

1 **Unique Inflammatory Mediators and Specific IgE Levels Distinguish Local from**
2 **Systemic Reactions After AVA Vaccination**

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18 Running Title: Biomarkers associate with AEs following AVA vaccination

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23 **Summary**

24 Although the U.S. National Academy of Sciences concluded that anthrax vaccine
25 adsorbed (AVA) has an adverse event (AE) profile similar to other adult vaccines, 30-70%
26 of queried AVA vaccinees report AEs. AEs appear to correlate with certain demographic
27 factors, but the underlying immunologic pathways are poorly understood. We evaluated a
28 cohort of 2,421 AVA vaccinees and found 153 (6.3%) reported an AE. Females were
29 more likely to experience AEs (OR=6.0 [95% C.I. 4.2-8.7], $p<0.0001$). Individuals 18-29
30 years of age were less likely to report an AE than individuals aged 30 years or older
31 (OR=0.31 [95% C.I. 0.22-0.43], $p<0.0001$). No significant effects were observed for
32 African, European, Hispanic, American Indian, or Asian ancestry after correcting for age
33 and sex. Additionally, 103 AEs were large local reactions (LLRs), whereas 53 AEs were
34 systemic reactions (SRs). In a subset of our cohort vaccinated 2-12 months prior to
35 plasma sample collection (n=75), individuals with LLRs (n=33) had higher protective
36 antigen (PA)-specific IgE levels than matched, unaffected vaccinated individuals (n=50,
37 $p<0.01$). Anti-PA IgE was not associated with total plasma IgE, hepatitis B-specific IgE, or
38 anti-PA IgG in individuals who reported an AE or in matched, unaffected AVA vaccinated
39 individuals. IP-10 was also elevated in sera of individuals who developed LLRs ($p<0.05$).
40 Individuals reporting SRs had higher levels of systemic inflammation as measured by C-
41 reactive protein ($p<0.01$). Thus, LLRs and SRs are mediated by distinct pathways. LLRs
42 associate with a vaccine-specific IgE response and IP-10, whereas SRs demonstrate
43 increased systemic inflammation without a skewed cytokine profile.

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46 Introduction

47 *Bacillus anthracis*, the causative agent of anthrax infection, forms long-lived
48 spores, which have previously been prepared and released as a biological weapon (1-4).
49 Anthrax vaccine adsorbed (AVA), the only anthrax vaccine licensed in the United States,
50 is administered to at-risk personnel to provide pre-exposure protection. AVA contains cell-
51 free culture supernatant of *B. anthracis* adsorbed on an adjuvant, aluminum hydroxide (5-
52 7). The primary immunogen of AVA is protective antigen (PA), a component of anthrax
53 toxin that is non-toxic alone. In many animal models, the magnitude and toxin
54 neutralization ability of the humoral response to PA correlates with protection against *B.*
55 *anthracis* spore challenge (8-12). Since AVA became mandatory for select populations in
56 1998, concerns have arisen regarding the relatively high rate of adverse events (AEs),
57 especially in women (13, 14). Among AVA vaccinees, 0.5-1% report systemic AEs and 4-
58 10% report local AEs to health care professionals (13, 15), similar to other adult
59 vaccinations (15-17). However, when AVA vaccinees are queried after each vaccination,
60 30-70% report AEs (18), similar to various vaccines with higher rates of AEs (16).

61 While the etiology of vaccination-induced AEs is uncertain, local reactions may
62 occur due to non-specific activation of the immune system in response to large doses of
63 aluminum adjuvants, mercurothiolate, formaldehyde, or various toxoids (19). In AVA
64 vaccination specifically, intramuscular rather than subcutaneous administration may
65 reduce the rate of local AEs but does not seem to influence the rate of systemic AEs (20).
66 Additionally, certain types of reactions may associate with sex, ethnicity, and body mass
67 index (14, 16, 17). Despite the results of these studies, little is known about the
68 measurable correlates or immune mechanisms that may underlie adverse events to AVA.
69 In this study, we investigated demographic predictors of AEs, as well as serological

70 markers in individuals who reported large local reactions (LLRs) and systemic reactions
71 (SRs) to AVA. An understanding of the immunologic pathways that associate with AEs
72 may help identify those at risk of AEs and minimize AEs in the future.

