

1 Human dendritic cell sequestration onto the *Necator americanus* larval sheath during ex-
2 sheathing: a possible mechanism for immune privilege

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8 Running title: *Necator americanus* interaction with human dendritic cells

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15 **ABSTRACT**

16 Despite the profound health implications of *Necator americanus* (*N. americanus*) infection in
17 humans, many aspects of its interaction with the host immune system are poorly understood.
18 Here we investigated the early events at the interface of *N. americanus* larvae (L3) and human
19 dendritic cells (DCs). Our data show that co-culturing DCs and the larvae triggers ex-sheathing
20 of hookworms rapidly where a majority of DCs are sequestered onto the larval sheath allowing
21 the ex-sheathed larvae to migrate away unchallenged. Intriguingly, DCs show negligible
22 interaction with the ex-sheathed larvae, alluding to differences between the surface chemistry
23 of the larva and its sheath. Furthermore, blocking of two key C-type lectin receptors on DC
24 surface (i.e. DC-SIGN and mannose receptor) resulted in inhibition of ex-sheathing process and
25 DC sequestration, highlighting the importance of C-type lectins on DCs in the induction of the
26 ex-sheathing. Analyses of DC phenotype and cytokine profile after co-culture with the *N.*
27 *americanus* larvae showed an immature phenotype as evidenced by the low expression of the
28 maturation markers and cytokines. These data provide new insights into early events at the
29 interface of human DCs and *N. americanus* larvae and could explain how L3 evade immune
30 recognition upon initial interaction with DCs.

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33 **KEYWORDS:** *Necator americanus*, dendritic cells, larval sheath, exsheathment, C-type lectin
34 receptors, mannose receptor, immune modulation, immune evasion, hookworm

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36

37 **KEY FINDINGS**

- 38 • Interaction between *Necator americanus* larvae and human DCs induces rapid ex-
39 sheathing of larvae
- 40 • DCs are sequestered around the larval sheath whilst the ex-sheathed larval cuticle
41 remains unchallenged
- 42 • The ex-sheathing process seems to be mediated by C-type lectins on the surface of
43 DCs
- 44 • DCs sequestration around the sheath and unchallenged migration of larvae could
45 explain the inefficiency of immune responses against *Necator americanus*

46

47 **INTRODUCTION**

48 Despite its profound health implications, chronicity and significant public health burden in
49 developing countries, many aspects of human *N. americanus* infection, particularly early events
50 at the interface with the host immune system, are under researched (Quinnell *et al.* 2004, Loukas
51 and Prociv, 2001, Hotez *et al.* 2008). These insidious parasites infect and re-infect, following
52 which no efficient immunological memory develops in the host, rendering chemotherapeutic
53 treatment as the method of choice, which is also inefficient due to the high prevalence of re-
54 infection.

55 Efforts in developing more effective therapeutic approaches could be helped by a better
56 understanding of the initial interactions between *N. americanus* larvae and key components of
57 the innate immune system such as dendritic cells (DCs). DCs are sentinels of the immune
58 system and act as a bridge between the innate and adaptive immune systems (Savina and
59 Amigorena, 2007). DCs are abundant in all barrier tissues (e.g. skin and airway epithelium) and
60 equipped with a range of pattern recognition receptors (PRRs) on their surface (e.g. Toll-like
61 and C-type lectin receptors (CLRs)) that can recognise various pathogen associated molecular

62 patterns (PAMPs)(Salazar *et al.* 2013). Interestingly previous studies have identified a range of
63 lectins isolated from plants capable of binding to sugars present on the *N. americanus* L3 sheath,
64 including mannose, fucose, heparan sulphate and galactose (Kumar and Pritchard, 1992a)
65 which could potentially act as ligands for CLRs on DCs.

66 During its life cycle *N. americanus* has many opportunities to interact with the host DCs
67 (Quinnell *et al.* 2004, Geiger *et al.* 2007). As part of their armoury of PRRs, DCs express a
68 range of CLRs with specificity for the recognition of glycosylated proteins (Thompson *et al.*
69 2011, Salazar *et al.* 2013). Amongst CLRs expressed by DCs are dendritic cell-specific
70 intercellular adhesion molecule-3 grabbing non-integrin (DC-SIGN) and mannose receptor
71 (MR), both of which have been implicated in key DC functions including mediating immune
72 responses to different pathogens as well as immune modulation (Garcia-Vallejo and van Kooyk,
73 Geijtenbeek *et al.* 2002, Wollenberg *et al.* 2002, Salazar *et al.* 2013, Emara *et al.* 2011, Emara
74 *et al.* 2012, Royer *et al.* 2010). However, the biological relevance of the glycosylated *N.*
75 *americanus* sheath in the context of interaction with CLRs on DCs has not been investigated.

