1 **Longitudinal characterization of phenotypic profile of T cells in chronic hepatitis**

2 **B identifies immune markers predicting HBsAg loss**

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29   **ABSTRACT**

30   The current desirable endpoint of treatment against chronic hepatitis B virus infection

31   (cHBV) is to achieve a functional cure, which is defined as HBsAg loss (sAg-L) with

32   or without anti-HBs seroconversion. However, the immunological features that are

33   associated with functional cure have not been studied in detail. Here, a total of 172

34   cHBV patients including 31 sAg-L patients, and 24 healthy individuals were examined

35   for their T cell phenotypic profile and HBV-specific T cell responses by flow cytometry.

36   sAg-L patients showed distinct CD4 and CD8 T cell phenotype fingerprints compared

37   to those of HBsAg-positive patients, as indicated by the upregulation of CD25, CD40L

38   and CTLA-4 expression on CD4 T cells; HLA-DR, CD95 and PD-1 on both CD4 and

39   CD8 T cells; as well as a potent HBcAg-specific CD8 T cell response. The changes in

40   the T cell phenotype in sAg-L patients began during rapid HBsAg decrease upon

41   treatment onset, were maintained after sAg-L. HLA-DR expression on T cells was

42   positively correlated with the level of HBsAg reduction and the magnitude of the

43   HBcAg-specific T cell responses in cHBV patients. Importantly, increased HLA-DR

44   and CTLA-4 expression on CD4, as well as HLA-DR and TIM-3 expression on CD8 T

45   cells were identified as predictive factors for HBsAg loss within 48 weeks of therapy

46   in cHBV patients. The onset of HBsAg decrease and subsequent loss in cHBV patients

47   on treatment is associated with significant alterations of both CD4 and CD8 T cell

48   phenotypes. Characterization of the T cell phenotype in cHBV patients possessed

49   greater predicative value for sAg-L.

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INTRODUCTION

The World Health Organization has implemented a Global Strategy on Viral Hepatitis to declare that the elimination of hepatitis B virus (HBV) is possible [1]. Currently, most clinical practice guidelines recommend that the optimal endpoint of chronic HBV infection (cHBV) treatment is to achieve functional cure, which is defined as HBsAg seroclearance with or without anti-HBs seroconversion [2-4]. This is based on the observation that the HBsAg seroclearance is associated with a beneficial effect on disease progression and reduced risk of hepatocellular carcinoma (HCC) development [5,6]. Spontaneous HBsAg seroclearance is common in acute HBV infection in adults, but it is a rare clinical event in untreated chronic HBV infected patients with annual clearance rates ranging from 0.7% to 2.26% [6,7].

T cells are believed to play an irreplaceable role in HBV clearance and determine the outcome of HBV infection as demonstrated in the HBV chimpanzee model [8,9]. Therefore, T cells from patients at different stages of HBV infection have been intensively characterized for their phenotypic profile and function [10-13]. Available data indicate that the quantity and function of HBV-specific T cells are associated with the outcome of HBV infection. Strong HBV-specific CD4 and CD8 T cell responses were frequently detected in patients who spontaneously cleared the virus during acute HBV infection, but were not found in cHBV patients [14,15]. During cHBV infection, an association between increased HBcAg/HBeAg-specific T cell responses and HBeAg seroconversion was also reported [16]. Moreover, the recovery of HBV-specific T cell

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74 responses was observed in cHBV patients after long-term effective therapy with

75 nucleos(t)ide analogues (NUCs), and in patients who achieved a functional cure either

76 spontaneously or under antiviral treatment [17]. A recent study also demonstrated that the

77 presence of functional HBV-specific T cells may serve as a potential immune system

78 biomarker to safely discontinue NUC therapy in CHB patients [18]. Thus, a better

79 understanding of HBV-specific T cell biology is believed to have significant

80 implications for guiding clinical practice in cHBV treatment and for developing

81 effective immunotherapy to cure cHBV. However, current available data on T cell

82 responses related to cHBV functional cure mainly come from cross-sectional studies,

83 and a longitudinal analysis of the T cell phenotype and function during the course of

84 HBsAg loss (sAg-L) and seroconversion in cHBV patients is still lacking. In this study,

85 T cell phenotype profiles and HBV-specific T cell responses were longitudinally

86 analyzed in a group of cHBV patients prior to, during and after achieving functional

87 cure upon treatment and compared with HBsAg-positive patients. Furthermore, T cell

88 features correlating with rapid HBsAg decrease, loss and seroconversion in cHBV

89 patients were characterized.

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96   **RESULTS**

97   ***Alteration of the T cell phenotype in sAg-L patients and patients experiencing rapid***

98   ***HBsAg decrease***

99   First, we characterized *ex vivo* whether immune cell populations and the T cell

100   phenotype in PBMCs changed in sAg-L patients compared to HBsAg retained (sAg-R)

101   patients and healthy controls by flow cytometry (as depicted in figure S1). No

102   significant differences in absolute numbers and frequencies of B cells, monocytes, and

103   dendric cells (DCs) were observed between sAg-L, sAg-R patients, and healthy controls

104   (HC). The absolute numbers of total T cells as well as $CD4^+$ T cells in sAg-R and sAg-

105   L patients were significantly lower than those in HC. sAg-R but not sAg-L patients also

106   showed significantly lower frequencies of total T cells as well as absolute numbers of

107   $CD8^+$ T cells than HC. sAg-L patients showed significantly higher $CD8^+$ T cell

108   frequencies than sAg-R patients and HC, and lower $CD4^+$ T cell frequencies than HC

109   (Figure S1C). Next, we analyzed the T cell phenotype by staining cell surface and

110   intracellular markers associated with T cell activation (CD69, HLA-DR, CD95, CD25

111   and CD40L), exhaustion (PD-1, TIM-3 and CTLA-4) and effector function (granzyme

112   B and CD107a). As shown in figure 1A, both CD4 and CD8 T cell phenotype

113   fingerprints of sAg-L patients were distinct from those of sAg-R patients and HC.

114   Statistical analysis of the percentage of cells expressing each marker revealed that $CD4^+$

115   T cells of sAg-L patients had significantly increased expression of the activation

116   markers HLA-DR, CD25 and CD95 compared to those from sAg-R patients and HC

117   (Figure 1B). HLA-DR expression on $CD8^+$ T cells in sAg-L patients was also

118 significantly increased compared to sAg-R patients and HC (Figure 1C). Consistent

119 with previous reports [15,19,20], the expression of the T cell exhaustion marker PD-1 on

120 both CD4$^+$ and CD8$^+$ T cells in sAg-R patients was significantly higher than in HC.

121 Interestingly, sAg-L patients showed a further increase in PD-1 expression on both

122 CD4$^+$ and CD8$^+$ T cells compared to sAg-R patients. The CD40L and CTLA-4

123 expression on CD4$^+$ T cells and CD95 expression on CD8$^+$ T cells in sAg-L patients

124 were significantly higher than in sAg-R patients, but not in HC (Figure 1B and 1C). No

125 significant differences in CD69, Tim-3, granzyme B, and CD107a expression on both

126 CD4$^+$ and CD8$^+$ T cells were observed between sAg-L, sAg-R patients, and HC (Figure

127 S2). Moreover, no significant differences in CD4$^+$ and CD8$^+$ T cell phenotypes were

128 observed between sAg-L patients with or without seroconversion to anti-HBs (Figure

129 S3).

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131 It has been shown that a lower HBsAg level is associated with a higher rate of HBsAg

132 loss [21,22]. Therefore, we subsequently examined whether cHBV patients experiencing a

133 rapid HBsAg decrease (sAg-RD) also had an alteration of the T cell phenotype

134 compared to cHBV patients with no decrease of serum HBsAg levels (sAg-ND) during

135 the observational period. Nineteen patients in the sAg-R group experienced more than

136 30% decrease in HBsAg levels from the baseline within 6 months (sAg-RD30), while

137 30 sAg-R patients showed no decrease of HBsAg levels (Table S4). As shown in Figure

138 2, sAg-RD30 patients showed significant increases in HLA-DR, Tim-3 and CD107a

139 expression on both CD4$^+$ and CD8$^+$ T cells compared to sAg-ND patients. CD40L and

140   CTLA-4 expression on CD4$^+$ T cells as well as CD69 expression on CD8$^+$ T cells in

141   sAg-RD30 patients were also significantly higher than those in sAg-ND patients

142   (Figure 2A). No significant differences in CD25, CD95, PD-1, and granzyme B

143   expression on T cells were observed between the two groups of patients (Figure S4).

144   The differences in the T cell phenotype became even more evident when we examined

145   a cohort of patients with a more profound HBsAg decrease (greater than 50% decrease,

146   sAg-RD50). sAg-RD50 patients showed significant increases in HLA-DR, CD95,

147   CD25, TIM-3, CTLA-4, and CD107a expression on CD4$^+$, as well as HLA-DR and

148   TIM-3 expression on CD8$^+$ T cells compared to sAg-ND patients (Figure 2A). No

149   significant differences in CD69, CD40L, PD-1 and granzyme B expression on T cells

150   were observed between the two groups of patients (Figure S4). Consistent with these

151   results, the levels of HLA-DR expression on CD4$^+$ and CD8$^+$ T cells positively

152   correlated with the ratio of HBsAg reduction, but not with the quantities of HBsAg, in

153   cHBV patients (Figure 2B and S5B). In addition, CD95, CD40L, CTLA-4 and CD107a

154   expression levels on CD4 T cells, as well as TIM-3 and Granzyme B on CD8$^+$ T cells,

155   were positively correlated with the ratio of HBsAg reduction in cHBV patients (Figure

156   2B).

