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2 **Asymptomatic primary infection with Epstein-Barr virus: observations on young adult**

3 **cases**

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14 Running Head: Asymptomatic primary EBV infection

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19

20 **ABSTRACT**

21 Epstein-Barr virus (EBV) is typically acquired asymptomatically in childhood. By contrast,

22 infection later in life often leads to infectious mononucleosis (IM), a febrile illness

23 characterised by anti-EBV IgM antibody-positivity, high loads of circulating latently-infected

24 B cells, and a marked lymphocytosis caused by hyper-expansion of EBV-specific CD8$^+$ T cells

25 plus milder expansion of CD56^{dim} NKG2A$^+$ KIR$^-$ NK cells. How the two situations compare is

26 unclear due to the paucity of studies on clinically-silent infection. Here we describe five

27 prospectively-studied asymptomatic infections identified in a sero-epidemiological survey of

28 University entrants. In each case the key blood sample had high cell-associated viral loads

29 without marked IM-like CD8 lymphocytosis or NK cell disturbance. Two of the highest viral

30 load cases showed a coincident expansion of activated EBV-specific CD8$^+$ T cells but overall

31 CD8$^+$ T cell numbers were either unaffected or only mildly increased. Two slightly lower load

32 cases, which serology suggests may have been caught earlier in the course of infection, also

33 showed no T or NK cell expansion at the time. Interestingly, in another higher load case

34 where T and NK cell responses were undetectable in the primary infection bleed, EBV-

35 specific T cell responses did not appear until several months later, by which time virus loads

36 in the blood had already fallen. Thus some asymptomatic primary infections have very high

37 circulating viral loads and a cell-mediated immune response that is qualitatively similar to

38 IM but of lower magnitude. However, others may be quite different and ultimately could

39 reveal novel mechanisms of host control.

40

41 **IMPORTANCE**

42 Epstein-Barr virus (EBV) is transmitted orally, replicates in the throat and then invades the B

43 lymphocyte pool through a growth-transforming latent infection. While primary infection in

44 childhood is usually asymptomatic, delayed infection is associated with infectious

45 mononucleosis (IM), a febrile illness with high circulating viral loads and an exaggerated

46 virus-induced immune response involving both CD8$^+$ T cells and natural-killer (NK) cells. Here

47 we show that in five cases of asymptomatic infection, virus loads in the blood are as high as

48 in acute IM whereas cell-mediated responses, even where they resemble IM in timing and

49 quality, are never as exaggerated. We infer that IM symptoms arise as a consequence not of

50 the virus infection *per se*, but of the hyper-activated immune response. Interestingly, there

51 were idiosyncratic differences among asymptomatic cases in the relationship between viral

52 load and response kinetics, emphasising how much there is still to learn about primary EBV

53 infection.

54

55 **INTRODUCTION**

56 Epstein-Barr virus (EBV), a gamma-1 herpesvirus with B cell growth-transforming ability, is

57 widespread in all human populations and carried by most individuals as a seemingly

58 harmless infection (1). The virus is orally spread and is typically acquired from close family

59 contacts during infancy or early childhood, at which time infection is almost always clinically

60 silent. However, with rising standards of hygiene in the developed world, primary infection

61 is increasingly being delayed until the second or third decade. This often leads to infectious

62 mononucleosis (IM), an illness whose symptoms (fever, sore throat, cervical

63 lymphadenopathy and fatigue) range from mild to severe and may take between 1 and 6

64 weeks to resolve (2). The proportion of delayed primary infections that are symptomatic

65 varies from 25 to >80% in different reports, likely reflecting differences in the rigour of

66 follow-up and clinical monitoring (3-6). Such symptomatic cases have been extensively

67 studied; indeed what little is known about the virology and immunology of primary EBV

68   infection comes almost exclusively from IM patients. Thus, by the time IM symptoms appear

69   (typically 5-7 weeks after oral transmission), high levels of infectious virus can be detected

70   in throat washings, reflecting lytic virus replication in epithelial and probably also B cells at

71   oropharyngeal surfaces (7, 8). Also, by this time, the virus has already spread into the

72   general B cell pool as a latent infection. Many latently-infected B cells can be found both in

73   tonsillar lymphoid tissue, where proliferating EBV growth-transformed cells exist alongside

74   cells with varying degrees of more restricted latency (9, 10), and in the blood, where most if

75   not all cells have fully suppressed EBV growth-transforming protein expression, moved to a

76   resting state and entered the recirculating memory B cell pool (11). While virus shedding in

77   the throat may continue at a high level for several months post-IM (5, 12), the number of

78   latently-infected cells in the blood tends to fall quickly during the disease course itself, then

79   more gradually to reach a stable set-point over the following weeks and months (11, 13).

80   These events are coincident with a range of immune responses (14). Thus IM patients can

81   be diagnosed serologically through the presence of IgM, and rising IgG, antibody titres to

82   the lytic EB viral capsid antigen (VCA) in the absence of an IgG response to the latent nuclear

83   antigen EBNA1; thereafter the IgM response gradually subsides, the anti-VCA IgG titre

84   stabilises and, months later, the anti-EBNA1 IgG response appears (2). Acute IM blood is also

85   characterised by a large expansion of $CD8^+$ T cells with an activated effector ($CD38^+$ HLA-DR$^+$

86    $CCR7^-$ $CD45RA^-$), apoptosis-prone ($Bcl2^{low}$) phenotype. Many of these activated cells are

87   specific for immunodominant EBV antigens of the immediate early and early lytic cycle,

88   accompanied by smaller (sometimes delayed) responses against latent antigens (15, 16).

89   The CD8 expansion in the blood is most marked in clinically more severe IM (5) and

90   contracts slowly as symptoms resolve, generating a memory population of resting EBV-

91   specific cells. Some memory cells re-express the CCR7 lymphoid homing marker, facilitating

92   entry into tonsillar tissue and ultimately the establishment of a tissue-resident memory

93   population at this site (17, 18). There is also a comparatively weak (median <2-fold)

94   expansion of circulating NK cell numbers in acute IM, reflecting an increase in cells whose

95   phenotype (CD56^{dim} NKG2A$^+$ KIR$^-$) is intermediate between the less mature (CD56^{bright}

96   NKG2A$^+$ KIR$^-$) and more mature (CD56^{dim} NKG2A$^-$ KIR$^+$) subsets of NK cells present in blood

97   (19-21). At the same time circulating dendritic cells of the myeloid (mDC), and especially the

98   plasmacytoid (pDC), subsets are reduced in number (20, 22). Finally, a range of anti-viral

99   and/or pro-inflammatory cytokines and chemokines are reported to be elevated in IM

100 plasma, including interferon(IFN)β and IFNγ, interleukin (IL)1, IL6, IL10, IL12 and IL18,

101 tumour necrosis factor(TNF)α, monokine induced by IFNγ (MIG/CXCL9) and IFNγ-induced

102 protein 10 (IP-10/CXCL10) (23-26). Since many of these cytokines and chemokines can be

103 produced by multiple different cell types, it is difficult to say whether they derive from

104 infected cells per se or from cells activated as part of the immune response to infection.

105   The factors determining whether primary EBV infection is asymptomatic, or presents as

106 IM, are poorly understood. Clearly the age at which the virus is acquired is important. In that

107 context, the greater risk of IM among adolescents and young adults compared to children

108 has been variously ascribed to their greater chance of acquiring a high initial virus dose by

109 kissing (14), to the diminishing competence with age of early NK cell control over new virus

110 acquisition (19), and to the increasing breadth with age of T cell memory such that

111 responses to a new agent may be inflated by cross-reactive recognition from previously-

112 primed specificities (27). That said, the effect of age is not absolute because classical IM is

113 seen occasionally in paediatric cohorts (13, 19) and may indeed be under-recognised there.

114 Furthermore, epidemiologic studies have found a greater concordance of IM incidence

115 among monozygotic twins than among dizygotic twins and first degree relatives, strongly

116   implying a genetic element to IM risk that is superimposed upon environmental influences

117   (28, 29).

118   A major barrier to progress in this field is our almost complete ignorance of the virologic

119   and immunologic events that occur in asymptomatic primary infection. Some early studies

120   attempted to address these issues in paediatric cohorts but were largely limited to serologic

121   screening or to the limited cellular immunologic assays available at that time (30-32).

122   Several more recent reports have monitored EBV acquisition in African children but mainly

123   in circumstances where it was not only difficult to assess symptomatology but also where

124   confounding factors affecting immune competence, notably co-infection with HIV and/or

125   malaria, appeared to have predisposed to the high EBV loads observed (33-36). There are

126   many differences, therefore, between such complex scenarios and clinically silent EBV

127   acquisition in the non-immunocompromised host, particularly as occurs covertly in young

128   adults in the developed world. Only one previous study, based in Australia, has focused on

129   asymptomatic primary infection in these circumstances (37). During prospective screening

130   of volunteers for an early EBV vaccine trial, Silins et al serendipitously identified four healthy

131   anti-VCA IgM$^+$, anti-VCA IgG$^{+/-}$, anti-EBNA1 IgG$^-$ individuals, two of whom also had IM-like

132   EBV genome loads in the blood. Although not analysed for EBV-specific T cell or NK cell

133   responses, all four asymptomatic sero-convertors had peripheral blood mononuclear cell

134   (PBMC) counts in the normal range and only one showed evidence of IM-like clonal T cell

135   expansions by T cell receptor Vβ analysis (37). The present study set out to identify more

136   such individuals, in this case within a cohort of more than 400 medical students who

137   volunteered blood samples at the beginning of their University course and, in some cases,

138   annually over the following four years. All were monitored for serologic status with respect

139   to two herpesviruses, EBV and cytomegalovirus (CMV), and for EBV load in PBMCs. Overall,

140   using a combination of serologic and virologic parameters, six subjects were identified as

141   likely to be undergoing asymptomatic primary infection with EBV at the time a bleed was

142   taken; additional bleeds allowed prospective studies in five of these cases.

143

144   **RESULTS**

145   **Identifying cases of asymptomatic primary infection**

146   A total of 448 medical school entrants, all in good health and with no recent history of

147   infectious disease, volunteered an initial blood sample. Of these, 278 (62%) were found to

148   be anti-VCA IgG$^+$, suggestive of an already established EBV carrier state. Viral DNA was

149   detected in the majority but not all seropositive cases (Fig. 1); this reflects the limitation of

150   the assay when applied to DNA from 6 x 10^5 PBMCs (36), since in our hands almost all

151   seropositive individuals are detectably EBV genome-positive in the same assay using DNA

152   from 6 x 10^5 purified peripheral blood B cells (10). As seen before among virus carriers in the

153   UK population (10, 36), viral load values were spread across a very wide (10^4-fold) range

154   (Fig. 1). Indeed that range slightly overlaps the range of virus loads seen in acute IM

155   patients, though the IM median value is around 70-fold higher than the median for the virus

156   carrier state.

157   During the analysis of these initial bleeds, or subsequently during annual follow-ups, a

158   combination of viral load assays and serologic screening identified six individuals

159   (designated AS1-6) who had been fortuitously sampled while undergoing an asymptomatic

160   primary infection. Four cases came to light through being EBV genome-positive in the viral

161   load assay yet serologically anti-VCA IgG$^−$. Further antibody assays on the plasma samples

162   taken at this time showed that two of the above individuals (AS1, AS5) were anti-VCA IgM$^+$

163   and anti-EBNA1 IgG$^−$ at the time, while the other two (AS3, AS4) were still seronegative even

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164 for anti-VCA IgM. Interestingly all four individuals had high to very high EBV genome loads in

165 the blood (770-15,600 genomes per 10^6 PBMCs) but remained asymptomatic in the weeks

166 following the sample being taken. All four were studied prospectively either through

167 additional voluntary bleeds or through bi-annually collected samples.

