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Cyanovirin-N inhibits mannose-dependent *Mycobacterium-C*-type lectin interactions but does not protect against murine tuberculosis

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Abstract

Cyanovirin-N (CV-N) is a mannose-binding lectin that inhibits HIV-1 infection by blocking mannose-dependent target-cell entry via C-type lectins. Like HIV-1, *Mycobacterium tuberculosis* expresses mannosylated surface-structures and exploits C-type lectins to gain cell-access. Here we investigated whether CV-N, as for HIV-1, can inhibit *M. tuberculosis* infection. We found that CV-N specifically interacted with mycobacteria by binding to the mannose-capped lipoglycan lipoarabinomannan. Furthermore, CV-N competed with the C-type lectins DC-SIGN and mannose receptor for ligand binding and inhibited the binding of *M. tuberculosis* to dendritic cells but, unexpectedly, not to macrophages. Subsequent *in vivo* infection experiments in a mouse model demonstrated that CV-N, despite its activity, did not inhibit or delay *M. tuberculosis* infection. This outcome argues against a critical role for mannose-dependent C-type lectin interactions during initial stages of murine *M. tuberculosis* infection and suggests that, depending on the circumstances, *M. tuberculosis* can productively infect cells using different modes of entry.

INTRODUCTION

Tuberculosis (TB), caused by *Mycobacterium tuberculosis*, is the most deadly bacterial disease worldwide and kills ~1.7 million people each year (1). As the prevalence of drug-resistant *M. tuberculosis* is rising and the current vaccine for TB, i.e. *Mycobacterium bovis* BCG, shows a variable efficacy (2), new TB vaccines and drugs are urgently needed (1). *M. tuberculosis* uses a large repertoire of strategies to modulate the host immune response, several of which are associated with mannosylated cell-surface structures (3). Upon inhalation, *M. tuberculosis* enters the lungs and establishes an infection by invading alveolar

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Conflict of interest

The authors declare that no conflict of interest exists related to this work.

macrophages (M ϕ) and dendritic cells (DCs). For this, *M. tuberculosis* may use different receptors, including the mannose-binding C-type lectins macrophage mannose receptor (MMR) (4) and the dendritic cell-specific ICAM-3 Grabbing Non-Integrin (DC-SIGN) (5). Both DC-SIGN and the MMR recognize mannosylated structures, including mycobacterial cell-surface glycolipids such as mannose-capped lipoarabinomannan (ManLAM) and phosphatidylinositol mannosides (PIMs) (6–9). Yet, the exact role of DC-SIGN and the MMR in establishing and maintaining *M. tuberculosis* infection remains unclear, as these receptors not only mediate mycobacterial phagocytosis, but also seem to have a role in modulating *M. tuberculosis*-related immune pathology (3,10,11).

Cyanovirin-N (CV-N) is a mannose-binding lectin derived from the cyanobacterium *Nostoc ellipsosporum* extensively studied for its ability to block infections with HIV-1 (12–14). It does so by interacting with mannosylated HIV-1 gp120 and block target-cell entry via C-type lectins (12,15). In addition, CV-N has been reported to reduce infectivity of the Ebola and Hepatitis C viruses, also by targeting surface-exposed mannosylated proteins (16,17). As mentioned above, *M. tuberculosis* expresses a large number of mannosylated cell-surface structures which it may exploit to gain access to host immune cells (3,4,6,7). This suggests that CV-N, in analogy with its ability to block viral infections, may also abrogate infection with *M. tuberculosis*. Here we report our study investigating the ability of CV-N to inhibit *M. tuberculosis* infection.

METHODS

Bacteria

M. tuberculosis strains H37Ra, H37Rv, CDC1551, HN878, and double auxotroph mc²6020 (18), *M. bovis* BCG Copenhagen (19), *M. marinum* E11 (20), and *M. smegmatis* mc²155 were grown in Middlebrook 7H9 broth (Difco) with 10% Middlebrook albumin/dextrose/catalase enrichment (BBL) and 0.05% Tween-80 or on Middlebrook 7H10 agar (Difco) with 10% Middlebrook oleic acid/albumin/dextrose/catalase enrichment (BBL) at 37°C, or 30°C for *M. marinum*. Medium for *M. tuberculosis* mc²6020 was supplemented with 100 $\mu\text{g mL}^{-1}$ L-lysine and 25 $\mu\text{g mL}^{-1}$ D-pantothenic acid (Sigma-Aldrich). *Escherichia coli* DH5 α was grown on Luria Bertani (LB) agar or broth at 37°C.

Cyanovirin-N

Recombinant CV-N was expressed in *E. coli* BL21(DE3) cells as described previously (21,22). Cells were harvested by centrifugation (7500 $\times g$) and resuspended in 100 mL cold phosphate-buffered saline (PBS) (pH7.4) containing 10 mM benzamidine prior to lysis in a microfluidizer. The total cell lysate was centrifuged at 25,000 $\times g$, and the supernatant loaded directly onto a C18 cartridge from which CV-N was eluted with a step gradient from 0–80% CH₃CN in H₂O. Fractions containing CV-N (40–70% CH₃CN) were combined, lyophilized, and purified by reverse-phase-HPLC yielding milligram quantities of CV-N with greater than 95% purity. These fractions were subjected to size-exclusion chromatography after reconstitution in PBS adjusted to pH7.4 with 0.5 M NaOH before loading onto a Superdex75 16/60 column. Fractions corresponding to homogeneous, monomeric CV-N were combined. The periodate method was used to conjugate horseradish peroxidase (HRP; Sigma-Aldrich) to CV-N (23,24). Before use, CV-N-HRP was dialyzed overnight (o/n) against H₂O at 4°C.

