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1 **Advax adjuvant formulations promote protective immunity against aerosol**
2 ***Mycobacterium tuberculosis* in the absence of deleterious inflammation and reactogenicity**
3

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19 **Running title:** Advax adjuvant formulations for TB vaccination

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20 **Highlights**

21 - Advax adjuvant formulations improve pulmonary protection against aerosol
22 *Mycobacterium tuberculosis* infection
23 - Different combinations of adjuvant components markedly influence the level of
24 protection observed
25 - Protection is associated with the rapid influx of myeloid cells to the site of vaccination
26 and the induction of antigen-specific polyfunctional $CD4^+$ T cells in the lung.
27 - Advax formulations abrogate vaccine-site ulceration and inflammatory cytokine
28 production

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29 **Abstract**

30 The development of safe and effective adjuvants is a critical goal of vaccine development
31 programs. In this report, we defined the immunostimulatory profile and protective effect
32 against aerosol *Mycobacterium tuberculosis* infection of vaccine formulations incorporating
33 the semi-crystalline adjuvant δ-inulin (Advax). Advax formulated with CpG oligonucleotide
34 and the QS-21 saponin (Advax^{CpQS}) was the most effective combination, demonstrated by the
35 capacity of CysVac2/Advax^{CpQS} to significantly reduce the bacterial burden in the lungs of *M.*
36 *tuberculosis*-infected mice. CysVac2/Advax^{CpQS} protection was associated with rapid influx
37 of neutrophils, macrophages and monocytes to the site of vaccination and the induction of
38 antigen-specific IFN-γ^+/IL-2^+/TNF$^+$ polyfunctional CD4$^+$ T cells in the lung. When compared
39 to the highly potent adjuvant combination of monophosphoryl lipid A and
40 dimethyldioctadecylammonium bromide (MPL/DDA), Advax^{CpQS} imparted a similar level of
41 protective efficacy yet without the profound stimulation of inflammatory cytokines and
42 vaccination site ulceration observed with MPL/DDA. Addition of DDA to CysVac2/
43 Advax^{CpQS} further improved the protective effect of the vaccine, which correlated with
44 increased polyfunctional CD4$^+$ T cells in the lung but with no increase in vaccine
45 reactogenicity. The data demonstrate that Advax formulations can decouple protective
46 tuberculosis immunity from reactogenicity, making them ideal candidates for human
47 application.

48

49 **Keywords**

50 Tuberculosis, protective vaccine, adjuvant, Advax, reactogenicity

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51 **1. Introduction**

52 From prehistoric times to now, *Mycobacterium tuberculosis* has remained one of the most
53 successful human pathogens. Tuberculosis (TB) is the leading cause of disease from a single
54 infectious agent, causing an estimated 1.5 million deaths and infecting an estimated 10 million
55 individuals in 2018 (1). The current vaccine, *Mycobacterium bovis* Bacillus Calmette–Guérin
56 (BCG), is particularly effective at reducing the incidence of childhood TB, miliary TB and
57 meningitis (2) but overall BCG efficacy in the lungs varies greatly depending on age of
58 administration, localisation of TB infection, geographical area where vaccine is administered,
59 previous exposure to various mycobacteria and current immune status (3). The negative global
60 impact of BCG's variable efficacy is profound because the lowest levels of protection occur in
61 countries with the highest incidence of TB (4). WHO has estimated that this results in BCG
62 only preventing 5% of all potentially vaccine-preventable deaths caused by TB (5).
63 In the search for a better TB vaccine, purified protein subunits are the safest and most refined
64 design option and can be used in HIV-endemic areas, where it is not recommended to use live
65 vaccine strategies. One important consideration is the fact that *M. tuberculosis* exists in two
66 metabolic states, active or latent depending on whether infection is acute or chronic, and this
67 can also impact vaccination strategies (6) since the antigen expression profile differs markedly
68 between the two states (7, 8). Previous investigation of *M. tuberculosis* protein expression
69 during chronic infection revealed strong induction of the sulfate assimilation pathway (SAP),
70 the components of which proved to be highly immunomodulatory (9). This led to the
71 development of CysVac2, a fusion protein consisting of early-stage immunodominant Ag85B
72 antigen (Rv1886c) and the late-stage SAP protein, CysD (Rv1285)(10).
73
74 In combination with monophosphoryl lipid A (MPL) and dimethyldioctadecylammonium
75 bromide (DDA), CysVac2 induces potent T helper 1 (Th1) and T helper 17 (Th17) responses,

