

1 **Atypical B cells are a normal component of immune responses to vaccination and infection in**  
2 **humans**

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32 **Abstract**

33

34 The full diversity of the circulating human B cell compartment is unknown. Flow cytometry analysis  
35 suggests that in addition to naïve and memory B cells, there exists a population of CD11c<sup>+</sup>, CD27<sup>-</sup>  
36 CD21<sup>-</sup> “atypical” B cells, that are associated with chronic or recurrent infection and autoimmunity. We  
37 used single cell RNA-seq approaches to examine the diversity of both antigen-specific B cells and total  
38 B cells in healthy subjects and individuals naturally-exposed to recurrent malaria infections. This  
39 analysis revealed two B cell lineages: a classical lineage of activated and resting memory B cells, and  
40 an atypical-like lineage. Surprisingly, the atypical lineage was common in both malaria exposed  
41 individuals and non-exposed healthy controls. Using barcoded antibodies in conjunction with our  
42 transcriptomic data, we found that atypical lineage cells in healthy individuals lack many atypical B  
43 markers and thus represent an undercounted cryptic population. We further determined using antigen  
44 specific probes that atypical cells can be induced by primary vaccination in humans and can be recalled  
45 upon boosting. Collectively these data suggest that atypical cells are not necessarily pathogenic but can  
46 be a normal component of B responses to antigen.

## 47 **Introduction**

48

49 The majority of currently approved vaccines require the generation of an effective antibody response to  
50 provide long term immune protection (Plotkin, 2010) . An effective antibody response requires the  
51 formation of germinal centers (GCs) to produce somatically hypermutated and affinity matured long-  
52 lived plasma cells (LLPCs) that secrete high-affinity antibody as well as antigen-experienced  
53 “memory” B cells (MBCs) that are primed to produce a faster, larger and more effective response upon  
54 secondary exposure (Tangye et al., 2003). In humans, circulating human B cells have been classified  
55 based on the expression of the surface proteins CD38, CD27 and CD21. Plasma cells (PCs) express  
56 high levels of CD38 and CD27 (Horst et al., 2002) while CD27<sup>+</sup> CD21<sup>+</sup> B cells are considered to be  
57 MBCs (Klein et al., 1997; Tangye et al., 1998). These CD27<sup>+</sup> cells show high levels of affinity  
58 maturation and readily differentiate into antibody secreting PCs after stimulation compared to CD27<sup>-</sup>  
59 CD21<sup>+</sup> naïve cells (Good et al., 2009; Tangye et al., 2003). Populations of CD27<sup>+</sup>, CD21<sup>-</sup> B cells have  
60 also been described, which have previously been associated with an activated B cell phenotype,  
61 predisposed to differentiate into PCs (Avery et al., 2005; Lau et al., 2017). Conversely a subset of B  
62 cells that are CD27<sup>-</sup> CD21<sup>-</sup> have also been observed, originally in tonsils and later in peripheral blood  
63 (Ehrhardt et al., 2005; Fecteau et al., 2006). These cells, commonly referred to as atypical B cells  
64 (atBC), are observed at high frequencies in conditions of chronic antigen stimulation such as infection  
65 with HIV or malaria (Moir et al., 2008; Weiss et al., 2009) or autoimmune disease (Isnardi et al., 2010;  
66 Wei et al., 2007).

67

68 Because they are often found in chronic infection and autoimmune disease, atBCs are usually  
69 considered to be a population of anergic or exhausted B cells that arise due to chronic antigenic  
70 stimulation. In support of this, atBCs typically express high levels of inhibitory receptors such as those

71 belonging to the family of Fc-receptor-like (FCRL) molecules, as well as having muted BCR signaling  
72 and display little to no capacity to differentiate into PCs following BCR stimulation *in vitro* (Moir et  
73 al., 2008; Portugal et al., 2015; Sullivan et al., 2015). However, not all data indicate that atBCs are an  
74 exhausted population. While SLE patients with high disease scores carry high numbers of atBCs, a  
75 recent study suggested that these are short-lived activated cells, in the process of differentiating into  
76 PCs (Jenks et al., 2018). Similarly, it has been shown that BCRs used by atBCs specific to *Plasmodium*  
77 *falciparum* could also be found contributing to the anti-*P. falciparum* antibody response (Muellenbeck  
78 et al., 2013). Furthermore, studies in mice show that short-lived, recently activated B cells express high  
79 levels of CD11c<sup>+</sup> and have similar gene expression patterns to human atBCs (Kim et al., 2019; Perez-  
80 Mazliah et al., 2018).

81

82 To better understand the heterogeneity of the circulating B cell response in humans, and gain insight  
83 into the role of atBCs, we performed single cell RNA-seq on antigen-specific B cells from malaria  
84 exposed adults, we compared these data to single-cell RNA-seq on non-antigen specific B cells from  
85 both malaria-exposed and non-exposed individuals. Finally, we examined the phenotypic profile when  
86 antigen specific atBCs and MBCs arise in the response to vaccination. Collectively we found that even  
87 non-exposed individuals carry high numbers of cells that express an atBC transcriptomic signature. Our  
88 vaccination studies revealed that antigen-specific atBCs arise following a primary immune response  
89 and are able to respond upon secondary exposure, suggesting that atBCs may have functional role in  
90 the human humoral response.

## 91 **Results**

92

93 *Single cell RNA-seq reveals three distinct populations of antigen-experienced circulating B cells*

94

95 The initial studies focused on the transcriptional diversity of the circulating B cell populations in  
96 malaria-vaccinated and -exposed humans by single-cell RNA sequencing (scRNA-seq). Specifically,  
97 we isolated CD19<sup>+</sup>, CD20<sup>+</sup> B cells that (i) were class switched i.e. IgD<sup>-</sup> and (ii) bound specific antigens.  
98 *P. falciparum* circumsporozoite (PfCSP) specific cells were isolated from the peripheral blood of five  
99 Kenyan children 6.5 and 74 months after receiving the last dose of the CSP-based RTS,S vaccine. To  
100 further examine the response to natural exposure to malaria PfCSP specific B cells, as well as B cells  
101 specific for the *P. falciparum* merozoite surface protein-1 (PfMSP1) were also sorted from 6 adult  
102 Kenyans from an area of moderate to high malaria transmission (Figure 1A); details of study subjects  
103 are given in Table S1). Individuals in this population carry high numbers of circulating atBCs as  
104 described using the absence of CD21 and CD27 as markers (Aye et al., 2020) . We also sorted tetanus  
105 toxoid (TT) specific B cells from the adult subjects; which we have previously shown to have a more  
106 classical MBC phenotype (Aye et al., 2020). Because B cells specific for a given antigen are rare  
107 within an individual, only ~10-50 antigen specific cells could be sorted per sample. Accordingly, we  
108 used a modified version of the relatively low throughput Smart-seq2 protocol (Picelli et al., 2014) to  
109 obtain transcriptomes of the individual cells.

110

111 Following quality control steps, a total of 163 transcriptomes from the 11 individuals were obtained  
112 and pooled for analysis using the R package, *Seurat* (Butler et al., 2018). Unsupervised hierarchical  
113 clustering grouped the cells into 3 clusters (Figure 1B-C). Differentially expressed genes (DEGs) were  
114 identified for each cluster using the Wilcox test to calculate the difference between the average

115 expression by cells in the cluster against the average expression by all cells not in the cluster. DEGs  
116 with an average log-fold change higher than 0.25 were used for further analysis. Gene set enrichment  
117 analysis (GSEA) showed that cluster 1 had many DEGs associated with the atypical B cell (atBC)  
118 phenotype (Portugal et al., 2015; Sullivan et al., 2015), such as *FCRL5*, *FCRL3*, *ITGB2*, *ITGAX*,  
119 *TNFRSF1B*, *LILRB1*, *CD19* and *MS4A1* (Figure 1D; Figure S1A-B). Cluster 2 expressed the lymphoid  
120 homing gene *CCR7* and the antiproliferative *BTG1* gene (Figure 1D; Figure S1B) suggesting that this  
121 may be a quiescent resting/central B cell memory subset and therefore were classified as memory B  
122 cells (MBC), while GSEA analysis revealed that these cells had a transcriptional profile similar to  
123 naïve B cells (Figure 1D; Figure S1A). Cluster 3 expressed high levels of *CXCR3* and was found by  
124 GSEA to be somewhat enriched for genes associated with previously described activated B cells  
125 (ABCs) (Ellebedy et al., 2016) including high expression *CSK* and *CD52*, however a similar level of  
126 ABC gene enrichment was also seen in the cluster 1 (Figure 1D; Figure S1A-B) therefore we did not  
127 assign a designation to these cells.

128

129 Because cells were index sorted prior to sequencing, we could also measure the surface protein  
130 expression on each cell, allowing us to investigate the expression of CD27 and CD21, the markers  
131 traditionally used to distinguish different B cell types in humans. Strikingly, only 44.7% of cells in the  
132 atBC cluster had the CD27<sup>-</sup> CD21<sup>-</sup> phenotype typically used to describe atBCs. Similarly, only 41.2 %  
133 of cluster 3 cells had the CD27<sup>+</sup> CD21<sup>-</sup> phenotype of activated B cells. Finally, 37.5 % of MBCs were  
134 CD27<sup>+</sup> CD21<sup>+</sup> suggesting that there may be distinct transcriptional signatures that suggest greater  
135 heterogeneity than using the canonical cell surface markers used to delineate memory B cell subsets  
136 (Figure S1C-D).

137

138 We have previously reported V(D)J sequences for the adult cells reconstructed using VDJpuzzle  
139 software (Aye et al., 2020; Rizzetto et al., 2018) we further extended this analysis to cells from the  
140 children analyzed in this study to V(D)J and isotype sequence from a total of 121/163 cells. This  
141 analysis revealed that all populations, including MBCs had undergone somatic hypermutation (SHM),  
142 though levels of SHM were slightly lower in MBCs, and – consistent with previous reports (Aye et al.,  
143 2020; Murugan et al., 2018; Tan et al., 2018) – CSP-specific B cells (Figure 1E). This analysis further  
144 revealed no strong association between antibody subclass and any population of memory cells (Figure  
145 1F). In contrast when we subdivided the populations by antigen specificity we found that PfCSP-  
146 specific B cells were predominantly atBCs which may be consistent with continuous exposure to low  
147 levels of this antigen via repeated *P falciparum* infections (Figure 1G; Figure S1E). Surprisingly -  
148 given the association of malaria exposure with atypical B cells - most PfMSP1 specific cells mapped to  
149 cluster 3 rather than the atBC population; finally, Tetanus Toxoid specific cells were mostly MBCs  
150 which is consistent with the absence of ongoing antigenic exposure (Figure 1G; Figure S1E). Overall  
151 our data on antigen-specific cells enables us to identify 3 distinct populations of circulating B cells.