76 The antigen presenting cell function of dendritic cells directly depends on their ability
77 to migrate to the site of infection (Martin-Fontecha *et al.* 2009). Upon capturing pathogens,
78 DCs migrate to lymph nodes where processed antigens are presented to naïve T cells, in the
79 context of MHC molecules, leading to polarisation of T cells towards distinct functional subsets
80 such as Th1, Th2, Th17 and regulatory T cells (Smith-Garvin *et al.* 2009). Many
81 microorganisms have developed strategies (e.g. masking of PAMPs or inducing changes in
82 PRR expression) to evade efficient recognition by DCs (van Kooyk and Geijtenbeek, 2003). In
83 addition, any physical or chemical barrier that interfere with DC migrating to or from the site
84 of infection could also hamper mounting appropriate immune responses. This function is
85 indispensable for the maintenance of immune surveillance and tissue homeostasis as well as
86 initiating protective tolerogenic and pro-inflammatory responses (Imai *et al.* 2012).

87 In this study we have investigated the cross-talk between human DCs and *N. americanus*
88 larvae, in particular the biological significance of the glycosylated molecules on the *N.*
89 *americanus* L3 sheath in influencing DCs function and their interaction with the *N. americanus*
90 larvae.

91

92 **MATERIALS AND METHODS**

93 All materials were purchased from Sigma-Aldrich, U.K., unless otherwise stated

94

95 **Preparation and identification of *N. americanus* (L3) larvae**

96 Infective *N. americanus* larvae were cultured from faecal material derived from infected
97 individuals as described previously (Kumar *et al.* 1992). The larvae were deemed to be axenic
98 following microbiological analysis (FDAS, BioCity, Nottingham).

99

100 **Dendritic cell generation**

101 Monocyte derived dendritic cells (DCs) were generated from peripheral blood monocytes
102 which were obtained from human blood buffy coats after obtaining written informed consent
103 and approval of local Ethics Committee (National Blood Transfusion Service, U.K.,
104 2009/D055) as we have previously described (Chau *et al.* 2013). Briefly, the peripheral blood
105 mononuclear cells (PBMCs) were isolated via histopaque density gradient centrifugation.
106 Monocytes were then isolated out from the PBMC by incubating the suspension with CD14+
107 magnetic beads (Milteny Biotech, U.K.) obtaining a purity of >98% as we have described
108 before (Garcia-Nieto *et al.* 2010). Subsequently, purified CD14+ monocytes were cultured with
109 complete RPMI medium (10% Fetal Bovine Serum, 2Mm L-glutamine, 1%
110 Penicillin/Streptomycin and 1% non-essential amino acid solution) supplemented with 50
111 ng/mL GM-CSF and 250 IU/ml IL-4 in a 24 well plate for a period of 6 days to generate

112 immature DCs (Salazar *et al.* 2016).

113

114 ***N. americanus* (L3) incubation with DCs**

115 Immature DCs were cultured in complete RPMI media (10% Fetal Bovine Serum, 2Mm L-
116 glutamine, 1% Penicillin/Streptomycin and 1% non-essential amino acid solution) and
117 incubated with approximately 50 ensheathed *N. americanus* L3 larvae for 24-hours. During the
118 incubation period the samples were imaged using the ZOE™ Fluorescent Cell Imager (Biorad).

119

120 **CLRs blocking experiments**

121 To assess the potential involvement of specific CLRs in DC- hookworm interaction, immature
122 DCs were treated with either 20 µg/ml of blocking antibodies for DC-SIGN (clone H-200) and
123 MR (clone 15.2) or mannan (from *Saccharomyces cerevisiae*) (100µg/ml) for 25 minutes at
124 370C prior to addition of approximately 50 *N. americanus* larvae. The cells were then incubated
125 for a further 24-hours at 370C/5% CO₂ and the samples were imaged using the ZOE™
126 Fluorescent Cell Imager. This was compared to DCs treated with the appropriate isotype
127 controls (rabbit IgG and normal mouse IgG1) and DCs which were untreated; both conditions
128 were also incubated with the infective larvae. All antibodies purchased from Santa Cruz
129 Biotechnology.

130

131 ***N. americanus* (L3) incubation with conditioned DC media**

132 To assess the effect of DCs secretions on larvae ex-sheathing, DCs were stimulated with either
133 mannan (100µg/ml) or Lipopolysaccharide (LPS) (100ng/ml) from *Escherichia coli* (*E. coli*)
134 for 24-hours at 370C/5% CO₂. The cell free conditioned supernatant was added to
135 approximately 50 *N. americanus* larvae for 24-hours. Following the incubation period, the
136 samples were imaged using an inverted Microscope (Olympus CKX41, Olympus America) and

137 analysed with Lumenera Infinity Capture software. This was compared to the supernatant from
138 untreated DCs incubated with the infective larvae.

