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Screening for C-type lectin receptor (CLR)/bacteria interactions using a bovine CLR-Fc fusion protein library reveals recognition of *Pasteurella multocida* B:2 by MICL

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ABSTRACT

Pattern recognition receptors (PRRs) are an essential component of the innate immune system. Myeloid C-type-lectin receptors (CLRs) serve as PRRs and play a crucial role in pathogen recognition. While the role of CLRs has been mainly studied in mice and humans, their function in cattle is poorly understood. To address this gap, we generated a novel bovine CLR-hFc fusion protein library, enabling high-throughput screening of bovine CLR/pathogen interactions.

The functionality of the bovine CLR-hFc fusion proteins was validated with known CLR ligands using ELISA- and flow cytometry-based binding assays, by comparison of bovine CLRs with their murine, ovine and human orthologues. In a proof-of-principle pathogen binding study, we assessed CLR binding to *Pasteurella (P.) multocida,* a Gram-negative bacterial pathogen causing hemorrhagic septicemia in cattle. The bovine CLR myeloid inhibitory C-type lectin (MICL, Clec12A) was identified as a potential receptor for *P. multocida*, as it exhibited significant binding in flow cytometry binding assays. Cross-species analysis confirmed that murine and ovine MICL also binds *P. multocida*, suggesting an evolutionarily conserved recognition.

To explore MICL-dependent innate responses to *P. multocida*-derived factors, cytokine assays were performed using dendritic cells (DCs) from wild-type (WT) and MICL-deficient ($MICL^{-/-}$) mice. $MICL^{-/-}$ DCs produced higher levels of IL-6 and IL-12 upon stimulation with heat-killed *P. multocida*, suggesting a role for MICL in the down-modulation of innate responses.

The results highlight MICL as a receptor in the recognition of *P. multocida* and demonstrate the utility of the generated bovine CLR-hFc fusion protein library for pathogen screening.

1. Introduction

The innate immune system represents the first line of defence against various pathogens, such as bacteria, viruses, fungi, and parasites. Pattern recognition receptors (PRRs) in innate immunity are expressed by antigen-presenting cells including dendritic cells, macrophages and monocytes, and recognise pathogen-associated molecular patters (PAMPs) and damage-associated molecular patterns (DAMPs) (Geijtenbeek and Gringhuis, 2009). The superfamily of C-type lectin receptors (CLRs) is classified into 17 groups based on domain architecture and ligand-binding specificities. While the majority of CLRs recognise ligands in a Ca^{2+}-dependent manner, some CLRs bind to ligands independent of Ca^{2+} (Zelensky and Gready, 2005). Ligand specificity is primarily determined by the C-type-lectin-like domain (CTLD), which contains conserved amino acid motifs such as EPN, QPD or EPS (Zelensky and Gready, 2005). A crucial role of CLRs in innate immunity is the recognition of a variety of structures, including lipopolysaccharides, peptidoglycans, glycoproteins and inorganic structures like uric

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acid crystals and hemozoin (Neumann et al., 2014; Raulf et al., 2019). CLR signalling is mediated by immunoreceptor tyrosine-based activation motifs (ITAMs), immunoreceptor tyrosine-based inhibition motifs (ITIMs), or occurs ITAM/ITIM-independently, thus leading to activation of cellular functions, such as cytokine and chemokine release and antigen presentation, or down-modulation of effector functions (Billadeau and Leibson, 2002; Mayer et al., 2017).

While most studies focus on CLRs in mice and humans, their relevance in farm animals is poorly characterized (Lindenwald and Lepenies, 2020). Bovine CLRs such as Dectin-1 and Mincle exhibit structural and functional homology to their human and murine orthologues (Pedro et al., 2021). A study reported human DC-SIGN as an entry receptor for the feline coronavirus and suggested a crucial role for DC-SIGN in the development of feline infectious peritonitis (Regan and Whittaker, 2008). Canine Mincle contributes to the infection with canine parvovirus disease and affects the immune response (Neyestani et al., 2024). During infection with *Mycobacterium avium* subspecies *paratuberculosis* in sheep, Dectin-1 and Dectin-2, in conjunction with TLRs, play a role in mycobacterial recognition (Nalubamba et al., 2008). In cattle, Dectin-1 is involved in pro-inflammatory cytokine production after binding yeast β-glucans (Pedro et al., 2021).

Pasteurella multocida is a Gram-negative, non-motile, facultative anaerobic coccobacillus belonging to the *Pasteurellaceae* family (Kuhnert et al., 2000), causes a variety of diseases across multiple animal species and poses a risk for zoonotic infections of humans (Wilson and Ho, 2013). Differentiation of *P. multocida* strains was originally done by serological methods. Based on the capsular polysaccharide (CPS) structure, isolates can be classified into five serogroups (A, B, D, E and F) by an indirect hemagglutination assay (Carter, 1952) and the Heddleston gel diffusion precipitin test (Heddleston et al., 1972) allows a further categorization into 16 lipopolysaccharide (LPS) serotypes. Both criteria are usually combined for strain designation, e.g. B:2 (capsular serogroup B/LPS serotype 2). Meanwhile, the serological techniques have been largely replaced by multiplex PCR assays to determine capsular genotypes (Townsend et al., 2001) and eight unique LPS outer core biosynthesis loci L1 – L8 (Harper et al., 2015). In cattle, *P. multocida* strains are involved in two economically important diseases. Bovine respiratory disease complex (BRD) is a multifactorial disease affecting dairy, beef, and veal cattle worldwide (Ferraro et al., 2021). It is characterized by a combination of management and environmental factors that weaken host immunity and a co-infection with one or more of several viruses and bacteria. Bacterial pathogens typically found in BRD cases are *Pasteurella multocida*, *Mannheimia haemolytica*, *Histophilus somni* and *Mycoplasma bovis* (Gershwin et al., 2015). Serotype B:2 is associated with bovine haemorrhagic septicemia, a disease characterized by severe morbidity, peracute lethality and rapid, high mortality in cattle (Almoheer et al., 2022; Annas et al., 2014).

