

HIV causes global B-cell dysregulation and restricts HBV-specific B-cell development in an incident HBV cohort

Katherine Cascino, Thomas Liechti, Eric C. Seaberg, Kathleen E. Stevens, Steven M. Wolinsky, Mallory D. Witt, Robbie B. Mailliard, Mario Roederer, Justin Bailey, Chloe L. Thio, Andrea L. Cox

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Background. Functional B cell responses for both prevention and control of hepatitis B virus (HBV) infection remain poorly understood, including in the context of HBV/HIV co-infection. **Methods.** Here, we employed high-dimensional single cell analysis to assess global and hepatitis B surface antigen (HBsAg)-specific B cells in a longitudinal cohort of incident HBV from the Multicenter Aids Cohort Study (MACS), with a subset of the cohort living with HIV-1. **Results.** We observed that prior HIV infection has negative consequences for B cell function in early post-acute HBV infection, including increased frequencies of atypical memory (AtM) B cells and regulatory B cells (B_{regs}), expression of the activation marker CD86 on multiple B cell subsets in chronic HBV (CHB), and restricted expansion of HBsAg-specific B cells. In contrast, in HBV mono-infection, we observed no changes in the global B cell population from prior to infection and robust expansion of HBsAg-specific B cells. These expanded antigen-specific B cells resembled class-switched intermediate and resting memory (IM and RM) B cells, with activation phenotypes that may contribute to ongoing HBV control. **Conclusion.** HIV infection has a significant impact on B cell responses to subsequent HBV infection that may promote development of CHB in HBV/HIV co-infection. **Funding.** National Institute of Allergy and Infectious Diseases, Bill & Melinda Gates Foundation.

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4

5 **Authors**

6 Katherine Cascino^{1*}, Thomas Liechti^{2*}, Eric C. Seaberg³, Kathleen Stevens¹, Steven M.
7 Wolinsky⁴, Mallory D. Witt⁵, Robbie B. Mailliard⁶, Mario Roederer², Justin Bailey¹, Chloe
8 L Thio^{1‡}, Andrea Cox^{1‡}

9

10 ¹Department of Medicine, Johns Hopkins University School of Medicine, Baltimore,
11 Maryland, USA.

12 ²ImmunoTechnology Section, Vaccine Research Center, NIAID, NIH, Bethesda,
13 Maryland, USA.

14 ³Department of Epidemiology, Johns Hopkins University, Baltimore, Maryland, USA.

15 ⁴Division of Infectious Diseases, Department of Medicine, Feinberg School of Medicine,
16 Northwestern University, Chicago, Illinois, USA.

17 ⁵Lundquist Research Institute at Harbor-UCLA Medical Center, Torrance, CA, USA.

18 ⁶Department of Medicine, University of Pittsburgh School of Medicine, Pittsburgh,
19 Pennsylvania, USA.

20

21 ‡L's present address is: Section of Immunology, Division of Infection Control, Norwegian
22 Institute of Public Health, Oslo, Norway.

23

24 *These authors contributed equally

25 †Corresponding authors contributed equally: Chloe Thio, email: cthio@jhmi.edu; Andrea
26 Cox, email: acox@jhmi.edu. 855 N. Wolfe Street, Rangos Building, Suite 520, Baltimore
27 MD 21205. Phone: 410-502-3717.

28

29 The authors have declared that no conflict of interest exists.

30

31

32 **Abstract**

33

34 **Background.** Functional B cell responses for both prevention and control of hepatitis B
35 virus (HBV) infection remain poorly understood, including in the context of HBV/HIV co-
36 infection.

37 **Methods.** Here, we employed high-dimensional single cell analysis to assess global and
38 hepatitis B surface antigen (HBsAg)-specific B cells in a longitudinal cohort of incident
39 HBV from the Multicenter Aids Cohort Study (MACS), with a subset of the cohort living
40 with HIV-1.

41 **Results.** We observed that prior HIV infection has negative consequences for B cell
42 function in early post-acute HBV infection, including increased frequencies of atypical
43 memory (AtM) B cells and regulatory B cells (B_{regs}), expression of the activation marker
44 CD86 on multiple B cell subsets in chronic HBV (CHB), and restricted expansion of
45 HBsAg-specific B cells. In contrast, in HBV mono-infection, we observed no changes in
46 the global B cell population from prior to infection and robust expansion of HBsAg-specific
47 B cells. These expanded antigen-specific B cells resembled class-switched intermediate
48 and resting memory (IM and RM) B cells, with activation phenotypes that may contribute
49 to ongoing HBV control.

50 **Conclusion.** HIV infection has a significant impact on B cell responses to subsequent
51 HBV infection that may promote development of CHB in HBV/HIV co-infection.

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53 Foundation.

54 **Introduction**

55

56 After infection with hepatitis B virus (HBV), 95% of immunocompetent adults control the
57 infection and develop protective antibodies, whereas the remaining 5% develop chronic
58 HBV (CHB), a leading cause of liver disease and hepatocellular carcinoma (HCC)
59 globally. B lymphocytes play a crucial role in the prevention and control of HBV infection
60 through diverse effector functions, including the secretion of antibodies and cytokines (1).
61 Antibodies to the HBV surface antigen (anti-HBsAg) indicate control of an acute infection
62 and protect against infection when generated through vaccination (2, 3). Further, B cell-
63 depleting immunotherapy can lead to HBV reactivation even years after recovery from an
64 acute infection, highlighting the importance of B cells in maintaining control (4–6). Thus,
65 B cells are considered a target for generating a functional cure. However, there is a limited
66 understanding of what constitutes an effective B cell response to HBV.

67

68 There is some evidence of perturbations in the global B cell population in CHB, including
69 increased proportions of activated CD69⁺CD73⁺ B cells (7, 8) and atypical memory (AtM)
70 B cells, a CD21⁻CD27⁻ B cell population often associated with chronic antigenic
71 stimulation (9, 10). Expansion of these populations has negative consequences, including
72 hyperactivation and functional impairment thought to contribute to poor viral clearance.
73 Furthermore, B_{regs} emerge in CHB and are associated with increased HBV replication (11,
74 12). However, insights into the functional role of B cells in an effective immune response
75 to acute hepatitis B may be better gleaned from examining B cells early (prior to CHB
76 development) and those specific for HBsAg. Peripheral HBsAg-specific B cells are

77 present at similar proportions in acute, chronic and controlled infection, on HBV treatment,
78 and in vaccinated controls (9, 10, 13–15). However, in people with CHB, HBsAg-specific
79 B cells have decreased proliferative capacity and higher proportion of AtM (mean 30% of
80 HBsAg-specific population), with increased levels of inhibitory markers including PD-1,
81 BTLA, CD22 and FcRL5 (9, 10, 14, 15). While previous studies begin to define
82 phenotypes of HBsAg-specific B cells in CHB, it is not known if these phenotypic changes
83 result from chronic infection or are causally associated with failure to recover during the
84 acute infection.

85

86 Understanding the role of B cells in HBV recovery is also imperative in people who are
87 living with HIV-1 due to the high prevalence of HBV/HIV co-infection and the adverse
88 consequences of HIV on CHB (16). Because people with HIV are less likely to develop
89 anti-HBsAg and recover from an acute infection (16, 17) and are more likely to undergo
90 HBV reactivation (18, 19), understanding how HIV affects the HBV-specific B cell
91 response may offer insights to an effective B cell response. Whether HBV-specific B cells
92 are present and affected in HBV/HIV co-infection similarly to the global B cell
93 compartment in HIV mono-infection, such as demonstrating increased proportions of
94 activated subsets (plasmablasts, CD21⁻CD27⁺ activated memory (AM), AtM) (20) and
95 inflammatory/adhesion molecules (CXCR3, CD11c) (20, 21) on these subsets, remains
96 to be seen.

97

98 Because acute HBV infection is frequently asymptomatic, most reports on immune
99 responses in HBV have been confined to assessment of CHB samples isolated years

100 after acute HBV infection. This raises the question of whether immune signatures
101 observed in these cohorts are a *cause* of failed HBV control or a *consequence* of
102 prolonged HBV exposure. To address this knowledge gap, we assessed a unique
103 longitudinal cohort of participants living with or without HIV who acquired an incident HBV
104 infection. We developed a high-dimensional flow cytometry panel to assess global and
105 HBsAg-specific B cells in these participants (22). We interrogated B cell dynamics at the
106 single-cell and antigen-specific levels to improve our understanding of B cell
107 characteristics and the role of B cell immunity around the acute HBV infection period,
108 including in the context of HBV/HIV co-infection.

109

110 **Results**

111

112 *Demographic and clinical characteristics*

113

114 To investigate B cell characteristics associated with HBV outcome, we studied
115 cryopreserved peripheral blood mononuclear cells (PBMCs) from 76 men (44 with HIV-1
116 and 32 without HIV-1) with incident HBV infection in the Multicenter AIDS Cohort Study
117 (MACS), now the MACS-WIHS Combined Cohort Study (MWCCS) (23, 24). MACS
118 participants were enrolled if they were at risk or living with HIV-1, and were prospectively
119 followed at semi-annual study visits (see Methods). As previously reported for the overall
120 MACS cohort, a subset of men acquired HBV during follow up (25). Of men with incident
121 HBV infection, a higher proportion of men with HIV (MWH) not on active HBV-ART
122 developed CHB (17.5%; 95% CI 8.7-29.9%) than men without HIV (MWoH) (8.2%; 95%
123 CI 3.8-15.0%) (26). For this study, sampling procedures did not randomly select MACS
124 participants and; therefore, do not reflect similar HBV recovery probabilities (see
125 Methods). Rather, given the limited number of participants who progressed to CHB,
126 individuals who met the sampling requirements (see Methods) were *all* selected for
127 assessment in this study, followed by matching on controllers demographics. Of the 44
128 MWH, 31 controlled the HBV infection (70%) and 13 progressed to CHB (30%). Of the 32
129 MWoH, 23 controlled the HBV infection (72%) and 9 progressed to CHB (28%).
130 Demographic characteristics stratified by HIV-1 status are in Table 1.

131

132 For each participant, up to four time points were analyzed, including (i) pre-HBV infection,
133 (ii) acute HBV infection, (iii) early outcome, and (iv) late outcome, with the latest outcome
134 time points acquired less than two years from the estimated time of incident HBV infection
135 (Figure 1A). At each time point, we utilized a 24-color flow cytometry assay to define main
136 B cell subsets, including global unswitched and class-switched memory and HBsAg-
137 specific B cells (Supplemental Figure 1) (22).