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76 and confers a high level of protection in a mouse model of pulmonary TB infection (10).
77 However, in order to improve the safety profile and reduce local reactogenicity events we have
78 focussed on alternative adjuvant combinations, in particular the Advax family of adjuvants
79 (11). Derived from δ-inulin, a plant storage carbohydrate, Advax has been shown to have a
80 good tolerability and safety profile as an adjuvant and has been previously evaluated in
81 vaccines against diseases such as hepatitis B, anthrax, listeria, SARS and influenza (12). We
82 have previously demonstrated the immunogenicity and protective efficacy of adjuvanting
83 CysVac2 with Advax in combination with a TLR9 agonistic CpG oligonucleotide (13). In this
84 report we have further optimised this adjuvant formulation by evaluating the effects of
85 additional adjuvant components that target different immune pathways. The saponin, QS-21,
86 is a widely used, highly immunogenic adjuvant that induces strong antibody as well as cell-
87 mediated immune responses, particularly Th1 and $CD8^+$ T cell responses (14, 15). QS-21 has
88 been used in clinical trials for malaria, influenza, HIV, hepatitis B, Alzheimer's disease, and
89 cancers (16), and is a key component of the M72/AS01 TB vaccine (17). The current study
90 assessed combination adjuvants based on Advax to identify safe, well-tolerated regimens to
91 protect against *M. tuberculosis* infection.
92

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93 **2. Materials and Methods**

94 *2.1. Bacterial Strains and Growth Conditions*

95 *M. tuberculosis* H37Rv and *M. bovis* BCG Pasteur were grown at 37 °C in Middlebrook 7H9
96 medium (BD) supplemented with 0.5% glycerol, 0.02% Tyloxapol, and 10% albumin-
97 dextrose-catalase (ADC) or on solid Middlebrook 7H11 medium (BD) supplemented with oleic
98 acid–ADC.

99

100 *2.2. Antigens and adjuvants*

101 Protein antigens Ag85B (Rv1886c), CysD (Rv1285), CysVac2 were produced in recombinant
102 form from *Eschericia coli* as described previously (10). Advax formulations, including
103 CpG7909 and QS-21, were provided by Vaxine Pty Ltd (Adelaide, South Australia). DDA was
104 purchased from Sigma Aldrich, Australia, and MPL was purchased from Invitrogen, USA.

105

106 *2.3. Vaccination and infection of mice*

107 Female C57BL/6 (6–8 weeks of age) were purchased from the Animal Resources Centre
108 (Perth, Australia). Mice were maintained in specific pathogen-free condition and experiments
109 were performed with the approval of the Sydney Local Health District Animal Welfare
110 Committee (approval number 2013/047C) in accordance with relevant guidelines and
111 regulations. Animals were randomly assigned to experimental groups. For protection
112 experiments, mice were vaccinated subcutaneously (s.c.) at the base of the tail either once with
113 5×10^5 CFU of BCG Pasteur (200 µl in PBS), or i.m. three times at two-week intervals with
114 3 µg of recombinant protein formulated in Advax (1 mg) in combination with other
115 components: 10 µg CpG7909, 100 µg QS-21, 250 µg DDA, and/or 25 µg MPL. For intradermal
116 (i.d) immunisation, mice were anaesthetised by intraperitoneal injection with
117 ketamine/xylazine (80/10 µg/kg). Four microliters of protein and/or adjuvant (1 µg and/or

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118 150 μg, respectively), adjuvant alone or PBS were injected i.d. into each ear under a surgical
119 Leica M651 microscope (Leica, Wetzlar, Germany) using an ultrafine syringe (29G, BD
120 Biosciences) as described by Li *et al*. (18).
121
122 For *M. tuberculosis* challenge experiments, mice were infected with *M. tuberculosis* H37Rv
123 four weeks after their final vaccination via aerosol using a Middlebrook airborne infection
124 apparatus (Glas-Col). Mice received an infective dose of approximately 100 viable bacilli. Four
125 or 20 weeks later, lungs and spleens were harvested, homogenised and plated after serial
126 dilution on supplemented Middlebrook 7H11 agar plates. Colony forming units (CFU) were
127 determined approximately 3 weeks later and expressed as $\log_{10}$ CFU.
128
129 *2.4. Cytokine production assays*
130 Single cell splenocytes and lung cells suspensions were resuspended in buffered ammonium
131 sulfate to lyse erythrocytes and then washed and resuspended in RPMI 1640 (Life
132 Technologies) supplemented with 10% heat-inactivated foetal bovine serum (Scientifix,
133 Cheltenham, Australia), 50 μM 2-mercaptoethanol (Sigma Aldrich, Australia), and 100 U
134 ml^{-1} penicillin/streptomycin (Sigma Aldrich, Australia). Antigen specific IFN-γ producing
135 cells were detected by ELISPOT assay as described previously (19). All antigens were used at
136 a concentration of 10 $\mu g\ ml^{-1}$. For cytokine bead array assays (CBA), cells were stimulated
137 with antigens and supernatants collected after 72 hours. A cytometric bead-based multiplex
138 assay kit (BD Biosciences) was used to measure the concentration of IFN-γ, TNF, IL-2, IL-17,
139 IL-6 and IL-10 in accordance with the manufacturer's instructions. Data were acquired on a
140 BD LSR-Fortessa flow cytometer (BD) and then analysed using the FCAP Array Software
141 (BD, USA).
142