Previously, we developed CLR-human-IgG-Fc (CLR-hFc) fusion protein libraries from mouse, sheep, and mosquito (Lindenwald et al., 2020; Maglinao et al., 2014; Mayer et al., 2018; Schon et al., 2022). CLR-Fc fusion proteins allow for screening of CLR/ligand and CLR/pathogen interactions by methods such as ELISA, flow cytometry, fluorescence microscopy, and glycan array (Fischer et al., 2022). In this study, we generated a bovine CLR-hFc fusion protein library, evaluated the functionality of the bovine CLR-hFc fusion proteins by their binding to known CLR ligands and performed flow cytometry-based binding assays to identify putative receptors of *P. multocida.*

2. Materials & methods

2.1. Cloning and expression of the CLR-hFc fusion protein library

The construction of the bovine CLR-hFc fusion proteins was done as previously described (Lindenwald et al., 2020). Briefly, the extracellular domain of each CLR of interest, containing the C-type lectin-like domain (CTLDs), was analysed using the Simple Modular Architecture Research Tool (SMART) (European Molecular Biology Laboratory, EMBL) (Letunic et al., 2021), UniProt (2025) and hydropathy plots from Expasy (SIB Swiss Institute of Bioinformatics) (Wilkins et al., 1999). Primers specifically targeting the CTLD regions were designed with appropriate restriction enzyme sites and, when necessary, additional nucleotides to ensure the correct reading frame for protein expression (Table 1). Total RNA was extracted from peripheral blood mononuclear cells (PBMCs) isolated from heparinized blood samples of clinically healthy Holstein-Friesian cows (provided by the Clinic for Cattle, University of Veterinary Medicine Hannover, Foundation). PBMCs were isolated via Ficoll separation (Capricorn Scientific), and RNA was extracted using TRI reagent (Sigma-Aldrich). The quality of the isolated RNA was verified by gel electrophoresis using a 2 % agarose gel to visualise the 28S (~4.8 Kb) and 18S (~2 Kb) ribosomal RNA bands (Aranda et al., 2012). Afterwards, cDNA was synthesized using AMV reverse transcriptase (NEB, New England Biolabs). To amplify the cDNA fragment of interest, the Phusion High-Fidelity DNA Polymerase (NEB) was used. Following amplification, the PCR products were digested with the corresponding restriction enzymes (NEB) and ligated into a pFUSE-hIgG1-Fc2 expression vector (InvivoGen) encoding the human Fc (hFc) domain. Sequence verification was performed by Sanger sequencing using a Mix2Seq Kit (Eurofins). The theoretical molecular weights of the CLRs-hFc fusion proteins were determined using the Sequence Manipulation Suite tool (Stothard, 2000). The expression of bovine CLR-hFc as chimeric hFc-fusion protein was carried out in Chinese hamster ovary (CHO) FreeStyle S cells (Thermo Fisher Scientific) to ensure mammalian glycosylation. Transfection was performed using 25 kDa-linear polyethyleneimine (PEI) (Polysciences). Protein expression was verified by intracellular staining of the CHO cells using a polyclonal goat anti-human IgG (Fc)-PE (phycoerythrin) conjugated antibody (Jackson ImmunoResearch Labs) and subsequent flow cytometry analysis. After incubation for 5 days at 31 °C, 8 % CO_2 and 125 rpm, the cell supernatants were harvested. CLR-hFc fusion proteins were purified using HiTrap Protein G HP affinity chromatography columns (Cytiva) and concentrated with Spin-C UF Concentrators (Corning). The protein concentration was determined using the Micro BCA Protein Assay Kit (Thermo Fisher).

Most of the binding assays in this study were performed with CLR-hFc fusion proteins, in which the hFc fragment was fused to the C-terminus of the CTLD. To investigate whether the orientation of the CTLD affects ligand binding, we also included selected CLR-hFc fusion proteins, in which the hFc fragment was fused to the N-terminus of the CTLD (termed Dectin-1-N, SIGNR3-N, and SIGNR5-N). These constructs were cloned into the pFUSEN-hG1Fc expression vector (InvivoGen), allowing for the comparison of both C- and N-terminal CLR-hFc fusion proteins (Maglinao et al., 2014; Willment, 2022).

2.2. Coomassie staining and Western blot

To evaluate the quality, purity and molecular size of the CLR-hFc fusion proteins, CLR-hFc fusion proteins were separated by SDS-PAGE followed by Coomassie Brilliant Blue staining and Western blot. Detection of the CLR-hFc fusion proteins was conducted using a goat anti-human IgG (Fc-specific)-HRP (horse radish peroxidase) conjugated antibody (Jackson ImmunoResearch Labs), followed by signal development using the Clarity Western ECL Substrate (Bio-Rad Laboratories). Visualisation of the Coomassie gel and Western blot was performed using a ChemiDoc Imaging System (Bio-Rad Laboratories) and analysis was done using Image Lab 6.1 (Bio-Rad Laboratories).

2.3. Cultivation and inactivation of Pasteurella multocida

P. multocida capsular serogroup B:2 strain Carter 1309-1 was cultured in 30 ml Brain Heart Infusion (BHI) broth at 37 °C with continuous shaking at 200 rpm until an OD_{600} of 0.3 was reached. To determine the bacterial growth, serial dilutions of the culture were

Table 1
A-B: Bovine (a) and murine N-linked (b) CTLDs, restriction enzymes and respective forward and reverse primers.

A) CLR	Sequence	Restriction enzymes	Primers Forward (FW)/Reverse (RV)
Dectin-1 (Clec7A)	NM_001031852.1	EcoRI	FW: CGAGAATTCGAGATCCAGTTCAGGGAACAAC
		BglII	RV: CGAAGATCTTACTGACAACTTCTTCTCACAAATACTG
Dectin-2 (Clec6A)	XM_005207057.4	EcoRI	FW: CGAGAATTCGACACATGGCAACATTGGC
		BglII	RV: CGAAGATCTTAGGTAAATCTTCTTCATCTCACATATTGA
MICL (Clec12A)	XM_024991537.1 (Isoform D)	EcoRI	FW: CGAGAATTCGAATAAGCTACAAAATTTCAAAGAAGAAC
		NcoI	RV: CGACCATGGCTTTTCTTCCATCCAATATTTCACTC
	NM_001105345.2 (Isoform N)	EcoRI	FW: CGAGAATTCGAATAAGCTACAAAATTTCAAAGAAGAAC
		NcoI	RV: CGACCATGGCTTTTCTTCCATTCAATATTTCACTC
MCL (Clec4D)	NM_001193117.1	EcoRI	FW: CGAGAATTCGAAAGGAACGGGGTTCTT
		NcoI	RV: CGACCATGGCGTTGAATGCAGTTCCAGTT
DCIR (Clec4A)	NM_001191510.1	EcoRI	FW: CGAGAATTCGAAAGAAGAAAAGGCTACAAGAG
		NcoI	RV: CGACCATGGCTAAGTAGATCTTCATCATCTTGC
MDL-1 (Clec5A)	NM_001206203.1	EcoRI	FW: CGAGAATTCGCCCACAGAGAGCTCTGCA
		NcoI	RV: CGACCATGGCTTGGGCCTTCTTCTCACAG
DNGR1 (Clec9A)	XM_002687806.5	EcoRI	FW: CGAGAATTCGAAGCAGCAAGAAAAACTCA
		NcoI	RV: CGACCATGGCGGTAGAAGATCTTAGTGCGTAC
MGL-1 (Clec10A)	XM_002695798.6	EcoRI	FW: CGAGAATTCGCAGAGTGACCTGCAGACCC
		NcoI	RV: CGACCATGGCGTTGCCTTGGCTCAGTCCA
DC-SIGN (Clec4L)	NM_001145756.1	EcoRI	FW: CGAGAATTCGCAGAGAGATTCGGGAGACCGTGAGA
		NcoI	RV: CGACCATGGCGAGGACTGGGCAGGGAACCGA
Clec2	NM_001281914.1	EcoRI	FW: CGAGAATTCGCAAAACTACCTACAAGCTGAG
		NcoI	RV: CGACCATGGCAAGTAGATGTTCCACCTTTACTAC
Clec2B	XM_024992652.1	EcoRV	FW: CGAGATATCGAAAGAAGATAGGGGTGCT
		NcoI	RV: CGACCATGGCGTGCACTTTTTTCCTGC