138

139 For both MWOH and MWH, the acute time point consisted of three groups based on
140 serologic analysis and eventual outcome of HBV infection (controller 1, controller 2, and
141 CHB). Participants in the controller 1 and CHB groups were HBsAg positive with or
142 without positive HBV DNA while the controller 2 group was already HBsAg negative and
143 anti-HBc positive. Thus, controller 2 group both acquired and controlled their incident HBV
144 infection (as shown by HBsAg clearance) in the six months between pre-infection and
145 acute sampling (Supplemental Figure 2A and Table 2). There was no difference in time
146 between the pre-infection visit and the acute visit in any of the three acute groups
147 (controller 1: median=0.50 years, IQR=0.48-0.56 years; controller 2: median=0.52 years,
148 IQR=0.50-0.62 years; chronic: median=0.51 years, IQR=0.48-0.57 years; p-value = 0.25).
149 Due to lack of significant differences in expression of B cell characteristics despite their
150 differential serological status between controller 1 and controller 2 (Supplemental Figure
151 2, B-D), we combined the two controller groups for subsequent analysis.

152

153 *Global B cells are dysregulated in HIV-1 infection.*

154

155 We first determined HIV-1 dependent B cell perturbations pre-HBV infection in MWH by
156 comparing them to MWOH. Uniform Manifold Approximation and Projection based
157 dimensionality reduction (UMAP) revealed differences in the B cell compartment as
158 demonstrated by different increased density areas between MWOH and MWH (Figure
159 1B). Median fluorescence intensity (MFI) heatmap overlays for all panel markers are
160 shown (Supplemental Figure 3A). B cell annotation based on manual gating revealed that
161 areas of increased density among MWOH were represented by both unswitched B cell
162 populations (IgM⁺IgD⁺CD27⁺ marginal zone (MZ) and IgM⁺IgD⁻CD27⁺ IgM-only memory)
163 and class-switched populations (CD21⁺CD27⁻ intermediate memory (IM) and
164 CD21⁺CD27⁺ resting memory (RM)), while the area of increased density among MWH
165 consisted of IgD⁺CD27⁻ naïve (unswitched), AM and AtM (class-switched) subsets. We
166 next confirmed these dynamics by quantifying the frequencies of all traditional B cell
167 subsets pre-HBV infection for total B cells (Figure 1C) and class switched B cells (Figure
168 1D). MWH showed increased frequencies of transitional B cells (CD10⁺CD27⁻), B1 B cells
169 (CD24^{-/+}CD38^{-/int}CD27⁺CD43⁺), and plasmablasts (CD24⁻CD38^{+/++}) and decreased levels
170 of total memory B cells (MBC) compared to MWOH (Figure 1C). The class-switched MBC
171 compartment for MWH was skewed towards activation, indicated by fewer IM and RM B
172 cells and increased frequencies of AM and AtM B cells compared to MWOH (Figure 1D).
173 UMAP analysis based on these differential B cell subset abundances resulted in clear
174 separation of MWH and MWOH (Figure 1E). This segregation based on HIV-1 status
175 persisted post-HBV infection in the acute and early/late outcome stages, supporting that
176 HBV infection does not overcome all global B cell differences between MWH and MWOH
177 (Supplemental Figure 3B).

178

179 We further investigated the expression of functional markers on MBC subsets in MWH
180 pre-HBV infection (Figure 1F and Supplemental Figure 3C). These markers included
181 chemokine receptors (CXCR3, CXCR5), the co-stimulatory molecule CD86, regulatory
182 molecules (BTLA, CD39), inhibitory Fc receptor CD32, and inhibitory markers associated
183 with chronic infections (PD-1, FcRL5, CD11c, CD22) (22). Consistent with the HIV-1
184 literature (20, 21), in MWH, AM B cells expressed more CXCR3 and PD-1 than AtM, while
185 both AM and AtM expressed higher levels of FcRL5 and CD11c compared with IM and
186 RM (Figure 1F). These phenotypic expression patterns are also observed on AtM B cells
187 in other chronic infections (21). In contrast, CXCR5, CD86, BTLA, CD39, CD32 and CD22
188 showed higher expression on IM and/or RM B cells compared with AM and AtM (Figure
189 1F). We also examined expression patterns on MBC subsets of MWOH prior to HBV
190 infection (Supplemental Figure 3, D and E) and compared to MWH (Figure 1G). The
191 expression of activation/exhaustion markers (e.g. CD86, PD-1, CD11c) on IM and RM B
192 cells in MWH were increased compared to MWOH irrespective of HBV infection stage
193 (Figure 1G). Overall, the pre-HBV infection samples demonstrate a B cell compartment
194 skewed toward activation and/or exhaustion in MWH, consistent with previous reports.
195 Most of the phenotypic B cell differences between men with and without HIV were in the
196 IM and RM subsets and persisted throughout the HBV infection course.

197

198 *Limited global B cell characteristics correlate with HBV outcome.*

199

200 Having established baseline differences in global B cells based on HIV-1 infection, we
201 next sought to interrogate global B cell dynamics throughout the course of HBV infection
202 to identify features associated with HBV outcome. UMAP based on the abundance of all
203 manually gated B cell subsets across each time point, irrespective of HIV-1 status,
204 revealed considerable overlap by HBV outcome alone between controllers and CHB
205 (Figure 2A). Thus, overall B cell subset composition was not associated with control of an
206 HBV infection. Given the observed effect of HIV-1 on the B cell compartment, we
207 performed all subsequent analyses comparing HBV outcome by first stratifying on HIV-1
208 status.

209

210 To gain more granular insights into HBV-dependent immune dynamics, we compared all
211 manually defined immune characteristics (frequency and MFI; N=370) at each time point
212 between controllers and CHB, stratified by HIV-1 (See Methods, Table S1). Statistically
213 significant differences were observed most often during the post-acute period when
214 comparing CHB to controllers, and nearly all were in MWH. Of the characteristics
215 identified, the frequency of AtM B cells and CXCR5 expression on transitional B cells
216 were significantly higher and lower, respectively, at outcome time points in those with
217 CHB compared to controllers (Figure 2, B and C). In addition, expression of CD86 on
218 multiple unswitched and memory B cell populations was significantly higher in MWH who
219 developed CHB compared to controllers at both acute and outcome time points (Figure
220 2D and Supplemental Figure 2, C and D). Our results show that the differential immune
221 signature signified by increased CD86 expression occurs during acute HBV infection
222 between individuals who develop CHB versus control and persists in the post-acute

223 period. Additional signatures including increased frequency of AtM B cells and decreased
224 expression of CXCR5 on transitional B cells are observed only in the post-acute period.

225
226 For each of the characteristics identified above in MWH, we next stratified participants
227 progressing to CHB based on CD4 count and observed that those with low CD4 counts
228 (<350 cells/mL) had significantly higher frequencies of AtM B cells and CD86 expression
229 or lower CXCR5 expression compared to controllers (Figure 2E). CXCR5 expression on
230 transitional B cells was also significantly lower in CD4 low CHB participants when directly
231 compared with CD4 high (>350 cells/mL) CHB participants. Taken together, MWH who
232 progressed to CHB had higher frequencies of AtM B cells and CD86 expression on
233 multiple B cell subsets, and lower CXCR5 expression on transitional B cells at acute
234 and/or outcome time points. These observations were significantly more pronounced with
235 lower CD4 count. In contrast, MWOH had stable proportions of cells and expression of
236 these molecules throughout their HBV infection regardless of HBV outcome.

237
238 *Unsupervised clustering reveals a unique population of B_{reg} cells upregulated in CHB at*
239 *outcome in MWH.*

240
241 Manual gating analysis is limited to biological knowledge and does not take the full
242 dimensionality of the data into account (27). Thus, interesting immune phenotypes may
243 be missed. To capture the phenotypic heterogeneity of B cells, we used unsupervised
244 clustering (FlowSOM) from B cells in all 244 samples (17.5 million cells) to define 40
245 unique clusters (28). Overlay of the cluster annotation on UMAP of global B cells from all

246 time points revealed heterogenous B cell phenotypes based on their unique locations on
247 the UMAP (Supplemental Figure 4A). The average FlowSOM cluster frequency ranged
248 from <0.1% to >30% and a heatmap of all markers for each cluster is shown
249 (Supplemental Figure 4B).

250

251 Next, we assessed if FlowSOM cluster abundance differed based on HBV outcome at
252 any time point. We again delineated individuals based on HIV-1 status due to profound
253 HIV-dependent B cell perturbations. Multiple clusters significantly differed between HBV
254 controllers and CHB groups in either MWH or MWOH (N=12) (Figure 3A). Marker
255 expression for these clusters is shown (Supplemental Figure 4C) and the heatmap
256 revealed further clustering into three groups (Supplemental Figure 4D), driven by CD11c,
257 CD27 and CD10, respectively, as shown by principal component analysis (PCA)
258 (Supplemental Figure 4E).

259

260 While multiple clusters were significantly altered at a given time point, only two clusters,
261 #14 and #31, differed at multiple time points and were selected to further explore (See
262 Methods). Clusters #14 and #31 were significantly increased in CHB compared to
263 controllers at multiple time points for MWOH and MWH, respectively (Figure 3A, red
264 circles). Cluster #14 differed at non-consecutive acute and late outcome in MWOH and
265 was characterized by IgM⁺, IgD⁺, CD27^{lo}, CD21^{lo}, CXCR5⁺, CD11c⁺, and FcRL5⁺, likely
266 representing activated naïve B cells (Supplemental Figure 4C) (29). In contrast, cluster
267 #31 differed consecutively at both early and late outcome time points in MWH, supporting
268 a robust association with CHB (Figure 3B). Cluster #31 consisted of transitional-like B

269 cells (CD10⁺IgM⁺IgD⁺CD5^{hi}CD24^{hi}CD38^{hi}CD21⁻CD32⁻CD27⁻) with heterogenous
270 expression of functional markers (Figure 3, C and D). Amongst MWH, cluster #31
271 abundance was also significantly higher in those with low CD4 count progressing to CHB
272 compared to either those with high CD4 count progressing to CHB or those in the
273 controller group (Figure 3E). This combination of lineage markers in association with
274 progression to CHB suggests that cluster #31 represents regulatory B cells (B_{regs}).
275 Although production of IL-10 is the gold standard to identify B_{regs}, transitional B cells
276 expressing CD24, CD38, and CD5, as the cluster #31 cells do, have been previously
277 shown to secrete IL-10 and have been described as B_{reg} populations (30). Altogether,
278 FlowSOM cluster analysis reveals a unique B_{reg} population in MWH with significantly
279 higher abundance in CHB versus controllers and in MWH with low versus high CD4
280 counts.

