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1 **PROFOUNDLY REDUCED CD1c^{+} MYELOID DENDRITIC CELL HLA-DR AND**
2 **CD86 EXPRESSION AND INCREASED TNF PRODUCTION IN EXPERIMENTAL**
3 **HUMAN BLOOD-STAGE MALARIA INFECTION.**

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11

12 **Running Head:**

13 CD1c^{+} mDC MODULATION IN HUMAN EXPERIMENTAL MALARIA

14

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35 ## Abstract

36 Dendritic cells (DC) are sentinels of the immune system that uniquely prime naive cells and
37 initiate adaptive immune responses. CD1c (BDCA-1) myeloid DC (CD1c$^+$ mDC) highly
38 express HLA-DR, have a broad TLR repertoire and secrete immune modulatory cytokines.
39 To better understand immune responses to malaria, CD1c$^+$ mDC maturation and cytokine
40 production were examined in healthy volunteers before and after experimental intravenous *P.*
41 *falciparum* infection with 150 or 1,800 parasite-infected red blood cells (pRBC). Following
42 either dose, CD1c$^+$ mDC significantly reduced HLA-DR expression in pre-patent infection.
43 Circulating CD1c$^+$ mDC did not upregulate HLA-DR following pRBC or TLR ligand
44 stimulation and exhibited reduced CD86 expression. At peak parasitemia, CD1c$^+$ mDC
45 produced significantly more TNF while IL-12 production was unchanged. Interestingly, only
46 the 1,800 pRBC dose caused a reduction in circulating CD1c$^+$ mDC count with evidence of
47 apoptosis. The 1,800 pRBC dose produced no change in T cell IFN-γ or IL-2 production at
48 peak parasitemia or 3 weeks post treatment. Overall, CD1c$^+$ mDC are compromised by *P.*
49 *falciparum* exposure, with impaired HLA-DR and CD86 expression and have an increased
50 capacity for TNF but not IL-12 production. A first pre-patent *P. falciparum* infection is
51 sufficient to modulate CD1c$^+$ mDC responsiveness, likely contributing to hampered effector
52 T cell cytokine responses and assisting parasite immune evasion.

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57 **Introduction**

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59 Malaria caused by *Plasmodium* spp. remains a major global health problem, with
60 584,000 deaths in 2013 (1). Repeat *Plasmodium* infections are common. Among reasons
61 cited for lack of sterile protective immunity, is the ability of parasites to subvert host immune
62 responses. Early effects include impaired function of dendritic cells (DC) (2), the only cells
63 capable of priming naive T cells. DC are a heterogeneous population composed of several
64 subsets distinguished by phenotype, location and functional properties (3). Circulating $CD1c^+$
65 mDC represent ~20% of total blood DC (4), express Toll-like receptors (TLR) 1-7 (5) and
66 produce immune-regulatory cytokines (IL-12 and IL-10) (6-8) and the pro-inflammatory
67 cytokine TNF (9). $CD1c^+$ mDC express high levels of HLA-DR when compared to other
68 circulating DC subsets (8, 10), suggesting a specialised ability to initiate adaptive immune
69 responses.

70 We previously reported loss of total myeloid DC (mDC), and reduced phagocytosis
71 by total blood DC during pre-patent experimental human blood-stage *P. falciparum* infection
72 (11), but $CD1c^+$ mDC were not individually examined. In acute *P. falciparum* malaria, $CD1c^+$
73 mDC decline (12) and have reduced MHC class II (HLA-DR) expression in both
74 uncomplicated (13) and severe malaria (14). However, it remains to be determined whether
75 this impairment is evident in pre-patent blood-stage *P. falciparum* infection, the effect of
76 different pRBC inoculating doses and whether $CD1c^+$ mDC cytokine production is impacted
77 by *Plasmodium,* informing whether $CD1c^+$ mDC can contribute to protective host adaptive
78 immune responses.

79 $CD1c^+$ mDC cytokine production and TLR response in pre-patent *Plasmodium*
80 infection have not been previously evaluated. Key immune-modulatory cytokines produced
81 by $CD1c^+$ mDC include IL-12, TNF and IL-10. These cytokines facilitate immune priming

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82 and can influence whether the immune response promotes the onset of immunity or assists
83 immune escape. DC generated IL-12 can drive T cell IFN-γ secretion and promote cytotoxic
84 capacity (15), as well as facilitate the development of clinical immunity to malaria (16-19).
85 TNF can promote maturation and survival of DC in vitro (20, 21) but in circulating blood,
86 TNF is not sufficient for maturation of CD1c$^+$ mDC (9). The function and influence of TNF
87 production by CD1c$^+$ mDC in the immune response to malaria is unclear. IL-10 is a
88 regulatory cytokine that plays a key role in host survival, pathogen control and prevention of
89 hyper-inflammatory responses (22). In acute malaria infection, IL-10 has been implicated in
90 mediating DC apoptosis (12). We here sought to understand if CD1c$^+$ mDC produce these
91 cytokines and if pre-patent *P. falciparum* infection altered their production.

92 Experimental human *P. falciparum* infection of malaria-naive healthy volunteers is a
93 valuable model to evaluate immune cell maturation and function. Firstly, allowing
94 assessment of responses before exposure and at subsequent time-points after inoculation and
95 secondly, allows comparison of responses following infection with different doses of parasite
96 infected red blood cell (pRBC) (150 versus 1,800 pRBC) (23). Because of limited current
97 understanding of *Plasmodium* antigens processed by DC and presented in the context of
98 HLA-DR to CD4$^+$ T cells, we measured cytokine production ex vivo and after stimulation
99 with TLR ligands or pRBC. TLR are key pathogen recognition receptors involved in the
100 initiation of the innate immune response (24). Differential expression of TLR on DC confers
101 functional specialisation of DC subsets. CD1c$^+$ mDC express a broad TLR repertoire
102 including TLR2 and TLR4 (5). *P. falciparum* glycosylphosphatidylinositol (GPI) is known to
103 mediate inflammatory responses via TLR2 and TLR4 (25). Furthermore, changes in TLR
104 expression and responses to the disease manifestation of malaria emphasise a role for TLRs
105 in malaria pathogeneses (42-44). To better understand the response of CD1c$^+$ mDC in pre-

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106 patent *P. falciparum* infection we assessed CD1c$^+$ mDC directly ex vivo and after stimulation
107 of three TLR (TLR1/2, TLR4, TLR7) with appropriate agonists or pRBC.