B) CLR	Sequence	Restriction enzymes	Primers Forward (FW)/Reverse (RV)
Dectin-1-N (Clec7A)	NM_020008.4	BamHI	FW: CGAGGATCCCGACACAATTCAGGGAGAAATCC
		NheI	RV: CGAGCTAGCCAGTTCCTTCTCACAGATACTGTATGAAGA
SIGNR3-N (CD209d)	NM_130904.2	BamHI	FW: CGAGGATCCAAGGTCCCCAGCTCAGAGGTTC
		NheI	RV: CGAGCTAGCTTTGGTGGTGCATGATGAGGTT
SIGNR5-N (CD209a)	NM_133238.5	BamHI	FW: CGAGGATCCTACAAAATACCCAGTTCTCAGGAAGAAAAC
		NheI	RV: CGAGCTAGCCTTGCTAGGGCAGGAAGTTGAAAG

The respective primer pairs span the extracellular domain of the CLRs, including the CTLD domains.

prepared and plated onto Columbia sheep blood (CSB) agar plates (Thermo Fisher Scientific). The plates were incubated for 16–20 h (overnight) at 37 °C with 5 % CO_2. After incubation, colonies were counted to determine the number of bacteria.

For the flow cytometry-based binding assay, bacterial cultures were centrifuged at 4800×*g* for 10 min at 4 °C, washed with dPBS (PanBiotech) and centrifuged again. The bacterial pellet was then fixated with 4 % paraformaldehyde (PFA) in dPBS at room temperature for 20 min. Following fixation, bacteria were centrifuged, washed with dPBS and resuspended in dPBS. For cytokine enzyme-linked immunosorbent assays (ELISA), the bacteria were heat-killed at 60 °C in a water bath for 45 min. To confirm bacterial inactivation, heat-killed bacterial samples were plated on CSB agar to monitor bacterial growth.

2.4. ELISA-based binding assays

ELISA was used to determine the binding of selected CLR-hFc fusion proteins to immobilized known ligands. Binding of Dectin-1 and CD209-family was assessed by ELISA as previously described (Mayer et al., 2018). Briefly, medium-binding, half-area 96-well plates (Greiner Bio-One) were coated with 2 µg/well of zymosan (Sigma-Aldrich) for Dectin-1 binding and 2 µg/well of mannan (Sigma-Aldrich) for CD209 binding in 50 µl dPBS/well and incubated overnight at 4 °C. Following incubation, the residual coating solution was aspirated, and the wells were blocked with 1 % bovine serum albumin (BSA) (Gibco) in dPBS to prevent non-specific binding. After washing, plates were incubated with 250 ng/well of CLR-hFc fusion protein diluted in lectin-binding buffer (50 mM HEPES, 5 mM magnesium chloride and 5 mM calcium chloride; pH 7.4). Binding of the CLR-hFc proteins to the immobilized ligands was detected using *anti*-hFc-HRP antibody (Jackson ImmunoResearch Labs) [1:5000]. For signal development, an o-phenylenediamine dihydrochloride (OPD) substrate solution was used, consisting of 5 mg OPD tablet (Thermo Fisher Scientific) dissolved in 24 mM citrate buffer supplemented with 3 % hydrogen peroxide and 50 mM Na_2HPO_4 in dH_2O. The enzymatic reaction was terminated by the addition of 2.5 M sulfuric acid and the absorbance was measured at 495 nm using a Multiskan Go microplate spectrophotometer (Thermo Fisher). As specificity control for CLR-hFc binding, hFc was used as control protein. Moreover, controls without ligand addition were included to ensure that there was no or only marginal background binding of the CLR-hFc fusion proteins to the ELISA plates.

2.5. Flow-cytometry-based binding assays

Flow cytometry was used to determine the binding of selected CLR-hFc fusion proteins to known ligands in solution as previously described (Lindenwald et al., 2020; Mayer et al., 2018). For DNGR1, $1x10^6$ IPEG cells/ml were subjected to freeze-thaw treatment at −20 °C to expose cytoskeletal F-actin filaments as a known DNGR1 ligand. For Dectin-1, heat-killed *Candida albicans* (HKCA) (InvivoGen) at 1×10^7 cells/ml was used, while for MICL monosodium urate crystals (MSU) (InvivoGen) were used at 0.2 mg/ml, prepared by sonication for 5 min. The binding assay with the CLR-hFc fusion proteins was conducted in lectin-binding buffer. For MICL, DMEM (PanBiotech) supplemented with 10 % fetal calf serum (FCS) was used. For the bacterial binding assay, the bovine CLR-hFc fusion protein library was tested for binding to 4 % PFA-fixated *P. multocida* at 5×10^8 CFU/ml. The cross-species comparison among bovine, murine and ovine MICL was performed accordingly. Prior to binding, the bacteria were washed and stained with Syto61 (12 µM) (Thermo Fisher Scientific) at room temperature in the dark for 30 min to check cell viability. Binding assays were conducted in calcium-containing lectin binding buffer, and in calcium-free EDTA buffer (50 mM HEPES, 10 mM EDTA; pH 7.4) to assess Ca^{2+} dependency of the CLR interactions. The bacteria were incubated with the CLR-hFc fusion proteins for 1 h at 4 °C in the dark. In all experiments, CLR-hFc fusion proteins were used at 250 ng per sample. The bacteria were

identified in FSC/SSC density plots and gated further according to their Syto61-fluorescence. Bacteria were either labelled with a control (hFc) or the respective CLR-hFc fusion protein. Binding was detected after incubation with a fluorochrome-labelled secondary antibody (anti hFc-PE [1:200] or goat anti-human IgG (Fc)-Alexa Fluor (AF) 488, Jackson ImmunoResearch Labs [1:200]). The mean PE or AF fluorescence intensity (MFI) was recorded for all bacteria. For further details, see Fig. S1. For the flow cytometric analysis, the BD Accuri C6 Plus Flow Cytometer System (BD Biosciences) was applied and data were processed using BD Accuri C6 software (BD Biosciences).

