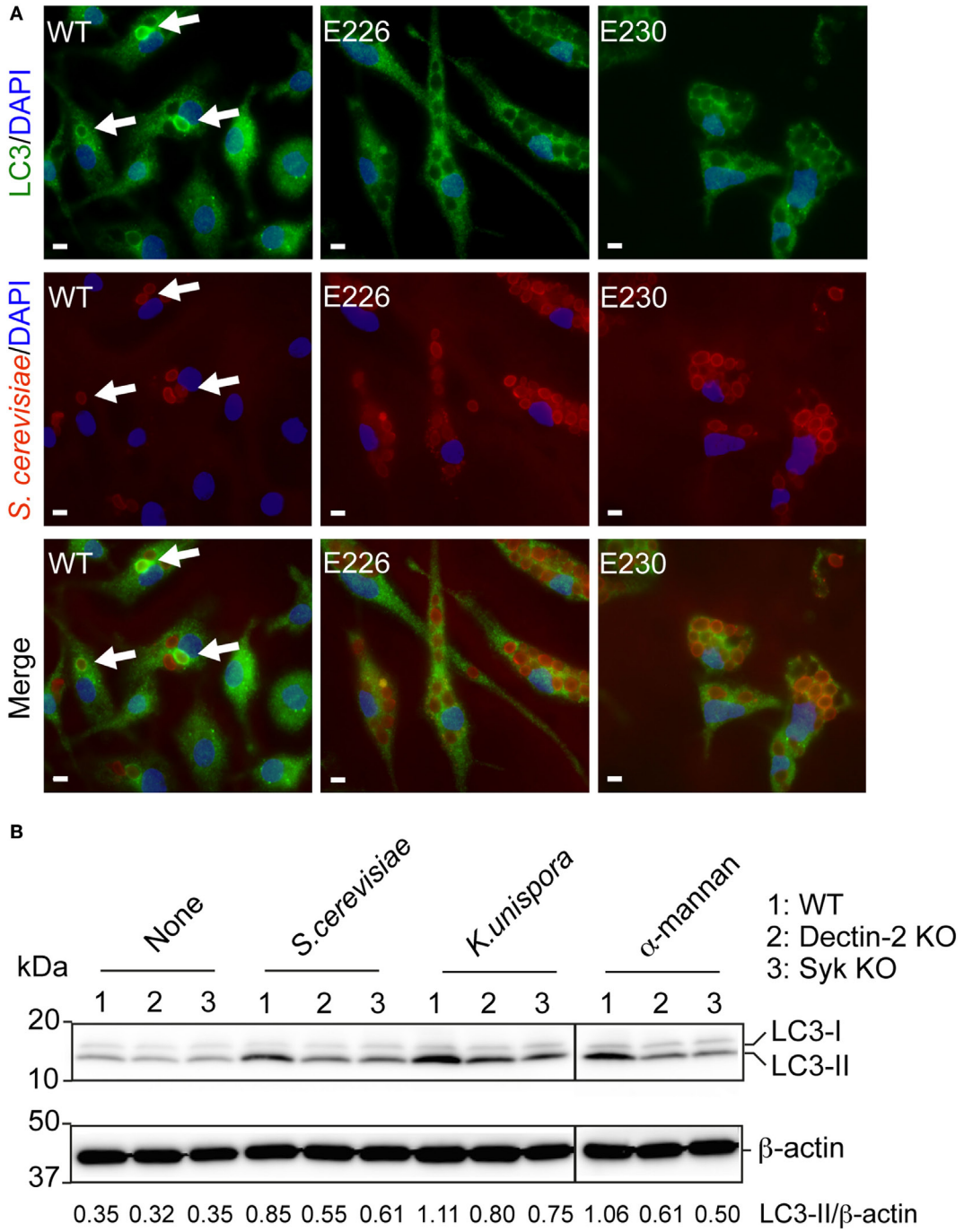

FIGURE 4 | Commensal yeasts induce LAP and LC3 activation. **(A)** WT, E226, and E230 BMDCs were incubated with Alexa555-labeled and PFA fixed *S. cerevisiae* at a MOI of 10 for 2 h. Cells were then fixed, permeabilized, and stained with anti-LC3 Ab and analyzed. White bar indicates 5 μm. Arrows show LC3 recruitment to *S. cerevisiae*. **(B)** WT, Dectin-2 KO, and Syk KO BMDCs were incubated with the indicated yeasts at a MOI of 5 or α-mannan for 1 h. Cells were lysed, and the proteins were separated by SDS-PAGE and blotted to a PVDF membrane. The membrane was probed by anti-LC3 Ab. The band intensity ratio of LC3-II over β-actin is shown in the bottom. Data shown are one representative experiment and reproducible at least two independent experiments.

and *S. cerevisiae* to understand molecular determinants that alter host C-type lectin specificity.

Together with previous studies, our data implies that LAP is a common mechanism employed by fungal recognition lectins. Dectin-1-mediated phagocytosis of *C. albicans* or particulated β-glucan induces LAP (19, 20). In this report, we have shown that Dectin-2 is capable of inducing LC3 activation, leading to LAP in response to commensal yeasts (**Figure 4**). Both Dectin-1 and Dectin-2 induce Syk activation (19) and ROS production (31), which is critical to induce LAP. Based on its association with

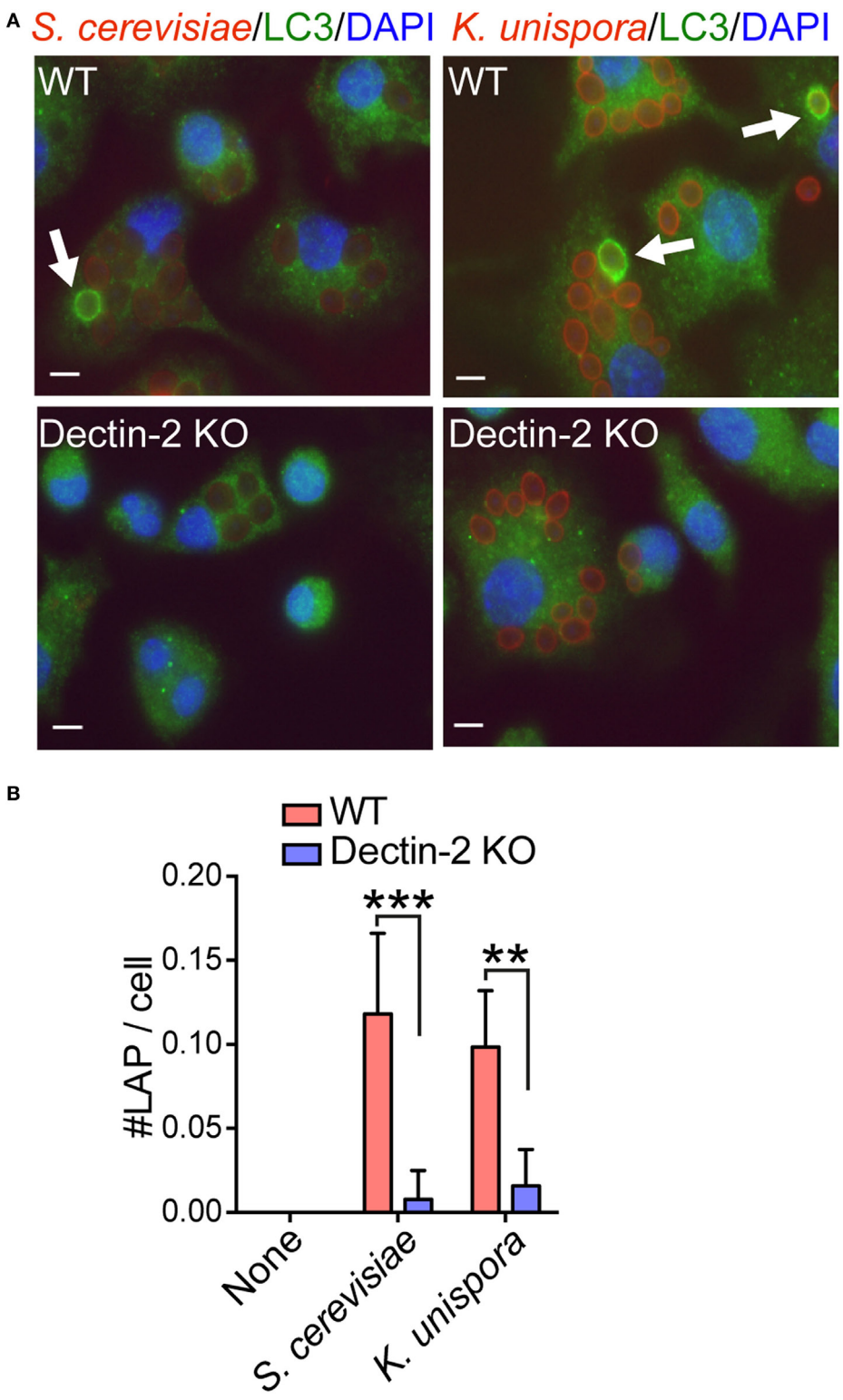

FIGURE 5 | Dectin-2-dependent LAP formation in response to commensal yeasts. **(A)** WT and Dectin-2 KO BMDCs were incubated with Alexa555-labeled and PFA-fixed *S. cerevisiae* and *K. unispora* at MOI 10 for 2 h. White bars indicates 5 μm. Arrows show LC3 recruitment to the yeasts. **(B)** The number of LAP in the cell was counted around 100 cells per each condition. Data shown are one representative experiment and reproducible in three independent experiments. Statistical analyses were performed by one-way ANOVA followed by Tukey's test.

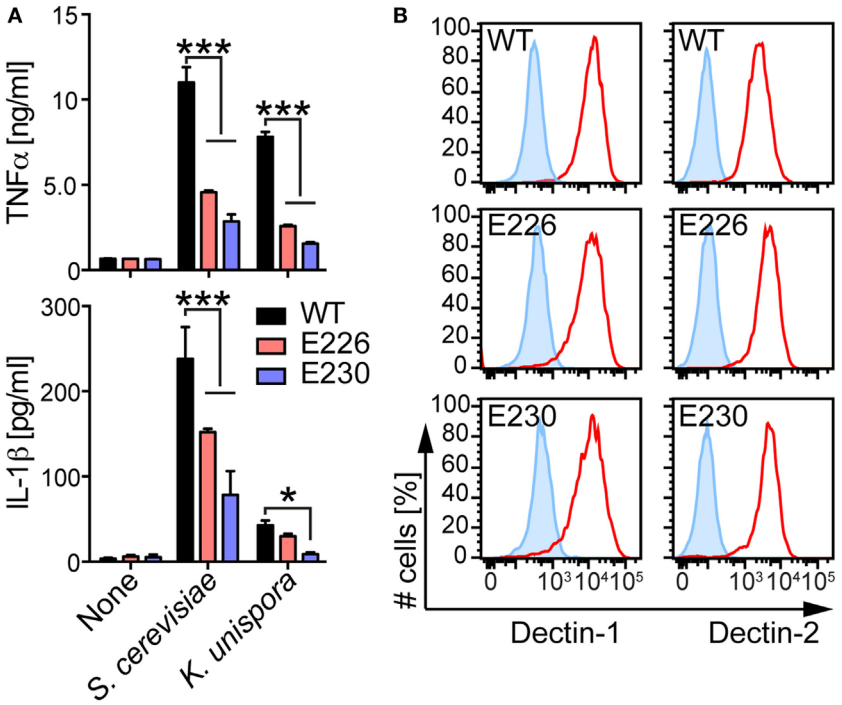

FIGURE 6 | LAP-dependent BMDC cytokine response to commensal yeasts. **(A)** WT, E226, and E230 BMDCs were incubated with the indicated yeasts at a MOI of 5. After 16 h incubation, the supernatant was harvested and the amount of TNFα and IL-1β was measured by ELISA. **(B)** WT, E226, and E230 BMDCs were stained with anti-Dectin-1 and 2 Abs (red) or the isotype control Abs (blue). The stained cells were analyzed by flow cytometry. Data shown are the mean of triplicates ± SD from one representative experiment and reproducible in three independent experiments. Statistical analyses were performed by one-way ANOVA followed by Tukey's test.

Syk, we speculate that other fungal recognition lectins such as Mincle and MCL/Dectin-3 are also linked to LAP (32, 33). For this regard, Mincle has been shown to be a phagocytic lectin for *C. albicans* (32). Beside the Syk-coupled receptors, several receptors not linked to Syk also induce LAP. Such receptors include TLRs (34) and TIM-4 which recognizes phosphatidylserine exposed on dead cells (35). TLRs induce ROS production (36), and MyD88 KO macrophages showed impaired ROS production in response to pathogenic bacteria (37). In case of TIM-4, it is not clear whether TIM-4 induce ROS production, or other molecular interactions involved in apoptotic cell recognition do so. Since many other lectins are also phagocytic such as Siglec-1, MR, and DC-SIGN (38, 39), it is of great interest to assess whether they induce ROS production and thereby LAP.

Our results of E226 BMDCs seemed opposite from a previous study showing that LC3β KO BMMs, which lack both autophagy and LAP, produced more TNFα and IL-1β in response to *C. albicans* (20). This might be attributed to the difference in the fungi used. Indeed, consistent with the previous report (20), we found that *C. albicans* induced more IL-1β production in E226 BMDCs compared with that in WT BMDCs (Figure S5 in Supplementary Material). Interestingly, E230 BMDCs showed no reduction in IL-1β response to *C. albicans*, indicating LAP is dispensable for IL-1β response to the fungal pathogen. TNFα response to *C. albicans* was moderately attenuated in E226 and E230 BMDCs (Figure S5 in Supplementary Material). These data indicate that LAP and autophagy pathways may play different roles in response to non-pathogenic yeasts and pathogenic fungi.

Several genetic tools have been utilized to distinguish the precise role of autophagy and LAP pathways. In this regard, autophagy-active and LAP-deficient mice, but not vise versa, exhibit auto-Ab production and kidney dysfunction, similar to the phenotype seen in systemic lupus erythematosus in humans (40), demonstrating a dominant role for LAP over autophagy in the autoimmunity. Furthermore, host–microbiota interaction through microbial OMVs was reported to induce LAP, rather than autophagy (41). Autophagy-active and LAP-deficient DCs failed to induce regulatory T cells in response to OMVs secreted from a commensal bacteria *Bacteroides fragilis* (41). In this report, we present evidence of a LAP-dependent host–mycobiota interaction. Using E230 mice that possess mutation in *Atg16l1* resulting in LAP-deficiency while preserving autophagy, we found that proinflammatory cytokine production of DCs in response to commensal yeasts is dependent on LAP, a response that was not achievable with LC3β KO cells (20). However, we cannot exclude the possibility that ATG16L1 has unknown functions apart from LAP formation, to account for the observed phenotype in E230 BMDCs.

What determines whether cells induce autophagy or LAP? One possible mechanism is the particle size; i.e., smaller size particle induces autophagy, whereas larger size particles are capable of inducing LAP. On the one hand, it is known that

TLR ligands such as Pam3 and LPS are capable of inducing autophagy; i.e., LC3 puncta formation in cells (42, 43). On the other hand, when presented with large particles, such as latex beads, these TLR ligands become a potent inducer for LAP (34). Both seem to occur through reactive oxygen species production (34, 42). Consistent with this observation, bacteria (diameter <1 μm) often induce autophagy. Recently, *Pseudomonas aeruginosa* has been shown to induce autophagy rather than LAP (44). Compared to bacteria, fungi are relatively large (diameter >1 μm). Several studies using *C. albicans* and *A. fumigatus* demonstrate that internalization of fungi induces LAP (18, 20). Consistent with these studies, our data demonstrate that commensal yeasts also induce LAP upon interaction with Dectin-2. It is, however, important to mention that autophagy is induced by *C. albicans* and is important for NF-κB activation and eventually fungal killing (45). When we stimulate BMDCs with the fungal polysaccharides, which are smaller than fungi, TNFα production in E226 and E230 BMDCs was indistinguishable with that in WT BMDCs (Figure S5 in Supplementary Material). Enhanced IL-1β production was observed in E226 BMDCs in response to β-glucan, suggesting the inhibitory role of autophagy in β-glucan-induced inflammasome activation. On the other hand, LAP was dispensable for β-glucan-induced IL-1β, as E230 BMDCs exhibited little change (Figure 5 in Supplementary Material). These data also imply that size of the ligands may determine the contribution of autophagy and LAP pathways. For future studies, the polysaccharide probes with defined sizes would be of great help to dissect precise role of autophagy and LAP in host–fungi interactions.

Overall, in this report, we have discovered novel intracellular machinery that enables Dectin-2 to induce cytokine production in DCs. Future study is required to understand the molecular and cellular mechanisms of how LAP and its associated proteins such as ATG16L1 are involved in the Syk-coupled cell signaling pathway.

AUTHOR CONTRIBUTIONS

All authors analyzed the data and contributed for preparation of the manuscript. DL, GB, and NK performed experiments, analyzed the data, and prepared the manuscript. AZ provided technical assistance for the work. AW provided technical support in the initiation of this project and established Dectin-1-BWZ cells. SJ, JD, and IR performed yeast analysis and provided technical assistance for yeast culture. YI and SS provided Dectin-2 KO mouse. XW and C-WC provided Syk KO mouse bone marrow. TK and TW provided intellectual support in autophagy pathway. UM supervised generation of E226 and E230 mice. TW and NK supervised and coordinated the work.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/article/10.3389/fimmu.2017.01397/full#supplementary-material.

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REVIEW
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Sensing Lipids with Mincle: Structure and Function

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Mincle is a C-type lectin receptor that has emerged as an important player in innate immunity through its capacity to recognize a wide range of lipidic species derived from damaged/altered self and foreign microorganisms. Self-ligands include sterols (e.g., cholesterol), and β-glucosylceramides, and the protein SAP130, which is released upon cell death. Foreign lipids comprise those from both microbial pathogens and commensals and include glycerol, glucose and trehalose mycolates, and glycosyl diglycerides. A large effort has focused on structural variation of these ligands to illuminate the structure–activity relationships required for the agonism of signaling though Mincle and has helped identify key differences in ligand recognition between human and rodent Mincle. These studies in turn have helped identify new Mincle ligands, further broadening our understanding of the diversity of organisms and lipidic species recognized by Mincle. Finally, progress toward the development of Mincle agonists as vaccine adjuvants providing humoral and cell-mediated immunity with reduced toxicity is discussed.

Keywords: Mincle, adjuvant, structure–activity relationship, glycolipids, C-type lectin receptor, cell-mediated immunity, innate immunity

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C-type lectin receptors (CLRs) comprise a large group of soluble and transmembrane receptors that possess a carbohydrate recognition domain (CRD) or a homologous domain (1). Transmembrane CLRs can function as pattern recognition receptors, enabling the recognition and internalization of a pathogen, its degradation, and subsequently presentation of component antigens, thereby providing innate immune protection and initiating adaptive immunity (2). Mincle is a transmembrane CLR that provides the capacity to recognize a broad range of self- and foreign molecules, as a part of innate immune sensing (**Figure 1A**) (3–6). Mincle has risen to prominence based on its identification as the key receptor involved in recognition of cord factor and trehalose dibehenate (TDB), trehalose-based lipids with powerful immune modulating properties (7–9). Cord factor is a component of complete Freund's adjuvant, an emulsion of inactivated and dried mycobacterial cells, and in purified form, can elicit the formation of focal collections of mononuclear phagocyte cells termed granulomas, as well as other serious side effects that have led to it no longer being used for human vaccination (10, 11). TDB is a synthetic analog of cord factor that is the focus of intense interest as an adjuvant for vaccination, particularly when co-formulated with the quaternary amine dimethyldioctadecylammonium (DDA) (12, 13).

Mincle is a type II transmembrane protein with a short cytoplasmic tail (14) and is expressed on various immune cells including dendritic cells, macrophages, and neutrophils. The extracellular domain binds ligands, causing signal transduction (**Figure 1B**). Ligand binding occurs at distinct sites, including the CRD and cholesterol and protein interaction sites. Signaling occurs through the immunoreceptor tyrosine-based activation motif-containing Fc receptor γ-chain molecule (15). This process initiates a signaling pathway involving spleen tyrosine kinase (Syk), Card9-Bcl10-MALT1 (16), and finally nuclear factor kappa light-chain enhancer of activated B cells (NF-κB) (2), a transcription

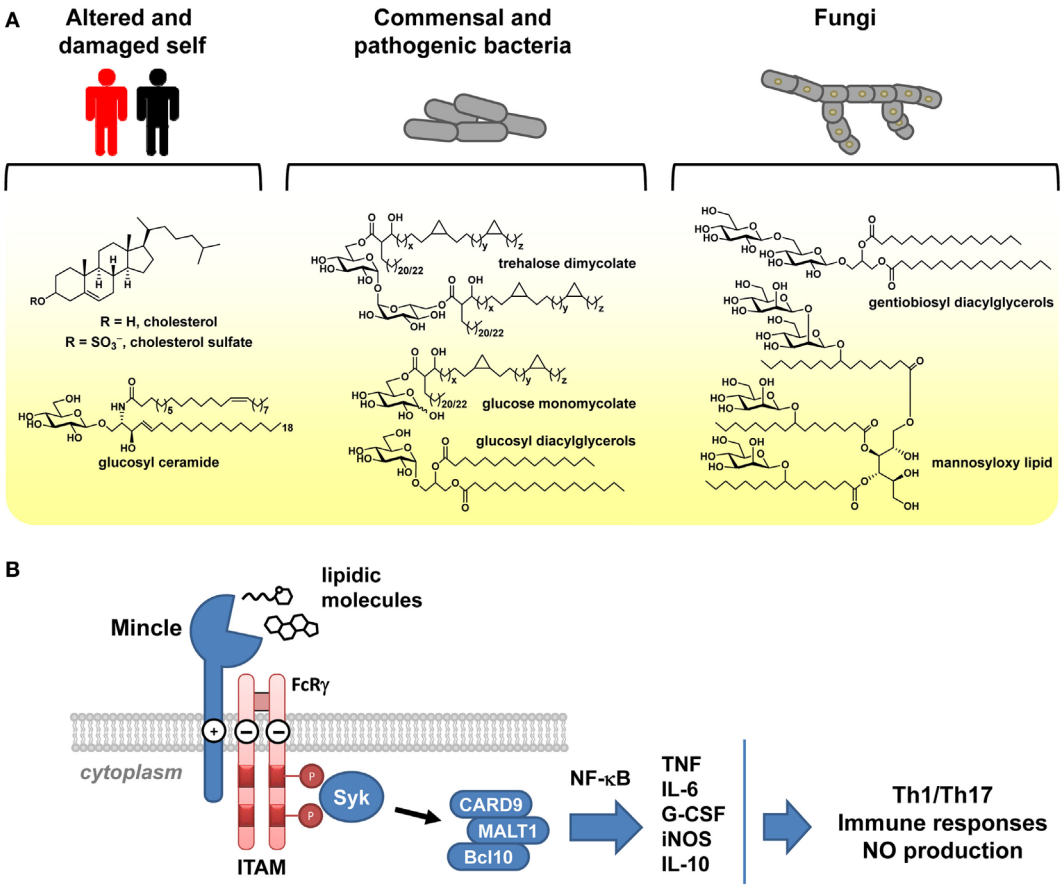

FIGURE 1 | Signaling by Mincle. **(A)** Examples of self and microbial lipidic species that signal through Mincle. **(B)** Signaling through Mincle involves an immunoreceptor tyrosine-based activation motif (ITAM) adaptor and leads to production of cytokines and chemokines, stimulating T-cell and immune responses.

factor that promotes the expression of cytokines including tumor necrosis factor (TNF), interleukin-6 (IL-6), granulocyte colony-stimulating factor (G-CSF), and interleukin-11 (IL-10), chemokines including macrophage inflammatory protein 2 (MIP-2 or CXCL2) and KC (CXCL1), as well as stimulating expression of inducible nitric oxide synthase (15, 16). The majority of these responses can be considered as pro-inflammatory; however, production of IL-10 is anti-inflammatory and can lead to down-regulation of IL-12p40 production and interference with pro-inflammatory cytokine secretion (17).

Cytokines produced upon agonism of Mincle signaling orient effector T helper (T_H) cell development into T_H subtypes, although the effects are complex and dependent on the nature of the host and pathogen (18). In mice, a protective T_H1 cell-mediated immunity is induced, and effects on T_H17 responses depend on the nature of the pathogen, with cord factor inducing a T_H17 response. In humans, effects are more complicated. When used as an adjuvant, TDB/DDA induces a T_H1 response (19). By contrast, assorted fungi such as *Fonsecaea* spp. escape T_H1-oriented immunity and instead induce a T_H2 cell-mediated immunity in an NF-κB-independent manner (20); cross-talk with Toll-like receptors can restore T_H1 immunity (21). Placed in context, these results are important as different T_H cell subsets have specific functions in adaptive immunity. T_H1 and T_H2 cells provide host immunity against intracellular and extracellular pathogens, respectively, particularly bacteria and protozoa, whereas T_H17 cells are pro-inflammatory T_H cells that are defined by the production of interleukin-17 (IL-17) and play a role in adaptive immunity at mucosal surfaces, especially against fungal pathogens (18).

Aside from the sole exception of the cell death-associated Sin3A-associated protein 130 (SAP130, *vide infra*), signaling through Mincle appears to be limited to water-insoluble glycolipids, with most *in vitro* cell-based studies using plate-bound or crystalline forms of the ligands (7, 22). It seems likely that effective signaling by lipidic species requires multimerization of Mincle at the cell surface and may mimic the presentation of glycolipids on the surface of mycobacterial and other microbial cells and in lipid vesicles (22). This phenomenon appears to be intimately connected with the ability of TDB and TDM as water-in-oil emulsions, or liposomal formulations with DDA, to act as adjuvants.

Mincle is a member of the large family of CLRs that enable recognition of a wide range of self- and foreign ligands

(2, 18, 23). Among the CLRs, Mincle is unique in its ability to recognize defined, low molecular weight species and especially glycolipids. As glycolipids are essentially ubiquitous species, there is large scope for Mincle to recognize such species from a wide range of organisms. Our knowledge of the repertoire of lipids that can agonize signaling through Mincle continues to grow, providing growing insight into structure–activity relationships. In addition, a growing range of synthetic lipids have been prepared and studied as agonists of Mincle signaling, further enriching our understanding of the structural features necessary for interaction with this receptor. Collectively, these data show that a remarkable breadth of lipidic species can signal through Mincle (4), suggesting that this receptor has a primitive-like capacity to recognize lipidic species that parallels the Toll-like receptors (24).

SENSING OF DAMAGED AND ALTERED SELF

An important role for Mincle in sterile (non-infected) inflammation has been identified through a range of effector molecules. An early report showed that Mincle is involved in the damaged cell response through recognition of SAP130 (15). Normally, this protein is sequestered within the cell but, upon cellular death, can be released. Binding to SAP130 was shown to occur outside the carbohydrate-binding region of the CRD.

More recently, several self-derived lipidic species have been discovered that signal through Mincle, which cause sterile inflammation (22, 25). Cholesterol crystals, which are present within atherosclerotic plaques during hypercholesterolemia, and within cholesterol granulomas, promote signaling through human Mincle (**Figure 2**) (22). Analysis of a range of cholesterol esters revealed that only free cholesterol (as either plate-bound or crystalline forms) can signal through human Mincle and that other endogenous steroids such as cortisone, progesterone, estradiol, testosterone, aldosterone, and dehydroepiandrosterone, cannot. Other sterols that can signal through human Mincle include the plant sterol sitosterol and the cholesterol intermediate desmosterol, but the yeast sterol ergosterol and the bile acid cholestanoic acid do not. These results suggest that a hydroxyl residue at C3 and an alkyl chain at C17 appear to be minimally, by not exclusively required for recognition by human Mincle. Recognition of cholesterol is limited to human Mincle; rat and mouse Mincle cannot sense cholesterol. Binding to human Mincle occurs through the cholesterol recognition/interaction amino acid consensus motif L^{127}SYKKPKMR^{135}, a sequence that is absent in mouse and rat Mincle. The R135L mutant of hMincle lost the ability to recognize crystalline cholesterol but maintained the ability to recognize TDM, suggesting that cholesterol recognition occurs at a site not identical to that of TDM. Only aggregate forms of cholesterol can induce signaling- soluble or membrane-associated forms are inactive- and it was suggested that signaling may be accompanied by multimerization of Mincle.

Cholesterol sulfate, which is present at high levels within the epidermal layer of the skin, has been identified to signal through Mincle and a role for Mincle in allergic skin inflammation has been established (25). Direct binding of cholesterol sulfate to immobilized human Mincle was demonstrated by surface

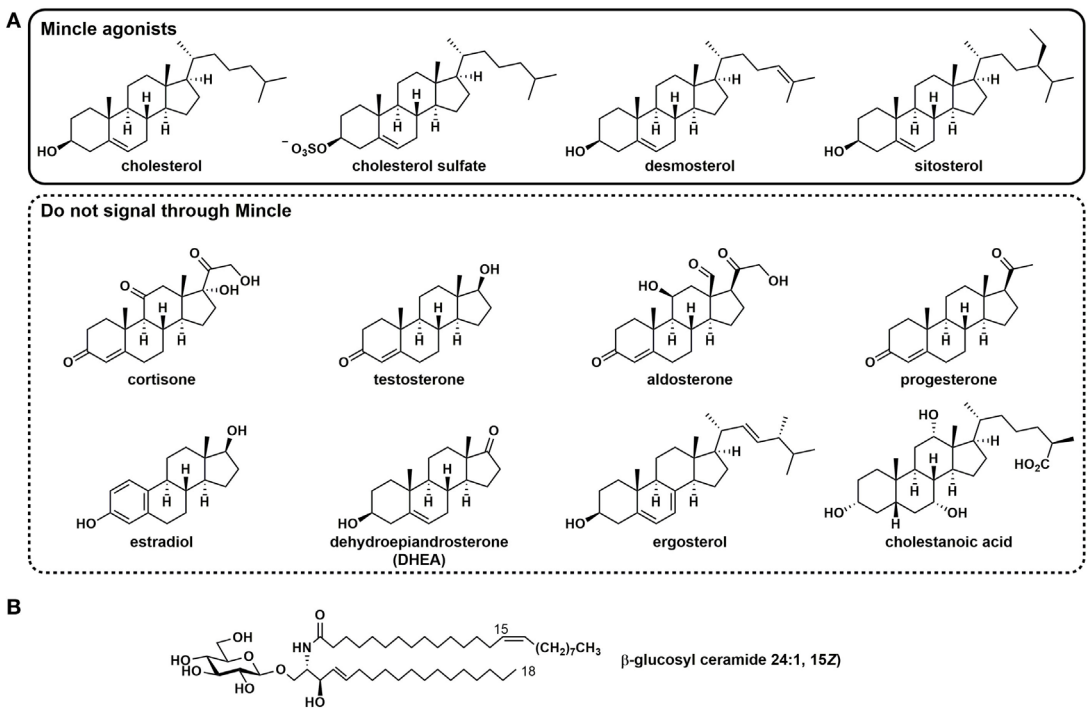

FIGURE 2 | Self-derived lipidic species that signal through human Mincle. **(A)** Sterols that signal through Mincle and assorted steroids that do not signal through Mincle. **(B)** The endogenous glycosyl sphingolipid and β-glucosyl ceramide.

plasmon resonance. Subcutaneous injection of cholesterol sulfate into mice resulted in a Mincle-dependent induction of a severe local inflammatory response with infiltration of neutrophils, monocytes, and eosinophils.

Fractionation of damaged cells led to the discovery that β-glucosylceramide (β-GlcCer), an important host glycosphingolipid, can signal through human and mouse Mincle (26). A range of species including those bearing homologous saturated fatty acyl ($C_{16:0}$,$C_{18:0}$,$C_{20:0}$,$C_{22:0}$,$C_{24:0}$) and one unsaturated ($C_{24:1}$) variants were detected. Aside from the release of β-GlcCer from cellular damage, this glycosphingolipid is important as it accumulates in Gaucher's disease, an inherited genetic defect in β-glucosylceramidase (GBA1) that is characterized by systemic inflammation. Mice in which bone marrow dendritic cells (BMDCs) were GBA1-deficient leading to the accumulation of β-GlcCer exhibited enhanced inflammatory responses suggesting that the inflammation-based pathologies of Gaucher's disease arise from Mincle-mediated processes; this was supported by the observation that no such augmentation was observed in $GBA1^{-/-} \times Mincle^{-/-}$ BMDCs. Notably, administration of GlcCer as an oil-in-water emulsion did not induce granuloma formation, suggesting that it may represent a safer compound for therapeutic applications than TDM.

SENSING OF MICROBIAL LIPIDS FROM PATHOGENS AND COMMENSALS

Mycolic Acid- and Corynomycolic Acid-Based Glycolipids

Natural mycolic acids are complex α-alkyl-β-hydroxy fatty acids with the specific structure dependent on the source organism. For example, *Mycobacterium tuberculosis* mycolic acids are C_{60-90} species that include a range of functional groups such as *cis*-alkenes and *cis*- and *trans*-cyclopropanes (CP), ketones, esters, and methoxy groups within the mero chain (27, 28). The equivalent species from corynebacteria are termed corynomycolic acids and are usually simpler, being C_{22-38} in length and containing only saturated and unsaturated species (29, 30).

Baird and co-workers synthesized a large range of homogeneous mycolic acids with structures as shown in **Figure 3A** (31). These have been elaborated to a range of homogeneous trehalose mono- and dimycolates, glucose monomycolates, and methyl arabinofuranoside monomycolates that bear authentic mycolic acids (**Figure 3B**), which were examined for their ability to stimulate the production of TNF-α and IL-6 in BMDCs from C57BL/6 mice. Generally, the order of potency was trehalose dimycolates > trehalose monomycolates > glucose monomycolates > arabinoside monomycolates. Most mycolic acid structures induced levels of cytokines similar to natural TDM from *M. tuberculosis*, and greater than that of TDB, except for a *bis*-dialkene TDM that was as potent as TDB. Concerning the lipid fine structure, TDM bearing a *trans*-CP-mero chain was more inflammatory than the equivalent *cis*-CP-mero-chain, while for the oxygenated mycolates, *cis*-isomers were more inflammatory than *trans*-isomers. Among the series of *cis*-isomers, *cis*-methoxy-TDM induced higher TNF-α levels compared to *cis*-alpha TDM or *cis*-keto TDM. These results provide a framework understanding of altered inflammatory activities of TDMs isolated from a range of *M. tuberculosis* mutants that vary in lacking *trans*-cyclopropanation [Δ*cmaA2* mutant (32)], oxygenated MA classes [Mtb Δ*mmaA4* (33)], and α-mycolate cyclopropanation [Mtb Δ*pcaA* mutant (34)], which display hyper- and hypoinflammatory responses.

Lang and co-workers showed that various corynebacteria, including *Corynebacterium diptheriae*, *C. ulcerans*, and

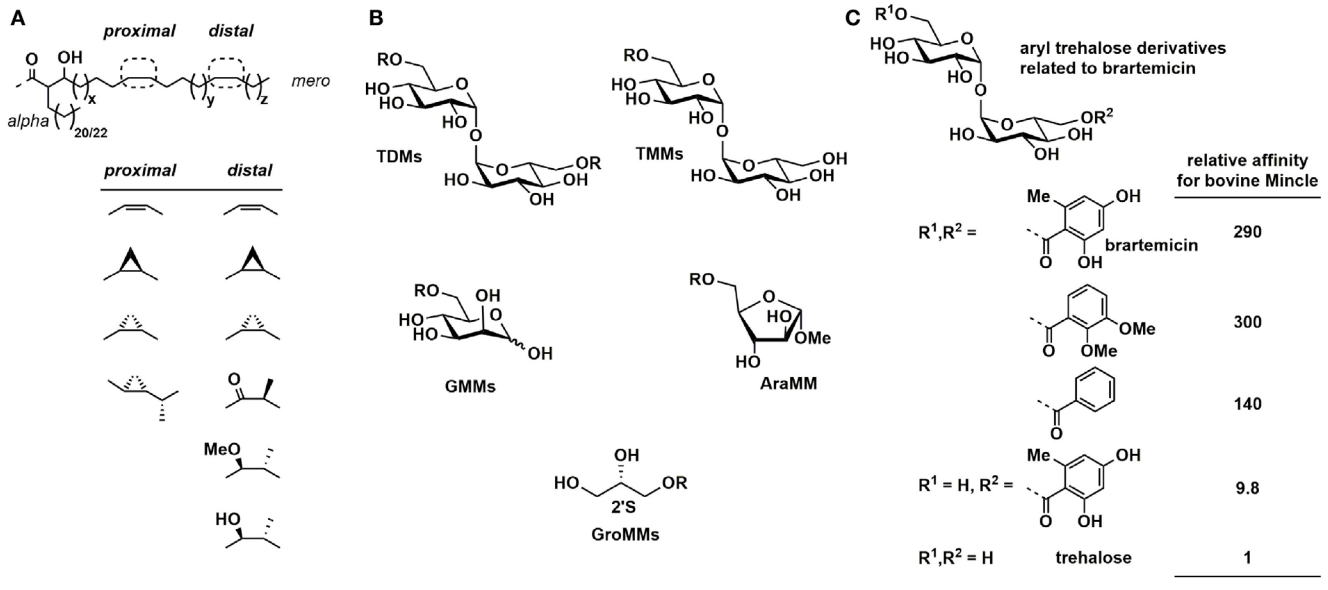

FIGURE 3 | **(A)** Chemically synthesized mycolic acid-based glycolipids: general structures of mycolic acids. **(B)** Mycolic acid-based glycolipid classes. **(C)** Relative affinities of brartemicin and analogs for binding to bovine Mincle.

C. glutamicum, could bind a murine Mincle-Fc fusion protein (35). Moreover, these bacteria could induce production of the cytokine G-CSF and nitrites by macrophages in a manner analogous to TDB in a Mincle-dependent manner. While the precise glycolipids responsible for these effects were not unequivocally identified, it is likely that they comprise corynomycolate esters of trehalose or glucose. Consistent with this conclusion, a *C. diptheriae* strain (DSM43989), which lacks the ability to produce cell wall mycolates including trehalose dicorynomycolate (TDCM) and trehalose monocorynomycolate (TMCM), or its glycolipid extracts, failed to bind the murine Mincle-Fc fusion and did not active macrophages (**Figure 4**). Williams and co-workers showed that TMCM, TDCM, and glucose monocorynomycolate (GMCM) derived from a synthetic C_{32}-corynomycolic acid representative of *C. glutamicum* could stimulate signaling in a reporter cell expressing human or murine Mincle, at levels similar to that of TDM from *M. smegmatis* (36).

Simplified Trehalose-Based Glycolipids

Prior to the discovery that cord factor can signal through Mincle, intense effort was applied to the development of structurally simpler and safer immunomodulators than whole heat-killed mycobacterial cells (11, 37). These studies demonstrated that adjuvant effects could be obtained with TDM, a molecule that had previously been identified as capable of inducing formation of granulomas that characterize lung infection with *M. tuberculosis*, and further studies showed that simplified cord factor analogs possessed useful adjuvant capabilities, often with better safety, particularly in the context of granuloma formation (10). As a part of efforts to develop less toxic analogs of TDM, TDB was invented (38). TDB is a purely synthetic simple analog

lipid structure	lipid name	trehalose diesters	trehalose monoesters	glucose monoesters	glycerol monoesters
representative structure shown	mycolic acid (MA)	very strong (h,m)	very strong (h) weak (m)	very strong (h,m)	strong (h) weak (m)
	Corynomycolic acid (CM)-C14C16	very strong (h,m)	very strong (h,m)	strong (h,m)	strong (h) weak (m)
	CM-C5C16	—	—	weak (h,m)	—
	CM-C23C16	—	—	strong (h,m)	—
	CM-PhC16	—	—	strong (h,m)	—
	C28	—	—	none (h)	—
	C22, behenate	strong (h,m)	moderate (h,m)	weak (h,m)	strong (h) weak (m)
	C18, stearate	moderate (h,m)	weak (m)	—	—
	C16, palmitate	moderate/weak (m)	weak (m)	none (h)	—
	β-C9C12	—	—	moderate (h)	—
	α-C14C18	—	—	strong (h)	—
	α-C4C12	—	—	moderate (h)	—
	α-C9C12	—	—	moderate (h)	—
	PEG-C18	moderate (m)	—	—	—

FIGURE 4 | Qualitative summary of structure–activity relationships for human (h) or mouse (m) Mincle-dependent signaling or cellular activation, by trehalose, glucose, and glycerol-based glycolipids. Very strong, strong, moderate, weak, or none refer to signaling through Mincle using reporter cell lines or by production of cytokines and nitric oxide by primary cell lines (usually bone marrow dendritic cells). "–" means not studied.

of TDM that has been widely employed in vaccination studies (39). In independent studies, TDM and TDB were shown to signal through Mincle (7, 9).

Stocker and co-workers studied the ability of trehalose diesters of simple straight chain fatty acyl lipids to activate murine bone marrow-derived macrophages (BMMs) (40). Short-chain saturated (C_4, C_7, and C_{10}) derivatives did not stimulate NO production, while C_{18}, C_{20}, C_{22}, and C_{26} were able to produce NO and the cytokines IL-6 and IL-1b, with a maximum activity for C_{22} (TDB). Trehalose C_{22} and C_{26} monoesters were also shown to activate BMMs but with lesser potency relative to the equivalent diesters; production of NO but not IL-6 was dependent on prestimulation with IFN-γ. Generally in these assays, the C_{22} monoester was more potent than the C_{26} monoester (41).

Huber and co-workers studied a series of trehalose mono- and diesters bearing C_{14} to C_{22} fatty acyl chains for their ability to activate BMMs and BMDCs (42). In terms of production of nitrite and G-CSF, the diesters were superior to the monoesters. Dose–response curves for trehalose monoesters and diesters confirmed the differences in potency in BMMs; an approximately 100-fold higher concentration of the monoester was required for robust production of nitrites yet still failed to induce G-CSF. Similar differences were seen in direct binding assays of a mouse Mincle-Fc to plate-coated mono- and diesters. These authors also studied a trehalose distearate (TDS) derivative that included a short PEG-spacer (PEG-C18). This compound exhibited significantly reduced potency for macrophage activation, relative to the distearate, yet still maintained activity greater than that of the stearate monoester.

Mycobacteria produce a wide range of other trehalose-based glycolipids. Decout et al. studied a range of glycolipids extracted from *M. tuberculosis* H37Rv (43). Trehalose derivatives that are acylated at the 2 and 3 positions such as diacyltrehalose and diacylglycolipid Ac_2GL (which is derived from Ac_2SGL by mild acid treatment) and at the 2, 3, and 6′ positions (triacyltrehalose) with various combinations of straight chain and methyl-branched fatty acids provide at best modest signaling through Mincle. Notably, the 2′-sulfated form of Ac_2GL, Ac_2SGL, does not signal through Mincle nor do a range of other trehalose-based glycolipids that lack a free 2-hydroxyl group. Collectively, these results reveal that the presence of a free trehalose 2-hydroxy group is critical for trehalose glycolipid signaling through Mincle.

Binding studies of trehalose analogs to the CRD of Mincle have by matters of practicality been limited to soluble derivatives, which typically do not signal through Mincle. Nonetheless, such binding data are important as it provides direct information on the affinity of the protein for the monomeric soluble glycolipid. Competition studies of binding of various soluble carbohydrate species to bovine Mincle have revealed key features required for tight binding to the CRD. Trehalose binds some 36-fold more tightly than methyl α-D-glucopyranoside, highlighting the importance of two sugar-binding subsites (44). Increasing the chain length of C_2–C_6 trehalose diesters resulted in a monotonic increase in enhancement of binding relative to trehalose of up to 250-fold (44, 45). Activity for a similar series of trehalose monoesters from C_4 to C_{12} increased monotonically up to 530-fold greater than trehalose. Surface plasmon resonance was used to probe binding of immobilized human Mincle CRD to soluble C_8, C_{10}, and C_{12} trehalose monoesters and showed monotonically increasing binding affinities (46). A plot of the binding affinities for the number of carbons per substituent for mono-acyl and di-acyl trehaloses versus log K_i was linear for a series of linear, iso-branched, and aromatic groups (45).

Drickamer and co-workers reported the synthesis of a glycan array containing the major carbohydrate structures present in the cell wall of *M. tuberculosis* and other mycobacteria (47). This array was probed with fluorescently labeled bovine Mincle, or alternatively by a secondary antibody. Bovine Mincle interacted with high selectivity only with trehalose-based structures, including immobilized trehalose monomycolate. Additionally, binding was seen for more complex glycan structures including a β-glucosyl-1,6-trehalose that comprises the core of several *M. smegmatis* lipooligosaccharides, and trehalose derivatives modified at the 4-position with additional β-glucosyl residues that comprise the glycan core of surface lipooligosaccharides from the opportunistic pathogen *M. kansasii*.

Brartemicin is a trehalose-based natural product from *Nonomuraea* sp. that has been reported to inhibit matrigel invasion of cancer cells (**Figure 3C**) (48). Brartemicin has been studied as a soluble analog of cord factor for binding to soluble bovine Mincle (49). In a competition binding assay, brartemicin and related analogs bound 300-fold tighter to Mincle than trehalose and trehalose dibenzoate bound around 140-fold more tightly. Interestingly, a monoester derivative bearing only one of the 2,4-dihydroxy-6-methylbenzoyl groups of brartemicin bound only 10-fold more tightly than trehalose, and *epi*-brartemicin, with an αβ-trehalose core, bound 3-fold more tightly than trehalose (i.e., 1/10th the affinity of brartemicin). It has not been reported whether brartemicin can signal through Mincle.

Glucose- and Glycerol-Based Glycolipids

The mycobacterial metabolite glycerol monomycolate (GroMM) has been shown to signal through human Mincle but not mouse Mincle (**Figure 4**) (50). Glycerol monobehenate (GroMB) also selectively signals through human Mincle but somewhat less potently than GroMM (50). Glycerol monocorynomyclate (GroMCM), a shorter-chain, C_{32}-corynomycolate analog, also signals through human Mincle selectively over mouse Mincle, and with similar potency to GroMM (36). Glycerol monoesters can exist in two stereoisomeric forms at the glycerol. In the case of GroMCM, the majority of the activity resides in the 2′*S*-isomer (36). Baird and co-workers have prepared a series of 2′*R*- and 2′*S*-GroMMs bearing authentic α-, keto-, and methoxy-mycolic acids (51). Consistent with the results for the GroMCMs, the 2′*R*-isomers did not show any significant effects in the stimulation of cytokines in BMDCs.

An early report noted that trehalose dimycolate, upon treatment with porcine trehalase, lost the ability to signal through Mincle (7). Although originally interpreted as indicating that GMM cannot signal through Mincle, it is now clear that this reduction of activity is not as a result of formation of GMM and may be as a result of contaminating esterase activity. Decout

et al. reported that GMM isolated from *M. tuberculosis* H37Rv was a powerful agonist of human and mouse Mincle reporter strains, with potency greater than that of TDM (43). Synthetic GMMs bearing authentic homogeneous mycolic acids were synthesized by Baird and co-workers and were able to activate BMDCs (31). A range of glucose monomycolate analogs have been synthesized. Detailed examination of the lipid structure while maintaining the α-alkyl-β-hydroxy motif has revealed that GMCM is a strong Mincle agonist and that activity is maintained upon increasing the length of the α-chain or incorporation of an aryl group into this chain (52). In contrast, shortening of the α-chain to a pentyl group resulted in the loss of signaling through Mincle (52).

More extensive analysis of the effect of structure on signaling through Mincle for GMM analogs reveals a complex dependence on structure. In contrast to the potent signaling seen for TDB, TMB, and GroMB, glucose monobehenate is only a very weak activator of human and mouse Mincle reporter cells (36, 43, 52). A β-branched C9C12 derivative signaled only weakly through human or mouse Mincle (52). Short, α-branched lipids were also able to signal through Mincle but short- to medium-chain derivatives were only moderate agonists (43). A longer chain α-C14C18 analog provided strong signaling through Mincle approaching the potency of GMCM, and the mannose analog also provided robust, albeit slightly weaker signaling through Mincle. This last result is interesting in the context that a mannose monomycolate derived from *Rhodococcus ruber* (*Nocardia rubra*) with an intermediate C_{45} mycolic acid did not induce granulomas in mice when delivered as a water-in-oil-in-water emulsion (53).

Overview of Glycolipid Recognition by Mincle

Several X-ray structures are available for ligands bound to Mincle from bovine (44, 45) or human (46) sources. These structures reveal binding of trehalose or simple trehalose derivatives in the CRD of Mincle. The CRD contains two carbohydrate-binding sites, with one appearing to act as a "primary" binding site and involving interactions of the O2 and O3 of a glucose residue with the Ca^{2+} ion; the secondary sugar-binding site does not interact with the Ca^{2+} ion. A lipophilic groove extends away from the 6 position of the glucose residue in the primary binding site that modeling studies suggest can accommodate two lipid chains (45). Taken together, structure–function relationship studies, crystallography, and molecular modeling data suggest that effective signaling through Mincle by glycolipids can involve interactions with one or both sugar-binding subsites and with one or two alkyl chains present on the same or different fatty acids and that the 2-hydroxy group must not be modified. Decout et al. have argued that at least three of these four binding sites should be occupied for effective signaling to occur (43). The fact that mono-acyl trehaloses appear to be less effective Mincle signaling agonists than the branched glycolipid GlcC14C18 suggests that the interactions with the secondary sugar-binding site are less important than those with a second alkyl chain in the second hydrophobic groove.

Glycosyl Glycerolipids

Yamasaki et al. screened 50 species of pathogenic fungi for their ability to signal through Mincle using a murine Mincle-GFP reporter strain and discovered signaling by a range of *Malassezia* spp., including *M. pachydermatis* and *M. dermatis* (54). In normal skin, *Malassezia* spp. are commensals; however, in atopic/eczema and psoriasis, these fungi can elicit inflammatory responses in skin lesions and can cause diseases such as tinea versicolor, atopic dermatitis, and lethal sepsis. Fractionation of a lipid extract from *M. pachydermatis* led to the identification of two classes of Mincle agonists (55). The first comprised a complex mannosyloxystearyl mannitol that possessed a potency similar to that of TDM (**Figure 1**). Limited degradation studies revealed that the mannosyloxystearic acid fragment was a weak Mincle agonist. The second class of Mincle agonist was a series of β-gentiobiosyl diglycerides (**Figure 5A**). Four lipoforms were isolated with the following substituent permutations (sn-1/sn-2): anteiso-C_{19}/anteiso-C_{15}, anteiso-C_{17}/anteiso-C_{15}, anteiso-C_{20}/anteiso-C_{15}, and anteiso-C_{19}/anteiso-C_{17}. All four lipoforms signaled weakly through mouse Mincle to similar degrees but did not signal through human Mincle. The *Malassezia* β-gentiobiosyl diglycerides have structures similar to that of the gentiobiosyl glycolipid anchor of lipoteichoic acids, a major constituent of the cell wall of Gram-positive bacteria; however, lipoteichoic acid does not signal though Mincle.

A related series of gentiobiosides were isolated by Brennan and co-workers from *M. tuberculosis* H37Ra (56). In order to determine whether these compounds can signal through Mincle, Williams and co-workers synthesized a series of related gentiobiosides representing those isolated, namely four lipoforms bearing C_{18}, iso-C_{17}, iso-C_{18}, and iso-C_{19} fatty acids (**Figure 5B**) (57). Like the *Malassezia* gentiobiosides, all four lipoforms signaled only weakly through mouse Mincle and did not signal through human Mincle. A range of analogs were synthesized to explore structure–activity relationships. The branched alkyl gentiobioside, the glycerol gentiobioside, and short-chain analogs bearing C_4 or C_8 fatty acyl chains did not signal through Mincle. However, an analog bearing two C_{12} chains provided moderate signaling through mouse Mincle and also provided weak signaling through human Mincle. Among the most potent of the analogs examined was a β-glucosyl diglyceride bearing iso-C_{17} fatty acids, which provided moderately strong signaling though mouse Mincle and moderate signaling through human Mincle. This compound was proposed to represent a biosynthetic precursor to the mycobacterial β-gentiobiosides, suggesting that human and mouse Mincle can preferentially recognize β-glucosyl diglycerides rather than their extended derivatives.

α-Glucosyl diacylglycerides from the pathogenic bacterium *Streptococcus pneumoniae* were shown by Yamasaki and co-workers to trigger Mincle reporter cell activation (**Figure 5C**) (58). These glycolipids are comprised of a range of lipoforms, including a $C_{16:0}/C_{18:1}$ species where $C_{18:1}$ is *cis*-vaccenic acid (59, 60). Model synthetic α-GlcDAGs bearing myristic ($C_{14:0}/C_{14:0}$) or stearic acid esters ($C_{18:0}/C_{18:0}$) could signal through Mincle, with the former more potent than the latter. Like β-gentiobiosyl

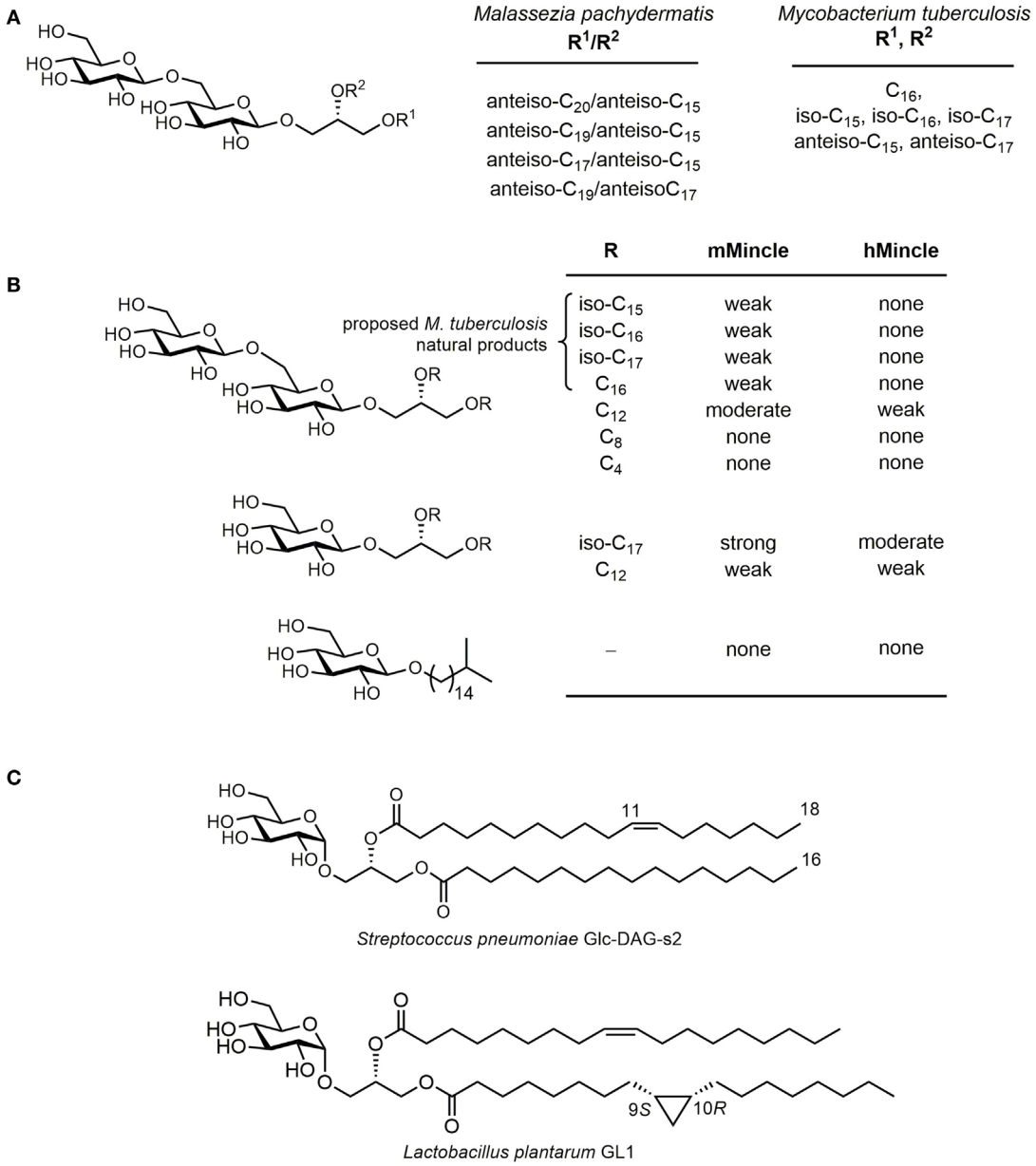

FIGURE 5 | Glycosyl diacylglycerols are a class of microbe-associated molecular patterns that signal through Mincle. **(A)** Structures of β-gentiobiosides isolated from *Malassezia pachydermatis* that weakly signal through mouse Mincle and related compounds from *Mycobacterium tuberculosis* H37Ra. **(B)** Structure activity relationships for signaling through mouse and human Mincle for β-glycosyl diglycerides using reporter cell assay. **(C)** Structures of α-glucosyl diglycerides from pathogenic and commensal bacteria that signal through Mincle.

diglycerides, α-glucosyl diacylglycerides are also lipid anchors of lipoteichoic acid, which do not signal through Mincle. Williams and co-workers synthesized an α-glucosyl diglyceride from the commensal bacterium *Lactobacillus plantarum* (61), which contained a CP fatty acid (dihydrosterculic acid) and oleic acid and demonstrated that this could activate human and mouse Mincle NFAT-GFP reporter cells, with similar potency (62, 63). A range of analogs were prepared that lacked cyclopropanation and/or unsaturation; all signaled though human and mouse Mincle with similar potency.

Collectively, these results suggest that α- and β-glucosyl diacylglycerides comprise a class of microbe-associated molecular patterns that can signal through Mincle.

Adjuvant Studies

Growing interest in the development of subunit vaccines for infectious diseases and cancer has intensified interest in the development of adjuvants (64, 65). Instead of the entire microbe, subunit vaccines include only the antigens that stimulate the body, in some cases being restricted to the specific antigen that

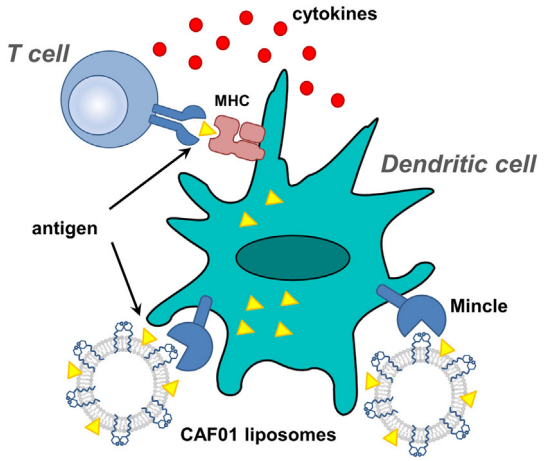

FIGURE 6 | Proposed action of CAF01-adjuvanted antigen vaccination. Liposomes, composed of dimethyldioctylammonium bromide liposomes co-formulated with TDB, are emulsified with antigen and used to stimulate dendritic cells.

is recognized by T cells or antibodies. Benefits of subunit vaccines include a lower potential for adverse reactions, the ability to be produced using recombinant DNA technology (recombinant subunit vaccines) or by chemical synthesis (molecular subunit vaccines), and the ability to systematically alter their structure (and therefore function) (66). However, subunit vaccines are inherently less immunogenic because they lack a full suite of cellular or viral immunogens and co-administration of an adjuvant is often required to stimulate a potent immune response.

A critical role for an oil delivery agent for trehalose glycolipid immunogens was demonstrated in the earliest studies of the ability of mycobacterial cells to act as adjuvants and immunostimulants. For example, complete Freund's adjuvant is an emulsion of inactivated and dried mycobacterial cells in various vegetable and mineral oils, which is emulsified with antigen using the surfactant mannide monooleate (11). To identify the bioactive component, Bloch obtained petroleum extracts of corded *M. tuberculosis* H37Rv or *M. bovis* Vallée to obtain a "cord factor" that alone was toxic to mice when injected as a suspension in paraffin oil (67). Subsequently, trehalose diesters have most commonly been studied as oil-in-water emulsions, with variation in the nature of the oil (mineral/paraffin, vegetable, squalene, or squalane), droplet size, and amount of Tween detergent (10). These changes can have large effects on immunogenicity as well as toxicity, with larger droplets often giving more severe reactions such as granulomas (68). Use of oil-in-water emulsions for intraperitoneal or intramuscular injection is believed to act through three different mechanisms: the establishment of a local depot at the site of injection allowing for sustained continuous release of the antigen; provision of a vehicle for transporting antigen through the lymphatic system; and interaction with immune cells such as antigen-presenting and phagocytic cells (11). The long duration of oil-in-water emulsions is striking: subcutaneous injection of squirrel monkeys or rats with radiolabeled hydrocarbon emulsified with mannide monooleate revealed that after 10 months nearly 30% of radiolabeled hydrocarbon was still located at the injection site (69).

Early studies of TDM analogs as immunomodulators have been reviewed (10). TDCM as an oil-in-water emulsion was shown to protect against bacterial challenge and suppress tumor growth. Typically, it was found to be similarly effective as TDM. Early studies with simple trehalose diesters utilized oil-in-water emulsions (using vegetable and mineral oils). TDB was found to be more effective than trehalose dipalmitate in suppressing ascetic tumor growth and protecting against bacterial infection. Typically, TDB was less effective than TMCM in suppressing tumor growth and protecting against bacterial infection; the reversed potency was seen for suppressing ascetic tumor growth in rats.

A breakthrough was made in the development of a two-component adjuvant comprised TDB and DDA, which can confer strong humoral and cell-mediated immune responses (39, 70). Alone, DDA acts as an adjuvant that can be co-formulated with antigens to elicit strong cell-mediated and moderate-to-strong humoral responses (71). DDA forms liposomes, but these are relatively unstable and aggregate. However, upon co-formulation with TDB, the liposomes exhibit greater stability and adjuvanticity, with optimum levels of IFN-γ production at 11% TDB; this formulation is termed CAF01 (70). Evidence that the adjuvancy arises from signaling through Mincle was obtained by showing that Mincle$^{-/-}$ mice could not be adjuvanted to H1 (Ag85B-ESAT-6) subunit vaccination by CAF01 (9). Immunization of mice with H1 in DDA–TDB liposomes induced a strong, specific T_H1 biased immune response characterized by substantial production of the interferon-γ cytokine and high levels of IgG2b isotype antibodies (39). More extensive studies of the CAF01 adjuvant has characterized it as a unique adjuvant, with low toxicity, and capable of providing a T_H1/T_H17 profile that is distinct to other approved (alum, squalene – MF59) and promising (IC31, GLA-SE) adjuvants and is effective in enhancing responses to *M. tuberculosis*, chlamydia, and HIV-derived peptides (12).

Given that no approved human adjuvants are available for induction of cellular immunity, CAF01 has attracted keen interest. A Phase I study was reported that enrolled healthy human volunteers who were vaccinated with the H1 and adjuvanted with CAF01 (19, 72). Two vaccinations elicited strong antigen-specific T-cell responses that persisted after 150 weeks, indicating the induction of a long-lasting memory response. CAF01 was shown to be a safe and tolerable T_H1-inducing adjuvant for human vaccination studies in where cellular immunity is required.

Other Mincle agonists have been investigated as CAF01-like adjuvants. Decout et al. examined the ability to adjuvant Ag85A immunization in mice (43). The glycolipids TDB, GlcC14C18, and ManC14C18 were formulated in a 1:25 ratio with DDA, which is suboptimal for TDB/DDA. For TDB/DDA, little effect on IL-2, IFN-γ, or IL-17 production was observed relative to DDA alone. Enhanced production of these cytokines was seen for ManC14C18 but without statistical significance. Significant increases in the cytokines were seen for GlcC14C18. DDA/GlcC14C18 provided superior production of Ag85A-specific

IgG2b titers, 80-fold greater than for DDA alone, while IgG1 titers were unaffected, a pattern characteristic of T_H1 response. A 25:1 DDA/GlcC14C18 formulation was as effective as the optimal 10:1 DDA/TDB formulation in the induction of protective immunity to *M. tuberculosis* infection, suggesting enhanced potency of the GlcC14C18 glycolipid.

Huber et al. have studied simple trehalose diesters for the ability to adjuvant *Chlamydia trachomatis* serovar D major outer membrane protein (42). TDS, trehalose monostearate (TMS), TDB, and trehalose PEG-C18 were formulated in an 11% ratio with DDA, which is optimal for TDB (70). Following immunization, TDB and TDS were more effective for the production of IL-17a and IFN-γ from splenocytes than TMS and PEG-C18 and produced higher numbers of antigen-specific IFN-γ^+ and IL-17a$^+$ CD44$^+$CD4$^+$ T cells. A study that evaluated the ability of TDP, TDS, and TDB to adjuvant H56 (Ag85B-ESAT-6-Rv2660c fusion protein) immunization of mice found all three glycolipids (as an 11% formulation in DDA) elicited comparable T-cell responses (73).

CONCLUSION

The discovery that Mincle is the key receptor involved in signaling by TDM and TDB has stimulated growing interest in the discovery of agonists for this receptor. A growing repertoire of natural lipidic species has been identified that belong to three major classes: sterols (e.g., cholesterol); trehalose, glucose, GroMMs, and related acylated species; and glycosyl diglycerides. Molecules from these classes are produced by an assortment of pathogenic and commensal microorganisms, suggesting the involvement of Mincle signalling in a broad range of infectious diseases and in a healthy gut microbiota. Rational design of Mincle agonists based on these structural templates is now possible and has led to the development of simplified structures that achieve similar or improved levels of immune stimulation as for natural cord factor. The dual humoral and cell-mediated immunity induced by CAF01 is supportive for its application as an adjuvant for vaccines directed at the treatment of infectious disease and holds promise for application in cancer immunotherapy, for example, in adjuvanting tumor-associated carbohydrate antigen vaccines. Finally, while attention to date has focused on developing small molecule agonists of Mincle signaling, future efforts should seek to discover small molecule antagonists of Mincle signaling in conditions of sterile inflammation, which could assist in probing the role of Mincle in diseases such as stroke (74), atherosclerosis (22), Gaucher's disease (26), skin allergies (25), and hepatitis (75).

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SW takes full responsibility for this article.

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Expression of Siglec-E Alters the Proteome of Lipopolysaccharide (LPS)-Activated Macrophages but Does Not Affect LPS-Driven Cytokine Production or Toll-Like Receptor 4 Endocytosis

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Siglec-E is a murine CD33-related siglec that functions as an inhibitory receptor and is expressed mainly on neutrophils and macrophage populations. Recent studies have suggested that siglec-E is an important negative regulator of lipopolysaccharide (LPS)-toll-like receptor 4 (TLR4) signaling and one report (1) claimed that siglec-E is required for TLR4 endocytosis following uptake of *Escherichia coli* by macrophages and dendritic cells (DCs). Our attempts to reproduce these observations using cells from wild-type (WT) and siglec-E-deficient mice were unsuccessful. We used a variety of assays to determine if siglec-E expressed by different macrophage populations can regulate TLR4 signaling in response to LPS, but found no consistent differences in cytokine secretion *in vitro* and *in vivo*, comparing three different strains of siglec-E-deficient mice with matched WT controls. No evidence was found that the siglec-E deficiency was compensated by expression of siglecs-F and -G, the other murine inhibitory CD33-related siglecs. Quantitative proteomics was used as an unbiased approach and provided additional evidence that siglec-E does not suppress inflammatory TLR4 signaling. Interestingly, proteomics revealed a siglec-E-dependent alteration in macrophage protein composition that could be relevant to functional responses in host defense. In support of this, siglec-E-deficient mice exhibited enhanced growth of *Salmonella enterica* serovar Typhimurium in the liver following intravenous infection, but macrophages lacking siglec-E did not show altered uptake or killing of bacteria *in vitro*. Using various cell types including bone marrow-derived DCs (BMDCs), splenic DCs, and macrophages from WT and siglec-E-deficient mice, we showed that siglec-E is not required for TLR4 endocytosis following *E. coli* uptake or LPS challenge. We failed to see expression of siglec-E by BMDC even after LPS-induced maturation, but confirmed previous studies that splenic DCs express low levels of siglec-E. Taken together, our findings do not support a major role of siglec-E in regulation of TLR4 signaling functions or TLR4 endocytosis in macrophages or DCs. Instead, they reveal that induction of siglec-E by LPS can modulate the phenotype of macrophages, the functional significance of which is currently unclear.

Keywords: Siglec-E, toll-like receptor 4, lipopolysaccharide, macrophage, dendritic cells, proteomics

INTRODUCTION

Innate immune cells express toll-like receptors (TLRs) which play critical roles in recognition of various pathogen-associated molecular patterns (PAMPs). Exposure of macrophages and dendritic cells (DCs) to PAMPS, such as Gram-negative bacterial lipopolysaccharide (LPS), which triggers through TLR4, can orchestrate a diverse gene expression program required for shaping the innate and adaptive arms of the immune response (2–4). These changes include the induction or repression of a wide range of genes that regulate pro-inflammatory cytokines, chemokines, inflammatory mediators, polarization, migration, and cell survival. These processes are tightly regulated and loss of control is associated with conditions, such as septic shock and inflammatory diseases (5–7).

Many immune cells express a variety of membrane proteins with cytosolic tyrosine-based inhibitory motifs (ITIMs) that negatively regulate signaling through activation receptors. One important class of such inhibitory receptors implicated in regulation of TLR signaling is the family of siglecs, defined as transmembrane sialic acid-binding Ig-like lectins (8). The CD33-related siglecs are a recently evolved subset that are mainly expressed in a complex manner by cells of the innate immune system. Most contain an ITIM and an ITIM-like motif in their cytoplasmic tails which, following tyrosine phosphorylation by Src-family kinases, are thought to be important for inhibitory signaling *via* recruitment and activation of protein tyrosine phosphatases SHP-1 and SHP-2 (9, 10). The sialic acid-binding sites of inhibitory siglecs on leukocytes are occupied by *cis*-interactions with sialic acids on the plasma membrane [reviewed in Ref. (8)]. Depending on the sialic acid carriers, these *cis*-interactions are likely to be important for regulating the functional responses of siglecs [reviewed in Ref. (11)]. Siglecs can also interact with sialic acid ligands *in trans*, for example, on encountering another cell or a pathogen expressing high-affinity/avidity ligands and this can trigger siglec-dependent signaling functions and endocytosis.

As a model system to understand the signaling functions of inhibitory CD33-related siglecs on myeloid cells, our laboratory has focused on murine siglec-E which is mainly expressed on neutrophils, tissue macrophages, and splenic DCs (12, 13). There have been several reports showing that siglec-E and its human homolog siglec-9 are important for regulation of TLR4-driven cytokine production in macrophages and DCs. In murine bone marrow-derived macrophages (BMDM), cross-linking of siglec-E with antibodies (Abs) reduced the production of TNF-α, IL-6, and RANTES in response to LPS stimulation (14). Overexpression of recombinant human siglec-9 in human THP-1 and mouse RAW264 macrophage cell lines downregulated the production of pro-inflammatory cytokines following LPS stimulation (15). Siglec-E expression has been shown to suppress pro-inflammatory cytokine production by macrophages in response to a sialylated strain of Group B *Streptococcus* (16) and treatment of murine macrophages with sialic acid-decorated nanoparticles was found to abrogate LPS-induced inflammation (17). More recently, Chen et al. reported direct interactions between TLRs and siglecs, including siglec-E (18). The same group also proposed that *cis*-interactions between siglec-E and TLR-4 are required for TLR4 endocytosis following uptake of *Escherichia coli* and are important for downregulating TLR4-mediated inflammatory responses (1, 18).

In this report, we further investigate the potential role of siglec-E in TLR4 signaling *via cis*-interactions using three different lines of WT and siglec-E-deficient mice. Consistent with previous studies, we show that siglec-E is strongly upregulated by low-dose (1 ng/ml) LPS leading to constitutive tyrosine phosphorylation and recruitment of the negative regulator SHP-1. However, we were unable to demonstrate a siglec-E-dependent effect on pro-inflammatory cytokine production by macrophages challenged with a high dose (100 ng/ml) of LPS, using a variety of approaches, including unbiased quantitative proteomics. Furthermore, we failed to see any expression of siglec-E on bone marrow-derived DCs (BMDCs) and were unable to reproduce the previous findings of siglec-E-dependent internalization of TLR4 in response to bacterial challenge. However, we could demonstrate by quantitative proteomics that the phenotype of siglec-E-deficient macrophages challenged with LPS was different from WT macrophages, suggesting that siglec-E contributes to the differentiation of macrophages exposed to LPS, but plays little or no role in directly regulating TLR4-dependent signaling by macrophages or DCs under physiological conditions.

MATERIALS AND METHODS

Materials

Dulbecco's phosphate-buffered saline (PBS) without Ca and Mg, fetal bovine serum (FBS) (qualified, heat inactivated, E.U.-approved), penicillin and streptomycin solution, Trypsin–EDTA solution, protein G Dynabeads, Microplate BCA Protein Assay, NuPAGE LDS Sample Buffer, NuPAGE® Novex 4–12% Bis-Tris gel, MOPS running buffer, and sample reducing agent, trypsin protease, Pierce MS Grade, TMT 10-plex™ Isobaric Reagent Label Set were from Thermo Fisher Scientific, Paisley, UK; SeraMag SpeedBead Carboxylate-Modified Magnetic Particles were from GE LifeSciences; Roche-COMPLETE Mini EDTA-Free Protease Inhibitor tablets, Roche-PHOSS-RO, PhosSTOP™ Trypan blue solution, anti-sheep IgG (whole molecule)-peroxidase and anti-rabbit IgG (whole molecule)–peroxidase Ab produced in goat, lipopolysaccharide from *E. coli* 0111:B4 were from Sigma; GM-CSF and IL-4 were from Peprotech, GolgiStop, CD16/CD32 (Fc block), V500 rat anti-mouse I-A/I-E (clone: M5/114; 562366) were from BD Bioscience, UK; anti-mouse TNF alpha PE (clone: MP6-XT22), anti-mouse CD11c PE-cy7 (Clone: N418), anti-mouse Ly-6G (Gr-1) Alexa Fluor® 488 (clone: RB6-8C5) were from eBioscience, UK; anti-*Salmonella* Typhimurium (clone: 1E6), anti-phosphotyrosine Ab (HRP) (Abcam clone: PY20-ab16389) were from Abcam, UK; APC anti-mouse CD11c Ab (clone: N418), PE-conjugated anti-siglec-E used in flow cytometry (clone: M1304A01), biotin anti-mouse TLR4 (CD284)/MD2 complex Ab (clone: MTS510), PE/Cy7 anti-mouse TLR4 (CD284)/MD2 complex Ab (clone: MTS510),

PE anti-mouse/human CD11b Ab (clone: M1/70), APC/Cy7 anti-mouse Ly-6G/Ly-6C (Gr-1) Ab (clone: RB6-8C5) were from Biolegend, UK; and anti-mouse SHP-1 Ab (clone: C-19) was from Santa Cruz. *E. coli* 0111:B4 LPS (Sigma) was used in all *in vitro* experiments. *E. coli*-GFP was obtained from the American Type Culture Collection (25922GFP). Affinity purified sheep anti-siglec-E Ab was produced in-house (12) and used for immunoprecipitation and immunoblotting and in flow cytometry experiments where indicated. Anti-mouse siglec-1 Abs SER-4 and 3D6 were produced in-house (19, 20).

Animals

Wild-type and siglec-E-deficient mice on C57BL/6J and Balb/c genetic backgrounds were generated as described previously (13, 21). Mice were bred and maintained under specific pathogen-free conditions within our own institutional colonies. WT and siglec-E-deficient mice were derived from heterozygous intercrosses and then maintained through homozygous crosses between WT mice and siglec-E-deficient mice. Periodically, the homozygous mouse colonies were refreshed by heterozygous intercrossing. Mice used in experiments were sex- and age-matched between the ages of 7 and 24 weeks. Animal experimentation was approved by the University of Dundee Animal Ethics Committee and carried out under UK Home Office Project License PPL60/3856.

Immunofluorescence Staining

Cryostat sections of liver samples were prepared, fixed in pre-cooled 100% methanol at −20°C, and blocked with 10% normal serum (Gibco) in 1% fish skin gelatin prior to addition of primary Abs and secondary Abs. Tissue sections were counterstained with 400 ng/ml DAPI (Sigma), mounted in media (DAKOCytomation, USA) and analyzed by confocal microscopy. Excitation wavelengths were 405, 488, and 555 nm, and emission wavelengths maxima were 493/519 and 557/574 nm.

Generation and Stimulation of BMDMs

Bone marrow cells were cultured in bacteriological plastic Petri dishes with DMEM media supplemented with penicillin and streptomycin, glutamine, 10% FBS, and either 20% L929 conditioned medium or M-CSF (25 ng/ml) for 7 days. BMDM were harvested with PBS supplemented with 3 mM EDTA and resuspended at a concentration of 1×10^6/ml. For priming, 10 ml of BMDM cell suspension were seeded in 100 mm dishes and treated with 1 ng/ml LPS.

Generation of BMDCs

Bone marrow cells were cultured in RPMI 1640 complete medium supplemented with penicillin and streptomycin, 10% FBS, 20 ng/ml recombinant mouse GM-CSF and 5 ng/ml IL-4 for 6 days or 10 ng/ml recombinant mouse GM-CSF and 1 ng/ml IL-4 for 12 days. The 12-day-cultured BMDC were stimulated for 24 h with 100 ng/ml LPS and analyzed by flow cytometry.

Isolation of Peritoneal Macrophages

Cells were isolated from the peritoneal cavity by lavage with 5 ml RPMI. Cells were washed in media prior to plating in 24-well plates in RPMI containing 10% FBS, penicillin, and streptomycin. After 2 h, non-adherent cells were washed away and adherent macrophages were treated with LPS in complete RPMI media for 48 h.

Flow Cytometry

Single cell suspensions were Fc-receptor-blocked for 30 min at 4°C with rat anti-mouse CD16/CD32 Ab in PBS with 1% FBS. Blocked cells were subsequently incubated with fluorophore-conjugated primary Abs for 60 min at 4°C, prior to washing in PBS containing 1% FBS and 2 mM EDTA. Following surface staining, cells were washed and analyzed by flow cytometry, or were fixed with 2% formaldehyde in PBS and then washed/permeabilized with BD perm/wash buffer (BD Biosciences), and stained with fluorophore-conjugated primary Abs for 60 min at 4°C. Cells were washed and intracellular fluorescence analyzed using a FACS Canto II flow cytometer and FlowJo software.

Intracellular TNF-α Production

1 ng/ml LPS-primed cells were stimulated with 100 ng/ml LPS for 7 h and monensin-containing GolgiStop (BD Biosciences) was added in the last 6 h of culture. After washing, cells were surface stained with biotinylated sheep anti-siglec-E Ab followed by streptavidin-APC. Cells were then fixed with the Cytofix/Cytoperm solution (BD Biosciences) and incubated with PE-conjugated anti-mouse TNF-α Ab diluted in BD Perm/Wash buffer (BD Biosciences). Cells were analyzed by flow cytometry using a FACS Canto II with FlowJo software.

Cytokine ELISAs

IL-6, RANTES, and IL-10 were measured in tissue culture supernatants and sera using ELISA kits according to the manufacturer's instructions and assay procedures (Peprotech).

Quantitative Real-time PCR

Total RNA was extracted using RNeasy mini kit (Qiagen). To quantify the gene expression, cDNA was synthesized using Omniscript RT kit (Qiagen). The sequences of the primers are shown below. Each PCR was performed in a 25 µl reaction mixture containing SYBR Green Universal master mix (Applied Biosystems). The final concentration of primers was 0.3 µM in each reaction. The thermal cycling conditions were as follows: 10 min at 95°C, followed by 40 cycles of 15 s at 95°C, 30 s at 60°C, and 30 s at 72°C.

Siglec-E	Forward	GTC TCC ACA GAG CAG TGC AAC TTT ATC
	Reverse	TGG GAT TCA ACC AGG GGA TTC TGA G
Siglec-F	Forward	CCA CAG GAC CAC CCT CTC CTC
	Reverse	GGA CTT TAG TTC CTG TGT CAT CTC CC
Siglec-G	Forward	GCT GCT ACC TGA TAA AGA CAG TGC C
	Reverse	TTT CCA ATT CCG AGC CAG GGA CC
GAPDH	Forward	CAA CTC CCA CTC TTC CAC CTT CG
	Reverse	GTA GGG AGG GCT CAG TGT TGG G

Treatment of Mice with LPS

Age- and sex-matched mice were injected intraperitoneally with 15 µg LPS [ultrapure *E. coli* 0111:B4 (Invivogen)]. After 3 h,

mice were euthanized, blood was collected by cardiac puncture and serum samples were prepared for use in ELISA. In some experiments, livers and spleens were harvested and frozen for immunofluorescence staining and microscopy.

Infection of Mice with *Salmonella*

Sex- and age-matched 9- to 15-week-old mice were infected by intravenous injection of *Salmonella enterica* serovar Typhimurium strain M525P suspensions in a volume of 0.2 ml PBS. Cultures were grown from single colonies in 10 ml LB broth incubated overnight without shaking at 37°C, then diluted in PBS to the appropriate concentration for inoculation. The infective dose was enumerated by plating dilutions onto LB agar plates. Mice were killed by exposure to a rising concentration of carbon dioxide, and death confirmed by cervical dislocation. Livers and spleens were aseptically removed and homogenized in sterile water using a Precellys 24 homogenizer. The resulting homogenate was diluted in a 10-fold series in PBS and LB agar pour plates were used to enumerate viable bacteria.

Infection of Macrophages with Bacteria for Bacterial Uptake, Bactericidal Activity, and TLR4 Endocytosis Assays

To assess bacterial uptake, cells were infected with either *S.* Typhimurium strain M525P or *E. coli*-GFP for 30 min. After infection, the cells were washed with PBS and analyzed by flow cytometry. For assessing bactericidal activity, the infected cells were further incubated for 60 min with medium containing 100 μg/ml gentamicin to kill extracellular bacteria. The medium was then replaced with 10 μg/ml gentamicin and bactericidal activity was measured by harvesting cells at different time points and analyzing the decaying *E. coli*-GFP signal by flow cytometry. To assess TLR4 levels, cells were infected with *E. coli*-GFP for 1 h, stained with anti-TLR4 Ab and analyzed by flow cytometry.

Siglec-E Co-Immunoprecipitation

Bone marrow-derived macrophages were primed with 1 ng/ml LPS for 3 days and lysed in 50 mM Tris–HCl, 150 mM NaCl, and 1% NP-40 with protease and phosphatase inhibitors. Lysates were subjected to immunoprecipitation with anti–siglec-E Ab. Immunoblots were probed with sheep anti-siglec-E Ab and Abs to SHP-1 and phosphotyrosine followed by HRP-conjugated secondary Abs followed by ECL autoradiography.

SP3 Processing for Quantitative Proteomics

Bone marrow-derived macrophages were primed with 1 ng/ml LPS for 3 days and stimulated with 100 ng/ml LPS for 7 h. Monensin-containing GolgiStop (BD Biosciences) was added for the last 6 h of culture. After washing, cells were lysed in lysis buffer (4% SDS, 50 mM TEAB pH 8.5, 10 mM TCEP), boiled and sonicated with a BioRuptor (30 cycles: 30 s on, 30 s off) before alkylation with 20 mM iodoacetamide for 1 h at room temperature in the dark. Lysates were subjected to the SP3 protein clean-up procedure (22), eluted into digestion buffer (0.1% SDS, 50 mM TEAB pH 8.5, 1 mM CaCl2) and digested with trypsin at a 1:50 (enzyme:protein) ratio. TMT labeling and peptide clean-up were performed according to the SP3 protocol. Samples were eluted into 2% DMSO, combined, and dried under vacuum. TMT samples were fractionated using offline high pH reverse-phase chromatography. Peptides were separated, concatenated to 22 fractions, dried and peptides redissolved in 5% formic acid and analyzed by LC-MS.

Proteomics Quantification and Bioinformatics Analysis

Four biological replicates from four independent biological samples were processed for proteomic analysis (22). The raw mass spectrometric data were loaded into MaxQuant (version 1.5.3.30) (23), using the Andromeda search engine software (24). Enzyme specificity was set to that of trypsin/P, allowing for cleavage of N-terminal to proline residues and between aspartic acid and proline residues. Other parameters used were as follows: (i) variable modifications—methionine oxidation, protein N-acetylation; (ii) fixed modifications, cysteine carbamidomethylation; (iii) database: Uniprot—mouse (downloaded 130501, 50800 sequences); (iv) labels: 10-plex TMT (v) MS/MS tolerance: FTMS- 50ppm, ITMS-0.5 Da; (vi) minimum peptide length, 7; (vii) maximum missed cleavages, 2; and (viii) and (ix) PSM and Protein false discovery rate, 1%. For bioinformatic analysis, Reporter ion intensities (corrected) results from MaxQuant were imported into Perseus software (version 1.5.1.6). The normalized corrected reporter ion intensities for each label were used to calculate ratios and all "Contaminant," "Reverse" and "Only identified by site" proteins were removed from the data. Proteins above twofold change [$\log2(2) = 1$], proteins with nominal p-value less than 0.05 [$-\log10(0.05) = 1.301$] were considered as differentially expressed proteins. All bioinformatics analyses were performed with the Perseus software of the MaxQuant computational platform (23–25). GO over representation enrichment analysis was done using WEB-based Gene SeT AnaLysis Toolket and geneontology database (26). The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium *via* the PRIDE (27) partner repository with the dataset identifier PXD008406.

Statistics

Statistical significance was determined using the two-tailed Student's t-test or non-parametric Mann–Whitney rank-sum test. All experiments were performed at least twice. p Values of <0.05 were considered significant.

RESULTS

Siglec-E Is Upregulated on Macrophages by LPS *In Vivo* and *In Vitro*, but Does not Regulate Production of Inflammatory Mediators

To study the physiological role of siglec-E in regulating LPS-TLR4-driven inflammatory responses, we used siglec-E-deficient

mice generated by three different approaches as described in our previous reports (13, 21). First, siglec-E KO1 mice (referred to as KO1) were generated in 129 embryonic stem (ES) cells following replacement of exons 1 and 2 with a neomycin cassette and backcrossed for more than 15 generations onto the C57BL/6J and Balb/c genetic backgrounds. Second, siglec-E "knockin" mice (referred to as R126D) were generated in C57BL/6 ES cells by introducing a targeted mutation, R126D, to destroy the sialic acid-binding site of siglec-E (13). R126D were shown previously not to express siglec-E protein at detectable levels due to effects on gene transcription (13). Third, siglec-E KO2 mice (referred as KO2) are a complete knock-out of siglec-E on a C57BL/6J background, generated by further crossing R126D mice with transgenic (Nes-cre)1Wme/J (Bal1 cre) mice to partially delete the loxP-flanked allele (21). As described previously, all mouse lines were born at normal Mendelian frequencies and were viable, with no alterations in leukocyte subpopulations compared with their matched WT controls.

On cryostat sections, siglec-E was shown to be expressed in tissue macrophages including liver Kupffer cells (**Figure 1A**) and splenic red pulp macrophages (data not shown) and was strongly upregulated on Kupffer cells following injection of mice with 15 μg LPS (**Figure 1**). As expected, siglec-E was undetectable in tissues of siglec-E-deficient mice (**Figure 1A**). To study the signaling functions of siglec-E in macrophages, we used BMDM grown in M-CSF or in L929 cell conditioned medium as a source of M-CSF. These cells expressed very low levels of siglec-E but this could be strongly increased by

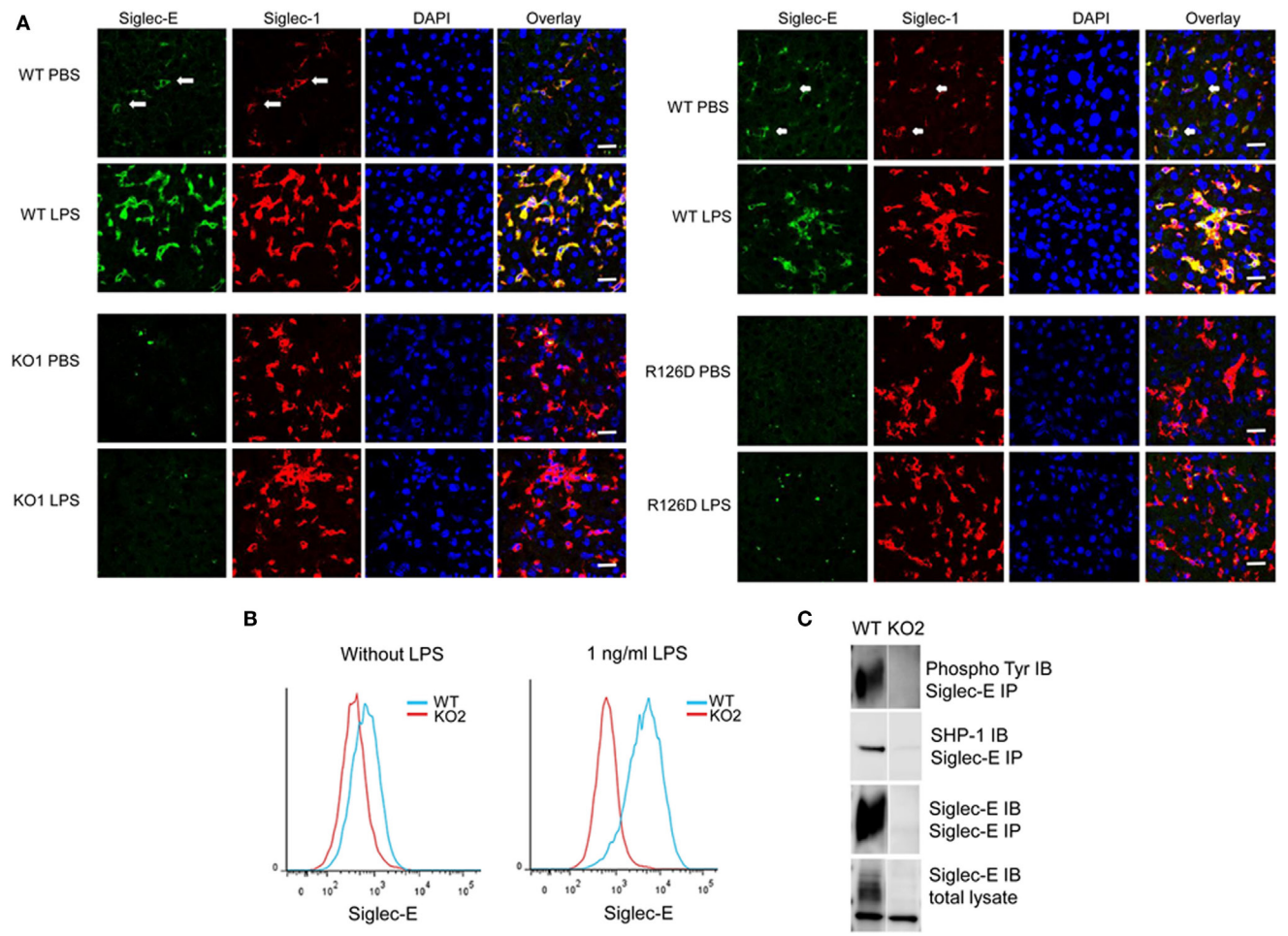

FIGURE 1 | Siglec-E expression on tissue macrophages and bone marrow-derived macrophages (BMDM) is upregulated following lipopolysaccharide (LPS) treatment. **(A)** Wild-type (WT), KO1, and R126D mice were injected with 15 μg LPS or PBS intraperitoneally. After 3 h, animals were euthanized and tissue samples collected and frozen. Liver cryostat sections were labeled with sheep anti-siglec-E Ab directly labeled with Alexa 488 and anti-siglec-1 Abs SER-4 and 3D6 followed by anti-rat Alexa 647. Sections were also stained with DAPI to reveal nuclei. Siglec-E is expressed on Kupffer cells, which co-express siglec-1, and is upregulated following LPS stimulation in WT but not KO1 or R126D mice. Green dots in the anti-siglec-E stained KO1 and R126D sections are due to non-specific binding of the antibody (Ab). The scale bar represents 10 μm. **(B)** Siglec-E is expressed at low levels on BMDM and strongly upregulated following 3 days culture in 1 ng/ml LPS. **(C)** Siglec-E is constitutively phosphorylated in LPS-stimulated BMDM. WT and KO2 BMDM were treated for 3 days with 1 ng/ml LPS and the upregulated siglec-E was immunoprecipitated (IP) using sheep ant-siglec-E Ab and immunoblotted (IB) for phosphotyrosine, SHP-1 and siglec-E.

cultivation for 3 days in low dose, 1 ng/ml LPS (**Figure 1B**). This low concentration of LPS was shown previously not to tolerize macrophages to a subsequent high-dose challenge of 100 ng/ml LPS (28). Immunoprecipitation of siglec-E from 1 ng/ml LPS-primed BMDM cells revealed that siglec-E was constitutively tyrosine-phosphorylated and associated with endogenous SHP-1 (**Figure 1C**).

To investigate whether siglec-E could inhibit TLR4-driven inflammatory responses, LPS-primed BMDM were challenged for 7 h with 100 ng/ml LPS, with GolgiStop (monensin) added for the last 6 h post LPS stimulation to trap secreted inflammatory proteins. This led to strong induction of TNF-α as measured by intracellular flow cytometry (**Figure 2A**). However, no differences were seen comparing WT and siglec-E-deficient macrophages. To determine whether siglec-E could modulate other TLR4-driven cytokine responses at the later time point of 48-h post LPS treatment, ELISA was used to measure IL-6, IL-10, and RANTES in tissue culture supernatants, but no significant differences were seen comparing WT and siglec-E-deficient BMDM (**Figure 2B**). Similar observations were made using resident peritoneal macrophages that constitutively express siglec-E (**Figure 2C**). Finally, we asked whether siglec-E-deficient mice exhibited exaggerated cytokine responses at 3 h following intraperitoneal injection of LPS. Surprisingly, we saw reduced IL-6 and IL-10 responses in the sera of KO1 mice, but no differences were seen in E126D mice (**Figure 3**).

To check whether other ITIM-containing CD33-related siglecs were expressed in LPS-treated macrophages to compensate for the loss of siglec-E expression, we performed quantitative RT-PCR on macrophage lysates and analyzed expression of mRNAs encoding siglecs-E, -F, and -G, which are the only ITIM-bearing CD33-related siglecs in mice (**Figure 4**). As a positive control for siglecs-F and -G, which are mainly expressed in eosinophils and B cells, respectively, we used mouse bone marrow cells that showed the expected signals. However, while siglec-E mRNA was strongly upregulated in LPS-treated WT macrophages, there was no evidence for upregulation of mRNAs for siglecs-F and -G which remained at low or undetectable levels (**Figure 4**). Therefore, the failure of siglec-E to suppress TLR4 signaling cannot be explained by compensatory upregulation of other related inhibitory siglecs.

Quantitative proteomics was next used as an unbiased approach to determine if additional LPS-induced inflammatory mediators could be regulated by expression of siglec-E in macrophages (**Figure 5**). A number of secretory inflammatory cytokines (TNF, Il16, Il1b, Il18, and Il19) and chemokines (Ccl2, Ccl3, Ccl4, Ccl5, Ccl6, Ccl7, Ccl9, Ccl12, Cxcl2, Cxcl3, Cxcl10, Cxcl16, and Cxxc1) were detected in the proteome following 100 ng/ml LPS treatment (Table S1 in Supplementary Material). However, apart from Ccl7 which was reduced in KO2 macrophages, none of the other upregulated cytokines and chemokines showed any significant differences comparing proteomes from WT and siglec-E-deficient BMDM (**Figure 5A**). Interestingly, the proteomics analysis revealed that WT and siglec-E-deficient BMDM exhibited many differentially regulated proteins not implicated in LPS-induced inflammation. Gene ontology enrichment analyses showed that these proteins were mainly associated with cellular features, such as membranes, vesicular transport, and cytoskeleton (**Figure 5B**).

Siglec-E-Deficient BMDM Are Not Defective in Bacterial Uptake and Killing

The proteomic differences between WT and siglec-E KO macrophages point to functions relating to endocytosis and endosomal/lysosomal trafficking which could be relevant to bacterial uptake and/or bactericidal activity of macrophages (29–31). This possibility was also consistent with *in vivo* observations that siglec-E-deficient mice showed increased bacterial loads following infection with *Salmonella* Typhimurium (**Figure 6**). Therefore, to test the hypothesis that siglec-E contributed to uptake and killing of bacteria by macrophages, *in vitro* infection studies were carried out using *S.* Typhimurium and *E. coli* (**Figure 7**). No differences in uptake of either bacteria were observed at 30 min after infection comparing WT and siglec-E-deficient BMDM (**Figure 7A**). In addition, no differences in bactericidal activity of macrophages were seen using *E. coli*–GFP and measuring loss of the GFP signal over a 6 h time course (**Figure 7B**).

Siglec-E Is Not Required for TLR4 Endocytosis in BMDM, BMDC, Splenic Macrophages, or Splenic DCs

Several studies have shown that LPS–CD14–TLR4–MD2 complexes undergo endocytosis, leading to macrophage desensitization and tolerance (32–35). The endocytosed TLR4 initially activates TRIF–TRAM signaling in the early endosome and is later channeled to lysosomes and degraded through the ubiquitin pathway to limit further signaling (36–38). Recent studies in DCs demonstrated a role for siglec-E in promoting TLR4 endocytosis and downregulating TLR4-mediated inflammatory responses following *E. coli* infection (1, 18). In view of our findings that siglec-E on macrophages does not seem to regulate TLR4 inflammatory signaling, we asked if siglec-E affects TLR4 endocytosis in macrophages. Following *E. coli*-GFP infection of BMDM, TLR4 underwent endocytosis as reported by others (**Figure 8A**). However siglec-E-deficient BMDM showed similar levels of TLR4 endocytosis (**Figure 8A**; Figure S1A in Supplementary Material). To check if the previously reported role of siglec-E in downregulating TLR4 was restricted to DCs, we also analyzed responses in BMDC (**Figure 8A**). Similar to BMDM, the BMDC showed strong downregulation of TLR4 on exposure to *E. coli*-GFP, but this was unaffected in cells prepared from siglec-E deficient mice (**Figure 8A**). We also asked whether siglec-E might regulate LPS-mediated TLR4 endocytosis. While low doses up to 1.0 ng/ml LPS did not affect TLR4 levels at the cell surface, higher doses such as 50 ng/ml led to reduced TLR4 expression that was similar in WT and siglec-E-deficient BMDM (**Figures 8B,C**; Figure S1B in Supplementary Material). Similar observations were made with splenic macrophages and splenic DCs incubated with *E. coli*-GFP though the overall levels of TLR4 endocytosis were low compared to *in vitro* cultured BMDM and BMDC (**Figure 8D**; Figures S1C,D in Supplementary Material).

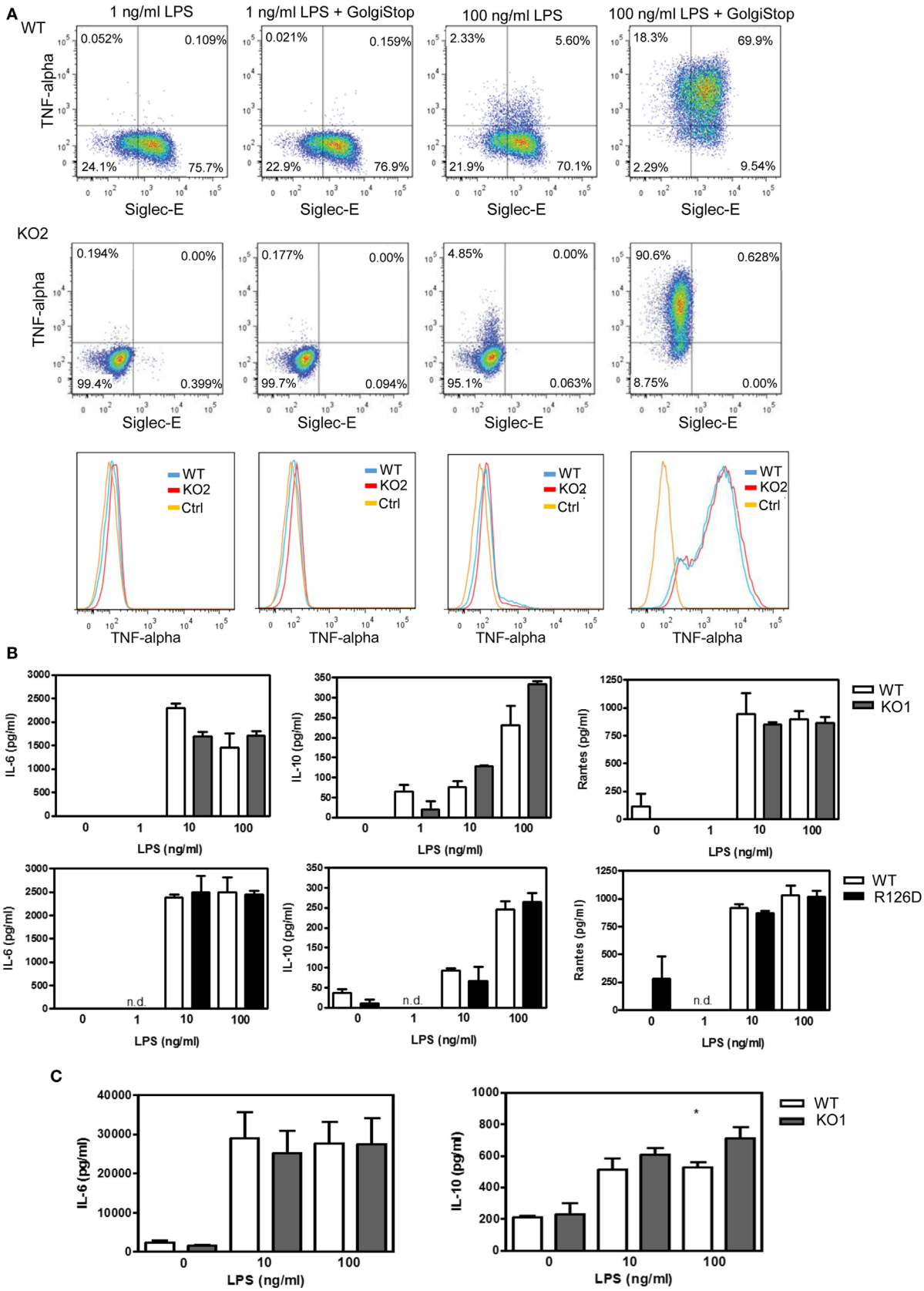

FIGURE 2 | Continued

FIGURE 2 | Analysis of cytokine production by wild-type (WT) and siglec-E-deficient macrophages following lipopolysaccharide (LPS) treatment. **(A)** WT and siglec-E KO2 bone marrow-derived macrophages (BMDM) were primed with 1 ng/ml LPS to induce siglec-E expression, treated with or without 100 ng/ml LPS ± GolgiStop to trap secretory proteins and analyzed by flow cytometry for TNFα. Three independent biological replicates were performed for each genotype. **(B)** BMDM from WT and siglec-E-deficient mice were cultured in the presence of LPS for 2 days. **(C)** Resident peritoneal macrophages from WT and siglec-E-deficient mice were cultured in the presence of LPS for 6 h. For **(B,C)**, cytokine levels in supernatants were assessed by ELISA. Data show means + 1 SD from a single experiment performed in triplicate and representative of two independent experiments. Statistical analyses were performed using Student's *t*-test. *indicates $p < 0.05$.

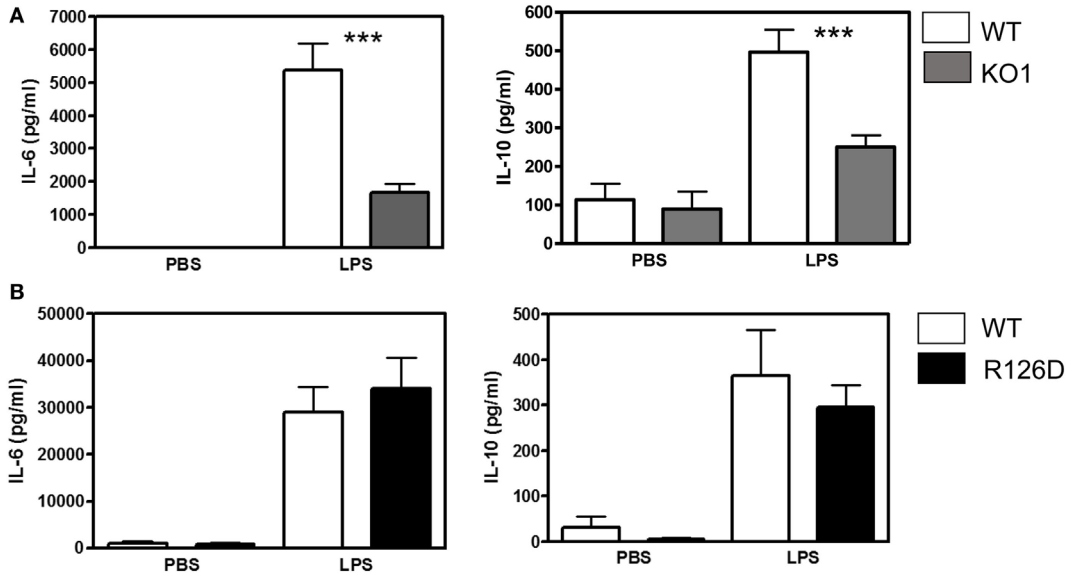

FIGURE 3 | Analysis of lipopolysaccharide (LPS)-dependent cytokine responses in siglec-E-deficient mice. **(A)** Wild-type (WT) and KO1 mice and **(B)** WT and R126D mice were injected with 15 µg ultrapure LPS or PBS intraperitoneally and blood collected at 3 h. IL-6 and IL-10 concentrations in sera were measured by ELISA. Data show means + 1 SD from pooled samples, derived from two independent experiments, with three to six mice in each experiment. Statistical analysis was performed using Mann–Whitney *t*-test, ***indicates significant differences between WT and KO1 mice, $p < 0.0005$.

Siglec-E Is Expressed by Splenic DC, Splenic Red Pulp Macrophages, and Splenic Granulocytes but Is Not Expressed on BMDCs

Previous studies have reported siglec-E-dependent functional responses using BMDC, but they did not demonstrate siglec-E expression by these cells (1, 18). To address this, we analyzed siglec-E levels in BMDC generated from BM progenitor cells cultured for 6 days in GM-CSF and IL-4 (**Figure 9**). As reported by others, the 6-day-cultured BM cells are a heterogeneous population containing granulocytes, macrophages, and DCs that can be readily distinguished using surface markers (39). In multiple experiments, we observed high siglec-E expression on Gr1-positive granulocytes, however, CD11c, MHCII double-positive DCs consistently lacked siglec-E expression (**Figure 9A**; Figure S1E in Supplementary Material). When 12-day-cultured BMDC were matured with LPS, there was clear upregulation of MHC class II, but more than 94% of the mature DCs (MHCII high cells) lacked siglec-E expression (**Figure 9B**).

In comparison to BMDC, CD11c, MHCII double-positive DCs extracted from the spleen expressed low levels of siglec-E as observed previously (13). By contrast, F4/80-positive splenic macrophages and Gr-1-positive granulocytes expressed much higher levels of siglec-E (**Figure 10**).

DISCUSSION

The focus of this study was to explore the role of siglec-E in regulating TLR4-induced inflammatory responses in macrophages. Our approach was to use siglec-E-deficient mice generated by gene targeting in ES cells, which is a conventional methodology to determine protein function in the immune system. Using three different strains of siglec-E-deficient mice and matched WT controls, we were unable to demonstrate an important role for siglec-E in regulating inflammatory cytokines downstream of TLR4 activation, both *in vitro* and *in vivo*. We showed that this was not due to compensation by other closely related inhibitory siglecs in the siglec-E-deficient macrophages. However, quantitative proteomics revealed differences in the levels of many proteins shared by WT and siglec-E-deficient macrophages following LPS activation and we cannot exclude the possibility that some of these differences could mask siglec-E dependent effects on TLR4 signaling.