108 Our data show CD1c$^+$ mDC are compromised during pre-patent blood-stage
109 *Plasmodium* infection, with reduced HLA-DR expression, at both infecting pRBC doses.
110 CD1c$^+$ mDC exhibited reduced CD86 expression and increased production of TNF, with no
111 change in IL-12 and no detectable IL-10 production. Furthermore, CD4$^+$ and CD8$^+$ T cell IL-
112 2 and IFN-γ cytokine responses at peak parasitemia and 3 weeks after curative treatment
113 remained stable from baseline. Taken together, results show modulation of CD1c$^+$ mDC by
114 *Plasmodium* associated with static effector T cell responses, which likely assists immune
115 evasion and parasite expansion.

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128 **Materials and Methods**

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130 **Infection cohorts:** 62 volunteers aged 19-41 years (median 25 [IQR 22-28] years,
131 34% female, 66% male), consented to participate in a phase Ib clinical trial testing the
132 efficacy of anti-malarial drugs. Volunteers enrolled in separate cohorts and received two
133 different sized parasite inoculums as determined by qPCR, an objective of exploratory drug
134 studies. The first cohorts (150 pRBC n=12, 20%), received intravenous inoculation of red
135 cells containing ~150 ring stage parasites, and subsequent cohorts received ~1,800 pRBC
136 (1,800 pRBC n=50, 80%). Antimalarial drugs were administered when a threshold of ≥1,000
137 parasites/mL was confirmed by qPCR (26, 27) [day 10 (n=1, 8%) or day 11 (n=11, 91%) post
138 150 pRBC and day 7 (n=19, 38%), day 8 (n=30, 60%) or day 9 (n=1, 2%) post 1,800 pRBC
139 inoculation] (**Figure 1A**). The protocol for the controlled human malaria infection studies has
140 previously been reported (27) details of the clinical trials and therapeutic response to the anti-
141 malarials in the current study will be reported elsewhere. Blood anti-coagulated with lithium
142 heparin was collected before inoculation and at the same time on days 7, 9, 10 and 11 (150
143 pRBC) and days 6, 7 and 8 (1,800 pRBC) (**Figure 1A**). Functional and flow cytometric
144 assays used fresh whole blood processed < 2 hours post collection. Full blood counts were
145 determined by an automated cell counter using EDTA blood (Beckman Coulter, USA).

146 **Ethics:** Studies were approved by the Human Research Ethics Committees of QIMR
147 Berghofer Medical Research Institute (P1479) and the Human Research Ethics Committee of
148 Menzies School of Health Research, Australia (HREC 10/1431). Written and informed
149 consent was obtained from all participants in the clinical trials. Clinical Trial Registrations;
150 ACTRN12611001203943; ACTRN12612000323820; ACTRN12612000814875;
151 ACTRN12613000565741; ACTRN12613001040752; NCT02281344.

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152 **CD1c$^+$ mDC enumeration:** 200 µL of blood was stained with surface antibodies
153 (lineage markers [CD3 (HIT3a), CD14 (HCD14), CD19 (HIB19), CD56 (HCD56)], HLA-
154 DR (L243), CD11c (B-Ly6), CD123 (6H6), CD1c (LI6I), CD16 (3G8) and CD141 (M80)),
155 RBC lysed with FACS lysing solution (BD Biosciences) and cells fixed with 1% (w/v)
156 paraformaldehyde in phosphate-buffered saline. Absolute numbers of CD1c$^+$ mDC were
157 determined by adding automated lymphocyte and monocyte counts (10^9 cells/L), dividing the
158 sum by 100, multiplying the percentage of CD1c$^+$ mDC, and multiplying the product by
159 1,000 to give the cell count per microliter. For the 150 pRBC cohort CD1c$^+$ mDC were
160 characterised as lineage negative (Lin$^-$), HLA-DR$^+$, CD11c$^+$, CD123$^-$, CD16$^-$ and CD141$^-$
161 mononuclear cells (**Supplementary Fig. 1A**) and for the 1,800 pRBC cohorts CD1c$^+$ mDC
162 were characterised as Lin$^-$, HLA-DR$^+$, CD11c$^+$, CD123$^-$ and CD1c$^+$ (BDCA-1)
163 (**Supplementary Fig. 1B**). Lymphocyte subsets were assessed in whole blood. T cells, B
164 cells and NK cells were gated by FSC and SSC properties, lack of CD14 expression and
165 differentiated by HLA-DR expression (**Supplementary Fig. 1C**).

166 **Apoptosis:** Intracellular active caspase-3 staining was assessed as previously
167 described (28). Briefly, 1,000 µL of blood was stained with surface antibodies, RBC were
168 lysed with FACS lysing solution (BD), cells were permeabilised using 1 x BD Perm/Wash™
169 (BD) and stained with active caspase-3 antibody (C92-605, BD USA).

170 **Antigen uptake:** CD1c$^+$ mDC phagocytosis was assessed by uptake of 1 mg/mL
171 FITC-dextran (Sigma, USA) after 60 min at 37°C or on ice as a control and expressed as delta
172 median fluorescence intensity (ΔMFI) (ie. [MFI of cells at 37°C]- [MFI control cells on ice]).

173 **T cell Activation:** T cell proliferation was assessed by expression of Ki-67 (B56). In
174 brief, 1 million PBMC were stained with surface antibodies (CD4 (SK4), CD3 (UCHT1) and

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175 CD8 (RPA-T8), washed with 2% FCS/PBS and permeabilised with 1x BD Perm/Wash™
176 (BD, USA) and stained with intracellular Ki-67.

177 **Intracellular cytokine staining:** Cytokine production was assessed in 300 µL (T
178 cells) or 1000 µL ($CD1c^+$mDC) of blood stimulated with TLR agonists; TLR1: Pam3CSK4
179 100 ng/mL and TLR2: HKLM 10^8 cells/mL, TLR4: *Escherichia coli* K12 LPS 200 ng/mL or
180 TLR7: Imiquimod 2.5 µg/mL (Sigma-Aldrich, USA), pRBC or un-parasitised RBC (uRBC)
181 at final concentration of 5 million ($CD1c^+$ mDC) or 1 million (T cells) pRBC or uRBC/ mL in
182 the presence of anti-CD28 and anti-CD49d antibodies at 1 µg/mL (BD, USA) for T cells.
183 Protein transport inhibitor (Brefeldin A, GolgiPlug, BD, USA) was added after 2h ($CD1c^+$
184 mDC) or 20h (T cells) at 37°C, 5% CO_2. At 6h or 24h, cells were stained to identify $CD1c^+$
185 mDC (including CD86 (IT2.2)) or T cells (CD4 (OKT4) and CD8 (SK1)). RBC were lysed
186 with FACS lysing solution (BD, USA), washed with 2% FCS/PBS, cells permeabilised with
187 1x Perm/Wash™ or Perm 2 (BD, USA) and stained with intracellular anti-TNF-α (MAB11),
188 IL-12/IL-23p40 (C11.5), IL-10 (JES3-9D7), or IFN-γ (B27) and IL-2 (MQ1-17H12) or IgG1
189 isotype controls.