2.6. Cultivation and differentiation of murine bone marrow-derived dendritic cells (BMDCs)

For the generation of bone marrow-derived dendritic cells (BMDCs), bone marrow cells from wild-type (WT) and MICL$^{-/-}$ mice were isolated. The cells were seeded at a density of 1×10^7 cells per T75 culture flask (Sarstedt) in IMDM medium (PanBiotech) complemented with 10 % fetal calf serum (FCS), 2 mM L-glutamine (Sigma-Aldrich) and 100 U/ml penicillin-streptomycin (Sigma-Aldrich). For the differentiation into DCs, 10 ml of supplemented IMDM (90 % IMDM complete medium supplemented with 10 % X63/GM-CSF cell supernatant) were used, followed by incubation at 37 °C with 5 % CO_2 for 8–9 days. On day two, 10 ml fresh medium was added, and a complete medium change was done on day four. Afterwards, unspecific antibody binding was blocked by incubating the cells with anti-CD16/32 (BioLegend) [1:100] and cells were stained with hamster anti-mouse CD11c-APC (BD Pharmingen) in combination with anti-mouse CD86-PE (Invitrogen) [1:200] or anti-mouse MHCII-FITC (BD Pharmingen) [1:200]. Subsequently, 1×10^5 cells/well were seeded in a 96 well TC-plate (Sarstedt). After a resting phase of 1 h, the cells were stimulated with heat-killed *P. multocida* using multiplicities of infection (MOI) of 1, 10 and 100 and 0.5 µg/ml lipopolysaccharide (LPS) (Sigma-Aldrich) as control for 24 h at 37 °C. Following incubation, the plate was centrifuged at 300×*g*, 4 °C for 5 min and the supernatants were collected for cytokine ELISA analysis.

2.7. Cytokine ELISA

Levels of interleukin (IL)-12, interleukin (IL)-6 and tumor necrosis factor-alpha (TNF-α) were quantified via ELISA following the manufacturer's protocols. The following kits were used: Mouse IL-6 and TNF-α DuoSet ELISA (R&D Systems) and mouse IL-12 p70 ELISA Development Kit (ABTS-based) (PeproTech). High binding, flat-bottom 96-well plates (Sarstedt) were used for all assays. The plates were coated with capture antibodies at the following concentration: IL-12 0.5 µg/ml, IL-6 2.0 µg/ml and TNF-α 0.8 µg/ml. Plates were incubated overnight at room temperature, followed by blocking with 1 % BSA (Gibco). Standards and experimental supernatants were added in triplicates according to the respective protocols. Detection was carried out using antibodies for IL-6 [1:60], TNF-α [1:60] and IL-12 [1:200], followed by Streptavidin-HRP [1:40] for IL-6 and TNF-α and Avidin-HRP [1:500] for IL-12. The substrate solutions were prepared with 0.01 g/ml 3,3′,5,5′-Tetramethylbenzidin (TMB) (AppliChem) for IL-6 and TNF-α and with 10 mg/ml ABTS (AppliChem) in phosphate-citrate-puffer supplemented with 30 % hydrogen peroxide for IL-12. The enzymatic reactions were stopped using 2.0 M sulfuric acid for IL-6 and TNF-α and the absorbance was measured at 450 nm with a wavelength correction at 570 nm. For IL-12, the absorbance was directly measured at 405 nm with a wavelength correction at 650 nm using the Infinite M1000 Microplate Reader (Tecan).

2.8. Statistical analysis

All data were analysed using GraphPad Prism Version 10 (GraphPad Software). Student's t-tests was applied to determine statistical significance (see figure legends for details). Asterisks indicate significant differences (n.s. = not significant, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$).

3. Results

3.1. Generation of bovine CLR-hFc fusion protein library

A bovine CLR-hFc fusion library was generated, comprising bDectin-1 (Clec7A), bDectin-2 (Clec6A), bMICL (Clec12A), bMCL (Clec4D), bDCIR (Clec4A), bMDL-1 (Clec5a), bDNGR1 (Clec9A), bMGL-1 (Clec10A), bDC-SIGN (Clec4L), bClec2 and bClec2B. Most purified CLR-hFc fusion proteins exhibited apparent molecular weights above their theoretical predictions (Figure 1A and B, Table 2), which suggests post-translational modification, such as glycosylation. Moreover, several CLR-hFc fusion proteins appeared as double bands, suggesting the presence of different glycoforms. Protein purity was determined by Coomassie staining (Fig. 1A) and the identity of the CLR-hFc fusion constructs was confirmed by Western blot (Fig. 1B), demonstrating a successful production of the bovine CLR-hFc fusion protein library.

3.2. Functional testing of selected bovine CLR-hFc fusion proteins

To verify the functionality of the generated bovine CLR-hFc fusion protein library, ELISA- and flow cytometry-based binding studies were performed with known ligands of CLR orthologues from other animal species. We compared the binding profiles of bovine CLR-hFc fusion proteins with ovine, murine and human CLR-hFc fusion proteins orthologues, as well as with selected CLR ligands. Previously, we generated an ovine CLR-hFc library and determined binding to known CLR ligands (Lindenwald et al., 2020). Hence, selected ovine CLR-hFc fusion proteins were included in the binding assays as additional controls and for comparison. The CD209 family, including bovine and human DC-SIGN (Clec4L), murine SINGR3, murine SIGNR3-N and SIGNR5-N, was tested for binding to mannan, an α-linked mannose-rich polysaccharide (Fig. 2A) (Cambi et al., 2008). All CLRs displayed significant binding, which indicates comparable ligand binding