152

153 *High throughput single-cell analysis identifies atBC, MBC and ABC populations in both malaria-*  
154 *exposed and non-exposed donors*

155

156 We next wanted to know if the 3 subsets of circulating B cells we identified were specific to malaria or  
157 reflected B cell memory in general. Moreover, we were concerned that the association of antigen with  
158 cell populations, while striking, could be a result of cells of different specificities coming from  
159 different donors and thus our analysis might be confounded by batch effects (Figure S1E-F). Finally,  
160 we wanted to sample a larger number of cells as the relatively small number of cells analyzed may not  
161 have allowed us to discern smaller populations of B cells. We therefore used the 10x Chromium



162 platform to sequence single CD20<sup>+</sup> CD19<sup>+</sup> IgD<sup>-</sup> memory B cells, regardless of antigen specificity,  
163 sorted from the PBMCs of two non-exposed donors (Non-Exp) and two malaria-exposed (Malaria-Exp)  
164 donors (Table S1). We also included barcoded antibodies specific for CD11c, CXCR3, CD21 and  
165 CD27 to perform Cellular Indexing of Transcriptomes and Epitopes by sequencing (CITE-seq) analysis  
166 linking surface protein expression to transcriptomic data (Stoeckius et al., 2017). We chose these  
167 markers based on our Smart-seq2 experiment and to reconcile our data with established markers.  
168 Finally, we used single cell immune profiling to obtain paired heavy and light chain V(D)J chain  
169 sequences for the BCR of each individual B cell.

170

171 After quality control steps, for the malaria-exposed donors, 1448 (Malaria-Exp 1) and 5719 (Malaria-  
172 Exp 2) cells with median genes per cell of 1576 and 1652 respectively were sequenced. While in the  
173 non-exposed individuals a further 2252 (Non-Exp 1) and 3561 (Non-Exp 2) cells were sequenced with  
174 median genes per cell of 1668 and 1535 respectively. Unsupervised clustering using *Seurat* was  
175 performed on each sample to identify any non-B cell clusters, to be removed before combining samples  
176 together (Figure S2A). Strikingly, in one of the exposed individuals (Malaria-Exp 1), we identified a  
177 cluster enriched for *CD5* and *BCL2* in which all cells expressed the same heavy and light chain  
178 immunoglobulin genes (*IGHV7-81* and *IGKV1-8*). We concluded that these cells might be from a  
179 premalignant B cell clonal expansion and were subsequently removed from further analysis (Figure  
180 S2A).

181

182 Following removal of non-B cell populations, we used *Seurat's* integration feature to remove batch  
183 effects between samples and combine all 4 into one integrated dataset (Figure 2A). Unsupervised  
184 clustering was then performed on this combined dataset of 12 621 cells, which revealed 11 conserved  
185 clusters (Figure 2A; Figure S2B). Three of these clusters which were more distantly related to the

186 others appeared to correspond to naïve B cells, PCs and a population of cells expressing high levels of  
187 proliferation markers (Figure 2B; Figure S2B). The PC cluster could be discerned by the high  
188 expression of the transcription factors *XBPI*, *IRF4* and *PRDMI* (Figure S3A), which are all associated  
189 with controlling PC differentiation and maintenance (Klein et al., 2006; Reimold et al., 2001; Shaffer et  
190 al., 2002). The naïve cluster was characterized by expression of *IGHD*, as well as *BACH2* and *BTG1*  
191 (Figure S3A) which are transcriptional repressors associated with cellular quiescence (Guehenneux et  
192 al., 1997; Muto et al., 1998; Tsukumo et al., 2013). The third cluster we designated proliferating (Prol)  
193 cells, due to their high expression of *CD69*, *IRF4*, *MYC* and *CD83* (Figure S3A).

194

195 DEGs were again identified in the same manner as with the Smart-seq 2 dataset. Visual inspection of a  
196 heatmap showing the top DEGs combined with phylogenetic analysis (Figure 2B; Figure S2B)  
197 suggested that the remaining 8 clusters could be grouped into 3 distinct “superclusters”. Similar to the 3  
198 clusters identified in our Smart-seq 2 analysis, GSEA showed that the 3 superclusters identified using  
199 the 10x Chromium correspond to atBC, MBC and ABC populations (Figure 2C; Figure S3B). Notably  
200 the ABC cells identified in the 10x Chromium analysis appeared to express a stronger ABC signature  
201 than the “cluster 3” cells from the Smart-seq2 analysis. To determine the relationship between our  
202 Smart-seq 2 clusters and those found using 10x Chromium, we combined both datasets using *Seurat*'s  
203 integration command (Figure S3C-D). This integrated dataset revealed that there was general  
204 consensus between the atBC and MBC clusters identified separately using the Smart-seq 2 and 10x  
205 Chromium methodologies. However only ~20% of the “cluster 3” Smart-seq2 cells were found to be  
206 ABCs in the integrated dataset. Rather, these cells clustered more closely with the MBC1  
207 subpopulation within the MBC supercluster (Figure S3C-D).

208

209 Similar to the Smart-seq2 atBCs, cells in the 10x Chromium atBC “supercluster” showed higher  
210 expression of atypical genes such as *ITGAX*, *FCRL5*, *TBET*, *LILRB1* and *CD19* (Figure 2D; Figure  
211 S3B). The presence of the three sub-clusters (designated atBC1, atBC2 and atBC3) showed that there  
212 was nonetheless some heterogeneity within this population, such as the lower expression of *ITGAX*,  
213 *FCRL5* and *TBET* in atBC2 and almost no expression of these markers in atBC3. In agreement with  
214 observations seen in mouse models suggesting that these cells are primed for antigen presentation  
215 (Rubtsov et al., 2015), we found that cells from the atBC supercluster upregulate genes associated with  
216 antigen presentation and processing (Figure 2E). The MBC supercluster, made up of the sub-clusters  
217 designated MBC1, MBC2 and MBC3, lacked a clear core gene signature with less than 10 positively  
218 expressed DEGs suggesting that these cells were in a state of quiescence. Consistent with the idea that  
219 these cells represent a recirculating, memory population, many were found to have high mRNA  
220 expression of the lymphoid homing receptors *CCR7* and *SELL* (Figure 2F). The ABC super-cluster,  
221 made from 2 clusters (ABC1 and ABC2), had high expression of the activated B cell genes such as  
222 *CD1C* and *CSK* (Figure 2F). *CXCR3* was not highly expressed in the ABC supercluster, but rather was  
223 most abundant on the MBC1 population, which is consistent with the fact that most of the Cluster 3  
224 *CXCR3* expressing cells identified in the Smart-seq2 analysis map to this population.

225  
226 *Pseudotime Analysis reveals two distinct lineages of circulating B cells*

227  
228 To determine the lineage relationships between the distinct populations identified by unbiased  
229 hierarchical clustering we used pseudotime analysis using the R package *Monocle 3* (Trapnell et al.,  
230 2014). Visual inspection of the resulting UMAP appeared to reveal 2 distinct, major branches of  
231 circulating B cells (Figure 3A). The first branch, made up of the more “classical” MBC2 and MBC3  
232 memory populations, and ABCs, had low progression along pseudotime (Figure 3A), indicating they

233 were more closely related to naïve cells, which marked the beginning of pseudotime. Strikingly,  
234 pseudotime appeared to form a loop, indicative of cells transitioning between states, such as activated  
235 (ABC1 and ABC2) and quiescent (MBC2 and MBC3) rather than forming terminally differentiated  
236 populations. The second, more “atypical” branch consisting of the atBCs, MBC1 and some MBC3s,  
237 had progressed further along pseudotime, suggesting they had differentiated further away from naïve  
238 precursors than had the “classical” branch. Pseudotime could again be seen to form a loop, suggesting  
239 that atBCs may also fluctuate between resting (MBC1 and MBC3) and activated (the atBCs) states. The  
240 proliferating cells appeared to form their own distinct branch, or lineage, separate from either ABCs or  
241 atBC. Interestingly, The PCs appeared entirely detached from the pseudotime pathway, indicating that  
242 an intermediate PC population could not be found amongst circulatory B cells.

243

244 Similar results were obtained when we used a diffusion mapping approach to examine lineage  
245 relationships. Two distinct branches of cells could be seen diverging from a central cluster of MBCs  
246 (Figure 3B). atBCs formed the first branch with atBC2s and atBC3s predominantly found at the base of  
247 the branch, closer to the MBCs and atBC1s found at the tip. The second branch was made of ABCs at  
248 the base extending to PCs at the tip suggesting that following immunization or infection, MBCs may  
249 follow either a classical activation pathway that ultimately leads to terminally differentiated antibody  
250 secreting PCs or an “atypical” pathway, culminating in the formation of Tbet<sup>+</sup> FCRL5<sup>+</sup> CD11c<sup>+</sup> atBCs.

251

252 *Atypical B cells are represented at high frequencies in all individuals, but are not necessarily CD27<sup>-</sup>*  
253 *CD21<sup>-</sup>*

254

255 Having identified 11 populations of circulating B cells by transcriptional profiling, we verified that all  
256 populations could be found in all individuals tested, albeit in slightly different proportions (Figure 4A;

257 Figure S4). The malaria-exposed donors had high numbers of atBC1 and atBC2 populations, but  
258 surprisingly the non-exposed donors also had significant numbers of atBCs (~20%) that were largely  
259 atBC3 (Figure 4A; Figure S4B). Non-exposed donors and malaria-exposed donors alike also  
260 additionally carried high numbers of the MBC1 population (~20%) which appears related to the atBC  
261 lineage (Figure 4A; Figure S4). This number of atBCs and related cells, as identified by transcriptomic  
262 techniques, contrasts with previous flow cytometry analysis which shows that non-malaria exposed  
263 healthy donors typically carry few (generally <5%) CD27<sup>-</sup>, CD21<sup>-</sup> atypical cells (Illingworth et al.,  
264 2013; Weiss et al., 2009).

265

266 To address this discrepancy, we used CITE-seq to correlate the cell surface levels of our candidate  
267 markers CD11c and CXCR3 as well as CD27 and CD21 to our transcriptomic data for each cell and  
268 cluster (Figure 4B). CITE-seq data was exported into flow cytometry analysis software (FlowJo) for  
269 further processing and presentation. CD11c was abundant on atBC1 and atBC2 cells which are  
270 common in malaria exposed individuals but was only found at low levels on the atBC3 population  
271 which was preferentially found in the non-exposed donors (Figure 4C). Among MBC populations we  
272 found that CD11c was more abundant on the MBC1 populations that appears related to atBC lineage.  
273 Analysis of CD21 and CD27 expression showed that while atBC1 cells were almost exclusively CD21<sup>-</sup>  
274 and CD27<sup>-</sup>, the other populations of atBCs had more heterogeneous expression of these markers  
275 (Figure 4D and E). Thus, the atBC3 population appears to be a cryptic atBC population which cannot  
276 be detected via conventional flow cytometry strategies. These data may support the conclusion from  
277 our pseudotime analysis that there is a spectrum of activation within atBCs, with CD21<sup>-</sup>, CD27<sup>-</sup>,  
278 FCRL5<sup>+</sup>, CD11c<sup>+</sup> B cells representing a more activated phenotype (atBC1 and 2) while other cells  
279 become more quiescent, losing expression of these markers while retaining a core gene signature  
280 (atBC3 and MBC1).

