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The missing link in EBV immune evasion

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1 **The missing link in EBV immune evasion: the BDLF3 gene induces ubiquitination**

2 **and downregulation of MHC class I and MHC class II**

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Abstract

The ability of Epstein-Barr virus (EBV) to spread and persist in human populations relies on a balance between host immune responses and EBV immune-evasion. CD8$^+$ cells specific for EBV late lytic cycle antigens show poor recognition of target cells compared to immediate early and early antigen-specific CD8$^+$ cells. This phenomenon is in part due to the early EBV protein, BILF1, whose immunosuppressive activity increases with lytic cycle progression. However, published data suggest the existence of a hitherto unidentified immune-evasion protein further enhancing protection against late EBV antigen-specific CD8$^+$ cells. We have now identified the late lytic gene, BDLF3, as the missing link accounting for the efficient evasion during late lytic cycle. Interestingly, BDLF3 also contributes to evasion of CD4$^+$ cell responses to EBV. We report that BDLF3 down-regulates expression of surface MHC class I and class II molecules in the absence of any effect upon other surface molecules screened, including CD54 (ICAM-1) and CD71 (Transferrin receptor). BDLF3 both enhanced internalization of surface MHC molecules and reduced the rate of their appearance at the cell surface. The reduced expression of surface MHC molecules correlated with functional protection against CD8$^+$ and CD4$^+$ T cell recognition. The molecular mechanism was identified as BDLF3-induced ubiquitination of MHC molecules and their subsequent downregulation in a proteasomal dependent manner.

Importance

Immune-evasion is a necessary feature of viruses that establish life-long persistent infections in the face of strong immune-responses. EBV is an important human pathogen whose immune evasion mechanisms are only partly understood. Of the EBV immune-evasion mechanisms identified to date, none could explain why CD8$^+$ T cell responses to late lytic cycle genes are so infrequent and, when present, recognize lytically-infected target cells so poorly relative to CD8$^+$ T cells specific for early lytic cycle antigens. The present work identifies an additional immune-evasion protein, BDLF3 that is expressed late in lytic cycle and impairs CD8$^+$ T cell recognition by targeting cell surface MHC class I molecules for ubiquitination and proteasomal dependent downregulation. Interestingly, BDLF3 also targets MHC class II molecules, to impair CD4$^+$ T cell recognition. BDLF3 is therefore a rare example of a viral gene that impairs both the class I and class II MHC antigen presenting pathways.

Introduction

46 Epstein-Barr virus (EBV) is a γ-herpesvirus found in more than 90% of the human

47 population. Primary infection with EBV is usually followed by establishment of lifelong

48 latent infection with occasional reactivation (1). The balance between host immune

49 responses, including $CD4^+$ and $CD8^+$ T cells, and viral immune evasion of these

50 responses is key to the spread and survival of EBV in human populations. Passive

51 evasion through the ability to establish antigenically silent latent infections is an

52 important characteristic of all herpesviruses, including EBV. In addition, active evasion

53 mechanisms are an important feature of herpesviruses. As these active evasion

54 mechanisms are predominantly observed during the lytic phase of the herpesvirus life-

55 cycle, they are presumed to be particularly important for enabling virus spread. There

56 have been a number of EBV encoded immune evasion genes identified that are

57 expressed in lytic cycle and target the MHC class I or class II antigen presentation

58 pathways (2, 3). Those genes responsible for interfering with MHC class I antigen

59 presentation include BGLF5, BNLF2a and BILF1 which each act upon different

60 elements of the MHC class I antigen presentation pathway (3-7). The EBV encoded

61 proteins BGLF5, BZLF1 and gp42 have been shown to interfere with MHC class II

62 antigen presentation (5, 8-10).

63 The above-mentioned MHC class I evasion genes encoded by EBV have been well

64 studied and shown to act via different mechanisms upon different elements of the MHC

65 class I antigen presentation pathway. Briefly, BGLF5 is a host shut off protein that has

66 been shown to induce the degradation of MHC class I mRNA, thereby reducing cell

67 surface MHC class I peptide presentation (5, 11). BILF1 is known to target both cell

68 surface MHC class I molecules and those on route to the surface for degradation thus

69 reducing the presentation of peptides to CD8$^+$ T cells (7, 12, 13). Finally, BNLF2a

70 inhibits the function of the transporter associated with antigen processing (TAP), which

71 reduces the supply of peptides for loading on to MHC class I molecules, thus reducing

72 the level of MHC class I:peptide presentation to CD8$^+$ T cells (4, 14, 15).

73 Our group recently investigated the relevance of BGLF5, BNLF2a and BILF1 immune

74 evasion genes in the context of lytic virus infection (16). It was concluded that BGLF5 in

75 fact plays a minimal role in protecting EBV infected cells against T cell recognition, and

76 that BNLF2a plays an important role of protecting cells during the immediate early and

77 early stages of lytic cycle, contributing little protection at late stage lytic cycle (IE>E>>L)

78 (14, 16). BILF1 was shown to contribute minimal protection during immediate early

79 stage lytic cycle, a reasonable level of protection during early stage lytic cycle and a

80 more dramatic level of protection was observed during late stage lytic cycle (IE<E<<L)

81 (16). This investigation revealed a level of co-operation between EBV encoded MHC

82 class I immune evasion genes in order to protect cells from CD8$^+$ T cell recognition.

83 However, CD8$^+$ T cell responses to late lytic cycle antigens still recognize lytically-

84 infected target cells relatively poorly, even in the absence of BILF1 expression. (16, 17).

85 This implies that another as yet unidentified immune evasion gene or genes may be

86 functioning late in lytic cycle.

87 In comparison to what is known about the immune evasion of MHC class I antigen

88 presentation, the evasion of MHC class II antigen presentation by EBV is less well

89 understood. Over-expression of the host shut off protein, BGLF5 has been shown to

90 result in a reduced level of surface MHC class II (5). In addition, the immediate early

91 protein BZLF1 has been shown to interfere with MHC class II antigen presentation by

92 modulating the expression of cell surface invariant chains (8). A third EBV encoded

93 gene, BZLF2 (gp42), has been shown to interfere with MHC class II antigen

94 presentation to CD4$^+$ T cells by sterically hindering MHC class II interaction with the T

95 cell receptor, thus blocking CD4$^+$ T cell recognition (9, 10). To date, no other EBV

96 proteins have been identified as potential CD4$^+$ T cell immune evasion proteins.

97 The present study sought to identify novel candidate EBV genes responsible for

98 interfering with MHC class I antigen presentation during late phase lytic cycle, and thus

99 providing an explanation for the pronounced immune evasion observed at that stage in

100 the lytic cycle. Screening experiments revealed that the late lytic protein, BDLF3, whose

101 functions are unknown (18-20), was able to impair MHC class I antigen presentation.