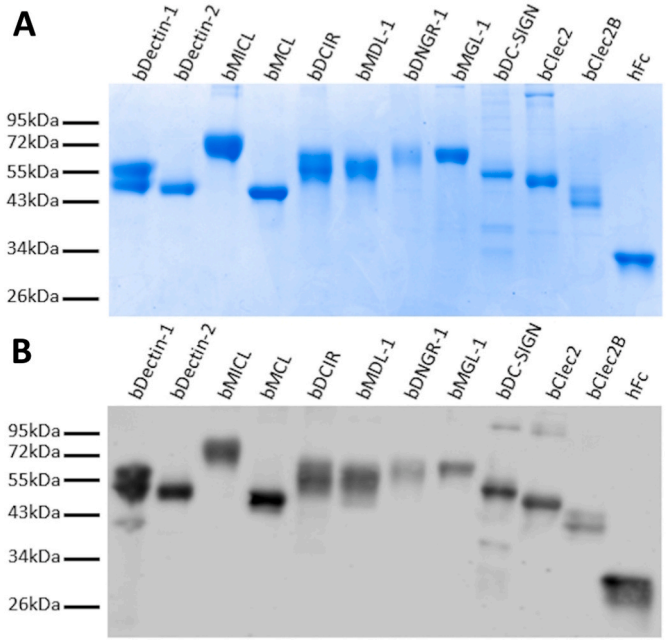

Fig. 1. Generation of the bovine CLR-hFc fusion protein library
Coomassie staining **(A)** and Western blot **(B)** analysis of the generated bovine CLR-hFc fusion protein library. For Coomassie staining, 1 µg, and for the Western blot, 300 ng of each CLR-hFc fusion protein was loaded. For further details, see Table 2.

Table 2
Expected size of bovine CLR-hFc fusion protein library.

CLR	Size (kDa)	CLR	Size (kDa)
Dectin-1 (Clec7A)	46	**DNGR1** (Clec9A)	47
Dectin-2 (Clec6A)	45	**MGL-1** (Clec10A)	54
MICL (Clec12A)	50	**DC-SIGN** (Clec4L)	48
MCL (Clec4D)	45	**Clec2**	46
DCIR (Clec4A)	45	**Clec2B**	41
MDL-1 (Clec5A)	43	**hFc**	26

preferences. For Dectin-1 (Clec7A), we included heat-killed *Candida albicans* (HKCA) and zymosan, a fungal β-1,3 linked glucose polysaccharide from *Saccharomyces cerevisiae* yeast, as ligands (Figure 2B and C) (Netea et al., 2006). Bovine and ovine Dectin-1 as well as murine Dectin-1-N exhibited significant binding to both HKCA and zymosan in comparison to the hFc control. For DNGR1 (Clec9A) (Fig. 2D), we examined binding to damaged IPEG cells, which contain intracellular filamentous actin (F-actin), a known ligand of the murine and ovine DNGR1 (Lindenwald et al., 2020). The results showed substantial binding also for bovine DNGR1. MICL from bovine, ovine and murine origin was tested for binding monosodium urate (MSU) crystals (Fig. 2E) (Neumann et al., 2014). Murine MICL exhibited strong binding to MSU but also ovine and bovine MICL displayed significant binding. In summary, the newly generated bovine CLR-hFc fusion protein library exhibited binding to a panel of known ligands, consistent with those recognized by murine, ovine and human orthologues.

3.3. Binding study with *Pasteurella multocida*

The bovine CLR-hFc fusion protein library was screened for binding to fixated *P. multocida* using a flow cytometry-based binding assay. Among the CLRs tested, bovine MICL displayed significant binding to *P. multocida* (Fig. 3A). In the absence of Ca^{2+}, bovine MICL showed no binding to *P. multocida* (Fig. 3B). While MICL has not been described as a