Monoclonal antibodies

Murine monoclonal antibodies (MAb) of the IgM class were used. MAb F30-5 recognizes the hexa-arabinan domain of LAM and binds both ManLAM and AraLAM (25,26). MAb 55.92.1A1 binds the mannose cap on LAM (25). F183-24 recognizes mainly $\alpha(1\rightarrow2)$ -linked

mannosyl residues as present in PIM₆ and with lower affinity the mannose cap on ManLAM as well (6,26). F30-5 and F183-24 were provided by Arend Kolk, Royal Tropical Institute, Amsterdam, the Netherlands. 55.92.1A1 was provided by Peter Willemsen, Central Institute for Animal Disease Control, Lelystad, the Netherlands.

SDS-PAGE/immunoblot

Bacterial cultures were grown until mid/late-log phase and disrupted with a Beadbeater (BioSpec). Supernatants were equalized for protein content using a BCA Protein Assay kit (Pierce). Cell lysates were run on a 12% SDS-PAGE gel in a tricine buffer (27), followed by transfer on a polyvinylidene fluoride membrane (Millipore) (28). Membranes were incubated in 1% bovine serum albumin (BSA) (Sigma-Aldrich) in PBS + 0.05% Tween-80 (PBST+BSA) followed by o/n incubations at room temperature (RT) with CV-N-HRP in PBST+BSA. For the immunoblots with MAbs: the membranes were incubated in 0.5% (w/v) blocking reagents (Boehringer) followed by o/n incubations at RT with the MAb in 1:1 v:v blocking reagents and PBST. As secondary antibody peroxidase-labeled goat-anti-mouse IgM (American Qualex) in 0.5% (v/v) normal goat serum was applied. After thorough washing, all membranes were developed using a mixture of 4-chloro-1-naphthol (Bio-Rad) and 3,3'-diaminobenzidine 4HCl (Sigma-Aldrich).

CV-N-HRP adhesion assay

Man- and AraLAM, polyacrylamide (PAA)-linked glycoconjugates (0.1 µg per well) (29), or bacterial suspensions ($A_{600}=1$; heat-killed at 85°C for 45 min) in NaCl-buffer were coated onto 96-well polyvinyl chloride-plates (BD Falcon, Biosciences) o/n at RT. ELISA-plates were washed with 20 mM Tris-HCl (pH7.5), 150 mM NaCl, 1 mM MgCl₂, 2 mM CaCl₂ (TSM) supplemented with 0.05% Tween-80 (TSMT). TSM + 1% BSA (Fluka) was used as blocking buffer. CV-N-HRP was titrated starting from 2.5 µg mL⁻¹ in TSM + BSA and left to incubate for 2h at RT. Binding of CV-N-HRP was detected using *o*-phenylenediamine dihydrochloride (OPD; Sigma-Aldrich) as coloring reagent and measuring absorption at 490 nm.

CV-N inhibition assay

The same protocol was used as described above, but plates were probed with serial dilutions of MMR-Fc (30) or a DC-SIGN-Fc construct (31) in the absence or presence of 50 mg/ml CV-N for 2 h at RT. Goat anti-human IgG-peroxidase (Jackson Laboratories) in 0.5% normal goat serum was used as secondary antibody.

Cells

Human peripheral blood mononuclear cells were isolated from buffy coats (Sanquin Bloodbank, Amsterdam, NL) by centrifugation on a Ficoll-Paque gradient (GE Healthcare), followed by selection for CD14⁺ monocytes using anti-CD14 magnetic beads and the Midi-MACS system (Miltenyi Biotech). Isolated monocytes were cultured in RPMI 1640 (Gibco) supplemented with 10% fetal calf serum (Gibco) (37°C, 5% CO₂) in presence of IL-4 (500 U mL⁻¹) and GM-CSF (800 U mL⁻¹) (PeproTech) to produce immature monocyte-derived dendritic cells (MoDC) (32) or in presence of 50 U mL⁻¹ GM-CSF for MoMφ. For MoMφ, the culture medium was replenished with GM-CSF on day three after isolation (33). Cells were used for further experiments on day 6 after isolation and tested for expression of DC-SIGN, MMR, and CD14 [MoDCs: DC-SIGN(high), MMR(high), CD14(low); MoMφ: DC-SIGN(low), MMR(high), CD14(high)] (Supplemental Fig. 3). RAW264.7 cells stably transfected or not with SIGNR1 or SIGNR3 were cultured in RPMI 1640 GlutaMAX (Invitrogen) supplemented with 10% FCS (Sigma-Aldrich) in the presence of 100 U mL⁻¹ penicillin/streptomycin (Invitrogen) and 10 µg mL⁻¹ Blastocidin when appropriate (34).

MoDCs and MoM ϕ binding assays

M. tuberculosis mc²6020 cultures were grown until mid-log phase ($A_{600}=0.6-0.8$), concentrated 10-fold in FITC-buffer (150 mM NaCl, 0.2 M Na₂CO₃ (pH9.2), 0.05% Tween-80), and incubated with 250 $\mu\text{g mL}^{-1}$ fluorescein isothiocyanate (FITC; Sigma-Aldrich) for 20 min at RT, after which the suspensions were washed and diluted in TSMT depending on the desired final multiplicity-of-infection (MOI). CV-N (or PBS) was added to the mycobacterial suspensions at a concentration of 200 $\mu\text{g mL}^{-1}$ and incubated for 1h at RT. After this, the bacteria were washed and diluted in RPMI 1640 medium containing 10% (v/v) FCS, and fluorescence of the different suspensions was quantified using the FLUOstar-Galaxy microplate reader (BMG). Mycobacteria were added to MoDCs and MoM ϕ which were either 10 min pre-incubated with AZN-D1 (50 $\mu\text{g mL}^{-1}$) (7), or mannan (2 mg mL⁻¹), respectively. After incubation for 45 min at 37°C, the percentage of fluorescent cells was determined using a Flow Cytometer (C6, Accuri) and analyzed using manufacturer's software (CFlow Plus version 1.0.208.2).

