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Ebola virus glycoprotein stimulates IL-18 dependent natural killer cell responses

Citation for published version:

Wagstaffe, HR, Clutterbuck, EA, Bockstal, V, Stoop, JN, Luhn, K, Douoguih, M, Shukarev, G, Snape, MD, Pollard, AJ, Riley, E & Goodier, MR 2020, 'Ebola virus glycoprotein stimulates IL-18 dependent natural killer cell responses', *Journal of Clinical Investigation*, vol. 130, no. 7, pp. 3936-3946.
<https://doi.org/10.1172/JCI132438>

Digital Object Identifier (DOI):

[10.1172/JCI132438](https://doi.org/10.1172/JCI132438)

Link:

[Link to publication record in Edinburgh Research Explorer](#)

Document Version:

Publisher's PDF, also known as Version of record

Published In:

Journal of Clinical Investigation

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1 **Ebola Virus Glycoprotein Stimulates IL-18 Dependent Natural Killer Cell**
2 **Responses**

3
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23 **Conflict of interest statement**

24 VB, JNS, KL, MD, GS are employees and potential stockholders of Janssen
25 Pharmaceuticals Inc. AJP chairs the UK Department of Health and Social Care's
26 (DHCSC) Joint Committee on Vaccination and Immunisation and the EMA Scientific
27 Advisory Group on vaccines, and he is a member of WHO's Strategic Advisory Group
28 of Experts. The views expressed in the publication are those of the author(s) and not
29 necessarily those of the DHSC, NIHR or WHO. MDS acts as an Investigator on behalf
30 of the University of Oxford on clinical research studies funded by vaccine
31 manufacturers including Janssen, Pfizer, GlaxoSmithKline, Novavax, Medimmune
32 and MCM. He receives no personal financial benefit for this work.

33

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35 for this manuscript.

36 **Abstract**

37 Background

38 NK cells are activated by innate cytokines and viral ligands to kill virus-infected cells;
39 these functions are enhanced during secondary immune responses and after
40 vaccination by synergy with effector T cells and virus-specific antibodies. In human
41 Ebola virus infection, clinical outcome is strongly associated with the initial innate
42 cytokine response, but the role of NK cells has not been thoroughly examined.

43 Methods

44 The novel 2-dose heterologous Adenovirus type 26.ZEBOV (Ad26.ZEBOV) and
45 modified vaccinia Ankara-BN-Filo (MVA-BN-Filo) vaccine regimen is safe and
46 provides specific immunity against Ebola glycoprotein, and is currently in phase 2 and
47 3 studies. Here, we analysed NK cell phenotype and function in response to
48 Ad26.ZEBOV, MVA-BN-Filo vaccination regimen, and in response to in vitro Ebola
49 glycoprotein stimulation of PBMC isolated before and after vaccination.

50 Results

51 We show enhanced NK cell proliferation and activation after vaccination compared
52 with baseline. Ebola glycoprotein-induced activation of NK cells was dependent on
53 accessory cells and TLR-4-dependent innate cytokine secretion (predominantly from
54 CD14⁺ monocytes) and enriched within less differentiated NK cell subsets. Optimal
55 NK cell responses were dependent on IL-18 and IL-12, whilst IFN- γ secretion was
56 restricted by high concentrations of IL-10.

57 Conclusion

58 This study demonstrates the induction of NK cell effector functions early after
59 Ad26.ZEBOV, MVA-BN-Filo vaccination and provides a mechanism for the activation
60 and regulation of NK cells by Ebola GP.

61 Trial registration

62 ClinicalTrials.gov Identifier: NCT02313077

63 Funding

64 U.K. Medical Research Council Studentship in Vaccine Research, Innovative
65 Medicines Initiative 2 Joint Undertaking, EBOVAC (Grant 115861) and Crucell Holland
66 (now Janssen Vaccines & Prevention B.V.), European Union's Horizon 2020 research
67 and innovation programme and European Federation of Pharmaceutical Industries
68 and Associations (EFPIA).

69 **Introduction**

70 Ebola virus infection causes a rapid onset, severe acute haemorrhagic fever (Ebola
71 virus disease, EVD) with mortality ranging from 25% to 90% depending on the
72 outbreak (1). Clinical development of effective vaccines remains a high priority as
73 regular disease outbreaks continue on the African continent, and there is still no
74 licensed product. Ebola vaccine development has focused on the viral glycoprotein
75 (GP), the only protein exposed on the surface of the mature virus particle; Ebola virus
76 GP is essential for viral entry into host cells and is highly immunogenic (2, 3). Studies
77 of a GP expressing recombinant vesicular stomatitis virus (rVSV) vaccine have shown
78 that immunity directed against this protein confers protection (4). A 2-dose vaccination
79 approach with adenovirus type 26 expressing the Zaire Ebola virus GP (Ad26.ZEBOV)
80 and modified vaccinia Ankara expressing ZEBOV, Sudan Ebola virus and Marburg
81 virus GP and Tai Forest Ebola virus nucleoprotein (MVA-BN-Filo), has been shown to
82 be safe and immunogenic in phase 1 clinical trials, eliciting robust and persistent
83 antibody concentrations and antigen specific T cell responses (5-9). The
84 Ad26.ZEBOV, MVA-BN-Filo vaccine regimen is currently being evaluated in Phase 2
85 and 3 clinical studies.

86 Innate immune dysregulation underlies the pathophysiology of EVD resulting in failure
87 to activate essential effector cell functions and consequent uncontrolled virus
88 replication, systemic virus dissemination and inflammation (2, 10). Ebola virus infects
89 macrophages and DCs impairing maturation and the type I IFN response due in part
90 to the presence of interferon inhibiting domains (IIDs) within viral proteins, VP24 and
91 VP35. In vitro studies with human peripheral blood mononuclear cells (PBMC) have
92 shown that DC maturation, type I IFN secretion and NK cell activation are all enhanced
93 when these Ebola virus IIDs are mutated (11, 12). Impairment of the type I IFN

94 response is accompanied by an excessive pro-inflammatory cytokine response (2, 13).
95 In vitro studies have shown that the Ebola virus GP is a potent ligand for TLR-4 and
96 induces activation of non-infected monocytic cell lines, monocyte-derived DCs and
97 macrophages to produce cytokines (14-18). Importantly, an initial type I IFN response
98 accompanied by modest and transient IL-1 β and TNF- α secretion correlated with
99 survival among EVD patients, whereas high IL-10 was associated with fatal outcome
100 (13, 19, 20). This indicates that the earliest interactions between the Ebola virus and
101 the host immune system are critical for determining the outcome of infection.

102 Non-clinical studies have suggested that, if they can be appropriately activated, NK
103 cells may potentially play a role in vaccine-induced protection from EVD. For example,
104 murine infection with Ebola virus fails to induce an NK cell response, whereas
105 treatment of mice with Ebola GP virus-like particles (VLPs) confers complete
106 protection against a lethal Ebola virus infection just 3 days later; this protection was
107 lacking after in vivo NK cell ablation (10). Furthermore, NK cell cytotoxicity and IFN- γ
108 secretion have been implicated in the prolonged survival of NK cell-sufficient mice
109 immunised with the rVSV-vectored Ebola virus GP vaccine compared with NK cell-
110 depleted mice (21). In humans, upregulation of the activation markers NKG2D and
111 CD38 on NK cells was noted within 24 hours of vaccination with the rVSV-ZEBOV
112 vaccine (22). When taken together with evidence from non-human primates of partial
113 protection against live virus within 3 days of vaccination and full protection within 7
114 days, this suggests that NK cells may be able to mediate rapid and effective protection
115 against Ebola virus (4, 23). Moreover, after vaccination, NK cells may synergise with
116 anti-GP antibodies to clear virus-infected cells via antibody-dependent cellular
117 cytotoxicity (ADCC) (24, 25).

118 Here, we evaluate the effect of the 2-dose Ad26.ZEBOV, MVA-BN-Filo vaccination
119 regimen on accessory cell cytokine secretion, NK cell phenotype and NK cell effector
120 function both ex vivo and in response to restimulation in vitro with soluble Ebola virus
121 GP (EBOV GP). We find that vaccination with Ad26.ZEBOV, MVA-BN-Filo induces
122 proliferation and activation of less differentiated NK cell subsets as measured ex vivo.
123 We also find that stimulation of PBMC (collected either before or after vaccination)
124 with EBOV GP induces TLR-4 dependent secretion of high concentrations of
125 inflammatory cytokines, mainly from CD14⁺ monocytes and accessory cell-dependent
126 NK cell activation. EBOV GP induced NK cell activation was inhibited by neutralising
127 antibodies to IL-18 (and IL-12) and was enhanced by IL-10 receptor blockade. These
128 studies further our understanding of innate immune responses to Ebola virus GP
129 stimulation and suggest NK cells could potentially play a role in early Ad26.ZEBOV,
130 MVA-BN-Filo vaccine regimen-induced immune responses.