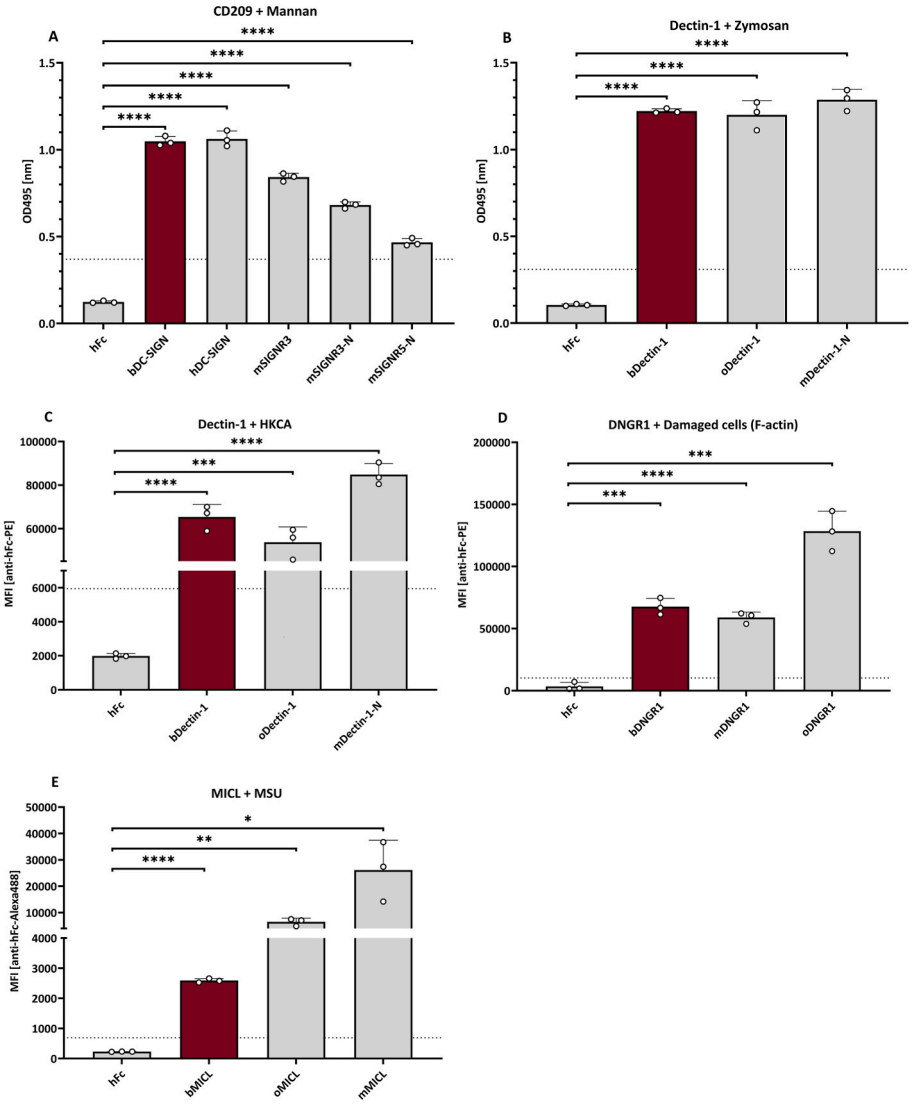

Fig. 2. Functional testing of selected bovine CLR-hFc fusion proteins
ELISA-based binding studies comparing bovine CLR-hFc fusion proteins with ovine, murine and human orthologues and selected known CLR ligands and hFc as control **(A–B)**. Mannan [40 μg/ml] was used as ligand for the CD209-family **(A)** and zymosan [40 μg/ml] for Dectin-1 **(B)**. Flow cytometry-based binding analysis comparing bovine CLR-hFc fusion proteins with their ovine and murine orthologues and selected known CLR ligands **(C–E)**. Dectin-1 binding was assessed with HKCA [1×10^7 cells/ml] **(C)**, DNGR1 was assessed using damaged IPEG cells [1×10^6 cells/ml] containing F-actin **(D)**. MICL binding was tested using MSU [0.2 mg/ml] **(E)**. Data is presented as mean ± SD. One representative experiment out of two independent experiments (n = 2, each performed in triplicate samples) is shown. Statistical analysis was performed using unpaired two-tailed Student's t-test. Asterisks indicate significant differences (n.s. = not significant, $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$).

classical Ca^{2+}-dependent CLR (Marshall et al., 2004; Tang et al., 2024), we observed a marked reduction of MICL binding to *P. multocida* in the absence of Ca^{2+}, suggesting Ca^{2+}-dependent recognition of *P. multocida* by MICL.

To investigate whether *P. multocida* is recognized by MICL from different animal species, binding of bovine MICL to *P. multocida* was compared to its murine and ovine orthologues. We hypothesized that *P. multocida* may be recognized by MICL of different animal species. Indeed, bovine, ovine, and murine MICL-hFc fusion proteins exhibited comparable and Ca^{2+}-dependent significant binding to *P. multocida* (Figure 4A and B).

3.4. Cytokine production in DCs after stimulation with heat-killed Pasteurella multocida

To determine whether MICL affects innate responses to *P. multocida*-derived factors, BMDCs from murine wild-type (WT) and MICL-deficient ($MICL^{-/-}$) mice were stimulated with heat-killed *P. multocida* at multiplicities of infection (MOI) 1, 10 and 100, as well as lipopolysaccharide (LPS), for 24 h. The production of the pro-inflammatory cytokines IL-12, IL-6, and TNF-α was quantified. The results revealed a significant increase of IL-12 secretion by $MICL^{-/-}$ BMDCs compared to WT BMDCs at MOI 10 and 100 (Fig. 5A). A similar trend was observed for IL-6, with significantly elevated levels detected in $MICL^{-/-}$ BMDCs at MOI 100 (Fig. 5B). In contrast, there were only minor differences in the TNF-α production between WT and $MICL^{-/-}$ BMDCs (Fig. 5C). Compared to WT DCs, $MICL^{-/-}$ DCs secreted significantly higher amounts of IL-12, IL-6, and TNF-α after stimulation with a TLR-4 agonist (LPS, Figure 5A–C), which may suggest a generally lower activation threshold of $MICL^{-/-}$ DCs, consistent with the role of MICL as an inhibitory receptor.

4. Discussion

To gain a deeper understanding of the role of CLRs in cattle, we established a bovine CLR-hFc fusion protein library as an *in vitro* screening tool. This approach is based on previously generated CLR-hFc libraries from other species (Lindenwald et al., 2020; Maglinao et al., 2014; Mayer et al., 2018; Schon et al., 2022). The extracellular CTLD, including the carbohydrate recognition domain (CRD), of selected bovine CLRs, was successfully fused to the Fc fragment of human IgG. Functional analyses confirmed ligand binding capacity similar to CLRs from mouse and sheep for several bovine CLR-hFc fusion proteins, including bDNGR1, bDC-SIGN, bDectin-1 and bMICL. The interaction between bDectin-1 and zymosan has already been described (Pedro et al., 2021); our results are consistent with this finding. Likewise, DNGR1 exhibited recognition of cellular damage, similar to its murine (Ahrens et al., 2012) and ovine (Lindenwald et al., 2020) orthologues. Moreover, consistent with studies showing human DC-SIGN binding to mannan (Netea et al., 2006), bovine DC-SIGN displayed binding to this ligand as well. The functionality of bMICL was also successfully validated using MSU, a well-characterized ligand of murine MICL (Neumann et al., 2014).

CLR-hFc protein libraries provide a valuable screening tool for studying CLR/pathogen interactions. The expression of CLR-hFc fusion proteins requires a correct construct design to preserve the full CRD within the CTLD and avoid frameshift mutations. Mammalian expression systems, such as CHO cells, are important for proper glycosylation, which can affect ligand-binding capacity (Bloem et al., 2013). Flow cytometry-based binding assays allow for binding studies in solution, including viability staining and gating strategies to discriminate debris from the pathogens. One challenge in the present study was the small size of *P. multocida,* requiring adjustments to the forward scatter (FSC) threshold set and the use of the viability stain Syto 61 to accurately distinguish bacterial populations.

To further investigate the impact of the Fc fragment as fusion partner of the CTLD on ligand binding, some of the murine CLR-hFc fusion proteins were designed by fusing the hFc fragment to the N-terminus of the CTLD instead of the C-terminus. To this end, we included murine Dectin-1-N, SIGNR3-N and SIGNR5-N, all of which retained their ability to bind to known ligands. These results suggest that the structural orientation has limited effect on ligand binding, at least for the respective CLR-hFc constructs.