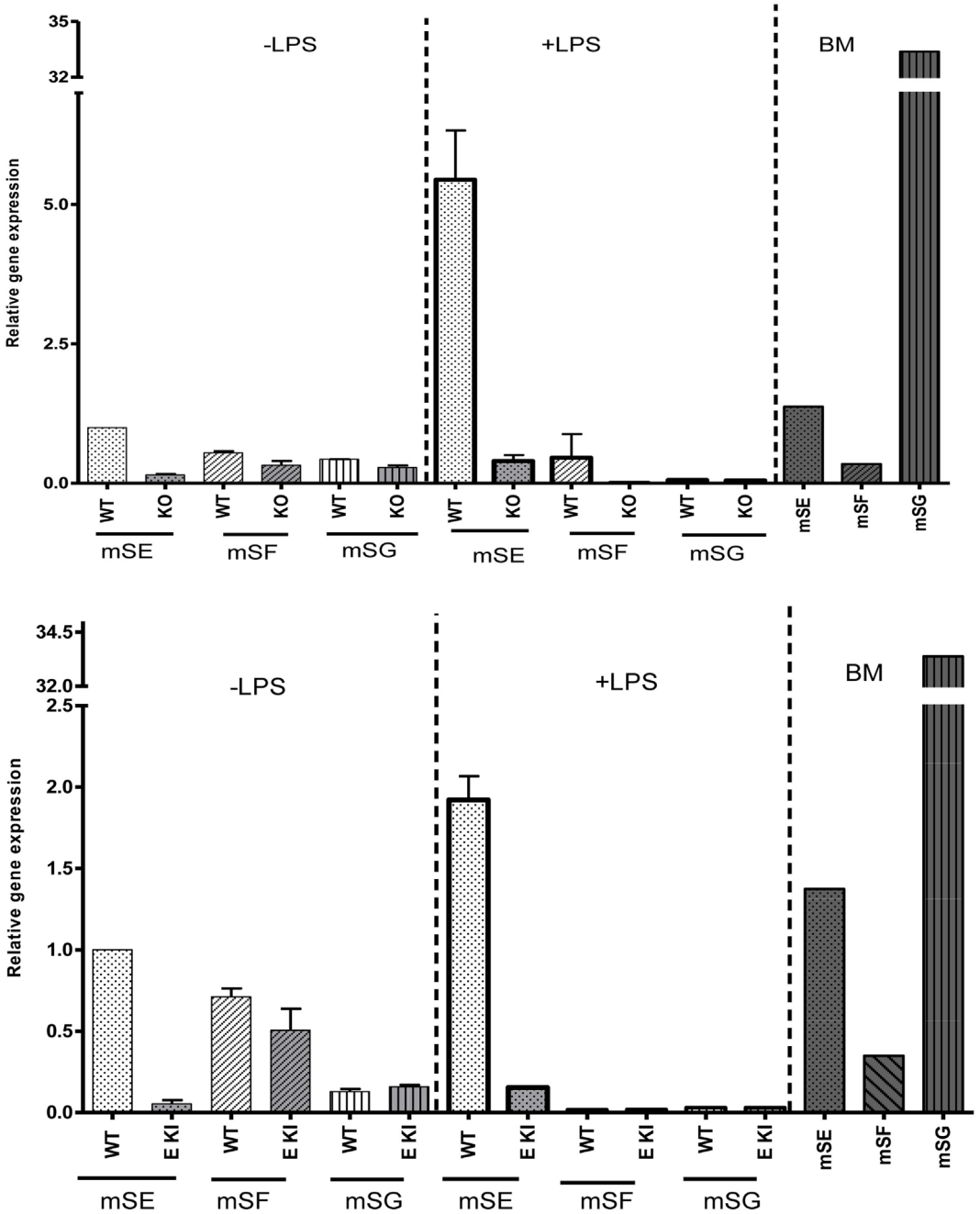

FIGURE 4 | Other inhibitory CD33-related siglecs do not compensate for siglec-E deficiency in bone marrow-derived macrophages (BMDM). Relative gene expression of siglec-E, siglec-F, and siglec-G in BMDM untreated or stimulated with 10 ng/ml lipopolysaccharide (LPS) for 48 h from wild-type and siglec-E-deficient mice. Gene expression is shown relative to GAPDH, using untreated WT BMDM as calibrator. WT bone marrow cells were used as a control for siglecs-E, -F, and -G as they are expressed in developing neutrophils, eosinophils, and B cells, respectively, and show the expected gene expression profiles.

In considering a potential role of siglec-E in regulating TLR4 signaling, it is important to distinguish *cis*-interactions of siglec-E with TLR4 (1, 18) and *trans*–interactions of siglec-E with other ligands. *Cis*-interactions occur through presentation of sialic acid ligands on neighboring glycoproteins and glycolipids. Their importance has been clearly documented in the case of CD22 and siglec-G on B cells using genetic and biochemical approaches where CD22 has been shown to associate with other CD22 molecules and siglec-G has been shown to associate with the B cell receptor complex [reviewed in Ref. (11)]. Such *cis*-interactions play a key role in regulating the threshold of B cell activation to antigens and preventing autoimmunity. The evidence that siglec-E associates *in cis* with TLR4 was based on pull-down experiments and overlays using recombinant forms of siglecs

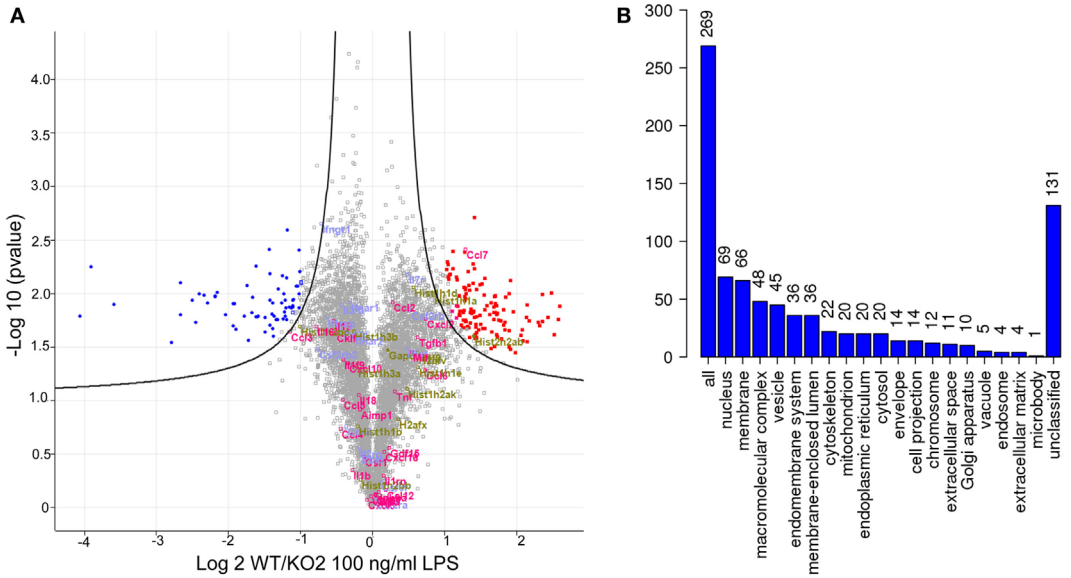

FIGURE 5 | Quantitative proteomics analysis of wild-type (WT) and KO2 bone marrow-derived macrophages (BMDM) activated by lipopolysaccharide (LPS). **(A)** Volcano plot showing proteomics data derived from four independent biological replicates. WT and KO2 BMDM cells were primed for 3 days with 1 ng/ml LPS and challenged with 100 ng/ml LPS + GolgiStop for 7 h and lysates subjected to quantitative proteomics. The inflammatory mediators are annotated in pink. Gapdh and histone variants are in green and cytokine receptors in blue. **(B)** GO analysis of proteins that are differentially regulated between WT and KO2 BMDM.

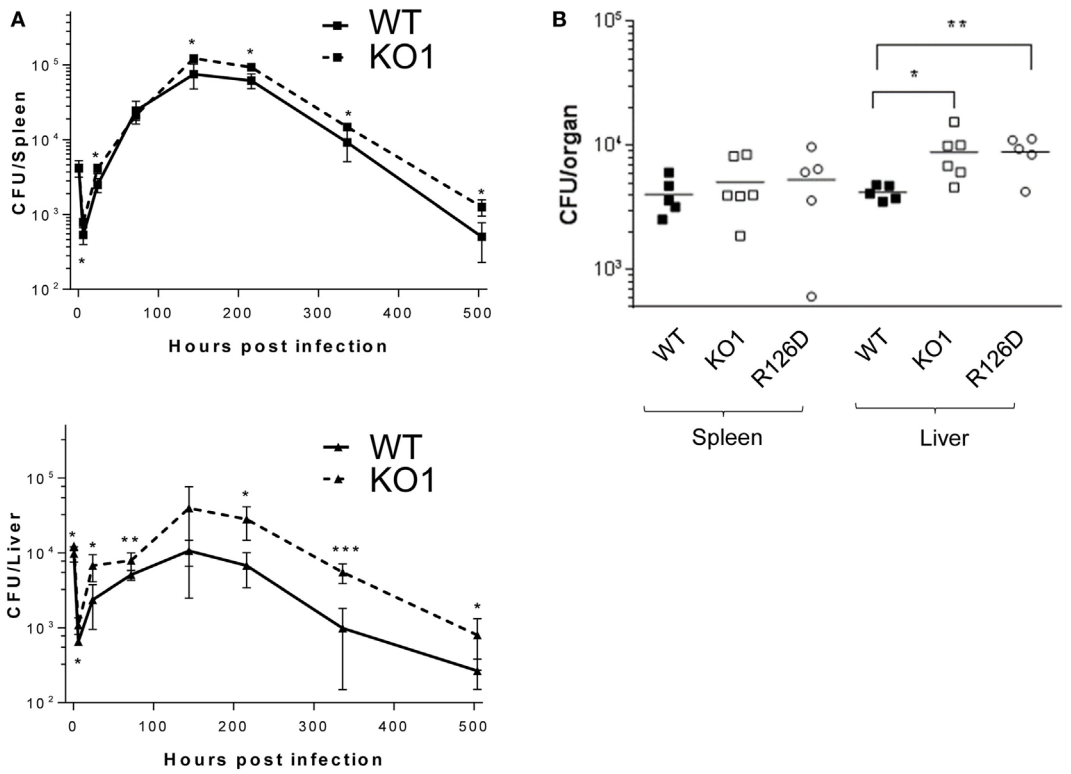

FIGURE 6 | Siglec-E-deficient mice show enhanced growth of *Salmonella* Typhimurium following intravenous infection. **(A)** Wild-type (WT) and KO1 mice on a Balb/c background and **(B)** WT and KO1 on a C57BL/6J background and R126D mice were infected with *S.* Typhimurium M525P and liver and spleen CFU determined from groups of 4–8 mice at the indicated time points. Data in **(A)** show mean values ± 1 SD and data in **(B)** show values for individual mice. Statistical analysis was performed by Student's *t*-test: $*p < 0.05$; $**p < 0.005$; $***p < 0.0005$.

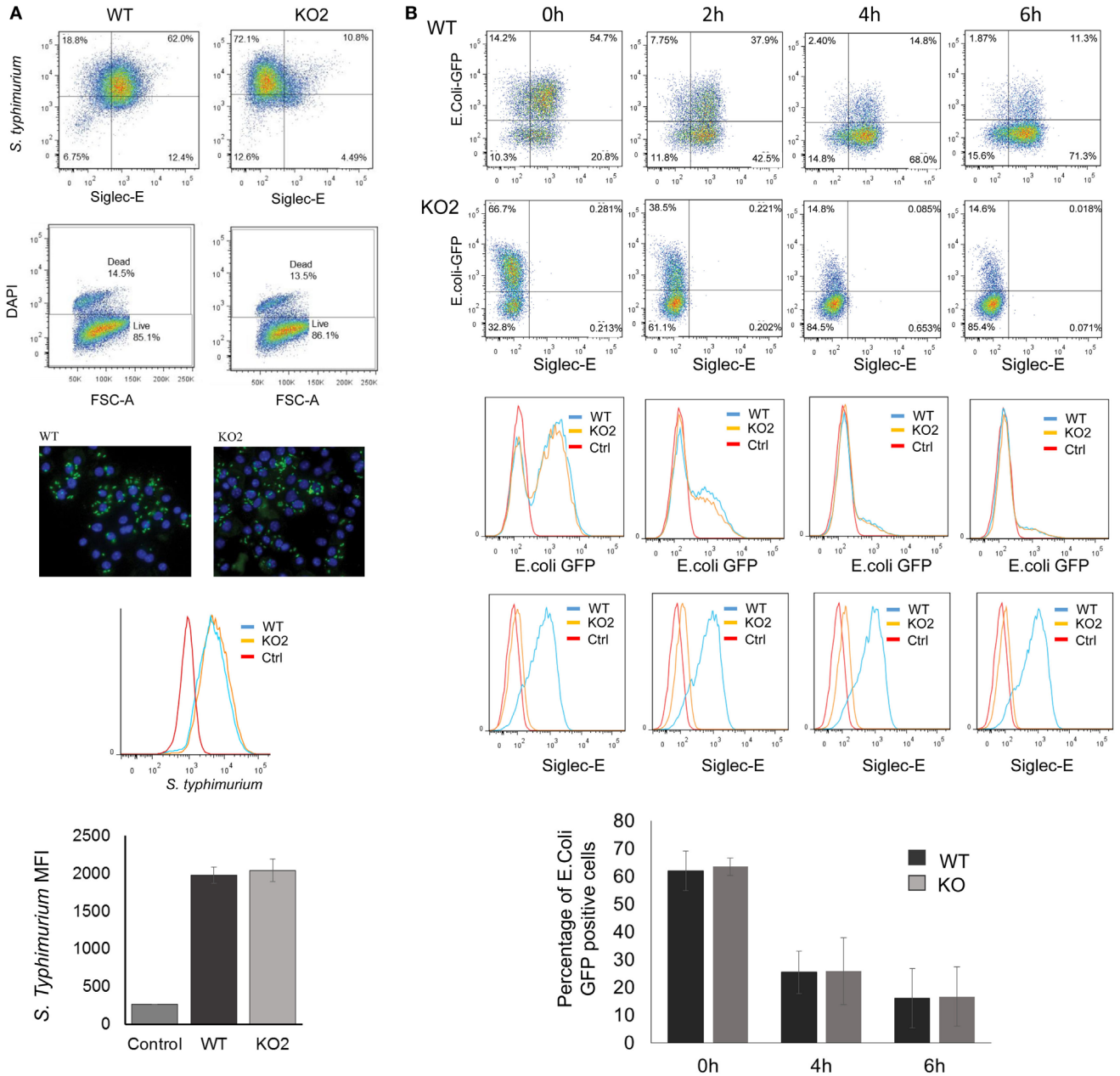

FIGURE 7 | Uptake and killing of bacteria by bone marrow-derived macrophages (BMDM) are not affected by siglec-E expression. **(A)** BMDM from wild-type (WT) and siglec-E-deficient mice were cultured for 3 days with 1 ng/ml LPS to upregulate siglec-E expression and incubated with *S.* Typhimurium M525P for 30 min at a ratio of 1:50 BMDM:bacteria. Bacterial uptake was analyzed by flow cytometry using anti-salmonella antibody (Ab) conjugated with Alexa-488 and siglec-E expression was measured using biotinylated sheep anti-siglec-E Ab followed by streptavidin-APC. **(B)** BMDM as above were incubated with *E. coli*-GFP for 30 min at a ratio of 1:20 BMDM:bacteria. Cells were washed and maintained in antibiotic media to kill extracellular bacteria and the bactericidal activity of BMDM was monitored at the indicated time points by flow cytometry. No differences were seen in bacterial uptake or killing activity. Bar charts in **(A,B)** show mean values + 1 SD from three independent experiments.

and TLRs, but no direct evidence that siglec-E associates with TLR4 *in situ* was provided (1). Experimentally induced *trans*-interactions of siglec-E occur following Ab cross-linking (14), exposure of macrophages to nanoparticles coated with sialic acid ligands (17), or to pathogens displaying a high density of sialic acids on their surface (16), leading to siglec-E-dependent suppression of TLR signaling. In these cases, the *trans*-ligand-induced clustering of siglec-E in the membrane is likely to drive strong ITIM phosphorylation and recruitment of effectors that could modulate TLR signaling through a number of pathways. Likewise, overexpression of siglecs in macrophage-like cell lines (15) could lead to non-physiological clustering and a

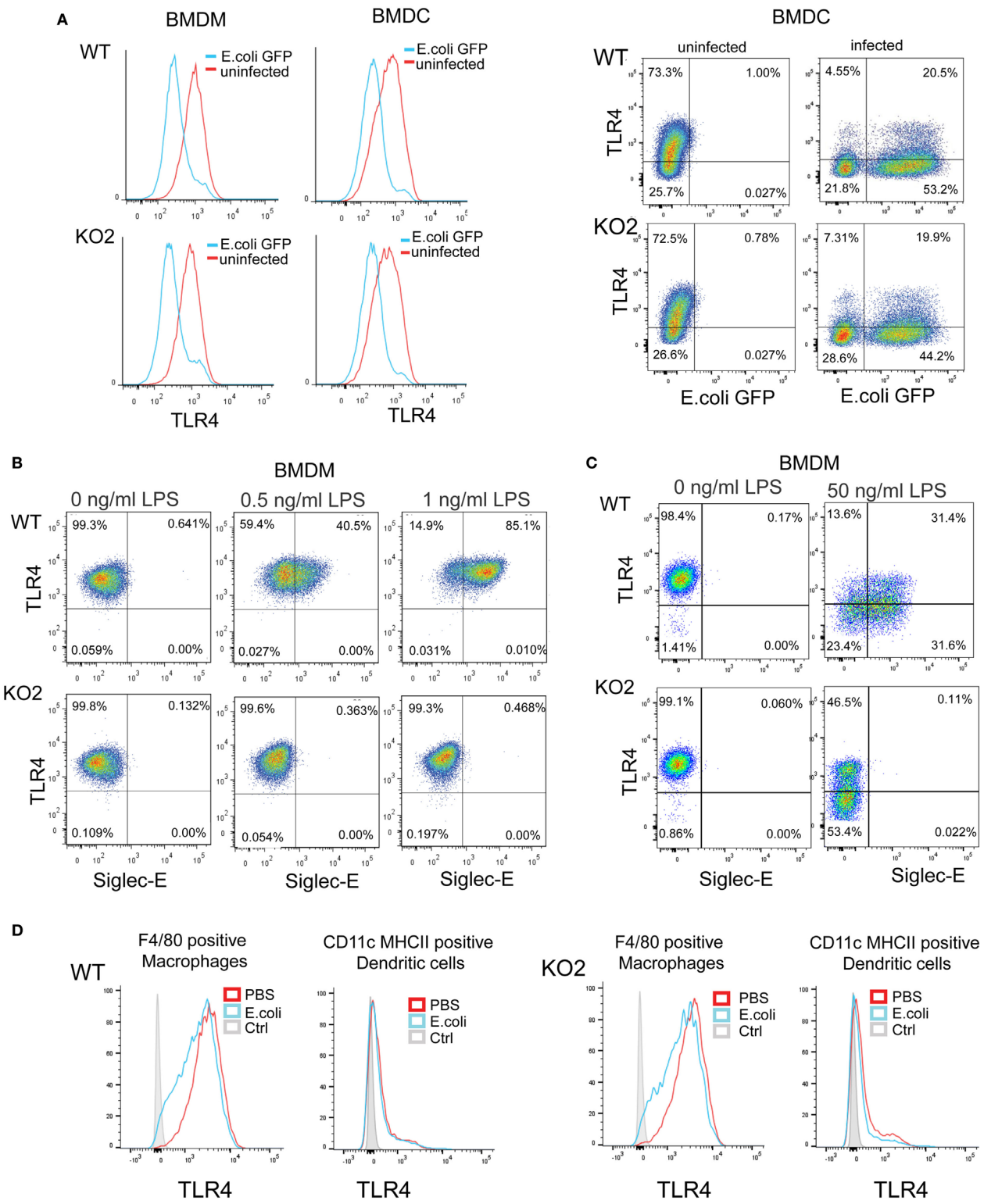

FIGURE 8 | Expression of siglec-E does not influence toll-like receptor 4 (TLR4) endocytosis in bone marrow-derived macrophages (BMDM) and bone marrow-derived dendritic cells (BMDC). **(A)** Wild-type (WT) and siglec-E-deficient BMDM cultured for 3 days in 1 ng/ml lipopolysaccharide (LPS) and BMDC were incubated with *E. coli*-GFP for 1 h a ratio of 1:10 and TLR4 and GFP expression determined by flow cytometry **(B)** WT and siglec-E-deficient BMDM were incubated with the indicated doses of LPS for 3 days and TLR4 expression determined by flow cytometry. **(C)** BMDM were incubated for 1 day without LPS or with 50 ng/ml LPS and TLR-4 and siglec-E levels determined by flow cytometry **(D)** Splenocytes from WT and siglec-E-deficient mice were incubated with *E. coli*-GFP at 1:10 ratio and gated on F4/80 positive cells and CD11c, MHCII double-positive cells and expression of TLR4 determined by flow cytometry. Two independent biological replicates were performed for each genotype.

similar outcome with respect to TLR-dependent signaling and suppression of pro-inflammatory cytokines. Based on the findings reported here, we propose that at physiological levels of siglec expression, stimulation of macrophages with LPS, in the absence of *trans*-ligand-induced siglec-E clustering, does not affect TLR4-dependent pro-inflammatory signaling. These observations are in line with a study showing that a sialic acid deletion mutant of Group B *Streptococcus* triggered similar amounts of TNF-α secretion in WT and siglec-E-deficient macrophages (16) and also with a study using lentiviral-mediated knockdown of siglec-E that did not affect the TLR4-triggered inflammatory response (17). In both cases, siglec-E inhibited LPS- or pathogen-induced inflammatory responses only *via trans*-interactions.

Our results contradict the findings of Chen et al. and Wu et al. who showed that BMDC from siglec-E-deficient mice exhibited strongly exaggerated IL-6 and TNF-α secretion following LPS and *E. coli* stimulation (1, 18). Furthermore, Wu et al. showed that TLR4 endocytosis induced by uptake of *E. coli* in DC populations was defective in cells prepared from siglec-E-deficient mice. This is at odds with findings presented here, where there was no difference in TLR4 endocytosis in both macrophages and DCs. Furthermore, we showed that siglec-E is not expressed on the vast majority of immature and mature BMDC, a key issue that was not addressed in their publications. Even splenic DCs expressed very low levels of siglec-E compared to macrophages and neutrophils, suggesting that siglec-E is not a major regulatory receptor in these cells. It is noteworthy that the siglec-E-deficient

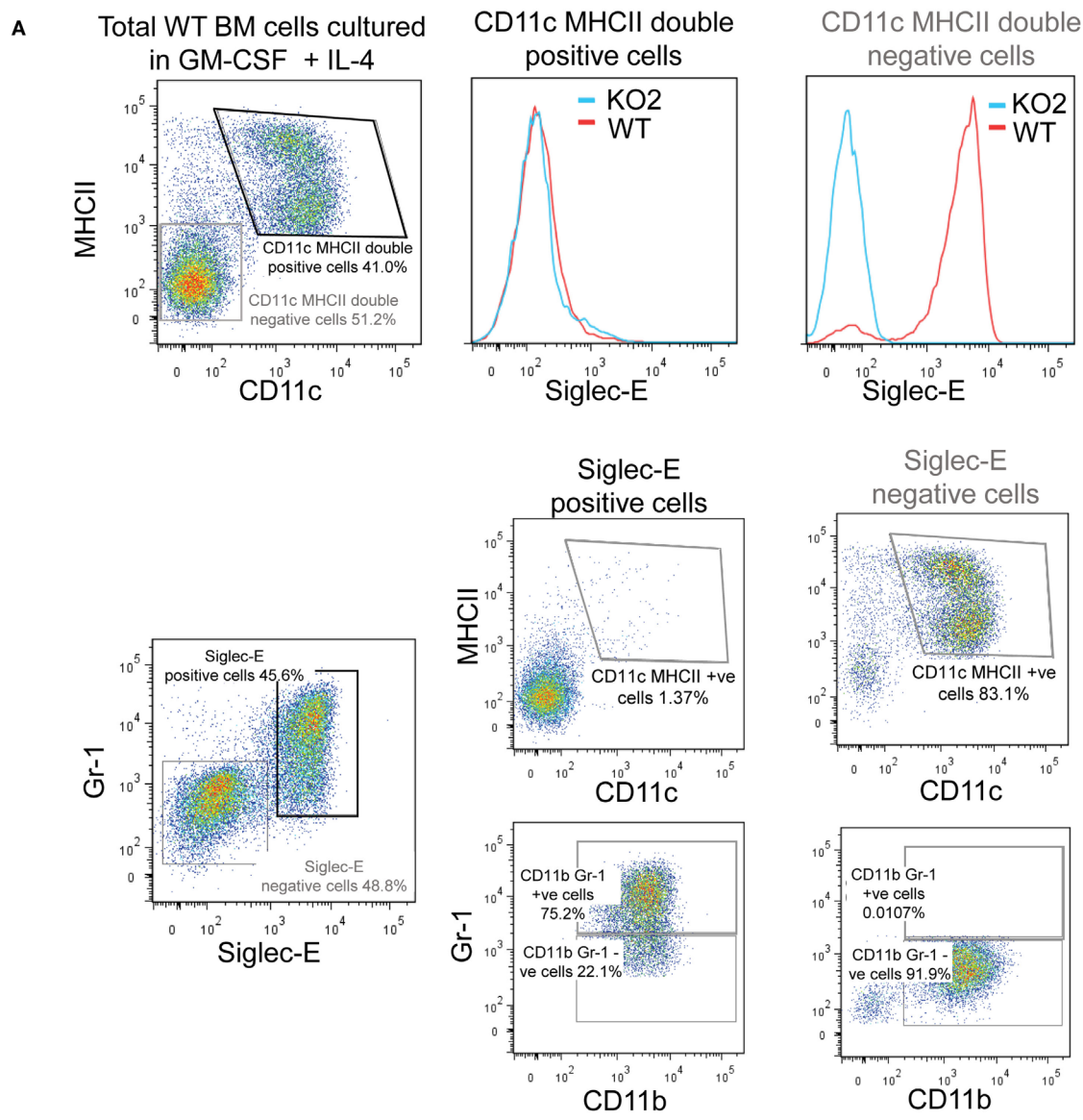

FIGURE 9 | Continued

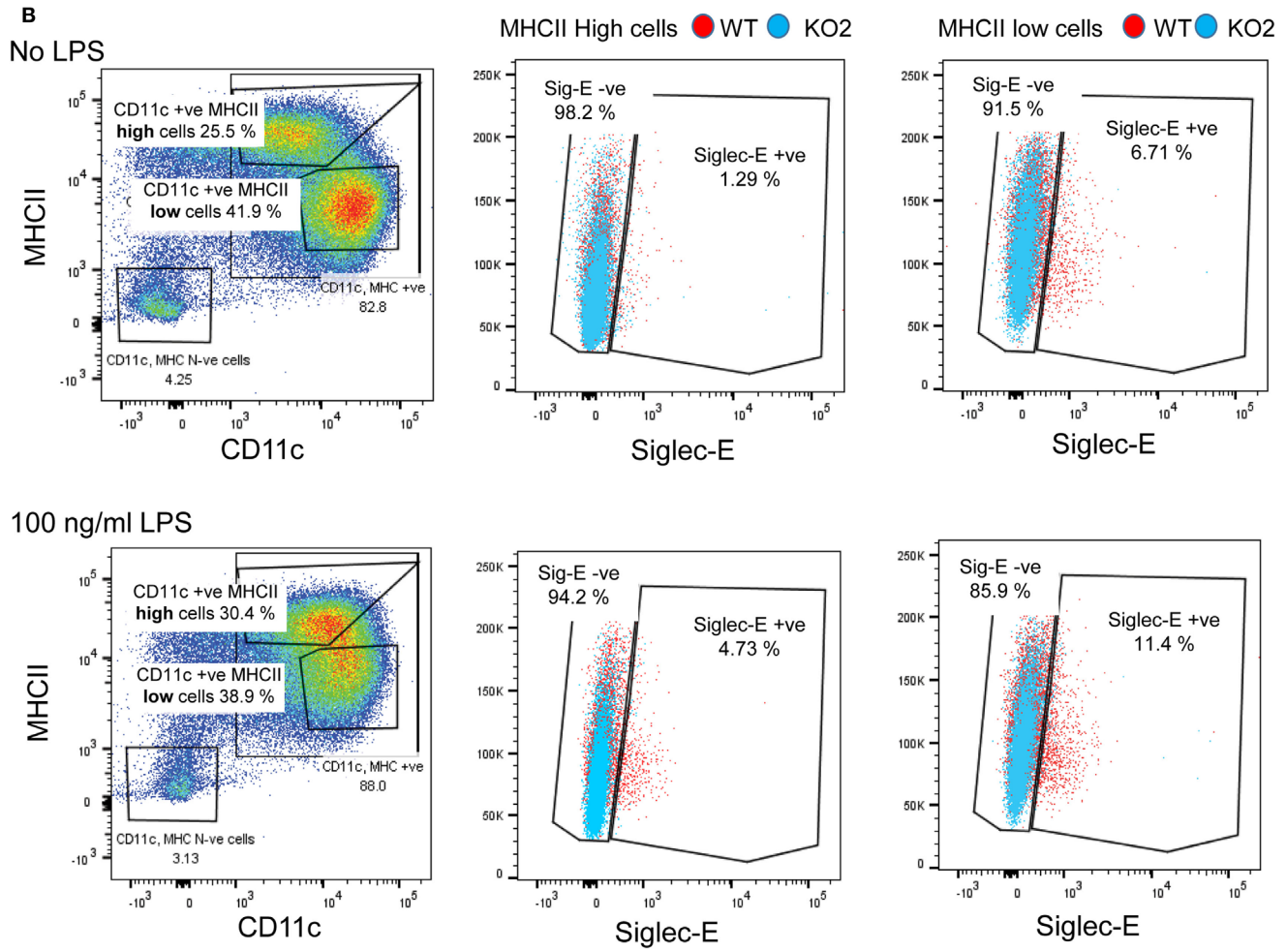

FIGURE 9 | Siglec-E is not expressed by bone marrow-derived dendritic cells (BMDC). **(A)** Wild-type (WT) and siglec-E-deficient bone marrow cells were cultured in 20 ng/ml recombinant mouse GM-CSF and 5 ng/ml IL-4 for 6 days to generate BMDC and analyzed by flow cytometry for siglec-E expression on CD11c, MHCII double-positive cells and Gr-1 positive cells **(B)** BMDC were cultured in 10 ng/ml recombinant mouse GM-CSF and 1 ng/ml IL-4 and matured for 24 h using 100 ng/ml lipopolysaccharide (LPS) or left untreated and analyzed by flow cytometry. CD11c positive BMDCs with high and low levels of MHC expression were gated and siglec-E levels analyzed on the different subsets. Two independent biological replicates were performed for each genotype.

mice used in the studies of Chen et al. and Wu et al. were generated from 129 ES cells and backcrossed for three generations onto a C57BL/6 line resulting in less than 90% of the genome being derived from the C57BL/6 background. However, of more concern is the likely large number of "passenger genes" derived from 129 mice flanking the siglec-E locus that differ in protein sequence between siglec-E-deficient mice and the matched WT mice that could have strongly influenced the results of their studies. It is well established that many phenotypes ascribed to genes of interest are actually due to polymorphic passenger gene effects (40). This could also explain why the 129 ES-derived KO1 mice used in the present study showed reduced IL-6 and IL-10 responses to LPS *in vivo* whereas no differences were seen in C57BL/6 ES-derived R126D mice. Further studies are required to reconcile these differences.

The most interesting outcome of our unbiased quantitative proteomics was the finding that WT and siglec-E-deficient macrophages challenged with high-dose LPS exhibit differences in levels of many proteins associated with membrane function, vesicular transport, and cytoskeleton. Although we did not see altered bacterial uptake or killing *in vitro*, these protein differences might regulate aspects of macrophage function *in situ* that are important for host defense to infection and explain the reduced numbers of *Salmonella* seen here in WT mice compared to siglec-E-deficient mice. An additional attractive hypothesis is that siglec-E contributes to TLR4-induced macrophage differentiation and/or polarization. The heterogeneity of resident macrophage populations in different tissues is well documented, although the physiological relevance of this phenotypic heterogeneity within different tissue microenvironments is not completely understood (41). Several studies have shown that TLR agonists can drive polarization and cellular reprogramming in macrophages, monocytes, hematopoietic stem, and progenitor cells (42, 43). Induction

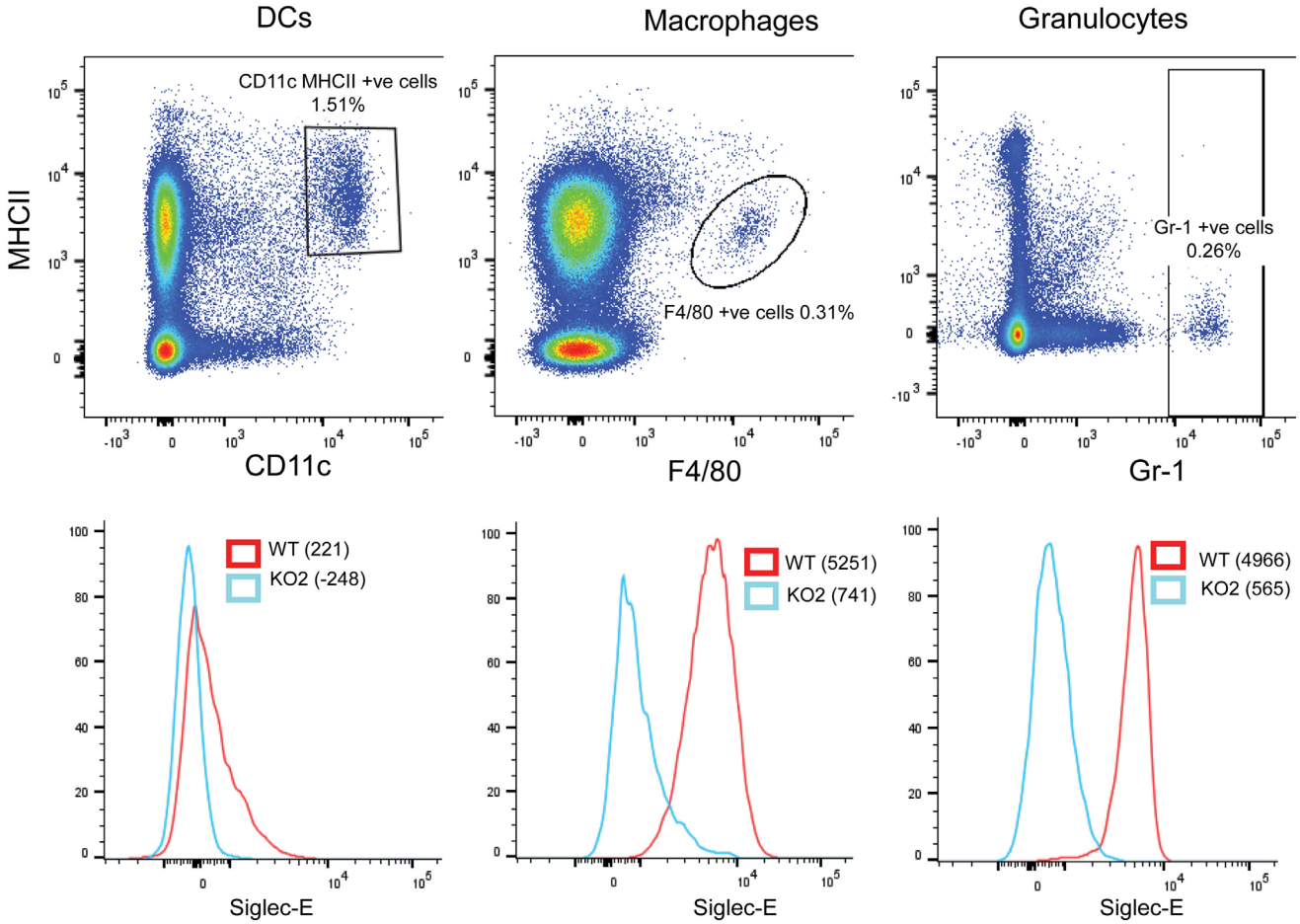

FIGURE 10 | Differential expression of siglec-E on splenic myeloid cell populations. Wild-type (WT) and siglec-E-deficient splenocytes were gated for dendritic cells (DCs) (CD11c, MHCII double-positive cells), macrophages (F480 positive cells), and granulocytes (Gr-1-positive cells) and analyzed for siglec-E expression by flow cytometry. Two independent biological replicates were performed for each genotype.

of siglec-E by TLR4 activation could contribute to this process, as we showed that the induced siglec-E is constitutively tyrosine phosphorylated and associated with protein tyrosine phosphatase SHP-1. Interestingly, SHP-1 has been shown to be one of the key players among several molecular pathways that control macrophage polarization (44). Therefore, siglec-E-mediated activation of SHP-1 in macrophages could target key downstream substrates that modulate macrophage differentiation and TLR reprogramming, leading to a siglec-E-dependent phenotype. Our future studies will attempt to investigate the significance of this putative differentiation pathway for host defense and homeostasis.

ETHICS STATEMENT

Animal experimentation was approved by the University of Dundee Animal Ethics Committee and carried out under UK Home Office Project License PPL60/3856.

AUTHOR CONTRIBUTIONS

MN designed the research, carried out experiments, analyzed results, and prepared the first draft of the manuscript. EM designed the research, carried out experiments, and analyzed results. HR designed the research, carried out experiments, and analyzed results. RS designed the research, carried out experiments, and analyzed results. ST analyzed results, and contributed to intepretation of data. PM designed experiments and interpreted results. PC led the research project, designed the experiments, interpreted the results, and wrote the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/articles/10.3389/fimmu.2017.01926/full#supplementary-material.

FIGURE S1 | Expression of siglec-E does not influence toll-like receptor 4 (TLR4) endocytosis in bone marrow-derived macrophages (BMDM) and bone marrow-derived dendritic cells (BMDC). **(A)** Wild-type (WT) and siglec-E-deficient BMDM were cultured for 3 days in 1 ng/ml lipopolysaccharide (LPS) and incubated with *E. coli*-GFP for 1 h at a ratio of 1:10 and TLR4 and GFP expression determined by flow cytometry. **(B)** BMDM were incubated for 1 day without LPS or with 50 ng/ml LPS and TLR4 levels determined by flow cytometry. **(C,D)** Splenocytes from WT and siglec-E-deficient mice were incubated with *E. coli*-GFP at 1:10 ratio and gated on F4/80 positive cells **(C)** and CD11c, MHCII double-positive cells **(D)** and TLR4 levels determined by flow cytometry. **(E)** Siglec-E is not expressed by BMDC. WT and siglec-E-deficient bone marrow cells were cultured in recombinant mouse GM-CSF and IL-4 for 6 days to generate BMDC and analyzed by flow cytometry for siglec-E expression on CD11c, MHCII double-positive cells. Staining for KO cells represents non-specific binding of the anti-siglec-E mAb. In **(A–E)**, data show means + 1 SD from two biological replicates.

TABLE S1 | Quantitative proteomics data comparing WT and siglec-E-deficient macrophages stimulated with 100 ng/ml LPS. Data show log2 intensity values for 6,791 proteins identified in four independent biological replicates. Proinflammatory factors that are not significantly different comparing WT and siglec-E-deficient macrophages are highlighted in orange and proteins that are altered significantly are in green. Those increased in WT versus siglec-E-deficient macrophages are in dark blue, whereas those decreased in WT versus siglec-E-deficient macrophages are in pale blue.

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REVIEW
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Identification and Biological Activity of Synthetic Macrophage Inducible C-Type Lectin Ligands

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The macrophage inducible C-type lectin (Mincle) is a pattern recognition receptor able to recognize both damage-associated and pathogen-associated molecular patterns, and in this respect, there has been much interest in determining the scope of ligands that bind Mincle and how structural modifications to these ligands influence ensuing immune responses. In this review, we will present Mincle ligands of known chemical structure, with a focus on ligands that have been synthetically prepared, such as trehalose glycolipids, glycerol-based ligands, and 6-acylated glucose and mannose derivatives. The ability of the different classes of ligands to influence the innate, and consequently, the adaptive, immune response will be described, and where appropriate, structure–activity relationships within each class of Mincle ligands will be presented.

Keywords: C-type lectin, Mincle, pathogen-associated molecular pattern, damage-associated molecular pattern, adjuvant, glycolipid

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INTRODUCTION

Macrophage inducible C-type (calcium-dependent) lectin (Mincle, Clec4e, ClecSf9) is a pattern recognition receptor that is involved in the innate immune response to pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs). First identified in 1999 by Matsumoto et al. as a downstream target of the nuclear factor (NF) that binds the interleukin (IL)-1 responsive element in the IL-6 gene (1), there has been much subsequent interest in elucidating the structure of exogenous and endogenous Mincle ligands. Mincle has been shown to sense dead cells through a protein component of small nuclear ribonucleoprotein (SAP130) (2) and to recognize yeast (e.g., *Candida albicans*) (3, 4) and fungi (e.g., *Malassezia*) (5); however, the first defined

Abbreviations: APCs, antigen-presenting cells; AraMM, arabinosyl monomycolate; BMDCs, bone marrow-derived dendritic cells; bMincle, bovine Mincle; BMMs, bone marrow-derived macrophages; CLRs, C-typecalcium-dependent, lectin receptors; CMA, corynomycolic acid; CRAC, cholesterol recognition/interaction amino acid consensus; CRD, carbohydrate recognition domain; DAMPs, damage-associated molecular patterns; DDA, dimethyldioctadecylammonium; ELISA, enzyme-linked immunosorbent assay; GBA1, β-glucosylceramidase; β-GlcCer, β-glucosylceramide; Glc-DAG, glucosyl diacylglycerol; GMB, glucose monobehenate; GMCMs, glucose monocorynomycolates; GMMs, glucose monomycolates; GroMB, glycerol monobehenate; GroMCM, glycerol monocorynomycolate; GroMM, glycerol monomycolate; hMincle, human Mincle; IFA, incomplete Freund's adjuvant; Ig, immunoglobulin; IL, interleukin; MCL, macrophage C-type lectin; Mincle, macrophage inducible C-type lectin; MIP-2, macrophage inflammatory protein 2; mMincle, mouse Mincle; moMφ, monocyte-derived macrophage; NF, nuclear factor; NFAT-GFP, nuclear factor of activated T cells-green fluorescent protein; PAMPs, pathogen-associated molecular patterns; rMincle, rat Mincle; SPR, surface plasmon resonance; TDB, trehalose dibehenate; TDCMs, trehalose dicorynomycolates; TDEs, trehalose diesters; TDM, trehalose dimycolate; TLRs, toll like receptors; TMCMs, trehalose monocorynomycolates; TMEs, trehalose monoesters; TMMs, trehalose monomycolates; TNF, tumor necrosis factor; WT, wild type.

non-proteinaceous Mincle ligand was identified in 2009 by Ishikawa et al. and found to be trehalose dimycolate (TDM) (6), the most abundant glycolipid in the cell wall of *Mycobacterium tuberculosis*. In the same year, Werninghaus et al. determined that C-type lectins, rather than toll like receptors (TLRs), recognize TDM (7), with the same group later demonstrating that Mincle is essential for the recognition and adjuvanticity of TDM and its related C22 linear analog, trehalose dibehenate (TDB) (8).

In humans and rodents, Mincle is expressed on a variety of cell types of the myeloid lineage (e.g., monocytes, macrophages, neutrophils, and dendritic cells) (1, 2, 9, 10) and some subsets of B cells (11), with the binding and subsequent activation of Mincle by ligands leading to the activation of the FcRγ-Syk-Card9-dependent pathway and NF-κB-mediated gene expression (**Figure 1A**) (2, 6–8). This activation results in the expression of many different inflammatory genes, ranging from cytokines (e.g., TNF-α, IL-6, IL-1β, and IL-12), to various chemokines and enzymes generating small molecule mediators like iNOS. Ultimately, these cellular mediators influence the adaptive immune response and T-helper cell differentiation. Moreover, the type of Mincle ligand can affect the ensuing immune response, and this has deemed Mincle agonists to be promising vaccine adjuvants (12–15) in the same way that TLR ligands show much potential in this respect (16, 17).

Insight into the binding site of Mincle ligands has gone some way into understanding how the different classes of Mincle ligand may bind to the receptor. Mincle shares high homology with other carbohydrate binding proteins (lectins), with the crystal structure of human Mincle (hMincle) and bovine Mincle (bMincle) being elucidated in 2013 by Furukawa et al. (18) and Feinberg et al. (19), respectively. The carbohydrate recognition domain (CRD) of Mincle (**Figure 1B**) is comprised of the common EPN motif (residues 169–171), which is often observed in C-type (calcium-dependent) lectin receptors (CLRs) and which has been shown to be indispensable for TDM recognition. Mincle uses this Ca^{2+} ion to bind the equatorial 3- and 4-OH groups of one glucose residue in trehalose (18, 19). An additional secondary binding site lacking Ca^{2+} accommodates the second glucose moiety of the trehalose disaccharide and this extra recognition provides increased binding affinity for trehalose by 36-fold compared to glucose (19). Moreover, Arg183 of hMincle is crucially involved in ligand recognition (18), and is in a suitable position to interact with hydroxyl groups on TDMs.

The regions surrounding the Ca^{2+} site in Mincle, however, are distinct from those in other CLRs. A series of hydrophobic pockets are found in Mincle, but not other CLRs, with the hydrophobic region of hMincle being composed of Val195, Thr196, Phe198, Leu199, Tyr201, and Phe202, which gives rise to an open-side groove to one side of the primary sugar binding site (18). Similarly, in bMincle, a shallow hydrophobic grove was also observed adjacent to the sugar binding site—albeit, to the other side of the EPN motif (19). As there is no crystal structure with bound TDM or related glycolipid, the exact mode of lipid binding is unknown; however, the aforementioned hydrophobic groove is the proposed recognition site for one of the lipids (18, 19). In addition to the major hydrophobic grove, there is also a second minor hydrophobic groove in Mincle, which is thought to be able to accommodate the second lipid in TDM (18, 19). When comparing hMincle and mouse Mincle (mMincle), there are subtle differences between the two species, however, on the whole, the two lectin domains contain highly identical folds and there is 85% sequence homology between the species (1). Notwithstanding, for certain classes of ligand (such as glycerol esters, *vide infra*), some Mincle species-specific activity is observed.

In addition to the carbohydrate-binding domain, Mincle also contains a second binding site, which has been shown to recognize DAMPs (2, 20). Less is known about Mincle binding of DAMPs; however, Yamasaki et al. suggested that SAP130 binds to a site other than the EPN motif (2), while Kiyotake et al. demonstrated that cholesterol crystals bind to hMincle *via* a cholesterol recognition/interaction amino acid consensus (CRAC)-like motif (**Figure 1B**) (20). Thus, it appears that Mincle has several binding domains that can accommodate a variety of PAMPs and DAMPs.

Several groups have used knowledge of the Ca^{2+} CRD of Mincle to better design Mincle ligands, or to understand how known Mincle ligands might be accommodated by the receptor (as described below). That said, it is not clear if Mincle binding alone correlates to a functional immune response. Thus, while knowledge of the Mincle binding site can serve to guide the design of potential Mincle agonists, functional studies whereby a variety of Mincle ligands are screened for their ability to activate the innate immune response are required in order to better identify promising Mincle ligands. Accordingly, this review focuses not only on the different classes of ligands that can bind and activate Mincle but also on the known structure-activity requirements for each class of ligands.

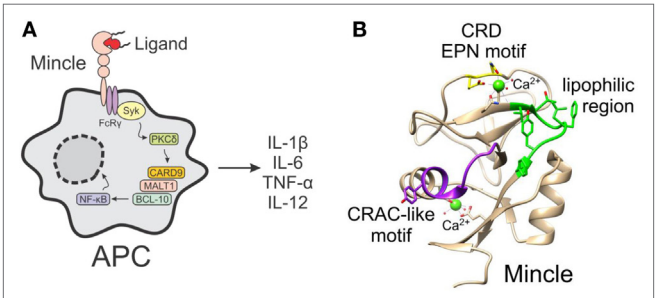

FIGURE 1 | **(A)** The activation of macrophage inducible C-type lectin (Mincle) on antigen-presenting cells by ligands leads to the induction of the FcRγ-Syk-Card9 pathway and NF-κB-mediated gene expression. **(B)** Crystal structure of human Mincle (18), with the carbohydrate recognition domain EPN motif indicated in yellow, the lipophilic region in green, and the cholesterol recognition/interaction amino acid consensus-like motif in purple.

TREHALOSE DIESTERS (TDEs)

Trehalose Dimycolates (TDMs)

Trehalose dimycolates (**1**, **Figure 2**), historically known as cord factor (21, 22), are the most abundant glycolipids in the cells wall of *Mycobacterium* species (23, 24). TDMs consist of

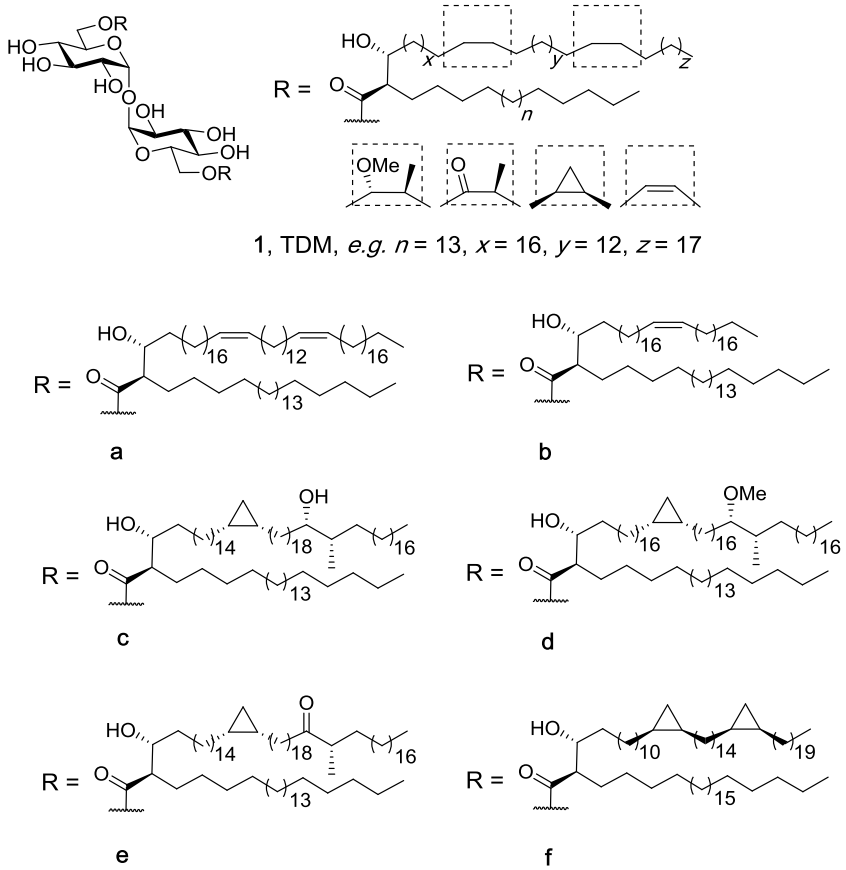

FIGURE 2 | Isolated trehalose dimycolate (TDM) (**1**) from natural sources, such as *Mycobacterium tuberculosis*, consists of a mixture of up to 500 different structures with a variety of side-chain functional groups and different lipid lengths (*n*, *x*, *y*, and *z*). Structures **1a–f** are representative homogenous TDMs synthesized by Baird et al. (27, 28).

an α,α′-trehalose core with the 6- and 6′-position esterified to mycolic acid which contain at least two chiral centers: an alkyl chain at the α-position and a hydroxyl group at the β-position, both with the *R*-configuration (25). The mycolic acid can be of varying length and contains functional groups consisting of either oxygenated mycolates (methoxy-, keto-, and epoxy-mycolates) or non-oxygenated mycolates (*cis*-cyclopropane, alkenes, and dienes) (25). In 1950, Bloch showed that cord factor was capable of activating the immune system (21), and since then, there has been much interest in understanding how the structural features of TDM relate to the immunomodulatory properties of the individual molecules (26, 27). To date, more than 500 closely related structures of TDM have been identified (25).

While the immunostimulatory activity of TDMs has been known for some time, Mincle was only recently identified as a receptor for TDMs. In 2009, Yamasaki et al. (6) used nuclear factor of activated T cells-green fluorescent protein (NFAT-GFP) reporter cells expressing Mincle to determine that Mincle is responsible for the direct recognition of TDM, and the simplified TDM analog TDB. Using Mincle deficient mice, Yamasaki et al. demonstrated that bone marrow-derived macrophages (BMMs) were no longer capable of producing tumor necrosis factor (TNF) and macrophage inflammatory protein 2 (MIP-2) when exposed to TDM and TDB, suggesting that this process is Mincle dependent. Yamasaki et al. also showed that this activity required the EPN motif, as recognition of TDM was eliminated in mutant Mincle$^{EPN\rightarrow QPD}$ NFAT-GFP reporter cells (6).

Simultaneously, Werninghaus et al. used Syk$^{-/-}$, Card9$^{-/-}$, and FcRγ$^{-/-}$ BMMs to determine that both TDM and TDB activate macrophages through an FcRγ-Syk-Card9-dependent pathway (7), with further studies by the Lang group showing that this process is both Mincle and FcRγ dependent (8).

The complexity of TDMs has meant that only a few groups have been able to synthesize and tease apart the immunomodulatory properties of the individual glycolipids. In 1978, Parant et al. (29) isolated mycolic acids ranging from C80 to C90 in length from mycobacteria as well as low-molecular-weight mycolic acids (C28 to C36) from *Corynebacterium diphtheria* and coupled these to trehalose. Immunization of mice with the different TDMs followed by infection with *Klebsiella pneumoniae* or *Lysteria monocytogenes* demonstrated that the low-molecular-weight mycolic acids were found to be equally potent at protecting the mice against infection as their longer-chain counterparts. However, as mixtures of compounds containing a variety of lipid lengths and

functional groups were ultimately tested in each case, detailed structure-activity relationships could not be derived.

Insight into the potential of the individual TDMs to lead to differences in the immune response was first possible due to the elegant synthetic endeavors of Baird et al. (28, 30–38). Initially, Al Dulayymi et al. synthesized a library of mycolic acids (28), whereby a key step in the total syntheses was the use of a Fráter–Seebach alkylation to install the α-branch and the β-hydroxyl group in the correct *anti*-configuration. The mycolic acids were then selectively coupled to the 6- and 6′-positions of α,α′-trehalose to give 16 TDMs (**1a**–**f**), including those containing diene (**1a**), alkene (**1b**), hydroxyl (**1c**), methoxy (**1d**), and keto (**1e**) functional groups in the meromycolate branch (28). In a first series of studies, the individual TDMs, as well as a mixture of isolated TDM and synthetic TDB, were tested for their ability to activate bone marrow-derived dendritic cells (BMDCs), as measured by the production of TNF-α (27). Here, it was shown that the diene TDM (**1a**), which lacks the two cyclopropane groups, had a lower inflammatory potential compared to isolated TDM, but induced similar levels of TNF-α compared to TDB. Moreover, the methoxy-functionalized TDM (**1d**) led to higher levels of TNF-α compared to the hydroxy-functionalized TDM (**1c**) suggesting that increased polarity and the potential for hydrogen bonding in the middle of the meromycolate branch does not increase binding and activation of Mincle. As the *cis*-methoxy TDM (**1d**) was found to induce similar levels of TNF-α compared to that of isolated TDM, this material was chosen as the lead compound.

Subsequent studies with the *cis*-methoxy TDM (**1d**) determined that this homogenous material caused BMDCs to induce similar levels of IL-6, IL-1β, and IL-12P40 to that produced by BMDCs in response to isolated TDM (27). Moreover, *cis*-methoxy TDM (**1d**) and isolated TDM both upregulated the surface expression of costimulatory molecules CD86, CD80, and MHC-II, with the immune response to TDM, TDB, and synthetic TDM (**1d**) being Mincle, FcRy, and Malt1 dependent. Synthetic TDM (**1d**) was also found to activate the NLRP3 inflammasome in a manner similar to isolated TDM, and *in vivo* studies, where mice were injected in the footpad with TDM (**1d**), isolated TDM or TDB as an emulsion [30% incomplete freunds adjuvant (IFA), glycolipid (10 μg/mouse), ovalbumin (50 μg/mouse)], showed that all three trehalose glycolipids caused similar footpad swelling, infiltration of granulocytes ($Ly6C^+$ and $Ly6G^+$), and cytokine and chemokine production (IL-1β, IL-12p35, TNF-α, and CXCL1).

Trehalose Dicorynomycolates (TDCMs)

Like TDM, TDCMs (e.g., **2**, **Figure 3**) consist of a trehalose core esterified at the 6- and 6′-positions, however instead of the more complex mycolic acids, TDCMs are esterified with corynomycolic acids, which consist of a shorter α-branch and a hydroxyl at the β-position, with both substituents in the *R* configuration (39). TDCMs were first isolated from *Corynebacteria diptheriae* in 1963 and were found to have carbon lengths ranging from C24 to C32 (40). Since this time, studies have demonstrated that TDCMs activate macrophages and cause tumor regression (41–43) and that TDCMs are less toxic than TDM (44). As a result, TDCM has been effectively used as an adjuvant in the Ribi Adjuvant system (45, 46). To date, however, only one study involving the use of a

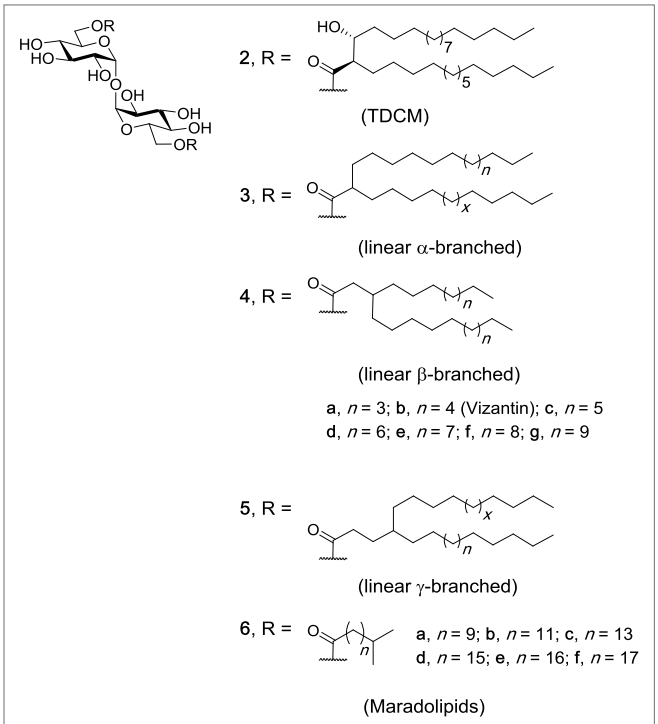

FIGURE 3 | Other branched trehalose diesters (TDEs). trehalose dicorynomycolate (TDCM) (**2**), first isolated from *Corynebacterium* species, occurs naturally as a mixture of chain lengths. Various TDCMs and dehydroxy derivatives have been synthesized, as have the maradolipids, which have been first isolated from the dauer larva stage of *Caenorhabditis elegans*. Of these branched TDEs, TDCM (**1**) and maradolipids (**6a–f**) have been confirmed to be macrophage inducible C-type lectin agonists (42, 43, 47, 48).

NFAT-GFP reporter assay has demonstrated that a representative TDCM (**2**) bound and activated hMincle and mMincle (47). Thus, while Nishizawa et al. synthesized a series of individual TDCMs and found that the immunoadjuvant activity of the TDCMs is associated with the (2*S*, 3*S*) and the (2*R*, 3*R*) diastereomers, and not the (2*R*, 3*S*) or (2*S*, 3*R*) diastereomers (42), and that the β-methoxy TDCM had similar activity to the β-hydroxy TDCM in a phagocytic assay (43), further studies are required in order to determine whether the immunomodulatory response to the aforementioned TDCMs is Mincle dependent.

Other Branched TDEs

In addition to the α-branched TDMs **1** and TDCMs **2**, a number of α- (**3**), β- (**4**), γ- (**5**), and iso-branched (**6**) TDEs have been synthesized over the years and their immunomodulatory properties explored (**Figure 3**). Examples include a series of α-, β-, and γ-branched TDEs (**3**, **4**, and **5**), including Vizantin (**4b**), as first prepared by Yamamotoet al. (43), with Vizantin and functionalized probes (e.g., fluorescent, bead-conjugated) later being synthesized by Oda et al. (49). However, to date, none of these ligands have been explored for their ability to bind and activate Mincle. Vizantin, which contains two linear alkyl chains at the β-position of the ester, is considered to be an analog of TDM and TDCM (43), and therefore might be expected to bind and activate

Mincle. Notwithstanding, Vizantin and its analogs have been found to bind to TLR-4. A simplified Vizantin monoester (*vide infra*) has been found to show weak activation of Mincle (50), further suggesting that Vizantin itself might be able to activate Mincle; however, this remains to be determined.

Iso-branched TDEs, also known as maradolipids, were first isolated in 2010 from the dauer larva stage of *Caenorhabditis elegans* (51) and synthesized shortly thereafter (52–54). Our group recently determined that maradolipids (**6a–f**, **Figure 3**) are Mincle-ligands (48). On the whole, the iso-branched maradolipids led to greater cytokine production (IL-6, IL-1β, IL-12, IL-12, MIP-2 by BMMs), and a faster immune response when compared to their linear counterparts. This is quite remarkable given the subtle structural difference between the linear and iso-branched TDEs. Moreover, only the iso-branched TDEs with a carbon chain of >C12 lead to a strong inflammatory response. This observation is in line with studies using the non-branched linear TDEs (*vide infra*).

Linear TDEs

Straight Chain TDEs

Trehalose dibehenate (TDB, **7h**, **Figure 4**), which contains behenic acid (C22) esterified to α,α′-trehalose, is one of the most simple and most studied Mincle ligands. As mentioned above, TDB was first identified as a Mincle ligand by Ishikawa et al. in 2009 (6), with contemporary studies by the Lang group illustrating that TDB engages the TLR-independent Syk/Card9-dependent pathway (7, 8). Although the receptor for TDB was only recently determined, there has been longstanding interest in this glycolipid with its first synthesis being described in 1978 (29), and with applications that include anti-tumor (55) and anti-bacterial activities (29, 56). Prior to the identification of TDB as a Mincle agonist, TDB was also incorporated into dimethyldioctadecylammonium (DDA) liposomes to generate the CAF01 adjuvant system which has been shown to improve T cell responses (57) and to induce protective immune responses in various disease models including influenza (58), tuberculosis, chlamydia, and malaria (59).

To explore the influence of lipid length on macrophage activation, our group synthesized a variety of linear TDEs (C4-C26, **7a–c,f–i**) and analyzed the ability of the diesters to activate BMMs, as determined by measuring NO production and the secretion of cytokines IL-6 and IL-1β (60). From this work, it was determined that a lipid length of >C12 was required to activate macrophages, with the greatest amount of IL-6, IL-1β, and NO being produced by the C22 (**7h**) and C26 (**7i**) TDEs.

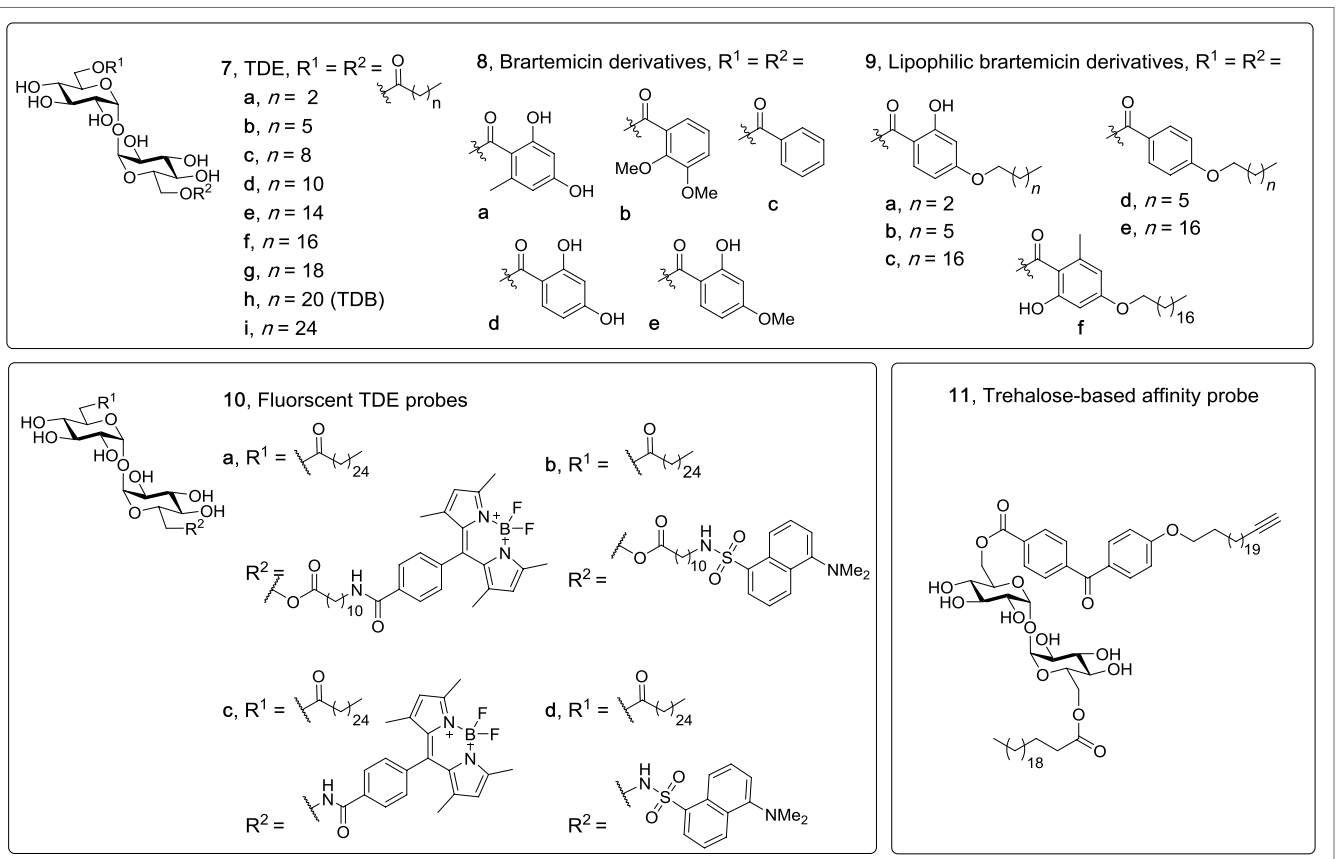

FIGURE 4 | Non-branched trehalose diesters (TDEs), including linear TDEs (60), brartemicin derivatives (61, 62), fluorescent TDE probes (63), and a trehalose based affinity probe (64). Of these derivatives, only the linear TDEs (**7d–i**), the lipophilic brartemicin derivatives (**9a–f**), and the fluorescent TDE probes (**10a–d**) have been shown to be macrophage inducible C-type lectin agonists.

In subsequent studies, we confirmed that this immune response was Mincle-dependent, and also demonstrated that the C12-TDE (**7d**) elicited the production of MIP-2 by BMMs, but not other pro-inflammatory cytokines (IL-1β, IL-6, IL-12), thereby providing the first example of a TDE with a chain length of ≤ C12 leading to a Mincle-dependent immune response and one that is less inflammatory in nature (48). In related studies, Kallerup et al. demonstrated that the incorporation of the C16 (**7e**) and C18 (**7f**) TDEs into DDA liposomes led to a similar *in vivo* immunological response to that elicited by TDB (**7h**)/DDA liposomes (65). Thus, it appears as if the subtleties of the *in vitro* responses to TDEs may have less of an effect *in vivo* and/or that the presentation of the glycolipids can alter their immunomodulatory properties, with the immunomodulatory DDA potentially having a greater influence on the overall immune response. Notwithstanding, further *in vitro* and *in vivo* studies using other Mincle agonists are required to test these ideas.

Brartemicin Analogs

The natural product brartemicin is a TDE with two substituted benzoates esterified to the 6- and 6′-positions (**8a**, **Figure 4**). Brartemicin (**8a**) was recently isolated from the culture broth of an actinomycete of the genus *Nonomuraea* and the natural product, along with synthetic analogs (66), were found to have an inhibitory effect on cancer cell invasion (67). Given the structural resemblance of brartemicin to other trehalose-based Mincle ligands, there has been much interest in determining whether brartemicin and/or analogs can bind and activate Mincle. In 2015, Jacobsen et al. determined that brartemicin is a potential ligand for Mincle by performing a series of competitive binding studies (61). Here, it was revealed that brartemicin (**8a**) and its 2,3-dimethoxy analog (**8b**) bound Mincle with a high affinity of $K_i = 5.5 \pm 0.9$ and 5.4 ± 0.3 μM, respectively. Removal of substituents on the benzene ring (**8c**) resulted in a relatively lower affinity of 11.3 ± 0.9 μM. In these studies, it was hypothesized that the aromatic interactions between the brartemicin derivatives and Phe197 and Phe198 in Mincle accounted for the improved binding affinity of brartemicin (**8a**). Furthermore, *epi*-(α,β′)-brartemicin was synthesized and tested but its ability to bind Mincle was completely abolished. Molecular docking of brartemicin in bMincle using the Glide algorithms further suggested that one of aromatic esters of brartemicin interacted with the hydrophobic grove (Leu172, Val173, Val194, Phe197, Phe198) adjacent to the Ca^{2+}-ion, while the second aromatic ester was orientated in the opposite direction with potential π-cation interactions with Arg182. In 2016, Feinberg et al. obtained a crystal structure of brartemicin and its analog binding to the extended CRD of bovine Mincle (68). Notwithstanding, no functional assays with the aforementioned brartemicin analogs were undertaken in either of these studies.

In more recent studies by our group, we synthesized a variety lipophilic brartemicin analogs (e.g., **9a–f**), including the natural product itself (**8a**) and other non-lipidated derivatives (**8c**,**d**), with the idea that a lipophilic tail would enhance Mincle binding (62). Surprisingly, we demonstrated that brartemicin (**8a**) did not bind either human or murine Mincle, as determined *via* an enzyme-linked immunosorbent assay (ELISA) using soluble Mincle-Ig fusion-proteins, and moreover, that **8a** did not activate NFAT-GFP reporter cells expressing mMincle or hMincle, or cause BMMs to produce IL-6, IL-1β, TNF-α, or MIP-2. In contrast, the medium chain length (C7) C7-brartemicin analogs (**9b,d**) showed better binding to hMincle and mMincle when compared to the longer chain length (C18) analogs (**9c**,**e**,**f**), however, the C18 brartemicin derivatives led to a stronger functional immune response (as determined by activation of the mMincle and hMincle NFAT-GFP reporter cell lines and cytokine production by BMMs), with activity being abolished when using Mincle$^{-/-}$ cells. This observation was intriguing and suggests that Mincle binding does not necessarily correlate to a functional immune response and that longer chain lipids are required for a robust immune response to trehalose-based Mincle ligands. Building on the computational studies of Jacobsen et al. (61), we then used site-directed mutagenesis to determine that Arg183 (in hMincle) was essential for the adjuvanticity of our lead brartemicin analog C18dMeBrar (**9c**). We subsequently explored the potential adjuvanticity of C18dMeBrar (**9c**), including *in vivo* immunization studies using the model antigen ovalbumin, and demonstrated that **9c** leads to excellent Th1 adjuvant activity better than that of TDB (**7h**).

TDE Probes

Functionalized TDE molecular probes have also been found to bind and activate Mincle, thereby demonstrating the capacity of Mincle to accommodate larger functional groups. In the first of such studies, we demonstrated that BODIPY- and dansyl-functionalized TDEs, **10a,c** and **10b,d** (**Figure 4**), respectively, activated macrophages in a Mincle-dependent manner (63). In earlier studies, we synthesized an affinity-based benzophenone and alkyne functionalized trehalose probe (**11**) as a tool to study TDB/protein interactions, and determined that probe **11** was able to activate BMMs (as measured by NO production), albeit to a lesser extent than TDB (64). While the activity of probe **11** was not assessed using Mincle$^{-/-}$ BMMs, it would seem likely that **11** bound Mincle and that the reduced activity of **11** compared to TDB might be due to the presence of the bulky benzophenone group near Mincle's EPN motif. This suggests that while the Mincle binding site can accommodate a variety of ligands, the positioning or type of electrophilic trap (in this case the benzophenone group) might need optimization in order to generate a more active affinity-based trehalose glycolipid probe.

TREHALOSE MONOESTERS (TMEs)

Linear TMEs, Trehalose Monomycolates (TMMs), and Trehalose Monocorynomycolates (TMCMs)

Several groups have investigated whether two lipophilic moieties attached to trehalose are required for effective Mincle binding and activation. An attraction of the use of monoesters of trehalose, as compared to their diester counterparts, is that monoesters can be comparatively easy to synthesize and may also have improved water solubility. Here, it is proposed that one lipid on the trehalose sugar binds to the major lipophilic groove in Mincle.

In seminal work, Furukawa et al. performed surface plasmon resonance (SPR) binding assays using a set of TMEs with a single acyl chain with different lengths [C8 (**12a**), C10 (**12b**), C12 (**12c**)] (**Figure 5**) and demonstrated that a minimum lipid length of C10 was required for lipid binding (18). No functional immune response to the TMEs, however, was determined. In the first study to demonstrate that TMEs could activate Mincle, our group synthesized the C22 (**12h**) and C26 (**12i**) TMEs and demonstrated that solubilized C22 and C26 TMEs led to the production of NO and IL-6 by BMMs in a Mincle-dependent manner (69). In our studies, NO and IL-6 production by BMMs in response to the C22 and C26 TMEs was comparable to that of the C22 TDB, thereby suggesting that only one lipid is sufficient to lead to robust Mincle binding and activation. Since these studies, naturally occurring TMEs have been identified in the lipid extracts of *C. elegans* larvae at the dauer and L3 stages, albeit to a lesser extent than TDEs (70).

In 2016, Huber et al. reported that TDEs were more potent than their corresponding TMEs when assessing the activity of the compounds using murine BMMs in plate-coating assays (71). Using a variety of TMEs containing different lipid lengths [C14 (**12d**), C16 (**12e**), C18 (**12f**), C20 (**12g**), C22 (**12h**)], the authors observed a significant decrease in both G-CSF and NO production when comparing TMEs to their corresponding TDEs. Notwithstanding, the differences between Lang's studies and ours could be explained by the subtleties in the types of assays used. Indeed, during the assessment of related TMEs (e.g., TMMs, TMCMs, *vide infra*), no significant difference between the monoesters and diesters was observed.

Several more elaborate TMEs, the TMMs, and the TMCMs, have also been tested for their ability to bind and activate Mincle. TMMs have been isolated from the wax D fraction of virulent human *M. tuberculosis* as mixtures of compounds (25); however, to better understand how functionalization along the mycolic acid group influences the immunomodulatory property of the compounds, 13 TMMs were synthesized, with 11 subsequently being tested for their immunomodulatory properties, including **13a**–**13e** (**Figure 5**) (27). All synthesized TMMs led to cytokine production by BMDCs in a plate-bound assay, with a dose–response analysis being performed with nine pairs of TMMs and TDMs composed of the same mycolate moiety. Here, the number of mycolate chains bound to trehalose influenced TNF-α and IL-6 production by BMDCs *in vitro,* with a lower cytokine response being observed following stimulation with the TMMs. This trend, however, was not seen in *in vivo* with representative TMM **13d** exhibiting similar immunostimulating potential as both TDM and TDB in an OVA immunization model. The immune response to TMM **13d** was determined to be Mincle dependent. When van der Peet et al. tested their synthesized TMCM (**14a**) using NFAT-GFP reporter cells expressing hMincle and mMincle, TMCM was found to be just as active as its diester counterpart (47). Taken together, these results suggest that a second lipid on the trehalose moiety might not be necessary to make an effective adjuvant and that subtle differences between the different immunomodulatory compounds might be observed when different immunological assays are used.

MONOSACCHARIDE ESTERS

Glucose Monoesters

Glucose monomycolate (GMM, **15a**, **Figure 6**) is an antigenic glycolipid that has been isolated from different species of

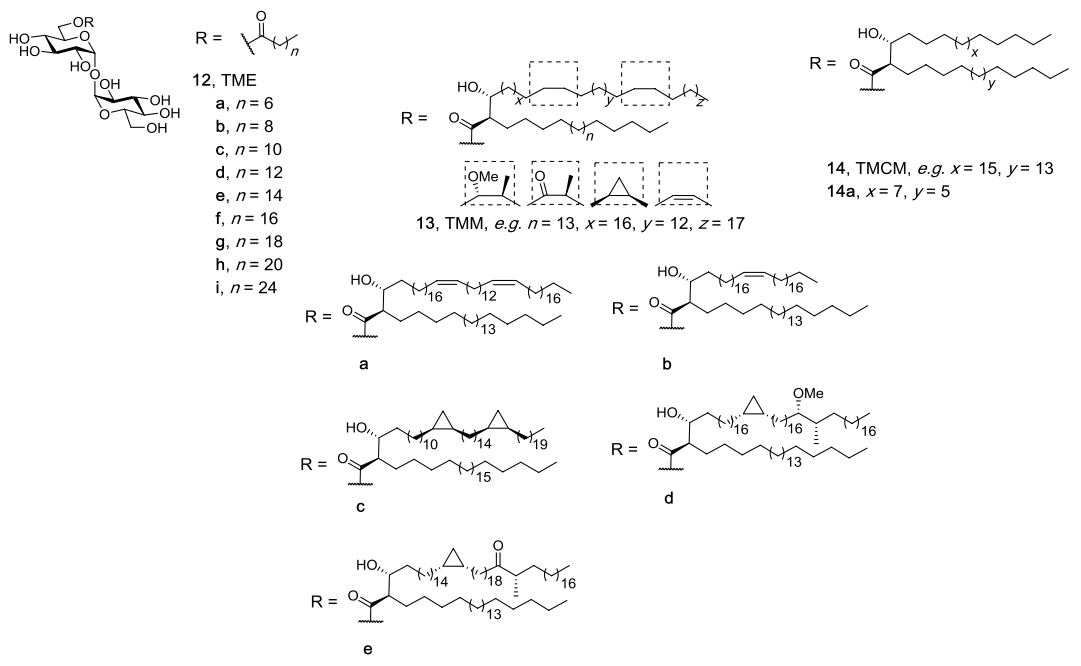

FIGURE 5 | Structures of the trehalose monoesters (69, 71), trehalose monomycolates (27, 28), and trehalose monocorynomycolates (47), of which representative family members (e.g., **12h**, **12i**, **13d**, and **14a**) have been determined to be macrophage inducible C-type lectin agonists.

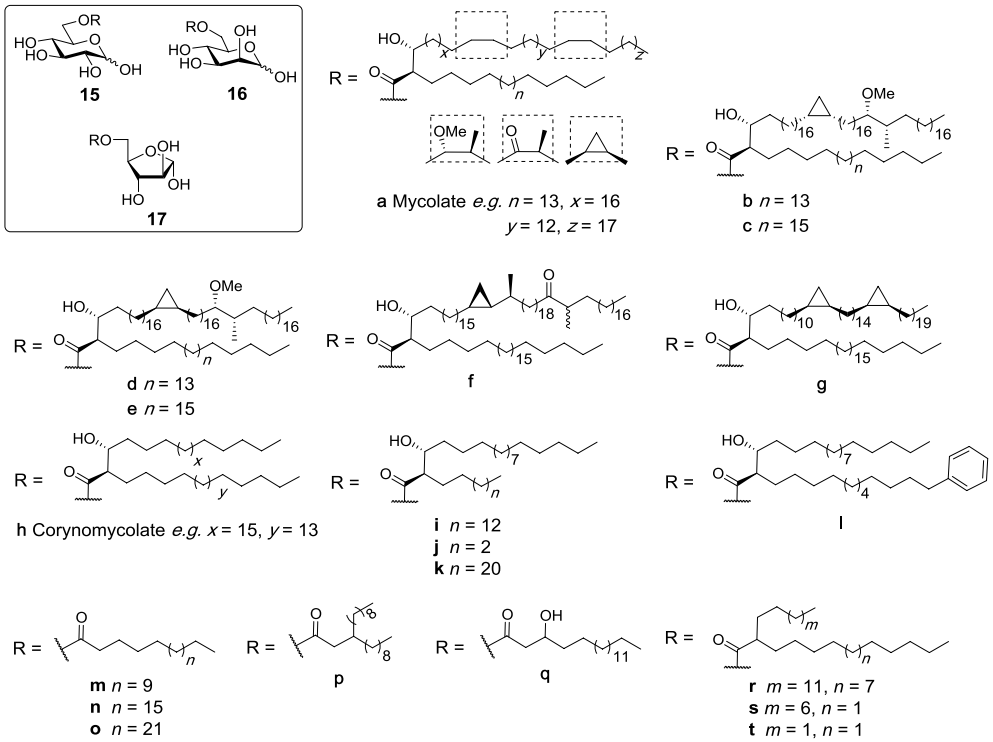

FIGURE 6 | Glucose monoesters have been isolated from different species of *Mycobacterium* and *Corynebacterium*, including glucose monomycolates (**15a–g**) and glucose monocorynomycolates (**15h–l**). These, and related monosaccharide monoesters (**15m–r**) have been examined for their ability to activate macrophage inducible C-type lectin (Mincle), with specific glucose monoesters (e.g., **15a**,**b**,**i–l**,**n**,**p–t**, **16a**,**h**,**r**, and **17b**) being selected and found to directly bind and activate Mincle (27, 47, 50, 75–77).

corynebacteria and mycobacteria including *M. tuberculosis* (72, 73), and was initially found to be presented to antigen-specific T cells by CD1b in humans (74). GMM can be generated by trehalase treatment of TDM, and GMM generated in this manner was initially not found to be a Mincle ligand (6). In contrast, more recent studies concluded that GMM is indeed a Mincle ligand (27, 47), whereby the lack of Mincle dependency in the first set of Mincle assays was attributed to the presence of esterases in the enzyme mixture, which may have cleaved the lipids (47).

Several syntheses of GMMs have been reported, with the first partially synthetic GMM being prepared by Prandi in 2012 in good overall yield (26%) *via* the esterification of benzyl 2,3,4-tri-*O*-benzyl-6-*O*-tosyl-β-D-glucopyranoside with a mixture of mycolic acids purified from *M. tuberculosis* (78). A series of distinct and enantiopure mycolic acids have also been used to prepare several homogeneous GMMs (**15b–h**, **Figure 6**) (75). Subsequent biological evaluation revealed that GMMs with a *cis*-methoxy mycolic acid (**15b**) induced production of TNF-α, IL-6, IL-12p40, and IL-1β from BMDCs in a dose-dependent manner, albeit with lower potency than TDB (27). Cytokine production was found to be Mincle dependent as activity was completely abolished in Mincle$^{-/-}$, FcRγ$^{-/-}$, and Malt1$^{-/-}$ mice. These observations were further confirmed using HEK-Mincle reporter cells, where GMM **15b** showed comparable activity to TDB. GMMs containing different mycolates (**15d–g**) were also able to stimulate BMMs to produce TNF-α at levels similar to that achieved *via* the stimulation of BMMs with TDB, however, no significant difference was observed between the different classes of mycolic acids. Further studies by the same authors revealed that synthetic GMM **15b** activated the NLRP3 inflammasome through the same mechanism as TDB, with the *in vivo* adjuvanticity of GMM (as determined by an OVA immunization model) demonstrating that GMM exhibited lower immunostimulatory potential than both TDM and TDB. Notwithstanding, the ability of GMM to activate both the innate and adaptive immune systems makes it an interesting adjuvant for further vaccination studies.

Glucose monocorynomycolate (GMCM, **15h**, **Figure 6**) is a simplified analog of TDCM previously isolated from *Corynebacterium glutamicum* when glucose was used as the carbon source (79). Recently, van der Peet et al. described the first synthesis of (+)-corynomycolic acid (CMA) through a previously reported boron mediated aldol reaction which allows for the generation of *anti*-configured lipids (47). The synthetic CMA was then coupled to benzyl 2,3,4-tri-*O*-benzyl-6-*O*-tosyl-β-D-glucopyranoside and deprotected to generate enantiopure GMCM **15i** in excellent yield. The synthetic GMCM was then compared to TDM, TDCM, TMCM, and glucose monobehenate (GMB, **15n**, *vide infra*) for Mincle activation using the NFAT-GFP reporter cell assay and was found to activate both human and mouse Mincle, albeit with lower potency than TDM, TDCM, and TMCM. Although GMCM (**15i**) was able to activate the reporter cell lines, GMB (**15n**) was inactive in this assay, highlighting the requirement of substituents

in the α- and β-positions of the lipid chain in glucose monoesters for strong Mincle activation. Direct binding of the active ligands to Mincle was confirmed using the human and mouse Mincle Ig fusion protein assay. The same group later synthesized derivatives of CMA in order to investigate the effects of changes in the lipid portion on Mincle activity (50). The synthetic GMCM analogs contained a shorter α-pentyl chain (**15j**), a longer α-tricosyl chain (**15k**), or a more complex α-phenyldodecyl chain (**15l**). A truncated version of Vizantin, which consists of glucose esterified to a β-branched achiral lipid (**15p**), was also synthesized. All four analogs were assessed for their Mincle activity and compared to TDM, TDB, GMCM and GMB using the NFAT-GFP reporter cell assay. Here, TDM, TDB, and GMCM (**15i**) signaled through both mouse and human Mincle with similar potencies, and surprisingly, GMB now showed weak signaling, albeit only at the highest concentration tested (1 nmol). The shorter α-branched chain GMCM analog **15j** and the simplified Vizantin analog **15p** also only showed notable Mincle activity at the highest concentration tested. Notwithstanding, the longer α-branched GMCM analog **15k** showed dose-dependent Mincle activity similar to GMCM **15i**, emphasizing the requirement of a longer α-branched chain for potent Mincle activity. The modified lipid analog **15l** also showed similar activity to GMCM **15i**.

Decout et al. recently synthesized simplified glucose C16 (**15m**), C22 (GMB, **15n**), and C28 (**15o**) monoesters (**Figure 6**) and compared their ability to bind and activate Mincle relative to synthetic TDM, TDB, and glucose monoesters with 3-hydroxyoctadecanoic acid (Glc3OHC18, **15q**) or 2-tetradecyloctadecanoic acid (GlcC14C18, **15r**) (77). HEK-Mincle reporter cells were used for assessing Mincle activity, where HEK cells expressing either human or mouse Mincle were coupled to an NF-κB–inducible reporter system using alkaline phosphatase. In comparison to the NFAT-GFP assay where GMB showed minimal activity (50), GMB showed no Mincle activity with HEK-Mincle cells and this lack of activity was also observed for the C16 and C28 monoesters. GlcC14C18 (**15r**), however, showed potent activity for both human and mouse Mincle similar to that seen for TDM, while Glc3OHC18 (**15q**) was unable to activate Mincle (77). Glc3OHC18 (**15q**) was also unable to bind a soluble form of hMincle, whereas GlcC14C18 (**15r**) showed direct binding, confirming the observed activity. GlcC14C18 (**15r**) was also able to induce the Mincle dependent production of pro-inflammatory cytokines TNF and IL-6 effectively by mouse BMMs and BMDCs and human monocyte-derived macrophages. The adjuvant properties of **15r** were then investigated *in vivo*, whereby incorporation of **15r** into DDA liposomes resulted in a significant increase in IL-2, IFN-γ, and IL-17 compared to TDB, whereby the latter did not cause a significant increase in cytokine levels. Moreover, **15r** was found to be less toxic than TDB, and also induced protective immunity in a mouse model of *M. tuberculosis* infection. Altogether, these results suggest that GlcC14C18 (**15r**) represents a potential new class of simple α-branched glycolipid Mincle ligands with promising adjuvant properties.

To understand more about the ability of the glucose monoesters to bind and activate Mincle, Decout et al. preformed a series of molecular dynamic studies with Glc3OHC18 (**15q**) and GMCM (**15h**) (77). Using the crystal structure of bovine Mincle, the authors demonstrated that the α-alkyl chain of **15h** bound to a short hydrophobic groove, which resulted in widening of the main hydrophobic groove. This new conformation better accommodated the main alkyl chain inside the cleft. In contrast, Glc3OHC18 (**15q**) lacked this interaction, thereby causing the lipid chain to sit on the surface instead of inside the hydrophobic groove. These studies suggested that the presence of a lipid containing an α-branch of at least four carbons at the 6-position of glucose is a requirement for Mincle signaling. To confirm this finding, synthetic GlcC9C12 (**15s**) and GlcC4C12 (**15t**) were evaluated for their ability to bind mMincle and hMincle. Both of the shorter analogs **15s** and **15t** were Mincle agonists, with EC_{50} values comparable to GlcC14C18 (**15r**) for mMincle and lower activity in hMincle. Next, mutagenesis studies were undertaken where the Mincle A174 residue that is involved in lipid recognition was changed to phenylalanine. This weakly improved the binding of **15r** and TDB, however, when the residue was changed to proline, the binding was decreased for **15r**. Taken together, these results suggest that the introduction of an aromatic side chain at position 174 in Mincle can slightly improve binding of the lipid in the main hydrophobic groove, without significantly interfering with binding of the 2-alkyl chain in the short hydrophobic groove.

Mannose Monoesters

As C-type lectins, such as Mincle, typically bind mannose and glucose residues (2), it has been proposed that Mincle may be able to bind mannose-derived glycolipids. To this end, Decout et al. synthesized mannose monomycolate (ManMM, **16a**, **Figure 6**), mannose monocorynomycolate (ManMCM, **16h**) and mannose 2-tetradecyloctadecanoate (ManC14C18, **16r**) (**Figure 6**) and assessed these derivatives for their ability to activate Mincle using HEK-Mincle reporter cells (77). All three compounds bound soluble hMincle and activated both mMincle and hMincle reporter cells, albeit with lower potency than their glucose counterparts (described above). Similar to GlcC14C18 (**15r**), ManC14C18 (**16r**) was also able to induce Mincle dependent pro-inflammatory cytokine production from BMMs and BMDCs and human monocyte-derived macrophages (moMφs). The incorporation of ManC14C18 (**16r**) into DDA liposomes also resulted in the production of IL-2, IFN-γ, and IL-17 *in vivo*; however, the result was deemed statistically insignificant and so no further testing was carried out with this ligand.

Arabinose Monoesters

Mohammed et al. reported the synthesis of six arabinose monomycolates from single synthetic mycolic acids (76), with several of these substrates being subsequently tested for their ability to induce cytokine production by BMDCs (27). Here, AraMM with a *cis*-methoxy mycolic acid (**17b**, **Figure 6**) induced the production of pro-inflammatory cytokines TNF-α, IL-6, IL-12p40, and IL-1β; however, cytokine production was lower for **17b** than that induced by TDB and *cis*-methoxy GMM **15b**. AraMMs containing different mycolic acids (**17c,f**) led to comparable levels of TNF-α production by BMMs to that induced by **17b**.

To explore the mode of action of the AraMMs, AraMM **17b** was used as a representative substrate, with TNF-α production

through stimulation with **17b** being found to be Mincle dependent and MyD88 independent (27). This finding was contradictory to a previous study where natural arabinose mycolates from *M. bovis* were reported to induce TNF-α through the MyD88 and TLR2 pathways (80). However, the natural arabinose mycolates consisted of a mixture of mono-arabinose mono-mycolates, tetra-arabinose tetra-mycolates, penta-arabinose tetra-mycolates, and hexa-arabinose tetra-mycolate; therefore, it is possible that some of these glycolipids signal through MyD88. The Mincle activity of **17b** was further confirmed by its ability to activate HEK-Mincle reporter cells, albeit only at a concentration 100-fold higher than that of TDB. AraMM **17b** also induced the production of IL-1β through NLRP3-dependent mechanisms similar to GMM and TDB, however, *in vivo* studies revealed that **17b** did not induce local inflammation and did not have adjuvant properties (27). Although the adjuvanticity of AraMM **17b** was modest compared to the other mycolic esters in this study, this work nonetheless represents the first report of Mincle being activated by pentose-derived glycolipids.

GLYCERIDES

Mono-Acyl Glycerides

Glycerol monomycolate (GroMM, **18a**, **Figure 7**) is a mycobacterial lipid that is perhaps best known for its ability to be presented to T cells by CD1b (81). In recent years, there has been much interest in the potential of GroMM (**18a**) and the simpler synthetic analog glycerol monocorynomycolate (GroMCM, **18b**) as vaccine adjuvants, with both glycolipids having *in vivo* adjuvanticity comparable to TDB when incorporated into DDA liposomes (82, 83). However, it was only in more recent studies that isolated GroMM (**18a**) was found to be capable of activating hMincle reporter cells in a dose-dependent manner similar to TDM, albeit with less potency (84). In contrast, this activity was absent in mMincle reporter cells, which recognized TDM. Similarly, synthetic glycerol monobehenate (GroMB, **18c**) was able to activate human but not mouse Mincle (84).

To better understand this species-specific activity, extracellular domain swaps between mouse and human Mincle were undertaken, leading to the conclusion that the hMincle ectodomain may be involved in GroMM recognition (84). Furthermore, site-directed mutagenesis studies were performed where the amino acid residues at positions 174–176 or 195–196 (or both) in mMincle were swapped with those of hMincle. The single mouse mutants showed activity with GroMM (**18a**), while the doubly mutated mouse Mincle showed no activity with either TDM or GroMM (**18a**) due to major conformational changes in the protein. *In vitro* assays revealed that **18a** was capable of inducing TNF-α production from hMincle transgenic mouse derived macrophages, but no induction was seen with non-transgenic mouse derived macrophages. This response was also confirmed *in vivo*, where injection of GroMM liposomes caused a local inflammatory response only in hMincle transgenic mice. Finally, TNF-α production induced by **18a** was completely abolished in the presence of an antibody against hMincle. Altogether, this study confirmed the first differential recognition of GroMM (**18a**) as ligand for human but not mouse Mincle.

The synthesis of four mycolate esters of *R*-glycerol (**19d**,**e**,**g**,**h**, **Figure 7**) and five esters of *S*-glycerol (**20e–g**,**i**,**j**) have been

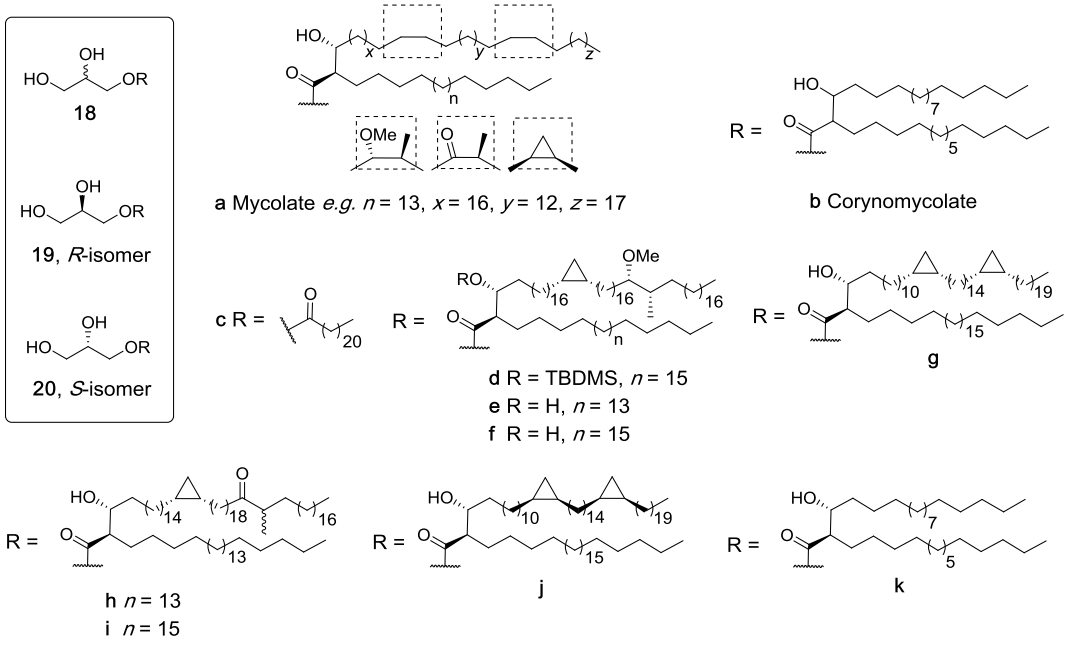

FIGURE 7 | Structures of synthetic glycerol monoesters that have been evaluated as macrophage inducible C-type lectin (Mincle) ligands (27, 47, 84, 85). Of these, **18a**, **18b**, **18c**, **19k**, and **20k** have been determined to be agonists for human Mincle and not murine Mincle.

described very recently and based on their structural similarity to the known glycerol-containing Mincle agonists, these mycolate esters of glycerol were suggested to be Mincle ligands (85). In these studies, however, *R*-GroMMs **19d**,**e**,**g**,**h** were unable to induce cytokine production by BMDCs, while no results for the *S*-isomers were reported. Notwithstanding, it has been suggested that such mycolate esters may play a key role in the stimulation of CD1b-restricted germline-encoded, mycolyl-reactive (GEM) T cells, a process that can also be modulated by the corresponding free mycolic acids (85).

In 2015, the enantiopure CMA esters of *R*- (**19k**) and *S*-glycerol (**20k**) were synthesized by van der Peet et al. and subsequently found to activate hMincle NFAT-GFP reporter cells in a dose-dependent manner similar to TDM (47). No significant activity was observed for reporter cells bearing mMincle. This result was in agreement with the previously described study by Hattori et al. where GroMM (**18a**) only signaled through human but not mouse Mincle (84). Moreover, the *S*-isomer **20k** had significantly higher activity for hMincle compared to the *R*-isomer. This was the first instance where the ability of Mincle to differentiate between isomers was demonstrated.

Glycosyl Glycerides

In 2009, Yamasaki et al. analyzed more than 45 species of pathogenic fungi using NFAT-GFP reporter cells expressing FcRγ with Mincle in search for exogenous Mincle ligands (5). Later studies found that Mincle only selectively recognized *Malassezia* species among all the species tested, with the Mincle ligands being subsequently identified (86). The ligands consist of glyceroglycolipids (**21a**, **Figure 8**) which are structurally similar to lipoteichoic acid (LTA) anchors, and which contain one glycerol and one gentiobiose (6-*O*-β-D-glucopyranosyl-glucopyranose). LTA, however, does not possess Mincle activity. In addition, a more complex Mincle ligand, compound **22**,

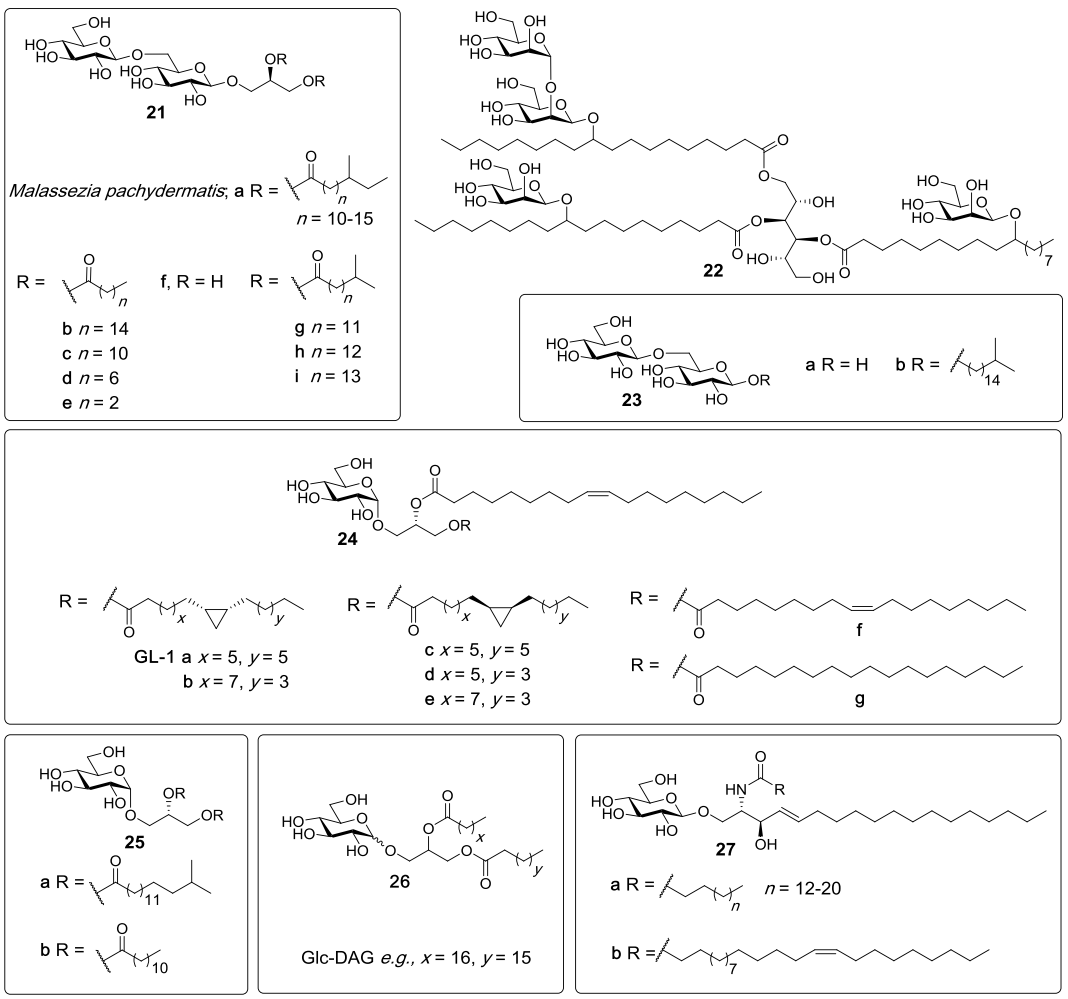

FIGURE 8 | Several glycosyl diacylglycerides have been isolated from bacterial species such as the *Mycobacterium tuberculosis*, *Streptococcus pneumoniae*, and *Lactobacillus plantarum*, as well as rom *Malassezia* sp. and shown to be macrophage inducible C-type lectin (Mincle) ligands (86–90). Mincle agonists include glyceroglycolipids (e.g., **21a**–**c**, **g**–**i**), mannoside (**22**), and glucosyl diglycerides (**24a**–**g**, **25a**,**b**, and **26**). β-Glucosylceramides (**27a**,**b**), isolated from dead human and mouse cells, have also been determined to be Mincle agonists (91).

which has a mannitol backbone and which is esterified to two 10-*O*-β-D-mannopyranosyl-10-hydroxy-octadecanoic acids and one 10-*O*-(β-D-mannopyranosyl-[1→2]-β-D-mannopyranosyl)-10-hydroxy-octadecanoic acid, was identified. Both **21a** and **22** had Mincle agonist activity almost as potent as TDM, as determined using the hMincle/mMincle NFAT-GFP reporter assay, and direct Mincle binding was confirmed using soluble Mincle-immunoglobulin (Ig) protein ELISAs. BMDCs were able to secrete TNF in response to **21a** and **22** in a Mincle-dependent manner.

In 2015, Richardson et al. synthesized a series of β-gentiobiosyl diacylglycerides (87), which were previously isolated from *M. tuberculosis* H37Ra (88), as well as additional β-gentiobiosyl analogs. The activity of synthetic *M. tuberculosis* β-gentiobiosyl diglycerides (**21b**,**g–i**, **Figure 8**) was then compared with TDM in an NFAT-GFP assay, whereby it was determined that all four compounds signaled only through mMincle, albeit weakly compared to TDM. Next, β-gentiobiosyl diacylglycerides with acyl chain lengths of C12 (**21c**), C8 (**21d**), and C4 (**21e**), gentiobiosyl glycerol (**21f**), free gentiobiose (**23a**), and iso-C17 gentiobioside (**23b**) were assayed using the Mincle NFAT-GFP reporter assay. Here, it was determined that only the C12 analog **21c** was able to activate mMincle, albeit less so than TDM, with the same ligand also leading to the weak activation of hMincle. Evidence for direct binding was obtained by a solubilized Mincle Ig fusion assay, where **21c** bound mMincle but not hMincle. To obtain a functional read-out, compounds **21b-e** were also tested for their ability to activate BMDCs through production of TNF and MIP-2. Consistent with the NFAT-GFP assay, significant cytokine production was only observed with **21c**. The authors proposed that the disaccharide moieties of the gentiobiosyl diglycerides bind to the Mincle CRD in the same manner as trehalose, with the first glucose residue engaging the Ca^{2+} in the primary site, and with the acyl chains binding to the hydrophobic groove adjacent to the sugar binding site.

Other glycosyl glycerides include the glucosyl diglycerides, which are a class of compounds first isolated in the mid 1960s from *Lactobacillus plantarum*—a bacterium that is part of the microbiota in the human mucosa (92). However, it was only in 2012 that our group structurally characterized GL-1 (**24a**) as the major glycolipid present in this bacterium (93). Subsequently, GL-1 (**24a**), and six analogs (**24b–g**) consisting of different lipids on the *sn*-1 glycerol position, were synthesized in seven steps from allyl α-D-glucopyranoside by Shah et al. and compared to TDM and TDB for Mincle activation using NFAT-GFP assays (89). Compounds **24a–g** showed almost identical dose-dependent Mincle activity for both mouse and human Mincle, however, this activity was lower than TDM and TDB. These results suggested that both fatty acid chains do not require a cyclopropane ring or an alkene to signal through Mincle.

Richardson et al. also explored the Mincle-binding capacity of iso-C_{17} glucosyl diglyceride (**25a**), with these studies being undertaken to probe the effect of the loss of the gentiobiosyl moiety on Mincle activation (87). Here, **25a** displayed superior signaling through both mouse and human Mincle compared to all the other gentiobiosyl analogs, including the most active C12 analog (**21c**), as determined using the NFAT-GFP reporter assay. The ability of **25a** to activate cells was also confirmed *via* the production of TNF and MIP-2 upon the stimulation of BMDCs with **25a**. Consequently, the glucosyl diglyceride with a C12 lipid (**25b**) was synthesized; however, this new analog showed reduced mMincle activity and no hMincle activity compared to its iso-C17 (**25a**) counterpart. These results suggest that iso-branched lipids are potentially required for more robust human but not mouse Mincle signaling and that only one glucose moiety is sufficient to achieve significant Mincle binding and activity.

In 2016, Behler-Janbeck et al. reported that Mincle expression peaked after lung infection with *Streptococcus pneumoniae,* with subsequent analysis revealing that glucosyl diacylglycerol (Glc-DAG, **26**) (**Figure 8**) was the glycolipid in *S. pneumoniae* that triggered Mincle reporter cell activation (90). Here, the identity of the natural ligand was confirmed by comparison to synthetic Glc-DAG. Studies revealed that Glc-DAG (**26**) was able to induce TNF-α and MIP-2 production by bone-marrow-derived phagocytes in a Mincle-dependent manner. A significantly higher mortality rate was observed in Mincle$^{-/-}$ mice compared to wild type (WT) mice in the focal pneumonia model (caused by *S. pneumoniae*) and not invasive pneumonia. Moreover, the antibacterial and dysregulated cytokine responses in mice challenged with focal pneumonia were normalized in Mincle$^{-/-}$ mice that were reconstituted with a WT hematopoietic system. Collectively, these results indicate that Mincle plays a crucial role in lung protective immunity, specifically in focal pneumonia caused by *S. pneumoniae*.

MONOSACCHARIDES FOR MINCLE AND MCL BINDING

Lee et al. investigated the binding specificities of many CLRs, including Mincle and macrophage C-type lectin (MCL), against different glycans that were tested as BSA-conjugated neoglycoproteins (94). Both Mincle and MCL had the highest binding affinity to mannose and fucose, with lower affinities for *N*-acetyl glucosamine (GlcNAc) and glucose (Glc). Interestingly, Mincle had higher binding for galactose (Gal) and *N*-acetyl galactosamine (GalNAc) than MCL. Mincle was able to bind Lewis^x-BSA, but MCL lacked this binding capacity. Moreover, both CTLs showed binding of lactose and *N*-acetyl lactosamine, but the binding affinity was less than Gal. Similarly, Glc disaccharides were bound less effectively than Glc, with the exception of MCL, which bound maltose better than Glc. The ability of the glycans to activate Mincle, however, was not determined.

β-GLUCOSYLCERAMIDE

In studies aimed at identifying endogenous Mincle ligands associated with cell death, Nagata et al. fractionated and analyzed the supernatant of dead cells using Mincle NFAT-GFP reporter cells (91). Subsequent mass spectrometry and NMR analysis revealed that the most active cell isolate fraction contained a mixture of six β-glucosylceramides (β-GlcCer) (**27a,b**); C16:0, C18:0, C20:0, C22:0, C24:0, and C24:1 (15Z) (**Figure 8**). When compared to TDM, synthetic β-GlcCer (C24:1) (**27b**) was able to activate

NF-κB reporter cells as well as NFAT-GFP reporter cells expressing either mMincle or hMincle, with the importance of the glucose moiety being highlighted since ceramide, lactosyl ceramide and galactosyl ceramide did not act as Mincle ligands. β-GlcCer did not activate MCL, Dectin-2, or DCAR. β-GlcCer (24:1) (**27b**) was also capable of inducing TNF and MIP-2 production through the stimulation of BMDCs and this induction was absent in Mincle$^{-/-}$ BMDCs. Moreover, cytokine production was dependent on Card9 activation and did not require TLR signaling through the MyD88 pathway. Altogether, these results confirm that β-GlcCer activates myeloid cells in a Mincle-dependent manner and is the first example of a carbohydrate-derived endogenous ligand being able to act as a potential adjuvant through Mincle-dependent mechanisms. Further studies indicated that β-glucosylceramidase (GBA1)-deficient mice had increased inflammation caused by cell death, but inflammation was lower in GBA1 and Mincle double-deficient mice suggesting that β-GlcCer induced inflammation may involve Mincle.