139

140 **Staining for cell surface markers and quantifying DC viability**

141 In order to prepare the DCs for phenotype analysis via flow cytometer, the cells were harvested
142 and washed twice in PBA buffer (5% Albumin solution from bovine serum, 0.1% Sodium azide
143 in PBS). The desired antibodies (e.g. CD11c, CD14 and CD83) were added to the pellet,
144 vortexed and incubated for 20 minutes, in dark at 4 °C. Nonreactive isotype-matched antibodies
145 and unstained cells were used to determine non-specific staining. The samples were then
146 washed with PBA and finally fixed with paraformaldehyde solution (0.5% in PBS). This was
147 stored at 4° C to be analysed within a 7 day period.

148 The viability of DCs were analysed using the ANNEXIN V– FITC Kit-Apoptosis
149 Detection Kit (Beckman Coulter) according to the manufacturer's protocol. Expression of
150 surface markers and the level of Annexin-V and Propidium Iodide expression in DCs were
151 assessed via flow cytometry analysis (Cytomics FC 500, Beckman Coulter) with a minimum
152 of 20,000 events collected for each sample. The data obtained were analysed using the Weasel
153 V.2.7.4 software. Median fluorescence intensity and percentage of positive cells for each
154 marker was determined and further evaluated using GraphPad Prism 6 analysis software.

155

156 **Cytokine expression**

157 The levels of cytokines were measured with ELISA Kits and were analysed according to the
158 manufacturer's protocol. All samples were analysed in two duplicates. Absorbance was
159 measured at 450 nm with SpectraMax Paradigm. IL-1 β (200-01B), TNF α (900-K25), IL-10
160 (900-K21), IL-12 (900-K96) and IL-6 (900-K16) were purchased from PeproTech and IL-8
161 (DY208) from R&D Systems.

162 **Statistical analysis**

163 The means and \pm SEM are shown. The statistical significance of the data was analysed and
164 evaluated using Student's t test with GraphPad Prism 6 analysis software. Statistical
165 significance was determined using the Holm-Sidak method with a p value of ≤ 0.05 .

166

167 **RESULTS**

168 **Ex-sheathing of *N. americanus* (L3) larvae upon co-culture with immature dendritic**
169 **cells**

170 To assess the consequence of a physical interaction between DCs and *N. americanus*, immature
171 DCs were incubated with 50 *N. americanus* L3 larvae. The interactions were imaged and
172 monitored using the ZOE™ Fluorescent Cell Imager (BioRad) for up to 24-hours. Microscopy
173 data showed that upon contact with the larval sheath, DCs are sequestered on the surface of
174 larval sheath which in turn triggered ex-sheathing, whereby the larvae discarded their outer
175 cuticle. Ex-sheathing in this study is defined by the initial breach and emergence of the larva
176 from its sheath. This phenomenon is observed at variable intervals after a minimum of 1 hour
177 incubation with DCs and complete ex-sheathing (i.e. full length larva leaving the cuticle) could
178 take up to 4 hours. Notably, DCs in direct contact with the sheath seem to attract a majority of
179 bystander cells leading to formation of large DC aggregates and sequestration of DCs around
180 the sheath, alluding to an adhesion cascade. Upon full ex-sheathing the larvae migrate away
181 from the sheath with the sequestered DCs, as well as free DCs, exhibiting negligible interaction
182 with the ex-sheathed larvae. The ex-sheathing of a single hookworm was examined,
183 documenting this novel interaction (Figure 1) (Supplementary Video 1).

184 Following 24 hours, DCs form dense aggregates around the sheath and remain un-
185 attracted to the exposed larvae. Despite highly dense cell aggregates surrounding discarded
186 sheaths they can still be visualised within some aggregates. (Figure 2).

187 **Blocking CLR_s on DC surface and *N. americanus* (L3) treatment with conditioned**
188 **media**

189 Previous research has distinguished *N. americanus* from other hookworm species based on its
190 distinct glycosylated surface chemistry, which binds a range of lectins derived from plants
191 (Kumar and Pritchard, 1992a). It was therefore reasonable to assume that sugars on the worm
192 sheath could mediate the interaction with DCs. To investigate this possibility, we blocked DC-
193 SIGN and MR, 2 major C-type lectins expressed by human DCs, using specific blocking
194 antibodies or mannan (to partially saturate MR and DC-SIGN) before incubation with *N.*
195 *americanus* larvae for 24 hours as described earlier. Our data clearly show almost complete
196 abrogation of DC aggregation around *N. americanus* larvae in the presence of either α -DC-
197 SIGN, α -MR or mannan compared to untreated DCs (Figure 3a). In parallel DCs treated with
198 the respective isotype control antibodies were also examined which illustrated no changes in
199 DC aggregation compared to untreated DCs (data not shown).