102 Unexpectedly, BDLF3 also impaired CD4$^+$ T cell recognition of MHC class II presented

103 peptides. The molecular mechanism for the effect of BDLF3 on antigen presentation

104 involved ubiquitination and proteasomal dependent downregulation of surface MHC

105 class I and class II molecules.

106

Materials and methods

Plasmids

The previously described (7) expression plasmid pCDNA3-IRES-GFP, a kind gift from Professor Emmanuel Wiertz (Utrecht Medical Center, Netherlands), was used to subclone and express a selection of EBV genes. The p509 expression plasmid for BZLF1, a kind gift from Professor Paul Farrell (Imperial College London, UK), has also been described (11) as has the cytoplasmic EBNA1 expression vector (21). The retroviral plasmid PLZRS-NGFR, also a kind gift from Professor Emmanuel Wiertz, was used to subclone PLZRS-BDLF3-NGFR and both were used in transient transfections to allow for in house sorting of transfected cells, on the expression of surface truncated nerve growth factor receptor (NGFR).

Cells, transfections and electroporations

The MJS (Mel JuSol) melanoma-derived cell line (22), and the EBV negative Burkitt lymphoma cell line DG75 (23) were maintained in RPMI 1640 supplemented with 10% fetal calf serum (FCS). CIITA-293 cells are HEK-293 cells stably expressing CIITA (24) and were a kind gift from Dr Andrew Hislop, University of Birmingham. These were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS. The myelogenous leukemia cell line K562, transduced to express either HLA-A2, -B35, -Cw1, -DR or –DQ (a kind gift from Professor Emmanuel Wiertz, Utrecht) were maintained in RPMI 1640 supplemented with 10% FCS plus 400µg/ml of geneticin (Invitrogen). EBV specific CD4^{+} and CD8^{+} T cell clones were grown in RPMI 1640

130 supplemented with 10% FCS, 5% human serum, 30% supernatant from the interleukin-

131 2 producing MLA 144 cell line (25) and 50 U/ml recombinant interleukin-2, as described

132 previously (17).

133 Transient transfection of MJS and 293-CIITA cells with plasmid DNA was performed

134 using lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

135 Transient expression of plasmid DNA using DG75 cells and K562 cells was performed

136 by electroporating cells at 290V and 950µF in 4mm gap cuvettes. In some experiments,

137 cells transiently transfected with NGFR expressing plasmids were positively selected for

138 the surface expression of NGFR using MACSelect NGFR-Transfected Cell Selection

139 kits, as per manufacturer's protocol (Miltenyi Biotec).

140

141 **Antibodies**

142 For immunoprecipitation experiments and for internalization/appearance assays,

143 unconjugated W6/32 and L243 murine monoclonal antibodies (MAbs) to human MHC

144 molecules were obtained from Biolegend: W6/32 (26) recognizes native β_2

145 microglobulin-associated MHC class I complexes (HLA-A, -B and –C alleles); and L243

146 for HLA-DR. For flow cytometry experiments, APC- and PE- conjugated antibodies to

147 HLA class I (W6/32), HLA-DR (L243), ICAM1/CD54 (HCD54) and transferrin

148 receptor/CD71 (TfR, CY1G4) were purchased from Biolegend. For western blotting,

149 mouse anti-ubiquitin antibody (P4D1) MAb was purchased from Biolegend. Goat

150 antibodies to calregulin were purchased from Santa Cruz Biotechnology. The BZ.1

151 murine MAb specific for the EBV BZLF1-encoded protein was generated by our

152 laboratory (27). The rabbit anti-BDLF3 (V8) serum was a kind gift from Dr. L.Hutt-

153 Fletcher (19).

154

Flow cytometry analysis of cell surface MHC class I and class II molecules

156 Cell surface expression of MHC class I and class II was determined by staining cells

157 with APC- or PE- conjugated anti-HLA class I or class II antibodies and detected on

158 BDbiosciences Accuri C6 Flow Cytometer. Data were analyzed using FlowJo software

159 (TreeStar).

160 The kinetics of internalization and appearance of cell surface MHC molecules were

161 determined essentially as described previously (12).To assay the kinetics of surface

162 MHC class I and class II internalization, MJS cells were incubated on ice with saturating

163 amounts of anti-MHC class I (W6/32) or anti-MHC class II (L243) MAbs. Cells were then

164 washed three times in phosphate-buffered normal saline (PBS) and placed in culture

165 medium at 37ºC for 60 mins. Aliquots of cells were taken at those times shown in

166 results, and were rapidly cooled to 0ºC to inhibit further membrane trafficking. The level

167 of W6/32 or L243 MAb remaining at the cell surface was then analyzed by staining cells

168 with APC-conjugated goat anti-mouse IgG2a antibody (Biolegend). Cells were analyzed

169 using flow cytometry.

170 To assay the kinetics of MHC class I and II appearance, MJS cells were again

171 incubated with saturating amounts of W6/32 or L243 for 60 mins on ice. Cells were

172 washed three times in PBS and then placed in warm culture medium at 37ºC for 60min,

173 cells were analyzed at times indicated in results. After cooling to 0ºC, to prevent further

174 appearance of molecules at the surface through membrane trafficking, cells were

9

175   stained with APC-conjugated W6/32 or APC-conjugated L243 and analyzed using flow

176   cytometry. These directly conjugated anti-MHC detection antibodies will only bind to

177   MHC molecules that have appeared since the excess unconjugated blocking antibody

178   was washed away immediately prior to beginning the incubations in warmed medium.

179   Note that the MHC molecules newly-arrived at the cell surface are likely to be a mixture

180   of de-novo synthesized molecules arriving at the surface for the first time, and recycled

181   molecules that had previously been endocytosed.

182

183   **Flow cytometric analysis of whole cell (intracellular) proteins**

184   Intracellular staining for HLA class I and class II was performed to quantify the total

185   cellular levels of these proteins. Washed pellets of 0.5×10^6 cells were first fixed using

186   100µl Ebiosciences intracellular (IC) fixative for 1h on ice, followed by permeabilization

187   using 100µl (0.2%) Triton X-100 and further 30 min incubation on ice. After washing in

188   PBS, cells were incubated with appropriate conjugated antibody for 1h at 37°C. Cells

189   were then washed in PBS and analyzed using flow cytometry.