STEROIDS

While carbohydrates are the most commonly recognized class of Mincle ligands, steroids have also been found to bind Mincle and induce a proinflammatory response. Cholesterol (**28a**, **Figure 9**) has been known to have inflammatory properties for some time; however, the first innate immune receptor for cholesterol, hMincle, was only identified in 2015 by Kiyotake et al. (20). Here, hMincle was identified as the receptor for plate-coated and crystalline cholesterol using a NFAT-GFP reporter assay, while NFAT-GFP reporter assays involving mMincle and rat Mincle (rMincle) were not activated by cholesterol. As part of their studies, Kiyotake et al. tested different cholesterol-based steroids and found that changes to the hydroxyl or alkene functionality or removal of the lipophilic alkyl chain abolished hMincle binding. However, hMincle tolerates slight changes in the lipophilic alkyl chain, with sitosterol (**28b**) and desmosterol (**28c**) also being hMincle agonists. Using RAW-Blue cells transfected with hMincle, it was determined that cholesterol crystals induce TNF and MIP-2, albeit at lower levels than TDM, with subsequent assays revealing that plate-coated cholesterol and cholesterol crystals upregulate inflammatory chemokines and cytokines in BMDCs.

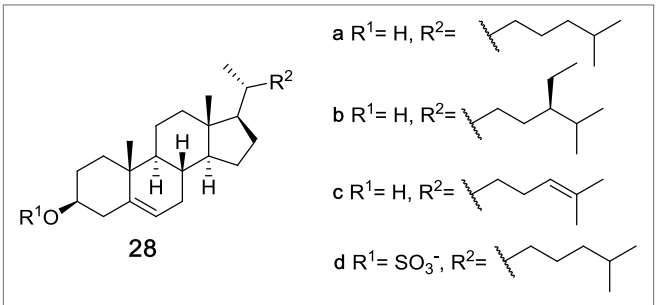

FIGURE 9 | Structures of steroid ligands that bind macrophage inducible C-type lectin through an alternative binding site containing a cholesterol recognition/interaction amino acid consensus-like motif (20, 95).

To better understand how cholesterol might activate Mincle, Kiyotake et al. drew insight from previous studies that reported that cholesterol can interact with proteins through their cholesterol recognition amino acid consensus (CRAC)-like motif (96). This sequence of amino acids is found in hMincle but not murine Mincle. To determine whether cholesterol binds the CRAC-like motif in Mincle, Kiyotake et al. thus created a NFAT-GFP reporter cell line with a mutation in the CRAC-like motif of hMincle (20). The mutant hMincle lost its ability to recognize cholesterol crystals but retained its capability to recognize TDM in comparable levels to that of WT Mincle, thereby demonstrating that cholesterol and TDM bind to different sites in hMincle.

The ability of cholesterol sulfate (**28d**) to act as a Mincle ligand was recently determined by Kostarnoy et al. in studies aimed at identifying the role of Mincle in skin allergies (95). In this work it was determined that Mincle is upregulated after skin tissue damage in mice, with the resulting proinflammatory cytokine response being Mincle dependent. Using a HEK293–mMincle–NF-κB–Luc reporter cell line, the endogenous Mincle ligand was then found to be cholesterol sulfate (**28d**), which exhibited activity comparable to that of TDB in the reporter cell line. Compound **28d** was also able to induce the secretion of pro-inflammatory cytokines IL-1α and IL-1β, KC, and MIP-1α and MIP-1β and this induction was abolished in Mincle$^{-/-}$ BMDCs. *In vivo* experiments revealed that injections with **28d** caused Mincle-mediated induction of local inflammation, with a contact hypersensitivity model subsequently being used to demonstrate that Mincle deficiency leads to a significantly lower inflammatory response and strongly suppressed clinical symptoms of allergy, suggesting that Mincle plays an important role in promoting skin allergies. The binding site for cholesterol sulfate (**28d**) in mMincle, however, was not determined in this study.

SUMMARY AND CONCLUSION

Macrophage inducible C-type lectin is able to accommodate a variety of ligands, however to date, most studies have focused on carbohydrate-derived ligands that bind to Mincle's CRD, which contains an EPN motif. As the major hydrophobic groove in Mincle is rather shallow and open-sided (18, 19), it is perhaps not surprising that Mincle can accommodate ligands that include both branched and linear chain glycolipids and other derivatives with bulkier (e.g., aromatic) functional groups. The presence of a second minor hydrophobic groove next to the EPN motif is also thought to be able to accommodate a second lipid, however, as illustrated by numerous examples (e.g., TMEs, glucose, and mannose monoesters), one lipid is often sufficient to lead to Mincle binding and activation. Mincle is also able to accommodate glycolipids with both α- and β-anomeric configurations.

When exploring the immunostimulatory properties of glycolipid Mincle ligands, subtle changes to functional groups along the lipid backbone have been found to affect the immune response. For example, studies with TDMs revealed slight differences in the ability of TDMs with different mycolic acids to activate BMDCs *in vitro*, whereby it was demonstrated that increased polarity in the middle of the meromycolate branch does not increase Mincle agonist activity (27). Similarly, iso-TDEs (maradolipids) led to

greater cytokine production by BMMs *in vitro* compared to their linear counterparts (48). The ability of Mincle to differentiate between isomers has also been demonstrated in studies with optically pure CMA esters of *R*- and *S*-glycerol, whereby the *S*-isomers was better able to activate reporter cell lines expressing hMincle (47). In other instances, however, changes in functional groups along the lipid backbone did not lead to differences in the adjuvanticity of Mincle ligands, as illustrated in studies with GMMs and with glucosyl glycerides (27, 89).

More marked differences in the immunomodulatory potential of similar classes of Mincle agonists have been observed when changes were made to the length of the lipid chains. This was first demonstrated in studies with linear TDEs, with longer lipids leading to a stronger pro-inflammatory response *in vitro* (48, 60). A similar trend was observed with lipidated brartemicin derivatives, whereby it was also demonstrated that π-cation interactions were essential for the activity of the lead compound (62). The effect of lipid length on the immunomodulatory potential of Mincle agonists other than trehalose derivatives has also been demonstrated with, for example, longer chain GMCM and β-gentiobiosyl diacylglycerides derivatives showing enhanced Mincle activity compared to their shorter-chain counterparts (50, 87).

With regard to the glucose monoester and glucosyl glyceride Mincle agonists, lipid branching influences activity. It has been suggested that the presence of an α-branched lipid containing at least four carbons at the 6-position of glucose is required for Mincle activation (47). Moreover, iso-C17 gentiobioside, which contains only one lipophilic chain, was unable to activate Mincle, yet a similar derivative (gentiobiosyl diglyceride) with two C12 lipid chains was a Mincle agonist (87). In the case of the glucosyl diglycerides it has been suggested that iso-branched lipids are required for more robust human but not mouse Mincle signaling (87). In contrast, certain non-glycosylated Mincle agonists containing no lipid branching (e.g., GroMB) can activate Mincle, albeit only the human isoform (84). These results highlight the complexity and challenges of predicting which compounds might be the most potent Mincle agonists and illustrate the need to assess the immunomodulatory potential of each ligand class. Notwithstanding, lipid length and configuration appear to be important considerations when designing Mincle agonists.

There has also been interest in assessing the relative immunostimulatory properties between different classes of Mincle agonist. Such analysis can only be undertaken when the different classes of Mincle ligands are compared in the same assay, and thus, a comparative study of the different Mincle ligands to date is lacking. Notwithstanding, a number of studies offer some clues in this direction. For example, glucose esters show greater immunomodulatory potential than arabinose esters (27), with an earlier study demonstrating that glucose esters showed greater adjuvant capacity compared to analogous monoesters, with one GMCM (GlcC14C18) having better adjuvant activity in an OVA immunization model when compared to TDB (77). In another study, however, a glucosyl monomycolate derivate showed similar adjuvant activity to TDB in an *in vivo* OVA immunization model (27). Lipidated brartemicin derivatives have also shown enhanced *in vivo* adjuvant activity compared to TDB (62). In studies that identified the first endogenous ligand for Mincle, β-GlcCer, it was also demonstrated that ceramide, lactosyl ceramide and galactosyl ceramide were not Mincle agonists (91).

The most comprehensive studies aimed at comparing the relative immunostimulatory properties of Mincle agonists, however, relate to comparative merits of TMEs and their diester counterparts. Needless to say, the overarching conclusions from such studies are not always clear. *In vitro*, some studies have demonstrated that linear non-branched TDEs are more immunostimulatory than their diester counterparts (71), while other studies point to no significant difference between the two classes of compound (69). In studies with TMCM, there was no significant difference in immune response *in vitro* of this compound compared to its diester counterpart (47), while studies with TDMs and TMMs revealed that the TDMs lead to greater cytokine production *in vitro*, yet the *in vivo* responses to both classes of glycolipids were similar (27).

It is difficult to explain why the Mincle agonist may give different immune profiles when tested *in vitro* or *in vivo*, and indeed, why differences might be observed upon subtle changes to *in vitro* models (e.g., plate-coated glycolipids compared to glycolipids in solution). To further illustrate this point, a C18 lipidated brartemicin derivate was found to give a similar immunomodulatory profile to TDB in several types of *in vitro* assay, yet the brartemicin derivative exhibited enhanced *in vivo* adjuvanticity in vaccination studies using ovalbumin as a model antigen (62). Conversely, a representative GMM was observed to lead to similar levels of cytokine production by BMMs when compared to cytokine production elicited by TDB, yet *in vivo*, GMM exhibited lower immunostimulatory potential than TDB (27). Moreover, computational docking and binding studies can assist in the rational design of Mincle agonists (77), although studies with brartemicin derivatives suggest that the strength of Mincle-binding may not always correlate to a functional immune response (62). Taken together, these findings suggest that *in silico* and *in vitro* profiling are useful tools to screen libraries of potential Mincle agonists but point to a need for several *in vitro* assays, with ultimately an *in vivo* model being performed to better determine the potential of lead Mincle agonists.

In addition to carbohydrate-derived Mincle agonists, there are also a number of cholesterol derivatives that activate Mincle, with plate-coated and crystalline cholesterol (and some derivatives thereof) being found to activate hMincle (but not mMincle or rMincle) (20), while cholesterol sulfate was shown to be an agonist for mMincle (95). In the former studies, cholesterol was determined to bind to the CRAC motif in Mincle, while the binding site of cholesterol sulfate was not determined. These studies also highlight potential species-specific activity across different classes of Mincle ligand. While Mincle has high sequence homology across a variety of species (1), with many classes of ligands (e.g., trehalose glycolipids) activating different Mincle homologs, species-specific activity has been observed in studies with GroMM (84) and with CMA esters of glycerol (47), which are ligands for hMincle but not mMincle, in addition to the aforementioned studies with cholesterol (20).

In summary, much has been discovered in the less than 10 years since TDM was identified as the first non-proteinaceous Mincle ligand, with many classes of glycolipids subsequently

being determined to be Mincle agonists. Several lipids and cholesterol derivatives have also been found to be Mincle agonists. Thus, the potential scope for Mincle agonists appears vast, with some ligands already exhibiting promise as vaccine adjuvants or immunotherapeutics. Nevertheless, much remains unanswered in terms of better understanding the key structural features required for Mincle binding and activation. This, in turn, will require the continued collaboration between synthetic and computational chemists, immunologists, and microbiologists, so that the potential of Mincle ligands for the prevention or treatment of disease can best be realized.

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METHODS
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C-Type Lectin Receptor (CLR)–Fc Fusion Proteins As Tools to Screen for Novel CLR/Bacteria Interactions: An Exemplary Study on Preselected *Campylobacter jejuni* Isolates

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C-type lectin receptors (CLRs) are carbohydrate-binding receptors that recognize their ligands often in a Ca^{2+}-dependent manner. Upon ligand binding, myeloid CLRs in innate immunity trigger or inhibit a variety of signaling pathways, thus initiating or modulating effector functions such as cytokine production, phagocytosis, and antigen presentation. CLRs bind to various pathogens, including viruses, fungi, parasites, and bacteria. The bacterium *Campylobacter jejuni* (*C. jejuni*) is a very frequent Gram-negative zoonotic pathogen of humans, causing severe intestinal symptoms. Interestingly, *C. jejuni* expresses several glycosylated surface structures, for example, the capsular polysaccharide (CPS), lipooligosaccharide (LOS), and envelope proteins. This "Methods" paper describes applications of CLR–Fc fusion proteins to screen for yet unknown CLR/bacteria interactions using *C. jejuni* as an example. ELISA-based detection of CLR/bacteria interactions allows a first prescreening that is further confirmed by flow cytometry-based binding analysis and visualized using confocal microscopy. By applying these methods, we identified Dectin-1 as a novel CLR recognizing two selected *C. jejuni* isolates with different LOS and CPS genotypes. In conclusion, the here-described applications of CLR–Fc fusion proteins represent useful methods to screen for and identify novel CLR/bacteria interactions.

Keywords: C-type lectin receptors, *Campylobacter jejuni*, innate immunity, flow cytometry, confocal microscopy, ELISA assay, screening tools, Dectin-1 receptor

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INTRODUCTION

C-type lectin receptors (CLRs) are pattern recognition receptors and are known to sense pathogen-associated molecular patterns as well as danger-associated molecules. Upon ligand recognition, CLRs trigger a variety of functions, including the production of inflammatory mediators, the phagocytosis of pathogens, or intracellular signaling (1, 2). The carbohydrate recognition domain (CRD) mediates

the binding of CLRs to their specific ligands. One well-described example for a CLR–ligand pair is the CLR Mincle and its ligand trehalose-6,6'-dimycolate (TDM), a unique glycolipid present in the cell wall of mycobacteria (3, 4). Crystal structure analyses of the bovine (5) and human (6) Mincle CRD revealed that the two glucose moieties and one acyl chain of TDM and its synthetic analog trehalose-6,6'-dibehenate interact with Mincle. However, for the majority of CLRs, their glycan ligands and binding mode to their respective ligands are still incompletely understood.

The main function of CLRs is the recognition of highly conserved glycans and glycoproteins located on the surface of pathogens including viruses, parasites, fungi, and bacteria (3, 7–9). However, CLRs may also sense endogenous danger signals released by damaged and necrotic cells (10–13). Dectin-1 is a CLR that is predominantly expressed by monocytes, dendritic cells (DCs), and macrophages (14). It has been described to bind to β-1,3-glucans present in the cell wall of several fungal pathogens. Dectin-1 was shown to recognize various fungal pathogens such as *Pneumocystis carinii*, *Candida albicans, Aspergillus fumigatus*, and *Cryptococcus neoformans* (15).

Fc fusion proteins are established tools to identify novel receptor–ligand interactions. To date, CLR–Fc fusion proteins have been successfully used to screen for novel CLR/pathogen interactions, as demonstrated for fungal interactions, such as the recognition of *P. carinii* by Mincle (16). In addition, several previously unknown CLR/bacteria interactions were identified using CLR–Fc fusion proteins, including the Mincle/*Streptococcus pneumonia*e (*S. pneumoniae*) interaction (17) or the SIGNR3/*Lactobacillus acidophilus* interaction (18). Moreover, CLEC5A (MDL-1) was found to be an important receptor for *Listeria monocytogenes* that impacts macrophage and neutrophil functions in *Listeria*-induced innate immunity (19). Recently, CLR–Fc fusion proteins were used to identify novel CLRs that interact with mycobacteria. Here, CLEC9A was identified as a receptor that was crucial for the regulation of signal transduction and cytokine production during *Mycobacterium tuberculosis* infection (20). Besides the identification of pathogen-derived CLR ligands, CLR–Fc fusion proteins also allow to screen carbohydrate libraries for novel CLR ligands. Identified ligands can then be further evaluated for their utility to target CLRs on antigen-presenting cells (21–24). Indeed, the ligation of cell surface CLRs can induce various responses such as phagocytosis, cell adhesion, cytokine and chemokine release, as well as antigen presentation (25), rendering CLRs promising targets for immune modulation.

The enteropathogen *Campylobacter jejuni* (*C. jejuni*) expresses various virulence factors that allow for motility, adhesion, and invasion of host tissue, leading not only to acute self-limiting gastrointestinal illness but also to autoimmune disorders like Guillain–Barré syndrome (26, 27). Diarrheal *Campylobacter* species can colonize the intestines of many different host species, ranging from farm animals such as cattle and chicken to humans. Interestingly, they persistently colonize most nonhuman species without overt symptoms, verging on commensalism, while they cause acute intestinal disease in humans (28). *C. jejuni* is genetically quite variable and exists as generalist variants able to colonize various host species equally well (29, 30) and as specialist variants, which usually have only one preferred host species (31). In addition, each individual *C. jejuni* strain has the intrinsic property to vary its phenotype, for instance, by phase variation and contingency genes (32, 33). *Campylobacter* sp. are the only bacteria to date which express functional *N*- and *O*-glycosylation modules (34). Surface-exposed glycolipids such as the capsular polysaccharide (CPS) (35) and the lipooligosaccharide (LOS) (36) of the outer membrane play a pivotal role in host interaction and evasion by *C. jejuni*. In addition, *C. jejuni* expresses several cell-envelope-located *O*- and *N*-linked glycoproteins (37, 38). Recent studies identified CLRs that are involved in *C. jejuni* recognition (39, 40). In one study, hMGL–Fc was shown to interact with *C. jejuni*-derived glycoproteins (39). Another study used a murine CLR–hFc fusion protein library and showed LMIR5 to bind to *C. jejuni* (40). These studies point to a role of CLRs in host interplay and modulating the host immune response against *C. jejuni*.

This "Methods" paper presents a combination of innovative techniques to screen for and study CLR/bacteria interactions, using *C. jejuni* as a representative example. All applied methods are based on CLR–hFc fusion proteins in which the extracellular part of the respective murine (m) or human (h)CLR containing the CRD has been fused to the Fc fragment of human IgG_1 molecules, thus leading to dimer formation. ELISA-based methods allow for a high-throughput prescreening for potential CLR interactions with bacteria, followed by flow-cytometric analyses of identified candidates as a confirmatory method. To visualize and confirm binding of CLRs to bacteria *in situ*, confocal microscopy can be applied and was used in this study to visualize the binding of Dectin-1 to *C. jejuni*.

MATERIALS AND METHODS

C. jejuni Strains, Culture Conditions, and Preparation of Bacteria for Interaction Studies

Campylobacter jejuni strains used were from two strain collections (29, 41) assembled in Germany between 2011 and 2016. We selected two different, but related, generalist strains that are from two frequent *C. jejuni* lineages that can colonize well with various animal species including humans and that cause frequent diarrheal diseases in the latter. Strain MHH-24 is equivalent to isolate FBI-Zoo 06025 (ST22) from Ref. (29) and was isolated from raw milk (cattle), and strain MHH-19, a human enteritis isolate, is equivalent to isolate FBI-Zoo 07079 (ST19) from the more recent case–control study (41). MHH-19 has well-recognized genetic determinants for capsule and LOS types (own unpublished data); however, strain MHH-24 has not been typeable so far by molecular methods for LOS or CPS (own unpublished data). Both strains' LOS and CPS glycans or other surface glycans have not been characterized biochemically so far. For the plate assay and FACS-based assay, heat-fixed bacteria were used, which allow for better staining of the bacteria with the fluorescent Syto61 dye (Thermo Scientific), while for immunofluorescent individual bacterial labeling, PFA-fixed bacteria were prepared, which permit a superior surface preservation of the cells and better storage capacity. Bacteria were grown on blood agar plates (Columbia agar, supplemented with 5% sheep blood, Oxoid, Germany)

and diluted in sterile 1x PBS at an OD_{600} of 1. Heat fixation was performed at 65°C in a heating block for 5 h. Heat-inactivated bacteria were stored at 4°C for a maximum of 3 weeks. Fresh 2% PFA as an alternative fixing agent for immunofluorescent labeling was prepared in 100 mM sterile-filtered potassium phosphate buffer, pH = 7.0, and bacteria were fixed twice for 1 h at room temperature (RT), with centrifugation (6.000 × *g*, 10 min, RT) and one change of fixing agent in between. Afterward, the bacteria were centrifuged again and resuspended in a sterile solution of 0.1% glycine in PBS to quench the fixing agent, which prevents nonspecific attachment of proteins or cell clumping. Ultimately, the bacteria were resuspended in pure, sterile 1× PBS (pH = 7.4) after a final centrifugation step and stored at 4°C until further use, with a high storage capacity of several months.

Generation of CLR–hFc Fusion Proteins

The production of the CLR–hFc fusion proteins was performed as previously described (23). Briefly, RNA was isolated from murine spleen and reverse-transcribed into cDNA using a reverse transcriptase (New England Biolabs, Ipswich, MA, USA). Polymerase chain reaction was applied to amplify the cDNA encoding the extracellular part of each CLR using specific primers (**Table 1**). The respective cDNA fragments were ligated into a pFuse-hIgG1-Fc expression vector (InvivoGen, San Diego, CA, USA). Next, CHO-S cells were transiently transfected with the vector construct using MAX reagent (InvivoGen). CLR–hFc fusion proteins were purified after 4 days of transfection from the cell supernatant using HiTrap protein G HP columns (GE Healthcare, Piscataway, NJ, USA). To confirm the purity of each CLR–hFc fusion protein, the protein was analyzed by dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and subsequent Coomassie staining as well as Western blot using an anti-human IgG- horseradish peroxidase (HRP) antibody (Dianova, Hamburg, Germany).

TABLE 1 | DNA sequences of primers used for amplification of the extracellular domain of the respective CLRs.

CLR	Primer
mCLEC12A	FW 5'- GAATTCTTTGGCAACAGAAATGATAA-3'
	RV 5'- AGATCTGCCATTCAACACACTTTCCA-3'
mDectin-1	FW 5'- GAATTCTTCAGGGAGAAATCCAGAGG-3'
	RV 5'- AGATCTTGAAGAAGTATTGCAGATTTGGTT-3'
mDectin-2	FW 5'- CCATGGAGAAAACATCATTCCAGCCCC-3'
	FW 5'- GAATTCCTGGAGCACCAGTGAGCAGAAC-3'
mCLEC9a	FW 5'-GAATTCGGGCATCAAGTTCTTCCAGGTATCC-3'
	RV 5'-CCATGGTGCAGGATCCAAATGCCTTCTTC-3'
mDCAR	FW 5'- CCATGGAACTTGACAGGTACCATTCATT-3'
	RV 5'- AGATCTTAAGTTTATTTTCTTCATCTGAC-3'
mSIGNR3	FW 5'- GAATTCCATGCAACTGAAGGCTGAAG-3'
	RV 5'- AGATCTTTTGGTGGTGCATGATGAGG-3'
mMGL-1	FW 5'- CCAGTTAAGGAGGGACCTAGGCAC-3'
	RV 5'- AGCTCTCCTTGGCCAGCTTCATC-3'
mMDL-1	FW 5'- GAATTCCCCCACGGAGAGCTACGGAACCA-3'
	RV 5'- CCATGGTGGCATTCATTTCGCAGATCCA-3'
hDC-SIGN	FW 5'- GAATTCCATGCAACTGAAGGCTGAAG-3'
	RV 5'- GATCTTTTGGTGGTGCATGATGAGG-3'
hL-SIGN	FW 5'- GAATTCCTATCAAGAACTGACCGATTTG-3'
	RV 5'- CCATGGATTCGTCTCTGAAGCAGGC-3'

Western Blot

After protein separation using SDS-PAGE, the proteins were transferred to a nitrocellulose membrane for 1 h at 5 V. The membrane was blocked for 1 h with 5% milk powder in TBS and 0.1% Tween-20 (TBS-T) followed by a 1-h incubation with an anti-human IgG antibody conjugated to HRP (Dianova). The membrane was washed three times with TBS-T, for 5 min each. Detection of the CLR–hFc fusion proteins was performed using the Amersham ECL Western blotting detection reagent (GE Healthcare).

ELISA-Based Binding Studies

A half-area microplate (Greiner Bio-One GmbH, Frickenhausen, Germany) was coated with 3×10^8 CFU/ml heat-inactivated *C. jejuni* for 3.5 h at RT. Non-adherent bacteria were washed away, and the plate was blocked with buffer containing 1% BSA (Thermo Fisher Scientific/Invitrogen, Darmstadt, Germany) in 1x PBS for 2 h at RT. After washing the wells, 200 ng of each respective CLR–hFc fusion protein in lectin-binding buffer (50 mM HEPES, 5 mM $MgCl_2$, and 5 mM $CaCl_2$) was added to the bacteria and incubated for 1 h at RT. Then, a 1:5.000-diluted HRP-conjugated goat anti-human IgG antibody (Dianova) was added for 1 h at RT. Finally, the substrate solution [*o*-phenylenediamine dihydrochloride substrate tablet (Thermo Fisher Scientific), 24 mM citrate buffer, 0.04% H_2O_2, 50 mM phosphate buffer in H_2O] was added to the samples, and the reaction was stopped with 2.0 M sulfuric acid. Data was collected using a Multiskan Go microplate spectrophotometer (Thermo Fisher Scientific) at a wavelength of 495 nm. Four independent experiments were performed with technical triplicates each.

Flow Cytometry-Based Binding Studies

To detect the bacteria and exclude them from debris, $3–6 \times 10^7$ CFU/ml heat-inactivated *C. jejuni* were stained with 1 μM of the DNA-staining dye Syto61 (Thermo Fisher Scientific) and incubated for 30 min at RT. Subsequently, samples were incubated for 1 h with 200 ng of the respective CLR–hFc fusion protein in lectin-binding buffer. After washing once with lectin-binding buffer, the bacterial pellet was stained with a PE-conjugated goat anti-human Fc (Dianova) antibody solution and incubated for 25 min at 4°C. Finally, flow-cytometric analysis was performed using an Attune NxT Flow Cytometer (Thermo Fisher Scientific). Data analysis was performed using the FlowJo Software (FlowJo, Ashland, OR, USA). As a control, hFc protein was used to exclude the nonspecific binding of *C. jejuni* to the Fc part of the CLR–Fc fusion proteins. Besides the use of the hFc protein, the secondary antibody alone served as an additional negative control. Three independent experiments were done with technical duplicates each.

Confocal Fluorescence Microscopy-Based Binding Studies

Cover slides (Thermo Fisher Scientific) were cleaned with 70% ethanol and coated with poly-L-lysine solution (Sigma-Aldrich, St. Louis, MO, USA) for 30 min at 60°C. 6×10^7 CFU/ml *C. jejuni*

isolate MHH-19 fixed with 2% PFA was washed with 1x PBS and incubated overnight (o.n.) with 500 ng Dectin-1–hFc and hFc in lectin-binding buffer at 4°C. After washing two times with lectin-binding buffer, samples were incubated for 2 h with 1:200-diluted goat anti-human Fc Alexa Fluor (AF) 488-conjugated antibody (Dianova) at 4°C. Next, samples were washed with 1x PBS, applied onto poly-L-lysine-coated cover slides, and incubated for 45 min at 37°C. In addition, a sample with *C. jejuni* and the secondary antibody only was used as a negative control. Finally, the cover slides were mounted on microscopic slides (Roth, Karlsruhe, Germany) with proLong™ gold antifade mountant containing DAPI (Thermo Fisher Scientific), sealed and visualized using a TCS SP5 confocal inverted-base fluorescence microscope (Leica, Nussloch, Germany) equipped with a HCX PL APO 63 × 1.4 oil immersion objective. To avoid the detection of artifacts, PFA-fixed bacterial samples were inspected visually by a high-magnification microscopy (100× lens magnification) for clumps before performing hFc fusion protein co-incubation. Only bacterial preparations without any visible clumps were further used. Three independent experiments were performed, each with three randomly selected pictures.

Statistical Analysis

All data are presented as mean ± SD. Unpaired, one-tailed Student's *t*-test was applied to determine the significance between CLR candidates and the hFc control. Data were analyzed using the GraphPad Prism software (version 7.02).

RESULTS

Generation and Detection of CLR–hFc Fusion Proteins Used in This Study

The generation of CLR–hFc fusion proteins required several steps (**Figure 1A**, 1–4). The first step was the cloning of the cDNA fragment encoding for the extracellular part of each CLR (containing the CRD) and its fusion to the Fc fragment of human IgG1 in the pFuse-hIgG1-Fc expression vector (1). Next, mammalian CHO-S cells were transfected with this vector construct (2). The use of a mammalian cell line such as CHO-S cells ensured that soluble CLR–hFc fusion proteins were secreted into the supernatant that carried mammalian-type glycosylation. Finally, the supernatant was harvested, followed by purification of the respective fusion

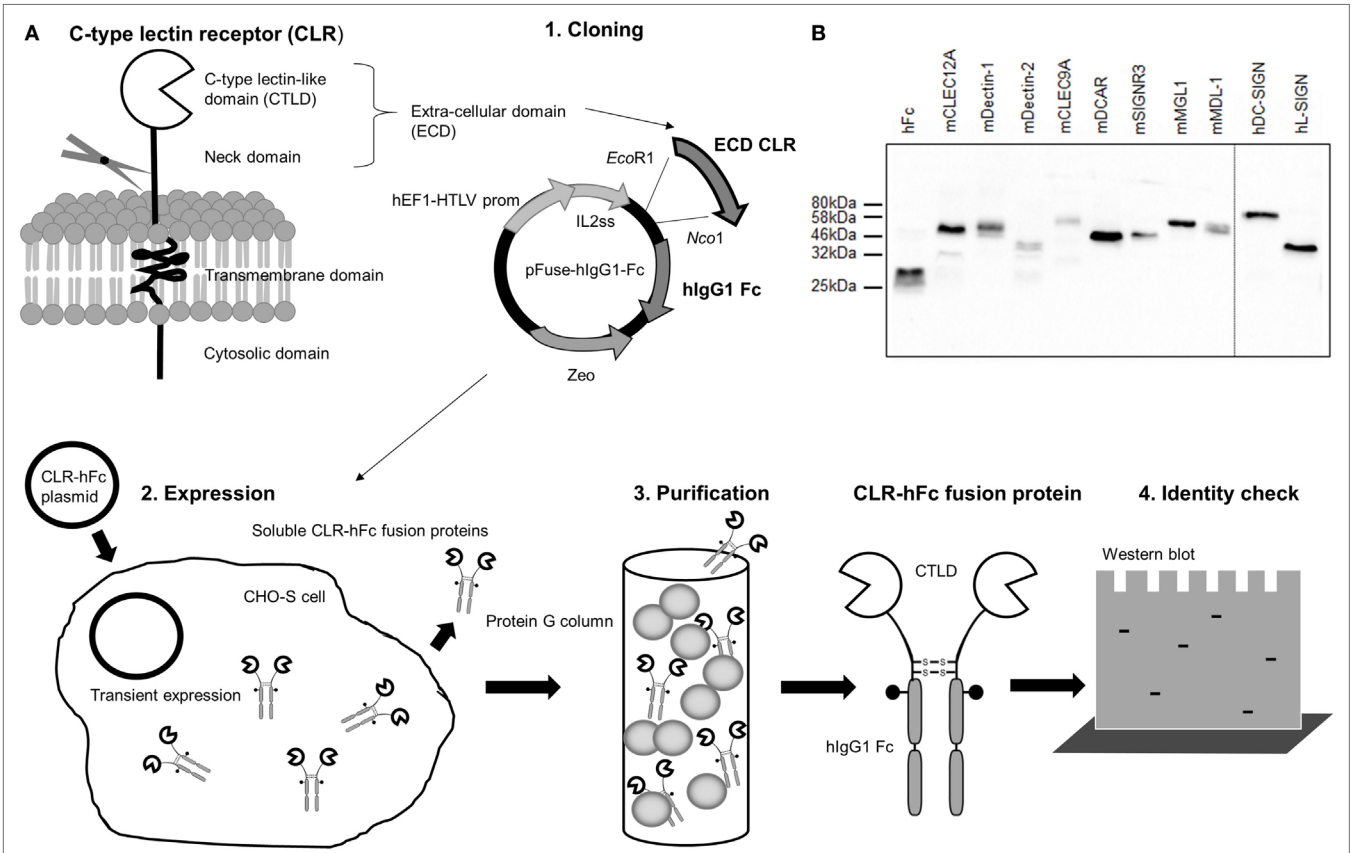

FIGURE 1 | **(A)** Overview of the generation and production of C-type lectin receptor (CLR)–hFc fusion proteins. (1) First, the cDNA encoding for the carbohydrate recognition domain of the respective CLR is cloned into the expression vector and thereby fused to the cDNA fragment encoding for human IgG1-Fc. (2) CHO-S cells are transiently transfected with the expression vector construct, and the respective soluble CLR–hFc fusion proteins are secreted. (3) The cell supernatant is purified, and (4) the presence of the CLR–Fc fusion proteins is detected. **(B)** Western blot of selected CLR–hFc fusion proteins. To confirm the successful CLR–hFc fusion protein production, supernatants of transiently transfected cells were purified using protein G columns, and detection by Western blot was performed using a horseradish peroxidase-conjugated anti-hFc antibody.

proteins using protein G columns (3). To confirm the presence and purity of the respective CLR–hFc fusion proteins after purification, SDS-PAGE and subsequent Coomassie staining and a Western blot were performed (**Figure 1B**). Bands at the expected size of the respective CLR–hFc fusion protein showed the presence of each CLR–hFc fusion protein.

Prescreening Using an ELISA-Based Assay

In a first screening, the binding of the CLR–hFc fusion proteins to two different *C. jejuni* isolates (MHH-19 and MHH-24) was tested using an ELISA-based method. After immobilization of the heat-inactivated bacteria on the ELISA plate and incubation with the respective CLR–hFc fusion proteins, their interaction with the *C. jejuni* isolates was determined by colorimetric detection (**Figure 2A**). Several controls were included, such as the incubation of the CLR–hFc fusion proteins on non-coated wells (data not shown) or with the hFc protein alone to exclude unspecific binding of the Fc fragment to *C. jejuni*. In general, the CLR–hFc fusion proteins exhibited a similar binding pattern to both *C. jejuni* isolates (**Figure 2B**). No binding was observed for DCAR–hFc and L-SIGN–hFc. All other CLR–hFc fusion proteins displayed weak to strong binding to *C. jejuni* in the ELISA-based assay and were considered as potential receptors for the *C. jejuni* isolates MHH-19 and MHH-24.

Confirmatory Test Using a Flow Cytometry-Based Assay

To verify and extend the results from the ELISA-based detection method, a flow cytometry-based protocol to screen for CLR/bacteria interactions in solution was established. To this end, *C. jejuni* was incubated with CLR–hFc fusion proteins, and subsequent binding was detected upon staining with a PE-conjugated anti-hFc antibody (**Figure 3A**). The gating strategy is displayed in **Figure 3B** and is based on the gating of bacteria in the forward-scatter/side-scatter plot, followed by gating on Syto61-positive events. Incubation with the Dectin-1–hFc fusion protein led to a marked shift in the fluorescence intensity, indicating the binding of Dectin-1–hFc to *C. jejuni*. For both *C. jejuni* isolates, no binding was observed for staining with the hFc fragment or with the secondary antibody alone. The lack of binding of the hFc fragment to *C. jejuni* illustrates the specificity of the recognition of the *C. jejuni* isolates by Dectin-1–hFc. The analysis of the binding studies shows that both tested *C. jejuni* isolates were significantly recognized by Dectin-1–hFc and, to a lesser extent, by CLEC12A–hFc (**Figure 3C**). All other CLR–hFc fusion

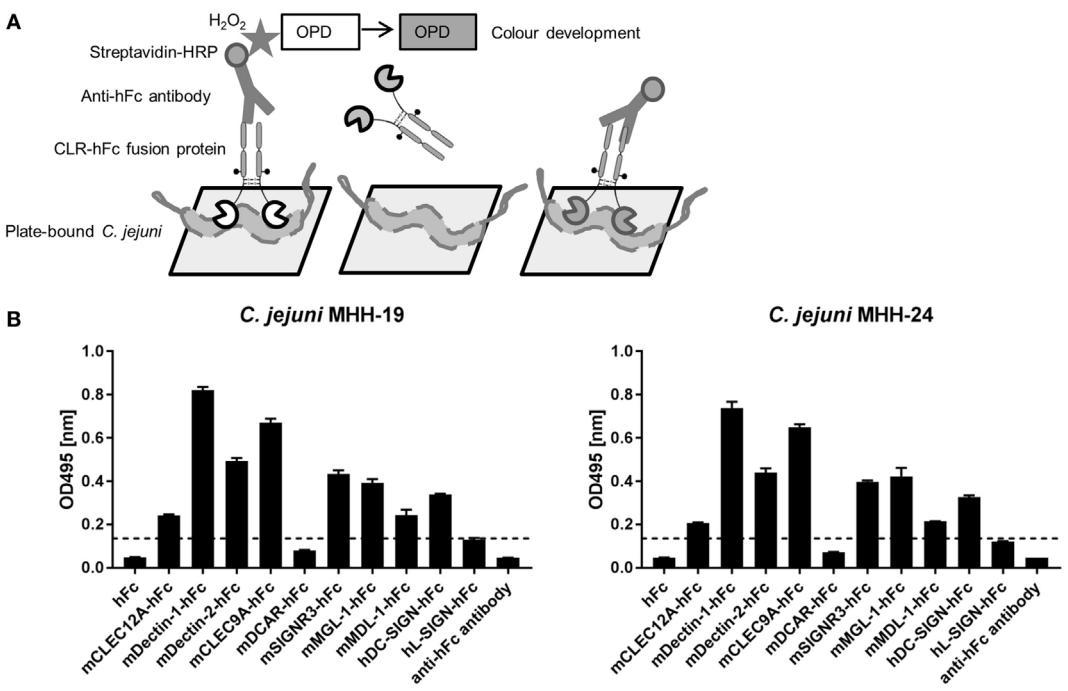

FIGURE 2 | **(A)** Schematic representation of the ELISA-based-binding study using the C-type lectin receptor (CLR)–hFc fusion proteins. Immobilized *C. jejuni* was incubated with the respective CLR–hFc fusion proteins. The detection of bound fusion proteins was performed using a horseradish peroxidase (HRP)-conjugated anti-hFc antibody and subsequent colorimetric detection (details are given in Section "Materials and Methods"). **(B)** Several CLR–hFc fusion proteins were analyzed for their interaction with heat-inactivated immobilized *C. jejuni*. This prescreening showed no or only marginal binding of DCAR–hFc and L-SIGN–hFc. The CLR–hFc fusion proteins CLEC9A, DC-SIGN, Dectin-1, Dectin-2, MDL-1, MGL-1, CLEC12A, and SIGNR3 exhibited weak to marked binding to both *C. jejuni* isolates. Representative data from one out of four independent experiments are shown (technical triplicates for each condition). The dashed line represents the defined threshold for CLR/bacteria interactions in the ELISA-based assay. Student's *t*-test was performed to compare all CLR–hFc fusion proteins with absorbance above the threshold to the hFc control alone. For both *C. jejuni* isolates, a highly significant binding (****$p \leq 0.0001$) was observed for the CLR–hFc fusion proteins mCLEC12A–hFc, mDectin-1–hFc, mDectin-2–hFc, mCLEC9A–hFc, mSIGNR3–hFc, mMGL-1–hFc, mMDL-1–hFc, and hDC-SIGN–hFc.

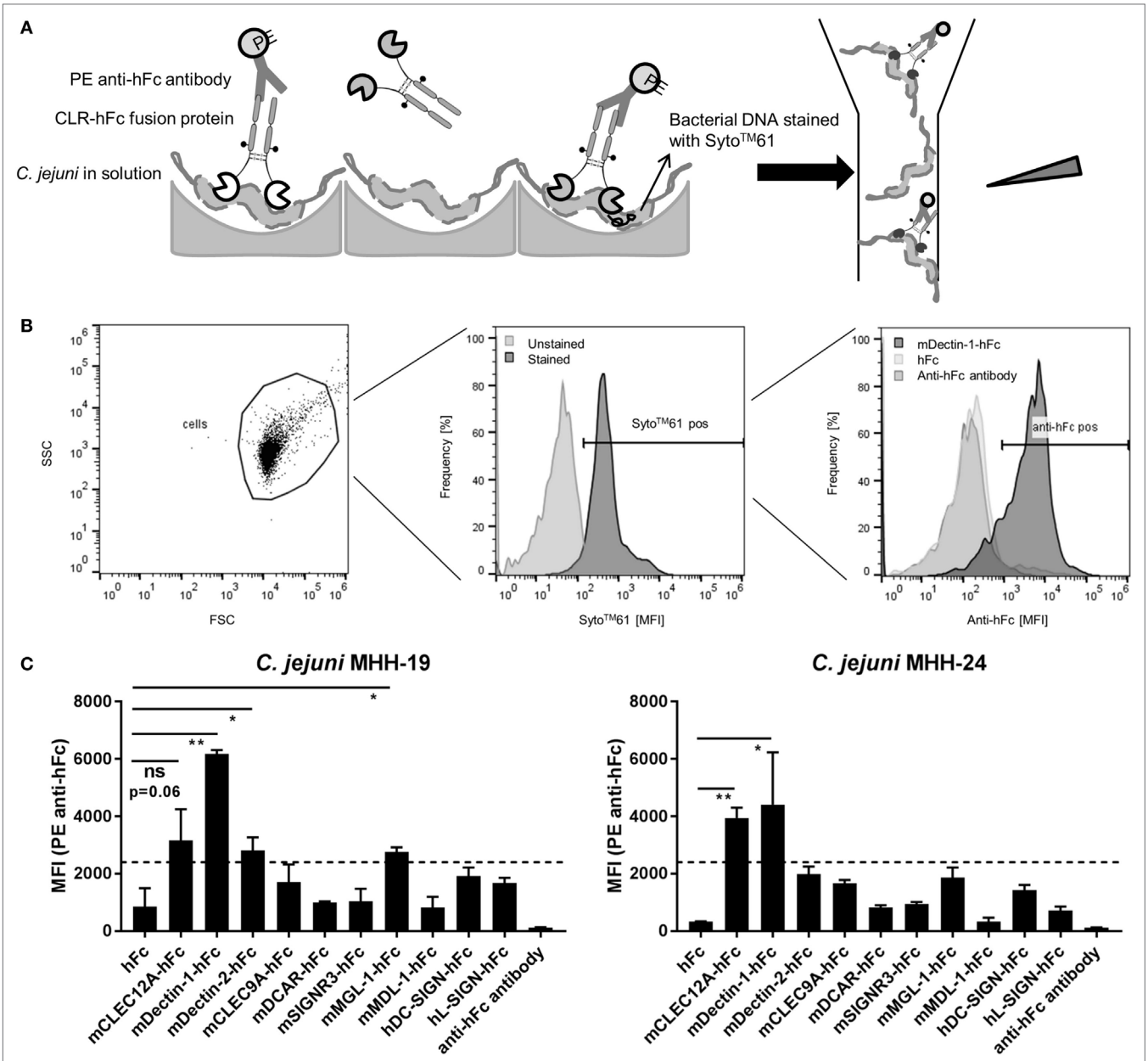

FIGURE 3 | **(A)** Overview of the flow cytometry-based protocol to detect bacterial recognition by C-type lectin receptor (CLR)–hFc fusion proteins. *C. jejuni* was incubated with the respective CLR–hFc fusion proteins in solution. Binding was detected using an anti-hFc antibody. Subsequently, samples were analyzed by flow cytometry based on their morphological and fluorescence properties. **(B)** Histoplots show the gating strategy to detect the binding of CLR–hFc fusion proteins to *C. jejuni* in a representative experiment. Cells were discriminated from debris by gating on forward scatter (FSC) and side scatter (SSC). *C. jejuni* was further gated on DNA staining, indicated by Syto61 positivity (pos.). Finally, Syto61-positive bacteria were analyzed for Dectin-1–hFc binding. hFc and the secondary antibody alone were used as controls. **(C)** Representative data from one out of three independent experiments of the flow cytometry-based CLR–hFc-binding studies to the *C. jejuni* isolates MHH-19 and MHH-24 (technical duplicates each). Similar binding profiles for both isolates were observed. While Dectin-1–hFc and CLEC12A–hFc exhibited binding to *C. jejuni*, all other tested CLR–Fc fusion proteins displayed marginal to no binding. The dashed line represents the defined threshold for CLR/bacteria interactions in the flow cytometry-based assay. Data are expressed as mean ± SD. Student's *t*-test was performed to compare the binding of CLR–Fc fusion proteins above the threshold to hFc alone. For all statistical analyses, *p*-values of <0.05 were considered to be significant: ns = not significant, $^{*}p \leq 0.05$, $^{**}p \leq 0.01$.

proteins included in the flow cytometry-based assay exhibited no or only marginal binding to both *C. jejuni* isolates. Interestingly, no binding of CLEC9A–hFc to both *C. jejuni* isolates was detected using the flow cytometry-based assay. This finding may either suggest a false-positive result in the ELISA-based assay or may be due to internal *C. jejuni* ligands that are not accessible in the flow cytometry-based assay. In summary, the CLR–hFc fusion proteins exhibited a similar binding profile to both *C. jejuni* isolates in the

flow cytometry-based assays and revealed Dectin-1 as a novel candidate receptor for *C. jejuni* recognition.

Visualization of CLR/Bacteria Interactions Using Confocal Microscopy

To visualize the identified Dectin-1–hFc interaction with *C. jejuni in situ*, confocal microscopy after immunofluorescent labeling was applied. The incubation of *C. jejuni* with Dectin-1–hFc and hFc control protein was performed in solution. Subsequently, bacteria were immobilized on poly-L-lysine-coated cover slides, and individual CLR/*C. jejuni* interactions were visualized using a confocal laser-scanning microscope (**Figure 4A**). The results confirmed Dectin-1 binding to *C. jejuni*, whereas no binding was observed for hFc (**Figure 4B**) and the secondary antibody alone (data not shown). Furthermore, merging the fluorescence

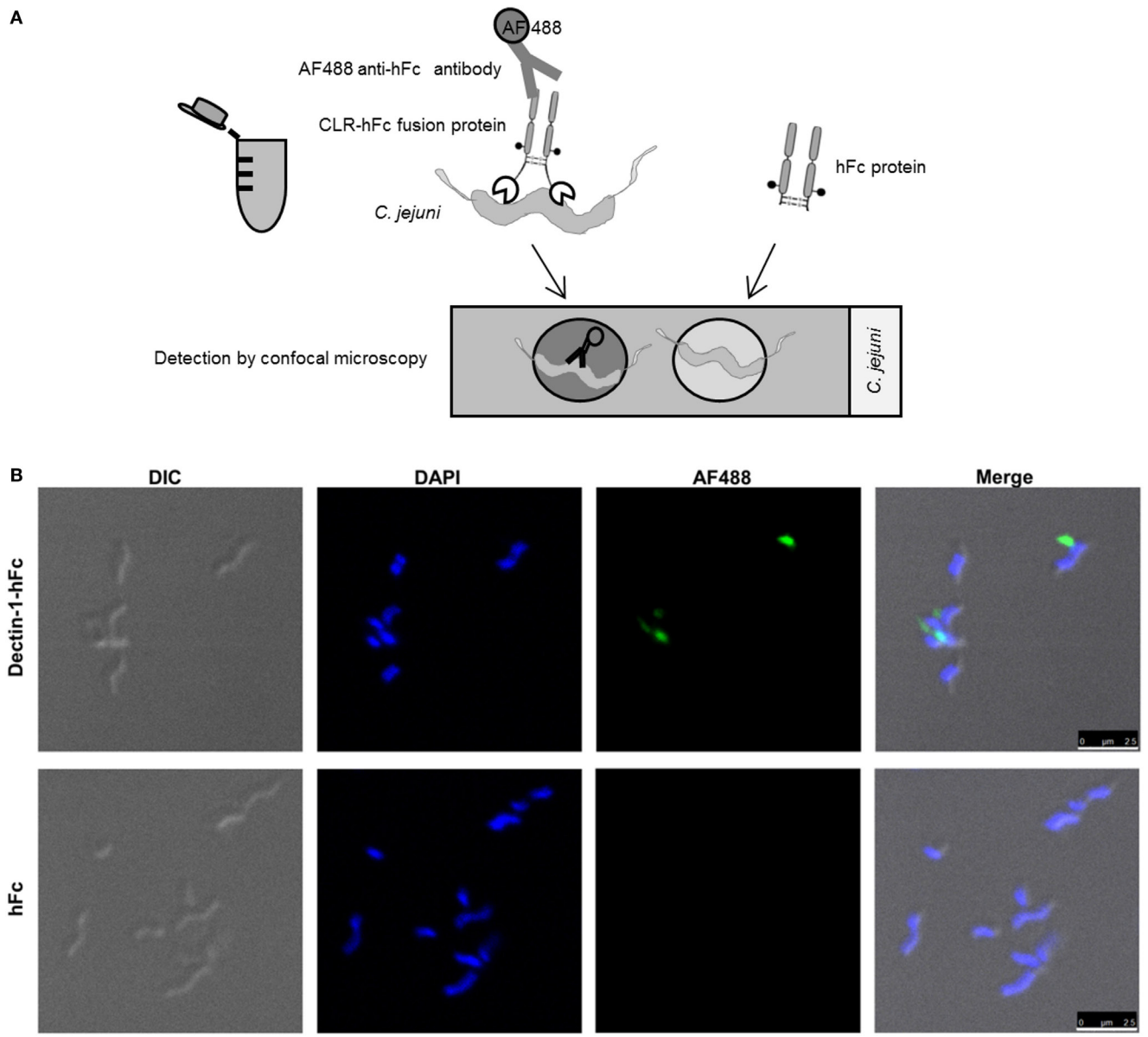

FIGURE 4 | **(A)** Schematic representation of the C-type lectin receptor (CLR)–hFc fusion protein-binding studies using confocal microscopy. Bacteria were incubated with the CLR–hFc fusion proteins. Bound CLR–hFc fusion proteins were detected by an Alexa Fluor (AF) 488-conjugated anti-hFc antibody. To exclude unspecific binding, bacteria were also incubated with hFc as control protein or with the secondary antibody alone. Bacteria were transferred onto cover slips, mounted onto microscopic slides, and visualized using confocal microscopy. **(B)** Bacteria were incubated with Dectin-1–hFc (upper panels) or hFc protein (negative control, lower panels). Differential interference contrast (DIC) was included to visualize the bacterial cell wall. Bacterial DNA was stained with DAPI (shown in blue). The binding of the Dectin-1–Fc fusion protein to *C. jejuni* was detected using an AF488-conjugated secondary antibody (shown in green). While Dectin-1–hFc recognizes *C. jejuni*, the hFc control and the secondary antibody alone showed no signal in the AF488 channel. The experiment was repeated three times with three randomly selected pictures each, and similar results were revealed. Scale bar indicates 2.5 µm.

channels and the differential interference contrast showed that the Dectin-1–hFc signal colocalized with patches on the bacterial cell periphery. This finding suggests that Dectin-1–hFc recognizes a cell-envelope component of *C. jejuni*. In conclusion, the combination of ELISA-, flow cytometry-, and confocal microscopy-based methods highlights the utility of CLR–Fc fusion proteins to identify novel CLR/bacteria interactions as demonstrated here using *C. jejuni* as an example. The functional role of the *C. jejuni* recognition by Dectin-1 can now be further elucidated in future studies.

DISCUSSION

This article presents three distinct methods to detect and verify novel CLR/bacteria interactions. Often, the identification of novel CLR/pathogen interactions is the first step to unravel the interplay of the host innate immune system with bacterial pathogens. Each of the methods can be applied for different purposes and has certain advantages and drawbacks (presented in **Table 2**). The ELISA-based method allows for a high-throughput screening of bacteria collections. Due to the possibility of false-positive results caused by protein aggregation on the ELISA plate, this method is mainly suitable for an initial prescreening using the whole CLR–hFc fusion protein library and requires confirmation by additional methods. To confirm initially identified CLR/bacteria interactions, flow-cytometric analysis represents a useful method that has several advantages. First, binding takes place in solution, thus avoiding protein aggregation on the ELISA plate. Second, the flow cytometry-based method offers the possibility to discriminate between bacteria and debris using an appropriate gating strategy. Third, it allows for narrowing down the localization of ligands to the bacterial surface, whereas the ELISA-based method may lead to a partial lysis of bacteria, thus releasing internal ligands. By contrast, confocal (fluorescence) microscopy offers the opportunity of visualizing CLR/bacteria interactions for single-bacteria *in situ*, thus enabling colocalization studies to further characterize the bacterial ligand. All described methods can be easily applied to other Gram-positive and Gram-negative bacterial species.

TABLE 2 | Advantages and drawbacks of the ELISA-based, flow cytometry-based, and confocal microscopy-based methods to detect novel CLR/bacteria interactions.

Method	Advantages	Drawbacks	Main purpose
ELISA	- High-throughput screening possible - Fast screening	- False-positive results possible due to protein aggregation on the plate - Requires pure pathogen samples	Prescreening for CLR/bacteria interactions
Flow cytometry	- Semi-quantitative comparisons possible - Exclusion of debris due to appropriate gating - Information on a large number of cells for statistical analyses	- Restricted to detectable events in SSC and FSC - Limited to ligands present on the surface of pathogens (can also be an advantage)	Confirmation of CLR/bacteria interactions
Microscopy	- Colocalization studies possible - Visualization of CLR interactions with single bacteria (detection of intra-strain variation) - Extracellular and intracellular staining possible (preserved structure)	- Time-consuming - Requires advanced staining protocols	Direct visualization of CLR/bacteria interactions

Campylobacter jejuni is an interesting candidate for screening glycan-binding factors, since the bacteria are heavily glycosylated with various different glycan species and possess active genes for variable *O*- and *N*-glycosylation, providing abilities to glycosylate capsule, LOS, and proteins (34). In addition, strain-specific differences between various *C. jejuni* strains exist concerning surface determinants and glycosylation (42), based, for instance, on strain-specific genetic differences and phase variation (32). Even in each individual *C. jejuni* strain, a high-variation potential of the bacterial surface phenotype, for instance, LOS, capsule, or additional LOS glycosylation, exists (33, 43–45), which provides an interesting field of future study. Applying the CLR–hFc fusion protein library to screen for binding to preselected, molecularly typed *C. jejuni* isolates from two frequent generalist lineages, we identified Dectin-1–hFc as a promising candidate receptor for *C. jejuni*. Phongsisay et al. screened *C. jejuni* lysates using a murine CLR–hFc fusion protein library (40). In this previous study, the murine CLR LMIR5 was described to interact with *C. jejuni*, whereas other tested CLR/*C. jejuni* interactions remained negative. In bacterial lysates, components such as glycolipids are released and better accessible to potential receptors as in intact live or heat-inactivated bacteria. Thus, the CLR/*C. jejuni* interactions identified in the respective study may also include internal ligands that are not detected when intact bacteria are immobilized on the plate for ELISA-based detection or used in solution for the flow cytometry-based assay. In addition, the use of different *C. jejuni* strains or growth under different culture conditions may impact the recognition by CLRs. Since the surface interaction of the bacteria with host lectins might be more relevant for the colonization and infection process *in vivo*, we employed heat-inactivated intact bacterial cells instead of bacterial lysates for our screening procedures. In our present study, both isolates used in our study, which are genetically related, but not identical, exhibited a similar CLR-binding profile. It will be interesting to compare more *C. jejuni* strains including distantly related isolates, generalists, and specialists (30, 31) for lectin binding. In addition, phase variation, which is a common genetic mechanism used by *C. jejuni* to modulate its surface properties (32), may play a role in CLR recognition. Indeed, phase variation between strains and within the population of one *C. jejuni* isolate (33) may affect cell wall components, as has already been shown for the glycosylation of LOS (43), capsule (44, 45), and for other bacterial properties (32). In this context, it is worth noting that in our confocal microscopy *in situ* approach, bacterial cells showed an individual variation

in Dectin-1 binding or the absence of binding. Phenotypical variation of this trait within the bacterial population has not been revealed in any earlier study on *C. jejuni* and might be explained by single-cell variation of a Dectin-1-binding surface determinant. This presents a very interesting opportunity for further study of individual bacterial intra-strain variation. In an earlier study, human MGL was shown to recognize *C. jejuni* through binding to *C. jejuni*-derived *N*-glycosylated proteins (39). For murine MGL-1, we observed only weak binding to *C. jejuni*, which may be due to experimental or strain differences, or to the different binding profiles between murine and human MGL isoforms. While two different orthologs (mMGL-1 and mMGL-2) are found in mice, humans only express one MGL isoform (hMGL). It is known that mMGL-2 displays a similar binding profile as hMGL which may account for the marginal binding observed for the mMGL-1 ortholog in our study (46, 47).

In this study, we have identified mouse Dectin-1 as a candidate receptor for the innate recognition of *C. jejuni*. To date, Dectin-1 has mainly been described as CLR-recognizing fungal pathogens. For instance, Dectin-1 binds to *C. albicans*, *A. fumigatus*, and *C. neoformans* (15). The Dectin-1 ligand recognized in the context of fungal infection is β-1,3-glucan (48), present in the cell wall of several fungi. Nevertheless, also parasites such as *Leishmania infantum* (49, 50) and *P. carinii* (51) were described to be sensed by Dectin-1. Interestingly, also an interaction of Dectin-1 in cooperation with TLR2 was shown for several *Mycobacterium* species (52). In *C. jejuni*, α-1,4-glucan has been reported as a capsule component (53).

The identification of candidate CLRs that play a role in bacterial recognition presents the first step to identify a distinct bacterial ligand for the respective receptor and may help to understand the interaction of bacteria with the host innate immune system. Identified CLR candidates can be further investigated for their relevance *in vitro* and *in vivo*. Using a comprehensive CLR–hFc library, Rabes et al. demonstrated that Mincle recognizes *S. pneumoniae* in a Ca^{2+}-dependent manner (17). This work was extended by a recent study showing that Mincle recognizes *S. pneumoniae*-derived glucosyl-diacylglycerol in a serotype-specific fashion (54). To date, several CLR ligands have been identified by the use of CLR–Fc fusion proteins. For instance, one study revealed Mincle–hFc as a receptor sensing mannose and glucose-rich glycolipids extracted from *Malassezia pachydermatis* (55). In addition, the identification of distinct glycan ligands of CLRs offers the possibility for glycan-based CLR targeting to deliver vaccine antigens into antigen-presenting cells and to induce subsequent adaptive immune responses (22, 56–58). This approach has already been applied successfully to vaccine design using carbohydrate-based adjuvants (22, 59, 60). Besides CLR–Fc fusion protein libraries, reporter cell lines expressing the respective CLR are used to identify novel CLR–pathogen interactions and CLR ligands (61, 62). In addition, such reporter cell lines also allow for investigating if the identified CLR ligands act as potential agonists or antagonists. In conclusion, this "Methods" paper combines three different screening and confirmatory methods for the detection of CLR–hFc fusion protein binding by pathogens. It also highlights the utility of CLR–hFc fusion proteins to screen for novel CLR/bacteria interactions as a first step toward the identification of distinct bacterial CLR ligands and characterization of their biological functions.

AUTHOR CONTRIBUTIONS

SM, CJ and BL designed the research; SM and RM performed the research; JM and KE contributed to new reagents/analytical tools; SM, RM, CJ, and BL analyzed data; and SM, CJ, and BL wrote the paper with the help of the other authors.

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Dectin-1-Syk-CARD9 Signaling Pathway in TB Immunity

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One of the first steps toward mounting an effective immune response to *Mycobacterium tuberculosis* (Mtb) is recognition of the pathogen through pattern-recognition receptors (PRRs) expressed by innate immune cells. Activation of the PRR Dectin-1 by an unknown mycobacterial ligand triggers an intracellular signaling cascade involving numerous proteins, including spleen tyrosine kinase, protein kinase C-delta, and caspase recruitment domain family member 9, some of which have been shown to influence host immune response to TB infection. Here, we review the role of Dectin-1 signaling pathway in anti-mycobacterial immunity and discuss its contribution in the control of Mtb infection, and potential applications in TB vaccine adjuvanticity.

Keywords: tuberculosis, *Mycobacterium tuberculosis*, dectin-1, C-type lectin receptors, innate immunity, TB immunity, pattern-recognition receptors, pathogen-associated molecular patterns

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INTRODUCTION

A critical function of the innate immune system is differentiating between cells or components which are "self," and those of pathogenic microorganisms (1). Innate immune cells express pattern-recognition receptors (PRRs) which recognize evolutionarily conserved microbial molecules, known as pathogen-associated molecular patterns (PAMPs) (2). Recognition *via* PRRs such as C-type lectin receptors (CLRs), NOD-like receptors, and toll-like receptors (TLRs) enable these cells to initiate responses to a broad range of potential pathogens.

When an innate immune cell, such as an alveolar macrophage, recognizes *Mycobacterium tuberculosis* (Mtb), a unique complement of PRRs will be activated. This triggers a signaling cascade within the innate cell, which results in the expression of immune modulators tailored to that pathogen. These immune modulators may trigger local inflammatory responses and provide the costimulation required for the activation and proliferation of adaptive immune cells, such as $CD4^+$ T cells (2).

Understanding the role that PRR signaling plays in immunity to mycobacteria has been a recent research focus. Recent studies have shown that some receptors that activate the spleen tyrosine kinase (Syk)/caspase recruitment domain family member 9 (CARD9) signaling pathway may contribute to anti-mycobacterial defense. Several CLRs that recognize mycobacteria are known to utilize this pathway, including Dectin-1, Dectin-2, Mincle, and Clecsf8 (3, 4). These receptors are briefly discussed below, and the rest of the review will discuss the importance of Dectin-1–Syk–CARD9 signaling in orchestrating anti-mycobacterial immunity.

Syk/CARD9-COUPLED CLRs IN TB IMMUNITY

Dectin-2 recognizes mycobacterial mannosylated lipoarabinomannan (ManLAM) (5). This interaction results in the recruitment of immunoreceptor tyrosine-based activation motif (ITAM)-linked FcRγ, which links to Syk and CARD9, resulting in a cascade of downstream signaling and cellular

activation (5, 6). Dectin-2 also induces production of anti- and pro-inflammatory cytokines IL-2, TNF, MIP-2, IL-6, and IL-10, in DCs stimulated with ManLAM and BCG. The interaction of this CLR with ManLAM has also been shown to induce T-cell responses (5). Dectin-2 deficiency results in increased pathological damage in mice infected with *M. avium* (5). Although Dectin-2 has been shown to recognize pathogenic Mtb strain, H37Rv, the *in vivo* protective role of this receptor against this strain is yet to be demonstrated.

Mincle interacts with mycobacteria *via* trehalose 6,6′ dimycolate (TDM) (7, 8), the most abundant glycolipid on the cell wall of the bacilli (9). Like Dectin-2, this receptor is also coupled to the adaptor molecule, FcRγ, which initiates Syk-mediated cellular responses (9, 10). Mincle has been shown to trigger pro-inflammatory cytokine production and nitric oxide (NO) in macrophages stimulated with TDM or its synthetic analog, trehalose 6,6-dibehenate (TDB). TDB also induces Mincle-driven adaptive Th1 and Th17 responses when used as an adjuvant to subunit vaccines in mice (7, 8, 11). Despite these contributions to protective responses, Mincle has been shown to be dispensable for the control of Mtb infection *in vivo* (12), although contradicting results have been reported (3, 4, 9).

Clecsf8 (MCL) is another FcRγ-coupled receptor that recognizes mycobacterial TDM (13). This CLR is known to positively regulate the expression of Mincle through a protein–protein complex interaction (14). Clecsf8-mediated cellular responses, which are dependent on the Syk/CARD9 complex, include phagocytosis, pro-inflammatory cytokine production, DC maturation, T-cell priming, and respiratory burst (14–16). Clecsf8-deficient mice are more susceptible to Mtb infection with increased lung bacillary loads, enhanced pathological damage with excessive neutrophilic infiltration, and early mortality (17). Clecsf8 polymorphisms are associated with TB susceptibility in humans (17).

Other Syk-coupled CLRs have been reported to recognize mycobacterial ligands. These have been reviewed elsewhere (4, 18), and they include DCAR, which recognizes glycolipds called PIMs (19), and SIGNR3, a DC-SIGN mouse homolog that recognizes ManLAM (20).

DECTIN-1 STRUCTURE AND FUNCTION

Dectin-1 was initially identified by subtractive cDNA cloning, using mRNA extracted from murine DCs (21). This PRR is expressed on various myeloid cells, including macrophages and other mononuclear cells, as well as a subpopulation of T cells (22). Consistent with its role in pathogen surveillance (23), Dectin-1 is highly expressed by immune cells residing in the mucosa of the lung (22) and gut (24). Dectin-1 is a glycosylated transmembrane receptor (type II) composed of two functional domains (see **Figure 1**). An extracellular C-type lectin domain (CTLD) binds β-glucans, polysaccharides that occur as (1 → 3)-β-D-linked glucose polymers, mainly found on the surface of fungi, plants, and some bacteria (25). Dectin-1 may also bind ligands other than β-glucans (21), as evidenced by the observation that mycobacteria, which do not contain β-glucans on their cell wall, seem to interact with Dectin-1 through a yet to be identified ligand (26). The second functional domain is found on the intracellular tail region, which houses an ITAM-like motif, called hemITAM (23).

DECTIN-1 SIGNALING AND IMMUNE RESPONSES

Dectin-1 initiates intracellular signaling *via* its hemITAM motif (**Figure 2**). Following ligand engagement by the CTLD, Src kinases mediate the tyrosine phosphorylation of the hemITAM domain (23), creating a docking site for Syk, which initiates a series of intracellular signaling cascades resulting in activation of the transcription factor nuclear factor κB (NF-κB) (23, 27, 28). Dectin-1 can also drive non-canonical activation of NF-κB by utilizing the serine-threonine kinase Raf-1 (29).

By influencing gene expression, Dectin-1 activation can lead to numerous downstream cellular responses, including expression of cytokines such as TNF-α, IL-2, IL-10 and IL-12, and CXCL2 (28, 30, 31). Dectin-1-mediated activation can also induce phagocytosis (32) and respiratory burst (33). Furthermore, Dectin-1 signaling has been shown to orchestrate adaptive immunity. DCs activated by Dectin-1 agonists are capable of differentiating naïve $CD4^+$ T cells to a T helper- (Th-)1 or Th17 phenotype both *in vitro* and *in vivo* (34). Dectin-1-activated DCs can also induce the maturation and proliferation of $CD8^+$ T cells *in vitro*: the Dectin-1 agonist curdlan was found to act as an adjuvant for cytotoxic T lymphocyte cross-priming *in vivo*, which elicited potent responses capable of protecting mice from experimental tumor challenges (35). Thus, Dectin-1 signaling serves as a link that generates appropriate adaptive responses following immune recognition (36).

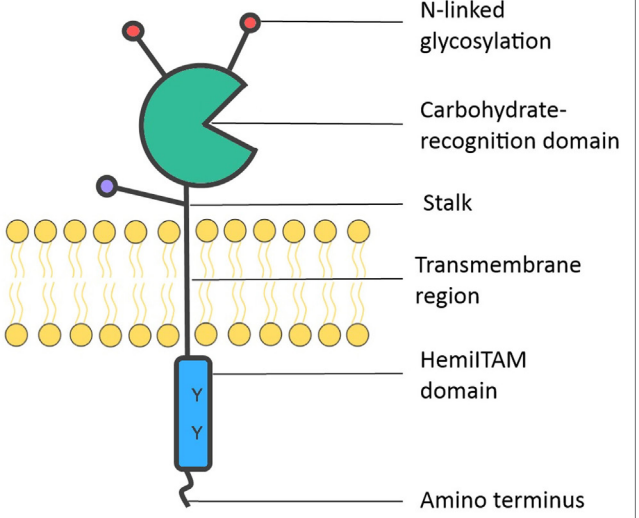

FIGURE 1 | Dectin-1 structure. Dectin-1 consists of an extracellular C-type lectin domain, which is the carbohydrate-recognition domain (CRD) that binds ligands such as β-glucans. The CRD is attached by a stalk to a transmembrane region. Dectin-1 undergoes N-linked glycosylation on the CRD (mice) or stalk region (humans), shown in red and blue, respectively. The intracytoplasmic region comprises an immunoreceptor tyrosine-based activation motif- (ITAM)-like motif, or hemITAM, which initiates intracellular signaling.

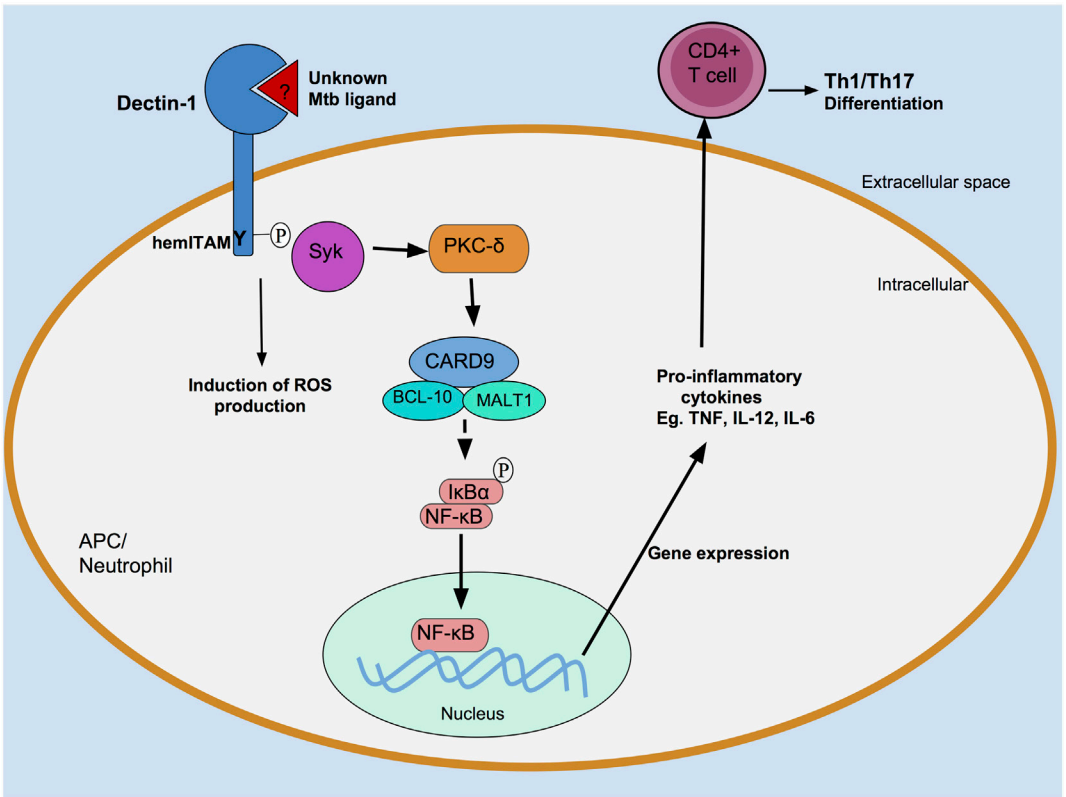

FIGURE 2 | Recognition of *Mycobacterium tuberculosis* (Mtb) by Dectin-1. An unknown Mtb ligand is recognized by Dectin-1, which is then tyrosine phosphorylated at its hemITAM residue by Src kinases (data not shown). This creates a docking site for spleen tyrosine kinase (Syk). Syk associates with a caspase recruitment domain family member 9 (CARD9)/B-cell lymphoma 10 (BCL-10)/mucosa-associated lymphoid tissue lymphoma translocation protein 1 (MALT1) protein complex, resulting in activation of the transcription factor nuclear factor κB (NF-κB). Pro-inflammatory cytokine production follows, which induce an adaptive T cell response. Dectin-1-dependent signaling can also occur in neutrophils, where they induce reactive oxygen species (ROS) production.

Dectin-1 does not appear to initiate protective responses in isolation, but acts synergistically with other receptors such as the TLRs. For instance, Dectin-1 stimulation was found to augment TLR-2-mediated production of cytokines in murine macrophages and DCs (30, 31). In addition, Ferwerda et al. showed that stimulation of human peripheral blood mononuclear cells (PBMCs) with a Dectin-1 ligand, as well as ligands for TLR-2 or -4, led to a synergistic increase in TNF-α production compared with Dectin-1 stimulation alone (37). Shin et al. extended this investigation to mycobacteria by infecting murine macrophages with *M. abscessus* (Mab), an environmental non-tuberculous *Mycobacterium* that can cause opportunistic infections in humans (38). The authors observed that Mab stimulation of macrophages initiated a physical colocalization between Dectin-1 and TLR-2 that was required for pro-inflammatory cytokine production (38). The mechanism underlying this apparent interaction remains to be elucidated (23, 39). In contrast to these findings, a study by Rothfuchs et al. showed that Dectin-1 inhibition significantly diminished the production of IL-12p40 by DCs lacking TLR-2 (40). This suggests that Dectin-1 signaling is not necessarily dependent on TLR-2. Notably, these conflicting studies described the effect of different microorganisms and stimuli on different cell types.

DECTIN-1 RECOGNITION OF MYCOBACTERIA

Dectin-1 has been well characterized as a major fungal β-glucan receptor (41). Interestingly, Dectin-1 has also been shown to be involved in the innate immune recognition of mycobacteria (26), which do not contain β-glucans (40). Yadav and Schorey established this by infecting murine bone marrow-derived macrophages (BMDMs) with non-pathogenic *M. smegmatis*. They found that the production of TNF-α by BMDMs was decreased by approximately 60% in the presence of Dectin-1-blocking antibodies (26). Moreover, they showed that Dectin-1 was required for the production of IL-6, G-CSF, and RANTES by BMDMs (26). However, when the authors performed infection using more virulent mycobacterial strains, such as Mtb strain H37Rv, they observed a significant decrease in the production of TNF-α compared with non-pathogenic strains, and this minimal TNF-α production was not reliant on Dectin-1 (26). This suggested that the role of Dectin-1 in orchestrating immune responses to pathogenic mycobacteria is somewhat limited. In contrast to this finding, a later study by Rothfuchs et al. reported that Dectin-1 does interact with pathogenic mycobacteria (40). These authors

demonstrated that murine splenic DCs (spDCs) infected with pathogenic Mtb produced significantly less IL-12p40 when treated with laminarin, a competitive inhibitor of Dectin-1 (40). Thus, Dectin-1 is involved in the recognition of the important human pathogen Mtb as well as less virulent mycobacteria. Rothfuchs et al. went on to show that pharmacological Syk inhibition reduced the capacity of spDCs to produce IL-12p40 upon Mtb exposure, suggesting that this response was Syk dependent (40). The authors also elegantly confirmed the presence of a Dectin-1 ligand on mycobacteria, by showing that a Dectin-1–Fc fusion protein (42) was capable of binding live *M. bovis* (40). These and other related studies established the role of Dectin-1-mediated responses in non-human innate immune cells. The role of this CLR in human cells was investigated more recently.

Although they are not traditionally considered to be immune cells, airway epithelial cells may serve an important function in mediating pulmonary immune responses. These cells express PRRs such as Dectin-1, albeit to a lesser extent than myeloid cells. Lee et al. reported that Mtb could induce Dectin-1 expression in human A549 airway epithelial cells in a TLR-2-dependent fashion (43), and such expression contributed to the production of reactive oxygen species (ROS), antimicrobial peptides, and pro-inflammatory cytokines by these cells (43). Another investigation into mycobacteria-induced Dectin-1 signaling in human cells was undertaken by Zenaro et al. These authors observed that Mtb infection induced the maturation of monocyte-derived DCs (MDCs), as well as the Dectin-1-dependent production of IL-1β, IL-6, IL-23, and TNF-α (44) by these cells. In addition, DCs activated with a Dectin-1 agonist stimulated naïve $CD4^+$ T cells to secrete IFN-γ and IL-17 (44). Another study has demonstrated that simultaneous activation of neonatal MDCs with agonists of Dectin-1 and TLRs promote synergistic production of IL-12p70 (45). In human PBMCs stimulated with Mtb, Dectin-1 and TLR-4 are the main receptors driving IL-17A production (46). Interestingly, administration of Dectin-1 agonist, curdlan, together with a mycobacterial antigen TB10.4, induced Th1 and Th17 responses in neonatal mice infected with Mtb (45). A recent study by Bisiaux et al. investigated cell-specific activation and pro-inflammatory responses in human whole blood stimulated with BCG (47). Interestingly, Dectin-1/2 responses were predominantly activated in neutrophils when compared with monocytes and lymphocytes populations. This work also demonstrated that induction of ROS production by BCG was decreased by neutralization of Dectin-1/2 and TLR-2/4 in both neutrophils and monocytes (47). In agreement with these findings, a recent report has shown that Mtb can induce DC maturation by generating ROS production through Dectin-1/TLR-2 (48). These *in vitro* findings indicated that Dectin-1 signaling is involved in the stimulation and activation of neutrophils and antigen-presenting cells (APCs), which lead to adaptive anti-mycobacterial immune responses.

To explore the significance of Dectin-1 *in vivo*, Marakalala et al. investigated Dectin-1-deficient mice and wild-type (WT) controls infected with aerosolized Mtb (49). The kncokout mice had significantly and reproducibly decreased (~0.5 log) pulmonary bacillary burdens (49). However, both the Dectin-1-deficient and WT mice developed similar histological signs of pneumonia, suggesting that there was still profound inflammatory activation in Dectin-1-deficient mice despite their decreased bacterial burden. Indeed, Dectin-1 deficiency did not result in any significant and reproducible changes in the pulmonary cytokine expression profiles compared with WT controls (49). The authors also found that Dectin-1 deficiency did not significantly affect mouse survival 150 days of post-infection. Marakalala et al. concluded that Dectin-1 does not play a major role in immunity to Mtb *in vivo* and acknowledged the potential significance of the mechanisms underlying the decreased bacillary burden conferred by Dectin-1 deficiency.

THE FUNCTION OF Syk in PRR SIGNALING AND IMMUNITY TO Mtb

Spleen tyrosine kinase is an intracellular signal transducer that performs diverse biological functions, including innate immune recognition (50). Syk can be activated by associating with the phosphorylated ITAM or hemITAM motif of CLRs *via* one of its two SH2 domains. In the case of the Dectin-1/Syk pathway, Syk activates protein kinase C-delta (PKCδ) which mediates the phosphorylation of CARD9 (51). This enables CARD9 to associate with B-cell lymphoma 10 and the paracaspase mucosa-associated lymphoid tissue lymphoma translocation protein 1 (MALT1) to form a trimolecular structure capable of canonically activating NF-κB (52, 53). Such manipulation of gene expression allows Syk to exert its secondary messenger functions, triggering ROS production (33), accelerated phagocytosis (28), and the production of pro-inflammatory cytokines, such as IL-1β, IL-6, IL-12, and TNF-α (18). Mature IL-1β production requires further post-translational modification of pro-IL-1β by a caspase-1-containing oligomer called an inflammasome (50). The NLPR3 inflammasome is activated in a Syk-dependent mechanism by mycobacteria that express the virulence factor ESAT6, and such activation has been linked to macrophage necrosis (54). There is therefore mounting evidence that this signaling molecule may be relevant in immune responses to mycobacterial infections.

Spleen tyrosine kinase is involved in a multitude of biological functions, and therefore essential for normal survival in mice (55). This makes it difficult to determine the effect of Syk deficiency on mycobacterial immunity *in vivo*, for example, using a gene knockout study in mice. Better research tools are required to understand the role of Syk in mycobacterial infections *in vivo* and in humans.

PKCδ AND IMMUNITY TO Mtb

As already described, CLRs which activate Syk are linked to the CARD9–BCL10–MALT1 pathway. Strasser et al. showed that the linker molecule PKCδ is activated by Syk and mediates the phosphorylation of CARD9 (51). The role of this kinase in signal transduction prompted Parihar et al. to investigate the relevance of PKCδ function following Mtb infection (56). They observed that mice deficient in PKCδ (either through gene knockout or pharmacological inhibition) were less resistant to Mtb, displaying more severe lung pathology, excessive pro-inflammatory

cytokine production, increased bacterial burdens, and increased mortality compared with their WT or untreated controls. Using gene expression profiles from Mtb-infected humans, the authors showed that PKCδ abundance was temporally associated with progression to TB disease. In addition, the authors analyzed lung specimens from TB patients and found that PKCδ was highly expressed in necrotic and cavitary granulomas (56). These data indicate that PKCδ is an important determinant of Mtb infection in humans and mice.

CARD9 AND IMMUNITY TO Mtb

CARD9 is an adaptor protein involved in the Dectin-1/Syk signaling cascade and is expressed in DCs and macrophages (52). The adaptor picks up on signals from multiple receptor classes, such as ITAM-based receptors (including CLRs) and TLRs (27). The role of CARD9 in Mtb immunity was evaluated by Dorhoi et al. using a mouse model of Mtb H37Rv infection (57). These authors demonstrated that CARD9-deficient mice had a reduced ability to control bacterial replication, developed severe lung pathology, and displayed increased mortality compared with their littermate controls (57). Infected CARD9-deficient mice developed acute pneumonia, and histological examination of the lungs revealed the presence of necrotic foci and an inflammatory infiltrate with profound neutrophil accumulation. Moreover, lung specimens from CARD9-deficient mice showed increased apoptotic cell death and secondary necrosis compared with WT controls (57). There is evidence to suggest that the mobilization of neutrophils from the bone marrow to peripheral tissues is dependent on the cytokines G-CSF and CXCL1 (58), and these cytokines were significantly elevated in the sera of CARD9-deficient mice compared with their littermate controls (57). The investigators also demonstrated that neutrophils deficient in CARD9 were unable to produce the regulatory cytokine IL-10 when challenged with Mtb, leading to deregulated pulmonary inflammation (57). These data led the authors to conclude that CARD9 is required for properly regulated innate immune cell activation during Mtb infection.

To further explore the mechanism of CARD9 function in TB, Dorhoi et al. infected APCs with Mtb H37Rv and found that deficiency in the CARD9 adaptor did not affect NO synthesis by the APCs nor did it affect the phagocytosis or destruction of Mtb following IFN-γ activation (57). However, CARD9-deficient BMDMs produced significantly less TNF-α, IL-1β, IL-6, IL-12, and CCL5 compared with WT controls (57). Given the reduced levels of IL-12 in their *in vitro* experiments, one might have expected to see impaired Th1 responses *in vivo* (as IL-12 contributes to Th-1 polarization). However, no such aberrations in T cell recruitment and activation were observed in the lungs of infected CARD9-deficient mice (57). Similarly, the authors did not observe any deficit in Th17 responses in CARD9-deficient mice (57), even though an earlier study demonstrated that the Syk/CARD9 pathway is required for developing Th17 responses to *Candida albicans* (34). Nevertheless, adequate T-cell responses were not able to overcome the pathology induced by defects in innate immune cell inflammatory activation. These results suggest an essential role for CARD9 in anti-mycobacterial immunity.

THE Syk/CARD9 PATHWAY IN TB VACCINOLOGY

To generate a protective immune response to Mtb, antigen-specific Th1 cells are required (59). In addition, the generation of Th17 cells secreting IL-17 has been shown to augment a protective host response by stimulating the influx of effector cells to the areas of infection (60). An effective vaccine to Mtb would probably need to activate APCs in such a way that they "instruct" T cell differentiation to Th1 and Th17 phenotypes. One novel vaccination strategy involves using recombinant Mtb antigens as subunit vaccines; however, these vaccines have not been successful on their own, perhaps because in themselves they do not activate APCs. This necessitates the use of vaccine adjuvants capable of triggering helpful innate cellular responses to guide adaptive immunity. Potential adjuvants include the mycobacterial PAMPs TDM and its synthetic analog TDB, which induce protective Th1 and Th17 immunity by activating APCs *via* the Syk–CARD9–BCL10–MALT1 pathway (11). The major PRRs that bind TDM are Mincle (7, 8) and Clecsf8 (13, 17), suggesting that signaling initiated by either one or both of these CLRs *via* the Syk/CARD9 pathway may be essential for protective immunity to Mtb.

CONCLUSION AND FUTURE DIRECTIONS

An important step toward a deeper understanding of Dectin-1–Syk–CARD9 signaling will be identifying the mycobacterial PAMP recognized by Dectin-1. This would help elucidate how Mtb interacts with human innate immune cells, and how this contributes to or lessens pathology. β-Glucans are currently the only known Dectin-1 ligand, yet Dectin-1 recognizes mycobacteria, which do not express β-glucans. The nature of the ligand will therefore broaden our knowledge of Dectin-1 PAMP recognition.

Dectin-1 itself may not be essential for Mtb immunity, but some of the downstream signaling molecules such as CARD9 evidently are. The effect of other signaling molecules, like Syk and PKCδ, in Mtb responses remains to be fully elucidated. In addition, future studies should look at how CLRs that utilize this signaling pathway interact, perhaps by performing gene knockouts of multiple receptors in mice. Although Dectin-1 appears to play a minor role in *in vivo* Mtb immunity, components of Dectin-1/Syk signaling can induce protective downstream host responses, and this pathway remains a potential target for vaccine adjuvants.

AUTHOR CONTRIBUTIONS

MW wrote the manuscript. All authors planned the manuscript content, analyzed the literature, wrote parts of, and edited the manuscript.

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MINI REVIEW
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C-Type Lectin-Like Receptors As Emerging Orchestrators of Sterile Inflammation Represent Potential Therapeutic Targets

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Over the last decade, C-type lectin-like receptors (CTLRs), expressed mostly by myeloid cells, have gained increasing attention for their role in the fine tuning of both innate and adaptive immunity. Not only CTLRs recognize pathogen-derived ligands to protect against infection but also endogenous ligands such as self-carbohydrates, proteins, or lipids to control homeostasis and tissue injury. Interestingly, CTLRs act as antigen-uptake receptors *via* their carbohydrate-recognition domain for internalization and subsequent presentation to T-cells. Furthermore, CTLRs signal through a complex intracellular network leading to the secretion of a particular set of cytokines that differently polarizes downstream effector T-cell responses according to the ligand and pattern recognition receptor co-engagement. Thus, by orchestrating the balance between inflammatory and resolution pathways, CTLRs are now considered as driving players of sterile inflammation whose dysregulation leads to the development of various pathologies such as autoimmune diseases, allergy, or cancer. For examples, the macrophage-inducible C-type lectin (MINCLE), by sensing glycolipids released during cell-damage, promotes skin allergy and the pathogenesis of experimental autoimmune uveoretinitis. Besides, recent studies described that tumors use physiological process of the CTLRs' dendritic cell-associated C-type lectin-1 (DECTIN-1) and MINCLE to locally suppress myeloid cell activation and promote immune evasion. Therefore, we aim here to overview the current knowledge of the pivotal role of CTLRs in sterile inflammation with special attention given to the "Dectin-1" and "Dectin-2" families. Moreover, we will discuss the potential of these receptors as promising therapeutic targets to treat a wide range of acute and chronic diseases.

Keywords: C-type lectin-like receptors, sterile inflammation, autoimmune diseases, tissue injury, cancer

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INTRODUCTION

C-type lectin receptors (CLRs) are a large family of transmembrane and soluble receptors that contain one or more carbohydrate-recognition domain able to recognize a wide variety of glycans on pathogens or on self-proteins. The hallmark of classical CLRs is the dependence on Ca^{2+} for glycan recognition. However, many other CLRs lack the coordinated Ca^{2+} ions and are therefore referred as C-type lectin-like molecules. These C-type lectin-like receptors (CTLRs) are still able to recognize carbohydrates but independently of Ca^{2+} but also recognize more diverse ligands

such as lipids and proteins (1). Of particular interest for their role in coupling both innate and adaptive immunity, are the CTLR genes of the "*Dectin-1*" and "*Dectin-2*" families localized on the telomeric region of the natural killer cluster of genes (2, 3). These two groups of CTLRs are expressed mostly by cells of myeloid lineage such as monocytes, macrophages, dendritic cells (DCs), and neutrophils. CTLRs not only serve as antigen-uptake receptors for internalization and presentation to T cells but also trigger multiple signaling pathways leading to NF-κB, type I interferon (IFN), and/or inflammasome activation (1–4). This leads, in turn, to the production of pro- or anti-inflammatory cytokines and chemokines, subsequently fine tuning adaptive immune responses. CTLRs can signal either directly, through integral signaling domains, or indirectly, by associating with adaptor molecules. As illustrated in **Figure 1**, activation of immune-receptor tyrosine-based activation motif (ITAM) directly or *via* adaptor proteins such as FcγR, leads to the recruitment of SYK family kinases and the formation of the Card9/Bcl10/Malt1 complex that downstream activates NF-κB pathway and various cellular responses. By contrast, activation of immune-receptor tyrosine-based inhibition motif (ITIM) induces the recruitment and activation of protein tyrosine phosphatases such as SHP-1 and SHP-2 and the dephosphorylation of motifs (1). Consequently, ITIM signaling can inhibit cellular activation mediated by other immunoreceptors to tightly regulate immune response. Such checkpoints allow to prevent uncontrolled immune responses that may lead to harmful, or even fatal, consequences. In addition, some CTLRs were also reported to signal *via* SYK-independent pathway through the serine/threonine kinase RAF-1 to drive particular Th differentiation (5). Besides, by integrating simultaneous signals from other pattern recognition receptors (PRRs), CTLRs can exert synergistic or antagonistic response to achieve appropriate biological responses (6). This cross talk is regulated by the level and localization of their expression, by their interaction and by their collaborative or conflicting signaling (6, 7). To date,

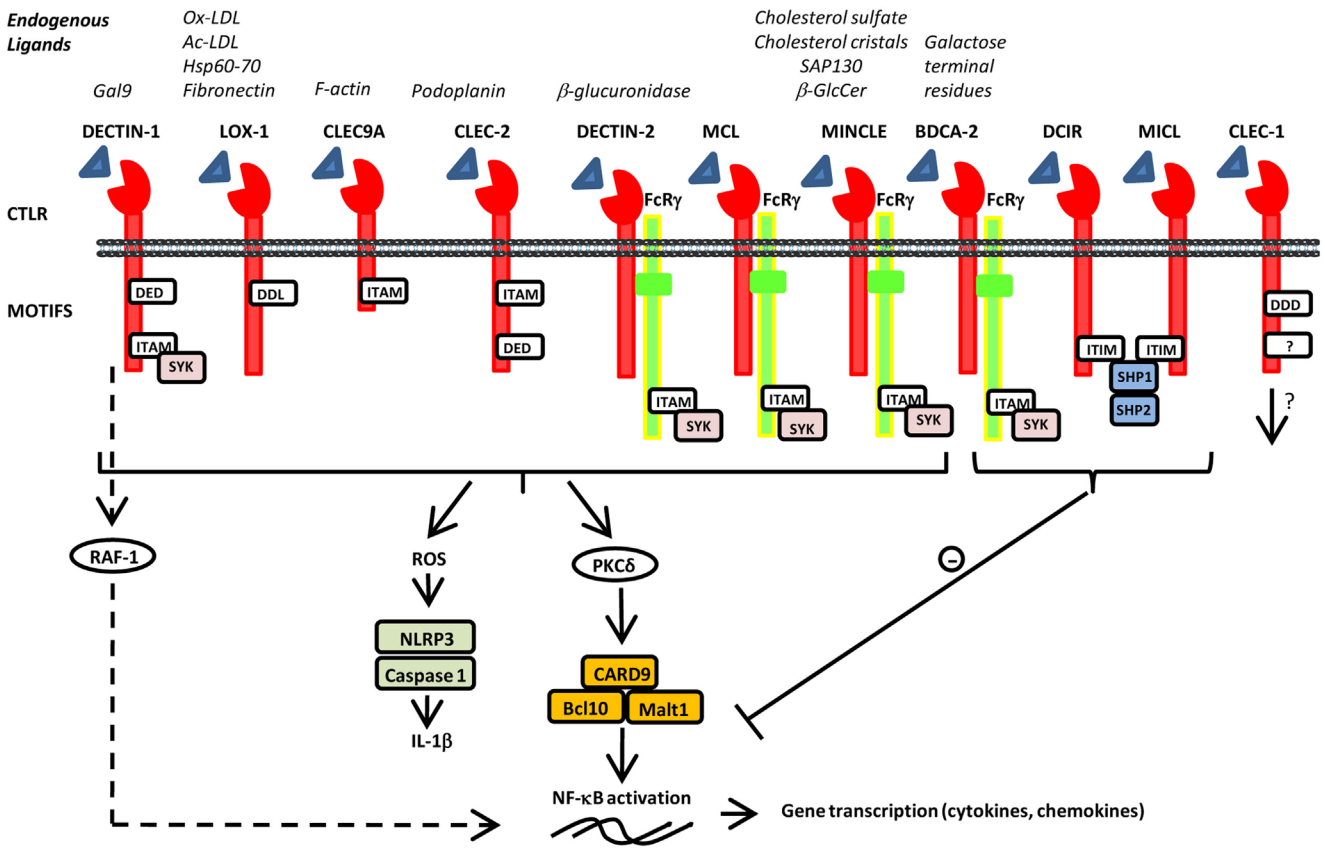

FIGURE 1 | Schematic representation of various C-type lectin-like receptors (CTLRs) and selected endogenous ligands and signals. CTLRs are composed of an extracellular C-type lectin-like domain able to recognize various endogenous ligands and signal directly, through integral motifs in their cytoplasmic tails or indirectly through association with FcRγ. They can also contain a tri-acidic domain DED or DDD important for phagocytosis. Activation of immune-receptor tyrosine-based activation motif (ITAM) leads to the recruitment and activation of SYK family kinases. Subsequent activation of the CARD9–Bcl10–Malt1 complex through PKδ induces NF-κB activation and gene transcription of various cytokine and chemokines. Furthermore, SYK induces reactive oxygen species production and inflammasome activation *via* NLRP3 and Caspase 1 leading to IL-1β production. Alternative pathway of signalization independently of SYK has been reported for dendritic cell-associated C-type lectin-1 (DECTIN-1) *via* RAF-1 to finely regulate NF-κB activation. By contrast, activation of immune-receptor tyrosine-based inhibition motif (ITIM) induces the recruitment and activation of protein tyrosine phosphatases such as SHP-1 and SHP-2 and the dephosphorylation of motifs to inhibit cellular activation mediated by other immunoreceptors.

CTLRs "Dectin" families were best known for their involvement in host defense as referred in these excellent reviews (1–4, 8, 9). However, over recent years, these receptors have gained growing interest for their ability to respond also to a wide variety of endogenous ligands (**Figure 1**). Identification of self-glycans, lipids, or proteins expressed or released by modified or damaged cells reinforced the hypothesis for their implication in sterile inflammation whose dysregulation foster the development of wide range of diseases (10). In this mini review, we aim to focus on some of the CTLRs of the "Dendritic cell-associated C-type lectin (Dectin)" families, discussing the recent discoveries on their implication in the control of tissue injury, autoimmune diseases, or tumorigenesis. In addition, we will underscore their therapeutic potential and impact on human health.

"(DECTIN-1)" FAMILY

DECTIN-1 (Alias CLEC7A, CLECSF12, CANDF4, CD369, BGR)

The CTLR, DECTIN-1 has been reported to be enhanced by pro-inflammatory conditions (11, 12) and to be a potent inducer of Th1 and/or Th17 responses in response to pathogens (2). Thereby, pathogenic ligands of DECTIN-1 are currently used to bolster immune responses notably in cancer. For example, administration of β glucans was shown to inhibit tumor growth in murine carcinoma models (13–15), in human melanoma, neuroblastoma, mastocytosis, and lymphoma xenograft models (16, 17) and in ovarian (18, 19), breast (20), lung (14, 21–23), and gastric cancer (19, 24). Mechanistically, β glucans were shown to convert immunosuppressive macrophages into an M1-like antitumoral phenotype (25), to promote NK (26) and $CD8^+$ T cell cytotoxicity (27) as well as a decrease in myeloid-derived suppressor cells and regulatory T cells (13, 28). Interestingly, Zhao et al. recently reported that β glucans upregulate particularly the expression of TNFSF15 and OX40L in DCs in mice, thus promoting efficient Th9 priming and potent anti-melanoma response following vaccination (29). On the contrary, some investigations have described an inhibitory function of DECTIN-1 in sterile inflammation notably during hepatic fibrosis and hepatocellular carcinoma (30). Authors showed that DECTIN-1 inhibit TLR4 signaling and downstream inflammation such as TNFα, IL-6, and chemokines secretion (30). Moreover, DECTIN-1 was reported to be associated with mechanisms of peritumoral immune tolerance by programming suppressive macrophages in pancreatic ductal adenocarcinoma (31). Strikingly, they showed that blockade of DECTIN-1 or its endogenous ligand Galectin-9, both strongly expressed on infiltrating myeloid cells and tumor, delayed tumor progression and extended mice survival. A similar tolerogenic signal of DECTIN-1 has been shown in myeloid cells in response to mucus in the intestine through interaction with Galectin-3 (32). In addition, DECTIN-1-deficient mice were described to exacerbate inflammation in a model of colitis suggesting an important role of DECTIN-1 in gut homeostasis (33). Therefore, DECTIN-1 seems to act as double-edged swords on the regulation of inflammation. Such discrepancy may depend of the type of the response, the nature and the property of the ligands, and of the complex signal network that integrates diverse engaged PRRs.

LOX-1 (Alias OLR-1, CLEC8A)

The CTLR lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) is particularly expressed by endothelial cells and platelets and is upregulated during inflammatory and pathological conditions (34–36). By recognizing oxidized and acetylated low-density lipoproteins, LOX-1 is largely described to play critical functions in vascular diseases, including atherosclerosis (37). However, recent investigations have revealed that LOX-1 is also expressed by human macrophages (38) and DCs (39), and its triggering increases secretion of IL-6 to potentiate B-cell class-switch (39). Moreover, LOX-1 enhances CCR10, APRIL, and BAFF secretion for plasma cell differentiation and migration to mucosal site. In line with these findings, targeting influenza histocompatibility antigen-1 to LOX-1 elicits antigen-specific protective antibody response to virus in macaques suggesting a good candidate for vaccine development (39). Besides, several studies have reported a high expression of LOX-1 in various tumors including gastric (40), colorectal (41), and prostate (42) cancers, which correlates with a poor prognosis in patients (40). Functionally, LOX-1 was shown to promote tumor angiogenesis (42), metastasis (43), and the migration and invasion of gastric cancer cells by notably driving epithelial–mesenchymal transition (40). Interestingly, LOX-1 was recently identified to be particularly expressed by potent polymorphonuclear myeloid-derived suppressor cells from blood and tumor of patients with non-small cell lung or head neck cancer and to be associated with worse survival (44). In fact, LOX-1 expression seems to be upregulated following endoplasmic reticulum stress that occurs during hypoxia or nutrient deprivation inside tumors. These data render this marker an attractive therapeutic target as well as a diagnostic tool for cancer screening (41).

CLEC-1 (Alias CLEC1, CLEC1A)

Although the C-type lectin-like receptor-1 (CLEC-1) was identified a long time ago (45, 46), the downstream signaling and ligand(s) remain uncharacterized (8, 47). We and others described CLEC-1 expression in human and rodent by myeloid cells such as monocytes, DC, and macrophages but also by endothelial cells (8, 9, 46, 48). CLEC-1 expression is decreased by pro-inflammatory stimuli and is enhanced by TGFβ (8, 9, 48). Interestingly, CLEC-1 was found to be expressed mostly intracellular particularly in human endothelial cells and neutrophils (8, 9), suggesting the requirement of particular conditions for cell-surface expression or for recycling from intracellular pools (7). Alternatively, CLEC-1 may play a role in intracellular organelles. Using CLEC-1-deficient rodents, we showed that disruption of CLEC-1 signaling enhances *Il12p40* subunit expression in DCs and accordingly exacerbates downstream $CD4^+$ Th1 and Th17 responses following *in vivo* immunization with exogeneous antigens (9, 48).

CLEC-2 (Alias CLEC2, CLEC2B, CLEC1B)

C-type lectin-like receptor 2 (CLEC-2) is found on platelets and DCs and is largely described for its interaction with its

endogeneous ligand podoplanin expressed by lymphatic endothelial cells, myeloid cells, and fibroblast reticular cells (2). The CLEC-2/Podoplanin axis was shown to be critical in platelet activation (49), lymph node microarchitecture (50, 51), reticular network (52), and vascular integrity. Besides, this interaction promotes tumor cell-induced platelet aggregation, tumor growth, and metastasis (53–56) in various types of cancer including brain, lung, and larynx (57–60). Furthermore, CLEC-2 is enhanced by inflammation, promotes DC migration (61) and together with LPS enhances the production of the anti-inflammatory cytokine IL-10 suggesting also a role in the resolution of inflammation (62).

MICL (Alias CLEC12A, DCAL-2, CLL1, CLL-1, KLRL1)

Myeloid inhibitory C-type lectin-like receptor (MICL) is expressed predominantly by granulocytes and monocytes, and its expression is downregulated by pro-inflammatory stimuli (63–65). MICL recruits inhibitory phosphatases and again seems to differently shape T-cell responses according to the cross talk with simultaneous PRR signals. Chen and colleges demonstrated that co-engagement of MICL with TLR4 suppress IL-12 expression in human DCs and downstream Th1 polarization whereas co-engagement with CD40 does the opposite (66). Interestingly, putative endogenous ligands of MICL were identified on various mouse tissues in steady-state conditions, proposing a role for MICL in the control of homeostasis and self-tolerance (65). Corroborating this notion, an inhibitory function for MICL has been put in light in an *in vivo* model of induced rheumatoid arthritis (67). In an original way, MICL was proposed to modulate myeloid cell activation threshold by acting as an autoantigen during arthritis development (67).

CLEC9A (Alias DNGR1, DNGR-1, CD370)

CLEC9A is selectively expressed on the mouse subsets of $CD8\alpha^+$ and $CD103^+$ DCs, and on their human $BDCA3^+$ DCs counterparts (68). CLEC9A expression is lost further TLR-induced maturation. Importantly, CLEC9A by recognizing F-actin released by necrotic cells is capable of internalizing bound dead cell-associated antigens for cross-presentation to $CD8^+$ T cells (69–71). Thereby, CLEC9A has been demonstrated to be a powerful target for peptide vaccination to boost antitumor immunity (72, 73). Interestingly, it has recently been shown that necrotic debris that accumulated during atherosclerosis development, trigger through CLEC9A, the downregulation of the anti-inflammatory cytokine IL-10 and the disease progression (74).

"DECTIN-2" FAMILY

DCIR (Alias CLEC4A, CLECSF6, CD367, LLIR)

DC immunoreceptor (DCIR) is expressed on monocytes, neutrophils, DC, and plasmacytoid DCs, and its expression is decreased by pro-inflammatory stimuli (75). The human genome encodes only a single DCIR gene, whereas the mouse genome presents four DCIR-like genes (DCIR1–4) (76). DCIR *via* its canonical ITIM domain is largely recognized to exert inhibitory cross talk with other PRRs to maintain immune homeostasis and prevent excessive detrimental inflammation and immunopathogenesis (77–79). DCIR inhibits TLR8-induced IL-12 and TNFα production in human moDCs following cross-linking with monoclonal antibody (79). Furthermore, DCIR1 KO mice develop a late spontaneous autoimmune disease associated with elevated levels of autoantibodies, are more susceptible to collagen-induced arthritis, and aggravated experimental autoimmune encephalomyelitis (80, 81). These effects were described to be mediated at least by unrestrained growth of DC population in these mice. However, in support for a role of DCIR in tempering DC activation, a recent study demonstrated that DCIR2 selectively expressed by mouse $CD8\alpha^-$ DCs, strongly moderates pro-inflammatory and downstream T-cell responses (82). *In vivo*, DCIR2-deficient mice are more susceptible to endotoxic shock and aggravate experimental autoimmune encephalomyelitis development by increasing both Th1 and Th17 differentiation. Authors demonstrated that putative endogeneous ligands of DCIR are expressed also on cell surface of DCs. In line with these data, DCIR2 was described in DCs to sustain STAT-1 type I IFN signaling leading to a reduction of IL-12p70 production and Th1 differentiation in response to endogeneous ligand(s) released during cell culture (83). Therefore, by regulating also the IFN responses, DCIR may be a critical player in the control of a number of inflammatory diseases. Interestingly, DCIR was also reported to bind to commensal intestinal microbes (84). However, DCIR-deficient mice only exhibit a slightly increased severity of colitis in a dextran sulfate sodium model (84).

DECTIN-2 (Alias CLEC6A, CLEC4N, CLECSF10)

Several studies suggest a role for DC-associated C-type lectin-2 (DECTIN-2) in the inhibition of sterile inflammation. DECTIN-2 is enhanced in pro-inflammatory conditions and was shown notably to bind to a putative ligand on regulatory $CD4^+CD25^+$ T cells to mediate ultraviolet radiation-induced tolerance (85, 86). In addition, DECTIN-2 recognizes glycan mannose on the lysosomal enzyme β-glucuronidase, known to moderate arthritis pathogenesis by preventing accumulation of pro-inflammatory glycosaminoglycans within inflamed joint tissue (87–90). Thus, β-glucuronidase released by dead myeloid cells following tissue damage may act *via* DECTIN-2 as an inhibitory loop in DCs to temper inflammation (91). Besides, polymorphism of this enzyme was reported to be associated with mucopolysaccharidoses characterized by a pro-inflammatory response (92). In addition, a role for DECTIN-2 in suppression of liver metastasis has been highlighted by its ability to phagocytose cancer cells *via* CD11b F4/80 Kupffer cells during extravasation step (93).

BDCA-2 (Alias CLEC4C, BDCA2, CD303, CLECSF11, CLECSF7)

Interestingly, blood DC antigen-2 (BDCA-2) is the most specific marker for human plasmacytoid DC but intriguingly is not

expressed in mice (94). Expression of BDCA-2 is downregulated following maturation (95). Surprisingly, unlike many other ITAM-coupled receptors, signaling through BDCA-2 inhibits activation of the NF-κB pathway and the production of type I IFNs and cytokines in response to TLR9 ligands or following recognition of galactose terminal residues notably expressed on tumor cells (94, 96, 97). BDCA-2 engagement was also shown to block TRAIL-mediated cytotoxic activity (98). In an interestingly way, BDCA-2 was suggested to function as an Fc receptor by binding glycans on immunoglobulins G (99) and thus, dampens down inflammation in response to rising levels of serum immunoglobulins G.

MINCLE (Alias CLEC4E, CLECSF9)

Macrophage-inducible C-type lectin (MINCLE) is an ITAM-coupled CTLR that forms a heterodimer with the macrophage C-type lectin (MCL). MINCLE expression is enhanced after exposure to pro-inflammatory stimuli or cellular stresses and is translocated to the cell surface *via* interaction with the stalk region of MCL (100–104). MINCLE was shown to activate in DCs both NF-κB and inflammasome to greatly enhance IL-1β expression in synergy with TLR7/8 (R848) or following *in vivo* immunization with Freund adjuvant (105, 106). MINCLE senses self-damage by recognizing "unfamiliar" glycolipids that are not present in the extracellular milieu under normal, healthy conditions. For example, MINCLE was reported to bind crystalline cholesterol present in atheriosclerotic plaques that are associated with inflammation and macrophage infiltrates (107). Likewise, as depicted in **Figure 2**, MINCLE is enhanced on plasmacytoid DCs following skin damage and by recognizing cholesterol sulfate, induces IL-1 α and β secretion, and promotes skin allergy and allergic contact dermatitis (108). Moreover, MINCLE was also reported to bind to the ubiquitous intracellular metabolite β-glucosylceramide released by damaged cells to promote production of pro-inflammatory cytokines by myeloid cells (109). In an opposite way, recent investigations have revealed that MINCLE rather than purely inducing pro-inflammatory responses can also promote the expression of the anti-inflammatory cytokines IL-10 (110). In addition, MINCLE was reported to counter regulate pro-inflammatory signaling pathways mediated by DECTIN-1 to temper IL12p35 production (6, 111). Therefore, MINCLE seems to also exert opposite role on immune responses depending of the ligands and PRR interference. This dual effect is illustrated by the recognition by MINCLE of the spliceosome-associated protein 130 (SAP130), a component of small nuclear ribonucleoprotein released during non homeostatic cell death. On one hand, MINCLE/SAP130 axis was shown to be involved in the pathogenesis of inflammation during tissue damages (112) or ischemia/reperfusion (113, 114) and to contribute to the development of experimental autoimmune uveoretinitis (115). This pro-inflammatory side of MINCLE is supported by a high expression of MINCLE in patients with rheumatoid arthritis (116) and by the link to arthritis of the rat chromosome 4q42 encoding *Mincle* (117). On the other hand, in the context of cancer, MINCLE/SAP130 axis was reported to be pro-tumorigenic in mouse and human pancreatic ductal adenocarcinoma (118). Both MINCLE and SAP130 released by programmed necrosis are highly expressed in mouse and human carcinoma and as depicted in **Figure 2**, this interaction leads to an immunosuppressive reprogramming of infiltrating myeloid cells (118). Future research is required to provide insight as to how MINCLE needs to integrate with other PRR signals to differently define the type of immune response.