281

282 We further used this analysis to investigate the utility of our markers for identifying MBC and ABC  
283 populations. ~50% of the MBC1 and MBC2 populations were CD27<sup>+</sup>, CD21<sup>+</sup>, but only ~20% of the  
284 ABC populations resembled the activated CD27<sup>+</sup>, CD21<sup>-</sup> phenotype. MBC3 also looked somewhat  
285 activated as ~30% of cells were CD27<sup>+</sup> CD21<sup>-</sup> (Figure 4D and E). This is consistent with the  
286 observation that this population does appear to express some activation genes, albeit at low levels  
287 (Figure 2F-Figure S2). As expected from our gene expression data CXCR3 was not a useful marker for  
288 the ABC populations, rather CXCR3 was most abundant on MBC1 cells consistent with the *CXCR3*-  
289 expressing “cluster 3” cells from the Smart-seq2 data set mapping to this population. CXCR3 was also  
290 found on the atBC2 population which is consistent with this being a marker of “activated” atBCs.  
291 Overall these data show that that CD27 and CD21 poorly mark different B cell memory subsets,  
292 however they suggest that - while imperfect - CD11c is a useful maker of the atBC lineage.

293

294 *Subsets of circulating B cells do not segregate with Ig subclass or V region usage*

295

296 In murine models different memory B cell subsets may be defined by their Ig-subclass, including in  
297 malaria infection (Krishnamurty et al., 2016; Pape et al., 2011). We therefore performed analysis of the  
298 VDJ and constant region usage of the heavy chains of our different B cells populations. The switched  
299 subclasses (IgM, IgG1, IgG2, IgG3, IgG4, IgA1, IgA2 and IgE) were found in all non-naïve B cell  
300 populations, with the exception of IgE, of which only 2 cells were found across all donors (Figure 5A).  
301 The population we identified as naïve was largely IgM<sup>+</sup> with some cells expressing IgD<sup>+</sup> further  
302 supporting this designation. In both malaria-exposed donors, IgG3 was overrepresented in the atypical  
303 memory B cell compartment, which is consistent with previous reports (Knox et al., 2017; Obeng-

304 Adjei et al., 2017). Overall, these data suggest that the transcriptional signatures are distributed across  
305 all Ig subclasses.

306

307 We further examined the variable region sequence of each cell to determine the level of SHM and  
308 clonal relationships based on identical *IGHV* CDR3's with matching *IGLV* or *IGKV*. Similar to the  
309 constant region, V region usage was similar between all donors and B cell clusters (Figure S5A-B). All  
310 clusters, with the exception of the “naïve” cells showed significant SHM, confirming that these cells  
311 were antigen experienced, post-GC B cells (Figure 5B). The degree of SHM differed significantly  
312 based on the cell population and donor (Figure 5B). Notably across all populations, non-exposed  
313 donors apparently carried lower levels of SHM than malaria-exposed donors, perhaps indicating lower  
314 lifetime pathogen burden (Figure 5B). However, most V(D)J databases are based on Europeans and  
315 may reflect allelic differences between populations of African and European ancestry. In 3/4 donors,  
316 ABC2 and PCs had higher levels of SHM compared to either atBC populations or MBC populations  
317 (Figure 5B-C), which may be consistent with these populations being related as indicated by diffusion  
318 mapping analysis (Figure 3C). Finally, 1-5% of all BCRs sequenced were shared between 2 or more  
319 cells in each sample (Figure 5D). In all individuals, expanded clones could be found, in most cases  
320 these expanded clones were found within clusters, however clones could also be found shared between  
321 clusters, including across superclusters indicating that a single clone can potentially adopt multiple cell  
322 fates (Figure 5D).

323

324 *Flow cytometry analysis reveals heterogeneity in atBC populations from malaria exposed individuals*

325

326 To extend the analysis of circulating B cells beyond the original 4 donors, we performed flow  
327 cytometry analysis of B cells from the PBMCs of 11 malaria-exposed and 7 non-exposed individuals

328 (Table S1). Because the pseudotime analysis suggested that both classical and atypical lineages can  
329 cycle between activated and resting states we also included CD71 as a marker of B cell activation  
330 (Ellebedy et al., 2016). To identify atBCs we used a panel of CD11c, FCRL5, CD27, CD21 and T-bet,  
331 though none of these markers would be likely to capture the “cryptic” atBC3 population found in non-  
332 exposed healthy adults which did not express any obvious candidate surface markers based on our  
333 transcriptomic and CITE-Seq analysis.

334

335 Consistent with our own transcriptomic and CITE-seq data as well as the data of others (Portugal et al.,  
336 2015; Weiss et al., 2009), we found that CD11c<sup>+</sup> cells were considerably enriched in malaria-exposed  
337 donors (Figure 6A-B). We were able to identify a small population (~5%) of CD11c<sup>+</sup> B cells among the  
338 non-exposed donors which were CD19<sup>hi</sup>, CD20<sup>hi</sup> and FCRL5<sup>hi</sup>, but only a few of these cells were  
339 CD21<sup>-</sup>, CD27<sup>-</sup> T-bet<sup>hi</sup> (Figure 6C-E). The CD11c<sup>+</sup> cells from malaria-exposed donors expressed high  
340 levels of T-bet and were mostly CD21<sup>-</sup> CD27<sup>-</sup> (Figure 6D-E). This supports the finding that there may  
341 be a spectrum of atBCs, which may also explain why these cells have been identified and characterized  
342 in slightly different ways in different pathologies (Jenks et al., 2018; Moir et al., 2008; Weiss et al.,  
343 2009). In both non-exposed and malaria-exposed donors around 30% of B cells were CD71<sup>+</sup> CD11c<sup>-</sup>  
344 (Figure 6A-B) which is consistent with the proportion of ABCs identified by single cell RNA-seq.  
345 However, CD71 was also expressed on a high proportion of the CD11c<sup>+</sup> B cells. This further supports  
346 the observations from the pseudotime analysis that atypical cells may fluctuate between activated and  
347 resting states. Further analysis found that these CD71<sup>+</sup> atBCs expressed high levels of CXCR3 (Figure  
348 6E), further supporting the observation from CITE-seq data that CXCR3 is a marker of activated  
349 atBCs.

350

351 *CD11c<sup>+</sup>B cells arise in the primary response and respond to booster immunization*



352

353 Our pseudotime analysis indicated that the majority of circulating B cells can be separated into two  
354 distinct lineages, classical or atypical, with cells in each lineage able to fluctuate between activated and  
355 resting states. While the diffusion map results suggest that the classical lineage gives rise to antibody  
356 secreting PCs, the role of the atypical lineage in the immune response is still not understood. In the past  
357 it has been suggested that atBC are an exhausted or dysfunctional cell population that arise following  
358 chronic antigen exposure (Portugal et al., 2015; Sullivan et al., 2015). We therefore wanted to examine  
359 a situation in which we could (i) track antigen specific cells after primary exposure and (ii) continue to  
360 follow those cells upon antigen re-exposure. We would thus be able to determine the point in an  
361 immune response at which atBCs arise as well measure their longevity and their capacity to be recalled.  
362 To meet these criteria, we tracked B cells specific for PfCSP in a cohort of malaria-naïve individuals  
363 who were given three doses of a whole *P. falciparum* sporozoite vaccine (PfSPZ) at 8-week intervals  
364 (Ishizuka et al., 2016; Lyke et al., 2017). These sporozoites are irradiated so do not establish ongoing  
365 infection, though there is previous evidence of antigen persistence (Cockburn et al., 2010). Blood  
366 samples were obtained at the time of each vaccination and 1 wk and 2 wks post each vaccination and  
367 flow cytometric analysis was performed to identify and characterize PfCSP-specific B cells (Figure 7A;  
368 Figure S6A). We were further able to identify PfCSP specific PCs in these individuals (Figure S6A).

369

370 By 1 week after immunization the number of CSP specific B cells had begun to increase, though most  
371 remained CD71<sup>-</sup>, CD11c<sup>-</sup> (Figure 7A-C). However, by 2 weeks post immunization, significant  
372 populations of CD71<sup>+</sup> and CD11<sup>+</sup> B cells could be seen, with many of the CD11c<sup>+</sup> B cells co-  
373 expressing CD71, matching those seen in malaria-exposed individuals (Figure 7A-C). By 8 weeks post  
374 immunization distinct CD71<sup>+</sup> and CD11c<sup>+</sup> B cell populations were seen, but the CD11c<sup>+</sup> B cells no  
375 longer expressed high levels of CD71 further supporting the idea that this is a marker of recent

376 activation on atBCs (Figure 7C). Importantly, both CD71<sup>+</sup> and CD71<sup>-</sup> CD11c<sup>+</sup> B cells expressed high  
377 levels of CD19, CD20 and low levels of CD21 and CD27 further supporting the fact that these cells  
378 represent a bona-fide atBC population (Figure S6B-C).

379

380 We further tracked these B cell populations following boosts after 8 and 16 weeks (Figure 7A-C).

381 There was a significant expansion of the all B cell populations at the first boost, while magnitude of the  
382 response did not change at the second boost. During the first boost the CD71<sup>+</sup> CD11c<sup>-</sup> population  
383 peaked after 1 week; more rapidly than during the primary response (Figure 7C), however the total  
384 CD11c<sup>+</sup> population continued to expand and only peaked 2 weeks after each boost. Notably the kinetics  
385 of the atBC population are distinct from those of the PC population which is only detectable 1 week  
386 after each boost, which further suggests that in normal conditions atBCs are not pre-PCs. Finally, 16  
387 weeks after the final boost (wk 34) we examined the number and phenotype of the different cell  
388 populations. At this “memory” timepoint most cells were double negative, but residual populations of  
389 CD11c<sup>+</sup> and CD71<sup>+</sup> B cells could still be detected (Figure 7A-C).