190

191   **T cell function assays**

192   'RAK' CD8^{+} T cell clones specific for the **RAK**FKQLL peptide originating from BZLF1

193   protein, and 'SNP' CD4^{+} clones specific for the **SNP**KFENIAEGLRVLLARSH epitope

194   from ENBA1 protein, were generated as previously described (16). Targets for RAK-

195   specific CD8^{+} T cells were generated by co-transfection of MJS cells with BZLF1 and

196   control-GFP or BDLF3-GFP expression plasmids. At 24h post transfection, cells were

197   used as targets for RAK specific CD8^{+} T cell clones. T cell recognition was determined

198 by interferon gamma (IFN-γ) enzyme-linked immunosorbant assay (ELISA) using a

199 previously described protocol (16). Targets for SNP-specific CD4$^+$ T cell clones were

200 generated by transfection of MJS cells with the cytoplasmic EBNA1 expression plasmid,

201 EBNA1ΔNLS, which generates a target protein that is efficiently processed via the MHC

202 antigen presentation pathway (21). At 24h post-transfection cells were re-seeded and

203 24h later these cells were transfected with control-NGFR or BDLF3-NGFR expression

204 plasmids. After a further 24h, cells were harvested and sorted as described above and

205 the recognition of these target cells by SNP-specific CD4$^+$ T cell clones was determined

206 by IFN-γ ELISA.

207

208 **Immunoprecipitation**

209 Positively selected control-NGFR and BDLF3-NGFR expressing MJS cells (2x10^6) were

210 used for surface MHC class I and class II immunoprecipitation. Cells were incubated for

211 two hours on ice with anti-HLA class I Mab (W6/32) or anti-HLA class II Mab (L243),

212 then washed and lysed using 400µl of NP-40 buffer (0.5% Nonidet P-40, 5mM MgCl$_2$

213 and 50mM Tris-HCl, pH7.5) with protease inhibitor cocktail (Sigma P8340) at 4ºC for 45

214 min. Nuclei and insoluble debris were removed by centrifugation, and the supernatants

215 were incubated with 20µl Dynabeads Protein A and 20µl Dynabeads Protein G

216 (Invitrogen) at 4ºC overnight. Beads were then washed four times with NET buffer (0.5%

217 NP-40, 150mM NaCl$_2$, 5mM EDTA and 50mM Tris-HCl, pH 7.5) and the precipitated

218 proteins were eluted by boiling in reducing sample buffer for 5 min. Finally, samples

219 were separated by SDS-PAGE on 4-12 % Bis-Tris NuPage mini-gels with

220 morpholinepropanesulfonic acid (MOPS) electrolysis buffer (Invitrogen).

221 **Western blotting**

222 Total cell lysates were denatured in reducing sample buffer and then sonicated and

223 heated to 100ºC for 5 min. Solubilized proteins equivalent to $2x10^5$ cells/20µl sample

224 were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on to 4-12%

225 acrylamide gradient bis-Tris NuPage minigels with MOPS running buffer (Invitrogen).

226

227 **Results**

228 **The late lytic gene BDLF3 is identified as an immune evasion protein**

229 As previously demonstrated, EBV encodes a number of immune evasion genes that co-

230 operate to afford the protection of EBV infected cells against recognition by CD8$^+$ T cells

231 during lytic cycle. However, it has been hypothesized that an as yet unidentified EBV

232 lytic gene may be responsible for the ultimate protection of EBV infected cells during

233 late lytic cycle (16). In order to identify other potential EBV immune evasion proteins

234 involved in protecting infected cells against CD8$^+$ T cell recognition, more than 25 EBV

235 genes expressed during lytic cycle were transiently expressed in MJS cells using

236 bicistronic plasmid vectors that co-expressed GFP with the test gene (Supplementary

237 Fig. 1A). The expression of GFP protein allowed for the identification of transfected cells

238 using flow cytometry. At 24h post-transfection, flow cytometric analysis was used to

239 analyse surface expression levels of MHC class I on GFP positive cells. Of all EBV lytic

240 genes included in this screen the only protein that reproducibly affected surface levels

241 of MHC class I was BDLF3 (Fig. 1A). Fig. 1A shows a representative selection of these

242 screens. As a control, the level of surface MHC class II was also analyzed in this screen

243 (Supplementary Fig. 1B). Interestingly, BDLF3 also affected the surface expression of

244 MHC class II (Fig. 1B).

245 BDLF3 has previously been classified as a late expressed lytic protein (19). We

246 confirmed the late expression kinetics using the EBV positive cell line, AKBM (9) that

247 can be induced in to lytic cycle by cross linking of the B cell receptor. Following

248 induction, aliquots of induced AKBM cells were taken at 0h, 1h, 6h, 12h, 24h, and 48h

249 time points and immunoblotting was performed in order to detect the expression of

250 BDLF3 and the immediate early protein BZLF1. As shown in Fig. 1C, BDLF3 protein

251 expression is detected weakly at 12hr post induction but with stronger expression seen

252 at 24hr. These expression kinetics are consistent with previous findings (19) and

253 suggests that BDLF3 could potentially be the 'missing link' immune evasion protein

254 responsible for interfering with MHC class I antigen presentation during late stage lytic

255 cycle to protect these cells against CD8$^+$ T cell recognition. In addition, the effect on

256 expression of MHC class II molecules raised the possibility that BDLF3 also plays a role

257 in CD4$^+$ T cell immune evasion. To confirm that the levels of BDLF3 protein expression

258 in our transfected cells were physiologically relevant, BDLF3 protein expression levels

259 in the transfected MJS cells was compared with BDLF3 expression level in induced

260 AKBM cells after adjusting for the percentage of GFP$^+$ cells in MJS cells and

261 percentage of VCA$^+$ cells in induced AKBM cells. Quantification of western blots

262 (Supplementary Fig. 2) showed that the expression of BDLF3 in MJS was around 70%

263 of that expressed in induced AKBM cells.

264 In order to confirm that BDLF3 acts specifically on MHC class I and MHC class II, MJS

265 cells transiently expressing BDLF3 were harvested at 24hr post-transfection and were

266 analyzed in more detail using flow cytometry to detect surface levels of MHC class I,

267 MHC class II, and other cell surface proteins. The results in Fig. 2A indicated that

268 BDLF3 expressing cells (dashed line) exhibit a 60% decrease in surface MHC class I

269 mean fluorescence intensity (MFI) compared to control cells (solid black line). A similar

270 result was observed for surface levels of MHC class II on BDLF3 positive cells (Fig. 2B),

271 where there was a reduction of 50% in MHC class II MFI. Importantly, the levels two

14

272 other surface proteins tested, Transferrin receptor (TfR; Fig. 2C) and ICAM1, (Fig. 2D)

273 were not affected by the expression of BDLF3.

274 Since B cells are the natural reservoir for EBV, we next investigated the phenotype of

275 BDLF3 in B cells. To this end, the effect of transient BDLF3 expression on surface MHC

276 molecules on the EBV negative B cell line, DG75 was investigated using flow cytometry.

