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β-Glucan receptors on IL-4 activated macrophages are required for hookworm larvae recognition and trapping

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Abstract

Recent advances in the field of host immunity against parasitic nematodes have revealed the importance of macrophages in trapping tissue migratory larvae. Protective immune mechanisms against the rodent hookworm *Nippostrongylus brasiliensis* (Nb) are mediated, at least in part, by IL-4-activated macrophages that bind and trap larvae in the lung. However, it is still not clear how host macrophages recognize the parasite. An *in vitro* co-culture system of bone marrow-derived macrophages and Nb infective larvae was utilized to screen for the possible ligand–receptor pair involved in macrophage attack of larvae. Competitive binding assays revealed an important role for β-glucan recognition in the process. We further identified a role for CD11b and the non-classical pattern recognition receptor ephrin-A2 (EphA2), but not the highly expressed β-glucan dectin-1 receptor, in this process of recognition. This work raises the possibility that parasitic nematodes synthesize β-glucans and it identifies CD11b and ephrin-A2 as important pattern recognition receptors involved in the host recognition of these evolutionary old pathogens. To our knowledge, this is the first time that EphA2 has been implicated in immune responses to a helminth.

INTRODUCTION

Hookworms (*Necator americanus* or *Ancylostoma duodenale*) cause a major neglected tropical disease affecting approximately 700 million people worldwide.[1] Hookworms typically infect people living in impoverished conditions without access to adequate sanitation, and infection is characterized clinically by anemia, malnutrition in pregnant women and impaired cognitive development in children.[1] In endemic areas, hookworm burden tends to increase with age. Unfortunately, no effective vaccines exist, and current control strategies using chemotherapy rely on a small range of drugs, which must be administered at least every 6 months.[2] New strategies of control are urgently needed.

In experimental models of murine hookworm infection, protective immune responses can develop and have been shown *in vivo* to require IL-4-activated

macrophages (M(IL-4)),[3-5] which can trap and immobilize tissue invasive larvae, as well as aiding in rapid tissue repair. While it is known that arginase-1 (Arg-1) expression by macrophages is needed to provide immunity,[3-6] the exact mechanisms involved in macrophage-mediated trapping of larvae remains unclear, as do the pathways allowing macrophages to recognize hookworm larvae.

Antigen presenting cells, including macrophages, express an array of pattern recognition receptors (PRR) that enable them to recognize diverse pathogens including bacteria, viruses and fungi.[7] PRRs include Toll-like receptors (TLRs), as well as intracellular nucleotide-binding domain and leucine-rich-repeat-containing family (NLRs), retinoic acid-inducible gene I (RIG-I)-like receptors and finally C-type lectin receptors (CLRs). Because helminths trigger a type 2 immune response, there have been extensive studies of which PRR could be involved in their recognition; however, no consensus has yet been reached. C-type lectin receptors have been proposed as the most likely receptors involved in helminth recognition, due to the high glycan content of the parasite cuticle (free glycans, glycoproteins and glycolipids) and excretory secretory products.[8] Indeed, CLRs have been shown to mediate immunomodulatory activities and/or detection of a subset of helminths, including *Schistosoma mansoni*, *Fasciola hepatica*, *Trichuris muris* and *Trichinella spiralis*.[8-16]

C-type lectin receptors can both be soluble or transmembrane proteins, and can recognize a variety of carbohydrates, and to a lesser extent, proteins and lipids. CLRs are particularly diverse, with over 1000 proteins identified to date. They share a similar structure, containing at least one C-type lectin binding domain. Many classifications of the CLRs and CLR-like receptors exist, mostly based either on the recognition of common ligands or on their downstream signaling pathways. Pathogen-specific carbohydrates can be recognized specifically by a given CLR: such as mannose from fungi or Mycobacterium by CD206; α-mannan from fungi by Mincle; or β1-3 glucans from fungi and Mycobacterium by dectin-1. However, this "monogamous" view of receptor-specificity has recently been challenged by the discovery that CLRs can bind to a diverse variety of complex ligands as well as associate to other PPRs to recognize their ligands.[17]

The current study investigated a potential involvement of CLRs and their ligands in the recognition and trapping of infective third-stage larvae (L3) of the rodent hookworm, *N. brasiliensis* (Nb), by macrophages. Using a newly established *in vitro* assay of larval trapping, we demonstrate that M(IL-4) can recognize hookworm cuticle through two main β-glucan receptors: CD11b and EpHA2.

RESULTS

Recognition of hookworm larvae by macrophages can be competitively inhibited by mannan and laminarin

We and others have previously reported the importance of M(IL-4) in providing immunity to mice following secondary infection with *N. brasiliensis*.[3,4] However, to date, it is unclear how M(IL-4) are able to recognize Nb larvae. To investigate this, bone marrow-derived macrophages (BMDMs) were co-cultured in the presence of the infectious larvae (L3). After 24 h of co-culture, the recognition of larvae by macrophages was assessed by calculating the percentage of larvae with 10 or more macrophages bound to their surface. In keeping with earlier studies,[3,4] we observed that macrophage recognition of Nb L3 was enhanced after IL-4 polarization, compared with unstimulated macrophages (M0; Supplementary figure 1a). We then focused on deciphering the possible involvement of PRRs in the recognition of Nb L3 by macrophages. The nematode cuticle is rich in glycans, and CLRs have been hypothesized to be important for nematode recognition by immune cells.[18] In order to identify such CLRs on our macrophages, we performed competitive binding experiments in which bone marrow-derived M(IL-4) were co-cultured with Nb L3, together with an excess of specific CLR ligands. We used fucose as the ligand for the mannose receptor (MR; Figure 1a), N-acetylgalactosamine (NAD-Gal) as the ligand for the macrophage galactose C-type lectin 1 and 2 (MGL; Figure 1b), α-mannan as the ligand for MR or dectin-2 (Figure 1c), and laminarin as the ligand for dectin-1 (Figure 1d). Addition of fucose or NAD-Gal did not affect the binding of M(IL-4) to Nb L3 (Figure 1a, b. In contrast, addition of α-mannan or laminarin led to a dose-dependent reduction in the percentage of larvae attacked by M(IL-4) (Figure 1c, d). We thus further explored the potential recognition of hookworm by macrophage receptors recognizing α-mannan or laminarin.