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158   Next, we analyzed the correlation between the T cell phenotype and serum ALT and

159   HBV-DNA levels. We found that CD95 and CD25 expression on CD4$^+$ T cells, as well

160   as HLA-DR and PD-1 expression on CD4$^+$ and CD8$^+$ T cells, were positively correlated

161   with serum ALT levels (Figure 2C). No correlations between the T cell phenotype and

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162   serum HBV-DNA levels were observed (Figure S5D).

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164   Overall, these data demonstrated that cHBV patients experiencing a rapid HBsAg

165   decrease or loss have altered T cell phenotypes compared to HBsAg retaining patients

166   mainly associated with cell activation.

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168   ***Association of HBV-specific T cell responses with alteration of T cell phenotype in***

169   ***sAg-L patients***

170   During the natural history of cHBV, the quantity and function of HBV-specific T cells

171   are shown to correlate with HBV control [23]. Thus, we next examined how HBV-specific

172      $CD4^+$ and $CD8^+$ T cell response change in our patient cohorts and how this correlated

173   with T cell phenotypes. The PBMCs from cHBV patients were stimulated for 10 days

174   with overlapping peptide pools spanning the HBcAg or HBsAg to induce HBV-specific

175      $CD4^+$ and $CD8^+$ T cell expansion. Subsequently, the frequencies of IFN-γ, TNF-α or

176   IL-2-producing HBV-specific CD4 or CD8 T cells were detected by FACS. sAg-L

177   patients showed significantly increased frequencies of IFN-γ, TNF-α, or IL-2-

178   producing HBcAg-specific $CD8^+$ T cells, but not $CD4^+$ T cells, compared to sAg-R

179   patients (Figure 3B). Although sAg-L patients that seroconverted to anti-HBs showed

180   higher frequencies of IFN-γ, TNF-α, or IL-2-producing HBcAg-specific $CD8^+$ T cells

181   than non-seroconverters, the differences were not statistically significant (Figure S6A).

182   We also observed that sAg-RD50, but not sAg-RD30 patients showed significant

183   increased frequencies of IFN-γ-producing HBcAg-specific CD4 T cells compared to

184   sAg-ND patients (Figure 3C). No significant increases in the frequencies of IFN-γ,

185   TNF-α, or IL-2-producing HBsAg-specific CD8$^+$ T cells were observed in sAg-L

186   patients compared to sAg-R patients (Figure S7A). Moreover, the correlation between

187   HBV-specific T cell responses and T cell phenotype was analyzed. We demonstrated

188   that HLA-DR, TIM-3, CTLA-4 and granzyme B expression on CD4$^+$ T cells was

189   positively correlated with HBcAg-specific CD4$^+$ T cell responses, while HLA-DR,

190   CD95 and CD25 expression on CD8$^+$ T cells positively correlated with HBcAg-specific

191   CD8$^+$ T cell responses (Figure 3D). These results suggest that the activated phenotype

192   of T cells during rapid HBsAg decrease and loss is associated with increased HBcAg-

193   specific CD8$^+$ T cell responses.

194

195   ***Alteration of the T cell phenotype in sAg-L patients is associated with IFN-α***

196   ***treatment***

197   Accumulating clinical experience and increasing data from clinical trials have

198   demonstrated that peg-IFN-α-containing therapy in cHBV significantly increased the

199   rates of functional cure in selected cHBV patient cohorts [24]. Our data also shows an

200   increased percentage of patients receiving peg-IFN-α-containing therapy in the sAg-L

201   group compared to the sAg-R group (58.1% vs 9.9%, Table S1). Therefore, we next

202   examined whether the treatment strategy with IFN-α had an influence on the T cell

203   phenotype. cHBV patients receiving peg-IFN-α-containing treatment (either alone or

204   in combination with NUCs) showed significant increases in HLA-DR, CD95, CD25,

205   CD40L, PD-1, TIM-3, and CTLA-4 expression on CD4 T cell, as well as HLA-DR,

206   PD-1 and TIM-3 expression on CD8$^+$ T cells, compared to patients receiving NUC

207   treatment alone or no treatment (Figure 4A). Patients receiving peg-IFN-α-containing

208   treatment also showed significantly higher frequencies of IFN-γ, TNF-α, or IL-2-

209   producing HBcAg-specific CD8$^+$ T cells than patients receiving NUC treatment alone

210   (Figure 4B). These results suggest that alterations of the T cell phenotype in sAg-L

211   patients is partially associated with peg-IFN-α treatment, which is known to exhibit

212   immunomodulatory function and known to be beneficial for achieving HBsAg loss in

213   cHBV patients.

214

215   ***Kinetics of the T cell phenotype during rapid HBsAg decrease, loss, and***

216   ***seroconversion***

217   Next, we characterized the kinetics of the T cell phenotype alteration during rapid

218   HBsAg rapid decrease and loss by analyzing the longitudinal data obtained from 7 sAg-

219   ND, 5 sAg-RD50, and 6 sAg-L patients (Figure 5). These patients were followed for up

220   to 60 weeks, monitored for virologic and clinical markers, and sampled 2-8 times for T

221   cell phenotype and HBV-specific T cell response analysis. No significant differences in

222   CD69, HLA-DR, CD25, CD40L, granzyme B, and CD107a expression on CD4$^+$ and

223   CD8$^+$ T cells were observed between the sAg-ND, sAg-RD50, and sAg-L groups at

224   baseline. Interestingly, the sAg-L group showed significantly higher CD95 and CTLA-

225   4 expression on CD4$^+$ T cells and TIM-3 expression on CD8$^+$ T cells compared to the

226   sAg-ND group at baseline (Figure 5A and 5B). The phenotypes of CD4 and CD8 T cells

227   in the sAg-ND patient group remained relatively stable during the entire observational

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228   period. In contrast, the phenotypes of CD4^{+} and CD8^{+} T cells in both the sAg-RD50

229   and sAg-L patients group showed significant alterations during the follow-up period

230   (Figure 5A and 5B). The expressions of HLA-DR, CD25, PD-1, and CTLA-4 on CD4^{+}

231   T cells and HLA-DR on CD8^{+} T cells from sAg-RD50 patients was significantly higher

232   than in sAg-ND patients during the follow-up, which was the period of rapid HBsAg

233   reduction (Figure 5A and 5B). Consistently, significant increases in the expression of

234   these markers were also observed in sAg-L patients both before and after HBsAg loss

235   during the follow-up period. Furthermore, sAg-L patients showed significant

236   upregulation of CD95 on CD4^{+} T cells and PD-1 on CD8^{+} T cells only before but not

237   after HBsAg loss compared to sAg-ND patients (Figure 5A and 5B). An upregulation

238   of CTLA-4 and TIM-3 on CD4^{+} T cells, as well as TIM-3 on CD8^{+} T cells was observed

239   in sAg-L patients only before but not after HBsAg loss (Figure 5A and 5B). Next, the

240   kinetics of the HBcAg-specific CD4^{+} and CD8^{+} T cell response were also analyzed. No

241   significant differences in HBcAg-specific CD4^{+} and CD8^{+} T cell responses were

242   observed between the three groups at baseline. The HBcAg-specific CD8^{+} T cell

243   responses in sAg-ND and sAg-RD50 patients remained relatively stable during the

244   entire observation period. sAg-L patients showed increases in HBcAg-specific CD8^{+} T

245   cell responses only at later time points of the observation period (37-48 weeks of

246   follow-up; Figure 5C). An increase of the HBcAg-specific CD8^{+} T cell responses in

247   sAg-L patients was observed only before but not after HBsAg loss; however, the

248   difference was not statistically significant (Figure 5C).

249

250   Next, the kinetics of T cell phenotype and serum HBsAg level were plotted individually

251   for representative patients from each group. As shown in figure 6A, sAg-ND patients

252   showed a minor alteration of the T cell phenotype and weak or undetectable HBcAg-

253   specific CD8 T cell responses. An association of altered T cell phenotypes, as mainly

254   represented by increasing HLA-DR expression on both $CD4^+$ and $CD8^+$ T cells, with a

255   decrease in serum HBsAg levels was observed in sAg-RD50 patients. However,

256   $HBcAg$-specific $CD8^+$ T cell response was also barely detected in sAg-RD50 patients

257   during rapid HBsAg decrease (Figure 6B). In sAg-L patients, an alteration of the T cell

258   phenotype was observed during the course of HBsAg loss. Additionally, HBcAg-

259   specific $CD8^+$ T cell responses were more frequently detected in sAg-L patients during

260   and after HBsAg loss (Figure 6C). The correlation of the T cell phenotype change with

261   the HBsAb titer was also kinetically analyzed in 6 cHBV patients with seroconversion

262   to anti-HBs. In most of the cases, the upregulation of HLA-DR expression on $CD4^+$

263   and $CD8^+$ T cells was accompanied by an increase in HBsAb levels (Figure S9D).