277 In this instance, the control of ICAM1 could not be included as its expression on DG75

278 cells is negligible; therefore the effect of BDLF3 on expression of CD19 was analyzed

279 along with TfR expression. As shown in Fig. 2E-H, results similar to those seen in MJS

280 cells were obtained. BDLF3 expressing DG75 cells showed a 43% reduction in the MFI

281 of MHC class I (Fig. 2E) and a 30% reduction in MHC class II (Fig. 2F) compared to

282 control cells not expressing BDLF3. There was no observed effect of BDLF3 on the

283 expression of surface TfR (Fig. 2G) or CD19 (Fig. 2H).

284

285 **BDLF3 induces downregulation of all screened MHC class I and MHC class II**

286 **alleles**

287 Since some viral immune evasion genes, including BILF1, have been shown to

288 preferentially target specific HLA class I alleles (13), we next sought to investigate the

289 HLA-specificity of BDLF3. To do so, MHC class I negative K562 cells engineered to

290 stably express HLA-A2, -B35 or –Cw1 were transiently transfected to express either

291 BDLF3 or a control vector. At 24h post-transfection the surface level HLA-A2, -B35 and

292 –Cw1 on positively transfected cells was detected using flow cytometry. As shown in

293 Fig. 3A, cells expressing BDLF3 showed a decrease in the cell surface level of HLA-A2

294 (upper histograms) (27% reduction in MFI), HLA-B35 (middle histograms) (34%

295 reduction in MFI) and HLA-Cw1 (lower histograms) (26% reduction in MFI) compared to

296 control cells. A similar approach was then used to test the specificity of BDLF3 for HLA

297 class II alleles. Here, HLA class II negative HEK-293 cells engineered to stably

298 expressing CIITA, thus driving the surface expression of HLA-DR and -DQ, were

299 transiently transfected to express BDLF3 or control vector. Similar to that seen for HLA

300 class I alleles, BDLF3 induced a reduction in both HLA-DR (47% reduction in MFI) (Fig.

301 3B, upper histograms) and HLA DQ (32% reduction in MFI) (Fig. 3B, lower histograms)

302 compared to control transfected cells. In all examples the level of surface TfR remained

303 similar between BDLF3 and control transfected cells (data not shown). These results

304 indicate that BDLF3 is not selective in down-regulating HLA molecules but instead acts

305 more broadly to down regulate all HLA class I and HLA class II molecules.

306

307 **BDLF3 mediated reductions in surface MHC class I and class II confers protection**

308 **against both CD8$^+$ and CD4$^+$ T cell recognition**

309 Since BDLF3 induces a reduction in the level of surface MHC class I and class II

310 molecules, we next investigated whether BDLF3 expression provided protection against

311 recognition by EBV-specific CD8$^+$ and CD4$^+$ T cells. In order to address this, the HLA-

312 B8 positive cell line MJS cells, were co-transfected with BZLF1 and either BDLF3-GFP

313 or control-GFP vector plasmids (Fig. 4A). At 24h post-transfection these cells were used

314 as targets in a T cell assay with CD8$^+$ T cell clones restricted through HLA-B8 and

315 specific for the peptide RAKFKQLL, contained within the BZLF1 antigen. T cell

316 recognition was measured as IFN-γ release using IFN-γ ELISA. As shown in one

317 representative experiment (n=3) in Fig. 4A, the expression of BDLF3 resulted in a

318 significant decrease in IFN-γ release by RAK-specific T cell clones, from ~1100pg/ml to

319 ~500pg/ml. The BDLF3-mediated reduction in BZLF1-specific CD8^{+} T cell recognition

320 was not due to any change in the expression of BZLF1 target protein expression (Fig.

321 4B).

322 In order to investigate the ability of BDLF3 to protect cells against recognition by CD4^{+} T

323 cells, a similar method was employed. Here, MJS cells stably expressing the HLA class

324 II allele DR51 were transfected to express cytoplasmic EBNA1 for 48h and either

325 BDLF3-NGFR or control-NGFR vector for a further 24hr (Fig. 4C). Cells were then

326 sorted on expression of NGFR and subsequently used as targets for a CD4^{+} T cell

327 clones specific for the HLA-DR51 restricted epitope SNPKFENIAEGLRVLLARSH,

328 contained within EBNA1. As shown in Fig. 4C, the expression of BDLF3 resulted in a

329 decrease in T cell recognition (IFN-γ release) by SNP-specific CD4^{+} T cell clones from

330 ~1300pg/ml to ~900pg/ml, compared to control cells. The BDLF3-mediated reduction in

331 EBNA1-specific CD4^{+} T cell recognition was not due to any change in the expression of

332 EBNA1 target protein expression (Fig. 4D). Thus, in a similar pattern to those results

333 seen for BDLF3 protection against CD8^{+} T cell recognition, BDLF3 induced reduction in

334 surface MHC class II molecules also correlated with protection against CD4^{+} T cell

335 recognition.

336   These data show that BDLF3 induced reduction in cell surface MHC class I and class II

337   is functional in protecting BDLF3 expressing cells against recognition by both CD8^{+} and

338   CD4^{+} T cells.

339   **BDLF3 downregulates surface MHC molecules more dramatically than total MHC**

340   **molecules**

341   To explore the mechanism of MHC class I and class II downregulation by BDLF3, we

342   first asked whether the total cellular pool of MHC molecules was affected, or whether

343   surface MHC molecules were selectively targeted. To this end, flow cytometry of

344   intracellular staining of fixed and permeabilized cells was used to detect the level of

345   whole cell MHC molecules compared to surface MHC molecules detected on

346   impermeable viable cells. As expected, surface level MFI of MHC class I and class II

347   were both reduced by approximately 50% on cells expressing BDLF3 compared to

348   control cells (Fig. 5A, left-hand column). This difference was found to be significant (Fig.

349   5B, white bars). Interestingly, there was only a slight decrease of 10% in the MFI of

350   whole cell MHC class I and MHC class II compared to control cells (Fig. 5A, right

351   column) and this small reduction was not statistically significant when the results from

352   three independent experiments were pooled and analyzed (Fig. 5B, grey bars). To

353   confirm these findings by an independent method, control GFP expressing MJS and

354   BDLF3-GFP expressing MJS cells were purified using Mo-flow cell sorter and the

355   expression of total MHC-I and MHC-II was examined by western-blot. The result

356   showed no significant difference (supplementary Fig.3), confirming the result from

357 intracellular flow cytometry data. Importantly, BDLF3 had no effect on surface or whole

358 cell levels of ICAM1 expression (Figs. 5A and 5B, bottom panel).

359 These data show that BDLF3 affects the levels of surface MHC molecules more

360 dramatically than it affects whole cell MHC molecules, suggesting that BDLF3 exerts its

361 function predominantly on surface MHC class I and II rather than the intracellular

362 fraction of these molecules.