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143 *2.5. Intracellular cytokine staining and flow cytometry*

144 For intracellular cytokine staining, cells were stimulated for 3-4 hours in the presence of
145 CysVac2 (10 μg ml^{-1}) and then for up to 12 hours with brefeldin A (10 μg ml^{-1}). Two million
146 cells were incubated with anti-CD32/CD16 (eBioscience, San Diego, CA) in FACS wash
147 buffer (PBS/2% FCS/0.1%) for 30 min to block Fc receptors, then washed and incubated for
148 30 min with either anti-CD3-PerCPCy5.5 (clone 145-2C11), anti-CD4-Alexafluor 700 (clone
149 RM4-5), anti-CD8a-allophycocyanin (APC)-Cy7 (clone 53-6.7), or anti-CD44- fluorescein
150 isothiocyanate (FITC) (clone IM7, BD). Fixable Blue Dead Cell Stain (Life Technologies) was
151 added to allow dead cell discrimination. Cells were then fixed and permeabilized using the BD
152 Cytofix/Cytoperm^{TM} kit according to the manufacturer's protocol.

153

154 Intracellular staining was performed using the following antibodies: anti-IFN-γ-phycoerythrin
155 (PE)-Cy7 (clone XMG1.2), anti-TNF-APC (clone MP6-XT22, Biolegend, San Diego, CA),
156 anti-IL-2-PE (clone JES6-5H4) (BD) or anti-IL-17A-Pacific Blue (clone TC11-18H10,
157 Biolegend). For surface staining of ear samples preparations, cells were stained with anti-
158 CD64-PE (clone X54-5/7.1.1), anti-MHCII-AF700 (clone M5/114.15.2), anti-CD45.2-BV510
159 (clone 104), anti-CD11c-PECy7 (clone HL3), anti CD11b-APC-Cy7 (clone M1/70), anti-
160 CD326-APC (clone G8.8), anti-Ly6G-PB (clone 1A8), Ly6C-PerCPCy5.5 (clone AL-21).
161 All samples were acquired on a BD LSR-Fortessa flow cytometer (BD), and analysed using
162 FlowJo^{TM} analysis software (Treestar, Macintosh Version 9.8, Ashland, OR). A Boolean
163 combination of gates was used to calculate the frequency of single-, double- and triple-positive
164 $CD3^{+}CD4^{+}$ cell subsets. The gating strategy for intracellular cytokine staining is described in
165 ref. (10).

166

167 *2.6. Statistical Analysis*

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168 The significance of differences between experimental groups was evaluated by one- or two-
169 way analysis of variance (ANOVA), with pairwise comparison of multi-grouped data sets
170 achieved using Tukey or Dunnet post hoc tests.
171

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172 **3. Results**

173 *3.1. Advax^{CpQS}/CysVac2 vaccination induces long-term protection against aerosol M.*
174 *tuberculosis infection*

175 We first examined how adjuvant formulations impacted on protective efficacy against *M.*
176 *tuberculosis*. Mice were immunised with BCG or combinations of the CysVac2 antigen and
177 Advax with CpG7909 (Advax^{CpG}) or CpG plus QS-21 (Advax^{CpQS}). CysVac2 formulated in
178 MPL/DDA was used as a protective positive control formulation (10). Four weeks after
179 immunisation, mice were challenged with aerosol *M. tuberculosis* and bacterial loads in the
180 lungs determined. All adjuvant combinations and BCG protected mice against *M. tuberculosis*
181 infection four weeks post-challenge when compared to unvaccinated mice, with the greatest
182 effect observed with MPL-DDA/CysVac2 (Fig. 1A). At 20 weeks post-infection, the protective
183 effect of BCG had waned, a finding we and others have observed previously (10). At this
184 timepoint, only Advax^{CpQS}/CysVac2 vaccination resulted in significant protection, reducing
185 bacterial load in the lungs by approximately 0.52 $\log_{10}$ CFU compared to unvaccinated mice
186 (Fig. 1B). Thus, Advax^{CpQS}/CysVac2 was able to promote protective immunity against *M.*
187 *tuberculosis* at both acute and chronic timepoints post-infection.