73

74 **Materials and Methods**

75 **Human subjects**

76 US military personnel (n=2,421) who had received AVA vaccination at least once
77 provided informed written consent and vaccination history, sex, age, and race information.
78 Institutional Review Board approval was obtained from the Oklahoma Medical Research
79 Foundation, Walter Reed National Military Medical Center Vaccine Healthcare Centers
80 (VHC) Network/Allergy-Immunology, and Womack Army Medical Center/Fort Bragg
81 Regional VHC. Peripheral blood samples were collected, and plasma, sera, and blood
82 smears were stored until testing. Criteria for experiencing an AE included new onset of
83 headache; myalgia and/or arthralgia; fatigue that limited daily activities; large local
84 swelling, redness or soreness; or another new-onset medical condition that affected the
85 ability to work, sleep, exercise, and/or enjoy leisure activities (Supplemental Tables 1 and
86 2). Subsequent serologic studies were performed in individuals who had experienced an
87 adverse event within the last year.

88 **Anthrax protective antigen IgG and IgE ELISAs**

89 Ninety-six-well microtiter plates (Corning, Lowell, MA) were coated with 1µg/well of
90 recombinant PA (List Biological Laboratories, Campbell, CA). For each sample, plasma
91 was diluted 1:10, 1:100, 1:1000, and 1:10,000 and added to the plate, followed by
92 conjugate (anti-human IgG, Jackson ImmunoResearch, West Grove, PA; anti-human IgE,
93 Genway Biotech, San Diego, CA), with washing after each step. Anti-PA IgG ELISAs were

94 developed using para-nitrophenylphosphate substrate (Sigma-Aldrich, St. Louis, MO);
95 anti-PA IgE ELISAs were developed with 3,3', 5,5'-tetramethylbenzidine (TMB) and stop
96 solution (SureBlue TMB and TMB Stop Solution, Kirkegaard and Perry Laboratories,
97 Gaithersburg, MD). The optical density (OD) at 405 or 450nm was detected using a Dynex
98 MRX II microplate reader (Dynex Technologies, Chantilly, VA). Concentration of anti-PA
99 IgG and IgE were calculated from standard curves of the reference sera AVR801 (BEI
100 Resources, Manassas, VA) (21) and human IgE (Athens Research and Technology,
101 Athens, GA), respectively.

102 **Total plasma IgG and IgE**

103 Total plasma IgG and IgE were assessed by commercial ELISAs (Bethyl
104 Laboratories, Montgomery, TX and Genway Biotech, San Diego, CA, respectively) per
105 manufacturer protocol. Briefly, total plasma IgG was detected using anti-human IgG
106 followed by additions of blocking solution, diluted plasma sample (1:100,000) or
107 standards, and conjugate, with washing between each step. Total plasma IgE was
108 detected by diluting plasma (1:200), transferring to pre-coated plates, and adding supplied
109 conjugate. Total IgG and IgE ELISAs were developed with supplied TMB substrate and
110 stop solutions. Plasma IgG and IgE concentrations were determined using assay-specific
111 7-point calibration curves generated with the manufacturer-supplied standard. A value of
112 1.0µg/mL was assigned for concentrations below the limit of detection.

113 **High sensitivity C-reactive protein (hs-CRP) testing**

114 CRP levels were measured in freshly thawed sera by a high sensitivity commercial
115 ELISA (Biomerica, Irvine, CA). Sera were diluted 1:100 and added to a pre-coated plate,
116 followed immediately by addition of conjugate. After incubating, the serum-conjugate
117 mixture was removed by washing, and the plate was developed with TMB and stop

118 solution (Biomerica, Irvine, CA). Serum CRP concentrations were calculated against a 5-
119 point standard curve.

120 **Blood smear assessment**

121 Blood smears were made at time of sample collection and stored at room
122 temperature with minimal light exposure. One hundred leukocytes per smear were
123 counted and characterized by an ASCP-licensed Medical Laboratory Technician (C. A.
124 Velte, MT[ASCP]).

125 **Multiplex bead-based assays for cytokines, chemokines and other soluble** 126 **mediators**

127 Fifty cytokines, chemokines, and other soluble mediators were assessed by xMAP
128 multiplex assays (Panomics/Affymetrix, Santa Clara, CA), as previously described (22).
129 Multiplex soluble mediator data were analyzed on the BioPlex 200[®] array system (Bio-Rad
130 Technologies, Hercules, CA) with a lower boundary of 100 beads per analyte per sample.
131 Median fluorescence intensity (MFI) for each analyte was used to interpolate
132 concentration values from 5-parameter logistic nonlinear regression standard curves.
133 Analytes below the detection limit were assigned a value of 0.001pg/mL. A known control
134 serum was included on each plate (Cellgro human AB serum, Cat#2931949, L/N#M1016).
135 Well-specific validity was assessed by AssayCheX[™] QC microspheres (Radix
136 BioSolutions, Georgetown, TX, USA) to evaluate non-specific binding. Mean inter-assay
137 coefficient of variance (CV) of multiplexed bead-based assays for cytokine detection has
138 previously been shown to be 10-14%, (1, 2, 23, 24) and a similar average CV (11%) was
139 obtained across the analytes in this assay using healthy control serum. Intra-assay
140 precision of duplicate wells averaged <10% CV in each 25-plex assay. Soluble mediators
141 were eliminated from further analysis if >80% of samples had out of range counts or if