Mannose receptor, dectin-2 and FcγR are all redundant for the recognition of Nb larvae by M(IL-4)

The complete repertoire of helminth glycans is still unknown to date, but recent advances in helminth glycomics have demonstrated that nematodes express numerous glycans rich in mannose residues.[19] α-Mannan can be recognized by several receptors including MR and dectin-2. Dectin-2 expression has been shown to be reduced after IL-4 stimulation in human monocytes and in dendritic cells,[20] whilst IL-4 activation causes the upregulation of MR expression on macrophages, indicating a possible involvement of this receptor in

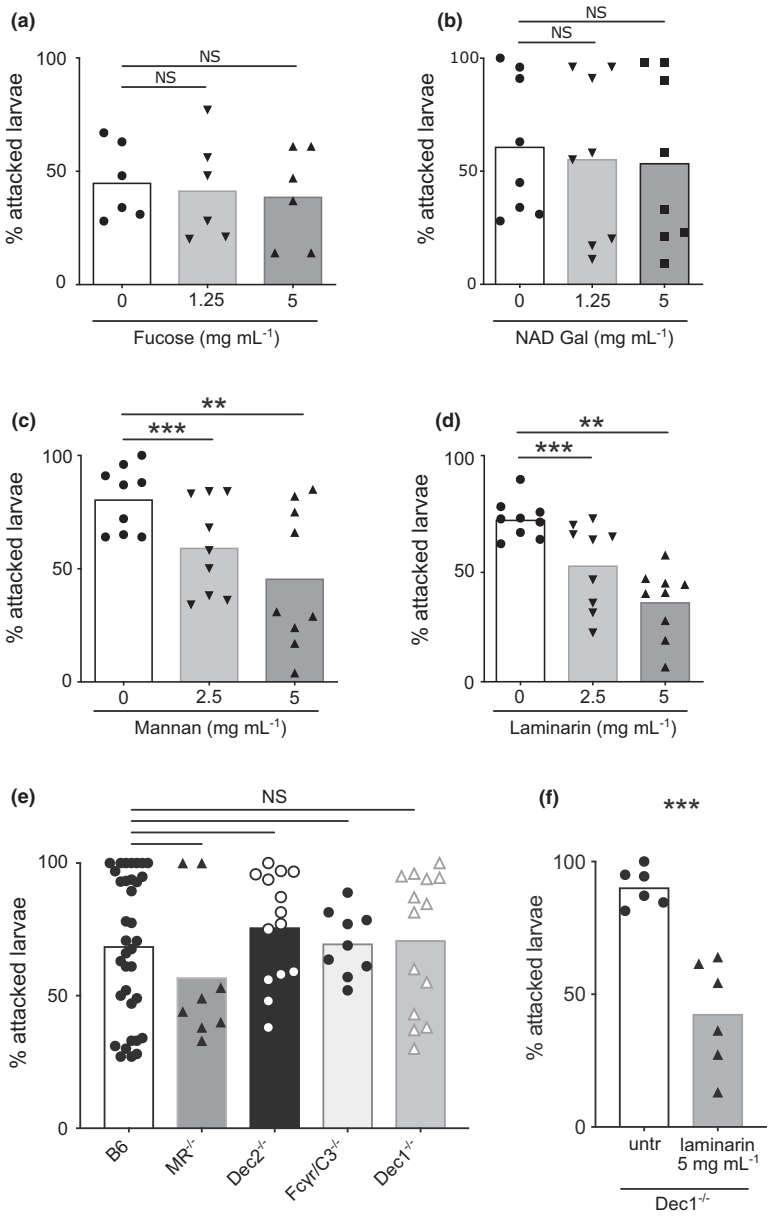

Figure 1. Competitive binding assays reveal the CLR ligands, mannan and laminarin, reduce Nb larval recognition by M(IL-4). Bone marrow derived macrophages were generated from **(a–d)** C57BL/6 (B6) wild-type, **(e)** mannose receptor deficient ($MR^{-/-}$), dectin-2 deficient ($Dec\text{-}2^{-/-}$), Fcγ receptor and complement 3 double deficient ($FcgR/C3^{-/-}$), or **(f)** dectin-1 deficient ($Dec\text{-}1^{-/-}$) mice. All macrophages were stimulated with IL-4 (10 ng mL^{-1}) and pre-incubated at 37°C for 1 h with various CLRs ligands at different concentrations as indicated (laminarin sourced from Sigma). Nb L3 were then added to the culture for 24 h. The percentage of larvae attacked by macrophages was then quantified by microscopy and manual counting of live and motile larvae. All experiments were performed 2 times independently, with $n = 2$ mice per experiment, and at least 3 technical replicates. **(e)** is pooled from 3 independent repeats. NS, not significant; untr, untreated; $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$.

hookworm recognition.[21] The MR has already been reported to recognize various helminths, such as the blood-fluke *S. mansoni*, the liver-fluke *F. hepatica* and the nematodes *T. muris*, *T. spiralis* and *A. suum*.[22] To assess the potential involvement of these receptors in M(IL-4) recognition of Nb larvae, we utilized M(IL-4) generated from the bone marrow of wild-type (WT), $MR^{-/-}$ or $dectin2^{-/-}$ mice. We also included mice lacking Fc receptor gamma (FcγR) signaling chain ($Fc\gamma R/C3^{-/-}$ mice) as dectin 2 associates with this receptor to mediate downstream signals, as does Mincle and MCL; CLRs that are also able to recognize mannose residues.[19,23]

Interestingly, no significant difference in the percentage of attacked Nb larvae was observed between WT and any of the gene-deficient mice (Figure 1e). This suggests that mannose residues expressed by Nb larvae are recognized by a yet unknown mannan receptor, or that functional redundancy exists between MR, dectin-2 and/or other FcγR-dependent CLRs for larval recognition.