264

265   ***Predicting HBsAg loss in cHBV patients by T cell surface markers***

266   Our data demonstrate that the alteration of T cell phenotype might occur prior to HBsAg

267   loss, therefore, we next evaluated the predictive performance of HLA-DR, CD25, CD95,

268   PD-1, TIM-3, and CTLA-4 expression levels on T cells, as well as the frequencies of

269   IFN-γ-producing HBcAg-specific $CD8^+$ T cells in predicting HBsAg loss within 48

270   weeks of follow-up. This time frame was chosen, because all incidents of HBsAg loss

271   in our patient cohort occurred within this period. The ROC curve analysis was

272   performed in a total of 54 cHBV patients with HBsAg less than 600 IU/ml. The results

273   showed that HLA-DR or CTLA-4 expression on CD4$^+$ T cells, as well as HLA-DR, or

274   TIM-3 expression on CD8$^+$ T cells were significant predictors for 48-week HBsAg loss

275   (Figure 7). The AUC of CD4$^+$ T cell HLA-DR expression was 0.773 (p=0.031, [95%

276   confidence interval, 0.607-0.939]), the optimal cutoff value was 7.35% (sensitivity=100%

277   and specificity=55.30%). The AUC of CD4$^+$ T cell CTLA-4 expression was 0.785

278   (p=0.024, [95% confidence interval, 0.608-0.963]), the optimal cutoff value was 1.49%

279   (sensitivity=83.30% and specificity=70.20%) (Figure 7A). The AUC of CD8$^+$ T cell

280   HLA-DR expression was 0.807 (p=0.005, [95% confidence interval, 0.637–0.977]), the

281   optimal cutoff value was 12.10% (sensitivity=83.33% and specificity=72.34%). The

282   AUC of CD8$^+$ T cell TIM-3 expression was 0.883 (p=0.002, [95% confidence interval,

283   0.705–1.000]), the optimal cutoff value was 3.26% (sensitivity=66.70% and

284   specificity=97.90%) (Figure 7B). A previous study has reported that serum HBsAg

285   level is a predictor for HBsAg loss [22]. However, our result showed that the AUC of

286   HBsAg for predicting 48-week HBsAg loss was only 0.135 (p=0.018, [95% confidence

287   interval, 0.000-0.467]) (Figure 7C). Therefore, these results suggest that CD4$^+$ T cell

288   HLA-DR and CTLA-4 expression, and CD8$^+$ T cell HLA-DR and TIM-3 expression

289   possess a predictive value for 48-week HBsAg loss in cHBV patients.

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294 **DISCUSSION**

295 In the current study, we longitudinally characterized T cell phenotype profile and HBV-

296 specific response during HBsAg rapid decrease, loss and seroconversion. Importantly,

297 all analyses were performed directly *ex vivo*, since freezing and thawing T cells before

298 analysis may induce profound changes in their functionality [18]. Our findings reveal that

299 the courses of HBsAg rapid decrease and loss in cHBV patients is associated with

300 significant alteration of both CD4 and CD8 T cell phenotypes. We identify that T cells

301 upregulate the expression of a panel of surface molecules, including CD25, CD40L,

302 and CTLA-4 expression on CD4 T cells, and HLA-DR, CD95, and PD-1 expression on

303 both CD4 and CD8 T cells. The expression of HLA-DR, CD95, CD40L, CTLA-4,

304 CD107a on CD4 T cells, and HLA-DR, TIM-3, granzyme B on CD8 T cells is positively

305 corelated with the extent of HBsAg decrease. The expression of HLA-DR, TIM-3,

306 CTLA-4, granzyme B on CD4 T cells and HLA-DR, CD95, CD25 on CD8 T cells were

307 positively corelated with the intensity of HBcAg-specific T cell response. HLA-DR and

308 CTLA-4 expression on CD4, as well as HLA-DR and TIM-3 expression on CD8 T cells

309 were identified as predictive factors for HBsAg loss within 48 weeks in cHBV patients.

310

311 Functional cure is a rare clinical event in cHBV patients, with an annual clearance rate

312 of 0.33% taking on average more than 50 years to clear the virus [25,26]. Previous studies

313 have shown that improved peg-IFN-α-containing treatment strategies, such as "adding-

314 on" or "switching to" peg-IFN-α in patients who achieved long-term effective

315 virological remission by NUCs, may significantly increase the HBsAg loss rates to

316   more than 20%[24,27,28]. In line with these reports, an increased rate of receiving peg-IFN-

317   α-containing therapy in sAg-L patients than sAg-R patients was observed in the patient

318   cohort of this study. Peg-IFN-α has been shown to have both direct antiviral and

319   immunomodulatory effects for the treatment of CHB, and it is conceivable that

320   treatment outcome may be mostly triggered by the immunomodulatory effects of Peg-

321   IFN-α on the innate and adaptive immune responses [29]. The mechanisms responsible

322   for the peg-IFN-α mediated restoration of anti-HBV immune functions are not fully

323   understood. Treatment with peg-IFN-α in CHB patients has been shown to lead to a

324   significant functional augmentation of NK cells, however, quite different effects were

325   observed on T cells [30]. IFN-α therapy led to a striking reduction of CD8+ T cells and

326   showed no effect on the restoration of frequency and early effector functions of HBV-

327   specific CD8+ T cells [30,31]. In contrast to these findings, we observed significant

328   increases in HBV-specific CD8 T cell responses in patients who received IFN-α

329   treatment compared to those without. In addition, patients who received IFN-α

330   treatment demonstrated a more active phenotype of global T cells than IFN-α untreated

331   patients. This is probably due to the majority (> 70%) of the IFN-α treated patient in

332   our study also received NUCs treatment, while all patients in previous studies [30,31] only

333   received IFN-α monotherapy. It has been shown that HBV-specific CD8+ T-cell

334   functions could be restored, at least in part, after long-term treatment with NUCs [17].

335   Therefore, it is assumed that peg- IFN-α could improve the immunomodulatory action

336   of NUCs and vice versa [32,33]. Our observation supports this assumption and indicates

337   that IFN-α therapy may also be favorable for the T cell arm of immune system to control

338 HBV infection in long-term NUC treated patients.

339

340 Our data demonstrates that the courses of HBsAg rapid decrease and loss are associated

341 with increasing global T cell activation as indicated by the upregulation of HLA-DR,

342 CD25, and CD95 on T cells. This is in line with a recent report showing that global T

343 cells of patients with subsequent HBsAg loss showed a more activated phenotype

344 compared to patients with retained HBsAg [34]. Interestingly, we observed that markers

345 usually used to represent CD8 T cell exhaustion statues during chronic viral infection,

346 such as PD-1 and TIM-3, were also upregulated on CD8 T cells during the course.

347 Previous studies have shown that PD-1 is not a definitive marker for complete

348 functional exhaustion but can also be expressed on at least partially functional T cells

349 in the context of a chronic infection [18,35-37]. Patients with partial immune control of HBV

350 infection display higher levels of intrahepatic PD-1$^+$ CD39$^+$ tissue-resident CD8+ T

351 cells that possess the capacity to mount immediate and strong cytokine responses [38].

352 PD-1 has also been shown to prevent virus-specific CD8+ T cells from terminal

353 exhaustion and to contribute to the survival of memory T cell populations [39].

354 Interestingly, a recent study has demonstrated that almost all HBcAg- and HBV

355 polymerase-specific CD8 T cells in cHBV patients were PD-1 positive [40]. Similar

356 results were observed by us in an HBV replication mouse model (data not shown).

357 Taken together, the increases in frequencies of PD-1+ T cells during the course of

358 HBsAg loss may indicate the expansion and activation of HBV-specific T cell

359 populations, but not enhanced T cell exhaustion. Also, the upregulation of PD-1 and

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360 TIM-3 expression on T cells during acute-resolving HBV infection has been reported

361 [41-43], and higher TIM-3 expression on CD8 T cells was found to be associated with

362 elevated serum anti-HBs production in HBV-carrying mice [44]. Together with the

363 observation of increases in HBcAg-specific CD8 T cell response, our data suggest that

364 T cells may experience similar course of activation during HBs loss and seroconversion

365 in chronic HBV infection as do in acute HBV infection.

366

367 The presence of functional HBV-specific T cells is considered as a potential

368 immunological biomarker to evaluate the immune status of cHBV patients and to guide

369 clinical practice, such as safe NUC therapy discontinuation [18]. However, the process of

370 detecting HBV-specific CD8 T cell responses in cHBV patients is relatively

371 complicated to be broadly applied for diagnostic purposes. In this study, we have

372 identified that HLA-DR expression on CD8 T is positively correlated with the intensity

373 of HBcAg-specific CD8 T cell response, and thus it might serve as an easy surrogate

374 detection marker for HBcAg-specific CD8 T cell response in cHBV patients. Besides,

375 we have also identified HLA-DR expression on both CD4 and CD8 T cells, as well as

376 CTLA-4 expression on CD4 T cells and TIM-3 expression on CD8 T cells, as candidate

377 immunological biomarkers for predicting HBsAg loss within 48 weeks. Previous

378 studies have indicated that a lower HBsAg level and HBV DNA level at baseline and

379 older age may predict HBsAg loss and seroconversion [45,46]. Therefore, it would be

380 interesting to investigate whether combining both virological and immunological

381 biomarkers possess a greater power to predict HBsAg loss and seroconversion.

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382

383   There are several limitations of the current study. First is the lack of access to hepatic

384   tissue samples, and thus we cannot compare the phenotypes of intrahepatic T cells with

385   their peripheral counterparts. However, previous reports have shown that the

386   immunologic changes of T cells and NK cells in peripheral blood could closely mirror

387   those in the liver in cHBV patients [47,48]. Second is the lack of analyzing phenotypes of

388   HBV-specific T cells. Due to the low frequency of HBV-specific T cells present in

389   cHBV patients, phenotype analysis of such rare cell populations requires large volume

390   of peripheral blood and applying a pMHC tetramer-based technic to enrich the cells

391   [35,40]. We were unable to perform such an analysis due to the limitation of sample volume

392   we were allowed to take from patients. It would be important to compare whether the

393   phenotype change of global T cells represents that of the HBV-specific T cells.

394

395   In the current study, we present the longitudinal characteristics of T cell phenotypes and

396   responses during the course of HBsAg rapid decrease, loss and seroconversion in the

397   peripheral blood of cHBV patients and identified potential immunological biomarkers

398   to predict HBsAg loss. This knowledge may assist in developing assays to precisely

399   evaluate immune status and to improve treatment strategies for achieving functional

400   cure in cHBV patients.