142 $\geq 20\%$ of samples had normalized MFI values < 0 . Plate-to-plate consistency was
143 determined by comparing controls from the individual plates. Soluble mediators were also
144 eliminated if the normalized MFI values of the controls differed significantly across plates
145 (adjusted p -value < 0.0001). The following cytokines did not meet these quality control
146 standards and were excluded from further analyses: IL-1 β , IL-4, IL-6, IL-10, TGF- β , IFN- β ,
147 IL-13, IL-23, IFN- γ , TNF- α , IFN- $\alpha 2$, IL-17a, IL-2RA, MIG, PAI, RANTES, MCP-1, eotaxin,
148 VEGF-A, TNFR1, TNFR2, resistin, and sVCAM. The following cytokines met the quality
149 control standards and were retained for analysis: IL-2, IL-5, IL-7, IL-12, G-CSF, IL-1 α , IL-
150 1RA, IL-15, IL-21, ICAM-1, NGF- β , leptin, SCF, SDF-1, MIP-1 α , MCP-3, sFasL, IP-10,
151 PDGF, MIP-1 β , LIF, TRAIL, GRO α , sE-selection, and sCD40L.

152 **Lethal toxin neutralization assay**

153 The ability of plasma to neutralize lethal toxin (LT) was assessed as previously
154 described (25-27) using RAW264.7 macrophages (ATCC, Manassas, VA). Plasma
155 samples were diluted 1:100 in culture medium and incubated with LT (500ng/mL PA,
156 500ng/mL lethal factor [LF]) for 1h at room temperature before adding to cell cultures.
157 Control wells included cells alone, cells with PA only, LF only, or LT. Viability was
158 assessed by addition of 10 μ L of WST-8 (CCK8, Dojindo Molecular Technologies,
159 Rockville, MD). The Optical Density (OD) at 450nm was detected when the ODs of cells-
160 only wells were 3.0. *In vitro* neutralization of LT was determined as the percent viability in
161 treated relative to cells-only wells.

162 **Statistical analysis**

163 Analyses included tests for correlation (Spearman), population differences (Mann-
164 Whitney U), and differences of proportion (Chi-square with Yate's Correction), as
165 appropriate. For cytokine comparisons, p -values were adjusted for multiple comparisons

166 by the false discovery rate method. Analyses of predictors of AEs were performed for
167 each variable independently; the variables analyzed were sex, race, and age at AE
168 occurrence. Number of vaccinations and the time since last vaccination were not
169 analyzed, as vaccination schedules were often stopped after an AE. Age at last
170 vaccination is approximate and was calculated from age at sample draw. All analyses of
171 proportion compared the population of interest to all other individuals (*e.g.*, individuals of
172 Asian descent versus all non-Asian individuals). Multivariate analyses were performed by
173 generalized linear models. In analyses of association, trends in data were visualized with
174 a linear regression line. Within the subset of the cohort that was used for serological
175 measurements, each individual who reported a specific AE (an LLR or SR) was cohort-
176 matched with two vaccinated individuals without reported events (VIs) such that the
177 number of vaccinations, time since last vaccination, age, race, and sex were consistent
178 between the group of AEs (LLRs or SRs) and the group of compared VIs. VIs were
179 matched within 6 months and 2 vaccinations. Due to individuals not matching person-to-
180 person on demographic and vaccination history factors, comparisons of the median
181 values were performed using unpaired statistical tests.

182

183 **Results**

184 **Sex, ethnicity, and age are associated with AEs**

185 More than two thousand consented human subjects who received at least one
186 dose of the currently licensed U.S. anthrax vaccine were included in this study (n=2,421).
187 Vaccination history and self-reported demographic information are presented in Table 1.
188 Of these, 153 individuals (6.3%) reported reactions that were classified either as LLRs or
189 SRs. Individuals were removed from the vaccination protocol after experiencing an AE;

190 therefore, individuals were unlikely to report more than one AE. Of all AEs, 103 (67.3%)
191 were LLRs, defined as local redness or swelling of >2 inches in diameter, nodule, or
192 numbness or burning at the vaccination site lasting >24h. Forty-five AEs (32.7%) were
193 SRs, defined as >24h of headache, muscle or joint pain, fatigue, or other systemic
194 response that interfered with work or recreation.