Phagocytotic uptake

M. tuberculosis mc²6020 was pre-incubated with CV-N at a concentration of 200 $\mu\text{g mL}^{-1}$ or PBS for 1h at RT. After this, the bacteria were washed and diluted in RPMI 1640 medium containing 10% (v/v) FCS. Human MoDCs, RAW264.7 cells, or RAW cells transfected with SIGNR1 or SIGNR3 were co-incubated for two hours in presence of serum with *M. tuberculosis* at a MOI of 5. After two hours, 200 $\mu\text{g mL}^{-1}$ amikacin was added to kill extracellular bacteria and incubated for another two hours. Cells were washed three times, lysed with 1% Triton X-100 in PBS for 5 minutes at 37°C after which the mycobacteria were plated in dilution series onto 7H10 agar plates. CFUs were counted after 3 weeks incubation at 37°C.

Statistical analysis

In vitro cell binding studies: all values were expressed as means of at least three independent experiments (each carried out in triplicate) \pm standard error of mean (n = 3). The unpaired Student *t* test was used to identify significant differences between groups ($p < 0.05$).

In vivo experiments

M. tuberculosis H37Rv was grown to an A_{650} of 0.4. Cells were harvested, resuspended in PBST, and filtered through a 5 μm -filter to obtain a single-cell suspension ($A_{650}=0.67$). Of this, 80 μL was added to 320 μL PBS supplemented or not with CV-N (100 and 500 $\mu\text{g mL}^{-1}$; CV-N:mycobacteria ratio similar to pretreatment of MOI5-suspensions in the *in vitro* assays and five-fold higher, respectively). After 2h pre-incubation at RT, cells were diluted with 10 mL of PBST and used for aerosol infection of mice. Eight-week-old C57Bl/6 mice were infected with ~100 *M. tuberculosis* colony forming units (CFUs) using a BioAerosol nebulizing generator (CH Technologies Inc., Westwood, NJ) for 10 min. Mice from separate groups were euthanized at days 0, 7, 14 and 28. Organs (lungs, spleens and livers) were homogenized in 7H9 Middlebrook supplemented medium and dilutions plated on 7H11 Middlebrook agar plates supplemented with 10% OADC (35).

The Animal Care and Use Committee (ACUC) of the National Institute of Allergy and Infectious Diseases, Division of Intramural Research, with permit number NIH IRP, PHS Assurance A4149-01, approved the animal study protocol LCID-3E under which all animal experiments were performed.

RESULTS

CV-N specifically interacts with *M. tuberculosis*

To examine the ability of CV-N to interact with mycobacteria, three different mycobacterial species were coated onto 96 well-plates and probed with serial dilutions of HRP-coupled CV-N (CV-N+HRP). Besides *M. tuberculosis* auxotrophic strain mc²6020, the closely related vaccine strain *M. bovis* BCG strain Copenhagen and the non-pathogenic species *M. smegmatis* strain mc²155 were also tested. *Escherichia coli* strain DH5 α was included as a negative control. As shown in Fig. 1A, CV-N strongly bound to *M. tuberculosis* and *M. bovis* BCG, whereas it showed only weak binding to *M. smegmatis* and *E. coli* (Fig. 1A). To investigate possible intra-species differences, CV-N-binding to a panel of different *M. tuberculosis* strains was also tested. As shown in Fig. 1B, CV-N bound equally well to all four isolates tested suggesting that binding of CV-N was species rather than strain specific. Overall, these data demonstrate that CV-N binding varies between different mycobacterial species and that CV-N strongly binds to species belonging to the *M. tuberculosis*-complex.

ManLAM is the primary CV-N ligand

Next we investigated which mycobacterial structures were recognized by CV-N. It has been reported that CV-N recognizes $\alpha(1,2)$ -linked mannosyl residues (21,22), which are also present in the mannose cap of ManLAM, and in the phosphatidylinositol hexamannoside PIM₆. First, total cell lysates of *M. tuberculosis* mc²6020, *M. bovis* BCG Copenhagen, the fish-pathogen *M. marinum* strain E11, and *M. smegmatis* mc²155 were examined on SDS-PAGE/immunoblot with CV-N+HRP. Staining was observed at the position of ManLAM for *M. tuberculosis*, *M. bovis* BCG, and *M. marinum*, but not for *M. smegmatis* which is known to express a phosphatidylinositol-capped version of LAM (PILAM) (Fig. 2A and Supplemental Fig. 1A). In addition, CV-N staining at the position of PIM₆ was observed in all four species although with varying intensity (Fig. 2A and Supplemental Fig. 1A).

To obtain further evidence that ManLAM and PIM₆ were indeed CV-N ligands, lysates prepared from wild-type *M. bovis* BCG and isogenic mutant strains with specific defects in ManLAM and/or PIM₆ biosynthesis were subjected to SDS-PAGE/immunoblot and probed with CV-N+HRP. As shown in Supplemental Fig. 1B, the mutant strains, as compared to wild-type *M. bovis* BCG, showed a different immunoblot profile. In case of the *M. bovis* BCG $\Delta capA$ strain, which expresses LAM devoid of mannose caps (25), staining at the position of LAM was no longer visible (Supplemental Fig. 1B). On the other hand, probing a *M. bovis* BCG $\Delta pimE$ mutant, which synthesizes PIM₂ and PIM₄, but not PIM₆ (6), demonstrated that the reactivity at the position of PIM₆ was absent. Finally, with the $\Delta capA\Delta pimE$ double mutant strain, devoid of both ManLAM and PIM₆, staining for both structures was absent (Supplemental Fig. 1B). To gain insight into the relative contribution of ManLAM and PIM₆ for binding of CV-N, the wild-type and mutant *M. bovis* BCG strains were analyzed for CV-N binding by whole-cell ELISA. As shown in Fig. 2B, mutation of either *pimE* or *capA* led to a reduction in CV-N binding whereas binding to the $\Delta capA\Delta pimE$ double mutant was almost absent. However, when compared to each other, the abrogating effect of the *capA* mutation was much stronger than the mutation in *pimE*, suggesting that the mannose caps on ManLAM are the dominant CV-N ligands. This finding is consistent with our earlier observation that *M. smegmatis*, which is devoid of ManLAM but does express PIM₆, shows minimal CV-N binding (Fig. 1A).