390

391 Finally, we wanted to know if our findings could extend beyond *Plasmodium* to another vaccination  
392 setting, also involving acute exposure to antigen. We therefore examined how these cells responded in  
393 a recall response to the inactivated influenza vaccine (IIV) (Koutsakos et al., 2018). Using recombinant  
394 rHA probes to two IIV antigens: A/California/07/09-H1N1 (A/Cal09-H1) or B/Phuket/3073/2013  
395 (BHA; Yamagata lineage) we were able to identify influenza specific B cells from the peripheral blood  
396 of subjects prior to immunization (Figure 7D; Figure S6D). CD71<sup>+</sup> ABCs, CD11c<sup>+</sup> atBCs and double  
397 negative MBCs to both influenza antigens could be found at baseline in most individuals as expected,  
398 reflecting past exposure to influenza virus infection or vaccination (Figure 7D-F). Following  
399 immunization, CD71<sup>+</sup> and CD11c<sup>+</sup> cells expanded alongside the double negative population. CD71<sup>+</sup>

400 CD11c<sup>+</sup> and CD11c<sup>-</sup> B cells peaked first, in this case 2 weeks after immunization particularly against  
401 the B/Phuket-HA strain, while CD11c<sup>+</sup> B cells had a more sustained expansion (Figure 7D-F). Again,  
402 influenza specific CD11c<sup>+</sup> cells found after vaccination expressed high levels of CD19, CD20 and  
403 FCRL5, and high proportions of these cells were CD21<sup>-</sup> and CD27<sup>-</sup> consistent with them being an  
404 atypical population (Figure S6E-F). All together these data reveal that atBC arise during the primary  
405 response and that these cells appear to participate in recall responses upon re-exposure to antigen,  
406 countering the suggestion that these cells are a dysfunctional or exhausted population.  
407

## 408 Discussion

409

410 Atypical B cells, which have been conventionally defined based on specific cell surface markers have  
411 been found in excess in many pathological conditions, in particular chronic or repeating infections and  
412 autoimmunity. This association with disease, along with the difficulty of re-stimulating these cells *in*  
413 *vitro* has led to the assumption that these are an exhausted or even pathological population. Our  
414 observations using single cell transcriptional analysis that atBC are more abundant than expected in  
415 both healthy non-exposed and malaria-exposed individuals lead us to test the hypothesis that these cells  
416 are a stable lineage that arise in response to antigenic stimulation. Accordingly, we tracked these cells  
417 in controlled conditions of vaccination and further showed that these cells arise during the primary  
418 immune response and can be recalled normally on multiple re-exposures. Importantly these cells are  
419 not merely recently activated B cells as they have a distinct gene signature from previously described  
420 ABCs. Thus, while - in accordance with previous literature - we have used the term “atypical” to  
421 describe this lineage, the data presented here suggests that atBCs contribute to antigen-specific primary  
422 and recall antigen specific responses, and thus are a typical part of the B cell response to antigen.

423

424 It has been hypothesized that atBCs are exhausted (Portugal et al., 2015; Sullivan et al., 2015) or  
425 recently activated cells (Jenks et al., 2018; Perez-Mazliah et al., 2018). However, our observations that  
426 atBC are generated during the primary response to sporozoite vaccination and can be recalled following  
427 booster immunizations, either to sporozoite or influenza antigens would suggest that atBC are not  
428 necessarily exhausted cells and can in fact participate in normal immune responses. Furthermore, our  
429 single cell RNA-seq data clearly differentiates atBC from previously described ABCs. Nonetheless  
430 there is also evidence that atBCs themselves can be activated which may resolve some of the  
431 discrepancies seen in the literature. Specifically, we have shown that CD71<sup>+</sup> atBC appear early in the

432 response and abate quickly, giving rise to a more conventional CD71<sup>-</sup> atBC population. These CD71<sup>+</sup>  
433 atBCs likely represent a population of recently recalled cells. Interestingly, while the first study to  
434 describe ABCs cells used CD71 as the primary marker for ABC (Ellebedy et al., 2016), CD11c<sup>+</sup> B  
435 cells, which we have shown can express CD71 were not excluded. Thus, this bulk sorted population  
436 may have contained some atBCs which may explain why in our smaller Smart-seq2 dataset, the atBC  
437 cluster was found to be enriched for ABC genes. We did however observe that the antigen-specific  
438 atBC population does diminish overtime in our sporozoite vaccinated individuals. One explanation for  
439 this is that atBCs are not a bona fide memory population that are as long-lived as the MBCs. However,  
440 the presence of a sizeable CD11c<sup>-</sup> FCLR5<sup>-</sup> CD27<sup>+</sup> atBC (atBC3) as well as MBC1 populations in  
441 healthy non-exposed individuals suggests that there may exist a pool of “cryptic”, quiescent MBCs  
442 primed to differentiate into CD11c<sup>+</sup> atBCs following re-exposure.

443

444 We initially described a tripartite division in our description of the circulating B cell subsets. This is  
445 based on the unsupervised clustering of our single cell RNA-seq datasets revealing distinct  
446 “superclusters” of circulating B cells: Activated (ABC), Memory (MBC) and Atypical (atBC).  
447 However, an alternative classification informed by pseudotime analysis and our data from vaccination  
448 cohorts would suggest a division of two distinct lineages, or pathways of MBCs and their  
449 corresponding activated populations. For example, in the classical pathway, MBC2 are quiescent cells  
450 that, following antigenic stimulation, transition into proliferating ABCs that can go on to either  
451 terminally differentiate into PCs or reseed the MBC pool. A previous study showed that up to 60% of  
452 ABC clones sequenced at day 7 could be found in the MBC subset 90 days post vaccination, revealing  
453 that ABCs can downregulate their activation markers and become MBCs over time (Ellebedy et al.,  
454 2016). Under this model the ABC2 and MBC3 populations likely represent a spectrum of cells that  
455 were either recently activated or entering a state of quiescence, rather than defined B cell fates.

456 Interestingly, our VDJ analysis revealed that B cell clones could be found in both cell lineages,  
457 indicating that the progeny of a single B cell may adopt both cell fates

458

459 While our data show that atBC are part of a normal B cell response, the function of these cells remains  
460 elusive. Early studies revealed that it was difficult to differentiate atBCs into antibody secreting cells  
461 under standard conditions *in vitro* (Moir et al., 2008; Portugal et al., 2015; Sullivan et al., 2015).

462 However bulk RNA-seq analysis of atBC-like cells from SLE patients showed higher expression of  
463 genes associated with PC maintenance in this populations lead to the hypothesis that atBCs represent a  
464 precursor PC population (Jenks et al., 2018). Our analysis however, using single cell RNA sequencing  
465 techniques, could not find any evidence of these genes being upregulated in any of our atBC  
466 populations. We also observe that our atBC population in sporozoite vaccinated individuals continues  
467 to expand even 2 weeks after booster immunization while PC populations peaked 1-week post-  
468 immunization. A reconciliation of these conflicting results may be that in pathogenic conditions such as  
469 SLE, atBCs can be driven to become pathogenic antibody secreting cells. Consistent with this the  
470 TLR7 pathway is implicated in SLE development and it has been found that including TLR7 agonists  
471 within the stimulating condition can help differentiate atBCs into PCs (Jenks et al., 2018; Perez-  
472 Mazliah et al., 2018; Rivera-Correa et al., 2019; Rubtsova et al., 2013).

473

474 A potential role for atBC may be specifically in the clearance of viral infection, as research in murine  
475 models has found that T-bet<sup>+</sup> CD11c<sup>+</sup> B cells, are required for effective clearance of viral infection  
476 (Barnett et al., 2016; Rubtsova et al., 2013). Our study has focused on relatively inflammatory B cell  
477 stimuli such as malaria infection and attenuated pathogen vaccines. It may be that atBC are  
478 preferentially formed in these conditions rather than subunit vaccination in less inflammatory adjuvants  
479 such as alum and thus would be expected to play a role in control of infection. Finally, it has been

480 proposed that atBCs are potent antigen presenting cells (Rubtsov et al., 2015). In agreement with this  
481 we did find that human atBC do appear to have higher expression of MHC II as well as the co-  
482 stimulatory molecule CD86 and components of the MHC Class II antigen processing pathway,  
483 indicating that they present more antigen than other B cell types. B cells have recently been shown to  
484 be required for the priming of Tfh cells in the context of malaria infection, though the role of  
485 individual subsets was not studied (Arroyo and Pepper, 2020). Intriguingly another mouse study also  
486 revealed that ablation of CD11c<sup>+</sup> B cells lead to the collapse of germinal centers after one  
487 immunization (Baumjohann et al., 2013). Thus, these data suggest that atBCs could represent a  
488 population of specialized antigen presenters, although further investigation is needed to confirm this  
489 hypothesis.

490

491 Here we provide an atlas of the B cell subsets circulating in human blood. Our powerful single-cell  
492 RNA-seq analysis combined with CITE-seq technologies and VDJ profiling enables us to reconcile our  
493 data with existing classifications of B cell memory based on flow cytometry markers or Ig-subclass.  
494 We have been able to resolve some key controversies in the human B cell literature, most notably by  
495 finding that atBCs are more abundant than previously expected in healthy donors and showing that  
496 these cells can be induced by primary exposure to acute antigen. Thus, our data suggests that atBC are  
497 a critical and typical component of the humoral immune response.

498 **Author Contributions**

499

500 Conceptualization (HJC, RA, AHI, FMN, IAC). Data curation (HJC, XL, TDA, FMN, IAC). Formal  
501 Analysis (HJC, RA, XL, XG, TDA, FL). Funding acquisition (KK, RAS, FMN, IAC). Project  
502 Administration (KEL, SLH) Investigation (HJS, RA, AHI, RV, EN, OK, JM, MK, THON, MN, PM,  
503 AAB, NK, SC). Methodology (AE, FL). Resources (AKW, SJK, FL, KK, RAS, FMN). Software (FL).  
504 Supervision (SJK, FL, KK, RAS, FMN, IAC). Visualization (HJS, XL, IAC). Writing – original draft  
505 (HJS, IAC). Writing – review & editing (FL, KK, RAS, FMN).

506

507

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509

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530

531

532 **Conflict of interest statement**

533

534 S.C., N.K., B.K.L.S., and S.L.H. are salaried employees of Sanaria Inc., the developer and owner of  
535 PfSPZ Vaccine and the investigational new drug (IND) application sponsor of the clinical trials. S.L.H.  
536 and B.K.L.S. have a financial interest in Sanaria Inc. All other authors declare no conflict of interest.

537 **Materials and Methods**

538

539 *Human samples and ethics statement*

540 All research was conducted according to the principles of the Declaration of Helsinki, which included  
541 the administration of informed consenting in the participant's local language. Studies in Australia were  
542 further performed in accordance with the Australian National Health and Medical Research Council  
543 (NHMRC) Code of Practice.