168   To search for other examples of occult primary infection, we retrospectively screened

169 248 plasma samples from the above anti-VCA IgG$^+$ cohort and looked for cases with an anti-

170 VCA IgM$^+$, anti-EBNA1 IgG$^-$ profile. Interestingly 17 samples were anti-EBNA1 IgG$^-$ but, of

171 these, 15 were also anti-VCA IgM$^-$. We infer that these 15 anti-VCA IgM$^-$, anti-VCA IgG$^+$,

172 anti-EBNA1 IgG$^-$ cases (EBV load range 26-4536 genomes per 10^6 PBMCs, median value 312)

173 reflect either recent but not current primary infections or the small fraction of virus carriers

174 (estimated at around 3%) who remain anti-EBNA1 IgG$^-$ in the longer term (38). More

175 importantly, we identified two individuals with an anti-VCA IgM$^+$, anti-EBNA1 IgG$^-$ profile

176 indicative of primary infection, both of whom had remained well in the weeks following the

177 key bleed. One, AS2, also had a very high load (11,350 genomes per 10^6 PBMCs) and,

178 because additional bi-annual samples were available, could be studied prospectively. The

179 other, AS6, who had only a relatively low load (148 genomes per 10^6 PBMCs) had not given

180 additional samples and our observations were limited to just the one bleed. Figure 1

181 compares the viral load data for all six AS cases at the time of asymptomatic infection with

182 the values seen in long term virus carriers (anti-VCA IgG$^+$) and acute IM patients; the viral

183 loads in the AS1-6 group were significantly higher than those seen in the carrier state and, in

184 three cases, were well within the acute IM range.

185   For comparison, the same serologic assays were used to screen 21 acute IM patients

186 whose EBV loads are also included in Fig. 1; all 21 were anti-VCA IgM$^+$ and 14 were already

187 anti-VCA IgG$^+$, whereas all but one were anti-EBNA1 IgG$^-$ (data not shown). Note therefore

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188   that the serological profiles in four of our asymptomatic cases (AS1, 2, 5 and 6: anti-VCA

189   IgM$^+$, anti-VCA IgG$^{+/-}$, anti-EBNA1 IgG$^-$) resemble those typically seen in acute IM. By

190   contrast the profile seen in the two other cases (AS3, AS4: anti-VCA IgM$^-$, anti-VCA IgG$^-$,

191   anti-EBNA1 IgG$^-$) is atypical and implies that these individuals were bled at an earlier stage

192   of infection before development of an IgM response. We also determined the CMV sero-

193   status of the AS cohort and their acute IM comparators, given the possibility that pre-

194   existing CMV carriage might influence the course of primary EBV infection (39, 40). All six AS

195   individuals were CMV-seronegative at the time of EBV acquisition and, in the five

196   prospectively studied cases, remained so throughout the whole study period. The majority

197   (18/21) of IM cases were likewise CMV-seronegative at the time of acute IM.

198

199   **Prospective studies on AS1 to AS5: viral load, antibody and T cell responses**

200   AS1 gave samples on four occasions, initially in October 2007 and then again 5, 17 and 55

201   months after that date. This individual was identified at the first bleed with a very high viral

202   load (15,600 genomes per 10^6 PBMCs) and a serological picture associated with primary

203   infection, i.e. anti-VCA IgM$^+$, anti-VCA IgG$^-$, anti-EBNA1 IgG$^-$ (Table 1). Surprisingly the viral

204   load was still high at the 5 month bleed, by which time both anti-VCA IgG and anti-EBNA1

205   IgG antibody responses had developed, whereas by the later bleeds the viral load had fallen

206   to a stable level (albeit at the high end of the virus carrier range) and the serological picture

207   was that of a long-term virus carrier i.e. anti-VCA IgM$^-$, anti-VCA IgG$^+$ and anti-EBNA1 IgG$^+$.

208   In contrast to the situation typically seen in acute IM, AS1's lymphocyte count was not

209   raised at the time of primary infection and there was no dramatic expansion of CD8$^+$ T cells.

210   However, the 30% CD8$^+$ T cell representation among the lymphocyte population was higher

211   than seen in later bleeds from this individual. Furthermore almost 40% of the circulating

212   CD8$^+$ T cells in the primary infection bleed had an activated CD38$^+$ HLA-DR$^+$ phenotype,

213   suggesting that a mild expansion had occurred. Since AS1 was HLA-B*0702-positive, this

214   allowed the initial and subsequent bleeds to be examined for EBV-specific CD8$^+$ T cells using

215   tetramers for three EBV-coded B*0702 -restricted epitopes, two lytic (RPR, TPS) and one

216   latent (RPP), here listed in their typically observed order of dominance (RPR>RPP>TPS). The

217   RPR-specific response accounted for >6% of all CD8$^+$ T cells in the blood at the time of

218   primary infection (Fig. 2, Table 1) and, just as seen for EBV-specific effectors in acute IM (15,

219   16), the great majority of these RPR-specific cells were in the CD45RA$^-$ CCR7$^-$ subset and had

220   an activated CD38$^+$ HLA-DR$^+$ phenotype with associated down-regulation of Bcl2 (Fig. 2).

221   Overall, therefore, around 14% of the CD8$^+$ T cell activation that occurred during primary

222   infection of AS1 could be explained by a response to this one B*0702 -restricted epitope.

223   Five months later, the general CD8$^+$ T cell activation had largely resolved and the RPR

224   epitope response had contracted to a lower level with loss of activation markers (Fig. 2,

225   Table 1). By that time, responses to the subdominant RPP and TPS epitopes had developed,

226   with the latter still having remnants of an activated phenotype (Table 1). Subsequent bleeds

227   showed a further slow fall in the size of all three B*0702 epitope responses and a complete

228   loss of activation markers.

229   AS2 was EBV-negative both by viral load and serology at the first bleed in October 2009

230   but two years later was caught at a time of very high viral load (11,350 genomes per 10^6

231   PBMCs) and an anti-VCA IgM$^+$, anti-VCA IgG$^+$, anti-EBNA1 IgG$^-$ antibody profile typical of a

232   primary infection (Table 1). When sampled a further two years on, the viral load had fallen

233   to a value just above the normal carrier median and the serological picture, anti-VCA IgM$^-$,

234   anti-VCA IgG$^+$, anti-EBNA1 IgG$^+$, was now typical of the long-term carrier state. In this case

235   the lymphocyte count at the time of primary infection was slightly raised (by a factor of 1.5-

236   to 1.8-fold) over earlier and later values. Furthermore, % CD8$^+$ T cell representation in the

237   lymphocyte population was increased almost 2-fold, 45% of those CD8$^+$ T cells were

238   activated and at least some of those T cells were EBV-specific (Table 1). Thus, tetramer

239   staining identified a highly activated (CD38$^+$ HLA-DR$^+$ Bcl2^{low}) CD8$^+$ T cell response to the

240   HLA-A*0201-restricted EBV lytic cycle epitope YVL (Fig. 3). These YVL-specific cells made up

241   2.5% of all CD8$^+$ T cells in the blood at the time and >5% of the activated population. Two

242   years later, YVL-specific cells were detectable in lower numbers and had lost their activation

243   markers. During primary infection the YVL response was also accompanied by smaller, but

244   similarly activated, responses to two other A*0201-restricted lytic epitopes, GLC and LLI

245   (Table 1); these again entered memory as smaller populations. No detectable response ever

246   developed to CLG, a weak A*0201-restricted latent cycle epitope.

247   Corresponding findings for two further individuals, AS3 and AS4, are summarised in Table

248   2. These were distinct from the above cases in that both were identified with viral loads that

249   were moderately high (770 and 1,030 genomes per 10^6 PBMCs, respectively) yet at a time

250   when both were still anti-VCA IgM$^-$, anti-VCA IgG$^-$, anti-EBNA1 IgG$^-$. This implies that

251   (relative to AS1 and AS2) these two individuals had been caught slightly earlier in the course

252   of infection, although clearly EBV had already entered the circulating B cell pool. At this time

253   AS3 (HLA-A*0201- and B*0702-positive) showed no evidence of raised lymphocyte counts,

254   of any expansion or activation in the CD8$^+$ T cell pool or of any A*0201- or B*0702 EBV

255   epitope reactivity (Table 2). A T cell response did subsequently occur however, since in

256   bleeds taken 2 and 4 years later tetramer staining detected small non-activated memory

257   populations to several A*0201- and B*0702 -restricted epitopes; these same later bleeds

258   also confirmed EBV loads falling, eventually to below detectable levels, and conversion to a

259   typical virus- carrier serostatus (anti-VCA IgM$^-$, anti-VCA IgG$^+$, anti-EBNA1 IgG$^+$). In the case

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260 of AS4 (HLA- B*0702-positive), total lymphocyte and CD8$^+$ T cell numbers were likewise

261 unchanged at the time of primary infection, relative to those seen in earlier and later

262 bleeds, and there was minimal activation of the general CD8$^+$ T cell pool. Interestingly

263 however, tetramer staining did detect a small population of cells specific for the B*0702-

264 restricted RPR epitope, 35% of which was activated. Responses to the sub-dominant B*0702

265 epitopes RPP and TYS were not yet present, but two years later all three reactivities were

266 detectable in memory; by this time, the viral load in PBMCs had fallen below detectable

267 levels and the serologic picture was typical of a long-term virus carrier (Table 2).

268 Donor AS5 was sampled on 5 occasions, initially in November 2007 and then again 3, 16,

269 26 and 54 months after that date. This individual (like AS1) was identified at the first bleed

270 with a very high viral load (13,450 genomes per 10^6 PBMCs) and a serological picture (anti-

271 VCA IgM$^+$, anti-VCA IgG$^-$, anti-EBNA1 IgG$^-$) associated with primary infection (Table 3). In this

272 case the viral load in the blood had fallen well into the normal carrier range within 3

273 months, remained at that level at 16 months, then became undetectable at later bleeds.

274 However, the serologic profile was slow to change; AS5 still remained anti-VCA IgM$^+$, anti-

275 VCA IgG$^-$, anti-EBNA1 IgG$^-$ at 3 months, had become anti-VCA IgM$^-$, anti-VCA IgG$^+$, anti-

276 EBNA1 IgG$^-$ at 16 months, and did not acquire the typical anti-VCA IgM$^-$, anti-VCA IgG$^+$, anti-

277 EBNA1 IgG$^+$ carrier profile until the 26 month bleed. Interestingly, the T cell response was

278 also slow to develop (Table 3). There was no sign of expansion or activation in the circulating

279 CD8$^+$ T cell population either at the time of primary infection or 3 months later. Total

280 lymphocyte counts were similar at these times but then increased to a higher level that was

281 maintained over the last three bleeds, though without any change in % CD8$^+$ T cells. As AS5

282 was HLA-A*0201-positive, we stained successive samples using A*0201 tetramers with

283 relevant EBV epitopes. Responses to the lytic epitopes GLC (Fig. 4), YVL and LLI were absent

284   in the first two bleeds but were first detected as small populations, still bearing some

285   residual evidence of an activated phenotype, at 16 months post-infection (Table 3). Their

286   numbers had fallen at 26 months, by which time a partly-activated response to the sub-

287   dominant latent epitope CLG (Fig. 4) was also in evidence, and all four responses had

288   assumed typical long-term memory levels by the last bleed.