Binding of CV-N to ManLAM is dependent on the length of the mannose cap

The mannose caps of ManLAM contain one to three mannosyl residues. To investigate the influence of mannose cap length on CV-N binding, a range of polyacrylamide (PAA)-coupled glycoconjugates resembling the LAM-mannose cap structures were probed with

CV-N+HRP. Glycoconjugates (man)₁ara-PAA, (man)₂ara-PAA and (man)₃ara-PAA are equivalent for short to long mannose caps on ManLAM respectively, and the structure of (ara)₆-PAA is equivalent to the uncapped Ara₆ motif found in LAM (29). As shown in Fig. 2C, CV-N recognized both (man)₂ara-PAA and (man)₃ara-PAA, while the single mannose-structure (man)₁ara-PAA and the hexa-arabinose motif were not bound (Fig. 2C). In a similar assay, ManLAM and non-capped LAM (AraLAM) were also tested. Consistent with the data for the glycoconjugates, CV-N recognized ManLAM but not AraLAM (Fig. 2D). Hence, the presence of at least a dimannoside cap is essential for recognition by CV-N.

CV-N inhibits the binding of MMR-Fc and DC-SIGN-Fc to ManLAM and *M. tuberculosis*

ManLAM is a ligand for the human C-type lectins DC-SIGN and the MMR (7,29,36). To test whether CV-N can compete with DC-SIGN and the MMR for ligand binding, glycoconjugate (man)₃ara-PAA, purified ManLAM, or whole *M. tuberculosis* cells were coated onto a 96-well plate and probed with a DC-SIGN-Fc or MMR-Fc construct in the presence or absence of CV-N. Binding of both DC-SIGN-Fc and MMR-Fc to the coating was inhibited by CV-N (Fig. 3A and 3B). Binding of the highest concentration of DC-SIGN-Fc to (man)₃ara-PAA, ManLAM, and *M. tuberculosis*, was reduced by CV-N by 68±17% (mean±SEM), 71±16%, and 44±6%, respectively (Fig. 3A). Similarly, binding of MMR-Fc to (man)₃ara-PAA, ManLAM, and *M. tuberculosis* was reduced by CV-N by 86±1%, 87±3%, and 87±5%, respectively (Fig. 3B). Noteworthy, a clear difference in affinity for the three ligands between the lectin-Fc constructs could be observed, with MMR-Fc exhibiting a relatively high affinity for (man)₃ara-PAA (Fig. 3B). Furthermore, CV-N inhibits binding of DC-SIGN-Fc and MMR-Fc to *M. tuberculosis* in a dose-dependent manner (Fig. 3C and D, respectively).

CV-N inhibits binding of *M. tuberculosis* to DCs but not to macrophages

As the MMR is reported to be an important receptor for mycobacteria on Mφ (4), and DC-SIGN the major receptor for mycobacteria on DCs (5), our next goal was to investigate whether CV-N was also able to block binding of *M. tuberculosis* to intact Mφ and DCs. Fluorescently labeled mycobacteria were pre-incubated with CV-N or PBS, after which the bacilli were washed to remove free CV-N and added to human monocyte-derived (Mo)Mφ or MoDCs. Binding of *M. tuberculosis* was monitored by flow cytometry. While pre-treatment with CV-N reduced binding of *M. tuberculosis* to MoDCs by ~40% at both MOIs tested (Fig. 3E), no significant inhibition was observed for binding to the MoMφ (Fig. 3F), although the MoMφs expressed significant amounts of MMR (Supplemental Fig. 3). To determine whether the binding of *M. tuberculosis* to MoMφ was indeed mannose independent, an excess of mannan was added extracellularly. As shown in Fig. 3F, addition of mannan did not reduce the binding of *M. tuberculosis* to the macrophages, indicating that the MMR, at least under these circumstances, was dispensable for *M. tuberculosis* binding. Conversely, although binding of CV-N pre-treated *M. tuberculosis* to MoDCs could still be further blocked by DC-SIGN-blocking antibody AZN-D1 (Fig. 3E), the level of inhibition never exceeded that of AZN-D1 combined with PBS-treated bacteria (~8%), suggesting that CV-N directly competed with DC-SIGN for binding to the same mannose-containing structures. Finally, to confirm that CV-N can also inhibit binding of *M. tuberculosis* to MoDCs for an extended period of time in the presence of serum (i.e. complement), we co-incubated MoDCs for up to four hours with *M. tuberculosis* pre-treated or not with CV-N in the presence of serum, after which the infection load was determined by counting CFUs. As shown in Supplemental Fig. 2A, CV-N pre-treatment of *M. tuberculosis* significantly reduced the amount of CFUs retrieved from the MoDCs suggesting that CV-N inhibits both DC-SIGN-dependent binding and subsequent phagocytotic uptake of *M. tuberculosis* by human MoDCs, also in the presence of serum.