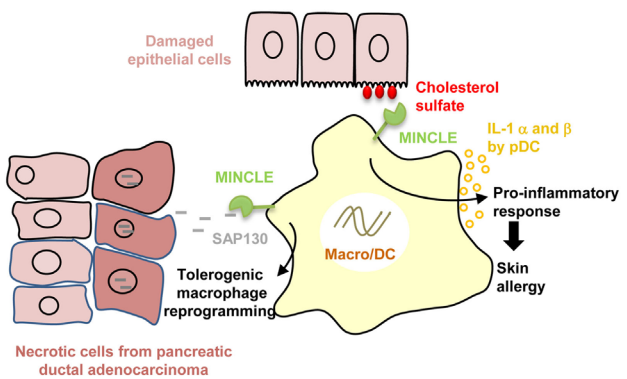

FIGURE 2 | Dual role of macrophage-inducible C-type lectin (MINCLE) in disease pathogenesis. Recognition of cholesterol sulfate by MINCLE whose expression is increased in plasmacytoid dendritic cells (DC) following skin damage, induces IL-1 α and β secretion, and promotes skin allergy and allergic contact dermatitis. By contrast, MINCLE recognition of spliceosome-associated protein 130 (SAP130) released by necrotic cancer cells leads to tolerogenic tumor-infiltrating macrophage reprogramming in pancreatic ductal adenocarcinoma.

THERAPEUTIC POTENTIAL OF CTLRs

Therefore, by their capacity to present antigen and ensure the balance between cellular activation and suppression, CTLRs have emerged as challenging pharmacological targets to treat a wide variety of diseases governed by sterile inflammation including cancers, autoimmune diseases or allergy (1, 119). Ligands such as carbohydrate structures, antibodies, or mimetic peptides could be therapeutically exploited as agonists or antagonists of CTLR signaling. As previously mentioned, the DECTIN-1 agonist β-glucans is used to elicit of potent antitumor immune responses in various types of cancer (14, 16–24). Furthermore, CTLRs such as DEC-205 (120, 121) or CLEC9A (71) have been exploited for the *in vivo* delivery target of vaccine antigens in cancer (122). In addition, synthetic ligands of MINCLE were generated to specifically enhance immune response (102). Besides, several specific antibodies generated against cancer-specific highly glycosylated podoplanin were shown to efficiently block the CLEC-2/Podoplanin interaction, subsequent platelet aggregation and tumor metastasis (123–128). Importantly, a particular antibody that reacts with podoplanin-expressing cancer cells but not with the one from normal cells has been successfully generated and will be useful for molecular targeting therapy against podoplanin-expressing cancer cells only (126).

However, since CTLRs have overlapping ligands that induce distinct and even contrasting immune responses, antibodies targeting specific CTLRs could be more appropriate. Also, inhibitors such as recombinant peptide spanning the CTLR binding region and modulating the receptor–ligand interaction could be considered. Only a few drug-like molecules have been developed for the CTLR family (129) but studies indicate high *in silico* druggability scores as well as high experimental hit rates from peptide fragment screenings (130, 131).

To conclude, CTLR modulation seems to represent promising strategy for disease management although attempts at identifying endogenous ligands as well as efforts to elucidate their role in sterile inflammation are still warrant.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and approved it for publication.

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AmpliSeq Screening of Genes Encoding the C-Type Lectin Receptors and Their Signaling Components Reveals a Common Variant in *MASP1* Associated with Pulmonary Tuberculosis in an Indian Population

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Tuberculosis (TB) is a multifactorial disease governed by bacterial, host and environmental factors. On the host side, growing evidence shows the crucial role that genetic variants play in the susceptibility to *Mycobacterium tuberculosis* (Mtb) infection. Such polymorphisms have been described in genes encoding for different cytokines and pattern recognition receptors (PRR), including numerous Toll-like receptors (TLRs). In recent years, several members of the C-type lectin receptors (CTLRs) have been identified as key PRRs in TB pathogenesis. Nevertheless, studies to date have only addressed particular genetic polymorphisms in these receptors or their related pathways in relation with TB. In the present study, we screened the main CTLR gene clusters as well as CTLR pathway-related genes for genetic variation associated with pulmonary tuberculosis (PTB). This case-control study comprised 144 newly diagnosed pulmonary TB patients and 181 healthy controls recruited at the Bhagwan Mahavir Medical Research Center (BMMRC), Hyderabad, India. A two-stage study was employed in which an explorative AmpliSeq-based screening was followed by a validation phase using iPLEX MassARRAY. Our results revealed one SNP (rs3774275) in *MASP1* significantly associated with PTB in our population (joint analysis $p = 0.0028$). Furthermore, serum levels of MASP1 were significantly elevated in TB patients when compared to healthy controls. Moreover, in the present study we could observe an impact of increased MASP1 levels on the lectin pathway complement activity *in vitro*. In conclusion, our results demonstrate a significant association of *MASP1* polymorphism rs3774275 and MASP1 serum levels with the

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development of pulmonary TB. The present work contributes to our understanding of host-Mtb interaction and reinforces the critical significance of mannose-binding lectin and the lectin-complement pathway in Mtb pathogenesis. Moreover, it proposes a *MASP1* polymorphism as a potential genetic marker for TB resistance.

Keywords: C-type lectin receptor, MASP1, pulmonary tuberculosis, complement, AmpliSeq

1. INTRODUCTION

Tuberculosis (TB) remains a major global health problem affecting millions of people each year and ranking as the first leading cause of death from an infectious disease worldwide (1). *Mycobacterium tuberculosis* (Mtb), a highly successful intracellular pathogen, is transmitted typically through aerosols into the respiratory system, thereby developing an infection. It has been well established that both innate and adaptive immune responses are required for host control of tuberculosis infection (2). In TB pathogenesis, the host cellular immune response determines whether an infection is arrested as latent or persistent infection or progresses to the next stages, i.e., the active TB infection (3). Efficient cell-mediated immunity hinders tuberculosis infection by permanently arresting the infection at latent or persistent stage, but if the initial infection in the lung is not controlled or if the immune system becomes weakened, Mtb can cause active pulmonary and to a lesser extend extrapulmonary tuberculosis (4).

Several pattern recognition receptors (PRRs) expressed on various immune cells play a major role in the recognition of Mtb and transduce signals either directly *via* receptor ligation or through various adaptor molecules to initiate an appropriate immune response (5). The PRR family of Toll-like receptors (TLRs) has been well described for their contribution to the Mtb-associated immune responses (6–9). In addition, the family of C-type lectin receptors (CTLRs) has been recently discovered to also recognize Mtb, leading to a considerable modulation of Mtb-induced immune responses and have secured a prominent and ongoing spot in TB research. Potent Mtb associated molecular patterns, including trehalose-6,6-dimycolate (TDM) and mannose-capped lipoarabinomannan (ManLAM), are recognized by CTLRs such as Mincle, MCL, and Dectin-2 (10–13). Moreover, Dectin-1 has been shown to be important for generating reactive oxygen species and other proinflammatory responses (14–16), while the mannose-binding lectin (MBL) interacts with Mtb directly to activate the lectin pathway of the complement system (17). Therefore, CTLRs binding to Mtb are associated with the induction or the modulation of several important signaling pathways such as the Syk-CARD9-Bcl10-MALT1 pathway, phagosome maturation, and complement activation (18–22).

Susceptibility to Mtb has a definite genetic component and host-genetic variation is thought not only to determine infection outcome, but also the risk of disease progression (3). Therefore, variants of genes involved in innate host-defense mechanisms have been associated with host susceptibility to TB (23). Various genome-wide association studies and candidate-gene studies demonstrate that several single nucleotide polymorphisms (SNPs) in certain genes are associated with TB susceptibility (24–26). In particular, SNPs in TLRs and their pathway adaptors have been widely associated with TB (27, 28). Additionally, particular SNPs in genes of the CTLR family have been investigated in case–control studies and found to be associated with TB susceptibility as reviewed in Goyal et al. (11). These include variants in the genes encoding for MBL and MASP2 (29, 30), which play a major role in the activation of the lectin complement pathway (Figure S1 in Supplementary Material). However, comprehensive studies addressing susceptibility to Mtb in association with genetic variants in the entire set of CTLR genes and their related pathways have not been performed so far. Here, we aimed to identify specific SNPs in the genes of CTLRs or/and in the genes of the related pathway adaptors that may have an impact on TB susceptibility and/or disease severity in a well-phenotyped Indian population (31, 32) from Hyderabad, where the TB prevalence is very high (33). An AmpliSeq-based approach was used as innovative technique in a two-stage process to screen for relevant polymorphisms in 33 genes. In this study, we identified an intronic SNP in the MBL-associated serine protease (*MASP1*) gene, an important component of the lectin pathway of the complement, associated with pulmonary tuberculosis (PTB) infection in our population.

Identification of genetic variations among genes of the CTLR pathways that influence the susceptibility to TB may lead to a better understanding of the pathogenesis and the development of novel strategies for the prevention and treatment of this significant infectious disease.

2. MATERIAL AND METHODS

2.1. Subject and Samples

We carried out a case–control study to determine whether common variants in genes involved in CTLR-dependent responses might be associated with the development of PTB in an Indian population. For that purpose, 144 PTB case patients and 181 unrelated healthy controls were recruited at the Mahavir Hospital and Research Center in Hyderabad (India) between July 2011 and November 2013. Criteria for inclusion of cases were: (i) admission in the Hyderabad Directly Observed Treatment, Short-course (DOTS) program at Mahavir Hospital, and (ii) new diagnosis of pulmonary sputum smear positive TB disease. The diagnostic criterion for PTB was defined as the presence of one of the following: at least 2 initial sputum smear examinations positive for Acid-Fast Bacilli (AFB) or sputum examination positive for AFB and radiographic abnormalities consistent with active PTB (34). Criteria for inclusion as healthy control were: (i) absence of apparent acute or chronic pulmonary diseases

or diseases of other origin, (ii) clinically in good health at the time of enrollment, and (iii) a negative history of TB disease. All subjects were from the same geographical origin, and residing in Hyderabad.

2.2. Ethics Statement

All study participants gave written informed consent in accordance with the Declaration of Helsinki. The study was approved by the institutional ethics committee for bio-medical research at the Bhagwan Mahavir Medical Research Center, Hyderabad, India (date: March 11, 2011).

2.3. Study Design

This study was divided in two phases: a genomic stage with an AmpliSeq-based discovery approach followed by a validation through iPLEX MassARRAY genotyping of candidate SNPs. In Phase 1 (discovery), we used 40 samples from each cases and controls group for Next Generation Sequencing (NGS)-profiling of selected regions (detailed in the AmpliSeq Library Preparation section below). For this initial phase, the 40 case samples were chosen based on the severity of the disease (determined by the chest X-rays and sputum microscopy). The population characteristics are provided in **Table 1**. After NGS, association analysis was performed at allele and genotype level. Candidate SNPs were then subjected to a second phase (validation), comprising the genotyping of all remaining cohort samples for the candidate SNPs obtained from the explorative approach. The genotyping was performed using the MassARRAY iPLEX Platform (Agena Bioscience).

2.4. DNA Extraction

Peripheral blood samples were collected from 144 PTB patients and 181 unrelated healthy controls. DNA was isolated from blood samples using QIAamp DNA Blood Mini Kit (Qiagen; Hilden-Germany) following manufacturer's instructions. DNA samples were stored at −20°C until further usage.

2.5. Discovery Phase

2.5.1. AmpliSeq Library Preparation

The targets for this study included the C-type lectin receptor genes encoded in two gene clusters of chromosome 12: the Dectin-1 cluster (221 kb, comprising *MICL*, *CLEC2*, *CLEC9A*, *CLEC12B*, *CLEC1*, *Dectin-1*, and *LOX1*), and the Dectin-2 cluster (812 kb, comprising *BDCA2*, *DCIR*, *Dectin-2*, *MCL*, and *MINCLE*). Entire genes (including introns and exons) as well as several intergenic regions (including 1 kb of the 5′ flanking regions of all genes) of both clusters were selected for sequencing. In addition, an extensive literature search was performed to ensure the inclusion of SNPs in other CTLRs or adaptors in their signaling pathways that have been already associated with PTB and/or fungal infections as well as lung infections (35–48) as targets in our AmpliSeq panel (see Table S1 in Supplementary Material for final targets list). This selection was supplemented with Tag-SNPs, SNPs that were informative of common gene variation, of the other important CTLR receptors/adaptors. Tag-SNPs lists were extracted from the UCSC Genome Browser (https://genome.ucsc.edu/), using the Affymetrix Genome-Wide Human SNP Array 6.0 (Assembly GRCh37/hg19) as reference. All targeted regions were encoded in a bed file for megaplex primer pair design using the `AmpliSeq Designer` version 3.0.1 (Thermo Fisher Scientific, USA). The design resulted in 83% effective coverage of the targeted regions. The final AmpliSeq design (Table S1 in Supplementary Material) comprised 1,470 amplicons, with expected amplicon sizes between 125 and 275 bp, divided in two pools of 739 and 731 amplicons.

DNA-AmpliSeq libraries were prepared using the Ion AmpliSeq™ Library Kit 2.0 (Thermo Fisher Scientific, USA), following manufacturers' instructions. In brief, 10 ng of DNA (for each pool) from 80 samples (40 cases/40 controls) were used as input for the HiFi-amplification with the designed primer mix. Resulting PCR products were subjected to partial primer digestion using FuPa reagent and subsequently ligated to barcoded Ion adapters (Ion Xpress™ Barcode Adapters Kit; Thermo Fisher Scientific, USA). The Ion Library Equalizer Kit was used to normalize library concentration to 100 pM, and AmpliSeq libraries were pooled for sequencing.

TABLE 1 | Summary of case–control study characteristics.

Parameter	Cases (n = 144)	Controls (n = 181)	p-Value
Age (years)	27 ± 11	31 ± 10	0.0008
Gender (M/F)	71/73	103/78	0.1808
BMI (kg/m^2)	16 ± 2.6	24 ± 4.7	<2.2e−16
Smoking (yes/no)	32/112	29/151	0.1980

Statistical analysis was performed using T-test (for age and BMI) and exact Fisher-test (for gender and smoking).

2.5.2. Sequencing

Library template pools were clonally amplified on Ion Sphere particles using the Ion PI™ Template OT2 200 Kit v2 on the instrument Ion OneTouch™ 2 System (Thermo Fisher Scientific, USA). The sequencing chips were prepared using the Ion PI Sequencing 200 Kit v2 (Thermo Fisher Scientific, USA), and sequenced on an Ion Proton Sequencer (Thermo Fisher Scientific, USA). In total, 80 samples were multiplexed on 2 chips for sequencing. The raw sequence data in bam format have been stored in the Sequence Read Archive (SRA) at National Center for Biotechnology Information (NCBI), and can be accessed at NCBI homepage (https://www.ncbi.nlm.nih.gov/; accession number: SRP123407).

2.5.3. SNP Identification

The quality of the raw data in fastq format was checked using `FastQC` (http://www.bioinformatics.babraham.ac.uk/projects/fastqc) and, thereafter, adapter sequences and low-quality regions (Phred Q score < 20) were trimmed using `cutadapt` (49). The trimmed reads for each sample were mapped onto the hg19 reference genome with `Bowtie2` (50).

To identify SNPs, the Variant Caller plugin of the `Partek Genomics Suite` 6.6 (Partek Inc., St Louis, MO, USA) was used. SNPs were then kept for further analyses according to a minimal sequencing depth (>20 reads/sample), Minor Allele Frequency (MAF > 0.02) and the existence of Hardy-Weinberg Equilibrium (HWE) in control samples. All known biallelic SNPs passing these filters were then subjected to association analyses.

2.5.4. Association Study

In order to control population stratification in the discovery phase, we used the LASER (Locating Ancestry from SEquence Reads) v. 2.01 software (51). All entries corresponding to the superpopulation code SAS (South Asian) were obtained from the 1000 Genomes Project (ftp://ftp.1000genomes.ebi.ac.uk/vol1/ftp/release/20130502/). These included datasets from the following populations: Gujarati (GIH), Punjabi (PJL), Bengali (BEB), Sri Lankan Tamil (STU), and Telugu (ITU). All known SNPs annotated by the 1000 Genomes Project were retrieved as vcf files and filtered for the amplicon regions covered in our AmpliSeq approach using VCFtools (52) and for a MAF > 0.05 using the GenomeAnalysisToolKit (GATK v. 3.2) (53, 54). The filtered SNP list was then pruned using PLINK (55) in order to exclude less informative SNPs in linkage disequilibrium (LD). A total of 161 resulting SNPs from the reference populations were then used as input for LASER (51) to define the PCA space and derive the background coordinates for ancestry adjustment.

Using PLINK (55), we used logistic regression models for association analysis of genotypes under the assumption of an additive inheritance model, using the first coordinate to adjust for population stratification. Success of stratification correction was empirically assessed *via* quantile-quantile plots and statistical inflation estimates (lambda) using the gap package (56, 57) for R (58); R Development Core Team, 2013; http://www.R-project.org/. Chi-square tests were also applied to test for allele frequency differences.

2.6. Validation Phase

2.6.1. iPLEX MassARRAY Genotyping

All SNPs with a statistical significance for association in the discovery ($p < 0.2$), and a nominal significance ($p < 0.05$) in the allele frequency distribution between groups were kept for follow-up studies in the validation phase. In this stage, 245 additional samples (141 controls and 104 TB patients) from the cohort were analyzed by iPLEX MassARRAY at Agena Bioscience GmbH (Hamburg). Statistical analysis of all samples was performed using logistic regressions with PLINK (55).

2.7. Meta-Analysis of Association Results

METAL (59) was used to combine the *per* SNP results from association studies in the discovery and validation phases. For this, the joint analysis used the *p*-values across the two phases taking sample size and direction of effect into account.

2.8. Measurement of MASP1, MASP3, and MAp44 Levels in Serum

The serum concentrations of MASP1, MASP3, and MAp44 in 106 healthy controls and 99 TB patients were measured using commercially available ELISA kits following the manufacturer's instructions (Human MASP1 ELISA Kit, Cloud-Clone Corp., Human MASP3 ELISA Kit, Hycult biotech Inc., Human MAp44 ELISA Kit, Hycult biotech Inc.). Diluted serum samples were incubated in the coated plates for the recommended time period and the amount of protein sandwiched was detected by a conjugated antibody and subsequent measurement of absorbance at 450 nm.

2.9. Measurement of Lectin Pathway Complement Activity

Serum from healthy donor blood samples was obtained by short centrifugation at 3,000 *g* at 4°C for 10 min. The serum MASP1 and MBL levels were measured by ELISA (Human MASP-1 ELISA Kit, Cloud-Clone Corp., Hycult Biotech Human MBL ELISA kit). To investigate the effect of increased MASP1 levels on complement function, recombinant human MASP1 (Creative BioMart MASP1-137H) was added at different concentrations (+13% rhMASP1, +26% rhMASP1, or +52% rhMASP1) to the donor serum, and complement activity after MBL pathway activation was measured using a commercially available ELISA kit (Complement system MBL pathway WIESLAB®) following manufacturer's instructions. Briefly, six diluted serum samples were measured in duplicate along with blank, positive and negative controls, and incubated at 37°C for 1 h. After washing, the formation of terminal complement complex C5b-9 was detected using conjugated antibody and absorbance was measured at 405 nm on a microplate reader (TECAN SpectraFluor Plus).

3. RESULTS

3.1. Discovery Study (Phase I)

In the discovery phase of this study, we screened the Dectin-1 and Dectin-2 gene clusters, as well as other CTLR-relevant genomic regions for potential variants that might be associated with pulmonary tuberculosis in an Indian population. Our AmpliSeq design covered 83% of the targeted regions, and yielded over six hundred known SNPs that passed the filters for sequencing depth, MAF and HWE (Table S2 in Supplementary Material). Since a heterogeneous ancestral background can be presumed for our study population (Figure S2A in Supplementary Material), we corrected for potential stratification effects using the first coordinate derived by the LASER v2.1 software (51).

No inflation of association results was evident based on quantile-quantile plots and lambda results (*lambda* = 1.004; see Figure S2B in Supplementary Material). After association analysis, we selected 18 common variants as candidate SNPs for follow up studies in the next phase (Table S3 in Supplementary Material). These included 2 exonic SNPs in *CD207* (chr. 2), 1 SNP in *MASP1* (chr. 3), 1 SNP in *SFTPA1* (chr. 10), and 14 SNPs in CTLRs of the Dectin-clusters in chromosome 12, including an intronic variant in *CLEC7A* (*Dectin1*), a missense variant in *CLEC1B*, and several variants in *CLEC12A* (*MICL*) and *CLEC12B*. All these variants showed differences at nominal significance level in their allele frequency distribution between cases and controls, and yielded top *p*-values (cutoff $p < 0.2$) when addressing their genotype distribution after ancestry adjustment using logistic regression models (Table S3 in Supplementary Material).

3.2. Validation (Phase II): Rs3774275 in MASP1 Is Significantly Associated with TB

Phase II of the study consisted in a MassARRAY-based genotyping of a total of 245 independent samples (141 controls and 104 TB patients) addressing the aforementioned candidate variants. Primer design failed for four of the 18 selected variants, which

were excluded from the validation by the MassARRAY. SNP rs374147676 was monomorphic in the samples from this phase and was removed from further analyses. Among the remaining 13 common variant candidates, only one SNP (rs3774275; *MASP1*) was nominally significant in phase II ($p = 0.0340$, **Table 2**), showing the same direction of effects as in phase I.

Joint analysis of phases I and II confirmed our findings, with a highly significant association of rs3774275 with pulmonary tuberculosis (joint analysis OR = 0.61 95%CI = 0.43–086, $p = 0.0028$; see **Table 2**). The G allele of rs3774275 showed a protective effect (G allele frequency: 39% controls vs. 28% TB patients; **Table 3**). The GG genotype was twice more frequent in the healthy group (15%) than in the TB group (7%) (see **Table 3**).

3.3. Increased MASP1 Levels in Serum of Tuberculosis Patients

Next, we measured the concentrations of MASP1, MASP3 and MAp44 in the serum. Our results show that the mean concentration of MASP1 was significantly higher in TB patients (median $(\tilde{x}) = 8.68$ μg / ml; mean $(\bar{x}) = 9.06$ μg / ml) than in healthy donors ($\tilde{x} = 6.68$ μg / ml; $\bar{x} = 6.99$ μg / ml ; see **Figure 1A**). We could also observe an increase in MAp44 and a decline in MASP3 levels in the serum of cases when compared to controls, although these differences did not reach statistical significance after adjusting for BMI (see **Figure 1A**). The increase of MASP1 in TB patients might suggest an important role of this protein in the immune response against Mtb.

When the MASP1 serum concentrations were analyzed by genotype in each group, we could observe a slightly higher concentration of MASP1 in healthy donors with a GG-genotype ($\tilde{x} = 7.74$ μg / ml; $\bar{x} = 8.63$ μg / ml) as compared to the other 2 genotypes in the same group ($\tilde{x} = 6.13$ μg / ml; $\bar{x} = 6.73$ μg / ml ; see **Figure 1B**). Furthermore, the GG-genotype also exhibited higher MAp44 levels in the control group. However, none of the observed genotype-dependent differences reached statistical significance, probably due to the sample size and the proportion of GG-genotype in our population. Nevertheless, the association of rs3774275 with the MASP1 serum concentration has also been documented in other studies (38, 60), where a GG-genotype has been linked with an increased amount of MASP1, ranging between 11 and 13% over the other genotypes. In our population, we observed a similar increase in the GG-genotype of the healthy study group. Median values of MASP1 were 20% higher for the GG-genotype when compared to the other two genotypes (**Figure 1B**). Interestingly, the MASP1 serum levels of the healthy GG-group were comparable to the concentrations observed in PTB patients.

We next examined radiographic abnormalities in chest X-rays from the TB patients (31) to determine the severity of the disease and to analyze whether the concentrations of MASP1, MASP3, or MAp44 might correlate with the progression of tuberculosis. The radiographic features here analyzed included the number of cavities, the extent of alveolar infiltrates, and the presence of pleural effusion or lymph nodes. These characteristics and the overall percentage of lung affected were previously reported to correlate with TB severity (31). Nevertheless, we could not detect any correlation between the severity-criteria analyzed with neither MASP1 nor MASP3 or MAp44 levels (**Figure 2**). We also failed to observe a clear correlation of MASPs levels with the BMI of the patients (Figure S3 in Supplementary Material).

3.4. MASP1 Levels Influence the Lectin Pathway Complement Activity in vitro

Next, we tested whether small increases in MASP1 concentration, such as those observed in our study, could have any impact on the lectin pathway complement activation. We performed an *in vitro* assay in which we added recombinant human MASP1 to serum samples, and measured the MBL pathway activity using a commercially available ELISA kit. Our results showed a significant increase of the lectin pathway complement activity ($p < 0.05$) after addition of 13% more rhMASP1 to the serum samples (see **Figure 3**). This suggests that even a small increase in MASP1 concentration is sufficient to improve the efficiency

TABLE 2 | List of common variants subjected to validation through MassARRAY, showing the results of the association analysis after phase II, as well as the joint analysis performed integrating data from phases I and II.

SNP_ID	Location				Type of mutation	Alleles		Association (*p*-values)		Effect
	Gene	Chr	Position (GRCh38)	Gene location		Test allele	Other	Phase II MassARRAY	Meta-analysis	
rs741326	*CD207*	2	70831704	Exonic	Missense	**A**	G	0.6727	0.1292	Risk
rs2080390	*CD207*	2	70831095	Exonic	Synonym.	**T**	C	0.7578	0.1726	Risk
rs3774275	*MASP1*	3	187247480	Intronic	Intron var.	**G**	A	**0.0340***	**0.0028****	Protective
rs1914663	*SFTPA1*	10	79612197	Intronic	Intron var.	**T**	C	0.9470	0.2902	Protective
rs76427726	*CLEC12A*	12	9950609	Intronic	Intron var.	**C**	T	0.0995	0.5581	Risk
rs35333643	*CLEC12A*	12	9957832	Intronic	Intron var.	**G**	A	0.2096	0.9281	Risk
rs148864420	*CLEC12A*	12	9959987	Intronic	Intron var.	**A**	C	0.2497	0.8767	Protective
rs648985	*CLEC12A*	12	9963978	Intronic	Intron var.	**C**	G	0.7202	0.5305	Protective
rs2961541	*CLEC12A*	12	9964134	Intronic	Intron var.	**C**	T	0.9937	0.3519	Protective
rs193214822	*CLEC12A*	12	9971188	Intronic	Intron var.	**T**	G	0.2497	0.8767	Protective
rs114421141	*CLEC12B*	12	10007247	Intronic	Intron var.	**C**	T	0.8407	0.4977	Protective
rs79967076	*CLEC12B*	12	10004170	Intronic	Intron var.	**A**	G	0.6343	0.6586	Protective
rs112915340	*CLEC12B*	12	10018224	Intronic	Intron var.	**G**	T	0.9492	0.4278	Protective

*Our results showed one SNP (rs3774275 in MASP1) significantly associated with pulmonary tuberculosis (*p < 0.05; **p < 0.01). Effect of test allele is also shown.*

of the MBL-dependent complement activity against pathogens. However, higher concentrations of rhMASP1 did not further increase the complement activation in our *in vitro* system.

TABLE 3 | Distribution of allele and genotype frequencies for SNP rs3774275 (*MASP1*) between controls and tuberculosis patients.

Rs3774275 allele frequencies (n = 321)			
Allele	**All subjects**	**Healthy controls**	**Cases (TB patients)**
A	421 (65.6%)	219 (60.8%)	202 (71.6%)
G	221 (34.4%)	141 (39.2%)	80 (28.4%)
Rs3774275 genotype frequencies (n = 321)			
Genotype	**All subjects**	**Healthy controls**	**Cases (TB patients)**
A/A	137 (42.7%)	66 (36.7%)	71 (50.3%)
A/G	147 (45.8%)	87 (48.3%)	60 (42.6%)
G/G	37 (11.5%)	27 (15.0%)	10 (7.1%)

Shown are the counts and percentages (in brackets) of the 321 samples genotyped for this variant (genotyping of four samples failed).

4. DISCUSSION

In the present work, we used an AmpliSeq-based approach to screen for TB-associated polymorphisms in several genes belonging to the C-type lectin receptor family or their related signaling pathways. Coupling of this NGS approach with a MassARRAY validation phase allowed the identification of a polymorphism in *MASP1* (rs37742752) that was significantly associated with disease susceptibility. Further analysis revealed increased MASP1 levels in serum of tuberculosis patients, constituting the first reported association between tuberculosis and this MBL-associated serine protease.

Previous studies were able to identify tuberculosis-associated variants in a few CTLR genes in different populations, such as for *MRC2*, *MBL*, or *MASP2* in Chinese populations, *DC-SIGN* variants in African populations, and several variants of *SPA-1*, *SPA-2*, or *MBL* in diverse populations (29, 30, 61). In our study, we targeted these polymorphisms and expanded the sequencing approach to a total of 33 genes involved in CTLR signaling. We identified a significantly associated polymorphism in the *MASP1*

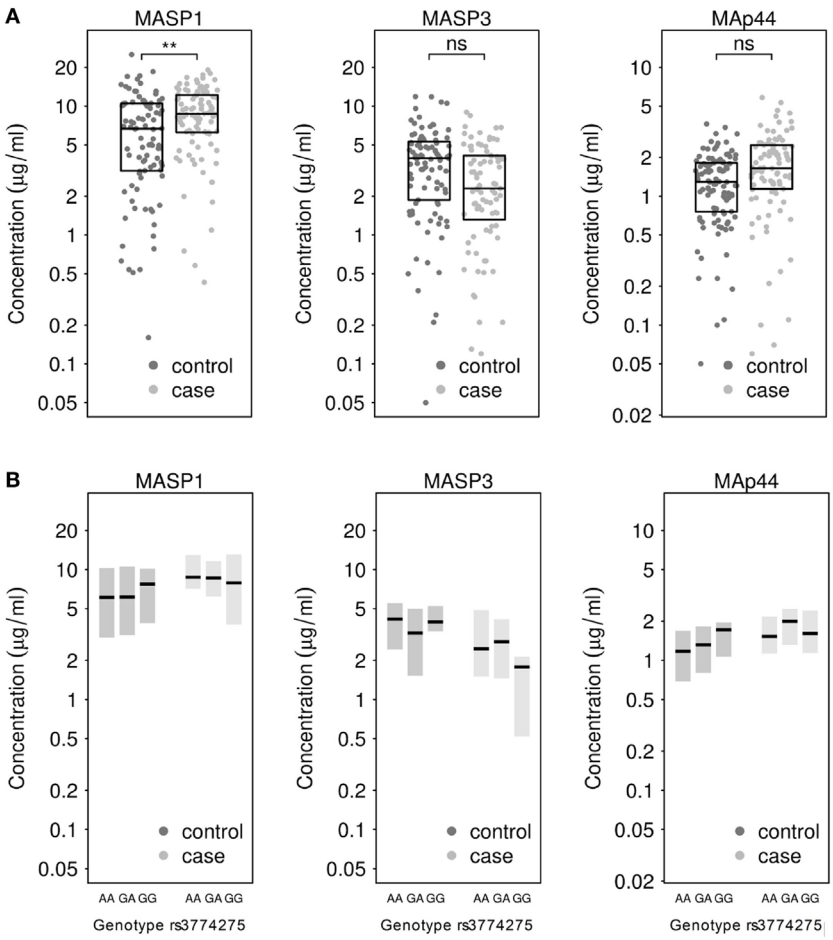

FIGURE 1 | **(A)** Measurement of MASP1, MASP3, and MAp44 levels in serum of healthy controls and tuberculosis patients. Results after adjusting for BMI show significantly higher levels of MASP1 in tuberculosis patients (*T*-test; $^{**}p < 0.01$) when compared to healthy controls. **(B)** Genotype-dependent distribution of MASP1, MASP3, and MAp44 levels across control and case samples. Shown are median values and 25–75th percentile box plots.

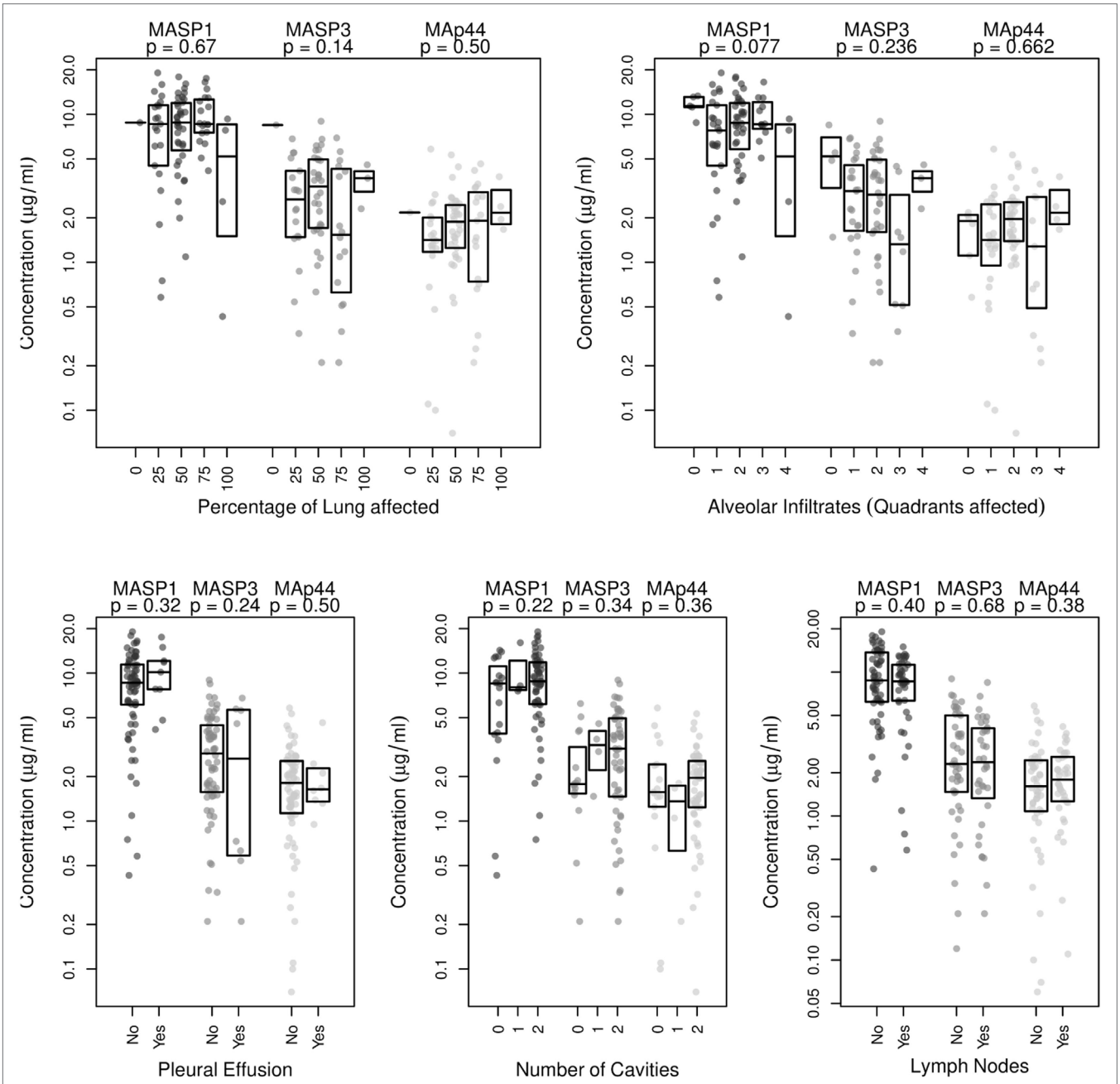

FIGURE 2 | Correlation analysis of MASP1, MASP3, and MAp44 serum concentrations with disease severity, as measured by examination of chest X-rays. Shown are the *p*-values obtained after linear regression analysis for each of the following criteria analyzed: Percentage of lung affected, number of quadrants showing alveolar infiltrates, presence of pleural effusion, number of cavities observed and presence of lymph nodes. Shown are box plots with median values and *p*-values obtained for the correlation with eachMASP1-splicing product.

gene, which has not been targeted in previous case-control studies addressing TB susceptibility. MASP1 plays a key role in the activation of lectin pathway of complement (Figure S1 in Supplementary Material). Mtb recognition *via* MBL leads to the activation of associated MASP1 homodimers, which catalyze the activation of MASP2 (17, 62, 63). MASP1 and MASP2 together cleave the C2 and C4 components of the complement and the cleavage products form C3 convertase. MASP1 is responsible for 60% of C2a production needed to generate C3 convertase (63; Figure S1 in Supplementary Material), which further creates a membrane attack complex on bacterial surface ultimately killing the cell, while the by-products of the cascade such as C3b may act as opsonins enhancing the bacterial phagocytosis (22). While the role of C-type lectin MBL polymorphisms in tuberculosis have been studied in several populations (29, 30, 35, 64), not many studies have focused on the other components of the lectin

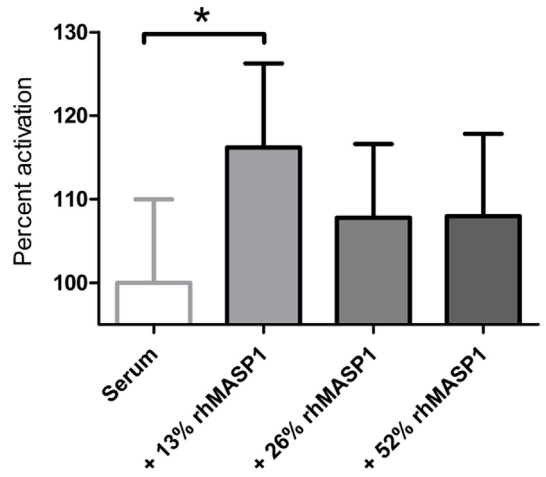

FIGURE 3 | Measurement of lectin pathway complement activation in serum samples ($n = 6$) after addition of rhMASP1. As measured by an MBL pathway complement activation system, an increase in rhMASP1 leads to a slight enhancement of the complement activation in the serum samples (repeated measures ANOVA with Dunnett *post hoc* test; $^*p < 0.05$).

pathway. A recent work found no association between *MBL*, *Ficolin-1*, *Ficolin-2*, or *MASP2* variants and TB susceptibility (65). In contrast, Chen et al. could observe a significant impact of *MBL* and *MASP2* polymorphisms upon TB susceptibility in a Chinese population (29, 30). However, the important factor MASP1 has not been included in any studies addressing the genetic predisposition to TB so far. Our study demonstrates for the first time that *MASP1* polymorphism rs3774275 is associated with TB susceptibility.

The SNP rs3774275 is located in the mutually exclusive splicing region in intron 8 of the MASP-1/3 gene and is responsible for the alternative splicing and the regulation of serum levels of MASP1 protein and its splice variants MASP3 and MAp44 (38). Two independent studies by Ammitzboll et al. and Krogh et al. recently showed that rs3774275 is associated with MASP1 serum concentrations (38, 60). In both studies, they observed that the G allele was related to higher MASP1 levels (38, 60). In our study, we also observed higher MASP1 levels in the GG-genotype of the control population, but failed to reach statistical significance, probably due to the small sample size for this minor genotype. However, we were able to detect significantly higher levels of MASP1 in TB patients when compared to healthy controls. As shown by correlation of MASP levels with radiographic evaluations, this increase seems to be independent of the severity of TB.

For long time, MASP2 had been considered the main effector of the lectin pathway of the complement and has been associated with several infectious diseases including Hepatitis C virus (HCV) infection, *Pseudomonas* infection, leprosy, as well as TB (29, 30, 66). Recent investigations on the mechanism of complement lectin-pathway activation suggest that MASP1 plays an even more central role than MASP2 (63). Nevertheless, MASP1 has not yet been studied in association to many infectious diseases. Some studies in HCV infection have demonstrated a high association between MASP1 activity and severe hepatic fibrosis (67, 68). In another study, a synonymous mutation in *MASP1* in the MASP3 serine protease domain was associated with early *Pseudomonas aeruginosa* colonization in cystic fibrosis patients (69). Our study is now the first study to demonstrate an association between MASP1 serum levels and pulmonary TB.

Recent research on MASP1, dissecting its physiological function, has revealed a much broader spectrum of its action than previously assumed. MASP1 is a promiscuous receptor and is shown to bind several ligands. MASP1 can not only activate the complement lectin pathway but also triggers cellular processes such as activation of signaling pathways. Megyeri et al. demonstrated that MASP1 could activate the NFκB, p38-MAPK and Ca^{2+} signaling in endothelial cells *in vitro* by cleaving surface protease activated receptor-4 (PAR-4) (62). Moreover, the p38-MAPK activation in endothelial cells by rMASP1 led to IL-6 and IL-8 secretion along with other cytokines *in vitro* that were able to recruit neutrophils (70). PARs are also expressed on lung epithelium (71), and therefore it may be speculated that high levels of MASP1 may help induce a similar response in lung tissue, activating cellular responses and recruiting phagocytes which may together contribute to bacterial clearance.

The pathognomonic increase of serum MASP1 observed in TB patients in our study reflects the important role that this serine protease plays in the immune response against Mtb. Interestingly, the rs37742752-GG-genotype, which is more frequent in healthy controls, correlates with elevated MASP1 expression as shown in this study and elsewhere (38, 60). Indeed, the MASP1 levels of healthy controls with the GG-genotype ($\bar{x} = 8.63\ \mu g / ml$) were comparable to those observed in TB patients ($\bar{x} = 9.06\ \mu g / ml$). It may be hypothesized that intrinsic upregulation of MASP1 (due to genetic predisposition), as observed in healthy controls with the GG-genotype, could play a protective role against infection with Mtb or the development of active TB in latently infected individuals. In the latter case, elevated MASP1 levels might contribute to prevent reactivation of latent Mtb. In this study, we could demonstrate a correlation between MASP1 levels and lectin complement activation *in vitro*. It is likely that, as the amount of MASP1 in the serum increases, more MASP2 is activated and more C2 and C4 molecules are cleaved (see Figure S1 in Supplementary Material), which in turn leads to the formation of more C5b9 complexes and higher opsonization rates. Our results implicate that even a small increase in the amount of MASP1 (+ 13%) can significantly enhance the lectin pathway activity. However, we could not observe any dose dependency when higher concentrations of rhMASP1 were added to the system. The observed saturation might be explained by the interdependency between MASP1 and MASP2 in the activation of the lectin complement system. This activation might reach a plateau when MASP2 becomes the limiting factor, since it is responsible for the cleavage of C4—a process that cannot be engaged by MASP1 (see Figure S1 in Supplementary Material). Further *in vivo* experiments, as well as *in vitro* assays with blood samples of TB patients, are needed to confirm the effect of increased MASP1 levels on the lectin complement pathway and to investigate its potential impact on the phagocytosis and killing of Mtb.

In the present study, we used a customized AmpliSeq approach to screen for relevant polymorphisms in 33 genes. Although this approach led to the identification of a TB-associated polymorphism in *MASP1*, which was strongly supported by the findings in the validation phase, certain limitations of the study have to be acknowledged. One polymorphism that was included in phase II of the study after NGS analysis resulted to be monomorphic during validation. This result might be related to the limitations of the semiconductor sequencing technology. Although the High-Q chemistry of Ion Torrent has largely improved the sequencing output with regard to coverage, noise, and read quality (72), false positives are still possible at loci in proximity of homopolymers. Thus, AmpliSeq approaches should always be coupled with post-validation procedures. An additional challenge of this association study was the potential stratification of the population analyzed. Stratification in the Indian population is expected due to historical ethnic, religious and language barriers existing in the community, which might exert important genetic effects and should be addressed in association studies such as this one (73). Thus, in this study we used the LASER software to correct for potential stratification effects. Ancestry adjustment resulted in a lambda value near 1 and no inflation of the association results (see Figure S2B in Supplementary Material). Finally, although rs3774275 showed a strong association with TB, it is still unclear how this SNP might be linked to other genetic variants or interrelates with other predisposing factors in this multifactorial disease. Moreover, due to the lack of control for asymptomatic Mtb infection in the control group of our study, it cannot be determined whether the rs3774275-GG-genotype confers protection against Mtb infection or resistance against the development of active tuberculosis disease. Considering that over 90% of those who are infected with Mtb remain asymptomatic (74), latently infected individuals should be expected among our control group. Indeed, latent TB prevalence rate in endemic TB countries has been estimated to be as high as 79% (75). Further studies with controls classified for asymptomatic infection will help to clarify the type of protection that is associated with the *MASP1* variant rs3774275.

In summary, in this study we investigated whether genetic variants of CTLR-related genes were associated with TB susceptibility. Our two-stage study allowed the identification of one *MASP1* polymorphism (rs3774275) significantly associated with PTB. MASP1 had been considered the underdog of the lectin-dependent complement activation until recently, when a more prominent role of this protein has been dissected in the lectin pathway (63). Our results, which suggest an important role of *MASP1* variants in tuberculosis, were reinforced by the observation of elevated MASP1 serum levels in PTB patients. The present work contributes to our understanding of host-Mtb interaction and highlights the critical role of the lectin-complement pathway in Mtb pathogenesis.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the committee for bio-medical research at the Bhagwan Mahavir Medical Research Centre with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the institutional ethics committee for bio-medical research at the Bhagwan Mahavir Medical Research Centre, Hyderabad, India (date: March 11, 2011).

AUTHOR CONTRIBUTIONS

TK, SG, CF, and HS conceived and designed the study and experiments. TK and SG wrote the manuscript. TK and SG conducted the experiments. TK, SG, MS, DD, LB-A, RS, CF, and HS analyzed the data. SG, AH, RM, GS, and VV recruited the study cohort and collected samples. NA supervised and coordinated the study in India. All authors reviewed and edited the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/articles/10.3389/fimmu.2018.00242/full#supplementary-material.

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Galectins in Intestinal Inflammation: Galectin-1 Expression Delineates Response to Treatment in Celiac Disease Patients

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Galectins, a family of animal lectins characterized by their affinity for N-acetyllactosamine-enriched glycoconjugates, modulate several immune cell processes shaping the course of innate and adaptive immune responses. Through interaction with a wide range of glycosylated receptors bearing complex branched N-glycans and core 2-O-glycans, these endogenous lectins trigger distinct signaling programs thereby controling immune cell activation, differentiation, recruitment and survival. Given the unique features of mucosal inflammation and the differential expression of galectins throughout the gastrointestinal tract, we discuss here key findings on the role of galectins in intestinal inflammation, particularly Crohn's disease, ulcerative colitis, and celiac disease (CeD) patients, as well as in murine models resembling these inflammatory conditions. In addition, we present new data highlighting the regulated expression of galectin-1 (Gal-1), a proto-type member of the galectin family, during intestinal inflammation in untreated and treated CeD patients. Our results unveil a substantial upregulation of Gal-1 accompanying the anti-inflammatory and tolerogenic response associated with gluten-free diet in CeD patients, suggesting a major role of this lectin in favoring resolution of inflammation and restoration of mucosal homeostasis. Thus, a coordinated network of galectins and their glycosylated ligands, exerting either anti-inflammatory or proinflammatory responses, may influence the interplay between intestinal epithelial cells and the highly specialized gut immune system in physiologic and pathologic settings.

Keywords: celiac disease, galectin-1, galectins, glycans, gut inflammation, inflammatory bowel disease

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INTRODUCTION: DECIPHERING GLYCOCODES IN IMMUNITY

Complex sugar structures play essential roles as hardware for storage of biological information, which can be deciphered by endogenous glycan-binding proteins or lectins (1). The singular role of lectins in translating glycan-containing information into a myriad of cellular responses invigorated further studies aimed at understanding their expression patterns and molecular mechanisms of action.

Galectins, a family of lectins with affinity for N-acetyllactosamine (LacNac) residues, have diverse roles in shaping the course of innate and adaptive immunity and tailoring inflammatory responses, thereby modulating tumor immunity and autoimmune reactions (2, 3). In this perspective article, we review current knowledge on the role of galectins in inflammatory intestinal disorders, and present new findings on the regulated expression of galectin (Gal)-1 in intestinal tissue of celiac disease (CeD) patients.

GALECTINS

Galectins, evolutionarily conserved glycan-binding proteins, play key roles in multiple immune cell processes. Either through protein-glycan or protein–protein interactions, these lectins function within the extracellular milieu by interacting with various glycosylated receptors, or work inside the cells by controlling distinct signaling pathways and modulating intracellular processes (3, 4).

To date, 15 members of the galectin family have been identified in vertebrates, which were classified into three groups based on their molecular architecture: (a) "proto-type" galectins, comprising a single polypeptide chain with one carbohydrate recognition domain (CRD) that is able to dimerize (Gal-1, -2, -5, -7, -10, -11, -13, -14, and -15); (b) "chimera-type" Gal-3, which consists of a C-terminal CRD linked to an N-terminal peptide, and (c) "tandem repeat-type" galectins composed of a single polypeptide chain exhibiting two CRDs in tandem connected by a linker peptide (Gal-4, -6, -8, -9, and -12) (4, 5). While some members of the family (e.g., Gal-1 and Gal-3) are widely distributed among different tissues and species (6–8), others have more restricted tissue localization. For example, Gal-7 is preferentially found in the skin (9, 10), Gal-12 is mostly expressed in adipose tissue (11, 12), Gal-5 is restricted to rat reticulocytes (13, 14) and Gal-10 is found in human but not mouse eosinophils (15).

Once synthesized, galectins may remain within the intracellular compartment and participate in protein-protein interactions to regulate intracellular events (16, 17). For example, both Gal-1 and -3 participate in pre-mRNA splicing (18) whereas Gal-10 modulates functionality of human $CD25^+$ Treg cells (19). However, despite the lack of a classical secretory signal peptide, most galectins are released through an unconventional route to the extracellular compartment (20). Secreted galectins can specifically decipher biological information encoded in complex saccharide structures (particularly LacNac-enriched complex branched N-glycans and core 2 O-glycans), and convey this biochemical information into functional cellular responses (3, 17). Although saccharide structures are widely distributed in a range of glycoconjugates, individual galectins may co-opt a particular set of glycosylated receptors, generated by the coordinated action of glycosyltransferases and glycosidases which are differentially regulated in distinct target cells (4, 21, 22). Notably, one-CRD galectins can dimerize *via* the back sides of their CRDs, whereas chimera-type Gal-3 can pentamerize *via* its non-lectin N-terminal domain, and tandem-repeat galectins can oligomerize (17). Thus, through formation of multivalent galectin–glycan complexes, galectins can promote cross-linking, reorganization, and clustering of glycosylated receptors thereafter regulating their activation and signaling (23, 24). Within the immune compartment, galectin–glycan complexes may control signaling thresholds of relevant receptors such as the T-cell receptor (25), pre-B cell receptor (26), and cytokine receptors (27) among others, thereby modulating lymphoid and myeloid regulatory programs.

GALECTINS: KEY PLAYERS IN THE INFLAMMATORY RESPONSE

Compelling evidence highlights major roles for galectins in controlling innate and adaptive immune responses. These lectins may influence the capacity of innate immune cells [e.g., neutrophils, dendritic cells (DCs), monocytes/macrophages, eosinophils, and mast cells] to respond to chemotactic gradients, migrate across endothelial cell surfaces, synthesize and release pro- or anti-inflammatory cytokines, and recognize, engulf, and kill microbes and damaged cells (28). In this regard, some galectins trigger innate immune responses, while others influence the resolution of acute inflammation (28). Galectins can also tailor adaptive immunity by influencing T-cell signaling and activation, modulating T-cell survival, controlling the suppressive function of regulatory T cells (Tregs), altering the cytokine balance and regulating B-cell maturation and differentiation (3). Both the specificity of the CRD as well as glycan presentation in the corresponding receptors make distinct contributions to the specific effects of individual galectins, selectively mediating different biological processes. The final balance of their synchronized actions contributes to activation, polarization, and resolution of adaptive immune responses (29). Although the specific immunoregulatory activities of each individual galectin is beyond the scope of the present work, and are described elsewhere (3, 29), some of the most relevant activities displayed by Gal-1, the central core of the present article, are summarized herein. This endogenous lectin, composed of two subunits of 14.5 kDa, functions as a regulatory signal which undermines acute inflammatory responses by controlling neutrophil adhesion, function and turnover (30, 31) and modulating monocyte and macrophage activation and polarization (32–35). Moreover, Gal-1 influences DC maturation, immunogenicity, and migration (36–40). Interestingly upon exposure to this lectin, DCs acquire an IL-27-dependent regulatory function leading to IL-10-mediated T-cell tolerance, suppression of T-helper (Th)1 and Th17 responses, promotion of tumor-immune escape and suppression of autoimmune neuroinflammation (40).

Regarding the T-cell compartment, Gal-1 controls T-cell viability, blunts Th1- and Th17-mediated responses and skews the balance of the immune response toward a Th2 cytokine profile (17, 41–43). Interestingly, we found that Th1- and Th17-differentiated

cells express the repertoire of cell surface glycans that are critical for Gal-1 binding and induction of apoptosis; whereas Th2 cells are protected from this lectin through α2,6-sialylation of surface glycoproteins (43). Remarkably, Gal-1 also controls the immunosuppressive activity of Tregs and promotes their differentiation (44–46). Finally, by influencing B-cell development, differentiation, signaling and survival, Gal-1 also controls B-cell function (47–50).

The essential role of Gal-1 in the control of inflammation has been widely demonstrated in experimental models of autoimmunity, allergy and cancer (29, 51–53). In cancer settings, Gal-1 contributes to create immunosuppressive microenvironments, allowing tumor cell evasion of immune responses (46, 54–63). On the other hand, in experimental models of autoimmune disease including collagen-induced arthritis (64), myelin-oligodendrocyte glycoprotein$_{35–55}$-induced encephalomyelitis (43, 65), diabetes (66), uveitis (67), and orchitis (68), Gal-1 elicits a broad spectrum of immunoregulatory activities leading to the resolution of chronic inflammation. The mechanisms underlying these immunosuppressive effects recapitulate those observed *in vitro* and *in vivo* including T-cell dysfunction and inhibition of proinflammatory cytokines (43, 58, 64, 69, 70), induction of tolerogenic DCs (40), expansion of Foxp3$^+$ and Foxp3$^-$ Tregs (60, 67) and generation of alternatively activated "M2-type" macrophages (71).

GALECTINS IN THE GUT: A SWEET PATH AT THE CROSS-ROADS OF TOLERANCE AND INFLAMMATION

Despite the broad immunoregulatory activities of galectins, only few studies have uncovered the role of these lectins in gut immune homeostasis and the implications of these findings in intestinal inflammation. Interestingly, Gal-1, -2, -3, -4, and -9 are typically expressed in particular gut areas: whereas Gal-1 is mainly present in the lamina propria (LP), Gal-2, -3, -4, -7, and -9 are constitutively expressed within the epithelial compartment of the mouse intestine (72, 73). Epithelial cells (ECs) of small and large intestine express high levels of Gal-3 and Gal-4, although Gal-2 is only found in the large intestine (72). Interestingly, while Gal-3 may interact with commensal bacteria possibly influencing their colonization capacity (74), Gal-4 and Gal-8 mediate bacterial recognition and killing (75). Notably, Gal-1 is broadly expressed in small bowel enterocytes and may influence their viability (76). Moreover, studies reporting the galectin signature of human intestinal cells were mainly focused on pathologic conditions. Thus far, Gal-1, -3, -4, and -9 have shown to be homogeneously expressed across different sections of the large intestine (77).

GALECTINS IN INTESTINAL INFLAMMATORY DISEASES

Inflammatory Bowel Diseases (IBD)

Crohn's disease (CD) and ulcerative colitis (UC) represent the two main forms of IBD, chronic relapsing inflammatory conditions that affect the gastrointestinal tract. Despite some shared clinical features, these diseases can be distinguished by differences in risk factors, and clinical, anatomical, histological, and immunological features (78–80). Both conditions may involve an aberrant activation of mucosal T-cells against the commensal microbiota and deregulation of the EC compartment, thus compromising normal intestinal function and promoting an exuberant inflammatory response (81, 82). Whereas CD is characterized by an overactivation of mucosal Th1 and/or Th17 cells (with the concomitant secretion of IFN-γ, IL-17, and IL-22), UC patients exhibit a marked Th2 bias (with higher levels of IL-5 and IL-13) (79, 80, 83).

In a murine model of acute and chronic 2,4,6-trinitrobenzenesulfonic acid (TNBS)-induced colitis, treatment with recombinant Gal-1 (rGal-1) resulted in improvement of the clinical, histopathological, and immunological manifestations of the disease. Further analysis revealed increased apoptosis of TNBS-specific CD4$^+$ T-cells in the LP, decreased percentage of activated T-cells and diminished levels of proinflammatory and Th1-type cytokines, effects that were accompanied by normalization of the mucosal architecture (69). Accordingly, Gal-1 was found to be upregulated in inflamed areas of IBD patients when compared with non-inflamed areas of the same patient or with control subjects. Indeed, expression of common mucosal-associated galectins (Gal-1, -3, -4, -9) was found dysregulated in these inflamed tissues, suggesting that alteration in galectin expression pattern may represent an endogenous compensatory mechanisms likely aimed at limiting the inflammatory process and restoring mucosal homeostasis (77). Notably, the viability of human and mouse enterocytes was also controlled by Gal-1 in human IBD biopsies and in murine models of intestinal inflammation. Interestingly, proinflammatory stimuli promoted Gal-1 binding to EC which in turn influenced their survival and secretion of proresolving cytokines, thereby protecting the intestinal epithelium from inflammatory responses (76, 84). Thus, through elimination of antigen-experienced T-cells, modulation of proinflammatory cytokines or direct stimulation of epithelial-derived anti-inflammatory factors, Gal-1 contributes to the resolution of gut inflammation (**Figure 1**).

Notably, other members of the galectin family could also be involved in controlling intestinal inflammation (**Figure 1**). Gal-3 may function as a proinflammatory mediator that aggravates dextran sulfate sodium (DSS)-induced colitis through promotion of an M1 macrophage phenotype (85). Deletion of Gal-3 gene in mice or pharmacological inhibition of this lectin promoted macrophage polarization toward a M2 phenotype in colonic tissue (85). In line with these observations, peritoneal macrophages lacking Gal-3 are more prone to undergo apoptosis than their wild-type counterparts, strongly suggesting a role for Gal-3 as a proinflammatory mediator in the peritoneal cavity (86). Notably, in IBD patients Gal-3 levels are reduced in active inflamed areas, probably aimed at limiting the inflammatory process and restoring mucosal homeostasis (77, 87–89). In contrast, a protective role for this lectin was suggested in both the DSS-induced and the T-cell transfer colitis models, through suppression of IL-6 production by colonic LP fibroblasts or

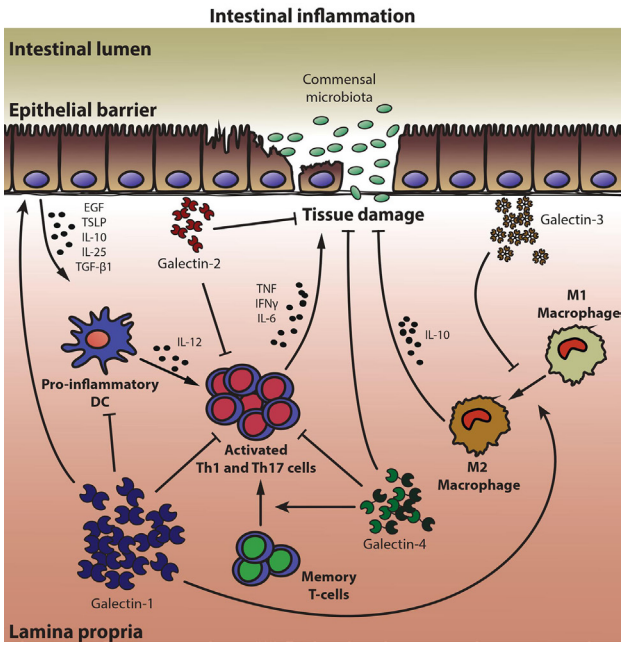

FIGURE 1 | Multifunctional roles of galectins in mucosal intestinal inflammation. A coordinated network of galectin family members, which exerts either anti-inflammatory or proinflammatory responses, conditions epithelial barrier maintenance and immune gut homeostasis. Galectin-1 (Gal-1) promotes the secretion of growth factors and anti-inflammatory cytokines by epithelial cells (ECs), induces apoptosis of activated Th1 and Th17 cells, inhibits secretion of proinflammatory cytokines by dendritic cells (DCs) and T lymphocytes, and favors an anti-inflammatory (M2) macrophage phenotype. Similar to Gal-1, Gal-2 displays several anti-inflammatory properties, but also promotes wound healing and tissue regeneration in ECs. In animal models of colitis, Gal-3 shows mostly proinflammatory functions, inhibiting the polarization of macrophages toward an M2 phenotype, whereas Gal-4 exhibits both anti- and proinflammatory properties within the intestinal inflamed mucosa, depending on the experimental setting analyzed. By blocking production of proinflammatory cytokines, Gal-4 prevented inflammation and favored epithelial regeneration. However, through binding to memory T-cells, Gal-4 led to T-cell activation and perpetuated intestinal inflammation.

by induction of Foxp3$^+$ Tregs (90, 91). These discrepancies could be explained not only by differences in experimental models (92), but also by dissimilar roles of endogenous versus exogenous Gal-3 during different stages of the inflammatory response (16, 29).

Similarly, Gal-4 has been shown to act either as an anti-inflammatory or as a proinflammatory factor in IBD. An anti-inflammatory function for both Gal-4 and Gal-2 was described, which contributed to ameliorate mucosal inflammation in the DSS colitis model through mechanisms involving apoptosis of activated mucosal LP T-cells and diminished proinflammatory cytokine secretion (93, 94) (**Figure 1**). Within the EC compartment, Gal-2 and Gal-4 (but not Gal-1) promoted wound-healing (95). Gal-4 may also function as a glycoprotein trafficking carrier, which generates an apical endocytic-recycling pathway *via* complex-type N-glycans (96, 97). Notably, during IBD progression, local inflammation was also associated with dysregulated

TABLE 1 | Analysis of duodenal biopsies from control subjects, untreated CeD patients, and CeD patients subjected to gluten withdrawal.

Characteristics	CeD	CeD-GFD
Number of cases and gender (female/male)	10 (8/2)	10 (7/3)
Median age, years (range)	32 (18–56)	37 (24–67)
Median time on a GFD, years (range)	–	4 (2–14)
Number of cases with positive serology		
IgA tissue transglutaminase > 20 UA/mL	10	3
Severity of histologic damage		
(Marsh 3 classification) number of patients	10	2

Demography, histological, and serology data of untreated (at diagnosis) celiac disease patients (CeD) and of CeD treated with gluten withdrawal (CeD-GFD) whose duodenal biopsies were employed for the study.
Patients were diagnosed with CeD according to conventional clinical, serological and histological criteria (108, 117). Control subjects: non-celiac subjects with negative CeD serology and normal duodenal histology, n = 10. Patients and controls were informed in detail about the study, and written consent was obtained. The protocols were approved by Ethics Committees of Hospital "Carlos B. Udaondo."

expression of glycosyltransferases, leading to exposure of altered glycan structures on memory CD4$^+$ T-cells (98). In fact, downregulation of core 2 β1,6-*N*-acetylglucosaminyltransferase 1 (C2GnT1) allowed Gal-4-O-glycan interactions resulting in expansion of memory CD4$^+$ T-cells, enhanced IL-6 production and perpetuation of intestinal inflammation (98, 99). Notably, inflamed IBD mucosa could be distinguished from control tissue and from other types of intestinal inflammatory conditions by a specific galectin signature, as revealed by a multivariate-linear discriminant analysis of Gal-1, -3, -4, and -9 in IBD patient biopsies (77).

Celiac Disease

Oral tolerance to dietary antigens is a key active process in which immune responses to innocuous antigens, commensal bacteria, and pathogens are suppressed (100). In CeD, intolerance to indigestible wheat gluten peptides results in chronic intestinal inflammation associated with an extensive Th1 and Th17 responses (101). Similar to most chronic inflammatory diseases, CeD has a multifactorial etiology involving environmental factors as well as genetic components. Among them, HLA-DQ2 and HLA-DQ8 have been identified to confer susceptibility to CeD development (102–104). In genetically susceptible individuals, intestinal inflammation is triggered when ingested gliadin (proline-rich and glutamine-rich gluten proteins) found in wheat, rye, barley, and oats (105, 106) is partially processed and presented to CD4$^+$ T-cells that infiltrate the LP of the small intestine. Thus HLA-DQ2/8 molecules may orchestrate a gluten-specific CD4$^+$ T-cell response (107).

Celiac disease patients on a gluten-containing diet show increased levels of serum antibodies specific for gliadin and tissue transglutaminase, an enzyme that plays a key role in disruption of tolerance to gluten, among other antigens (108, 109). To date, the only known effective treatment for CeD is a lifelong gluten-free diet (GFD) (109), which allows the complete recovery of intestinal structure and function, and normalization of serum antibodies (110). In spite of considerable progress in our understanding of the mechanisms

underlying CeD development and progression, there is no clear answer to how breaking mucosal tolerance to gluten turns a controlled local immune response into chronic inflammation and epithelial destruction (111).

Although the involvement of galectins in IBD has been well documented, their relevance in CeD development and progression is poorly understood. In this regard, a significant increase in Gal-10 expression has been correlated with mucosal damage and number of eosinophils in duodenal lesions of CeD patients (112). In addition, despite some discrepancies, evidence suggest a role for Gal-9 in human and mouse food allergy, a broad entity with some common features with CeD (113–115).

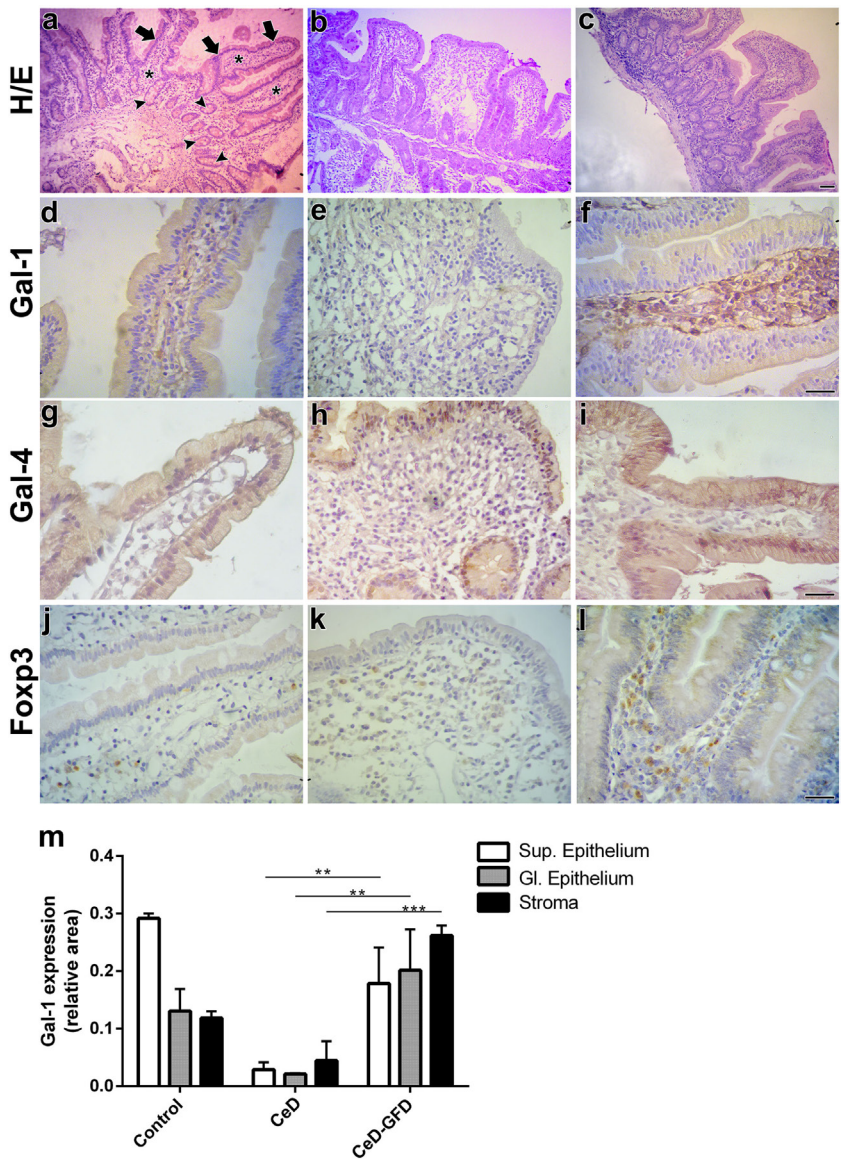

FIGURE 2 | Expression of galectin-1 (Gal-1), Gal-4, and Foxp3 in response to gluten-free diet (GFD) in duodenal biopsies from celiac disease (CeD) patients. Representative micrographs of control subjects, untreated CeD patients and CeD patients subjected to gluten withdrawal (CeD-GFD patients) are shown. **(A–C)** Hematoxylin/eosin (H/E) staining of paraffin-embedded sections of duodenal biopsies from **(A)** control subjects, **(B)** CeD patients, and **(C)** CeD-GFD patients. Arrows indicate the superficial epithelium, and arrowheads indicate the glandular epithelium while asterisks denote the stroma. Bar = 20 μm. **(D–F)** Immunohistochemical analysis of Gal-1 expression in duodenal biopsies from control subjects **(D)**, CeD patients **(E)**, and CeD-GFD patients **(F)**. Bar = 20 μm. **(G–I)** Immunohistochemical analysis of Gal-4 expression in duodenal biopsies from control subjects **(G)**, CeD patients **(H)**, and CeD-GFD patients **(I)**. Bar = 20 μm. **(J–L)** Immunohistochemical analysis of Foxp3$^+$ cells in biopsies from control subjects **(J)**, CeD patients **(K)**, and CeD-GFD patients **(L)**. Bar = 20 μm. **(M)** Quantification of Gal-1 expression determined by immunohistochemistry. Bars represent immunostained area corresponding to superficial (Sup) and glandular (Gl) epithelium, and stroma, in paraffin sections from duodenal biopsies from controls, untreated CeD patients and CeD-GFD patients. Evaluation of staining intensity was performed with the Image J software (NIH, Bethesda, MD, USA). One-way ANOVA Tukey test was used for multiple comparisons. $^{**}p < 0.01$, $^{***}p < 0.001$.

TABLE 2 | Analysis of duodenal biopsies from control subjects, untreated CeD patients, and CeD patients subjected to gluten withdrawal.

Antigen	Primary antibody	Secondary antibody
Gal-1	In-house rabbit anti-Gal1 antibody (1:500) (64)	anti-rabbit biotinylated antibody (1:130) (Amersham Pharmacia, Buckinghamshire, UK)
Gal-4	Goat anti-Gal-4 antibody (1:75) (Santa Cruz Biotech, Dallas, TX, USA)	anti-goat biotinylated antibody (1:180) (Amersham Pharmacia, Buckinghamshire, UK)
Foxp3	Rabbit anti-Foxp3 antibody (1:50) (Abcam, Cambridge, UK)	anti-rabbit biotinylated antibody (1:130) (Amersham Pharmacia, Buckinghamshire, UK)

Antibodies used for immunohistochemical analysis of Gal-1, Gal-4, and Foxp3 expression in duodenal biopsies from control subjects and patients.
Four intestinal biopsies from the second duodenal section from each patient and control were collected.

GALECTIN-1 EXPRESSION DELINEATES RESPONSE TO GFD IN CeD PATIENTS

Since several immunoregulatory mechanisms are dysregulated in mucosal tissue of CeD patients (108) and Gal-1 displays broad tolerogenic and anti-inflammatory activities in mucosal tissues (29), we evaluated the expression of this lectin in biopsies of CeD patients with or without gluten withdrawal (**Table 1**).

Hematoxylin/eosin staining of duodenal biopsies showed that, unlike the conserved LP structures observed in control subjects (**Figure 2A**), CeD patients exhibited atrophic villi with enlarged hyperplastic crypts and increased intraepithelial lymphocytes infiltration (**Figure 2B**). Mucosa from CeD patients after GFD (CeD-GFD patients) presented considerably recovered villi (**Figure 2C**). In control biopsies, Gal-1 labeling (**Table 2**) was mainly localized in stromal cells, while most ECs exhibited weak positive staining (**Figure 2D**). Biopsies from CeD patients exhibited a poorly labeled stromal fibrillar network, while atrophic epithelia showed no considerable staining. Subepithelial and periglandular infiltrating cells appeared negative for Gal-1 (**Figure 2E**). Duodenal biopsies from CeD-GFD patients exhibited a substantial increase in Gal-1 immunoreactivity, especially in the interstitium of the recovered villi. Numerous subepithelial fibroblast-like cells, as well as round nucleus-containing cells scattered in the LP compatible with macrophages, and a few lymphocytes were Gal-1-positive. Notably, ECs recovered their Gal-1 weak positive staining (**Figure 2F**). Moreover, no significant differences were observed in the expression of Gal-4 (**Table 2**)—a galectin family member mostly expressed in ECs of the intestinal tract—in biopsies from CeD patients before or after gluten withdrawal (**Figures 2G–I**).

Overall, while control duodenal biopsies showed moderate Gal-1 staining, and both epithelium and stroma from untreated CeD patients were poorly labeled, CeD-GFD biopsies showed a dramatic increase in Gal-1 immunoreactivity ($p < 0.001$; **Figure 2M**), which correlated with normalization of duodenal mucosal structure. Interestingly, the expression of stromal Gal-1 in these patients was not only recovered but also increased in intensity compared with control biopsies (**Figures 2D–F,M**).

To further characterize the underlying inflammatory response and given the association of Gal-1 with induction of $Foxp3^+$ Tregs, we analyzed the expression of this transcription factor in inflammatory infiltrates (**Table 2**). Though less accurate in defining human Tregs than mouse Tregs (116), determination of Foxp3 staining is typically considered a reliable indicator of the suppressive tissue microenvironment. An increased number of $Foxp3^+$ cells was observed in CeD-GFD patients (**Figures 2J–L**), which positively correlated with Gal-1 expression, suggesting activation of a circuit of immunosuppressive events leading to restoration of mucosal homeostasis. Further studies should be aimed at addressing the immunosuppressive potential of this tolerogenic circuit in functional assays.

Our findings suggest that, in response to gluten withdrawal, upregulation of Gal-1 might contribute to restrain the chronic inflammatory response, thus allowing the onset of the recovery process leading to remission of mucosal damage and reestablishment of villi structure. In addition, decreased Gal-1 expression observed in untreated CeD patients compared to control individuals may suggest a role for this lectin in controlling gut homeostasis under physiologic conditions. Interestingly, modulation of Gal-1 expression during CeD development appeared to be specific as no differences were found in the expression of Gal-4, suggesting selective regulation of individual galectins during mucosal inflammation.

CONCLUSION

The delicate balance between host immunity and tolerance allows the maintenance of gut homeostasis avoiding detrimental intestinal inflammation. Data presented here, resulting both from published information (**Figure 1**) and new observations (**Figure 2**), highlight the role of galectins as active players of complex regulatory circuits operating in intestinal mucosal tissue to preserve immune and epithelial homeostasis. While galectins (particularly Gal-1, -2, -3, -4, and -9) may be critical in preserving intestinal homeostasis, an initial set up in which galectins' expression is altered or the intestinal glycome is reprogrammed may influence development of intestinal inflammation.

To gain insight into the role of Gal-1 in CeD patients, we demonstrated here an increase in Gal-1 expression following GFD that was accompanied by an increased frequency of $Foxp3^+$ cells. The coordinated action of both immunosuppressive mechanisms may occur as synchronized events to generate a tolerogenic milieu in mucosal tissue of treated patients. Since tolerance to gluten peptides would be hard to reestablish under sustained inflammatory conditions, the antigen challenge-free time window (achieved by gluten withdrawal) may allow the development of these immunosuppressive pathways. The subsequent resolution of the inflammatory response may foster the onset of the recovery process, leading to remission of mucosal damage and reestablishment of villi structures.

In line with findings observed in other intestinal inflammatory conditions (76, 77, 84), our observations support the use of Gal-1 agonists to treat severe mucosal inflammation. In addition, Gal-1 may serve as a potential biomarker to follow up CeD progression.

Challenges for the future will embrace the rational manipulation of the Gal-1-glycan axis toward attenuating immune responses in CeD. Studies in *Lgals1*$^{-/-}$ mice will be necessary to determine a putative role of Gal-1 and its specific ligands in supporting mucosal tolerance to gluten. Moreover, the ability of rGal-1 to suppress intestinal inflammation should also be evaluated in experimental CeD models. In this regard, evidence stemming from the study of experimental models of autoimmunity, chronic inflammation, fetomaternal tolerance, and tumor growth provides fundamental insights into the critical role of this lectin and its specific glycosylated ligands in maintaining and restoring immune tolerance and homeostasis, thus encouraging future implementation of Gal-1-based therapies in CeD patients.

ETHICS STATEMENT

Patients and controls were informed in detail about the study, and written consent was obtained. The protocols were approved by Ethics Committees of Hospital "Carlos B. Udaondo."

AUTHOR CONTRIBUTIONS

VS acquired data, analyzed and interpreted data, and wrote the manuscript. AQ developed methodology, analyzed and interpreted data, and revised the manuscript. LM analyzed and interpreted data and revised the manuscript. SN analyzed data, managed patients, and revised the manuscript. AC and ES, and EM managed patients and revised the manuscript. KM analyzed and interpreted data and wrote the manuscript. JB and CM conceived and designed the study, analyzed and interpreted data, and revised the manuscript. GR conceived and designed the study, analyzed and interpreted data, and wrote the manuscript.

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Targeting *Mycobacterium tuberculosis* Antigens to Dendritic Cells *via* the DC-Specific-ICAM3-Grabbing-Nonintegrin Receptor Induces Strong T-Helper 1 Immune Responses

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Tuberculosis remains a major global health problem and efforts to develop a more effective vaccine have been unsuccessful so far. Targeting antigens (Ags) to dendritic cells (DCs) *in vivo* has emerged as a new promising vaccine strategy. In this approach, Ags are delivered directly to DCs *via* antibodies that bind to endocytic cell-surface receptors. Here, we explored DC-specific-ICAM3-grabbing-nonintegrin (DC-SIGN) targeting as a potential vaccine against tuberculosis. For this, we made use of the hSIGN mouse model that expresses human DC-SIGN under the control of the murine CD11c promoter. We show that *in vitro* and *in vivo* delivery of anti-DC-SIGN antibodies conjugated to Ag85B and peptide 25 of Ag85B in combination with anti-CD40, the fungal cell wall component zymosan, and the cholera toxin-derived fusion protein CTA1-DD induces strong Ag-specific $CD4^+$ T-cell responses. Improved anti-mycobacterial immunity was accompanied by increased frequencies of Ag-specific IFN-γ^+ IL-2^+ TNF-α^+ polyfunctional $CD4^+$ T cells in vaccinated mice compared with controls. Taken together, in this study we provide the proof of concept that the human DC-SIGN receptor can be efficiently exploited for vaccine purposes to promote immunity against mycobacterial infections.

Keywords: DC-specific-ICAM3-grabbing-nonintegrin, tuberculosis, vaccine, dendritic cells, Ag85B

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INTRODUCTION

Tuberculosis (Tb) remains one of the leading causes of death worldwide with an estimated 10.4 million people becoming infected per year (1). Currently, the only available vaccine against Tb is *Mycobacterium bovis* Bacillus Calmette-Guérin (BCG); however, it is only partially effective: it provides protection against severe forms of Tb in infants but is unable to prevent the development

Abbreviations: *Mtb*, *Mycobacterium tuberculosis*; Tb, tuberculosis; DCs, dendritic cells; CLR, C-type lectin receptor; Ag, antigen; DC-SIGN, DC-specific-ICAM3-grabbing-nonintegrin; BMDCs, bone-marrow-derived dendritic cells; GM-CSF, granulocyte-macrophage colony-stimulating factor; KLRG1, killer cell lectin-like receptor G1.

of adult pulmonary Tb, the most prevalent form of the disease (2, 3). Thus, there is an urgent need to develop novel vaccine strategies that are safe and effective and can prevent all forms of Tb in different age groups.

Protection against Tb has long been attributed to $CD4^+$ T cells and in particular to IFN-γ-secreting T-helper 1 (Th1) cells (4). However, recent knowledge suggests that additional pathways could also play important roles in vaccine-induced immunity against Tb. In this respect, IL-23-driven Th17 cells were shown to contribute to the generation of antigen (Ag)-specific Th1 cells and the protection against *Mycobacterium tuberculosis* (*Mtb*) following vaccination with BCG (5) and proved to be key effector cells in different parenteral and mucosal subunit-based Tb vaccine models (6–9). Furthermore, BCG- or environmental mycobacteria-induced regulatory T cells (Tregs) have been proposed as one of the reasons for the delayed onset of adaptive immunity observed in Tb and to limit the generation of sterilizing immunity (10, 11).

Dendritic cells (DCs) are specialized Ag-presenting cells that play a central role in initiating and regulating adaptive immunity (12). Owing to their potent Ag presentation capacity and ability to generate distinct T-cell responses, efficient and specific delivery of Ags to DCs is the cornerstone for generating Ag-specific effector and memory cells against tumors or pathogens (13, 14). Administration of autologous DCs exogenously loaded with tumor-Ags was the first DC-based vaccine developed (15). Since then, a few other *ex vivo* DC vaccines have been generated and tested in clinical trials. However, they show low clinical responses and have high production costs, making them unavailable for mass vaccination in developing countries which hold the highest Tb burden (16, 17). To overcome these limitations, a new concept of directly targeting endocytic receptors on DCs by Ag-coupled antibodies or glycosylated molecules was developed as a more effective strategy. Moreover, this type of approach allows the targeting of specific DC subsets while maintaining the natural environment of the cells (13, 17, 18).

C-type lectin receptors (CLRs) are an important family of calcium-dependent lectins that are structurally related through the expression of at least one carbohydrate recognition domain (CRD). Many CLRs are abundantly but also uniquely expressed on the surface of specific DC subsets, where they mediate pathogen recognition and internalization of Ags (19, 20). Due to these properties, CLRs represent ideal candidates for targeting purposes. Pioneer studies in this field focused on the use of antibodies against DEC-205 (CD205) conjugated to OVA to elicit resistance against OVA-modified pathogens and tumors (21–23). However, expression of DEC-205 in humans is not only restricted to DCs (24), thus carrying the possibility of inadvertently targeting other cell types. In contrast, human DC-specific-ICAM3-grabbing-nonintegrin (DC-SIGN, CD209) is predominantly present on the surface of immature monocyte-derived DCs and at lower levels on mature monocyte-derived DCs and macrophages in the skin, mucosal tissues, and secondary lymphoid organs (25, 26). Contrary to humans, who only express DC-SIGN, mice possess eight DC-SIGN homologs in their genome. Sequence analysis of the DC-SIGN receptor family in humans and mice has demonstrated that it underwent substantial divergence between both species. Thus, none of the murine DC-SIGN homologs presents the same functions (glycan specificity, internalization and intracellular trafficking, intercellular adhesion and signaling) as the human DC-SIGN, making the study of this receptor in mice challenging (27, 28). To circumvent this issue, we generated and made use of the hSIGN mouse model which expresses human DC-SIGN under the control of the murine CD11c promoter and thus expresses the human receptor predominantly on DCs (29). We previously demonstrated that DC targeting *via* injection of anti-DC-SIGN antibodies into hSIGN mice induces strong and durable Ag-specific $CD4^+$ and $CD8^+$ T-cell responses capable of mediating protection against infection with OVA-expressing *Listeria monocytogenes* (30). Thus, this study provided powerful evidence that targeting of DC-SIGN *in vivo* results in protection against intracellular pathogens.

Targeting of DCs *via* anti-CLR antibodies is also known to induce tolerance unless an adjuvant is co-delivered (21, 31, 32). Given that adjuvants have the ability of skewing the type of response upon vaccination by the induction of different T-helper subsets, selection of the proper adjuvant system is critical for targeting approaches. In the current study, we aimed to develop a new vaccine strategy against *Mtb* based on targeting DCs through the use of anti-human-DC-SIGN antibodies conjugated to Ag85B, a subdominant but highly immunogenic protein from *Mtb* (33), and peptide 25 (P25) (covering the amino-acid residues 240–254) of Ag85B, a major Th1 epitope (34). We provide here the proof of concept that immunization with anti-DC-SIGN antibodies conjugated to *Mtb* Ags can effectively induce anti-mycobacterial immunity *in vivo*. Furthermore, we characterize the type of response elicited by different adjuvant systems.

MATERIALS AND METHODS

Mice

hSIGN mice were described previously (29) and P25ktk mice (35) were obtained from Jackson Laboratories. P25ktk mice were further crossed to CD45.1 mice. Sex- and age-matched mice between 12 and 18 weeks were used in all experiments. All animals were bred and maintained under specific pathogen-free conditions at the animal facility of TWINCORE, Center for Experimental and Clinical Infection Research (Hannover, Germany) or the Helmholtz Center for Infection Research (HZI, Braunschweig, Germany). All animal experiments were approved by the Veterinary Institute of LAVES (Lower Saxony State Office for Consumer Protection and Food Safety, permit numbers: 12/0732 and 17/2472) considering the German Animal Welfare Act.

Conjugation of anti-DC-SIGN Antibodies

The conjugated monoclonal anti-DC-SIGN (αDC-SIGN) antibodies (clone: AZN-D1, IgG1) to Ag85B protein (αDC-SIGN:Ag85B) and $Ag85B_{240–254}$ peptide (P25) (αDC-SIGN:P25) were prepared as previously described (25). Briefly, the antibodies or an isotype control antibody were conjugated to the different proteins using the crosslinking agent sulfosuccinimidyl-4-(N-maleimidomethyl)-cyclohexane-1-carboxylate according to the manufacturer's protocol (sulfo-SMCC; Pierce).

Flow Cytometry

The following antibodies and reagents were purchased from Thermo Fisher Scientific/eBioscience: anti-CD4 (GK1.5), anti-CD4 (RM4–5), anti-CD45.1 (A20), anti-IFN-γ (XMG1.2), anti-CD11c (N418), anti-MHC-II (M5/114.15.2), anti-CD86 (GL1), anti-IL-17A (eBio17B7), anti-CD44 (IM7), anti-IL-2 (JESG-SH4), anti-TNF-α (MP6-XT22), anti-IL-10 (JES5-16E3), anti-FoxP3 (FJK-16s), anti-KLRG1 (2F1), anti-CD127 (A7R34), and Brefeldin A. Cellular aggregates were excluded by gating singlets using SSC-A versus SSC-W. Dead cells were excluded by LIVE/DEAD® Fixable Aqua Dead (Thermo Fisher Scientific/Invitrogen) cell staining. For intracellular cytokine staining, cells were fixed with 0.5% Paraformaldehyde (Roth) overnight and permeabilized in PBA-S buffer (0.5% Saponin (Roth) and 0.25% BSA (Roth) in PBS). Intranuclear FoxP3 staining was performed using the Fixation/Permeabilization kit (Thermo Fisher Scientific/eBioscience) according to manufacturer's instructions. Data acquisition was performed using a LSRII (BD, Biosciences) or a CyAn™ ADP (Beckman Coulter) flow cytometer. Data analysis was performed with FlowJo software (Tree Star).

Adjuvants

Zymosan was purchased from Sigma-Aldrich and prepared as indicated by the manufacturer's instructions. The non-toxic CTA1-DD adjuvant was kindly provided by Prof. N. Lycke (Department of Clinical Immunology, Göteborg University, Sweden).

In Vitro T-Cell Proliferation Assay

Granulocyte-macrophage colony-stimulating factor (GM-CSF)-derived bone-marrow-derived dendritic cells (BMDCs) were generated from BM cells using a standard protocol. Briefly, BM cells were cultured for 7 days in complete RPMI [10% FCS (Biochrom), 10 mM Hepes (Gibco), 50 µM β-mercaptoethanol (Gibco), 100 U/mL penicillin, and 100 µg/mL streptomycin (Biochrom)] supplemented with 5% culture supernatant of a GM-CSF-producing cell line (36). On day 7, 25.000 BMDC/well were incubated with αDC-SIGN:Ag85B or αDC-SIGN:P25 at the indicated concentrations in the presence of αCD40 (1 µg/mL; clone 1C10) for 24 h, washed and incubated in a 1:8 ratio with $CD4^+$ T cells obtained from the spleen and lymph nodes of P25ktk mice, enriched by negative magnetic selection using the Dynabeads™ Untouched™ Mouse CD4 Cells isolation kit (Thermo Fisher Scientific/Invitrogen) following the manufacturer's instructions. After enrichment, cells were labeled with the CellTrace Violet Cell Proliferation Kit (Thermo Fisher Scientific/Invitrogen). The purity of enrichment was checked by flow cytometry and resulted higher than 85%. Co-cultures were then incubated in complete RPMI medium for 4 days at 37°C in 96-well round bottom plates (Greiner Bio-One/Cellstar). At day 4, cells were stimulated with PMA (0.1 µg/mL) and ionomycin (1 µg/mL). After 2 h of stimulation, Brefeldin A was added for additional 2 h before staining for flow cytometric analysis.

In Vitro Stimulation of BMDCs

GM-CSF-derived BMDCs were generated as mentioned above and stimulated with αCD40 (1 µg/mL) or different concentrations of CTA1-DD or zymosan for 24 h followed by staining for surface activation markers for flow cytometric analysis. LPS (100 ng/mL; *E. coli* Serotype 055:B5; Merck/Sigma Aldrich) was used as positive control. Culture supernatants were collected and ELISA assays were performed to determine IL-6, IL-23, IL-1β, and IL-10 production following the manufacturer's instructions (R&D System).

In Vivo T-Cell Priming

2×10^6 CellViolet-labeled congenic $CD45.1^+$ $CD4^+$ P25ktk T cells were adoptively transferred intravenously (i.v.) into WT or hSIGN mice. One day later, mice were immunized with αDC-SIGN:P25 (2 µg/mouse), αDC-SIGN:Ag85B (2 µg/mouse) or isotype control (2 µg/mouse) in the presence of αCD40 (10 µg/mouse), CTA1-DD (10 µg/mouse) or zymosan (200 µg/mouse). Five days after transfer, spleens were removed and stimulated with PMA (0.1 µg/mL) and ionomycin (1 µg/mL). After 2 h of stimulation, Brefeldin A was added for additional 2 h before staining for flow cytometric analysis.

Vaccination with αDC-SIGN Antibodies

WT or hSIGN mice were immunized with αDC-SIGN:P25 (10 µg/mouse) in combination with αCD40 (10 µg/mouse), CTA1-DD (10 µg/mouse) or zymosan (200 µg/mouse) intraperitoneally (i.p.) three times with a 2-week interval between each immunization. Unvaccinated controls received saline solution (PBS).

Experimental Infections

Vaccinated mice were challenged 42 days after the first immunization by i.v. administration of 2×10^6 colony-forming units (CFUs) of *M. bovis* BCG overexpressing Ag85B (*M. bovis* BCG-Ag85B), kindly provided by Dr. Joel Ernst (NYU School of Medicine, USA). *M. bovis* BCG-Ag85B was grown at 37°C in Middlebrook 7H9 broth (BD Bisociences) supplemented with 10% Middlebrook oleic acid–albumin–dextrose–catalase (OADC) enrichment medium (Difco Laboratories), 0.05% of Tween 80 (Roth) and 0.002% glycerol (Roth). After 5 days of infection, mice were sacrificed and spleens were collected in sterile bags (Nasco) containing 1 mL of WTA buffer [0.01% Tween-80 and 0.05% BSA (Roth)] and mechanically disrupted. Viable bacterial loads were determined by plating serial dilutions onto Middlebrook 7H11 agar (BD Biosciences) supplemented with 10% OADC (Difco Laboratories) and 0.5% glycerol (Roth). Colonies were counted after 2 to 3 weeks of incubation at 37°C.

Statistical Analysis

Data analysis was performed using GraphPad Prism Software 5.0. Statistics were calculated using one-way or two-way ANOVA as indicated in figure legends. *P*-Values were considered significant as follows: $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

RESULTS

Targeting *Mtb* Ags to DCs *via* DC-SIGN Induces Strong $CD4^+$ T-Cell Responses

We previously showed that αDC-SIGN antibodies conjugated to OVA induce strong and persistent Ag-specific $CD4^+$ and

$CD8^+$ T-cell responses which promote rapid clearance of OVA-expressing *Listeria monocytogenes* infection (30). Thus, we proposed DC targeting *via* DC-SIGN as a promising strategy for vaccination protocols against intracellular pathogens. In order to determine whether this strategy could be effective against *Mtb*, we conjugated αDC-SIGN antibodies to Ag85B (αDC-SIGN:Ag85B) and $Ag85B_{240–254}$ peptide (P25) (αDC-SIGN:P25) and tested their capacity to induce T-cell activation *in vitro*. To achieve this, BMDCs were prepared from WT or hSIGN mice and pulsed with different concentrations of αDC-SIGN:Ag85B or αDC-SIGN:P25 in the presence of αCD40 as an adjuvant. The capacity of antibody-targeted WT and hSIGN BMDCs to promote T-cell responses was then evaluated by co-culturing them with CellViolet-labeled $CD4^+$ T cells purified from P25ktk mice, carrying a transgenic TCR that specifically reacts to P25 in the context of MHC class-II presentation (35). After 4 days of co-culture, cell proliferation and cytokine production were measured by flow cytometry as parameters of T-cell activation. Both αDC-SIGN:Ag85B and αDC-SIGN:P25 in combination with αCD40 were able to promote Ag presentation by BMDCs generated from hSIGN but not WT mice, as evidenced by their ability to induce the proliferation of $CD4^+$ P25ktk T cells (**Figures 1A,C**). Moreover, both conjugated antibodies also promoted the production of IFN-γ in the proliferating $CD4^+$ P25ktk T cells, indicating that targeting DCs *via* DC-SIGN not only results in Ag presentation to $CD4^+$ T cells *in vitro* but also in efficient cytokine production (**Figures 1B,D**).

We next determined whether targeting Ags to DCs *via* human DC-SIGN also leads to enhanced T-cell responses *in vivo*. To this aim, we adoptively transferred CellViolet-labeled congenic $CD45.1^+$ $CD4^+$ P25ktk T cells into WT or hSIGN-recipient mice and 1 day later immunized them with either αDC-SIGN:Ag85B, αDC-SIGN:P25, isotype or vehicle control in combination with αCD40. Four days after immunization, mice were sacrificed and the proliferation as well as cytokine production of the transferred T cells was determined by flow cytometry after gating on $CD45.1^+$ $CD4^+$ T cells in the splenic cell population. Treatment of hSIGN mice with either αDC-SIGN:Ag85B or αDC-SIGN:P25 led to significant expansion and IFN-γ production of the transferred $CD45.1^+$ $CD4^+$ P25ktk T cells. In contrast, in WT mice only a marginal expansion of Ag-specific $CD4^+$ T cells was observed upon immunization, indicating that the conjugated Ags were preferentially delivered to DCs *via* the DC-SIGN receptor. Similar results were obtained after the administration of an isotype antibody in combination with αCD40 while the vehicle control failed to induce T-cell proliferation (**Figures 1E,F**). Thus, *in vitro* and *in vivo* targeting of DCs through αDC-SIGN antibodies conjugated to *Mtb* Ags efficiently promotes Ag presentation, proliferation and IFN-γ production by $CD4^+$ T cells.

CTA1-DD and Zymosan Induce DC Activation and Cytokine Production

In recent years, it has been demonstrated that vaccine-induced immunity against *Mtb* not only depends on the generation of Th1 cells but also on other T-helper subsets such as Th17 cells (5, 7). Thus, adjuvant selection for new vaccine candidates is critical. Cholera toxin (CT) is a potent cyclic adenosine monophosphate (cAMP)-based adjuvant which can effectively prime Th17 cells *via* stimulation of $CD11b^+$ DCs (37). Unfortunately, CT is not approved for human vaccination due to its high toxicity. To solve this problem, a fusion protein between the A1 catalytic domain of CT and two immunoglobulin-binding D regions from *Staphylococcus aureus* protein A called CTA1-DD was developed with the same adjuvant activity of CT but without its toxicity (38). Through a different mechanism, the *Saccharomyces cerevisiae* cell-wall component zymosan also promotes strong Th17 cell responses using TLR-dependent and -independent pathways (39–41). Therefore, we evaluated the ability of these adjuvants to broaden the spectrum of T-helper responses elicited by our αDC-SIGN antibodies. We first tested whether CTA1-DD and zymosan were able to induce DC maturation and cytokine production. For this, we generated BMDCs from WT and hSIGN mice and incubated them with αCD40 (1 μg/mL), LPS (100 ng/mL) and different concentrations of CTA1-DD and zymosan. After 24 h, upregulation of the activation marker CD86 was measured by flow cytometry and cytokine production by ELISA. CTA1-DD only mildly enhanced CD86 surface expression by WT and hSIGN DCs (**Figures 2A,B**) but was able to increase the percentage of $CD86^{hi}$ $MHC\text{-}II^{hi}$ DCs of both genotypes at the highest concentration tested (**Figure 2C**). Moreover, CTA1-DD also promoted the secretion of IL-6, IL-23 and IL-1β in a dose-dependent manner while inducing low levels of IL-10 (**Figures 2D–G**). In contrast, zymosan significantly upregulated CD86 expression (**Figures 2A,B**) and increased the percentage of $CD86^{hi}$ $MHC\text{-}II^{hi}$ DCs of both genotypes at all concentrations tested (**Figure 2C**). Overall, zymosan induced the highest cytokine levels (**Figures 2D–G**). Surprisingly, αCD40 proved a poor stimulus for DC activation, at least at the studied concentration (**Figures 2A–G**). As expected, the TLR4 agonist LPS served as positive control promoting DC maturation and cytokine production (**Figures 2A–G**). In addition, no marked differences were observed between WT and hSIGN BMDCs in regard to their ability to upregulate CD86 expression and secrete pro- and anti-inflammatory cytokines, demonstrating that human DC-SIGN expression does not affect DC function/activation. Taken together, these results indicate that both CTA1-DD and zymosan are capable of promoting DC activation and cytokine production.

Administration of αDC-SIGN:P25 with CTA1-DD or Zymosan Promotes Effective T-Cell Priming

We next determined whether CTA1-DD and zymosan in combination with the αDC:SIGN conjugates could also induce specific T-cell responses *in vivo*. Given the similar performance observed in our preliminary results, we opted to focus on the αDC-SIGN:P25 antibody for further experiments. To investigate this, we performed adoptive transfer assays as described above using CellViolet-stained congenic $CD45.1^+$ $CD4^+$ P25ktk T cells transferred into WT or hSIGN-recipient mice. One day later, we injected αDC-SIGN:P25 in the presence of CTA1-DD or zymosan. Expansion and cytokine production by the transferred cells was evaluated 4 days later by flow cytometry. Immunization of hSIGN

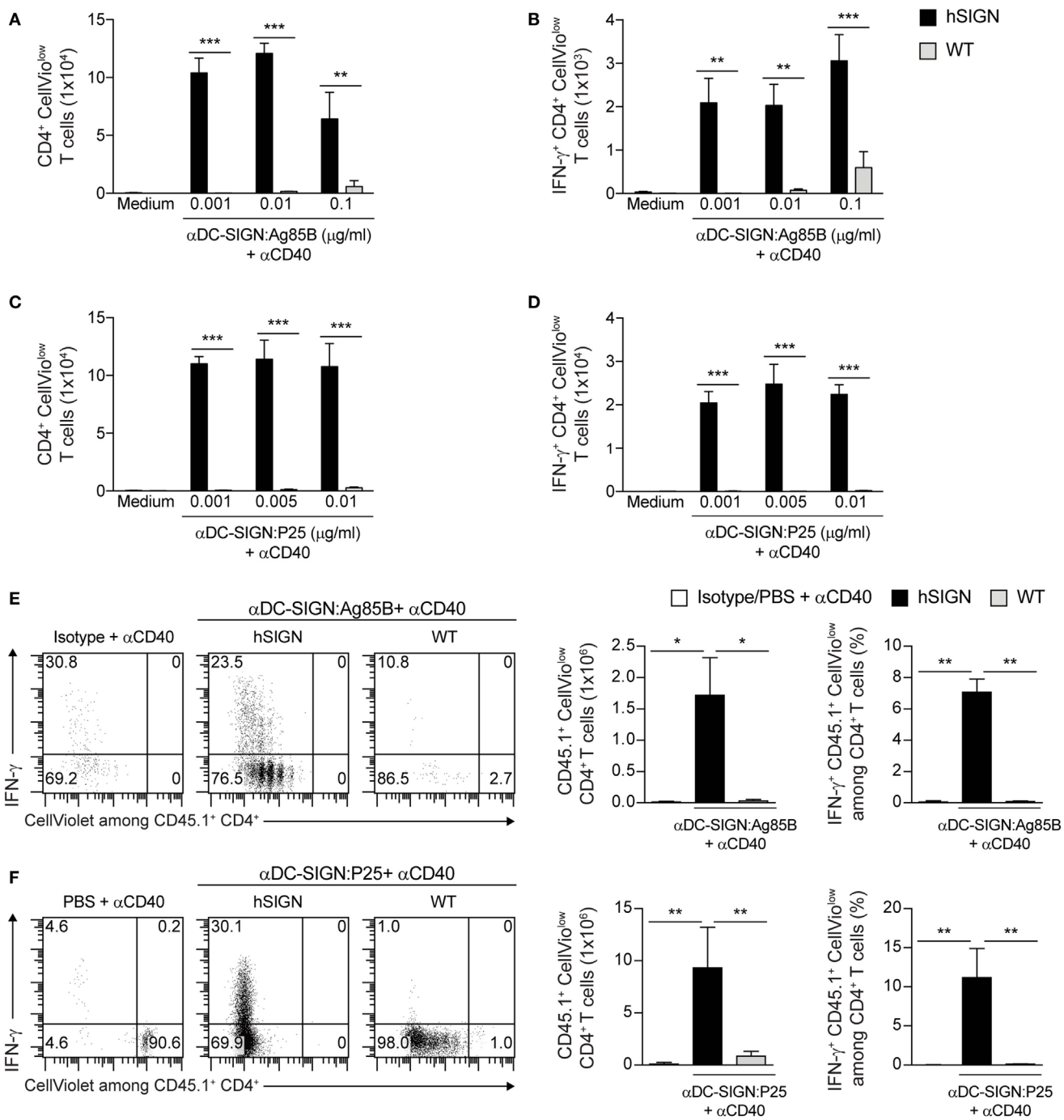

FIGURE 1 | *In vitro* and *in vivo* targeting of *Mycobacterium tuberculosis* antigens to DC *via* DC-specific-ICAM3-grabbing-nonintegrin (DC-SIGN) induces strong CD4+ T-cell responses. **(A–D)** CellViolet-labeled CD4+ P25ktk T cells were co-cultured with WT or hSIGN BMDC targeted with increasing doses of **(A,B)** αDC-SIGN:Ag85B or **(C,D)** αDC-SIGN:P25 in the presence of αCD40 (1 μg/mL). After 4 days of co-culture **(A,C)** *in vitro* proliferation of CD4+ T cells and **(B,D)** intracellular IFN-γ production were determined by flow cytometry after restimulation with PMA/ionomycin. Bar graphs represent the **(A,C)** number of proliferating CellVio^{low} CD4+ cells and **(B,D)** number of proliferating IFN-γ+ CellVio^{low} CD4+ cells. Error bars represent SD of triplicate wells from one of three experiments. $^{**}p < 0.01$ and $^{***}p < 0.001$; two-way ANOVA with Bonferroni's *post hoc* test. **(E,F)** CellViolet-labeled CD45.1+ CD4+ P25ktk T cells were adoptively transferred into WT or hSIGN mice. One day later, mice were immunized with **(E)** αDC-SIGN:Ag85B (2 μg), **(F)** αDC-SIGN:P25 (2 μg) or an isotype control (2 μg) **(E)** or vehicle **(F)** in the presence of αCD40 (10 μg). Four days post-immunization **(E,F)** *in vivo* cell proliferation and intracellular IFN-γ production were determined by flow cytometry in splenocytes after *ex vivo* restimulation with PMA/ionomycin. Shown are representative flow cytometry plots depicting the percentage of IFN-γ+ CellVio^{low} among CD45.1+ CD4+ T cells. Bar graphs represent the total number of proliferating CD45.1+ CellVio^{low} CD4+ cells and percent of proliferating IFN-γ+ CD45.1+ CellVio^{low} among all live CD4+ T cells. Error bars represent SD of 3–5 mice per group from one of two experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$; one-way ANOVA with Bonferroni's *post hoc* test.

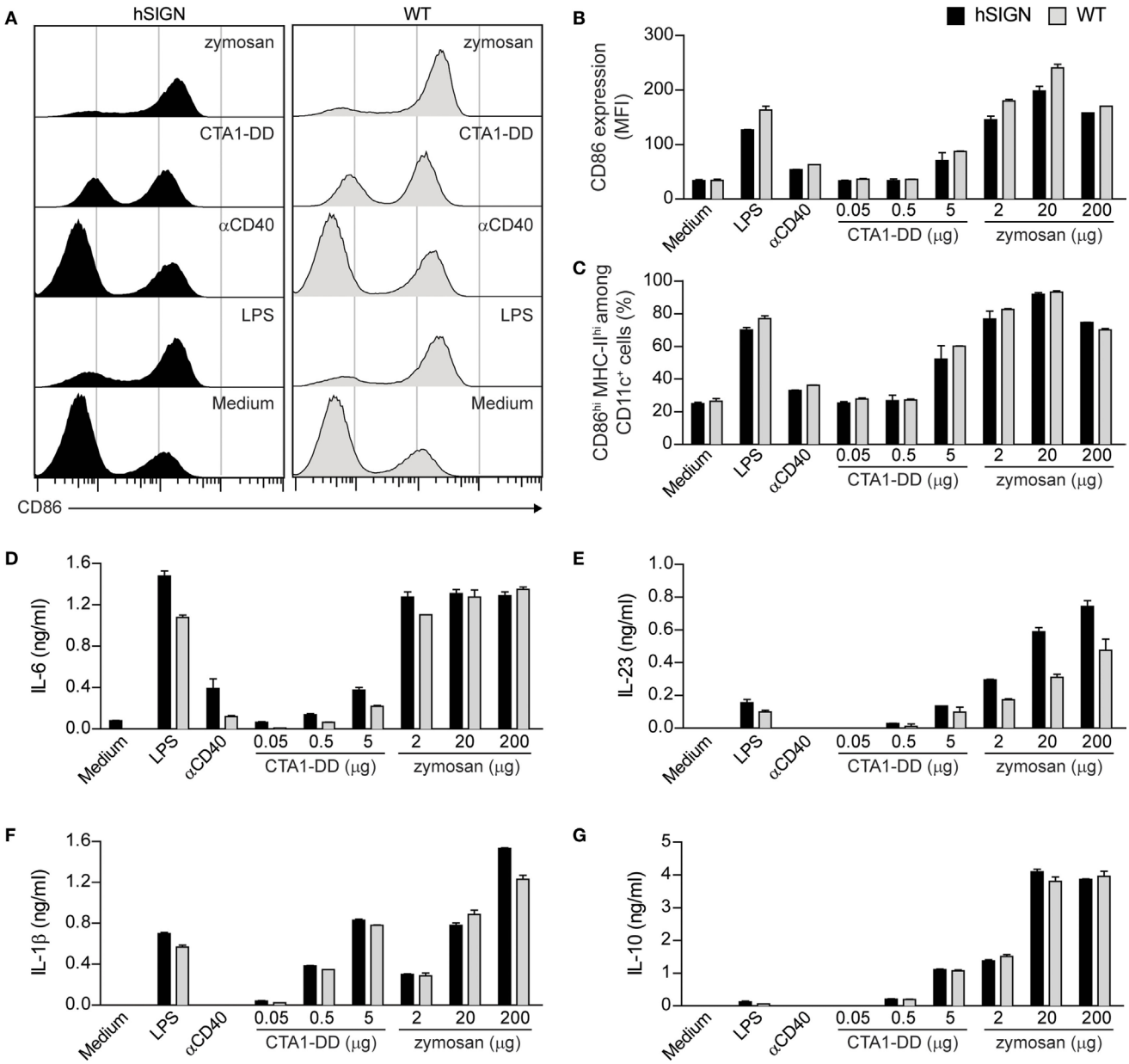

FIGURE 2 | CTA1-DD and zymosan induce DC activation and cytokine production. **(A–G)** WT or hSIGN BMDC were treated with αCD40 (1 μg/mL) and different concentrations of CTA1-DD and zymosan. LPS (100 ng/mL) was used as a positive control. After 24 h stimulation, **(A–C)** CD86 and MHC-II surface expression was determined by flow cytometry and **(D)** IL-6, **(E)** IL-23, **(F)** IL-1β and **(G)** IL-10 production by ELISA. **(A)** Representative histograms from one of three independent experiments. **(B)** Bar graphs represent mean fluorescence intensity (MFI) of CD86 and **(C)** percent of $CD86^{hi}$ $MHC\text{-}II^{hi}$ among $CD11c^{+}$ cells. Error bars represent SD of triplicate wells from one of three experiments.

but not WT mice with αDC-SIGN:P25 plus either CTA1-DD or zymosan significantly induced expansion and IFN-γ production of the transferred $CD45.1^{+}$ $CD4^{+}$ P25ktk T cells (**Figures 3A–C**). However, only zymosan was able to significantly enhance IL-17A production by the transferred $CD4^{+}$ T cells (**Figure 3D**). Thus, targeting DC-SIGN using a P25-conjugated αDC-SIGN antibody co-delivered with CTA1-DD or zymosan promotes the generation and proliferation of Ag-specific Th1 cells. Furthermore, zymosan can, in addition, slightly prime Th17 cells.