200 To elucidate whether soluble factors produced by DCs play a role in the observed ex-
201 sheathing, DCs were stimulated with mannan (100 μ g/ml) (to simulate CLR ligation) and the
202 conditioned media was collected after 24 hours. The *N. americanus* larvae were then incubated
203 with the conditioned media for 24 hours as described. In parallel, media from unstimulated DCs
204 was collected and incubated with the larvae as a control. The hookworms did not ex-sheath in
205 culture with neither the conditioned media nor media collected from un-stimulated DCs,
206 suggesting that a cell mediated interaction is required for the induction of ex-sheathing (Figure
207 3b).

208

209 **DC surface phenotype in response to *N. americanus* (L3)**

210 To better understand the effect of *N. americanus* larvae on DCs function we assessed DCs
211 phenotype after 24 hours incubation with *N. americanus* larvae. In control cultures DCs were

212 stimulated with 100 ng/mL LPS to induce maturation. In this study we report that DCs retrieved
213 from co-culture with viable axenic larvae maintained an immature phenotype as evidenced by
214 a lack of up-regulation in maturation markers CD80, CD83, CD86, CD40 and HLA-DR. In
215 addition, there was a significant downregulation in CD206 expression (Figure 4). Subsequently,
216 the ability of DCs to acquire a mature phenotype in response to co stimulation with LPS in the presence
217 of *N. americanus* larvae was assessed. These data showed DCs treated with *N. americanus*
218 larvae will remain responsive to LPS stimulation (Figure 5). In all these experiments we
219 monitored DC viability using Annexin-V and PI staining and did not observe any significant
220 changes in DCs viability upon co-culture with *N. americanus* larvae (Figure 6).

221

222 **DC cytokine expression in response to *N. americanus* (L3) and LPS stimulation**

223 Following the stimulation of DCs with either the infective L3 *N. americanus* larvae, LPS or
224 both, supernatant samples were collected at 24 hours and were analysed for IL6, 8, 10 and 12
225 using ELISA. Our data show that while axenic *N. americanus* larvae on its own do not induce
226 any cytokine production by DCs, they seem to suppress LPS induced cytokine production
227 however these changes were not statistically significant (p value ≤ 0.3) (Figure 7).

228

229 **DISCUSSION**

230 Infection with *N. americanus* has remained a major health problem with significant health
231 implications. The high prevalence of reinfection, due to inefficient protective immunity, makes
232 disease eradication a challenge. Therefore better understanding of how the immune system
233 interacts with infective larvae could pave the ways for the rational design of novel treatment
234 strategies. This study provides new insights into early immunological events at the interface of
235 human DCs and *N. americanus* larvae and could explain the lack of efficient immune response
236 during early stages of infection.

237 The ex-sheathing of *N. americanus* larvae has been observed during the initial stages of
238 infection as well as in the presence of human sweat (Pasuralertsakul and Ngrenngamlert, 2006,
239 Hawdon *et al.* 1993, Matthews, 1982), however the factors initiating ex-sheathing and the
240 benefit of this to the parasite are yet to be fully understood (Loukas and Prociv, 2001). From
241 previous literature, it is known that the larval sheath does not accompany the hookworm post
242 the skin stages of infection and progression into the blood circulation (Kumar and Pritchard,
243 1992b); implying the ensheathed larvae encounter immune cells in the skin *in vivo*.

244 Our data show for the first time that immature DCs bind to the ensheathed larvae,
245 initiating the *N. americanus* to ex-sheath and mechanically migrate away from this site leaving
246 behind its sheath. DCs are sequestered onto the discarded sheath and continue to form
247 aggregates, exhibiting no interest in binding to the exposed larval cuticle allowing its
248 unchallenged movement away from DCs. Migration of immature DCs from the site of infection
249 to draining lymph nodes, where they interact with naïve T cells, is a key step in initiating an
250 efficient immune response (Heuze *et al.* 2013). Therefore, it is reasonable to suggest that DC
251 sequestration on larval sheath and no interaction between DCs and ex-sheathed larvae could
252 play a major role in the lack of immune recognition/activation at early stages of infection.

253 The differential interaction between DCs and the larval sheath versus the larvae allude
254 to a disparity between the surface chemistry of the sheath and the larvae. While the exact nature
255 of differences in the surface properties of the larvae and sheath are yet to be fully characterised,
256 these data clearly indicates the presence of distinct chemical signatures on the *N. americanus*
257 sheath that attract DCs. Interestingly earlier work by authors has identified a range of sugars on
258 the L3 sheath, including mannose, fucose, heparan sulphate and galactose (Kumar and
259 Pritchard, 1992a). These sugars could be clear targets for a range of C-type lectin receptors
260 expressed on the surface of immature DCs that are part of DCs armoury of Pattern Recognition
261 Receptors (PRRs) (Thompson *et al.* 2011).