190 FACS data was acquired using a FACSCanto™ II and LSRFortessa™ 4 (BD) and
191 data analysed using Kaluza® 1.3 (Beckman Coulter, USA) or FlowJo (FlowJo, LLC, USA).

192 ***P. falciparum* infected pRBC:** *P. falciparum* K1 (from MR4, part of BEI Resources
193 Repository, NIAID, NIH: *P. falciparum* K1, MRA-159, deposited by DE Kyle) was cultured
194 in human RBC as previously described (29). In brief, culture supernatant was washed in
195 sterile PBS and schizonts and trophozoites isolated using density centrifugation. Washed
196 culture supernatant was layered on 63% Percoll and 27% Percoll and centrifuged at 2000 rpm
197 for 20 min, no brake. Upper (and if necessary lower) layers were removed and schizonts and
198 trophozoites collected from 63%-27% interface and washed with PBS. Thin smears checked

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199 purity and enumerated pRBC or uRBC. Stocks were frozen in glycerol 30% freezing media at
200 a final concentration of 150 x 10^6 pRBC/mL and added to assays immediately, post thaw.

201 **Statistics:** Statistical analyses used GraphPad Prism 6 (Graphpad Software Inc.,
202 USA). Wilcoxon matched-pairs test compared longitudinal data. Tests were two-tailed and
203 considered significant if *p*-values <0.05.

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205 **Results**

206

207 **Effect of infecting pRBC dose on CD1c$^+$ mDC.** Circulating CD1c$^+$ mDC were
208 examined in volunteers prior to and during experimental *P. falciparum* infection with 150 or
209 1,800 pRBC. Mean parasitemia determined by PCR for subjects infected with either dose is
210 shown in figure 1B. Subjects administered 150 pRBC, retained circulating CD1c$^+$ mDC at
211 peak parasitemia (day 11 median parasitemia 2,555/mL [IQR 781-4,753]), with a decline 24
212 hours after treatment (**Fig. 1C**). We observed no change in circulating lymphocytes; CD4$^+$ T
213 cells, CD56$^+$ NK cells or CD20$^+$ B cells, while monocytes significantly increased on day 7
214 (**Supplementary Table 1**). In contrast, subjects administered 1,800 pRBC had a significant
215 decline in circulating CD1c$^+$ mDC from day 7 (median parasitemia 4,577/mL [IQR 1,645-
216 10,066], n=21 **Fig. 1D**), with the decline persisting day 8 (median parasitemia 9,073/mL
217 [IQR 4,755-21,857], n=14**)** and 24 hours after drug treatment (**Fig. 1D**). The higher
218 inoculation dose caused the decline of circulating lymphocytes; CD4$^+$ T and CD56$^+$ NK cells
219 on day 8 and 24 hours after treatment, but did not impact monocyte counts (**Supplementary**
220 **Table 1**). Circulating B cells reduced only 24 hours after anti-malarial drug therapy
221 (**Supplementary Table 1**).

222 Circulating CD1c$^+$ mDC were examined to determine if there was active caspase-3
223 staining. Caspase-3 is an executioner caspase, essential to intrinsic and extrinsic apoptotic
224 pathways and apoptosis occurs upon cleavage of caspase-3 (30). Among subjects
225 administered 150 pRBC, no active caspase-3$^+$ was detected on day 10-11 in CD1c$^+$ mDC
226 (median 0.3% [IQR 0-0.7 %] caspase-3$^+$, **Supplementary Fig. 2**) at median parasitemia of
227 2,555/mL [IQR 781-4,753]. In contrast, on day 7-8 after 1,800 pRBC infection, there was a
228 trend to increased active caspase-3$^+$ expression by CD1c$^+$ mDC (median 5% [IQR 1.5-7.3 %]
229 caspase-3$^+$, **supplementary Fig. 2,** *p*=0.08), at median parasitemia of 6,877/mL [IQR 4,182-

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230 20,398]. In the 1,800 pRBC cohort, one participant with 17 times more active caspase-3$^+$
231 staining was excluded (outlier: caspase-3$^+$ on day 7: 85% versus cohort median of 5%). When
232 originally included in the analysis a significant increase in active caspase-3$^+$ expression after
233 1,800 pRBC infection was observed (p=0.04).

234 **Reduced HLA-DR and CD86 expression on circulating CD1c$^+$ mDC.** We next
235 examined the impact of infecting pRBC dose on HLA-DR expression by CD1c$^+$ mDC. In
236 accord with previous reports, HLA-DR expression was significantly higher on CD1c$^+$ mDC
237 when compared to other human blood DC subsets (**Fig. 2A**). In cohorts inoculated with either
238 150 or 1,800 pRBC, there was a significant reduction in CD1c$^+$ mDC HLA-DR expression at
239 peak parasitemia (day 10-11 for 150 pRBC, median parasitemia 2,555/mL [IQR 781-4,753]
240 and day 7-8 for 1,800 pRBC, median parasitemia 4,577/mL [IQR 1,645-10,066], **Fig. 2B**). In
241 a sub-group of participants (n=14), longitudinal HLA-DR MFI was assessed, 24 hours before
242 and after anti-malarial drug treatment (**Supplementary Figure 3A**). Before peak parasitemia
243 there was no reduction in HLA-DR MFI and at 24 hours post drug treatment reduced HLA-
244 DR MFI failed to recover (**Supplementary Figure 3B+C**). There was no change in
245 monocyte HLA-DR expression at peak parasitemia following 1,800 pRBC infection (**Fig.**
246 **2C**).

247 The impact of 150 or 1,800 pRBC on circulating CD1c$^+$ mDC phagocytosis was
248 examined using FITC-dextran particles. There was no significant change in particle uptake
249 after 150 pRBC infection (**Fig. 2D**) and a trend towards reduced uptake on day 7 after 1,800
250 pRBC infection (**Fig. 2D**). Importantly, there was a significant positive association between
251 HLA-DR expression and FITC-dextran uptake following 1,800 pRBC (**Fig 2F**) and a trend
252 suggesting weak association for the 150 pRBC cohort (**Fig. 2E**). These data imply that CD1c$^+$
253 mDC HLA-DR expression is proportional to phagocytic capacity.

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254 In the 1,800 pRBC cohort, at baseline and peak parasitemia, HLA-DR expression was
255 further assessed ex vivo and after TLR stimulation to determine the capacity of CD1c$^+$ mDC
256 to respond to external stimuli (**Fig. 3A**). Before infection, CD1c$^+$ mDC drastically increased
257 HLA-DR expression upon TLR1/2 or TLR4 and moderately upon TLR7 or *P. falciparum*
258 infected pRBC stimulation (**Fig. 3B, top panel**). In contrast, at peak parasitemia, CD1c$^+$
259 mDC failed to upregulate HLA-DR in response to any TLR or pRBC (**Fig 3B, bottom**
260 **panel**). HLA-DR expression was significantly impaired directly ex vivo and across all
261 stimulatory conditions tested (p=0.03, day 0 versus day 7 for all conditions). In contrast,
262 monocytes from the same blood sample retained the ability to upregulate HLA-DR at peak
263 parasitemia following stimulation with *P. falciparum* infected pRBC or TLR ligands (**Fig.**
264 **3C, bottom and top panel**). The similar magnitude of HLA-DR expression by monocytes on
265 day 0 and day 7 (peak parasitemia) highlights the assays reproducibility.