Vaccination with αDC-SIGN:P25 Plus αCD40, CTA1-DD, or Zymosan Does Not Induce Th17 Cells but Prevents the Expansion of Tregs

Having demonstrated the potential of αDC:SIGN antibodies to target DC and induce strong Ag-specific T-cell responses, we decided to evaluate the αDC-SIGN:P25 antibody in a standard vaccination protocol. For this, we immunized WT or hSIGN

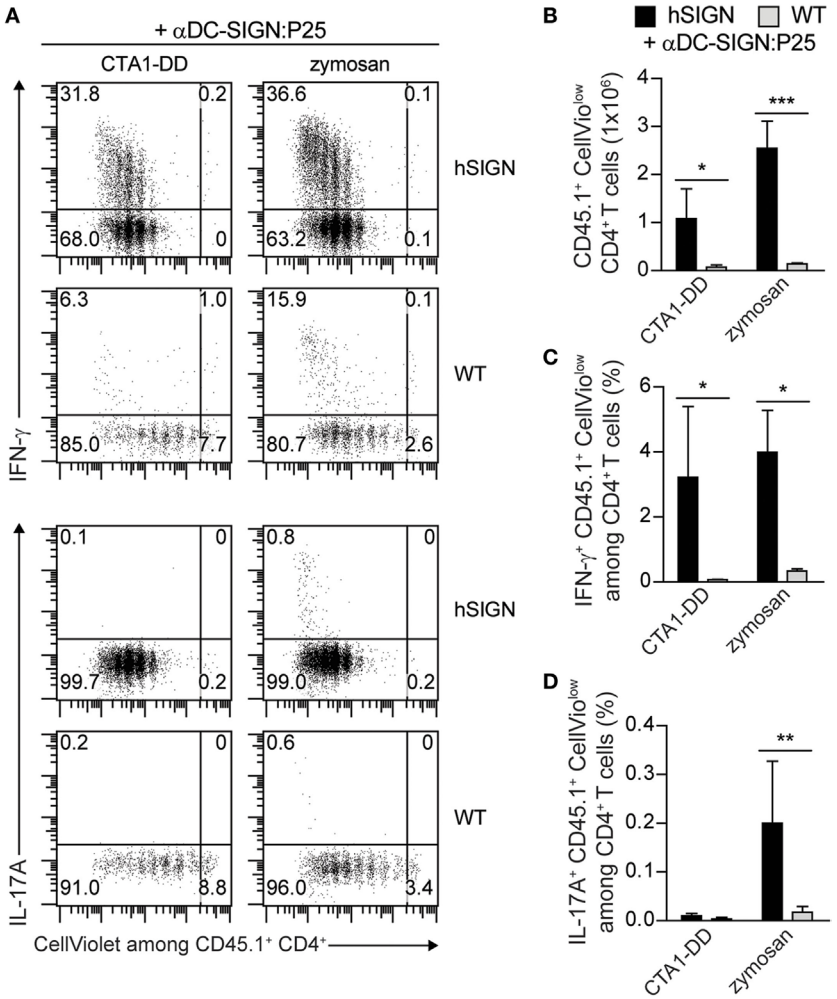

FIGURE 3 | Administration of αDC-SIGN:P25 with CTA1-DD or zymosan promotes T-cell priming. **(A–D)** CellViolet-labeled CD45.1$^+$ CD4$^+$ P25ktk T cells were adoptively transferred into WT or hSIGN mice. One day later, mice were immunized with αDC-SIGN:P25 (2 μg) plus CTA1-DD (10 μg) or zymosan (200 μg). Four days post-immunization **(A,B)** *in vivo* cell proliferation and intracellular **(A,C)** IFN-γ and **(A,D)** IL-17A production were determined by flow cytometry in splenocytes after *ex vivo* restimulation with PMA/ionomycin. **(A)** Representative flow cytometry plots depicting the proliferation and percentage of IFN-γ$^+$ (upper panels) or IL-17A$^+$ (lower panels) CellVio^{low} among CD45.1$^+$ CD4$^+$ T cells. Bar graphs represent the **(B)** total number of proliferating CD45.1$^+$ CellVio^{low} CD4$^+$ cells and percent of proliferating **(C)** IFN-γ$^+$ or **(D)** IL-17A$^+$ CD45.1$^+$ CellVio^{low} among all live CD4$^+$ T cells. Error bars represent SD of three mice per group from one of two experiments. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$; two-way ANOVA with Bonferroni's *post hoc* test.

mice i.p. at days −42, −28, and −14 prior to *M. bovis* BCG-Ag85B infection with αDC-SIGN:P25 (10 μg) plus either αCD40 (10 μg), CTA1-DD (10 μg) or zymosan (200 μg). As control group, mice were treated with vehicle (PBS). At day 0, mice were infected i.v. with 2×10^6 *M. bovis* BCG-Ag85B, a strain that overexpresses Ag85B. Five days later, bacterial loads and intracellular cytokine production after *ex vivo* restimulation with the cognate peptide P25 were determined in spleens (**Figure 4A**). Contrary to our short-term vaccination experiments, we could not detect any differences in bacterial burden nor a significant increase in the percentage of P25-specific IL-17A-producing CD4$^+$ T cells in hSIGN mice vaccinated with the αDC-SIGN:P25 antibody plus any of the adjuvants used (**Figures 4B,C**). These results seem to indicate that in the tested settings the αDC-SIGN:P25 antibody is unable to promote a persistent Th17 response and does not impact bacterial growth. On the other hand, limited vaccine efficacy is thought to be linked to the expansion of Tregs upon BCG administration (10). Therefore, vaccine candidates should avoid induction of anti-inflammatory immune responses. Thus, we also evaluated whether vaccination with αDC-SIGN:P25 plus αCD40, CTA1-DD or zymosan could increase the population of IL-10-producing T cells or FoxP3$^+$ Tregs in spleens. hSIGN mice vaccinated with CTA1-DD showed a significant increase in the percentage of P25-specific IL-10$^+$ CD4$^+$ T cells only compared with unvaccinated controls (**Figure 4D**). Yet, we could not detect differences in the percentage of IL-10-producing CD4$^+$ T cells in any of the other experimental groups (**Figure 4D**). Regarding Tregs, hSIGN mice vaccinated in the presence of zymosan showed a significant decrease in FoxP3$^+$ CD4$^+$ T cells compared with PBS-treated controls (**Figure 4E**). None of the

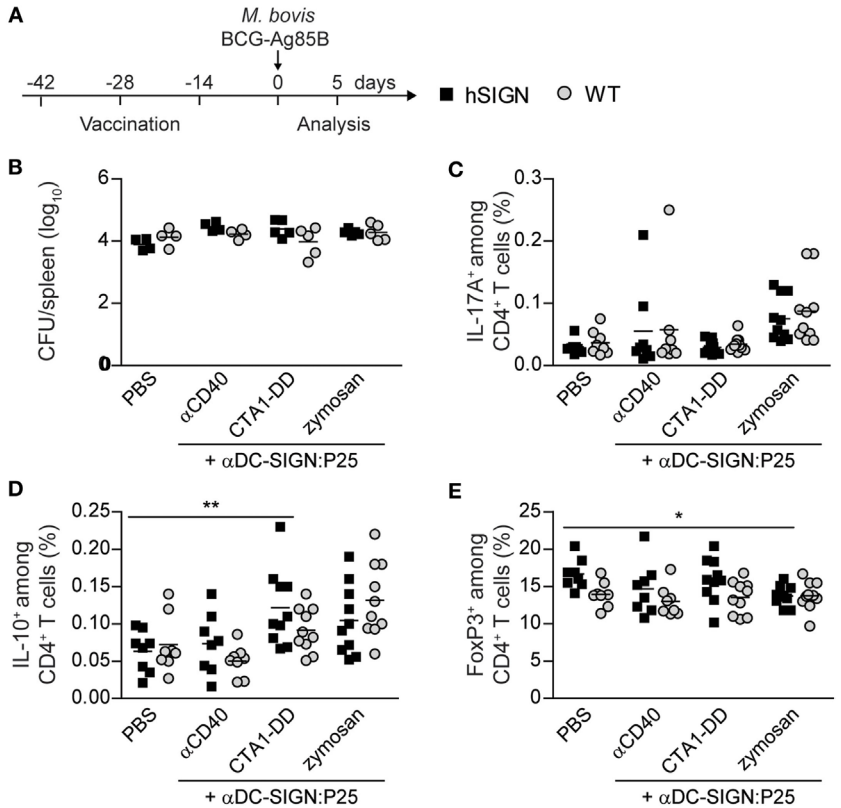

FIGURE 4 | Vaccination with αDC-SIGN:P25 plus αCD40, CTA1-DD or zymosan does not induce Th17 cells but prevents the expansion of regulatory T cells. **(A)** Experimental scheme. WT or hSIGN mice were vaccinated i.p. at days −42, −28, and −14 with vehicle (PBS) or αDC-SIGN:P25 (10 μg) plus αCD40 (10 μg), CTA1-DD (10 μg) or zymosan (200 μg). At day 0, mice were challenged i.v. with 2×10^6 *Mycobacterium bovis* BCG-Ag85B. Five days later, bacterial burden, intracellular cytokine production after *ex vivo* restimulation with P25 (30 μg/mL), and FoxP3 expression were determined in spleen. **(B)** Graph represents the logarithmic transformation of the number of colony-forming units (CFUs) per spleen of vaccinated mice. **(C–E)** Graphs represent percent of **(C)** P25-specific IL-17A$^+$, **(D)** IL-10$^+$ and **(E)** FoxP3$^+$ among CD4$^+$ T cells of vaccinated mice. Each symbol represents an individual mouse and results are pooled from two experiments with 4–5 mice per group. $^*p < 0.05$ and $^{**}p < 0.01$; two-way ANOVA with Bonferroni's *post hoc* test.

other adjuvant systems employed showed differences in this population compared with controls (**Figure 4E**), suggesting that Tregs are not significantly expanded during our vaccination approach.

Vaccination With αDC-SIGN:P25 Plus αCD40, CTA1-DD, or Zymosan Induces Pro-inflammatory Cytokine Production

T-helper 1 cells have a preponderant role in mounting protective immune responses against Tb (42, 43). Polyfunctional T cells, defined as able to produce multiple pro-inflammatory cytokines, such as IFN-γ, IL-2 and TNF-α, have been identified as correlates of protection in the mouse model of Tb (44, 45). We therefore tested the ability of the αDC-SIGN:P25 antibody in combination with αCD40, CTA1-DD or zymosan to induce this type of responses after vaccination and *ex vivo* restimulation with P25 (**Figure 5A**). hSIGN mice vaccinated with αDC-SIGN:P25 plus αCD40 only showed a mild increase in the percentage of Ag-specific IFN-γ and IL-2-producing cells among CD44$^+$ CD4$^+$ T cells (**Figures 5B,C**). In the case of CTA1-DD, we could observe a significant increase in the percentage of IFN-γ, IL-2 and TNF-α-producing cells among CD44$^+$ CD4$^+$ T cells in hSIGN mice compared with WT and unvaccinated controls (**Figures 5B,D**). Moreover, we could observe a slight but significant increase in the percentage of Th1-type polyfunctional CD4$^+$ T cells, which co-secrete IFN-γ, IL-2 and TNF-α and are indicators of vaccine-induced immunity against Tb (**Figures 5B,D**). Concerning zymosan, the generation of IFN-γ, IL-2 and TNF-α-producing cells among CD44$^+$ CD4$^+$ T cells was significantly enhanced in hSIGN mice with respect to WT and unvaccinated controls and percentages of Th1-polyfunctional T cells were also significantly higher (**Figures 5B,E**). It is important to note that we obtained the strongest immune responses with zymosan. Taken together, these results suggest that DC targeting *via* αDC-SIGN:P25 in combination with CTA1-DD or zymosan induces immunity against *Mtb via* the generation of Th1-type cells and polyfunctional CD4$^+$ T cells.

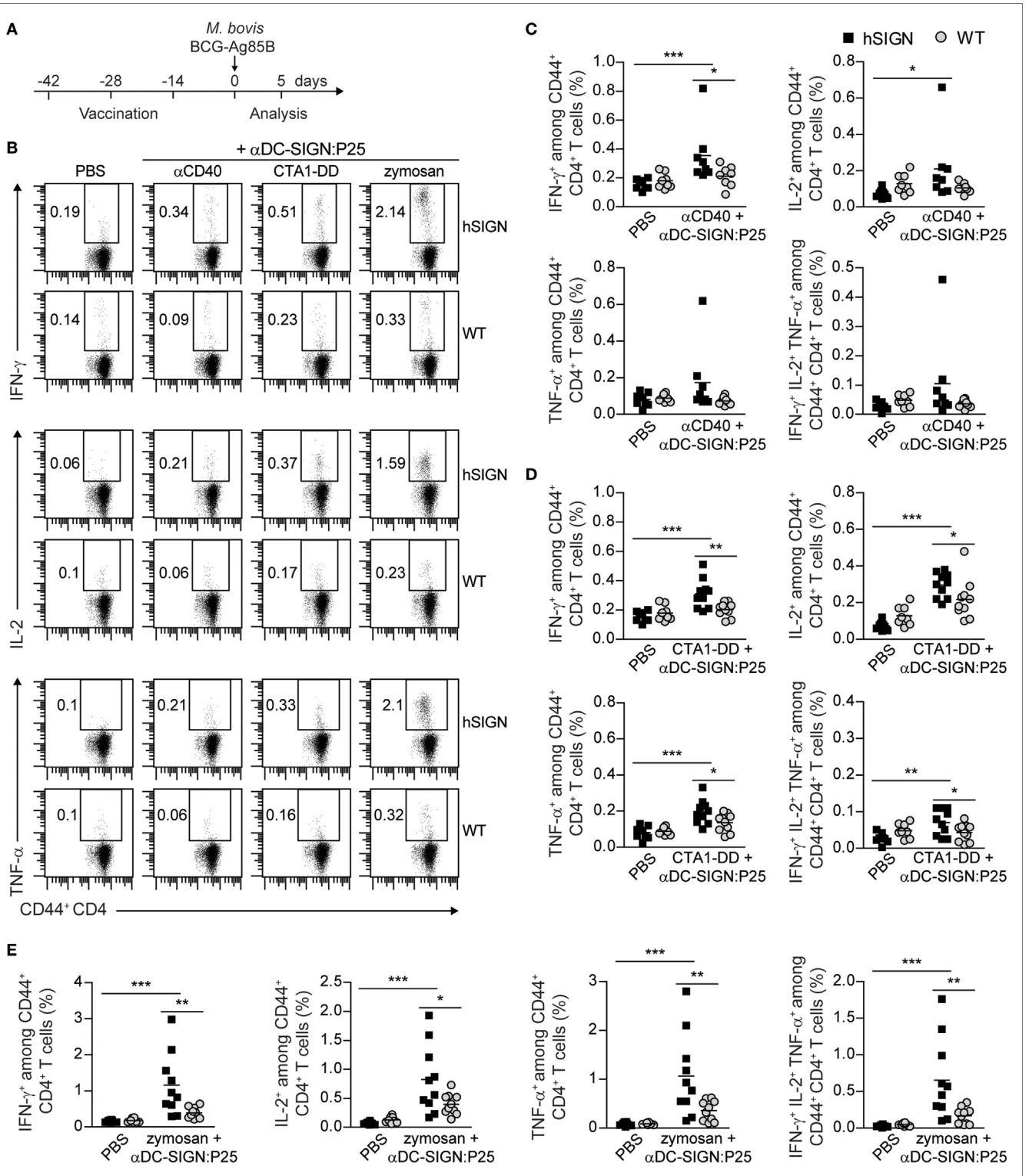

FIGURE 5 | Vaccination with αDC-SIGN:P25 plus αCD40, CTA1-DD or zymosan induces pro-inflammatory cytokine production. **(A)** Experimental scheme. WT or hSIGN mice were vaccinated i.p. at days −42, −28, and −14 with vehicle (PBS) or αDC-SIGN:P25 (10 μg) plus αCD40 (10 μg), CTA1-DD (10 μg) or zymosan (200 μg). At day 0, mice were challenged i.v. with 2×10^6 *Mycobacterium bovis* BCG-Ag85B. Five days later, mice were sacrificed and splenocytes were isolated and stained for intracellular cytokine production after *ex vivo* restimulation with P25 (30 μg/mL). **(B)** Representative flow cytometry plots depicting the percentage of IFN-γ$^+$ (upper panels), IL-2$^+$ (middle panels) and TNF-α$^+$ (lower panels) among CD44$^+$ CD4$^+$ T cells. **(C–E)** Graphs represent percent of IFN-γ$^+$, IL-2$^+$, TNF-α$^+$ and polyfunctional (IFN-γ$^+$ IL-2$^+$ TNF-α$^+$) among CD44$^+$ CD4$^+$ T cells of mice vaccinated with **(C)** αCD40, **(D)** CTA1-DD and **(E)** zymosan. Each symbol represents an individual mouse and results are pooled from two experiments with 4–5 mice per group. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$; two-way ANOVA with Bonferroni's *post hoc* test.

αDC-SIGN:P25 Plus Zymosan Generates Hyperactivated CD4+ T Cells

In recent years, several reports have shown that the state of differentiation and polarization of Th1 cells is important to determine their ability to control *Mtb* infection. Indeed, it has been demonstrated that less-polarized CD4+ T cells are more beneficial in terms of long-term protection against this pathogen (46–49). Thus, we analyzed the CD4+ T-cell memory and activation profile generated by the αDC-SIGN:P25 antibody in combination with the different adjuvants according to the experimental scheme in **Figure 6A**. hSIGN mice vaccinated with αDC-SIGN:P25 plus zymosan showed a significant increase in the percentage of both effector memory (KLRG+ CD127+) (**Figures 6B,C**) and terminally differentiated effector (KLRG1+ CD127−) CD4+ T cells in the blood (**Figures 6B,D**). On the contrary, αCD40 and CTA1-DD did not induce an increase in those populations. These results correlate with the fact that zymosan proved to be the strongest stimulus for the activation of CD4+ T cells and indicate that this adjuvant induces a rapid response upon infection with a tendency toward a more differentiated phenotype.

DISCUSSION

The development of an effective vaccine against Tb remains an unresolved public health issue. Of the many vaccine candidates in the clinical trial pipeline, so far none has proven to provide protection against infection or sterilizing immunity. In this study, we explored the potential of DC targeting *via* human DC-SIGN as a novel vaccine strategy against Tb. Using anti-human-DC-SIGN antibodies conjugated to Ag85B and P25 we could demonstrate that the CLR DC-SIGN can be efficiently targeted in DCs which results in the proliferation and IFN-γ production of P25-specific

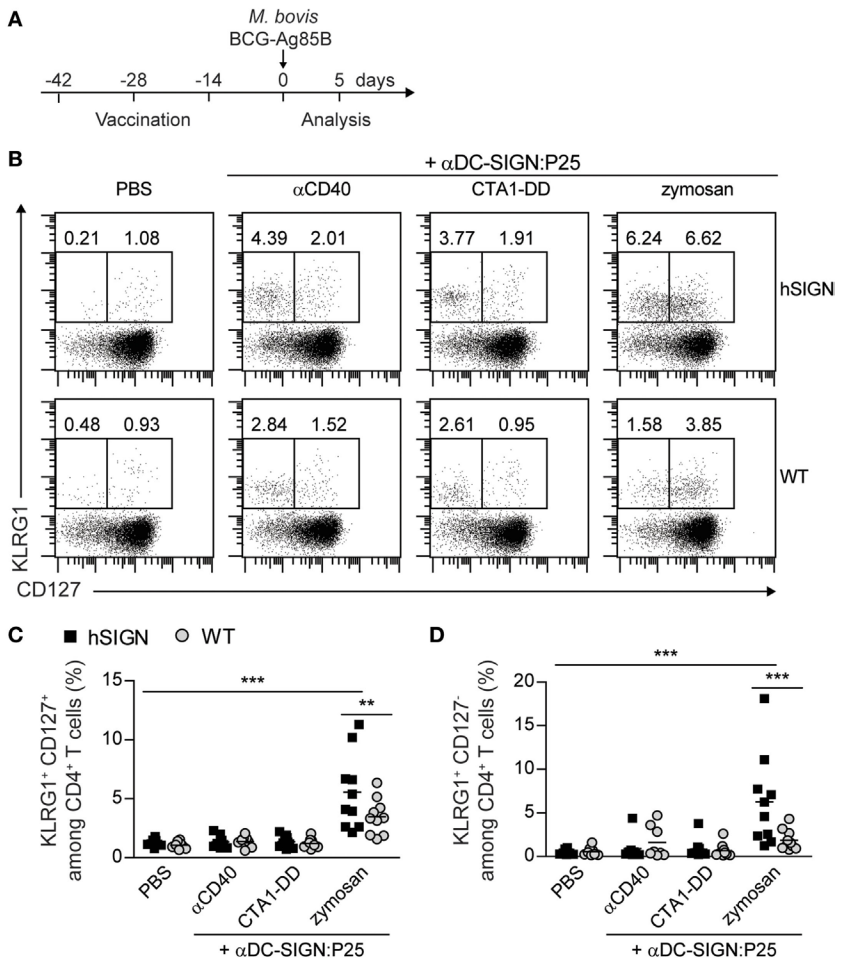

FIGURE 6 | αDC-SIGN:P25 plus zymosan generates hyperactivated CD4+ T cells. **(A)** Experimental scheme. WT or hSIGN mice were vaccinated i.p. at days −42, −28, and −14 with vehicle (PBS) or αDC-SIGN:P25 (10 μg) plus αCD40 (10 μg), CTA1-DD (10 μg) or zymosan (200 μg). At day 0, mice were challenged i.v. with 2×10^6 *Mycobacterium bovis* BCG-Ag85B. Five days later, mice were sacrificed and blood was isolated and stained for killer cell lectin-like receptor G1 (KLRG1) and CD127 surface expression. **(B)** Representative flow cytometry plots depicting percentage of KLRG1+ CD127+ and KLRG1+ CD127− among CD4+ T cells. **(C,D)** Graphs represent percent of **(C)** KLRG1+ CD127+ and **(D)** KLRG1+ CD127− among CD4+ T cells of vaccinated mice. Each symbol represents an individual mouse and results are pooled from two experiments with 4–5 mice per group. $^{**}p < 0.01$ and $^{***}p < 0.001$; two-way ANOVA with Bonferroni's *post hoc* test.

$CD4^+$ T cells *in vitro* and *in vivo*. We also demonstrated that the type of T-cell response elicited by the anti-DC-SIGN antibodies and its strength is influenced by the adjuvant system used for the delivery. Finally, we show that anti-mycobacterial immunity based on Th1 and polyfunctional $CD4^+$ T cells is achieved in a vaccination protocol with the anti-DC-SIGN antibody conjugated to P25.

We here provide evidence that human DC-SIGN is a potent targeting receptor that has the ability of inducing strong immune responses. First, in our *in vitro* results we show that low amounts of anti-DC-SIGN antibodies (0.001–0.1 μg/mL) are enough to induce Ag presentation and secretion of IFN-γ. This represents an advantage to *ex vivo* DC vaccines, where not only higher amounts of protein are needed but also the generation of autologous DCs is time consuming and expensive. Second, we also demonstrate that the presence of human DC-SIGN on DCs is sufficient to promote Ag presentation and the induction of an immune response upon immunization with anti-DC-SIGN antibodies *in vivo*. Though some response can be observed in WT and isotype-treated mice, probably due to the binding of the antibodies to Fc receptors or other receptors in an unspecific manner, the response in hSIGN mice is much stronger with high percentages of IFN-γ-producing cells. This corresponds with the fact that the IgG1 murine AZN-D1 antibody used for our targeting purposes has been shown to induce fast clathrin-dependent internalization of DC-SIGN upon binding and to direct Ags to late endosomal compartments facilitating efficient Ag presentation (50). Third, we have previously demonstrated that conventional DCs are the main target of the anti-DC-SIGN antibodies in this kind of vaccination approach. hSIGN mice express the human DC-SIGN receptor under the control of the CD11c promoter. Though CD11c is not exclusively expressed on DCs, we have previously shown that the transgene is predominantly expressed on $CD11c^{high}$ $SiglecH^-$ conventional DCs and can be detected in spleen, lymph nodes and lungs of hSIGN mice *via* immunohistochemistry (29, 30). Furthermore, the complementarity determining regions of AZN-D1 were grafted onto a human IgG2/IgG4 composite antibody with the objective of generating a humanized antibody which was later conjugated to the model Ag keyhole limpet hemocyanin (KLH) resulting in 100-fold more efficient targeting of human DCs compared with *ex vivo* Ag loading (51). Hence, extrapolating DC-SIGN targeting into a human vaccine could be an achievable aim. In this regard, it would be interesting to evaluate which subsets of DCs are the main targets for this vaccination approach.

Our results demonstrate that DC-SIGN targeting can generate P25-specific Th1 and polyfunctional T cells without significantly inducing IL-10-producing T cells or expanding the Treg population. Protective immunity against *Mtb* is known to rely on IFN-γ-secreting Th1 cells. The fact that Th1 cells play a central role in protection against Tb is based on several factors: (i) mice lacking $CD4^+$ T cells, IFN-γ and IL-12 signaling or T-bet are highly susceptible to infection (4); (ii) individuals with genetic deficiencies in IFN-γ and IL-12 signaling are unable to control mycobacterial infections (52); (iii) HIV patients co-infected with Tb have increased risk of developing active disease (53). Apart from Th1 cells, other potential correlates of protection have been identified in the mouse model of Tb. The frequency and quality of polyfunctional $CD4^+$ T cells was shown to correlate with protective immunity when comparing five different Tb vaccine models (live-attenuated, subunit, viral vectored, plasmid DNA and combination vaccines) (44). In contrast, Tregs have been shown to negatively influence vaccine efficacy (10). In this respect, our targeting strategy has the added advantage of avoiding the induction of these types of counteractive immune responses.

T-helper 17 cells have also been shown to provide protection against Tb either by recruiting Th1 cells to the infected lung or even by IFN-γ-independent mechanisms (5, 7, 54). Zymosan is a strong Th17-inducer stimulus, used in different IL-17-dependent experimental models such as arthritis (55). Upon treatment with zymosan, BMDCs were shown to promote the differentiation of naïve T cells into IL-17-producing cells *in vitro* and to support the induction of experimental autoimmune encephalomyelitis (EAE) symptoms in mice (40). In contrast, CTA1-DD was designed as a mucosal adjuvant capable of inducing powerful antibody responses (38). We demonstrated that both of these adjuvants were able to induce the activation of BMDCs and the secretion of IL-6, IL-23, and IL-1β, all important cytokines necessary for the induction of Th17 responses (56, 57). The fact that we could not observe differences in the percentages of P25-specific Th17 cells after vaccination could be due to the low amounts of IL-17A-producing cells generally observed in our experimental system. Likewise, in our previous reports *M. bovis* BCG-infected mice showed only marginal Th17 percentages at 21 days post-infection (58). This suggests that in our settings this T-cell subset is underrepresented, probably due to the restricted flora in our animal facility (SPF conditions). In this sense, there is evidence that the diversity of the intestinal microbiota and the presence of specific segmented filamentous bacteria can influence the amount of intestinal Th17 cells in mice from the same strain but from different facilities (59, 60). Thus, testing our vaccination system in mice with a more diverse microbiota would be important to define the importance of Th17 for anti-DC-SIGN-mediated immunity.

Several studies suggest that the activation status as well as the migration capacity of Th1 cells themselves is important to generate long-term protection. Distinct phenotypes based on the expression of programmed death-1 (PD-1) and killer cell lectin-like receptor G1 (KLRG1) were described among $CD4^+$ T cells during *Mtb* infection. Thus, $PD\text{-}1^+$ $CD4^+$ T cells exhibit superior proliferation capacity though produce lower levels of IFN-γ, while $KLRG1^+$ $CD4^+$ T cells secrete high amounts of IFN-γ but show a terminally differentiated phenotype with reduced ability to proliferate (46). These $KLRG1^+$ T cells express CX3CR1, are mainly found in the vasculature, and have diminished capacity to migrate into the lung parenchyma in contrast to $KLRG1^-$ $PD\text{-}1^+$ $CXCR3^+$ cells which are mainly found in this tissue (48). As a consequence of their less-differentiated profile, $KLRG1^-$ cells are able to persist and maintain anti-mycobacterial immunity after *Mtb* infection (47, 48, 61). In our experimental approach, the combination of the anti-DC-SIGN antibody conjugated to P25 with zymosan resulted in the strongest immune response in terms of cytokine production and also showed increased percentages of total $KLRG1^+$ $CD4^+$ T cells 5 days post-infection. These

results suggest that zymosan induces a rapid increase of terminally activated cells but whether this is consequence of the vaccination or the ongoing infection remains unclear. Also, whether zymosan or CTA1-DD can generate Ag-specific KLRG1$^-$ CD4$^+$ T cells with the ability to persist in the long term is yet to be evaluated. Therefore, it would be interesting to assess the memory profile of the P25-specific CD4$^+$ T cells generated upon vaccination with anti-DC-SIGN antibodies previous to challenge.

In spite of generating P25-specific CD4$^+$ T cells against *Mtb*, we were unable to detect differences in the bacterial burden between unvaccinated and vaccinated mice. In our approach, we challenged the immunized mice with Ag85B-overexpressing BCG strain as a mycobacterial infection model. The rational behind this choice was based on the fact that Ag85B is expressed at lower levels in BCG compared to *Mtb*, where this protein is one of the major components of the bacterial culture filtrate (62). However, this model has a limitation in the sense that it is cleared much faster than the reference BCG Pasteur strain (data not shown). Hence, aerosol challenge with virulent *Mtb* would provide a better indication of whether DC-SIGN targeting has protective capacity against infection. In this sense, it would also be interesting to test whether the intranasal immunization route influences the type of response elicited by our anti-DC-SIGN antibodies. The mucosal administration of *M. bovis* BCG has shown to provide superior protection and favor the generation of lung-resident memory T cells (63, 64). Furthermore, upon intranasal administration of CTA1-DD in combination with QuilA-containing immune-stimulating complexes (ISCOMs) and the fusion protein Ag85B-ESAT-6, this adjuvant was shown to provide protection against Tb in the lung (38, 65, 66). Another advantage of the use of an antibody-based targeting strategy is the possibility of coupling different Ags to them. A broader range of *Mtb* epitopes can be covered in this manner, either CD4$^+$ or CD8$^+$, and therefore result in an improved immune response. CD8$^+$ T-cell responses have also been shown to mediate protection through their ability to secrete high amounts of IFN-γ and TNF-α and kill *Mtb*-infected macrophages (67, 68). Thus, mucosal administration of anti-DC-SIGN antibodies conjugated to different CD4$^+$ and CD8$^+$ epitopes plus CTA1-DD or zymosan could support the long-term generation of *Mtb*-specific Th1, Th17 and CD8$^+$ T cells along with other desirable memory T-helper subsets.

To conclude, we demonstrate here the potential of targeting human DC-SIGN to generate Ag-specific immune responses against *Mtb*. Even though further studies are needed in order to optimize this vaccination approach, we conclude that CLR targeting is a powerful tool that can be exploited to modulate immune responses and design novel vaccine strategies against global-health-threatening intracellular pathogens.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the German Animal Welfare Act. The protocol was approved by the Veterinary Institute of LAVES (Lower Saxony State Office for Consumer Protection and Food Safety, permit numbers: 12/0732 and 17/2472).

AUTHOR CONTRIBUTIONS

Conceptualization: LB and TS. Investigation: LV, PS, MG, MS, JB and MM. Resources: NL, DB, HL, HK and YK. Writing and visualization: LV, PS, TS and LB. Supervision and project administration: TS and LB. Funding acquisition: TS and LB.

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REVIEW
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C-Type Lectin Receptors in Antiviral Immunity and Viral Escape

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C-type lectin receptors (CLRs) are important pattern recognition receptors involved in recognition and induction of adaptive immunity to pathogens. Certain CLRs play an important role in viral infections as they efficiently interact with viruses. However, it has become clear that deadly viruses subvert the function of CLRs to escape antiviral immunity and promote infection. In particular, viruses target CLRs to suppress or modulate type I interferons that play a central role in the innate and adaptive defense against viruses. In this review, we discuss the function of CLRs in binding to enveloped viruses like HIV-1 and Dengue virus, and how uptake and signaling cascades have decisive effects on the outcome of infection.

Keywords: C-type lectin receptors, antiviral immunity, antigen presentation, type I IFN, complement opsonized HIV-1

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INTRODUCTION

Mucosa and skin are targets for invading viruses and are therefore important sites where adaptive immunity is initiated. Dendritic cells (DCs) and macrophages guard these tissues and detect the invading pathogens by pattern recognition receptors (PRRs) and lead to initiation of immunity and elimination of the pathogens (**Figure 1**). DCs are professional antigen presenting cells (APCs) that capture pathogens for degradation and antigen presentation, whereas macrophages are a first line of defense that destroy pathogens *via* degradation but are also able to activate memory T cells (1–3). PRRs are crucial for these functions of DCs and macrophages, as PRRs recognize conserved molecular structures to distinguish between the different types of pathogens, called pathogen-associated molecular patterns (PAMPs) (4). Distinct classes of PRRs recognize a wide range of PAMPs and induce different transcriptional programs leading to tailored immune responses. Furthermore, pathogens will often trigger several PRRs, leading to crosstalk between these receptors, which provide immune cells with another important level of control to tailor the adaptive immune response to the pathogen. Several classes of PRRs exist, such as Toll-like receptors (TLRs), Rig-I-like receptors (RLRs), and C-type lectin receptors (CLRs). Here, we will focus on CLRs that recognize carbohydrate structures, which are able to independently induce immunity or provide powerful signals *via* crosstalk to modulate responses that are triggered by other PRRs (5). As the immune system is in a never-ending arms race with viruses, many viruses have devised strategies to evade recognition and antiviral immune responses to successfully infect the host. In this review, we describe the complex role of CLRs in the immune processes that are essential in the defense against viruses. In addition, we discuss how certain viruses target specific CLRs to suppress or avoid antiviral immunity.

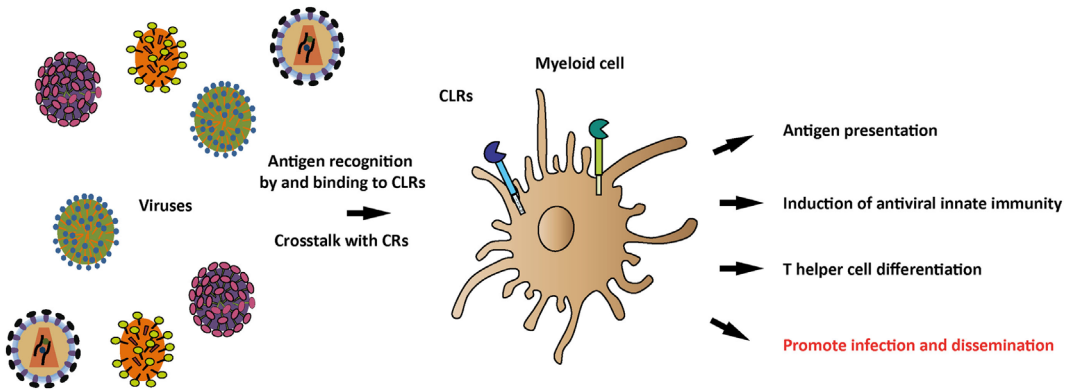

FIGURE 1 | Recognition of viral antigens by C-type lectin receptors (CLRs) and induction of antiviral immune responses. Various CLRs on antigen-presenting myeloid cells recognize a plethora of viruses through their carbohydrate-recognition domains (CRDs) and subsequently induce a tailored immune response, depending on the specific CLR and viral antigens. Viral antigens that trigger the CLR can modulate myeloid cell functions, thereby affecting antigen presentation, antiviral innate immune responses, and T helper differentiation. Once viral antigens are recognized by CLRs, crosstalk between CLRs and complement receptors (CRs) can occur, thereby further shaping the antiviral immune response. Additionally, CLRs play a role in viral recognition, internalization, and dissemination.

CLRs INTERACT WITH VIRUSES, LEADING TO VIRUS DEGRADATION OR TRANSMISSION

Virus Recognition

Innate immune cells like monocytes, macrophages, DCs, and Langerhans cells (LCs) express CLRs that act as PRRs. Most of these CLRs bind carbohydrate moieties in a calcium-dependent manner using conserved carbohydrate recognition domains (CRDs) (**Figure 2**). CLRs are important for recognition and capture of pathogens as these PRRs have a high affinity for their ligands, which results in internalization of the pathogens. Internalization often leads to degradation *via* lysosomes, which has been shown for the DC-specific ICAM-3 grabbing non-integrin (DC-SIGN; CD209) and DEC-205 (6, 7), or the binding induces degradation *via* autophagy as shown recently for langerin (8). Therefore, the outcome of CLR recognition depends on the specific CLR and the cell type on which it is expressed.

Lysosomal Degradation and Virus Transmission

After binding of pathogens by CLRs, the routing (intracellular transport) of antigen has various outcomes depending on the CLR and the immune cell. The Mannose Receptor (MR) is expressed by macrophages and DCs, and is involved in antigen processing and presentation. MR recognizes mannose, *N*-acetylglucosamine, and fucose that are often found on the surfaces of viruses, bacteria, and parasites (9). Upon binding, the pathogen is internalized and targeted to lysosomes for degradation. Subsequently, MR recycles back to the cell surface, for the next round of internalization, resulting in high amounts of internalized pathogens (10). However, several studies have shown that viruses such as HIV-1 and Dengue virus (DENV) target MR to evade degradation (11). Besides MR, the CLR DC-SIGN also plays an important role in virus binding and internalization. DC-SIGN recognizes mannose and fucose structures (12–14). HIV-1 internalization within DCs is dependent on the association between gp120 and DC-SIGN and this interaction can deliver HIV-1 to lysosomes where they are degraded (6). However, strikingly, a major part of DC-associated HIV-1 evades the degradation pathway by trafficking to a tetraspanin (CD81)-enriched protective environment from where infectious particles are specifically released to T lymphocytes upon DC–T cell contact (15). Thus, a virus that is taken up by DCs can enter two pathways: either routed to the endocytic pathway, resulting in viral degradation and antigen presentation or diverted to a transmission pathway and thereby avoids degradation. It is unknown how these pathways are related and which factors determine the fate of the virus. Langerin is a CLR expressed exclusively on LCs and is important for antigen capture and internalization, which induces Birbeck granules (BG) formation and routing of antigen into organelles (16). Langerin has a role in antiviral protection as immature LCs capture HIV-1 *via* langerin, leading to TRIM5α-mediated autophagic degradation of HIV-1, which prevents LC infection (8, 17). Thus, CLRs are important in the final fate of the virus, which can be either routing for degradation or dissemination.

Antigen Presentation

Next to routing of antigen in lysosomal pathways for degradation, antigen presentation on major histocompatibility complex (MHC) molecules is an important anti-viral immune mechanism. Many antigens taken up by various CLRs such as DC-SIGN, DEC-205, DCIR, or dectin-1 are routed into MHC class II compartments, where the antigens are loaded for presentation to CD4 + T cells (18–21). These MHC class II molecules are released from late endocytic compartments and accumulate at the cell surface (22). The expression of most CLRs is generally downregulated upon cell maturation (23). Mature DCs have a reduced capacity to take up antigen, which is reflected by lower levels of CLRs, but are more efficient in stimulating T cells through stabilizing MHC class I and class II at the cell surface (24). An exception represents

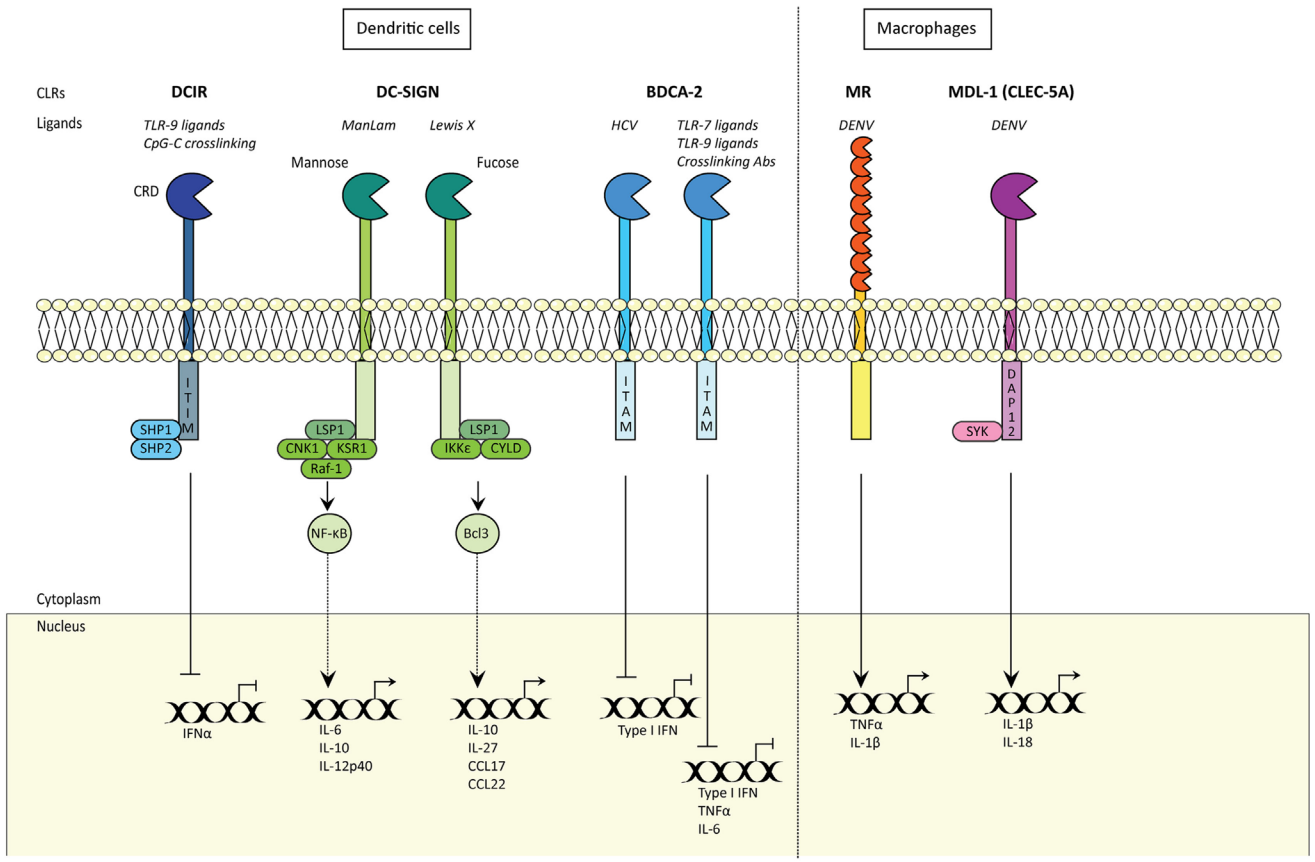

FIGURE 2 | C-type lectin receptors (CLRs) shape innate and adaptive immune responses. CLRs induce innate and adaptive immune responses. Certain CLRs contain ITIM domains and signal *via* SHP1 and SHP2 phosphatases, whereas other CLRs signal *via* their ITAM motif. DC-SIGN signaling is carbohydrate specific (CRD) and either signals *via* Raf-1 signalosome or IKKε and de-ubiquitinase CYLD, with distinct outcomes. MDL1 signals *via* DAP12 and Syk. CRD; carbohydrate recognition domain; DCIR, DC immunoreceptor; TLR9, Toll-like receptor 9; ITIM, immunoreceptor tyrosine-based inhibitory motif; SHP1, SH2-domain-containing protein tyrosine phosphatase 1; DC-SIGN, DC-specific ICAM-grabbing non-integrin; IFNα, interferon alpha; LSP1, lymphocyte-specific protein 1; CNK1, Connector Enhancer of KSR1; KSR1, kinase suppressor of Raf-1; IKKε, IκB kinase subunit-ε; IL6, interleukin 6; CCL17, Chemokine (C-C motif) ligand 17; NF-κB, nuclear factor kappa beta; Bcl-3, NF-κB family member Bcl-3; KSR1 and CNK1 are adaptor proteins; BDCA-2, blood DC antigen 2; HCV, hepatitis C virus; ITAM, immunoreceptor tyrosine-based activation motif; TNFα, tumor necrosis factor alpha; Type I IFN, type I interferon; MR, mannose receptor; DENV, Dengue virus; MDL-1, myeloid DAP12-associating lectin-1.

DEC-205, as the receptor is upregulated in mature plasmacytoid DCs (pDCs) (25). Importantly, antigen presentation by DEC-205 is not affected by this as endocytosis of an antigen targeted to DEC-205 *via* an antibody still leads to antigen presentation and CD4 + T cell induction in immature as well as activated pDCs (25). Furthermore, CLR Siglec-1 is upregulated upon DC maturation (26). It is unclear whether this affects antigen presentation, but the enhanced expression of Siglec-1 enhances HIV-1 transmission (27). Since CLR internalization enhances antigen presentation, several strategies have been designed to target these CLRs with antibodies or antigens for vaccination purposes.

Virus Transmission

Virus internalization can lead to antigen presentation but, strikingly, several CLRs have been shown to protect the virus and promote viral transmission and dissemination (15, 28–30). Virus transmission in the context of DCs can be in *cis*, which depends on productive infection of DCs or in *trans*, where CLRs function as attachment receptors that facilitate capture and transmission without infection of the DC. DC-SIGN is an important CLR on DCs involved in transmission of viruses to susceptible target cells (15), facilitating virus dissemination throughout the host. After DC-SIGN mediated endocytosis of HIV-1, these virions can be kept in multivesicular bodies to enable the release of HIV associated with exosomes. This trans-infection pathway in DCs helps with dissemination of HIV-1 to CD4 + T-cells (31, 32). DC-SIGN also interacts with flaviviruses *via* mannose glycans present on viral envelope glycoproteins and is used by DENV, Hepatitis C virus (HCV) (33), Sindbis virus (34), and the West Nile virus (WNV) for cellular attachment and infection of immature DCs (35, 36). Studies have shown that HCV particles bind to DC-SIGN and are targeted to non-lysosomal compartments in immature DCs, where they are protected from lysosomal degradation and transmit the virus to hepatocytes (33). DC-SIGN functions as an

attachment receptor for many different viruses, such as DC-SIGN also mediates Cytomegalovirus (CMV) and Ebola virus (EBOV) enters into DCs and facilitates transmission to susceptible cells in *cis* and *trans* (37, 38). This function is not exclusive for DC-SIGN. Capture of HIV-1 by MR on macrophages also results in protection of the virus and transmission to T-cells (29). Another CLR involved in virus transmission is DCIR. The DCIR expression was detected on DCs, monocytes, macrophages, B-lymphocytes, and granulocytes (23) such as DC-SIGN, DCIR captures HIV-1 and promotes infection in *cis* and *trans* of CD4 + T-cells from immature DCs (28). Inhibiting HIV-1 binding to DCIR on DCs significantly decreases exosomal release of HIV-1 (39), which might be the mechanism for transmission. In contrast, langerin prevents HIV-1 transmission by LCs *via* autophagosomal degradation of HIV-1 (8). When langerin function is impaired, LCs become infected and subsequently transmit HIV-1 to T cells in *cis* (3, 9). In contrast, langerin also functions as an attachment and an entry receptor for influenza A virus and thereby promotes viral dissemination (40). It is unclear how Influenza A virus escapes autophagosomal degradation by langerin. Thus, several viruses have devised strategies to subvert the internalization route of CLRs in order to promote viral dissemination. It is interesting that even though viruses such as HIV-1 escape from degradation in DCs, these DCs still present HIV-1 derived antigens in the context of MHC-II to CD4 + T cells (41). These data suggest that capture by DCs and simultaneous presentation of antigens to CD4 + T cells might enhance the destruction of virus-specific T cells. Moreover, internalization routes of CLRs might be not exclusive for antigen presentation or virus protection, and both routes can occur simultaneously.

Cross-Presentation

Besides activation of CD4 + T helper (T_H) subsets, CLRs have also been implied in cross-presentation. *Via* this process, DCs can elicit cytotoxic T cell responses by presenting exogenous antigens *via* MHC class I (42). There are at least two pathways that are generally referred to as "cytosolic" and "vacuolar" (42). Upon endocytosis, antigen processing for MHC class I loading either takes place in endocytic compartments or the cytosol (43–45). Several mechanisms are involved in the transfer of antigens from endosomes into the cytosol and include unfolding of proteins, members of the ER-associated degradation machinery like p97 and the pore-building protein Sec61 as has been reviewed by Schuette and Burgdorf (46). "Cross-priming" describes the subsequent stimulation and expansion of naïve CD8 + T cells to initiate cytotoxic immune responses and memory T cells (47). Even though the exact mechanisms are still under investigation, it is evident that CLRs facilitate uptake that leads to cytosolic exposure and cross-presentation. The BDCA3 + CD141 + DCs excel at cross-presentation and have recently been suggested as the main cross-presenting DC subset in humans, closely resembling mouse CD8 + DCs (48–50). Interestingly, this DC subset expresses CLR CLEC9A (DNGR-1) that efficiently internalizes antigens for cross-presentation (51–53). CLEC9A binds to F-actin (54), which is exposed in necrotic cells and CLEC9A might be involved in cross-presentation of antigens from necrotic cells that have, for example, been infected by viruses. Indeed, murine CLEC9A-deficient DCs are unable to facilitate cross-presentation of Vaccinia virus antigens upon infection (55). CLEC9A might facilitate not only antigen presentation but also activation of CD8 + T cells by presenting signals of tissue damage (55). Moreover, CLEC9A also promotes cross-presentation of dead cell-associated antigens by altering the route of internalization as CLEC9A co-localizes with the phagocytosed necrotic cargo, which diverts the cargo toward the recycling endosomal route for MHC class I presentation (56). Upon infection with the highly immunogenic Herpes simplex virus, cytotoxic CD8 + T cell responses were reduced in mice lacking CLEC9A (56). In humans targeting antigens by CLEC9A antibodies to immature BDCA3 + DCs leads to cross-presentation and induction of antigen-specific CD4 + and CD8 + T cells, whereas DC maturation and cytokine production are not affected (57). Antigens targeted to DCIR on different DC subsets result in efficient cross-priming and cross-presentation (58). DC-SIGN is also able to route viruses or antigens into the cross-presentation pathway as HIV-1 antigens are presented to MHC class I *via* DC-SIGN capture (59, 60). Upon internalization and processing of HIV-1 *via* DC-SIGN, viral exogenous antigens are cross-presented on MHC class I, thereby inducing anti-HIV cytotoxic T cell (CTL) responses (6). Interestingly, the group around Moris et al suggests that the virus in this case is not routed toward lysosomes but is processed by another, proteasome-dependent pathway. Whether langerin is capable of inducing cross-presentation is under debate. Internalization of synthetic long peptides through langerin on LCs enhances cross-presentation (61, 62). Whereas, activated LCs become infected by measles virus (MV) and therefore present newly formed virions *via* MHC class I to MV-specific CD8 + T cells (63). Targeting of MV or MV-infected cells to langerin does not result in cross-presentation, suggesting that langerin routing into BG is not linked to the cross-presentation route (63). MR promotes cross-presentation by routing its cargo into a distinct, low degradative, early endosome subset (7, 64). Importantly, due to poly-ubiquitination of its cytoplasmic tail (65) as well as recruitment of p97 to the endosomal membrane (66), MR might not only internalize antigens but also export the antigens from the endosomal compartment into the cytoplasm (67). The mechanisms of cross-presentation are currently studied extensively in the context of tumor immunology with CLRs as attractive targets (62, 68, 69). However, their impact is also crucial for anti-viral immune responses, and their proven and proposed roles during antigen uptake and presentation depict that CLRs have an important role in connecting these processes.

SHAPING ANTIVIRAL INNATE IMMUNE RESPONSES BY CLRs

PRR signaling in DCs is vital to the induction of innate and adaptive immune responses to viruses. Type I IFN responses are paramount in limiting viral replication and therefore form a strong innate immune defense mechanism against invading viruses (70–73). Moreover, type I IFN responses also modulate adaptive immunity thereby further tailoring immunity to the pathogen. Different CLRs possess the capacity to activate various

innate signaling pathways that give rise to specific types of cellular immune responses (**Figure 2**) (74). However, viruses contain the capacity to alter CLR-induced signaling, thereby inhibiting induction of type I IFN responses (75).

Type I IFN Responses in DCs

Sensing of viral structures *via* a variety of PRRs induces an antiviral program to help viral infections (76–78). This innate antiviral program consists predominantly of various IFNα subtypes and IFNβ, which has been reviewed extensively elsewhere (79). IFNα and IFNβ are both produced by DCs, but IFNα is predominantly secreted by pDCs (80–82). Membrane-bound TLRs trigger signaling cascades leading to phosphorylation of interferon-regulatory factor 3 (IRF3) and IRF7 activate transcription of *IFNA* and *IFNB* genes (82, 83). IRF3 and IRF7 are crucial in inducing type I IFN, albeit in a different manner. IRF3 is indispensable for the first production of type I IFN and predominantly activates IFNβ signaling, which then initiates transcription of IRF7 thereby strongly inducing IFNα. IRF7 in contrast to IRF3 is not constitutively expressed by DCs, and is upregulated by IFN signaling. Therefore, IRF7-induced IFNα is under control of IRF3 activation and thus IFNβ production (4, 78, 82, 83). Therefore, IFNβ comprises the very first line of antiviral defense as it forms the first wave of type I IFN. Secreted IFNα/β proteins bind the heterodimeric transmembrane IFN receptors (IFNARs) on the cell surface (78). Upon receptor ligation, intracellular signaling leads to induction of *IFNA/B* gene transcription and transcription of interferon stimulated genes (ISGs) Depending on the cell type, IFN dosage and timing of the first wave of IFN exposure, 50–1000 ISG can be identified among 200–500 different cell types (84). ISGs form an important antiviral type I IFN component, since they can interfere with viral replication steps or serve as viral restriction factors (70–73). Thus, upon transcription of *IFNA/B* genes a self-enhancing antiviral program is induced that can strongly inhibit viral replication. In addition, the production of type I IFN is essential to adaptive immunity as these cytokines control proliferation, differentiation, activation and maturation of monocytes, DCs, and macrophages (85). Therefore, type I IFN plays a crucial role in the induction of antiviral immunity.

Shaping of Innate Immune Responses by CLRs

C-type lectin receptors are important in inducing and modulating immunity (**Figure 2**). Some CLRs contain immunoreceptor tyrosine-based activation motifs (ITAMs), hemi-ITAMs, or immunoreceptor tyrosine-based inhibitory motifs (ITIMs), whereas some do not contain any obvious signaling motifs (75). Dectin-2, DCAR, and MDL-1 belong to the ITAM-containing CLRs, whereas DCIR contains an ITIM. Dectin-1 belongs to the hemi-ITAM group, and other CLRs such as DC-SIGN, MR, and DEC-205 do not contain any known ITAMs or ITIMs (75). DCIR is expressed by DCs and macrophages, and contains an ITIM that mediates inhibitory signals by recruiting phosphatases SH2-domain-containing protein tyrosine phosphatase 1 (SHP1) or SHP2 after receptor ligation (86). Endocytosis of DCIR on DCs does not affect TLR4 and TLR8-dependent DC maturation (87). However, DCIR inhibits TLR8-dependent IL-12 and TNFα production, whereas TLR2, TLR3, and TLR4-induced cytokine levels are unaffected (87). DCIR is also expressed on macrophages, and its activation inhibits CpG-ODN-induced expression of pro-inflammatory cytokines IL-1β and IL-6 (88). Although DCIR inhibits IFNα (19), it also serves an opposing role by sustaining type I IFN responses (89) as murine DCs deficient in DCIR have decreased STAT1 phosphorylation upon *Mycobacterium tuberculosis* infection, indicating that DCIR sustains STAT1 and therefore type I IFN responses (89). Notably, DCIR expression inhibits IL12p70 production and DC-dependent T_H1 skewing (89). Thus, DCIR depending on the DC subset can inhibit or sustain type I IFN responses depending on the pathogen and cell-type. Whether DCIR is able to sustain type I IFN responses in humans and against viruses remains elusive. DCIR contributes to DC capture and dissemination of HIV-1, whereas murine DCIR has shown to be involved in the internalization of Chikungunya virus (CHIKV) (28, 90). Whether DCIR, in this context, functions as a PRR and is able to sustain type I IFN responses remains elusive. HIV-1 induces DCIR expression on T cells, which increases viral entry and replication (91) whereas DCIR serves a protective role in CHIKV infection (90). Thus, depending on the virus and on the origin of the host cell, DCIR appears to either protect against or enhance viral infection of the host cell. BDCA-2 (CLEC4C, CD303) is expressed by pDCs and therefore widely used to identify pDCs (92). BDCA-2 inhibits type I IFN responses in pDCs in response to CpG oligonucleotides, Influenza virus, or DNA-autoantibody complexes (93, 94). Similar to Dectin-2 and DCAR, BDCA-2 contains the capacity to engage with the transmembrane adaptor FcεRIγ (95) and induce ITAM-dependent signaling (96). BDCA-2 crosslinking with antibodies on pDCs inhibits both type I IFN and pro-inflammatory cytokine responses after TLR7 and TLR9 triggering (96). Interestingly, BDCA-2 on pDCs interacts with HCV glycoprotein E2, which similarly blocks type I IFN responses (97). MR binding to DENV on macrophages induces pro-inflammatory cytokines TNFα and IL-1β, causing decreased endothelial integrity and leading to more severe disease symptoms (11, 98). As MR expression is increased during DENV infection *via* IL-4, this suggests that MR-induced cytokines might be important in sustained high cytokine responses (99, 100). Recent findings have shown that when macrophages are stimulated with vitamin D3, IL-4-induced MR upregulation is attenuated and thus leads to decreased ligation of viral particles to macrophages (101). MDL-1 (CLEC-5A) also interacts with DENV and induces Syk-dependent pro-inflammatory IL-1β and IL-18 responses upon DENV-induced inflammation (102). Ligation of MDL-1 activates the NLRP3 inflammasome and pyroptosis (102). In macrophages, MDL-1 is exploited by Japanese encephalitis virus, which uses this inflammatory axis to induce virus-dependent inflammation of host cells (103). Moreover, MDL-1 binding to Influenza A virus (104, 105) contributes to Influenza A virus pathogenicity through induction of pro-inflammatory responses in a murine model (105). HIV-1 binding to DC-SIGN induces signaling by DC-SIGN that affects TLR-mediated signaling. Endosomal degradation of HIV-1 triggers TLR8-dependent NF-κB and initiates transcription of integrated HIV-1 (106, 107). Notably, DC-SIGN signaling by HIV-1 induces phosphorylation

of NF-κB allowing propagation of transcription and production of viral proteins (108). Thus, DC-SIGN-dependent endocytosis and signaling is exploited by HIV-1. Furthermore, a recent study showed that HIV-1 targets DC-SIGN on primary DC subsets to suppress type I IFN responses induced by the HIV-1 sensor DDX3 (109). Similarly, MV exploits DC-SIGN signaling to suppress type I IFN responses as DC-SIGN signaling blocks phosphatases that are crucial in activation of MV-sensors RIG-I and MDA5 (110). Thus, both HIV-1 and MV exploit DC-SIGN signaling to inhibit antiviral type I IFN responses.

CLRs IN T HELPER CELL POLARIZATION

Efficient pathogen-specific T cell responses require differentiation of CD4 + T cells into various T_H cell subsets (**Figure 1**). Distinct T_H cell subsets each have specialized roles in the defense against invading pathogens. A T_H1 response is directed against intracellular pathogens, whereas T_H2 cells produce IL-4, IL-5, and IL-13 to combat extracellular pathogens (111). Follicular T_H cells (T_{FH}) are crucial for efficient B cell responses by the formation of germinal centers in the lymph node. In these germinal centers, T_{FH} stimulate B cell proliferation and isotype class-switching *via* the production of IL-21 (112, 113). CLRs crosstalk with other PRRs to induce specific cytokine expression profiles thereby directing T_H cell polarization (114). DC-SIGN distinguishes between mannose- and fucose-containing antigens (14, 115, 116). Notably, differential recognition of mannose and fucose structures by DC-SIGN results in the induction of disparate intracellular pathways that are controlled by the composition of the signalosome bound to DC-SIGN (112, 117, 118). DC-SIGN is continuously bound by adaptor protein LSP1 in combination with a signalosome complex KSR1, CNK, and kinase Raf-1 (**Figure 2**) (117). Activation of DC-SIGN by mannose-containing pathogens such as HIV-1 or MV leads to activation of Raf-1. Raf-1 triggers a signaling pathway that induces a specific phosphorylation of NF-κB, thereby enhancing the transcription of pro-inflammatory cytokines IL-6 and IL-12 (**Figure 2**) (74, 119). High mannose structures are prevalent on the surface of many viruses including HIV-1, EBOV, HCV, DENV, CMV, and SARS coronavirus (SARS-CoV) (74). Therefore, the interaction of these viruses with DC-SIGN might enhance the induction of T_H1 responses (120). In contrast, activation of DC-SIGN by fucose-expressing pathogens dissociates the Raf-1 signalosome from LSP1 and recruits IκB kinase subunit-ε (IKKε) and the de-ubiquitinase (CYLD) (**Figure 2**) (112, 118). CYLD activation results in accumulation of ubiquitinated Bcl3 in the nucleus, which forms p50–p50–Bcl3 complexes that inhibit IL-12 production and enhances T_H2-associated cytokines IL-10, and CCL17 and CCL22 (**Figure 2**) (118). Indeed fucose-containing pathogens induce T_H2 polarization *via* DC-SIGN activation (118). Furthermore, fucose-induced signaling by DC-SIGN also modulates IFNR signaling, which is paramount to the induction of IL-27 and T_{FH} cells (112). Mostly parasites express fucose, suggesting that DC-SIGN is important in the defense against parasites that require T_H1 and T_{FH} responses. Interestingly, a few viruses, such as DENV, expose fucose-structures on their surface. However, it remains unclear whether these viral fucose–DC-SIGN interactions induce T_H2 responses. It might be possible that other CLRs also induce T_H2 fucose-dependent differentiation. CLRs such as langerin and MR also interact with LSP1, suggesting that the signaling properties of these receptors are similar to DC-SIGN. Besides DC-SIGN, other receptors are also involved in T cell polarization. MR is known to inhibit the production of TLR4-induced IL-12 secretion in DCs (121). Among DCs, DCIR and myeloid inhibitory C-type lectin-like receptor (MICL) both down-regulate TLR-induced IL-12 secretion *via* activation of intracellular ITIM motifs (87, 122). In addition, BDCA2 downregulates TLR-mediated IL-6 production by preventing recruitment of MyD88 to the intracellular domains of TLRs in pDCs (93). Therefore, activation of one or more of these CLRs might skew toward a T_H2 balance. However, most of these studies used bacterial ligand such as LPS to investigate cytokine response upon CLR stimulation. Additional research is needed to investigate whether these receptors still exhibit these signaling modulating functions upon viral infections.

CROSSTALK BETWEEN CLRs AND COMPLEMENT RECEPTORS (CRs)

C-type lectin receptors are also involved in complement-dependent clearance of pathogens as well as induction of immunity. The complement system can be activated through three distinct pathways, named the classical, the lectin, and the alternative pathway (123). The three different cascades are responsible in activating complement factor C3b that deposits on diverse surface formations of pathogens and apoptotic cells inducing opsonization, inflammation, phagocytosis, elimination of the pathogen, and lastly, the induction of adaptive immune responses. Interestingly, the soluble CLR MBL is involved in the lectin pathway and recognizes carbohydrates such as mannose, glucose, L-fucose, *N*-acetyl-mannosamine (ManNAc), and *N*-acetyl-glucosamine (GlcNAc) on a wide range of pathogens (124). The recognition of carbohydrates is mediated *via* the CRDs and oligomerization of MBL enables high avidity binding to repetitive carbohydrate ligands. Once bound, MBL has the capacity to modify the efficiency of uptake by the expression of other phagocytic receptors. MBL further activates complement, due to its association with Mannose-binding lectin-Associated Serine Proteases, or act directly as an opsonin (125, 126), which results in deposition of complement on the pathogen surface that leads to uptake *via* CRs (126, 127). MBL has been found to bind directly to virions from a number of different virus families, including HIV, SARS-CoV, EBOV, DENV, and WNV. For instance, MBL binds directly to HIV-infected cells (128) and HIV-1 particles that lack gp120 do not bind MBL, supporting the idea that gp120 and gp41 directly bind to C1q and guide the interaction between the whole virus and MBL (129, 130). Despite these findings, the role of MBL in HIV pathogenesis is still unclear. Moreover, complement also affects CLR function. When entering the host, complement is spontaneously deposited on the surface of HIV-1 since gp120 and gp41 contain a C1q binding site (131). Over the past years, a lot of studies have supported the idea that HIV-1 envelope glycoprotein gp41 functions as a viral ligand for gC1qR

(132–135), the receptor for the globular heads of C1q complex, which modulates a plethora of immunological functions, such as infection and inflammation. The HIV-1 transmembrane glycoprotein gp41 has been shown to interact with the C1q complex (136, 137), causing the activation of the classical pathway of the complement system (**Figure 3**). Furthermore, different studies have shown that MBL interacts with HIV-1. Expression of viral proteins, gp120 and gp41, seems to be crucial for the HIV-1-MBL interaction. However, the binding is presumably to the *N*-linked glycans on the gp120 (**Figure 3**), due to the fact that it is highly glycosylated and in contrast there are only few potential *N*-linked carbohydrate sites on gp41 (138, 139). MBL interaction with the envelope glycoprotein of different HIV-1 strains mediate several downstream antiviral effects, in particular the complement activation, independent of C1q and antibodies, inducing the lectin pathway and enhancing the opsonization and viral elimination (128, 140). Complement is important early in infection, when HIV-1 specific antibodies are still absent. Complement is active at the mucosal site and in the seminal fluid, which suggests that the virus is opsonized with C3b prior to transmission (141). Opsonization reduces the accessibility of the viral envelope protein gp120, and interferes with the CLR interaction of the virus with DCs (142). In addition, complement opsonization of HIV-1 causes a significantly higher productive infection of DCs, which is caused by binding to the CR3, whereas non-opsonized HIV-1 is bound *via* gp120 to DC-SIGN (143) (**Figure 3**). However, complement is a two-edged sword and besides its clearance and neutralization activity, it also enhances viral spread and maintenance. Thus, complement-coated HIV-1 accumulates in different parts of the host and is able to bind CR-expressing immune cells, for example DCs, macrophages, NK cells, B cells, or even follicular DCs, thereby enhancing infectivity and dissemination (130, 144). In addition, novel complement activation pathways have been identified such as interaction of C1q with CLR SIGN-R1 expressed on macrophages (145), and cells with a DC-like phenotype (146). It has been widely accepted that infection of DCs by HIV-1 and DC-mediated transmission of the virus to T cells is mediated by CLRs (143), but this is not the case for opsonized virus. The productive infection caused by HIV-C is associated with an activation of DC responses characterized by up-regulation of

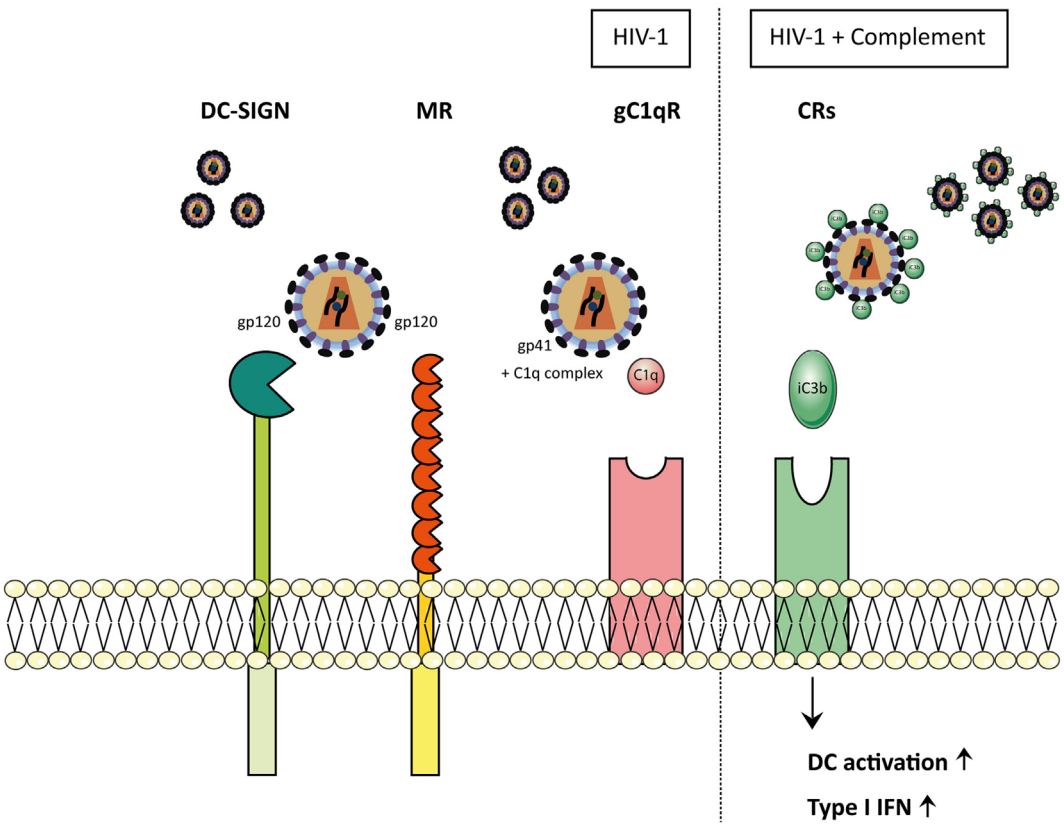

FIGURE 3 | HIV-1 particles opsonize-dependent recognition on myeloid cells. In physiological conditions, when HIV-1 enters the body, it is either non-opsonized (HIV) or complement-opsonized (HIV-C). Depending on the opsonization pattern of the virions, interactions with the receptors differ. Non-opsonized virus (HIV-1) interacts with CLR receptors, such as DC-SIGN and mannose receptor (MR) *via* the glycoprotein gp120. Furthermore, non-opsonized virus (HIV-1) is able to establish an interaction between gp41 and C1q complex, allowing the final binding to gC1qR. MR and C1qR cause the activation of the complement system *via* the lectin pathway and the classical pathway, respectively, inducing complement-mediated opsonization of the virions. Once HIV is coated by C3b fragments, it is able to bind to the complement receptors (CRs). Dendritic cells (DCs) exposed to complement-opsonized HIV-1 showed increased activation as well as up-regulation of type I IFNs.

maturation (CD83, CCR7), co-stimulatory function (CD40 and CD86), together with HLA-DR and HLA-ABC. Furthermore, innate type I IFN responses are enhanced that are also involved in T cell activation (CXCL9, CXCL10, CXCL11), suggesting that there is also an improved antiviral response (147) (**Figure 3**).

CONCLUDING REMARKS

Some CLRs are important in shaping innate and adaptive immunity to different viruses. Many viruses interact with CLRs and the elicited immune responses are induced by triggering of CLRs in combination with PRRs. Although the intracellular signaling pathways have not been clearly defined for most CLRs, it is becoming evident that CLRs are very efficient in modulating signaling by other PRRs. This seems to be a recurring feature that is important in tailoring adaptive immunity to the pathogens. However, certain viruses have subverted these CLRs to inhibit antiviral immunity. In particular, several CLRs have been shown to inhibit type I IFN responses, which is crucial for a strong effective antiviral immune response. Moreover, although CLRs are currently main players in immunotherapy strategies to enhance antigen presentation to tumor antigens, the intracellular routing of CLRs is subverted by many viruses for viral transmission. These pro-virus functions might be natural functions of the CLRs and it is important to further understand these functions and why CLRs require these in inducing immunity. It is likely that these CLRs recognize other pathogens and that the subsequent immune responses require limiting amounts of type I IFN responses or pro-inflammatory cytokines. The elucidation of the mechanisms behind these manipulations is crucial to target CLRs in order to combat viral infections or to prevent viral invasion and is also important to understand inflammatory as well as auto-immune diseases.

AUTHOR CONTRIBUTIONS

MB, JE, LH, NH, BN, MS: wrote sections of the manuscript, contributed equally. TG: wrote sections of the manuscript, supervision and final responsibility. All authors contributed to manuscript revision, read and approved the submitted version.

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C-Type Lectin Receptors in Asthma

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Asthma is a heterogeneous disease that affects approximately 300 million people worldwide, largely in developed countries. The etiology of the disease is poorly understood, but is likely to involve specific innate and adaptive responses to inhaled microbial components that are found in allergens. Fungal-derived allergens represent a major contributing factor in the initiation, persistence, exacerbation, and severity of allergic asthma. C-type lectin like receptors, such as dectin-1, dectin-2, DC-specific intercellular adhesion molecule 3-grabbing nonintegrin, and mannose receptor, recognize many fungal-derived allergens and other structurally similar allergens derived from house dust mites (HDM). In some cases, the fungal derived allergens have been structurally and functionally identified alongside their respective receptors in both humans and mice. In this review, we discuss recent understanding on how selected fungal and HDM derived allergens as well as their known or unknown receptors shape allergic airway diseases.

Keywords: C-type lectin receptors, allergens, dectin-1, fungi, sensitization

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INTRODUCTION

Over the past few decades, it has become widely accepted that fungi can contribute negatively to many aspects of human health and the particular focus on this review is allergic airway diseases. Two-thirds of asthmatics display an atopic response to multiple allergens, with at least 6–8% of the world's population suffering due to exposure to mold and other microbial components (1, 2). Fungal sensitization plays a role in patients with respiratory allergic disease through initiation, persistence, and exacerbation of allergic asthma. There is a correlation in the number of patients sensitized to fungi and the severity of asthma (3). The exact estimate of the prevalence of fungal sensitization among asthma patients is unclear, partly because exposure is universal, variable in time and intensity (4).

Asthmatics can be sensitized to multiple fungal species, and early exposure in the first 3 months of life has been associated with increased risk of developing asthma in children (5). The most common fungal species associated with sensitization, include *Alternaria alternata*, *Penicilium* spp., *Cladosporium* spp., *Aspergillus* spp., and *C. albicans* (6). Sensitization to fungi occurs everywhere, both indoors and outdoors, and this is linked to asthma exacerbations (7). Allergic bronchopulmonary aspergillosis (ABPA) is a disease caused by bronchial colonization with *Aspergillus* spp. and it affects approximately 0.7–3.5% of asthmatics (2). The disease features closely resemble those of severe asthmatics sensitized to fungi and both groups of patients have significantly better outcomes if treated with antifungals, such as itraconazole and voriconazole (3, 8, 9). In a study conducted in North India, the prevalence of ABPA and fungal hypersensitivity among acute asthma patients was estimated at 38.6 and 50.9%, respectively, and the overlap between diseases was estimated at 75.9% using a skin prick test (10).

TABLE 1 | Common allergens found in fungi and house dust mites (HDM) and their binding receptors.

Allergen	Source	Receptor	Localization	Reference
β-glucan	HDM *A. fumigatus* *A. versicolor* *C. cladosporioides*	Dectin-1	Macrophage, monocytes, subsets of DCs, epithelium, basophils	(108, 117, 118)
α-mannans	HDM *A. fumigatus* *C. neoformans*	Dectin-2	Macrophage, monocytes, subsets of DCs, basophils	(46, 48, 53–55)
Derp-1 Derp-2 BG-60	HDM HDM	DC-specific intercellular adhesion molecule 3-grabbing nonintegrin	Monocyte-derived DCs	(63–65)
Derp-1 Derp-2	HDM HDM Cockroach	Mannose receptor	DCs, macrophages	(76, 77)
Derp-1 Derf-1 Glycoprotein 55 and 45	HDM HDM *A. fumigatus*	Surfactant protein A and D	Alveolar type II cells	(84, 125)
Chitin	HDM *A. fumigatus*	Mannose receptor, dectin-1/TLR-2, NOD, RegIIIγ, FIBCD1	FIBCD1 (gut enterocytes), RegIIIγ (gut epithelium)	(30, 33, 36, 39)

Allergic bronchopulmonary aspergillosis is characterized by a Th2 cell infiltrate, increased pulmonary and blood eosinophilia, brownish black mucus plugs, total serum IgE levels over 1,000 ng/mL (416 IU/mL), *Aspergillus*-specific IgE and IgG1 antibodies (3). These characteristics are similar to allergic asthmatics sensitized to various allergens such as those derived from house dust mites (HDM), pollen, cockroach, etc. In fact many fungal sensitized asthmatics can be misdiagnosed as having ABPA with further tests usually required to rule out ABPA (2, 11). A considerable difference in fungal sensitized asthmatics is that they do not show signs of bronchial colonization by *A. fumigatus* and are immunologically sensitized to more than one fungal species, such as *A. alternata*, *P. notatum*, *C. herbarum*, *A. fumigatus, A. niger,* and *C. albicans* (3). Other useful methods for identifying ABPA, include computed tomography scan, chest radiographic lesions, and plasma levels of CCL17 which in combination with other criteria can help to stratify patients (12). This shows an overlap in clinical diagnosis, treatment, and fungal spores responsible for initiating these diseases (11). Fungal spores are diverse, and ubiquitous in our environment, making it difficult to estimate an average number of spores humans inhale per day (13, 14).

The majority of fungal spores in outdoor environments belong to the phylum *Ascomycota* and *Basidiomycota* (2). Most fungi contain multiple and variable allergens derived from conidia, spores, hyphae, or hyphal fragments and these are easily inhalable and likely to be the causal factors in allergic asthma. Some of these allergens are released during lysis/breakdown of fungal spore/hyphae and some are secreted by the fungus (14). Although protease allergens from fungi represent a larger proportion and are the most studied of the fungal allergens, other molecules, which are part of the fungal cell wall like chitin, mannans, and β-glucans, represent an important group of fungal allergens (**Table 1**). In recent years, there has been a huge effort in understanding what host-factors recognize fungal species and how the immune response to these fungal patterns is initiated and intricately regulated. C-type lectins were discovered as major fungal recognition receptors and shown to play critical roles in innate immunity and directing adaptive responses. The focus of this review is to highlight how fungal-derived allergens and their respective receptors (mainly, but not limited to C-type lectins) shape asthma.

C-TYPE LECTIN RECEPTORS

C-type lectin-like receptors (CLRs) are carbohydrate, lipid, or protein binding proteins, identified by the unique structure of at least one C-type lectin-like domain (CTLD). The CLRs family of proteins is made of more than 1,000 members, organized into 17 groups with diverse functions, including cell adhesion, phagocytosis, complement activation, innate immunity, and others (15). CLRs recognize non-self, microbial pattern-associated molecular patterns (PAMPs) on surface of fungi, bacteria, viruses, parasites, and house dust mite allergens (16, 17). They can also recognize damaged self-antigens, damage-associated molecular patterns or tumor antigens, tumor-associated molecular patterns (16). CLRs mostly recognize extracellular ligands, but some have been reported to recognize endogenous intracellular ligands, for example, DNGR-1 senses F-actin (18). The recognition occurs through their extracellular structurally conserved CTLD and is tightly regulated by specific amino acid motifs, calcium ions, and the carbohydrate structure. Once engaged with their respective ligands they induce intracellular signal pathways coupled to spleen tyrosine kinase (Syk). Downstream, Syk phosphorylates a number of substrates, such as protein kinase c-delta (PKC-δ), which in turn phosphorylates CARD-9 resulting in the formation of a complex made up of CARD-9, Malt-1, Bcl10 (19–22). This leads to activation of NF-kB and induction of inflammatory responses including activation of anti-microbial responses

and cytokine production that direct both innate and adaptive responses (19, 23). Some Syk coupled CLRs possess an intracellular immunoreceptor tyrosine-based activation motifs (ITAM) on their signaling tail that acts as an adaptor which recruits and activates Syk (24). Other pathways that do not involve ITAMs have been reported to trigger Raf-1 activation *via* receptors such as DC-specific intercellular adhesion molecule 3-grabbing nonintegrin (DC-SIGN) and dectin-1. Some Syk coupled CLRs lack the intracellular signaling tail and rely on ITAM coupled FcRγ adaptors for engagement with Syk. CLRs can be classified according to their ability to interact and signal directly with Syk (cluster 1) or requirement for adaptor molecule FcRγ (cluster 2). Cluster 1 mainly consists of dectin-1-like CLRs, such as Clec-2 and DNGR1, and the second cluster mainly consists of dectin-2-like CLRs, such as MCL and Mincle (25). Not all CLRs are activating upon ligand binding and some CLRs have an integral immunoreceptor tyrosine-based inhibitory motif (ITIM) on their cytoplasmic tail, such as MICL (26).

Chitin Detecting CLRs

Chitin, a polymer of β-(1–4)-poly-*N*-acetyl-D-glucosamine repeating units is found in various organisms, including fungi, arthropods, helminth parasites, and mites. In fungi, chitin constitutes about 2% of the fungal cell wall dry weight, and forms part of the cell wall inner layer giving it its rigidity (27). How chitin is sensed by the host immune cells remains controversial and unresolved (28). To date the only chitin vertebrate-specific receptor identified is FIBCD1, a 55 kDa homotetrameric type II transmembrane protein expressed in gut enterocytes (29). Chitin sensing has been speculated to involve engagement of multiple PRRs, including TLR-2, dectin-1, TLR-9, NOD receptor, mannose receptor, and soluble C-type lectin RegIIIγ, in a polymer size, concentration, and acetylation status-dependent manner (30–32). Smaller chitin fragments (1–10 μm) promote anti-inflammatory IL-10 production in a MR-, NOD-, and TLR9-dependent manner, whereas intermediate fragments (40–70 μm) promote pro-inflammatory cytokine TNF-α in a dectin-1/TLR-2 manner both *in vitro* and *in vivo* (31, 32). Larger ones (50–100 μm) promote eosinophilic inflammation and alternatively activated macrophages *in vivo* (33–35). However, difficulties in purifying high quality chitin and mannan contaminated commercial chitin have left some speculations on whether contaminants could be partly responsible for multiple PRR engagement. *Aspergillus fumigatus* and crustacean-derived chitin was shown to initiate innate allergic responses by recruiting IL-4 positive immune cells, such as eosinophils and basophils (33, 35, 36). Chitin was shown to activate ILC2s by inducing IL-33, IL-25, and TSLP production by the epithelial cells (33). It was further shown that chitin induced alternative-activated macrophages (AAMs, M2) and eosinophilia an ILC2-dependent manner. Lack of IL-25, TSLP, and IL-33 resulted in normal lung ILC2 accumulation, but reduced secretion of IL-5 and IL-13 which lead to reduced AAM and eosinophilia (33). The role of chitin in Th2-mediated airway inflammation was also supported by another study that showed that chitin induced CCL2 production by epithelial cells, which in turn activated AAMs and eosinophilia in a CCR2-dependent manner (37). Mice constitutively overexpressing acidic mammalian chitinase (AMCase) (SPAM mice) in the lung, when challenged with chitin or *A. fumigatus* had attenuated eosinophilia, AAMs, and Th2-associated cytokines (33, 35, 36, 38). In addition, AMCase enzyme dead knock-in mice (AMCase-ED) or AMCase knock-in/knock-out (ChiaRed) mice are unable to clear chitin polymers and have heightened Th2 type and pro-fibrotic lung inflammation at steady state or when exposed to medium or large chitin particles, crude HDM (large particles chitin content) or filtered HDM (small particle chitin content) (38, 39). However, AMCase-deficient mice have given contradictory reports compared to AMCase-ED or ChiaRed mice (38, 39). AMCase-deficient mice did not show aberrant type 2 responses during ovalbumin or HDM-induced allergic asthma (40, 41). These contrasting findings between these mice remain to be reconciled by direct comparison of the strains. One key difference between the two strains is that AMCase-ED and ChiaRed mice are able to express AMCase protein which is capable of binding chitin, however, the chitinase activity is diminished (38, 39).

Much remains to be learnt about the mechanisms involved in chitin recognition and clearance. Currently, it is unclear whether the PRRs that have been implicated in recognition of small and medium sized chitin polymers to induce IL-10 and TNF-α will be the same receptors inducing Th2 type allergic asthma. None of the receptors identified so far as chitin recognition receptors which are specific to chitin apart from FIBCD1. FIBCD1 seems to be expressed specifically in the stomach and not lung tissue. Perhaps the role of this receptor as a chitin-specific receptor will become clearer once FIBCD1-deficient mice become available. In humans, an AMCase single nucleotide polymorphism exists displaying hyper chitinase activity (42). This AMCase isoform is common among American ethnic groups and has been associated with protection against asthma (42).

Dectin-2

Dectin-2 is a 28 kDa, type II transmembrane CTLR, containing a single extracellular CTLD connected by a neck region to its short cytoplasmic tail (43). Dectin-2 recognizes high mannose structures on the surfaces of a wide range of microbes and has also been shown to have a putative endogenous ligand on CD4 T cells (44, 45). Moreover, dectin-2 recognizes α-mannans in many species of fungi, including *C. albicans*, *Paraccoides brasiliensis*, *Histoplasma capsulatum*, and *Cryptococcus neoformans* (46, 47). Apart from fungal ligands, dectin-2 has also been shown to recognized HDM allergen (*Dermatophagoides farinae* and *D. pteronyssinus*) (48), although the exact nature of ligands have not been elucidated.

Dectin-2 has no obvious signaling motif on its short cytoplasmic tail and relies on the ITAM-bearing FcRγ chain for signal transduction and downstream effects, including NFkB activation, Syk recruitment, and cytokine production (45, 49). The FcRγ chain and its phosphorylation by Src kinases were shown to be important for the surface expression of dectin-2 and the subsequent downstream signals (45, 49).

In *C. albicans*, mice deficient in dectin-2 are susceptible to systemic infections and fail to mount Th17 responses critical for fungal clearance (45, 47, 50). In the cases of allergic asthma induced by *D. farinae* mites, dectin-2 was shown to be activated

by a yet unidentified ligand (51, 52). Dectin-2 activation during the sensitization stage in DCs triggers generation of cysteinyl leukotrienes, pro-inflammatory lipid mediators, Th2 cytokines, in a FcRγ-, Syk-, and CARD-9-dependent manner (51, 53). Blocking of dectin-2 with antibody during the HDM challenge stages showed a redundant role of the receptor in airway inflammation and AHR (54). In contrast, antibody-mediated blockage of dectin-2 and dectin-2-deficient mice sensitized with wild type *D. furinae*-pulsed DCs had attenuated pulmonary inflammation (55). Moreover, dectin-2 deficiency had little effect on Th2 cytokine production by lung-draining MLN and systemic IgE and IgG1 production, suggesting that dectin-2 mediates localized Th2 responses during the effector phase (54, 55). Indeed, the mechanisms of dectin-2/HDM-induced Th2 allergic airway responses has been shown to require FcRγ-dependent type 2 alarmin IL-33 secretion and its autocrine signaling receptor ST2, which is thought to promote lung DCs maturation (56). Recently, phosphorytidylinositol 3 kinase delta (PI3Kδ) signaling pathway was also shown to be required for the dectin-2/FcRγ-dependent cysteine leukotriene production and Th2 and Th17 allergic airway responses induced by *D. furinae* (57). Inhibition of PI3Kδ ameliorated HDM-induced allergic asthma, suggesting a targetable pathway in the treatment of asthma. Interestingly, PBMCs from asthmatic patients were shown to upregulate expression of dectin-2, suggesting an important role for this receptor in human disease (54). Despite dectin-2 being shown to be important in antifungal immunity and in asthma in mouse models, no single mutation associated with disease has been observed so far in humans.

DC-Specific Intercellular Adhesion Molecule 3-Grabbing Nonintegrin

DC-specific intercellular adhesion molecule 3-grabbing nonintegrin is a 44 kDa type II transmembrane protein receptor (58). DC-SIGN is arranged as a tetramer of autonomous CTLD that interact through alpha helix neck domain (59). It consists of an extracellular domain that contains a CRD arranged in a homodimeric cluster, a hinged domain followed by a transmembrane region and a cytoplasmic domain/signaling tail (**Figure 1**). The CRD recognizes a broad spectrum of mannose and fucose ligands

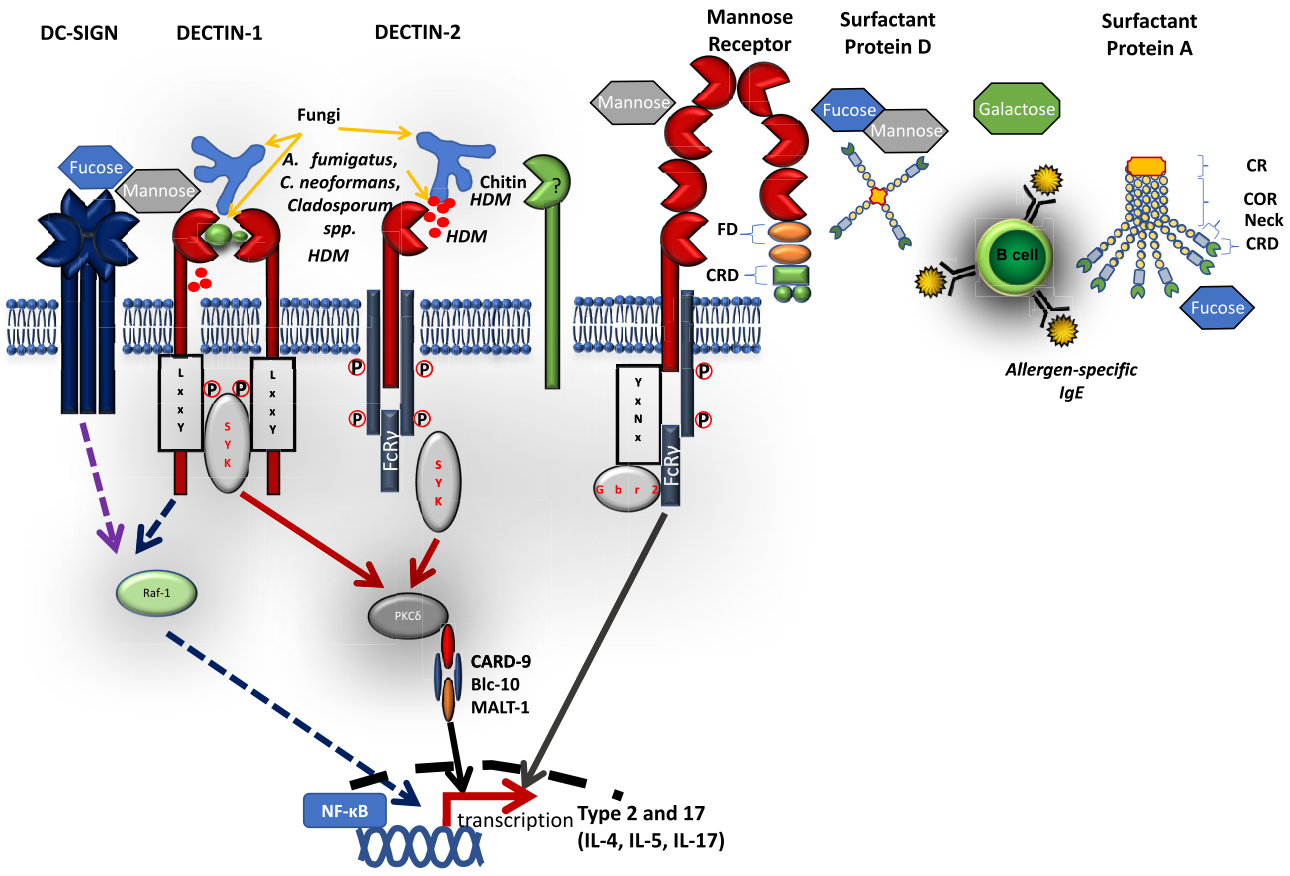

FIGURE 1 | Schematic diagram of selected C-type lectin-like receptors (CLRs) and their respective ligands. C-type lectin receptors bind to their respective extracellular microbial pattern-associated molecular patterns on cell wall surfaces of fungi and house dust mites. Most CLRs signal *via* a Syk-dependent pathway inducing reactive oxygen species, inflammatory cytokines, and antimicrobial peptides. Some CLRs, e.g., dectin-1 and DC-specific intercellular adhesion molecule 3-grabbing nonintegrin can signal *via* a Syk-independent pathway involving Raf-1. Some CLRs (such as dectin-2 and MR) cannot directly activate Syk, but require adaptor protein FcRγ attached to its intracellular immunoreceptor tyrosine-based activation motifs. Soluble CLRs, such as surfactant proteins (SP-A, SP-D) are arranged in either one or two polypeptide chains with a CRD connected to neck region, a collagen-rich domain, and cytoplasmic region. FD, fibronectin domain; CRD, carbohydrate recognition domain; COR, collagen-rich domain.

on various microbes, including viruses (HIV gp120 glycoprotein, measles viruses), parasites (*Leshmania* spp., *S. mansoni*), fungi (*C. albicans*), bacteria (*Helicobacter pylori*, *M. tuberculosis*, and *M. leprae*), and self-ligands, such as, ICAM-2, ICAM-3, and Mac-1 (58, 60). The carbohydrate structure recognition by CRD seems to be Ca^{2+}-dependent. Because of its broad specificity to sugar moieties, DC-SIGN has been shown to be an exploitable target for many pathogens as they use it to subvert host killing mechanisms (58, 59).

Although DC-SIGN lacks the consensus signaling motifs that interact with Syk kinases and other downstream adaptors, DC-SIGN still acts as a signaling receptor. Activation of DC-SIGN does not induce an immediate downstream signal, however, DC-SIGN has been shown to modulate signals initiated by toll-like receptors (61). Depending on the engaged ligand, e.g., fucose or mannose, the intracellular tail of DC-SIGN forms a ligand-specific signalomes with kinases including Raf-1. This enhances phosphorylation and acetylation of NFkB subunit p65 and heightened pro-inflammatory (Th1) or suppression of NFkB activation and induction of anti-inflammatory (IL-10 and Th2) gene transcription (61, 62).

Allergens containing protease activity have been shown to have multiple mechanisms of generating allergic inflammation. The major peanut allergen (Ara h 1), grass pollen allergen (BG-60), major dog allergen (Can f 1), and major dust mite allergens (Derp-1 and Derp-2) have been shown to activate DC-SIGN, initiating cytokine production and allergic inflammation biased toward Th2 polarization (63–65).

Binding of glycosylated Derp-2 to DC-SIGN on human DCs elicits TNF-α release. Furthermore, in DC/T-cell co-culture experiments Derp-1 pre-treated monocyte-derived DC induced GATA3 and IL-4 expression by naive T cells (66). This is also supported by studies showing that Derp-1 stimulation of human DCs leads to downregulation of DC-SIGN and bias toward Th2 polarization (63, 66). Moreover, DCs from atopic asthmatics have reduced DC-SIGN expression which correlates with data demonstrating downregulation of DC-SIGN expression by Derp-1 during the differentiation of immature monocyte-derived DCs (66).

The mechanism for Th2 differentiation by protease/DC-SIGN engagement are not fully understood, but are thought to be due to the loss of DC-SIGN expression through cleavage. This leads to reduced binding to its ligand ICAM-3 on naive T cells, which is thought to be important in Th1 polarization (67). Although protease cleavage of DC-SIGN is the most studied mechanism for Th2 type response induction, other studies suggests that non-proteolytic mechanisms involving selective DC-SIGN activation by fucose ligands may direct Th2 cell differentiation (62). Fucose from pathogens, such as *H. pylori* and soluble egg antigen, from *S. mansoni* was recently shown to directly inhibit TLR-4-induced pro-inflammatory cytokine production associated with Th1 polarization and activated anti-inflammatory cytokine IL-10 and Th2-associated chemokines CCL17 and CCL22. This fucose triggering of DC-SIGN required an initial TLR-4 priming, Raf-1 activation, and displacement of the mannose signalome on the signaling tail of DC-SIGN. Downstream, Bcl3, an NFkB subunit interferes with p50–p65 subunit dimerization and binds to p65 binding site and translocate to the nucleus, thereby repressing NFkB-dependent transcription of pro-inflammatory genes (62). Whether this fucose/DC-SIGN signaling and Th2 differentiation is a common mechanism for all fucose decorated allergens such as those derived from fungi or HDM, and how DC-SIGN selectively engages different carbohydrate, is currently unclear and requires further investigation.

Studies on DC-SIGN function *in vivo* are limited as there are no murine orthologs, although several murine homologs, such as SIGNR3 and SIGNR5, exist with gene-deficient mice available (59). However, the marked differences in signaling pathways downstream of DC-SIGN and its supposed murine orthologs puts into question their validity in experimental models.

Mannose Receptor

Mannose Receptor was one of the first CLR to be discovered and was shown to be involved in the clearance of glycoproteins (68, 69). Mannose receptor is a 175 kDa type I integral transmembrane glycoprotein that binds structures like L-fucose, D-mannose, or *N*-acetylglucosamine through Ca^{2+}-dependent mechanisms as well as structures, such as sulfated acidic glycans through the cysteine-rich domain (70). Like DC-SIGN, MR structure has 3 regions, a cysteine-rich domain, a fibronectin type II-like domain, and 8 CTLDs, followed by a transmembrane region and a hydrophilic internal cytoplasmic domain (**Figure 1**) (68, 70). The cytoplasmic domain participates in receptor internalization and recycling, whereas the fibronectin type II-like domain mediates collagen binding (68).

Mannose receptor has been shown to recognize a range of organisms, including *C. albicans* (71), *P. carinni* (72), HIV (gp120) (73), mycobacterium (74), *Leishmania*, and some bacterial species. MR has also been shown to bind endogenous self-antigens, including ligands in the B cell follicles in the spleen and thyroid (68, 75).

Although MR was one of the first studied CLR, its signaling pathways have been a matter of considerable interest, mainly because the cytoplasmic tail lacks an ITAM or ITIM motifs. The tyrosine residue on the 18th position of the MR cytoplasmic tail is thought to be important for receptor endocytosis (68). A recent study has identified the ITAM-bearing FcRγ as a key adaptor in human MR signaling (74). Upon ligand, mannose capped lipoarabinomannan (ManLAM) engagement, MR cytoplasmic tail interacts with FcRγ which when phosphorylated recruits another adaptor Grb2. Gbr2 recognizes specific motif (YxNx) on MR cytoplasmic tail and activates another complex Rac-1, Cdc42, and PAK-1. The formation of this complex eventually promotes phagocytosis. Once in the phagosome, Grb2 complex recruits a tyrosine phosphatase SHP-1 which upon phosphorylation interacts directly with Grb2 complex. SHP-1 interrupts the formation of MR-dependent phagosome–lysosome fusion upon *M. tb* infection and promotes *M. tb* growth inside phagosomes.

Similar to DC-SIGN, MR binds to a number of carbohydrate microbial cell surface ligands, such as mannans and proteolytic allergens, including HDM allergens (Derp-1 and Derp-2), German cockroach allergen (Bla g 2), major dog allergen (Can f 1), cat allergen (Fel d 1), and major peanut allergen (Ara h 1) (76–78). DCs from atopic asthmatics have elevated MR expression

and are efficient in uptake of Derp-1 HDM allergen compared to non-asthmatic controls (78). Furthermore, human MR-deficient DCs fail to induce a Th2 polarization in naive T cell/DC co-culture when stimulated with Derp-1. Unlike DC-SIGN, MR activation by protease does not involve cleavage of the receptor, however, it is thought to involve direct recognition by the CTLD-4–7 region (76, 77). This is further supported by studies demonstrating that human MR engagement with its ligand, Derp-1 initiates Th2 polarization through modulation of indoleamine 2,3-dioxygenase (IDO) activity, an immune modulator enzyme involved in tryptophan metabolism. In a mouse model using a cockroach allergen-induced allergic airway response, MR was shown to be important in allergen uptake and in regulation of Th2 allergic airway inflammation, as mice deficient of MR showed aberrant Th2 responses (79). Mechanistically, MR together with its intronic microRNA (miR-55-3p) was shown to regulate macrophage polarization from M2 to M1 upon cockroach allergen stimulation, resulting in protection against allergen-induced allergic airway inflammation *in vivo* (79). Currently, most of what we know about MR in allergic asthma has been done using human cells and knockdown approaches. Very little been done *in vivo* using known fungal derived ligands despite the fact that MR-deficient mice have been available for some time and can be a useful resource in understanding mechanisms of MR-mediated asthma (79). It is also important to note that there are considerable differences between mouse and human MR signaling, for example, SHP-1 is not robustly expressed in mice and does not interact with MR. Humanized mice expressing a human version of MR will bring into light some of the key regulatory roles of MR in allergic asthma.

Surfactant Proteins A and D

Lung surfactant proteins A and D (SP-A and SP-D) are soluble PRRs secreted mainly by alveolar type II cells (pneumocytes) and by other cells types, including non-ciliated bronchiolar cells, lung epithelial cells, and gastrointestinal and genitourinary tracts (80) SP-A and SP-D are part of the six human collectins described so far (80). They recognize sugar moiety patterns on the surface of various pathogens, including bacteria, fungi, and viruses with varying preference, i.e., SP-A preferentially binds monosaccharides, in contrast SP-D binds avidly to more complex saccharides (80). The structure of SP-A and SP-D is organized into four regions: an N-terminal region (which forms interchain disulfide bonds), a collagen-rich region containing (Gly-Xaa-Yaa) repeats, a neck peptide and a C-terminal CRD (which interacts with carbohydrate structures) (80). SP-A has two 35 kDa polypeptide chains (SP-A 1 and 2) arranged in a 635 kDa hexamer, SP-D has one 43 kDa polypeptide chain arranged in a tetramer of 520 kDa (**Figure 1**) (80, 81).

SP-A and SP-D have been shown to bind to HDM allergen Derp-1 and glycoprotein allergens from *A. fumigatus* in a carbohydrate-specific and Ca^{2+}-dependent manner (82, 83). This was shown to inhibit IgE binding to these glycoproteins, blocking allergen-induced histamine release from basophils and mast cells, and effectively reducing IgE hypersensitivity and allergic inflammation (84–86). SP-A and SP-D-deficient mice are inherently hypersensitive to pulmonary infections, showing increased eosinophilia and IL-13, a Th2-associated cytokine (86). The ability of SP-A and SP-D to block allergen-specific IgE and IgG1 can be explained by their potential to block B cell proliferation resulting in reduced antibody production. Another suggested mechanism is through production of IFN-γ by CD4 T cells, which shifts cellular responses from Th2 to Th1 responses (84, 85). SP-A was recently shown to play an important role in local lung macrophage proliferation by boosting IL-4Rα signaling, which was important for helminth expulsion (87). It is likely that similar mechanisms would exist for the induction of allergic asthma, but remain to be investigated. Genome-wide association studies have also linked single nucleotide polymorphisms in SP-A1 and SP-A2 gene to high susceptibility to ABPA, suggesting a key role of surfactants in fungal-induced asthma (88).

Dectin-1

Dectin-1 is a 28 kDa type II membrane protein with a single extracellular lectin-like carbohydrate recognition domain connected by a stalk to the transmembrane region followed by a cytoplasmic signaling tail (**Figure 1**) (89). Dectin-1 was identified to be a major β-(1,3)-D-glucan receptor in the early 2,000 through screening of mouse macrophage cDNA expression library with zymosan (a β-glucan-rich particle) (90). Other receptors have also been shown to bind β-(1,3)-D-glucan, including CR3, SCARF, lactosylceramide, CD36, and ephrin type-A receptor 2 (EphA2) (91, 92). β-(1,3)-D-glucan is a glucose polymer that is widely distributed across the biosphere predominantly found on the cell wall surfaces of plants (93) and fungi (such as *Candida* spp., *Aspergillus* spp., *Coccidiodes* spp., and *Pneumocystis* spp.) (90, 94–96). Dectin-1 has also been shown to bind an unidentified endogenous ligand in T cells (89).

Upon engagement with β-(1,3)-D-glucan, dectin-1 initiates intracellular signals *via* its cytoplasmic ITAM-like motif through phosphorylation of a tyrosine residue residing within the YxxxL motif. Phosphorylation of the membrane proximal single tyrosine-based motif is sufficient to induce activation and recruitment of Syk and this type of sequence has been termed ITAM-like motif or HemITAM (19, 24, 25). The hemITAM is thought to be important in dimerization of dectin-1 and the formation of a docking site for SH2 domains of Syk. Downstrean effects after Syk activation and recruitment are discussed above.

The wide distribution of β-(1,3)-D-glucans in nature and its importance in cell wall formation has brought considerable interests in its structure as a possible target for drug development or therapeutic potential. The β-(1,3)-D-glucan glucose polymer has variable molecular weight and degree of branching that can be triple helix, single helix, or random coil structured (97). In fungal cell wall, glucans compose about 40–50% of cell wall dry weight with at least 65–90% of the cell wall being β-(1,3)-D-glucans (98). β-(1,3)-D-glucans are covalently linked to β-(1,6)-glucans and chitin [β-(1,4)-linked polymer of *N*-acetylglucosamine] (98, 99). β-(1,3)-D-glucan serves as a backbone structure to which other structures are covalently bound such as N- and O-linked oligosaccharides, GPI anchored glycoproteins, and β-(1,6)-glucans forming a mesh of β-(1,3)-D-glucans sandwiched between inner chitin layer and outer mannan layer (27, 98). β-(1,3)-D-glucans (hereon referred to as β-glucans) have been shown to have

immunomodulatory activities, for instance anti-tumor effects by promoting leukocyte cytotoxic activities and hematopoiesis recovery after chemotherapy, protective effects against bacterial infections and wound healing (100). However, β-glucans have also been shown to have negative effects, including inducing formation of granulomas, triggering arthritis (101), and are implicated in airway inflammatory responses in human and animal models (102).

In humans, exposure to β-glucans has been associated with reduced FEV_1/pulmonary function (103), however, the same group found no or even opposite association in later studies (104). Epidemiological studies evaluating multiple parameters, including atopy, lung function FEV_1, skin symptoms, inflammation, and cytokines, generally found very few significant associations with exposure to β-glucan which confirms inconsistency in the results (105). Subjects with previous history of airway reactivity showed minor nose and throat irritation, but no effects on FEV_1 or airway responsiveness when challenged with aerosolized β-glucan (curdlan) (104, 106). In healthy volunteers, inhalation of low doses of soluble β-glucan (grifolan) showed decrease in TNF-α from PBMCs, but no effects on several markers of inflammation and pulmonary function (104). Human dectin-1 activation can enhance or inhibit Th2 polarization depending on the antigen presenting cell encountering β-glucan *in vitro* (107). A recent study reported that stimulation of plasmacytoid DCs promote Th2 polarization of naive T cells *in vitro* in a dectin-1-dependent manner, whereas stimulation of monocyte-derived DCs suppressed Th2 polarization and favored Th17 responses *in vitro* (107). Other studies have also supported the role of dectin-1 promoting Th2 polarization of naive T cells *in vitro*, by showing that β-glucans derived from HDM promoted secretion of Th2 type cytokines IL-4 and IL-13 by human DCs and CCL20 production by human bronchial epithelial cells, a chemokine associated with Th2 type immune response (64, 108, 109).

In animal models, β-glucans purified from various sources have been shown to contribute to allergic airway inflammation, mainly acting as adjuvants when topically co-administered with allergens (102, 110–114). A recent study showed that particulate β-glucans acted as mucosal adjuvants to HDM allergen when co-administered during priming phase and promoted a strong Th2 allergic airway response to HDM allergen (112). This is consistent with other studies that have shown adjuvant potential of β-glucans (derived from barley and baker's yeast and *C. albicans*) when co-administered with ovalbumin (OVA), promoting OVA-specific IgE, eosinophilia, and Th2 cytokines (110, 115). In these studies, dectin-1 either had no role in the adjuvant effects of β-glucans to HDM/OVA allergens or was not investigated (110, 112, 115). Dectin-1 has also been shown to regulate allergic asthma induced by fungal species depending on the surface exposure of β-glucans. In *A. versicolor* live conidia-induced asthma, dectin-1-deficient mice showed increased Th2 type allergic airway inflammation with increased IL-33, airway hyperresponsiveness, and reduced IL-17A (116). In the same study, *C. cladosporioides* live conidia-induced Th2 type, eosinophilic airway inflammation in a dectin-1-independent manner. Subsequent studies showed that the difference in β-glucan surface exposure between *A. versicolor* and *C. cladosporioides* was key in the distinct allergic airway profile and that dectin-1 suppressed this Th2 type allergic asthma by promoting IL-17A and IL-22 type immune responses (116, 117). In *A. fumigatus* live conidia allergic asthma chronic model, dectin-1 was shown to promote IL-22 and immunopathology, corroborating other findings showing a detrimental role of dectin-1 in fungal-induced allergic asthma (118). This type of allergic asthma is often seen in allergic models with neutrophilic inflammation that is usually unresponsive to high doses of corticosteroids (111, 119).