262 Amongst the CLRs expressed by DCs are DC-SIGN and mannose receptor (MR or
263 CD206) both of which have been implicated in mediating immune responses to different
264 pathogens as well as immune modulation (Garcia-Vallejo and van Kooyk, Geijtenbeek *et al.*
265 2002, Wollenberg *et al.* 2002, Salazar *et al.* 2013). Given the high expression of DC-SIGN and
266 MR on myeloid DCs and their prominent role in recognition of different pathogens, we
267 investigated their potential role in DCs interaction with *N. americanus* live larvae by blocking
268 MR and DC-SIGN. Our data illustrate that DCs treated with blocking antibodies against DC-
269 SIGN and MR or mannan (a ligand for both DC-SIGN and MR) (Salazar *et al.* 2013) exhibit a
270 significant decrease in induction of larvae ex-sheathing and forming DC aggregates around
271 larval sheath, compared to untreated DC. These data clearly indicate a role for MR and DC-
272 SIGN in mediating interactions between DC and the larval sheath. Additionally, the treatment
273 of *N. americanus* larvae with conditioned media from DCs stimulated mannan, an agonist for
274 both MR and DC-SIGN, showed that the hookworms do not ex-sheath in response to cell free
275 conditioned media, proposing a cell mediated interaction and that binding to CLRs on DC are
276 necessary for induction of hookworms ex-sheathing.

277 Previous studies have shown the ability of some pathogens to subvert DC-SIGN
278 function in order to evade immune detection and surveillance; these include viral pathogens,
279 such as HIV-1 via gp120 protein, and non-viral pathogens including Mycobacterium
280 tuberculosis (van Kooyk and Geijtenbeek, 2003, Ludwig *et al.* 2004). However, in the context
281 of this study, the *N. americanus* uses interaction with both DC-SIGN and MR to sequester DCs
282 onto its sheath. This could provide a plausible explanation for the poor T cell responses
283 observed *in vivo*, as the sequestration of DCs on larval sheaths could prevent necessary DC
284 migration to lymph nodes that is a prerequisite for developing an effective adaptive immune
285 response (Martin-Fontecha *et al.* 2009). These data provide strong rationale for further
286 experiments (e.g. targeting specific lectins on larvae surface and/or silencing MR and DC-SIGN

287 expression on DCs)(Royer *et al.* 2010, Emara *et al.* 2012) in order to fully elucidate the cross-
288 talk between the sugar moieties on larvae sheath and the CLR on DCs.

289 Another interesting observation arising from these experiments was a significant
290 reduction in MR expression upon co-culture with *N. americanus* larvae while all the other tested
291 surface markers did not change and DCs maintained an immature phenotype. The immature
292 phenotype of DCs is perhaps reflection of the axenic nature of larvae that are used in these
293 experiments which is unlikely to be the case *in vivo*. Nevertheless, the down-regulation of MR
294 expression on DCs in co-culture with *N. americanus* is of interest and further highlights the
295 presence of MR ligands on larval sheath. This observation is in line with other studies in
296 *Schistosoma mansoni* infection, revealing a novel pathway involving the internalization (and
297 decrease in MR expression) of helminth derived glycoproteins through the MR. This interaction
298 has been shown to interfere with DC protein synthesis, conditioning DCs to support a Th2
299 phenotype differentiation (Everts *et al.* 2012).

300 To further investigate whether the larvae are able to modulate DC responses to other
301 stimuli we also studied DC cytokine profile after co-stimulation with LPS from E.coli, which
302 is likely to be present during any *in vivo* exposure. Interestingly, DCs co-cultured with *N.*
303 *americanus* larvae maintained their ability to respond to LPS stimulation as evidenced by
304 upregulation in maturation markers. Furthermore, our data clearly show a general suppression
305 in LPS induced cytokine (IL-6, IL-8, IL-10 & IL-12) production in the presence of larvae which
306 was not due to any changes in DC viability. Although such suppression in cytokine production
307 was not statistically significant (p value ≤ 0.3), most likely due to small number of donors, it is
308 in line with the generation of non-immunogenic or poorly immunogenic DCs.

309 In summary, our data clearly suggest that the *N. americanus* larvae actively target DC-
310 SIGN and MR on DCs that leads to DC sequestration on the surface of larval sheath and
311 unchallenged migration of un-sheathed larvae enabling larvae to escape immune surveillance

312 and potentially promote pathogen survival (figure 8). These data provide new insights into the
313 early events at the interface of DCs and *N. americanus* larvae which could pave the way for the
314 rational design of new and more efficient intervention strategies against hookworm infection.