131 **Results**

132 **Robust NK cell responses to Ad26.ZEBOV, MVA-BN-Filo vaccination regimen**
133 **measured ex vivo.**

134 Vaccination with several anti-viral vaccines, including influenza, has been shown to
135 promote NK cell activation and a realignment of subsets associated with functional
136 differentiation (26-28). We therefore analysed the effect of Ad26.ZEBOV, MVA-BN-
137 Filo vaccination on NK cell activation and subset distribution. Ex vivo flow cytometric
138 analysis of CD56⁺CD3⁻ NK cells from pre-vaccination (visit 0), post-dose 1 (visit 1) and
139 post-dose 2 (visit 2) samples was performed. NK cells were divided into CD56^{bright},
140 CD56^{dim}CD57⁻ and CD56^{dim}CD57⁺ (or total CD56^{dim}) subsets (CD56^{bright} representing
141 the least differentiated and CD56^{dim}CD57⁺ the most differentiated subset) (29). The
142 expression of Ki67 (a cell cycle marker of proliferation), IL-2R α -chain (CD25, a
143 component of the IL-2R complex and marker of activation) and NK cell receptors
144 NKG2A and NKG2C was analysed for each subset (the flow cytometry gating
145 strategies are shown in Supplementary Figure 1a). Initially, samples from all five
146 vaccination groups (groups 1 and 2; MVA-BN-Filo on day 1 and Ad26.ZEBOV on
147 either day 29 or 57 respectively, groups 3, 4 and 5; Ad26.ZEBOV on day 1 and MVA-
148 BN-Filo on days 29, 57 or 15 respectively) were pooled for analysis.

149 When data for all vaccination groups were combined, there was a significant increase
150 in the representation of CD56^{bright} NK cells within total NK cells and a corresponding
151 decrease in the frequency of CD56^{dim} NK cells across vaccination visits (Figure 1a).
152 CD56^{bright} NK cells had the highest intrinsic capacity to proliferate, reflected in the
153 higher percentage expression of Ki67 in this subset (Figure 1b), followed by
154 CD56^{dim}CD57⁻ cells. There was a significant increase in the frequency of CD56^{bright}

155 Ki67⁺ and CD56^{dim}CD57⁻ Ki67⁺ NK cells between visit 1 and visit 2, suggesting that
156 proliferation of less differentiated NK cells may explain their increasing frequency (as
157 in Figure 1a). There was no significant change in the proportion of more highly
158 differentiated (CD56^{dim}CD57⁺) NK cells expressing Ki67 (Figure 1b).

159 Consistent with the expression of the inhibitory receptor NKG2A on less differentiated
160 NK cell subsets, a significant increase in frequency of NK cells expressing NKG2A
161 was observed at visit 2, with no significant change in expression of the corresponding
162 activating receptor, NKG2C (Figure 1c). There was a small but significant increase
163 between visits 1 and 2 in the percentage of CD56^{dim} (but not CD56^{bright}) NK cells
164 expressing CD25 (median 0.73% at visit 1; 0.86% at visit 2) (Figure 1d). The proportion
165 of CD25⁺ NK cells was positively correlated with the frequency of proliferating (Ki67⁺)
166 NK cells 21 days post-dose 2, further suggesting an association between NK cell
167 activation and proliferation in response to vaccination (Figure 1e). No effect of
168 vaccination was observed on the percentage or mean fluorescence intensity (MFI) of
169 NK cells expressing CD16 (the low affinity IgG receptor III, FcγRIII) (Supplementary
170 Figure 1b). These data indicate proliferation of less differentiated NK cells in response
171 to Ad26.ZEBOV, MVA-BN-Filo vaccination.

172 Overall, no significant changes in ex vivo NK cell phenotype and function were
173 observed after the primary vaccination but significant NK cell proliferation and CD25
174 expression were observed after the secondary vaccination but with a diversity of
175 responses among individuals. To investigate any effects of the order and/or interval of
176 the 2 doses, NK cell responses were reanalysed by vaccination group. Increasing
177 CD56^{bright} and decreasing CD56^{dim} NK cell frequencies after vaccination was indicated
178 by a trend in all groups except group 4 (Ad26.ZEBOV followed by MVA-BN-Filo at day
179 57) and reached significance by one-way ANOVA across vaccination visits in groups

180 3 and 5 only (Ad26.ZEBOV followed by MVA-BN-Filo at day 29 and 15 respectively)
181 (Supplementary Figure 2a, b). Furthermore, there was a significant increase in
182 CD56^{bright} Ki67⁺ and CD56^{dim} CD25⁺ NK cells between baseline and post-dose 2 in
183 group 4 only (Supplementary Figure 2c, d). These data suggest that the Ad26.ZEBOV,
184 MVA-BN-Filo vaccine regimen induced a more robust NK cell response than MVA-BN-
185 Filo, Ad26.ZEBOV regimen. However, these effects were small and this subgroup
186 analysis may lack statistical power due to small numbers of participants.

187

188 **NK cell CD107a and CD25, but not IFN- γ upregulation in response to EBOV GP**
189 **stimulation in vitro.**

190 To determine the effect of Ad26.ZEBOV, MVA-BN-Filo vaccination regimen on NK cell
191 responses to soluble EBOV GP, baseline, visit 1 and visit 2 PBMCs were cultured for
192 8 and 18 hours with 10 μ g/ml EBOV GP. Frequencies of NK cells expressing CD107a
193 and IFN- γ (at 8 hours) or CD25 and CD16 (at 18 hours) were analysed by flow
194 cytometry (gating strategies are shown in Figure 2a). There were no significant
195 differences in response to EBOV GP between vaccination groups (Supplementary
196 Figure 2e-g), therefore, all five vaccination groups were combined for analysis. In vitro
197 stimulation with EBOV GP induced a significant increase in the proportion of NK cells
198 expressing CD107a (Figure 2b) and CD25 (Figure 2c) at the cell surface compared
199 with unstimulated cultures (medium alone). EBOV GP stimulation had no effect on NK
200 cell IFN- γ (at 8 or 18 hours) or CD16 expression (Figure 2d, e). The effect of EBOV
201 GP on markers of NK cell function did not differ across vaccination visits (Figure 2b-e)
202 suggesting the effect of EBOV GP on NK cells is independent of vaccine-induced T
203 cell and antibody responses.

204 Given that there was no effect of vaccination on the NK response to EBOV GP, the
205 analysis of NK cell function by differentiation subset was restricted to the baseline data
206 set (Figure 3). This analysis revealed that IFN- γ secretion was restricted to the less
207 differentiated CD56^{bright} and CD56^{dim}CD57⁻ subsets and that significant induction of
208 IFN- γ by EBOV GP was detected only within the CD56^{dim}CD57⁻ subset (Figure 3a).
209 By contrast, CD107a and CD25 upregulation in response to EBOV GP was seen in all
210 NK cell subsets (Figure 3b, c), with a significantly higher CD25 expression in the
211 CD56^{bright} subset compared with CD56^{dim} subsets (Figure 3c). The majority of CD25⁺
212 NK cell events were CD56^{dim}CD57⁻ (60.5%) (Figure 3d). Overall, these data
213 demonstrate that EBOV GP induces markers associated with NK cell cytotoxicity
214 (CD107a) and activation (CD25), with a much lesser impact on IFN- γ secretion, and
215 that these responses are not enhanced by vaccination.

216

217 **High concentrations of inflammatory cytokines induced by EBOV GP in vitro.**

218 NK cells are able to respond to cytokines secreted from activated accessory cells in
219 response to viral stimuli. To quantify cytokine production in response to EBOV GP
220 stimulation, baseline and 21-day post-dose 2 vaccination PBMC samples were
221 stimulated with EBOV GP in vitro for 18 hours and cytokine concentrations in cell
222 supernatants were measured by Luminex. EBOV GP induced secretion of high
223 concentrations of IL-10, IL-1 β , IFN- α 2, GM-CSF, TNF- α and IFN- γ from PBMCs at
224 baseline and post-dose 2 samples compared with medium alone, where minimal
225 concentrations were observed (Figure 4). Particularly high concentrations of IL-10
226 (median 3142pg/ml at baseline), IL-1 β (median 1299pg/ml at baseline), GM-CSF
227 (median 465pg/ml at baseline) and TNF- α (median 5480pg/ml at baseline) were

228 measured in response to EBOV GP (Figure 4a, b, d, e). IFN- α 2 secretion was also
229 significantly enhanced by EBOV GP however the absolute concentrations of this
230 cytokine were low (median 6.1pg/ml at baseline) compared with the other myeloid cell-
231 derived cytokines (Figure 4c). Similarly, a low concentration of IL-12(p70) (maximum
232 6.6pg/ml) was detectable by Luminex in only a small number of individuals (13 of 71
233 at baseline and 9 of 71 at post-dose 2; not shown). Conversely, there was no increase
234 in IP-10 secretion over medium alone and IL-15 was not detected (not shown).

235 With the exception of a small but significant reduction in EBOV GP-induced TNF- α
236 concentration in cultures of post-dose 2 PBMCs (4555pg/ml post-dose 2; 5480pg/ml
237 at baseline) (Figure 4e), there was no overall effect of vaccination on cytokine
238 concentrations. When vaccination groups were analysed separately, concentrations
239 of GM-CSF in group 3, IFN- α 2 in group 4 and TNF- α in group 5 were significantly
240 reduced at visit 2 compared with baseline (Supplementary Figure 3c, d, e) suggesting
241 that reductions in cytokine responses were limited to Ad26.ZEBOV, MVA-BN-Filo
242 vaccine regimen. In summary, EBOV GP stimulated the release of high concentrations
243 of IL-10, IL-1 β , GM-CSF and TNF- α from PBMCs, indicative of myeloid cell activation,
244 with lower concentrations of IFN- α 2, IL-12 and IFN- γ detected.