195 Previous clinical trials have identified sex and race as factors associated with
196 developing an AE. Following vaccination, females are three times more likely to develop
197 swelling, pain and activity limitations compared to men. Interestingly, individuals of African
198 American descent were significantly less likely to report an AE when compared to non-
199 African Americans (14). To assess the influence of sex or other demographic factors on
200 the prevalence of AEs in this real-world cohort of AVA vaccinees, we determined the
201 relative ratio of total AEs (both LLRs and SRs) occurring in multiple demographic groups.
202 AVA-vaccinated females (52/231, 22.5%) were markedly more likely to develop an AE
203 than males (101/2190, 4.6%) ($p < 0.0001$, OR=6.0 [95% C.I. 4.2-8.7], Figure 1A). Hispanic
204 individuals appeared less likely to report an AE (3/149, 2.0%) than non-Hispanics
205 (160/2421, 7.0%) ($p = 0.03$, OR 0.27 [95% C.I. 0.09-0.86], Figure 1B). Individuals of Asian
206 descent appeared more likely to report an AE (5/26, 19.2%) than non-Asians (158/2421,
207 6.6%) ($p = 0.03$, OR 3.39 [95% C.I. 1.26-9.10], Figure 1B). However, the effects of Hispanic
208 ($p = 0.07$) and Asian ($p = 0.4$) ancestry were not significant after adjusting for age and sex.

209 Although AVA safety studies have been conducted with various age groups, an
210 evaluation of age as a risk factor for AEs has not been done. We observed a significant
211 increase of AEs in individuals over the age of 30. Specifically, individuals 18-29 years of
212 age were less likely to report an AE (69/1713, 4.0%) than anyone 30 years or older
213 (84/708, 11.9%) ($p < 0.0001$, OR 0.31 [95% C.I. 0.22-0.43], Figure 1C). Increased age was

214 incrementally associated with increased risk. Individuals 30-39 years were more likely to
215 report an AE when compared to all other age groups (11.4% vs. 5.0%, $p<0.0001$, OR 2.55
216 [95% C.I. 1.82-3.57]), as were individuals 40-49 years when compared to all other age
217 groups (12.3% vs. 6.0%, $p=0.01$, OR 2.20 [95% C.I. 1.25-3.87], Figure 1C). Finally, the
218 greatest difference was observed between the number of AEs reported among individuals
219 50 years of age or older compared with all other age groups (21.7% vs. 6.2%, $p=0.009$,
220 OR 4.22 [95% C.I. 1.55-11.54], Figure 1C). Notably, increased age was also associated
221 with increased number of vaccinations (Spearman $r = 0.37$, $p<0.0001$), suggesting that
222 older individuals simply may have more opportunities for a potential AE. After adjusting for
223 sex and number of vaccine doses with generalized linear models, adverse events
224 remained significantly more likely in individuals 30-39 years ($p<0.0001$, OR 3.25 [95% C.I.
225 2.17-4.86]), 40-49 years ($p<0.0001$, OR 4.95 [95% C.I. 2.92-8.38]), and 50 years or older
226 ($p<0.0001$, OR 9.68 [95% C.I. 4.29-21.85]) compared to individuals 18-29 years of age.
227 These results suggest that the risk for AE after AVA vaccination increases with age,
228 independent of the total number of vaccinations.

229 **Elevated anti-PA IgE levels are associated with LLRs**

230 IgE specific for tetanus and diphtheria toxoids (Td) is common after Td
231 vaccination in children, adolescents, and adults (28-30). Although rare, elevated levels of
232 IgE antibodies to vaccine component in HPV, Varicella, and Rubella have been correlated
233 to immediate allergic reaction (31). We therefore measured the level of IgE specific to PA
234 in this cohort. Individuals reporting an LLR ($n=33$) had higher median levels of PA-specific
235 IgE than their matched controls, termed vaccinated individuals (LLR-VIs, 88.3 vs.
236 60.5ng/mL, $p<0.01$, Figure 2A). In contrast, PA-specific IgE did not differ between

237 individuals reporting an SR and their matched controls (SR-VIs, 45.9 vs. 50.3ng/mL,
238 Figure 2A).

239 Due to the short half-life of IgE, a consistent time lapse between vaccination and
240 blood draws was maintained between individuals reporting an AE (LLRs or SRs) and their
241 matched controls (LLR-VIs and SR-VIs) (total n=123; Table 2). Sample collection
242 occurred 2-11 months (Avg= 6±1 month) after vaccination for all individuals reporting an
243 AE, and 1-15 months (Avg= 6±1 month) after vaccination for VIs. All comparisons were
244 performed between the subset of AEs (LLRs or SRs) and their matched VIs (LLR-VIs and
245 SR-VIs).