188

189 To investigate vaccine-specific immune responses associated with the protection observed in
190 Figure 1, mice were vaccinated and challenged as described above for Figure 1 and the
191 generation of polyfunctional cytokine-secreting CD4^{+} T cells was examined four weeks post-
192 infection. In Advax^{CpG}/CysVac2-, Advax^{CpQS}/CysVac2- and MPL-DDA/CysVac2-vaccinated
193 mice, the vaccine-specific responses were dominated by IFN-γ^{+}/IL-2^{+}/TNF^{+} and TNF^{+} CD4^{+}
194 T cell subsets (Fig. 2). BCG cytokine responses upon recall to CysVac2 were at background
195 levels. While a similar pattern of responses was observed for all CysVac2 vaccinated groups,
196 MPL-DDA/CysVac2 vaccination resulted in the highest proportion of polyfunctional CD4^{+} T

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197 cells (Fig. 2). Although a similar pattern was observed for Advax^{CpQS}/CysVac2-vaccinated
198 mice, the polyfunctional CD4^{+} T cell response to MPL-DDA/CysVac2 vaccination was
199 significantly higher, and this was most apparent when examining the frequency of TNF^{+}-
200 containing subsets (Fig. 2). Thus, Advax is able to potentiate protective immunity when
201 formulated with CysVac2, but without the heightened levels of inflammatory cytokine
202 responses observed with MPL/DDA.
203
204 *3.2. DDA can augment Advax^{CpQS}/CysVac2 protection against aerosol* M. tuberculosis
205 *infection without enhancing vaccine reactogenicity.*
206 Results in Figure 1 demonstrated that optimal protective immunity was observed with CysVac2
207 formulated in MPL/DDA, however this was associated with a strong inflammatory cytokine
208 readout. We thus investigated if Advax^{CpQS}/CysVac2 immunogenicity and protection could be
209 improved by addition of DDA. Vaccination of mice with Advax^{CpQS}-DDA/CysVac2 displayed
210 protection equivalent to BCG and MPL-DDA/CysVac2 in the lung (Fig. 2A), and was the only
211 CysVac2 formulation that significantly protected mice against systemic *M. tuberculosis*
212 infection in the spleen, compared to unvaccinated mice (Fig. 2B). Analysis of cytokine-
213 secreting CD4^{+} T cell subsets in the lung from vaccinated and challenged mice demonstrated
214 that addition of DDA to Advax^{CpQS}/CysVac2 markedly enhanced the triple positive IFNɣ$^{+}$/IL-
215 2^{+}/TNF^{+} population (Fig. 2C). However, the responses for other cytokine combinations were
216 lower than those observed with MPL-DDA/CysVac2, particularly for double positive
217 IFNɣ$^{+}$/TNF^{+} and single positive TNF^{+} producing CD4^{+} T cell subsets (Fig. 2B). When we
218 further examined the level vaccine-specific cytokines in the supernatants of splenocytes
219 restimulated with CysVac2, cytokine levels were highest in cells from MPL-DDA/CysVac2
220 mice, particularly for IL-2, TNF, IL-17A and IL-6. Splenocytes from Advax^{CpQS}/CysVac2
221 released relatively low levels of cytokines compared to MPL-DDA/CysVac2, with only IFN-ɣ