245

246 **Myeloid accessory cell cytokine-dependent NK cell activation.**

247 Vaccination independent activation of less differentiated, cytokine-responsive NK cell
248 subsets accompanied by high levels of myeloid cell-derived cytokine secretion, led us
249 to hypothesise that the NK cell response to EBOV GP is a function of indirect NK cell
250 activation. To test this hypothesis, we compared IFN- γ , CD107a and CD25 expression
251 in response to EBOV GP among PBMCs, purified NK cells and purified NK cells in the

252 presence of a 1:1 ratio of CD14⁺ monocyte-enriched cells from healthy (non-
253 vaccinated) control subjects (Figure 5a-c). Expression of CD107a, IFN- γ and CD25 in
254 the CD56^{bright} NK cell population (in which significant induction was measured) were
255 determined by flow cytometry as before. IFN- γ , CD107a and CD25 expression was
256 significantly reduced in purified NK cells compared with whole PBMC suggesting that
257 accessory cell-derived stimuli are required for optimal NK cell responses to EBOV GP
258 (Figure 5a-c). CD107a and CD25 responses were recovered in all individuals after
259 adding back the enriched CD14⁺ monocyte fraction (Figure 5b-c), suggesting this
260 population of cells supports NK cell function after EBOV GP stimulation; NK cell IFN-
261 γ expression was not consistently recovered after adding back CD14⁺ cells (Figure
262 5a).

263 To determine the precise nature of the accessory cell-dependent stimuli that drive NK
264 cell responses to EBOV GP, whole PBMCs from (non-vaccinated) control subjects
265 were stimulated with EBOV GP in the presence of neutralising antibodies to IL-2, IL-
266 12, IL-15, IL-18 and IFN- $\alpha\beta$ R2. The blockade of IL-18 significantly reduced the
267 frequency and MFI of NK cell CD25 expression (Figure 5d, e, Supplementary Figure
268 4a), with blockade of IL-12 also significantly reducing CD25 expression within the
269 CD56^{bright} NK cell subset (Figure 5f, g). CD107a expression was also impaired by IL-
270 18 blockade, reflected in the CD56^{bright} and CD56^{dim}CD57⁻ subsets (Figure 5h,
271 Supplementary Figure 4a). There was no effect of IL-12 or IL-18 blockade on NK cell
272 IFN- γ expression (Figure 5i, Supplementary Figure 4a). Conversely, neutralisation of
273 IL-2 or IL-15, or IFN- $\alpha\beta$ R2 blockade, had no significant effect on NK cell activation in
274 any NK cell subset (not shown). In summary, these data suggest optimal NK cell CD25
275 and CD107a expression in response to EBOV GP stimulation is dependent on myeloid
276 cell-derived IL-18 and IL-12.

277 As both IL-12 and IL-18 were not amenable to detection by Luminex assay of cell
278 culture supernatants, we next sought to measure these responses to EBOV GP using
279 high sensitivity ELISA for secreted IL-18 and flow cytometry for intracellular IL-12
280 (gating strategy shown in Supplementary Figure 5a). There was a significant increase
281 in IL-18 measured in supernatant after 18 hours stimulation with EBOV GP (median
282 47.6pg/ml, range 16.8-183.5pg/ml) (Figure 5j), which correlated significantly with
283 increasing NK cell CD25 expression (Figure 5k). We were able to detect IL-12(p40)⁺
284 cells by flow cytometry with significantly higher frequencies of IL-12(p40)⁺ cells in
285 CD14⁻CD11c⁺ myeloid DCs (mDC) (30), total CD14⁻ cells and CD14⁺ monocytes
286 compared with medium alone. The highest frequencies of IL-12(p40)⁺ cells were
287 observed in the CD14⁺ monocyte population (0.22%) (Figure 5L), consistent with the
288 recovery of NK cell CD107a and CD25 responses by purified NK cells in the presence
289 of this cell population.

290

291 **Regulation of NK cell IFN- γ production by EBOV GP induced IL-10.**

292 IL-10 is an essential immunoregulatory cytokine that is typically upregulated in
293 response to inflammation (31). Having detected very high concentrations of IL-10 in
294 supernatants of EBOV GP-stimulated PBMCs (Figure 4a) we explored the relationship
295 between IL-10 production and NK cell function. NK cell IFN- γ expression significantly
296 negatively correlated with IL-10 secretion in 18-hour cultures in both baseline ($r=-$
297 0.331, $p=0.0218$) (Figure 6a) and 21-day post-dose 2 PBMC ($r=-0.324$, $p=0.0157$; not
298 shown) suggesting that IL-10 induced by EBOV GP might restrict the NK cell IFN- γ
299 response. Therefore, PBMC from (non-vaccinated) control subjects were cultured for
300 18 hours with EBOV GP in the presence of a blocking monoclonal antibody to the IL-

301 10 receptor (IL-10R) or the appropriate isotype control antibody. IL-10R blockade
302 resulted in significantly higher frequencies of IFN- γ ⁺ (Figure 6b) and CD25⁺ (Figure
303 6c) NK cells (and a significant increase in CD25 MFI; median 349.5 with IL-10R
304 blockade; 110.5 with isotype control; p=0.0002; not shown) compared with isotype
305 control treated cultures. CD107a was significantly enhanced by IL-10R blockade in the
306 CD56^{dim}CD57⁺ NK cell subset only (Supplementary Figure 4b), and IL-10R blockade
307 particularly enhanced IFN- γ responses in CD56^{bright} and CD56^{dim}CD57⁻ NK cell
308 populations (Supplementary Figure 4b).

309 We also investigated whether serum components, such as IL-18 binding proteins, may
310 restrict IL-18-dependent responses to EBOV GP in some individuals. Overall, in vitro
311 NK cell responses to EBOV GP were minimally affected by high concentrations of pre
312 or post-Ad26.ZEBOV, MVA-BN-Filo vaccination serum (up to concentrations of 25%
313 v/v) except CD25 expression was partially inhibited in the CD56^{bright} NK cell population
314 (Supplementary Figure 6a-d). In contrast, induction of CD25 by exogenous IL-18 was
315 almost fully inhibited in the presence of high concentrations of serum, consistent with
316 a potential role for IL-18BP in limiting the activity of IL-18. However, NK cell activation
317 by a cocktail of IL-18 and IL-12 was only partially inhibited at high serum concentration
318 (Supplementary Figure 6f, g).

319 To determine the cellular source of the cytokines induced by EBOV GP, PBMC were
320 cultured with EBOV GP for 18 hours, stained for intracellular IL-10, GM-CSF and TNF-
321 α and analysed by flow cytometry (gating strategy shown in Supplementary Figure
322 5a). IL-10 was expressed predominantly in CD14⁺ monocytes (median 6.0%) with little
323 or no evidence of expression in B cells, mDCs, CD14⁻, NK cells or T cells (Figure 6e).
324 Back-gating confirmed that the majority of IL-10⁺ cells were CD19⁻CD14⁺ monocytes
325 (Figure 6f) which is consistent with the lack of recovery of IFN- γ responses in purified

326 NK cells co-cultured with CD14⁺ monocytes (Figure 5a). GM-CSF expression was also
327 essentially restricted to monocytes whereas the frequencies of TNF- α was similar in
328 mDCs and monocytes (Supplementary Figure 5b, c). In summary, monocytes are the
329 predominant source of inflammatory cytokines in response to EBOV GP in primary
330 peripheral blood and monocyte-derived IL-10 negatively regulates NK cell IFN- γ
331 secretion and CD25 expression. This immediate, robust IL-10 response could
332 potentially explain the lack of IFN- γ expression by NK cells in response to EBOV GP
333 both before and after vaccination (Figure 2).

334

335 **EBOV GP-induced NK cell activation is TLR-4 dependent.**

336 EBOV GP stimulates cytokine secretion in human monocytic cell lines and in vitro
337 generated monocyte-derived DCs and macrophages in a TLR-4-dependent fashion
338 (14-17). TLR-4 is expressed at high levels on human peripheral blood monocytes, as
339 well as other myeloid lineage cells including macrophages and granulocytes (32). We
340 therefore assessed the effect of blocking TLR-4 on cytokine secretion (measured by
341 Luminex) and NK cell activation (by flow cytometry) in response to EBOV GP within
342 PBMC from (non-vaccinated) control subjects. TLR-4 blockade significantly reduced
343 secretion of IL-10 (0.3 fold-reduction; 7 of 7 donors) (Figure 7a), IL-1 β , GM-CSF and
344 IFN- γ but had no overall effect on IFN- α 2 or TNF- α secretion (Figure 7b). Parallel
345 effects were observed among NK cells where there was a partial, but significant,
346 decrease in frequencies of IFN- γ ⁺ (median 49.6% decrease in frequency) and CD25⁺
347 (median 14.6% decrease in frequency) CD56^{bright} NK cells in the presence of TLR-4
348 blocking antibodies (Figure 7c, d). Overall, these data indicate that NK cell activation

349 by EBOV GP is mediated, at least in part, via ligation of TLR-4 on primary human
350 monocytes and the induction of cytokines.