281

282 *HBsAg-specific B cells expand in MWH who control HBV and comprise distinct clusters.*

283

284 We next interrogated HBsAg-specific B cell dynamics and phenotypes based on HBV
285 outcome given their importance in prevention and control of HBV. Representative manual
286 gating of HBsAg-specific B cells using a dual probe strategy is shown (Figure 4A). HBsAg-
287 specific B cells ranged from undetectable to 1.13% total B cells (median 0.013%).
288 Longitudinal sampling enabled us to uniquely assess the HBsAg-specific B cell dynamics
289 within each participant by calculating the fold-change of frequencies over baseline (pre-
290 HBV infection) (Figure 4B). We observed significant expansion in HBsAg-specific B cells

291 in MWOH only in participants who controlled their HBV infection. No expansion was
292 observed in MWOH who progressed to CHB, or in MWH, regardless of HBV outcome.

293

294 We also examined HBsAg-specific B cell frequencies cross-sectionally, similar to
295 previously published analyses (9, 10, 13, 14). Here, we observed that MWOH who
296 controlled their HBV infection showed significantly higher HBsAg-specific B cell
297 frequencies at outcome time points compared to pre- and acute infection while chronic
298 HBV infection did not show an increased abundance of HBsAg-specific B cells
299 (Supplemental Figure 5A). In contrast, MWH who ultimately controlled HBV infection had
300 significantly higher HBsAg-specific B cell frequencies compared to CHB only during acute
301 infection, not at outcome time points. When comparing MWOH to MWH, we observed
302 significantly increased HBsAg-specific B cell frequencies in MWOH during the acute stage
303 in those who progressed to CHB and during early outcome in the HBV controllers
304 (Supplemental Figure 5B). HBsAg-specific B cell frequencies were not altered in MWH
305 on treatment (N=8, Supplemental Figure 5C). Stratifying the three acute groups
306 (controller 1, controller 2 and chronic) revealed a significantly higher HBsAg-specific B
307 cell frequency in controller 2 compared to participants with subsequent chronic HBV
308 infection within MWH (Supplemental Figure 5D).

309

310 We then stratified the previously generated UMAP of total B cells based on time point,
311 HIV-1 status and HBV outcome and overlaid HBsAg-specific B cells (Figure 4C). We
312 observed the HBsAg-specific B cell expansion in MWOH occurred in specific UMAP
313 locations, suggesting unique B cell subsets. HBsAg-specific B cells were detected at

314 various frequencies in 39 of the 40 FlowSOM clusters (Supplemental Figure 5E). UMAP
315 and FlowSOM analysis revealed that expanded HBsAg-specific B cells in MWOH
316 controllers consist of multiple phenotypically distinct B cell subsets, primarily RM and IM
317 MBCs (Figure 4D; Supplemental Figure 4B for FlowSOM cluster marker expression
318 patterns).

319
320 Overall, unlike the global B cell analysis demonstrating HBV outcome-related differences
321 primarily in MWH with CHB, HBsAg-specific B cells expanded only in MWOH controllers.
322 These cells expanded within distinctive areas of the UMAP projection, indicating
323 potentially protective phenotypes that merited further interrogation.

324

325

326 *Expanded HBsAg-specific B cells are phenotypically heterogeneous.*

327

328 Next, we assessed if the frequency of HBsAg-specific B cells in any of the 39 FlowSOM
329 clusters differed between time points to identify specific phenotypes associated with
330 expansion upon HBV infection. Volcano plots stratified by HBV outcome and HIV-1
331 infection status revealed significant expansion of HBsAg-specific B cells in 12 FlowSOM
332 clusters only in HBV controllers (Figure 5A). No differences occurred in CHB
333 (Supplemental Figure 5F). All but two of the clusters identified were from MWOH and
334 significantly differed between pre-infection/acute vs outcome time points. This is
335 consistent with our data showing expansion of total HBsAg-specific B cells in MWOH at
336 the HBV infection outcome time points versus pre-infection. Grouping the 10 clusters that

337 significantly differed by at least two time points (See Methods) based on HIV-1 status
338 (MWOH, clusters #4, #9, #16, #23, #24, #27, #28, #33; MWH, clusters #10 and #15)
339 demonstrated significantly increased frequencies only during outcome time points (Figure
340 5B). The total number of B cells per sample were consistent across time points,
341 regardless of HIV-1 status and HBV outcome, indicating that HBsAg-specific B cell
342 frequencies did not merely reflect sampling differences (Supplemental Figure 5G).

343

344 We next sought to investigate the phenotypes of these clusters to gain in-depth insights
345 into the phenotypic landscape of antigen-specific B cell expansion in HBV infection
346 (Figure 5, C and D). Expression levels of all markers assessed in the panel were plotted
347 by histogram and compared with total B cells (cluster #0, grey histogram). For MWOH,
348 significantly expanded HBsAg-specific B cells consisted of clusters which resembled
349 IgM⁺IgD⁺CD21⁺CD27⁻ naïve B cells (cluster #4), IgM⁻IgD⁻CD21^{+/int}CD27⁺ IM and RM B
350 cells (clusters #16, #23, #24, #27 and #28) and IgM⁻IgD⁻CD21⁻CD27^{lo} AtM B cells (cluster
351 #9) (Figure 5C). Cluster #33 appeared to be a mixed cluster consisting of negative to
352 intermediate expression of all lineage markers assessed. For clusters upregulated in
353 MWH (clusters #10 and #15), both demonstrated lineage marker expression of
354 IgM⁺IgD^{int}CD21⁻CD27^{int/+} consistent with activated naïve and/or MZ B cells.

355

356 Further analysis of phenotypic markers demonstrated heterogeneous phenotypes
357 between the IM and RM B cell clusters from MWOH (Figure 5D). For example, clusters
358 #23 and #24 showed higher expression of CD86, CD11c and FcRL5, markers of both
359 activation and exhaustion, compared to clusters #16, #27 and #28. In addition, clusters

360 #16 and #27 are characterized by increased expression of lymph node trafficking marker
361 CXCR5 compared with #23 and #24. In contrast, clusters #10 and #15, which resemble
362 naïve and/or MZ B cell subsets in MWH, demonstrated expression of multiple inhibitory
363 and/or putative inhibitory markers including CD11c, FcRL5, CD22, and CD32 (Figure 5D).
364 Interestingly, no selected clusters expressed PD-1 and unlike the global B cell analysis,
365 there was limited CD86 expression in these clusters.

366

367 Overall, in MWH, HBsAg-specific B cell expansion was observed upon HBV outcome,
368 and these cells demonstrate heterogeneous phenotypes associated mainly with resting
369 and intermediate MBCs. For the two unswitched B cell clusters that minimally expanded
370 in MWH, their phenotypic marker expression is consistent with dysfunctional naïve-like
371 HBsAg-specific B cell subsets. As these clusters were identified in controllers, the
372 phenotypes may represent a sub-optimal B cell compensatory mechanism in people with
373 HIV-1 to manage subsequent infections.

374 **Discussion**

375

376 Here, we provide a comprehensive longitudinal assessment of global and HBsAg-specific
377 B cell dynamics in peripheral blood sampled prior to, during, and after established
378 outcome for acute HBV infection. Our study evaluates B cell function in the largest
379 longitudinal collection of participants with incident HBV. Importantly, over half of the
380 participants included were living with HIV-1 at the time of HBV infection, allowing us to
381 additionally interrogate B cell characteristics in the context of HBV/HIV co-infection. Prior
382 studies have described widespread HIV-associated B cell perturbations (20, 31–33),
383 which we confirmed in our cohort and followed through an acute HBV infection,
384 demonstrating that HIV-1 negatively affects HBV infection responses, both at the global
385 and the antigen-specific B cell level. Interestingly, no global B cell characteristics
386 significantly differed in MWOH based on HBV outcome. This suggests that in mono-
387 infection, global B cells remain minimally affected around the acute infection period,
388 consistent with observations that people with CHB can respond normally to vaccination
389 or subsequent infections (34, 35).

390

391 Multiple global B cell characteristics significantly differed based on HBV outcome between
392 CHB and controllers in MWH. We identified decreased expression of CXCR5 on
393 transitional B cells, increased frequencies of B_{regs}, elevated CD86 expression on multiple
394 unswitched and MBC subsets, and increased AtM B cell frequencies in individuals with
395 CHB. These differences were not present prior to HBV infection, developing in MWH only
396 after incident HBV, suggesting that baseline B cell differences did not pre-dispose to CHB

397 development and that precedent HIV-1 infection may drive adverse B cell changes over
398 the course of CHB.

399

400 The lower expression of CXCR5 on transitional B cells observed in our study may impair
401 replenishment of B cells. CXCR5 recruits mature B cells to lymph nodes to promote
402 entrance into germinal centers for mounting productive antiviral responses (36). CD10⁺
403 transitional B cells are elevated in chronic viral infections, potentially as a compensatory
404 reaction to the loss of naïve B cells and to increased B cell activation (37). However, the
405 role of CXCR5 on transitional B cells and its implications in HBV infection warrants further
406 investigation.

407

408 In addition, we observed higher frequencies of B cells with phenotypes consistent with
409 B_{regs} in CHB compared to controllers in MWH. While FlowSOM cluster #31 shows
410 similarities with transitional B cells based on CD10, CD24, and CD38, their unique
411 phenotypic characteristics suggest regulatory functions, as described previously. For
412 instance, CD5 expression has been associated with reduced T cell proliferation and
413 increased expression of IL-10 upon *in vitro* stimulation (38, 39). In addition, the reduced
414 expression CD21 and CD32 on cluster #31 matches the phenotypic characteristics of
415 CD24⁺CD38⁺ transitional B cells with regulatory functions (40). Thus, based on these
416 observations, we concluded that cluster #31 consists of B_{regs}. B_{regs} have been reported in
417 CHB mono-infection and contribute to a dampened CD8⁺ T cell response that may allow
418 for chronic infection to persist (11, 12). The CD8⁺ T cell response is crucial for control of
419 HBV and impaired CD8⁺ T cell activation may hamper the development of protective

420 cytotoxic T cell responses (41). Thus, the combination of HIV-1 infection induced T cell
421 perturbations (42) and the observed increase in B_{regs} may further hinder control of HBV
422 infection.