544

545 The malaria-immunology cohort and vaccination studies, under which the samples described were  
546 collected in Kenya, were approved by the Kenyan Medical Research Institute Scientific and Ethics  
547 Review Unit, Nairobi, and the use of these samples at the Australian National University was further  
548 approved by the Australian National University Human Research Ethics Committee (protocol number  
549 2014/102). The Kenyan adults are members of the KEMRI/Wellcome Research Programme's  
550 longitudinal malaria immunology cohort studies in Junju and Ngerenya villages (supplementary table  
551 1), 20 km apart from each other in Kilifi, Kenya.. In addition, we included samples from the  
552 RTS,S/AS01 phase 3 clinical trial. Blood was also drawn from healthy control Australian donors who  
553 were recruited at the Australian National University.

554

555 VRC 314 clinical trial (<https://clinicaltrials.gov/>; [NCT02015091](https://clinicaltrials.gov/ct2/show/study/NCT02015091)) was an open-label evaluation of the  
556 safety, tolerability, immunogenicity and protective efficacy of PfSPZ Vaccine. Subjects in the high  
557 dose cohort received a total of three doses of  $9 \times 10^5$  PfSPZ intravenously at week 0, 8 and 16. Blood  
558 was drawn at the time of each immunization, as well as 7d and 14 d after each immunization. Plasma  
559 and PBMCs were isolated from all samples at these timepoints. Full details of the study are described  
560 in (Lyke et al., 2017).

561

562 The investigation of B cell responses after IIV immunization was approved by the University of  
563 Melbourne Human Ethics Committee (ID 1443389.3) and the Australian Red Cross Blood Service  
564 (ARCBS) Ethics Committee (ID 2015#8). PBMCs were used from 8 donors taken on the day of IIV  
565 immunization, as well as 14 and 28 days later. Full details of the study are described previously  
566 (Koutsakos et al., 2018).

567

#### 568 *Flow cytometry*

569 For samples from Kenya and the IIV cohort PBMCs were thawed and washed in PBS with 2% heat-  
570 inactivated FBS. Cells were then stained with Live/Dead dye for 5 min in PBS before incubation with  
571 fluorescently labelled antibodies for a further 30 min. Details of all antibodies used are given in Table  
572 S2. Flow-cytometric data was collected on a BD Fortessa or X20 flow cytometer (Becton Dickinson)  
573 and analyzed using the software FlowJo (FlowJo). A BD FACs Aria I or II (Becton Dickinson) was  
574 used for sorting cells.

575

576 For VRC314 clinical trial specimens PBMCs were thawed into prewarmed RPMI media then washed  
577 with PBS. Cells were stained with Live/Dead dye for 15 minutes, washed in PBS with 2% heat  
578 inactivated FBS, and labelled with antibodies for an additional 30 minutes. Labelled cells were washed  
579 with PBS 2% FBS and fixed for 15 minutes in 0.5% PFA before and final wash and resuspension in  
580 PBS 2% FBS. Flow-cytometric data was collected on a BD X50 flow cytometer (Becton Dickinson)  
581 and analyzed using the software FlowJo (FlowJo).

582

#### 583 *Tetramer Preparation*

584 *Pf*MSP1, AMA1 and TT were biotinylated with the Sulfo-NHS-LC-Biotinylation Kit (ThermoFisher)  
585 at a ratio of 1:1 according to the manufacturer's instructions, biotinylated (NANP)<sub>9</sub> repeat region of *Pf*  
586 CSP was sourced from Biomatik (Ontario, Canada). Biotinylated antigens were incubated with  
587 premium-grade SA-PE and SA-APC (Molecular Probes) or SA-BV421 and SA-BB660 (Biolegend and  
588 BD Horizon) at a molar ratio of 4:1, added four times with 15 min incubation at room temperature.

589

#### 590 *Single cell RNA-seq using Smart-seq2*

591 Antigen-specific single cell RNA sequencing was performed using a Smart-seq 2 protocol (Picelli et  
592 al., 2014) with the following modifications. Cells were sorted into plates with wells containing 1 µl of  
593 the cell lysis buffer, 0.5 µl dNTP mix (10 mM) and 0.5 µl of the oligo-dT primer at 5 µM. We then  
594 reduced the amount reagent used in the following reverse transcription and PCR amplification step by  
595 half. The concentration of the ISPCR primer was also further reduced to 50 nM. Due to the low  
596 transcriptional activity of memory B cells, we increased the number of PCR cycles to 28. cDNA was  
597 then purified with AMPure XP beads at a bead to sample ratio of 0.8:1. Sequencing libraries were  
598 prepared using the Nextera XT Library Preparation Kit with the protocol modified by reducing the  
599 original volumes of all reagents in the kit by 1/5<sup>th</sup>. Another round of bead cDNA bead purification was  
600 performed using a bead to sample ratio of 0.6:1. Sequencing was performed on the Illumina NextSeq  
601 sequencing platform. Following sequencing, fastq files were passed through the program VDJpuzzle  
602 (Rizzetto et al., 2018) where reads were trimmed using Trimmomatic, then aligned to the human  
603 reference genome GRCh37 using tophat2. Gene expression profiles were then generated using  
604 cufflinks 2. As a further QC step, cells where reads were mapped to less than 30% of the reference  
605 genome were removed. All following downstream analysis for transcriptomic data was performed  
606 using *Seurat*. Details of all key reagents for single cell RNA-seq are given in Table S3.

607

608 *Single cell RNA-seq and CITE-seq using 10x Chromium*

609 Post-sorting, CD19<sup>+</sup> CD20<sup>+</sup> IgD<sup>-</sup> B cells were incubated with Total-seq C antibodies (Biolegend) for 30  
610 min and washed 3 times. The number of cells were then counted and 14 000 cells per sample were run  
611 on the 10X Chromium (10X Genomics). Library preparation was completed by Biomedical Research  
612 Facility (BRF) at the JCSMR following the recommended protocols for the Chromium Single Cell 5'  
613 Reagent Kit as well as 5' Feature Barcode and V(D)J Enrichment Kit for Human B cells. Libraries  
614 were sequenced using the Illumina NovaSeq6000 (Illumina). The 10X Cell Ranger package (v1.2.0,  
615 10X Genomics) was used to process transcript, CITE-seq and VDJ libraries and prepare them for  
616 downstream analysis. Details of all key reagents for single cell RNA-seq are given in Table S3.

617

618

619 **Quantification and statistics**

620

621 *Single Cell RNA-seq analysis*

622 The package *Seurat* (version 3.1) (Butler et al., 2018) was used for graph-based clustering and  
623 visualizations. All functions described are from *Seurat* or the standard R package (version 3.60) using  
624 the default parameters unless otherwise stated. Each sample was initially analyzed separately using the  
625 following procedures. Cells that expressed less than 200 genes and genes that were expressed in less  
626 than 3 cells were excluded, along with cells that had greater than 10% mitochondrial genes. Gene  
627 expression was normalized for both mRNA and CITE-seq assays using the `NormalizeData` function,  
628 then the 2000 most variable genes for each sample were identified using `FindVariableFeatures`. Next  
629 expression of all genes was scaled using `ScaleData` to linearly regress out sources of variation.  
630 Principal component analysis on the variable genes identified above was then run with `RunPCA`. Based  
631 on `ElbowPlot` results we decided to use 13, 20, 12 and 20 principal components (PCs) for the clustering

632 of samples Non-Exp 1, Non-Exp 2, Exp 1 and Exp 2 respectively using FindNeighbours. FindClusters  
633 was then run to identify clusters for each sample, using the resolutions .3, .4, .5 and .4 respectively.  
634 FindAllMarkers was then used to identify clusters of non-B cells. The remaining cells in each sample  
635 were then normalized and scaled again as above. Australian and Kenyan samples were combined  
636 together first using FindIntegrationAnchors and then Intergratedata to create two combined datasets, 1  
637 with both non-exposed samples and one with both malaria-exposed samples. These two combined  
638 samples were further combined using the commands to from one combined dataset containing all 4  
639 samples. The combined dataset was then scaled again as above and a PCA was run. Using FindClusters  
640 with a resolution of 0.8, we identified our 11 clusters. DEGs were identified using FindAllMarkers.  
641 The clustering was visualized with Uniform Manifold Approximation and Projection (UMAP)  
642 dimensionality reduction using RunUMAP and plotted using DimPlot with umap as the reduction.  
643 Phylogentic analysis was done using BuildClusterTree to report the hierarchical distance matrix  
644 relating an ‘average’ cell from each cluster. Log-normalized gene expression data was visualized using  
645 violin plots (VlnPlots) as well as onto -UMAP plots (FeaturePlot). Heatmaps were generated using  
646 DoHeatmap.  
647 For Smart-seq2 analysis, cells with greater than 10% mitochondrial genes were not excluded. 8 PCs  
648 were used as determine by ElbowPlot. For clustering a resolution of 0.8 was used.

649

#### 650 *Diffusion Map Analysis*

651 To create the diffusion map we utilized the R package *destiny* (Angerer et al., 2016). Our Seurat object  
652 was converted into a SingleCellExperiment object using as.SingleCellExperiment. The diffusion map  
653 was then generated using *Destiny*'s DiffusionMap command.

654

#### 655 *Pseudotime Analysis*

656 Pseudotime analysis was performed using the R package *Monocle 3*. Our Seurat Object was converted  
657 into monocle3 main data calls `cell_data_set`. The default *Monocle 3* workflow was then followed.

658

#### 659 *Gene Set Enrichment Analysis (GSEA)*

660 GSEA was done using javaGSEA through the Broad Institute. For each comparison, DEGs were  
661 ranked by log-fold change and pre-ranked analysis using 1000 permutations was used to examine  
662 enrichment in selected gene sets (SUPP table?).

663

#### 664 *VDJ Analysis*

665 To determine the antigen-specific BCR repertoire, we made use of VDJpuzzle (Rizzetto et al., 2018) to  
666 reconstruct full-length heavy and light chains from each cell from our Smart-seq2 dataset. From this we  
667 were able to determine V region usage and mutation frequency.

668 VDJ sequences from the 10x dataset were obtained using the `cellranger vdj` command. From this  
669 output, V region usage and mutation frequency could be determined.

670

#### 671 *Statistical Analysis*

672 Statistical analysis of flow cytometry data was performed in GraphPad Prism for simple analyses  
673 without blocking factors; all other analyses was performed in R (The R Foundation for Statistical  
674 Computing) with details of statistical tests in the relevant figure legends. Abbreviations for p values are  
675 as follows:  $p < 0.05 = *$ ,  $p < 0.01 = **$ ,  $p < 0.001 = ***$ ,  $p < 0.0001 = ****$ ; with only significant p  
676 values shown.