Some studies using HDM extracts to induce allergic asthma in animal models show requirement for dectin-1 signaling in Th2 type, eosinophilic, allergic airway asthma (109, 120). Dectin-1 is mainly expressed by CD11b DCs and recognizes HDM-derived β-glucan-like substance to initiate a Th2 polarization and migration of lung DCs to MLN by secretion of chemokines, such as CCL3 and CCR7 (120). Mechanistically, during HDM-induced allergic asthma, dectin-1 induces the production of prostaglandin E2 (PGE_2), which promotes M2 macrophage polarization and subsequently, Th2 type allergic asthma (121). It is difficult to reconcile studies using HDM extracts, co-administration of β-glucans with allergens and those using fungal species. The main challenge with commercial HDM extracts is that it is still unclear if β-glucans are contaminants derived from ingestion of fungi by mites or endosymbionts-derived molecules. Furthermore, commercial HDM extracts do vary largely in their content of these microbial PAMPs from batch to batch depending on preparation (122).

There are considerable inconsistencies in the results using β-glucans in both human and animal models as discussed above. The disparities could be due to the fact that differences in β-glucan source, content, solubility, conformation, choice, and strain of animal used, and whether human volunteers had previous fungal exposure, as discussed above. Possible clarification for such inconsistencies using β-glucan stems from a study, where it was shown that dectin-1 activation requires receptor clustering forming a "phagocytic synapse" (123). In this study, it was demonstrated that larger particulate β-glucans particles had greater ability to induce this dectin-1 phagocytic synapse (123). It is now easier to explain why β-glucan exposure has resulted in different phenotypes. Future studies on the effects of β-glucan in asthma or any other immunomodulatory activities should certainly consider the particulate size of β-glucan to induce the dectin-1 phagocytic synapse. In summary, it is clear from both animal and mouse studies that much needs to be reconciled on the role of dectin-1 and its ligand β-glucans in allergic asthma. Already a few SNPs have been identified in human dectin-1 gene (CLEC7A) which correlates with development of allergic asthma, highlighting the importance of this receptor in driving allergic asthma (124).

CONCLUDING REMARKS

It is becoming clear that fungal sensitization and fungal-associated components play a crucial role in the initiation, exacerbation, and severity of asthma. Evidence is now emerging showing that CLRs that recognize most fungal-derived components

are also crucial in shaping the development of allergic asthma. Many other fungal species, such as *A. alternate*, associated with allergic asthma have not been investigated in CLRs-deficient animals and the ligands and mechanisms of action remain elusive. Of significant interest is how some of these CLRs can be targeted as mucosal adjuvants in cases, where they play protective roles in asthma and drug targets, where they promote allergic asthma, especially difficult to treat asthma. The fact that CLRs can also collaborate with each other or other PRRs is likely to have huge implications in deciphering an already heterogeneous and complex disease like asthma. There is still a lot to be learnt about these interactions and how such collaborations can be used to harness a beneficial immune response toward pathogens or treatment strategies for asthma. Although there is correlation between human and mouse data, there is still a considerable discrepancy in findings using CLR-deficient mice in allergic asthma models and *in vitro* data using human cells. CLRs-deficient animals are a useful tool in understanding disease mechanisms and this requires improvements to better mimic human asthma. Technologies like humanized mice or mice expressing human version of CLRs will give insights into how CLRs shape allergic asthma. Finally, it is clear that some ligands still have ambiguous or unidentified CLRs despite being known for generations to play key roles in allergic asthma, and much work remains to generate new mouse tools to decipher these mysteries.

AUTHOR CONTRIBUTIONS

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REVIEW
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Flexible Signaling of Myeloid C-Type Lectin Receptors in Immunity and Inflammation

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Myeloid C-type lectin receptors (CLRs) are important sensors of self and non-self that work in concert with other pattern recognition receptors (PRRs). CLRs have been previously classified based on their signaling motifs as activating or inhibitory receptors. However, specific features of the ligand binding process may result in distinct signaling through a single motif, resulting in the triggering of non-canonical pathways. In addition, CLR ligands are frequently exposed in complex structures that simultaneously bind different CLRs and other PRRs, which lead to integration of heterologous signaling among diverse receptors. Herein, we will review how sensing by myeloid CLRs and crosstalk with heterologous receptors is modulated by many factors affecting their signaling and resulting in differential outcomes for immunity and inflammation. Finding common features among those flexible responses initiated by diverse CLR-ligand partners will help to harness CLR function in immunity and inflammation.

Keywords: lectin receptors, signaling, monocytes, macrophages, dendritic cells, innate immunity, inflammation

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DIVERSITY OF SIGNALING MODULES IN MYELOID C-TYPE LECTIN RECEPTORS (CLRs)

The expression of diverse pattern recognition receptors (PRRs), including differential expression of CLRs, provides different subsets of immune cells with a repertoire to interpret and respond distinctly to the information coming from the environment. Myeloid cells are central for initiation and regulation of innate and adaptive immunity or tolerance and the CLR repertoire essentially contributes to myeloid cell function. We previously proposed a classification of myeloid CLRs based on their intracellular signaling motifs (1). While signaling motifs allow to predict effector responses following sensing by CLRs, this canonical response is subjected to modulation by the physical nature, affinity, and avidity of the ligand (2). Based on their intracellular signaling motifs, myeloid CLRs can be classified into the following broad categories (**Figure 1**): immunoreceptor tyrosine-based activating motif (ITAM)-coupled CLRs, hemi-ITAM-(hemITAM)-bearing CLRs, immunoreceptor tyrosine-based inhibitory motif (ITIM)-containing CLRs, and a group of CLRs lacking typical signaling motifs (1, 3, 4).

Immunoreceptor tyrosine-based activating motif-coupled CLRs have a classical ITAM motifs in their intracellular tail, consisting of YXXL tandem repeats, or can interact with ITAM-containing adaptor proteins, as Fc receptor γ (FcRγ) chain or DNAX-activation protein 12 (DAP12) (5). The majority of them, including Dectin-2 (*CLEC6A* in human, *Clec4n* in the mouse), Mincle (*CLEC4E*), MCL (*CLEC4D*), BDCA-2 (human *CLEC4C*), DCAR (mouse *Clec4b1*), DCAR1 (mouse *Clec4b2*), and mannose receptor (MR) (*MRC1*, CD206) utilize the FcRγ chain adaptor, while MDL-1

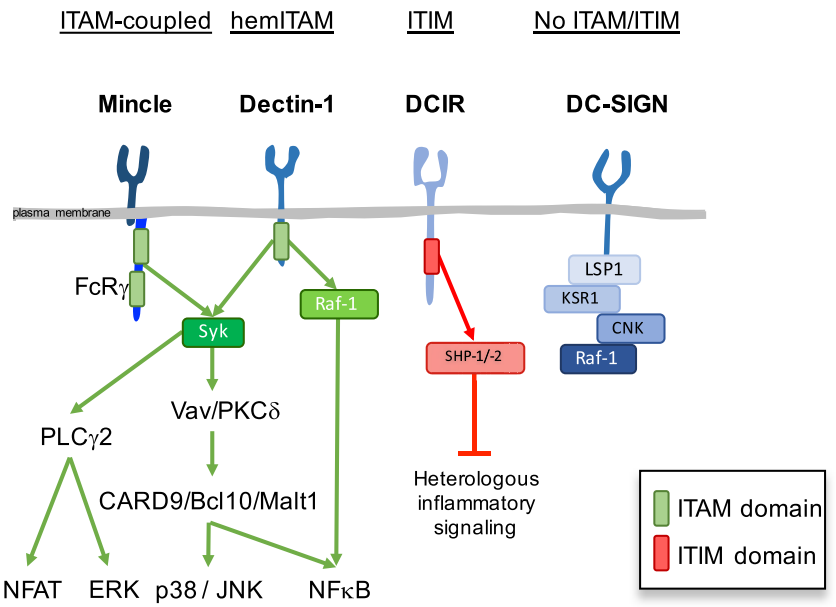

FIGURE 1 | Canonical signaling modules in myeloid C-type lectin receptors (CLRs). Based on canonical intracellular signaling motifs, myeloid CLRs can be classified into immunoreceptor tyrosine-based activating motif (ITAM)-coupled CLRs, hemi-ITAM-(hemITAM)-bearing CLRs, immunoreceptor tyrosine-based inhibitory motif (ITIM)-containing CLRs, and a group of CLRs lacking typical signaling motifs. Mincle, Dectin-1, DCIR, DC-SIGN, and their corresponding canonical signaling pathways and adaptors are depicted as prototypical examples of each category.

(*CLEC5A*) interacts with DAP12 (6–12). Hemi-ITAM-bearing CLRs contain a single tyrosine within an YXXL motif in their cytoplasmic domain (13, 14). Dectin-1 (*CLEC7A*), CLEC-2 (*CLEC1B*), DNGR-1 (*CLEC9A*), and SIGN-R3 (mouse *Cd209d*) belong to the hemITAM-based CLRs category (15–20).

These ITAM or hemITAM CLRs are considered activating receptors that couple to the spleen tyrosine kinase (Syk) (**Figure 1**) (15, 21, 22). Phosphorylation of the tyrosine(s) in the ITAM or hemITAM motifs generates docking sites for the SH2 domains of Syk, which undergoes a conformational change that permits autophosphorylation and activation (23). Mincle acts as a prototypical activating CLR after recognition of glycolipids in the cell wall of some fungal and bacterial pathogens (24–26). Through the full ITAM of the FcRγ chain adaptor, Mincle couples to Syk and activates Vav proteins and PKCδ, which lead to downstream activation of CARD9/Bcl10/Malt1 and MAPK pathways, thus resulting in the induction of several cytokines and chemokines, including TNF-α, macrophage inflammatory protein 2 (MIP-2; CXCL2), keratinocyte-derived chemokine (KC; CXCL1), and IL-6 (7, 27, 28). Production of inflammatory cytokines by myeloid cells, together with the generation of Th1 and Th17 responses, contribute to protective immunity upon recognition of some Mincle ligands (29–38).

Spleen tyrosine kinase activation downstream of the hemITAM-bearing CLR Dectin-1 leads to similar signaling pathways to those described for Mincle (**Figure 1**), with activation of the CARD9/Bcl10/Malt-1 module that promotes canonical NF-κB signaling (27, 28, 39). Dectin-1 can also activate MAPK (40, 41), NFAT through phospholipase C-γ2 (42, 43), and a Syk-independent non-canonical NF-κB activation relying on the activation of the Raf-1 kinase (44). These integrated pathways mediate production of reactive oxygen species (ROS) and cytokines, such as IL-1β, IL-6, IL-10, IL-12, TNF-α, and IL-23 to drive Th1 and Th17 differentiation, being essential for the development of antifungal immune responses (45–48). This axis is also activated in response to intestinal fungi, where Dectin-1 contributes to gut homeostasis (49).

Immunoreceptor tyrosine-based inhibitory motif-containing CLRs negatively regulate signaling initiated by kinase-associated heterologous receptors through the recruitment of tyrosine phosphatases, such as Src homology region 2 domain-containing phosphatase (SHP)-1 or -2 (**Figure 1**). Myeloid CLRs included in this group are human DCIR (*CLEC4A*), mDcir1 (*Clec4a2*), mDcir2 (*Clec4a4*), Clec12a (MICL, DCAL-2, KLRL1, CLL1), MAgH (*CLEC12B*), and Ly49Q (1, 50, 51). The ITIMs of both hDCIR and mDCIR1 have been shown to mediate inhibitory signaling through activation of the phosphatases, SHP-1 and SHP-2 (52–54). Activation of hDCIR on dendritic cells (DCs) leads to inhibition of TLR8-mediated IL-12 and TNF-α production and TLR9-induced IFN-α production (55, 56). Sensing endogenous ligands by DCIR modulates innate immunity to pathogens, such as *Plasmodium* or *Mycobacterium* (57, 58).

Myeloid CLRs that do not bear evident ITAM or ITIM domains include MMR (*MRC1*), DEC-205 (*LY75*), human DC-SIGN (*CD209*), mouse SIGN-R1 (*Cd209b*), Langerin (*CD207*), human MGL (*CLEC10A*), mouse Mgl1 (*Clec10a*), mouse Mgl2 (*Mgl2*), CLEC-1 (*CLEC1A*), human DCAL-1 (*CLECL1*), LOX-1 (*OLR1*), and LSECtin (*CLEC4G*). As an

example, DC-SIGN intracellular tail is associated with a signalosome composed of the scaffold proteins LSP1, KSR1, and CNK and the kinase Raf-1 in unstimulated DCs (59) (**Figure 1**). Similar to other CLRs in this group, DC-SIGN cannot promote DCs activation or cytokine secretion *per se*, but it rather modulates signaling by heterologous receptors (see below) or engages the endocytic machinery contributing to antigen processing and presentation to T cells (3).

Along this review, we will provide illustrative examples of how signaling pathways triggered by a CLR coupled to a particular canonical motif can vary depending on many factors. We will focus on Mincle, Dectin-1, DNGR-1, DCIR, and DC-SIGN as myeloid CLRs representative of each category of signaling motif. **Table 1** includes the signaling module coupled to each CLR surveyed in this review, common and gene names, category of flexible signaling source, signaling pathway involved, and the inflammatory outcome provided by such flexibility. In this **Table 1**, CLRs are grouped based on the signaling module they bear (left column) and graphically illustrates how the signaling pathways triggered by these receptors are more complex and versatile (right columns) than expected by their signaling modules.

SIGNALING FLEXIBILITY BEYOND THE CANONICAL MOTIFS

Motif Context and Receptor Location Modulate Signaling

Classifications of receptors based on intracellular structural motifs stand on the fact that those domains determine the molecular signaling pathways initiated after ligand recognition (1). However, in addition to the basic ITAM and ITIM motifs, subtle variations in the context of the canonical motifs profoundly affect the signal delivered. For example, DNGR-1 is a DC-specific hemITAM-bearing receptor that detects dead cells and promotes cross-presentation in sterile or infectious settings, without contributing to inflammation (**Figure 2A**), in contrast to the close-related Dectin-1 (19, 60–63). This deficiency to promote cytokine production through DNGR-1 hemITAM was linked to an isoleucine that precedes the tyrosine in DNGR-1 hemITAM and rescued by mutation to the glycine present in Dectin-1 hemITAM (60). Signaling flexibility can thus be intrinsically provided by the amino acid sequence of those motifs present in a CLR. In this regard, residues in the neck region of DNGR-1 allow the

TABLE 1 | Myeloid C-type lectin receptors (CLRs) surveyed in this review.

Signaling module	Common name	Gene name	Source of flexible signaling	Signaling pathway[a]	Flexibility outcome[b]
No immunoreceptor tyrosine-based activating motif (ITAM) or immunoreceptor tyrosine-based inhibitory motif (ITIM)	DC-SIGN	*CD209*	Homotetramerization	LSP1–KSR1–CNK–Raf-1	Intrinsic (80)
			Sensing self and non-self	LSP1–IKKε–Bcl3	Inhibitory (113–115)
				KSR1–CNK–Raf-1	Activating (59, 110)
			Heterologous modulation	(?)	Activating (148)
				Raf-1–MEK	Inhibitory (112)
			Heterotrimerization DC-SIGN/MR/MDL-1	DNAX-activation protein 12 (DAP12)	Activating (86)
ITAM	MDL-1	*CLEC5A*	Heterotrimerization DC-SIGN/MR/MDL-1	DAP12	Activating (86)
	Mannose receptor (MR)	*MRC1*	Heterotrimerization DC-SIGN/MR/MDL-1	DAP12	Activating (86)
			Inhibitory ITAM	Fc receptor γ (FcRγ)–Grb2–SHP-1	Inhibitory (12)
	Dectin-2	*CLEC6A*, *Clec4n*	Heterodimerization Dectin-2/MCL	FcRγ–spleen tyrosine kinase (Syk)–NF-κB p65	Activating (85)
	MCL	*CLEC4D*	Heterodimerization Mincle/MCL	FcRγ–Syk	Activating (81–84)
	Mincle	*CLEC4E*	Heterodimerization Mincle/MCL	FcRγ–Syk	Activating (81–84)
			Inhibitory ITAM	FcRγ–Syk–SHP-1	Inhibitory (91, 92)
			Sensing self	Retarded Syk	Inhibitory (104, 105)
				FcRγ–Syk	Activating (7, 100–103)
			Heterologous modulation	FcRγ–Syk	Activating (140, 141, 143)
				FcRγ–Syk–PKB–Mdm2	Inhibitory (146)
hemITAM	CLEC-2	*CLEC1B*	Homodimerization	Syk	Intrinsic (75, 76)
	DNGR-1	*CLEC9A*	Motif context	Syk	Intrinsic (19, 60–64)
	Dectin-1	*CLEC7A*	Subcellular location	Syk	Intrinsic (67–70)
			Ligand size-conditioned subcellular location	Syk–MAPK–reactive oxygen species	Intrinsic (71–74)
			Phosphatase association	SHP-1–PTEN–FcRγ SHIP-1	Inhibitory (93, 94)
				SHP-2–Syk	Activating (95)
			Heterologous modulation	Syk	Activating (124–126)
				PI3K–mTOR–HIF-1α	Activating (130–134)
				Syk–Pyk2–ERK–SOCS-1	Inhibitory (128)
ITIM	DCIR	*CLEC4A*, *Clec4a2*	Activating ITIM	IFNI–STAT1 SHP-2 hijacking (?)	Activating (58)
			Sensing self and non-self	(?)	Activating (57)
				SHP-2/SHIP-1	Inhibitory (108, 109)

[a]*Described in the indicated reference. In case it was not studied in depth, it might be incomplete.*

[b]*This column indicates the inflammatory balance provided by each source of signaling flexibility. "Intrinsic" refers to specific responses triggered by particular CLRs.*

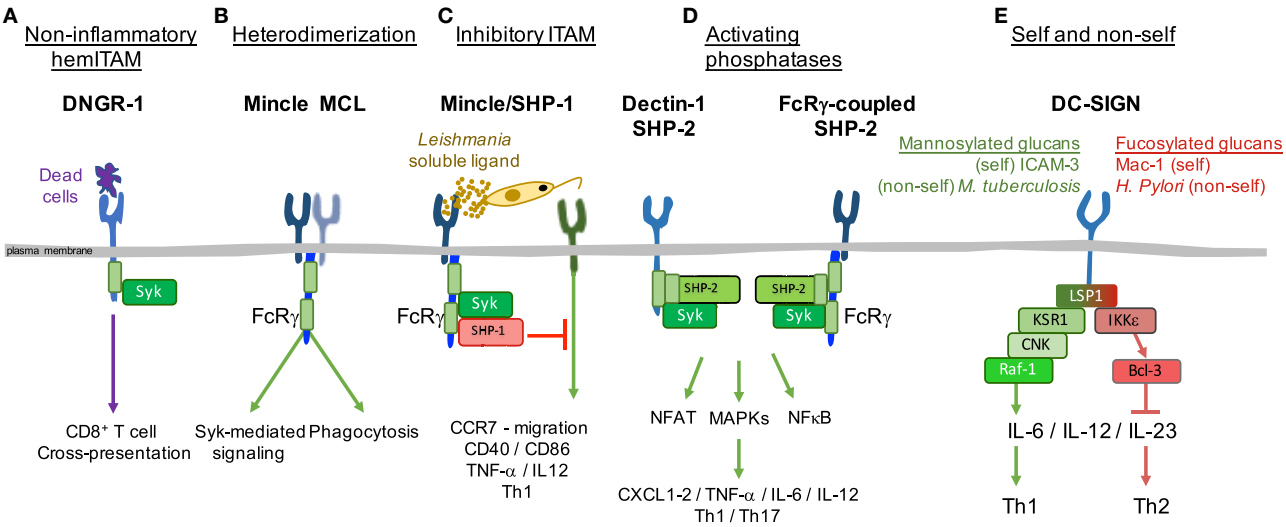

FIGURE 2 | Signaling flexibility downstream of C-type lectin receptors (CLRs). Signaling triggered downstream of CLRs goes beyond the canonical modules present in their intracellular domains and can be modulated by different processes. Some examples of such plasticity are represented. **(A)** DNGR-1 promotes cross-presentation of antigens to $CD8^+$ T cells, yet not directly contributing to inflammation. **(B)** Mincle and MCL dimerize, boosting phagocytosis, and spleen tyrosine kinase (Syk)-mediated inflammatory responses. **(C)** Sensing of a soluble ligand from *Leishmania* by Mincle triggers an inhibitory immunoreceptor tyrosine-based activating motif conformation downstream of Fc receptor γ (FcRγ), where SHP-1 dampens inflammatory responses triggered by heterologous receptors. **(D)** The phosphatase SHP-2 acts as a scaffold downstream of Dectin-1 and FcRγ-coupled CLRs, facilitating the recruitment of Syk and its inflammatory signaling. **(E)** Both self and non-self ligands share signaling pathways downstream of DC-SIGN depending on whether they are mannosylated or fucosylated glucans.

receptor to adopt different conformations that depend on pH and ionic strength, modulating its function as the receptor progresses through the endocytic pathway (64). Even the inflammatory response of mouse and human Dectin-1 to the same ligand varies because of minor interspecies variations in the signaling motif, with low valency ligands inducing proinflammatory genes through human but not mouse Dectin-1 (65).

Receptor location also affects CLR signaling and functions. A single CLR may be expressed in different cell types (66) as diverse isoforms that may differ in subcellular location. For example, two isoforms of Dectin-1 have been described to bind β-glucans (67); isoform A is characterized by the presence of a stalk region including an N-linked glycosylation site, which is missing in isoform B (68). This glycosylation determines the cell surface expression of isoform A, while non-glycosylated isoform B is retained intracellularly, thus conditioning the response to ligands (69) and the sensitivity to proteolytic cleavage (70).

The subcellular location of a CLR may not only depend on intrinsic features in its sequence, but also on the size of the particle where the ligand is recognized. For example, "frustrated" phagocytosis mediated by Dectin-1 in response to ligands exposed in large particles leads to enhanced cytokine response and ROS production compared with soluble ligands (71–73). Blockade of Dectin-1 internalization following ligand exposure leads to sustained MAPK activation (72), suggesting that endocytosis dampens Dectin-1 production of cytokines. Thus, formation of a phagocytic synapse by particulate β-glucan redistributes Dectin-1 and phosphatases along the cellular membrane, favoring proinflammatory signals including ROS production (73). In addition, the size of the ligand-containing particle and the consequent location of the receptor, can lead to qualitatively different responses. Dectin-1-mediated phagocytosis dampens the nuclear translocation of neutrophil elastase, controlling the extent of neutrophil extracellular traps (NET) formation in response to small pathogens (bacteria or yeast). Consequently, Dectin-1 blockade or deficiency leads to enhanced NETosis, as observed in response to non-phagocytic large pathogens (hyphae) (74).

Thus, the expected canonical response based on signaling modules can be altered both by slight modifications in motif context and the subcellular location of CLRs, taking into account that the latter may be affected by the size of the ligand recognized.

Multimerization of CLRs for Signaling

The signal transduction through several myeloid CLRs may also depend on their capacity to form dimers or multimers with other CLRs. CLRs bearing hemITAMs may require two phosphorylated tyrosines in a homodimer to bind Syk. It has been shown that CLEC-2 preexists as a dimer that aggregates following ligand binding (75, 76). The hemITAM motif of CLEC-2 is crucial for blood-lymph separation during development (77, 78). Of note, thrombus stability is dependent on CLEC-2 but not on the hemITAM, revealing a hemITAM-independent signaling for CLEC-2 (79).

DC-SIGN provides another example of homomultimerization, despite lacking ITAM or ITIM domains. This CLR appears assembled as a tetramer, allowing multiple interactions with diverse pathogens that differ in size, but also increasing ligand avidity (80). In addition, some CLRs form heterodimers, such as MCL and Mincle (11, 81). These two CLRs are interrelated as they both sense the mycobacterial glycolipid trehalose-6,6-dimycolate

(TDM), triggering an FcRγ-dependent pathway (11). Indeed, MCL and Mincle are co-regulated and depend on each other for their mutual surface expression (82, 83). However, the association of MCL with FcRγ in this complex is species-specific, being direct in mouse cells (11) but requiring Mincle in rat (81). Thus, the interaction between these CLRs would facilitate MCL signaling capacity *via* association with Mincle and translocation to the plasma membrane. On the other hand, Mincle would benefit the endocytic capacity of MCL (**Figure 2B**) and both receptors could increase affinity or specificity for their ligands (84). MCL also forms a heterodimeric pattern-recognition receptor with Dectin-2 (85), which has a high affinity for α-mannans on the surface of *Candida albicans* (*C. albicans)* hyphae.

Cooperative interaction is also found in the case of dengue virus binding with high affinity to MR and DC-SIGN, receptors that subsequently handle the virus to the lower affinity receptor CLEC5A, which mediates signal transduction (86).

All these examples illustrate how multimerization of CLRs, forming either homo- or hetero-complexes, facilitates a cooperative response to the ligand.

Is the Function of CLRs Inhibitory or Activating?

Another layer of complexity in CLR signaling stems from the ability of a single CLR to bind different ligands through its plastic C-type lectin domain. For instance, depending on their relative affinity or avidity, ligands may fine-tune signaling pathways downstream of ITAM motifs. Whereas the binding of high-avidity ligands to these receptors induces activating signals, the binding of low-avidity ligands leads to hypophosphorylation of the ITAM domain and preferential association of SH2-containing phosphatases like SHP-1, a configuration known as "inhibitory ITAM" (87). Although FcαRI receptor, which associates for signaling with the FcRγ chain, is the paradigmatic example of this inhibitory pathway (88–90), we have shown that CLRs associated with FcRγ chain may behave in the same fashion.

As an example, Mincle senses a soluble ligand derived from *Leishmania* that induces phosphorylation of SHP-1 coupled to FcRγ chain, inhibiting DC activation through heterologous receptors (**Figure 2C**) (91). In addition, SHP-1 contributes to deceleration of phagosome maturation upon TDM binding, suggesting an inhibitory signal downstream of Mincle during phagocytic processes (92). MR binds the FcRγ chain and, upon sensing *Mycobacterium tuberculosis*, recruits SHP-1 to the phagosome, thus limiting PI(3)P generation and delaying fusion with the lysosome, which promotes *M. tuberculosis* growth (12). Following treatment of DCs with curdlan or depleted zymosan (lacking TLR-stimulating properties), Dectin-1 signaling is modulated by the association of SHP-1 and PTEN to the FcRγ chain, hindering cytokine expression, DC maturation, and T-cell proliferation (93). ROS production downstream of Dectin-1 sensing of *C. albicans* is also tightly regulated by the SH2-domain containing inositol 5′ phosphatase (SHIP)-1 in response to Dectin-1 ligands (94). Thus, association of phosphatases to "activating" CLRs depending on the ligand nature, binding affinity, or avidity may contribute to maintenance of immune homeostasis.

Conversely, tyrosine phosphatases can contribute to activation. Contrary to SHP-1, the related tyrosine phosphatase SHP-2 acts as a scaffold, facilitating the recruitment of Syk to Dectin-1 or the adaptor FcRγ chain (95) (**Figure 2D**). In this way, DC-derived SHP-2 was crucial *in vivo* for the induction of TNF-α, IL-6, IL-12, and Th1 and Th17 anti-fungal responses upon *C. albicans* infection (95).

Immunoreceptor tyrosine-based inhibitory motif-coupled receptors can also deliver an activating signal. In a model of tuberculosis infection in non-human primates, DCIR deficiency impairs STAT1-mediated type I IFN signaling in DCs, leading to increased production of IL-12 and differentiation of T lymphocytes toward Th1. Thus, DCIR-deficient mice with increased Th1 immunity control *M. tuberculosis* better than WT animals, but also shown increased inflammation in the lungs mediated by TNF-α and inducible nitric oxide synthase (iNOS) (58). This study suggests that DCIR acts as an activating receptor for the STAT1-type I IFN signaling, and speculates that DCIR may function as a molecular sink binding unphosphorylated inactive SHP-2, therefore, limiting SHP-2′s capacity to deactivate STAT1.

The examples explained above illustrate a lack of correspondence between the canonical motif coupled to a CLR and the resulting signaling pathway. Association to kinases would lead to activating routes, while association to phosphatases would result in regulatory pathways, with some exceptions like the SHP-2-mediated CLR-induced activation (95). Association of kinases or phosphatases could be related to the strength of the initiating signal, with suboptimal phosphorylation leading to phosphatase binding to the hypo-phosporylated ITAM (inhibitory ITAM) (87). Due to the signaling flexibility offered by CLRs, a detailed empiric analysis for each CLR-ligand interaction in terms of type of ligand, concentration, and kinetics of exposition would be required to predict the signaling outcome.

Dealing With Self and Non-Self

C-type lectin receptors act as plastic receptors, some of them detecting self-ligands, other detecting non-self ligands, and many of them acting as dual receptors sensing self and non-self. It is possible that CLRs will behave as activating receptors when they sense non-self ligands, while CLRs bearing an ITIM motif will preferably bind self to dampen inflammation. However, in opposition to non-dangerous self, also known as "self-associated molecular patterns" (96, 97), Polly Matzinger proposed the existence of dangerous-self (damage-associated molecular patterns or DAMPs) exposed and/or released upon necrotic cell death (98, 99). In addition, tissue damage signals concomitant to infection can contribute to effector responses. Thus, DNGR-1 senses tissue damage concomitant with viral infections and facilitates antigen processing of viral antigens for cross-presentation to $CD8^+$ T cells, decoding the antigenicity rather than the adjuvanticity of the cargo (60–63). Some examples of CLRs dealing with self and non-self ligands are explained below.

Mincle is a plastic CLR promoting proinflammatory signals after sensing glycolipids in the cell wall of bacteria and fungi (24–26), but also sensing damaged self in the form of soluble SAP-130 following necrosis (7). Mincle sensing of β-glucosylceramide

(100) or cholesterol sulfate (101) promotes immunopathology (102, 103). Conversely, there are reports suggesting that Mincle sensing of SAP-130 can also drive immunosuppression (104). Moreover, human albumin abolishes innate immunity by directly binding Mincle receptor in the microglia after subarachnoid hemorrhage (105). Thus, Mincle is an example of CLR that deals with self and non-self ligands that may result in activating or inhibitory signals. However, the correlation of sensing self with an inhibitory response and sensing non-self with an activating response is not established. In this regard, non-self signals from pathogens may mimic self-inhibitory signals to escape immune surveillance, which could be the case for Mincle sensing of *Leishmania* (91).

DCIR is a myeloid CLR endowed with an ITIM motif that behaves as a self PRR. DCIR maintains the homeostasis of the immune system (106), since aged mice deficient for this CLR spontaneously develop several autoimmune disorders (107). Intravenous immunoglobulins bearing sialic acid induce a DCIR-mediated negative signal in DCs *via* SHP-2 and SHIP-1 that promotes Treg differentiation and dampens allergy (108). DCIR self-sensing can also occur in the context of infection, thus modulating the inflammatory response. DCIR-deficient mice exhibited severe inflammatory disease following Chikungunya virus infection (109). However, reduced adaptive T-cell responses in DCIR-deficient mice following cerebral malaria caused by *Plasmodium berghei* renders them more resistant (57). Since no evidence for direct interactions between DCIR and Chikungunya virus and *P. berghei* exists, we could hypothesize that DCIR may be recognizing DAMPs released during infection.

DC-SIGN illustrates how a single CLR deals differently with a variety of self and non-self ligands. DC-SIGN binds high mannose and fucose (LeX, LeY, LeA, LeB) that can be exposed in a variety of self receptors, such as ICAM-2, ICAM-3, CEACAM-1, Mac1 and CEA, or non-self proteins (structures in pathogens, including viruses, bacteria, fungi, and eukaryote parasites) (3, 110–115). Upon binding of mannosylated glucans, either self as those present on ICAM-3 (110) or non-self from *M. tuberculosis* (59), DC-SIGN couples to a LSP1–KSR1–CNK signalosome, leading to activation of Raf-1 and acetylation of the NF-κB p65 subunit, which results in enhancement of proinflammatory responses, including IL-12p70 and IL-6, although also promotes IL-10 transcription (59) (**Figure 2E**). In contrast, DC-SIGN recognition of fucosylated glucans as presented in self proteins, such as Mac1 (113) or non-self pathogens (*Helicobacter pylori*) (114), leads to dissociation of the LSP1-based signalosome and leaves just LSP1 associated with DC-SIGN. Phosphorylated LSP1 subsequently recruits IKKε and CYLD. IKKε activation inhibits CYLD deubiquitinase activity, facilitating nuclear translocation of ubiquitinated Bcl3 that represses TLR-induced proinflammatory cytokine expression, enhancing expression of IL-10 and Th2-attracting chemokines, and thus promoting Th2 polarization (114) (**Figure 2E**). In addition, IKKε collaborates with type I IFNR signaling to induce and activate the transcription factor ISGF3 that induces IL-27p28, a key cytokine for induction of T follicular helper cells (115). These results point to DC-SIGN as a dual receptor that, depending on the nature of the ligand, contributes to maintain homeostasis or initiates the immune response against some pathogens.

All these examples illustrate how a single CLR can trigger different signaling pathways depending on the recognition of self or non-self ligands. Current understanding of these processes is based on the study of individual CLRs. Deciphering common signaling patterns for self versus non-self sensing would allow harnessing immunity and inflammation by CLRs.

MODULATION OF HETEROLOGOUS SIGNALING BY MYELOID CLRs

In addition to the diverse response of a single CLR depending on the stimulus, it is fascinating how these signaling pathways interact with signals from heterologous receptors and lead to complex responses to stimuli that are simultaneously detected by several myeloid PRRs expressed in myeloid cells [see also Ref. (116, 117) for reviews focused on this topic]. In this section, we illustrate some examples of how myeloid CLRs cross-talk with surrounding heterologous receptors.

Dectin-1 Affects Simultaneous and Deferred Signaling Through Heterologous Receptors

Dectin-1 triggers a response after sensing infectious agents, such as diverse fungi and mycobacteria (118), *Salmonella typhimurium* (119) or *Leishmania infantum* (120). Dectin-1 may also promote proinflammatory signals following the detection of endogenous factors, such as vimentin from atherosclerotic plaques (121), galectin-9 from pancreatic carcinoma (122), or N-glucans on tumor cells (123). In addition to a prototypical activating CLR, Dectin-1 modulates signals simultaneously triggered through other PRRs. Dectin-1 cooperates with signals from TLR2/MyD88 to increase proinflammatory cytokine production (124–126). This synergy is exerted at the level of effector responses resulting in increased production of TNF-α, IL-12, and ROS (124) (**Figure 3A**, left). Dectin-1 also positively cooperates in the full activation of the NLRP3 inflammasome, participating in the priming and generation of pro-IL-1β and the induction of ROS required for NLRP3 activation (127). Conversely, Dectin-1 stimulation with depleted zymosan in bone marrow macrophages leads to Syk and Pyk2-ERK-dependent activation of SOCS-1 that downregulates IL-10 and IL-12p40 production induced by TLR9 stimulation (128) (**Figure 3A**, right). This effect would contribute to the Dectin-1 signature in priming Th17 responses (40, 128). In addition, Dectin-1 protects against chronic liver disease by suppressing TLR4 signaling. This effect is mediated by reducing TLR4 and CD14 expression, which are regulated by Dectin-1-dependent macrophage colony stimulating factor expression (129).

Apart from direct modulation of signaling pathways triggered simultaneously, Dectin-1 can leave a footprint that affects deferred signaling by heterologous receptors, a process named as trained immunity (130). Trained immunity after sensing of *C. albicans* or purified β-glucan *via* Dectin-1 results in enhanced protection to a lethal challenge with *Candida* and cross-protection

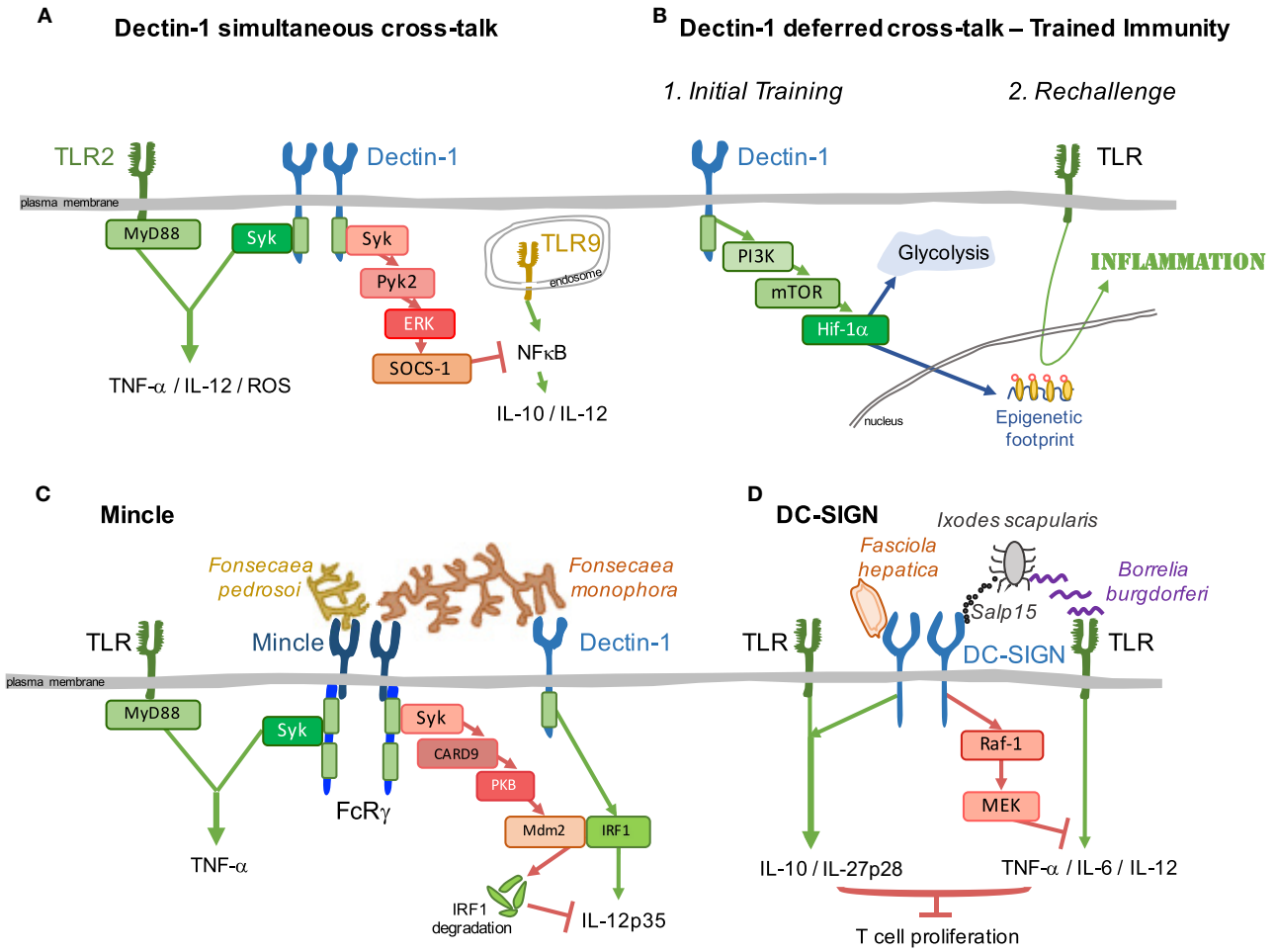

FIGURE 3 | Signaling crosstalk between myeloid C-type lectins and heterologous receptors. Signaling pathways initiated downstream of C-type lectin receptors (CLRs) interact with surrounding cascades triggered by heterologous receptors. Examples of such crosstalk are illustrated. **(A)** Dectin-1 coordinates with simultaneous signals from diverse TLRs to modulate the inflammatory response; this interaction can be either positive, as for TLR2/MyD88 (left), or negative, as for TLR9 through a Pyk2/ERK/SOCS-1-dependent pathway (right). **(B)** In addition, the axis Dectin-1/PI3K/mTOR/Hif-1α generates a switch toward glycolytic metabolism together with an epigenetic footprint, allowing for a "deferred" improved response to TLRs, boosting the inflammatory response. This process is known as trained immunity. **(C)** A full inflammatory response against *Fonsecaea pedrosoi* is achieved by synergistic stimulation between Mincle and ligands for TLRs coupled to the MyD88 adaptor (left). However, simultaneous recognition of *Fonsecaea monophora* by Mincle and Dectin-1 triggers a Mincle-PKB-Mdm2-dependent degradation of Dectin-1-activated IRF1, dampening the expression of protective IL-12p35 (right). **(D)** DC-SIGN recognition of *Fasciola hepatica* enhances TLR-induced IL-10 and IL-27p28 (left). Moreover, DC-SIGN sensing of the salivary protein Salp15 from the tick vector *Ixodes scapularis* dampens inflammatory responses triggered by *Borrelia burgdorferi* through TLRs (right). Both examples illustrate strategies to escape immune surveillance based on inhibition of T cell proliferation.

to *Staphylococcus aureus* infection (130, 131). This increased protection upon a later infection is linked to increased proinflammatory responses to delayed rechallenge with different TLR ligands, such as LPS or Pam_3Cys_4 (130) (**Figure 3B**), or bacteria, i.e., *Bacteroides fragilis, Escherichia coli, Staphylococcus aureus, Borrelia burgdorferi,* or *M. tuberculosis* (130, 132, 133). In monocytes, Dectin-1 signaling triggers the PI3K-Akt pathway, leading to activation of mTOR and HIF-1α (131). This leads to a shift from oxidative phosphorylation to aerobic glycolysis. Accumulation of fumarate, associated with glutamine replenishment of the TCA cycle, inhibits KDM5 histone demethylases, a key step for induction of monocyte epigenetic reprogramming that underlies the long-lasting effects of trained immunity (130, 134) (**Figure 3B**).

Apart from β-glucan or *Candida*, several other self and non-self ligands, such as chitin (135), BCG vaccine (136), and uric acid (137) induce trained immunity (137, 138). It would thus not be surprising that more CLRs could contribute to trained immunity. In this regard, although *C. albicans* mannans, potentially sensed by MR, Dectin-2, or Mincle (46), have shown not to prime human monocytes directly (130), they are essential for *C. albicans*-induced training (133). Furthermore, both Dectin-1 and MR are needed to trigger glycolysis upon *C. albicans* stimulation (139); this glycolytic switch constitutes a critical metabolic

step in trained immunity induction (131, 139). Trained immunity triggered by Dectin-1 and potentially other CLRs is thus a consequence of metabolic switch and epigenetic programming that affects deferred heterologous signaling.

Mincle-Triggered Regulatory Responses

As described before, Mincle triggers an FcRγ-mediated activating signal in response to different stimuli. In addition, Mincle engagement can deliver regulatory responses affecting signaling pathways triggered by heterologous PRRs, such as TLRs or other CLRs, for example, Dectin-1. This section will explore modulation of heterologous receptors by Mincle.

Mincle is induced following TLR activation (7). Following sensing of *Fonsecaea pedrosoi*, Mincle triggers an incomplete inflammatory response that requires synergistic TLR stimulation to induce a potent proinflammatory response (**Figure 3C**, left), needed to clear the infection in a mouse model of chromoblastomycosis (140). This cooperative activation through Mincle and TLRs is particularly effective in human newborn DCs. Co-stimulation using the Mincle agonist trehalose-6,6-dibehenate and the TLR7/8 agonist R848 led to enhanced caspase-1 and NF-κB activation, Th1 polarizing cytokine production and autologous Th1 polarization (141).

However, Mincle exhibits a dual role in promotion and subsequent resolution of inflammation. Mycobacteria express ligands for TLRs which induce expression of Mincle that can then detect TDM and contribute to inflammation. Mincle *via* the Syk/p38 axis can also lead to eIF5A hypusination that increases translation efficiency of iNOS, which is transcriptionally induced by TLR2 ligation (142). In this way, Mincle favors NO production that inhibits late-stage activation of NLRP3 inflammasome in TDM-induced inflammation, contributing to termination (142). Similarly, TLR2 sensing of *Corynebacterium* induces robust Mincle expression, which cooperatively detects corynebacterial glycolipids favoring production of granulocyte colony stimulating factor and NO (143).

Dectin-1 and Mincle are involved in the recognition of *Fonsecaea monophora*, a pleomorphic fungus also responsible for chromoblastomycosis (144, 145). Signaling triggered by Dectin-1 initiates protective immunity against the fungus by activating IRF1 and IL-12p35 transcription. However, these responses are dampened by the Mincle/Syk axis, in a process involving PI3K/PKB-mediated activation of the E3 ubiquitin ligase Mdm2, leading to degradation of IRF1 and repression of IL-12p35 production (**Figure 3C**, right). In this way, Mincle sensing of *F. monophora* dampens induction of protective Th1 immunity triggered by Dectin-1 (146). Mincle is also targeted by *Leishmania* parasites to evade the priming of Th1 immunity initiated by DCs. As explained above, Mincle recruits SHP-1 to an inhibitory ITAM configuration in the coupled FcRγ chain, and this results in inhibition of DC activation by heterologous receptors sensing *Leishmania* or LPS (91) (**Figure 2C**). Mincle ligation can also reduce TLR4-mediated inflammation, whereas Mincle deletion or knockdown results in exaggerated inflammation in response to LPS. This effect is mediated through the control of TLR4 correceptor CD14 expression (147).

Tailoring Immunity Through DC-SIGN

DC-SIGN engagement does not generally induce the expression of cytokines by itself, but rather modulates responses initiated by TLRs. Thus, glycans from the helminth *Fasciola hepatica* are recognized by DC-SIGN leading to enhanced TLR-induced IL-10 and IL-27p28, triggering a tolerogenic program that differentiates naive $CD4^+$ T cells into regulatory T cells (148) (**Figure 3D**, left). However, the interaction of DC-SIGN with the salivary protein Salp15 from the tick *Ixodes scapularis* dampens inflammatory responses triggered by *Borrelia burgdorferi*. Raf-1 activation downstream of DC-SIGN sensing Salp15 results in MEK-dependent decrease of IL-6 and TNF mRNA stability and impaired nucleosome remodeling at the IL-12p35 promoter, modulating TLR-induced DC activation and T cell proliferation (112) (**Figure 3D**, right).

All these examples clearly illustrate how signaling pathways triggered by CLRs can have an impact on responses mediated by surrounding heterologous receptors, adding an extra layer of complexity to our understanding of CLR-mediated responses.

CONCLUDING REMARKS

Classical sorting of myeloid CLRs based on the structure of the C-type lectin domain does not have functional significance. A more recent classification based on the presence of ITAM, hemITAM, or ITIM intracellular signaling motifs associated with the receptors has been useful as a starting point to predict the functional outcome of signaling CLRs (1). However, many factors may alter the expected canonical response. Minor variations in the context of the canonical motifs result in different signaling and effector outcomes (60, 65). Subcellular location depending on the isoform (69) or conformation of the receptor based on specific residues (64) also affects the function of the receptor. CLR signaling also depends on the size of the particle, where the ligand is recognized, affecting quantitatively the strength of the reaction (71–73) and also leading to qualitatively different responses (74, 149). Cooperative binding and signal transduction may be a consequence of multimerization. There are examples of homodimerization (75, 76) and formation of hetero-complexes (11, 81, 84–86). Hetero-complexes result in a mutual benefit for involved receptors, combining avidity for the ligand, capacity for endocytosis and/or signal transduction capabilities.

The plasticity of the C-type lectin domain allows binding to different ligands that, depending on their relative affinity or avidity, may trigger activating or inhibitory signaling pathways downstream of the same motifs. For example, low-avidity ligands drive a Syk-dependent association with SHP-1 to the ITAM domain (87, 88, 90), with a growing list of examples illustrating CLRs coupled to the FcRγ chain (12, 91–93). Conversely, tyrosine phosphatases may contribute to activation (95) and ITIM-containing CLRs may trigger activating signals (58). These results evidence the fine regulation of signaling though a single receptor based on differential interaction with diverse ligands, leading to the hypothesis that sensing self-ligands through CLRs could drive tolerance while non-self ligands could provoke immunity. However, dangerous-self could rather contribute to immunity and some non-self ligands could inhibit immune response for

evasion, making the final outcome of a single response rather unpredictable. In addition, the concerted sensing of complex ligands by a variety of PRRs leads to complex integrated responses. CLRs may affect signals of heterologous receptors that are simultaneously triggered, either enhancing or modulating the response (59, 91, 115, 124–126, 128, 142, 146). Of note, Dectin-1 induces a metabolic switch and epigenetic programming that affects deferred heterologous signaling (130, 131). In conclusion, understanding how different signaling pathways triggered by CLRs and heterologous receptors act in concert during sensing self and non-self remain a fascinating endeavor.

Research in the field of CLRs has gained much attention considering the diversity of members, ligands, expression pattern on clinically relevant cellular populations and their relevant function on the initiation, and regulation of immunity and inflammation. Some of these features have been illustrated here and offer multiple possibilities to harness CLR-triggered responses. However, CLR manipulation may lead to unexpected outcomes and needs to be tested empirically. In addition, deciphering molecular signatures common to signaling pathways triggered by CLRs in response to different ligands will help to understand their precise role in immunity and inflammation.

AUTHOR CONTRIBUTIONS

CF, SI, PS-L, MM-L, and DS conceived and wrote the manuscript. CF did the figures that were edited by all the authors.

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CLEC10A Is a Specific Marker for Human CD1c$^+$ Dendritic Cells and Enhances Their Toll-Like Receptor 7/8-Induced Cytokine Secretion

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Dendritic cells (DCs) are major players for the induction of immune responses. Apart from plasmacytoid DCs (pDCs), human DCs can be categorized into two types of conventional DCs: CD141$^+$ DCs (cDC1) and CD1c$^+$ DCs (cDC2). Defining uniquely expressed surface markers on human immune cells is not only important for the identification of DC subpopulations but also a prerequisite for harnessing the DC subset-specific potential in immunomodulatory approaches, such as antibody-mediated antigen targeting. Although others identified CLEC9A as a specific endocytic receptor for CD141$^+$ DCs, such a receptor for CD1c$^+$ DCs has not been discovered, yet. By performing transcriptomic and flow cytometric analyses on human DC subpopulations from different lymphohematopoietic tissues, we identified CLEC10A (CD301, macrophage galactose-type C-type lectin) as a specific marker for human CD1c$^+$ DCs. We further demonstrate that CLEC10A rapidly internalizes into human CD1c$^+$ DCs upon binding of a monoclonal antibody directed against CLEC10A. The binding of a CLEC10A-specific bivalent ligand (the MUC-1 peptide glycosylated with N-acetylgalactosamine) is limited to CD1c$^+$ DCs and enhances the cytokine secretion (namely TNFα, IL-8, and IL-10) induced by TLR 7/8 stimulation. Thus, CLEC10A represents not only a candidate to better define CD1c$^+$ DCs—due to its high endocytic potential—CLEC10A also exhibits an interesting candidate receptor for future antigen-targeting approaches.

Keywords: dendritic cell subpopulations, CLEC10A, lineage marker, antigen targeting, cDC2, CD1c$^+$ dendritic cells

INTRODUCTION

Dendritic cells (DCs) are potent antigen-presenting cells capable of inducing and regulating adaptive immune responses (1). They continuously sample pathogenic and harmless antigens from their surroundings. This is followed by the migration into lymphoid organs, accompanied by antigen processing and the presentation of the uptaken antigens as peptide MHC complexes to T cells. Tolerogenic T cell reactions are induced if danger signals are missing. In contrast, additional danger signals (such

as TLR-mediated signals) allow for the full maturation of DCs and thereby the induction of life-long immunity against invading pathogens (1, 2).

Dendritic cells can be distinguished into plasmacytoid DCs (pDCs) and conventional DCs. pDCs are known for their ability to secrete high amounts of type 1 interferon in response to viral infections and a lower T cell stimulatory capacity (3–5). In mice and man, two major conventional DC subpopulations have been described. Murine conventional DCs can be separated into $CD8^+CD11b^-$/$CD103^+CD11b^-$ DCs (also named cDC1) and $CD8^-CD11b^+$ DCs (also named cDC2) (6–8). The first excel in cross-presentation of antigens to cytotoxic $CD8^+$ T cells as well as T_H1 $CD4^+$ T cell responses, whereas the latter induce preferentially T_H2 and T_H17 responses (9–14). According to their expression profiles, human conventional DCs can be distinguished into $CD141^+$ and $CD1c^+$ DCs. $CD141^+$ DCs share the expression of BATF3, IRF8, and XCR1 with murine $CD8^+CD11b^-$/$CD103^+CD11b^-$ DCs, whereas $CD1c^+$ DCs are homologous to murine $CD8^-CD11b^+$ DCs in regard to their expression of SIRPα and IRF4 (15–18).

Due to their unique ability to induce naïve T cell responses, DCs are suitable targets for immunotherapeutic approaches for the therapy of cancer as well as infectious diseases. One promising strategy is the *in vivo* delivery of antigens to DCs using antibodies directed against endocytic surface receptors (19). Hereby, it is possible to induce protective as well as therapeutic immune responses (19–27). In order to harness DCs for antigen-targeting approaches, it is necessary to identify endocytic receptors specifically expressed on DCs. One suitable subclass of such endocytic receptors are C-type lectin receptors (CLRs). In mice, the specific expression of the CLRs DEC205 and DCIR2 allowed for the distinct targeting of the conventional DC subsets, leading to $CD8^+$ or $CD4^+$ T cell responses, respectively (9, 20, 28). In humans, DEC205 and DCIR (a homolog of murine DCIR2) are not only expressed by one specific DC subset, thereby hindering the direct translation into the human system (15, 29–31). Recently, CLEC9A was identified as a uniquely expressed CLR on murine $CD8^+CD11b^-$/$CD103^+CD11b^-$ DCs and human $CD141^+$ DCs (21, 22, 32–35). However, a potential targeting receptor specifically expressed on human $CD1c^+$ DCs is still missing.

Transcriptional data of human primary DC subpopulations suggest that the type 1 CLR CLEC10A [CD301, macrophage galactose-type C-type lectin (MGL), and CLECSF14] might be an interesting candidate expressed on human $CD1c^+$ DCs (15, 17, 36) and human $CD103^+SIRP\alpha^+$ DCs, the equivalent of $CD1c^+$ DCs in the human gut (16). Although transcriptomic analyses of human primary monocytes revealed human CLEC10A mRNA expression in intermediate monocytes ($CD14^{++}CD16^+$), only very low protein expression could be detected in these cells (37). Originally, human CLEC10A was identified as a CLR expressed on immature monocyte-derived DCs (moDCs), but not or to a lower extend on mature moDCs (38). It was further demonstrated that the carbohydrate recognition domain of CLEC10A recognizes galactose/*N*-acetylgalactosamine (Tn antigen) (38). In mice, two homologs of CLEC10A can be distinguished (CD301a/MGL1 and CD301b/MGL2), which show different expression patterns and binding specificities (39, 40). MGL1 was specifically present on macrophages, whereas MGL2 is expressed on DCs (41). Taking together, these data imply CLEC10A as a useful marker to distinguish DC subsets (16).

The unique transcriptional expression pattern of CLEC10A in human lymphohematopoietic tissues prompted us to dissect CLEC10A protein expression in blood, thymus, and spleen. We here show a $CD1c^+$ DC specific, but organ-irrespective expression profile of CLEC10A. Our data further indicate rapid receptor internalization upon binding of an anti-CLEC10A antibody or a natural ligand (with Tn glycosylated MUC-1 peptide) of the receptor. Furthermore, this ligand in combination with the TLR7/8 ligand R848 led to an enhanced secretion of the cytokines IL-8, IL-10, and TNFα by $CD1c^+$ DCs. Due to its unique expression profile and its endocytic activity, we are here proposing that CLEC10A might be not only a very good marker for the distinction of human $CD1c^+$ DCs from monocytic lineages in lymphoid tissues but also a suitable receptor for the *in vivo* delivery of antigens to human $CD1c^+$ DCs.

MATERIALS AND METHODS

Human Tissue Preparation

Leukocyte reduction cones were retrieved from anonymous healthy adult donors. Thymus samples were retrieved from cardiac surgeries of otherwise healthy children. The sources of spleen samples were patients requiring therapeutic splenectomy. All samples were received under local ethical committee approvals (Ethikkommission der Friedrich-Alexander-Universität Erlangen-Nürnberg), and informed written consents were obtained in accordance with the Declaration of Helsinki.

All tissues were freshly processed as described earlier (15). In brief, thymic and splenic tissues were chopped into small pieces using forceps and scalpel. Then, the tissue was transferred into C-tubes (Miltenyi Biotec), filled with 5 ml RPMI1640, further mechanically disrupted using a Gentle MACS tissue dissociator (Miltenyi Biotec), and enzymatically digested with 400 U/ml collagenase D (Serva) and 100 µg (spleen) or 300 µg (thymus) deoxyribonuclease I (Sigma). After filtering the cell suspension twice, cell suspension of splenic and thymic tissue as well as the leukocyte enriched fraction of human blood was diluted with RPMI1640 and a density gradient centrifugation using Human Pancoll ($\rho = 1.077$ g/ml; Pan Biotech) was performed as described earlier. After the centrifugation, the interphase containing the mononuclear cells was collected, washed twice with RPMI1640, and used for experiments.

Microarray Analysis

Published microarray data were analyzed for relative expression of CLEC10A (15). Microarray data are available in the Gene Expression Omnibus database (www.ncbi.nlm.nih.gov/gds) under the accession number GSE77671. Transcriptome data of whole Human Genome Oligo microarray (Agilent) of human $CD1c^+$ DCs, $CD141^+$ DCs, and pDCs from three blood, spleen, and thymus donors as well as blood monocytes, B cells, and $CD4^+$ and $CD8^+$ T cells were used. Raw values generated by automated feature extraction have been RMA background corrected and quantile normalized using R (Windows, x64, 3.3.1) (42). Relative

expression values were plotted taking advantage of the gplots package of R (43).

Flow Cytometry

Flow cytometric analyses of single cell suspensions of blood, spleen, and thymus were performed on a BD LSRFortessa and analyzed using FlowJo software (Treestar). 5×10^6 (blood) to 8×10^6 (thymus, spleen) cells were stained with the following antibody cocktail to discriminate different immune cell populations for 15 min at 4°C: CD1c-APC/Cy7 (L161; BioLegend), CD3-BUV395 (UCHT1; BD Biosciences), CD4-BV510 (OKT4; BioLegend), CD8-APC (RPA-T8; eBioscience) or CD8-BUV737 (SK1; BD Biosciences), CD11b-PE/Cy5 (M1/70; BioLegend), CD11c-PE/Cy7 (3.9; BioLegend), CD14-A700 (HCD14; BioLegend), CD19-BV605 (SJ25C1; BioLegend), CD20-BV605 (2H7; BioLegend), CD56-BV421 (5.1H11; BioLegend), CD123-BV650 (6H6; BioLegend), CD141-BV711 (1A4; BD Bioscience), CD303-PerCP/Cy5.5 (201A; BioLegend), CLEC10A-PE or -APC (H037G3; BioLegend), HLA-DR-PE-CF594 (G46-6; BD Bioscience), and NKp46–BV421 (9E2; BioLegend). As appropriate isotype control for CLEC10A-PE and -APC, the cells were stained with a PE- or APC-labeled mouse IgG2a isotype control (MOPC-173; BioLegend). For some experiments, CLEC10A was stained using 10 μg/ml of an FITC-coupled MUC-1 peptide (βAla-GVTSAPDTRPAPGSTAPPAHGVT-NH_2) that was glycosylated with N-acetylgalactosamine (Tn antigen) at Serine 4 and Threonine 15 [referred to as MUC-1-$(Tn)_2$] or a non-glycosylated FITC- βAla-MUC-1 peptide as control (referred to as MUC-1). Synthesis and characterization of the compounds have been described earlier (44). After staining for 30 min on ice, the cells were washed and stained for different immune cell populations as before. After washing the cells, they were resuspended in PBS + 2% human sera + 0.1 mg/ml 4′,6-diamidino-2-phenylindole and acquired using a BD LSRFortessa.

Internalization Assay

For the internalization assay, 5×10^6 cells were stained with anti-CLEC10A-PE (H037G3; BioLegend) or an appropriate isotype control (mouse IgG2a, MOPC-173; BioLegend) for 15 min on ice. Then, the cells were incubated for different time points at 37°C (5, 15, 30, and 60 min) or kept on ice for 60 min. In a next step, surface CLEC10A was stained with an anti-PE antibody (polyclonal goat IgG; Novus Biologicals) and after washing with an anti-goat-A647 (polyclonal donkey IgG; Southern Biotech). In a last step, the cells were stained for the discrimination of different immune cell populations as described under flow cytometry and samples acquired using a BD LSRFortessa.

Generation of moDCs

PBMCs were isolated by gradient-density centrifugation. 5×10^7 PBMCs in 10 ml DMEM were adhered to a human IgG-coated tissue culture dish (BD Falcon) and incubated for 1 h at 37°C. Then, the medium was replenished with 10 ml moDC medium [RPMI-1640/containing 1% penicillin/streptomycin, 1% L-glutamine, 1% HEPES, and 2% human sera type AB (Lonza)]. On the next day, the medium was replenished with fresh moDC medium containing 800 U/ml GM-CSF and 50 U/ml IL-4. On day 3 and day 5, 4 ml of fresh moDC medium was added containing 800 U/ml GM-CSF/50 U/ml IL-4 and 400 U/ml GM-CSF/25 U/ml IL-4, respectively. On day 6, half of the cells were matured using a maturation cocktail consisting of 13.2 ng/ml IL-1β, 10,000 U/ml IL-6, 10 ng/ml TNFα, and 1 μg/ml prostaglandin E_2 for 24 h. IL-1β, IL-6, and TNFα were purchased from PeproTech and prostaglandin E_2 from Sigma Aldrich.

Enrichment of DCs

In order to perform cell sorts and for internalization experiments using thymic DCs, DCs were enriched prior to the experiments. Therefore, blood DCs were enriched with the EasySep Pan-DC Pre-Enrichment Kit (Stemcell Technologies) as described in the manual of the manufacturer. Briefly, PBMCs were diluted in PBS + 2% human sera + 1 mM EDTA and stained with anti-human CD32 (Fc gamma RII) Blocker (Stemcell Technologies) and the EasySep Pan-DC Pre-Enrichment Cocktail for 45 min at room temperature (RT). Then, magnetic Dextran Rapidspheres were added, and the cells were incubated for 10 min at RT followed by incubation for 5 min inside "The Big Easy" EasySep Magnet (Stemcell Technologies). The unlabeled DCs were poured off into a new tube and stained for cell sorts.

Thymic DCs were enriched using biotinylated antibodies against thymocytes (CD7, clone eBio124-1D1; eBioscience), T cells (CD3, SK7, BioLegend; CD8, RPA-T8, BioLegend), B cells (CD19, HIB19; BD Bioscience), and NK cells (CD56, MEM-188; BioLegend), and MojoSort Streptavidin Nanobeads (BioLegend). The cell suspension was stained with the cocktail of biotinylated antibodies in PBS + 2% human sera + 1 mM EDTA for 30 min on ice. Then, the cells were washed, resuspended in PBS + 2% human sera + 1 mM EDTA, and MojoSort Streptavidin Nanobeads added. After incubation for 15 min on ice, the tube was transferred into "The Big Easy" EasySep Magnet and incubated for 5 min at RT. Next, the unlabeled DCs were poured off into a new tube and used for further experiments.

Cell Sorting of Human DC Subpopulations

Dendritic cell-enriched cell suspensions were stained with an antibody cocktail consisting of the following antibodies in PBS + 2% human sera for 30 min on ice: CD1c-APC/Cy7 (L161; BioLegend), CD3-BUV395 (UCHT1; BD Bioscience), CD11b-A700 (M1/70; BioLegend), CD11c-PE/Cy7 (3.9; BioLegend), CD14-A700 (HCD14; BioLegend), CD19-V450 (HIB19; BD Bioscience), CD20-eFluor 450 (2H7; eBioscience), CD56-BV421 (5.1H11; BioLegend), CD123–BV605 (6H6; BioLegend), CD141-BV711 (1A4; BD Bioscience), CD303-PerCP/Cy5.5 (201A; BioLegend), HLA-DR-PE-CF594 (G46-6; BD Bioscience), and NKp46-BV421 (9E2; BioLegend). After washing the cells, they were resuspended in PBS + 2% human sera + 0.1 mg/ml 4′,6-diamidino-2-phenylindole and cell sorted using a BD FACSAria II cell sorter into $CD1c^+$ DCs ($CD3^-CD11b^-CD14^-CD19^-CD20^-CD56^-CD123^-NKp46^-HLA$–$DR^+CD11c^+CD1c^+$), $CD141^+$ DCs ($CD1c^-CD3^-CD11b^-CD14^-CD19^-CD20^-CD56^-CD123^-NKp46^-HLA\text{-}DR^+CD11c^+CD141^+$), pDCs ($CD1c^-CD3^-CD11b^-CD14^-CD19^-CD20^-CD56^-NKp46^-HLA\text{-}DR^+CD123^+CD303^+$), and monocytes ($CD3^-CD19^-CD20^-CD56^-CD123^-NKp46^-HLA\text{-}DR^+CD11b^+CD14^+$). The purity of sorted cell populations was reanalyzed and routinely above 95%.

Stimulation of Sorted DCs

Cell-sorted DC subpopulations and monocytes were resuspended in DC medium (RPMI1640 + 5% human sera + 5% Pannexin NTA + 5% Pannexin NTS + 1% glutamine + 1% sodium pyruvate + 1% penicillin/streptomycin) to a concentration of 2×10^5 cells/ml. Cells were cultured in sterile 96-well plates (v-bottom) and stimulated either with one of the TLR ligands pIC (5 μg/ml) or R848 (5 μg/ml), the CLEC10A ligand MUC-1-$(Tn)_2$ (10 μg/ml) or a non-glycosylated MUC-1 control peptide (10 μg/ml), or a combination of both. TLR ligands were purchased from Invivogen and reconstituted in water. After 12 h of stimulation, the cells were analyzed by flow cytometry for the expression of CLEC10A (CLEC10A-APC, clone: H037G3, BioLegend) and activation markers (CD40-PE, clone: 5C3, BioLegend; CD86-PE-CF594, 2331(FUN-1), BD Biosciences) together with sorted DCs and monocytes of the very same donor, which were kept on ice until analysis. Supernatants of stimulated cells were harvested and stored at −80°C until analysis with the LEGENDplex Human Anti-Virus Response Panel (BioLegend). Acquisition of the samples was performed using a BD LSRFortessa and data analyzed using FlowJo (CLEC10A, CD40, CD86) or LEGENDplex software (VigeneTech; cytokine secretion).

Intracellular Signaling in Sorted CD1c+ DCs

$CD1c^+$ DCs were sorted from PBMCs of healthy donors, resuspended in DC medium to a final concentration of 2×10^5 cells/ml and stimulated either with 10 μg/ml MUC-1-$(Tn)_2$ or non-glycosylated MUC-1 in combination with 5 μg/ml R848 and incubated at 37°C for different time points (0, 5, 15, 30, and 60 min). Afterward, the cells were washed and fixed with Cytofix/Cytoperm Buffer (BD Bioscience) for 20 min on ice. Then, the cells were washed and permeabilized with Perm Buffer III (BD Bioscience) for 30 min on ice. After washing, the cells were stained with BV421 Mouse Anti-Human NF-κB p65 (pS529), Alexa Fluor® 647 Mouse anti-JNK (pT183/pY185), Alexa Fluor® 488 Mouse Anti-CREB (pS133)/ATF-1 (pS63), PE-CF594 Mouse Anti-p38 MAPK (pT180/pY182), PE-C7 Mouse anti-ERK1/2 (pT202/pY204), and PE Mouse anti-Human IKKγ (pS376) for 30 min at RT. All antibodies were purchased from BD Bioscience. The cells were washed and then acquired using a BD LSRFortessa.

RESULTS

CLEC10A Is Specifically Expressed on Human CD1c+ DCs in Various Lymphoid Tissues

In our recently performed transcriptional analysis of human lymphohematopoietic organ DC subpopulations (blood, thymus, and spleen), we demonstrated a strong expression of the CLEC10A mRNA in $CD1c^+$ DCs in all tested tissues (**Figure 1A**) (15). In line with an earlier report about intermediate monocytes ($CD14^{++}CD16^+$), we could confirm a low CLEC10A mRNA expression in monocytes (**Figure 1A**) (37). To prove that CLEC10A is also expressed on protein level, we performed flow cytometric analyses on single cell suspensions of human blood. We found that the $CLEC10A^+$ lineage (CD3, CD14, CD19, CD20, CD56, and NKp46) negative cells consisted almost exclusively of $CD1c^+$ DCs (**Figures 1B,D**). In contrast, the $CLEC10A^-$ lineage negative cells consisted mainly of $CD16^+$ DCs, $CD141^+$ DCs, and pDCs (**Figures 1B,C**). In addition, a low amount of $CD1c^+$ DCs were represented in the $CLEC10A^-$ DC compartment (**Figure 1C**). We further extended our analyses to thymus and splenic tissue and analyzed the expression of CLEC10A on other cells of the immune system (**Figure 2**). Remarkably, also in these lymphoid tissues CLEC10A demonstrated a selective expression on human $CD1c^+$ DCs comparable to the blood (**Figures 2A,B,D,F**). Only to a low extent we found CLEC10A protein expression on thymic B cells and blood as well as splenic monocytes/macrophages (**Figure 2**). Notably, no expression could be detected on $CD141^+$ DCs, pDCs, T cells, or NK cells (**Figure 2**). Taken together, we found that CLEC10A was expressed on about 80% of $CD1c^+$ DCs in all tested tissues, while it was only expressed on about 5% of blood monocytes, 20% of thymic B cells, and 20% of splenic monocytes/macrophages (**Figures 2C,E,G**). In summary, our data suggest a prevalent expression profile of CLEC10A protein on human $CD1c^+$ DCs.

CLEC10A Is Rapidly Internalized Into Human CD1c+ DCs

As a potential receptor for antigen-targeting approaches, it is necessary that antibodies bound to its specific receptor can internalize into the cell. To investigate the endocytic activity of the CLEC10A receptor, we performed an internalization assay. We therefore stained single cell suspensions of blood and thymic tissue with a PE-coupled anti-CLEC10A antibody. After binding of the antibody, the cells were washed and incubated at 37°C for different time points to allow the internalization of the antibody bound to CLEC10A. To distinguish between antibodies that were internalized from remaining antibodies at the surface of the cells, we stained the cells with a secondary antibody recognizing the PE fluorochrome coupled to the anti-CLEC10A antibody. For controlling the internalization efficacy, the secondary surface-bound antibody was visualized by a tertiary A647-labeled antibody directed against the Fc part of the secondary antibody. In cells that were kept on ice, the PE-labeled primary anti-CLEC10A antibody remained on the surface and thereby the cells displayed a strong staining for the A647-labeled tertiary antibody (**Figure 3**). With increasing incubation time, the A647 signal was reduced (**Figures 3A–C**), whereas the PE signal of the primary PE-labeled anti-CLEC10A antibody was stable over the whole incubation time (**Figure 3D**). Our data indicate that already after 5 min at 37°C large amounts of the PE-labeled anti-CLEC10A antibody were internalized into $CD1c^+$ DCs. We did not observe other cells, which expressed or internalized the antibody except for a low internalization into thymic B cells (**Figure 3**). Interestingly, whereas blood $CD1c^+$ DCs completely internalized the anti-CLEC10A antibody within 30 min (**Figure 4B**), about 50% of the signal remained on the surface of thymic $CD1c^+$ DCs (**Figure 3C**). Thus, our data suggest a strong endocytic activity of CLEC10A upon antibody binding.

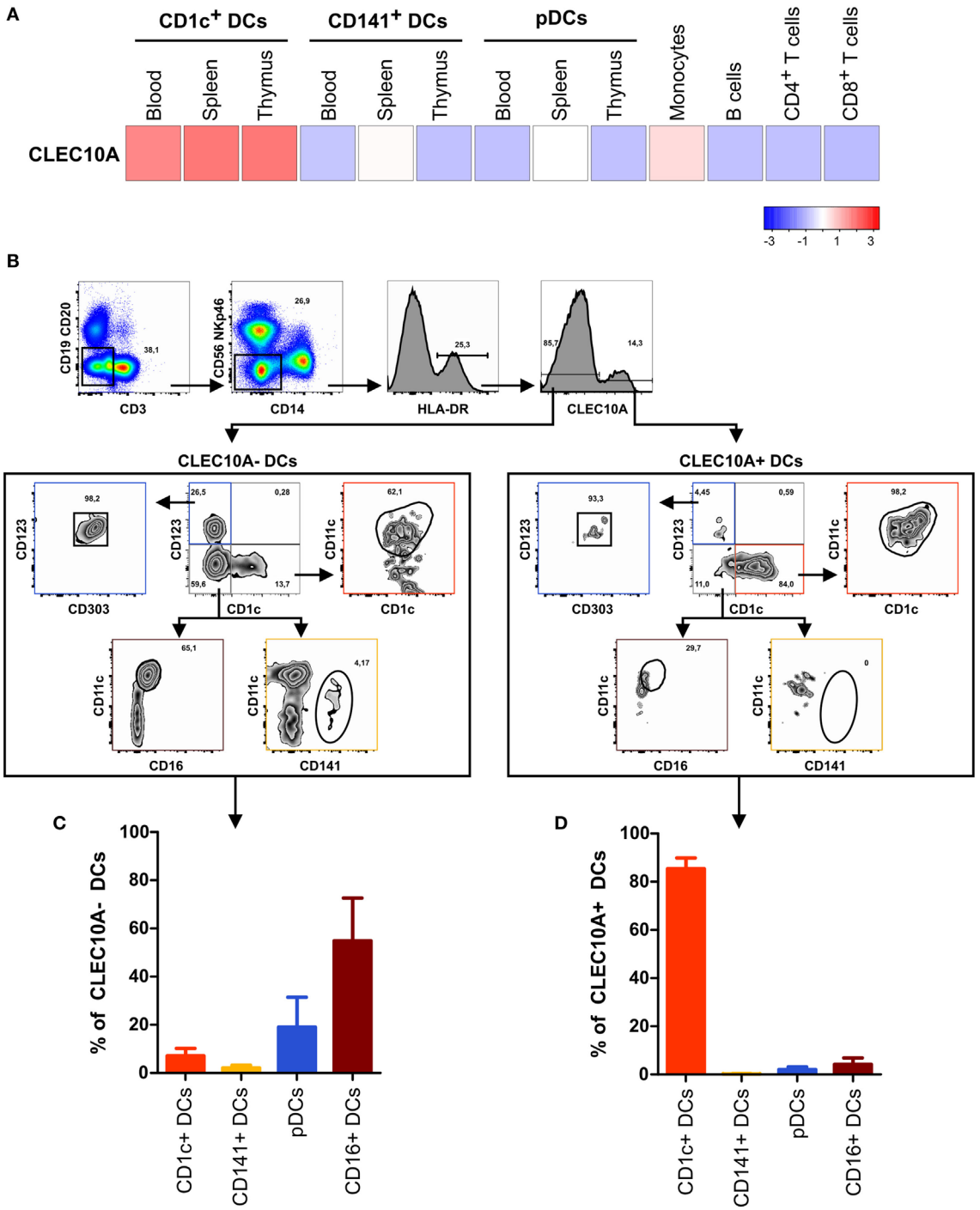

FIGURE 1 | The CLEC10A+ dendritic cell (DC) compartment is predominantly composed of CD1c+ DCs in human blood. **(A)** The expression of CLEC10A was analyzed by microarray analysis. Previously published microarray data (15) were used (GEO accession number GSE77671). For these microarray data, DC subpopulations from each three blood, spleen, or thymus donors as well as blood monocytes, B cells, CD4+ and CD8+ T cells were sorted by flow cytometry, and whole human genome microarray (Agilent) analyses were performed (15). The heat map shows the relative expression of CLEC10A transcripts. **(B–D)** PBMCs of a healthy donor were stained with a PE-labeled anti-CLEC10A antibody as well as antibodies against different immune cell populations and analyzed by flow cytometry. **(B)** After exclusion of doublets and dead cells (not shown), lineage+ (CD3, CD14, CD19, CD20, CD56, and NKp46) cells were excluded. In the remaining cells, HLA-DR+ cells were selected and CLEC10A+ and CLEC10A− cells gated. The CLEC10A+ and CLEC10A− cells were then analyzed for the proportion of CD1c+ DCs (CD1c+CD11c+CD123−), CD141+ DCs (CD141+CD11c+CD1c−CD123−), plasmacytoid DCs (pDCs) (CD123+CD303+CD1c−), and CD16+ DCs (CD11c+CD16+CD1c−CD123−). One representative donor is shown. **(C,D)** The percentages of CD1c+ DCs, CD141+ DCs, pDCs, and CD16+ DCs of **(C)** CLEC10A− and **(D)** CLEC10A+ cells were calculated and plotted as bar graphs (mean ± SD; $n = 3$).

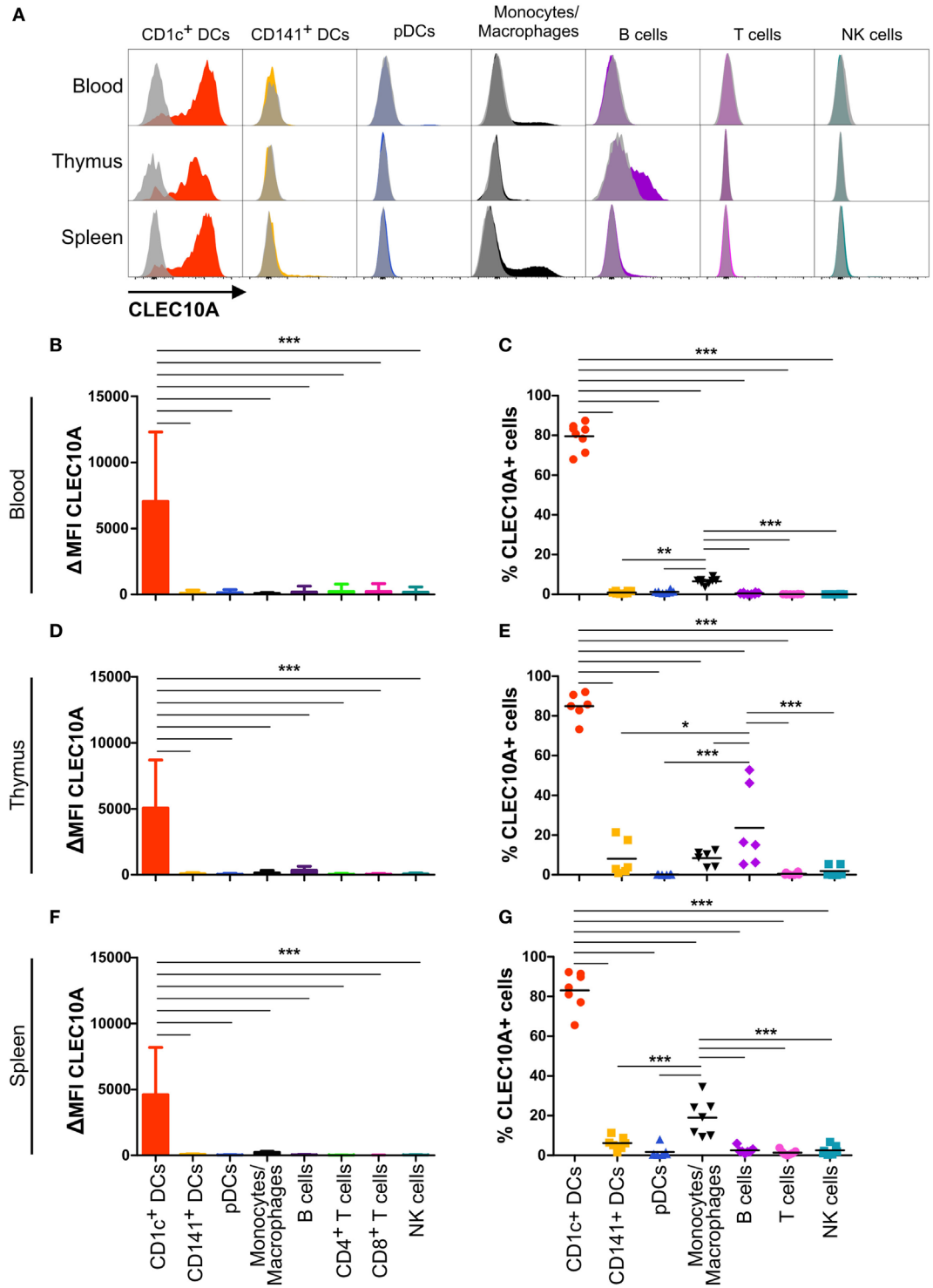

FIGURE 2 | CLEC10A is mainly expressed on human CD1c+ dendritic cells (DCs) in different tissues in the steady state. The expression of CLEC10A was analyzed by flow cytometric analysis on human DC subpopulations in blood, thymus, and spleen. **(A)** Depicted are overlay histograms of single cell suspensions of one representative donor each of blood, thymus, and spleen analyzed by flow cytometry for an anti-CLEC10A antibody (colored) and appropriate isotype control (gray, transparent). Bar graphs in **(B,D,F)** show the ΔMFI for CLEC10A (median ± SD, blood $n = 8$; thymus $n = 6$; spleen $n = 7$). **(C,E,G)** In each leukocyte population, the CLEC10A+ cells were determined using an appropriate isotype control and plotted as a scatter plot with mean as horizontal line. Significant differences in the **(B,D,F)** expression of CLEC10A or **(C,E,G)** percentages of CLEC10A+ cells were calculated by one-way ANOVA with Bonferroni posttest using GraphPad Prism (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$).

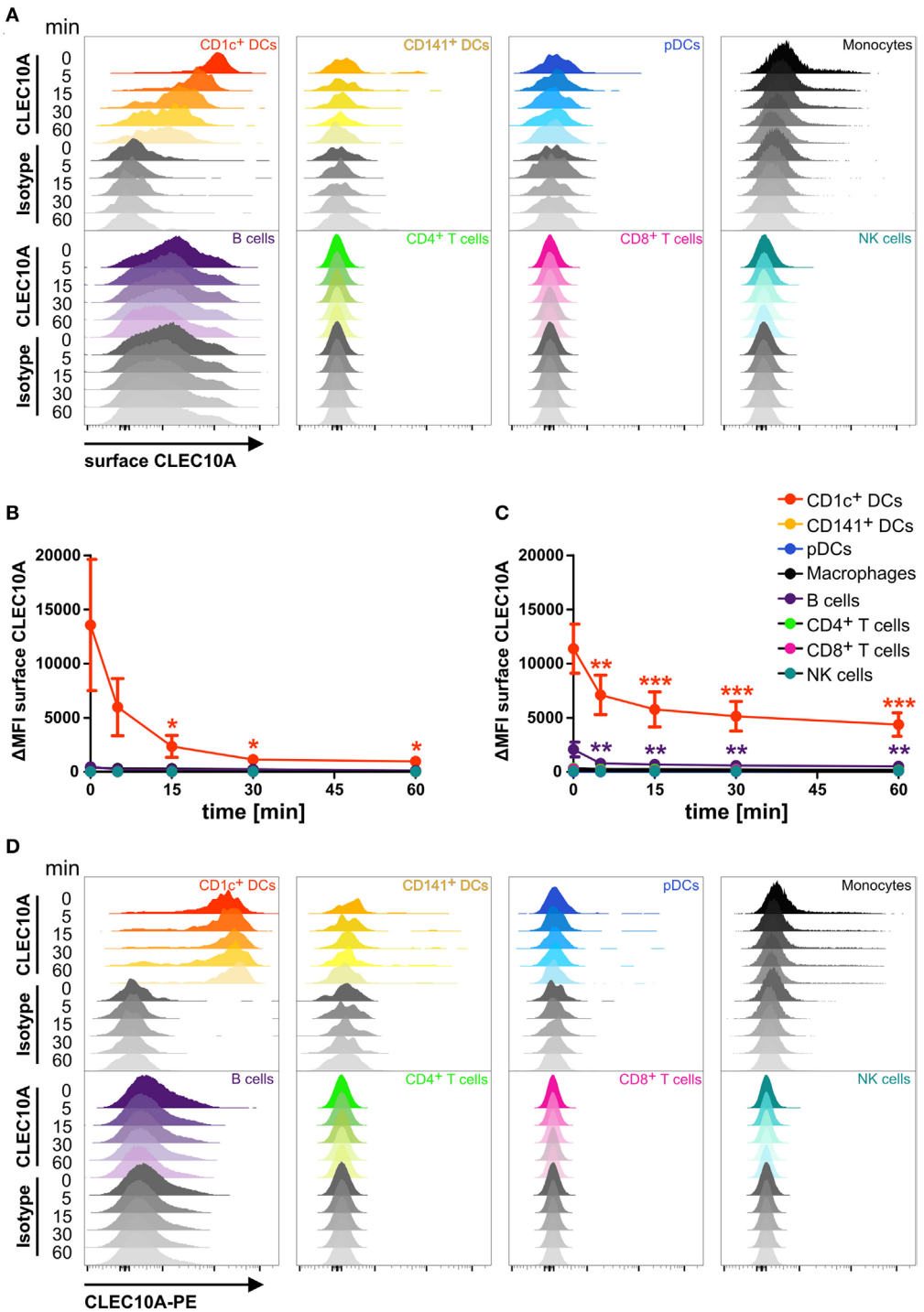

FIGURE 3 | CLEC10A is rapidly internalized into blood and thymic CD1c+ dendritic cells (DCs). Single cell suspensions of blood **(A,B,D)** and thymus **(C)** were incubated with a PE-coupled anti-CLEC10A antibody or an appropriate isotype control at 4°C for 15 min, washed, and resuspended in PBS containing 2% human sera. Subsequently, the cells were incubated at 37°C for different time points (0, 5, 15, 30, and 60 min) to allow for the internalization of the primary PE-coupled antibody. Afterward, the cells were stained for surface CLEC10A by an anti-PE secondary antibody (polyclonal goat IgG) and an A647-labeled tertiary anti-goat antibody. Then, the cells were stained for different immune cell populations. 2.5×10^6 cells were acquired using a BD LSRFortessa and analyzed using FlowJo. **(A)** Overlay histograms show surface expression of CLEC10A for CD1c+ DCs (red), CD141+ DCs (yellow-orange), plasmacytoid DCs (pDCs) (blue), monocytes (black), B cells (purple), CD4+ T cells (green), CD8+ T cells (pink), and NK cells (cyan). **(B,C)** PBMCs **(B)** and thymic **(C)** cells were analyzed as in **(A)**. Plot shows mean ± SD of surface CLEC10A ($MFI_{CLEC10A} - MFI_{isotype\ control}$) of three **(C)** or five **(B)** donors. Statistical analysis was performed using one-way ANOVA with Dunnett posttest for each cell population (ΔMFI 0 min as control; $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$). **(D)** Overlay histograms show the PE-signal of the primary PE-coupled anti-CLEC10A antibody or the appropriate isotype control for the same donor as in **(A)**.

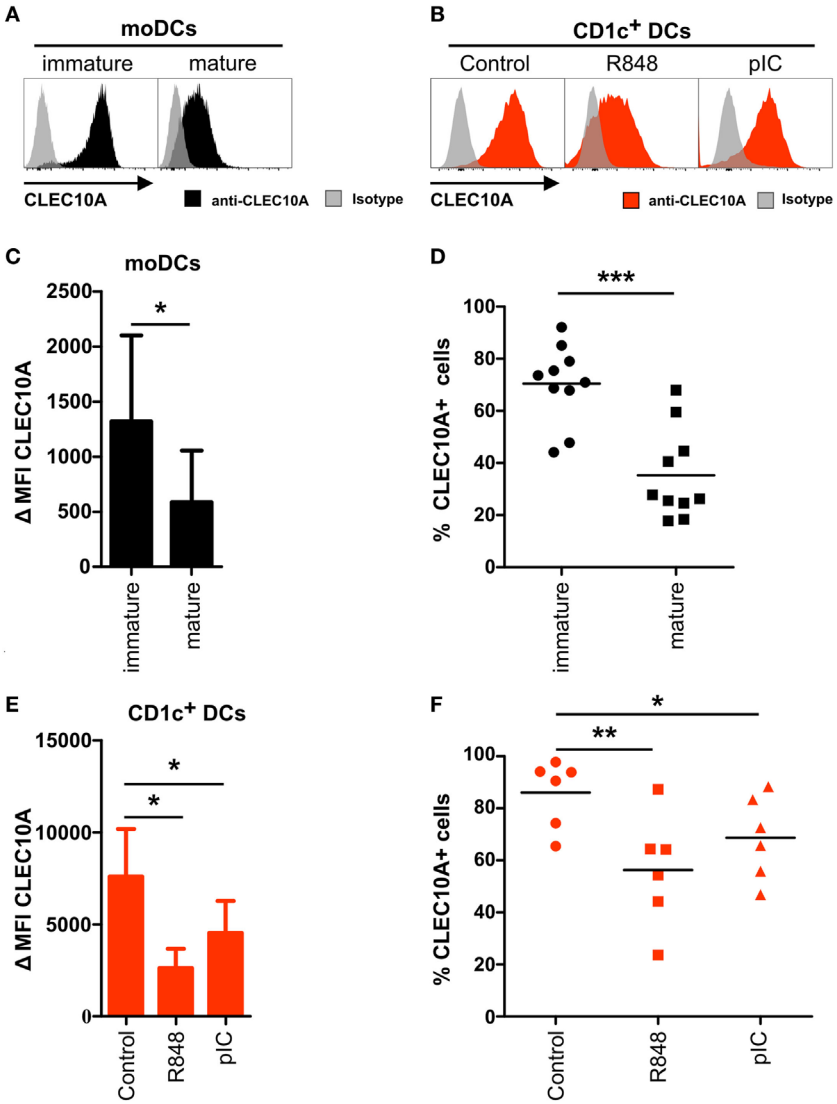

FIGURE 4 | CLEC10A is downregulated on activated human CD1c+ dendritic cells (DCs). **(A,C,E)** Monocyte-derived DCs were generated from blood monocytes of a healthy donor. After 6 days, half of the cells were matured using a maturation cocktail consisting of IL-1β, IL-6, TNFα, and PGE_2 for 24 h. **(B,D,F)** Blood CD1c+ DCs (red) were sorted from PBMCs of a healthy donor and subsequently stimulated either with R848 (5 μg/ml), pIC (5 μg/ml), or cultured in DC medium for 12 h at 37°C. Afterward, the cells were stained with an APC-labeled anti-CLEC10A antibody or an appropriate isotype control and analyzed using a BD LSRFortessa and FlowJo software. **(A,B)** Overlay histograms demonstrate a representative donor for **(A)** moDCs (black: CLEC10A; gray-transparent: isotype control) and **(B)** CD1c+ DCs (red: CLEC10A; gray-transparent: isotype control). **(C,E)** Bar graphs show the expression of CLEC10A [mean ± SD of ΔMFI ($MFI_{CLEC10A}$ − $MFI_{Isotype\ control}$)] on **(C)** moDCs ($n = 10$) and **(E)** CD1c+ DCs ($n = 6$). **(D,F)** CLEC10A+ **(D)** moDCs (black symbols) and **(F)** CD1c+ DCs (red symbols) were determined using an appropriate isotype control and blotted as scatter plots. Significant differences in the **(C,E)** expression of CLEC10A or **(D,F)** percentages of CLEC10A+ cells were calculated using *t* test (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$).

CLEC10A Is Downregulated on Activated CD1c+ DCs

Human CLEC10A was identified as a CLR expressed on immature moDCs. To place our data into the context of previous findings, we performed flow cytometric analyses on immature and mature moDCs. Accordingly, we could detect a low to intermediate surface expression of CLEC10A on immature moDCs (**Figures 4A,C**). In line with earlier published data, displaying an absent expression of CLEC10A-mRNA in mature moDCs (38), we found that after stimulation of moDCs with a maturation cocktail consisting of IL-1β, IL-6, TNFα, and prostaglandin E2, the expression of CLEC10A was significantly reduced, but not absent, on mature moDCs (**Figures 4A,C,D**).

To investigate potential expression changes of CLEC10A on activated human CD1c+ DCs, we stimulated sorted human blood CD1c+ DCs with TLR ligands R848 (TLR7/8) or pIC (TLR3) and analyzed the cells by flow cytometry. In line with the results obtained with moDCs and independent on the used stimulus,

12 h upon TLR ligand stimulation we found a reduced surface expression of CLEC10A on blood CD1c+ DCs (**Figures 4B,E**), in which TLR7/8 stimulation was superior to TLR3 stimulation, as the first demonstrated a stronger reduction of the CLEC10A surface expression. Comparable to data generated from moDCs, TLR stimulated CD1c+ DCs remained partially positive for CLEC10A (**Figure 4F**). Our data suggest a typical behavior of CLEC10A compared to other type I CLRs such as DCIR, DC-Sign, or CLEC9A upon cell stimulation.

CD1c+ DCs Specifically Bind Glycosylated Ligands for CLEC10A

In order to better define the CLEC10A-specific expression profile, we performed FACS staining experiments applying a previously described bivalent ligand of CLEC10A (44). The use of such natural ligands is not only of importance for the identification of interaction partners and cell activation, natural ligands can also be used for the delivery of antigens to endocytic receptors as it was demonstrated for the chemokine ligand receptor pair XCL1and XCR1 (45, 46). Thus, PBMCs of healthy donors were incubated either with 10 µg/ml of an FITC-coupled MUC-1 peptide (sequence: βAla-GVTSAPDTRPAPGSTAPPAHGVT-NH_2) that was glycosylated with N-acetylgalactosamine (Tn antigen) at Serine 4 and Threonine 15 (referred to as CLEC10A ligand MUC-1-$(Tn)_2$) or with 10 µg/ml of a non-glycosylated FITC-coupled MUC-1 peptide (referred to as MUC-1) (44). After ligand staining, the cells were incubated with antibodies for the detection of different immune cell populations and analyzed by flow cytometry. Analogously to the expression profile we have seen upon application of an anti-CLEC10A antibody, we detected a specific binding of the glycosylated form of MUC-1 [MUC-1-$(Tn)_2$] to CD1c+ DCs (**Figure 5**). No other immune cell populations, such as T cells, B cells, NK cells, monocytes, nor CD141+ DCs, and pDCs demonstrated an interaction with the glycosylated MUC-1-$(Tn)_2$ (**Figures 5A,B**).

As we found that upon TLR ligand activation of CD1c+ DCs the expression of CLEC10A was downregulated, we next investigated CLEC10A ligand binding on activated CD1c+ DCs. Therefore, we sorted CD1c+ DCs together with CD141+ DCs, pDCs, and monocytes from PBMCs of healthy donors and stimulated them simultaneously with the glycosylated or the non-glycosylated MUC-1 peptide [MUC-1-$(Tn)_2$ or MUC-1] in combination with one of the TLR ligands R848 or pIC. We found that CD1c+ DCs

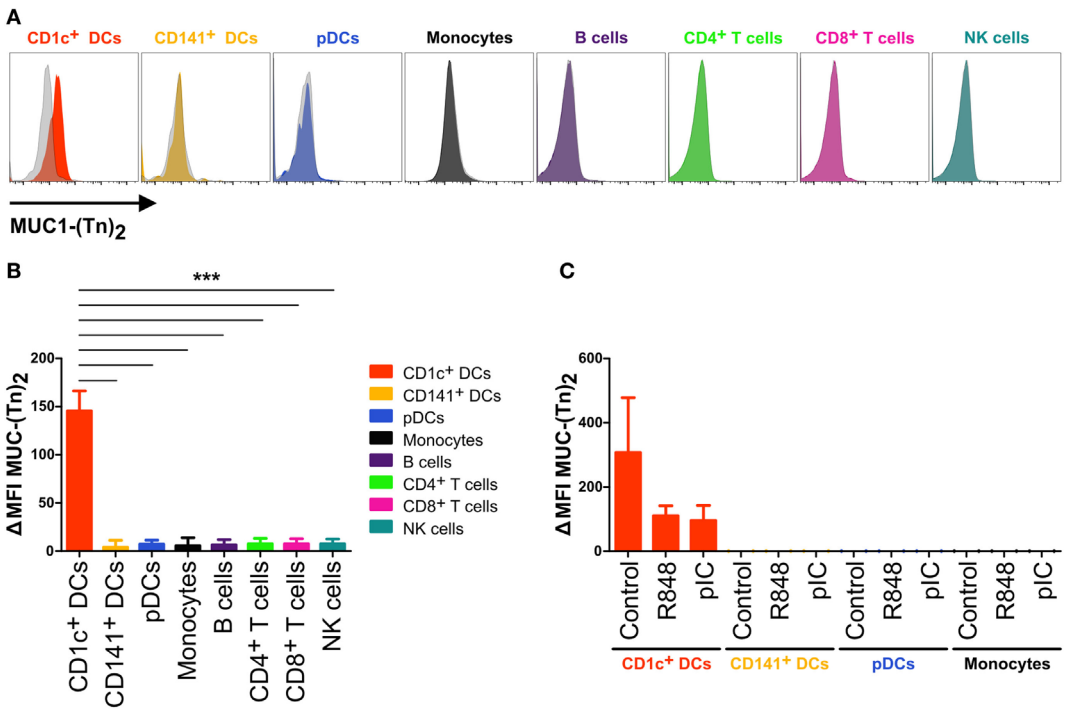

FIGURE 5 | CD1c+ dendritic cells (DCs) bind specifically to glycosylated ligands for CLEC10A. **(A,B)** PBMCs of healthy human donors were incubated with 10 µg/ml of a glycosylated or non-glycosylated MUC-1 peptide. After washing with FACS buffer twice, the cells were stained for different immune cell populations. 1.5×10^6 cells were acquired using a BD LSRFortessa and analyzed using FlowJo software. **(A)** Overlay histograms demonstrate the binding of glycosylated MUC-1-$(Tn)_2$ (colored) and non-glycosylated MUC-1 (gray-transparent) to CD1c+ DCs (red), CD141+ DCs (yellow-orange), plasmacytoid DCs (pDCs) (blue), monocytes (black), B cells (purple), CD4+ T cells (light-green), CD8+ T cells (pink), and NK cells (cyan). **(B)** Bar graphs show the ΔMFI (ΔMFI = MFI MUC-1-$(Tn)_2$ – MFI MUC-1) for the binding of Tn antigen to CLEC10A for the same cell populations as in **(A)**. Significant differences in the binding of MUC-1-$(Tn)_2$ to cell populations were calculated using one-way ANOVA with Bonferroni posttest (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$). **(C)** CD1c+ DCs (red), CD141+ DCs (yellow-orange), pDCs (blue), and monocytes (black) of PBMCs of healthy donors were sorted using a FACS Aria II and subsequently incubated with 10 µg/ml FITC-coupled MUC-1-$(Tn)_2$ or MUC-1 and stimulated with either 5 µg/ml R848, 5 µg/ml pIC, or left untreated. After 12 h at 37°C, the cells were analyzed for the binding of MUC-1-$(Tn)_2$ by flow cytometry. Bar graphs show the ΔMFI of MUC-1-$(Tn)_2$.

incubated without a TLR ligand bound high amounts of MUC-1-$(Tn)_2$, whereas CD1c$^+$ DCs stimulated with either R848 or pIC showed a lower binding of MUC-1-$(Tn)_2$ (**Figure 5C**). This is in accordance with the induced downregulation of CLEC10A upon stimulation with the TLR ligands R848 and pIC (**Figure 4**).

Stimulation of CD1c$^+$ DCs With CLEC10A Ligands Enhances TLR-Induced IL-8, IL-10, and TNFα Secretion

As some CLRs have been described to induce signaling upon binding of their ligands, we were interested to understand whether stimulation of CD1c$^+$ DCs with the CLEC10A ligand MUC-1-$(Tn)_2$ would induce signaling and thereby activation and cytokine secretion by the DCs. Therefore, CD1c$^+$ DCs were sorted from PBMCs of healthy donors and incubated with glycosylated MUC-1-$(Tn)_2$ or the non-glycosylated MUC-1 in the presence or absence of the TLR ligands R848 or pIC. In the absence of further TLR ligand stimulation, we found no activation of CD1c$^+$ DCs by the glycosylated MUC-1-$(Tn)_2$ or the non-glycosylated MUC-1 (**Figure 6A**). As shown earlier (47, 48), stimulation with R848 or pIC induced high expression of the co-stimulatory molecules CD40 and CD86, which was not enhanced by binding of MUC-1-$(Tn)_2$ to CLEC10A (**Figure 6A**). We further investigated, if this glycosylated ligand might influence the cytokine secretion of CD1c$^+$ DCs, as it was shown that signaling *via* CLEC10A can enhance TLR-induced secretion of cytokines by moDCs (49). We therefore analyzed the supernatants of CD1c$^+$ DCs for IL-6, IL-8, IL-10, IL-12p70, IL-23, and TNFα. Neither the incubation with the glycosylated MUC-1-$(Tn)_2$ nor the non-glycosylated MUC-1 alone induced strong cytokine secretion by CD1c$^+$ DCs (**Figure 6B**). However, we found a slightly, but statistically significant increased secretion of the cytokines IL-8, IL-10, and TNFα after stimulation with glycosylated MUC-1-$(Tn)_2$ in combination with R848, but not pIC (**Figure 6B**). In contrast, glycosylated MUC-1-$(Tn)_2$ did not enhance IL-6, IL-12p70, or IL-23 secretion induced by TLR7/8 or TLR3 stimulation (**Figure 6B**).

Since the combination of MUC-1-$(Tn)_2$ and R848 enhanced the secretion of cytokines by CD1c$^+$ DCs, we wondered, if key signaling pathways were influenced in CD1c$^+$ DCs. We therefore incubated the cells with the glycosylated MUC-1-$(Tn)_2$ or the non-glycosylated MUC-1 in the presence or absence of the TLR ligand R848 as described earlier. We performed intracellular FACS staining for the detection of phosphorylation in signaling cascade proteins and of transcription factors, such as NFκB p65, p38 MAPK, IKKγ, JNK, ERK-1/2, or CREB/ATF-1. Although all of the tested proteins are important components in intracellular signal transduction, we could not detect any differences in their phosphorylation states after costimulation of CD1c$^+$ with MUC-1-$(Tn)_2$ and R848 (**Figure 7**).

DISCUSSION

Here, we investigated the expression of CLEC10A in various immune cell populations and found a selective presence of this CLR on CD1c$^+$ DCs. Our data indicate that CLEC10A is rapidly internalized upon antibody binding. Although MUC-1-$(Tn)_2$, a natural ligand for CLEC10A, specifically binds to this DC subset, signaling events were not directly induced. However, we detected a slightly enhanced secretion of selective cytokines induced by the TLR7/8 ligand R848 upon coadministration of MUC-1-$(Tn)_2$.

CLEC10A belongs to the family of CLRs. CLRs are responsible for the recognition of sugar structures on bacteria and cancer cells (40, 50–52). For CLEC10A, the specific ligand N-acetylgalactosamine was recently described (41, 44). Although CLEC10A was originally identified on human moDCs (38), we previously found a high mRNA expression in human CD1c$^+$ DCs isolated from blood, spleen, and thymus (**Figure 1**) (15), which is in accordance to other published transcriptomic data (16, 17, 36). This led us to the investigation of CLEC10A protein levels on immune cell populations. Thereby, we found a selective CLEC10A protein expression on human CD1c$^+$ DCs. While protein expression of CLEC10A on a human subpopulation of blood CD1c$^+$ DCs was already reported by Lundberg et al. (53, 54), we here show that CLEC10A is expressed on the majority of blood as well as lymphoid tissue CD1c$^+$ DCs. In contrast to Lundberg et al., we demonstrate that CLEC10A is specifically expressed on CD1c$^+$ DCs, but not on CD141$^+$ DCs. Our data are further supported by the transcriptional profiling of human lymphohematopoietic DC subpopulations [**Figure 1**; (15)] as well as our ligand binding assays using the CLEC10A ligand MUC-1-$(Tn)_2$ on various immune cell populations (**Figure 5**). Although we and others found a CLEC10A mRNA expression in monocytes in lymphohematopoietic tissues, we could detect no or a very moderate protein expression (**Figure 2**) (37). The same was true for CD141$^+$ DCs and pDCs (**Figures 1** and **2**). We here suggest that the lectin receptor CLEC10A might be a useful marker for a more delicate gating of CD1c$^+$ DCs, resulting in a better separation from monocyte-related cells.

In dependency on the antibody clone used, recent studies showed different results on the expression of CLEC10A on immune cells. Wong et al. compared the transcriptome of classical, non-classical, and intermediate monocytes and found a specific expression of the mRNA in intermediate monocytes compared to the other two populations of monocytes (37). However, the protein expression of CLEC10A on intermediate monocytes was only marginal and neither the mRNA nor the protein expression was compared to other cell populations of the immune system (37). In our experiments, we identified a small population of CLEC10A$^+$ monocytes in the blood (**Figure 2**), which might represent the described intermediate monocytes of Wong et al (37). In another study, Li et al. generated an antibody against CLEC10A (here named DC-ASGPR) (55). In contrast to our obtained data, the anti-DC-ASGPR antibody (clone 49C11) bound to all myeloid DCs (HLA-DR$^+$ CD11c$^+$ CD123$^-$), CD14$^+$ monocytes, and CD19$^+$ B cells (55), while we could not observe binding to blood B cells and the majority of monocytes (**Figure 2**). Moreover, our data reveal that only CD1c$^+$ DCs were positive for CLEC10A, while other CD11c$^+$ DCs such as CD16$^+$ DCs and CD141$^+$ DCs were represented among the CLEC10A$^-$ DCs (**Figure 1**). As Valladeau et al. reported that short and long isoforms of CLEC10A (DC-ASGPR) exist (56), we here speculate that the antibody used in our study (clone: H037G3) and the antibody used by Li et al. (49C11) might recognize different isoforms

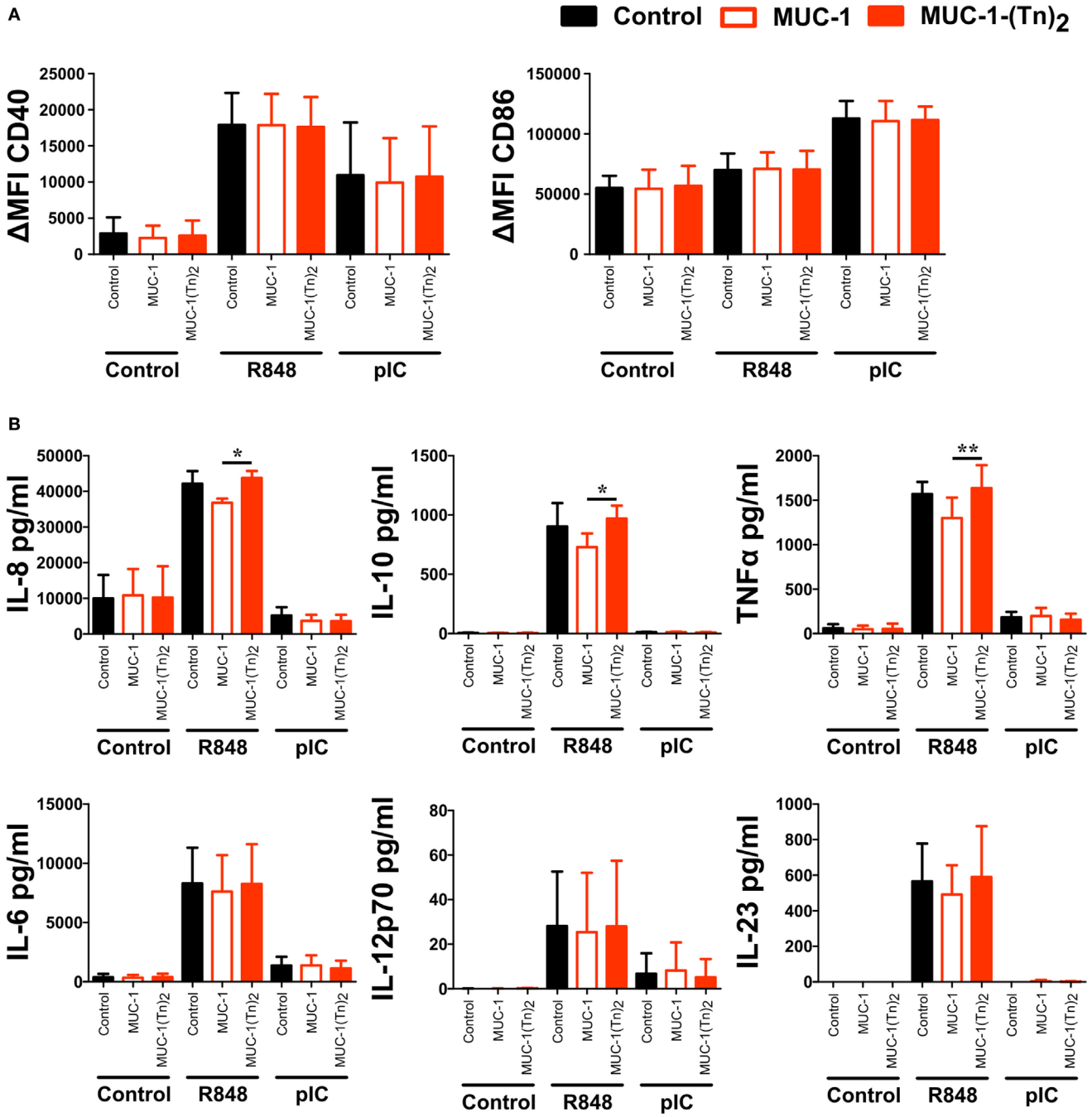

FIGURE 6 | Stimulation of CD1c+ dendritic cells (DCs) with MUC-1-$(Tn)_2$ enhances TLR-induced secretion of IL-8, IL-10, and TNFα. CD1c+ DCs were sorted from PBMCs of healthy donors and stimulated with 10 μg/ml MUC-1-$(Tn)_2$ or MUC-1 in the presence of 5 μg/ml R848 or 5 μg/ml pIC or in medium alone. After 12 h of stimulation, **(A)** cells were analyzed for the expression of the co-stimulatory molecules CD40 and CD86 and **(B)** the supernatant analyzed for the secretion of the cytokines IL-8, IL-10, TNFα, IL-6, and IL-12p70 with the LEGENDplex Human Anti-Virus Response Panel (BioLegend). Bar graphs show **(A)** ΔMFI ± SD ($n = 4$) and **(B)** mean ± SD ($n = 4$). p values were calculated using GraphPad Prism (paired t-test; $^*p < 0.05$ and $^{**}p < 0.01$).

of CLEC10A, which could explain the observed expression differences. The system of different CLEC10A isoforms expressed on either DCs or monocytes/macrophages would also reflect a high similarity to the murine system with CD301a and CD301b expressed on macrophages and DCs, respectively (41, 57). In summary, as the natural ligand of CLEC10A (44) demonstrated a similar binding behavior as the CLEC10A antibody (**Figure 5**) and as our and other's transcriptomic analyses (**Figure 1**) revealed a strong specificity of CLEC10A for CD1c+ DCs, the receptor CLEC10A is approved to be a good marker for CD1c+ DCs.