Recognition of Nb larvae by M(IL-4) does not require dectin-1

Laminarin is a β-(1-3)-glucan that can competitively inhibit β-glucan binding to the dectin-1 receptor.[24] The properties of laminarin have recently been shown to be dependent on their commercial source (and their level of purity).[25] To further explore the role of laminarin in preventing hookworm recognition by M(IL-4), we repeated the binding assay in the presence of highly purified and endotoxin free laminarin (Invitrogen). As previously, laminarin led to a dose-dependent reduction in the percentage of larvae attacked by M(IL-4) (Supplementary figure 1b).

We then assessed whether the recognition of Nb by M (IL-4) was dectin-1 dependent. No difference in the percentage of attacked larvae was observed between WT and dectin-1 deficient M(IL-4) (Figure 1e). To determine whether another β-(1-3)-glucan receptor was involved, we added excess laminarin to dectin-1 deficient M(IL-4) macrophages and we observed a significant reduction in the percentage of attacked larvae, similar to that observed for M(IL-4) derived from WT mice (laminarin treatment normalized to untreated: 0.49 ± 0.21 for WT, 0.47 ± 0.22 for Dec1$^{-/-}$; Figure 1f). This observation suggested that receptor(s), other than dectin-1, present on bone marrow derived M(IL-4) can recognize larval glucans.

Because the absence of a role for dectin-1 was unexpected, we further investigated whether the laminarin reduction of hookworm recognition by M(IL-4) was physiologically relevant. Indeed, bone marrow-derived M (IL-4) are reported to be phenotypically distinct from macrophages *in vivo*. We thus repeated these co-culture experiments with pulmonary macrophages isolated from infected animals. As observed with the bone marrow derived macrophages, the percentage of larvae attacked by lung macrophages *ex vivo* was reduced by laminarin co-incubation (Supplementary figure 1c), proving the *in vivo* relevance of the observed phenomenon.

In order to rule out a possible toxic effect of laminarin on M(IL-4), we investigated whether the cellular viability and polarization were altered following overnight incubation of these cells with laminarin. We used calcein, a non-fluorescent dye that emits fluorescence only upon enzymatic conversion by live cells, as a readout of cellular viability. No difference in the percentage of viable cells could be observed between laminarin treated and untreated M(IL-4) (Supplementary figure 2a). We next assessed whether macrophage polarization by IL-4 was affected by laminarin exposure, given that macrophage polarization by IL-4 enhances their binding to Nb L3 (Supplementary figure 1a). As expected, M(IL-4) displayed slightly increased protein expression for arginase-1 and CD11b, and strongly increased the expression of CD206 compared with M0. However, addition of laminarin to M(IL-4) did not interfere with their ability to express these markers of IL-4 induced polarization (Supplementary figure 2b).

Next, to be sure that laminarin was interfering with a receptor–ligand pathway, rather than through modulating macrophage polarization, we pre-treated M(IL-4) for 24 h with laminarin, then subjected these cells to extensive washing to remove unbound laminarin. Washed M(IL-4) were then co-cultured with Nb L3 and the binding of the cells to the larvae was assessed 24 h later (Supplementary figure 2c). No significant difference in the adherence M(IL-4) to larvae was observed between those cells subjected to laminarin pre-treatment and washing compared with untreated macrophages. Lastly, we sought to determine whether laminarin could be masking another non-glucan larval PAMP motif by binding non-specifically to the larvae. To address this question, larvae were pre-incubated with laminarin for 24 h followed by a wash of the unbound laminarin, and then incubated with M(IL-4) for 24 h, as before. A significant, albeit minor, reduction in macrophage binding was observed (untreated L3 70.96% ± 7.09; versus laminarin-treated L3 62.8% ± 8.73; Supplementary figure 2d). This suggests that laminarin can indeed bind to larvae but that this binding contributes poorly to the phenotype we observed during co-incubation of laminarin with L3 and M(IL-4).

Recognition of Nb larvae by M(IL-4) requires β-glucan and can be abrogated by glucanase

Altogether, these data suggest that laminarin interferes with M(IL-4) larval recognition by competing for a receptor involved in the recognition and adherence to Nb. Furthermore, a CLR other than dectin-1 capable of recognizing β-glucans, or molecules with a similar 3D structure is involved in this process. To further substantiate this hypothesis, we assessed the impact of other β-glucans using competitive binding assays. The β-glucans employed included glucan phosphate (a β-(1-3)-D glucan from *Saccharomyces cerevisiae*), curdlan (a β-(1-3) particulate glucan) and cellobiose (a β-(1-4) glucan polymer). We also assessed the α-glucans pullulan and dextran, to determine whether the branching on the glucan was important for

macrophage recognition. All β-glucans tested were able to reduce the binding of M(IL-4) to Nb L3 in a dose-dependent manner (Figure 2a–c). In each case the extent of reduction observed for the highest dose of the β-glucan was comparable to that previously seen for laminarin (glucan phosphate at 1 mg mL^{-1} 51.4% ± 19.7; curdlan at 100 μg mL^{-1} 55.2% ± 24.5; cellobiose 10 mg mL^{-1} 44.1 ± 25.0; laminarin at 5 mg mL^{-1} 50.7 ± 20.0). In contrast, high doses of the α-glucans, pullulan and dextran, failed to have an impact on M(IL-4) binding to Nb L3 (Figure 2d).

As the presence of β-glucans on the surface of Nb larvae has not been characterized to date, we first investigated their presence on the larvae. To this aim, we stained larvae with aniline blue, a dye that specifically emits fluorescence about 500 nm when bound to carbohydrates with β-(1-3) linkages.[26,27] We observed specific fluorescent staining on the whole length of the cuticle of stained larvae, as well as some more discreet fluorescence inside the parasite (intestine and pharynx; Figure 2e).