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222 and TNF increased compared to background levels (Fig. 3). The addition of DDA to
223 Advax^{CpQS}-DDA/CysVac2 did not appreciably increase T cell cytokine responses. An increase
224 in IL-17A was observed but this difference was not statistically significant (Fig. 3G). Finally,
225 we examined reactogenicity of vaccines by determining the frequency of vaccine site ulceration
226 across experiments. For all mice vaccinated with MPL/DDA, 12% developed ulceration (38 of
227 320 mice), with a minimum time to ulcer formation of 23 days. For all groups vaccinated with
228 Advax, we observed no ulceration, irrespective of the adjuvant combinations used (0 of 221
229 mice). Thus overall, these results demonstrated that the Advax^{CpQS}-DDA/CysVac2
230 combination can afford strong protective efficacy against both pulmonary and systemic *M.*
231 *tuberculosis* infection but without deleterious inflammation and ulceration at the site of
232 injection.
233
234 *3.3. Cellular influx at site of injection after Advax/CysVac2 delivery*
235 Early innate responses to vaccines shape the subsequent adaptive immune responses and are
236 important for both persistence of memory and effector T cell functions. We used an i.d. delivery
237 to the dermis of the ear to investigate cellular recruitment induced by CysVac2 vaccination
238 (10), together with the gating strategy outlined in Supplementary Fig. 1. The day 2 response
239 after vaccination with Advax^{CpQS}/CysVac2 and Advax^{CpQS}-DDA/CysVac2 vaccination was
240 dominated by neutrophils, which were significantly elevated compared to unvaccinated or
241 MPL-DDA/CysVac2 vaccinated mice (Fig 4A, 4B). NK cells, macrophages and, to a lesser
242 extent, monocytes were elevated with Advax^{CpQS} formulations, however the addition of DDA
243 reduced this response (Fig 4A, 4B). At day 4 post-vaccination, macrophages were the most
244 abundant cell type at the vaccination site, and these were most apparent in mice vaccinated
245 with Advax^{CpQS}/CysVac2 and Advax^{CpQS}-DDA/CysVac2 (Fig 4C). Neutrophils, monocytes
246 and NK cells remained elevated in groups adjuvanted with Advax^{CpQS} compared to MPL/DDA,

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247 however for NK cells in particular, responses were markedly reduced for mice vaccinated with
248 Advax^{CpQS}-DDA/CysVac2. The numbers of T cells, DCs and B cells were not significantly
249 different between groups (not shown). These results show that the protective effect of Advax-
250 adjuvanted vaccines correlates with the early influx of distinct leukocyte populations to the site
251 of injection.

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252 **4. Discussion**

253 Successful vaccine development is a balance between effectively stimulating the immune
254 system to mount a protective response while minimising adverse reactions upon administration.
255 In this study, a fusion protein subunit vaccine consisting of CysD and Ag85B, termed CysVac2,
256 was assessed in combination with various Advax adjuvant formulations. In accordance with
257 previous results (10), vaccination with MPL-DDA/CysVac2 induced robust polyfunctional
258 $CD4^+$ T cell responses and protection against pulmonary *M. tuberculosis* infection in mice, on
259 par with that afforded by BCG (Fig. 1A-B). However, MPL-DDA/CysVac2 vaccination was
260 also associated with significant side-effects (injection site ulcer formation) in comparison to
261 the Advax adjuvant formulations tested. This corresponded to increased inflammatory cytokine
262 readouts in groups administered with MPL-DDA (Fig. 3C, E) and is consistent with the known
263 reactogenicity of MPL-containing adjuvants (20). Furthermore, MPL has been shown to drive
264 terminal differentiation of effector T cells and reduce protective capacity on antigen recall (48),
265 suggesting MPL containing vaccines may not be ideal for vaccines seeking to provide long-
266 term protection against mycobacterial infection. (21). We found that CysVac2 formulated with
267 Advax combined with CpG and the saponin QS-21 afforded significant protection against
268 pulmonary *M. tuberculosis* challenge, which was maintained at extended time-points post
269 vaccination, unlike MPL-DDA (Fig. 1). QS-21 is also associated with dose-limiting side-
270 effects when used alone (22), however we observed no adverse events with the
271 $Advax^{CpQS}$/CysVac2 vaccine in mice. This further supports the excellent safety profile of
272 Advax-adjuvanted vaccines observed in pre-clinical and clinical studies (11).
273

274 Further optimisation of the $Advax^{CpQS}$/CysVac2 formulation was achieved through
275 incorporation of DDA, a potent immunostimulatory compound approved for use in human
276 clinical trials (NCT00922363). DDA has long been recognised as an efficient adjuvant for TB