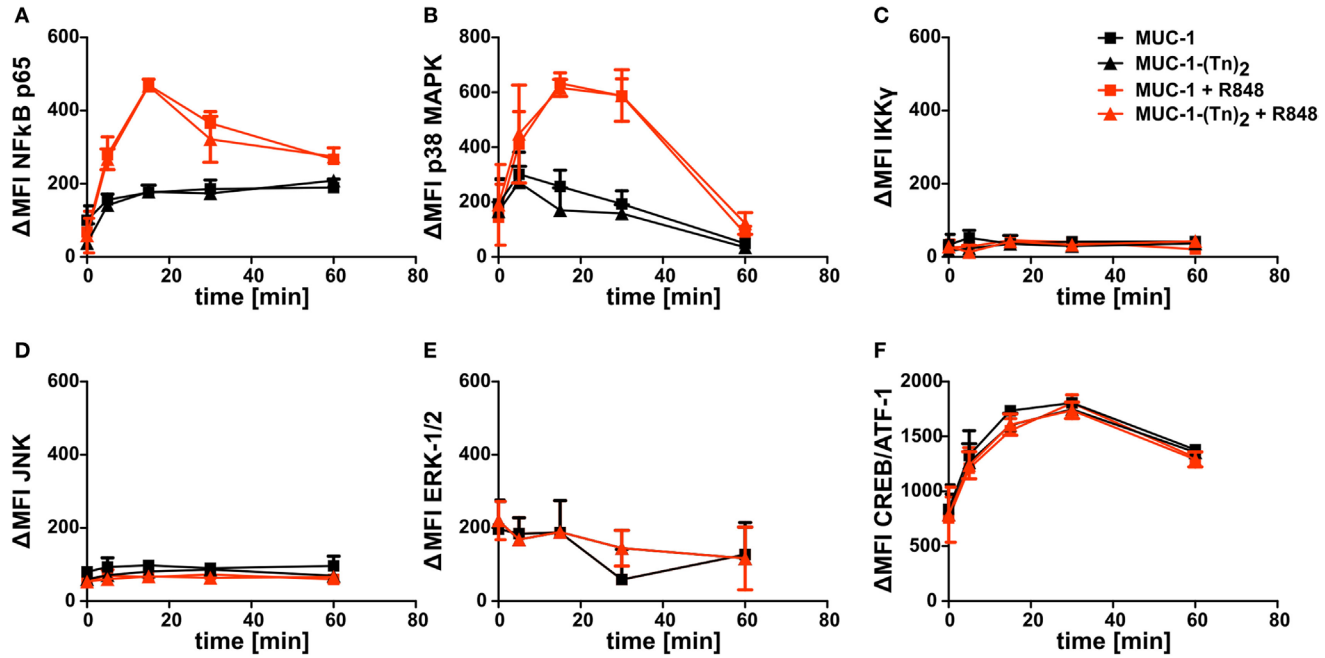

FIGURE 7 | Intracellular signaling in CD1c+ dendritic cells (DCs) induced after stimulation with a ligand for CLEC10A. CD1c+ DCs were sorted from PBMCs of healthy donors. Subsequently, cells were stimulated with MUC-1-$(Tn)_2$ (triangles) or non-glycosylated MUC-1 (squares) as control together with (red curves) or without 5 μg/ml R848 (black curves) for different time points (0, 5, 15, 30, and 60 min) at 37°C. Afterward, the cells were fixed and permeabilized using BD Perm Buffer III and intracellularly stained for phosphorylated **(A)** NFκB p65, **(B)** p38 MAPK, **(C)** IKKγ, **(D)**, JNK, **(E)** ERK-1/2, and **(F)** CREB/ATF-1 with BD Phosflow antibodies. Diagrams show ΔMFI ± SD for two donors. $\Delta MFI = MFI_{antibody} - MFI_{isotype}$.

Interestingly, data of Breton et al. indicate that, in addition to fully differentiated CD1c+ DCs, CD1c+ DC-committed pre-cDCs also selectively expressed the mRNA of CLEC10A (17). In contrast, non-committed as well as CD141+ DC-committed pre-cDCs lacked the mRNA expression for CLEC10A (17). Although the protein expression has to be confirmed, these data suggest the usage of CLEC10A as a selective lineage marker in the development of CD1c+ DCs (**Figure 1**).

As most *in vivo* studies on immunological questions are performed in the murine system, it is also of major importance to identify the most similar antigen-presenting cells in mice in man. For the CD8+/CD103+ subset in mice and the CD141+ DCs as their potential human counterpart, XCR1 and CLEC9A have been suggested as universal subset-defining markers for DCs, which potentially excel in cross-presentation of engulfed antigens (33, 34, 58–61). However, there is no such marker described for murine CD11b+ DCs and human CD1c+ DCs, which is not also expressed by monocytic lineages (such as CD172a, also known as SIRPα). In mice, two homologs of CLEC10A exist, named MGL1 (CD301a) and MGL2 (CD301b), respectively. These two CLRs exhibit different expression patterns as MGL1 was found on macrophages in the skin, whereas MGL2 was expressed on dermal CD11b+ DCs (57). Human CLEC10A is more closely related to murine MGL2 as both can recognize similar ligands (40, 44). Although there are controversial reports on the protein expression of MGL2 on murine splenic CD11b+ DCs (16, 41, 51), we here propose that CLEC10A might not only be a suitable lineage but also a cross-species marker.

In addition to the selective expression of CLEC10A on CD1c+ DCs, we further demonstrated that CLEC10A internalizes rapidly into CD1c+ DCs upon binding of a specific antibody (**Figure 3**). A similar behavior has been reported for DEC205 (9, 62, 63). The rapid uptake is a prerequisite for the usage of CLEC10A as a potential antigen-targeting receptor. Antigen targeting takes advantage of fusing a receptor-specific antibody with an antigen of choice, such as viral, bacterial, or cancer antigens. This technique has successfully been used for the induction of immune responses against various antigens (9, 19–22, 24, 28, 64). However, till date there was no suitable CD1c+ DC-specific targeting receptor described. We here propose CLEC10A as a potential antigen-targeting receptor for human CD1c+ DCs.

In future studies, it will be important to analyze the compartmentalization of CLEC10A by the bound antigen-targeting antibody or the interacting natural ligand. Other lectin receptors such as the murine and human DEC205 receptors localize to late endosomes after uptake into the cell (63, 65). As the efficiency of crosspresentation in human DCs is partially dependent on the antigen routing to late or early endosomes, it will be important to follow the internalization of the CLEC10A receptor, its ligands, or its respective antigen-targeting antibodies (65–67). Importantly, we found no intracellular activation upon binding of the anti-CLEC10A antibody to its receptor (data not shown), supporting our previous findings that internalization and signaling might be

separate events (28). As CLEC10A was recently demonstrated to leap into HLA I and II rich compartments in human immature moDCs (68), we propose that targeting of CLEC10A using antigen-targeting antibodies will lead to a presentation of antigens, both on HLA I and II.

Moreover, we investigated the influence of a natural ligand of CLEC10A, MUC-1-$(Tn)_2$, on CD1c$^+$ DCs. As expected, MUC-1-$(Tn)_2$ bound solely to CD1c$^+$ DCs, but not to other immune cells (**Figure 5**). In our analyses, we have not observed a stimulatory capacity of the ligand alone on CD1c$^+$ DCs. However, an additional costimulation of sorted CD1c$^+$ DCs with a ligand for CLEC10A and the TLR7/8 ligand R848 induced a slightly increased secretion of the cytokines IL-8, IL-10, and TNFα in comparison to R848 alone (**Figure 6**). This is in line with a study by van Vliet et al., which also showed an enhanced secretion of IL-10 and TNFα after stimulation of moDCs with TLR2 ligands in combination with either anti-CLEC10A antibodies or CLEC10A ligands (49). Furthermore, Li et al. showed that stimulation of IFN-DCs, which were generated from blood monocytes using GM-CSF and IFNα, with an anti-CLEC10A antibody induced ERK and p38 phosphorylation and secretion of IL-10 (55). In contrast to the study by van Vliet et al., an additional TLR stimulation was not necessary. This might be due to the culture with IFNα, which led to a sufficient activation of moDCs (49, 55). In contrast to these studies, we could not observe any additional phosphorylation of ERK in CD1c$^+$ DCs upon stimulation with ligands for CLEC10A by Phosflow analyses with or without TLR stimulation (**Figure 7**) (49, 55).

As the TLR7/8 ligand R848 alone induces high secretion of IL-10 by CD1c$^+$ DCs (**Figure 6**), the additional stimulation of CLEC10A could lead to only subtle changes in ERK phosphorylation, which we could not detect with Phosflow analysis. Furthermore, the induced signaling by a TLR is influenced by the localization of the TLR (69, 70). Therefore, the different localization of TLR7/8 and TLR2 could lead to the activation of different signaling molecules. Even TLR ligands targeting the same receptor induce different signaling events dependent on the targeted ectodomain of the TLR. Although both R848 and RNA40 activated TLR8, only R848 induced phosphorylation of ERK1 (71). Additionally, the used cell type influences the induced signaling after TLR stimulation, too (72). Since moDCs are more similar to human inflammatory DCs than to CD1c$^+$ DCs (73), this might additionally explain the observed difference in TLR signaling.

Stimulation of CD1c$^+$ DCs with the TLR3 ligand pIC together with glycosylated MUC1-$(Tn)_2$ did not induce enhanced cytokine secretion, although the CD1c$^+$ DCs showed a high expression of costimulatory molecules (**Figure 6**). Overall, the detected cytokine levels were notably lower compared to stimulation with the TLR7/8 ligand R848. This is in accordance with published data by other groups, who also showed a differential cytokine pattern induced by TLR3 and TLR7/8 stimulation (47, 48, 74). While TLR7/8 stimulation induces inflammatory cytokines, such as IL-6, IL-12, and TNFα, stimulation with pIC results mainly in secretion of IFNλ as well as chemokines, such as RANTES and IP-10 (47, 48, 74). As TLR3, in contrast to TLR7/8, signals independent of MyD88 *via* TRIF and IRF3, we assume that this difference in cytokine secretion is attributed to the differential signaling pathways that are induced by TLR3 and TLR7/8 (75).

Overall, CLEC10A showed a specific expression on CD1c$^+$ DCs in different lymphohematopoietic tissues rendering it as a unique marker for this subset to clearly separate it from monocytic lineages. CLEC10A did not only bind its natural ligand but also internalized rapidly upon binding of a specific antibody. Furthermore, ligands for CLEC10A alone did not induce activation or cytokine secretion by CD1c$^+$ DCs. This is a favorable feature for future immunotherapeutic approaches, since thereby antigen targeting of CLEC10A could not only be used to induce immunity but also tolerance under non-stimulatory conditions (22, 76–78).

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the local ethical committee (Ethikkommission der Friedrich-Alexander-Universität Erlangen-Nürnberg) with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the local ethical committee (Ethikkommission der Friedrich-Alexander-Universität Erlangen-Nürnberg).

AUTHOR CONTRIBUTIONS

LH performed the experiments with participation by GFH, SB, and LHa; and JJL, FG-M, and S-IN provided MGL ligands. GH provided microarray data. CL analyzed microarray data. AH, AP, and RC ensured human tissue sample supply. GH and CL contributed to the review of the manuscript. LH, FN, and DD contributed to the data analysis and interpretation as well as discussions. LH and DD designed the study; and LH, CL, and DD wrote and revised the manuscript.

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Mouse DC-SIGN/CD209a as Target for Antigen Delivery and Adaptive Immunity

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The efficacy of vaccination studies aimed at targeting antigens to human DC-SIGN (hDC-SIGN) have been notoriously difficult to study *in vivo*, as eight dendritic cell-specific intercellular adhesion molecule-3 grabbing non-integrin (DC-SIGN) homologs have been described in mice. CD209a/SIGNR5 has been coined as the mouse DC-SIGN (mDC-SIGN) ortholog, based on its expression and location in the genome. Nonetheless, which properties of hDC-SIGN are covered by mDC-SIGN is poorly investigated. One of the most important functions of DC-SIGN is the induction of adaptive immunity. As such, the aim of this study is to determine the capability of mDC-SIGN to induce adaptive immune responses. Here, we show that mDC-SIGN is expressed on GM-CSF cultured bone marrow-derived dendritic cells (BMDCs) and macrophages. However, mDC-SIGN is an internalizing receptor which, unlike hDC-SIGN, quickly resurfaces after internalization. Binding of OVA-coupled anti-mDC-SIGN antibody by BMDCs leads to quick internalization, processing, and presentation to antigen-specific $CD8^+$ and $CD4^+$ T cells, which can be boosted using the TLR4 ligand, monophosphoryl lipid A. In the homeostatic condition, mDC-SIGN is mostly expressed on myeloid cells in the skin and spleen. A subcutaneous injection of fluorescent anti-mDC-SIGN reveals specific targeting to mDC-SIGN$^+$ skin dendritic cells (DCs) and monocyte-derived DCs *in situ*. A subcutaneous vaccination strategy containing OVA-coupled anti-mDC-SIGN antibody generated antigen-specific polyfunctional $CD8^+$ T cell and $CD4^+$ T cell responses and a strong isotype-switched OVA-specific antibody response *in vivo*. We conclude that mDC-SIGN shows partly overlapping similarities to hDC-SIGN and that targeting mDC-SIGN provides a valuable approach to investigate the immunological function of DC-SIGN *in vivo*.

Keywords: CD209a, dendritic cell-specific intercellular adhesion molecule-3 grabbing non-integrin, SIGNR5, vaccination, dendritic cell, antigen delivery

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INTRODUCTION

The human innate immune receptor dendritic cell-specific intercellular adhesion molecule-3 grabbing non-integrin (DC-SIGN) recognizes pathogen- and host-derived glycoproteins (1). In addition, it mediates antigen internalization, processing, and presentation of antigens to T cells, which are functional hallmarks of antigen-presenting cells (APCs). Therefore, targeting antigens to

human DC-SIGN (hDC-SIGN) has been shown to induce adaptive immune responses *in vitro* (2). In humans, DC-SIGN can be found on immature dendritic cells (DCs) and macrophages in peripheral tissues like the placenta and lung (3), as well as mature DCs in lymphoid tissue (4), but not on other APC subsets, including plasmacytoid DCs or Langerhans cells (5). DC-SIGN can also be found on DCs and M2-like macrophages in tumor tissue (6, 7) and on inflammatory macrophages in rheumatoid arthritis synovium (8). Interestingly, DC-SIGN expression is particularly high on monocyte-derived dendritic cells (moDCs) and dependent on IL4 (9). Although the physiological relevance of moDCs in humans is still unclear, in mice these cells have shown to contribute to antigen presentation and T cell activation (10). Although eight DC-SIGN-related receptors are described in mice, the absence of a clear murine ortholog has hampered the *in vivo* validation of hDC-SIGN and has so far been performed with mice that express hDC-SIGN driven by the CD11c promoter (11). Subsequent targeting of antigens in this model has demonstrated the potency of hDC-SIGN on $CD11c^+$ DCs to internalize, process, and present antigen to T cells (12, 13). For example, targeting of DC-SIGN in combination with genetic depletion of regulatory T cells was sufficient to induce long-term tumor regression in B16 melanoma-bearing mice (14). A similar strategy induced high levels of antigen-specific $CD8^+$ and $CD4^+$ T cells, which protected mice from *Listeria monocytogenes* (15). While it is evident that hDC-SIGN is an effective gateway to strong adaptive immunity, its expression on all $CD11c^+$ cells limits its translational value as an *in vivo* model for antigen targeting.

Of the eight mouse homologs, SIGNR5/CD209a has been coined as mouse DC-SIGN (mDC-SIGN) because of similar expression patterns and localization in the genome (16). Several reports have shown mDC-SIGN to be mostly expressed by moDCs, which are present in steady-state muscle (17) and skin (18) or develop from circulating monocytes after pro-inflammatory signals like GM-CSF (19), LPS (20), or even T cell activation (21). While $mDC\text{-}SIGN^+$ moDCs have been shown to be potent inducers of adaptive T cell immunity, it still remains unclear whether mDC-SIGN itself is able to mediate antigen uptake and presentation to T cells.

Here, we show data that support the paradigm that mDC-SIGN shares expression patterns *in vitro* and *in vivo* with hDC-SIGN, as well as functional properties, including endocytic capacity and antigen presentation to $CD8^+$ and $CD4^+$ T cells *in vitro*. Combining targeting of antigen to mDC-SIGN and a potent adjuvant *in vivo* generates antigen-specific $CD8^+$ and $CD4^+$ T cells and increased antibody responses. In particular, targeting antigen to mDC-SIGN induces significantly higher antigen-specific humoral responses.

MATERIALS AND METHODS

Mice

Mice transgenic for hDC-SIGN, OT-I, and OT-II on the C57BL/6 background have been described previously (11, 22, 23). The transgenic and wild-type C57BL/6 mice were bred at the animal facility of VU University (Amsterdam, Netherlands) under specific pathogen-free conditions and used at 8–16 weeks of age. Female and male mice were equally divided among groups, unless stated otherwise. All experiments were approved by the Animal Experiments Committee of the VU University and performed in accordance with national and international guidelines and regulations.

Flow Cytometry Facilities and Reagents

All flow cytometry experiments were performed at the O_2 Flow Facility at VU University (Amsterdam, Netherlands) using an X20 Fortessa flow cytometer (BD Biosciences) and ImageStreamX (Amnis Corp.) imaging flow cytometer. All antibodies were purchased from Biolegend, Miltenyi, and eBioscience (ThermoFisher), specifically: anti-CD4 (Clone GK1.5), anti-CD8 (Clone H35-17.2), anti-CD11b (Clone M1/70), anti-B220 (Clone RA3-6B2), anti-Ly6C (Clone HK1.4), anti-CD11c (Clone N418), anti-NK1.1 (Clone PK136), anti-CD45 (Clone 30-F11), anti-CD3 (Clone 145-2C11), anti-CCR2 (Clone SA203G11), anti-GR1 (Clone RB6-8C5), anti-CCR7 (clone 4B12), anti-mDC-SIGN (Clone MMD3), anti-MHCII (Clone M5/114.15.2), anti-CD16/32 (Clone 93), and Fixable viability dye-eFluor 780 (Thermo Fisher). $OVA_{257–264}$-H2-Kb-PE tetramers were a kind gift from Dr. J. W. Drijfhout at the LUMC, Leiden, Netherlands.

Imaging Flow Cytometry and Sample Preparation

Bone marrow-derived dendritic cells (BMDCs) were cultured as described by Lutz et al. (24). Because of the high number of cells needed for image flow cytometry, no *ex vivo* isolated DCs could be used in these experiments. BMDCs were incubated with anti-mDC-SIGN:AF488 antibody (clone MMD3) for 1 h, either on 4°C or 37°C. Cells were washed with PBS twice and fixed for 15 min using cold 4% PFA. After washing twice, the fixed cells were resuspended in PBS. Cells were analyzed on the ImageStream X100 (Amnis-Merck Millipore) imaging flow cytometer as previously described (25). A minimum of 15,000 cells were acquired per sample. The internalization score was calculated as previously described (25). Briefly, cells were acquired on the basis of their area. Analysis was performed with single cells after compensation (with a minimum of 5,000 cells). For standard acquisition, the 488-nm laser line was set at 100 mW. First, a mask was designed based on the surface of cells in the bright field image. This mask was then eroded to exclude the cell membrane. Finally, the resulting mask was applied to the fluorescence channel. The internalization score was then calculated on this mask using the Internalization feature provided in the Ideas v6.0 software (Amnis-Merck Millipore). Internalization can be interpreted as a log-scaled ratio of the intensity of the intracellular space vs. the intensity of the entire cell. Cells that have internalized antigen typically have positive scores, while cells that show the antigen still on the membrane have negative scores. Cells with scores around 0 have similar amounts of antigen on the membrane and in intracellular compartments.

Mouse Tissue Collection, Digestion, and FACS Staining

Mice were sacrificed and skin-draining lymph nodes (LNs), spleen, skin, and blood were obtained for further analysis. Skin-draining LNs were verified by the presence of migratory DCs after 100 µl

adjuvant [25 μg agonistic CD40 (in house 1C10) in 1:1 AddaVax (InVivoGen)] injection subcutaneously in the skin. For antigen-tracking experiments, skin biopsies were taken using 8-mm sterile dermal biopsy punches (KAI Medical) 2 h after injection of fluorescently labeled antibody with adjuvant. LNs, spleen, and skin were cut small using sterile scissors in 385 μg/ml liberase TL (2WU) and incubated at 37°C for 20 min. Enzymes were deactivated using ice-cold RPMI 1640 complete (10% FCS, 1% 50 U/ml penicillin, 50 μg/ml streptomycin, HEPES/EDTA). After digestion, cells were run through a 100-μm cell strainer and extensively washed before FACS staining. Cells were stained for 30 min at 4°C using only directly labeled primary antibodies and in the presence of 1 μg/ml anti-CD16/32 antibody. After extensive wash with PBS, labeled cells were fixed with 1% PFA at 4°C for 15 min, washed, and measured on the flow cytometer.

Flow Cytometry Analyses

Flow cytometry data were analyzed first using FlowJo analysis software. First, files were compensated using UltraComp eBeads (Thermo Fisher) microspheres labeled with the appropriate antibodies. Compensation was additionally verified using fluorescence-minus-one (FMO) controls for every single fluorochrome for every tissue type (equally pooled per group) on experimental samples. Next, first gating was performed on a stable flow (time vs. cell count), subsequently on viability dye-negative/CD45-positive cells and finally on single cells (FSC-A/FSC-H). The resulting cells were concatenated and exported per experimental group into an FCS. file and uploaded to the Cytobank online analysis platform (https://www.cytobank.org/). Using the ViSNE module, we generate tSNE plots per tissue type based on the following input and analysis settings: all cells (concatenated) per condition used up to 300,000 total, number of iterations = 3,000, Perplexity = 50, Theta = 0.5. Cells were clustered by MHCII, CD11b, CD11c, B220, NK1.1, Ly6C, GR-1, and CD3 expression. Next, we identified and manually gated subpopulations as represented by the tSNE clustering analysis (Figure S2 in Supplementary Material), color-coded, and overlaid the subpopulations as represented in the graphs (**Figure 4A**; Figure S2 in Supplementary Material). After defining gating strategies, the individual experimental samples were similarly gated in FlowJo and statistics were exported to GraphPad Prism 6 for visualization. Histograms were generated in FlowJo by comparison of "fluorescence minus one" (FMO; all antibodies minus one) or isotype antibody as negative control.

Antigen Presentation Assays

Bone marrow-derived dendritic cells were cultured as described by Lutz et al. (24). OTI and OTII transgenic mice were sacrificed, and spleens were mechanistically run through a 100-μm cell strainer. Red blood cells were lysed using ACK lysis buffer (0.15 M NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA) and washed before purification using MagniSort Mouse CD4- or CD8-negative isolation kits according to the manufacturer's instructions (eBioscience/Thermo Fisher). Purified $CD4^+$ and $CD8^+$ T cells were labeled using 2μM CFSE and counted before co-culture. BMDCs and purified T cells were co-cultured for 3 days at 37°C, stained, and measured on an X-20 Fortessa flow cytometer. To avoid overestimation of CFSE-based proliferation results (26), we adopt the % responding cells metric. Results are calculated and represented as percentage responding cells ("calculated cells at the start of culture"/"number of cells that went into division" × 100). Total # of cells at start of culture = #G0 + (#G1/2) + (#G2/4) + (#G3/8) + (#G4/16) + (#G5/32) + (#G6/64). Number of cells that went into division = "Total # of cells at start of culture" − G0.

Generation of Ovalbumin-Coupled Antibody

In this study, we have used two clones of mDC-SIGN-binding antibodies (MMD2 and MMD3) with identical properties to visualize the receptor and investigate functional characteristics of the mDC-SIGN molecule (27). Anti-DC-SIGN (clone AZN-D1, clone MMD2) and IgG2c (clone 6.3; SouthernBiotech) antibodies were conjugated to ovalbumin (OVA; Calbiochem, Darmstadt, Germany) using the cross-linking agent sulfosuccinimidyl-4-(*N*-maleimidomethyl)-cyclohexane-1-carboxylate or Alexa Fluor 488 NHS Ester (20,000; Thermo Fisher) according to the manufacturer's protocol (Pierce). Antibody conjugates were separated from reaction-reductants using PD-10 desalting columns (Pierce, Rockford, IL, USA). The concentration of OVA and antibody was determined using the bicinchoninic acid assay (Pierce) and ELISA. The products were tested for endotoxins using the Limulus Amebocyte Lysate assay and a level of <0.125 EU/ml was deemed acceptable.

Vaccination

Mice were subcutaneously injected with endotoxin-free 25 μg anti-mDC-SIGN:OVA or anti-mDC-SIGN:AF488 with 25 μg agonistic CD40 antibody (in house, clone 1C10) in 1:1 PBS/AddaVax emulsion according to the manufacturer's instructions (InvivoGen) in a maximum volume of 100 μl. For functional readouts, spleens and blood were collected 7 days after vaccination. For antigen-tracking experiments, organs were harvested 2 and 12 h after vaccine injection. For mDC-SIGN phenotyping experiments, C57BL/6 mice were sacrificed and organs harvested as described.

OVA-Specific Antibody Determination

To determine the antigen-specific antibody response to the ovalbumin, NUNC Maxisorp 96-well plates (Thermo Fisher) were coated with 10 μg/ml purified ovalbumin (Sigma-Aldrich) for 24 h at 4°C in coating buffer in PBS. Next, plates were washed extensively with PBS/Tween 0.05% and additionally blocked with 1% PBS/BSA. A dilution range of serum, obtained from vaccinated mice (on day 7 after vaccination) through a heart puncture, was incubated over night at 4°C. After washing, samples were incubated with anti-mouse IgG-biotin (and anti-IgG1, 2, 3 isotypes) antibodies for 1 h at RT and after wash incubated with HRP-conjugated streptavidin for 1 h at RT. Then after washing, the ELISA plate was developed using TMB substrate buffer. Reaction was stopped when properly developed using 2N H_2SO_4 and extinction was measured at 450 nm using an iMark microplate reader (Bio-Rad). Serum dilution of 1:400 showed the most consistent and reproducible signal to noise ratio. All samples were normalized with PBS as blanco. Secondary antibodies (1:2,000)

used: IgG1 115-065-205 (Jackson ImmunoResearch), IgG2a m32215 (Invitrogen), IgG2b ab97248 (Abcam), IgG3 1100-08 (ITK), IgM 62-6840 (Zymed), IgG 315-065-006 (Dianova), and Streptavidin-HRP p0397 (Dako).

Statistics

Statistics were performed using GraphPad Prism 6 software. For the comparison of two groups, a Student's *t*-test was used. For more than two groups, a two-way analysis of variance was used followed by a Tukey *post hoc* analysis to compare means between two groups. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$, data represented as mean ± SEM.

RESULTS

First, to study the functional characteristics of mDC-SIGN, we examined its expression on cultured DCs *in vitro* by flow cytometry. We generated BMDCs, as previously described (24), using bone marrow from hDC-SIGN transgenic mice (11). GM-CSF-cultured BMDCs roughly generate two populations of APCs distinguished by CD11c and MHC class II expression, GM-DCs ($CD11c^+MHCII^{high}$), and GM-Macs ($CD11c^+MHCII^{int}$) (28). Both classically GM-CSF-cultured BMDCs and macrophages showed clear expression of mDC-SIGN and hDC-SIGN (**Figure 1**). No difference in mDC-SIGN expression between WT and hDC-SIGN transgenic was observed, while hDC-SIGN was absent on wild-type BMDCs, as expected (data not shown). Interestingly, mDC-SIGN is higher expressed on GM-Macs compared with GM-DCs cultured from bone marrow precursors.

To investigate whether mDC-SIGN behaves as an internalizing receptor like hDC-SIGN, we determined the endocytic capacity of BMDCs by imaging flow cytometry. Given the sequence similarity of the different family members in the DC-SIGN family, we selected a set of monoclonal antibodies that have been previously demonstrated to specifically recognize either mDC-SIGN (MMD2/MMD3) or hDC-SIGN (AZN-D1) (4, 20, 27). Fluorescently labeled mDC-SIGN antibody (α-mDC-SIGN-AF488) recognizes mDC-SIGN, which is Fab dependent as it cannot be blocked by pre-incubation with IgGs or FC-block for 30 min (**Figure 2A**), as previously described (20). Using imaging flow cytometry, we can discriminate between membrane-bound fluorescence and intracellular fluorescence and thereby follow internalization of the receptor (**Figure 2B**). To analyze the capacity of mDC-SIGN antibody to internalize *in vitro*, we incubated GM-CSF cultured BMDCs with anti-mDC-SIGN antibody at either 4 or 37°C for an hour. We observed clear internalization of the fluorescently labeled mDC-SIGN antibody after 1 h incubation at 37°C compared with 4°C, when cells are metabolically inactive (**Figure 2C**; red line and blue line, respectively). Interestingly, no obvious accumulation of fluorescent signal is observed when the fluorescently labeled antibody is continually present for an hour (**Figure 2D**). This suggests that mDC-SIGN is either not recycled within this time window to accumulate more antibody intracellularly or the degradation of antibody is balanced by continuous uptake. To examine this, we tested whether mDC-SIGN was available on the membrane for antibody binding after 1 h of internalization with mDC-SIGN targeting antibody. Pulse-chase experiments revealed quick degradation of the fluorochrome after pulse (**Figure 2E**; blue line), which could not be blocked by pre-incubation with the same antibody (black line). When mDC-SIGN-AF488 fluorescently labeled antibody was incubated for 1 h and allowed to internalize at 37°C, unbound membrane-bound mDC-SIGN molecules were still available for staining with a second mDC-SIGN-eFluor660 antibody (**Figure 2F**, green

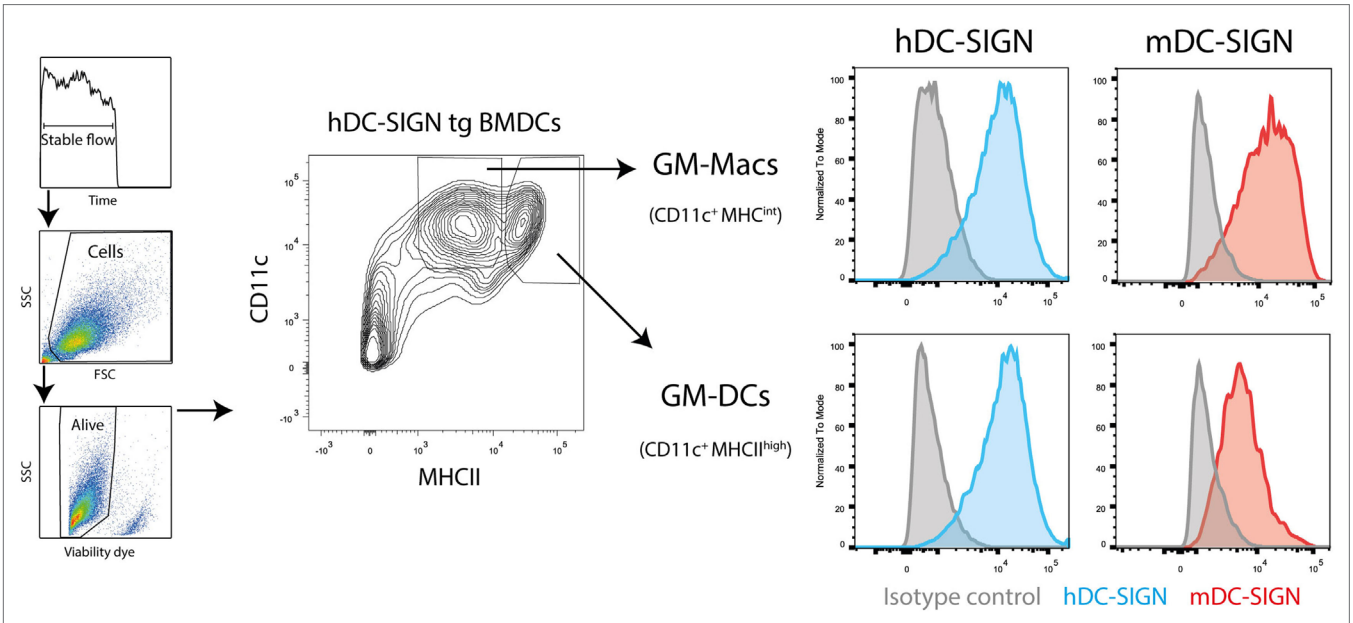

FIGURE 1 | Mouse dendritic cell-specific intercellular adhesion molecule-3 grabbing non-integrin is expressed by GM-DCs and GM-Macs cultured *in vitro* cultures. Both GM-CSF-cultured (differentiation for 7 days) bone marrow-derived macrophages (GM-Macs) and dendritic cells (GM-DCs) express mDC-SIGN. Data are representative of two individual experiments.

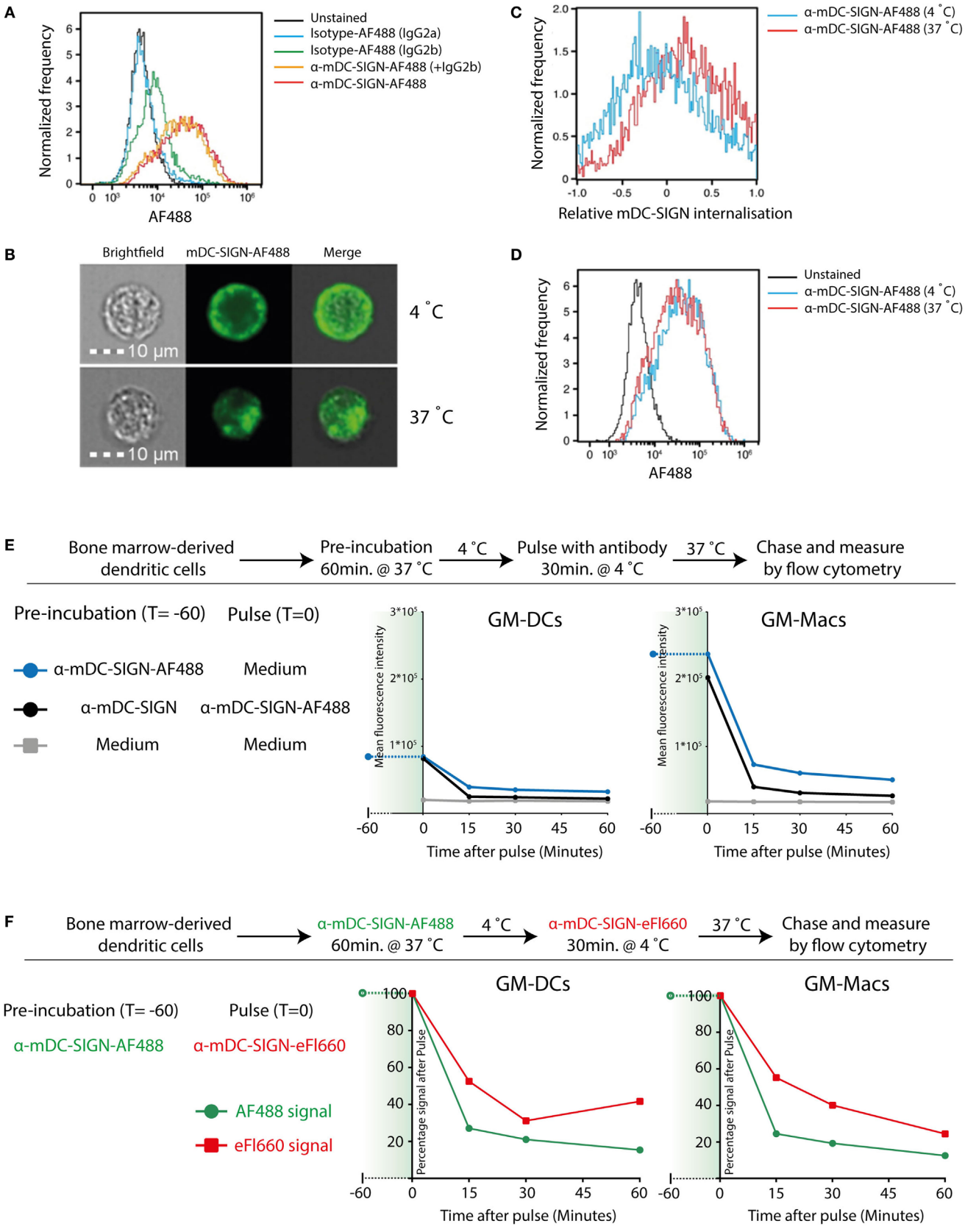

FIGURE 2 | Continued

FIGURE 2 | Mouse DC-SIGN (mDC-SIGN) on GM-DCs and GM-Macs is a quickly internalizing receptor for antigen processing. **(A)** Anti-mDC-SIGN binding to bone marrow-derived dendritic cells (BMDCs) cannot be blocked by isotype-IgG pre-incubation. **(B)** Example of ISX Image Stream data of mDC-SIGN-AF488 binding and internalization on BMDCs. **(C)** Upon binding, mDC-SIGN is quickly internalized at 37°C for 1 h. **(D)** Anti-mDC-SIGN-AF488 fluorescence does not increase after 1 h at 37°C, suggesting either a balance in uptake and degradation or an absence of continued uptake. Experiments representative of two individual experiments. **(E)** Pulse-chase experiments show that pre-incubation of BMDCs with unlabeled anti-mDC-SIGN (clone MMD2) for 1 h at 37°C does not abrogate pulse binding of labeled anti-mDC-SIGN-AF488 (clone MMD3). In addition, fluorescent signal is quickly reduced as the antibody is internalized and degraded in both GM-DCs and GM-Macs. **(F)** BMDC labeling 1 h with anti-mDC-SIGN-AF488 (clone MMD3) before pulse staining with anti-mDC-SIGN-eFluor660 (clone MMD3) shows similar availability of membrane-bound mDC-SIGN molecules after internalization by the first AF488-labeled antibody.

lines and red lines, respectively). Also, the secondary antibody was internalized and degraded in a similar fashion to the mDC-SIGN-AF488 antibody. The fact that mDC-SIGN was apparently available for antibody binding after 1 h of internalization shows that mDC-SIGN is either recycled or newly synthesized. Taken together, these data suggest that mDC-SIGN has endocytic capacity as previously described for hDC-SIGN (29), but quickly resurfaces on the cell membrane for binding and uptake.

Since hDC-SIGN is able to route internalized antigen to MHC class I and II complexes for presentation to T cells (5), we investigated the antigen-presenting capacity of mDC-SIGN$^+$ DCs *in vitro*. GM-CSF cultured hDC-SIGN transgenic BMDCs express both mDC-SIGN and hDC-SIGN (**Figure 1**), allowing the comparison between these receptors using the same bone marrow culture. hDC-SIGN BMDCs pulsed with mDC-SIGN:OVA or hDC-SIGN:OVA targeting antibody were able to internalize, process, and present antigen to OTI CD8$^+$ or OTII CD4$^+$ T cells (**Figure 3**). In addition, the TLR4 agonist and known inducer of cross-presentation to CD8$^+$ T cells (30), monophosphoryl lipid A (MPLA), significantly boosted antigen presentation to CD4$^+$ and CD8$^+$ T cells, with a more pronounced effect on CD8$^+$ T cells (**Figure 3**). The antigen presentation capacity of mDC-SIGN using wild-type BMDCs resulted in comparable results, whereas T cell activation by hDC-SIGN:OVA was abolished in WT BMDCs lacking hDC-SIGN (Figure S1 in Supplementary Material). Hence, both mDC-SIGN and hDC-SIGN are capable of antigen presentation to CD4$^+$ T cells and cross-presentation to CD8$^+$ T cells *in vitro*, which can be boosted by TLR4 activation through MPLA. These data clearly support mDC-SIGN as an endocytic receptor for antigen presentation to T cells, similar to hDC-SIGN.

Next, we aimed to explore mDC-SIGN expression on the major immune subsets in the skin, spleen, LN, and blood of C57/Bl6 mice. Using a 12-color flow cytometry panel including directly labeled antibodies against CD45, CD3, B220, NK1.1, Ly6C, GR-1, CD11b, CD11c, MHCII, CCR2, mDC-SIGN, and a viability dye, we could dissect the major immune population present in blood, spleen, skin, and skin-draining LN (i.e., lateral inguinal LN). To distinguish the populations, we applied tSNE unsupervised clustering as previously described (31), using the online analysis platform Cytobank. The output in **Figure 4** represents all alive CD45$^+$ cells with high-dimensional data in a two-dimensional plot (tSNE1 vs. tSNE2). Cells that are similar in marker expression are clustered together in space. This approach prevented us from overlooking subpopulations of immune cells in the tissue, while developing proper manual gating strategies (Figure S2 in Supplementary Material). In addition, by manually gating we could assimilate individual subsets into a clear immune composition of the tissue (**Figure 4**). The immune composition of the skin seems to be largely dominated by skin macrophages/monocytes [CD11b$^+$Ly6C$^-$GR-1$^-$ (orange)], dermal T cells [NK1.1$^-$ CD3^{int} (brown)], and CD11c$^-$ APCs [CD11b$^+$Ly6C$^-$GR-1$^-$CD11c$^-$MHCII$^+$ (green)]. Interestingly, the CD11b$^+$ DCs and the CD11c$^-$ APCs could only be distinguished by CD11c expression and could not be further subdivided based on the markers used in this panel. Hence, they are not distinctly separated in the tSNE analysis, suggesting that these cell types are similar based on the markers used. The immune composition of LNs, spleen, and blood is mainly dominated by lymphoid cells like B-, T-, and NK cells (gray, purple, and pink, respectively). Subsequent gating showed low but clear mDC-SIGN expression mostly on Ly6C^{high} monocytes (CD11b$^+$Ly6C^{high}GR-1^{int}), Ly6C$^-$ monocytes/macrophages (CD11b$^+$Ly6C$^-$), and CD11b$^+$ and CD11b$^-$ DCs (MHCII$^+$CD11c$^+$) in the skin and spleen (**Figure 4A**). Additional gating strategies to identify splenic CD11b$^-$ and CD11b$^+$ DCs (**Figure 4**; in purple and red) resulted in similar expression levels (Figure S3 in Supplementary Material). Lymphoid cell types and granulocytes like neutrophils and eosinophils were generally devoid of mDC-SIGN, although T-, NK-, and NKT cells showed very low levels of mDC-SIGN staining in the spleen. Expression on DCs was clearly present in the spleen, but significantly lower in the LN, while mDC-SIGN was absent on immune cells in blood or low on blood monocytes (**Figure 4B**).

To investigate the potential of mDC-SIGN$^+$ APCs to facilitate adaptive immunity, we injected fluorescently labeled mDC-SIGN targeting antibody and isotype control antibody subcutaneously in the skin combined with adjuvant (agonistic CD40 in MF59/AddaVax emulsion) (32). Using similar gating strategies as previously shown, injected mDC-SIGN targeting antibody can be found on mDC-SIGN$^+$ APCs in the skin within 2 h after injection (**Figure 5A**). Notably, fluorescent IgG2c isotype control antibody was mostly bound by CD11b$^+$Ly6C$^-$CD11c$^-$MHCII$^+$ monocytes/macrophages and to some extend by CD11b$^+$ DCs, but not CD11b$^-$ DCs and moDCs (**Figure 5A**). Of note is the high level of signal from targeted APCs *in situ* compared with *ex vivo* stained skin APCs, which likely reflects the rapid turnover rate of the mDC-SIGN molecule, leading to signal accumulation in the 2 h after injection of the antibody. To distinguish between LN-homing DCs and APCs unable to migrate to LNs, we stained CCR7 on isolated APCs, 2 h after subcutaneous antibody injection. We could verify CCR7 expression on all DC subsets, including CD11b$^+$Ly6C$^+$MHCII$^+$ moDCs, although expression levels were low (**Figure 5B**). To analyze the fate of the mDC-SIGN targeting antibody in the draining LNs, we isolated skin-draining LNs 12 h after subcutaneous injection of fluorescently labeled mDC-SIGN targeting antibody and analyzed the content of targeted DCs. CD11b$^-$ DCs, moDCs, and CD11b$^+$ DCs contained

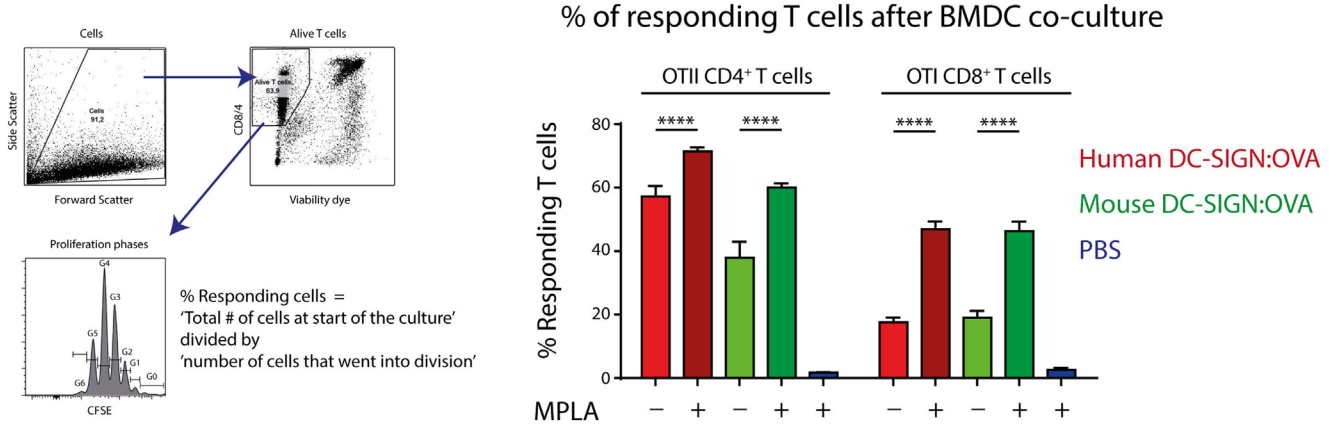

FIGURE 3 | Antigen targeting to mouse- and human DC-SIGN (hDC-SIGN) on bone marrow-derived dendritic cells (BMDCs) leads to internalization, processing, and presentation to antigen-specific $CD8^+$ and $CD4^+$ T cells. GM-CSF cultured hDC-SIGN BMDCs pulsed with 1 μg/ml α-mDC-SIGN:OVA or α-hDC-SIGN:OVA (1 h 37°C, with Fc block) presents processed antigen to CFSE-labeled OTI $CD8^+$ or OTII $CD4^+$ T cells after 3 days of co-culture. Also, the TLR4 ligand, monophosphoryl lipid A (MPLA) consistently boosts antigen presentation. T cell proliferation is represented as percentage responding cells ("calculated cells at the start of culture"/"number of cells that went into division" × 100). Data represented as mean ± SEM, co-cultured in triplicates (analysis of variance with Tukey *post hoc* $^{****}P < 0.0001$), representative of three individual experiments.

significantly more skin-injected mDC-SIGN targeting antibody compared with IgG control antibody (**Figure 5C**). Direct targeting of injected antibody to the skin-draining LN did not occur within 2 h after injection (Figure S4 in Supplementary Material), suggesting that these cells are derived from the periphery and are not labeled through direct drainage to the LN. Importantly, labeling was the most proficient in $CD11b^+$ DCs and moDCs (**Figure 5C**).

To determine whether *in vivo* targeting of antigen to mDC-SIGN induces adaptive immunity, subcutaneous vaccination using mDC-SIGN targeting antibody-coupled to ovalbumin (OVA) protein, with AddaVax-containing adjuvant was assessed. After 7 days, spleens and serum from s.c. vaccinated mice were collected to measure the generation of the adaptive immune response. A single dose of adjuvanted mDC-SIGN targeting antibody resulted in the generation of *de novo* antigen-specific $CD8^+$ T cells (**Figure 6A**) and $CD4^+$ T cells (**Figure 6B**). Antigen (OVA)-specific $CD8^+$ T cells obtained high polyfunctionality as measured by intracellular cytokine staining after peptide re-stimulation (**Figures 6A,B**). Indeed, up to 50% of antigen-specific $CD8^+$ T cells are TNFα, IFNγ, and IL-2 triple-producers upon antigen-specific re-stimulation. Interestingly, while targeting mDC-SIGN did not show significant differences in the quantity or quality (i.e., cytokine production) of the T cell responses compared with the whole protein ovalbumin, clear differences were observed in the humoral response in mDC-SIGN-OVA vaccinated mice 7 days after vaccination. OVA-specific IgG antibody titers (IgG1, IgG2a/b/c, and IgG3) after 7 days were particularly high when the antigen was targeted to mDC-SIGN (**Figure 6C**). Notably, the bias for B cell responses was not dependent on Fc-mediated uptake in the skin as IgG2c-OVA contributed significantly to the induction of T cell responses, while significantly less OVA-specific antibody responses were measured (Figure S4 in Supplementary Material). As such, mDC-SIGN$^+$ APC targeting in the skin induces strong humoral responses while retaining cellular responses, further potentiating vaccination potential.

DISCUSSION

The C-type lectin receptor DC-SIGN has been described as a crucial innate immune receptor involved in a plethora of immunological processes, including the recognition of pathogen-derived ligands and self-glycoproteins, intracellular signaling, antigen processing and presentation, and activation of T cells (1). However, research on its physiological role *in vivo* has been hampered because the lack of a true ortholog in the murine genome (16). Nonetheless, CD209a (also known as SIGNR5) has been coined as mouse DC-SIGN (mDC-SIGN) because of its overlapping expression patterns and localization in the genome. Here, we show expression of mDC-SIGN on GM-CSF cultured bone marrow-derived macrophages (GM-Macs) and dendritic cells (GM-DCs) *in vitro*. Interestingly, a detailed study on the identity and transcriptome of GM-DCs and GM-Macs has suggested their similarity with *in vivo* migratory DCs and skin-resident monocyte-derived DCs/macrophages, respectively (28). This appears to be in line with our *in vivo* data that mDC-SIGN can be found on both skin-resident DCs and skin-resident monocytes. More importantly, *in vivo* GM-CSF-dependent mDC-SIGN$^+$ Mo-DCs seem to arise from specific FcγIIIR$^+$MHCII$^+$mDC-SIGN$^+$ monocytes (19). Nonetheless, while hDC-SIGN can be found on GM-CSF cultured moDCs and skin-resident $CD14^+$ macrophages, the expression of hDC-SIGN$^+$ on migratory DCs remains to be demonstrated, suggesting a possible discrepancy in expression patterns between mouse and hDC-SIGN.

Expression of mDC-SIGN seems to be most pronounced on APCs in organs like the spleen and skin, where pathogens are most likely to be encountered. This may suggest an endogenous role for mDC-SIGN as pattern-recognition receptor, similar to hDC-SIGN. However, hDC-SIGN functions as an important pattern-recognition

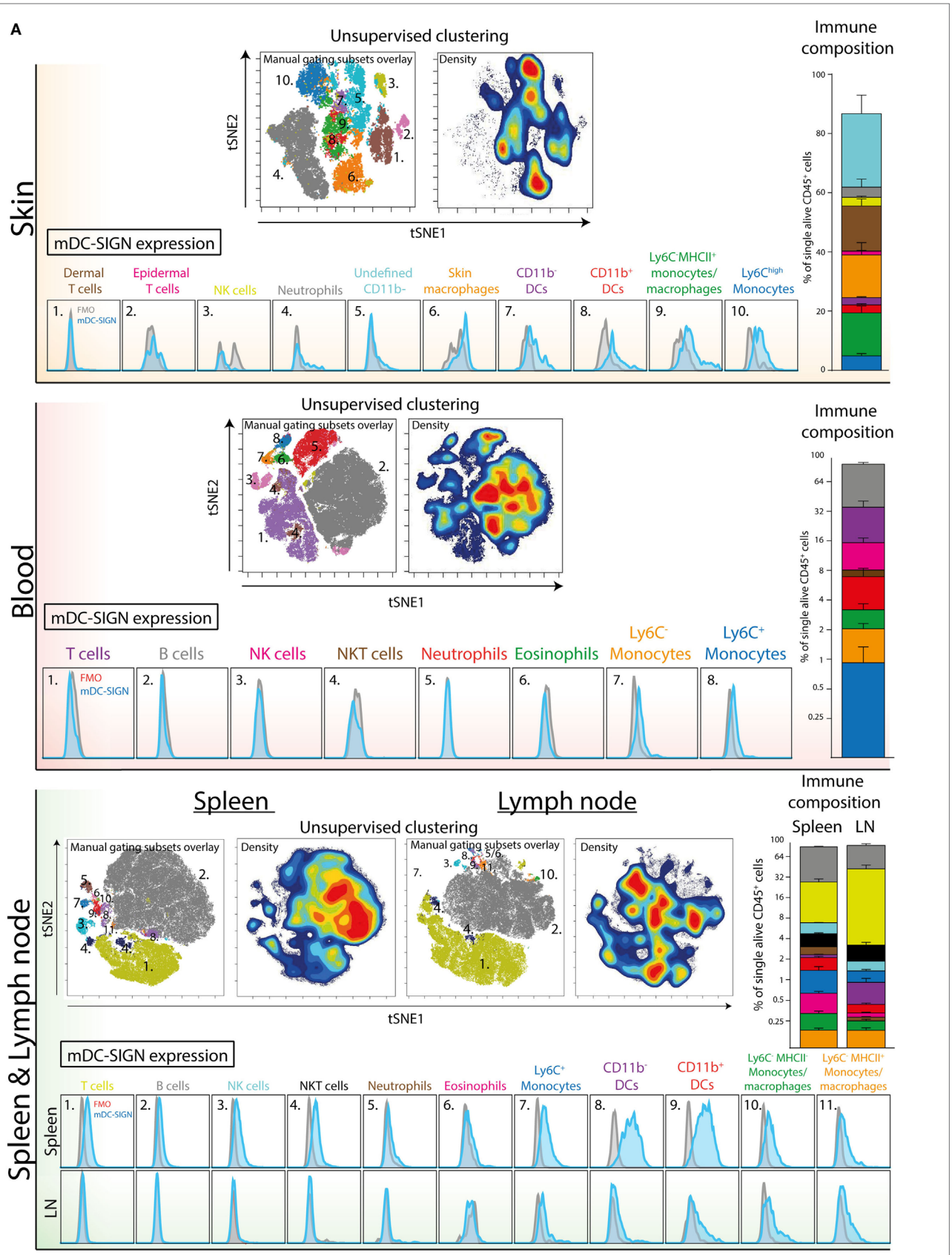

FIGURE 4 | Continued

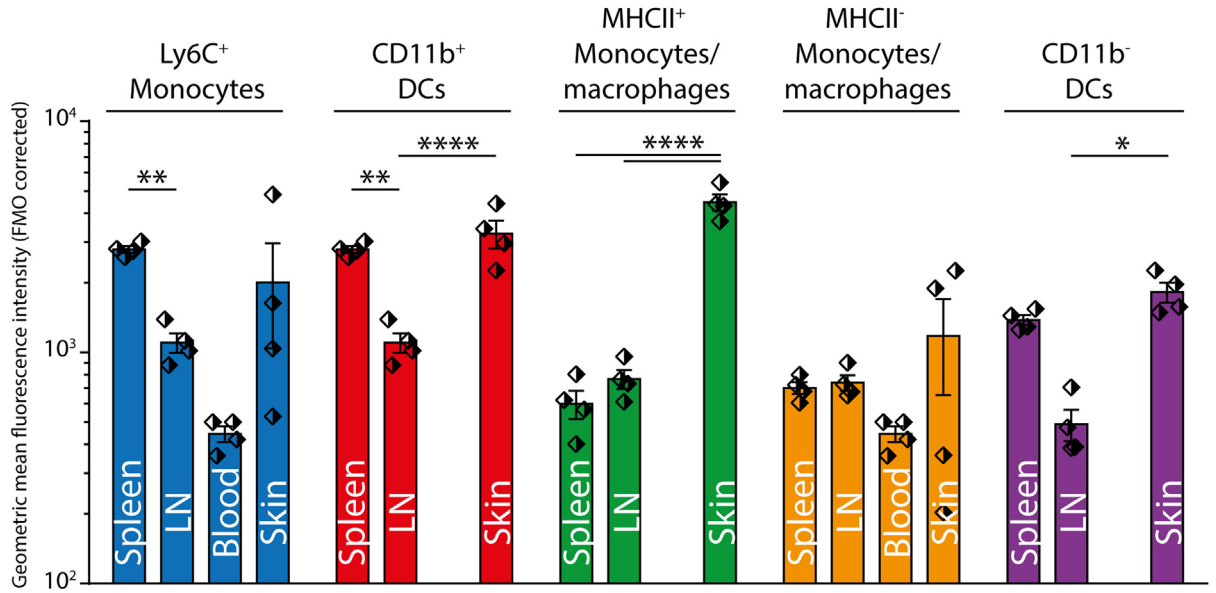

FIGURE 4 | Unsupervised clustering (tSNE) of total CD45+ cells distinguishes organ-specific immune cell subsets expressing mDC-SIGN. **(A)** Unsupervised clustering (tSNE) of alive single CD45+ cells based on high-dimensional flow cytometry into two dimensions allows the distinction of immune subsets and reconstruction of cell frequency (density plot) within the immune composition. Expression of mDC-SIGN (blue histogram) is generally expressed by monocytes/macrophages in the skin and spleen. Dendritic cells in the spleen express clear levels of mDC-SIGN, but expression is less pronounced in the skin-draining lymph nodes (LNs). **(B)** mDC-SIGN is highly expressed on myeloid cells and in particular monocytes/macrophages and DCs in the skin. Also, expression is high on CD11b− and CD11b+ DCs in the spleen, compared to the LN. Data in the bar graphs represented as mean ± SEM (*N* = 4, representative of two individual experiments).

receptor *via* its carbohydrate recognition domain, allowing the binding of particular sugar structures on pathogens like HIV-1, Ebola virus, *Mycobacterium tuberculosis*, *Candida albicans*, *Schistosoma mansoni*, *and Helicobacter pylori* (33). By contrast, mDC-SIGN does not seem to share the exact ligand specificity as hDC-SIGN (34), but has not been carefully studied in detail. Still, fucosylated Lewis x antigen, a known hDC-SIGN ligand, mediated suppression and tolerance to transplantation through mDC-SIGN+ monocyte-derived macrophages (35). In addition, a recent study on mDC-SIGN functioning during schistosome egg infection revealed the capacity of mDC-SIGN to signal *via* Raf-1 depending on its carbohydrate recognition domain and affect DC functioning (36). Therefore, studies aimed at investigating DC-SIGN functioning *in vivo* that rely on recognition of specific glycan structures should take the ligand-binding specificity into consideration, as well as the capacity of mDC-SIGN to affect intracellular signaling. Interestingly, among the other mouse homologs, CD209b (or SIGNR1) has been most widely investigated and shares glycan-binding specificity, including Lewis antigens, with hDC-SIGN (1). Also, the glycan-binding properties of CD209b are vital to the immunological response to *C. albicans* (37), influenza (38), and pneumococcal polysaccharides (39). However, its expression by subcapsular macrophages in LNs and marginal zone macrophages more resembles the hDC-SIGN homolog L-SIGN (40).

Regardless of endogenous ligand specificity, we provide evidence that mDC-SIGN is an internalizing receptor capable of internalizing antigen, resulting in antigen presentation to T cells. However, where hDC-SIGN has been shown to be a slow-recycling receptor (29), mDC-SIGN shows quick recovery and membrane expression after internalization. Therefore, the mode of mDC-SIGN molecule membrane homeostasis is differently regulated compared with hDC-SIGN. A current limitation of the study is the use of antibodies to target a receptor, which can add Fc-mediated effects to the experimental outcome of the results. However, since many murine SIGNR molecules exist with overlapping ligand specificity, investigating the function of mDC-SIGN *in vivo* can currently only be done using highly specific antibodies. Nonetheless, our data support the paradigm that mDC-SIGN displays functional homology to hDC-SIGN, sharing expression *in vitro* and *in vivo*, endocytic capacity, and antigen presentation to CD8+ and CD4+ T cells. This is of particular interest, since C-type lectins like DC-SIGN have been implicated in T cell functioning in humans (41). In mice, mDC-SIGN+ moDCs and macrophages have been shown to control T cell-mediated responses to transplantation tolerance (35), cerebral malaria (42), murine schistosomiasis (43), LPS-induced system infection (20), and experimental colon inflammation (44). Also, mDC-SIGN targeting antibody to skin mDC-SIGN+ APCs and skin mDC-SIGN+ monocytes induced antigen-specific CD8+ and CD4+ T cell responses. It remains to be defined which subset migrates to the draining LN to contribute to T cell activation, especially since moDCs are not assumed to

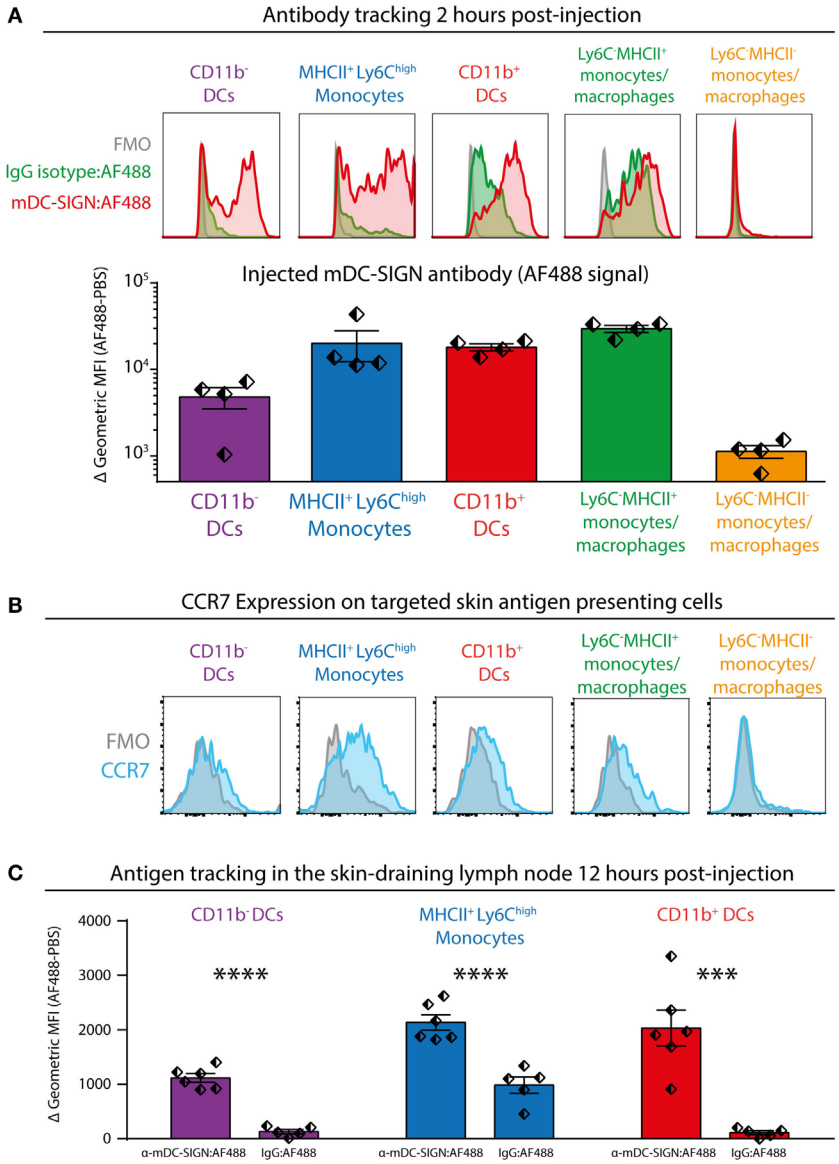

FIGURE 5 | Subcutaneous injection of anti-mDC-SIGN antibody targets skin mDC-SIGN$^+$ antigen-presenting cells (APCs) primarily in the skin. **(A)** 2 h after subcutaneous injection of fluorescently labeled anti-mDC-SIGN in adjuvant (MF59/AddaVax with agonistic anti-CD40 antibody) shows targeting of skin APCs. mDC-SIGN antibody shows particular targeting of MHCII$^+$Ly6C^{high} monocytes, CD11b$^-$ and CD11b$^+$ dendritic cells. Red/green = fluorescent signal, gray = fluorescence-minus-one (FMO) negative control. **(B)** Expression of CCR7 on *ex vivo* isolated skin-derived antigen-presenting cells (APCs) 2 h after subcutaneous injection of anti-mDC-SIGN antibody. **(C)** Anti-mDC-SIGN-AF488 antibody-labeled APCs can be found in the skin-draining lymph node (LN) 12 h after injection. Data represented as mean ± SEM, analysis of variance with Tukey *post hoc* ***$P < 0.001$, ****$P < 0.0001$, representative of two individual experiments.

possess high migratory potential out of peripheral tissues (45). Nonetheless, targeting mDC-SIGN led to high antigen-specific antibody responses, suggesting a potency for mDC-SIGN$^+$ APCs to induce germinal center B cell responses. Notably, while conventional DCs initiate T cell responses, monocyte-derived DCs specifically boost the T follicular helper program that is needed to induce potent germinal center responses *in vivo* (46). Human moDCs have been shown to direct follicular helper T cell differentiation and subsequent T-cell-dependent IgG production by B cells *in vitro* through DC-SIGN (47). Therefore, it is possible that through targeting antigen to mDC-SIGN the mDC-SIGN$^+$ moDCs provide additional Tfh programming and subsequent B cell responses. Alternatively, targeted deletion of CD11b$^+$ DCs, an mDC-SIGN$^+$ DC subset in the murine skin readily targeted in the presented vaccination strategy, has shown to reduce humoral responses to vaccination (48). As such, the specific contribution of specific mDC-SIGN$^+$ APC subsets remains to be defined.

It is this potency by DC-SIGN$^+$ cells to control adaptive immunity that has stimulated research that targets this receptor for therapeutic purposes. Indeed, recent progress has been made in

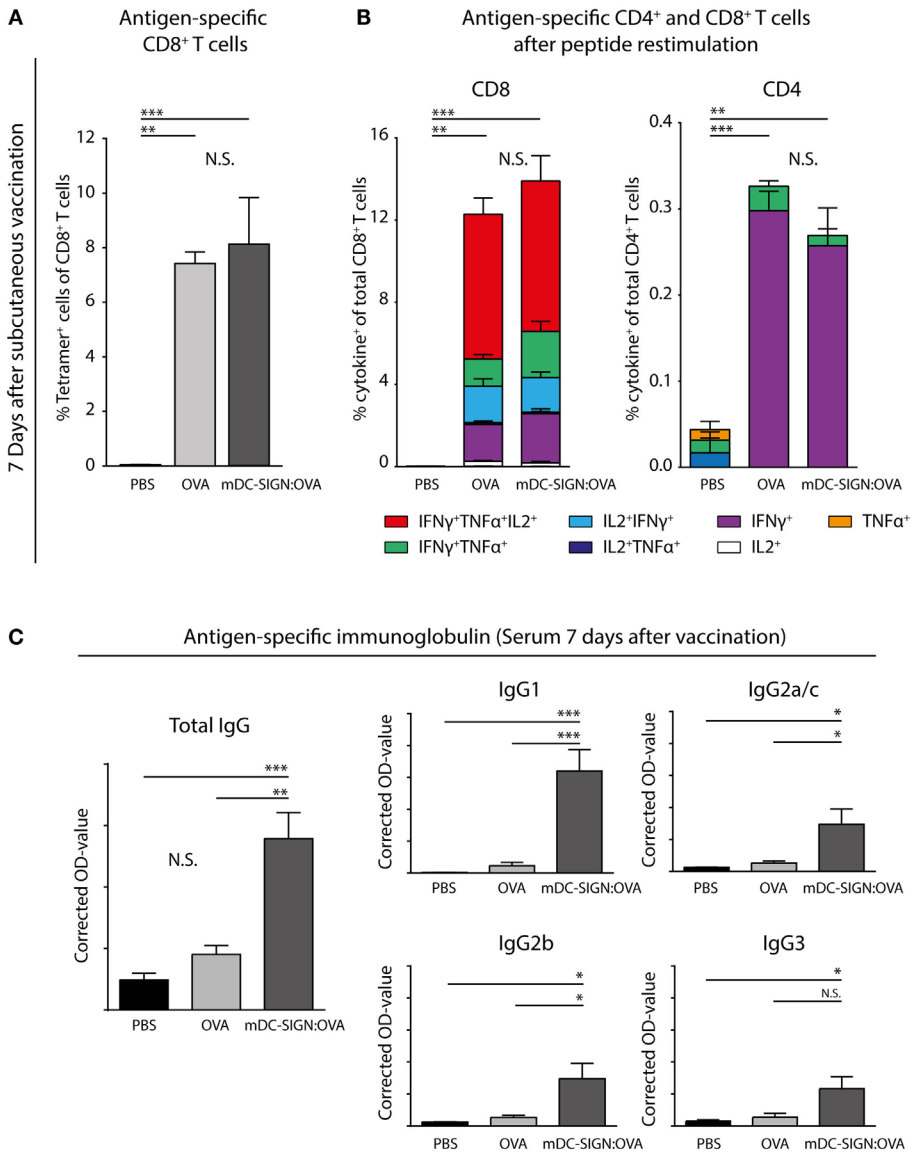

FIGURE 6 | One subcutaneous dose of mDC-SIGN-OVA in adjuvant leads to antigen-specific CD8+ and CD4+ T cell responses and an enhanced isotype-switched OVA-specific antibody response. **(A)** Antigen-specific CD8+ T cells in splenocytes 7 days after vaccination as measured by H2-kb-SIINFEKL tetramer staining. **(B)** T cell re-stimulation through incubation with cognate antigen and intracellular cytokine staining reveals polyfunctional antigen-specific CD8+ and CD4+ T cell responses. **(C)** Antigen-specific antibody capture ELISA reveals antigen-specific immunoglobulin production in the serum of mice vaccinated with mDC-SIGN-OVA (7 days after vaccination; 1:400 serum dilution). All data represented as mean ± SEM ($N = 5$ per group). Two-way analysis of variance with Tukey *post hoc*; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$. Graphs are representative of two individual experiments.

using hDC-SIGN to target tumor-associated antigens to DCs for T cell activation (13, 14, 49, 50). Indeed, in the hDC-SIGN:CD11c humanized mice, targeting OVA to hDC-SIGN on DCs elicited antigen-specific CD8+ T cell responses capable of eradicating OVA-expressing melanoma tumors (14). Since hDC-SIGN is expressed by all CD11c+ cells in this model and does not reflect the natural situation in humans, targeting antigen to mDC-SIGN provides a novel method to investigate hDC-SIGN-generated adaptive immune responses. Most notably, targeting moDCs through DC-SIGN could contribute to vaccine efficacy through the generation of antibody responses. The hDC-SIGN transgenic mouse model also does not express hDC-SIGN on cells that do not express CD11c, including CD11c− APC and Ly6C+MHCII+ monocytes, which express mDC-SIGN. In conclusion, using mDC-SIGN as an hDC-SIGN homolog will allow further study of DC-SIGN-initiated adaptive immune responses in the context of a complex immune system *in vivo* and will aid preclinical DC-SIGN-targeting vaccination strategies. Notwithstanding, since the mDC-SIGN receptor internalization/resurfacing characteristics and ligand specificity is different from hDC-SIGN, there is still a need to investigate the hDC-SIGN molecule in its functional tetrameric form and under its proper genomic control *in vivo*.

ETHICS STATEMENT

All experiments were approved by the Animal Experiments Committee of the VU University and performed in accordance with national and international guidelines and regulations.

AUTHOR CONTRIBUTIONS

SS provided contribution to the conception and design of the work, acquisition, analysis, and interpretation of data for the work, drafting of the work and revising it critically, and agreed to be accountable for all aspects of the work. LK, MC, and HK were involved in the acquisition and analysis and interpretation of the data. JO and JJ-G were involved in the drafting of the work, and contributions to the conception and interpretation of the work. JH was involved in the analysis and interpretation of the work, drafting and critically reading of the manuscript. YK was involved in the design of the work, drafting and intellectual content of the document, and accountable for the work to accuracy and integrity. All authors provided approval for publication of the content.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fimmu.2018.00990/full#supplementary-material.

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REVIEW
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The Interaction of Human Pathogenic Fungi With C-Type Lectin Receptors

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Fungi, usually present as commensals, are a major cause of opportunistic infections in immunocompromised patients. Such infections, if not diagnosed or treated properly, can prove fatal. However, in most cases healthy individuals are able to avert the fungal attacks by mounting proper antifungal immune responses. Among the pattern recognition receptors (PRRs), C-type lectin receptors (CLRs) are the major players in antifungal immunity. CLRs can recognize carbohydrate ligands, such as β-glucans and mannans, which are mainly found on fungal cell surfaces. They induce proinflammatory immune reactions, including phagocytosis, oxidative burst, cytokine, and chemokine production from innate effector cells, as well as activation of adaptive immunity *via* Th17 responses. CLRs such as Dectin-1, Dectin-2, Mincle, mannose receptor (MR), and DC-SIGN can recognize many disease-causing fungi and also collaborate with each other as well as other PRRs in mounting a fungi-specific immune response. Mutations in these receptors affect the host response and have been linked to a higher risk in contracting fungal infections. This review focuses on how CLRs on various immune cells orchestrate the antifungal response and on the contribution of single nucleotide polymorphisms in these receptors toward the risk of developing such infections.

Keywords: pattern recognition receptor, C-type lectin receptor, *Candida*, *Aspergillus*, pathogenic fungi, single nucleotide polymorphisms

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INTRODUCTION

Fungi are ubiquitously present in the environment and as commensals in humans; therefore, innate immunity needs to continuously work against the constant exposure. Pattern recognition receptors (PRRs) found on cell surfaces and as soluble forms in body fluids can recognize microbe-specific molecules; the so-called pathogen-associated molecular patterns (PAMPs). PRRs are expressed on immune cells and also on epithelial cells. The interaction of PRRs with PAMPs induces cell- and receptor-specific cellular host responses involving both, the innate and the acquired immune system. There are mainly four different kinds of PRR families, including the toll-like receptors, Nod-like receptors, C-type lectin receptors (CLRs), and RIG-I-like receptors (1). CLRs can recognize carbohydrates by virtue of having a C-type lectin-like domain (2). The domain consists of a conserved double loop structure and a long, structurally, and evolutionarily flexible loop which is involved in Ca^{2+}-dependent carbohydrate binding (3). The characteristic fungal cell wall feature is its richness in carbohydrates and, therefore, they serve as the candidate targets for recognition by CLRs. It is widely accepted that CLRs play major role in antifungal immunity compared to other PRRs (1, 4).

C-type lectin receptors can be found as soluble forms in the serum and other body fluids or as transmembrane receptors on various immune cells, such as macrophages, dendritic cells (DCs), neutrophils, and various other cell types (**Table 1**) (5). Although CLRs have been divided based on their domain organization and phylogenetic features (3, 6), a broader classification of transmembrane receptors is possible based on the type of signaling mechanisms employed by them (7). One such mechanism is the signaling of CLRs *via* immunoreceptor tyrosine-based activation motifs (ITAMs). The ITAM motif (consensus sequence YxxL/I) recruits and phosphorylates Syk kinase on receptor ligation. Signaling *via* Syk typically leads to NF-κB activation *via* the complex consisting of caspase recruitment domain-containing protein 9 (CARD9) singalosome, a trimeric CARD9, B cell lymphoma/leukemia 10, and the mucosa-associated lymphoid tissue lymphoma translocation protein 1. Syk activation ultimately induces subsequent proinflammatory responses, as well as other responses, such as phagocytosis and reactive oxygen species (ROS) and reactive nitrogen species (RNS) production (8, 9). Some CLRs do not have their own cytoplasmic ITAMs. Such receptors couple with ITAM containing adaptor molecules like FcRγ to emanate signaling (10, 11). Dectin-1 is another non-classical CLR bearing a hemITAM motif (consensus sequence YxxL) and the ligand binding is Ca^{2+} independent (12). A second signaling mechanism with contrary effects to those elicited by ITAM signaling is employed by CLRs containing a cytoplasmic immunoreceptor tyrosine-based inhibitory motif (ITIM). Here, receptor ligation leads to the phosphorylation of tyrosine within the ITIM motif (consensus sequence I/V/L/SxYxxI/L/V) and the recruitment of SHP-1, SHP-2, and/or SHIP-1 phosphatases which exert an inhibitory effect by dampening the proinflammatory response (13, 14). Finally, some CLRs do not contain any known signaling motifs and, therefore, only little is known about their signaling mechanisms, such as LOX-1, MR, and langerin.

Fungal pathogens have a huge influence on human life, since they can infect the human body and cause various diseases from superficial infections to invasive and systemic infections. Infections of the skin and nails are the most common fungal diseases which affect ~25% of the general population worldwide (83). Invasive fungal infections have a lower incidence than superficial infections; however, they are of greater concern

TABLE 1 | C-type lectin receptors and their respective ligands involved in fungal recognition by different human cell types.

Receptor	Fungus	Ligand	Cell Type	Reference
Dectin-1	*Aspergillus fumigatus, Malassezia* spp., *Saccharomyces cerevisiae*, *Fonsecaea pedrosoi*, *Pneumocystis carinii*, *Cryptococcus neoformans*, *Paracoccidioides brasiliensis*, *Histoplasma capsulatum*, *Coccidioides posadasii*, *T. mentagrophytes*, *Candida albicans*, *Talaromyces marneffei*, *Fusarium solani*, *Exserohilum rostratum*, *Cladosporium cladosporioides*, *T. asahii*, and *Sporothrix schenckii*	β-1,3-glucan	hDC, macrophages, bronchial epithelial cells, monocytes, neutrophils, mast cells, dendritic cells (DCs), and pulmonary epithelium	(12, 15–32)
Dectin-2	*A. fumigatus*, *Malassezia* spp., *F. pedrosoi*, *H. capsulatum*, *T. rubrum*, *C. albicans*, *C. glabrata*, and *M. audouinii*	α-mannans	DCs, macrophages	(11, 33–37)
MR	*A. fumigatus*, *S. cerevisiae*, *P. carinii*, *C. neoformans*, *P. brasiliensis*, *C. albicans*, *C. parapsilosis*, *T. marneffei*, and *S. schenckii*	gp43 (*P. brasiliensis*), mannoproteins (*C. neoformans*), and α-mannans	Corneal epithelial cells, alveolar macrophages (AMs), DCs, monocytes, and keratinocytes	(38–47)
SP-A, SP-D	*A. fumigatus*, *S. cerevisiae*, *Pneumocystis* spp., *C. neoformans*, *Histoplasma* spp., and *Coccidioides* spp.	β(1→6)-glucan, gpA and gp120 (*P. carinii*), glucuronoxylomannan, and mannoprotein (*C. neoformans*)	Isolated from lung lavage fluids	(48–54)
MBL	*A. fumigatus*, *P. carinii*, *C. neoformans*, and *C. albicans*		Purified from plasma	(55–58)
DC-SIGN	*A. fumigatus*, *S. cerevisiae*, *C. neoformans*, *C.albicans*, *T. marneffei*, and *C. topicum*	Galactomannans (*A. fumigatus*), mannoprotein (*C. neoformans*), and N-linked mannans	DCs, AMs	(5, 39, 59–64)
Mincle	*A. fumigatus*, *Malassezia* spp., *F. pedrosoi*, *P. carinii*, and *C. albicans*	α-mannose, glyceroglycolipid and mannosyl fatty acids (*Malassezia* spp.), MSG/gpA (*P. carinii*)	Corneal epithelial cells, monocytes, macrophages, neutrophils, myeloid DCs, and some B-cell subsets	(36, 65–69)
MCL	*C. neoformans*, *C. albicans*	α-mannans	Plasmacytoid dendritic cells	(70, 71)
CR3	*A. fumigatus*, *M. furfur*, *S. cerevisiae*, *P. brasiliensis*, *H. capsulatum*, and *C. albicans*	pH-regulated Ag 1 (*C.albicans*)	Neutrophils, macrophages, natural killer cells, and monocytes	(5, 72–78)
Lox-1	*A. fumigatus*		Corneal epithelial cells	(79, 80)
Langerin	*M. furfur*, *S. cerevisia*, C. albicans, *C. glabrata*, *C. krusei*, *C. parapsilosis*, and *C. tropicalis*	β-glucan	Langerhans cells	(81)
MelLec	*A. fumigatus*	1,8-dihydroxynaphthalene-melanin	Endothelial cells, macrophages	(82)

because they are associated with high morbidity and mortality. They are mostly caused by opportunistic fungal pathogens that take advantage of a debilitated immune system to proliferate in the human host and cause disease (84). Among the fungal species, only several 100 species are associated with human fungal diseases and just a minor number of species cause the most common invasive infections in immunocompromised individuals (85). The most notorious genera that are responsible for more than 90% of all reported fungal-related deaths are *Cryptococcus*, *Candida*, *Aspergillus*, and *Pneumocystis* (84). This increased prevalence of fungal infections has motivated the study of host–pathogen interactions in order to understand the protective and nonprotective mechanisms of antifungal immune responses in the human body. Investigation of the fungal recognition by the innate immune system led to the discovery of CLRs, the best-characterized PRRs for fungi. CLRs recognize carbohydrate polymers (mannan, glucans, and chitins) present in the fungal cell wall, resulting in the induction of innate and adaptive immunity to clear the pathogen (**Figure 1**; **Table 1**) (86).

In the following sections, we will summarize the current knowledge about the interaction of important human pathogenic fungi with CLRs. We further include information on CLR-associated single nucleotide polymorphisms (SNPs) and their effect on the susceptibility to fungal infections.

OPPORTUNISTIC INVASIVE MYCOSES

Aspergillus spp.

Aspergillus species (*Aspergillus* spp.) are ubiquitous molds commonly found in the soil. They produce a large number of conidia, which are released and dispersed into the air by wind leading to a deep penetration into the respiratory tract upon inhalation (87). These conidia are effectively cleared from the lungs of immunocompetent individuals. However, patients with a compromised immunity are at risk of developing an acute invasive aspergillosis (AIA). AIA is characterized by hyphal invasion of lung tissues and even dissemination to other organs (87). *Aspergillus fumigatus* (*A. fumigatus*) accounts for about 65% of all invasive infections

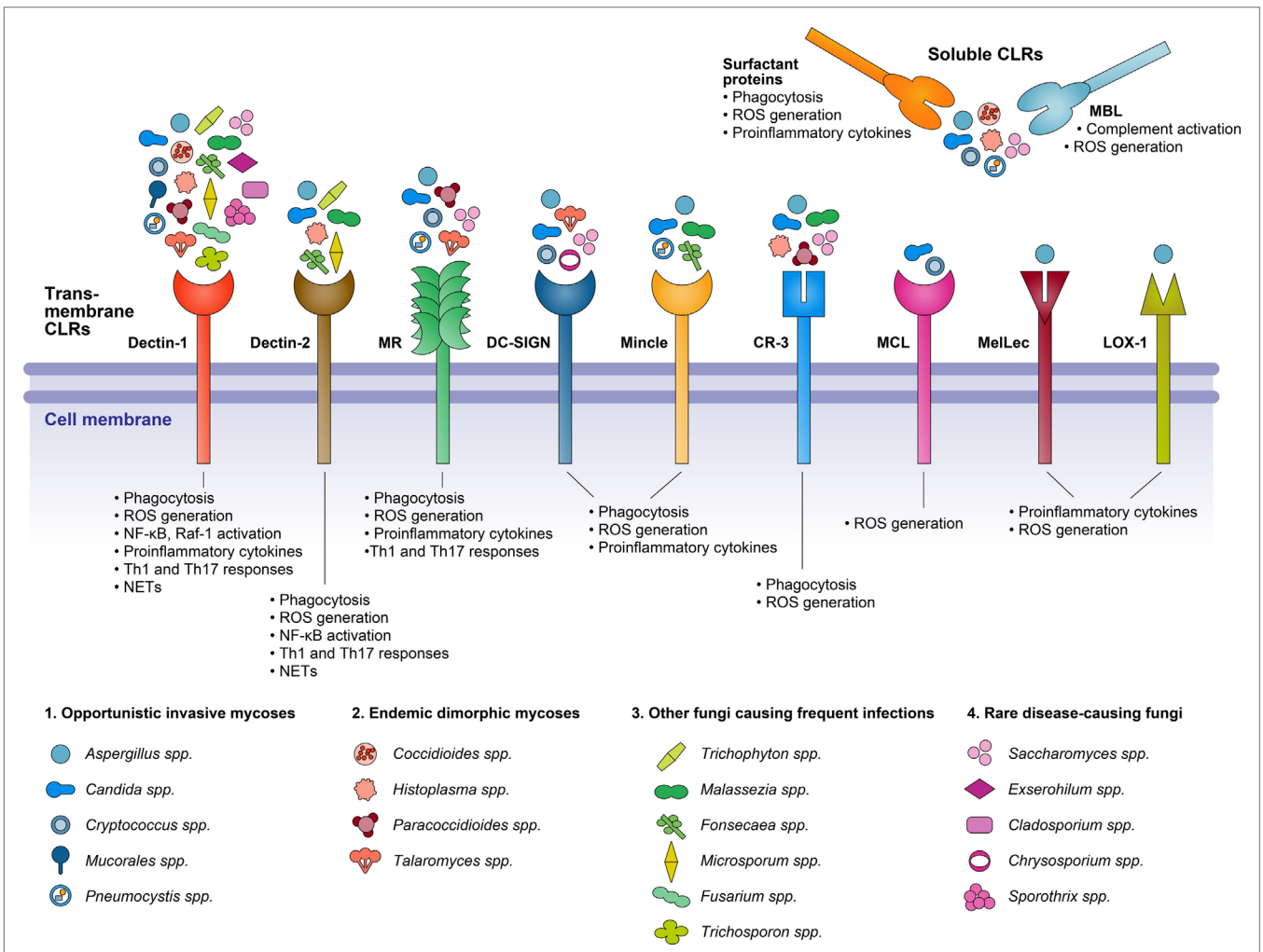

FIGURE 1 | A diagrammatic representation of C-type lectin receptors (CLRs) involved in the recognition of various fungal species, and the resepective cellular responses triggered on receptor–ligand binding.

in humans and is the most frequently encountered *Aspergillus* spp. in pulmonary infections. *A. flavus*, *A. niger*, *A. terreus*, and *A. nidulans* are less frequent causes of infections (87). The primary innate immune response is mediated mainly by macrophages, DCs, and neutrophils, taking place after *Aspergillus* spp. encounters these cells. Several of the *Aspergillus* cell wall components, such as β-glucans, chitins, and mannans act as ligands that are recognized by CLRs. Ligation results in the activation of cellular immune responses, such as phagocytosis, extracellular trap formation, conidial killing, and the production of proinflammatory and anti-inflammatory cytokines, such as TNF-α, IFN-α, IL-6, and IL-18 (88–91). Fungal recognition by specific CLRs can depend on morphological changes of *Aspergillus* spp., since different growth forms expose diverse PAMPs at variable amounts on their surface. For example, the surface of the *Aspergillus* dormant conidia does not present β-glucan, but is accessible for receptor recognition after the loss of hydrophobic cell wall components (outer layer of rodlets/hydrophobins and melanin) during the swelling of conidia and the development of germ tubes (89, 92, 93). Several CLRs are involved in the recognition of *Aspergillus* spp. such as the transmembrane receptors Dectin-1, Dectin-2, MR, DC-SIGN, and the soluble collectins MBL and the lung surfactant proteins (SP) SP-A and SP-D (94) (**Table 1**). The most studied *Aspergillus* receptor is Dectin-1. It is present on the surface of myeloid cells recognizing β-1,3-glucan, a common component of the cell wall of several fungi (15). However, another *Aspergillus* cell wall-associated polysaccharide, the galactosaminogalactan, has also been identified as a ligand and prevents host inflammatory responses *in vitro* and *in vivo*, in part by avoiding cell wall β-glucans recognition by Dectin-1 (95). Several observations suggest a significant role for Dectin-1 in protective immunity against *A. fumigatus* (96–98). *A. fumigatus* also induces the expression of cytokines (TNF-α and IL-12) and genes related to fungal recognition and phagocytosis in immature human DCs (99). The transcription of Dectin-1 in response to *A. fumigatus* likely occurs *via* granulocyte-macrophage colony stimulating factor (GM-CSF)/PU.1, where GM-CSF potentiates the expression of PU.1, which carries out transcription of Dectin-1 augmenting Dectin-1 protein expression and responsiveness in THP-1 cells (100, 101). In HEK293T cells, the activation of AP-1 by heat-killed swollen conidia was inhibited by treatment with Syk inhibitor, indicating that the Syk signaling pathway is required for AP-1 activation in a Dectin-1-dependent manner (102). Silencing of Dectin-1 in murine macrophages resulted in a reduced expression of proinflammatory cytokines, and an observed inhibition of phagocytosis (103). Likewise, *A. fumigatus* conidia and germ tubes stimulated NF-κB activation, mediated the secretion of proinflammatory cytokines involved in the recruitment of neutrophils, and led to ROS production by human monocyte-derived macrophages, murine macrophages, and alveolar macrophages (AMs) (89, 92, 93). Clinical studies showed that individuals who developed AIA during the course of chemotherapy often displayed a defective expression of Dectin-1. The frequency of Dectin-1-expressing monocytes was reduced in patients with AIA compared to controls (65.6 vs. 87.5%) (104). This important role of Dectin-1 was confirmed by transfecting murine AMs with a vector encoding full-length Dectin-1 (105). Results demonstrated that Dectin-1 overexpression enhanced the generation of proinflammatory cytokines TNF-α and IL-1β, and enhanced the killing ability of macrophages during *A. fumigatus* exposure (105). The epithelial lining of human airways is another important spot for host-pathogen interactions, and Dectin-1 is also expressed in lung tissues (12). One study found that *A. fumigatus* induces the expression of Dectin-1 *via* TLR-2 in human bronchial epithelial cells, resulting in the stimulation of proinflammatory responses and ROS generation in response to *A. fumigatus* indicating its important role in the innate immune response in non-phagocytic cells (106). Furthermore, some findings indicate that the pulmonary infection of mice with *A. fumigatus* induces concurrent Th1 and Th17 responses that depend on Dectin-1 (107). With regards to *Aspergillus*-induced fungal keratitis, recent findings demonstrated that Dectin-1 is expressed in the cornea of rat and mice, where it is involved in the detection of invading fungi (108–110). Also Dectin-2 triggers a response to *A. fumigatus* infection. Human plasmacytoid dendritic cells (pDCs) recognize *A. fumigatus* hyphae *via* Dectin-2, resulting in cytokine release and extracellular trap (pET) formation (88). The noticeable Dectin-2 expression of AMs in human lung during *A. fumigatus* invasion suggests a prominent contribution to antifungal defenses in pulmonary aspergillosis (90). Moreover, Dectin-2 ligation leads to NF-κB activation and ROS production in response to *A. fumigatus* infection in human macrophages (111). An *A. fumigatus*-specific ligand has not been described until now, but Dectin-2 binds to high-mannose structures distributed in several fungal species, including *Aspergillus* spp. (33). Collectins, such as SP-A, SP-D, and MBL also bind to *A. fumigatus* (55, 112). One study about the contribution of MBL in the antifungal defense in invasive pulmonary aspergillosis (IPA) showed that in murine models of IPA, rhMBL-treated (recombinant human MBL) mice showed 80% survival compared to untreated IPA mice. A clear increase of TNF-α and IL-1α in treated IPA mice and a significant decrease in pulmonary fungal hyphae and IL-10 could be observed (113). *In vitro*, there was an enhanced uptake of *A. fumigatus* conidia by polymorphonuclear neutrophil (PMNs) in the presence of rhMBL, indicating a protective role of this receptor during IPA, possibly through MBL-mediated lectin complement activation (113). SP-A and SP-D also enhanced agglutination and binding of conidia to AMs and neutrophils and increased the phagocytosis, oxidative burst, and killing of *A. fumigatus* conidia by human neutrophils and AMs (91). The SP-D-mediated protective mechanism is dependent on calcium-activated protein phosphatase calcineurin (114) and include enhanced phagocytosis by recruited macrophages and neutrophils and enhanced local production of the Th1 cytokines TNF-α and IFN-γ in the supernatant from mice lung cell suspension (115). Corneal epithelial cells also express SP-D and in the setting of fungal keratitis *A. fumigatus* may induce these cells to express inflammatory cytokines *via* the SP-D and NF-κB pathway (116, 117). Since β(1→6)-glucan is a ligand for SP-D and since many fungi, including *Aspergillus* spp., have this carbohydrate structure in their cell wall compositions, it is expected that SP-D recognizes all *Aspergillus* spp. (48).

Several other CLRs ligate *Aspergillus* spp., but for each only a few data are available. DC-SIGN, another transmembrane

receptor of the CLR family expressed on the surface of DCs, contributes to the binding of *A. fumigatus* conidia in human DC (59). However, DC-SIGN is also expressed in AMs and lung tissue, suggesting a contribution of DC-SIGN in the initial stages and in fungal spreading during AIA (60). Galactomannans appear to be the main DC-SIGN ligand on the cell wall of *A. fumigatus* conidia (60). Additionally, CR3 influences adaptive responses to *Aspergillus*. Blocking of CR3 significantly reduced *Aspergillus*-induced Th1 and Th17 responses independently from complement activation, demonstrating that CR3 might play a significant role in the adaptive host defense against *A. fumigatus* (72).

In fungal keratitis models, LOX-1 was increased in *A. fumigatus* infected corneas of C57BL/6 mice and human corneal epithelial cells, indicating a possible role of this receptor in controlling the infection (79, 80). In addition, Mincle and MR may play a role in the early innate immune response of the corneal resistance, since their expression increased significantly during the initial period of *A. fumigatus* infection, along with an increased expression of TNF-α and IL-1β in human and rat cornea (38, 65). *A. fumigatus*-specific ligands for these receptors have not been described up to now.

Recently, the CLR Clec1a, also called melanin-sensing C-type lectin receptor (MelLec), has been decribed to play an important role in the detection of *A. fumigatus* through recognition of the naphthalene-diol unit of 1,8-dihydroxynaphthalene-melanin in conidial spores of *A. fumigatus*. MelLec is ubiquitously expressed by CD31+ endothelial cells in mice and is required for protection against disseminated infection with *A. fumigatus*. MelLec is also expressed by myeloid cells in humans and a SNP within the coding region of this receptor (rs 2306894) was identified that significantly increased the susceptibility of stem-cell transplant recipients to AIA (82). AIA is of great interest for immunogenetic studies due to its high prevalence. A moderately large number of studies have investigated the association of SNPs and other genetic variations of different CLRs in order to get some benefit for preventive strategies. For Dectin-1, the *CLEC7A* rs3901533 (T/T) and rs7309123 (G/G) genotypes and the presence of Y238X (rs16910526) polymorphism resulted in a significantly increased risk of AIA in a Caucasian population (118–120). Two SNPs of *CD209* encoding DC-SIGN (rs735239 and rs735240) are associated with a higher susceptibility to fungal keratitis in the northern Han Chinese population (121). Association analysis revealed that carriers the *CD209* rs4804800 (G), rs11465384 (T), rs7248637 (A), and rs7252229 (C) alleles and the variant *CD209*-139A/G (rs2287886) in the Caucasian population had a significantly increased risk of contracting IPA (118, 122).

Several studies show that distinct alleles, genotypes, and genotype arrangements of *SFTPA2* and *MBL2* may contribute to a susceptibility of the host to aspergillosis. A significant association of *SFTPA2* 1649G and *SFTPA2* 1660G and *MBL2* 1011A alleles with allergic bronchopulmonary aspergillosis patients suggests that defects in these innate immune molecules may lead to an increased genetic susceptibility to allergic airway inflammation and asthma (123–126). Another study implies that the presence of the T allele and CT genotype at position 868 of *MBL2*, the CC genotype at position 1649 of *SFTPA2*, and its combination with the CC or CT genotype on position 868 of MBL gene increases susceptibility specifically to chronic cavitary pulmonary aspergillosis in the Caucasian population (127). It was demonstrated that the presence of the codon 52 mutation (W/M52) within the MBL gene was particularly common in patients with chronic necrotizing pulmonary aspergillosis. Since the mutation results in changes in the protein structure, it is likely that a reduced amount of active protein is available for pathogen clearance (128).

Overall, Dectin-1 plays an important role in the local immune response during aspergillosis by inducing the expression of proinflammatory cytokines. Dectin-1 is the best-characterized CLR for the recognition of *A. fumigatus*, since it recognizes β-1,3-glucan, which is a major component of the inner cell wall of this fungus. Even a single polymorphism results in a significantly increased risk of contracting AIA, indicating the importance of this receptor in the contribution to antifungal defenses. With regards to the other CLRs recognizing *Aspergillus* spp., more studies are required in order to establish a concrete role of them during an AIA.

Candida spp.

The most common species of *Candida* responsible for causing human diseases is *Candida albicans*. It is an opportunistic pathogen that commensally colonizes not only the skin but also the gastro-intestinal and urino-genital mucosal surfaces mostly in yeast form in healthy individuals. In cases of immunosuppression or weakening, the yeast forms can convert into virulent hyphae that can cause either muco-cutaneous infection or disseminate to internal organs causing candidaemia (129). In addition to phenotypic switching between yeast and hyphal forms, *C. albicans* virulence factors include adhesion properties, secreted lipases, and aspartyl proteases (130). Other clinically relevant *Candida* species include *C. krusei*, *C. glabrata*, *C. tropicalis*, *C. parapsilosis*, and others (131). Among the *Candida* species-recognizing CLRs are Dectin-1, Dectin-2, MCL, Mincle, MR, DC-SIGN, CR3, MBL, and Langerin.

Dectin-1 is a type II transmembrane receptor expressed on several antigen-presenting cells of myeloid origin, including macrophages, monocytes, neutrophils, mast cells, DCs, as well as pulmonary epithelium (12, 16). Like *A. fumigatus* and other fungi, Dectin-1 also recognizes *C. albicans* by binding to β-1,3-glucan (**Table 1**) (132).The binding of β-1,3-glucan to Dectin-1 is Ca^{2+}-independent (133). Notably, Dectin-1 recognition of the yeast form of *C. albicans* induces responses such as phagocytosis and oxidative burst in mouse phagocytes, ultimately resulting in the clearance of the yeast cells; in contrast, filamentous forms may mask the β-glucans by mannans and affect certain β-glucan-mediated responses (17, 134, 135). However, *C. albicans* germ tubes can be resognized by Dectin-1 in Syk-dependent mechanism and initiate Th-17 response (136).

Dectin-1 recognition of *C. albicans* or its ligand β-1,3-glucan initiates several distinct immune responses. β-1,3-glucan-Dectin-1 binding leads to NFκB-mediated ROS production and proinflammatory cytokine release, such as IL-12, TNFα, and IL-6, *via* the Syk-CARD9 pathway in mouse DCs and macrophages, as well as in human intestinal cells. The response is enhanced in co-operation with TLR-2 (137–141). Interestingly, *C. albicans* activation of Dectin-1 can also result in anti-inflammatory

responses, like IL-10 release by macrophages and peripheral blood mononuclear cells (PBMCs) or the production of IL-1 receptor antagonist (IL-1Ra) (142–144). Furthermore, ligation of Dectin-1 on APC by *C. albicans*, but also by other fungi and even the endogenous ligand galectin-9 drives T cell differentiation into a TH2/TH17 response (145–148). PKCδ is essential for CARD9-dependent NFκB activation (149). *C. albicans* also induces mast cell activation in rat and mice that leads to a differential cytokine production depending upon the fungal morphology, and induces phagocytosis and nitric oxide production in a TLR-2 and Dectin-1-dependent manner (150, 151). A similar co-operation of Dectin-1 with TLR-2 and TLR-4 can be observed in human mononuclear cells, PBMCs, and macrophages on stimulation with β-1,3-glucan (152, 153). The Dectin-1-Syk-CARD-9 pathway can also activate IRF5 to produce IFN-β (154) or ERK to generate proinflammatory responses against *C. albicans* (155). Moreover, Dectin-1 also mediates the β-1,3-glucan-medidated opsonization-independent phagocytosis by human neutrophils and retinal microglia (156, 157). The Syk-dependent pathway is also involved in β-1,3-glucan-containing phagosome maturation and recruitment of TLR-9 in RAW cells (158). Additionally, Dectin-1 involvement with *C. albicans* activates many other signaling pathways. Dectin-1 binding with *C. albicans* can activate NFAT transcription factors induce IL-2, IL-10, and IL-p70 release in collaboration with TLR-2 in mouse DCs (15, 159). β-1,3-glucan-induced human DCs activate NFκB *via* Syk as well as Raf-1 *in vitro*. In fact, Raf-1 activation represses Syk-induced RelB activity, although not completely, and increases p65 transactivation activity to induce IL-12p40 and IL-1β production (160). Several studies based on human and mouse cell lines have demonstrated that Dectin-1 is important in activating the inflammasomes such as the noncanonical caspase-8 inflammasome that promotes Th-17 responses which are essential for antifungal immunity (136, 161–166). Th17 responses are important against cutaneous infection, while Th1 responses are directed against systemic infection (134). Dectin-1 also co-operates with other CLRs such as SIGNR1 in mouse macrophages enhancing the oxidative burst against *C. albicans* (167). Some studies have also demonstrated a Dectin-1-dependent CR3 activation on mouse neutrophils and subsequent killing of *C. albicans* by these cells (168, 169). Human neutrophils release neutrophil extracellular traps in response to *C. albicans in vitro*, triggered by the ROS production on recognition of β-glucan by Dectin-1 and CR-3 (170, 171). Interestingly, Dectin-1 stimulation with *C. albicans* or β-1,3-glucan can also generate certain immunomodulatory responses, e.g., IL-10 production and reduction in ROS production *via* SHIP-1 activation in mouse GM-CSF-derived bone marrow cells (172). Additionally, *C. albicans*-Dectin-1 engagement induces human as well as mouse granulocytic myeloid-derived suppressor cells to dampen the pathogenic hyperinflammatory NK and Th17 responses (173). Moreover, a recent study has also demonstrated a role of Dectin-1 in adaptive immunity by controlling CD4+ T cell responses in the murine gut (174). All these results indicate how *C. albicans* can influence the immune responses by engaging the same receptor on different cell types.

Mouse knockout studies have shown contrasting results. Dectin-1 deficient mice display defective macrophage activation with impaired subsequent inflammatory responses and present enhanced fungal burden and dissemination after *C. albicans* infection (163, 175). However, further mouse studies implied that Dectin-1 deficiency probably plays a minor role in systemic *Candida* infection but may control the mucosal infections (176). The differences in the results may be attributed to different mouse and *C. albicans* strains used in experiments (177). Dectin-1-deficient mice also are more susceptible to *C. glabrata* infections and show impaired inflammatory responses (178).

Polymorphisms in Dectin-1 have been studied with respect to *Candida* infections. The first study to report an early-stop-codon mutation Y238X in a family with recurrent vulvovaginal candidiasis (RVVC) among four women demonstrated that the monocytes and neutrophils from homozygotes lack Dectin-1 expression and are defective in cytokine production such as IL-17 upon *C. albicans* stimulation *in vitro*. However, phagocytosis and killing of fungi is normal (179, 180). Another report demonstrated that heterozygotes for Y238X receiving hematopoietic stem cell transplantation display an increased incidence of gastrointestinal *Candida* colonization. The monocytes of homozygotes show less IL-1β production and lack of TLR-2-Dectin-1 synergism complementing the previous *in vivo* and *in vitro* studies describing the role of Dectin-1 in *Candida* infections (181). In an HIV-infected African population, a mutation I223S has been associated with a lower IFNγ response to *C. albicans* stimulation of whole blood and tends to provide protection against oropharyngeal candidiasis (182).

The hyphal form of *C. albicans* can be recognized by Dectin-2, which binds to high-mannose structures such as α-mannans in a cation-dependent manner and is expressed predominantly on macrophages and DCs (**Table 1**) (11, 33). Several studies in mouse DCs and macrophages have demonstrated that Dectin-2 mediates its signaling *via* the ITAM-bearing adapter FcRγ and the Syk-CARD9 pathway but the subsequent responses seem to differ depending upon the cell type, fungal morphology, and methodologies (11, 34, 183, 184). Nevertheless, *C. albicans* recognition by Dectin-2 can induce phagocytosis, proinflammatory cytokine production, such as IL-6, IL-23, TNFα, and IL-12 as well as protective Th-17 responses (34, 183). In addition, Dectin-2-deficient mice show decreased survival and high kidney fungal burden after 10 days of infection with *C. albicans* (34). Indeed, it was later shown in two independent studies that Dectin-2-deficient mice are also susceptible to *C. albicans* and *C. glabrata* systemic infections, showing high fungal burdens in kidneys and reduced neutrophilic phagocytosis (185, 186). Similar to Dectin-1, Dectin-2 can also induce type I IFN responses in mouse macrophages and DCs by activating IRF5 in response to *C. albicans* (154). PLCγ2 is essential for Dectin-2-mediated NF-κB, MAPK and ROS activation in mouse macrophages when infected with hyphal *C. albicans* (187). Zhu et al. showed that Dectin-2 and MCL (Dectin-3) heterodimers recognize *C. albicans* α-mannans more effectively than either receptor alone and that MCL-deficient mice are highly susceptible to systemic candidiasis (70). However, most of these studies have been performed in mouse models and provide a picture of Dectin-2 and MCL roles in murine *Candida* infections, and studies regarding

their role in the human host and the impact of mutations in the human receptors will be needed in order to complete the picture.

Mannose receptor is a mannan-binding lectin found on phagocytic cell surfaces and recognizes *C. albicans* α-mannans (**Table 1**) (39). Early studies have demonstrated the involvement of MR in cytokine release, non-opsonic phagocytosis, and killing of *Candida* spp. by phagocytic cells (**Figure 1**) (40, 188, 189). Human DCs phagocytose and kill *Candida via* MR leading to subsequent responses such as Th1 immunity and ROS production (39, 190–192). Dectin-1 engagement with *C. albicans* on mouse macrophages induces surface MR shedding which could be the reason for downregulation of MR surface expression observed on rat macrophages after *C. albicans* ingestion (193, 194). Dectin-1 was later shown to be the main phagocytic receptor, while MR is recruited to phagosomes in mouse macrophages in later stages and mediates the secretion of immunomodulators such as TNFα and MCP-1 (195). Indeed, MR-deficient mouse macrophages are able to take up and phagocytose *C. albicans* normally (195, 196). However, in human phagocytic cells, MR induces Th17 responses upon stimulation with *C. albicans in vitro* by inducing IL-1β and prostaglandin E2 production, which is enhanced by Dectin-1/TLR-2 synergism (162, 197–199). Neumann et al. reported the formation of unique MR-induced pseudopodial protrusions called fungipods in human monocyte-derived DCs in response to *C. albicans* yeast, which may have role in fungal phagocytosis. This response is species-specific with *C. parapsilosis* showing stronger fungipod formation compared to *C. albicans* and *C. tropicalis* (41). In fact, the innate immune recognition of *C. parapsilosis* complex and *C. albicans* by human PBMCs differ with respect to the receptors involved and the induced cytokine production; for example, MR is important for TNFα and IL-1β production upon *C. parapsilosis* stimulation (200). Interestingly, IFNγ stimulation of human monocyte-derived DCs and macrophages increases the candidacidal activity of these cells by increasing non-opsonic phagocytosis and ROS production which is related to a reduced expression of MR (201, 202). So far, no genetic studies have been performed to understand the significance of MR polymorphisms in *Candida* infections.