266 Expression of the co-stimulatory maturation marker CD86 was next examined on
267 CD1c$^+$ mDC (**Fig. 4A**) and monocytes in the 1,800 pRBC cohort. At peak parasitemia (day 7)
268 CD86 expression on circulating CD1c$^+$ mDC was significantly reduced, directly ex vivo and
269 following TLR stimulation (**Fig. 4B**). In contrast, circulating monocytes had unaltered CD86
270 expression at peak parasitemia ex vivo or in response to TLR ligands or pRBC (**Fig. 4C**).
271 CD86$^+$ and CD86$^-$ CD1c$^+$ mDC were more closely examined for HLA-DR expression. As
272 expected CD86$^+$ CD1c$^+$ mDC expressed significantly more HLA-DR compared to CD86$^-$
273 CD1c$^+$ mDC, at baseline and at peak parasitemia (**Fig. 4D**). At peak parasitemia, HLA-DR
274 expression was significantly reduced on both the CD86$^+$ and CD86$^-$ CD1c$^+$ mDC ex vivo and
275 across stimulatory conditions (**Fig. 4E+F**). Results show that CD86 expression (albeit
276 reduced) can be induced however, HLA-DR is clearly impaired on these DC even post TLR
277 stimulation.

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278 **Increased TNF and stable IL-12 production by circulating CD1c$^+$ mDC.** In
279 participants infected with 1,800 pRBC, the ability of CD1c$^+$ mDC to produce TNF, IL-12 and
280 IL-10 was simultaneously assessed in response to TLR ligands and *P. falciparum* infected
281 pRBC (**Fig. 5A)**. At peak parasitemia CD1c$^+$ mDC significantly increased TNF production in
282 response to *P. falciparum* infected pRBC stimulation (**Fig. 5B).** CD1c$^+$ mDC also
283 significantly increased TNF production upon combined TLR1/2 or TLR4 but not TLR7
284 stimulation (**Fig. 5C**). Ex vivo (nil or uRBC) cells showed no statistically significant change
285 in spontaneous TNF production between baseline and peak parasitemia (**Fig. 5B**). There was
286 no consistent change in CD1c$^+$ mDC IL-12 in response to pRBC (**Fig. 5D**) or any TLR
287 stimulation (**Fig. 5E**).

288 The frequency of CD1c$^+$ mDC co-producing TNF and IL-12 in response to TLR1/2 or
289 TLR4 remained stable from day 0 to day 7 (TLR1/2 day 0 11% [IQR 9-19%], day 7 12%
290 [IQR 10-20%], p=0.5 and TLR4 stimulation day 0 17% [IQR 10-23%], day 7 24% [IQR 21-
291 28%], p=0.09). No intracellular IL-10 was detected in CD1c$^+$ mDC either directly ex vivo or
292 after stimulation at baseline or peak parasitemia, despite validation of the assay with IL-10
293 detection in monocytes.

294 **TNF is produced by CD86$^+$ and CD86$^-$ CD1c$^+$ mDC in *P. falciparum* infection.**
295 The phenotype of TNF-producing CD1c$^+$ mDC was further evaluated by assessment of CD86
296 expression and is represented in pie charts (**Supplementary Fig. 4**). For all stimulations,
297 except TLR7, the majority of TNF producing CD1c$^+$ mDC expressed CD86. At peak
298 parasitemia, CD1c$^+$ mDC which lacked CD86 increased TNF production after TLR1/2, TLR4
299 or TLR7 stimulation (p=0.06 **Supplementary Fig. 4**).

300 **Stable CD4$^+$ and CD8$^+$ T cell cytokine responses.** To determine if *P. falciparum*
301 impacted T cell cytokine production, CD4$^+$ and CD8$^+$ T cell responses were evaluated

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302 following 1,800 pRBC infection. There was no significant change in IFN-γ or IL-2
303 production by $CD4^+$ or $CD8^+$ T cells (**Fig. 6**) from baseline to peak parasitemia, or 3 weeks
304 after anti-malarial drug treatment. Furthermore, there was no change in Ki-67 expression by
305 $CD4^+$ T cells (%Ki-67 day 0; 1.4 [IQR 0.4-2.3] and 2.0 [0.5-2.3] at peak parastiemia) or
306 $CD8^+$ T cells (%Ki-67 day 0; 0.7 [IQR 0.5-2.4] and 1.2 [0.7-1.8] at peak parastiemia)
307 between baseline and peak parasitemia.

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309 **Discussion**

310 This study demonstrates that a single experimental human *P. falciparum* blood-stage
311 infection leads to downregulation of HLA-DR and CD86 expression on circulating CD1c$^+$
312 mDC following either a 150 or 1,800 pRBC inoculating dose. The *Plasmodium*-impacted
313 CD1c$^+$ mDC do not upregulate HLA-DR in response to TLR or pRBC stimulation, display an
314 increased propensity for TNF production and impaired phagocytic capacity. Interestingly,
315 monocytes were not similarly impacted and maintained HLA-DR and CD86 expression
316 suggesting *Plasmodium* modulation of HLA-DR is not generic. Taken together, pre-patent *P*
317 *falciparum* infection subverts CD1c$^+$ mDC phenotypically and functionally, compromising
318 their ability to prime adaptive immune responses.

319 CD1c$^+$ mDC express significantly more HLA-DR than other DC subsets suggesting a
320 specialised role in antigen uptake and presentation. In pre-patent *P. falciparum* infection,
321 reduced HLA-DR expression on CD1c$^+$ mDC occurred at comparable parasite densities in
322 both 150 and 1,800 pRBC cohorts (day 10-11 and day 7-8 respectfully), indicating parasite
323 biomass rather than infection duration impacted HLA-DR expression. HLA-DR expression
324 was an indication of phagocytosis ability as there was a significant association between
325 CD1c$^+$ mDC HLA-DR expression and FITC-dextran uptake. Further investigation is required
326 to verify if similar data are obtained with phagocytosis of *P. falciparum* infected pRBC The
327 persistent reduction of HLA-DR despite stimulation with different TLR agonists or *P.*
328 *falciparum* infected pRBC, indicates impaired HLA-DR expression was not reversible nor
329 stimulus specific. In the rodent *P. yoelii* model, intact pRBC induce a similar general
330 inhibition of TLR responsiveness in DC (31). At peak infection, impaired antigen presentation
331 by splenic DC has been shown in vivo, in the rodent *P. chabaudi* model (32). In addition, loss
332 of total mDC HLA-DR is described in infection with helminths (33), *Salmonella* (34) herpes

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333 simplex virus (35) and severe sepsis (36), suggesting this is not a *Plasmodium* specific
334 phenomenon. As with helminth infection where reduced HLA-DR on mDC results in
335 impaired T cell proliferation and activation (33), we show absence of T cell activation,
336 manifest by lack of increased Ki-67 or IFN-γ, IL-2 production, with altered HLA-DR^{lo} CD1c^{+}
337 mDC following *P. falciparum* infection.