246 Notably, median PA-specific IgG levels did not differ between the LLR and LLR-VI
247 groups (86.5 vs. 72.0µg/mL) or between the SR and SR-VI groups (37.2 vs. 73.3µg/mL,
248 Figure 2B). Similarly, the ability of plasma to neutralize LT *in vitro* did not differ between
249 LLR and LLR-VI groups (50.3 vs. 24.9% viability, Figure 2C) or between SR and SR-VI
250 groups (50.7 vs. 63.2% viability, Figure 2C). However, in the LLR group, anti-PA IgE
251 levels were associated with anti-PA IgG levels (Spearman's $r=0.53$, $p<0.01$) and number
252 of vaccinations (Spearman's $r=0.41$, $p<0.02$), suggesting increased vaccination may
253 induce both PA-specific IgG and IgE.

254 In general, the highest levels of toxoid-specific IgE after toxoid vaccination may be
255 found in atopic individuals (19). To determine if PA-specific IgE levels reflected in overall
256 immunoglobulin levels, we measured total plasma IgE and total plasma IgG. Total plasma
257 IgE levels were below the limit of detection in 53% (35/66) of LLR-VIs, but similar between
258 the remaining LLR-VIs and individuals with LLRs (Figure 2D). Median total plasma IgE
259 levels were non-significantly higher in the LLR group versus LLR-VIs (85.3 vs. 1.0µg/mL,
260 $p=0.07$), but did not differ between the SR and SR-VI groups (154.8 vs. 36.0µg/mL, Figure

261 2D). Anti-PA IgE levels were associated with total IgE levels, but only in the LLR group
262 (Spearman's $r=0.34$, $p<0.05$). Median total plasma IgG levels were very similar across all
263 groups (Figure 2E). Additionally, complete blood counts performed on blood smears from
264 31 individuals with LLRs and 31 matched LLR-VIs showed no significant differences in
265 levels of basophils (Figure 2F), eosinophils (Figure 2G), or any other leukocyte type
266 evaluated.

267 **IP-10 levels are associated with LLRs**

268 Multiple studies have previously demonstrated that increased levels of particular
269 cytokines are associated with increased allergic symptoms (32, 33). We therefore
270 hypothesized that levels of soluble inflammatory mediators would associate with
271 occurrence of an AE. To test this hypothesis, we determined levels of 50 soluble
272 mediators by multiplex bead-based assays in freshly thawed plasma from all individuals
273 with LLRs, SRs, and matched VIs. After correcting for multiple comparisons, levels of
274 interferon- γ -inducible protein of 10kDa (IP-10; also known as CXCL10) remained
275 significantly associated with experiencing an LLR. In addition, median IP-10 levels were
276 increased in the LLR group compared to LLR-VIs (MFI=251.3 versus 183.2, FDR-adjusted
277 $p=0.03$), while no differences in IL-4, IL-5 or eotaxin levels were noted.

278 **C-reactive protein levels are elevated in patients with SRs**

279 We hypothesized that widespread inflammation would be associated with elevated
280 CRP levels in individuals reporting an SR. Indeed, we found that mean CRP levels were
281 significantly higher in the SR group than in the SR-VIs (6.88 vs. 1.86mg/L, $p=0.02$). Mean
282 serum levels of CRP did not differ between the LLR group and the LLR-VIs (3.88 vs.
283 3.05mg/mL).

284

285 **Discussion**

286 The possible use of *B. anthracis* as a biological warfare agent necessitates a safe,
287 effective anthrax vaccine. In 1998 the Department of Defense implemented mandatory
288 AVA vaccination of the U.S. military and certain civilian personnel, making adverse events
289 (AE) to AVA vaccination an important consideration for this population. In this study, we
290 assessed whether AVA-related AE could be predicted by vaccination history,
291 demographic factors, or serological factors in otherwise healthy adult vaccinees.

292 Demographic factors correlate with AEs; in the Vaccine Adverse Event Reporting
293 System (VAERS), AEs after AVA vaccination typically occurred among individuals 30
294 years of age and older (34). Likewise, our results show that the rate of AEs incrementally
295 increases with age, independent of other known risk factors. In addition, multiple studies
296 have demonstrated that females have an increased incidence of AEs following AVA
297 vaccination (18, 34). A 2003 study of U.S. Army medical personnel reported 3.9% of men
298 were temporarily unable to perform one or more of their normal duties due to an AE,
299 compared with 5.8% of women. AEs were most common following the initial vaccination,
300 affecting 6.0% of males and 12.2% of females (35). Among U.S. soldiers deployed in
301 Korea and served by the same clinic, a higher percentage of females reported an AE after
302 the first (male=39.6%, female=67.9%), second (male=39.7%, female=66.1%) and third
303 (male=31.5%, female=59.9%) vaccinations (18). Consistent with these studies, a higher
304 proportion of AVA-related VAERS reports were from females even though more males
305 were immunized with AVA in the military healthcare system (34). Our results highlight the
306 need for additional studies to elucidate the causes of AEs in female AVA vaccinees and
307 AVA vaccinees over the age of 30, and to identify modified vaccination schedules or other
308 measures to reduce the rate of AEs in at-risk populations.