CV-N does not inhibit *M. tuberculosis*-infection in a murine aerosol infection model

As CV-N is able to inhibit interaction of *M. tuberculosis* with DC-SIGN(-Fc) and MMR(-Fc), the effect of CV-N on *M. tuberculosis* infection was next examined *in vivo*. For this, mice were infected with ~100 CFUs of *M. tuberculosis* H37Rv pre-incubated or not with CV-N using a validated aerosol infection model (35). Mice were sacrificed at days 0, 7, 14 and 28 and CFUs were enumerated in the lungs, liver and spleen. As shown in Fig. 4, no differences in CFU counts were observed between mice infected with untreated or CV-N-pre-treated *M. tuberculosis*. Also when a higher infection dose was applied (~10-fold higher), no significant differences in infection with CV-N-treated or untreated bacteria were observed (Supplemental Fig. 4). To investigate whether the lack of an *in vivo* effect was due to the fact that mouse and human DC-SIGN/MMR do not behave in the same way or that inhibiting mannose-dependent interactions has no major impact on *M. tuberculosis* host-cell interaction, a phagocytotic uptake assay was performed using the murine alveolar macrophage-like cell line RAW264.7 stably transfected or not with the murine DC-SIGN homologs SIGNR1 or SIGNR3. As shown in Supplemental Fig. 2B, the contribution of SIGNR1 and SIGNR3 to the uptake of mycobacteria by the macrophages appeared minor under these conditions, and more importantly, CV-N only induced a small, statistically non-significant, reduction in phagocytosis in all three cell lines tested. This result is consistent with the *in vivo* data and suggests that mannose-dependent interactions are (at least partially) dispensable for *M. tuberculosis* infection of murine macrophages. Furthermore, it suggests that, unlike human DC-SIGN, expression of murine SIGNR1 or SIGNR3 does not necessarily enhance the rate of infection. Thus, in conclusion, at least in this mouse model system, CV-N pre-coating of *M. tuberculosis* did not delay or alter the outcome of infection most likely because mannose-dependent interactions do not play an important role during early *M. tuberculosis* infection in the murine infection model.

DISCUSSION

Mannosylated mycobacterial cell-surface structures have been associated with various immunomodulating properties of mycobacteria (3,37). Binding of these structures to host C-type lectins, including DC-SIGN and MMR, has been suggested to modulate the host immune response and to assist in establishing *M. tuberculosis* infection (3-5,7,10,36,38,39). In this study, we demonstrate that CV-N, a mannose-binding lectin, specifically interacts with *M. tuberculosis* and reduces the interaction with host mannose-binding C-type lectins but that this inhibition does not alter the outcome of short-term *M. tuberculosis* infection in C57Bl/6 mice.

First, we tested the ability of CV-N to interact with different mycobacterial species. CV-N displayed enhanced binding to *M. tuberculosis* as compared to the vaccine strain *M. bovis* BCG, whereas it showed only marginal binding to non-pathogenic *M. smegmatis* (Fig. 1A). This indicates a possible relationship between the amount of surface-exposed mannosyl residues and the concomitant recognition by CV-N.

The observed binding pattern of CV-N shows similarity to that of host-cell receptors DC-SIGN and MMR (6,9,29,36): CV-N recognized the $\alpha(1,2)$ -linked mannosyl residues in PIM₆ and in the mannose cap of ManLAM, but not the $\alpha(1,2)$ -linked mannosyl side branches present on the mannan core domain of LAM/lipomannan or the less mannosylated PIMs PIM₄ and PIM₂ in which $\alpha(1,2)$ -linked mannosyl residues are missing (Fig. 2 and Supplemental Fig. 1). However, some differences between the binding patterns of DC-SIGN/MMR and CV-N may also exist: the MMR has been suggested to be a phagocytic uptake receptor on MoM ϕ for virulent *M. tuberculosis* strains Erdman and H37Rv, but not for strain H37Ra (4), while CV-N, at least in our assay system, did not discriminate between strains H37Rv and H37Ra (Fig. 1B). Furthermore, genetically uncapping LAM by mutating

capA in combination with the absence of PIM₆ (*pimE*-mutation) almost completely abrogated CV-N binding (Fig. 2B). In contrast, the same set of mutations was previously reported not to disturb the binding of DC-SIGN to *M. bovis* BCG (6,25). The reason for this discrepancy is unclear, but may be related to differences in specificity.

CV-N induced a significant reduction of binding of *M. tuberculosis* to MoDCs (Fig. 3E), but residual DC-SIGN-dependent binding was still present. One possible explanation is the presence of DC-SIGN-ligands that are not targeted by CV-N. One good candidate is capsular α -glucan. This extracellular glucose polymer was recently demonstrated to be a DC-SIGN ligand (40). Testing of CV-N for binding to α -glucan demonstrated that this structure does not bind CV-N (data not shown). Therefore, α -glucan could be responsible for the residual binding of *M. tuberculosis* to MoDCs in the presence of CV-N. In our assay system, binding of *M. tuberculosis* to MoM ϕ was lower as compared to binding to MoDCs. Furthermore, neither CV-N nor mannan inhibited the binding of *M. tuberculosis* to MoM ϕ (Fig. 3F). In contrast to DCs, for which DC-SIGN is the dominant receptor for binding of mycobacteria (5), M ϕ express several receptors all of which are reported to bind mycobacteria (41). Since CV-N did not block the *M. tuberculosis*-MoM ϕ interaction, binding of *M. tuberculosis* in our assay system was most likely mediated by receptors that do not depend on the recognition of mannosyl residues, e.g. complement receptors (4). This is in sharp contrast with previous studies which suggested a major role for the MMR as uptake receptor on M ϕ for *M. tuberculosis* (3,4,36). We do not have a clear explanation for this discrepancy other than differences in the assay systems. Of note, as *M. tuberculosis* induces DC-SIGN expression in human alveolar M ϕ (42), uptake by these cells in humans may still be inhibited by CV-N.