423

424 The co-stimulatory receptor CD86 is upregulated upon B cell activation and is a crucial
425 co-stimulatory molecule for optimal T cell activation (43, 44). Viral infections lead to the
426 expansion of CD21-T-bet⁺ B cells, which express high levels of CD86 and have similar
427 phenotypes to AM and AtM B cells (45). T-bet⁺ B cells persist in chronic viral infections
428 such as HIV-1 and CHB and are thought to be both a source of antiviral immunoglobulin
429 (9, 45) but are also associated with B cell exhaustion in the context of chronic antigen
430 exposure (21, 46). In CHB, T-bet expression is increased in AtM B cells with higher
431 frequencies of inflammatory homing markers CD11c and CXCR3 expression (9). While
432 we did not measure T-bet, the combination of increased abundance of AtM B cells and
433 elevated expression of CD86 on various B cell subsets within MWH with CHB may reflect
434 the expansion of an activated, antiviral B cell population driven toward exhaustion by
435 aberrant trafficking to inflamed sites such as the liver in CHB.

436

437 AtM B cells are present in circulation of healthy individuals (3-5% total B cells) and may
438 represent a short-lived lineage that can expand upon infection or vaccination (47, 48).
439 However, during persistent antigen exposure, they can expand to over 50% and appear
440 terminally differentiated and functionally inferior, with upregulation of exhausted-like
441 inhibitory markers and less somatic hypermutation than their RM counterparts (21, 49,
442 50). Consistent with a negative functional consequence, we observed significantly

443 increased AtM B cell frequencies in MWH with CHB compared to controllers. Of note,
444 AtM B cell expansion has been reported in multiple long-term CHB mono-infection cohorts
445 (9, 10, 51), while we see expansion only in HBV/HIV co-infection. Previous studies were
446 conducted with participants many years after developing CHB while our samples were
447 collected relatively shortly after acute infection (within 2 years, mean time 1.66 years).
448 The discrepancy between our data and previous reports may reflect prolonged antigenic
449 exposure in HBV mono-infection. Clinically, infection with HIV-1 prior to HBV exposure
450 increases an individual's risk of developing CHB six-fold (16, 17) and within the MACS
451 cohort, MWH not on active HBV-ART had a higher proportion of those with incident HBV
452 develop CHB (17.5%) compared to MWOH (8.2%) (26). In addition, HIV-1 co-infection
453 accelerates CHB associated end-stage liver disease, cirrhosis and HCC (52). Thus, AtM
454 expansion in HIV-1 co-infection, as well as the other phenotypes we observed only in
455 MWH, may reflect acceleration of a process that occurs over a longer period in CHB
456 without HIV-1 co-infection. This hypothesis is supported by our results in MWH with low
457 CD4 counts showing significantly more pronounced immune perturbations in CHB.

458

459 A key feature of our B cell panel is the inclusion of HBsAg probes to identify ex vivo
460 HBsAg-specific B cells longitudinally. The analysis of HBsAg-specific B cells also
461 revealed a negative impact of HIV-1 on HBV, with limited HBsAg-specific B cell expansion
462 in MWH. This failure of HBsAg-specific B cell expansion may restrict the anti-HBsAg
463 response in co-infection. In support of this finding, clinical studies demonstrate that adults
464 with HIV-1 mount a limited anti-HBsAg response to standard recombinant HBV
465 vaccination compared with healthy individuals (53). In addition, HIV-1 infection leads to

466 higher rates of reactivation of controlled HBV infection (18, 19), which may be partially
467 attributable to the fact that people with HIV-1 are unable to sufficiently expand their
468 HBsAg-specific B cell pool to maintain long-term HBV control. Although MWH failed to
469 expand HBsAg-specific B cells, our cohort consisted of 31 participants who controlled
470 their incident HBV infection. This suggests that in these individuals, additional immune
471 populations such as effector T cell functions may be involved in HBV control. New
472 evidence suggests TCF-1⁺CD127⁺PD-1⁺ precursor to exhaustion (Tpex) HBV-specific
473 CD8⁺ T cells may contribute to control in the context of HIV/HBV co-infection (54).

474

475 In contrast, we found that in MWOH, HBsAg-specific B cells did expand, with significantly
476 increased frequencies in controllers within 6 months to 2 years after acute infection. We
477 did not see this B cell expansion during acute infection, when productive immune
478 responses against HBV are first presumably being mounted, suggesting an overall delay
479 in HBsAg-specific B cell induction and highlighting the potential importance of HBV-
480 specific T cell responses during acute HBV control. A technical limitation to the HBsAg
481 probe approach is that in samples with endogenous anti-HBsAg bound to HBsAg (acute
482 and CHB samples), our ability to fully quantify HBsAg-specific B cells as soon as antibody
483 is produced was likely negatively affected. However, if the detection of HBsAg-specific B
484 cell expansion in MWOH controllers were solely due to loss of HBsAg, we would also
485 expect to see similar expansion in MWH controllers.

486

487 Unlike previous reports that characterize HBsAg-specific B cells in CHB (9, 10, 13–15),
488 we focused our phenotypic analysis on intra-participant expanded HBsAg-specific B cells

489 to interrogate potential signatures of productive antigen-specific responses in HBV.
490 Expanded HBsAg-specific B cells were predominantly in MWOH controllers at outcome
491 and consisted mainly of class-switched (non-IgM expressing) IM and RM subsets. IM and
492 RM antigen-specific B cells have been shown to expand upon control of SARS-CoV-2
493 infection (55) and are important for recall response to reinfection (56). In addition, HBsAg-
494 specific B cells in CHB with treatment-associated HBsAg loss or seroconversion
495 (functional cure) exhibit higher proportions of CD21⁺CD27⁺ RM B cells, suggesting this
496 population is important for maintaining HBV control (14, 15). High-dimensional single-cell
497 approaches like the method we employed enables a more granular assessment of B cell
498 subsets. For example, despite their shared memory B cell annotation, expanded IM and
499 RM subsets revealed heterogenous phenotypes, suggesting the definition of memory B
500 cells may go beyond conventional CD21 and CD27 expression patterns. We observed a
501 group of clusters with either less (#16 and #27; CXCR5⁺, CD86⁻, PD-1⁻, FcRL5⁻, CD11c⁻)
502 or more (#23 and #24; CXCR3⁺, CD86⁺, BTLA⁺, FcRL5⁺, CD11c⁺) activated signatures.
503 Interestingly, these activated clusters appear phenotypically similar to AtM B cells based
504 on the increased expression of FcRL5 and CD11c, and downregulation of CXCR5.
505 Therefore, our data suggests an activation and differentiation continuum within the
506 HBsAg-specific B cell compartment which may be required for productive and sustained
507 HBV control in MWOH.

508

509 This study had multiple limitations due to the cohort studied. First, the findings described
510 here were generated from an all-male cohort and may differ in women as immune
511 responses are known to differ by sex. Second, since the participants came to semi-annual

512 visits, sampling at the peak of their acute infection was not always feasible and the time
513 between the acute infection and the sampling is not uniform. Third, all samples were
514 obtained from peripheral blood draws, limiting our assessment to circulating B cells rather
515 than the liver, the site of HBV infection. However, previous studies have identified
516 phenotypes of circulating B cells to be present but enriched in the liver in HBV infection
517 (9). Finally, the limited cell numbers available for each participant precluded subsequent
518 in vitro testing to further interrogate functional consequences of the phenotypic signatures
519 described by the ex vivo analysis.

520

521 Altogether, these results identify HIV-associated B cell alterations that elucidate potential
522 mechanisms for previously identified negative effects of HIV-1 on HBV control. Multiple B
523 cell subsets associated with dysregulation in antiviral responses were significantly
524 upregulated in CHB in MWH alongside failure to expand an antigen-specific B cell
525 response. In contrast, global B cells in HBV mono-infection showed no differences by
526 HBV outcome and HBsAg-specific B cells expand during HBV control. These antigen-
527 specific B cells consist mainly of IM and RM subsets with heterogenous activation
528 phenotypes. Our observations support a role for B cells in HBV control of acute infection
529 and warrant further functional examination to fully characterize HBsAg-specific B cells in
530 acute and ongoing HBV control.

531

532 **Methods**

533 ***Sex as a Biological Variable***

534 All participants in this study were XY male and identified as men. This choice was guided
535 by the access and availability of samples in the all-male MACS cohort, however, men
536 demonstrate a higher incidence of CHB and HCC with poorer prognosis compared to
537 women (57, 58).

538

539 ***Study Participants***

540 Male participants followed semi-annually in the Multicenter AIDS Cohort Study (MACS)
541 (25) (now part of the MACS-WIHS Combined Cohort Study) (23) who had, or were at risk
542 for, HIV-1 infection and had an incident HBV infection during follow-up, were considered
543 for inclusion. In the MACS, biological specimens including plasma and PBMCs are
544 collected and stored at each study visit. Incident HBV was identified through retrospective
545 serologic analysis, as previously described (24). Briefly, incident HBV infection was
546 defined in participants who were negative for HBsAg, anti-HBc, and anti-HBs who
547 seroconverted to anti-HBc positivity during follow-up. The estimated date of incident HBV
548 infection was defined as the midpoint between the last negative and first positive anti-
549 HBc or -HBsAg test result, whichever was positive first (24). The outcome of the incident
550 HBV infection, HBV recovery (controller) or CHB, was defined based on serologic testing,
551 as previously described (26). Briefly, CHB was defined by at least two positive HBsAg
552 tests ≥ 6 months apart and controller was defined as at least one visit negative for HBsAg
553 and positive for anti-HBc at a visit within 18 months after the incident infection.