309 Several mechanisms have been suggested to lead to AEs. One of the
310 mechanisms is linked to an increase in IgE (31, 36). Our findings show an increase in PA-
311 specific IgE in individuals who develop LLRs but not SR. IgE has a half-life of two days;
312 therefore, relatively elevated levels of IgE in patients reporting LLR vs LLR-VI is intriguing.
313 Furthermore, this study reports a 46% increase of anti-PA IgE in individuals suffering from
314 LLRs. An increase in IgE levels of this magnitude is likely to be biologically and clinically
315 significant, and may relate to the etiology of the LLRs. For instance, a similar increase in
316 HIV-1 specific IgE correlates with reduced viral production (37), decreased opportunistic
317 infections and a better quality of life for individuals infected with HIV (38). Proving a direct
318 causal relationship between an antigen-specific IgE and an AE is difficult and further
319 investigations are necessary to determine a possible mechanism (39).

320 Both SRs and LLRs are likely mediated by chemokines. In this study, IP-10 was
321 also associated with LLRs. Various cell types produce IP-10 (CXCL10) upon stimulation
322 with the pro-inflammatory cytokine IFN- γ (40, 41). In addition to recruiting leukocytes to
323 sites of inflammation (42, 43), IP-10 interferes with *B. anthracis* spore germination and
324 causes extensive cell wall and cell membrane disruption *in vitro* (44). Moreover,
325 neutralization of IP-10 increases host susceptibility to pulmonary *B. anthracis* infection *in*
326 *vivo* (45). Previous studies have identified IP-10 as one of the molecules correlated with
327 trivalent influenza vaccine-associated AE; however, despite consistent clinical features
328 shared by the patients, a biological mechanism could not be identified (46).

329 A recent study showed increased levels of C-reactive protein (CRP), but not of IP-
330 10, in 17 individuals (not stratified by AEs) within 7 days after AVA administration (47). In
331 the same study, individuals receiving an AVA formulation with CPG 7909 adjuvant had
332 early increases in both CRP and IP-10. We observed increased CRP levels months after

333 vaccination in individuals with SRs. Others have noted a moderate increase in CRP
334 accompanies other pro-inflammatory molecules in healthy term infants and adults
335 following Hepatitis B and influenza vaccine administration respectively (48, 49). However,
336 these studies reported that CRP levels decrease 48-72h after vaccination. Here, we
337 observe elevated CRP levels 2-11 months post vaccination in individuals reporting SRs
338 compared to their paired controls (SR-VIs). It is plausible that one or more of the AVA
339 components is capable of instigating CRP production in these individuals for an extended
340 period of time. Consequently, prolonged up-regulation of pro-inflammatory molecules,
341 including CRP, likely contributed to a systemic adverse reaction.

342 Together, these results support a role for IP-10 and CRP in LLRs and SRs
343 respectively, after AVA vaccination. We have identified likely relationships between types
344 of adverse events and their possible triggers which could be applied to future vaccine
345 design and current vaccination guidelines. Using demographic information, along with
346 serological markers, the ability to differentiate individuals likely to have a specific type of
347 an adverse reaction may enable future vaccination guidelines to recommend less frequent
348 immunization or co-administration of anti-inflammatories for individuals at risk of an
349 adverse event. However, further studies should be done to identify the direct relationship
350 between CRP and SRs, as well as IP-10, elevated PA-specific IgE levels and LLRs.

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374

375 **Conflict of Interest**

376 All authors declare that there are no conflicts of interest. All authors materially participated
377 in the research, contributed to manuscript development, and approved the final
378 manuscript.

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541 **Figure Legends**

542 **Figure 1: AVA-related AEs associate with female sex and increased age.** Self-
543 reported demographic data were analyzed for associations with prevalence of adverse
544 events (AEs). In individual comparisons of sex, ethnicity, and age, males (A), Hispanic
545 individuals (B), and individuals less than 30 years of age (C) were less likely to experience
546 AEs than other demographic groups. Conversely, females (A), individuals of Asian
547 descent (B), and individuals of 30-39, 40-49, and 50 or more years of age (C) were more
548 likely to experience an adverse event. Numbers within bars indicate the number of
549 unaffected vaccinated individuals (VIs) within each demographic group. ** $p < 0.01$, * $p < 0.05$
550 against all other demographic groups by Chi-square with Yate's correction. In multivariate
551 analysis, sex and age, but not race, remained significantly associated with AEs. EA-
552 European American, AA- African American, H- Hispanic, A- Asian.