338 *P. falciparum* compromised CD86 expression on CD1c^{+} mDC, albeit to a lesser
339 degree than HLA-DR, suggesting different mechanisms and/or recovery of CD86 than MHC
340 class II. Interestingly, both CD86^{+} CD1c^{+} mDC and CD86^{-} CD1c^{+} mDC showed impaired
341 HLA-DR expression. Reduction in HLA-DR and CD86 expression on CD1c^{+} mDC after
342 blood-stage infection contrasted the stable HLA-DR and CD86 expression during
343 experimental *P. falciparum* sporozoite infection (37). The method of *P. falciparum*
344 inoculation, intravenous pRBC versus intradermal sporozoites is a possible explanation for
345 the difference. In acute HIV infection (38) and pancreatitis patients (39), CD86 expression is
346 reduced on lymphoid tissue migrating DC and circulating monocytes. In vitro, *P. falciparum*
347 pRBC inhibit monocyte-derived DC maturation HLA-DR and CD86 expression via contact-
348 dependent (40) or contact-independent mechanisms at high concentrations (41). Our data
349 support these in vitro studies and demonstrate *P. falciparum* compromises CD1c^{+} mDC CD86
350 expression in vivo, already at a very low parasite density.

351 The cytokine profile of CD1c^{+} mDC in *P. falciparum* infection has not previously
352 been reported. In pre-patent *P. falciparum* infection, we characterised a pro-inflammatory
353 cytokine profile, with stable IL-12, absent IL-10 and increased TNF production. Despite
354 compromised HLA-DR and CD86 expression, CD1c^{+} mDC increased TNF production upon
355 TLR1/2, TLR4 or pRBC stimulation. At peak parasitemia, HLA-DR^{lo} CD86^{-} CD1c^{+} mDC
356 increased their proportion of TNF production. The consequences of enhanced TNF remain to
357 be determined. TNF can promote DC maturation and survival in vitro (20, 21). However, pro

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358 inflammatory cytokines such as TNF are not sufficient for full functional maturation of DC
359 (9), defined as the DC's ability to induce effector T cell responses (42). Increased TNF
360 responses in the current study concur with increased PBMC TNF production (plus IL-1β, IL-
361 6 and IL-10) upon TLR1/2 and TLR4 stimulation following experimental *P. falciparum*
362 sporozoite infection (43). Up-regulation of TLR expression may explain the increased TNF
363 production, as acute malaria increases mDC and monocyte TLR2 and TLR4 expression (44,
364 45). Our IL-12 data support previous reports of absent TLR responses in healthy $CD1c^{+}$ mDC
365 after single TLR stimulation (TLR2,3,4,7,8 or 9) (9). The apparent inability of $CD1c^{+}$ mDC
366 to increase IL-12 production may impact adaptive immunity as IL-12 is essential for priming
367 of naive $CD4^{+}$ T cells (15). The design of our study with drug cure of parasitemia at a pre-
368 determined threshold of 1,000 parasites/mL precluded examination of associations between
369 TNF production and clinical symptoms or subsequent parasite biomass.

370 The decline in circulating $CD1c^{+}$ mDC in pre-patent *P. falciparum* infection has not
371 previously been reported. Total blood mDC are reduced in experimental infection (11) and
372 $CD1c^{+}$ mDC reduce in acute malaria (12, 46), but importantly in endemic areas, there is
373 preservation of blood $CD1c^{+}$ mDC in asymptomatic carriers of *P. falciparum* with patent
374 parasitemia (46). Interestingly, after the 150 pRBC infection circulating $CD1c^{+}$ mDC were
375 stable. Caspase-3 staining of $CD1c^{+}$ mDC following the 1,800 but not 150 pRBC infection
376 indicates apoptosis, which might partially explain differences in peripheral mDC stability.
377 Differences between 1,800 and 150 pRBC cohorts suggest the inoculating concentration has
378 considerable impact on the viability of circulating $CD1c^{+}$ mDC. Migration is another likely
379 explanation for DC loss from the periphery, as reduced plasmacytoid DC in uncomplicated
380 malaria is attributed to expression of the lymph node migration chemokine CCR7 (47).

381 How malaria compromises HLA-DR and CD86 expression remains to be determined.
382 Complexities in MHC class II antigen-processing pathways provide extensive opportunities

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383 for pathogen interference (48). Indeed, *Salmonella* can reduce HLA-DR surface expression
384 by enhancing ubiquitination of MHC class II (49). MARCH I, a ubiquitin ligase is capable of
385 mediating ubiquitination of MHC Class II (50). It is proposed that TLR stimulation of
386 monocyte-derived DC down-regulates MARCH I, and HLA-DR accumulates on the cell
387 surface (51). However, in early *P. falciparum* infection we show reduced HLA-DR
388 expression on CD1c^{+} mDC with sustained repression upon TLR stimulation. Additional
389 studies are required to identify the causes of HLA-DR down-regulation and further assess the
390 role of CD1c^{+} mDC in the generation and maintenance of effector T cell responses and
391 malaria immunity.

392 CD1c^{+} mDC perform a crucial role in the early steps of the adaptive immune
393 response, they process and present antigens (via MHC class I and II), deliver co-stimulation
394 (CD86), signal via TLR and produce and secrete cytokines to initiate or shape cellular
395 adaptive immune responses. Here we report alterations in each of these vital functions by *P.*
396 *falciparum*, reduced HLA-DR expression (and failed upregulation), reduced CD86 and
397 increased TNF but not IL-12. *P. falciparum* even at low parasitemia rapidly and
398 comprehensively compromises CD1c^{+} mDC maturation and functionality, potentially
399 contributing to the failed enhancement of T cell cytokine responses and immune evasion by
400 *P. falciparum*. Candidate malaria vaccines should avoid these deleterious responses and
401 instead aim to mature and activate CD1c^{+} mDC function.