289   To allow comparison between these asymptomatic EBV infections and classical IM, we

290   selected four IM patients (IM 221, 232, 253 and 265) who were either HLA-A*0201 or HLA-

291   B*0702-positive and from whom acute and follow-up samples were available for

292   retrospective analysis. These samples were assayed alongside the asymptomatic infection

293   samples using the same reagents, and the results are summarised in Table 4. At the time of

294   acute IM, all four patients had high viral loads (1,270-21,650 genome copies/ml), while the

295   three from whom acute phase sera were available had an antibody profile consistent with

296   primary infection (anti-VCA IgM$^+$, anti-VCA IgG$^+$, anti-EBNA1 IgG$^-$). All four had elevated

297   lymphocyte counts (3.3 - 8.8 x 10^6 cells/ml) with a high percentage (60-76%) of CD8$^+$ T cells

298   and with 77-95% of those CD8$^+$ T cells having an activated phenotype. In later bleeds, the

299   viral load fell (in three of four cases), the antibody profile had become anti-VCA IgM$^-$, anti-

300   VCA IgG$^+$, anti-EBNA1 IgG$^+$, and the lymphocyte count normalised, as did the %CD8$^+$ T cells

301   and the CD8 activation phenotype. Staining with the above panel of A*0201- and B*0702-

302   tetramers showed activated CD8 responses to the relevant epitopes in acute phase,

303   followed by the resolution of these activated responses down to resting memory levels

304   (Table 4). Note that in acute phase these particular epitope-specific responses were

305   relatively small (only comprising up to 7% total CD8$^+$ T cells), reflecting the fact that neither

306   HLA-A*0201 nor B*0702 are strong EBV response alleles and are frequently outcompeted by

307   more dominant restricting alleles in the genotype, most likely by HLA-B*0801 in the cases of

308   IM221 and IM232 (15, 16).

309   The data in Fig. 5 show how the percentage of CD8$^+$ T cells within the lymphocyte

310   population changed over time for these IM patients and for the asymptomatic cases AS1-5;

311   red dotted lines denote the time of primary infection. The scatter plots (right) show, for

312   each of the IM patients and for AS1-5, the ratio of the % CD8$^+$ T cells seen in primary

313   infection versus the mean % value seen in the same individual in bleeds taken >6 months

314   post-infection. The significant difference between the two sets of results make it clear that

315   the gross expansion of CD8$^+$ T cells typically seen in IM was not observed during

316   asymptomatic infection. Note that a similar picture emerged from the other case of

317   asymptomatic primary infection, AS6, from whom only the primary infection bleed was

318   available. There was again no obvious lymphocytosis in the blood, the CD8$^+$ T cell population

319   constituted only 25% lymphocytes and completely lacked activation markers (data not

320   shown).

321

322   **Prospective studies on AS1 to AS5: NK cell responses**

323   Given recent reports of mild NK cell expansion in IM (19) accompanied by differentiation of

324   immature CD56^{bright} cells to an intermediate CD56^{dim} NKG2A$^+$, KIR$^-$ phenotype (19-21) , we

325   established staining protocols that could identify the total NK cell population and the

326   relevant NK subsets (Fig. 6A). We then used these protocols to analyse the sequential

327   samples from the IM and AS donors. Fig. 6B shows the individual data and summary plots in

328   a format that follows the lay-out introduced in Fig. 5.

329   Studying the acute and post-IM PBMCs from five prospectively studied IM patients

330   (including the four used in tetramer staining above) confirmed the recently reported trends

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331 (19-21). Note that the size of the CD56$^+$ NK cell population, when expressed as a % of total

332 lymphocytes, was typically around 2-fold lower in acute IM than in later post-IM bleeds;

333 however, this reduced percentage in acute phase is a side effect of the dominant CD8$^+$ T cell

334 expansion and does not reflect a real fall in numbers. Indeed, when allowance is made for

335 the 3- to 4-fold increase in total PBMCs seen in IM, absolute NK cell numbers were 1.5-to 2-

336 fold higher in acute phase. Staining with the NK cell subset markers further showed that the

337 proportion of CD56^{bright} cells within the NK cell population was lower in acute phase than

338 post-IM, while that of CD56^{dim} NKG2A$^+$ KIR$^-$ cells was higher (Fig 6B), consistent with a

339 preferential expansion of the latter subset in IM (19-21).

340   By contrast, corresponding data on sequential bleeds from donors AS1-5 showed, in most

341 cases, no evidence of elevated CD56$^+$ NK cell representation within the lymphocyte

342 population during primary infection and no change in relative representation of the

343 CD56^{bright} and CD56^{dim} NKG2A$^+$ KIR$^-$ subsets (Fig. 6B). The one partial exception was AS3,

344 where the percentage of CD56$^+$ cells within the (non-expanded) PBMC pool at primary

345 infection was unusually high compared to the more typical values seen in later bleeds.

346 However, this apparent expansion had occurred without enrichment of the CD56^{dim} NKG2A$^+$

347 KIR$^-$ NK subset (Fig. 6B). Furthermore, in their primary infection bleeds, the AS donors

348 showed no evidence of NK cell activation using two markers, increased HLA-DR$^+$ expression

349 and loss of Bcl2, that in our hands were typically seen in NK cells in acute IM (data not

350 shown). The summary scatter plots (Fig. 6B, centre right and bottom right) show %

351 representation of the CD56^{bright} and CD56^{dim} NKG2A$^+$ KIR$^-$ NK cell subsets at the time of

352 primary infection relative to that seen in later bleeds from the same individuals. The IM and

353 AS scatter plots are significantly different, reflecting the absence of a detectable IM-like NK

354 cell response at the time the five asymptomatic primary infections were sampled.

355

356 **Prospective studies on AS1 to AS5: DC frequencies and plasma cytokines**

357 The final sets of experiments asked to what extent two other haematological features of

358 acute IM were detectable in asymptomatic primary infection, namely the almost complete

359 loss of pDCs and partial loss of mDCs recently reported in IM blood (20, 22), and the well

360 documented increase in circulating levels of various anti-viral and/or pro-inflammatory

361 cytokines and chemokines during the acute disease (23-26).

362   To examine DC populations, we established a staining protocol that identifies both pDCs

363 (defined as live lineage⁻ HLA-DR⁺ CD11c^{low} CD123^{high} cells) and mDCs (defined as live lineage⁻

364 HLA-DR⁺ CD11c^{high} CD123⁻ cells) (Fig 7A). Applying this to prospective bleeds from our five

365 reference IM patients, we found the % representation of pDCs and mDCs was very low (Fig.

366 7B, left). This was particularly the case for pDCs with a median value of 0.062% mononuclear

367 cells, 8-fold lower than the levels reached post-IM, whilst mDCs underwent a similar but

368 less marked decrease with a median value of 0.065%, 4.5-fold lower than observed post-IM.

369 A change of this magnitude cannot be wholly explained by the diluting effect of CD8⁺ T cell

370 expansion in IM blood but must reflect an actual depletion of the circulating pDC and mDC

371 population. Corresponding data for AS1-5 revealed a mixed picture (Fig. 7B). Both AS1 and

372 AS2, the two asymptomatic infections characterised by high virus loads and an activated

373 CD8⁺ T cell response, gave results suggestive of pDC and mDC depletion. By contrast the

374 other three asymptomatic infections occurred without obvious effects on circulating DC

375 numbers. The summary scatter plots (Fig. 7B, right) illustrate the rather tight patterns of DC

376 depletion seen in acute IM relative to post-IM values versus the much more heterogeneous

377 picture seen in the AS subjects.

378   Finally, using a Luminex assay, we screened longitudinal plasma samples from the IM and

379   AS cases for concentrations of 11 cytokines/chemokines reported to be elevated in IM. We

380   found increased levels of IL18, IP10 and MIG to be the most sensitive and consistent

381   indicators of analyte dysregulation in acute IM, with peak concentrations being greatest for

382   MIG and IP10. Fig. 8 shows data from five IM patients, with IL18, MIG and IP10 levels in

383   acute phase being much higher than those present in the weeks and months post-IM.

384   Corresponding results from the individual AS subjects are shown alongside; the picture was

385   again heterogeneous. Both AS1 and AS2 had clear increases in MIG at the time of primary

386   infection, accompanied by a small elevation of IL18 above base-line levels (note the reduced

387   scale for IL18 and MIG concentrations compared to those seen in IM); in addition AS1

388   showed an increase in IP10. By contrast, AS3 and AS4 showed rising levels over time but no

389   clear evidence of changes specifically linked to EBV infection. Most interesting were the

390   data from AS5, where there was long delay between virus acquisition and a virus-specific T

391   cell response; in this case, increased levels of MIG and IP10 were observed coincident with

392   the cellular response and not with infection per se.

393

394   **DISCUSSION**

395   Our understanding of primary EBV infection in its usual asymptomatic form is very limited

396   because of the difficulty of studying a clinically silent event. As a result much is inferred

397   from the study of IM, a disease which appeared in affluent societies as an unexpected

398   consequence of delayed primary infection and which, in evolutionary terms, is a novelty.

399   Whether IM is a legitimate model for the successful control of primary EBV infection, or an

400   artefact of inefficient control, remains to be determined. The fact that not all delayed

401   primary infections are clinically manifest as IM (3-6) affords an opportunity to address this

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402   issue, providing one can identify asymptomatic cases. An earlier study (37) serendipitously

403   identified four IgM anti-VCA$^+$ individuals during serologic screening of volunteers for an EBV

404   vaccine trial. In the present work we took advantage of a study in which entrants into a UK

405   Medical School were screened annually or biannually throughout their degree course for

406   EBV and CMV status. This allowed us to identify six individuals undergoing sub-clinical

407   primary infection with EBV at one point during their studies; four of these were identified as

408   anti-VCA IgM$^+$ by serologic screening (as in ref 37) while two were found to be EBV DNA-

409   positive yet still anti-VCA IgM$^-$. In two cases (both anti-VCA IgM$^+$) we were able to arrange

410   additional bleeds in the months following infection; in three other cases we only had access

411   to those samples taken biannually before or after the event, and in one case we only had

412   the primary infection bleed. Such constraints are clearly a limitation since changes are

413   occurring rapidly during primary infection and one's snapshot of the virus-host interaction

414   will be highly dependent on the time of sampling. Nevertheless the present work is only the

415   second published analysis of asymptomatic EBV acquisition in adulthood and the only one

416   using a wide range of immunological tools to allow direct comparison with individuals of a

417   similar age who developed acute IM.

418   The first of several important questions concerned virus loads in asymptomatic infection.

419   Here the message was clear. Three individuals, AS1, AS2 and AS5, with an EBV-antibody

420   profile equivalent to that seen in acute IM (anti-VCA IgM$^+$, anti-VCA IgG$^{+/-}$, anti-EBNA1 IgG$^-$)

421   had circulating EBV genome loads well within the acute IM range, whereas a fourth, AS6

422   (anti-VCA IgM$^+$, anti-VCA IgG$^+$, anti-EBNA1 IgG$^-$), had a relatively low load, although it may

423   be that we had missed an earlier peak of infection in this particular case. The other two

424   individuals, AS3 and AS4, had loads that were just below the IM range; however, the fact

425   that neither individual had yet mounted a detectable anti-VCA IgM response suggests that

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426   they may have been caught relatively early in the course of infection, with EBV load in the

427   blood possibly still rising. These findings reinforce those in the earlier report of

428   asymptomatic adult infections, where two of three anti-VCA IgM[+] individuals tested had

429   elevated IM-like viral loads in PBMCs (37). Together the two studies make it clear that, while

430   in acute IM the severity of symptoms is reported to correlate directly with virus load in the

431   blood (5), the virus load per se cannot be the driver of symptoms since most asymptomatic

432   infections identified to date display similarly high loads.