553
554 **Figure 2: AVA-related LLRs are associated with anti-PA IgE.** PA-specific IgE was
555 assessed by ELISA (A), along with anti-PA IgG (B), total plasma IgE (D) and IgG (E). The
556 ability of plasma to neutralize LT (C) was assessed by *in vitro* assay, and percent of
557 leukocytes that were basophils (F) and eosinophils (G) were assessed in blood smears by
558 a trained medical technician. Individuals who reported a LLR had higher levels of anti-PA
559 IgE than their unaffected, matched, vaccinated counterparts. There were no significant
560 differences between the groups in any other measure. Each symbol represents a single
561 individual; error bars indicate mean and standard deviation. ** $p < 0.01$ between indicated
562 groups by Mann-Whitney U. LLR- large local reaction, LLR-VIs- large local reaction
563 vaccinated individual, SR- systemic reaction, SR-VIs- systemic reaction vaccinated
564 control, PA- Protective Antigen, LT- Lethal Toxin.

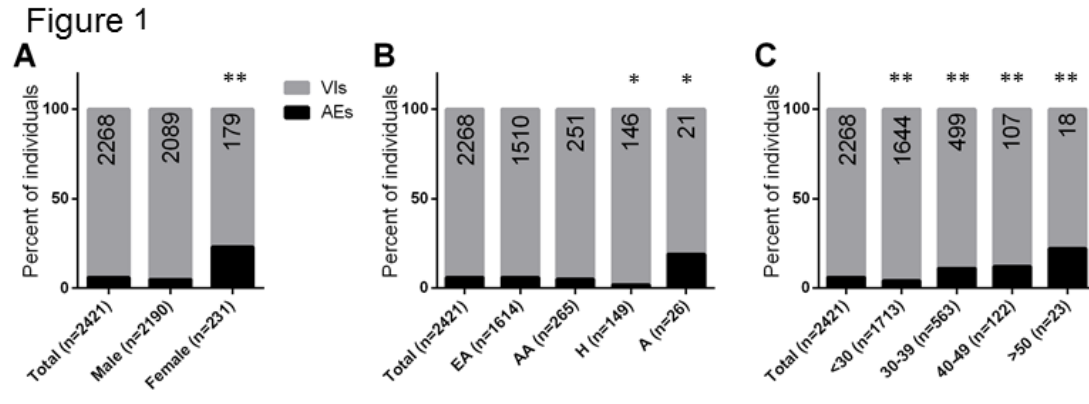


Figure 2

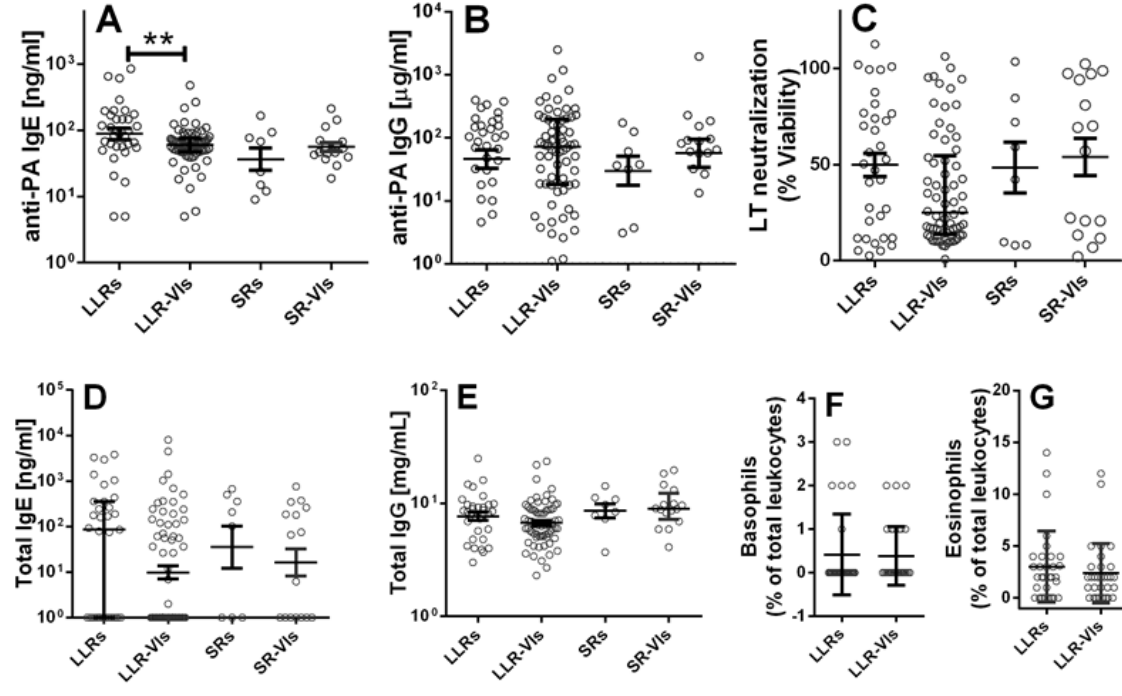


Table 1: Demographic, vaccination history, and vaccine response information for vaccinated individuals.