554

555 Men with incident HBV were included in this study if PBMCs were available prior to
556 incident HBV infection (pre-infection, -0.5 to 0 years from estimated incident infection), at
557 the first visit positive for anti-HBc or HBsAg (acute infection, 0 to 0.5 years), and/or 0.5-2
558 years after the estimated date of incident infection (early outcome, 0.5 to 1.5 years; late
559 outcome, 1.5 to 2 years) (Figure 1A; Table 1 and Table 2). We matched all men with
560 known CHB who had PBMCs available at these time points to men with known HBV
561 recovery (controllers) at a minimum 1:1 (CHB to controller, 4 groups), but where possible,
562 we matched 1:2 (10 groups), 1:3 (8 groups) or 1:5 (1 group) based on age, HIV-1 status,
563 CD4 T cell count (for MWH), and time samples were stored in the repository. We included
564 76 men of whom 54 were HBV controllers and 22 had CHB. Of the 76 men included, 44
565 were living with HIV-1 and 32 were not for all time points assessed. For most participants
566 included in the study, seropositivity for HBsAg, anti-HBc, anti-HBs and HBV DNA levels
567 are available, as well as antiretroviral therapy for those participants receiving HIV-1
568 treatment (N = 8) (Table 1 and 2). HBV DNA was measured only at the acute HBV time
569 point. All participants on treatment remained so throughout the entire time course
570 assessed here. HIV viral load and CD4 counts were also measured at all time points and
571 did not significantly differ by HBV infection status. Not all participants had samples
572 available for all 4 time points (21 participants lacked one of two outcome samples, 9
573 participants lacked samples from both outcome time points, and 4 participants did not
574 have a pre-infection sample available). A total of 262 samples were included for
575 processing and staining.

576

577 ***Sample Processing and Flow Cytometry Staining***

578 PBMCs were cryopreserved in FBS + 10% DMSO and stored in liquid nitrogen until use
579 in this study. PBMC processing and flow staining were performed using the pipeline
580 previously described (59). Briefly, cryopreserved PBMCs were thawed in 37°C pre-
581 warmed RPMI + 10% FBS (R10) using the Thawsome adaptor (Medax International, Inc.)
582 (60). Cells were washed once in 5mL R10, then centrifuged at 860 x g for 3 min and
583 transferred to V-bottom 96-well plates for immediate B cell panel staining.

584

585 All flow cytometry antibodies and staining reagents used for this study are in
586 Supplemental Table 2 and are further described in detail in Cascino et al. 2020 (22). The
587 panel was designed to define comprehensive B cell subsets (Supplemental Figure 1). For
588 the antigen-specific B cell probe, we obtained recombinant small hepatitis B surface
589 antigen (HBsAg) genotype C, manually conjugated to one of two fluorochromes, Dylight
590 550 or Dylight 650, from Gilead (22, 61).

591

592 Antibody staining cocktail containing all but the antigen-specific B cell probes were
593 prepared in advance and stored at 4°C before staining the next day. Immediately prior to
594 B cell staining, 0.2ug/50uL reaction of each antigen-specific B cell probe was added and
595 centrifuged at 15,000 x g for 5 min to remove dye aggregates. PBMCs were washed twice
596 in 200uL phosphate buffered saline (PBS) prior to staining with Fixable Live/Dead Aqua
597 (Thermo Fisher Scientific) for 20min at 4°C. Samples were then washed twice in 200uL
598 PBS and stained with 50uL staining cocktail for 30min at 4°C in the dark. After incubation,
599 samples were washed twice with 200uL staining buffer (PBS + 0.5% bovine serum
600 albumin (BSA)) and subsequently fixed for 20min with 2% paraformaldehyde (PFA) at

601 4°C. After fixation, cells were washed once with staining buffer, resuspended in 250uL
602 0.5% PFA, and stored at 4°C in the dark until acquisition the next day on a BD
603 FACSymphony A5 cytometer using FACS DIVA software (version 9.3.1). The flow
604 cytometers were standardized to ensure consistent and reproducible performance
605 according to Perfetto et al. (62, 63).

606

607 The total 262 samples were processed in four batches. Matched participants were
608 randomized to one of the four batches and all samples for those participants were
609 included in the same batch to mitigate potential issues with batch effects. PBMCs from
610 the same blood draw from an unexposed healthy individual were measured in all
611 experiments as a technical control to assess reproducibility and for data normalization
612 (59).

613

614 ***Flow cytometry data pre-processing***

615 Flow cytometry data pre-processing is critical to remove low quality data and prevent
616 artificial data output. We used the R-implemented (R version 4.0.0) algorithm FlowAI
617 (version 1.18.5) (59) for automated removal of irregular events and outliers, which can
618 arise from fluctuations in flow cytometer performance. In 222 out of 262 samples, FlowAI
619 detected minor irregular events (<10%), which were removed automatically. For the
620 remaining 40 samples with more than 10% irregular events, we manually inspected the
621 raw data to prevent excessive data loss (64). 20 of these samples required manual
622 removal of irregular events.

623

624 Subsequently, correction for spectral overlap (compensation) was performed in FlowJo
625 10.1.7 (BD Biosciences) using single-stained beads. Persisting compensation
626 inaccuracies were corrected manually.

627

628 We performed preliminary gating of viable CD19⁺CD20⁺ B cells with FlowJo 10.9 and
629 exported a new set of fcs files for subsequent analysis of immune cell characteristics. The
630 precision of flow cytometric analysis depends on Poisson statistics and thus requires
631 sufficient cell counts. To this end, four samples were excluded from analysis due to limited
632 B cell counts (<5,000 B cells). 14 additional samples were removed from analysis to avoid
633 duplication of samples in each of the four longitudinal groups (pre-infection, acute, early
634 outcome, late outcome) from a single participant. A total of 244 samples were included
635 for analysis moving forward.

636

637 ***Manual flow cytometry analysis***

638 Manual gating was performed using FlowJo 10.9. Markers were divided in two groups
639 based on their purpose to either define immune cell subsets (lineage markers) or
640 functional markers/characteristics (Supplementary Table S2). Manual gating strategy can
641 be found in Supplemental Figure 1 and Cascino et al. (22).

642

643 Three different parameters were extracted for subsequent analysis: frequency of immune
644 cell populations, frequency of cells expressing functional markers and geometric mean
645 fluorescence signal (MFI).

646

647 ***Unsupervised data analysis***

648 Based on the technical control samples we observed batch-to-batch variation between
649 the four experiments. Our manual gating analysis accommodates for batch variation by
650 applying cut-off gates on a per-experiment basis. However, non-biological inter-assay
651 data variation will negatively impact the accuracy of unsupervised data analysis (27, 59).
652 Therefore, we normalized the flow cytometry raw data using CytoNorm (65). To this end,
653 we concatenated all files per experiment to train the CytoNorm model, which was applied
654 to the aggregated files as described (66). The normalized flow cytometry data was used
655 for subsequent unsupervised data analysis with concatenated fcs files. Samples were
656 identifiable by sample-specific keywords. R (version 4.2.1) programming language with
657 RStudio (2023.06.0 Build 421) interface was used for unsupervised data analysis. Raw
658 data (fcs files) containing concatenated B cells were imported using the flowCore (version
659 2.10.0) package. Data were transformed using the estimateLogicle function from the
660 flowCore package.

661
662 For dimensionality reduction algorithm UMAP (version 0.2.10.0) (67) and FlowSOM
663 clustering analysis we combined all HBsAg-specific B cells (N = 5,321) with 1000
664 randomly selected B cells per sample (total 244k B cells from 244 samples; 109k B cells
665 from MWH, 135k B cells from MWOH) to generate UMAP projection and define cluster
666 abundances for total and antigen-specific B cells from all time points. HBsAg-specific B
667 cells were defined based on co-staining of the two B cell probes. A cut-off of 1.8 and 1.9
668 was used for logicle-transformed signal from Dylight650 and Dylight550 conjugated
669 probes, respectively (Figure 4, C and D). For UMAP, default settings were used and all

670 markers except the HBsAg-specific B cell probes and viability/dump markers were used.
671 To visualize expression levels on the UMAP, logicle-transformed fluorescence intensity
672 values were normalized based on 1% and 99% percentile. Figure 1B is based only on pre-
673 infection total B cell composition between MWOH and MWH. Here, UMAP was visualized
674 based on 800 and 1000 randomly selected B cells per MWH (N = 40) and MWOH (N =
675 32) to obtain equal numbers of B cells between HIV-1 infection status (n = 32,000 cells
676 per group). In Figure 1E, 2A and Supplemental Figure 3B, UMAP algorithm based on 16
677 non-normalized manually gated B cell subset frequencies were used to define unique
678 immune compositions between MWH and MWOH (Figure 1E and Supplemental Figure
679 3B) and controller and CHB (Figure 2A) where each dot represents one sample.

680

681 For visualization of HBsAg-specific B cells in either UMAP or biaxial raw data, one
682 participant was removed due to large expansion of HBsAg-specific B cells which affects
683 the visualization of rare HBsAg-specific B cells from other samples. The HBsAg-specific
684 B cells from this participant were included for statistical analysis.

685

686 Clustering of B cells from all 244 samples (N = 17,432,605) was performed using
687 FlowSOM (version 2.4.0). FlowSOM parameters included a cluster grid of 20 x 20 and in
688 a subsequent step 40 metaclusters were defined. Fluorescence signals from all markers
689 (N =21) except for viability dye and the two antigen-specific B cell probes were used to
690 compute FlowSOM clustering. Manual gating designation was extracted from FlowJo
691 workspace using the GetFlowJoLabels function from the FlowSOM package.

692

693 The heatmap showing median fluorescence intensity for each marker per cluster
694 (Supplemental Figure 4B) was created with the pheatmap package (version 1.0.12) and
695 input data was normalized using 1% and 99% percentile normalization. The ggplot2
696 (version 3.4.4) package was used for data visualization. Overlay histograms depicting
697 expression of markers per cluster was performed using the ggridges package (version
698 0.5.5). To this end, 10,000 B cells were randomly selected across all samples to visualize
699 expression on total B cells.

700

701 ***Statistical analysis***

702 Statistical comparisons were performed in GraphPad Prism 10 or R. Comparison
703 between conditions were performed using non-parametric tests with or without correction
704 for multiple testing as indicated in figure legends. A p-value less than 0.05 was considered
705 significant except for analysis in Figure 5B where we visualized FlowSOM clusters, which
706 had at least one significant difference with a p-value < 0.01 to highlight the differences
707 that remained statistically significant using a more conservative type 1 error threshold.