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277 subunit vaccines (23, 24) and is able to create a "depot" effect upon administration, which
278 allows for undispersed antigen delivery to the draining lymph nodes to efficiently prime the
279 adaptive immune response (25). The addition of DDA to Advax^{CpQS}/CysVac2 resulted in a
280 significantly greater induction of cytokine producing CD4^{+} T cells in the lung, although notably
281 less TNF producing CD4^{+} T cells compared to MPL-DDA formulations (Fig. 2 and 3),
282 highlighting the reduced inflammatory cytokine induction by Advax adjuvant combinations
283 (26). In particular, the addition of DDA to Advax^{CpQS} increased induction of triple positive
284 IFN-γ^{+}/TNF^{+}/IL-2^{+} producing CD4^{+} T cells in the lung (Fig. 2C). A greater proportion of
285 double positive IFN-γ^{+}/TNF^{+} and IL-2^{+}/TNF^{+} CD4^{+} T cells were also observed in Advax^{CpQS}-
286 DDA/CysVac2-vaccinated mice (Fig. 3C), with the latter population having been shown to
287 correlate with vaccine-induced protection against chronic *M. tuberculosis* infection (27).
288
289 When examining the effect of the different vaccine adjuvant formulations at the site of
290 injection, Advax-containing vaccines resulted in a significant influx of macrophages and
291 neutrophils to the dermis (Fig. 4A). Neutrophils are indicative of acute inflammation but also
292 important in the activation of macrophages (28, 29) and direct killing of *M. tuberculosis* (30).
293 The strong neutrophil response to Advax adjuvant may be due to the preferential phagocytosis
294 of carbohydrates exhibited by neutrophils and enhanced by the structure of δ-inulin (31, 32).
295 Neutrophils play a key role in macrophage recruitment (33) and this may explain the high levels
296 of macrophages at the site of injection at 4 days post-immunisation in Advax groups (Fig. 4C).
297 In contrast, MPL/DDA induced lower neutrophil and macrophage recruitment after CysVac2
298 vaccination. The greatest levels of NK recruitment to the vaccination were also observed in
299 Advax^{CpQS}/CysVac2-vaccinated mice (Fig. 4B-C). BCG vaccination has been demonstrated to
300 induce trained immunity in NK cells and is postulated to play a role in the non-specific benefits
301 of BCG vaccination such as decreased neonatal mortality (34). It is interesting to note that the

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302 addition of DDA to the Advax^{CpQS}/CysVac2 formulation abrogated this NK response. This
303 differs to the effect of MPL/DDA on NK cell recruitment when injected intraperitoneally (35)
304 and may reflect difference in NK function at distinct anatomical sites. Nonetheless, as
305 Advax^{CpQS}-DDA/CysVac2 was the most protective formulation, this suggests that NK cells
306 may not play a major role in the early induction of immunity after vaccination with this
307 formulation.

308

309 In conclusion, this study demonstrates that potent protective immunity can be induced by the
310 multistage CysVac2 fusion protein vaccine when administered with adjuvant combinations
311 based on the non-inflammatory δ-inulin Advax platform. As Advax adjuvant formulations have
312 been tested in both animals and humans without any major local or systemic reactogenicity
313 (36-39), the vaccine formulations described in this study warrant further investigation to
314 determine their potential to protect against *M. tuberculosis* in humans.

315

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316 **Authorship contribution statement**

317 D.Q., N.P., W.B. and J.T. conceived and designed the study. D.Q., C.C., G.N and R.P.
318 performed the experiments. All authors analysed and interpreted the data. D.Q. and J.T. wrote
319 the first draft of the manuscript. All authors reviewed and approved the final manuscript
320 version.

321

322 **Declaration of Competing Interest**

323 N.P. is an inventor on patents over Advax and has interests in Vaxine Pty Ltd, which owns
324 interests in the Advax patents. W.B. and J.T. are inventors on patents over CysVac2. The
325 authors declare that they have no known competing financial interests.

326

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446 **Figure Legends**

447 **Figure 1. Protection against aerosol *M. tuberculosis* by Advax formulations.** Female
448 C57BL/6 mice (n=5-7 per group) were vaccinated once s.c. with BCG (5 x 10^5 CFU), three
449 times s.c. with 3 µg CysVac2 formulated in MPL-DDA, or three times i.m. with 3 µg CysVac2
450 formulated in $Advax^{CpG}$ or $Advax^{CpQS}$. Four weeks after vaccination, mice were challenged by
451 aerosol infection with *M. tuberculosis* (~100 CFU). Four weeks (A) and 20 weeks (B) post-
452 challenge, the bacterial loads in the lung were determined and are shown as means ±SEM.
453 Lung cells were restimulated *ex vivo* with CysVac2 in the presence of brefeldin A for the
454 detection of intracellular IFN-γ, IL-2 or TNF via flow cytometry (C). The results are shown as
455 means ±SEM for cytokine-producing $CD4^+$ T cells. The significance of differences between
456 the groups was determined by ANOVA (*P<0.05, **P<0.01, ***P<0.001, ****P<0.0001).
457 Data are representative of two independent experiments.