433   We would stress that these elevated EBV loads refer to cell-associated virus genomes and

434   almost certainly reflect high frequencies of latently-infected cells present within the

435   circulating B cell pool. We could not prove this directly in the present work because the

436   limited size of the AS blood samples precluded isolation of the B cell fraction. However

437   many previous studies in subjects with high PBMC loads, whether IM patients,

438   immunocompromised transplant recipients or healthy individuals, have shown that EBV is

439   carried selectively in B cells and as a latent, not lytic, infection (10, 11, 41, 42). This speaks

440   against virus replication as a major contributor either to cell-associated virus loads or,

441   through virus shedding, to cell-free viral DNA in plasma. In this latter context, even in the

442   most acute cases of IM with huge B cell loads, viral genomes are only detectable in plasma

443   transiently and at very low levels (12, 43). At the same time, it is important to recognise that

444   studies on PBMCs and/or circulating B cells tell us nothing about the oropharyngeal

445   infection, in particular about the level of infectious virus shedding in the throat. We did not

446   take throat washings in the present work. Recently, however, two prospective studies of

447   EBV-seronegative students sampled both sites at regular intervals and found that, in those

448   individuals who subsequently developed IM, virus shedding did not become detectable until

449   very close to or at the time symptoms appeared (5, 6), suggesting that there is little

19

450   oropharyngeal replication occurring in the long prodromal phase. This reinforces the idea

451   that oropharyngeal B cells are the first target of orally-transmitted virus and that foci of lytic

452   replication in permissive epithelium are dependent upon seeding from locally-infected B

453   cells. Interestingly, in contrast to virus load in the blood, levels of virus replication in the

454   throat of IM patients bear no relationship to the severity of symptoms (5) ; indeed such

455   discordance is also apparent post-IM, with oral shedding typically remaining high long after

456   the resolution of symptoms (5, 12, 17).

457   We next examined aspects of the cell-mediated response in asymptomatic infection. In

458   their earlier study, Silins et al (37) found no evidence that their subjects had raised

459   lymphocyte counts at the time of infection; however they were unable to look at the key

460   features affecting the blood picture in IM, namely CD8$^+$ T cell activation and the induction of

461   a massive EBV antigen-specific CD8$^+$ T cell response (15, 16). In the present work we found

462   that three of our five prospectively studied subjects had an on-going virus-specific CD8

463   response at the time EBV was first detected, and in two of those individuals (AS1 and AS2),

464   this was coincident with significant levels of activation in the CD8$^+$ T cell pool; however,

465   overall CD8$^+$ T cell numbers never rose to the levels seen in IM. In the case of AS1, while

466   total lymphocyte counts were not raised at the time of infection, the percentage of CD8$^+$ T

467   cells was higher than in subsequent bleeds and almost 40% of those CD8$^+$ T cells were

468   activated. Furthermore, tetramer staining showed that around 15% of that activation could

469   be explained by the response to one EBV lytic cycle epitope, while a bleed taken six months

470   later revealed a delayed response to two further epitopes. In the case of AS2, a somewhat

471   larger CD8$^+$ T cell expansion was observed, sufficient to cause a detectable (but still <2-fold)

472   increase in total lymphocyte counts. Some 45% of the expanded CD8 population was

473   activated and the activated population contained CD8$^+$ T cell responses to three EBV lytic

474   epitopes. Both AS1 and AS2 therefore show elements of an IM-like CD8$^+$ T cell response but

475   in less exaggerated form. How much of the overall CD8$^+$ T cell activation can be ascribed to

476   EBV-specific cells in such cases remains a moot point. Some of this may reflect "bystander

477   activation", as acute EBV infection has been shown to induce an activated phenotype

478   (though not proliferation) in pre-existing CD8$^+$ T cell memory to unrelated viruses (44).

479   However we anticipate that the EBV-specific response is a major contributor because our

480   tetramer assays, focusing on a small number of epitopes restricted through just one or two

481   HLA alleles, clearly underestimate the true size of that response.

482   In contrast to the above cases, AS3 and ASS4 showed no evidence of any lymphocytosis

483   or general CD8$^+$ T cell expansion/activation in the first virus-positive bleed. However, since

484   both individuals may have been sampled relatively early in the course of infection and were

485   not re-sampled until much later, it may be that a subsequent expansion occurred and was

486   missed. This remains a possibility but, at least in the case of AS4, an EBV-specific CD8$^+$ T cell

487   response was already detectable without disturbance of the CD8$^+$ T pool as a whole. AS3

488   and AS4 may therefore represent positions at the other end of the asymptomatic spectrum,

489   i.e. cases where control of the infection occurs without obvious impact on the CD8 pool as a

490   whole. Most interesting was the case of AS5 who, despite already sustaining high EBV loads

491   in the blood, showed no lymphocytosis, no CD8 expansion/activation and no detectable

492   EBV-specific CD8$^+$ response either during acute infection or in a blood sample taken three

493   months later. It was not until the 16 month bleed that an EBV-specific CD8$^+$ T cell response

494   was detected, at which time cells reactive to three lytic epitopes were present, some still

495   with a phenotype suggesting relatively recent activation. Later bleeds detected these same

496   CD8$^+$ T cell reactivities in memory, joined in the 26 month bleed by a recently activated

497   response to a sub-dominant latent epitope. Note that there is a parallel between this long-

498   delayed T cell response to infection and the same individual's unusually slow IgG (i.e. T cell-

499   dependent) antibody response to the virus; thus AS5 was still anti-VCA IgG⁻ 3 months after

500   the primary infection bleed and still anti-EBNA1 IgG⁻ after 16 months.

501   Several lines of evidence both from IM patients (5, 45) and from children with primary

502   immune deficiencies (46, 47) suggest that, besides T cells, the NK cell system has a role to

503   play in restraining EBV infection. As to the mechanism of control, early interest came from

504   the finding that CD56^{bright} NK cells (the dominant NK cell subset in lymphoid tissues) have

505   the potential to delay EBV-induced B cell transformation in vitro by producing IFNγ (48).

506   However, more recent work studying EBV infection in the humanised mouse model suggests

507   that NK cells target lytic rather than growth-transforming latent infections (49). This accords

508   with in vitro evidence that latently-infected B cells only become susceptible to NK cell

509   recognition and killing upon entry into lytic cycle (50). Interestingly, the CD56^{dim} NKG2A$^+$

510   KIR⁻ subset that best mediates such lytic cycle recognition in vitro is also the subset that is

511   preferentially expanded in IM blood (19). This has prompted the hypothesis that NK cells

512   play an important role early in the host response to primary EBV infection by controlling

513   lytic virus replication, thereby limiting the amount of infectious virus entering the B cell

514   system and also reducing the yield of lytic antigens that are the main drivers of the primary

515   CD8$^+$ T cell response (49). By this argument, since IM is a disease associated with high virus

516   loads and an exaggerated T cell response, it may arise as a result of impaired NK cell control;

517   conversely, asymptomatic infection would be associated with efficient NK cell responses. In

518   the present study of asymptomatically infected individuals, we therefore extended our

519   analysis to include the circulating NK cell population and its subsets. One possibility was

520   that, compared to the small expansion in NK cell numbers and slight increase in CD56^{dim}

521   NKG2A$^+$ KIR⁻ representation reported in IM (19), these indicators of an active NK cell

522   response would be magnified in asymptomatic infection. Using protocols that confirmed the

523   reported NK cell changes in IM blood, we found (in at least four of the five individuals

524   studied) no clear evidence for expansion either of total NK cells or of the CD56^{dim} NKG2A$^+$

525   KIR$^-$ subset. The one partial exception was AS3 (one of the two cases possibly caught early

526   post-infection) where percentage representation of total NK cells was increased in the

527   lymphocyte population at the time EBV was first detected; however this was not

528   accompanied by any sign of NK cell activation or subset shift. Taken overall our findings

529   suggest that, contrary to the above prediction, asymptomatic infection is not characterised

530   by an NK cell response that is magnified compared to that seen in IM. However, we would

531   add two caveats to this conclusion. Firstly it is possible that the key NK cell responses are

532   only transiently reflected in the blood and have simply been missed because of the irregular

533   monitoring of our AS cases, especially in those individuals who (unlike patients with acute

534   IM) have not yet become anti-VCA IgM$^+$. Secondly, the composition of NK populations in the

535   blood does not necessarily reflect what is occurring at the presumed site of NK cell action, in

536   oropharyngeal tissues.

537   Little is known about EBV's interaction with other innate immune cell lineages. In this

538   regard the potential importance of myeloid cells, and particularly DCs, is interesting because

539   the virus encodes a unique lytic cycle protein BARF1 which blocks colony stimulating factor

540   CSF-1, a cytokine promoting myeloid cell proliferation/function (51). Moreover, in the

541   macaque model with the EBV-related rhesus-lymphocryptovirus, deletion of the rhesus-

542   BARF1-coding sequence (hence loss of BARF1's immune evasive effects) led to lower acute

543   viral loads (52), implying that the early events of virus infection in vivo are indeed subject to

544   immune pressure from the myeloid lineage. Reasoning that the recently described loss of

545   pDCs and mDCs from acute IM blood (20, 22) might reflect active mobilisation of such an

546   innate response towards the site of infection, as observed in other viral systems (53, 54), we

547   asked whether the same fall in circulating DCs was also detectable in asymptomatic cases.

548   Interestingly both AS1 and AS2, the two asymptomatic infections that were most IM-like in

549   viral load and T cell response kinetics, did indeed show lower pDC and mDC subset

550   representation (as a % of PBMCs) in the primary infection bleed compared to later blood

551   samples. By contrast, AS5 showed no significant change despite equally high circulating EBV

552   genome loads; this is at least consistent with the idea that, in this individual, not only the

553   adaptive but also parts of the innate immune response were delayed.

554   The work was then extended to another reported feature of acute IM, the increase in

555   several pro-inflammatory cytokines/chemokines in plasma (23-26). Screening a range of

556   these candidates, we found that many were elevated in some but not all IM samples; this

557   likely reflects the transient nature of some cytokine/chemokine elevations in IM, with levels

558   rising and falling at particular times even within the disease course itself. By contrast, we

559   identified three markers that appeared less susceptible to such variability, being

560   consistently increased across our panel of acute IM samples: these were IL18, known to

561   induce IFNγ production (55), and IP10 and MIG, both induced by IFNγ (56). Going on to

562   study these markers in the asymptomatic infections, we found some interesting changes

563   though the magnitude of change was always less marked than in IM. Thus the acute

564   infection bleeds of AS1 and AS2 showed slight rises over base-line in IL18 and larger

565   increases in MIG and (for AS1) also in IP10. By contrast, both AS3 and AS4 showed no such

566   effect, possibly because they were sampled relatively early in the course of infection. More

567   interestingly, cytokine levels were also not increased in the high load, acute infection bleed

568   of AS5; however both MIG and IP10 were significantly elevated (compared to earlier and

569   later levels) in the bleed taken at 16 months, just after the peak of the virus-specific CD8^{+} T

570   cell response. This chimes with recent work on the changing transcriptional profile of PBMCs

571   over the course of IM, where upregulation of IFNγ pathway genes strongly correlated with

572   CD8$^+$ T cell expansion rather than other parameters (57). We provisionally conclude that, for

573   those cytokines/chemokines that are most markedly elevated during primary EBV infection,

574   the cytokine storm is not a product of the virus infection itself but of the exaggerated

575   immune response to infection.