708

709 For the exploratory analyses in Figures 1G, 2B-D, 3 A and B, and 5A, we tested the
710 difference of 40-370 unique immune characteristics between CHB and HBV controllers in
711 four time points (Supplemental Table 1). Thus, our sample size is not sufficiently large for
712 multiple testing correction with such comprehensive lists of distinct immune
713 characteristics. Therefore, we focused our analysis on specific immune characteristics as
714 follows. For Figure 1G, we show all phenotypic immune characteristics with a p-value <
715 0.05 by Wilcoxon ranked-sign test (BTLA, FcRL5 and CD22 demonstrated no significant

716 differences). For the remaining figures, immune characteristics selected to highlight in the
717 figures were only those characteristics that significantly differed in consecutive time points
718 (Figures 2, B and C; Figure 3, A and B; Figure 5A) and/or between multiple immune
719 characteristics (manually gated frequency and MFI; Figure 2D). Finally, for the analysis
720 in Supplemental Figure 2B, we first assessed the differences between the two controller
721 groups and CHB at the acute time point using Kruskal-Wallis rank test. Those significant
722 characteristics were then compared between the acute groups by Wilcoxon signed-rank
723 test in Supplemental Figure 2C which primarily revealed CD86 expression to be
724 significantly different. Thus, we focused our analysis in Supplemental Figure 2D on CD86
725 expression in various B cell subsets to visualize significance of CD86 expression between
726 all three acute groups (controller 1, controller 2 and chronic) as determined by Wilcoxon
727 signed-rank test.

728

729 In Figure 5, frequency of HBsAg-specific B cells within the 40 B cell clusters was
730 calculated. Wilcoxon signed-rank test without correction for multiple testing was used to
731 identify significant differences in HBsAg-specific B cell abundance within FlowSOM
732 clusters between time points and per HIV infection status and HBV outcome (Figure 5A
733 and Supplemental Figure 5F). For multiple clusters (#2, #17, #19, #22, #32, #34, #35,
734 #36, #38 and #39), differences could not be assessed due to insufficient HBsAg-specific
735 B cells between the two groups in at least one sampling time point.

736

737 12 FlowSOM clusters were identified in Figure 5A with at least one significant difference
738 ($p < 0.01$). Of these clusters, we selected to further analyze 10 clusters of interest in

739 Figure 5B under the following criteria: the two clusters significantly upregulated for MWH
740 (clusters #10 and #15) and only the 8 clusters significantly upregulated at two or more
741 time point comparisons (clusters #4, #9, #16, #23, #24, #27, #28 and #33). In Figure 5B
742 we then performed Wilcoxon signed-rank test with Bonferroni correction for multiple
743 testing to determine statistically significant differences in longitudinal HBsAg-specific B
744 cell abundances in our clusters of interest. Of note, the remaining clusters from Figure 5A
745 (#13 and #18) did not show any significant differences between time points after
746 Bonferroni correcting for multiple testing.

747

748 Finally, to incorporate all available longitudinal data in comparisons of expression levels
749 across multiple timepoints, we fit generalized estimating equation (GEE) models using
750 SAS proc GENMOD which accounts for the resulting correlated data structure (Figure 2E
751 and Figure 3E). In these GEE analyses, we modeled the natural-log transformed immune
752 expression data as the dependent variable and chose to account for the correlated data
753 using an autoregression correlation structure after examining model fit results.

754

755 **Study approval**

756 All participants provided written informed consent for this study. This study was approved
757 by the Institutional Review Board at Johns Hopkins University.

758

759 **Data Availability**

760 Data values for all graphs are provided in the Supporting Data Values file.

761

762 **Author Contributions**

763 KC and TL (co–first authors) contributed equally to designing and conducting
764 experiments, acquiring and analyzing data, and preparing figures for publication. KC had
765 primary responsibility for planning, scheduling experiments, and drafting the manuscript
766 and is therefore assigned as lead co–first author. ES contributed to data interpretation.
767 KS contributed to flow cytometry panel design. SW, MW and RM contributed to sample
768 acquisition and manuscript review. MR provided facilities and contributed to manuscript
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771 review.

772

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828

829

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831

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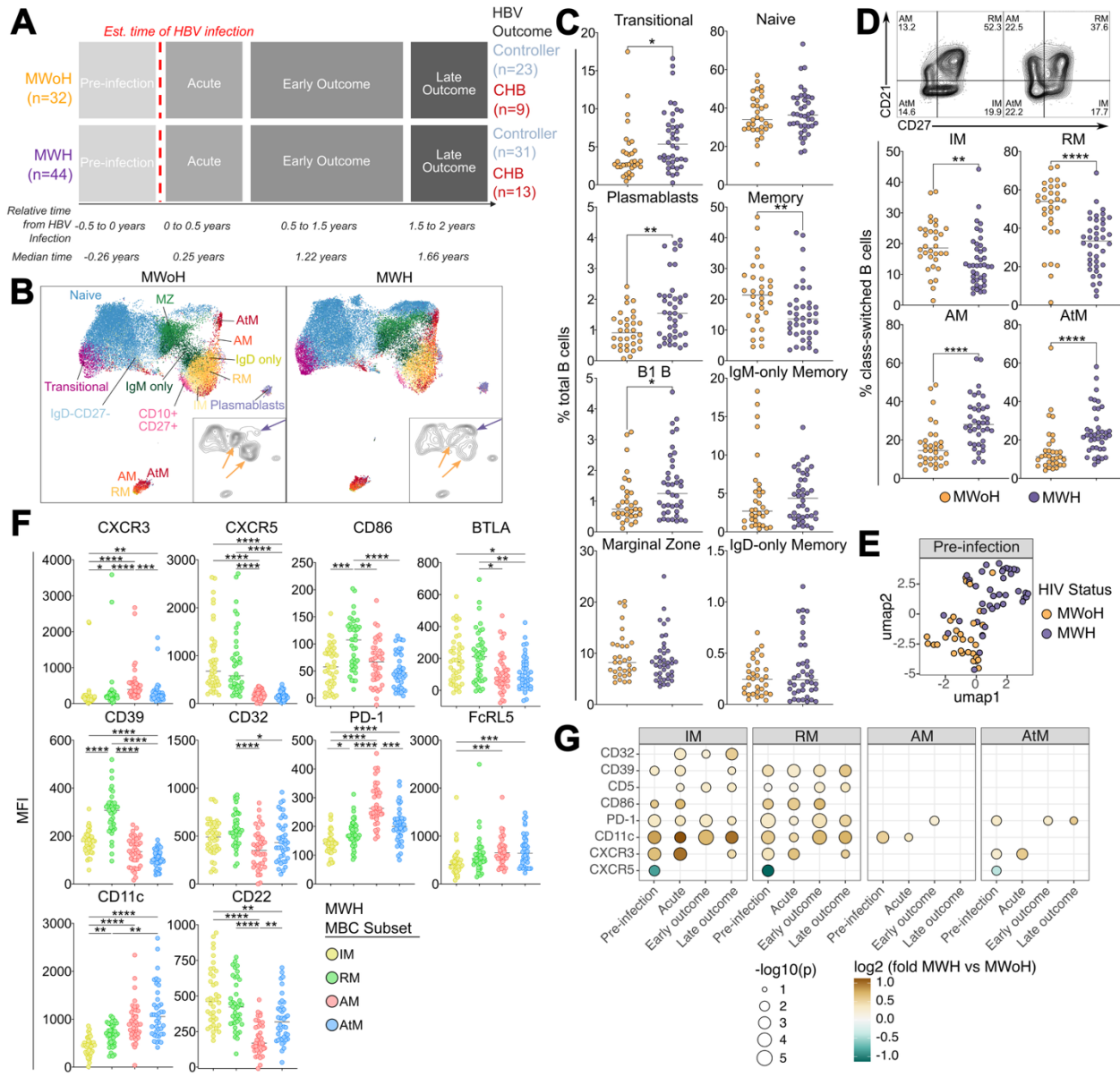
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978 **Figures**