401

402

403

METHODS

Subjects

A total of 24 healthy controls (HC), 141 HBsAg retained (sAg-R) cHBV patients and cHBV patients with 31 HBsAg loss (sAg-L) were recruited at the Department of Infectious Diseases, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology from June 2016 to March 2019. The diagnosis and phase classification of chronic HBV infection were based on the EASL 2017 Clinical Practice Guidelines on the management of hepatitis B virus infection [3]. All patients tested negative for HIV, HCV, hepatitis E virus, and hepatitis delta virus. Patients with alcoholic liver disease, autoimmune disease, malignancy, or serious illness of other systems were excluded. Informed consent was obtained from each patient, and the study protocol was approved by the local medical ethics committee of Union Hospital, Tongji Medical College, Huazhong University of Science and Technology in accordance with the guidelines of the Declaration of Helsinki. Twenty-four healthy volunteers were enrolled as controls.

Characteristics of study cohort

Demographic profiles and detailed patient characteristics are listed in Table S1 and Table S2. All patients in the sAg-R group remained serum HBsAg positive during the observation period, and 12 of them had been longitudinally monitored for their T cell phenotype profile and HBV-specific T cell response for 60 weeks. In the sAg-L patient group, 6 patients were serum HBsAg positive at the start of the study and experienced

20

426 HBsAg loss during the observation period, among 3 of them showed HBsAg

427 seroconversion. Twenty-five patients were already serum HBsAg negative when

428 enrolled and 13 of them showed HBsAg seroconversion during the observation period.

429 There were no significant differences in age, ALT levels, and sex between the sAg-R

430 and sAg-L patient groups. A significantly higher percentage of patients in the sAg-L

431 group compared to the sAg-R group (58.07% vs 9.93%) had received interferon α

432 (IFNα) treatment either alone or in combination with NUCs for more than 24 weeks.

433 For cHBV patients, 107 of them received NUCs monotherapy treatment, 32 received

434 interferon α (IFN-a) combined or monotherapy treatment and the others received no

435 antiviral treatment.

436

437 *Peripheral blood mononuclear cells (PBMCs)*

438 PBMCs of healthy controls and patients were isolated using Ficoll density gradient

439 centrifugation (DAKEWE Biotech, Beijing) and were freshly used for flow cytometry

440 analysis.

441

442 *Flow cytometry*

443 Surface and intracellular staining for flow cytometry analysis were performed as

444 described previously[49]. The antibodies used for surface and intracellular staining are

445 listed in Table S3. For surface staining, cells were incubated with relevant

446 fluorochrome-labeled antibodies for 20 min at 4°C in the dark. For intracellular

447 cytokine staining, cells were fixed and permeabilized using the Intracellular Fixation &

448   Permeabilization Buffer Set (Invitrogen, USA) and stained with FITC -anti-IFN-γ, PE-

449   anti-IL-2, or APC-anti-TNF-α (eBioscience, San Diego, USA). Freshly isolated cells

450   were used for all assays, and approximately 20 000-40 000 T cells were acquired for

451   each sample using a BD FACS Canto II flow cytometer. Data analysis was performed

452   using Flow Jo software V10.0.7 (Tree Star, Ashland, OR, USA). Cell debris and dead

453   cells were excluded from the analysis based on scatter signals and Fixable Viability Dye

454   eFluor 506 (Thermo Fisher Scientific).

455

456   *Analysis of the HBV-specific CD8$^+$ T cell response in patients*

457   The HBV-specific CD8$^+$ T cells were detected after antigen-specific expansion as

458   previously described[50]. Briefly, PBMCs were resuspended in complete medium (RPMI

459   1640 containing 10% fetal calf serum, 100U/ml penicillin, 100μg/ml streptomycin, and

460   100μM HEPES (4-[2-hydroxyethyl]-1-piperazine ethanesulfonic acid buffer) and

461   stimulated with overlapping peptide pools covering the entire sequences of HBcAg or

462   HBsAg (genotype B and C, GeneBank accession number: AF121243 and AF 112063),

463   anti-CD28/CD49d (0.5μg/ml; BD Biosciences), and recombinant interleukin-2

464   (20U/ml; Hoffmann-La Roche). Fresh medium containing IL-2 was added twice per

465   week. On day 10, the cells were tested for the expression of IFN-γ, TNF-α and IL-2

466   after re-stimulation with corresponding peptide pools by intracellular cytokine staining

467   and subsequent flow cytometry analysis.

468

469   *Statistical analysis*

22

470 Statistical analysis was performed using the SPSS statistical software package (version

471 22.0, SPSS Inc., Chicago, IL, USA). The Shapiro-Wilk method was used to test for

472 normality. Parametric analysis methods were used when the data was normally

473 distributed; otherwise, nonparametric tests were employed. Unpaired t test, paired t test,

474 one-way ANOVA, Pearson product-moment correlation coefficient, log-rank test, and

475 analysis of covariance (ANCOVA) were used where appropriate. The values of the

476 selected parameters for predicting HBsAg loss in cHBV patients were assessed by

477 receiver operating characteristic (ROC) and area under the ROC curve (AUC). All

478 reported P values were two-sided, and a P value less than 0.05 was considered

479 statistically significant.

480

481 **REFERENCES**

482   1   Trautmann, L. *et al.* Upregulation of PD-1 expression on HIV-specific CD8+ T cells leads to
483   reversible immune dysfunction. *Nature medicine* **12**, 1198-1202 (2006).

484   2   Terrault, N. A. *et al.* Update on prevention, diagnosis, and treatment of chronic hepatitis B:
485   AASLD 2018 hepatitis B guidance. *Hepatology* **67**, 1560-1599, doi:10.1002/hep.29800 (2018).

486   3   European Association for the Study of the Liver. Electronic address, e. e. e. & European
487   Association for the Study of the, L. EASL 2017 Clinical Practice Guidelines on the management
488   of hepatitis B virus infection. *Journal of hepatology* **67**, 370-398,
489   doi:10.1016/j.jhep.2017.03.021 (2017).

490   4   Hou, J. *et al.* Guideline of Prevention and Treatment for Chronic Hepatitis B (2015 Update).
491   *Journal of clinical and translational hepatology* **5**, 297-318, doi:10.14218/JCTH.2016.00019
492   (2017).

493   5   Yuen, M. F. *et al.* HBsAg Seroclearance in Chronic Hepatitis B in Asian Patients: Replicative Level
494   and Risk of Hepatocellular Carcinoma. *Gastroenterology* **135**, 1192-1199,
495   doi:https://doi.org/10.1053/j.gastro.2008.07.008 (2008).

496   6   Simonetti, J. *et al.* Clearance of hepatitis B surface antigen and risk of hepatocellular carcinoma
497   in a cohort chronically infected with hepatitis B virus. *Hepatology* **51**, 1531-1537,
498   doi:10.1002/hep.23464 (2010).

499   7   Liu, J. *et al.* Incidence and determinants of spontaneous hepatitis B surface antigen
500   seroclearance: a community-based follow-up study. *Gastroenterology* **139**, 474-482,
501   doi:10.1053/j.gastro.2010.04.048 (2010).

502   8   Thimme, R. *et al.* CD8(+) T cells mediate viral clearance and disease pathogenesis during acute
503   hepatitis B virus infection. *Journal of virology* **77**, 68-76 (2003).

504   9   Asabe, S. *et al.* The size of the viral inoculum contributes to the outcome of hepatitis B virus
505   infection. *Journal of virology* **83**, 9652-9662, doi:10.1128/JVI.00867-09 (2009).

506   10   Bertoletti, A. & Gehring, A. J. The immune response during hepatitis B virus infection. *The*
507   *Journal of general virology* **87**, 1439-1449, doi:10.1099/vir.0.81920-0 (2006).

508   11   Chisari, F. V., Isogawa, M. & Wieland, S. F. Pathogenesis of hepatitis B virus infection.
509   *Pathologie-biologie* **58**, 258-266, doi:10.1016/j.patbio.2009.11.001 (2010).

510   12   Webster, G. J. *et al.* Longitudinal analysis of CD8+ T cells specific for structural and nonstructural
511   hepatitis B virus proteins in patients with chronic hepatitis B: implications for immunotherapy.
512   *Journal of virology* **78**, 5707-5719 (2004).

513   13   Loggi, E. *et al.* Virus-Specific Immune Response in HBeAg-Negative Chronic Hepatitis B:
514   Relationship with Clinical Profile and HBsAg Serum Levels. *PLOS ONE* **8**, e65327,
515   doi:10.1371/journal.pone.0065327 (2013).

516   14   Maini, M. K. *et al.* Direct ex vivo analysis of hepatitis B virus-specific CD8(+) T cells associated
517   with the control of infection. *Gastroenterology* **117**, 1386-1396, doi:10.1016/s0016-
518   5085(99)70289-1 (1999).

519   15   Boni, C. *et al.* Characterization of hepatitis B virus (HBV)-specific T-cell dysfunction in chronic
520   HBV infection. *Journal of virology* **81**, 4215-4225 (2007).

521   16   Tsai, S. L. *et al.* Acute exacerbations of chronic type B hepatitis are accompanied by increased
522   T cell responses to hepatitis B core and e antigens. Implications for hepatitis B e antigen
523   seroconversion. *The Journal of clinical investigation* **89**, 87-96, doi:10.1172/JCI115590 (1992).

524   17   Boni, C. *et al.* Restored function of HBV-specific T cells after long-term effective therapy with
525   nucleos(t)ide   analogues.   *Gastroenterology*   **143**,   963-973   e969,
526   doi:10.1053/j.gastro.2012.07.014 (2012).

527   18   Rivino, L. *et al.* Hepatitis B virus-specific T cells associate with viral control upon nucleos(t)ide-
528   analogue therapy discontinuation. *The Journal of clinical investigation* **128**, 668-681,
529   doi:10.1172/JCI92812 (2018).

530   19   Evans, A. *et al.* Programmed death 1 expression during antiviral treatment of chronic hepatitis
531   B: Impact   of   hepatitis   B   e-antigen   seroconversion.   *Hepatology*   **48**,   759-769,
532   doi:10.1002/hep.22419 (2008).