Screening of the generated bovine CLR-hFc fusion protein library against *P. multocida* identified bovine Myeloid inhibitory C-type lectin (MICL, CLEC12A) as a putative receptor that displayed binding to heat-killed *P. multocida*. MICL is expressed by innate immune cells, including granulocytes, macrophages, and DCs and contains an ITIM, which attenuates inflammatory signaling pathways such as cytokine release to down-regulate immune responses (Gabay, 2006). MICL is known to recognise DAMPs, such as monosodium urate crystals (MSU) (Neumann et al., 2014), and was reported to bind to hemozoin during malaria (Raulf et al., 2019). It was shown that MICL is involved in viral and parasitic infections as well as autoimmune diseases, including rheumatoid arthritis, and experimental autoimmune encephalomyelitis (Barra et al., 2025; Kattner et al., 2023; Li et al., 2019; Raulf et al., 2019; Redelinghuys et al., 2016; Sagar et al., 2017; Wakim et al., 2015). MICL also plays an important role in the development of cerebral malaria (Raulf et al., 2019). In contrast, knowledge about the function of MICL in

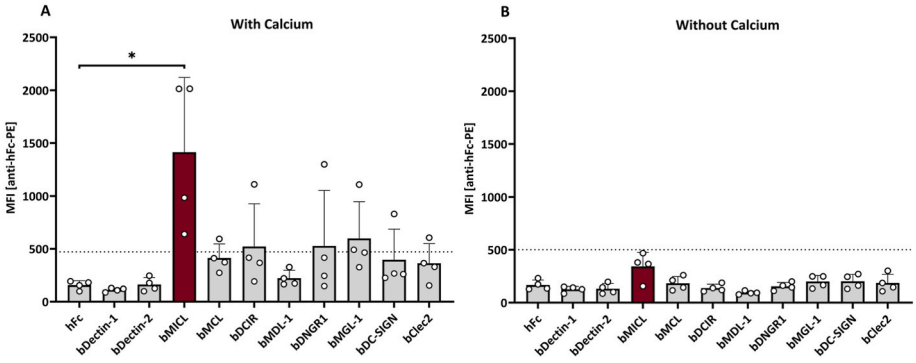

Fig. 3. Binding of *Pasteurella multocida* to bovine CLR-hFc fusion proteins
A flow cytometry-based binding assay was performed to assess the binding of bovine CLR-hFc fusion proteins to *P. multocida* [5×10^8 CFU/ml]. Bacteria were stained with Syto61 [12 μM]. 250 ng of each bovine CLR-hFc fusion protein (bDectin-1, bDectin-2, bMICL, bMCL, bDCIR, bMDL-1, bDNGR1, bMGL-1, bDC-SIGN, bCLEC2) and hFc as control were tested for binding *P. multocida*. Binding was detected using *anti*-hFc-PE [1:200]. To evaluate calcium dependency, the binding assay was conducted in the presence **(A)** and absence **(B)** of calcium. Data is presented as mean ± SD. Cumulative data from four independent experiments (n = 4, each performed as a single measurement) is shown. Statistical analysis was performed using paired two-tailed Student's t-test. Asterisks indicate significant differences (n. s. = not significant, $*p < 0.05$).

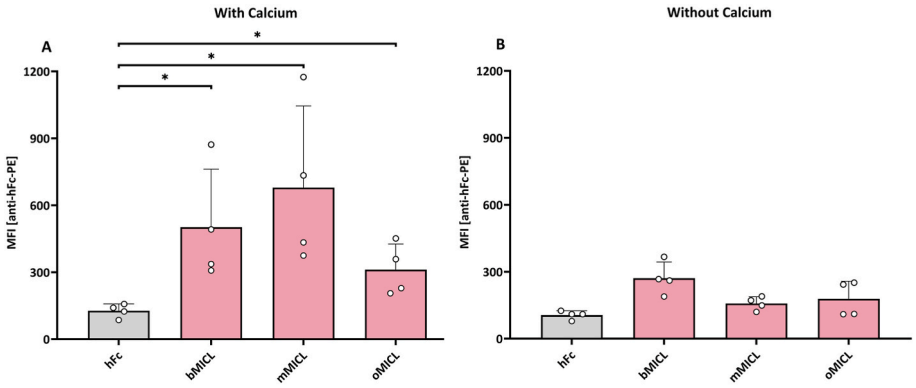

Fig. 4. Cross-species comparison of MICL binding to *Pasteurella multocida*
A flow cytometry-based binding assay was conducted to evaluate the interaction of MICL-hFc fusion proteins from different species with *P. multocida* [5×10^8 CFU/ml]. Bacteria were stained with Syto61 [12 μM]. Bovine, murine and ovine MICL and hFc as control were tested (250 ng per sample). Binding was detected using the detection antibody *anti*-hFc-PE [1:200]. To evaluate calcium dependency, the binding assay was conducted in the presence **(A)** and absence **(B)** of calcium. Data is presented as mean ± SD. Cumulative data from four independent experiments (n = 4, each performed as a single measurement) is shown. Statistical analysis was performed using paired one-tailed Student's t-test. Asterisks indicate significant differences (n.s. = not significant, $*p < 0.05$).

bacterial infections is scarce. Recent studies reported a relevance of MICL in binding mycolic acid from *Mycobacterium tuberculosis* (Nishimura et al., 2023), antibacterial autophagy during *Salmonella* Typhimurium infection (Begun et al., 2015), recognition of *Mycoplasma mycoides* (Lindenwald et al., 2020) and *Legionella pneumophila* (Klatt et al., 2023).

To explore species specificity, we included murine and ovine MICL orthologues in our analysis. We hypothesized that ligand recognition by MICL may be evolutionarily conserved across species, which was supported by the observation that murine and ovine MICL also bound to *P. multocida*. Similar results were recently observed for bovine Mincle in comparison with human and murine Mincle, for which amino acid sequence homologies of around 70 % were found (Holder et al., 2023). While MICL has been described as a non-classical CLR, it was also shown to recognise bacterial structures, such as mycolic acid in *M. tuberculosis* (Nishimura et al., 2023) and a yet unknown ligand in *L. pneumophila* (Klatt et al., 2023). In this study, we observed that MICL senses the bacterium *P. multocida*. The capsule of *P. multocida* contains structures which may have the potential to interact with MICL. For instance, the capsular serogroup B structurally consists of units of N-acetyl-β-D-mannosaminuronic acid, N-acetyl-β-D-glucosamine and β-D-fructofuranose (Richardson et al., 2023), which should be evaluated for MICL binding in further studies.

To assess the cytokine response triggered by the recognition of *P. multocida*-derived factors by MICL, we stimulated DCs from wild-type and MICL$^{-/-}$ mice with varying MOI of heat-killed *P. multocida*. Since murine MICL bound to *P. multocida*, we used this assay as a surrogate test for determining the MICL dependency of the early DC cytokine response to *P. multocida*-derived factors. We observed a significant increase in IL-12 and IL-6 levels in MICL$^{-/-}$ DCs compared to wild-type DCs, which is in accordance with its putative role as inhibitory CLR (Marshall et al., 2006). In contrast, only minor differences in TNF-α levels were observed suggesting that MICL may differentially affect signalling pathways leading to cytokine production. It is unlikely that a single CLR acts as a sole receptor for *P. multocida*, as this bacterium displays a variety of PRR ligands. Indeed, additional PRRs, such as Toll-like receptors (TLRs), are involved in *P. multocida* sensing and substantially contribute to cytokine production. In a rabbit infection study with *P. multocida* capsular serogroup A, TLR2 and TLR4 activation was observed leading to the upregulation of pro-inflammatory cytokines such as IL-1β, IL-6, IL-8, and TNF-α (Yang et al., 2022).