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582 **Fig 1. Parasitemia and CD1c^{+} mDC absolute counts in participants infected with 150**
583 **pRBC (white) or 1,800 pRBC (grey). A.** Schematic of clinical trial cohorts, 150 pRBC
584 (black) and 1,800 pRBC (grey), on days specified PCR, full blood counts and immunological
585 assays were performed, arrows indicate day of anti-malarial treatment and n represents
586 number of volunteers treated. **B.** Parasitemia was determined by qPCR in participants in
587 infected with 150 pRBC (white circles) or 1,800 pRBC (grey circles). The dotted line
588 indicates the pre-determined parasite treatment threshold of 1,000 parasites/mL. Brackets
589 represent the day of anti-malarial treatment; after infection with 1,800 pRBC, day 7 (n=19),
590 day 8 (n=30), day 9 (n=1) or 150 pRBC, day 10 (n=1), day 11 (n=11). Mean parasitemia +/-
591 standard error is presented. **C.** The absolute number of circulating CD1c^{+} mDC after 150
592 pRBC infection in 12 participants (24 hours after drug treatment p=0.04) (exception being 6
593 individuals on day 7 and day 10). **D.** The absolute number of circulating CD1c^{+} mDC after
594 1,800 pRBC infection in 21 participants (day 7 p=0.05; day 8 p=0.04; 24 hours after drug
595 treatment p=0.0002) (exception being 7 individuals on day 6 and 14 individuals) on day 8
596 and post anti-malarial drug treatment (Rx). Box plot show the minimum, maximum, median
597 and interquartile range for data from all subjects.

598 **Fig 2. HLA-DR expression on CD1c^{+} mDC. A.** HLA-DR expression on DC subsets; CD1c^{+}
599 mDC (n=21), CD16^{+} mDC (n=26), CD141^{+} mDC (n=33) and plasmacytoid DC (n=33) in
600 participants at baseline (day 0). **B.** CD1c^{+} mDC HLA-DR MFI % baseline (day 0) after 150
601 pRBC (12 participants) or 1,800 pRBC (21 participants) and peak parasitemia (day 10-11 or
602 day 7-8, respectively). **C.** Monocyte HLA-DR MFI % baseline following 1,800 pRBC
603 infection in 6 participants. **D.** Uptake of particulate antigen by CD1c^{+} mDC after 150 pRBC
604 infection (12 participants, left graph or 1,800 pRBC infection (7 participants, right graph).
605 ΔMFI of FITC dextran uptake (calculated as [MFI for cells incubated at 37 ^{o}C] – [MFI for

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606 cells incubated on ice]). **E.** Association between day 11 (peak parasiteamia) % baseline HLA-
607 DR MFI and FITC-dextran uptake following 150 pRBC. **F.** Association between day 7 (peak
608 parasiteamia) % baseline HLA-DR MFI and FITC-dextran uptake following 1,800 pRBC
609 infection. Statistics were calculated using the Wilcoxon matched-paired test and linear
610 regression. Abbreviations: MFI, median fluorescence intensity.

611 **Fig 3. CD1c$^+$ mDC HLA-DR expression after TLR or pRBC stimulation. A.**
612 Representative histograms of HLA-DR MFI on whole blood CD1c$^+$ mDC in one individual
613 on day 0 and day 7. **B.** CD1c$^+$ mDC HLA-DR expression in 6 participants on day 0 (top
614 graph) and day 7 (bottom graph), dotted line shows the median HLA-DR MFI on day 0 for
615 the control (NIL) condition (median=17.6). **C.** Monocyte HLA-DR expression in 6
616 participants on day 0 (top graph) and day 7 (bottom graph), dotted line shows the median
617 HLA-DR MFI on day 0 for the control (NIL) condition (median=4). Abbreviations: MFI,
618 median fluorescence intensity, uRBC, uninfected red blood cell; pRBC, parasitised red blood
619 cell.

620 **Fig 4. CD1c$^+$ mDC CD86 expression at peak parasitemia. A.** Representative gating
621 strategy for CD86 on whole blood CD1c$^+$ mDC in one individual on day 0 and day 7. **B.** The
622 paired frequency of CD86$^+$ CD1c$^+$ mDC in 6 participants, day 0 (baseline) and day 7 (peak-
623 parasitemia); ex vivo (NIL), post TLR and uRBC or pRBC stimulation. **C.** The paired
624 frequency of CD86$^+$ CD14$^+$ monocytes in 6 participants day 0 (baseline) compared to day 7
625 (peak parasitemia). **D.** HLA-DR expression on CD86$^+$ compared to CD86$^-$ CD1c$^+$ mDC, day
626 0 (baseline) and day 7 (peak parasitemia). Mann-Whitney test was used for comparison
627 between cell subsets. **E.** HLA-DR expression on CD86$^+$ CD1c$^+$ mDC, day 0 (baseline) and
628 day 7 (peak parasitemia) in 6 participants. **F.** HLA-DR expression on CD86$^+$ CD1c$^+$ mDC,

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629 day 0 (baseline) and day 7 (peak parasitemia) in 6 participants. The Wilcoxon matched-paired
630 test was used for comparison between day 0 and day 7, * significance p= 0.03.

631 **Fig 5. CD1c$^+$ mDC cytokine responsiveness to TLR or pRBC stimulation. A.**
632 Representative staining of blood CD1c$^+$ mDC for intracellular cytokines. CD1c$^+$ mDC were
633 identified as negative for lineage markers (CD14, CD3, CD19 and CD56), HLA-DR$^+$ and
634 CD1c$^+$. Intracellular cytokine production by CD1c$^+$ mDC on day 0 (IL-12, TNF and IL-10) in
635 two conditions, ex vivo (NIL) (top panel) and TLR4 (bottom panel). **B.** TNF production on
636 day 0 and day 7 ex vivo (NIL) and post uRBC or pRBC (p=0.03) stimulation. **C.** TNF
637 production on day 0 and day 7 post TLR1/2 (p=0.02), TLR4 (p=0.0002) or TLR7 stimulation.
638 **D.** IL-12 production on day 0 and day 7 ex vivo (NIL) and post uRBC or pRBC stimulation.
639 **E.** IL-12 production on day 0 and day 7 post TLR stimulation. The Wilcoxon matched-paired
640 test was used for comparison between day 0 and day 7, * p<0.05, *** p=0.0002. Line graphs
641 show data for all subjects (n=14) (exception being 8 individuals for TLR1/2; 10 individuals
642 for TLR7; 6 individuals for uRBC and pRBC). Abbreviations: FSC, forward scatter; SSC,
643 side scatter; uRBC, uninfected red blood cell; pRBC, parasitised red blood cell.

644 **Fig. 6. Cytokine production by T cells post uRBC or pRBC stimulation on day 0, day 7**
645 **and day 28. A.** CD4$^+$ T cell IFN-γ production. **B.** CD8$^+$ T cell IFN-γ production. **C.** CD4$^+$ T
646 cell IL-2 production. **D.** CD8$^+$ T cell IL-2 production. The Wilcoxon matched-paired test was
647 used for comparison between day 0 and day 7, day 0 and day 28, 19 participants.