533   20   Chen, J. *et al.* Intrahepatic levels of PD-1/PD-L correlate with liver inflammation in chronic
534   hepatitis B. *Inflammation research : official journal of the European Histamine Research
535   Society ... [et al.]* **60**, 47-53, doi:10.1007/s00011-010-0233-1 (2011).

536   21   Brunetto, M. R. *et al.* Hepatitis B virus surface antigen levels: a guide to sustained response to
537   peginterferon alfa-2a in HBeAg-negative chronic hepatitis B. *Hepatology* **49**, 1141-1150,
538   doi:10.1002/hep.22760 (2009).

539   22   Tseng, T. C. *et al.* Serum hepatitis B surface antigen levels predict surface antigen loss in
540   hepatitis B e antigen seroconverters. *Gastroenterology* **141**, 517-525, 525 e511-512,
541   doi:10.1053/j.gastro.2011.04.046 (2011).

542   23   Bertoletti, A. & Ferrari, C. Adaptive immunity in HBV infection. *Journal of hepatology* **64**, S71-
543   83, doi:10.1016/j.jhep.2016.01.026 (2016).

544   24   Ren, H. & Huang, Y. Effects of pegylated interferon-alpha based therapies on functional cure
545   and the risk of hepatocellular carcinoma development in patients with chronic hepatitis B. *J
546   Viral Hepat* **26 Suppl 1**, 5-31, doi:10.1111/jvh.13150 (2019).

547   25   Kim, G. A. *et al.* HBsAg seroclearance after nucleoside analogue therapy in patients with
548   chronic hepatitis B: clinical outcomes and durability. *Gut* **63**, 1325-1332, doi:10.1136/gutjnl-
549   2013-305517 (2014).

550   26   Chevaliez, S., Hezode, C., Bahrami, S., Grare, M. & Pawlotsky, J. M. Long-term hepatitis B
551   surface antigen (HBsAg) kinetics during nucleoside/nucleotide analogue therapy: finite
552   treatment   duration   unlikely.   *Journal   of   hepatology*   **58**,   676-683,
553   doi:10.1016/j.jhep.2012.11.039 (2013).

554   27   Qiu, K. *et al.* Systematic review with meta-analysis: combination treatment of regimens based
555   on pegylated interferon for chronic hepatitis B focusing on hepatitis B surface antigen
556   clearance. *Alimentary pharmacology & therapeutics* **47**, 1340-1348, doi:10.1111/apt.14629
557   (2018).

558   28   Hu, P. *et al.* HBsAg Loss with Peg-interferon Alfa-2a in Hepatitis B Patients with Partial Response
559   to Nucleos(t)ide Analog: New Switch Study. *Journal of clinical and translational hepatology* **6**,
560   25-34, doi:10.14218/JCTH.2017.00072 (2018).

561   29   Thimme, R. & Dandri, M. Dissecting the divergent effects of interferon-alpha on immune cells:
562   time to rethink combination therapy in chronic hepatitis B? *Journal of hepatology* **58**, 205-209,
563   doi:10.1016/j.jhep.2012.11.007 (2013).

564   30   Micco, L. *et al.* Differential boosting of innate and adaptive antiviral responses during
565   pegylated-interferon-alpha therapy of chronic hepatitis B. *Journal of hepatology* **58**, 225-233,
566   doi:10.1016/j.jhep.2012.09.029 (2013).

567   31   Penna, A. *et al.* Peginterferon--α does not improve early peripheral blood HBV-specific T-cell

568   responses in HBeAg-negative chronic hepatitis. *Journal of hepatology* **56**, 1239-1246,
569   doi:10.1016/j.jhep.2011.12.032 (2012).

570   32   Vigano, M., Invernizzi, F., Grossi, G. & Lampertico, P. Review article: the potential of interferon
571   and nucleos(t)ide analogue combination therapy in chronic hepatitis B infection. *Alimentary*
572   *pharmacology & therapeutics* **44**, 653-661, doi:10.1111/apt.13751 (2016).

573   33   Vigano, M., Grossi, G., Loglio, A. & Lampertico, P. Treatment of hepatitis B: Is there still a role
574   for interferon? *Liver international : official journal of the International Association for the Study*
575   *of the Liver* **38 Suppl 1**, 79-83, doi:10.1111/liv.13635 (2018).

576   34   Rinker, F. *et al.* Hepatitis B virus-specific T cell responses after stopping nucleos(t)ide analogue
577   therapy in HBeAg-negative chronic hepatitis B. *Journal of hepatology* **69**, 584-593,
578   doi:10.1016/j.jhep.2018.05.004 (2018).

579   35   Wieland, D. *et al.* TCF1(+) hepatitis C virus-specific CD8(+) T cells are maintained after cessation
580   of chronic antigen stimulation. *Nature communications* **8**, 15050, doi:10.1038/ncomms15050
581   (2017).

582   36   Im, S. J. *et al.* Defining CD8+ T cells that provide the proliferative burst after PD-1 therapy.
583   *Nature* **537**, 417-421, doi:10.1038/nature19330 (2016).

584   37   Paley, M. A. *et al.* Progenitor and terminal subsets of CD8+ T cells cooperate to contain chronic
585   viral infection. *Science* **338**, 1220-1225, doi:10.1126/science.1229620 (2012).

586   38   Pallett, L. J. *et al.* IL-2(high) tissue-resident T cells in the human liver: Sentinels for hepatotropic
587   infection. *J Exp Med* **214**, 1567-1580, doi:10.1084/jem.20162115 (2017).

588   39   Odorizzi, P. M., Pauken, K. E., Paley, M. A., Sharpe, A. & Wherry, E. J. Genetic absence of PD-1
589   promotes accumulation of terminally differentiated exhausted CD8+ T cells. *J Exp Med* **212**,
590   1125-1137, doi:10.1084/jem.20142237 (2015).

591   40   Schuch, A. *et al.* Phenotypic and functional differences of HBV core-specific versus HBV
592   polymerase-specific CD8+ T cells in chronically HBV-infected patients with low viral load. *Gut*
593   **68**, 905, doi:10.1136/gutjnl-2018-316641 (2019).

594   41   Zhang, Z. *et al.* Dynamic programmed death 1 expression by virus-specific CD8 T cells correlates
595   with the outcome of acute hepatitis B. *Gastroenterology* **134**, 1938-1949, 1949 e1931-1933,
596   doi:10.1053/j.gastro.2008.03.037 (2008).

597   42   Zhang, Z. *et al.* Dynamic decrease in PD-1 expression correlates with HBV-specific memory CD8
598   T-cell development in acute self-limited hepatitis B patients. *Journal of hepatology* **50**, 1163-
599   1173, doi:10.1016/j.jhep.2009.01.026 (2009).

600   43   Wu, W. *et al.* Tim-3 expression on peripheral T cell subsets correlates with disease progression
601   in hepatitis B infection. *Virology journal* **8**, 113, doi:10.1186/1743-422X-8-113 (2011).

602   44   Wu, W. *et al.* Blockade of Tim-3 signaling restores the virus-specific CD8(+) T-cell response in
603   patients with chronic hepatitis B. *European journal of immunology* **42**, 1180-1191,
604   doi:10.1002/eji.201141852 (2012).

605   45   Chan, H. L., Wong, G. L., Tse, C. H., Chan, H. Y. & Wong, V. W. Viral determinants of hepatitis B
606   surface antigen seroclearance in hepatitis B e antigen-negative chronic hepatitis B patients.
607   *The Journal of infectious diseases* **204**, 408-414, doi:10.1093/infdis/jir283 (2011).

608   46   Chen, Y. C., Jeng, W. J., Chu, C. M. & Liaw, Y. F. Decreasing levels of HBsAg predict HBsAg
609   seroclearance in patients with inactive chronic hepatitis B virus infection. *Clinical*
610   *gastroenterology and hepatology : the official clinical practice journal of the American*
611   *Gastroenterological Association* **10**, 297-302, doi:10.1016/j.cgh.2011.08.029 (2012).

612   47   Zhang, Z. *et al.* Hypercytolytic activity of hepatic natural killer cells correlates with liver injury
613   in chronic hepatitis B patients. *Hepatology* **53**, 73-85, doi:10.1002/hep.23977 (2011).

614   48   Fisicaro, P. *et al.* Antiviral intrahepatic T-cell responses can be restored by blocking programmed
615   death-1 pathway in chronic hepatitis B. *Gastroenterology* **138**, 682-693, 693 e681-684 (2010).

616   49   Yang, S. *et al.* MMP2/MMP9-mediated CD100 shedding is crucial for inducing intrahepatic anti-
617   HBV CD8 T cell responses and HBV clearance. *Journal of hepatology*,
618   doi:10.1016/j.jhep.2019.05.013 (2019).

619   50   Kefalakes, H. *et al.* Adaptation of the hepatitis B virus core protein to CD8(+) T-cell selection
620   pressure. *Hepatology* **62**, 47-56, doi:10.1002/hep.27771 (2015).

27

Authors contributions:

Conceived and designed the experiments: SEX, DZ, JL, XZ. Performed the experiments: SEX, DZ, BYL, MYL, WP, JYH. Analyzed and interpreted the data: SEX, DZ, JL, XZ. Contributed reagents/materials/analysis tools: HW, MJL, DLY, JL, XZ. Drafted the manuscript: SEX, MJL, KS, UD, DL, XZ, JL.