The effect of CV-N surface-coating on *M. tuberculosis* infection *in vivo* was tested in a murine aerosol infection model. Results from two independent experiments (Fig. 4 and Supplemental Fig. 4), revealed that CV-N, although capable of inhibiting mannose-dependent *M. tuberculosis*-C-type lectin interactions *in vitro*, did not block or delay the infection. As CV-N is also known to be active *in vivo* in a vaginal gel used in macaques (13), this suggests that mannose-dependent interactions, in this model system, were (at least partially) dispensable during the early stages of infection. This finding is consistent with earlier reports in which it was demonstrated that mutants defective in producing mannose caps on LAM and/or unable to produce PIM₆ behave similar to wild-type strains both *in vitro* and *in vivo* (6,25). Whether this finding is specific for the murine infection model or is also true in other models, most importantly humans, remains to be seen. Possibly, a protective effect by CV-N cannot be observed in this model system as the murine C-type lectins differ too much from the human versions, in particular DC-SIGN (43) (compare Fig. 3E with Supplemental Fig. 2B). Furthermore, CV-N did not fully block *Mycobacterium*-DC-SIGN interactions leaving open the possibility that the residual binding, possibly via capsular α -glucan, was enough to support cell-entry via mannose-binding C-type lectins.

In conclusion, we demonstrated here that coating mycobacterial mannosylated surface structures with an exogenous compound other than antibodies abrogates mannose-dependent *Mycobacterium*-C-type lectin interactions. This makes CV-N, and CV-N-like molecules, an interesting class of compounds. However, as demonstrated in the *in vivo* infection experiments, inhibition of these mannose-dependent interactions did not alter the outcome of infection. This finding suggests that mannose-dependent interactions are, at least partially, dispensable during the early phases of infection. Important remaining questions are then what are the preferred routes for *M. tuberculosis* host-cell entry *in vivo* and more importantly, does entry via different routes lead to different outcomes of infection? Further addressing these issues will not only answer the important question whether or not targeting mycobacterial cell-surface structures, for instance through carbohydrate-based antibody

vaccines, and thereby altering the cellular uptake route can be used to combat *M. tuberculosis*, but may also provide further insight into the mechanisms explaining the success of *M. tuberculosis* as a very potent human adversary.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Nonstandard abbreviations

ara	arabinosyl residue
AraLAM	non-capped LAM
CV-N	cyanovirin-N
DCs	dendritic cells
DC-SIGN	dendritic cell-specific ICAM3-grabbing non-integrin
HIV	human immunodeficiency virus
LAM	lipoarabinomannan
man	mannosyl residue
ManLAM	mannose-capped LAM
MMR	macrophage mannose receptor
Mϕ	macrophages
MoMϕ-DCs	monocyte-derived M ϕ -DCs
PAA	polyacrylamide
PIM_x	phosphatidylinositol mannosides (<i>x</i> = number of mannose residues)
TB	tuberculosis