315

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319

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326 **REFERENCES**

- 327 CHAU, D. Y., JOHNSON, C., MACNEIL, S., HAYCOCK, J. W. & GHAEMMAGHAMI, A. M. 2013. The
328 development of a 3D immunocompetent model of human skin. *Biofabrication*, 5, 035011.
- 329 EMARA, M., ROYER, P. J., ABBAS, Z., SEWELL, H. F., MOHAMED, G. G., SINGH, S., PEEL, S., FOX, J.,
330 SHAKIB, F., MARTINEZ-POMARES, L. & GHAEMMAGHAMI, A. M. 2011. Recognition of the
331 major cat allergen Fel d 1 through the cysteine-rich domain of the mannose receptor
332 determines its allergenicity. *J Biol Chem*, 286, 13033-40.
- 333 EMARA, M., ROYER, P. J., MAHDAVI, J., SHAKIB, F. & GHAEMMAGHAMI, A. M. 2012. Retagging
334 identifies dendritic cell-specific intercellular adhesion molecule-3 (ICAM3)-grabbing non-
335 integrin (DC-SIGN) protein as a novel receptor for a major allergen from house dust mite. *J*
336 *Biol Chem*, 287, 5756-63.
- 337 EVERTS, B., HUSSAARTS, L., DRIESSEN, N. N., MEEVISSSEN, M. H., SCHRAMM, G., VAN DER HAM, A. J.,
338 VAN DER HOEVEN, B., SCHOLZEN, T., BURGDORF, S., MOHRS, M., PEARCE, E. J., HOKKE, C. H.,
339 HAAS, H., SMITS, H. H. & YAZDANBAKHS, M. 2012. Schistosome-derived omega-1 drives Th2
340 polarization by suppressing protein synthesis following internalization by the mannose
341 receptor. *J Exp Med*, 209, 1753-67, s1.
- 342 GARCIA-NIETO, S., JOHAL, R. K., SHAKESHEFF, K. M., EMARA, M., ROYER, P. J., CHAU, D. Y., SHAKIB, F.
343 & GHAEMMAGHAMI, A. M. 2010. Laminin and fibronectin treatment leads to generation of
344 dendritic cells with superior endocytic capacity. *PLoS One*, 5, e10123.
- 345 GARCIA-VALLEJO, J. J. & VAN KOOYK, Y. The physiological role of DC-SIGN: A tale of mice and men.
346 *Trends in Immunology*, 34, 482-486.
- 347 GEIGER, S. M., CALDAS, I. R., MC GLONE, B. E., CAMPI-AZEVEDO, A. C., DE OLIVEIRA, L. M., BROOKER,
348 S., DIEMERT, D., CORREA-OLIVEIRA, R. & BETHONY, J. M. 2007. Stage-specific immune
349 responses in human *Necator americanus* infection. *Parasite Immunol*, 29, 347-58.
- 350 GEIJTENBEEK, T. B., ENGERING, A. & VAN KOOYK, Y. 2002. DC-SIGN, a C-type lectin on dendritic cells
351 that unveils many aspects of dendritic cell biology. *J Leukoc Biol*, 71, 921-31.

352 HAWDON, J. M., VOLK, S. W., ROSE, R., PRITCHARD, D. I., BEHNKE, J. M. & SCHAD, G. A. 1993.
353 Observations on the feeding behaviour of parasitic third-stage hookworm larvae.
354 *Parasitology*, 106 (Pt 2), 163-9.

355 HEUZE, M. L., VARGAS, P., CHABAUD, M., LE BERRE, M., LIU, Y. J., COLLIN, O., SOLANES, P.,
356 VOITURIEZ, R., PIEL, M. & LENNON-DUMENIL, A. M. 2013. Migration of dendritic cells:
357 physical principles, molecular mechanisms, and functional implications. *Immunol Rev*, 256,
358 240-54.

359 HOTEZ, P. J., BRINDLEY, P. J., BETHONY, J. M., KING, C. H., PEARCE, E. J. & JACOBSON, J. 2008.
360 Helminth infections: the great neglected tropical diseases. *The Journal of Clinical*
361 *Investigation*, 118, 1311-1321.

362 IMAI, K., MINAMIYA, Y., KOYOTA, S., ITO, M., SAITO, H., SATO, Y., MOTOYAMA, S., SUGIYAMA, T. &
363 OGAWA, J.-I. 2012. Inhibition of dendritic cell migration by transforming growth factor- β 1
364 increases tumor-draining lymph node metastasis. *Journal of Experimental & Clinical Cancer*
365 *Research : CR*, 31, 3-3.

366 KUMAR, S., LAOUAR, L., PRITCHARD, D. I. & LOWE, K. C. 1992. A novel method for the isolation of
367 nematode larvae using pluronic F-68-treated cellulose strips. *J Parasitol*, 78, 550-2.

368 KUMAR, S. & PRITCHARD, D. I. 1992a. Distinction of human hookworm larvae based on lectin-binding
369 characteristics. *Parasite Immunol*, 14, 233-7.

370 KUMAR, S. & PRITCHARD, D. I. 1992b. Skin penetration by ensheathed third-stage infective larvae of
371 *Necator americanus*, and the host's immune response to larval antigens. *Int J Parasitol*, 22,
372 573-9.