636   **Figure legends**

637   **Figure 1. Characterization of T cell phenotype profiles in cHBV patients with**

638   **HBsAg loss.** (A) The mean percentage of CD4 and CD8 T cells expressing different T

639   cell surface markers of healthy controls (HC), HBsAg retained patients (sAg-R) and

640   HBsAg loss (sAg-L) were calculated by z-score and are depicted by radar plots. (B)

641   Expression of HLA-DR, CD25, CD95, PD-1, CD40L, and CTLA-4 on CD4 T cells of

642   HC, sAg-R and sAg-L patients were analyzed by flow cytometry. (C) Expression of

643   HLA-DR, CD95, and PD-1 on CD8 T cells of HC, sAg-R and sAg-L patients were

644   analyzed by flow cytometry. Error bars, mean ± s.e.m.; *P < 0.05; **P < 0.01; ***P <

645   0.001; ****P < 0.0001. Abbreviation: ns, not significant.

646

647   **Figure 2. Characterization of T cell phenotype profiles in cHBV patients with**

648   **HBsAg rapid decrease.** (A) Expression of HLA-DR, CD25, CD40L, TIM-3, CTLA-4,

649   and CD107a on CD4 T cells, and CD69, HLA-DR, TIM-3 and CD107a on CD8 T cells

650   were analyzed by flow cytometry and were compared between cHBV patients who

651   experienced more than 30% decrease of HBsAg levels (sAg-RD30, n=19), patients who

652   experienced more than 50% decrease of HBsAg levels compared to the baseline within

653   6 months (sAg-RD30, n=8)and patients with no decrease of serum HBsAg levels (sAg-

654   ND, n=30). (B) Pearson correlation analysis between expression of corresponding T

655   cell markers and the extent of HBsAg reduction was performed in CHB patients. (C)

656   Pearson correlation analysis between the expression of corresponding T cell markers

657   and ALT levels was performed in CHB patients. Error bars, mean ± s.e.m.; *p<0.05,

658   **p<0.01, ****p<0.0001, ns-not significant (p>0.05).

659

660 **Figure 3. Characterization of HBV specific T cell responses in cHBV patients with**

661 **HBsAg loss and rapid decrease.** PBMCs from chronic hepatitis B patients were

662 stimulated with overlapping peptide pools covering the entire sequences of HBcAg or

663 HBsAg for 10 days. Cells were analyzed for IFN-γ, TNF-α, and IL-2 production by

664 intracellular cytokine staining. (A) Gating strategy of intracellular cytokine staining

665 analysis. (B) HBV-specific T cell responses were compared between HBsAg retained

666 patients (sAg-R) and HBsAg loss (sAg-L) patients. (C) HBV-specific T cell responses

667 were compared between sAg-ND, sAg-RD30 patients and sAg-RD50 patients. (D)

668 Pearson correlation analysis between expression of corresponding T cell markers and

669 frequencies of IFN-γ-producing HBcAg-specific T cells was performed in CHB

670 patients. Error bars, mean ± s.e.m.; *p<0.05, **p<0.01, ****p<0.0001, ns-not

671 significant (p>0.05).

672

673 **Figure 4. Characterization of T cell phenotype and HBcAg-specific T cell**

674 **responses in cHBV patients received IFN-α treatment.** T cell phenotypes (A) and

675 HBcAg-specific T cell responses (B) were compared between cHBV patients received

676 NUC monotherapy (cHBV-NUC), IFN-α contained therapy (cHBV-IFN), and no

677 therapy (CHB-NT). Error bars, mean ± s.e.m.; *p<0.05, **p<0.01, ****p<0.0001, ns-

678 not significant (p>0.05).

679

680 **Figure 5. Kinetic analysis of T cell phenotypes and HBcAg-specific T cell responses**

681 **in cHBV patients during HBsAg rapid decrease and loss.** The phenotypes of CD4 T

682   cells (A), CD8 T cells (B), and HBcAg-specific T cell responses (C) were longitudinally

683   analyzed during the observation period. Left: Data were pooled and compared at either

684   baseline (green background) and follow-up period (pink background) between cHBV

685   patients with no HBsAg decrease (sAg-ND), HBsAg rapid decrease (sAg-RD50), and

686   HBsAg loss (sAg-L). Right: Kinetic changes of T cell phenotype and HBcAg-specific

687   T cell responses were demonstrated at indicated time points for sAg-ND, sAg-RD50,

688   and sAg-L patients. Error bars, mean ± s.e.m.; *$p<0.05$, **$p<0.01$, ****$p<0.0001$, ns-

689   not significant ($p>0.05$).

690

691   **Figure 6. Kinetic analysis of T cell phenotypes and HBcAg-specific T cell responses**

692   **in individual cHBV patients.** (A) Data from 5 representative patients with no HBsAg

693   reduction. (B) Data from 5 patients who experienced greater than 50% decrease of

694   HBsAg levels than the baseline within 6 months. (C) Data from 6 patients who

695   experienced HBsAg loss.

696

697   **Figure 7. Predictive factors for HBsAg loss within 48 weeks in cHBV patients.** ROC

698   curve and AUC were calculated for HLA-DR and CTLA-4 expression of CD4 T cells

699   (A), HLA-DR and TIM-3 expression of CD8 T cells (B) and HBsAg (C) by using R