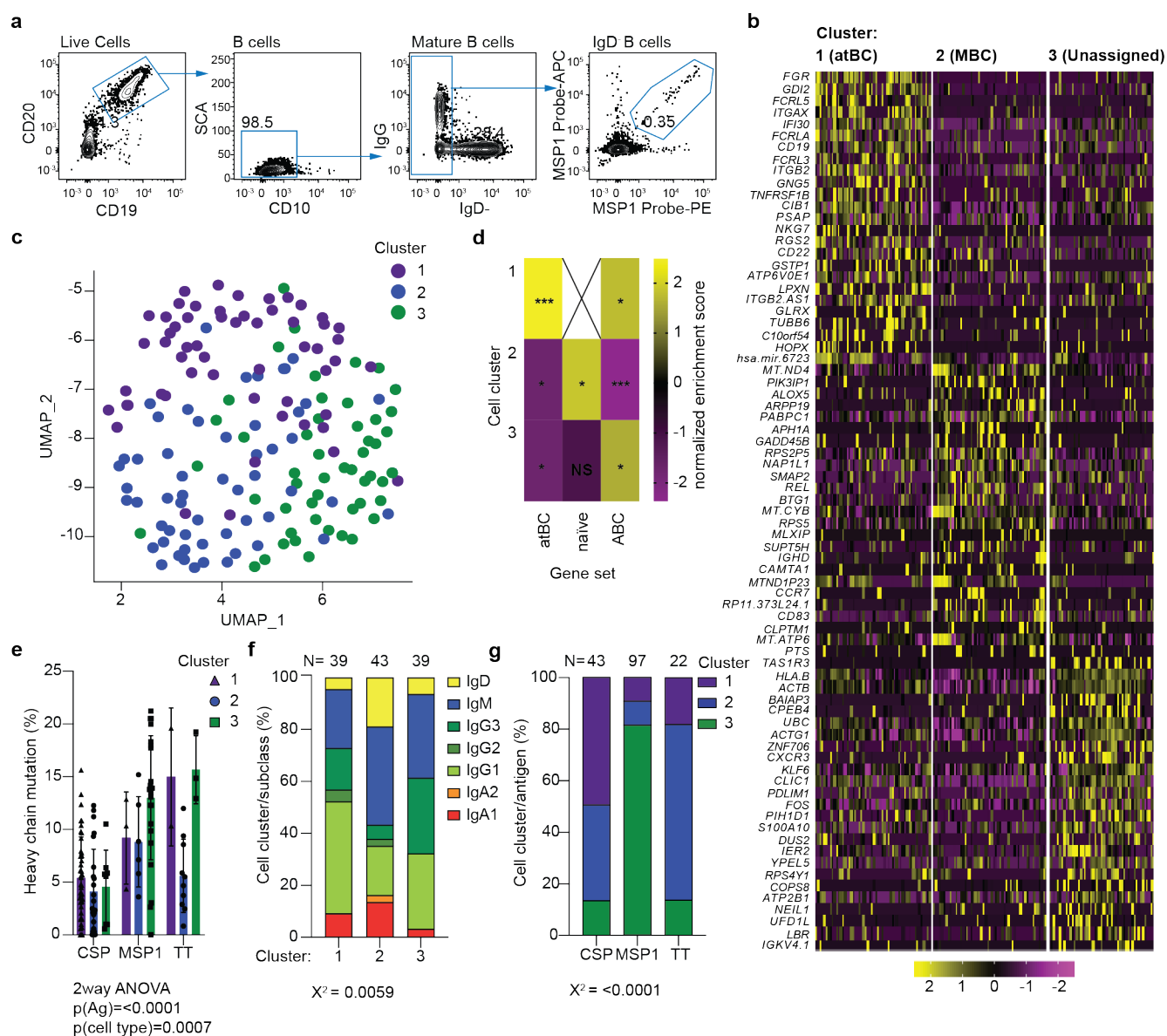
677

#### 678 *Data Deposition*

679 Single cell RNA-seq data are deposited at NCBI BioProject accession number PRJNA612353:

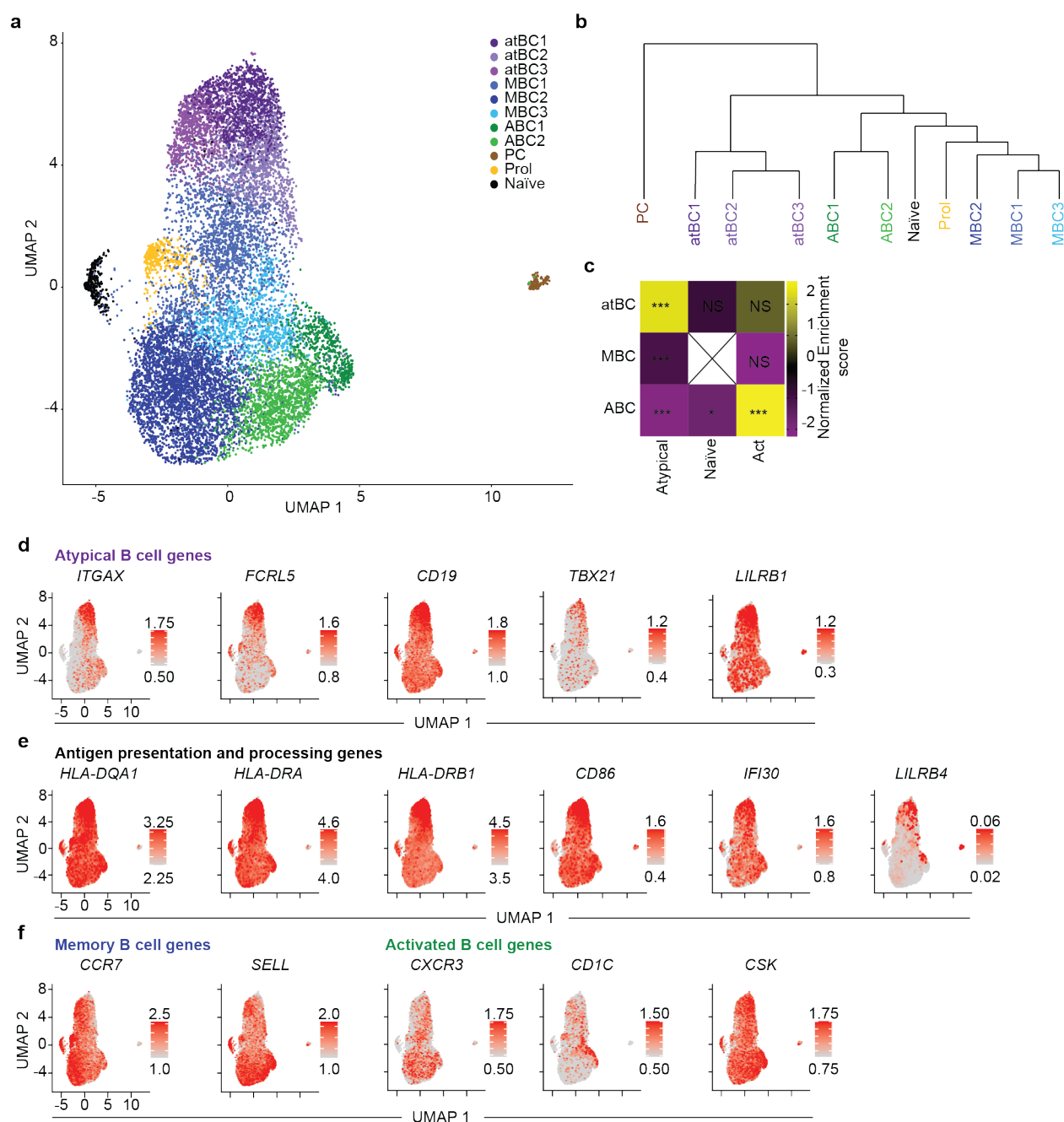
680 <https://dataview.ncbi.nlm.nih.gov/object/PRJNA612353?reviewer=bf7ee45b186vstua0d23qud1nk>





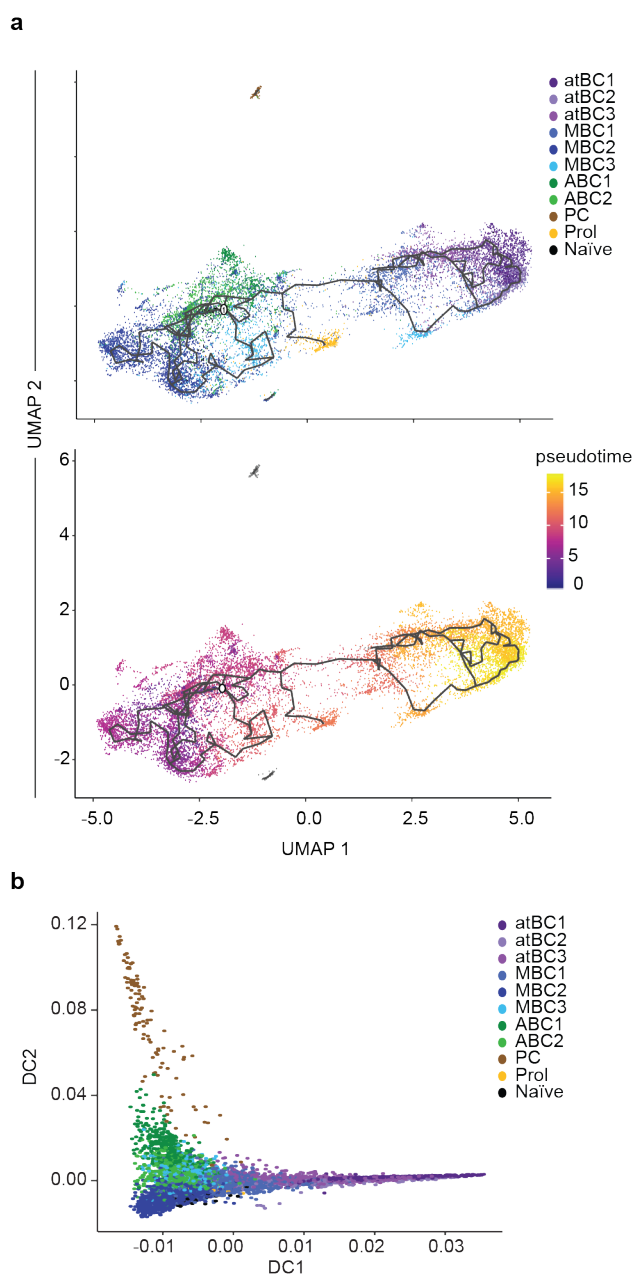
681

682 **Figure 1: Three distinct populations of antigen-experienced B cells revealed by single cell RNA-**  
 683 **seq** CSP, MSP1 and TT specific B cells were index, single cell sorted from malaria vaccinated and  
 684 exposed donors; transcriptomic information for each cell was generated using Smart-seq2  
 685 methodology. **A.** Representative flow cytometry plots showing the gating strategy used to sort mature  
 686 IgD<sup>-</sup> antigen-specific B cells. **B.** Heatmap showing the expression of the top 25 DEGs (row)  
 687 for each cell (column). **C.** Unsupervised clustering of circulating antigen-specific B cells  
 688 visualized using UMAP. Each point represents a cell and is colored by cluster. **D.** Heatmap  
 689 displaying the normalized enrichment scores of multiple GSEA comparing each cluster vs  
 690 previously published gene sets from atBCs, naïve B cells and ABCs **E.** Percentage of  
 691 antigen-specific cells that were found in each cluster, analysis was by chi-squared test on  
 692 the absolute values which are given above each bar. **F.** Percentage of antibody isotype  
 693 usage by each cluster, analysis was by chi-squared test on the absolute values which are  
 694 given above each bar. **G.** Percentage of mutations found in the heavy chain V(D)J  
 695 region of each antigen-specific cell per cluster, analysis was by 2-way ANOVA including  
 696 each subject as a blocking factor, bars represent mean  $\pm$  s.d.. Where the exact p value is  
 not quoted \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ .