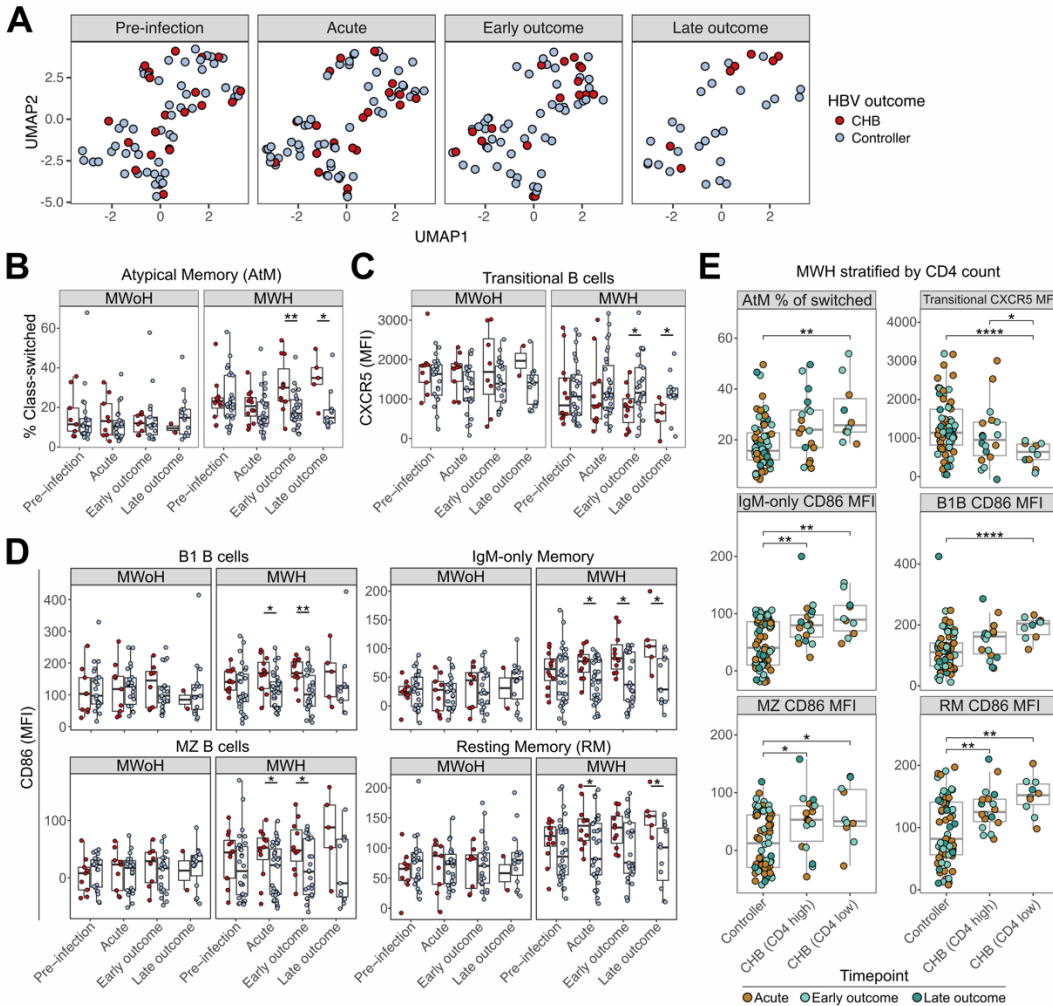


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980 **Figure 1. Global B cell subsets are significantly altered in MWH.**
 981 **(A)** Schematic overview of longitudinal HBV cohort. **(B)** Concatenated flow cytometry data
 982 depicted as UMAP projection of all B cells from pre-infection samples and plotted for
 983 MWOH or MWH. Each dot represents a single B cell. All markers except the
 984 dump/viability and HBsAg probes were used to compute UMAP. Contour plots depict
 985 distribution of events on UMAP based on the equally sampled pre-infection data. Yellow
 986 and purple arrows indicate areas of up- and down-regulation in MWOH and MWH,
 987 respectively. **(C)** Frequencies of B cell subsets from MWOH (orange) and MWH (purple).
 988 **(D)** Representative MWOH participant (left plot) and MWH (right plot) from the pre-
 989 infection time point were examined for expression of MBC subset markers CD27 and
 990 CD21. Frequencies of MBC subsets from all MWOH and MWH are shown below. **(E)**
 991 UMAP projection based on the composition of all B cells shown in C and D. Each dot

992 represents one subject at pre-infection time point. **(F)** Mean fluorescence intensity (MFI)
993 of all phenotypic markers on MBC subsets based on CD21/CD27 expression profile from
994 MWH pre-HBV infection. **(G)** Bubble plot highlights p-values of all significant phenotypic
995 marker expression (MFI) on MBCs between MWOH and MWH when compared using
996 Wilcoxon signed-rank test. Dot size is based on $-\log_{10}$ transformed and unadjusted p-
997 values and dot color is based on \log_2 transformed (upregulated in MWH, brown; MWOH,
998 blue) fold change. **(B-F)** Data from pre-infection samples for MWOH (n=32) and MWH
999 (n=44). **(C-D)** Data compared using Mann-Whitney test and **(F)** Kruskal-Wallis test
1000 corrected for multiple comparisons, each dot represents a single measurement from each
1001 subject assessed at the pre-infection time point. Bars represent median values. *, P <
1002 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001.

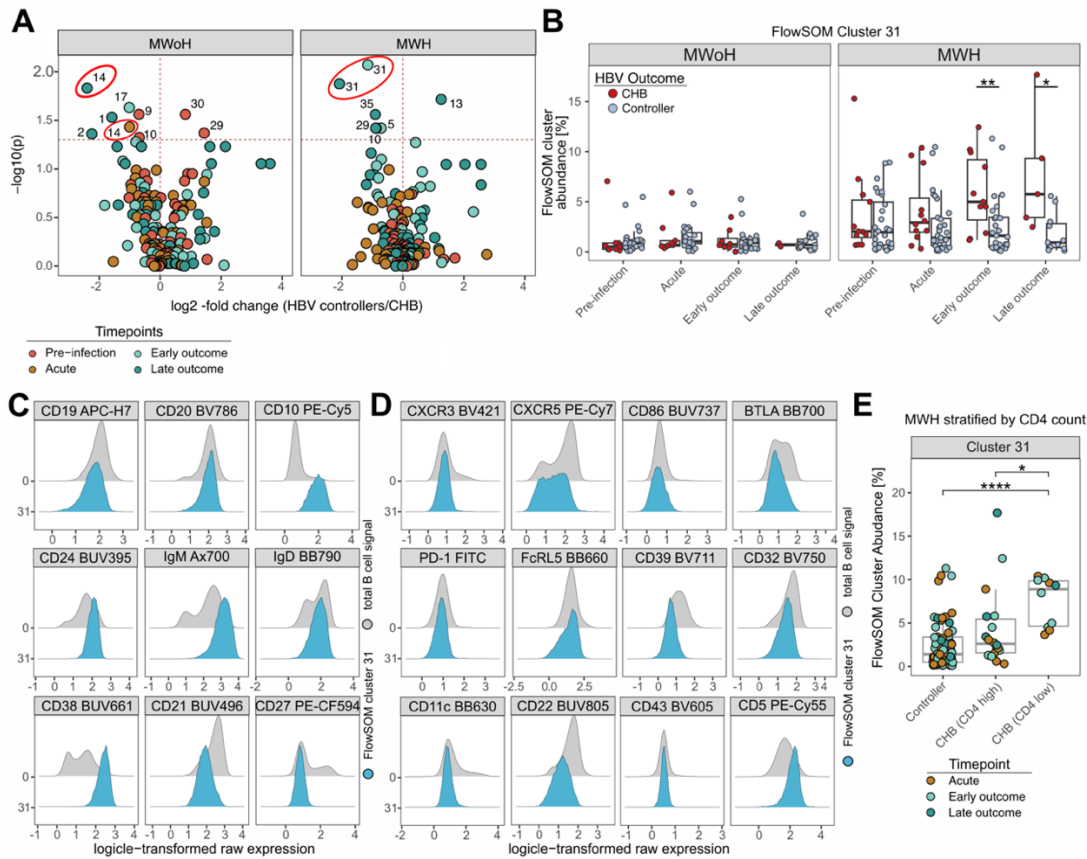
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Figure 2. Differences by HBV outcome in global B cell populations are minimal and observed only in HIV-1 co-infection.

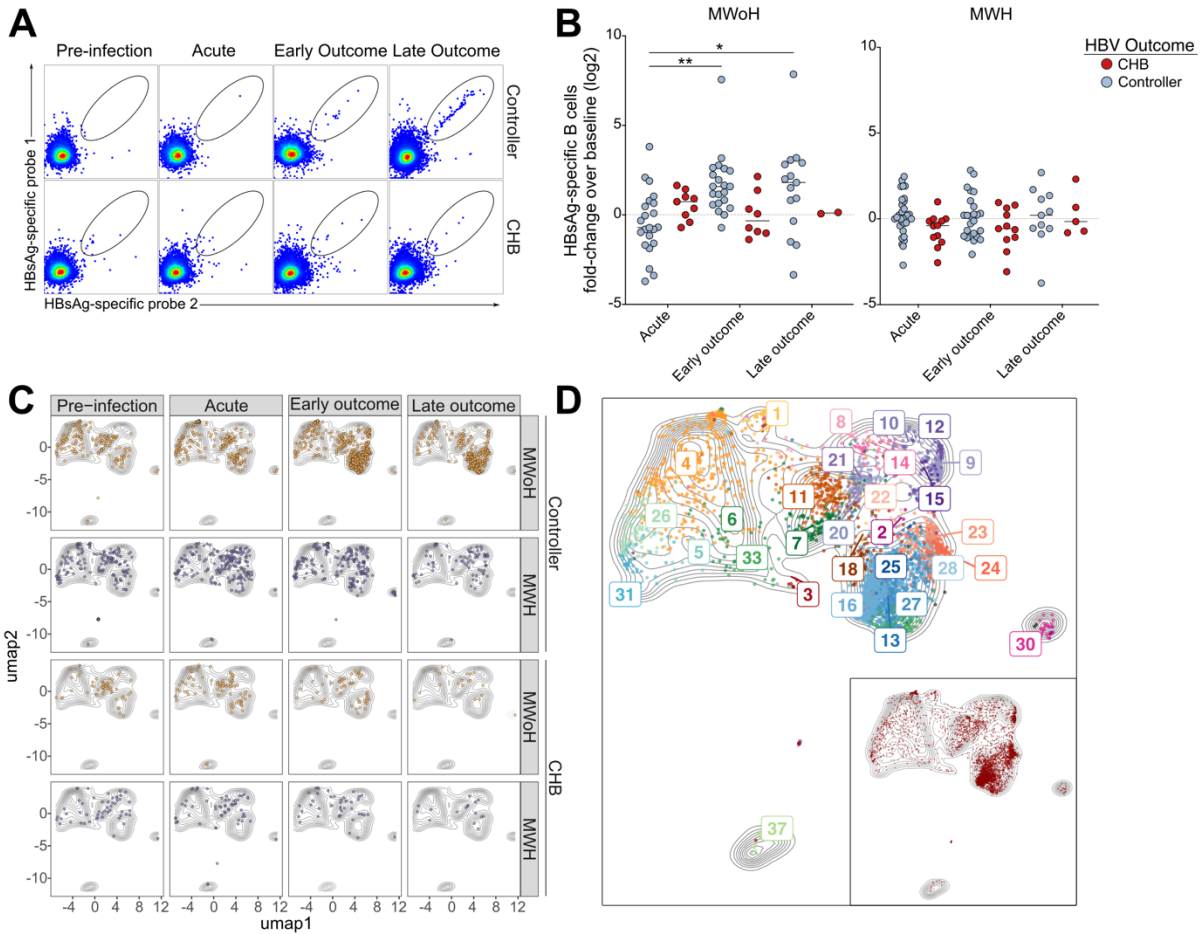
(A) UMAP projection of B cells shown in Figure 1, C and D from all MWOH and MWH at each time point (CHB, red; controller, blue). At each time point, each participant is represented by one dot. (B-D) Abundance of AtM (% class-switched), and expression (MFI) of CXCR5 (transitional B cells) and CD86 (B1B, IgM-only Memory, MZ and RM B cells) between HBV outcome in MWH and MWOH are shown. At each time point, each participant is represented by one dot (MWOH: pre-infection, controller n=23, CHB n=9; acute, controller n=22, CHB n=9; early outcome, controller n=21; CHB n=8; late outcome, controller n=15, CHB n=2; MWH: pre-infection, controller n=28, CHB n=12; acute, controller n=31, CHB n=12; early outcome, controller n=25; CHB n=11; late outcome, controller n=11, CHB n=5). Boxplots show median, quartile and minimum/maximum. Data compared using Wilcoxon signed-rank test. P-values are unadjusted for multiple comparisons. (E) Manually gated B cell characteristics compared between MWH controllers, MWH CHB with low (<350 cells/mL) and high (>350 cells/mL) CD4 counts using a GEE regression model. Each dot represents one sample, colored by time point (orange, acute; light green, early outcome; dark green, late outcome). p-values were obtained from the GEE model. *, P < 0.05; **, P < 0.01; ****, P < 0.0001.