458

459 **Figure 2. DDA augments Advax-mediated protective immunity against aerosol *M.***
460 ***tuberculosis*.** C57BL/6 mice (n=5-7 per group) were vaccinated once s.c. with BCG (5 x 10^5
461 CFU), three times s.c. with 3 µg CysVac2 formulated in MPL-DDA, or three times i.m. with 3
462 µg CysVac2 formulated in $Advax^{CpG}$ or $Advax^{CpQS}$. Four weeks after vaccination, mice were
463 challenged by aerosol infection with *M. tuberculosis* (~100 CFU). Four weeks post-challenge,
464 the bacterial loads were determined in the lung (A) and the spleen (B), and are shown as mean
465 ±SEM. Lung cells were restimulated *ex vivo* with CysVac2 in the presence of brefeldin A for
466 the detection of intracellular IFN-γ, IL-2 or TNF via flow cytometry (C). The results are shown
467 as mean ±SEM for cytokine-producing $CD4^+$ T cells. The significance of differences between
468 the groups was determined by ANOVA (*P<0.05, **P<0.01, ***P<0.001, ****P<0.0001).
469 Data are representative of two independent experiments.

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471 **Figure 3. Reduced release of inflammatory cytokines after vaccination with Advax**
472 **formulations.** C57BL/6 mice (n = 4-7 per group) were vaccinated once s.c. with BCG (5 x 10^5
473 CFU), three times s.c. with 3 µg CysVac2 formulated in MPL-DDA, or three times i.m. with 3
474 µg CysVac2 formulated in Advax^{CpQS} or Advax^{CpQS} with DDA. Four weeks after infection,
475 spleen cells were harvested and restimulated *ex vivo* with CysVac2 over 72h. Cell supernatants
476 were taken for the detection of secreted IFN-γ (A), TNF (B), IL-2 (C), IL-17A (D), IL-6 (E) or
477 IL-10 (F) by CBA analysis in triplicate. The results are shown as mean ±SEM for mice in each
478 group. The significance of differences between the groups was determined by ANOVA
479 (*P<0.05, **P<0.01, ***P<0.001, ****P<0.0001). Data are representative of two independent
480 experiments.
481
482 **Figure 4. Leukocyte subsets recruited to the site of injection with adjuvanted CysVac2**
483 **formulations.** C57BL/6 mice (n = 3) were i.d. injected in the ears with 1 µg CysVac2
484 formulated in PBS, Advax^{CpQS}, Advax^{CpQS}-DDA or MPL-DDA and the proportions and
485 number of cell populations examined (A). Representative flow cytometry plots of neutrophil
486 and monocyte/macrophage populations at day 2. The total number of neutrophils, monocytes,
487 NK cells and macrophages are shown at two (B) and four (C) days post-injection and shown
488 as mean ±SEM for the absolute numbers of the different cell types. The significance of
489 differences between the groups was determined by ANOVA (*P<0.05, **P<0.01, ***P<0.001,
490 ****P<0.0001).
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Figure 1