Finally, to directly confirm whether β-glucan recognition was implicated in macrophage recognition of Nb L3, we sought to degrade β-glucans on the surface of larvae directly. Larvae were incubated for 30 min with β-(1-3)-D-glucanase (also known as laminarase) to degrade β-(1-3) and, to a lesser extent, β-(1-3; 1-4) linkages. Following enzymatic digestion and thorough washing, larvae were co-cultured with M(IL-4) as before. Cleaving surface carbohydrates in this way led to a significant reduction in the percentage of larvae attacked by M(IL-4) compared with buffer controls suggesting that β-(1-3)-glucan linkages are essential in macrophage recognition of Nb L3 (Figure 2f).

The β-glucan receptors EphA2 and CD11b both contribute to the ability of M(IL-4) to recognize Nb L3

The integrin CD11b plays a role in various adhesion reactions[28] and is also a receptor for complement C3bi.[29] CD11b has previously been implicated in the recognition of hookworm larvae by M(IL-4)[4,30] and its surface expression has been shown to be upregulated in human macrophages following IL-4 stimulation.[31] Our own studies also indicated a slight upregulation of CD11b on the surface of M(IL-4) compared with M0 derived from murine bone marrow (Supplementary figure 2b). Interestingly, CD11b has a β-glucan binding site on its C-terminal domain,[32] thus we sought to determine whether it played a role in the M(IL-4)–Nb interactions reported in our study. We first confirmed that CD11b deficient M(IL-4) exhibited a decreased ability to bind to Nb larvae (Figure 3a). To confirm, the phenotype observed is not due to a defect in the development/activation of $CD11b^{-/-}$ macrophages, we used the anti-CD11b antibody clone M1/70 (Supplementary figure 3a) that has been reported to block the β-glucan binding site of CD11b.[33] This blockade at the time of the co-culture was sufficient to decrease the percentage of larvae attacked by WT M(IL-4) (Supplementary figure 3a), proving that CD11b is likely involved directly in the recognition of Nb L3. Of note, not only was the total percentage of larvae bound by M(IL-4) affected, but we also observed that fewer macrophages were bound to each larva (Supplementary figure 3b). Surprisingly, however, the addition of laminarin to M(IL-4) derived from CD11b deficient ($CD11b^{-/-}$) mice led to a further reduction of the ability of these macrophages to attack the larvae (Figure 3b, left-hand panel). To further substantiate a contribution of CD11b to M(IL-4) recognition of Nb β-glucans we performed a side-by-side comparison of WT and $CD11b^{-/-}$ M(IL-4), with or without the addition of laminarin (Figure 3b, right-hand panel). Laminarin treatment led to a smaller reduction in larval attack by M(IL-4) when macrophages were derived from $CD11b^{-/-}$ mice compared with WT mice (laminarin treatment at 5 mg mL^{-1} normalized to untreated: 0.37 ± 0.21 for WT versus 0.86 ± 0.18 for $CD11b^{-/-}$ M(IL-4)). These data indicate that the β-glucan-binding site of CD11b accounts for part, but not all, of the ability of M(IL-4) to recognize Nb larvae.

These observations suggested that other β-glucan binding CLRs may contribute to the recognition of Nb by M(IL-4). EphA2, originally described as an oral epithelial cell PRR, has also been shown to bind to laminarin.[34] More recently, EphA2 was described to be expressed by macrophages and neutrophils[35,36] We therefore assessed whether EphA2 was involved in the recognition of Nb larvae by M(IL-4). Similar to $CD11b^{-/-}$ M(IL-4), M(IL-4) from EphA2-deficient ($EphA2^{-/-}$) mice exhibited a reduced ability to adhere to Nb L3 (Figure 3c). Again, like $CD11b^{-/-}$ M(IL-4), laminarin treatment of $EphA2^{-/-}$ M(IL-4) led to a reduction in macrophage attack of the larvae (Figure 3d), but a side-by-side comparison of WT and $EphA2^{-/-}$ M(IL-4) indicated that addition of laminarin to the $EphA2^{-/-}$ cultures did not reduce the larvae attack as efficiently as it did for WT M(IL-4): (laminarin treatment normalized to untreated: 0.19 ± 0.1 for WT versus 0.31 ± 0.22 for $EphA2^{-/-}$ M(IL-4)).

Several CLRs have been reported to dimerize or act in a synergistic manner.[37] As such, we investigated whether EphA2 and CD11b interaction was important for Nb L3 recognition by macrophages. M(IL-4) from WT and $EphA2^{-/-}$ were co-treated with anti-CD11b antibody and incubated with larvae (Figure 3e). As before, antibody treatment reduced the percentage of larvae attacked; however, there was no significant difference between the