363

364 BDLF3 induces rapid internalization and delayed appearance of MHC molecules

365 Since BDLF3 predominantly targets surface MHC molecules, we next examined

366 whether it targets those MHC molecules already at the cell surface or those trafficking to

367 the cell surface. We therefore compared the kinetics of MHC class I and class II

368 internalization and appearance at the cell surface of BDLF3 expressing cells using flow

369 cytometry. Representative examples are shown of MHC class I (Fig. 6A, upper) and

370 class II (Fig. 6A, lower) internalization assays, where the percentage of MHC class I and

371 class II remaining on the surface of BDLF3 expressing and control cells was measured

372 over 60 minutes. Cells expressing BDLF3 showed lower levels of surface MHC

373 remaining at the cell surface at each time point indicated, such that by 60min there were

374 respectively 20% and 13% less surface MHC class I and MHC class II on BDLF3

375 expressing cells compared to control cells. These data indicate that BDLF3 induces a

376 more rapid rate of both MHC class I and MHC class II internalization.

377 When a similar assay was used to measure the rate of MHC class I and class II surface

378 appearance, BDLF3 expressing cells conversely showed a decreased rate of both MHC

379 class I and class II surface appearance at each time point compared to control cells

380 (Fig. 6B). By 60 min, the appearance of MHC class I and class II on BDLF3 expressing

381 cells at time point 60min was reduced by 50% and 47% respectively in comparison to

382 control cells. This BDLF3-mediated reduction in the rate of appearance of MHC at the

383 surface (Fig. 6B) was noticeably greater than the accelerated rate of endocytosis

384 (Fig.6A).

385 It should be noted that in all experiments rate of TfR internalization or appearance

386 remained similar between control and BDLF3 expressing cells (data not shown). These

387 data indicate that BDLF3 is able to both enhance endocytosis of MHC molecules at the

388 cell surface and interfere with the trafficking of intracellular MHC molecules to the cell

389 surface.

390

391 **BDLF3 downregulation of surface MHC molecules involves ubiquitination and the**

392 **proteasomal pathway**

393 We next sought to identify the mechanism by which BDLF3 is able to enhance

394 internalization and delay the appearance of surface MHC class I and MHC class II

395 molecules. Our initial experiments were designed to identify which pathway BDLF3

396 might utilize in order to reduce the expression of surface MHC molecules. To this end,

397 we incubated BDLF3 expressing cells with proteasomal and lysosomal inhibitors. In the

398 absence of drug treatment, cells expressing BDLF3 showed lower levels of surface

399 MHC class I and II expression compared to control cells (Fig. 7A) as expected.

400 However, when incubated with the proteasomal inhibitor, MG132, BDLF3 expressing

401 cells showed no such reduction in surface MHC class I and MHC class II levels

402 compared to control cells (Fig. 7B). Part abrogation of BDLF3 phenotype by MG132

403 was observed after 4hr treatment, but the effect of MG132 treatment was maximal after

404 16h (Supplementary Fig. 4). Similar results were seen when cells were treated with a

405 second proteasomal inhibitor, bortezomib (Supplementary Fig. 5), whereas treatment

406 with a lysosomal inhibitor, bafilomycin (Supplementary Fig. 6), did not prevent BDLF3

407 induced downregulation of surface MHC molecules. These data indicate that BDLF3

408 induced downregulation of surface MHC molecules is dependent upon the proteasomal

409 pathway.

410 As BDLF3 downregulates surface MHC molecules through increased internalization and

411 delayed appearance (Fig. 6), we next examined what effect proteasomal inhibition might

412 have on the kinetics of MHC class I and class II internalization and appearance at the

413 cell surface of BDLF3 expressing cells. The results showed that MG132 completely

414 abrogated the effect of BDLF3 on both the rate of internalization (Fig 7C) and the rate of

415 appearance (Fig.7D), demonstrating an essential role of the proteasome in BDLF3

416 induced MHC molecule downregulation.

417

418 Given the essential role that the proteasome plays in BDLF3 induced reduction of

419 surface MHC molecules, and that ubiquitination is an important component of the

420 proteasomal pathway, we next assessed whether BDLF3 induces ubiquitination of

21

421 surface MHC molecules. To this end, MJS cells were transfected with various

422 expression vectors including BDLF3, control vector and ubiquitin (Fig. 7E, F). These

423 cells were then incubated with or without the proteasomal inhibitor, MG132. At 24h post-

424 transfection, surface MHC class I or MHC class II molecules were immunoprecipitated

425 from BDLF3 expressing or control cells and resulting immunoblots were probed with

426 ubiquitin-specific antibodies. As shown in Fig. 7, poly-ubiquitinated high molecular

427 weight bands appeared in immunoblots for immunoprecipitated MHC class I (Fig. 7E)

428 and MHC class II (Fig. 7F) in BDLF3 expressing cells treated with MG132. These

429 ubiquitin-reactive bands were less pronounced both in control cells treated with MG132

430 and in BDLF3-expressing cells not treated with MG132.

Discussion

This study reveals the identity and mechanism of novel immune evasion gene, BDLF3, which induces downregulation of not only cell surface MHC class I but also MHC class II, to the extent that antigen recognition by both CD8^{+} and CD4^{+} virus-specific T cells is functionally impaired. The BDLF3 protein was first identified a number of years ago as the glycoprotein gp150, which is located at the cell membrane and in the virion, is not essential for EBV replication, and hitherto had no known function (18-20). Our study now allows a function to be assigned to the BDLF3 protein.

The identification of BDLF3 as an immune evasion protein has an important impact on our knowledge of the T cell response to lytic EBV antigens and the protection of EBV infected cells from recognition by these T cells. EBV lytic cycle involves the synchronous expression of more than 60 viral proteins, many of which elicit strong CD4^{+} and CD8^{+} T cell responses, but various immune-evasion mechanisms enable EBV to persist as a lifelong infection. For CD8^{+} T cell responses to lytic cycle antigens, there is a pattern of immunodominance that correlates with the efficiency of antigen presentation during lytic cycle. An earlier study by our group revealed that the known immune evasion genes, BGLF5, BNLF2a and BILF1 act in co-operation to afford protection to EBV infected cells against lytic specific CD8 T[+] cell recognition. However, the ultimate protection that is seen in late phase lytic cycle could not be fully explained by the action of these known evasion genes (16). The identification of BDLF3, which is expressed during late stage lytic cycle, as a potent inhibitor of the MHC class I antigen presentation pathway makes it a prime candidate for the 'missing link' immune evasion

453 protein responsible for protecting EBV infected cells from CD8$^+$ T cell responses during

454 late stage lytic cycle.