351 **Discussion**

352 In the 2014-2016 Ebola virus outbreak in West Africa, almost 30,000 cases of EVD
353 were reported with more than 11,000 deaths (33). In 2019, Ebola virus continues to
354 be a considerable global health concern, with the second largest outbreak on record
355 currently ongoing in the Democratic Republic of the Congo (34). Detailed
356 understanding of the immune response to Ebola virus infection and mechanisms of
357 protection induced by Ebola virus vaccines would assist in efforts of prevention and
358 containment of future outbreaks. We analysed the effect of the heterologous 2-dose
359 Ad26.ZEBOV, MVA-BN-Filo vaccine regimen on human NK cell phenotype ex vivo
360 and primary human innate cell function in response to soluble EBOV GP in vitro. We
361 demonstrate NK cell activation, proliferation and expansion of less differentiated NK
362 cells and found that, independently of vaccination, CD14⁺ monocytes are key
363 responders to Ebola virus GP, rapidly producing a range of inflammatory cytokines in
364 a manner that is partially dependent on TLR-4. Subsequent NK cell activation and
365 function, dependent on myeloid cell-derived IL-12 and IL-18, was almost completely
366 abrogated by the very high levels of IL-10 secreted as part of the acute myeloid cell
367 response to EBOV GP in vitro.

368 Activation and proliferation of NK cells after vaccination has been demonstrated with
369 both inactivated and live attenuated vaccines. Jost et al demonstrated upregulation of
370 CD69 and CD25 and increased numbers of CD56^{bright} NK cells at day 4 post-influenza
371 vaccination (26) and Marquardt et al. observed heightened NK cell Ki67 expression
372 (peaking at day 10) after yellow fever vaccination (27). We have previously
373 demonstrated increased percentages and proliferation of CD56^{bright} NK cells at day 3
374 and up to 4 weeks after influenza vaccination (28). Our ex vivo data demonstrate
375 activation of less differentiated NK cells by a vectored, Ebola GP-expressing vaccine.

376 We detected heightened CD56^{bright} NK cell proliferation up to 78 days after first
377 vaccination (21 days post-dose 2) and an increase in the proportion of CD56^{bright} NK
378 cells from as early as day 15 post-dose 1 until at least 21 days post-dose 2. Increased
379 expression of CD25 by NK cells post-vaccination may indicate the potential for T cell
380 derived IL-2 to contribute to NK cell proliferation and activation (28, 35, 36).

381 The pathogenesis of EVD is closely linked to the very high levels of pro-inflammatory
382 cytokines induced by the infection (13, 19, 20). We show for the first time within primary
383 human PBMC cultures, that Ebola GP stimulated the secretion of high levels of IL-1 β ,
384 GM-CSF and TNF- α independently of vaccination. This inflammatory response was
385 accompanied by an equally rapid and potent IL-10 response and somewhat lower
386 levels of IL-12, IL-18 and IFN- α 2. These data - in a highly relevant *ex vivo* system –
387 corroborate previous observations from human cell lines and *in vitro* generated
388 monocyte-derived DCs and macrophages (11, 14, 16, 18). The relatively low levels of
389 NK cell and T cell-activating cytokines together with the abundance of IL-10 suggest
390 the generation of a tightly regulated cytokine environment within hours of exposure to
391 soluble EBOV GP. Rapid production of IL-10 in response to a potent pro-inflammatory
392 stimulus is a well-described feature of the human homeostatic response; in preventing
393 a life-threatening cytokine storm, this can also influence the emerging adaptive
394 response (31). Indeed, pro and anti-inflammatory cytokines both indirectly correlate
395 with survival after EVD indicating that IL-10 itself, although associated with anti-
396 inflammatory properties does not predict protection from disease (13).

397 Innate, pro-inflammatory cytokine responses are also regulated by specific cytokine-
398 binding serum proteins, including IL-18 binding protein (IL-18bBP) (37). We observed
399 that high concentrations (up to 25% v/v) of serum (pre- or post-vaccination) inhibited
400 the NK cell CD25 response to rIL-18 (as expected) but had rather little effect on the

401 response to cytokine cocktails (e.g. rIL-18 plus rIL-12) or EBOV GP suggesting that
402 while IL-18BP may limit the effects of IL-18 it may have less impact on the much lower,
403 synergistic, combinations of cytokines induced by, for example, a viral infection or on
404 the cell-contact mediated events at the NK cell-monocyte synapse. Additionally, our
405 data demonstrate reduction of CD25 and degranulation responses in post-dose 2
406 vaccination serum compared with pre-vaccination serum in some individual vaccinees,
407 consistent with a potential role for vaccine-induced antibody in blocking EBOV GP-
408 TLR-4 interactions at higher serum concentrations.

409 CD14⁺ monocytes were the main source of both inflammatory and anti-inflammatory
410 cytokines within hours of EBOV GP stimulation. Both types of monocyte response and
411 the downstream NK cell response were TLR-4-dependent confirming prior studies
412 showing Ebola virus GP is recognised by TLR-4 inducing inflammatory cytokine
413 secretion (14, 16, 17, 38). We demonstrate indirect, innate cytokine-dependent NK
414 cell effector function in response to Ebola virus GP in human PBMC in vitro culture,
415 independent of prior Ad26.ZEBOV, MVA-BN-Filo vaccination. IL-18 and to a lesser
416 extent, IL-12, from myeloid accessory cells were required for optimal NK cell
417 degranulation and CD25 upregulation. This innate response, which is particularly
418 enriched in less differentiated NK cell subsets, is consistent with the proliferation and
419 activation of the least differentiated, CD56^{bright} NK cells after vaccination itself
420 (measured ex vivo). This suggests that, as seen in vitro, expression of Ebola GP by
421 vaccination could potentially stimulate innate, cytokine-dependent NK cell activation
422 in vivo.

423 Innate NK cell activation in response to EBOV GP, with an apparent lack of
424 enhancement of NK cell responses post-vaccination, is in complete contrast to
425 previous observations with yellow fever, BCG and influenza vaccination (27, 28, 39).

426 It is well established that enhanced NK cell responses after vaccination, are mediated
427 in part by IL-2 from antigen-specific T cells and vaccine induced antibody (27, 28, 35,
428 39-41). Despite evidence of moderate induction of IL-2⁺IFN- γ ⁺TNF- α ⁺ triple positive T
429 cells and the presence of 1% post-Ad26.ZEBOV, MVA-BN-Filo vaccination serum (5),
430 there was no enhancement of the NK cell response, or downregulation of CD16 in
431 response to EBOV GP post-vaccination compared with baseline. Plausibly, the lack of
432 post-vaccination NK cell enhancement in vitro may be linked to the effects of
433 monocyte-derived IL-10. A system-wide analysis of the immune response to the rVSV-
434 ZEBOV Ebola vaccine suggested negative regulation by inflammatory monocytes
435 (22), additionally, IL-10 blockade restored antigen-specific T cell-derived IL-2-
436 dependent activation of NK cells in other viral infection models (42, 43).

437 In summary, we have characterised the NK cell response to the novel 2-dose
438 Ad26.ZEBOV, MVA-BN-Filo vaccination regimen. We also demonstrate that the
439 robust TLR-4-dependent, monocyte-derived, innate cytokine response to Ebola GP
440 both stimulates and regulates the NK cell effector response. This study contributes to
441 our understanding of immune responses induced by Ebola vaccines and demonstrates
442 that innate cytokine responses induced by Ebola GP may be integral to the induction
443 and regulation of NK cell function after vaccination.

444 **Materials and Methods**

445 **Study participants and samples**

446 Cryopreserved PBMCs (with corresponding serum samples) from healthy adults, aged
447 18 to 50 years (median 39 years), were obtained from participants enrolled in the
448 EBL1001 single-centre, randomised, placebo-controlled, observer blind trial
449 conducted in Oxford, U.K. as described (ClinicalTrials.gov Identifier: NCT02313077)
450 (5). Participants were randomised into four groups, with a fifth group subsequently
451 added by a protocol amendment, to receive the Ad26.ZEBOV, MVA-BN-Filo vaccine
452 according to one of five vaccination schedules (Table 1). The vaccine comprises
453 monovalent Ad26.ZEBOV expressing the GP of the Ebola Zaire virus (Mayinga
454 variant) (Janssen Vaccines and Prevention B.V., The Netherlands) and multivalent
455 MVA-BN-Filo expressing the GP of the Sudan and Zaire Ebola viruses and Marburg
456 virus together with Tai Forest virus nucleoprotein (Bavarian Nordic, Denmark). Groups
457 1 and 2 received MVA-BN-Filo on day 1 and Ad26.ZEBOV on either day 29 or 57
458 respectively; groups 3, 4 and 5 received Ad26.ZEBOV on day 1 and MVA-BN-Filo on
459 days 29, 57 or 15 respectively.