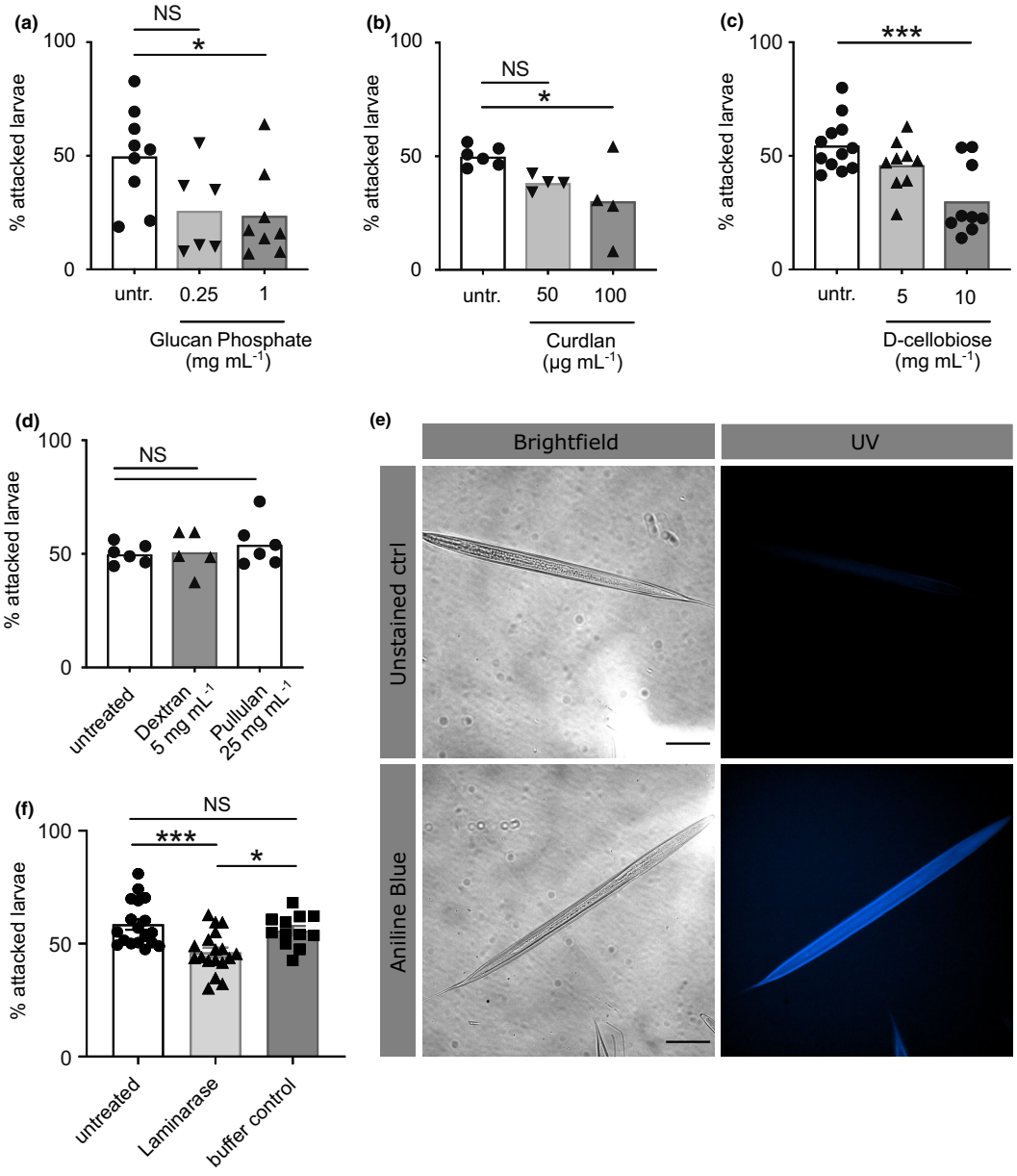

Figure 2. β-Glucans, but not α-glucans interfere with M(IL-4) macrophage recognition of Nb L3 via non-classical CLR pathways. **(a-d, f)** Bone marrow derived macrophages were generated from C57BL/6 (B6) wild-type mice. Macrophages were stimulated with IL-4 (10 ng mL^{-1}) and **(a–d)** pre-incubated at 37°C for 1 h with various CLRs ligands at different concentrations as indicated in the figure. **(f)** Nb L3 were incubated with laminarase (β-(1→3)-D-glucanase), or 0.05 M Na acetate buffer control for 30 min, then extensively washed and **(a–d, f)** Nb L3 were then added to the culture for 24 h. The percentage of larvae attacked by macrophages was then quantified by microscopy and manual counting of live and motile larvae. Data are pooled from two independent experiments with technical triplicates. **(e)** Larvae were stained or not with aniline blue and imaged with a DAPI filter. Representative of 25 images, with three larvae per image. NS, not significant; untr, untreated; $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$.

pre-treated WT or $EphA2^{-/-}$ M(IL-4), suggesting that EphA2 recognizes larvae in a manner dependent on, or redundant to, CD11b (Figure 3e, left-hand panel). However, further addition of the β-glucan laminarin to anti-CD11b-treated $EphA2^{-/-}$ macrophages still led to an, albeit minor, reduction of macrophage binding to Nb L3 (Figure 3e, right-hand panel). This likely reflects the complexity of glucans expressed by the parasite and the redundancy in the host recognition receptors. Taken together, these competitive inhibition assays suggest that M(IL-4) recognizes and adheres to Nb larvae via β-glucan recognition pathways including CD11b and EphA2.