455 Another important feature of BDLF3 is its ability to induce MHC class II downregulation

456 and evade CD4$^+$ T cell recognition. Evidence is accumulating in the literature showing

457 that viruses target multiple points on the MHC class II antigen presentation pathway,

458 including: suppression of CIITA (28, 29), diversion or degradation of DR molecules

459 during membrane transport (30) and direct targeting of the CD74 (invariant chain)

460 chaperone of DR (8). Two viral genes expressed during EBV lytic cycle have been

461 reported to manipulate MHC class II antigen presentation pathway. BZLF1 induces a

462 marked downregulation of surface CD74 to impair antigen presentation and CD4$^+$ T cell

463 recognition (8), and Gp42 sterically inhibits interactions between TCR on the CD4$^+$ T

464 cell with MHC-II peptide complexes (10). The identification of BDLF3 as a novel MHC

465 class II evasion gene from EBV indicates that, similarly to interference with MHC class I

466 antigen presentation, interference with MHC class II antigen presentation very likely

467 involves the cooperative action of multiple evasion genes.

468 The fact that BDLF3 is an EBV late lytic cycle protein, suggests that it would help to

469 provide enhanced protection of the virus-producing cells prior to release of mature

470 virions. In addition, as BDLF3 can be detected in the EBV virion (31), this raises the

471 possibility that BDLF3 can act immediately after new infections of B cells to modulate

472 recognition by existing EBV specific CD4$^+$ T cells. Considering the important role of

473 MHC class II molecules and gp42 in EBV infection, and also the observation that

474 BDLF3 knock-out EBV virus particles can infect epithelial cells better than B cells (18),

475 we can propose a potential role of BDLF3 in the EBV infection. BDLF3 expression

476 results in decreased MHC class II expression at the surface of lytically replicating cells,

477 reducing the amount of MHC class II available to bind gp42. We would therefore predict

478 that BDLF3 knockout virions may contain less envelope gp42 than wild type EBV, with a

479 consequent enhanced ability to infect epithelial cells.

480 Our data indicate that the mechanism of BDLF3 interference with the appearance of

481 MHC molecules at, and internalization from, the cell surface, involves ubiquitination of

482 MHC molecules and proteasome-dependent pathways. The targeting of MHC

483 molecules for ubiquitination has been described for other viral immune evasion proteins,

484 but the mechanism of action of BDLF3 is clearly distinct. An example is the K3 and K5

485 proteins encoded by Kaposi's sarcoma-associated herpesvirus (KSHV), which function

486 as two membrane-bound E3 ubiquitin ligases and have been shown to facilitate the

487 rapid endocytosis and subsequent degradation of MHC class I by inducing

488 ubiquitination (32-37). Unlike K3 and K5, there is no evidence to show that BDLF3 itself

489 is an E3 ubiquitin ligase, therefore it is very likely that BDLF3 functions via a different

490 mechanism. Indeed in terms of the MHC class I downregulation, BDLF3 can affect all

491 MHC class I alleles we studied, whereas K5 affects HLA-A and –B but has a weak

492 effect on HLA-C, while K3 downregulates all HLA class I alleles (33). More importantly,

493 KSHV K3 and K5 can downregulate a range of other surface proteins including B7-2,

494 CD54 (ICAM-1), CD1d, CD31 (PECAM-1), IFN-γR1, MICA/B, BST-2, ALCAM, Syntaxin-

495 4 (38-42). In contrast we found that BDLF3 targets MHC-I and MHC-II, but not CD54

496 (Fig. 2) or MICA/B (data not shown). Another distinguishing feature of BDLF3 is that

497 whilst the surface levels of MHC class I and class II were reduced by around 50%, there

498   was a minimal decrease of whole cell MHC class I and MHC class II (Fig. 5A, and

499   Supplementary Fig. 3). That BDLF3 can induce a 50% reduction in surface MHC

500   molecules against a reduction of less than 10% of the whole cell MHC molecules

501   reflects the fact that there is relatively large reservoir of MHC class I and MHC class II

502   inside the cells. Therefore, the 10% reduction in whole cell MHC class I and II could

503   represent the complete degradation of the 50% of MHC class I and II that is lost from

504   the surface in the presence of BDLF3.

505   Whilst BDLF3 does not function as ubiquitin E3 ligase, it does nevertheless

506   downregulate surface MHC class I and MHC class II through inducing ubiquitination.

507   How might this be? One possible explanation is that it may recruit other cellular E3

508   ubiquitin ligase proteins such as members of the membrane-associated RING-CH

509   (MARCH) proteins, the cellular orthologues of K3 and K5. These proteins have been

510   implicated in the regulation of cell surface molecules including MHC class I, MHC class

511   II, ICAM and transferrin receptor (36, 43-46). Indeed, the overexpression of MARCH-IV

512   and MARCH-IX proteins induces ubiquitination and rapid internalization of MHC class I

513   (43). Numerous MARCH proteins have been identified that target MHC class I or MHC

514   class II although, at the time of writing, no single known MARCH protein induces the

515   ubiquitination of both MHC class I and class II without affecting other surface markers

516   that are left unaffected by BDLF3 (47). Our preliminary experiments have not been able

517   to demonstrate co-immunepreciptation of MHC molecules with BDLF3 (data not shown).

518   Thus BDLF3 may recruit an as of yet unidentified MARCH family protein or perhaps

519   several of these proteins. If so, then the specificity of BDLF3 would be due to the target

520 molecules of these recruited proteins. Future work will be aimed at resolving these

521 possibilities.

522 Considering the data that we have obtained for BDLF3, and the features that distinguish

523 the effects of BDLF3 from previously characterized immune evasion proteins, we

524 postulate that its mechanism of action is broadly as follows. As BDLF3 reduces the rate

525 of appearance of MHC molecules at the cell surface to a greater extent than it increases

526 the rate of endocytosis (Fig.6) we suppose that the reduced rate of appearance must be

527 due at least in part to an effect on de novo synthesized MHC molecules trafficking to the

528 surface. Therefore, BDLF3 targets for ubiquitination both de novo synthesized and

529 recycling endocytosed MHC molecules. These ubiquitinated MHC molecules are

530 directed for proteasomal degradation or, in the presence of proteasomal inhibitors,

531 accumulate at the cell surface. The finer details of the biochemical mechanisms, and

532 the identity of the ubiquitin ligases involved, remain to be resolved.

533 The acquisition of immune evasion proteins has played a critical role in the evolution of

534 viruses. It is interesting to note that an EBV homolog, the marmoset lymphocryptovirus

535 (maLCV), which naturally infects new world nonhuman primates, lacks BDLF3. This

536 may be relevant to the fact that serological studies reveal maLCV infection in

537 marmosets to be much less ubiquitous than EBV in humans (48, 49). It might therefore

538 be speculated that the acquisition of BDLF3 immune evasion functions is a later

539 evolutionary event that contributes to the success of EBV in successfully colonizing the

540 vast majority of the human population.