576   In summary, the present study helps to resolve some of the questions that surround

577   asymptomatic primary infection, notably with respect to latent virus load in the blood,

578   which can be as high as in IM, and the cell-mediated immune response, which may be

579   qualitatively similar to IM but never matches its size. However, our observations also

580   emphasise the need for further, more intense, studies and highlight the challenges such

581   studies face. In particular, one does not know the point at which asymptomatic infection

582   samples are directly comparable with those from IM because the kinetics of infection (virus

583   acquisition, oropharyngeal replication, entry into the circulating B cell pool) may be

584   different in the two situations. Furthermore the present results, albeit from a limited study,

585   strongly suggest that asymptomatic infections themselves do not follow a uniform kinetic

586   path. For example, AS1 was similar to IM patients in mounting a prompt CD8$^+$ T cell

587   response to primary infection followed by T cell-dependent IgG antibody response to both

588   VCA and EBNA1; by contrast in AS5, who was caught with an equally high virus load at the

589   time of primary infection, the responses were delayed by at least several months.

590   Intriguingly, these response kinetics did not correlate with anticipated changes in latent

591   virus load; thus, despite mounting a prompt response, AS1 still had a high load in the blood

592   at six months post-infection (though it fell to normal levels after that time), whereas the

593   virus load in AS5 had fallen significantly within three months in the apparent absence of any

594   T cell-mediated response.

595   Interpreting these findings is difficult without a proper understanding of the early events

596   that occur in the oropharynx following oral transmission of the virus (8, 9, 17, 20). Important

597   prospective studies identifying subjects in the 5-7 week prodromal phase of IM have shown

598   that high virus loads in both throat washings and blood do not appear until around the

599   onset of symptoms (20); the authors suggest that a slow smouldering infection of the local B

600   cell system may take weeks before expanding to a magnitude that drives an immune hyper-

601   reaction (20). With respect to that hyper-reaction, evidence both from X-linked

602   lymphoproliferative disease studies (58-60) and from the humanised mouse model (49)

603   strongly suggest that lytically-infected B cells, as opposed to mucosal epithelium, are the

604   main drivers of both the NK and highly-expanded CD8^{+} T cell responses seen in IM. The

605   balance between lytic and latent B cell infection is finely drawn and it seems possible that, if

606   the local environment within the oropharynx favours latency, then the general B cell system

607   might be colonised without putting the NK and T cell systems on high alert. The inference is

608   that, compared to IM, asymptomatic infections would be characterised by lower

609   oropharyngeal replication of the incoming virus, hence a lower lytic antigen load. More

610   broadly, perhaps the most important lesson from this work is that not all asymptomatic

611   infections necessarily follow the same course; while some may be IM-like in the timing and

612   quality of immune responses, others may be quite different and ultimately reveal novel

613   mechanisms of host control.

614

615   **MATERIALS AND METHODS**

616   **Study cohort**

617   Initial blood samples were collected from a total of 448 medical students recruited either

618   between October-December 2007 or between October-December 2009 when entering their

619   degree course. Subject to consent, follow-up samples were collected in year 2

620   (February/March), year 3 (December/January) and year 5 (February-May) of their studies.

621   Additional samples were requested from particular individuals if subsequent assays revealed

622   recent EBV infection. This study was approved by the West Midlands (Edgbaston) Research

623   Ethics Committee (REC reference 07/H1208/40) and participants gave written informed

624   consent in accordance with the Declaration of Helsinki.

625

626   **Sample collection and preparation**

627   PBMCs were isolated from a 20 ml heparinised blood sample by Ficoll/Hypaque density

628   gradient centrifugation. In each case, an aliquot of 1-2 x 10^6 PBMCs was frozen for later DNA

629   extraction to determine HLA genotype and EBV viral genome loads, while the remaining

630   PBMCs were cryopreserved as duplicate ampoules in RPMI1640, 20% FCS, 10% DMSO.

631   Plasma was frozen down to identify EBV and CMV serostatus. These samples were studied

632   in parallel with cryopreserved PBMC and frozen plasma samples collected prospectively

633   from IM patients during acute phase and at later times after resolution of symptoms (16,

634   61). Full blood counts to determine lymphocyte numbers were performed at the

635   Haematology laboratory, Queen Elizabeth Hospital, Birmingham.

636

637   **Determining EBV and CMV serostatus**

638   Serial dilutions of plasma were assayed for the presence of IgM and IgG antibodies to EBV-

639   VCA by indirect immunofluorescence as described (62); as VCA-positive and negative target

640   cells, the Akata-BL and EBV-loss Akata-BL lines were used in the IgM assay, and the P3HR1

27

641 and BJAB lines in the IgG assay. IgG antibody titres to EBNA1 were determined using a

642 commercially available diagnostic ELISA according to the manufacturer's instructions

643 (Diamedix, Miami FL). CMV serostatus was determined as described (63).

644

645 **Determining EBV viral genome load in PBMC**

646 Genomic DNA was isolated from PBMC pellets using the GenElute™ Blood Genomic DNA Kit

647 (Sigma-Aldrich) according to manufacturer's instructions. DNA was eluted into 80 µL buffer,

648 quantified using a Nanodrop and stored at -20°C. A multiplex qPCR assay which

649 simultaneously amplifies EBV BALF5 (DNA Pol) and cellular beta-2-microglobulin sequences

650 was used to determine the EBV genome load (64); four replicate samples of 500 ng DNA

651 (each equivalent to 1.5×10^5 cells) were assayed for each PBMC preparation. All standards

652 and samples were tested in triplicate and the data analysed using SDS v1.4 software.

653

654 **Flow cytometric analysis of EBV-specific and total T cell populations**

655 Tetramers were used to identify and analyse the surface marker phenotype of EBV-specific

656 $CD8^+$ T cells. In addition to CD3 and CD8, cell surface CD45RA and CCR7 were used to

657 identify the naïve ($CD45RA^+$ $CCR7^+$), central memory ($CD45RA^-$ $CCR7^+$), effector memory

658 ($CD45RA^-$ $CCR7^-$) and revertant memory ($CD45RA^+$ $CCR7^-$) T cell subsets. Staining for cell

659 surface HLA-DR and CD38 and for intracellular Bcl2 was used to identify activated (HLA^-DR^+

660 $CD38^+$ $Bcl2^{low}$) T cells. Tetramer staining identified $CD8^+$ T cells specific for the following

661 epitopes derived from individual EBV lytic or latent cycle proteins (see below) and restricted

662 either through HLA-A*0201: YVLDHLIVV (lytic, BRLF1), GLCTLVAML (lytic, BMLF1), LLIEGIFFI

663 (lytic, BaRF1), CLGGLLTMV (latent, LMP2); or through HLA-B*0702: RPRATWIQEL (lytic,

664 BaRF1), <u>TPS</u>VSSSISSL (lytic, BFRF3), <u>RPP</u>IFIRRL (latent, EBNA3A) (14). Epitopes are henceforth

665 designated by their initial three residues (underlined).

666   Tetramer and antibody staining was performed on cryopreserved PBMCs as follows. Cells

667 were thawed and stained with LIVE/DEAD fixable Aqua Dead Cell Stain (Molecular Probes,

668 ThermoFisher Scientific) for 15 min at room temperature, washed and stained with

669 tetramer-PE for 15 min at 37°C. Following two further washes, surface staining with anti-

670 CD14-Pacific Green (Clone SJ25-C1), anti-CD19-Pacific Green (Clone TüK4) (both from

671 Molecular Probes, ThermoFisher Scientific), anti-CD3-BV786 (Clone SK7), anti-CD8-APC-H7

672 (Clone SK1), anti-CD45RA-BV605 (Clone HI100), anti-CD45RO-BV711 (Clone UCHL1) (all from

673 BD Biosciences) , anti-CD38-PerCP-Cy5.5 (Clone HIT2), anti-HLA-DR-APC (Clone L243) (both

674 from BioLegend) and anti-CCR7-FITC (Clone 150503 from R&D Systems) was performed in

675 Brilliant Stain Buffer (BD Biosciences) for 30 min at 4°C. Following fixing and

676 permeabilisation using the Cytofix/Cytoperm kit (BD Biosciences), intracellular staining with

677 anti-Ki67-BV421 (Clone B56) and anti-Bcl-2-PE-CF594 (Clone 563601) (both from BD

678 Biosciences) was performed in Brilliant Stain and Perm/Wash buffer (both from BD

679 Biosciences) for 30 min at 4°C.

680   Flow cytometry data were acquired with an LSR Fortessa X20 Analyser (BD Biosciences)

681 with standard filter sets. Data were analysed using Kaluza 1.3 software (Beckman Coulter)

682 and figures were created using FlowJo software (Treestar). CD3^{+} T cells were gated on

683 within the single, viable, CD14^{−} CD19^{−} lymphocyte population before CD8^{+} T cells and CD8^{+}

684 tetramer^{+} cells were selected for further analysis of surface and/or intracellular marker

685 expression.

686

687 **Flow cytometry-based analysis of NK cells and DCs**

688   PBMCs were thawed and washed twice in R10 medium (RPMI-1640 (Sigma Aldrich)

689   containing 10% FBS, 50 IU/ml penicillin and 50 µg/ml streptomycin). Cells were counted and

690   resuspended in R10 at a concentration of 2×10^6 cells/ml. NK cells were identified by

691   staining 2×10^5 PBMCs with Live/Dead Violet Amine Dye (Life Technologies); anti-CD19

692   Pacific Blue (PB) (Clone LT19, AbD Serotec); anti-CD3 PB (Clone SP34-2), anti-CD14 PB (Clone

693   M5E2) and anti-CD56 PECy7 (Clone B159) (all from BD Bioscience) and gating on live,

694   lineage-negative lymphocytes expressing CD56 (Fig. 6A). Receptor expression on CD56^{+} NK

695   cells and subsets thereof was evaluated using the following antibodies: anti-CD158a/h biotin

696   (KIR2DL1/S1 Clone 11PB6, Miltenyi), anti-CD158b (KIR2DL2/L3/S2 Clone CH-L, BD

697   Bioscience); anti-CD158e1/e2 (KIR3DL1/S1 Clone Z27) and anti-NKG2A PE (Clone Z199) (both

698   from Beckman Coulter), and anti-HLA-DR-APC (Clone L243) (BioLegend). Streptavidin Pacific

699   Orange (Life Technologies) was used to detect anti-CD158a/h biotin. Intracellular expression

700   of Ki67 and Bcl2 was assayed as described above for T cells.

701   DC subsets were identified by staining 5×10^5 PBMCs with Live/Dead Violet Amine Dye

702   (Life Technologies), anti-HLA-DR Peridin chlorophyll protein (PerCP) (Clone L243, BD

703   Bioscience), anti-CD123 PECy7 (Clone 7G3, eBioscience), anti-CD11c APC-H7 (custom

704   conjugate, ReaMetrix), anti-CD3 (Clone SP34-2), anti-CD14 PB (Clone M5E2), anti-CD16 PB

705   (Clone 3G8) all from (BD Bioscience); anti-CD19 PB (Clone LT19), anti-CD20 (Clone 2H7)

706   (both from AbD Serotec); and anti-CD56 PB (custom conjugate, ReaMetrix), gating on live,

707   lineage negative mononuclear cells expressing HLA-DR^{+} cells, then identifying pDC and mDC

708   subsets within this population on the basis of differential expression of CD123 and CD11c

709   (Fig. 7A).

710   Flow cytometry data were acquired with a Cyan ADP Analyser (Beckman Coulter) with

711   standard filter sets. Data were analysed using FlowJo software (v8.8.6; Treestar).

712

Plasma cytokine assays

713

714 Plasma levels of IFNβ, IFNγ, IL1β, IL6, IL10, IL12, IL18, TNFα, MIG/CXCL9 and IP-10/CXCL10

715 were determined by Luminex assay as described (65).