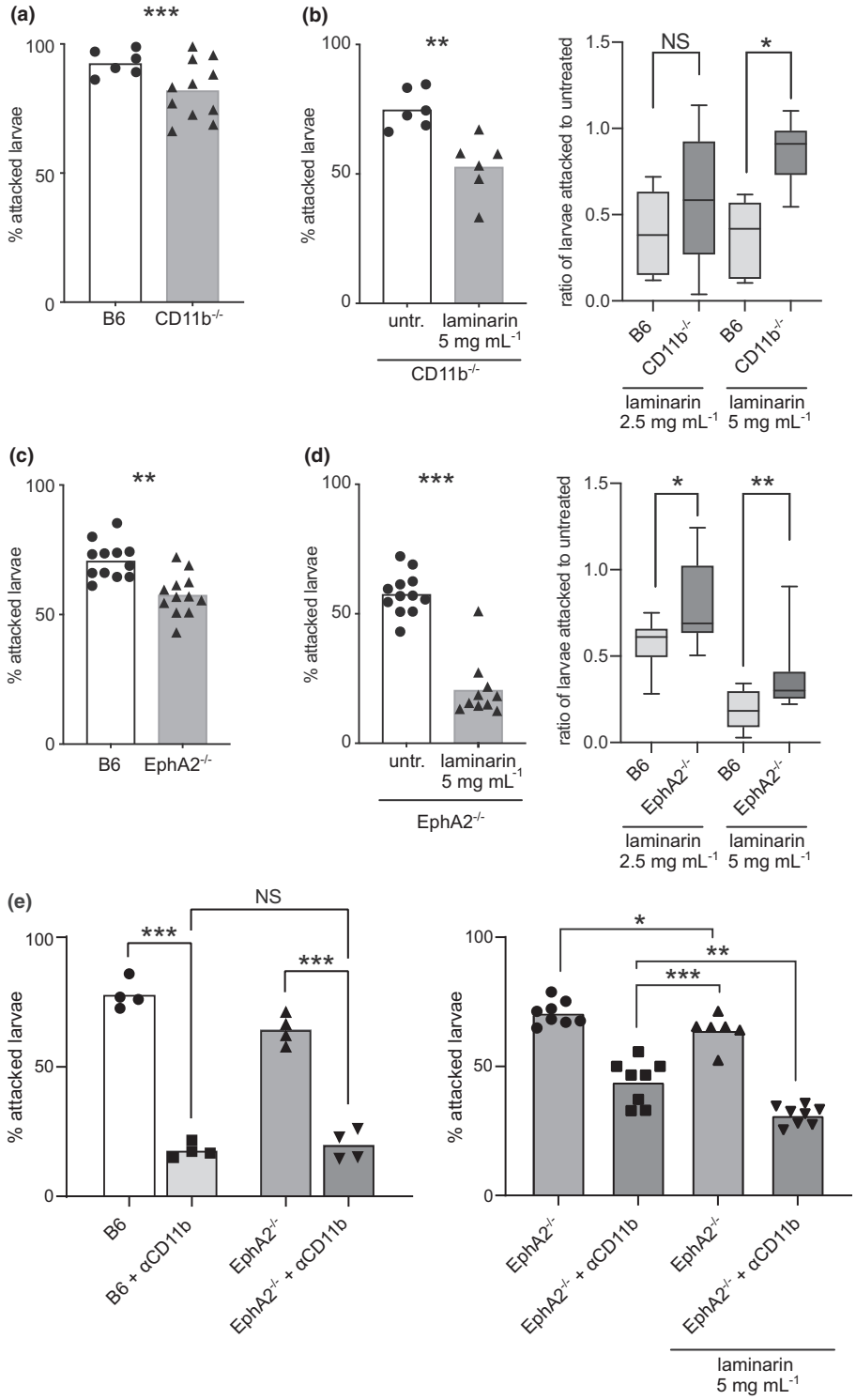

Figure 3. M(IL-4) recognize larval β-glucans on the Nb cuticle through expression of CD11b and EphA2 receptors. **(a–e)** Bone marrow derived macrophages were generated from C57BL/6 (B6) wild-type, CD11b deficient ($CD11b^{-/-}$) or EphrinA2 deficient ($EphA2^{-/-}$) mice. Macrophages were stimulated with IL-4 (10 ng mL^{-1}). **(a–d)** Nb L3 were then added to the culture for 24 h. The percentage of larvae attacked by macrophages was quantified 24 h later by microscopy and manual counting of live and motile larvae. To compare the effect of laminarin between macrophage genotypes, the percentage of attacked larvae by macrophages was normalized to unstimulated macrophages (no laminarin, UNS). Data are pooled from 2–4 independent experiments with technical triplicates. **(e)** Represents two independent experiments with technical quadruplicates and five total biological replicates. NS, not significant; untr, untreated; $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$.

Although our data show a clear role for these two receptors, there may be other receptors, as yet untested or uncharacterized, that contribute to the recognition of Nb larvae by M(IL-4).

DISCUSSION

It has long been known that nematodes drive the activation of type 2 immune responses, which ultimately leads to their expulsion and killing. However, in contrast to other pathogens, no pathogen-associated molecular patterns for nematode parasites have been described to date, and the upstream trigger of the type 2 immune cascade remains poorly understood. The 3-dimensional structures of glycans found at the surface of nematodes have previously been implicated in the initiation of a type-2 immune response;[18] however, the involvement of glycans in parasite recognition and killing by host myeloid cells has not been interrogated so far. Here, we describe that a variety of β-glucans can compete for the binding of IL-4-polarized macrophages to the surface of Nb larvae. On the basis of these data, we further postulate that hookworm larvae contain putative β-glucan(s) or are associated with microorganisms expressing such β-glucan (s) at the cuticle surface. Our work additionally identifies CD11b and EphA2 receptors as mediating, at least in part, the ability of IL-4-polarized macrophages to recognize the postulated larval β-glucan(s).

CD11b has previously been shown to be important for hookworm recognition by macrophages, but the ligand that this receptor recognizes has not been directly investigated in other studies, and instead was hypothesized to involve recognition of complement.[4,30] Using different β-glucans to interfere with the macrophage recognition, we have been able to show that several β-(1-3)-glucans, as well as the β-(1-4)-glucan cellobiose, were able to limit the binding of M(IL-4) to hookworm larvae. Unfortunately to date, the glycomics of nematodes remains poorly defined[38] impairing our ability to identify the molecule at the surface of the parasite involved in this recognition. It is possible that the PAMP recognized by macrophage PRRs is not a β-(1-3)-glucan, but rather a molecule sharing a similar helical 3D structure. However, our observations that treating larvae with β-(1-3)-glucanase (laminarase) could abrogate macrophage recognition of Nb suggests that intact surface glucans are required for macrophage recognition. Laminarinase displays substrate specificity towards β-(1-3) linkages and mixed β-(1-3;1-4) linkages – but also to a lesser extent β-(1-6)- and β-(1-4)-linked polymers,[39] and as such we cannot conclude which β-linkage(s) is(are) required in this recognition.

To date, β-(1-3)-glucans and their associated synthesis machinery have been reported to be present in fungi, bacteria, yeast, protozoa, seaweeds, mushrooms and plants, but not in higher animals (D Williams, University of East Tennessee, personal communication).[40,41] β-(1-3)-Glucan polymers are utilized by such organisms for energy storage and as a major structural polysaccharide. We have searched the genome of *N. brasiliensis* for such machinery, but we have not been successful in identifying a β-(1-3)-glucan synthase. The diversity of the machinery of β-glucan synthesis between different species is, however, quite important[42,43] and as such the fact that we have not identified β-(1-3)-glucan synthase in the *N. brasiliensis* genome does not indicate that this parasite cannot produce it. While searching other nematode genomes, we found a protein in *Trichuris trichuria* associated with the regulation of β-(1-3)-glucans (TTRE_0000844001) as well as a cellulose synthase (a β1-4 glucan, TTRE_0000741801). A cellulose synthase was also predicted in *Trichinella patagoniensis* (T12_15222). Altogether this demonstrates that it is possible that nematodes could synthetize β-(1-3) or β-(1-4) glucans.