700   package "pROC".
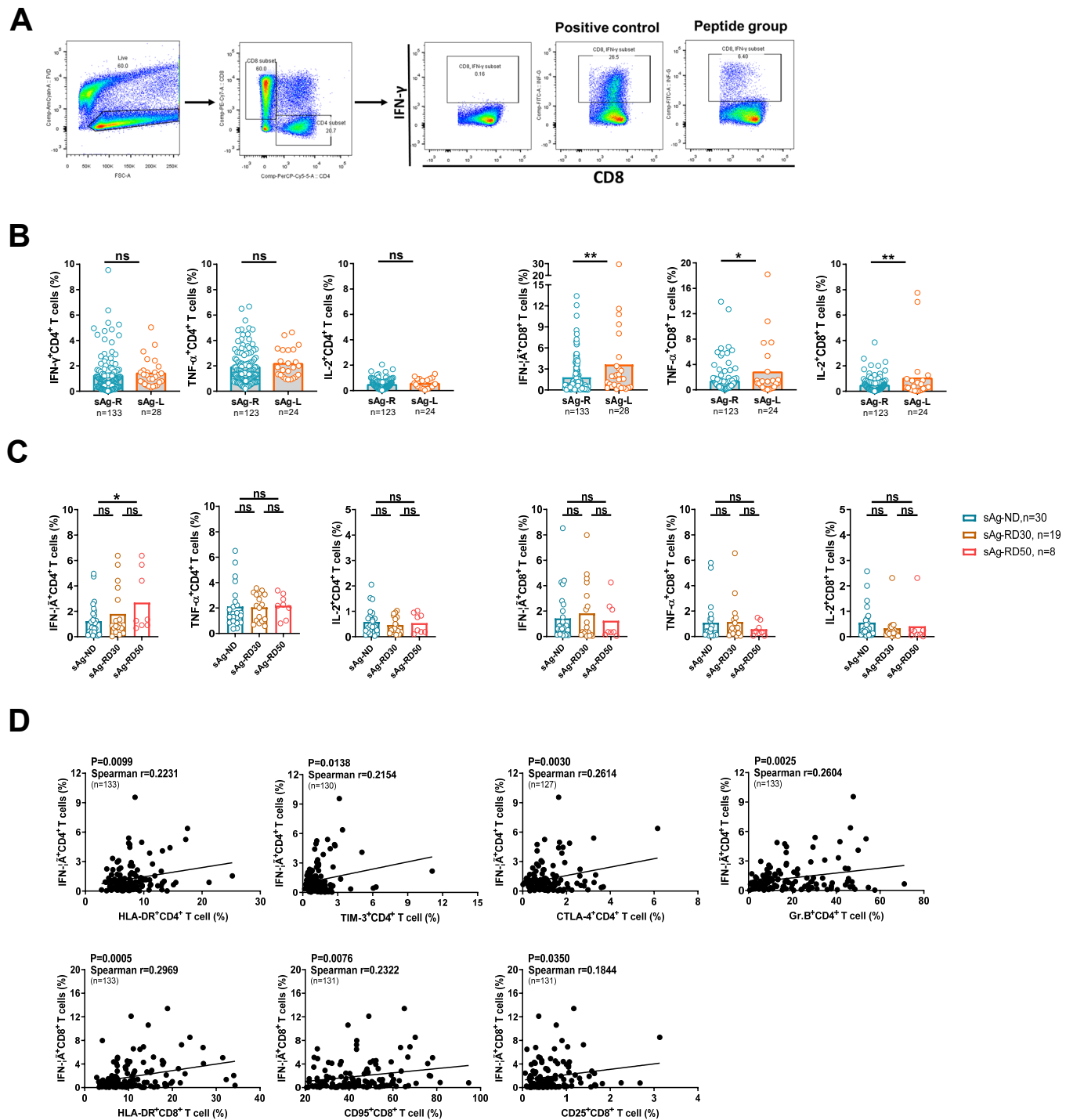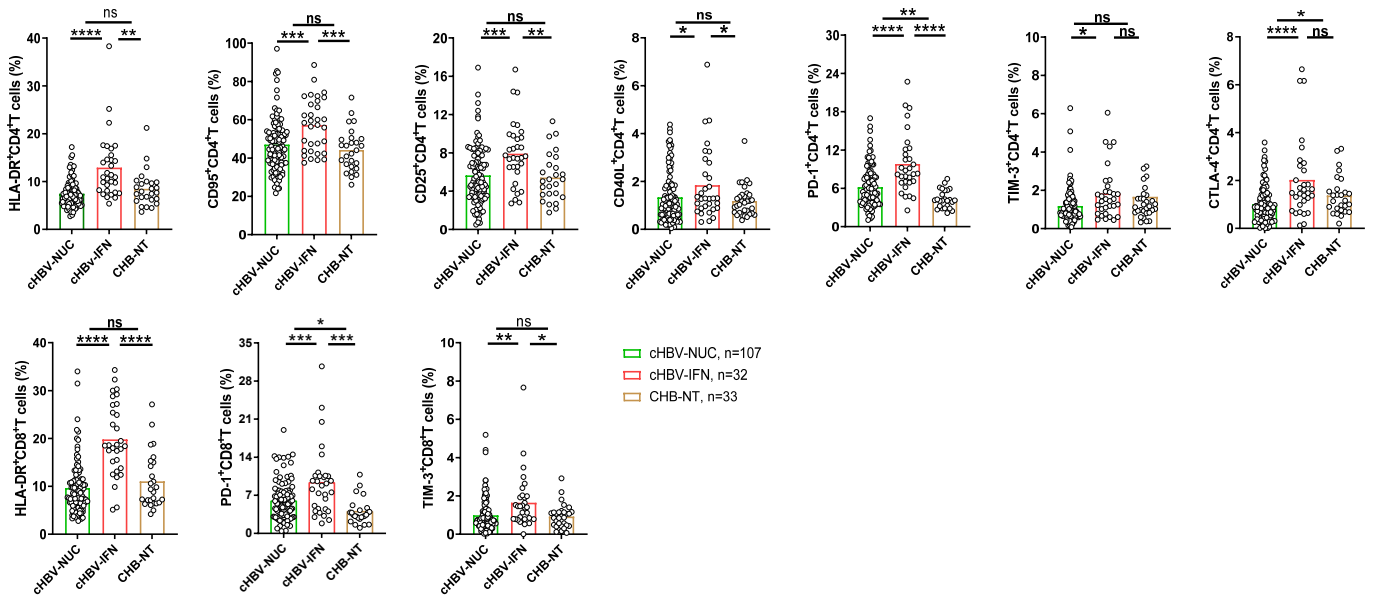
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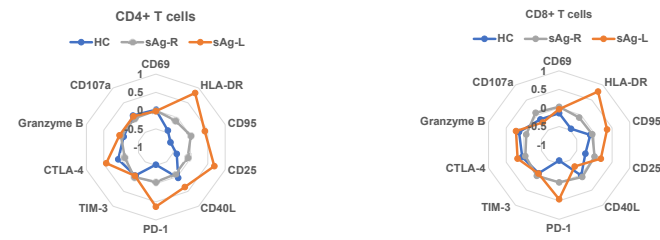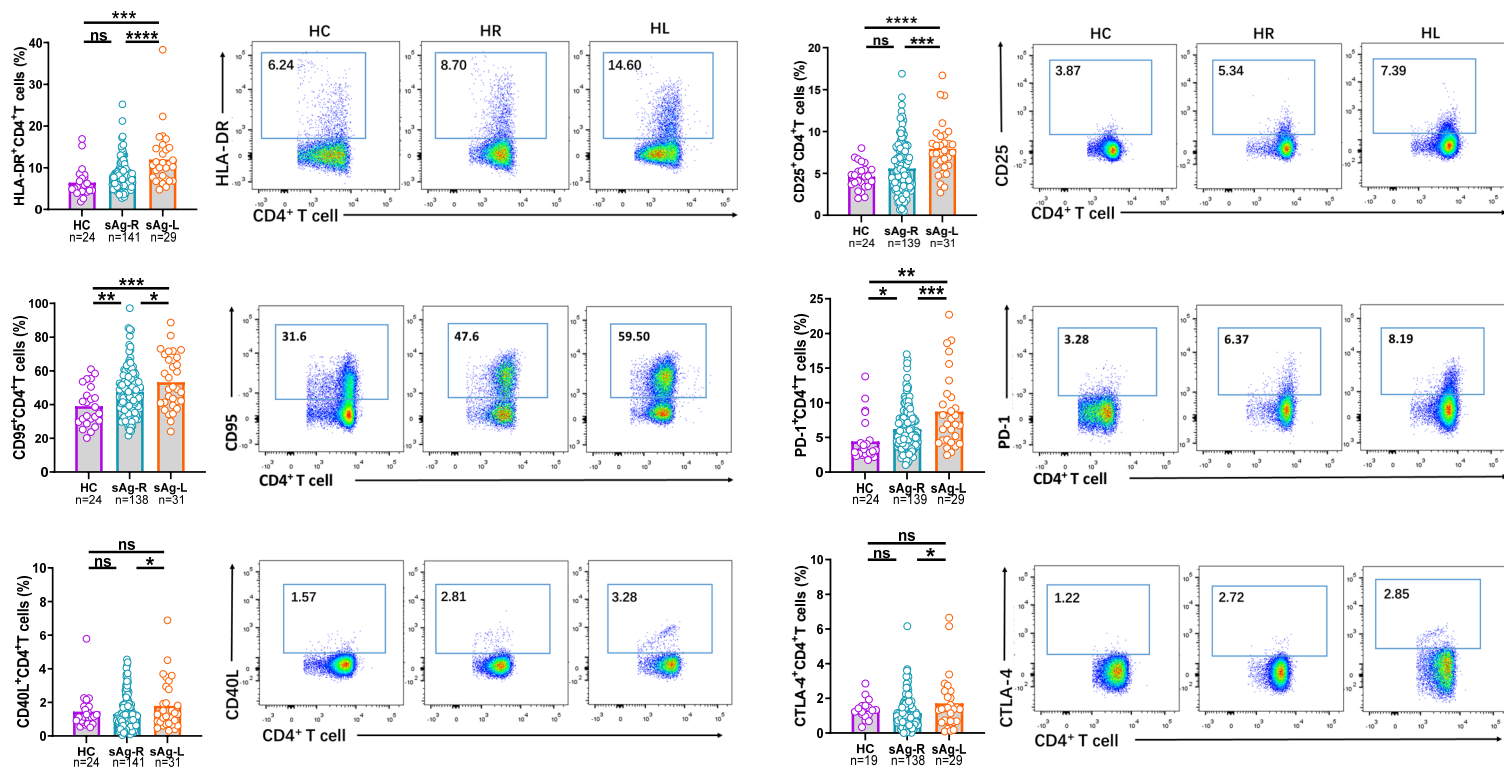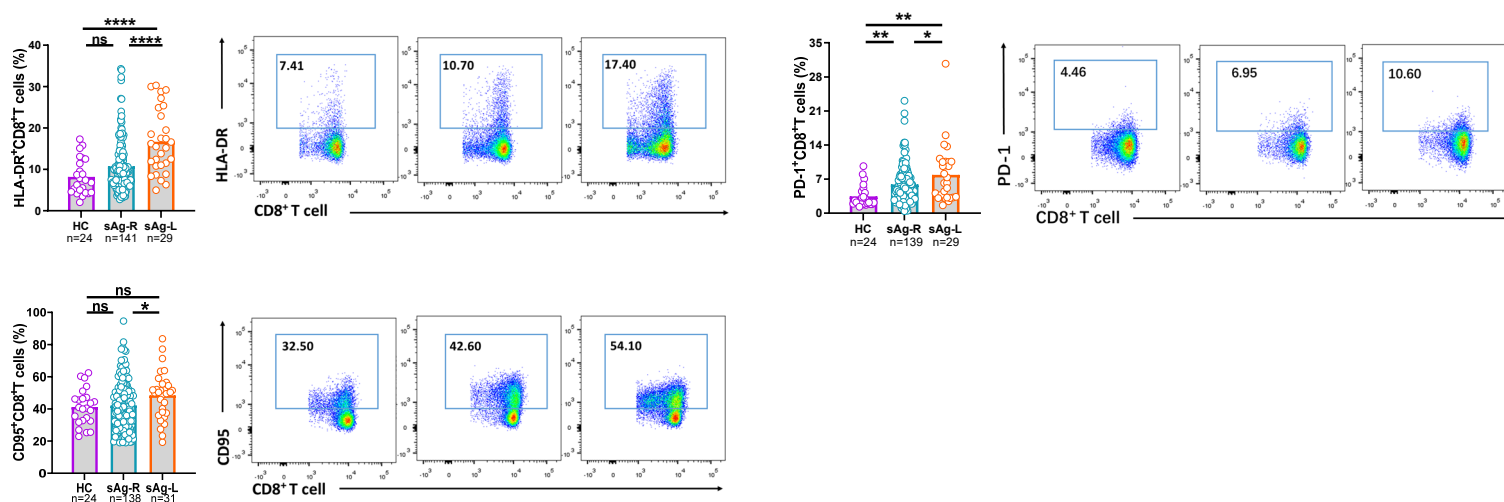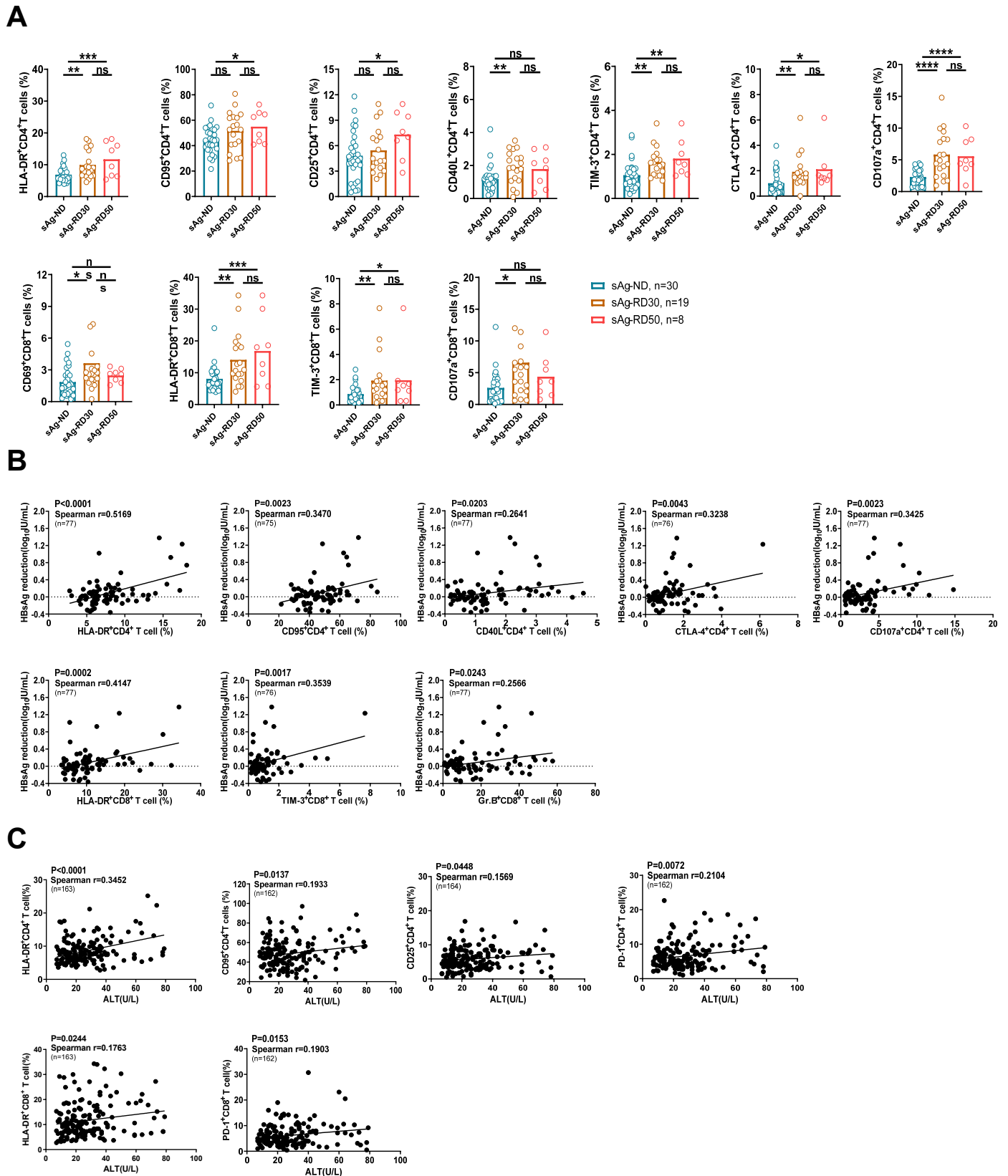