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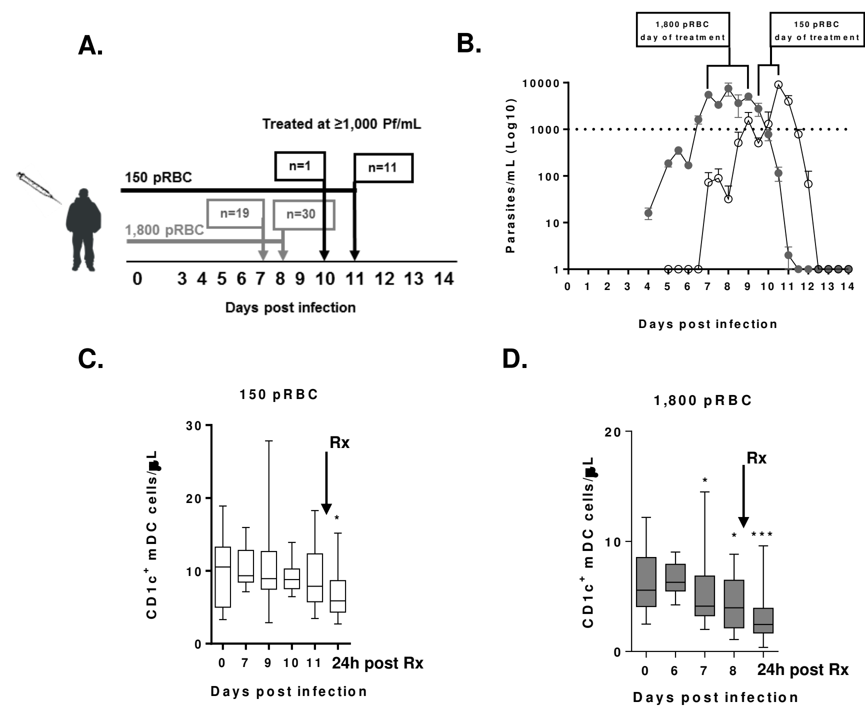

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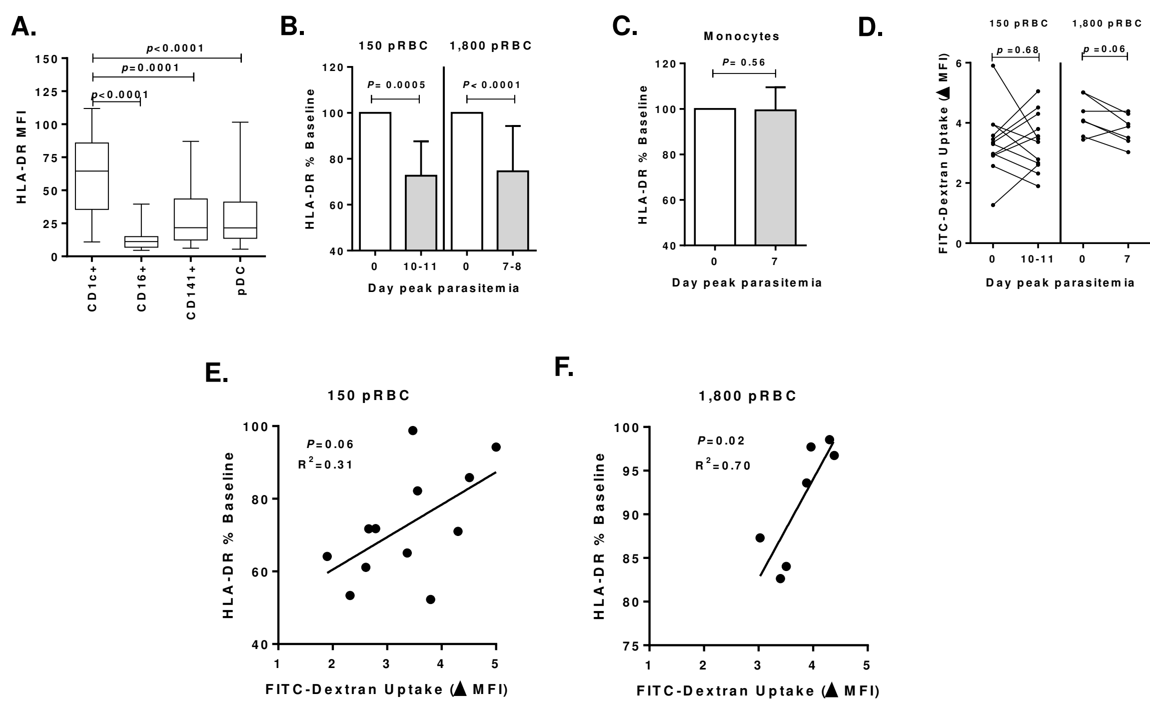

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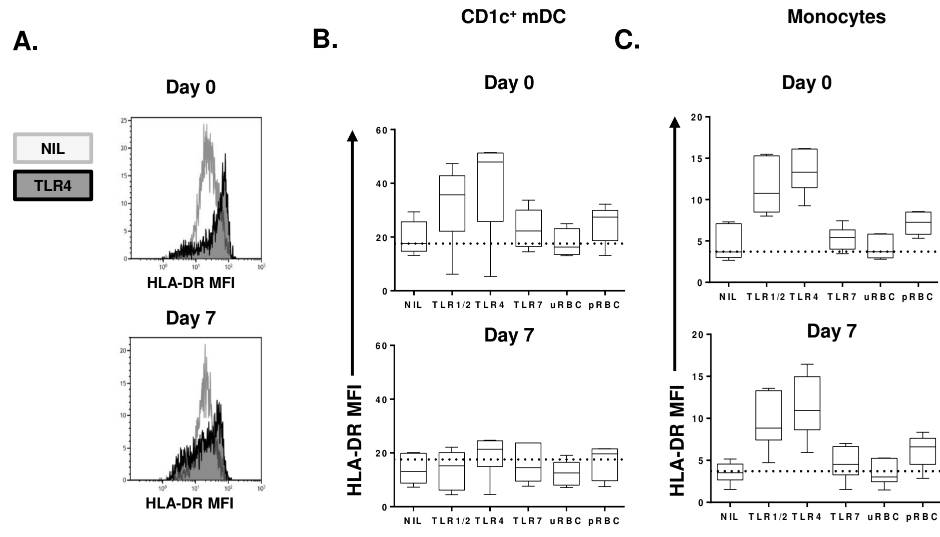