Given such synthases have not been predicted in many nematode genomes, it is important to note that these identified sequences might come from bacteria or fungi associated with the parasite. While the micro- and mycobiome of the cuticle of Nb is to date uncharacterized, several helminths have indeed been shown to harbor bacteria (with symbiotic or opportunistic relationships) internally or on the cuticle surface.[44] Thus, it is interesting to speculate that fungal and bacterial organisms coating the surface of the larvae could be important to the immune recognition of Nb. Intriguingly, this could explain why we observed important variability in the efficacy of macrophage binding from one batch of larvae to another.

To date, there are no reports of direct dectin-1 involvement in the recognition of nematodes, but it has recently been shown that *Heligmosomoides polygyrus* L3 products trigger the activation of the COX/PGE2 pathway in a dectin-1-dependent manner.[45] Furthermore, dectin-1 has also been shown to contribute, together with dectin-2, MR and DC-SIGN in the recognition of egg antigens from the trematode *Schistosoma*.[46] Here, we show that dectin-1 is not required for M(IL-4) binding to hookworm larvae, despite the ability of exogenous β-glucans to inhibit this interaction. As such our results could indicate that the primary structure of some β-glucans is different in nematodes compared with those present in pathogenic fungi or potentially present in trematodes. Such a hypothesis would be in keeping with the well-known differences in the primary structure of glycans depending on their origin (bacteria, fungi, seaweeds),[47] and with the importance of the branching structure and stereochemistry of β-glucans recognized by dectin-1.[47]

EphA2 has recently been identified as a non-classical PRR with the capacity to recognize β-glucans from the pathogenic fungi, *Candida*, and noted for its expression by epithelial cells and neutrophils.[34-36] The Eph family is one of the largest families of tyrosine kinases in the mammalian genome and has been associated with cell migration and cancer; however, the functions of its 14 receptors are largely unknown in the immune system.[48] To our knowledge, this is the first report indicating a role for EphA2 in mediating immune recognition of a helminth. Whether recognition by this receptor can occur in cell types other than macrophages, such as neutrophils, dendritic or epithelial cells, or whether EphA2 is important for the initiation of a type 2 response will be of interest in future studies. Recently EphB2 has been shown to form a co-receptor with dectin-1 to recognize *Candida albicans* ligands and a diverse variety of ligands including curdlan, α-mannan and β-glucan. Intriguingly, while our competitive binding assays also highlighted the importance of these three ligands in the immune recognition of *N. brasiliensis*, this was independent of dectin-1. EphA2 and CD11b may cooperate in a similar manner in order to program anti-helminthic responses. EphA2 has been shown previously to be expressed by macrophages present in atherosclerosis plaques;[35] however, reports concerning EphA2 upregulation following IL-4 stimulation in macrophages are not available. Notably, EphA2 deletion does not alter macrophage polarization.[35]

Although both CD11b and EphA2 were observed to contribute to the recognition of hookworm larvae by M (IL-4), macrophages lacking either of these receptors were still able to bind to larvae even if to a really limited extent. Simultaneous blocking of both receptors did not give rise to an additive effect, suggesting a potential association of the two receptors in the recognition.[49,50]

Macrophages also express other β-glucan receptors, such as the scavenger receptors CD36, scavenger receptor A and scavenger receptor class F member 1.[46,47] Even though our data did not reveal a crucial contribution of either MR or FcγR-dependent receptors in gene-knockout models, it is possible that mannan-recognizing receptors are functionally redundant for pattern recognition yet can collaborate with other β-glucan receptors in a similar manner to the synergy observed between dectin-1 and TLR2 in the recognition of *C. albicans* β-glucans.[51] Such synergy could take the form of spatial organization at the cell surface or intracellular signaling pathway convergence.

While recent research has focused primarily on the detection of type 2 allergens and antigens following damage,[52] it has become increasingly evident from the recent literature that various cell types, including tuft cells,[53] nerves[54] and other innate immune cells[4,30,55,56] can directly recognize allergens and helminths. Here we describe for the first time β-glucan binding receptors as being important for the recognition of hookworms by IL-4 polarized macrophages. We also document the ability of EphA2 to contribute to this process, thereby expanding the array of PRRs reported to be involved in helminth sensing. Taken together these findings could open the door for new avenues of research for vaccines against helminths as well as for understanding other type-2 driven diseases.

METHODS

Mice

C57BL/6 mice were bred and maintained under specific pathogen-free conditions at the Ecole Polytechnique Fédérale de Lausanne (Switzerland) or by the Monash Intensive Care Unit Facility at Monash University, AMREP campus Melbourne (Australia). Dectin-1$^{-/-}$, Dectin-2$^{-/-}$ and mannose receptor$^{-/-}$ mice were bred and maintained under specific pathogen-free conditions at the University of Lausanne (Epalinges, Switzerland). EphA2$^{-/-}$ mice were bred and maintained under specific pathogen free conditions at Brisbane (Australia). All animal experiments were approved by the office of Affaires Vétérinaires (Epalinges, Canton Vaud, Switzerland) with Authorization 2238 according to the guidelines set by the Service de la Consummation et des Affaires Vétérinaires Federal (Canton Vaud, Switzerland) or by the AEC committee of the Alfred campus, Melbourne Australia with authorization number E/1843/2018/M.