716

Statistical tests

717

718   All graphical data and statistical analyses were generated using Prism software version 5

719 (GraphPad Software Inc., San Diego, Calif.). The statistical significance of differences

720 between values measured at the first viraemic time point and time points after 6 months in

721 each group of subjects was determined using a Mann-Whitney non-parametric test; p values

722 <0.05 were considered significant.

723

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947

948

949 **Table 1** Longitudinal viral loads, CD8 responses and serological profiles of donors AS1 and

950 AS2

						EBV CD8 responses			EBV serology		
Donor[a]	Time (months)[b]	EBV load[c]	Lymphocytes (10^6/ml)	CD8+ (%)[d]	Activated CD8+ (%)[e]	EBV tetramer	Tetramer+ of CD8+ (%)	Activated tetramer+ (%)	EBV VCA IgM titre	EBV VCA IgG titre	EBNA1 IgG index value
AS1	0	15600	1.3	30	39.0	RPR	6.41	85.0	1/20	negative	negative
						RPP	0	0			
						TPS	0	0			
	+5	6020	1.7	25	2.1	RPR	0.92	0.4	1/20	1/320	3.9
						RPP	0.47	5.9			
						TPS	0.20	21.6			
	+17	847	3.4	22	0.5	RPR	0.56	0.8	negative	1/320	16.3
						RPP	0.34	0			
						TPS	0.14	9.7			
	+55	140	1.6	18	0.4	RPR	0.11	0	negative	1/320	12.8
						RPP	0.08	0			
						TPS	0.06	0			
AS2	-27	0	2.3	27	1.6	YVL	0	0	negative	negative	negative
						GLC	0	0			
						LLI	0	0			
						CLG	0	0			
	0	11350	4.1	46	45.0	YVL	1.13	96.0	1/5	1/80	negative
						GLC	0.38	94.0			
						LLI	0.09	72.0			
						CLG	0	0			
	+25	350	2.8	27	0.5	YVL	0.07	3.1	negative	1/320	14.5
						GLC	0.09	3.9			
						LLI	0.02	0			
						CLG	0	0			

951 [a]Donor HLA types: AS1 A1, A3, B7, B13; AS2 A2, A11, B40, B44

952 [b]Time in months since primary infection

953 [c]EBV genome copies per 10^6 PBMCs

954 [d]CD8$^+$ as % total lymphocytes

955 [e]HLA-DR$^+$ CD38$^+$ CD8$^+$ cells as % total CD8$^+$ cells

956

957 **Table 2** Longitudinal viral loads, CD8 responses and serological profiles of donors AS3 and

958 AS4

Donor[a]	Time (months)[b]	EBV load[c]	Lymphocytes (10[6]/ml)	CD8+ (%)[d]	Activated CD8+ (%)[e]	EBV CD8 responses			EBV serology		
						EBV tetramer	Tetramer+ of CD8+ (%)	Activated tetramer+ (%)	EBV VCA IgM titre	EBV VCA IgG titre	EBNA1 IgG index value
AS3	0	770	1.9	18	0.5	RPR	0	0	negative	negative	negative
						TPS	0	0			
						RPP	0	0			
						YVL	0	0			
						GLC	0	0			
						LLI	0	0			
						CLG	0	0			
	+27	420	1.9	21	1.0	RPR	0.26	0	negative	1/40	2.8
						TPS	0.05	0			
						RPP	0.05	0			
						YVL	0.06	0			
						GLC	0.06	7.7			
						LLI	0	0			
						CLG	0	0			
	+51	0	1.8	24	0.2	RPR	0.12	0	negative	1/40	5.1
						TPS	0.01	0			
						RPP	0.02	0			
						YVL	0.03	0			
						GLC	0.06	0			
						LLI	0	0			
						CLG	0	0			
AS4	-9	0	1.9	24	0.4	RPR	0.00	0.0	negative	negative	negative
						TPS	0.00	0.0			
						RPP	0.00	0.0			
	0	1030	1.8	25	1.9	RPR	0.33	35.3	negative	negative	negative
						TPS	0.00	0.0			
						RPP	0.00	0.0			
	+26	0	2.0	26	1.2	RPR	0.07	0.0	negative	1/80	30.1
						TPS	0.01	0.0			
						RPP	0.50	1.5			

959 [a]Donor HLA types: AS3 A1, A2, B7, B57; AS4 A24, A25, B7, B18

960 [b]Time in months since primary infection

961 [c]EBV genome copies per 10[6] PBMCs

962 [d]CD8$^+$ as % total lymphocytes

963 [e]HLA-DR$^+$ CD38$^+$ CD8$^+$ cells as % total CD8$^+$ cells

964

965 **Table 3** Longitudinal viral loads, CD8 responses and serological profiles of donor AS5

Donor[a]	Time (months)[b]	EBV load[c]	Lymphocytes (10^6/ml)	CD8+ (%)[d]	Activated CD8+ (%)[e]	EBV tetramer	Tetramer+ of CD8+ (%)	Activated tetramer+ (%)	EBV VCA IgM titre	EBV VCA IgG titre	EBNA1 IgG index value
AS5	0	13450	1.4	26	0.5	YVL	0	0	1/20	negative	negative
						GLC	0	0			
						LLI	0	0			
						CLG	0	0			
	+3	180	1.4	31	0.3	YVL	0	0	1/10	negative	negative
						GLC	0	0			
						LLI	0	0			
						CLG	0	0			
	+16	160	3.9	32	2.8	YVL	0.69	6.9	negative	1/160	negative
						GLC	0.73	9.1			
						LLI	0.73	4.3			
						CLG	0	0			
	+26	0	3.3	32	1.3	YVL	0.26	5.0	negative	1/160	2.9
						GLC	0.46	2.1			
						LLI	0.20	1.5			
						CLG	0.06	21.0			
	+54	0	3.4	30	0.6	YVL	0.23	0	negative	1/160	1.7
						GLC	0.31	0			
						LLI	0.16	0			
						CLG	0.04	0			

966 [a]Donor HLA type: A2, A31, B40, B44

967 [b]Time in months since primary infection

968 [c]EBV genome copies per 10^6 PBMCs

969 [d]CD8+ as % total lymphocytes

970 [e]HLA-DR+ CD38+ CD8+ cells as % total CD8+ cells

971

972 **Table 4** Longitudinal viral loads, CD8 responses and serological profiles of acute IM cases

Donor[a]	Time (months)[b]	EBV load[c]	Lymphocytes (10^6/ml)	CD8+ (%)[d]	Activated CD8+ (%)[e]	EBV CD8 responses			EBV serology[f]		
						EBV tetramer	Tetramer+ of CD8+ (%)	Activated tetramer+ (%)	EBV IgM VCA titre	EBV IgG VCA titre	IgG EBNA1 index value
IM221	0	1270	3.3	76	83.1	YVL	2.10	97.4	1/40	1/80	negative
						GLC	0.07	57.6			
						LLI	1.92	90.9			
						CLG	0	0			
	+19	640	1.1	35	10.8	YVL	0.46	16.1	negative	1/160	19.4
						GLC	0.20	7.1			
						LLI	0.46	11.0			
						CLG	0.13	17.4			
	+29	130	0.7	32	8.2	YVL	0.54	14.5	negative	1/80	16.8
						GLC	0.26	17.4			
						LLI	0.29	17.2			
						CLG	0.06	10.5			
IM232	0	19290	8.8	74	80.3	YVL	0.05	87.2	Not done		
						GLC	0.34	96.4			
						LLI	0	0			
						CLG	0	0			
	+15	49	1.8	29	0.9	YVL	0.06	0	negative	1/160	1.7
						GLC	1.80	1.8			
						LLI	0.02	0			
						CLG	0.03	0			
	+24	111	1.5	25	0.6	YVL	0.05	7.1	negative	1/640	2.1
						GLC	0.75	1.5			
						LLI	0	0			
						CLG	0	0			
	+76	0	2.5	28	0.4	YVL	0.07	0	negative	1/640	3.5
						GLC	1.36	0.3			
						LLI	0.01	0			
						CLG	0.03	0			
IM253	0	5010	5.0	76	95.3	RPR	7.07	98.7	1/320	1/160	negative
						TPS	0	0			
						RPP	0.44	99.5			
	+7	8740	0.9	37	6.1	RPR	1.77	2.3	negative	1/160	16.0
						TPS	0.74	26.3			
						RPP	2.23	4.1			
	+19	5800	0.7	28	6.9	RPR	1.03	2.7	negative	1/80	31.5
						TPS	0.55	8.2			
						RPP	1.39	4.1			
IM265	0	21650	3.3	60	76.9	RPR	3.62	91.5	1/40	1/80	negative
						TPS	0	0			
						RPP	1.21	99.5			
	+5	5420	0.6	26	2.2	RPR	0.70	2.7	negative	1/80	4.8
						TPS	0.25	18.5			
						RPP	0.30	17.7			
	+19	1310	1.2	27	1.4	RPR	0.23	4.8	negative	1/160	21.0
						TPS	0.24	3.1			
						RPP	0.29	7.7			

973 [a]Donor HLA types: IM221 A1, A2, B8, B44; IM232 A2, A3, B8, B27; IM253 A3, A24, B7, B37; IM265 A23, A26, B7, B44

974 [b]Time in months since primary infection

975 [c]EBV genome copies per 10^6 PBMCs

976 [d]CD8+ as % total lymphocytes

977 [e]HLA-DR+ CD38+ CD8+ cells as % total CD8+ cells

978 [f]IM221, IM253 CMV-seronegative; IM265 CMV-seropositive; IM232 not tested

979 **FIGURE LEGENDS**

980 **Fig 1** EBV genome loads in long term virus carriers and cases of primary infection. Virus

981 loads are reported as EBV genome copies per 10^6 PBMCs for 276 anti-VCA IgG$^+$ virus

982 carriers, six cases of asymptomatic primary infection (AS1-6) from the same student cohort,

983 and 21 acute IM patients included for reference. Median viral loads (horizontal bars) for the

984 232 of 276 anti-VCA IgG$^+$ virus carriers with detectable EBV DNA, for AS1-6, and for the 21

985 IM patients were 103, 6190 and 7350 EBV genome copies per 10^6 PBMCs, respectively. Data

986 points below the dotted line identify the 44 anti-VCA IgG$^+$ virus carriers who had

987 undetectable EBV loads. Not shown are data from 166 anti-VCA IgG$^-$ individuals in the same

988 student cohort; all 166 had, as expected, undetectable viral loads.

989

990 **Fig 2** Longitudinal analysis of the B*0701/RPR lytic epitope response in donor AS1. PBMCs

991 were obtained from donor AS1 at the time of asymptomatic infection (AS1.1) and again 5,

992 17 and 55 months later (AS1.2, AS1.3 and AS1.5 respectively) and screened for responses

993 against the B*0701/RPR lytic epitope derived from BaRF1. The left hand column shows the

994 CD8/tetramer staining profiles of the CD3$^+$ T cell population. The remaining plots show the

995 phenotypic profiles of the whole CD8$^+$ T cell population (grey dots) and of the RPR-specific

996 CD8$^+$ T cells (red dots) after co-staining for HLA-DR and CD38, for Bcl2 and the RPR-tetramer,

997 and for CCR7 and CD45RA. Red numbers show the % distribution of RPR-specific cells across

998 the four quadrants.

999

1000 **Fig 3** Longitudinal analysis of the A*0201/YVL lytic epitope response in donor AS2. PBMCs

1001 were obtained from donor AS2 27 months before infection (AS2.1), at the time of

1002 asymptomatic infection (AS2.3) and again 25 months later (AS2.5) and screened for

1003   responses against the A*0201/YVL lytic epitope from BRLF1. The left hand column shows

1004   the CD8 tetramer staining profiles of the CD3^{+} T cell population. The remaining plots show

1005   the phenotypic profiles of the whole CD8^{+} T cell population (grey dots) and of the YVL-

1006   specific CD8^{+} T cells (red dots) after co-staining for HLA-DR and CD38, for Bcl2 and the YVL-

1007   tetramer, and for CCR7 and CD45RA. Red numbers show the % distribution of YVL-specific

1008   cells across the four quadrants.