Candida albicans yeast and hyphae are also recognized by the collectin MBL (56, 203). Several studies have demonstrated the binding of *Candida* spp. to MBL followed by activation of the complement system and subsequent opsonophagocytosis of fungi by phagocytic cells *in vitro* (204–206). Li et al. demonstrated MBL-dependent opsonophagocytosis of *C. albicans* by human neutrophils but without complement activation. This response was coupled with intracellular Dectin-1-dependent ROS production (207). Parenteral administration of MBL increased the resistance of mice in a model of disseminated candidiasis (56). In fact, mice deficient in MBL-A and MBL-C (mice homologs to human MBL) are more susceptible to systemic *Candida* infection (208). MBL is expressed in the mouse gut and its blocking or elimination leads to increased *C. albicans* colonization (209). MBL can also modulate the *C. albicans*-triggered TLR-generated proinflammatory signals by THP-1 cells (210). MBL concentrations are greatly affected by promoter polymorphisms in the *MBL2* gene and the resulting lower MBL levels are linked to the risk to develop several infectious diseases (128, 211, 212). Reduced levels of MBL were observed in the cervicovaginal lavage of RVVC patients, while the levels were higher in VVC patients compared to healthy controls (213–215). Moreover, MBL deficiency is also associated with the development of abdominal yeast infection in peritonitis patients (216). However, MBL serum levels and genotypes were not associated with intra-abdominal candidiasis in a Swiss cohort (217). The RVVC patients also have a higher frequency of *MBL2* mutations compared to both VVC and healthy groups (214). Furthermore, the *MBL2* codon 54 allele B is associated with a higher susceptibility to RVVC as observed in Belgian, Latvian, and Brazilian women (215, 218, 219). A recent meta-analysis of five different studies also concluded the correlation of allele B of codon 54 to be associated with both RVVC and VVC (220). Only a couple of studies have addressed the polymorphisms in components of MBL complement pathway in development of invasive fungal infections (221, 222). The polymorphisms in MBL complement pathway components have been associated with other infectious diseases as well, such as tuberculosis and leprosy (223, 224) and further studies exploring the effects of mutations in complement proteins on the pathogenesis of fungal infections are still needed.

Another CLR, DC-SIGN, can recognize the N-linked mannans in the *C. albicans* cell wall (39, 225). It mediates the internalization of conidia by human DCs, which are abundantly present in mucosal tissue (**Table 1**) (61), although human DCs exhibit less-efficient phagocytic activity compared to monocytes and macrophages (226). Gringhuis et al. showed that *C. albicans* stimulation can modulate TLR-dependent pathways by Raf-1 activation in human DCs. There is also evidence for possible anti-inflammatory effects upon DC-SIGN ligation. However, data so far are derived from non-fungal ligands (227). The fungus-induced IL-10 production is mediated through coactivation of DC-SIGN and TLR signaling pathways (228). The mouse homolog of DC-SIGN, SIGNR1 works in co-operation with Dectin-1 and TLR-2 in mouse macrophages to induce responses such as oxidative burst and TNFα production *in vitro* (167, 229). Knockout mouse studies and genetic studies may enlighten us more regarding the importance of this CLR in these fungal infections.

Candida albicans is also recognized by Mincle, another CLR expressed on several immune cells (**Table 1**), although the related ligand has not yet been discovered (230). An *in vivo* study demonstrated a non-redundant role of Mincle against *Candida* infection as Mincle-deficient mice were highly susceptible to systemic candidiasis. Mincle induces TNFα production in mouse macrophages upon *C. albicans* hyphae stimulation *in vitro* but is not important for phagocytosis, indicating that Mincle has a role in initial macrophage binding and early responses to the fungus (66). A similar effect was observed in human monocytes where the stimulation with *C. albicans* yeast leads to TNFα production but is related to poor yeast uptake. However, human neutrophils expressing Mincle show a fungicidal activity correlated with phagocytosis of the yeast (231). Taken together, more studies are needed to discover the *C. albicans* ligand(s) for Mincle and the related pathways as well as *in vitro* and *in vivo* immune responses and further work is necessary to elucidate the role of Mincle polymorphisms in fungal infections.

CR3 is an important CLR expressed on neutrophils, macrophages, NK cells, and monocytes and is involved in adhesion and phagocytosis of *C. albicans* (5, 73, 232). CR3 has a role in *C. albicans* hyphae recognition as the human lymphocyte adhesion of *C. albicans* hyphae was abrogated upon blocking CR3 with monoclonal antibodies (233, 234). Soloviev et al. showed that *C. albicans* releases a soluble CR3-binding mannoprotein called pH-regulated Ag 1 (Pra1), which mediates CR3-dependent adhesion and migration of THP-1 cells and neutrophils toward *C. albicans* (74, 235). Pra1 is expressed highly and exclusively on *C. albicans* hyphae (236). Soluble Pra1 was found to be beneficial for fungal survival as it inhibits the human neutrophil activation upon stimulation with Pra1 overexpressing *C. albicans* hyphae (235). Additionally, a CR3 knockout mouse study demonstrated that these mice show an increased susceptibility toward *C. albicans* systemic infection (168, 237) and their neutrophils display impaired adhesion, migration, and oxidative burst when challenged with *C. albicans in vitro* (237). Several studies have shown β-glucan to be another potential ligand for CR3 (238–240). Dectin-1 and CR3 co-localize on yeast *C. albicans* phagocytic cups in mouse peritoneal macrophages (195). Further studies support the interaction of Dectin-1 and CR3 in *C. albicans* infection as mentioned earlier. Dectin-1 activates CR3 for recognition of *C. albicans* yeast components and together they induce neutrophil cytotoxic responses in mice (168, 169).

Langerhans cells (LCs), found in epidermis and mucosal linings, express Langerin, which can bind to β-glucans and recognizes Candida spp., including *C. albicans*, *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, and *C. krusei*, among others, *in vitro* (81, 241, 242). Some studies have shed light on the roles of LCs in *Candida* infections (243, 244), but specific studies regarding the role of Langerin are lacking.

Taken together, *Candida* is recognized by a number of CLRs, each of which is able to generate fungal-specific immune responses. While Dectin-2 and CR3 can recognize and respond to the fungal hyphae, Dectin-1, MR, and DC-SIGN mainly recognize the conidial forms. For Mincle, MCL and Langerin little is known and they need to be further investigated for their roles in *Candida* infections. Moreover, more studies need to focus on the genetic component of the effect of CLRs on *Candida* infections.

Cryptococcus spp.

Cryptococcosis is a worldwide distributed and invasive fungal infection that is caused by species of the genus *Cryptococcus*. Nearly 100 species have been described within this genus so far, but *Cryptococcus neoformans* and *C. gattii* species are considered to be the only disease-causing fungi (245). Although cryptococcosis is predominantly a disease of immunocompromised patients (AIDS-defining illness), a recent outbreak showed the capacity of some lineages of the fungus to act as primary pathogens in healthy individuals (246). Within the lung, *Cryptococcus* spp. can cause pneumonia in immunosuppressed patients, and the latent infection can then disseminate to other tissues, most particularly the central nervous system (CNS), where this fungus causes an infection of the meninges accompanied by elevated intracranial pressure and without a rapid treatment it becomes fatal (246).

The interaction of *Cryptococcus* with CLRs is poorly understood. Initial data demonstrated that *C. neoformans* binds to soluble collectin MBL and the ingestion of the acapsular form is inhibited by both soluble mannan and β-1,3-glucan, showing that ingestion of acapsular *C. neoformans* takes place *via* mannose and β-glucan receptors in murine macrophages (247).

The role of Dectin-1 is still controversial, since no significant differences were observed in the clinical course and cytokine production between Dectin-1-deficient and control mice in a cryptococcosis model (248), but another study found that *C. neoformans* spores are phagocytosed by murine AMs *via* Dectin-1 (18). The role of Dectin-2 is also poorly understood. Some results show that it may not be required for the production of Th1 and Th17 responses, proinflammatory cytokines or for the clearance of *C. neoformans* in Dectin-2 knockout mice (249). Dectin-3 seems to have a role as it was demonstrated that human and murine pDCs have a direct Dectin-3-dependent anti-cryptococcal activity by inhibiting the growth of *C. neoformans via* ROS production (71). A *Cryptococcus*-specific ligand for this receptor has not been described yet.

Mannose receptor also has a role in *Cryptococcus* infection. After a pulmonary infection with *C. neoformans*, MR knockout mice died significantly earlier than wild-type mice and had higher lung fungal burdens (250). This receptor was required for the presentation of *C. neoformans* antigens to T lymphocytes by primary DCs, since blocking this receptor reduced both uptake of *C. neoformans* and lymphocyte proliferation (251). Some data suggest that mannoproteins, secreted by *C. neoformans,* might be the ligands for MR, as T cell stimulation is inhibited either by competitive blockade of MR in APCs or by removal of carbohydrate residues from mannoproteins. These results imply a capacity of mannoproteins to bind MR and to be processed by APCs to stimulate primary T cells (42). However, multiple receptors on DC could recognize this ligand, since DC-SIGN was also determined to have an affinity for mannoproteins. Further, MR and DC-SIGN both colocalize with mannoproteins, supporting a role for each in mannoprotein capture (62).

The pulmonary surfactant proteins, SP-A and SP-D bind to both encapsulated and acapsular *C. neoformans* (49, 252) and SP-D binds to the high-molecular weight polysaccharide glucuronoxylomannan and mannoproteins on the fungal cell wall (50). However, some data suggest that these receptors actually increase the susceptibility to *C. neoformans* infection. SP-A inhibits the IgG-dependent phagocytosis of *C. neoformans* by AMs and SP-A$^{-/-}$ mice exhibit wild-type vulnerability to *C. neoformans*; SP-D$^{-/-}$ mice are even protected during *C. neoformans* infection and display decreased fungal burden compared to wild-type mice. SP-D$^{-/-}$ AMs also demonstrate an enhanced ability to kill *C. neoformans* cells (253, 254). Indeed, SP-D increases vulnerability to *C. neoformans* infection by stimulating *C. neoformans*-driven pulmonary IL-5 and eosinophil infiltration (255). SP-D may also play a role in protecting *C. neoformans* cells during the early stages of infection by opsonization. It was found that SP-D increases phagocytosis of hypocapsular *C. neoformans* by murine macrophages and enhances fungal survival allowing to gain access to specific intracellular compartments where it can grow (256). Another study reports that both, the presence of

capsules and a wild-type cell wall design, prevent MBL binding to *C. neoformans* (257).

Last but not the least, complement activation by *Cryptococcus* spp. was demonstrated in the presence of MBL *in vitro* (57). A *Cryptococcus*-specific ligand for this receptor has not been described up to now.

Given that Dectin-1, Dectin-2, SP-A, and SP-D studies showed controversial results and their interactions with *Cryptococcus* are poorly understood, further studies are necessary.

Mucorales spp.

Mucormycosis is the second most-common form of invasive mold infections. The disease is characterized by vessel thrombosis and tissue necrosis resulting from extensive angioinvasion and further dissemination (258). The members of the *Mucorales* order of Zygomycetes are among the leading causes of mucormycosis in immunocompromised individuals apart from more common fungal genera, such as *Candida* or *Aspergillus*, with mortality rates ranging from 50 to 100% (259). Among *Mucorales* spp., although rare, *Rhizopus oryzae* accounts for 70% of mucormycosis infections. Mainly phagocytotic cells play an important role in restricting the infection (260). The studies on mechanistic details of fungal recognition by CLRs and their role in pathogenesis are still lacking. One study reported that patients with mucormycosis showed reduced expression of Dectin-1 on monocytes compared to healthy controls (104). However, further investigations into the role of C-type lectins and their polymorphisms in this infection are needed.

Pneumocystis spp.

The genus *Pneumocystis* includes a variety of ubiquitous fungi that colonize and infect several mammalian host species. The species *P. jirovecii* particularly infects humans, whereas *Pneumocystis carinii* (*P. carinii*) and *P. murina* are associated with rats and mice, respectively. In the immunocompromised host, *Pneumocystis* pneumonia (PCP) is fatal if untreated. However, infection of an immunocompetent host can result in a self-limited mild or subclinical lower respiratory tract infection (261).

The first studies demonstrated an interaction of *Pneumocystis* cell wall isolates with macrophage β-glucan receptors, which induced a potent stimulation of TNF-α release in rat AMs in response to *P. carinii* (262, 263). During *P. carinii* infection, the expression of Dectin-1 is upregulated in macrophages of immunocompetent rat models (264). According to some studies, Dectin-1 is required for the protection against *P. carinii* infection, since Dectin-1-knockout mice are more sensitive to infection than infected wild-type mice, and production of ROS is completely abolished in Dectin-1-knockout macrophages incubated with *P. carinii* (176). Probably, the expression levels of Dectin-1 in AMs are under the control of the transcription factor PU.1 during a PCP infection, where the GM-CSF appears to play a major role in the regulation of PU.1 expression (265). Phagocytosis of *P. carinii* and generation of hydrogen peroxide by murine AMs is mediated by Dectin-1, since the blockage of Dectin-1 inhibits the binding and killing of *P. carinii* (266). The binding of Dectin-1 to *Pneumocystis* was tested by creating recombinant Dectin-Fc fusion proteins which bind *P. carinii* and enhance murine macrophage-dependent killing. These findings demonstrate that Dectin-1 binds β-glucan from *Pneumocystis,* enhancing host recognition and clearance of *P. carinii* (19). *P. carinii* β-glucan cell wall component challenge of rat alveolar epithelial cells resulted in a prominent nuclear translocation of p65 NF-κB with a subsequent increase in MIP-2 and TNF-α mRNA production. However, rat alveolar epithelial cells do not require Dectin-1 for MIP-2 production, which rather involves the participation of the alternative lactosylceramide β-glucan receptor (267, 268).

Pneumocystis carinii also enhances soluble MR production in human and murine macrophages (269). In human AMs, phagocytosis of *Pneumocystis* is mediated through MR and depends on Cdc42 and especially RhoB activation (270). *Pneumocystis* also stimulates NF-κB nuclear translocation in human AMs, which is mediated primarily through MR (43). A recombinant soluble MR-Fc fusion protein binds *P. carinii* and leads to an increased uptake by hPMNs (271). The role of MR was confirmed by the fact that binding and uptake of cultured *P. carinii* by human and rat AMs is reduced 90% by using competitive inhibitors of MR, emphasizing the role of the AMs in the first-line host defense (272). Other studies suggest that a reduced AM MR-mediated binding of *P. carinii* may contribute to the susceptibility of HIV-infected individuals to this pathogen (273). However, it was demonstrated that IL-8 release by human AMs following the stimulation with *Pneumocystis* requires the co-expression of MR and TLR-2, since the IL-8 release is reduced significantly upon blocking of TLR-2 and silencing of MR gene (274). These results support the idea that MR on human AMs may suppress the production of proinflammatory cytokines and may serve to regulate the innate inflammatory responses to *Pneumocystis* infection in the lungs (275). A *Pneumocystis*-specific ligand for MR has not been described up to now.

Some results indicate that SP-A and SP-D can modulate the virulence of *P. murina* and *P. carinii* during development of infection in SP-D- and SP-A-deficient and immunosuppressed mice. They attenuate the production of proinflammatory cytokines and ROS and RNS, indicating that both receptors are local effector molecules in the lung host defense against *Pneumocystis in vivo* (276–280). These results are supported by the fact that SP-A and SP-D can bind *P. carinii*, acting as opsonins and enhancing their phagocytosis by AMs (281–284). However, some data suggest that the increased SP-A and SP-D mediated aggregation of *P. carinii* fungal particles interferes with AM recognition and thus the SPs may contribute to the pathogenesis of *P. carinii* pneumonia (285–287). This view is supported by the fact that SP-A in immunosuppressed mice acts as a therapeutic agent in the beginning of *Pneumocystis* infection, but not in the middle or late stages of the infection (288). SP-D strongly interacts with gpA, the main glycoprotein antigen on the surface of *P. carinii*. The interaction of SP-D with *P. carinii* gpA is mediated by the carbohydrate recognition domain (CRD) of this collectin (51, 289). Similarly, the CRD of SP-A mediates binding to the main surface glycoprotein gp120 of *P. carinii* (52, 290).

Binding of MBL to *P. carinii* is followed by the activation of the respiratory burst, indicating that the MBL in serum has opsonizing properties and might contribute in controlling fungal spread

from the lungs (58). A *Pneumocystis*-specific ligand has not been described up to now for this receptor.

Mincle binds whole *P. carinii* and a surface glycoprotein called MSG/gpA, a *Pneumocystis* cell wall component, which is expressed at enhanced levels during infection (67). Moreover, Mincle$^{-/-}$ mice exhibit significantly higher *P. murina* burdens with elevated levels of TNF-α, IL-6, and IL-1Ra during infection, indicating that Mincle functions as an important signaling receptor in host defense against *Pneumocystis* infection (67).

Little is known about polymorphisms affecting *Pneumocystis* recognition, however, one study analyzed 53 HIV patients having CD4 counts <200 μL, in order to find a correlation between MBL and PCP. Of these 53 patients, 30 had PCP at admission, and 23 did not. Genotypes related with a low production of MBL were significantly more common in the PCP group than in the non-PCP group. Serum MBL levels were significantly higher in the non-PCP group. Genetic variations influencing MBL production also affect the susceptibility to PCP in HIV-advanced infection patients, and may be considered as a risk factor for PCP (291).

Overall, CLRs seem to be of importance for orchestrating the *Pneumocystis*-induced immune response. However, *Pneumocystis* cannot easily be propagated in culture, which has delayed the understanding of its pathobiology. Efforts to study *Pneumocystis* have been greatly limited by the inability to maintain *ex vivo* culture of the organism. Early attempts to isolate and propagate *P. jirovecii*, have been moderately successful, however, none of these models garnered sufficient recognition to become a standard method for the isolation of *Pneumocystis* (292). Nonetheless, studies of organisms isolated directly from the infected lung of patients or immunosuppressed research animals still allow for some insight into the pathobiology of *Pneumocystis* (293).

ENDEMIC DIMORPHIC MYCOSES

Coccidioides spp.

There are two species of *Coccidioides* (*C. immitis* and *Coccidioides posadasii*) that cause human disease. They have similar phenotypes and pathogenicities, but differ in genotype and geographic distribution. They are the etiologic agents of coccidioidomycosis, which ranges from asymptomatic infections to pneumonia and severe disseminated disease. These organisms are found in the soil, especially in low-moisture environments, so preventing exposure can be difficult due to the ubiquitous risk of dust inhalation by individuals living in endemic areas. The pathogenesis of coccidioidomycosis is complex and can be asymptomatic but also cause extrapulmonary dissemination (294).

The reasons of the complexity of the pathogenesis of coccidioidomycosis are not well understood; however, some data suggest the main involvement of Dectin-1. Some results suggest that an alternative splicing of the Dectin-1 gene enhances the susceptibility of C57BL/6 mice to coccidioidomycosis, regulating the cytokine responses of macrophages and mDCs to spherules, the pathognomonic structure of this fungus (295). RAW 264.7 macrophages overexpressing Dectin-1 produced more TNF-α than control macrophages in respond to *C. posadasii* spherules. Also, macrophages overexpressing Dectin-1 and activated with purified β-glucan from *C. posadasii* spherules produced a significantly higher level of TNF-α than control macrophages, indicating a role of β-glucan from *C. posadasii* as a ligand for Dectin-1 (20). Moreover, Dectin-1 activation is essential to leading the adaptive immune response toward Th1 and Th17 pathways, thus leading to the resolution of infections in mice (35, 296).

Other results suggest that there is an association between low serum MBL levels and symptomatic coccidioidomycosis, but in order to understand the role of MBL in the pathogenesis of this fungal disease, further studies are necessary (297). SP-A and SP-D also bind coccidioidal antigens (53). Deficiencies of MR and Dectin-2, either alone or in combination, affect cellular responses to formalin-killed spherules *in vitro* but do not make C57BL/6 mice more vulnerable to pulmonary coccidioidomycosis (298). A *Coccidioides*-specific ligand for these receptors has not been described up to now.

In conclusion, further studies are necessary to elucidate interactions of *Coccidioides* with CLTRs, since few receptors and no ligands have been studied. However, Dectin-1 seems to have an important role in this infection.

Histoplasma spp.

Fungi of the genus *Histoplasma* cause histoplasmosis and are found throughout the world, but are most common in North America and Central America. *Histoplasma capsulatum* is a member of this group of fungal pathogens that cause respiratory and disseminated disease in mammals. It grows as a saprobic conidia-producing mycelium in the environment, and when the aerosolized mycelium fragments and conidia are inhaled, they reach the lower respiratory tract causing disease even in immunocompetent hosts (299).

Little is known about the receptors recognizing *Histoplasma* and its signaling response. However some CLRs, such as Dectin-1, Dectin-2, and some collectins are involved in *Histoplasma* immunity (**Table 1**). Dectin-1 and Dectin-2 exert several contributions to the development of antifungal Th1 and Th17 cells and vaccine resistance in mice against *H. capsulatum* (35, 300). CR3 and Dectin-1 act together to induce murine macrophages to TNF and IL-6 responses through a Syk-JNK-AP-1-dependent mechanism (75). Some data show that CR3 participates in phagocytosis and cytokine responses, but Dectin-1 takes part in cytokine production only on murine macrophage (21). *Histoplasma* pathogenic yeast cells secrete Eng1, a β-glucanase that hydrolyzes β-(1,3)-glycosyl linkages, which reduces levels of surface-exposed β-glucans on yeast cells, thereby enabling *Histoplasma* yeasts to escape detection by Dectin-1. *Histoplasma* yeasts deficient for Eng1 show an enhanced binding to Dectin-1 and an increased TNF-α and IL-6 production in murine macrophages and DCs (301, 302). Also, SP-A and SP-D demonstrate potent antifungal properties, since they cause a dose-dependent decrement in yeast viability, which is associated with an increase in the permeability of the yeast cells. Mice lacking SP-A manifest a modestly higher fungal burden in lungs than wild-type littermates (54). A *Histoplasma*-specific ligand for these receptors has not been described up to now.

Together, these studies indicate minor roles for CLRs in the control of *Histoplasma* infections, but further studies are needed to understand the significance of CLRs in *Histoplasma* infections.

Paracoccidioides spp.

Paracoccidioides spp. is the causal agent of paracoccidioidomycosis (PCM), a systemic mycosis endemic to Latin America. It comprises two species: *Paracoccidioides brasiliensis* and the recently described *P. lutzii* (303). Manifestations of PCM include subclinical or asymptomatic infection. The symptomatic disease causes an acute/subacute or a chronic form, the latter involving the lungs as well as other organs. PCM is acquired after inhalation of infectious propagules in the environment, leading to a primary pulmonary infection (303).

Few studies have investigated the role of CLRs on *Paracoccidioides* infection. Human monocytes display a decrease in Dectin-1 expression as soon as 30 min after stimulation with *P. brasiliensis* (22). There is a trend toward an increased Dectin-1 mRNA expression in response to *P. brasiliensis* and this receptor is able to induce a balanced production of TNF-α, IFN-γ, IL-12, and IL-10 in human neutrophils and monocytes (22, 304, 305). By binding to Dectin-1, *P. brasiliensis* induces neutrophil extracellular trap (NET) release that is responsible for trapping yeast cells, promoting their immobilization, as well as contributing to their extracellular killing (306). Moreover, the fungal infection of Dectin-1$^{-/-}$ mice results in enhanced tissue pathology and mortality rates. The deficiency of Dectin-1 has also reduced the production of Th1, Th2, and Th17 cytokines and the activation and migration of T cells to the site of infection (307). Altogether, these results suggest the participation of Dectin-1 in *P. brasiliensis* recognition, internalization, and consequent activation of the immune response against the fungus. A *Paracoccidioides*-specific ligand for Dectin-1 has not been described up to now, however, it is known that Dectin-1 binds glucan structures that are distributed in a wide range of fungal species.

The gp43 glycoprotein is the main antigenic component secreted by *P. brasiliensis*. gp43 binds to TLR2, TLR4, and MR receptors and all three receptors influenced a high production of IL-10 and TNF-α in human monocytes (44). The specific blockade of MR and CR3 impaired fungal recognition and modified the production of cytokines (308). The CR3 receptor may participate in phagocytosis of *P. brasiliensis* conidia through both opsonic and non-opsonic mechanisms, since treatment of murine macrophages with anti-CR3 and α-methyl-d-mannoside, a competetive inhibitor of the binding of mannose, decreased phagocytosis of *P. brasiliensis* (76). In the same way, the mannose-binding lectin complement pathway was demonstrated to play a key role in complement activation by *P. brasiliensis* (309). A *Paracoccidioides*-specific ligand for this receptor has not been described until now.

Overall, interactions between host immune cells and *Paracoccidioides* spp. are mediated by the recognition of Dectin-1 which controls internalization by phagocytes as well as lymphocyte proliferation during *P. brasiliensis* infection. However, further studies are necessary, since few receptors and no ligands have been studied.

Penicillium spp.

Penicillium species are rarely considered as human pathogens except *Talaromyces (Penicillium) marneffei,* which can cause opportunistic infections, called penicilliosis, in immunocompromised patients, especially in HIV positive persons, but also in old and new born (310). The infection is most prevalent in South-east Asia and is characterized by symptoms, such as weight loss and fever, skin lesions, generalized lymphadenopathy and hepatomegaly, and respiratory signs such as hemoptysis (310, 311). The main virulence factor of *T. marneffei* is its temperature-dependent dimorphic growth, owing to which it grows as mycelium at 25°C while at 37°C it grows as yeast (311). *In vitro* experiments have shown that *T. marneffei* can be recognized by Dectin-1, DC-SIGN, and MR (**Figure 1**) (23, 45, 63). Koguchi and colleagues demonstrated that blocking MR with antagonists reduces the osteopontin production from PBMCs upon *T. marneffei* stimulation and suggested a mannoprotein as the possible ligand (45). Indeed, MR was later found to be involved in adhesion and phagocytosis of the fungus by human monocyte-derived DCs, while DC-SIGN only mediated adhesion (63). IL-12p40 production by bone marrow-derived dendritic cells (BMDCs) upon stimulation with *T. marneffei* is abrogated in Dectin-1 knockout mice (23). Together, these studies suggest that CLRs modulate the immune response to *T. marneffei*; however, further studies are needed to dissect the *in vivo* role of CLRs and their polymorphisms in *T. marneffei* infections.

OTHER FUNGI CAUSING FREQUENT INFECTIONS

Trichophyton spp.

Dermatophytosis is one of the most common mycoses worldwide. Compromising keratinized tissues and characterized by establishing chronic inflammatory processes, it is highly resistant to standard antifungal therapies. The main etiological agent in humans is *Trichophyton rubrum*. It establishes infection after being inoculated in the host tissue, where it survives through the degradation of dead cells, consuming keratin and other host components (312).

Only few studies analyzed the roles of CLRs in *Trichophyton* spp. infections. Dectin-1 is involved in mediating inflammation induced by trichophytin, a *T. mentagrophytes* antigen with β-glucans and zymosan as the main components (24). *T. rubrum* hyphae are recognized by Dectin-1 and Dectin-2 in murine DCs, triggering production of inflammatory cytokines, mainly IL-1β and TNF-α. This inflammatory process is able to promote the clearance of the pathogen *in vivo* without the involvement of lymphocytes. Even though IL-17 is induced, it is not essential for infection resolution (313). Moreover, trichophytin enhances the Dectin-1 expression in mice, and the blockage of Dectin-1 inhibits the increased IFN-γ production in cervical lymph node cells from mice *in vitro* (314). Dectin-2 preferentially binds to hyphae of various fungal species, including *T. rubrum* (11).

Malassezia spp.

The fungal genus *Malassezia* comprises yeast species that are part of the normal skin microbiota. However, *Malassezia* spp. can be

involved in skin disorders, such as pityriasis versicolor, seborrheic dermatitis, atopic eczema, and folliculitis (315). *Malassezia* spp. may also cause invasive infections in infants and in immunocompromised individuals. The clinical spectrum ranges from asymptomatic infections to life-threatening sepsis and disseminated diseases (316).

Various *Malassezia* spp. (*M. japonica*, *M. slooffiae*, *M. furfur*, and *M. sympodialis*) induce a Dectin-1-dependent NLRP3 inflammasome activation with a subsequent IL-1β secretion in human APCs. This activation is dependent on Dectin-1, since the blocking of Dectin-1 decreased the IL-1β secretion upon *M. furfur* exposure (25). A *Malassezia*-specific ligand has not been described until now, but β-1,3-glucan is most likely present on their surface.

Several *Malassezia* spp. (*M. pachydermatis* and *M. furfur*) are recognized by Mincle and Dectin-2 through different ligands. A glyceroglycolipid and mannosyl fatty acids linked to mannitol are two Mincle ligands, and an O-linked mannobiose-rich glycoprotein is a ligand for Dectin-2. Both receptors cooperatively contribute to the TNF and IL-10 production in BMDCs from mice in response to *Malassezia spp* (36). Other results indicate that Mincle also recognizes *Malassezia* spp. (*M. pachydermatis*, *M. dermatis*, *M. japonica*, *M. nana*, *M. slooffiae*, *M. sympodialis*, *M. furfur*, and *M. pachydermatis*) through α-mannose but not mannan. Mincle may recognize a particular distribution of α-mannosyl residues on *Malassezia* spp. and use this to discriminate them from other fungi, inducing inflammatory responses (TNFα and IL-10) in murine macrophages (317).

Langerin plays a role in pathogen recognition by facilitating pathogen uptake and processing for antigen presentation (318). Langerin on primary LCs isolated from human epidermis interact strongly with *M. furfur via* β-glucan structures and the interaction can lead to the phagocytosis of the fungus (81). *M. furfur* is also recognized through MR and CR3 on THP-1 cells (77).

Fonsecaea spp.

Fonsecaea spp. are found in soil and plants. Although they are considered a worldwide-distributed fungus, they are frequently found in tropical regions (319). *Fonsecaea pedrosoi* is a frequent causative agent of chromoblastomycosis (or chromomycosis), a chronic fungal disease limited to the skin and subcutaneous tissues. Initial lesions are habitually erythematous papules, which progressively enlarge to morphologies, such as verrucous nodules, cauliflower-like tumors, and psoriasis-like plaques (319).

Dectin-1, Dentin-2, and Mincle have a role in the recognition of this fungus (**Table 1**). Murine macrophages stimulated by co-culturing with muriform cells (the parasitic form of *F. pedrosoi*) show an elevated expression of the Dectin-1, and by blocking Dectin-1, the phagocytosis of muriform cells, was impaired, demonstrating that muriform cells are recognized by Dectin-1 *in vitro* (26). *F. pedrosoi* spores trigger Dectin-1 and Dectin-2 signaling and induce IL-6 production, but only the Dectin-2 signaling pathway promotes the differentiation of Th17 cells, indicating that the adaptive immune response to *F. pedrosoi* spores in this murine infection model is determined by Dectin-2 (37). A *Fonsecaea*-specific ligand has not been described until today.

Mincle acts as a major receptor involved in the innate immune response to *F. pedrosoi* through the Syk/CARD9 pathway in murine BMDCs (68). However, another study identified Mincle as a suppressor of antifungal defenses by suppressing IL-12. The absence of IL-12 leads to impaired Th1 responses. Dectin-1 binding of *F. monophora* activates the transcription factor IRF1, which is crucial for the *IL12A* transcription. However, simultaneous binding of *F. monophora* to Mincle induces a Mdm2 (E3 ubiquitin ligase)-dependent degradation pathway *via* Syk-CARD9-mediated PKB signaling, that leads to the loss of nuclear IRF1 activity, therefore, blocking *IL12A* transcription (320). A *Fonsecaea*-specific ligand has not been described up to now for this receptor.

Regarding Mincle, it is difficult to ascertain a particular role for this CLR in *Fonsecaea* infection, since it is not clear if this receptor is mainly involved in the recognition and subsequent clearance of this fungus or acts as a suppressor of antifungal defenses and is exploited for immuno evasive strategies.

Microsporum spp.

Microsporum causes skin infections or dermatophytosis characterized by severe scalp itching and patchy scaly scalp skin which is highly contagious. The pathogenic species include mainly *M. cani*, *M. gypseum*, and *M. hominis* (321). The fungi secrete a number of enzymes and immunomodulators such as keratinolytic subtilase and keratinolytic metalloprotease as well as other cell wall glyco-proteins, endoproteases, and exoproteases (322). *M. cani* activates the NLRP3 inflammasomes in THP-1 cells. The production of IL-1β and its precursor are decreased in Dectin-1, Syk, and CARD-9 knockdown cells (323). Soluble Dectin-2 can bind the filamentous *M. audouinii* (11). Further research on CLR ligands, their recognition, and corresponding immune response in *Microsporum* infection are lacking.

Fusarium spp.

Fusarium species can cause superficial, locally invasive infections in immunocompetent individuals, or disseminated infections in immunocompromised patients. The infection is called fusariosis and is characterized by keratitis, onychomycosis, fungimia with or without organ involvement, and other symptoms depending upon the fungal species, port of entry, and host immune status. In humans, *Fusarium solani* and *F. oxysporum* are responsible for most cases of infections by these species (324). *Fusarium* spp. secrete various mycotoxins as well as certain proteases and collagenases, which modulate the immune response and destroy tissue (324).

Dectin-1 expression is highly elevated in the corneal tissue from patients infected with *F. solani* when compared to healthy non-infected individuals (325). Human corneal epithelial cells secrete defensive antimicrobial peptides in response to heat-killed *F. solani* or zymosan and this effect was Dectin-1- and TLR-2-dependent (326). Another more recent study addressed the Dectin-1-dependent CXCL-8 release from the human bronchial epithelial cell line BEAS-2B in response to *F. proliferatum* and showed that the chemokine release is decreased to various degrees by inhibiting Dectin-1, Syk, MAPKs, PI3K, and NFκB, respectively (27). The expression of SP-D increased in rat corneal cells after *F. solani* infection but its further role in murine models as well in humans is still to be established (327).

Trichosporon spp.

Trichosporon are ubiquitous dimorphic fungi, which also exist as commenals on the skin and in the gastrointestinal tract in humans. They induce superficial infections such as white piedra characterized by the presence of irregular nodules on the affected hair, as well as invasive infections such as allergic pneumonitis and trichosporonosis (invasive mycoses) especially in immunocompromised patients and those with hematological malignancies (328, 329). *T. asahii*, *T. asteroids*, and *T. mucoides* are the major causes of trichosporonosis and opportunistic infections (330). Only a little is known about the interaction of these fungi with CLRs. Dectin-1 binds *T. asahii via* β-glucan recognition (28). Dectin-1-deficient mice with *T. asahii* induced hypersensitivity pneumonitis show decreased Th-17 cell populations and less monocytes/MDMs compared to wild-type mice (28).

RARE DISEASE CAUSING FUNGI

Saccharomyces spp.

Classically, *Saccharomyces* spp. are considered safe, non-pathogenic organisms. Within this genus, *Saccharomyces cerevisiae* is the most important species (331). However, due to its ubiquity and long association with humans, *S. cerevisiae* has been implicated as a causative agent of infections in immunocompromised individuals, those with underlying diseases or medical conditions (332). Several cases of life-threatening invasive infections with *S. cerevisiae* resulting in pneumonia, liver abscess, and sepsis have been reported (333).

Saccharomyces cerevisiae cells cause a subtle upregulation of Dectin-1 from the moment of initial recognition in human DCs (334). Most studies have been performed by evaluating pure soluble and particulate β-glucans such as β-1,6-branched and β-1,3-D-glucan found in the *S. cerevisiae* cell wall (335), which can be directly recognized by Dectin-1 (29, 336). β-glucan induces Dectin-1 signaling pathways for the activation of TNFα in both human and mouse macrophages. The signaling pathways involve RTKs, ROS production, and NF-κB activation (337, 338). The Dectin-1-dependent response is essential for immunomodulatory effects on DC activation and macrophage phagocytosis. It induces the expression of immuno-regulatory cytokines, such as IL-10, TGF-β1, and IL-2 and can promote both Treg and Th17 responses (339–341). Moreover, the Dectin-1 response was investigated by observing the direct phagocytosis of β-glucan-coated particles by RAW macrophages expressing a GFP-Dectin-1 fusion protein. As expected, the β-1,3-beads induced a higher TNF-α response and a GFP-Dectin-1 recruitment to the phagosome, indicating that Dectin-1 recruitment is specific to β-1,3-glucan (342). In general, binding of particulate β-glucans to Dectin-1 triggers phagocytosis (343, 344). However, phagocytosis of β-glucan-bearing particles by human neutrophils is CR3-dependent, with a very minor role for Dectin-1, if any (78). Like *C. albicans*, also *S. cerevisiae* components in form of Zymosan are able to induce anti-inflammatory responses such as IL-10 release in human and mouse DCs and macrophages (345, 346).

Also, Langerin and DC-SIGN interact strongly with *S. cerevisiae* (64, 81). SP-D but not SP-A binds *S. cerevisiae,* and β(1→6)-glucan is a ligand for SP-D (48). Moreover, phagocytosis of unopsonized heat-killed yeast by murine macrophages is also mediated by MR (46).

Several studies have concluded that genetically determined low MBL concentrations in patients could be, at least in part, responsible for the enhanced immune reactivity to *S. cerevisiae* antigens (347–349). The analysis of *MBL2* polymorphisms revealed an association between three variants rs930508, rs1800450, and rs5030737, with a reduction in MBL serum levels in Crohn's disease patients (350). However, these results are in contrast with other reports in which such an association was not found. Therefore, the relationship between enhanced immune reactivity to *S. cerevisiae* antigens and MBL is still controversial (351, 352).

Despite its low pathogenicity, *S. cerevisiae* constitutes one of the better studied microorganisms, since it was developed as a model organism for several traits. Zymosan is mostly prepared from *S. cerevisiae* cell walls and consists of a glucan with repeating glucose units linked by β-1,3-glycosidic linkages, which have served as a model for recognition of microbes by the innate immune system for over 50 years (353). Many studies have been conducted testing and evaluating the zymosan interaction with human receptors and Dectin-1 emerged as the most important receptor for detecting *Saccharomyces* spp. However, for the other CLRs more studies are required in order to stablish a concrete role for them.

Exserohilum spp.

Exserohilum species are environmental fungi and, although rare, can lead to a number of human diseases such as skin and corneal infection, invasive disease, as well as allergic fungal sinusitis especially during impaired immunity, trauma, and atopy (354). Members of this genus are among the causes of phaeohyphomycosis which is characterized by the presence of dark septate mycelial elements in tissues (355). Not much work has been done to elucidate the mechanism of infection and immune responses against these fungi. A very recent work demonstrates the role of Dectin-1 in the recognition of *Exserohilum rostratum* (30). Mouse macrophages generate a Dectin-1 dependent TNF-α, IL-1β, MIP-1, and MIP-2 secretion in response to *E. rostratum* hyphae *in vitro* and the response is diminished in Dectin-1-deficient macrophages. However, wild-type and Dectin-1-deficient mice show no difference with respect to the type of inflammatory response and fungal control (30).

Cladosporium spp.

The *Cladosporium* genus consists of ubiquitous fungi that are mainly plant pathogens but few species such as those belonging to *Cladosporium cladosporioides* and *C. herbarum* complexes may cause infections in humans (356). The clinical manifestations range from keratitis, opportunistic phaeohyphomycosis including superficial or deep infections such as those of the CNS, to acnes (356–359). *Cladosporium* conidia are widely present in the air and have also been associated with respiratory allergy (360). The *C. cladosporioides* cell wall is rich in β-glucans but unavailable for recognition on live spores. A mouse *in vivo* study demonstrated that *C. cladosporioides* induces airway hyperresponsiveness and eosinophilia in a Dectin-1-independent manner (361).

Furthermore, the heat-induced availability of surface β-glucans is important for a Dectin-1-dependent pulmonary IL-17 response and Dectin-1$^{-/-}$ mouse DCs show a decreased IL-17 response upon stimulation with heat-killed *C. cladosporioides* (31). The ligands on live *Cladosporium* cell surfaces and the corresponding CLRs, as well the subsequent immune responses are still to be investigated.

Chrysosporium spp.

The members of this genus are saprophytic soil fungi and many species are keratinolytic (362). Several case reports of superficial infections affecting nails and skin as well as opportunistic infections in immunocompromised patients have been reported (362–365). Superficial infections are mainly caused by species, such as *C. keratinophilum*, *C. tropicum*, and *C. queenlandicum* (362). Invasive infections are rare but have been reported (366–368). A single study demonstrated that DC-SIGN recognizes *C. topicum* conidia probably by recognition of fungal cell wall mannans *in vitro* (59).

Sporothrix spp.

These fungi are usually found living as a saprophytes thriving on decaying vegetation or soil. Sporotrichosis, caused by dimorphic *Sporothrix* spp., is one of the most prevalent forms of subcutaneous mycoses with a worldwide distribution particularly in tropical and subtropical regions (369, 370). While different species of clinical interest have been identified, including *S. globosa*, *S. brasiliensis*, *S. Mexicana*, and *S. luriei*, the most commonly reported species in human clinical isolates is *Sporothrix schenckii* (371, 372). Infection results in cutaneous or subcutaneous lesions usually with compromised adjacent lymphatic vessels. Rarely, disseminated disease can also ensue and several publications report infection of the lung, the CNS, bones, and other organs, mostly in immunocompromised individuals (372–375).

In a rat co-infection model of *Tenia taeniaeformis* and *S. schenckii*, a high expression of Dectin-1 only occurs in cutaneous lesions of co-infected rats, but is dispensable for the clearance of *S. schenckii* (376). However, a more recent study shows an increased Dectin-1 expression in peritoneal macrophages from *S. schenckii*-infected mice. Furthermore, the antibody-mediated blockade of Dectin-1 inhibits the cytokine production in response to different stimuli by peritoneal macrophages. A Dectin-1 blockade additionally results in a decreased phagocytic uptake of *S. schenckii* yeast cells (32). Martínez-Álvarez et al. proved that Dectin-1 is crucial for the secretion of cytokines by human PBMCs during *S. schenckii* infection, but dispensable for the recognition of *S. brasiliensis*. The authors also reported that while MR appears to have only a minor role in the recognition of *S. schenckii* yeast-like cells, it mediates the production of proinflammatory cytokines by human PBMCs in response to conidia from this fungus as well as yeasts from *S. brasiliensis* (47). An earlier study on the contribution of MR in the recognition of *S. schenckii* evidenced the presence of mannose residues in the cell wall of *S. schenckii* conidia and yeasts. Nevertheless, MR seemed to be involved only in the phagocytosis of opsonized conidia (377). Taken together, Dectin-1 seems to be important for the generation of cytokines while MR mainly plays a role in the phagocytosis of this fungus.

CHALLENGES AND FUTURE DIRECTIONS

C-type lectin receptors recognize carbohydrate ligands in fungal cell walls and years of research have provided a huge body of evidence on their importance in modulating immune responses against fungal infections. However, there are still a number of gaps to be filled and areas that need attention from the scientific community. Almost all of the fungal infecitons are opportunistic, mainly concerning people with a compromised immune system, including HIV-infected individuals. Although they are a major high-risk group, many studies exclude HIV-infected patients and, therefore, this area of research seems to be largely unexplored. More studies focusing on HIV co-infection and the genetic make-up of HIV-infected individuals which affects their susceptibility toward developing specific fungal infections would be fundamental in developing tailored therapies for such cases. Fungi such as *Aspergillus* and *Candida* species interact with a number of CLRs upon infection, opening up opportunities to study the interactions between these receptors on a functional level [for example, the formation of heteromers as precdicted in case of Mincle and MCL (378)] as well as on a genetic level, including epistatic effects that the concerned genes have on each other. Another complex aspect that still needs to be studied in detail is how different cell types interact in an *in vivo* environment in order to control the infection. On the other hand, there are many fungal species such as *Paracoccidiodes*, *Fusarium*, etc., with few identified immune receptors, ligands, and/or virulence factors. Discovering these ligands and their corresponding receptors or new virulence factors will not only improve our understanding of fungal interactions with immune cells but also aid in developing vaccines and diagnostic or even therapeutic strategies against these fungi. Moreover, we now also know that CLRs not only recognize carbohydrate ligands but also lipids, proteins, and nucleic acids (3). We need to widen our views and explore this aspect more fully, since the fungal cell walls in addition to their manyfold carbohydrate structures also contain a number of lipids and proteins that may as well serve as potential ligands for CLRs (379). Moreover, some recent studies have demonstrated how differential immune responses are generated by different fungal isolates or strains depending on their individual pahogenic potential and virulence (380–383). Such studies suggest that a strain-specific comparison of immune response might be essential to fully understand the host–pathogen interactions. This knowledge would be helpful in generating data for individual case-specific therapies and, therefore, such studies need to be encouraged. Furthermore, it is often noted that many studies, such as *in vivo* mouse studies, provide contrasting results that lead to contradictions and questions. Therefore, the scientific studies need to be designed more carefully taking into account the strains of mice and the fungal species being studied, cell type or cell lines being used, and the type of *in vitro* or *in vivo* environment provided. Also, population structures and stratification in genetic studies should be addressed. The effects of each of these factors on the outcome of the results should be appropriately discussed in order to have a comprehensive view. In summary, there is a need to further enhance the understanding of how CLRs recognize pathogenic fungi in order to promote approaches to take advantage of this

knowledge for future therapeutic interventions. The ability of these receptors to bind a wide variety of pathogens which share the same ligand as fungi, makes them important molecules in the innate immune response. While there are many functional aspects of CLRs yet to be discovered, there is an increasing amount of information published over the past decade that already allows us to benefit from our knowledge on regulatory functions and new translational opportunities.

CONCLUSION

It is widely excepted that CLRs play a major role in modulating immune responses with respect to fungal infections, being able to recognize the carbohydrate moieties in the fungal cell walls. Fungal infections, though mainly opportunistic, can prove fatal in case of faulty diagnosis or treatment. The fungal recognition by CLRs mainly leads to proinflammatory responses and a subsequent activation of adaptive immunity *via* Th17 responses. However, negative or anti-inflammatory effects have also been noted and both types of responses are necessary to mount a specific immune response. A considerable body of work has been done with regards to frequent pathogens, such as *Candida*, *Aspergillus*, and *Cryptococcus*, etc., and genetic susceptibilities pertaining to fungal infections have been attributed to various mutations in CLRs. While some fungal infections are frequent, others are emerging into a major health problem with the continous increase in immuno-compromised patients. A thorough knowledge of the molecular mechanisms of fungal infections and the interaction of these fungi with their major receptors, the CLRs, can provide a basis to a better and more specific diagnosis and treatment regime. Also, knowledge about the host-related genetic factors, which can greatly affect the course and outcome of these infections, may advance a timely diagnosis and care for the patient.

AUTHOR CONTRIBUTIONS

HS conceived the review framework. SG and JC-B wrote the manuscript. EK and HS revised the manuscript. JC-B and SG created the figure. All authors have read and approved the final version of the manuscript.

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Toll-Like Receptor 4 Triggering Promotes Cytosolic Routing of DC-SIGN-Targeted Antigens for Presentation on MHC Class I

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DC-SIGN is an antigen uptake receptor expressed on dendritic cells (DCs) with specificity for glycans present on a broad variety of pathogens and is capable of directing its cargo to MHC-I and MHC-II pathways for the induction of $CD8^+$ and $CD4^+$ T cell responses, respectively. Therefore, DC-SIGN is a very promising target for the delivery of antigen for anti-cancer vaccination. Although the endocytic route leading to MHC-II presentation is characterized to a large extent, the mechanisms controlling DC-SIGN targeted cross-presentation of exogenous peptides on MHC-I, are not completely resolved yet. In this paper, we used imaging flow cytometry and antigen-specific $CD8^+$ T cells to investigate the intracellular fate of DC-SIGN and its cargo in human DCs. Our data demonstrates that immature DCs and toll-like receptor 4 (TLR4) stimulated DCs had similar internalization capacity and were both able to cross-present antigen targeted *via* DC-SIGN. Interestingly, simultaneous triggering of TLR4 and DC-SIGN on DCs resulted in the translocation of cargo to the cytosol, leading to proteasome-dependent processing and increased $CD8^+$ T cell activation. Understanding the dynamics of DC-SIGN-mediated uptake and processing is essential for the design of optimal DC-SIGN-targeting vaccination strategies aimed at enhancing $CD8^+$ T cell responses.

Keywords: dendritic cells, DC-SIGN, cross-presentation, imaging flow cytometry, toll-like receptor, MHC-I, T cell, proteasome

INTRODUCTION

Dendritic cells (DCs) are antigen-presenting cells (APCs) that reside in all tissues and use germ-line encoded receptors to sample the tissue environment for pathogens. Upon pathogen recognition, DCs migrate to secondary lymphoid tissues, while they mature and process the internalized antigen to initiate antigen-specific T cells leading to humoral and/or cellular immune responses. Among the different receptors used by DCs to detect pathogens are C-type lectin receptors (CLRs), a large family of receptors that recognize carbohydrates in a Ca^{2+}-dependent manner. Whereas some pattern-recognition receptors, such as toll-like receptors (TLRs), are specialized in activating intracellular signaling cascades to initiate DC maturation, CLRs primarily mediate pathogen endocytosis *via* internalization motifs present in their cytoplasmic domains (1, 2). This mechanism allows the

efficient processing of pathogens for loading on MHC class II and I molecules and presentation to $CD4^+$ and $CD8^+$ T cells, respectively. These capacities of CLRs make them potent targets for vaccine development, especially for the induction of cellular responses for cancer treatment. The first studies on the targeting of CLRs have been done using DEC205-specific antibodies (Abs). These studies showed that targeting antigens to DCs resulted in prolonged and increased T cell responses when administered with an adjuvant. Also the amount of antigen needed for the induction of this response *in vivo* was considerately lower than when free antigen was used (3). The CLR DC immunoreceptor (DCIR) containing an immunoreceptor tyrosine-based inhibitory motif and present on a variety of blood and skin DC subsets, also mediated increased $CD8^+$ T cells responses. This effect was further enhanced by the addition of a TLR 7/8 agonist (4). DC-SIGN is a type II membrane CLR discovered as a cell-adhesion receptor that supports primary immune responses (5) and enhances HIV infection of $CD4^+$ T cells (6). DC-SIGN is expressed on monocyte-derived DCs (moDCs) in peripheral tissue, $CD14^+$ dermal DCs in the dermal layers of the skin (7), and mature DCs in lymphoid tissues, however, DC-SIGN expression is lacking on follicular DCs and $CD1a^+$ Langerhans cells (8). The carbohydrate recognition domain (CRD) of DC-SIGN contains a Ca^{2+}-coordination site and has a dual specificity for high-mannose and Lewis-type carbohydrate structures (glycans), which gives the receptor the ability to recognize a broad variety of ligands (9), both on pathogens and self-glycoproteins (10). Lectin–glycan interactions have classically been considered to be of low affinity (11). As DC-SIGN is present in nano-clusters on the cell surface (12), the concept of avidity is of importance in the design of DC-SIGN-targeting compounds for *in vivo* vaccination strategies. We have explored the possibility of using DC-SIGN-targeting glycoconjugates for triggering of T cell responses (13–15) and demonstrated that DC-SIGN not only induces potent $CD4^+$ T cell responses by targeting antigen to the endo-lysosomal pathway (16) but also triggers $CD8^+$ T cell responses that can be boosted by supplementing a TLR4 stimulus. Unfortunately, the mechanism by which the intracellular routing initiated by DC-SIGN results in MHC-I presentation has not been fully identified. Understanding this mechanism will help in designing DC-SIGN-targeting vaccination strategies for the induction of anti-tumor immunity.

Dendritic cells are the most potent APC subset capable of priming naïve $CD8^+$ T cells with exogenous antigen, for the induction of immunity against antigens derived from tumors or pathogens that do not infect DCs (17, 18). Although processing and presentation of endogenous proteins in MHC-II is quite well characterized, the mechanisms by which exogenous antigens are processed and loaded in MHC-I for presentation to $CD8^+$ T cells (cross-presentation) are not fully understood. Cross-presentation efficiency and intracellular routing can differ depending on the mode of uptake, the antigen, and maturation status of the DC (19). To date two main routes of antigen cross-presentation have been described, namely the cytosolic and vacuolar pathway. In the vacuolar pathway, the exogenous antigens are processed by proteases and reloaded on MHC-I molecules without leaving the endosome. Cross-presentation *via* the vacuolar pathway has shown to be independent of TAP and degradation by the proteasome. By contrast, in the cytosolic pathway, the exogenous acquired antigens translocate to the cytosol and are processed by the proteasome, before they are loaded on MHC-I molecules. It remains elusive if loading of MHC-I is done by the endogenous MHC-I loading mechanism in the ER or by the possible recruitment of these MHC-I peptide loading complexes to phagosomes and endosomes (18, 19, 20).

Here, we used imaging flow cytometry to track DC-SIGN and its ligand in DCs and their co-localization with the different compartments involved in antigen processing and presentation. To further unravel the intracellular fate of the DC-SIGN ligand, we treated moDCs with different inhibitors of antigen processing. Our results demonstrate that DC-SIGN directs its cargo to early endosomal compartments, where the receptor–cargo complex partly dissociates. Since maturation status of the DCs can influence $CD4^+$ and $CD8^+$ T cell priming by means of co-stimulation and cytokine secretion, TLR agonists are often used as adjuvant to induce proper T cell responses. However, TLR stimulation can also influence antigen routing within the DCs, thereby changing the cross-presentation capacity (20). We observed that the cross-presentation capacity of DC-SIGN greatly depends on concomitant TLR4 triggering, which induces translocation of the ligand from the endosomes to the cytosol, where it can be efficiently routed for loading on MHC-I and subsequent $CD8^+$ T cell activation.

MATERIALS AND METHODS

Chemicals and Abs

The following reagents were used: *E. coli* lipopolysaccharide (LPS) (Sigma-Aldrich, MO, USA), monophosphoryl lipid A (MPLA) from *Salmonella enterica* (Invivogen), Paraformaldehyde (formaldehyde) aqueous solution (Electron Microscopy Sciences), Saponin (Sigma-Aldrich), and BSA (Roche). Abs used include: CD83-PE (Beckman Coulter), CD80-PE (clone L307.4, BD Biosciences), CD86-PE (clone 2331, BD Biosciences), EEA1-FITC (clone 14/EEA1, BD Biosciences), HLA-DM-PE (clone MaP.DM1, BD Biosciences), LAMP-FITC (clone H4A3, BD Biosciences), polyclonal rabbit-α-rab 11 (Invitrogen), HLA-A2-PE (BD Biosciences), CD107a-AF488 (BioLegend), CD107b-AF488 (BioLegend), Pacific Orange-labeled goat-α-rabbit IgG (Invitrogen), AF594-labeled goat-α-mouse IgG2a (Invitrogen), AF488-labeled goat-α-mouse IgG2b (Invitrogen), and biotin-labeled horse-α-mouse IgG (Vector Labs). CSRD (8), the polyclonal Ab against DC-SIGN, and AZN-D1 (5), a murine monoclonal IgG1 Ab against the carbohydrate recognition domain of DC-SIGN, were from our own stocks. DC-28 (21), the monoclonal IgG2a Ab against the stalk region of DC-SIGN was a kind gift of R. Doms (University of Pennsylvania). AZN-D1 was labeled with AF405 (Invitrogen) according to manufacturer's instructions. AZN-D1 coated fluorescent beads were made as previously described (22). Gp100 with a C-terminal cysteine was conjugated to AZN-D1 *via* thiol-mediated conjugation using the bifunctional linker SMCC [succinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate, Thermofisher Scientific, Breda]. Briefly, 5 mg AZN-D1 was activated with eight equivalents of SMCC in 50 mM phosphate buffer

pH 8.3 containing 10 mM EDTA and 100 mM NaCl. After desalting the activated AZN-D1 over Sephadex-25 desalting columns (GE Healthcare Life Sciences, Breda), 12 equivalents of gp100 is added and vortexed thoroughly. The reaction is incubated for 1 h at 37°C. Final product is purified over Superdex 75 column (10 × 300, GE Healthcare Life Sciences, Breda).

Cells

Monocytes were obtained from buffy coats of healthy donors, with informed consent (Sanquin, Amsterdam, reference: S03. 0023-XT). Monocytes were isolated through a sequential Ficoll/Percoll gradient centrifugation (purity, >85%) and cultured in RPMI 1640 (Invitrogen) supplemented with 10% FCS (Bio-Whittaker), 1,000 U/ml penicillin (Lonza), 1 U/ml streptomycin (Lonza), and 2 mM glutamine (Lonza) in the presence of IL-4 (262.5 U/ml; Biosource) and GM-CSF (112.5 U/ml; Biosource) for 4–7 days (23). MoDC differentiation and maturation was monitored by FACS analysis (Calibur, Fortessa BD Biosciences) of DC-SIGN, CD83, CD80, and CD86. Stable CHO/DC-SIGN transfectants (24) were maintained in RPMI 1640 medium containing 10% FCS, 1,000 U/ml penicillin, 1,000 U/ml streptomycin, 2 mM glutamine, and 1 mg/ml geneticin (Invitrogen).

Pulse-Chase Experiments

Approximately 10^6 moDCs were incubated for 20 min in 100 µl of ice-cold culture medium. AF405-labeled AZN-D1 10 µg/ml was added and incubated for 30 min on ice. Cells were washed once with ice-cold medium and then transferred to 37°C for indicated time points or kept on ice. At indicated time points, cells were washed with ice-cold PBS, fixed in ice-cold 4% PFA in PBS for 20 min, and then washed two times with ice-cold PBS. For intracellular stainings, cells were permeabilized in 0.1% saponin in PBS for 30 min at room temperature and then blocked with a solution containing 0.1% saponin, 2% BSA, and 1% goat serum in PBS. Primary and secondary antibody stainings were performed in PBS with 0.1% saponin and 2% BSA at room temperature. After staining, cells were kept at 4°C in PBS supplemented with 0.5% BSA and 0.02% NaN_3 until analysis.

Antigen Presentation to Human CD8$^+$ T-Cells

Immature moDCs were seeded in 96-well plates (Greiner) at 20×10^3 cells/well and incubated with the different antigens in the presence or absence of the TLR4 ligand MPLA (10 µg/ml). After 3 h, cells were washed three times with RPMI and co-cultured overnight with a $gp100_{280–288}$ TCR transduced CD8$^+$ HLA-A2 restricted T cell clone (25) (10^5 cells per well, E:T ratio 1:5). IFNγ in the supernatant was measured by sandwich ELISA according to protocol (Biosource). To determine the effect of proteasomal and endosomal inhibitors, moDCs (30×10^3 cells/well) were incubated with chloroquine (25 µM, Sigma), MG132 (10 µM, Selleck), epoxomicin (0.25 µM, Selleck), or cathepsin S inhibitor (5 µM, calbiochem) at 37°C for 30 min prior to the addition of antigen and the TLR4 ligand LPS (100 ng/ml). After 3 h, the moDCs were washed and co-cultured with a $gp100_{280–288}$ TCR transduced CD8$^+$ HLA-A2 restricted T cell clone (10^4 cells per well, E:T ratio 3:1) (25). Degranulation was analyzed by flow cytometry, *via* the membrane staining of CD107a and CD107b, as a measure for T cell activation.

Confocal Laser-Scanning Microscopy (CLSM)

Stained cells were allowed to adhere to poly-L-lysine-coated glass slides and mounted with anti-bleach reagent vinol. Samples were analyzed using a 63×/1.4 HCX PL APO CS oil objective on a TCS SP2 AOBS confocal microscope (Leica Microsystems GmbH). Images were acquired using LCS 2.61 (Leica Microsystems GmbH) and processed using Adobe Photoshop CS4 or ImageJ.

Live Cell Imaging

CHO/DC-SIGN cells were cultured on gelatin coated glass slides. AZN-D1 coated beads were added to the cells and followed for different time points. Cells were analyzed by means of a 3I Marianas™ digital imaging microscopy workstation (Zeiss Axiovert 200 M inverted microscope Carl Zeiss), equipped with a nanostepper motor (*Z*-axis increments 10 nm) and a cooled CCD camera (Cooke Sensicam, 1,280 × 1,024 pixels Cooke Co). Visualization was performed with a 40× air lens. The microscope, camera, and data viewing process were controlled by SlideBook™ software (version 4.0.8.1 Intelligent Imaging Innovations).

Imaging Flow Cytometry

Cells were acquired on the ImageStreamX (Amnis corp.) imaging flow-cytometer. A minimum of 15×10^3 cells was acquired per sample at 40× magnification at a flow rate ranging between 50 and 100 cells/s. Analysis was performed using the IDEAS v6.0 software (Amnis corp.). A compensation table was generated using the compensation macro built in the software and applied to the single staining controls. Proper compensation was then verified by visualizing samples in bivariate fluorescence intensity plots (Figure S1 in Supplementary Material). A template analysis file to gate for single optimally focused cells (Figure S2 in Supplementary Material) and applied to the experimental samples in order to export this population to a new compensated image file to allow merging all experimental samples within a single file for direct sample analysis. Ag/receptor internalization was investigated using a combination of a mask designed to detect the intracellular space and the internalization feature (Figure S3 in Supplementary Material).

Co-localization was calculated using the bright detail similarity R3 feature on a whole cell mask. Co-localization is calculated as the logarithmic transformation of Pearson's correlation coefficient of the localized bright spots with a radius of 3 pixels or less within the whole cell area in the two input images (bright detail similarity R3). Since the bright spots in the two images are either correlated (in the same spatial location) or uncorrelated (in different spatial locations), the correlation coefficient varies between 0 (uncorrelated) and 1 (perfect correlation). The logarithmic transformation of the correlation coefficient allows the use of a wider range for the co-localization score. In general, cells with a low degree of co-localization or no co-localization at all between two probes show scores below 1.

mRNA Isolation, cDNA Synthesis, and Real-Time PCR

After cell lysis, mRNA was isolated by mRNA Capture kit (Roche) and cDNA was synthesized with the Reverse Transcription System kit (Promega) following manufacturer's guidelines. cDNA was diluted 1:2 in nuclease-free water and stored at −20°C until analysis. Primers specific for human DC-SIGN (5′-aacagctgaga ggccttgga-3′, 5′-gggaccatggccaagaca-3′) and GAPDH (26) were designed with Primer Express 2.0 (Applied Biosystems) and synthesized at Invitrogen (Invitrogen). Primer specificity was computer-tested (BLAST, National Center for Biotechnology Information) and confirmed by dissociation curve analysis. Real-time PCR reactions were performed using the SYBR Green method in an ABI 7900HT sequence detection system (Applied Biosystems) as previously described (26).

Statistics

Unless otherwise stated, data are presented as the mean ± SD of at least three independent experiments. Statistical analyses were performed using the statistical package SPSS. Statistical significance was set at $P < 0.05$ and it was evaluated by the Mann–Whitney U test.

RESULTS

DC-SIGN Is Exclusively Localized at the Cell Membrane and Is Quickly Internalized Upon Receptor Ligation

We first tested the steady state localization of DC-SIGN on moDCs using imaging flow cytometry, a technology that allows for the quantification of morphological aspects of images acquired from large populations of cells. The localization of DC-SIGN on fixed moDCs was assessed *via* staining with the anti-DC-SIGN polyclonal Ab CSRD (8), which does not interfere with the carbohydrate-binding site of DC-SIGN (see **Figure 1A**). An internalization score higher than 0 indicates that the fluorescent signal is mainly localized inside the cell, whereas a negative internalization scores reflects exclusive membrane localization. When the intracellular and membrane localization are equal, the internalization score is set to 0. In the steady state, DC-SIGN in DCs is exclusively expressed on the cell membrane (**Figure 1B**), since more than 95% of the moDCs had a negative internalization score. We confirmed the exclusive membrane localization of DC-SIGN, using the left over cells from the imaging flow cytometry for CLSM imaging (Figure S4 in Supplementary Material). To investigate the kinetics of internalization, DC-SIGN was stably transfected in CHO cells, exposed to AZN-D1-coated fluorescent beads and followed by live cell widefield epifluorescence imaging. AZN-D1 is a monoclonal Ab against the carbohydrate-binding site of DC-SIGN and is known to trigger receptor internalization (see **Figure 1A**) (16). The still frames in **Figure 1C** show how a bead adheres to the surface of the cell within seconds and is quickly internalized, approximately 2 min after receptor ligation.

Because the mechanisms of internalization of particulate and soluble antigen may vary, we also investigated the internalization of AF405-labeled AZN-D1. First, moDCs were incubated in the presence of AF405-labeled AZN-D1 for 30 min at 4°C. Then the cells were transferred to 37°C for the indicated time points, washed, fixed, and analyzed by imaging flow cytometry. The maximum level of AZN-D1 internalization was already achieved at 7.5 min (**Figure 1D**), indicating that DC-SIGN internalization is a fast process. To investigate whether receptor internalization was dependent on the amount of antigen available, we repeated the pulse-chase experiment with a titration of AF405-labeled AZN-D1 and then fixed, permeabilized, and stained the receptor with CSRD (8). At 1 μg/ml, the amount of internalized receptor equaled the amount of receptor on the surface (internalization score 0) and total internalization was achieved using 5 μg/ml of ligand (**Figure 1E**).

Subsequently, we tracked both ligand and receptor in a time-course pulse-chase experiment using the AF405-labeled AZN-D1 Ab to model the ligand and staining with CSRD after fixation and permeabilization to track the receptor. Upon DC-SIGN triggering, both ligand and receptor were quickly internalized and DC-SIGN did not return to the membrane after internalization (**Figure 2A**). At an early time point (7.5 min), the internalization of receptor and ligand almost perfectly correlated, implying an interdependence of both processes (**Figure 2B**). When the co-localization score of the ligand and the receptor was assessed, we observed that the co-localization score was maximal at baseline ($t = 0$ min) and decreased very quickly once both ligand and receptor were internalized (**Figure 2C**), indicating that ligand and receptor partly dissociate. We also assessed the amount of ligand and receptor at the different time points during the experiment. The signal for the ligand decayed by almost 80% during the experiment (**Figure 2D**). By blocking vesicular degradation with chloroquine, we were able to significantly reduce ligand decay after 30 min (Figure S5 in Supplementary Material), indicating that the ligand gets (partly) processed in the endosomes. We stained for the receptor after fixation and permeabilization of the cells, allowing us to detect the total amount of intracellular and membrane associated DC-SIGN. We observed a reduction in receptor signal to approximately 50–60% of the starting amount (**Figure 2D**). The loss of signal indicates that DC-SIGN gets degraded and does not recycle to the membrane. This is supported by previous work of Tacken et al. (27) showing that in the presence of the protein synthesis inhibitor cycloheximide barely any newly synthesized DC-SIGN molecules re-emerged on the cell surface within 3 h following DC-SIGN mediated internalization. Even when DC-SIGN was targeted for a prolonged period of time, the surface expression of DC-SIGN was significantly decreased up to 2 days after Ab removal. Taken into account that the recycling of receptors is a fast process that often takes places within minutes after receptor internalization our results suggest that DC-SIGN is slowly degraded and not recycled, while the ligand of DC-SIGN quickly gets processed after internalization.

DC-SIGN Directs Its Cargo Through Early Endosomal Compartments Before Dissociation

To investigate the fate of both ligand and receptor, we measured the co-localization scores of both ligand (AZN-D1) and receptor

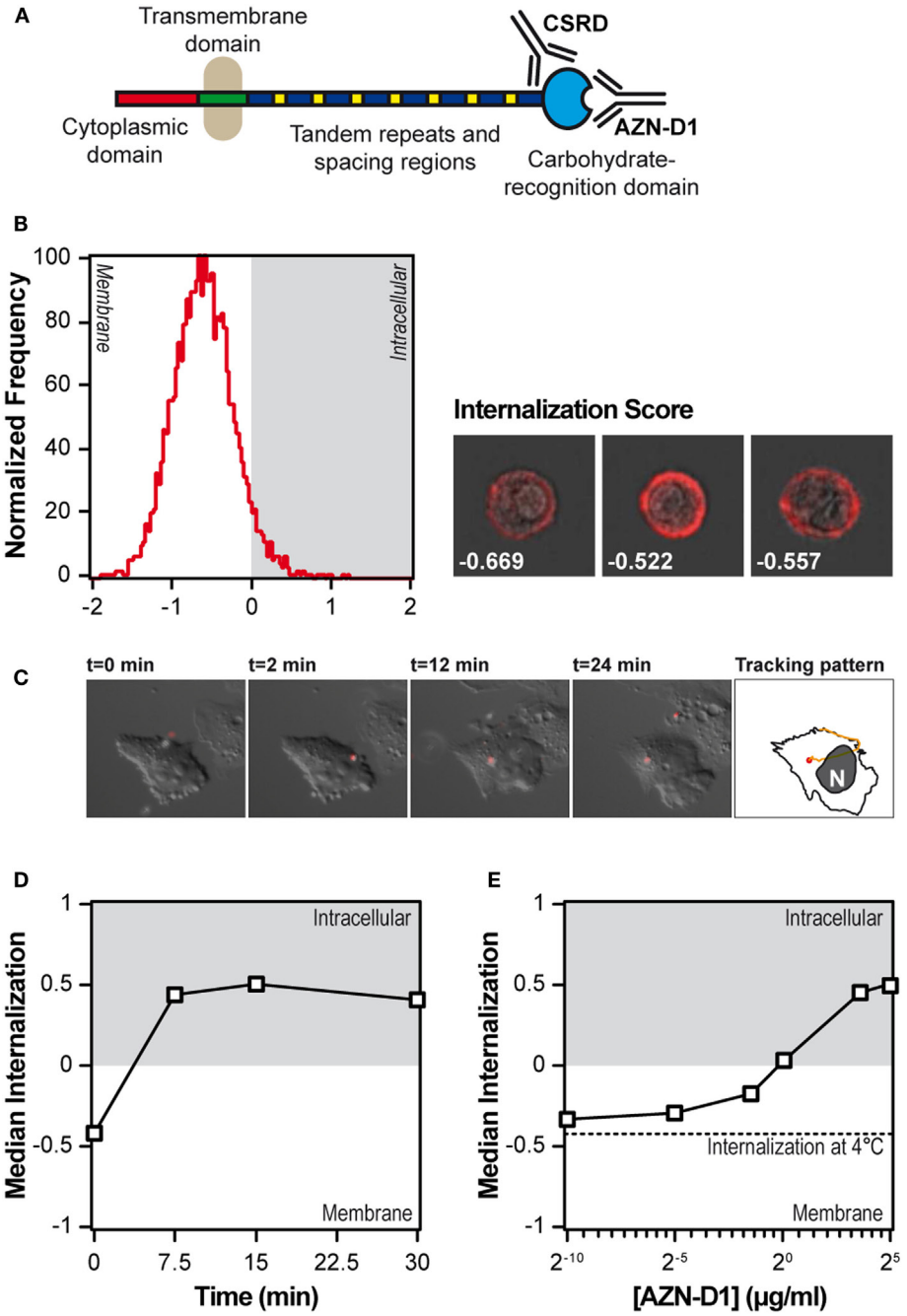

FIGURE 1 | DC-SIGN on immature monocyte-derived DCs (moDCs) is exclusively expressed at the extracellular membrane and quickly internalizes upon triggering. **(A)** Summary of the anti-DC-SIGN antibodies used in the present study. **(B)** Internalization score of resting immature moDCs, after fixation, permeabilization and staining with a polyclonal antibody against DC-SIGN (n > 5,000). Next to the histogram, three representative images are included with their respective internalization score. **(C)** Still frames of a live cell imaging experiment in which DC-SIGN-CHO cells were exposed to AZN-D1-coated fluorescent beads. The right-most frame shows the tracking pattern, representative of eight experiments. **(D)** Time-course of the median internalization score of moDCs triggered with AF405-labeled AZN-D1 (n > 5,000). **(E)** Effect of the AF405-labeled AZN-D1 concentration on the internalization score after 30 min at 37°C. The dotted line indicates the internalization of the highest antibody concentration after 30 min at 4°C (n > 5,000).

(CSRD) (see **Figure 1A**) with Abs commonly used to track endocytic compartments. Until approximately 30 min both ligand and receptor co-localized evenly with the early endosomal marker EEA1 (both scores around 1.05). Hereafter, the co-localization for the receptor dramatically decreased, whereas co-localization with the ligand slowly decreased, suggesting that ligand and receptor partly dissociate in early endosomes (**Figure 3A**). This is further supported by the LAMP1 (lysosomes) co-localization

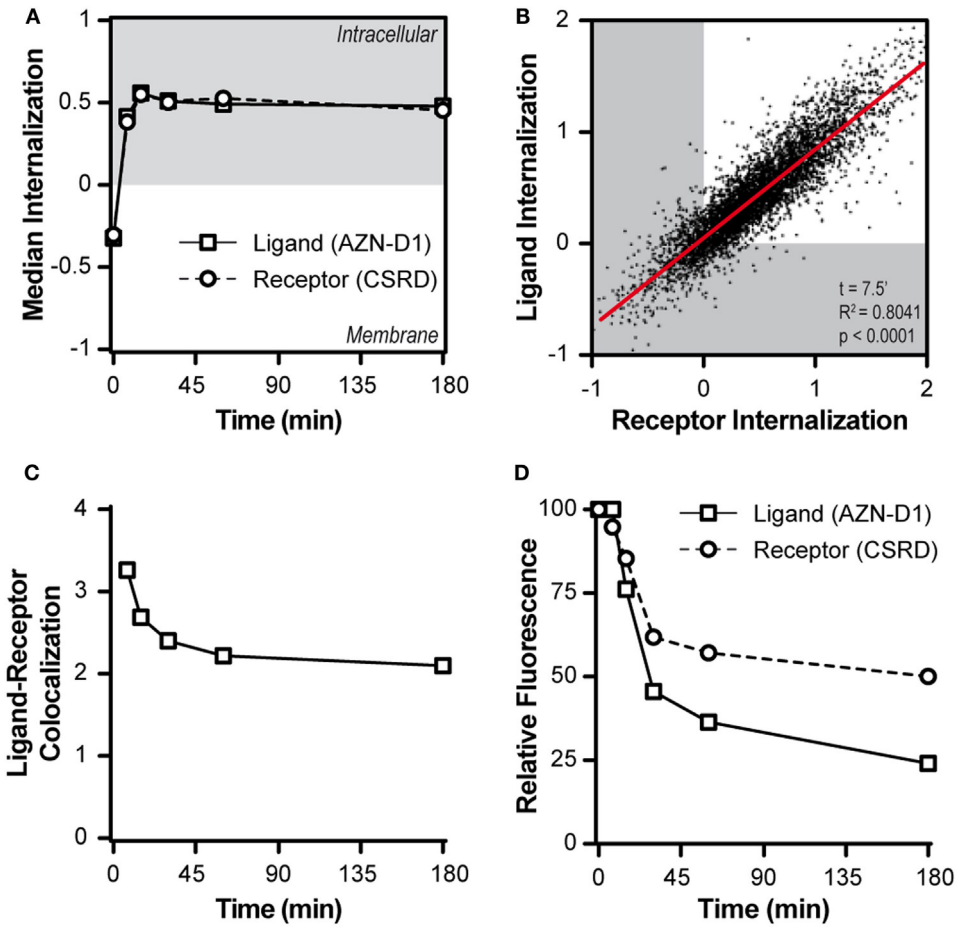

FIGURE 2 | DC-SIGN and its cargo quickly dissociate upon internalization. **(A)** Time-course of the median internalization score of monocyte-derived DCs triggered with AF405-labeled AZN-D1 and stained intracellular against DC-SIGN (n > 5,000). **(B)** Scatter plot of the internalization scores of both ligand and receptor 7.5 min after triggering with AF405-labeled AZN-D1 (n > 5,000). **(C)** Time-course of the median co-localization of AF405-labeled AZN-D1 and DC-SIGN (n > 5,000). **(D)** Time-course of the fluorescence signal intensity of both AF405-labeled AZN-D1 and DC-SIGN (n > 5,000).

scores, which show that the ligand (but not DC-SIGN receptor) reached the lysosomes before 30 min, while peaking at around 45 min (**Figure 3B**). In accordance, the MHC-II compartment co-localized with the ligand (at 30 min score 1.05), but not with the receptor (30–180 min score 0.6, **Figure 3C**). Interestingly, rab11 shows a moderate co-localization with the ligand (30 min score 1.05), but a poor co-localization with the receptor (score 0.6), suggesting that routing to this compartment is receptor-independent and may follow upon a stay at the early endosomes or the lysosomes (**Figure 3D**). The decay observed for the receptor in **Figure 2D** might be explained by quick lysosomal degradation, but since there is very little co-localization of the receptor with the lysosomal marker LAMP1, degradation could also already occur in the early endosomes.

Our data indicate an internalization model in which DC-SIGN mediates the internalization of its antigen ligand, which ends up in early endosomes where the receptor–ligand complex dissociates. The released cargo continues its way to lysosomes by the maturation of early endosomes, while a fraction of receptor–ligand complexes possibly translocate to the cytosol to initiate MHC-I loading.

Simultaneous DC-SIGN and TLR4 Triggering Affects DC-SIGN Internalization

TLR4 triggering is commonly used to address the effects of DC activation and maturation, a process that typically occurs upon pathogen recognition and that is necessary for proper antigen processing, presentation, and $CD8^+$ T cell priming (28). In addition, DC-SIGN triggering has been described to elicit a signaling cascade that modulates the TLR4-induced signaling (29, 30). We therefore investigated the consequences of DC maturation on DC-SIGN internalization. First, we investigated the effect of TLR4-mediated moDC activation on DC-SIGN expression levels. LPS treatment of moDCs resulted in a dramatic decrease in both DC-SIGN protein (10-fold) and mRNA (100-fold) after 18 h (**Figure 4A**). The decrease in DC-SIGN expression was not accompanied by an internalization of the

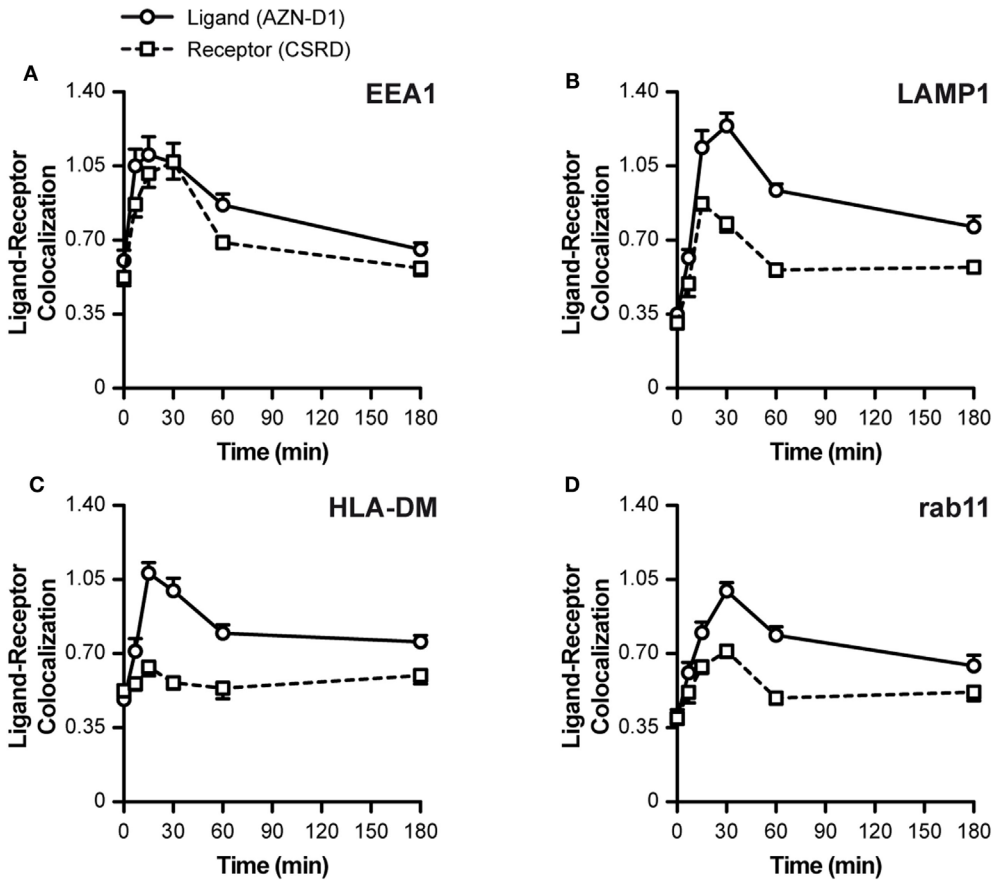

FIGURE 3 | Intracellular routing of DC-SIGN and its ligand. Monocyte-derived DCs were pulsed with AF405-labeled AZN-D1 for 30 min on ice and transferred to 37°C. Cells were fixed at indicated time points and stained with the CSRD antibody to localize DC-SIGN. Time-course of the co-localization scores of AF405-labeled AZN-D1 (mean ± SEM) and CSRD (DC-SIGN) with **(A)** EEA1, **(B)** LAMP1, **(C)** HLA-DM, and **(D)** Rab11 (n > 5,000).

receptor, as it was still located on the cell membrane (**Figure 4B**), indicating that DC-SIGN was lost by either shedding into the supernatant or by incorporation into exosomes, possibilities that have been previously described for DC-SIGN (31, 32). Still, simultaneous triggering of DC-SIGN and TLR4 (LPS at $t = 0$) or triggering of DC-SIGN on mature moDCs (overnight LPS treatment) had no consequences for the overall internalization rate, which proceeded as efficiently on mature moDCs as on immature moDCs (**Figure 4C**). Nevertheless, the fate of AZN-D1 ligand differed greatly between the simultaneous triggering of DC-SIGN and TLR4 (LPS at $t = 0$) and the triggering of DC-SIGN on matured moDCs (o/n LPS) (**Figure 4D**). While ligand degradation was similar in immature moDCs and moDCs that received LPS at $t = 0$, triggering of DC-SIGN on mature moDCs showed decreased ligand degradation. Less than 20% AZN-D1 degradation occurred in mature moDCs even after an extended incubation time (6 h), compared to 70–80% ligand degradation in immature moDCs and moDCs receiving LPS at $t = 0$ (**Figure 4D**). This was consistent with a reduced trafficking of AZN-D1 to the lysosomes upon overnight (o/n) treatment with LPS (**Figure 4E**).

Simultaneous Triggering of DC-SIGN and TLR4 Affects the Cross-Presentation Route in DCs

To evaluate the effect of TLR4 triggering on cross-presentation after DC-SIGN targeting, we compared the capacity of antigen pulsed immature moDCs and TLR4-stimulated moDCs ($t = 0$), to activate $CD8^+$ T cells. We excluded the DCs that were incubated o/n with a TLR4 stimulus, because of their greatly reduced DC-SIGN receptor surface expression. Therefore, pre-treatment with a TLR4 stimulus before antigen administration is not a favorable vaccine strategy when targeting DC-SIGN. As an antigen, we used a gp100 synthetic long peptide (SLP) (29-mer, VTHTYLEPGPVTANRQLYPEWTEAQRLDC) containing both the $gp100_{280–288}$ $CD8^+$ and $gp100_{45–59}$ $CD4^+$ T cell epitope conjugated to the DC-SIGN-targeting monoclonal antibody AZN-D1. A 3 h antigen pulse was followed by co-culturing of moDCs o/n with $gp100_{280–288}$ specific $CD8^+$ T cells, after which the released IFNγ was determined as a measure for T cell activation (**Figure 5A**). MoDCs that received TLR4 stimulus at $t = 0$ outperformed the immature moDCs in their capacity to activate $CD8^+$

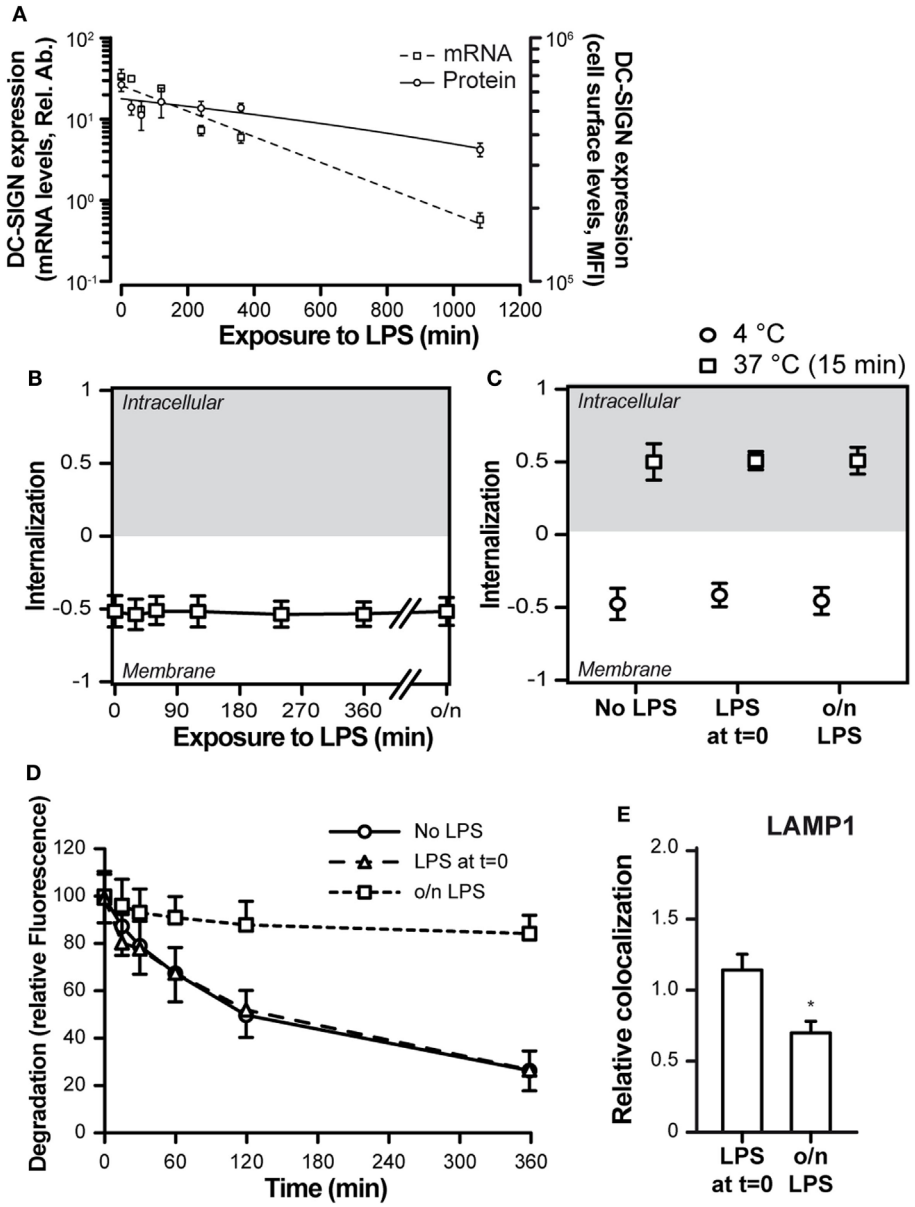

FIGURE 4 | Toll-like receptor 4 (TLR4) triggering affects the routing of DC-SIGN and its ligand. **(A)** Time-course of the expression levels of DC-SIGN at both the mRNA and protein level after TLR4 [lipopolysaccharide (LPS)] stimulation of monocyte-derived DCs. **(B)** Time-course of the internalization score of DC-SIGN after treatment with a TLR4 ligand (LPS). **(C)** Internalization score of AF405-labeled AZN-D1 after a 60-min incubation at 4°C or a 15-min incubation at 37°C. **(D)** Time-course of the fluorescence signal intensity of AF405-labeled AZN-D1 **(E)** Co-localization scores (relative to no LPS) of AF405-labeled AZN-D1 with LAMP1, 60 min after triggering. Mean ± SEM (n > 5,000). *P < 0.01 compared to no LPS.

T cells. Next, we determined the specificity of our DC-SIGN targeting Ab by pulsing immature DCs for 3 h with gp100/AZN-D1 conjugates, gp100/mIgG1 isotype control conjugates (functioning as a negative control), the 29-mer gp100 SLP, and the 9-mer minimal epitope that can directly bind to MHC-I. After a 3 h antigen pulse, $gp100_{280–288}$ specific $CD8^+$ T cells were added to the moDCs for 45 min and stained for degranulation markers (CD107a and CD107b). MoDCs pulsed with the gp100/AZN-D1 were able to activate antigen-specific $CD8^+$ T cells as measured by degranulation levels. By contrast, the gp100/mIgG1 conjugate induced no $CD8^+$ T cell activation. The SLP as a single non-targeted agent induced degranulation of more than 40% of the $CD8^+$ T cells, confirming the robustness of this assay (**Figure 5B**; Figure S6 in Supplementary Material). Therefore, the lower response induced by gp100/AZN-D1 is due to the limited amount of SLPs that can be conjugated to the antibody, rather than the sensitivity of the experiment.

The enhanced cross-presentation after TLR stimulation has been described to result from the induction of a different antigen-processing route (20, 33–36). To investigate if changes

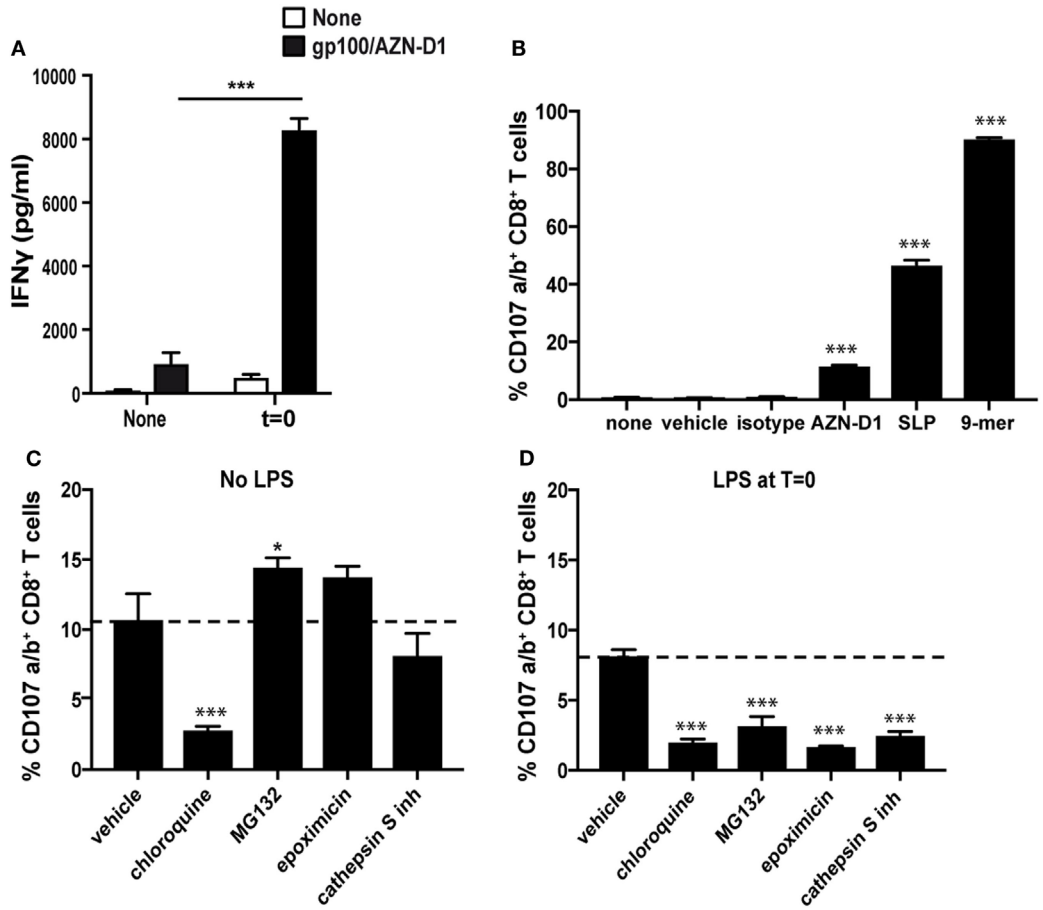

FIGURE 5 | Toll-like receptor 4 (TLR4) triggering facilitates antigen translocation to the cytosol. **(A)** Immature monocyte-derived DCs (moDCs) and moDCs that received a TLR4 stimulus at $t = 0$ [monophosphoryl lipid A (MPLA)] were pulsed with gp100/AZN-D1 for 3 h and subsequently co-cultured o/n with $gp100_{280-288}$ $CD8^+$ T cells. IFNγ secretion was analyzed by ELISA as a measure for T cell activation. **(B)** Immature moDCs were incubated with 0.1% DMSO (vehicle), gp100/AZN-D1 (10 μg/ml), gp100 synthetic long peptide (SLP) (10 μM), and the $gp100_{280-288}$ 9-mer minimal epitope (1 μg/ml) for 3 h. Thereafter, moDCs were co-cultured with $gp100_{280-288}$ $CD8^+$ T for 45 min and CD107a/b expression on the cell surface was analyzed as a measure for $CD8^+$ T cell activation. Groups are significantly different compared to none, vehicle, and isotype. **(C)** Immature moDCs and moDCs that received a TLR4 stimulus [lipopolysaccharide (LPS)] at $t = 0$ **(D)** were incubated 30 min prior and during the 3 h antigen (gp100/AZN-D1) pulse with 0.1% DMSO (vehicle), chloroquine (25 μM), MG132 (10 μM), epoxomicin (0.25 μM), and cathepsin S inhibitor (5 μM). Groups are significantly different compared to AZN-D1. Data represented in mean ± SD, one-way ANOVA was performed, experiments are representative of a $N = 2$ for graph A and B and a $N = 3$ for graph C and D (*$P < 0.05$, ***$P < 0.001$).

in DC-SIGN ligand routing after TLR4 stimulation is responsible for the observed increase in cross-presentation, we investigated the antigen-processing route by looking at $CD8^+$ T cell activation after DC antigen loading in the presence of relevant inhibitors. For this experiment, we incubated immature and LPS-stimulated moDCs with chloroquine for blocking of endosomal acidification, cathepsin S inhibitors to block endosomal antigen-processing or MG132 and epoxomicin to inhibit proteasomal degradation of antigens. Treatment with the inhibitors mentioned above showed only minor differences in viability (Figure S7 in Supplementary Material). Interestingly, the routing of antigen in immature and LPS-treated moDCs differed substantially (**Figures 5C,D**). While the activation of $CD8^+$ T cells by immature moDCs was not affected by the proteasome inhibitors MG132 and epoxomicin, LPS stimulated moDCs showed decreased $CD8^+$ T cell activation in the presence of these inhibitors. Also inhibition of the protease cathepsin S reduced cross-presentation by LPS-stimulated moDCs, while it did not affect cross-presentation of DC-SIGN targeted antigens in immature moDCs. This indicates that DC-SIGN-mediated uptake and proteolysis of antigen in the endosomes/lysosomes of immature moDCs is not dependent on the protease cathepsin S. By contrast, chloroquine, a drug that inhibits acidification of endosomes, significantly reduced $CD8^+$ T activation by both immature and LPS-stimulated moDCs. When we checked for HLA-A2 molecules on the cell surface after the inhibitor treatment, we observed a decrease of HLA-A2 expression on chloroquine treated moDCs, while the other inhibitors did not affect HLA-A2 expression (Figure S8 in Supplementary Material). However, the external loading of membrane expressed MHC-I molecules with the 9-mer minimal epitope in the presence of inhibitors did not result in a decrease in $CD8^+$ T cell activation (Figure S9 in Supplementary Material). Therefore it is difficult to

say if the observed decrease in CD8+ T cell activation after chloroquine treatment is related to the lower expression of HLA-A2.

Surprisingly, we did not observe any enhanced cross-presentation of TLR4-stimulated moDCs within the time frame of the degranulation assay (Figure S10 in Supplementary Material), which could possibly be explained by the short time window of antigen processing after the pulse. These results were further validated by measuring IFNγ secretion after an o/n culture with the gp100 specific CD8+ T cells (Figure S11 in Supplementary Material), confirming the enhanced CD8+ T cell activation by combining DC-SIGN targeting with a TLR4 stimulus. We observed a smaller inhibitory effect of the cathepsin S inhibitor on CD8+ T cell activation. This can be explained by the different time points and assays used to analyze the amount of CD8+ T cell activation. The secretion of IFNγ was measured in the supernatant after a co-culture of 16 h, while the percentage of degranulation was analyzed after a 45-min co-culture. In both experiments, the cells were treated with the inhibitors during the 3 h antigen pulse. Thereafter, cells were washed and co-cultured with the CD8+ T cells. Some of the inhibitors are reversible; therefore, the effect can decrease overtime explaining the difference in inhibitory capacity of the cathepsin S inhibitor (Figure S11 in Supplementary Material).

Together our results indicate that the combination of DC-SIGN targeting and TLR4 triggering leads to the escape of antigen to the cytosol, where it is further processed *via* the proteasome for cross-presentation.

DISCUSSION

In this study, we investigated the intracellular routing of the CLR DC-SIGN and its involvement in loading antigens on MHC-I through cross-presentation. While DC-SIGN targeting of antigen leads to cross-presentation by both immature and TLR4-stimulated DCs, we found a major contribution for TLR4 signaling, instigating an alternative intracellular cross-presentation route *via* the cytosol, which resulted in an increased capacity of moDCs to activate CD8+ T cells.

Targeting DC-SIGN with antigen conjugated Abs, glycan conjugated antigens or HIV virus, a natural ligand of DC-SIGN, results in efficient MHC-II and MHC-I loading and CD4+ T cell and CD8+ T cell activation, respectively (15, 16, 37–39). This makes DC-SIGN an attractive candidate for vaccine targeting strategies. Since vaccination strategies also rely on adjuvants inducing DC maturation, such as TLR agonists, understanding the intracellular fate of DC-SIGN and its ligand in both immature and TLR stimulated DCs is vital for vaccine development.

Previous studies have shown that pathogens and AZN-D1, both binding to the CRD, are taken up in a clathrin-dependent manner (40, 41), while DC-SIGN targeting *via* the neck region results in clathrin-independent internalization (42). Our data demonstrates that upon targeting the CRD with AZN-D1, DC-SIGN on immature moDCs is internalized within minutes and directed to early endosomes. At this stage, part of the DC-SIGN–ligand complexes begin to dissociate and proceed to late endosomes and lysosomes. Co-localization of the DC-SIGN ligand with the receptor was decreased after 15–30 min, which was followed in parallel with an increase of the antigen in the lysosomes. This indicates that once DC-SIGN ligands are dissociated from the receptor in the early endosomes, they are at least partly routed to maturing endosomes. Interestingly, the dissociation between the ligand and receptor occurs at the maturing endosomes, indicating that ligand and receptor follow different intracellular routes. Although DC-SIGN does not return to the membrane, we were not able to clarify its intracellular fate upon release from its ligand. Nevertheless, the clear fluorescence signal decay and previous work showing that prolonged DC-SIGN targeting with AZN-D1 significantly reduced the surface expression for up to 48 h (27) suggests that DC-SIGN is targeted for destruction. Since we observed that DC-SIGN poorly co-localizes with the lysosomes, we hypothesize that it is degraded by a different mechanism, which has not yet been identified. Endocytosis *via* DC-SIGN is regulated by a dileucine (LL) motif in the cytoplasmic tail of the receptor (16, 43) which might function as potential targets for ubiquitination. Different modes of ubiquitination exist that regulate among other protein degradation (44). Polyubiquination of the C-type lectin Mannose receptor facilitates antigen translocation from the endosomes to the cytosol (45), indicating that this process of receptor ubiquitination is a recognized mechanism, whereby CLRs redirect their cargo to the cytosol. Possibly DC-SIGN also uses this mode of action.

Our data shows an important role for the timing of the maturation stimulus when targeting antigens *via* DC-SIGN for cross-presentation to CD8+ T cells. Triggering of TLR4 has been described to lead to an enhanced cross-presentation of soluble antigen until approximately 16 h after stimulation, while fully matured DCs (LPS for >24 h) showed a decreased ability to cross-present antigen (20, 33). Apparently, 16 or 24 h of LPS stimulation can make a major difference for the ability of DCs to efficiently cross-present antigen. In our experimental setup, moDCs were stimulated with LPS o/n (16 h) and therefore should still be in the enhanced cross-presentation phase. In fact, we saw decreased shuttling of the ligand to LAMP1 positive compartments in line with the findings of Alloatti et al., who showed a decreased phago-lysosomal fusion after TLR triggering, resulting in decreased degradation, thereby supporting cross-presentation (33). While LPS did not affect the uptake capacity of the DC-SIGN receptor, its expression was dramatically decreased on both mRNA and protein level, which would result in an overall decrease in antigen uptake. Based on these data, administration of the adjuvant before providing the antigen *via* DC-SIGN targeting would not be a favorable vaccination strategy. Multiple studies have described that the enhanced efficiency of cross-presentation after TLR triggering is due to a change in antigen routing and processing, like enhanced translocation to the cytosol and increased activity of the proteasome (34–36). To investigate if a different route of antigen processing was responsible for the increase in cross-presentation, we blocked different molecules known to be important for MHC-I and MHC-II presentation in immature DCs and DCs that received a TLR4 stimulus at $t = 0$. We observed a striking difference in antigen routing as early as 3 h after antigen pulse. Both the immature and TLR4-stimulated moDCs were sensitive to chloroquine, a drug that inhibits endosomal acidification. Unexpectedly, chloroquine

had a reducing effect on MHC-I expression, making it difficult to conclude if the observed effect on $CD8^+$ T cell activation is due to the inhibition of cross-presentation or to the reduced expression of MHC-I on the cell surface. MoDCs that received a TLR4 trigger at $t = 0$ showed a significant dependence on proteasome activity, a mechanism not observed in immature DCs. Thus, our results suggest that following TLR4 triggering antigens translocate from the endosome to the cytosol, thereby entering the cytosolic pathway of cross-presentation. This route of MHC-I loading also has been described for natural DC-SIGN ligands (HIV-1 virions) (39). It has been described that TLR triggering can result in antigen translocation from endosomes to the cytosol. Dingjan et al. showed that upon LPS triggering the NOX2 complex in phagosomes produces reactive oxygen species resulting in lipid peroxidation, thereby inducing membrane damage and the release of antigen from these "leaky" endosomes (35). This was a rather quick process, already observed 30 min after LPS stimulation. Also a role for sec61 in the endosomal escape after TLR triggering has been reported (36). Our results stress the importance of appropriately timing the maturation stimulus when targeting antigens to DC-SIGN, as not only the antigen enters a more efficient route of cross-presentation, but also the fate of the DC-SIGN receptor is dependent on maturation status of the DC.

The recycling endosome is characterized, among others, by Rab11, which allows direct recycling to the plasma membrane, but also to the secretory pathway through the trans-Golgi network (46). Our data show that co-localization of the DC-SIGN ligand with Rab11 follows the same pattern as HLA-DM, a molecule associated with the MHC-II loading compartment. Since we cannot observe the return of DC-SIGN ligands to the plasma membrane and the MHC-II compartment originates from the trans-Golgi network, it is likely that Rab11 facilitates a connection between early endosomes and the MHC-II loading compartment without further contribution of lysosomal degradation.

The regulation of the internalization and intracellular routing of DC-SIGN on DCs is an important aspect for the rational design of antibody and glycan-based DC-SIGN-targeting vaccines (47). Based on this study, the use of DC-SIGN ligands in combination with TLR4 ligands would serve as excellent antigen targeting platforms to enhance the antigen cross-presentation in DC-based anti-tumor vaccination strategies.

AUTHOR CONTRIBUTIONS

All the authors were responsible for design of the experiments; data collection was performed by SH, SD, KB, PS, RM, and JG-V; all authors participated in the data analysis and interpretation; SH, SD, KB, SV, JG-V, and YK drafted and critically revised the article; all authors gave their approval for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fimmu.2018.01231/full#supplementary-material.

FIGURE S1 | Monocyte-derived DCs (moDCs) were fixed and measured by imaging flow cytometry. Once the compensation table was calculated for each of the staining sets, it was applied to the single staining samples that were acquired using the same settings as experimental samples. Proper compensation was then verified by visualizing samples in bivariate fluorescence intensity plots. Representative images are displayed underneath the corresponding dotplots.

FIGURE S2 | **(A)** After the application of the compensation table, cells were plotted in an area vs aspect ratio intensity bivariate scatter plot. Several populations could be identified. Population 1 was characterized by small area and high aspect ratio intensity. Images from the population 1 gate clearly show the events correspond to beads. Population 2 had an average area of approximately 100 square pixels and high aspect ratio intensity. Images from the population 2 gate show that these cells are small single cells with a large nucleus, suggesting these cells could be lymphocytes, a common contamination in Percoll-isolated monocyte-derived cell cultures. Population 3 had an area between 150 and 300 square pixels and an aspect ratio intensity higher than 0.6. These cells, the biggest population, represent dendritic cells in single cell suspension. The remaining populations (4 and 5) had a larger area and/or low aspect ratio intensity, suggestive of cell doublets and aggregates, as demonstrated in the corresponding imagery. **(B)** Gradient RMS on the brightfield channel 1 shows that the majority of the cells had a sharp contrast. Images have been selected with gradient RMS values across the whole range of gradient RMS values of the population. The threshold can then be manually set up in approximately 60.

FIGURE S3 | **(A)** First, a morphology mask is applied to the brightfield channel (channel 1). This mask takes the whole perimeter of the cell. Then, 5 pixels are eroded from this mask until the membrane of the cell is left out of the mask. The resulting mask is applied to the channel containing the probe of interest and a ratio of the intensity inside the mask relative to the total intensity of the cell is calculated. **(B)** Monocyte-derived DCs exposed to AZN-D1 for 30 min at 4°C show a membrane-bound pattern of staining, with a median internalization score of −0.985. When these cells are incubated at 37°C for 2 h, the probe is internalized and the internalization score increases to 1.002. A selection of cells with internalization scores ranging from −1 to 1 are depicted as a merge of the brightfield (1) and the AZN-D1 (7) channels.

FIGURE S4 | Cells used for **Figure 1A** were analyzed by confocal laser-scanning microscopy. Sagital, longitudinal, and transversal two-dimensional sections of a three-dimensional reconstruction are shown. Representative of 10 cells.

FIGURE S5 | Immature monocyte-derived DCs were pre-treated with chloroquine (50–25 μM) for 30 min at 37° and pulsed with AF-488 labeled AZN-D1 (10 μg/ml) for 30 min at 4°C. Next, they were washed and transferred to

37°C for 30 min followed by fixation. Degradation of the ligand was analyzed by flow cytometry, $N = 3$. Data are represented in mean ± SD, a two-way ANOVA was performed.

FIGURE S6 | Representative flow cytometry dot plots of $CD8^+$ T cell degranulation.

FIGURE S7 | Immature and lipopolysaccharide-stimulated ($t = 0$) monocyte-derived DCs were treated with the inhibitors: chloroquine (25 μM), MG132 (10 μM), epoxomicin (0.25 μM), cathepsin S inhibitor (5 μM), and 0.1% DMSO (vehicle) for 4 h and thereafter stained with a viability dye and analyzed by flow cytometry. Representative of a $N = 2$.

FIGURE S8 | Immature and lipopolysaccharide-stimulated ($t = 0$) monocyte-derived DCs were incubated with chloroquine (25 μM), MG132 (10 μM), epoxomicin (0.25 μM), cathepsin S inhibitor (5 μM), and 0.1% DMSO (vehicle) for 3 h at 37°C. Thereafter, cells were stained with an α-HLA-A2 antibody and the surface expression of HLA-A2 after inhibitor treatment was analyzed by flow cytometry. Representative of a $N = 2$.

FIGURE S9 | Immature and lipopolysaccharide-stimulated ($t = 0$) monocyte-derived DCs were treated with the inhibitors: chloroquine (25 μM), MG132 (10 μM), epoxomicin (0.25 μM), cathepsin S inhibitor (5 μM), and 0.1% DMSO (vehicle) 30 min prior and during the 3 h antigen pulse with the 9-mer minimal epitope of gp100 peptide. Followed by a co-culture with $gp100_{280-288}$ $CD8^+$ T cells for 45 min. The degranulation markers $CD107^{a/b}$ were stained as a measure of $CD8^+$ T cell activation, $N = 3$.

FIGURE S10 | Vehicle control of immature and lipopolysaccharide-stimulated ($t = 0$) monocyte-derived DCs. Representative of a $N = 3$.

FIGURE S11 | Immature and lipopolysaccharide-stimulated ($t = 0$) monocyte-derived DCs (moDCs) were incubated 30 min prior and during the 3 h antigen (gp100/AZN-D1) pulse with chloroquine (25 μM), MG132 (10 μM), epoxomicin (0.25 μM), cathepsin S inhibitor (5 μM), and 0.1% DMSO (vehicle). Thereafter, moDCs were co-cultured with $gp100_{280-288}$ $CD8^+$ T cells o/n, IFNγ secretion was analyzed by ELISA as a measure for T cell activation. Statistical analysis was performed by executing a one-way ANOVA. Groups are significantly different compared to AZN-D1.

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