460 Samples from 70 donors (non-placebo arms) were obtained from pre-vaccination
461 (baseline, visit 0), post-dose 1 (day 29, 57 or 15 depending on group; visit 1) and 21
462 days post-dose 2 (day 50, 78 or 36 depending on group; visit 2) (Table 1). Human
463 cytomegalovirus (HCMV) serology was conducted on the baseline serum sample of
464 each donor by HCMV IgG ELISA (Demeditec, Kassel, Germany); 26 of 70 volunteers
465 (37%) were HCMV seropositive, 44 were HCMV seronegative and two were
466 indeterminate. Additional non-vaccinated, healthy, adult volunteers (n=16) were
467 recruited for subsequent in vitro experiments from among staff and students at the

468 London School of Hygiene and Tropical Medicine (LSHTM) using an anonymised
469 volunteer database.

470 **In vitro cellular assays**

471 Cryopreserved PBMCs were thawed, washed in RPMI 1640 supplemented with
472 100U/ml penicillin/streptomycin and 20mM L-glutamine (Gibco, ThermoFisher) and
473 rested for 2 hours. The average cell yield after thaw was 5.8×10^6 per vial (58%
474 recovery). Fresh PBMC were isolated from heparinised whole blood using Histopaque
475 1077 (Sigma-Aldrich, Gillingham, U.K.) gradient centrifugation. All cells were counted
476 using Fastread counting slides (ImmuneSystems, U.K.). Trial PBMC were stained
477 immediately ex vivo or cultured in 96-well round-bottom plates in RPMI 1640
478 supplemented as above and with 1% autologous (pre, post-dose 1 or post-dose 2)
479 serum and 10 μ g/ml purified recombinant Ebola virus GP (EBOV GP), Mayinga variant,
480 prepared in Hek293F cells (Janssen Vaccines and Prevention B.V.) for 8 and 18 hours
481 at 37°C.

482 For additional 18-hour experiments, fresh PBMC from non-trial donors were stimulated
483 with 10 μ g/ml EBOV GP or cytokines alone; IL-12; 5ng/ml (PeproTech, London, U.K.)
484 and/or IL-18; 10 or 50ng/ml (R&D Systems, Oxford, U.K.) in RPMI supplemented as
485 above and with 5% FCS, or 1, 5, or 25% pooled pre or post-vaccination serum. The
486 following blocking antibodies or isotype control antibodies were used, all at 3 μ g/ml;
487 anti-IL-2 (Becton Dickinson (BD) Biosciences, Oxford, U.K.), anti-IL-10R (Biolegend),
488 rat IgG2a isotype control (eBiosciences, ThermoFisher), anti-IL-12 (BD Biosciences),
489 anti-IL-15 (eBiosciences), anti-IL-18 (MBL, U.S.A), mouse IgG1 isotype control
490 (eBiosciences). Anti-IFN- $\alpha\beta$ R2 (Merck Millipore, Watford, U.K.) and mouse IgG2a
491 isotype control (eBiosciences) were used at a final concentration of 1 μ g/ml. In vitro

492 blockade of TLR-4 was performed in the presence of 5µg/ml anti-TLR-4 rabbit
493 polyclonal anti-sera or isotype matched control reagent with irrelevant specificity
494 (Invivogen, U.K.).

495 To determine accessory cell dependency, NK cells and CD14⁺ monocytes were
496 purified by magnetic bead separation (MACS) using NK Cell Isolation Kit (Miltenyi
497 Biotec, Germany) (NK cells 90.2%±3.2% pure) and Pan Monocyte Isolation Kit
498 (Miltenyi Biotec) (monocytes 62.8%±11% pure with less than 1% NK cell
499 contamination), respectively. Cells were cultured as above for 18 hours in 5% FCS
500 (n=5). GolgiPlug (Brefeldin A; 1/1000 final concentration; BD Biosciences) and
501 GolgiStop (Monensin; 1/1500 concentration; BD Biosciences) were added to all in vitro
502 cultures for the final 3 hours of culture. Cells were then stained with fluorophore
503 labelled antibodies for flow cytometry and culture supernatants were collected and
504 stored at -80°C for cytokine analysis by Luminex/ELISA.

505 **Flow cytometry**

506 Cells were stained for surface markers including a viability marker (Fixable Viability
507 Stain 700; BD Biosciences) in FACS buffer (PBS, 0.5% FCS, 0.05% sodium azide and
508 2mM EDTA) for 30 minutes in 96-well round bottom plates after blocking Fc receptors
509 for 5 minutes with Fc Receptor (FcR) Blocking Reagent (Miltenyi Biotec). Cells were
510 then washed in FACS buffer, fixed and permeabilised using Cytofix/Cytoperm Kit (BD
511 Biosciences) or Foxp3/Transcription Factor Fixation/Permeabilisation Kit
512 (eBiosciences) according to the manufacturer's instructions. Cells were then stained
513 for intracellular markers with FcR blocking for 20 minutes and washed again. Finally
514 cells were resuspended in FACS buffer and analysed using a BD LSRII flow
515 cytometer. Cells were acquired using FACSDiva software and data were analysed

516 using FlowJo V10 (Tree Star, Oregon, U.S.A). FACS gates were set using
517 unstimulated cells or FMO controls. Samples with less than 100 NK cell events were
518 excluded from the analysis (<4% of samples evenly distributed across all groups).

519 The following fluorophore labelled antibodies were used: anti-CD3-V500 (clone
520 UCHT1) (BD Biosciences), anti-CD56-BV605 (clone HCD56), anti-IFN- γ -BV785
521 (clone 4S.B3), anti-IFN- γ -APC (clone 4S.B3), anti-CD25-BV785 (clone BC96), anti-
522 CD11c-BV785 (clone 3.9), anti-CD14-AF700 (clone 63D3), anti-GM-CSF-PE-Dazzle
523 (clone BVD2-21C11), anti-TNF- α -FITC (clone MAb11), anti-IL-10-PE (clone JES3-
524 9D7) (all Biolegend, London, U.K.). Anti-CD16-APC (clone CB16), anti-CD25-
525 PerCPCy5.5 (clone BC96), anti-CD57-e450 (clone TB01), Ki67-PerCP-eFluor710
526 (clone 20Raj1), anti-CD19-PECy7 (clone HIB19), anti-IL-12-eFlour660 (clone C8.6)
527 (all eBiosciences), anti-NKG2A-PE-Vio770 (clone REA110) (Miltenyi Biotec), anti-
528 NKG2C-PE (clone 134591) (R&D systems). Anti-CD107a-FITC (clone H4A3) (BD
529 Biosciences) was added to the culture at 2 μ l per 100 μ l for the whole culture period.

530 **Luminex and IL-18 ELISA**

531 Concentrations of GM-CSF, IFN- α 2, IFN- γ , TNF- α , IP-10, IL-1 β , IL-10, IL-12p70, IL-
532 15 in cell culture supernatants were determined by Luminex technology (Merck
533 Millipore) using the xPONENT 4.1 software for data acquisition. The concentration of
534 IL-18 was determined by ELISA (R&D Systems).

535 **Statistics**

536 Statistical analysis was performed using GraphPad Prism version 7.04 (GraphPad,
537 California, U.S.A.). Functional responses were compared using Wilcoxon signed-rank
538 test or one-way ANOVA Friedman test with Dunn's correction for multiple
539 comparisons. For correlation analysis, a linear regression model was fitted in prism

540 and r and p values were determined using Spearman's correlation analysis.
541 Significance levels are assigned as *p, <0.05, **p, <0.01, ***p, <0.001, and ****p,
542 <0.0001 for all tests.

543 **Study approval**

544 Written informed consent was received from all participants prior to inclusion in the
545 study. The trial protocol and study documents were approved by the National
546 Research Ethics Service (reference number 14/SC/1408) and the LSHTM Research
547 Ethics Committee (reference number 14383).

548 **Author contributions**

549 HRW and MRG designed and performed the experiments, analysed data, and wrote
550 the manuscript. VB, JNS and KL participated in the analysis of data and advised on
551 the manuscript. MD and GS participated in the conception and design of the work
552 described and advised on the manuscript. AP and EAC were coinvestigators on the
553 above trial and advised on the manuscript. MDS was the Chief Investigator on the
554 phase 1 clinical trial of Ad26.ZEBOV, MVA-BN-Filo and advised on the manuscript.
555 EMR wrote and advised on the manuscript.

556 **Acknowledgements**

557 This work was supported by a U.K. Medical Research Council Studentship in Vaccine
558 Research (HRW). This project has received funding from the Innovative Medicines
559 Initiative 2 Joint Undertaking, EBOVAC (Grant 115861) and Crucell Holland (now
560 Janssen Vaccines & Prevention B.V.). This Joint Undertaking receives support from
561 the European Union's Horizon 2020 research and innovation programme and
562 European Federation of Pharmaceutical Industries and Associations (EFPIA). AJP is
563 supported by the NIHR Oxford Biomedical Research Centre and is an NIHR Senior
564 Investigator. The views expressed in the publication are those of the author(s) and not
565 necessarily those of the NHS, the NIHR, MRC or European Union. MDS is supported
566 by the NIHR Oxford Biomedical Research Centre.

567 We also thank Carolynne Stanley for recruiting and obtaining consent from LSHTM
568 study subjects and for blood sample collection.