While our findings suggest that MICL may have immunomodulatory potential in *P. multocida* infection, a modulatory or stimulatory role of MICL may be context-dependent. For instance, a recent study showed that innate immune responses and recall T cell responses were enhanced in MICL$^{-/-}$ mice after mycobacterial infection. Thus, mycobacteria dampen host immune responses by hijacking the inhibitory receptor MICL through their mycolic acid (Nishimura et al., 2023), consistent with the role of MICL as an inhibitory receptor. In contrast, MICL signalling positively contributes to neuropathology in cerebral malaria and experimental autoimmune encephalitis, suggesting that this CLR may also exhibit context-dependent activation of initiated inflammatory processes (Raulf et al., 2019; Sagar et al., 2017).

While we determined cytokine responses in DCs from wild-type and MICL$^{-/-}$ mice, we did not include bovine antigen-presenting cells in this study, e.g. monocyte-derived DCs or macrophages, to verify a role for MICL in innate immune cells of cattle. This is a limitation of this work and should be addressed in future studies, for instance by MICL knockdown or CRISPR-Cas9-mediated gene editing of MICL in bovine cells. Thus, further investigations are needed to compare MICL binding among different *P. multocida* capsular serotypes, identify the distinct *P. multocida* ligand(s) recognized by MICL, and assess the initiated innate response upon MICL engagement by *P. multocida.*

5. Conclusion

Our study establishes a bovine CLR-hFc fusion protein library as a screening tool for studying CLR/ligand and/or CLR/pathogen interactions. In a proof-of-concept binding assay, we show the utility of the established bovine CLR-hFc fusion protein library for binding studies to pathogens, as exemplified by *P. multocida*. We demonstrate the binding of bMICL to *P. multocida*, suggesting a role for this CLR in bacterial recognition and initiated innate responses upon *P. multocida* sensing.

CRediT authorship contribution statement

Samira Christin Görig: Writing – review & editing, Writing – original draft, Investigation, Data curation. **Yeliz Gün:** Writing – review & editing, Resources. **Dimitri Leonid Lindenwald:** Writing – review & editing, Resources. **Jochen Meens:** Writing – review & editing, Resources. **Hans-Joachim Schuberth:** Writing – review & editing, Funding acquisition. **Bernd Lepenies:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

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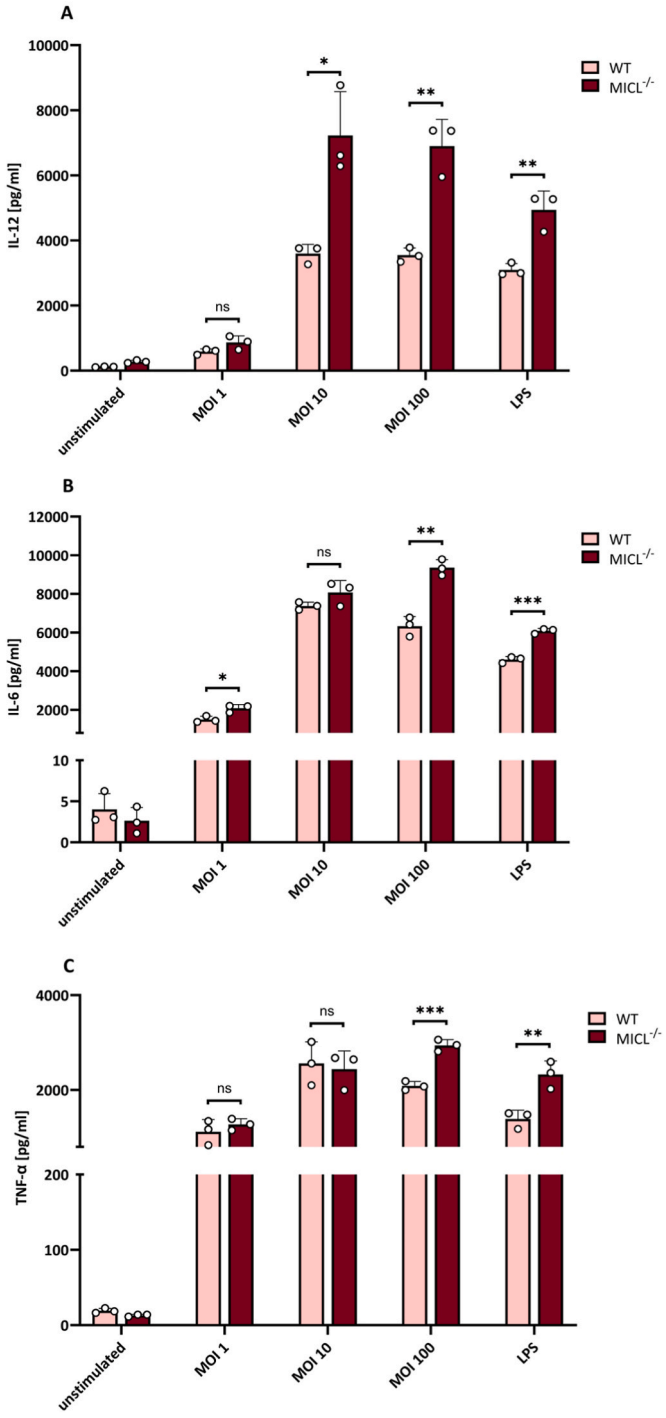

Fig. 5. Cytokine production of murine WT vs. MICL$^{-/-}$ DC after stimulation with heat-killed *Pasteurella multocida*

BMDCs from WT and MICL$^{-/-}$ mice were stimulated with heat-killed *P. multocida* using different MOI 1,10, 100 and LPS [0.5 μg/ml] as a positive control, for 24 h. The production of IL-12 **(A)**, IL-6 **(B)**, and TNF-α **(C)** was measured. Data are presented as mean ± SD. One representative experiment out of five independent experiments (n = 5, each performed in triplicate samples) is shown. Statistical analysis was performed using unpaired two-tailed Student's t-test. Asterisks indicate significant differences (n.s. = not significant, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$).

Declaration of competing interest

No author has any competing interests or conflicts of interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.dci.2025.105474.

Data availability

Data will be made available on request.

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