Parasites

Nippostrongylus brasiliensis was sourced from Graham Le Gros (New Zealand) and has been maintained by monthly passage through Lewis rats in École polytechnique fédérale de Lausanne or at Monash University. The iL3 larvae were prepared from 2-week rat fecal cultures as described previously.[57] Prior to *in vitro* experiments, iL3s were washed three times in phosphate-buffered saline (PBS, Sigma-Aldrich, Merck & Cie, Schaffhausen, Switzerland) and incubated for 1 h at 37°C in an antibiotic solution (penicillin/streptomycin 1000 U mL^{-1} (GIbcoTM, ThermoFisher Scientific, Reinach, Switzerland), gentamicin 300 U mL^{-1} (Sigma) in PBS). Larvae were cultured in complete RPMI medium (Gibco) supplemented with 10% FBS (Gibco), 2 mM L-glutamine (Gibco), 100 U mL^{-1} penicillin/streptomycin, 0.5 mg mL^{-1} gentamicin, 10 mg mL^{-1} tetracycline (Sigma).

Macrophage generation

For bone marrow-derived macrophages, marrow was flushed from the femur and tibia of C57BL/6 mice and passed through cell strainers (70 μm) in PBS. The cells (10^6 mL^{-1})

were cultured in 30% M-CSF (supernatant of L929 cell culture) supplemented medium (RPMI (Gibco), 10% FCS (Gibco), penicillin/streptomycin) for 7 days as described previously.[6] Macrophages were harvested between day 7 and 10 of culture for experiments.

For lung macrophages, C57BL/6 mice were infected with 250 L3 subcutaneously, then challenged with 500 L3 30 days later and killed 2 days post re-infection. The lungs were washed by bronchoalveolar lavage with 3 mL of PBS and then digested in media (DMEM (Gibco), 2 mg mL^{-1} collagenase (Sigma), DNase 12.5 U mL^{-1} (Roche, Sigma-Aldrich, Merck & Cie, Schaffhausen, Switzerland)) at 37°C on an orbital shaker for 45 min. The cells were passed through a cell strainer (70 μm) and seeded (2.5×10^5) directly in a flat-bottom 96-well plate. Non-adherent cells were washed away the following day and adherent macrophages were cultured in complete DMEM (10% FCS, 1% penicillin/streptomycin) for 2 days before co-culture with larvae.

Larval binding assay

Murine bone marrow-derived macrophages (BMDM) were stimulated overnight with IL-4 (10 ng mL^{-1}) and glucans when indicated in the text. The next day BMDM or lung macrophages were washed in PBS and plated at 1×10^5 in a flat bottom 96-well plate; with technical triplicates performed for each condition. The cells were then incubated with 100 Nb L3 larvae as described previously.[3] All co-cultures were performed at 37°C, 5% CO_2, for 24 h. The percentage of larvae attacked by macrophages were then quantified using a stereo microscope with manual counting of live and motile larvae.

Chemicals

Macrophages were stimulated with various compounds at the concentration described in the manuscript 1 h before the larvae were added to the culture. Laminarin was sourced from Sigma-Aldrich (#L9634) or from Invivogen (#tlrl-lam). Mannan (#M7504), NADGal (#A2795), pullulan (#P4516), dextran (#31392), fucose (#F8150) and laminarase (beta-(1 → 3)-D-glucanase, #67138, resuspended in 0.05 M Na acetate pH 5.0) were all purchased from Sigma-Aldrich. Glucan phosphate was kindly offered by Professor D Williams (University of East Tennessee). Curdlan Beta-1,3-glucan was sourced from Invivogen. CD11b blocking was performed using rat anti-CD11b (clone M1/70, Biolegend).

Flow cytometry

Bone marrow-derived macrophages were stained with the following fluorescently labeled mAbs: anti-CD206 FITC, anti-CD11b PE, anti-CD45 Alexa Fluor 700, anti-F4/80 allophycocyanin, (all from BioLegend). The cells were stained with Live Dead Stain Aqua (Life Technologies) or calcein-AM (Thermofisher) prior to surface staining. Samples were acquired on a BD LSRII flow cytometer (BD Biosciences).

Aniline staining

Aniline blue was kindly provided by the Monash University Histology platform. In brief, Nb L3 were fixed overnight at 4°C in 95% EtOH and acetic acid (3:1) to prevent auto-fluorescence and 250 Nb L3 were stained using Ko and Lin's method[26] while protected from light in 1 mL of aniline blue dye mix (40 volumes of 0.1% aniline blue in water, 21 volumes of 1 N HCl, and 59 volumes of 1 M glycine/NaOH buffer, pH 9.5) or buffer control at 50°C for 30 min with agitation. L3 were imaged on a Nikon-TiE inverted fluorescent microscope in the DAPI channel (395 nm/495 nm); 25 images were captured for each group, average of three larvae per image, and analyzed in FIJI.

Statistics

Bars represent the mean and the error bars represent the standard deviation. Agostino-Pearson tests were used to test for normality, and *t*-tests were used to compare two groups and ANOVA tests were used to compare three or more unmatched groups. Post-hoc tests were performed to study significant differences, only performed tests are indicated on graphs. *P*-values higher than 0.05 were considered non-significant (NS). *P*-values less than or equal to 0.05, 0.01, 0.001, 0.0001 are respectively represented by one, two, three or four asterisks (*). Statistical analyses were performed with GraphPad Prism 7 (GraphPad Software).

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

T Bouchery: Conceptualization; Formal analysis; Investigation; Methodology; Supervision; Writing – original draft; Writing – review & editing. **B Volpe:** Conceptualization; Formal analysis; Investigation; Methodology. **R Doolan:** Formal analysis; Investigation; Methodology; Writing – review & editing. **G Coakley:** Investigation; Writing – review & editing. **M Moyat:** Investigation; Writing – review & editing. **J Esser-von-Bieren:**

Conceptualization; Investigation; Supervision; Writing – review & editing. **LC Wickramasinghe:** Investigation; Methodology. **ML Hibbs:** Resources; Writing – review & editing. **J Sotillo:** Investigation; Resources. **M Camberis:** Resources. **G Le Gros:** Resources. **N Khan:** Resources; Writing – review & editing. **D Williams:** Conceptualization; Resources; Writing – review & editing. **N Harris:** Conceptualization; Funding acquisition; Supervision; Writing – review & editing.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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