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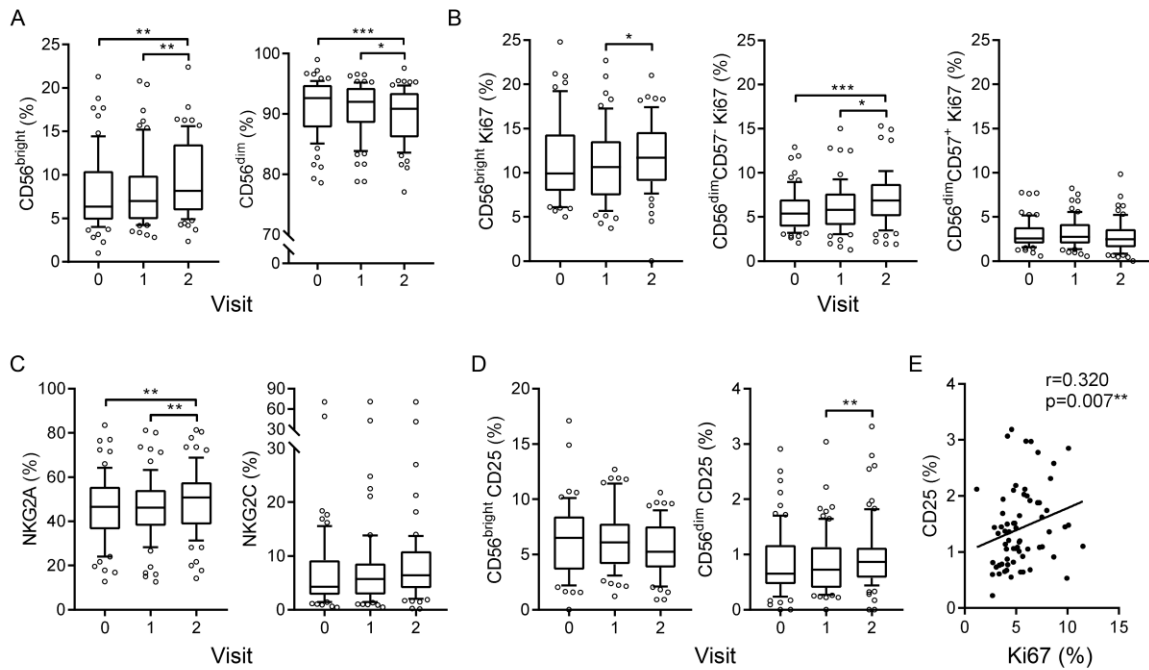
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718 **Figures and legends**

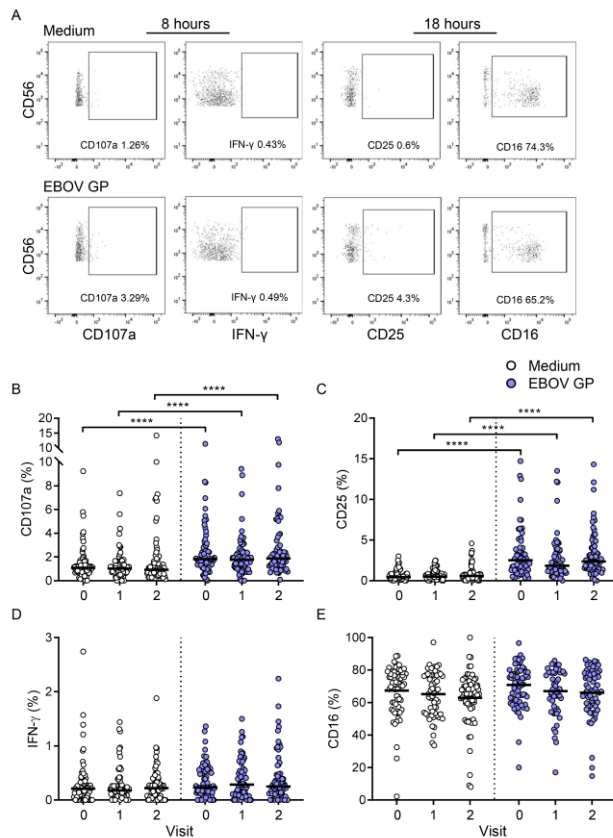


719

720 **Figure 1.** Robust NK cell responses to Ad26.ZEBOV, MVA-BN-Filo vaccination
 721 measured ex vivo.

722 NK cell phenotype at baseline (visit 0), visit 1 (day 29, 57 or 15 post-dose 1) and visit
 723 2 (21 days post-dose 2) was analysed ex vivo by flow cytometry (gating strategy is
 724 shown in Supplementary Figure 1), n=70. Frequencies of CD56^{bright} and CD56^{dim} (a),
 725 CD56^{bright} Ki67⁺, CD56^{dim}CD57⁻ Ki67⁺ and CD56^{dim}CD57⁺ Ki67⁺ (b), NKG2A⁺ and
 726 NKG2C⁺ (c), CD56^{bright} CD25⁺ and CD56^{dim} CD25⁺ NK cells (d) were determined. The
 727 correlation between total NK cell CD25 and Ki67 expression at 21 days post-dose 2
 728 (e) was also determined by Spearman's coefficient. Graphs show box and whisker
 729 plots with median, interquartile range (IQR) (box) and 10th-90th percentile (whiskers).
 730 Comparisons across vaccination visits were performed using one-way ANOVA with
 731 Dunn's correction for multiple comparisons. *p <0.05, **p <0.01, ***p <0.001.

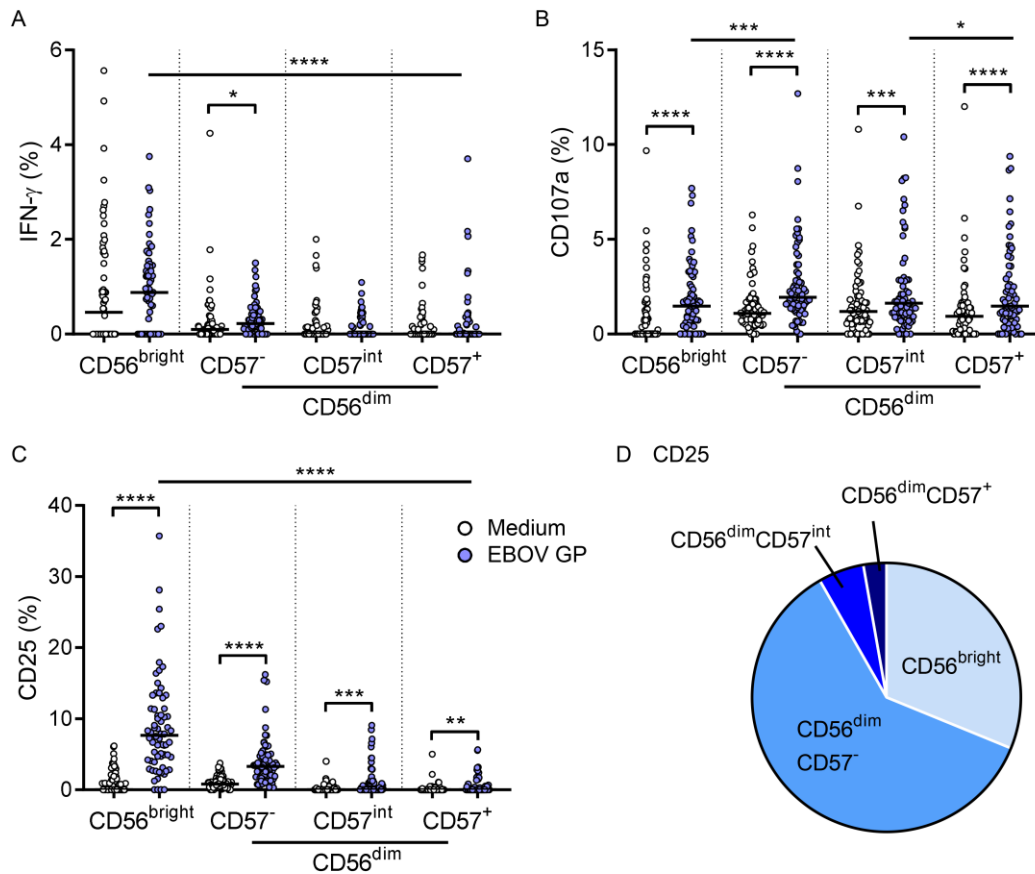
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734 **Figure 2.** NK cell CD107a and CD25, but not IFN- γ expression upregulation in
 735 response to EBOV GP stimulation in vitro.