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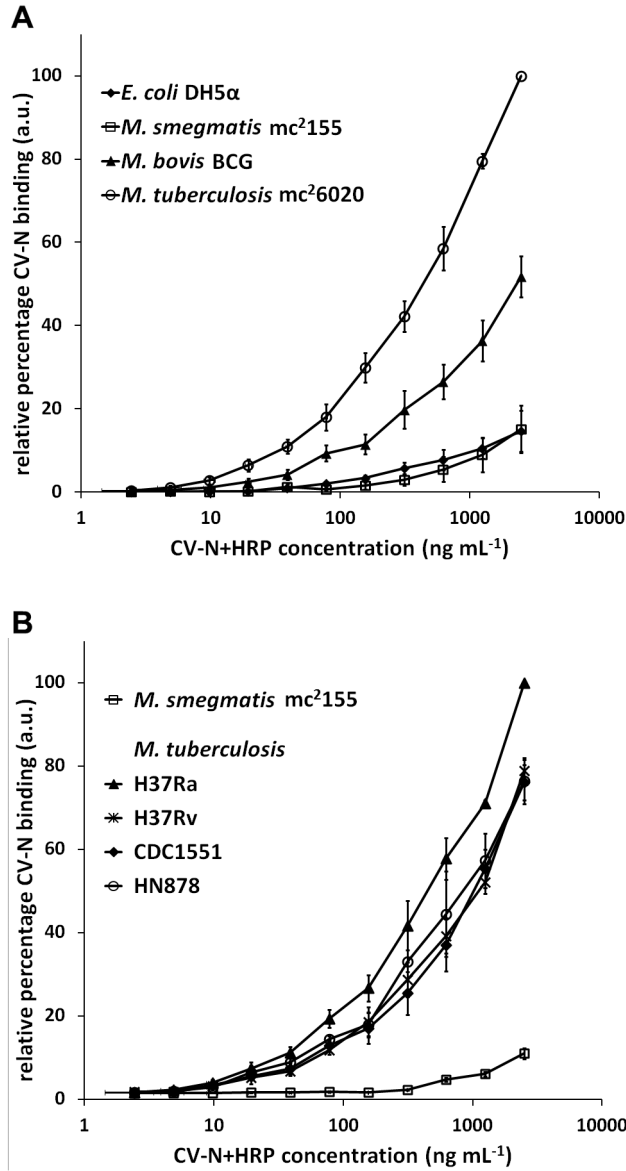
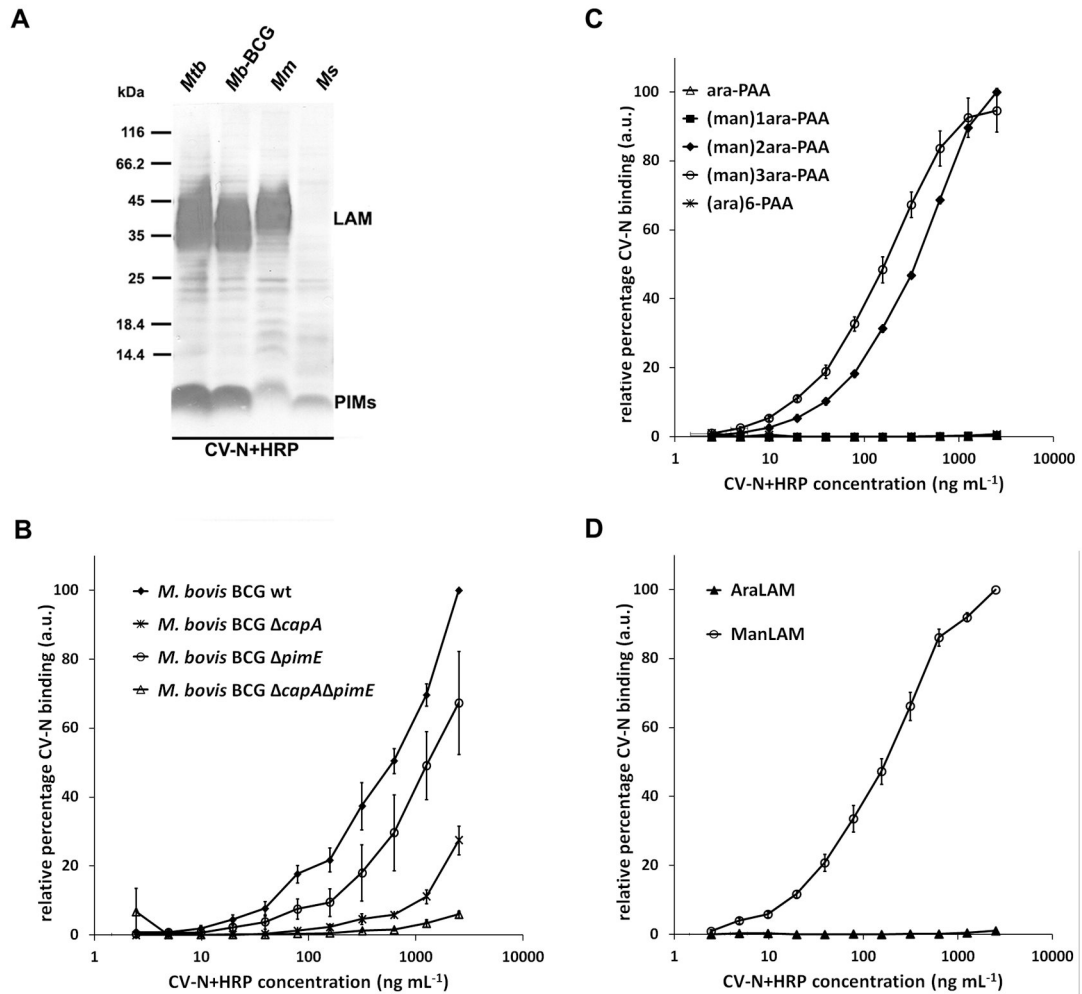
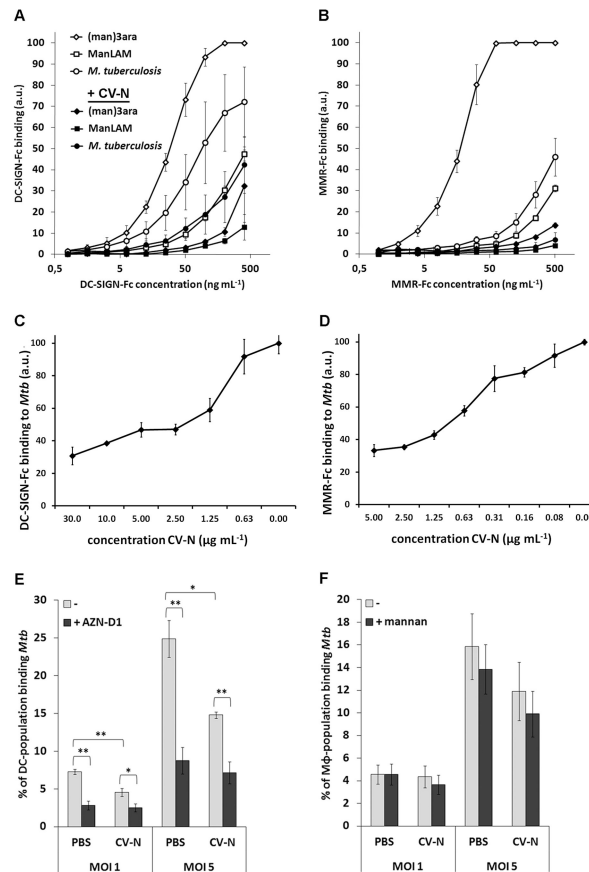


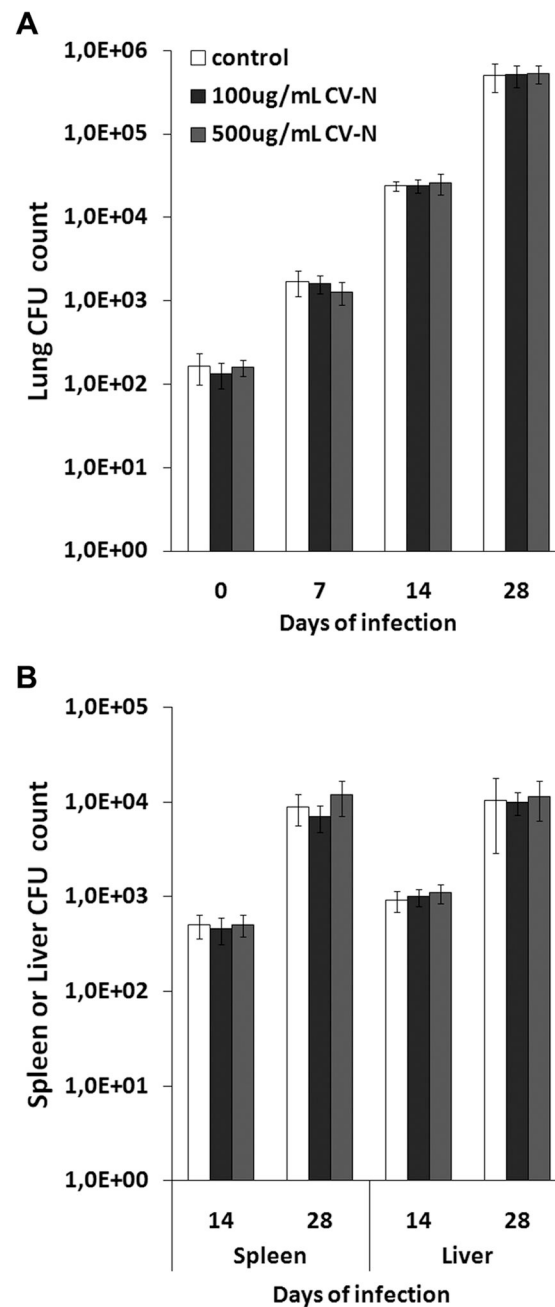
FIGURE 1. CV-N differentially recognizes various mycobacterial species. *A*, *M. tuberculosis* strain mc²6020, *M. bovis* BCG strain Copenhagen, *M. smegmatis* strain mc²155, and *E. coli* strain DH5α were coated on a 96-well plate and probed with a serial dilution of CV-N+HRP. CV-N binding was detected using OPD as coloring reagent and absorption was measured at 490 nm. *B*, In a similar assay, binding of CV-N to *M. tuberculosis* strains H37Rv, H37Ra, CDC1551, and HN878 was tested. In all cases, the data represent the average of three independent experiments plus the standard error of mean.