373 LOUKAS, A. & PROCIV, P. 2001. Immune responses in hookworm infections. *Clin Microbiol Rev*, 14,
374 689-703, table of contents.

375 LUDWIG, I. S., LEKKERKERKER, A. N., DEPLA, E., BOSMAN, F., MUSTERS, R. J. P., DEPRAETERE, S., VAN
376 KOOYK, Y. & GEIJTENBEEK, T. B. H. 2004. Hepatitis C Virus Targets DC-SIGN and L-SIGN To
377 Escape Lysosomal Degradation. *Journal of Virology*, 78, 8322-8332.

378 MARTIN-FONTECHA, A., LANZAVECCHIA, A. & SALLUSTO, F. 2009. Dendritic cell migration to
379 peripheral lymph nodes. *Handb Exp Pharmacol*, 31-49.

380 MATTHEWS, B. E. 1982. Skin penetration by *Necator americanus* larvae. *Z Parasitenkd*, 68, 81-6.

381 PASURALERTSAKUL, S. & NGRENNGARMLERT, W. 2006. The exsheathment of *Necator americanus*
382 infective larvae. *Southeast Asian J Trop Med Public Health*, 37 Suppl 3, 28-31.

383 QUINNELL, R. J., PRITCHARD, D. I., RAIKO, A., BROWN, A. P. & SHAW, M. A. 2004. Immune responses
384 in human necatoriasis: association between interleukin-5 responses and resistance to
385 reinfection. *J Infect Dis*, 190, 430-8.

386 ROYER, P. J., EMARA, M., YANG, C., AL-GHOULEH, A., TIGHE, P., JONES, N., SEWELL, H. F., SHAKIB, F.,
387 MARTINEZ-POMARES, L. & GHAEMMAGHAMI, A. M. 2010. The mannose receptor mediates
388 the uptake of diverse native allergens by dendritic cells and determines allergen-induced T
389 cell polarization through modulation of IDO activity. *J Immunol*, 185, 1522-31.

390 SALAZAR, F., HALL, L., NEGM, O. H., AWUAH, D., TIGHE, P. J., SHAKIB, F. & GHAEMMAGHAMI, A. M.
391 2016. The mannose receptor negatively modulates the Toll-like receptor 4-aryl hydrocarbon
392 receptor-indoleamine 2,3-dioxygenase axis in dendritic cells affecting T helper cell
393 polarization. *J Allergy Clin Immunol*, 137, 1841-1851 e2.

394 SALAZAR, F., SEWELL, H. F., SHAKIB, F. & GHAEMMAGHAMI, A. M. 2013. The role of lectins in allergic
395 sensitization and allergic disease. *Journal of Allergy and Clinical Immunology*, 132, 27-36.

396 SAVINA, A. & AMIGORENA, S. 2007. Phagocytosis and antigen presentation in dendritic cells.
397 *Immunol Rev*, 219, 143-56.

398 SMITH-GARVIN, J. E., KORETZKY, G. A. & JORDAN, M. S. 2009. T cell activation. *Annu Rev Immunol*,
399 27, 591-619.

400 THOMPSON, M. R., KAMINSKI, J. J., KURT-JONES, E. A. & FITZGERALD, K. A. 2011. Pattern Recognition
401 Receptors and the Innate Immune Response to Viral Infection. *Viruses*, 3, 920-940.

402 VAN KOOYK, Y. & GEIJTENBEEK, T. B. H. 2003. DC-SIGN: escape mechanism for pathogens. *Nat Rev*
403 *Immunol*, 3, 697-709.

404 WOLLENBERG, A., MOMMAAS, M., OPPEL, T., SCHOTTDORF, E. M., GUNTHER, S. & MODERER, M.
405 2002. Expression and function of the mannose receptor CD206 on epidermal dendritic cells
406 in inflammatory skin diseases. *J Invest Dermatol*, 118, 327-34.

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410 **Figure 1: The mechanical interaction of immature DCs with *Necator americanus*.**

411 The sheathed larva remains dormant during the initial incubation period where DCs are
412 sequestered onto its sheath (up to approx.1 hour). Subsequently the en-sheathed larva begins to
413 move dynamically until it finally breaches its sheath (A). Once the larvae begin ex-sheathing,
414 the process occurs within seconds as the sheathed hookworm exits the cuticle sheath swiftly.
415 For this individual larva ex-sheathing was initiated at the 4-hour time point. At the first sight of
416 ex-sheathing, images were captured sequentially with 10 second intervals (B: 10 seconds; C:
417 20 seconds). The ex-sheathed hookworm progressively migrates away from its discarded
418 cuticle sheath with sequestered DCs (H). Images are representative of 3 independent
419 experiments using DCs from 3 different donors and 3 larvae batches. The sheathed larva
420 remains dormant during the initial incubation period where DCs are sequestered onto its sheath
421 (up to approx.1 hour). Subsequently the en-sheathed larva begins to move dynamically until it
422 finally breaches its sheath (A). Once the larvae begin ex-sheathing, the process occurs within
423 seconds as the sheathed hookworm exits the cuticle sheath swiftly. For this individual larva ex-
424 sheathing was initiated at the 4-hour time point. At the first sight of ex-sheathing, images were
425 captured sequentially with 10 second intervals (B: 10 seconds; C: 20 seconds). The ex-sheathed
426 hookworm progressively migrates away from its discarded cuticle sheath with sequestered DCs
427 (H). Images are representative of 3 independent experiments using DCs from 3 different donors
428 and 3 larvae batches.