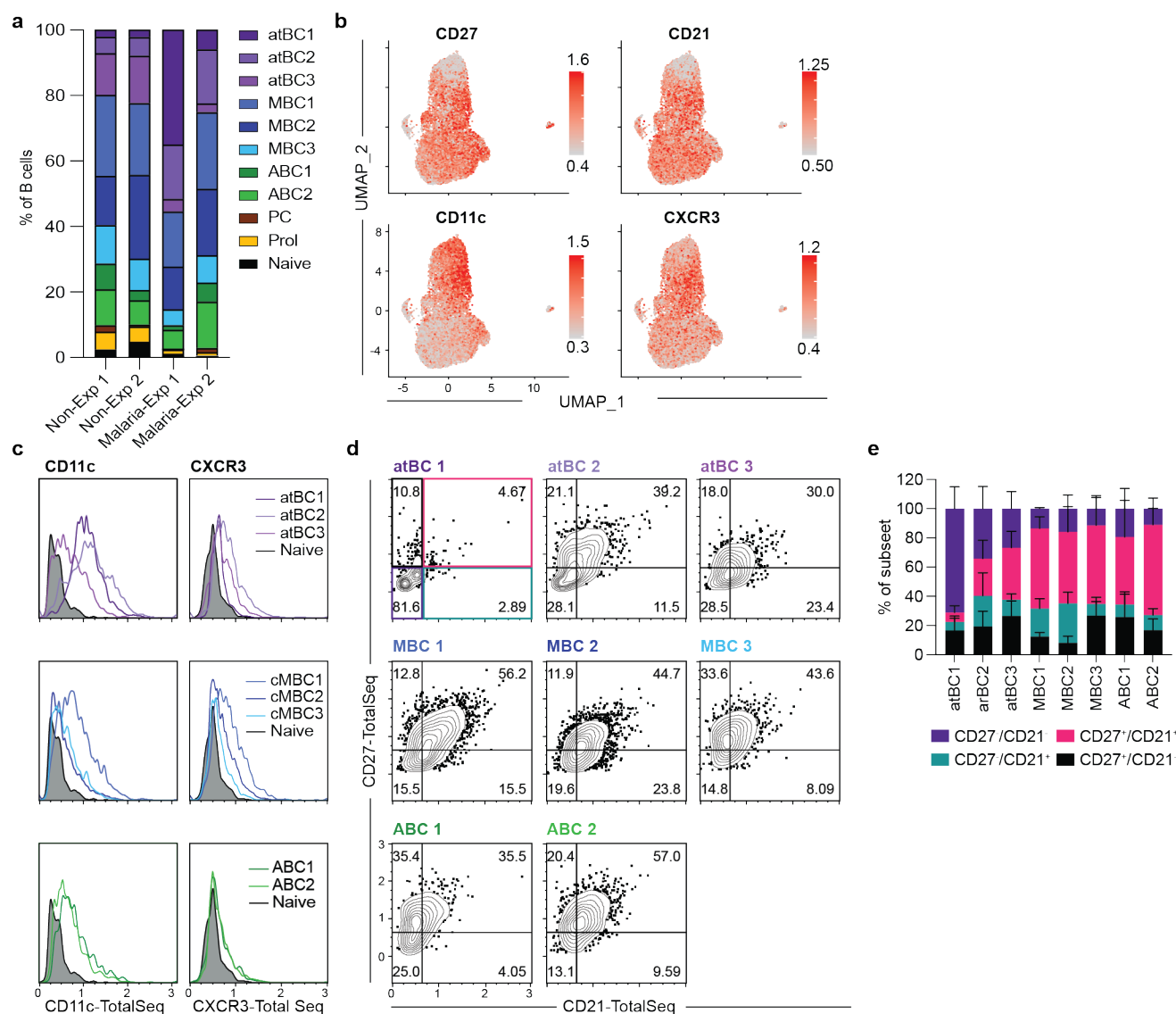


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698 **Figure 2: High throughput single cell analysis of reveals the full diversity of circulating B cell**  
 699 **populations.** Single B cells were sorted from 2 malaria exposed Kenyan individuals and 2 Australian  
 700 individuals and gene expression was assessed using 10x chromium methodology **A.** Unsupervised  
 701 clustering of circulating mature IgD<sup>-</sup> B cells pooled from all individuals visualized using UMAP. Each  
 702 cell is represented by a point and colored by cluster. **B.** Phylogenetic tree based on the ‘average cell’  
 703 from each cluster showing relationships in gene expression patterns between clusters. **C.** Heatmap  
 704 displaying the normalized enrichment scores of multiple GSEA comparing each cluster against  
 705 previously published gene sets. **D, E & F.** Expression of atBC (D), antigen presentation (E), MBC and  
 706 ABC (F) genes projected onto UMAP plots. Color was scaled for each marker with highest and lowest  
 707 log-normalized expression level noted. Where the exact p value is not quoted \* p<0.05, \*\*p<0.01,  
 708 \*\*\*P<0.001.



710 **Figure 3: Pseudotime analysis and diffusion mapping reveal 2 distinct lineages of circulating B**  
711 **cells. A.** Pseudotime analysis of circulating B cells generated visualized using UMAP. Each point  
712 represents a cell and is colored by cluster or progression along pseudotime. **B.** Diffusion map showing  
713 diffusion components (DC) 1 and 2, each cell is represented by a point and colored by cluster.



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**Figure 4: CITE-Seq analysis reveals a cryptic population of atBCs found predominantly in**

**Australian individuals** CITE-seq analysis to correlate expression of cell surface markers with gene-

expression was performed on cells from the four donors described in figure 2 **A**. Percentage of cells

from each individual found in each cluster. **B**. Surface protein expression measured by CITE-seq

projected onto UMAP plots. Color was scaled for each marker with highest and lowest log-normalized

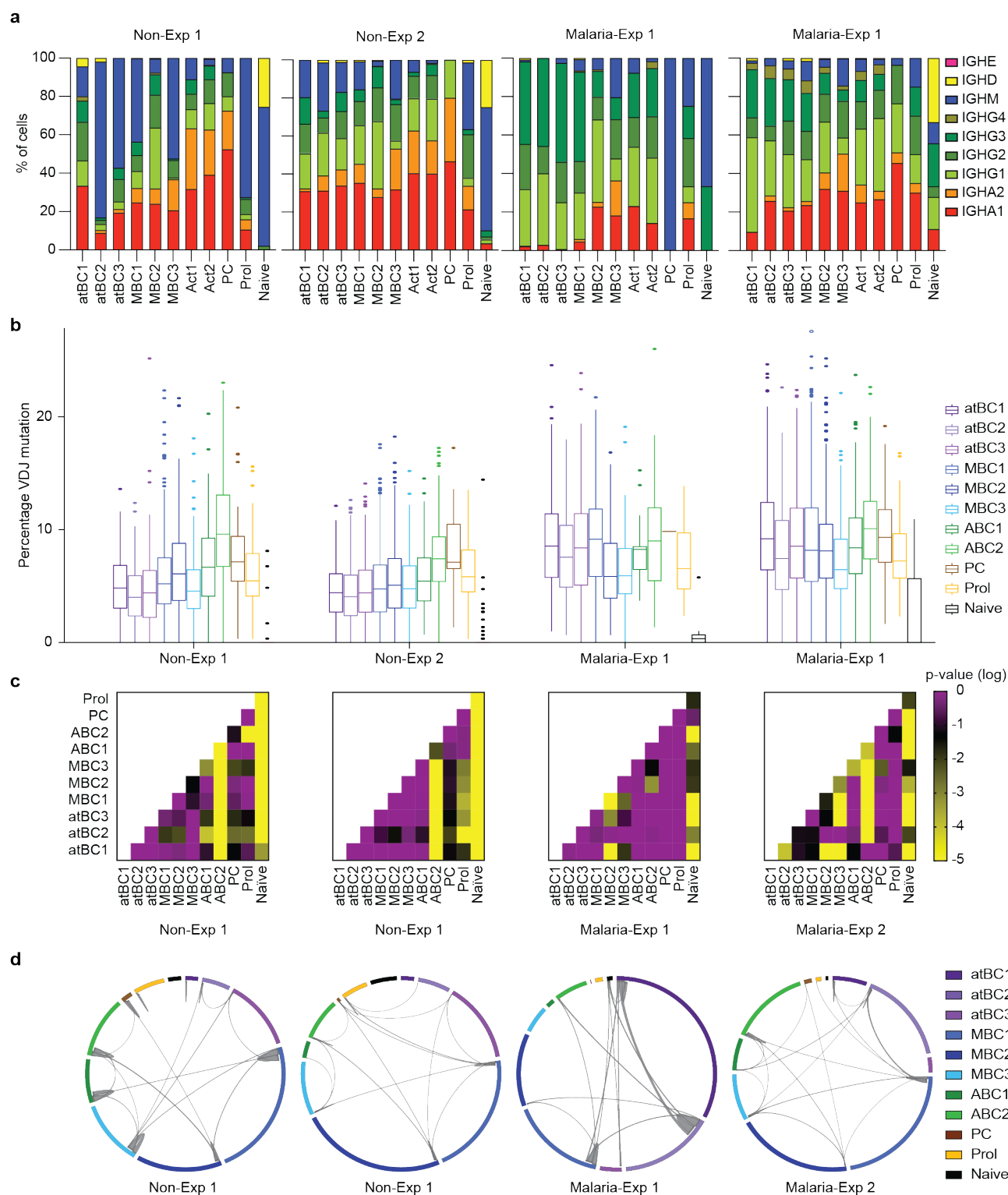
expression level noted. **C**. Histogram plots showing the expression of CD11c and CXCR3 for the

different atBC, MBC and ABC clusters, grey histogram represents expression on naïve B cells; data are

concatenated from all individuals. **D**. Contour plots showing the expression of CD27 and CD21 as

measured by CITE-seq; data are concatenated from all individuals **E**. Quantification of (D), data show