**FIGURE 2.**

CV-N binds the $\alpha(1,2)$ -linked mannosyl residues present in the mannose caps of ManLAM and in PIM₆. **A**, Cell lysates of *M. tuberculosis* mc²6020 (*Mtb*), *M. bovis* BCG (*Mb*-BCG), *M. marinum* E11 (*Mm*), and *M. smegmatis* mc²155 (*Ms*) were examined on SDS-PAGE/immunoblot with CV-N+HRP. CV-N+HRP binds to ManLAM of *M. tuberculosis*, *M. bovis* BCG, and *M. marinum*, but not to PILAM of *M. smegmatis*. Staining with CV-N+HRP is also seen at the position of PIMs on the immunoblot. See Supplemental Fig. 1A for staining of the same cell lysates on immunoblot with (Man)LAM- and PIM₆-specific antibodies. **B**, Whole cells of *M. bovis* BCG wild-type, $\Delta capA$ (deficient for the mannose cap on LAM), $\Delta pimE$ (deficient in biosynthesis of PIM₆), and double knockout $\Delta capA\Delta pimE$ were examined for CV-N+HRP binding in whole-cell ELISA. The average of three independent experiments is shown. See Supplemental Fig. 1B for concomitant SDS-PAGE/immunoblot with *M. bovis* BCG cell lysates. **C**, PAA-coupled glycoconjugates and, **D**, lipoglycans ManLAM and AraLAM were coated on a 96-well plate and probed with a serial dilution of CV-N+HRP. The average of three independent experiments is shown plus the standard error of mean. Note, in **C**, binding of CV-N+HRP to glycoconjugates (ara)₆-PAA, ara-PAA and (man)₁ara-PAA, was below detection levels (i.e. 0.0 in figure) in all three cases.

**FIGURE 3.**

CV-N inhibits binding of *M. tuberculosis* mc²⁶⁰²⁰ or mycobacterial mannosylated structures to DC-SIGN-Fc, MMR-Fc, and to MoDCs, but not to MoMφ. *A–B*, Whole *M. tuberculosis* mc²⁶⁰²⁰ cells, (man)₃ara-PAA, and ManLAM were coated on a 96-well plate and incubated with serial dilutions of either *A*, DC-SIGN-Fc (starting at 400 ng mL⁻¹), or *B*, MMR-Fc (starting at 500 ng mL⁻¹) in the presence or absence of 50 μg mL⁻¹ CV-N. The average of three independent experiments is shown plus the standard error of mean. *C–D*, CV-N inhibits binding of *M. tuberculosis* mc²⁶⁰²⁰ by *C*, DC-SIGN-Fc and *D*, MMR-Fc in a dose-dependent manner. Whole *M. tuberculosis* mc²⁶⁰²⁰ cells were coated on a 96-well plate and incubated DC-SIGN-Fc (400 ng mL⁻¹), or MMR-Fc (500 ng mL⁻¹) in the absence or presence of a dilution series of CV-N. Shown is one representative experiment with average of triplicates plus standard deviation. *E–F*, *M. tuberculosis* cells were fluorescently labeled, diluted to the correct MOI, pre-treated with PBS or 200 μg mL⁻¹ CV-N, washed, and added to *E*, MoDCs and *F*, MoMφ. MoDCs and MoMφ were used untreated, or after pre-incubation with the DC-SIGN-specific inhibitory antibody AZN-D1 or with 2 mg mL⁻¹ mannan, respectively. The percentage of the cell population binding *M. tuberculosis* was determined by flow cytometry. Shown is the average of results from five donors plus the standard error of mean. * $p < 0.05$; ** $p < 0.005$.

**FIGURE 4.**

Aerosolic infection of mice with untreated or CV-N-pre-treated *M. tuberculosis* H37Rv. Mycobacteria were pre-incubated with PBS ('untreated') or with CV-N at concentrations of either 100 $\mu\text{g mL}^{-1}$ or 500 $\mu\text{g mL}^{-1}$ (is comparable CV-N:bacteria ratio as used for pre-treatment of the MOI5-suspensions in the *in vitro* assays in Figure 3E-F, or five-fold higher, respectively). Mice were infected with the untreated or CV-N-treated *M. tuberculosis* via aerosolic solution on day 0. CFUs were counted in A, lung, and B, spleen and liver. The CFU count on day 0 and day 7 for spleen and liver was zero in all mice. Shown is median of five mice per group plus median absolute deviation.