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Figure legends

696 **Figure 1. Screening of EBV lytic genes to identify potential MHC class I immune**

697 **evasion genes.** MJS cells were transiently transfected with pCDNA3.1-IRES-GFP

698 plasmids encoding for a selection of EBV lytic genes. At 24h post transfection surface

699 levels of MHC class I (A) and MHC class II (B) on GFP positive cells were analysed

700 using two color flow cytometry. (C) AKBM cells were induced into lytic cycle by cross-

701 linking of B cell receptors for 1hr at 37ºC and analyzed at time points post-induction, as

702 indicated, using western blot. Levels of BZLF1 protein (upper blot), BDLF3 protein

703 (middle blot; stars indicate monomeric and trimeric BDLF3 protein) and, as a loading

704 control, calregulin (lower blot) are shown.

705 **Figure 2. BDLF3 expression induces the down regulation of surface MHC class I**

706 **and MHC class II.** MJS cells (A-D) and DG75 cells (E-H) were transiently transfected

707 with control-GFP or BDLF3-GFP plasmids. At 24h post transfection, two color flow

708 cytometry was used to measure surface levels of MHC class I (A,E), MHC class II (B,F),

709 TfR (C,G) and ICAM1 (D) or CD19 (H) in the GFP$^+$ populations of control-GFP (solid

710 line histogram), and BDLF3-GFP transfected cells (dashed line histogram). The grey

711 histogram denotes background staining obtained with an isotype control antibody.

712 **Figure 3. BDLF3 induces downregulation of all HLA class I and class II alleles.** (A)

713 The MHC class I negative cell line K562 transduced to stably express either HLA-A2, -

714 B35 or -Cw1 was electroporated with control-GFP or BDLF3-GFP plasmids. At 24h

715 post-transfection, two color flow cytometry was used to measure surface MHC class I

716 levels in the GFP$^+$ populations in the control-GFP transfected (solid line histogram) and

the BDLF3-GFP transfected cells (dashed line). (B) HEK-293 cells stably expressing

CIITA were transiently transfected with control-GFP or BDLF3-GFP plasmids. At 24h

post-transfection, two color flow cytometry was used to measure surface HLA-DR and

HLA-DQ levels in GFP[+] populations in the control-GFP transfected (solid line histogram)

and the BDLF3-GFP transfected cells (dashed line). The grey histogram denotes

background staining obtained with an isotype control antibody.

Figure 4. BDLF3 can inhibit EBV specific CD8^{+} and CD4^{+} T cell recognition. MJS

cells were co-transfected with p509 plasmid (BZLF1 expression vector) together with

control-GFP or BDLF3-GFP. At 24h post transfection, the MJS cells were co-cultured

with effector T cells, BZLF1 (RAK)-specific CD8^{+} T cell clone, for a further 18hr and the

supernatants were tested for the release of IFN-γ as a measure of T cell recognition. All

results are expressed as IFN-γ release in pg/ml and error bars indicate standard

deviation of triplicate cultures. (B) Total cell lysates were generated from the above

transfections, and analyzed by western blotting using antibodies specific for BDLF3,

BZLF1 or calregulin as a loading control. The asterisks adjacent to the BDLF3 blot

indicate monomeric and trimeric BDLF3 protein. (C) MJS-DR51 cells were first

transfected with EBNA1ΔNLS, allowed to recover in culture overnight, then were divided

to two groups and transfected with either BDLF3-NGFR or Control-NGFR. After a

further 24h, NGFR^{+}/BDLF3^{+} or control NGFR^{+} cells were sorted with magnetic beads

and used as targets for HLA-DR51 restricted EBNA1 (SNP) specific CD4^{+} T cell clones.

Recognition was measured as pg/ml of IFN-γ release by T cell clones. Error bars

represent standard deviation of the mean for triplicate assay replicates. Results are

739   representative of three independent experiments. (D) Total cell lysates were generated

740   from the above transfections, and analyzed by western blotting using antibodies specific

741   for BDLF3, EBNA1 or calregulin as a loading control.

742   **Figure 5. BDLF3 induces a more dramatic reduction in surface MHC class I and II**

743   **compared to whole cell levels.** (A) MJS cells were transiently transfected with control-

744   GFP or BDLF3-GFP plasmids. At 24h post transfection, two color flow cytometry was

745   used to measure the level of surface MHC class I (upper left), MHC class II (middle left)

746   and ICAM1 (lower left) in the viable GFP$^+$ populations of control-GFP transfected (solid

747   line histogram), and BDLF3-GFP transfected cells (dashed line histogram). The grey

748   histogram denotes background staining obtained with an isotype control antibody. In

749   parallel, these GFP$^+$ transfected MJS cells were analysed for whole cell levels of MHC

750   class I (upper right), MHC class II (middle right) and ICAM1 (lower right) using

751   intracellular staining of fixed and permealized cells. The results are representative of

752   repeated experiments. (B) Relative mean fluorescence intensity (MFI) of MHC class I,

753   MHC class II and ICAM1 in BDLF3-GFP$^+$ cells compared to control-GFP$^+$ cells were

754   calculated. Results are the combined data from three independent experiments. White

755   bars represent surface staining, grey bars represent whole cell staining. Differences that

756   reached significance (p<0.05) in a Student's Paired T test are denoted by an asterisk.

757   **Figure 6. BDLF3 induces more rapid internalization and delayed appearance of**

758   **both MHC class I and class II at the cell surface.** Internalization and appearance

759   assays were performed on MJS cells transiently expressing control-GFP or BDLF3-

760   GFP. The GFP$^+$ population was used to gate on BDLF3 expressing cells. Internalization

761 and appearance assays were performed on cells pre-treated on ice with saturating

762 amounts of anti-MHC class I antibody or anti-MHC class II antibody. Cells were then

763 washed and incubated at 37°C for up to 60 min. (A) For the internalization assay, viable

764 cells harvested at each time point were stained with APC-conjugated goat anti-mouse

765 IgG antibody, and analyzed using flow cytometry at the indicated times; this identifies

766 the pre-labeled antibody-bound MHC molecules that remain at the surface while

767 endocytosed labeled MHC molecules are not detected on the viable cells. The mean

768 fluorescence intensities of staining were averaged for triplicate samples, and then

769 normalized to the time 0 samples. (B) For the appearance assays, newly-arrived MHC-I

770 and MHC-II molecules, which were not prelabeled with unconjugated antibodies, were

771 detected by staining with APC-conjugated anti-MHC class I antibody or anti-MHC class

772 II antibody. The mean fluorescence intensities of staining were averaged for triplicate

773 samples, and then normalized to the time 0 samples. Results are representative of

774 three independent experiments.