1009

1010   **Fig 4** Longitudinal analysis of the delayed EBV lytic epitope responses in donor AS5. PBMCs

1011   were obtained from donor AS5 at the time of asymptomatic infection (AS5.1) and again 3,

1012   16, 26 and 54 months later (AS5.2, AS5.3, AS5.4 and AS5.5 respectively) and screened for

1013   responses against the A*0201/GLC lytic epitope from BMLF1 (**A**) and the A*0201/CLG latent

1014   epitope from LMP2 (**B**). In each case, the left hand column shows the CD8 tetramer staining

1015   profiles of the CD3^{+} T cell population. The remaining plots show the phenotypic profiles of

1016   the whole CD8^{+} T cell population (grey dots) and of the tetramer-specific CD8^{+} T cells after

1017   co-staining for HLA-DR and CD38, for Bcl2 and the relevant tetramer, and for CCR7 and

1018   CD45RA. Red numbers show the % distribution of GLC- or CLG-specific cells across the four

1019   quadrants.

1020

1021   **Fig 5** Longitudinal analysis of CD8^{+} T cells in IM patients and in cases of asymptomatic

1022   infection. Shown are the proportion of CD8^{+} T cells within the lymphocyte population over

1023   time for four IM patients (IM221, IM232, IM253, IM265; left hand graph) and for

1024   asymptomatic infection cases AS1-5 (individual graphs). In each case, the time of primary

1025   infection is indicated by the vertical red dotted line. The scatter plots (right hand panel)

1026   show the ratio of the proportion of CD8^{+} T cells at the time of primary infection to the mean

47

1027   proportion of CD8$^+$ T cells at time points more than 6 months later (1°/recovery) for both IM

1028   and AS groups.

1029

1030   **Fig 6** Longitudinal analysis of NK cell subsets in IM patients and in cases of asymptomatic

1031   infection. **(A)** Gating strategies for flow cytometric analysis to identify total NK cells (defined

1032   as live lineage$^-$ CD56$^+$ cells), CD56^{bright} cells, CD56^{dim} cells and CD56^{dim} NKG2A$^+$ KIR$^-$ cells

1033   within the lymphocyte population. The staining shown is from a representative

1034   asymptomatic subject (AS2) 27 months before detection of viraemia. **(B)** Longitudinal bleeds

1035   from five IM patients (IM221, IM225, IM232, IM253 and IM265) and asymptomatic infection

1036   cases AS1-5 were analysed by multi-parameter flow cytometry to determine the percentage

1037   of CD56$^+$ NK cells within lymphocytes, the percentage of CD56^{bright} NK cells within the

1038   circulating CD56$^+$ NK population, and the percentage of NKG2A$^+$ KIR$^-$ cells in the CD56^{dim} NK

1039   cell population. Combined IM and individual AS1-5 results are displayed as in Fig. 5, with the

1040   time of primary infection identified by a vertical red dotted line. The scatter plots (right

1041   hand column) show the ratio of the proportion of each subset at the time of primary

1042   infection to the mean proportion at time points more than 6 months later (1°/recovery) for

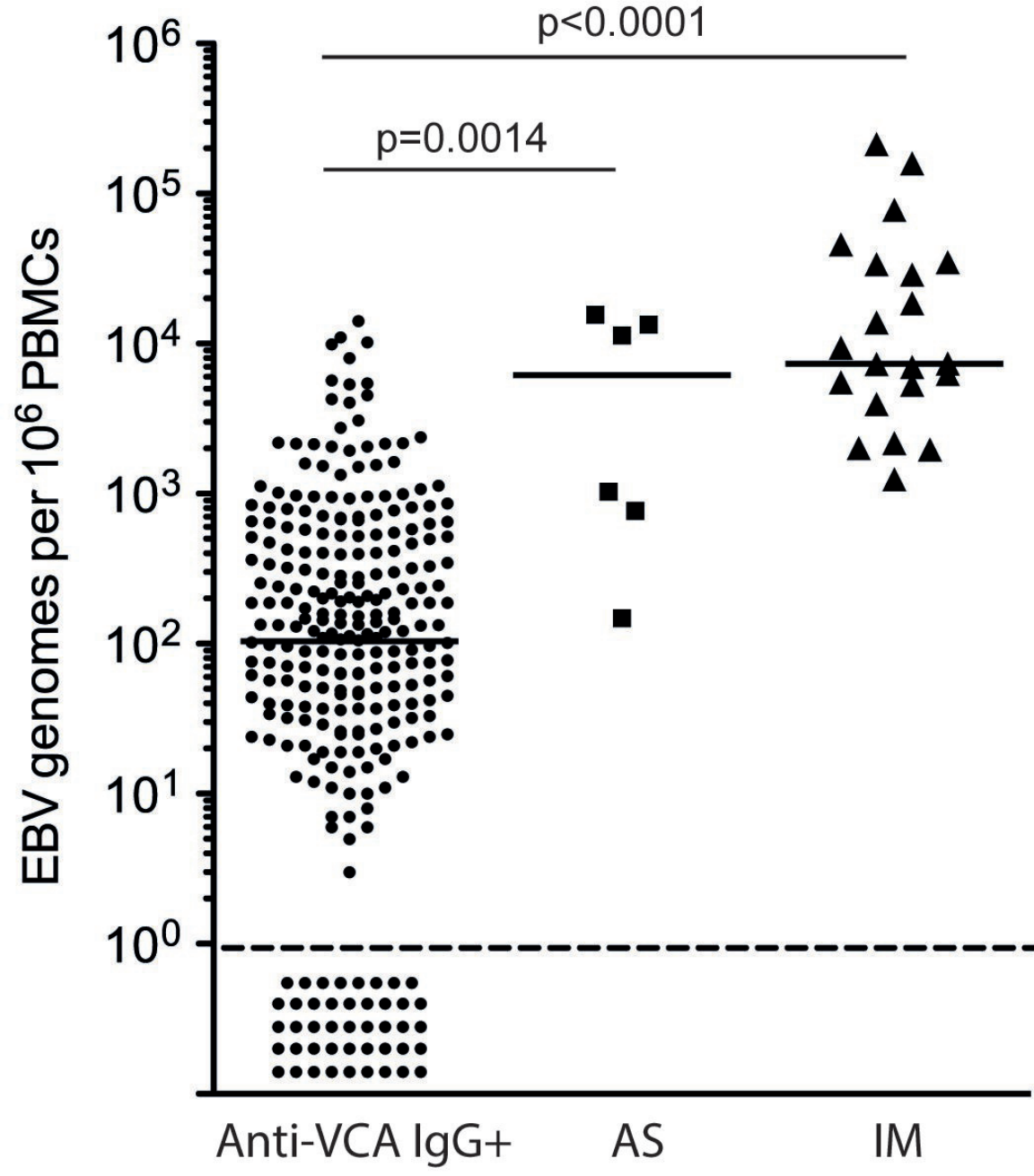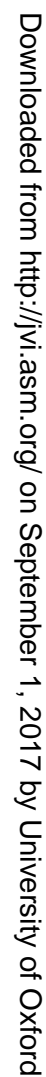1043   both IM and AS groups.

1044

1045   **Fig 7** Longitudinal analysis of DC subsets in IM patients and in cases of asymptomatic

1046   infection. **(A)** Gating strategy for flow cytometric analysis of DC populations to identify total

1047   mDCs (defined as live lineage$^-$ HLA-DR$^+$ CD11c^{high} CD123$^-$ cells) and pDCs (defined as live

1048   lineage$^-$ HLA-DR$^+$ CD11c^{low} CD123^{high} cells) within mononuclear cells. The staining shown is

1049   from a representative asymptomatic subject (AS2) 27 months before detection of viraemia.

1050   **(B)** Longitudinal bleeds from five IM patients (IM221, IM225, IM232, IM253 and IM265) and
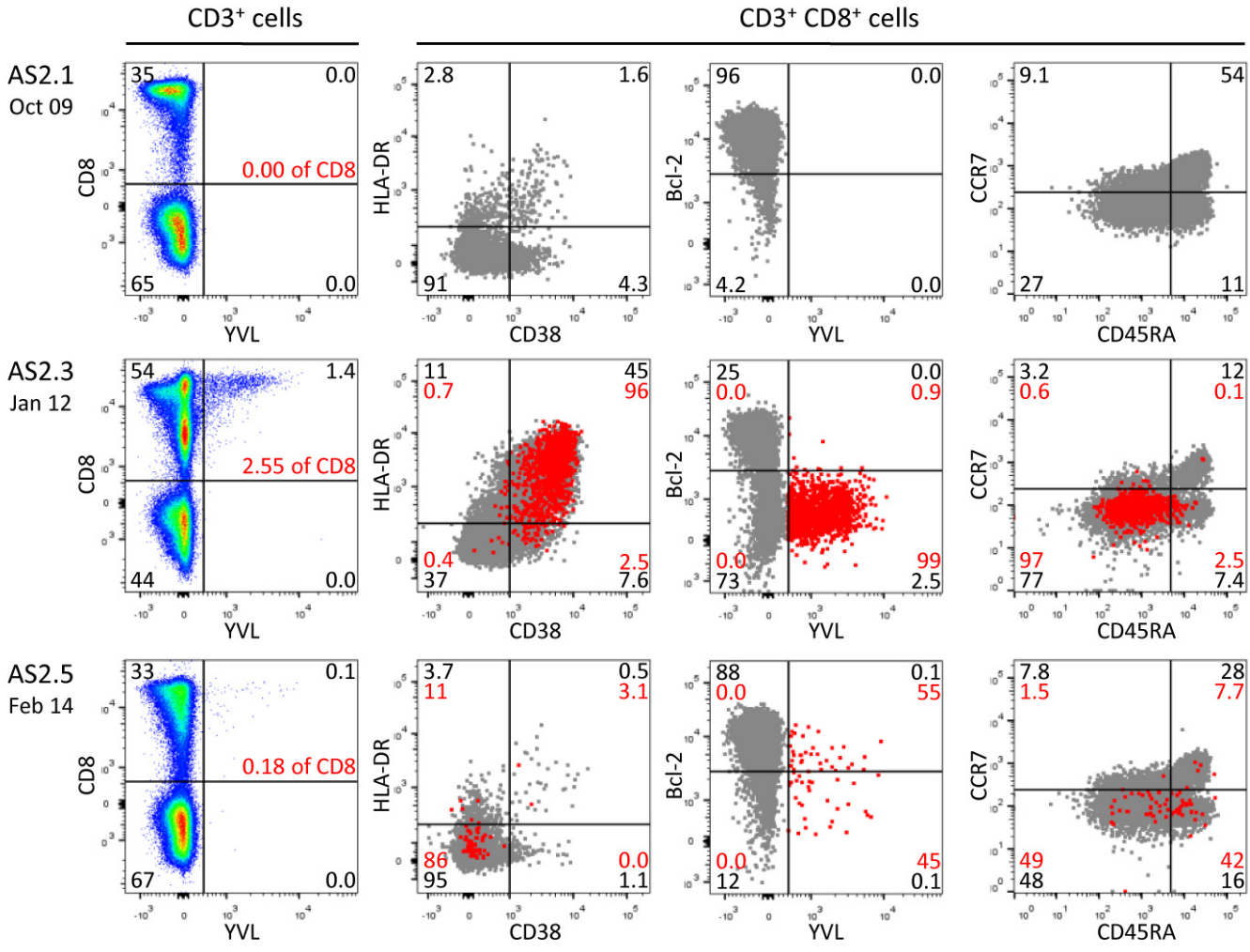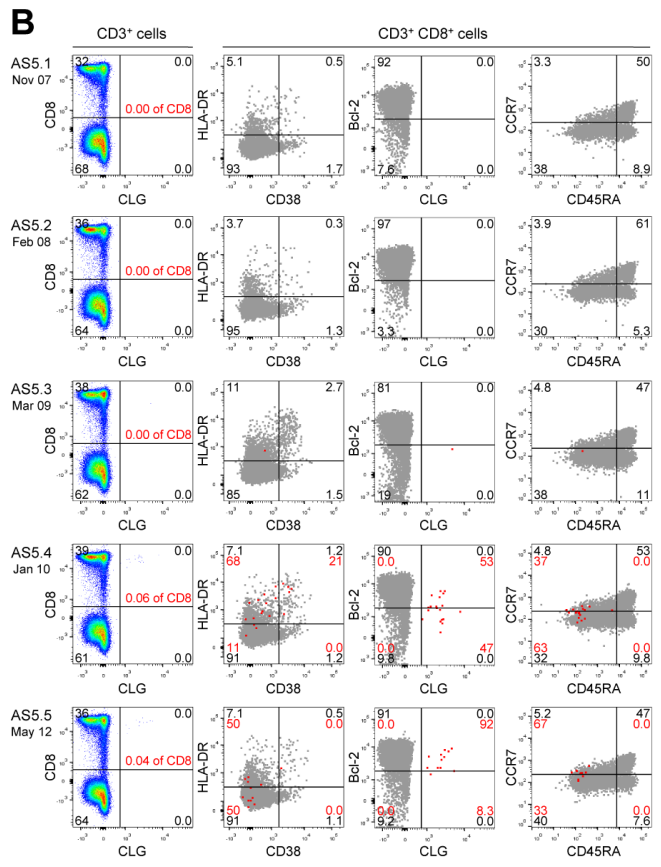
1051   asymptomatic infection cases AS1-5 were analysed by multi-parameter flow cytometry to