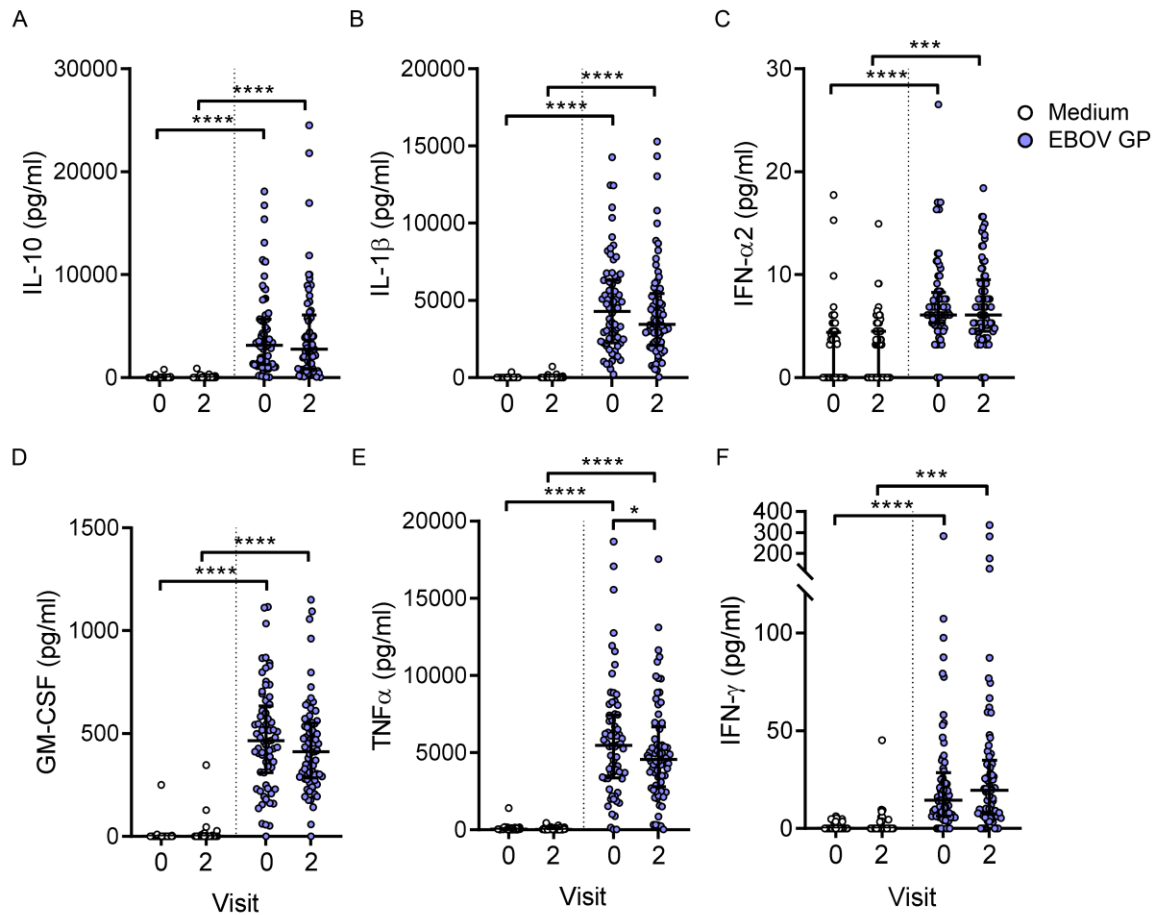
736 Whole PBMC from baseline (visit 0), visit 1 (day 29, 57 or 15 post-dose 1) and visit 2
 737 (21 days post-dose 2) were stimulated with EBOV GP or left unstimulated (medium)
 738 for 8 and 18 hours in the presence of 1% autologous serum, n=70. Cells were stained
 739 for NK cell activation markers and analysed by flow cytometry. Frequencies of CD107a
 740 and IFN- γ , measured at 8 hours or CD25 and CD16 measured at 18 hours, within total
 741 live CD3⁺CD56⁺ NK cells were gated using medium alone controls, plots shown from
 742 one representative donor (a). Graphs show NK cell CD107a (b), IFN- γ (c), CD25 (d)
 743 and CD16 (d) expression as one point per donor with a line representing the median.
 744 Comparisons across vaccination visits were performed using one-way ANOVA with
 745 Dunn's correction for multiple comparisons and between conditions by Wilcoxon
 746 signed-rank test. ****p <0.0001.



747

748 **Figure 3.** Less differentiated NK cells respond strongly to EBOV GP stimulation in
 749 vitro.

750 NK cell IFN- γ (a) and CD107a (b), measured at 8 hours and CD25 (c), measured at
 751 18 hours in response to medium alone and EBOV GP in baseline (visit 0) samples
 752 only was analysed according to NK cell differentiation subset determined by CD56 and
 753 CD57 expression (CD56^{bright}, CD56^{dim}CD57⁻, CD56^{dim}CD57^{intermediate} (int) and
 754 CD56^{dim}CD57⁺), n=70. The proportion of CD25⁺ NK cell events per subset determined
 755 by back-gating is also shown as a pie chart (d). Graphs show one point per donor with
 756 a line representing the median. Comparisons across NK cell subsets were performed
 757 using one-way ANOVA with Dunn's correction for multiple comparisons and between
 758 conditions by Wilcoxon signed-rank test. *p <0.05, **p <0.01, ***p <0.001, ****p
 759 <0.0001.



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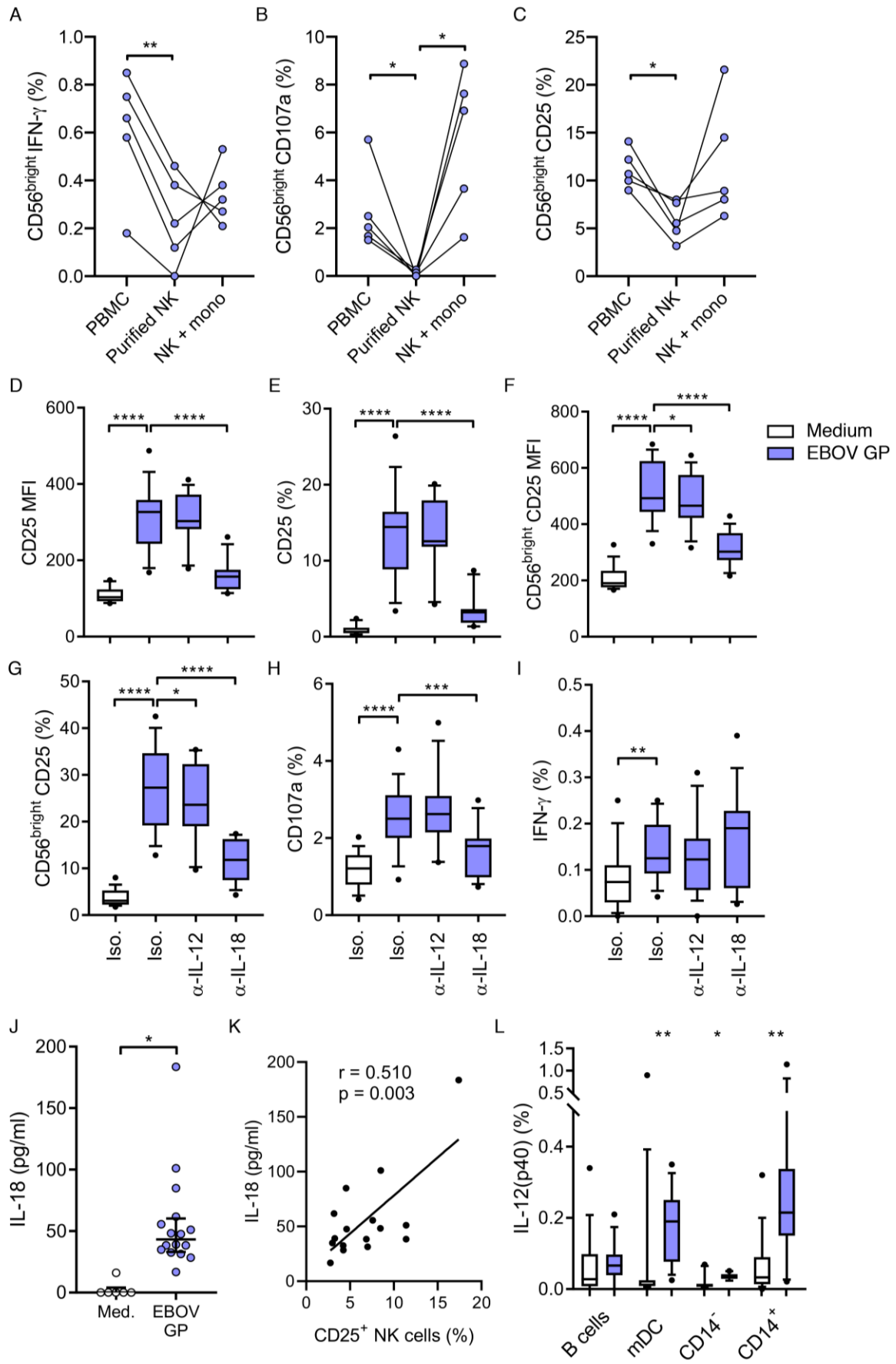
761 **Figure 4.** High concentrations of inflammatory cytokines induced by EBOV GP
 762 stimulation in vitro.

763 Supernatants were collected from baseline (visit 0) and post-dose 2 (visit 2) PBMC
 764 after 18 hours stimulation with EBOV GP or medium alone and concentrations of IL-
 765 10 (a), IL-1 β (b), IFN- α 2 (c), GM-CSF (d), TNF- α (e) and IFN- γ (f) were determined by
 766 Luminex, n=70. Graphs show one point per donor with the median and IQR.
 767 Comparisons were performed using one-way ANOVA with Dunn's correction for
 768 multiple comparisons. *p < 0.05, ***p < 0.001, ****p < 0.0001.