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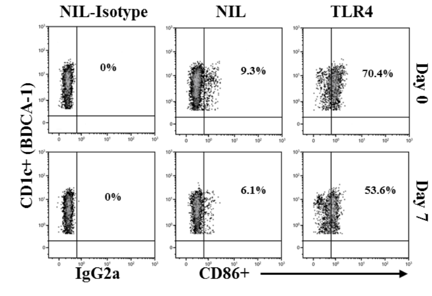

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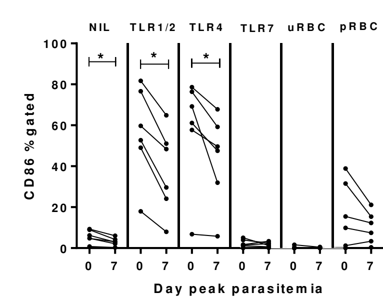

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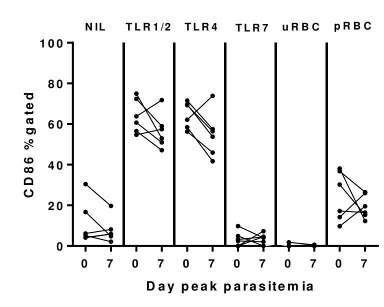

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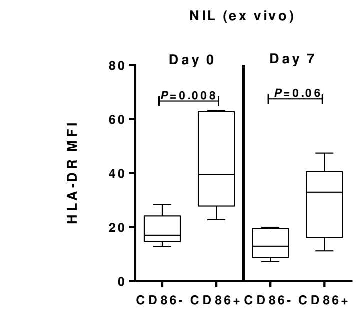

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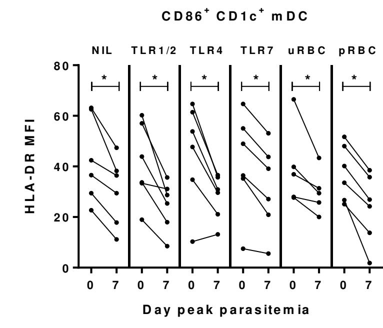

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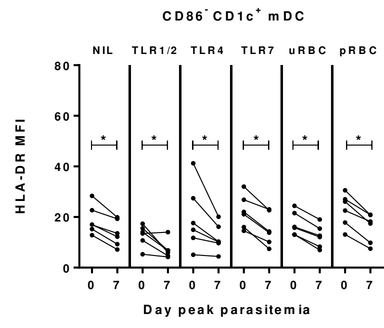

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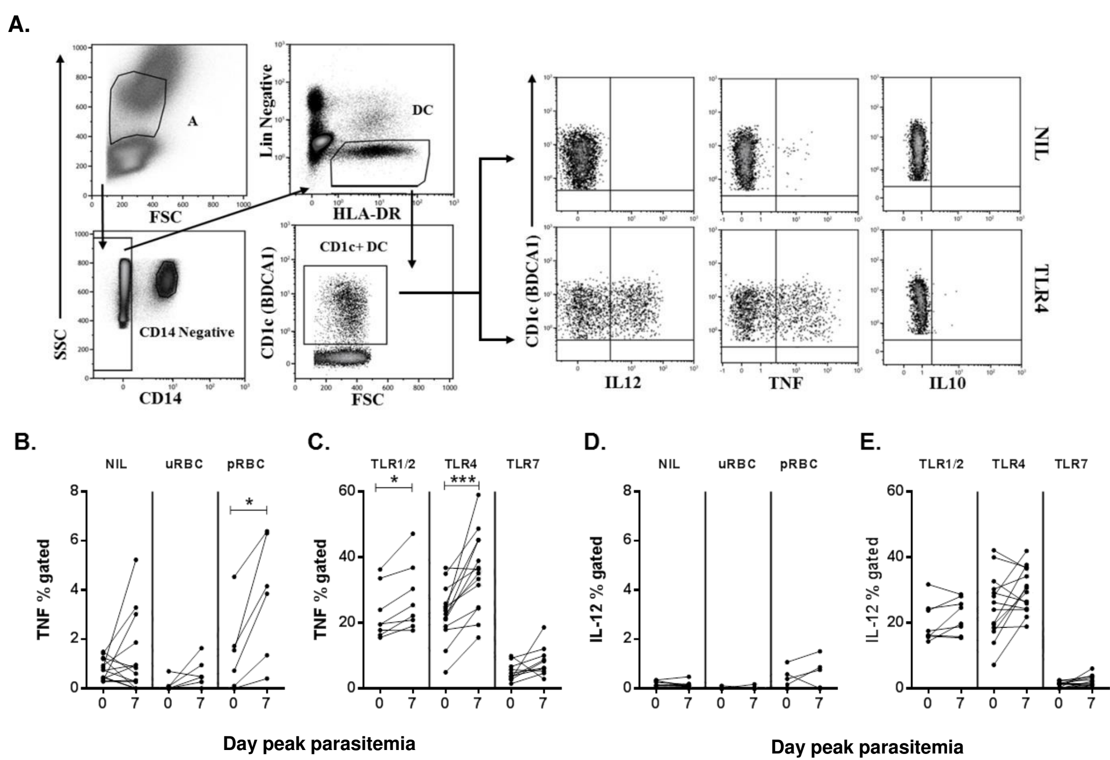

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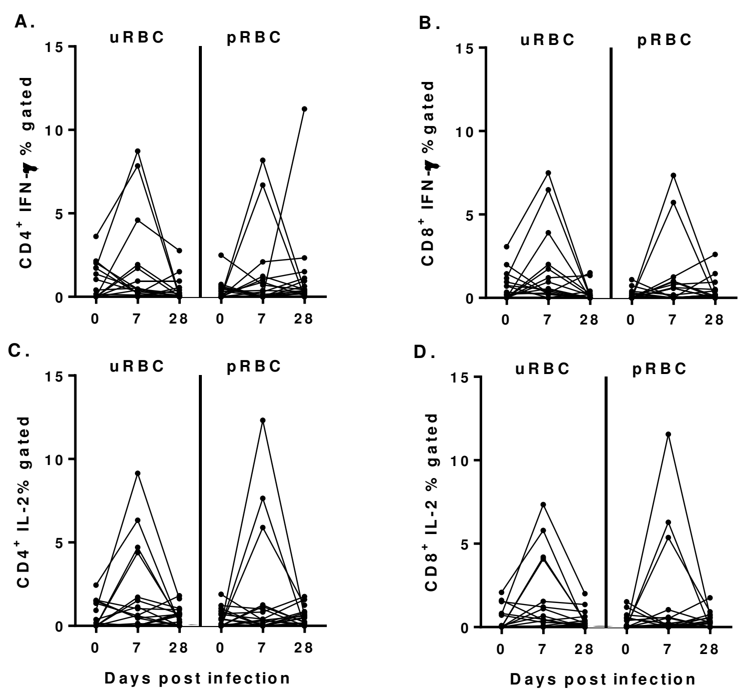

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