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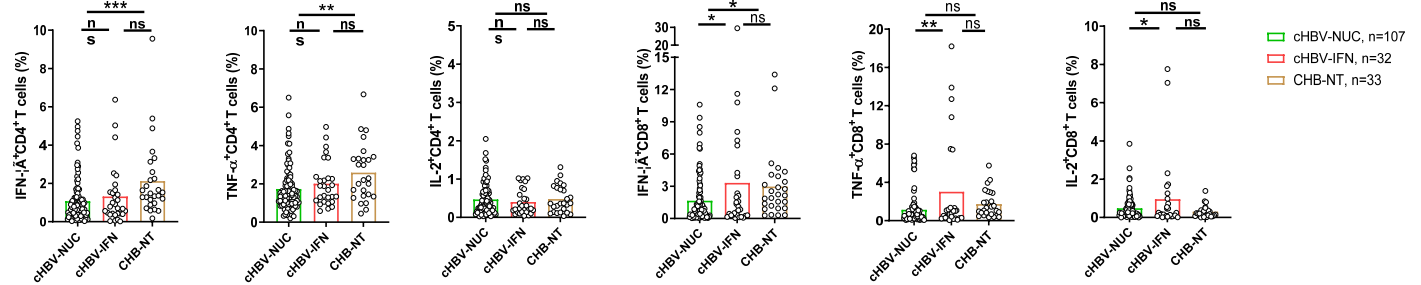
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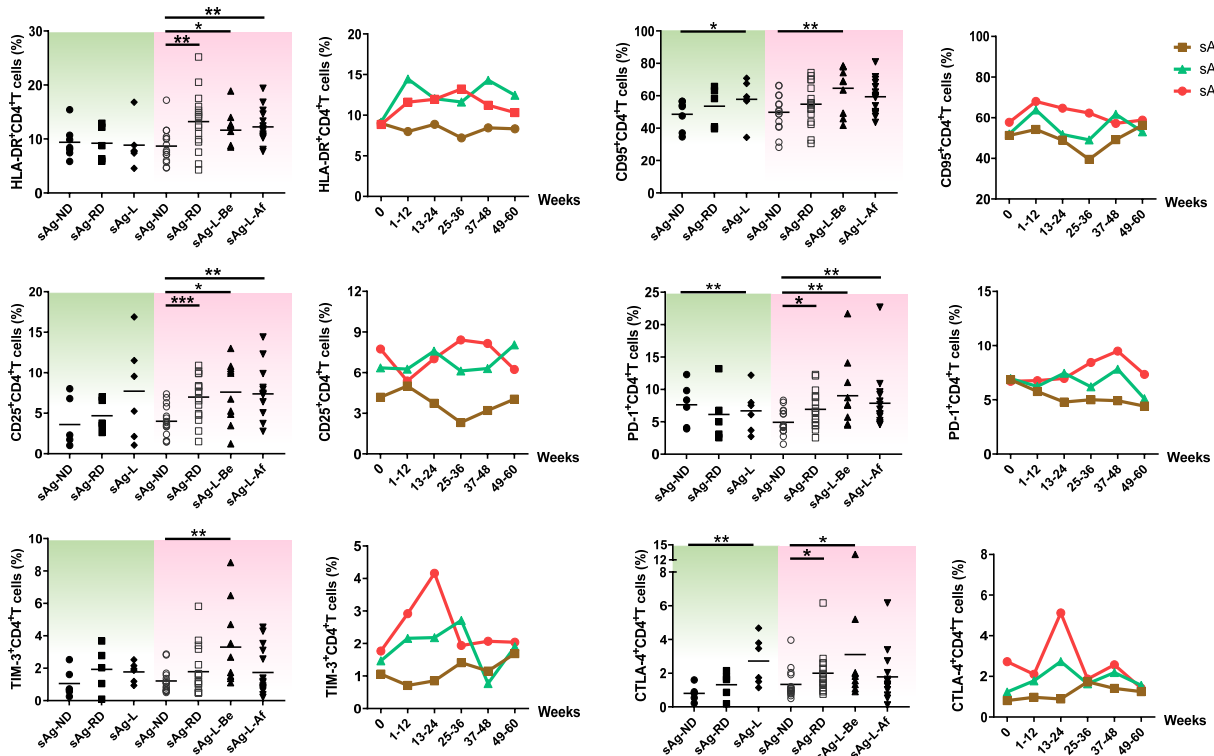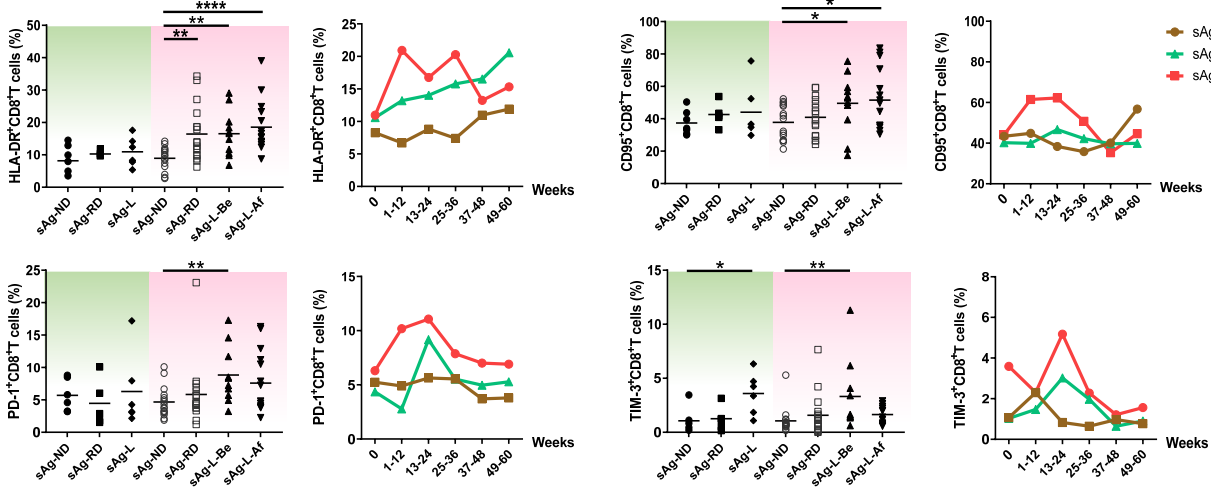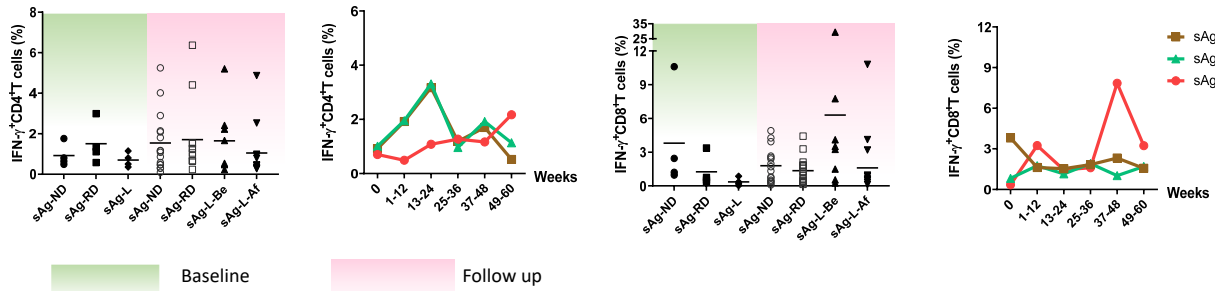
Figure 3

Figure 4

Figure 5

Figure 6

Figure 7