Characteristic	Entire Cohort (n=2421)	Unaffected Vaccinees (n=2268)	Vaccinees Reporting an Adverse Event (n=153)
Ethnicity			
EA (%)	1614 (66.6)	1510 (66.6)	104 (68.0)
AA (%)	265 (10.9)	251 (11.1)	14 (9.2)
H (%)	149 (6.2)	146 (6.4)	3 (2.0)
A (%)	26 (1.1)	21 (1.0)	5 (3.3)
Other (%)	367 (15.2)	340 (15.0)	27 (17.6)
Sex			
M (%)	2190 (90.5)	2089 (92.1)	101 (66.0)
Age at last vaccination			
Average (SD)	27.7 (6.8)	27.3 (6.6)	32.2 (8.0)
Median	25.7	25.5	31.6
Range	18-61	18-61	19-54
Years since last vaccination			
Average (SD)	1.2 (1.3)	1.2 (1.1)	2.3 (2.6)
Median	0.8	0.7	1.5
Range	0.0-17.0	0.0-11.0	0.0-17.0
Number of Vaccinations			
Average (SD)	4.9 (1.8)	4.9 (1.8)	4.9 (2.1)
Median	5	5	5
Range	1-13	2-13	1-10
Plasma anti-PA IgG (µg/mL)			
Average (SD)	153.9 (411.2)	155.3 (413.2)	132.8 (381.0)
Median	42.5	43.2	29.9
Range	0-5505.5	0-5505.5	0-3132.8
LT neutralization (% viability)			
Average (SD)	31.3 (29.6)	31.2 (29.6)	32.9 (30.2)
Median	16.3	16.2	19.2
Range	0.0-128.7	0.0-128.7	2.6-112.7

EA- European American, AA- African American, H- Hispanic, A- Asian, PA- Protective Antigen, LT- Lethal Toxin, M- male.

Table 2: Demographic, vaccination history, and vaccine response information for matched AE and VI groups.

Characteristic	LLRs (n=33)	LLR-VIs (n=66)	SRs (n=8)	SR-VIs (n=16)
Ethnicity				
EA (%)	20 (60.6)	39 (59.1)	5 (62.5)	12 (75.0)
AA (%)	2 (6.0)	6 (9.0)	1 (12.5)	2 (12.5)
H (%)	1 (3.0)	7 (10.6)	0 (0)	0 (0)
Mixed (%)	10 (30.3)	14 (21.2)	2 (25.0)	2 (12.5)
Sex				
M (%)	29 (87.9)	59 (89.4)	6 (75)	10 (62.5)
Age at sample collection				
Average (SD)	30.5 (6.6)	29.8 (6.6)	36.4 (7.2)	33.0 (6.2)
Median	30	28	37	34
Range	21-48	21-48	21-44	21-41
Years since last vaccination				
Average (SD)	0.5 (0.2)	0.5 (0.2)	0.6 (0.2)	0.6 (0.2)
Median	0.5	0.5	0.7	0.6
Range	0.2-0.9	0.2-1.3	0.3-0.9	0.1-0.9
Number of Vaccinations				
Average (SD)	4.8 (2.0)	5.0 (1.6)	5.5 (1.9)	5.3 (1.3)
Median	4	5	6	5.5
Range	1-9	3-8	3-8	3-8
Plasma anti-PA IgG (µg/mL)				
Average (SD)	115.6 (113.4)	172.4 (353.6)	59.1 (60.3)	199.3 (471.2)
Median	86.5	72.0	37.2	73.2
Range	0.1-395.8	1.1-2494.3	3.1-173.6	0.1-1951.1
LT neutralization (% viability)				
Average (SD)	49.8 (34.6)	36.9 (29.1)	48.4 (37.5)	54.0 (38.8)
Median	50.3	24.8	50.7	63.2
Range	2.6-112.7	0.6-106.3	7.9-103.6	2.0-102.4

AE- adverse event, VI- vaccinated individuals, LLR- large local reaction, LLR-VIs- large local reaction vaccinated individual, SRs- systemic reactions, SR-VIs- systemic reaction vaccinated control, EA- European American, AA- African American, H- Hispanic, A- Asian, PA- Protective Antigen, LT- Lethal Toxin, M- male.