775 **Figure 7. Treatment of BDLF3 expressing cells with a proteasome inhibitor**

776 **prevents down regulation of MHC class I and class II**. MJS cells were transiently

777 transfected with BDLF3-GFP or control-GFP plasmids, and then incubated in normal

778 medium (A) or with MG132 (5µM) supplemented medium (B). At 24h post-transfection,

779 two color flow cytometry was used to measure surface MHC class I (upper histograms),

780 surface MHC class II (middle histograms) and surface ICAM1 (lower histograms) in

781 GFP^{+} populations of control-GFP (solid line histogram), and BDLF3-GFP (dashed line

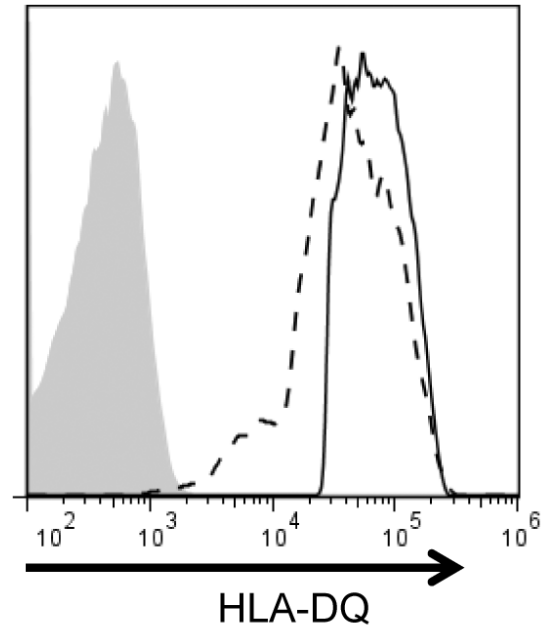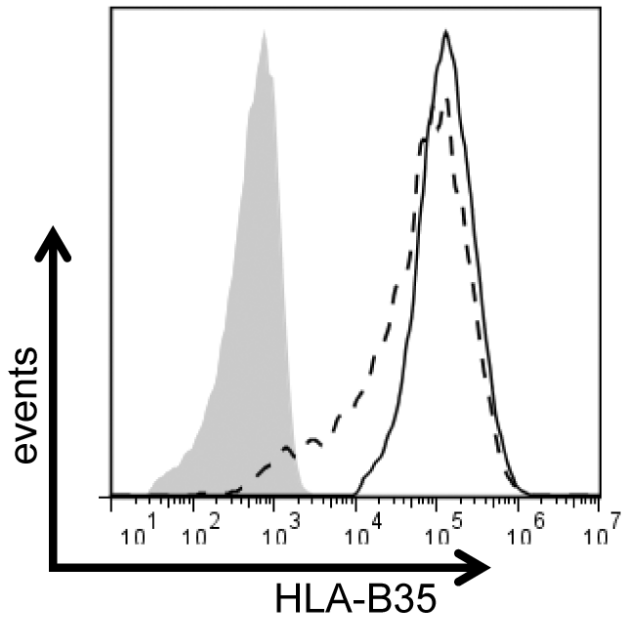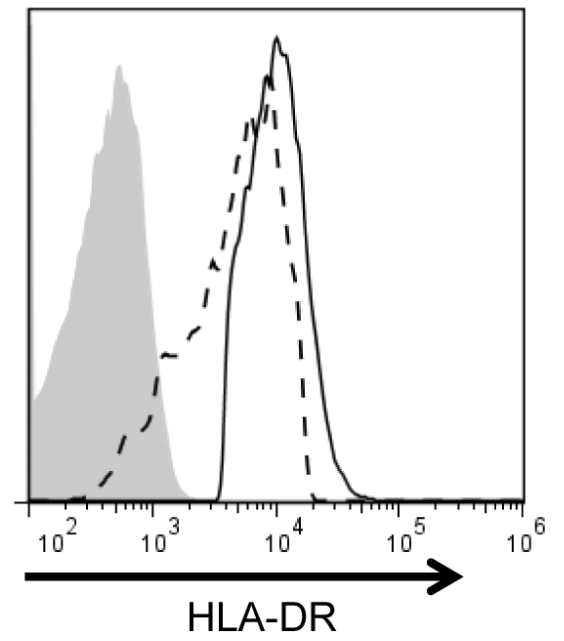782 histogram) transfected cells. The grey histogram denotes background staining obtained

with an isotype control antibody. Results are representative of repeated experiments (n>4). (C,D) MJS cells were transiently transfected with BDLF3-GFP or control-GFP plasmids, and then were incubated with MG132 (5µM). Following drug treatment, the rate of internalization (C) of MHC-I (top panel) and MHC-II (bottom panel), and the rate of appearance (D) of MHC-I (top panel) and MHC-II (bottom panel) were measured using the same method as in Fig. 6. (E, F) MJS cells were transfected with a ubiquitin expression plasmid plus either Control-NGFR or BDLF3-NGFR plasmids; these transfected cells were then divided in to two and incubated in normal medium or in medium supplemented with MG132. At 24h post transfection, NGFR[+]/BDLF3^{+} or control NGFR^{+} cells were sorted with magnetic beads, and surface MHC class I (C) or MHC class II (D) were immunoprecipitated, eluted, and then immunoblotted using anti-ubiquitin antibody (P4D1).

A

MHC Class I

B

MHC class II

Control-GFP   BXLF1-GFP   BDLF2-GFP   BDLF3-GFP

C

| | 0 | 1 | 6 | 12 | 24 | 48 | Post-induction (hr) |

39-
28-
BZLF1

97-

*

BDLF3

*

28-

51-
Calregulin

MJS

A MHC Class I

events

Fluorescence intensity

B MHC Class II

events

Fluorescence intensity

C TfR

events

Fluorescence intensity

D ICAM1

events

Fluorescence intensity

DG75

E MHC Class I

Fluorescence intensity

F MHC Class II

Fluorescence intensity

G TfR

Fluorescence intensity

H CD19

A

HLA-A2

HLA-B35

HLA-Cw1

B

HLA-DR

HLA-DQ

A

IFN-γ pg/µl

1500

1000

500

| BDLF3 | − | − | + |
| BZLF1 | − | + | + |

C

IFN-γ pg/µl

2000

1500

1000

500

| BDLF3 | − | − | + |
| EBNA1 | − | + | + |

B

97-

*

BDLF3

28-

*

39-

28-

BZLF1

51-

Calregulin

D

97-

* BDLF3

28-

*

51-

EBNA1

51-

Calregulin

A

Surface | Whole cell

MHC Class I

MHC Class II

ICAM1

B

Relative MFI

control   BDLF3   control   BDLF3
Surface MHC class I | Whole cell MHC class I

Relative MFI

control   BDLF3   control   BDLF3
Surface MHC class II | Whole cell MHC class II

Relative MFI

control   BDLF3   control   BDLF3
Surface ICAM1 | Whole cell ICAM-1