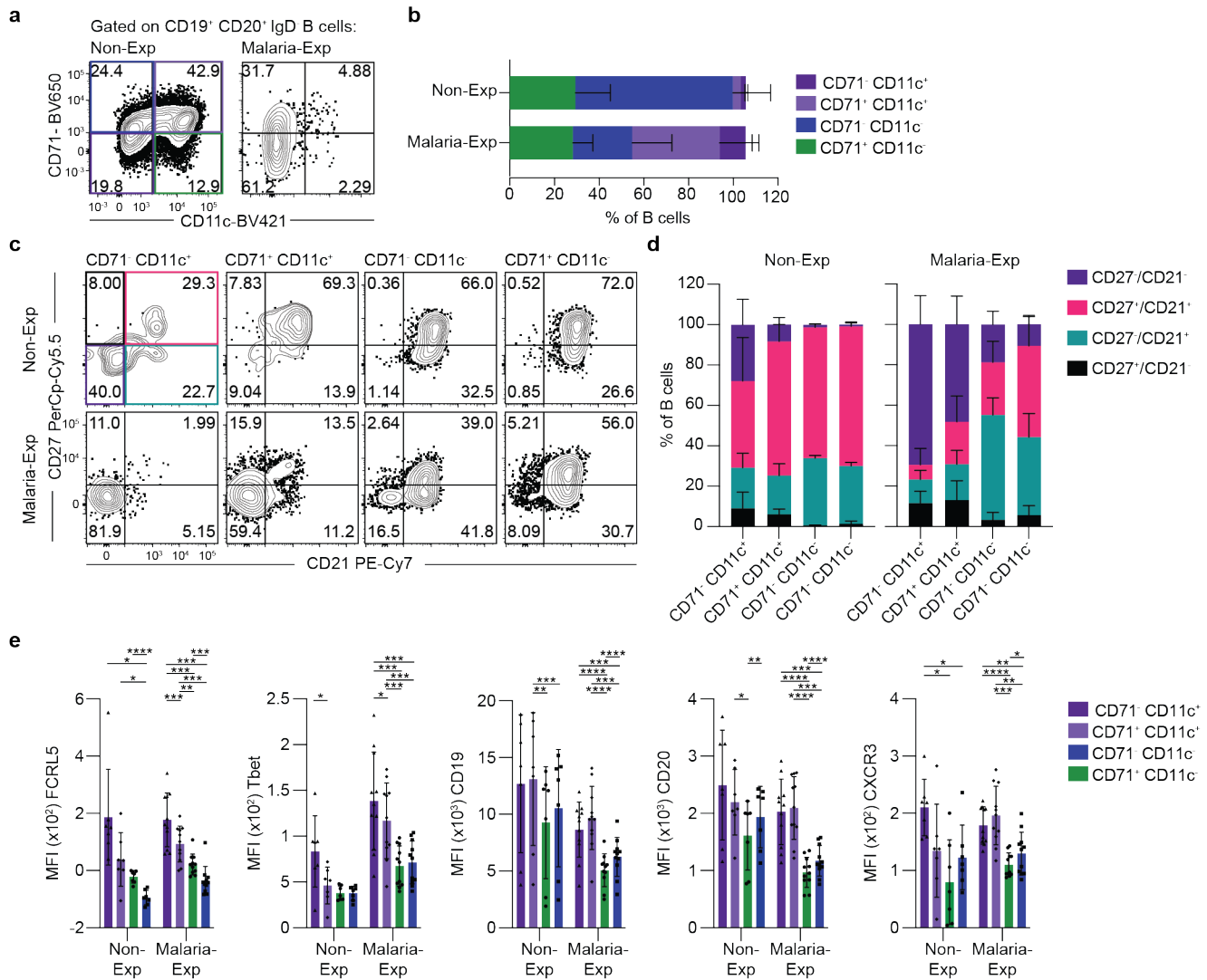
the mean proportion per individual  $\pm$  s.d..



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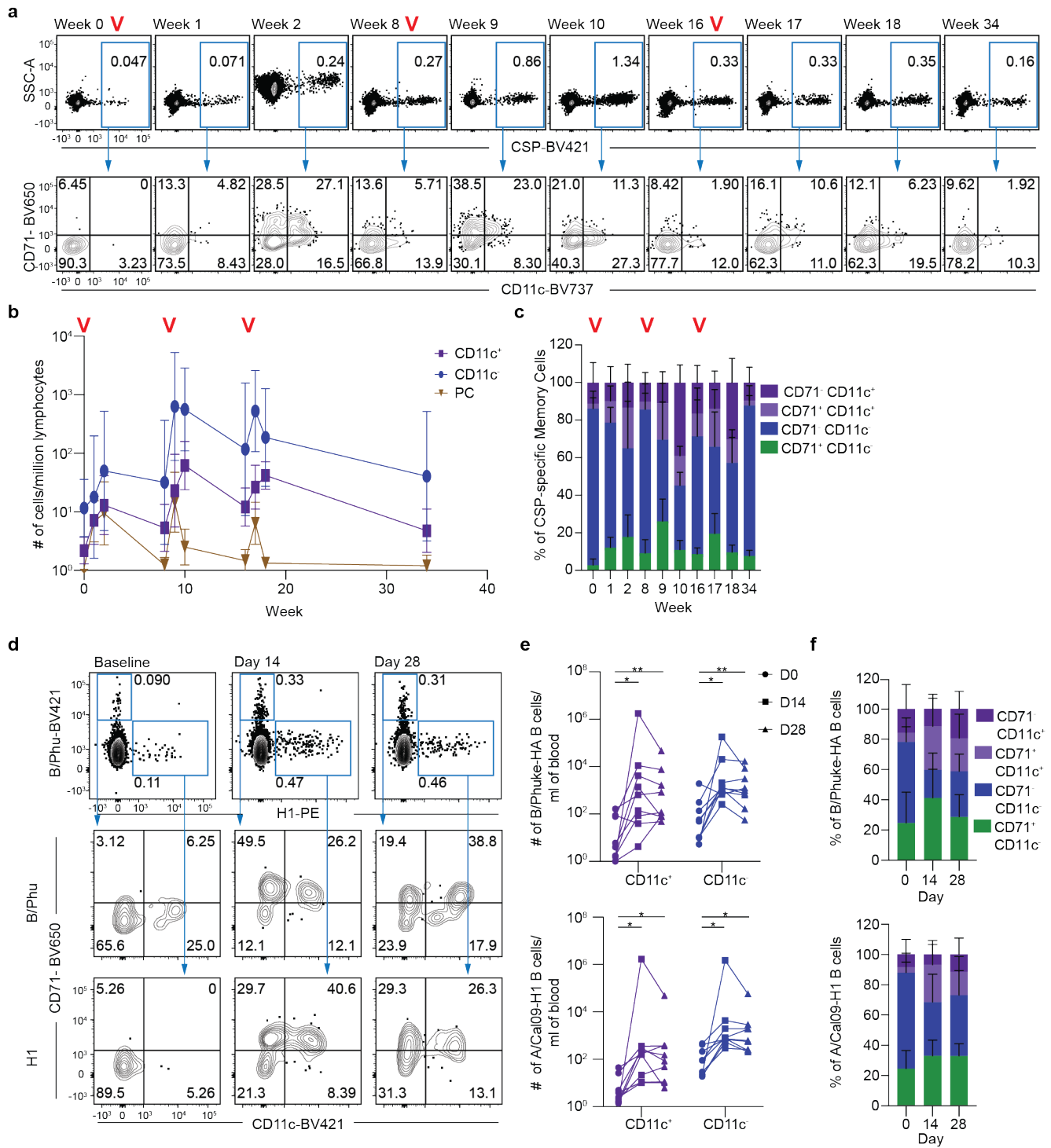
726 **Figure 5: Lack of association between BCR variable and constant regions with different B cell**  
 727 **subsets** V(D)J and constant region sequences for each cell from each donor described in figure 2 were  
 728 mapped to the individual transcriptomes and relationships analyzed **A.** percentage of isotype usage for  
 729 each cluster per individual. **B.** Percentage of mutations found in the heavy chain V(D)J region of cells  
 730 for each cluster in each donor, mean  $\pm$  s.d. shown. **C.** Heatmaps displaying the pairwise p-values from  
 731 Tukey's post-tests based on one-way ANOVA of the data in (B) to determine the association between

732 cell type and mutation frequency with subclass and individual also included in the model as fixed  
733 factors. **D.** Circos plots showing clonal B cell populations per individual, the thickness of the lines  
734 between or within clusters denotes the number of cells that belong to shared/expanded clones.



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736 **Figure 6: CD11c and CD71 identify atBC, ABCs and MBCs via flow cytometry** PBMCs from 7  
 737 Non-Exp and 11 Malaria-Exp donors were isolated and analyzed by flow cytometry for expression of  
 738 markers associated with different B cell populations **A.** Flow cytometry plots from representative  
 739 individuals showing the CD11c and CD71 expression on mature IgD<sup>-</sup> B cells. **B.** Quantification of (A)  
 740 showing the percentage of cells found in each cell type by country, bars represent mean  $\pm$ s.d.. **C.**  
 741 Representative flow cytometry plots showing the expression of CD27 and CD21 per cell type. **D.**  
 742 Quantification of (C) showing the percentage of cells separated by expression of CD27 and CD21  
 743 found in each cell type, bars represent the mean proportion  $\pm$ s.d.. **E.** The expression of surfaces  
 744 markers on each cell type, measured by MFI, analysis was done using 2-way ANOVA, bars represent  
 745 mean  $\pm$ s.d..



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747 **Figure 7: Antigen-specific atBCs arise during the primary response and can be effectively**  
 748 **recalled. A.** 15 individuals were vaccinated with 3 doses of  $9 \times 10^5$  PfSPZ at 8-week intervals, with  
 749 blood drawn at the indicated timepoints; panel shows representative flow cytometry plots from a single  
 750 individual of the gating of CSP-specific IgD<sup>-</sup> B cells and the CD71 and CD11c expression found in  
 751 these cells over time. Red “V”s indicate time points where booster immunizations were given. **B.**  
 752 Kinetics of the CSP-specific B cell response quantified by the number of cells per million lymphocytes.  
 753 **C.** The percentage of CSP-specific memory cells divided by CD11c and CD71 expression over time **D.**



754 9 individuals were vaccinated with inactivated influenza vaccine (IIV) with blood drawn at baseline  
755 and 14 or 28 days later; panel shows representative flow cytometry plots showing the number of either  
756 B/Phuket or H1-specific IgD<sup>+</sup> B cells and the CD71 and CD11c expression found in these cells over  
757 time. **E.** Kinetics of the influenza-specific B cell response quantified by the number of cells per mL of  
758 blood. **F.** The percentage of influenza-specific memory cells divided by CD11c and CD71 expression.

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