1052   determine the percentage of pDCs and mDCs within circulating mononuclear cells.

1053   Combined IM and individual AS1-5 results are displayed as in Fig. 5, with the time of primary

1054   infection identified by a vertical red dotted line. The scatter plots (right hand panels) show

1055   the ratio of the proportion of each subset at the time of primary infection to the mean

1056   proportion observed at time points more than 6 months later (1°/recovery) for both IM and
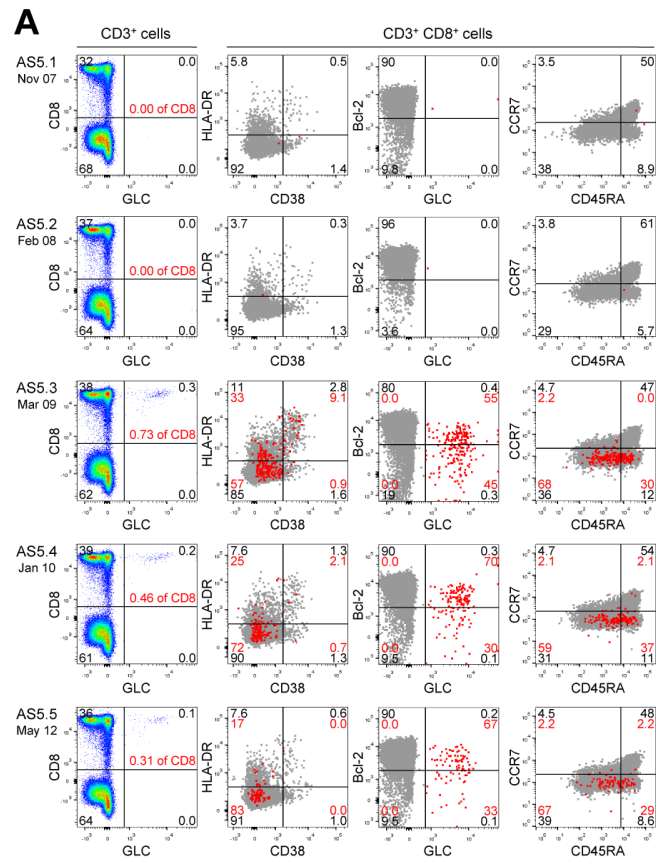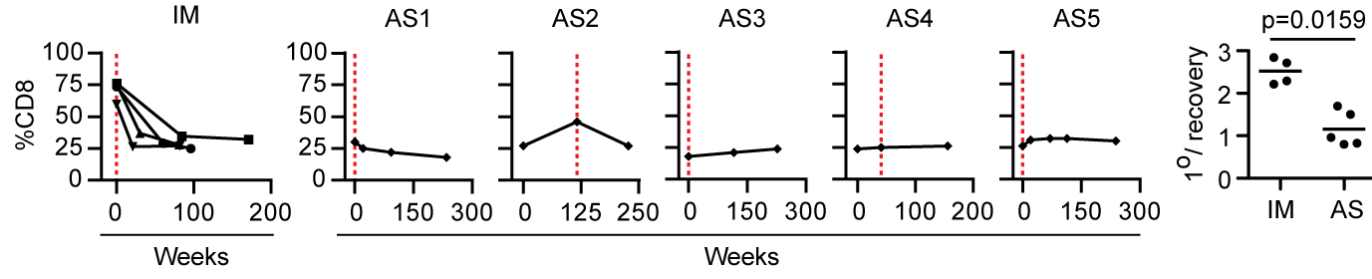
1057   AS groups.

1058

1059   **Fig 8** Longitudinal analysis of IL18, IP-10 and MIG in plasma samples from IM patients and in

1060   cases of asymptomatic infection. Longitudinal PBMC samples from five IM patients (IM221,

1061   IM225, IM248, IM253 and IM265) and asymptomatic infection cases AS1-5 were analysed

1062   for the concentrations of IL18, IP-10 and MIG in plasma. Combined IM and individual AS1-5

1063   results are arranged as in Fig. 5, with the time of primary infection identified by a red dotted

1064   line. The scatter plots (right hand panels) show the ratio of the quantity of each analyte at

1065   the time of primary infection to the mean quantity observed at time points more than 6

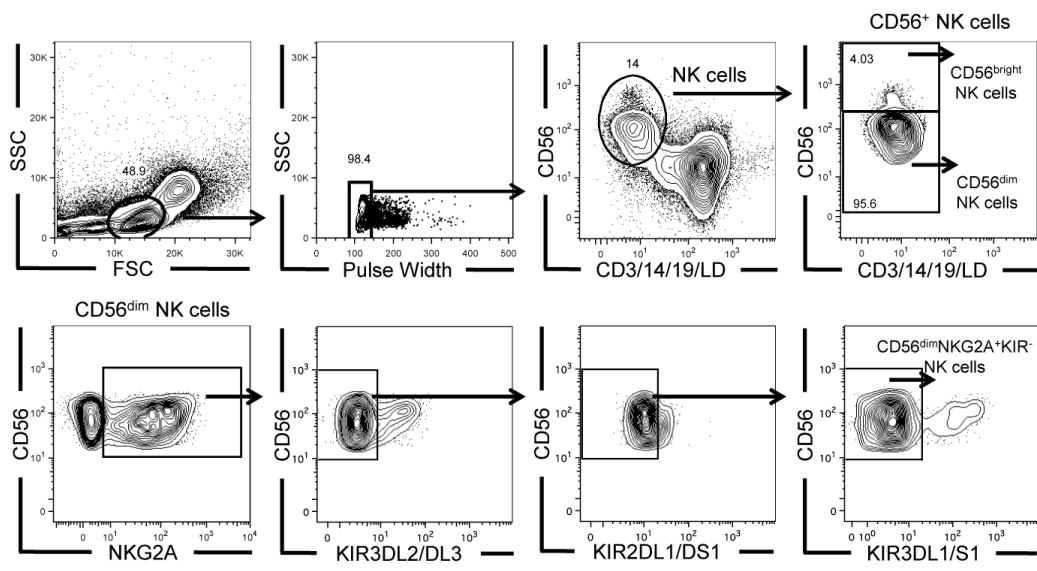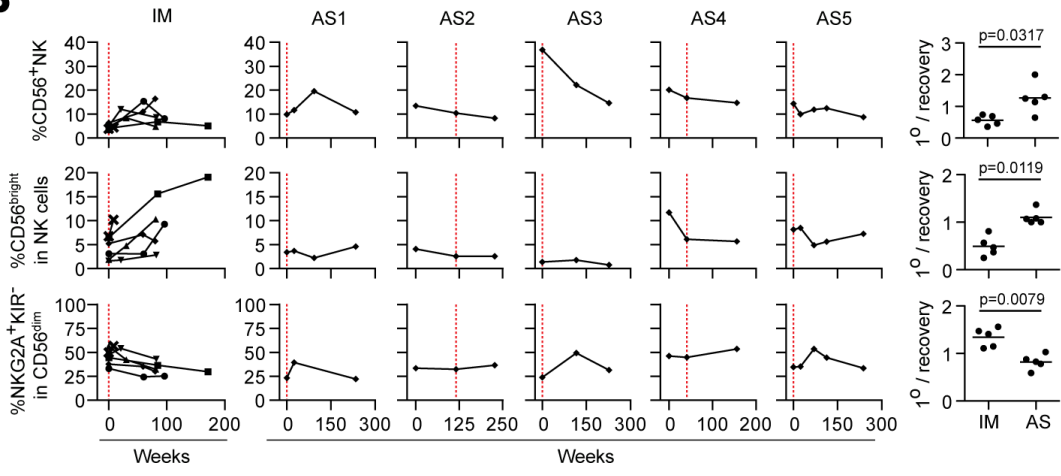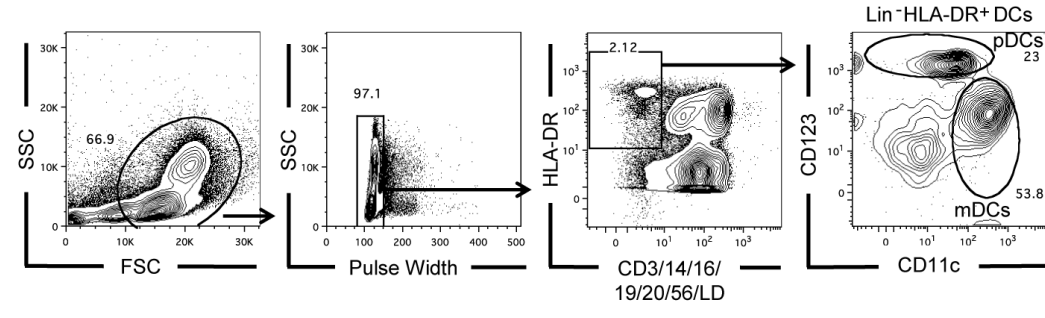1066   months later (1°/recovery) for both IM and AS groups.

49

CD3⁺ cells

CD3⁺ CD8⁺ cells

AS1.1
Oct 07

AS1.2
Mar 08

AS1.3
Mar 09

AS1.5
May 12

CD3+ cells   CD3+ CD8+ cells

IM AS1 AS2 AS3 AS4 AS5

%CD8

Weeks

Weeks

1°/ recovery

p=0.0159

IM   AS

A

CD56⁺ NK cells

NK cells

CD56bright NK cells

CD56dim NK cells

CD56dim NK cells

CD56dimNKG2A⁺KIR⁻ NK cells

B

A

B