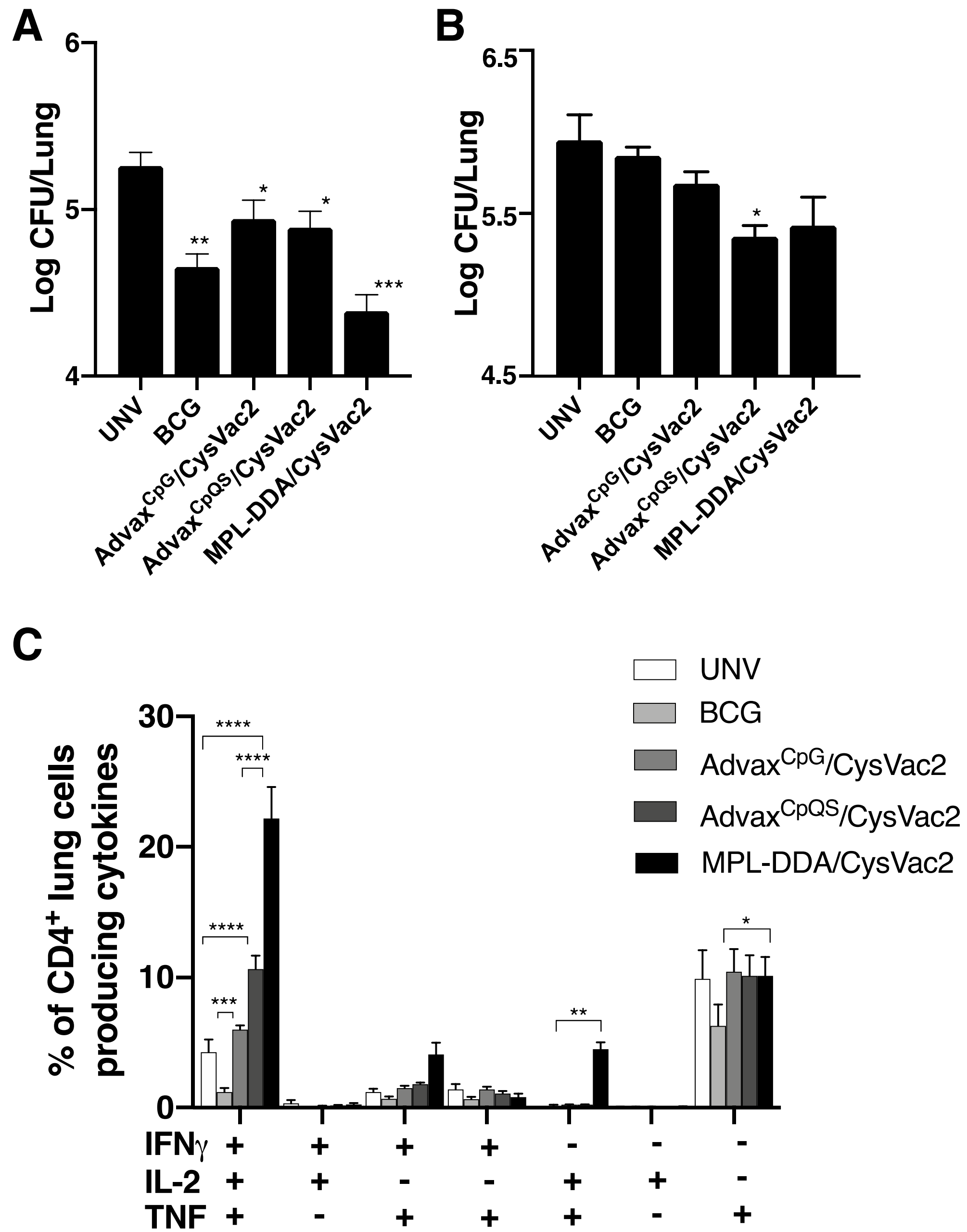

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Figure 2

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Figure 3

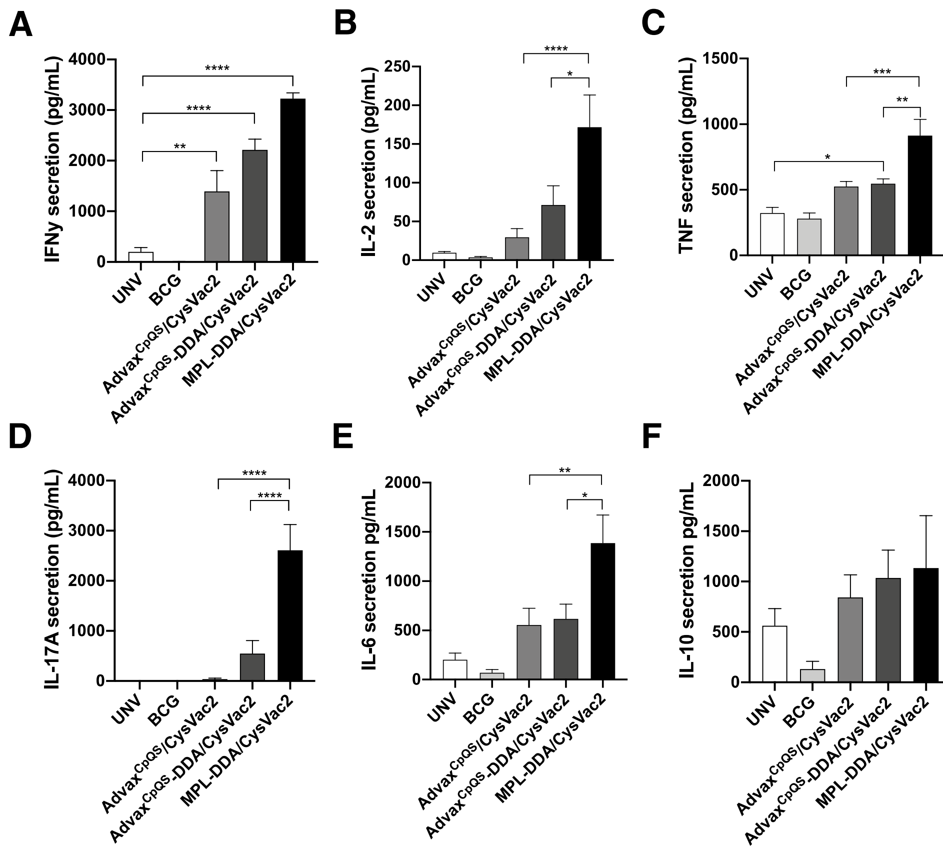

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Figure 4