429 **Figure 2. Dendritic cell sequestration around *N. americanus* larvae sheath.** Following 24-
430 hours majority of DCs form dense aggregates around discarded larval sheath. Data show
431 representative images of 6 independent experiments.

432 **Figure 3. The impact of blocking CLR on DCs interaction with *N. americanus*.** (A)
433 Microscopy data illustrates that DCs treated with either α -DC-SIGN, α -MR or mannan prior to
434 incubation with *N. americanus* exhibit a significant decrease in aggregation following 24-hour

435 incubation with the larvae compared to untreated DCs where DCs form aggregates around
436 larvae (also shown in figure 2). (B) *N. americanus* larvae do not ex-sheath in culture with ‘cell-
437 free’ conditioned media from DCs stimulated with mannan. Data show representative images
438 of 3 independent experiments.

439 **Figure 4. Dendritic cells maintain an immature phenotype upon interaction with *N.***
440 ***americanus*.** Dendritic cells were cultured in the presence of *N. americanus* larvae for 24 hours
441 followed by assessing the expression of co-stimulatory receptors/maturation markers CD40,
442 CD80, CD83, CD86, CD206 (mannose receptor), CD209 (DC-SIGN) and HLA-DR using flow
443 cytometry. Data show no changes in the expression of CD40, CD80, CD83, CD86 and HLA-
444 DR compared to un-stimulated cells which is in line with an immature phenotype. While there
445 are no changes in CD209 expression levels in response to *N. americanus* larvae, there is a
446 significant down regulation in CD206 expression. Cells stimulated with LPS show an increase
447 in the expression of maturation markers as expected. Data shown are mean values \pm SD of 3
448 independent experiments using blood samples from 3 different donors.

449 **Figure 5. *N. americanus* larvae does not modulate the dendritic cells response to LPS**
450 **stimulation.** Dendritic cells were simultaneously stimulated with LPS and *N. americanus*
451 larvae for 24 hours followed by assessing the expression of co-stimulatory receptors/maturation
452 markers CD40, CD80, CD83, CD86, CD206 (mannose receptor), CD209 (DC-SIGN) and
453 HLA-DR using flow cytometry. Data indicate that dendritic cells remain responsive to LPS
454 stimulation when co-stimulated with *N. americanus* larvae. Data shown are mean values \pm SD
455 of 3 independent experiments using blood samples from 3 different donors.

456 **Figure 6: The viability of dendritic cells post treatment with *N. americanus* larvae.**
457 Dendritic cells viability shows no significant changes after 24 hour treatment with the *N.*
458 *americanus* larvae with >80% viability quantified by measuring the expression levels of
459 Annexi-V and Propidium Iodide (PI). Cells in lower left quadrant are negative for both

460 Annexin-V and PI which indicates viability. Data shown is representative of 3 independent
461 experiments using blood samples from 3 different donors.

462 **Figure 7. The cytokine profile of dendritic cells in response to *N. americanus* in the**
463 **presence and absence of LPS stimulation.** *N. americanus* infective larvae on their own did
464 not induce production of selected pro (IL-6, IL-8, IL-12) or anti-inflammatory (IL-10)
465 cytokines. However, cytokine production in response to LPS seems to be ameliorated when
466 dendritic cells are stimulated with LPS and *N. americanus* *simultaneously*. Such suppression
467 was consistently observed in all cytokines tested but did not reach statistical significance
468 expression (p value= ≤ 0.3 Data shown are mean values \pm SD of 3 independent experiments
469 using blood samples from 3 different donors.

470 **Figure 8: The proposed mechanism of *Necator americanus* immune evasion strategy.**
471 Dendritic cells bind the *N. americanus* sheath via CLR_s which triggers the ex-sheathing of the
472 larvae. Dendritic cells are then sequestered onto the discarded sheath and the larvae escapes to
473 the vasculature unchallenged.

474

475 **Supplementary Video 1. The mechanical interaction of immature DCs with *N.***
476 ***americanus*.** This short film describes the novel interactions between DC and *N. americanus*.
477 Representative of 3 independent experiments.

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