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771



773 **Figure 5.** Myeloid accessory cell cytokine dependent NK cell activation.

774 Non-vaccinated control PBMC, purified NK cells or purified NK cells plus CD14⁺
775 monocyte enriched population (mono) were stimulated with EBOV GP, n=5 (a-c).
776 PBMC were also left unstimulated or stimulated with EBOV GP in the presence of
777 blocking antibodies against IL-12 and IL-18 or appropriate isotype control (Iso.), n=16.
778 NK cell function was analysed by flow cytometry. Graphs show CD56^{bright} IFN- γ ,
779 CD107a and CD25 expression (a-c), total NK cell CD25 MFI (d) or percentage (e) or
780 CD56^{bright} CD25 MFI (f) or percentage (g) and total NK cell CD107a (h) and IFN- γ
781 expression (i). Concentrations of IL-18 in culture supernatant and intracellular IL-12
782 expression were determined by ELISA and flow cytometry respectively, the
783 relationship between IL-18 and total NK cell CD25 expression was determined by
784 Spearman's coefficient (j-l). IL-12(p40)⁺ B cells (CD19⁺), myeloid DC (mDC; CD19⁻
785 CD14⁻CD11c⁺), total CD14⁻ and total CD14⁺ cells were gated as per gating strategy in
786 Supplementary Figure 5a. Graphs show box and whisker plots with median, IQR (box)
787 and 10th-90th percentile (whiskers) or one point per donor. Comparisons were
788 performed using Wilcoxon signed-rank test and correlations were determined using
789 Spearman's correlation. *p <0.05, **p <0.01, ***p <0.001, ****p <0.0001.

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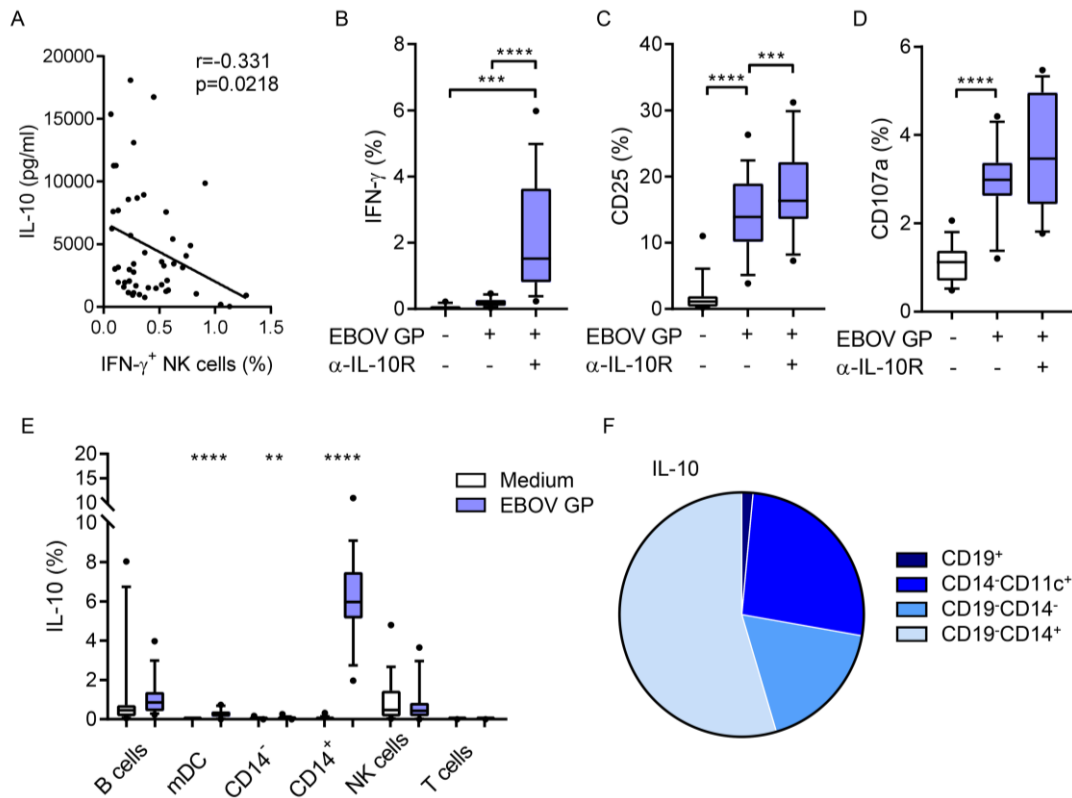
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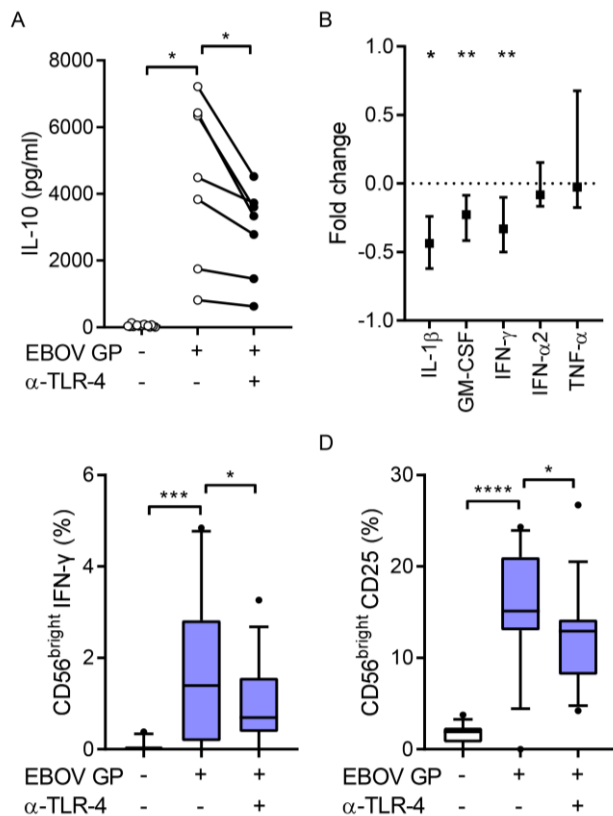
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797 **Figure 6.** Regulation of NK cell IFN- γ production by EBOV GP induced IL-10.

798 The correlation between NK cell IFN- γ secretion determined by intracellular staining
 799 and IL-10 secretion measured by Luminex in response to EBOV GP (in baseline trial
 800 samples) was determined by Spearman's coefficient, $n=70$ (a). Non-vaccinated control
 801 PBMC were stimulated in the presence of blocking antibodies against IL-10R or
 802 isotype control, $n=16$. Total NK cell IFN- γ (b), CD107a (c) and CD25 (d) expression
 803 was determined. Intracellular IL-10 was also measured by flow cytometry (gating
 804 strategy as per Supplementary Figure 5a) in B cells (CD19⁺), myeloid DC (mDC;
 805 CD14⁻CD11c⁺), total CD14⁻ and total CD14⁺ cells, NK cells (CD3⁻CD56⁺) and T cells
 806 (CD3⁺) (e). The proportion of IL-10⁺ events per cell type determined by back-gating is
 807 also shown as a pie chart (f). Graphs show box and whisker plots with median, IQR
 808 (box) and 10th-90th percentile (whiskers). Comparisons were performed using
 809 Wilcoxon signed-rank test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.



810

811 **Figure 7.** EBOV GP induced NK cell activation is dependent on interaction with TLR-
 812 4.

813 Non-vaccinated control PBMC were stimulated in the presence of blocking antibodies
 814 against TLR-4 or isotype control, n=16. Supernatants were collected and
 815 concentrations of IL-10, IL-1 β , GM-CSF, IFN- γ , IFN- α 2 and TNF- α were measured by
 816 Luminex. Graphs show IL-10 concentration as one dot per donor (n=7 with values
 817 below Luminex cut-off value of 10,000pg/ml) (a) and IL-1 β , GM-CSF, IFN- γ , IFN- α 2
 818 and TNF- α as fold change between isotype control and TLR-4 blockade (b).
 819 Expression of CD56^{bright} NK cell IFN- γ (c) CD25 (d) were determined after 18 hours by
 820 flow cytometry. Graphs show one point per donor (IL-10), median with IQR (remaining
 821 cytokines) or box and whisker plots with median, IQR (box) and 10th-90th percentile
 822 (whiskers). Comparisons between conditions were performed using Wilcoxon signed-
 823 rank test. *p <0.05, ***p <0.001, ****p <0.0001.

824 Table 1: Vaccination schedule of each group and samples received (PBMC and
 825 corresponding serum).

Samples received:				
Group (n)	Vaccine schedule	Baseline (Visit 0)	Post-dose 1 (Visit 1)	Post-dose 2 (Visit 2)
Group 1 (n=15)	MVA, Ad26	Day 1	Day 29	Day 50
Group 2 (n=15)	MVA, Ad26	Day 1	Day 57	Day 78
Group 3 (n=14)	Ad26, MVA	Day 1	Day 29	Day 50
Group 4 (n=14)	Ad26, MVA	Day 1	Day 57	Day 78
Group 5 (n=12)	Ad26, MVA	Day 1	Day 15	Day 36

826