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1028 **Figure 3. Unsupervised FlowSOM clustering reveals a B_{reg} population upregulated**
 1029 **in MWH who develop CHB.**

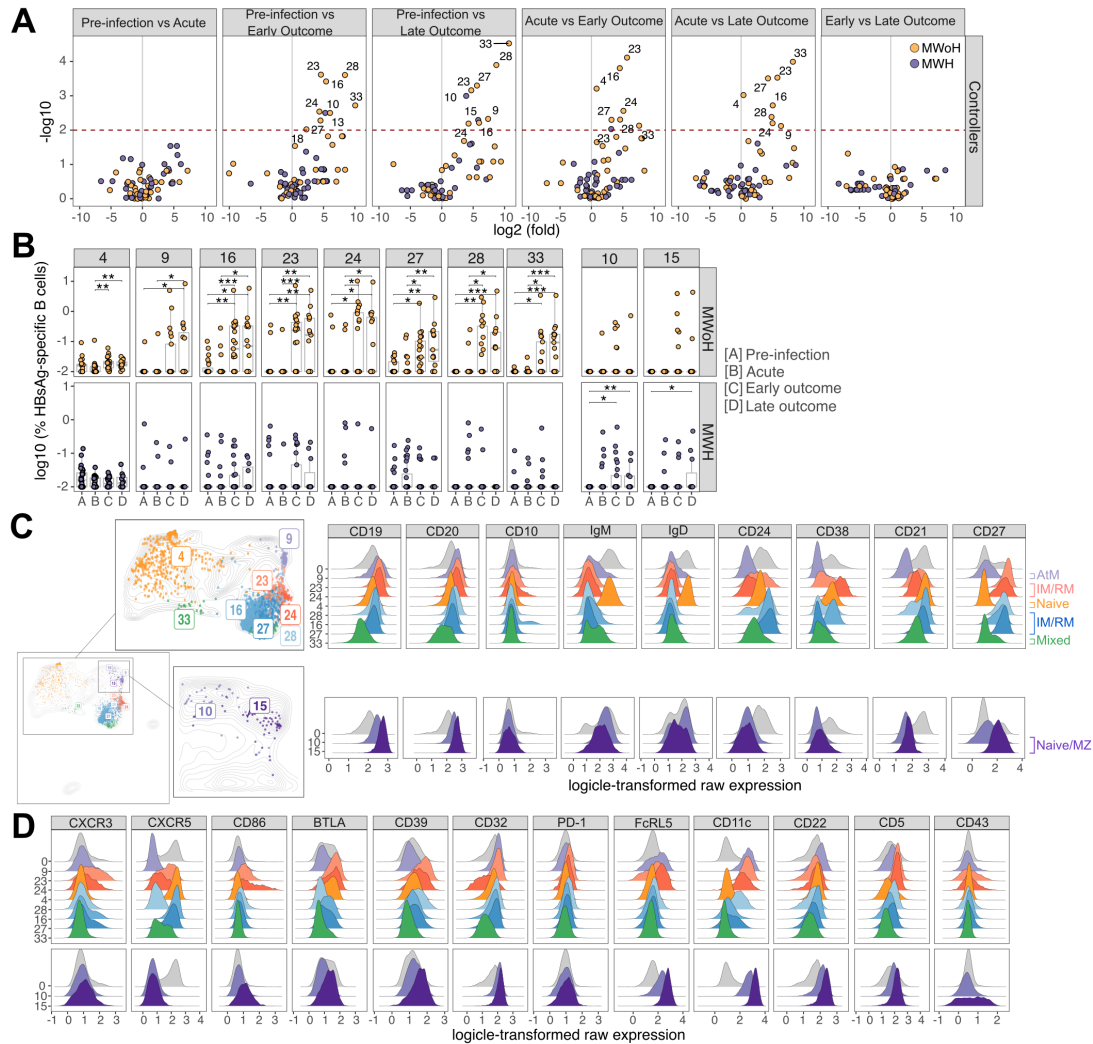
1030 **(A)** Comparison of FlowSOM cluster abundance by HBV outcome in MWOH and MWH
 1031 by Wilcoxon signed-rank test is shown. Volcano plot depicts $-\log_{10}$ transformed
 1032 unadjusted p-values and \log_2 fold-change of cluster abundance in controllers compared
 1033 to CHB. Upper right quadrant depicts clusters significantly upregulated in controllers while
 1034 upper left quadrant depicts clusters significantly upregulated in CHB. Each dot represents
 1035 a FlowSOM cluster colored by time point (red, pre-infection; orange, acute; light green,
 1036 early outcome; dark green, late outcome). **(B)** FlowSOM cluster 31 abundance in MWOH
 1037 and MWH stratified by HBV outcome is shown. Each dot represents one sample for each
 1038 participant at given time point (see Figure 2 legend for sample numbers). Boxplots show
 1039 median, quartile and minimum/maximum. Data compared using Wilcoxon signed-rank
 1040 test. P-values are unadjusted for multiple comparisons. **(C-D)** Histogram plots show
 1041 expression of all lineage markers **(C)** and phenotypic markers **(D)** for total B cells (grey)
 1042 compared with FlowSOM cluster 31 (blue). **(E)** FlowSOM cluster 31 abundance compared
 1043 between MWH controllers, MWH CHB with high CD4 counts (>350 cells/mL) and MWH
 1044 CHB with low CD4 counts (<350 cells/mL) using a GEE regression model. Each dot
 1045 represents one sample, colored by time point. p-values were obtained from the GEE
 1046 model. *, $P < 0.05$; **, $P < 0.01$; ****, $P < 0.0001$.



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Figure 4. HBsAg-specific B cells are expanded in MWOH upon control of acute HBV infection.

(A) Flow cytometry plots of manually gated HBsAg-specific B cells from two representative participants are depicted (MWOH controller, top row; MWOH CHB, bottom row). Dual staining strategy with HBsAg probes was used to define antigen-specific B cells. (B) Frequencies of HBsAg-specific B cells plotted as log₂ fold change over baseline. Data compared using Kruskal-Wallis test corrected for multiple comparisons. Each dot represents one participant's sample compared to baseline for each given time point (see Figure 2 legend for sample numbers). Bars represent median values. *, P < 0.05; **, P < 0.01. (C) UMAP projection of total B cells (contour plot, grey) overlaid with HBsAg-specific B cells (MWOH, yellow; MWH, purple) stratified by time point in controllers (top 2 rows) and CHB (bottom 2 rows). (D) UMAP projection of total B cells (contour plot, grey) overlaid with total HBsAg-specific B cells colored by FlowSOM cluster. Total HBsAg-specific B cells depicted in red on UMAP inlay. (C-D) Each dot represents a single HBsAg-specific B cell.



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Figure 5. Expanded HBsAg-specific B cells are phenotypically heterogeneous.

(A) Volcano plots depict log₂ fold-change and -log₁₀ transformed unadjusted p-values based on comparisons of HBsAg-specific B cell abundance by FlowSOM cluster between each possible combination of time points in HBV controllers. P-values calculated by Wilcoxon signed-rank test. Each dot represents a FlowSOM cluster, colored by HIV-1 infection status (MWOH, yellow; MWH, purple). (B) log₁₀ transformed frequency of HBsAg-specific B cells over time are plotted for both MWOH and MWH controllers in 10 clusters of interest. Top row consists of clusters upregulated in MWOH; bottom row are clusters upregulated in MWH. Abundances lower than 0.01% were adjusted to 0.01% for visualization but not statistical analysis purposes. Each dot represents one sample at a given time point. Data compared by Wilcoxon signed-rank test with Bonferroni correction for multiple testing. *, P < 0.05; **, P < 0.01; ***, P < 0.001. (C) Previously generated UMAP of total B cells overlaid with HBsAg-specific B cells from 10 clusters of interest are colored by FlowSOM cluster. HBsAg-specific B cells are plotted on zoomed in sections of total UMAP (top row: expanded in MWOH; bottom row: expanded in MWH). (C-D) Histogram plots show expression levels for all lineage markers (C) and phenotypic markers (D) assessed in the panel for each of the 10 FlowSOM clusters compared with total B cells (grey histograms).

1085 **Tables**

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Table 1. Characteristics of incident HBV infected participants from MACS cohort.

	Men without HIV	Men with HIV
Participants (no.)	32	44
Age at estimated HBV infection (yr)	33 (20-57)	37 (21-74)
HIV VL (copies/mL)^A	NA	18,549 (50-74,232) ^B
CD4 count (cells/mm³)^A	916 (362-1345) ^C	574 (160-1295)
HIV treatment (no.)^D	NA	8
HBV Outcome (no.)		
Controller	23	31
CHB	9	13
HBV VL (IU/mL)^E		
Controller	1.86e8 (0-2.96e9) ^F	2.58e6 (0-1.16e7) ^G
CHB	3.98e7 (1.01e7-8.74e7) ^C	1.56e8 (0-5.11e8) ^I

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All MACS participants are XY male and identify as men. ^AMeasured pre-HBV infection. ^DHIV treatments include: lam, tdf, lam/tdf, ftc/tdf, lam/ftc/tdf. ^EMeasured in acute HBV infection. Data missing for: 26^G, 11^B, 5^F, 3^I, and 2^C participants. Chronic HBV (CHB).

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Table 2. HBV disease state serology.

	HBsAg	Anti-HBcAg	Anti-HBsAg
Controller			
Pre-infection	-	-	-
Acute			
controller 1	+	-/+	-
controller 2	-	+	+
Early Outcome	-	+	+
Late Outcome	-	+	-/+
Chronic HBV (CHB)			
Pre-infection	-	-	-
Acute	+	-/+	-
Early Outcome	+	+	-
Late Outcome	+	+	-

1098 Plasma serology measures. Hepatitis B surface antigen (HBsAg), antibody against hepatitis B core antigen
 1099 (anti-HBcAg) and antibody against HBsAg (anti-HBsAg).

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