Mechanism of EBV inducing anti-tumour immunity and its therapeutic use

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Supplementary Information

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Supplementary Discussion

Depletion of germinal center B cells
We examined whether the T cells elicited by LMP1+B cells also kill in vivo CD40-activated B cells, such as germinal center (GC) B cells. GC cannot form in CL mice, because their LMP1-expressing peripheral B cells are eliminated by T cells. Therefore, we used inducible LMP1-expressing CD19-creERT2;LMP1STOP (ERT2-CL) mice, and found that GC B cells were markedly depleted around the peak of T cell response. Following T cell contraction, GC re-formed in these mice (Extended Data Fig. 3). Thus, GC B cells are apparently attacked by cytotoxic CD4 and CD8 T cells in LMP1 mice.

Reduction of GC B cells seems also to occur in patients with primary EBV infection. Another notable phenomenon in primary EBV infection is that the (GC-dependent) IgG type antibody response often peaks 1–3 months after the IgM response, in contrast to the 1–2 week lag time in other virus infections. It is tempting to speculate that GC disruption by the cytotoxic T cell response may underlie the delay in IgG response in primary EBV infection, which should be thoroughly investigated.

Pre-existing EBV immunity and feasibility of LMP1-based CD4 CTL production approach
All the 11 CLL patients in this study were found carrying IgG antibodies against EBV viral capsid antigen (data not shown), and thus had been previously infected with EBV, in line with the extremely high prevalence of this virus in humans. The successful generation of tumour antigen-specific CD4 CTLs from these patients, together with the finding that LMP1 protein itself is rarely targeted by human CD4 cells, indicate that even if a few LMP1 epitope-specific memory CD4 cells do exist in EBV-immune individuals, they will not outcompete tumour antigen-specific CD4 CTLs produced by the LMP1-based approach described here.

CD4 CTL combination with checkpoint blockade
CD4 CTL-based therapy has the ability to circumvent a major immune escape mechanism observed in many B cell tumours—the complete loss of MHC-I expression, as reported in > 70% of diffuse large B cell lymphoma (DLBCL) and classical Hodgkin lymphoma (cHL) cases. Complete loss of MHC-II occurs less frequently, and it is noteworthy that in cHL, clinical responses to PD-1 blockade were found to be associated with the expression of MHC-II, but not MHC-I on tumour cells. This suggests therapeutic importance of CD4 cells, and provides a strong rationale for combining CD4 CTL adoptive therapy with checkpoint blockade. Indeed, our results in the A20 mouse lymphoma model exemplify the synergistic effect of CD4 CTL combination with PD-1 blockade.

Therapeutic potential of CD4 CTLs in MHC-II negative tumours
Recent preclinical and clinical studies have highlighted the therapeutic potential of CD4 CTLs in several types of solid cancers. Of note, IFN-γ exposure can upregulate MHC-II on some solid tumours, like melanoma, which can then be directly attacked by cytotoxic CD4 cells. Furthermore, CD4 cells specific for TAAs and neoantigens are often readily detectable in tumour patients. This encourages developing CD4 CTL approaches capable of targeting multiple antigens in these tumours.
Supplementary References

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Statistics and reproducibility

Figure 1
Data in a–e are representative of two independent experiments.

Figure 2
a, Data show mean of n = 2 biologically independent samples per group. This experiment was performed once.
b, Data are representative of four independent experiments.
c, Upper right panel, n = 8 biologically independent animals per group. Data are pooled from five independent experiments. ***P = 0.0002 by unpaired two-tailed Mann-Whitney U test. Lower right panel, n = 3 (C) or 6 (CL) biologically independent animals. Data are pooled from two independent experiments. ****P < 0.0001 by unpaired two-tailed Student’s t test.
d, Upper right panel, n = 5 biologically independent animals per group. Data are pooled from three independent experiments. ****P < 0.0001 by unpaired two-tailed Student’s t test. Lower right panel, n = 3 (ERT2-C) or 4 (ERT2-CL) biologically independent animals. Data are pooled from two independent experiments. **P = 0.0052 by unpaired two-tailed Student’s t test.
e, Upper panel, Surv-Tetrm⁺, Day 4: n = 2 (ERT2-C) or 4 (ERT2-CL); Day 5: n = 5 (both groups); Days 7 and 38-42: n = 3 (ERT2-C) or 4 (ERT2-CL) biologically independent animals. Data are pooled from two (Days 4, 7 and 38-42) or three (Day 5) independent experiments. EphA2-Tetrm⁺, Days 4, 7 and 38-42: n = 2 (both groups); Day 5: n = 3 (ERT2-C) or 4 (ERT2-CL) biologically independent animals. Data at each time point are pooled from two independent experiments. Lower panel, Day 3: n = 2 (ERT2-C) or 3 (ERT2-CL); Day 4: n = 3 (both groups); Days 5 and 6-8: n = 4 (ERT2-C) or 5 (ERT2-CL); Day 38-42: n = 2 (ERT2-C) or 4 (ERT2-CL) biologically independent animals. Data are pooled from two (Days 3, 4 and 38-42), three (Day 5) or four (Day 6-8) independent experiments.
f-i, Data are representative of two (f, g, h) or three (i) independent experiments.
j, n = 2 (1019 MHC-II⁺) or 3 (1019 MHC-II⁺ Trp1) technical replicates. Data are representative of two independent experiments.

Figure 3
a, c, e, Data are representative of two independent experiments.
b, Data show mean of n = 2 (LMP1TM1m-A20) or 3 (LMP1-A20) technical replicates. Data are representative of two independent experiments.
d, Data show mean of n = 2 technical replicates per sample group. Data are representative of two independent experiments.
f, Data are representative of two independent experiments.
h, n = 4 (Naive CD4 and CD4 CTL) or 5 (No ACT) biologically independent animals. Data are pooled from two independent experiments. Statistics show differences between naive CD4 and CD4 CTL groups. Day 11: *P = 0.0492; Day 13: *P = 0.0323; Day 15: *P = 0.0176; Day 17: **P = 0.0064; Day 19: ***P = 0.0081; Day 21: *P = 0.0153; Day 23: **P = 0.0050; Day 25: **P = 0.0034; Day 27: ***P = 0.0010 by unpaired two-tailed Student’s t test.
i, n = 4 biologically independent animals per group pooled from two independent experiments, except for the Th0 CD4 control group where n = 3 from one experiment. **P = 0.0090 and *P = 0.0267 by unpaired two-tailed Student’s t test.
j, Data are representative of three independent experiments, with n = 4 biologically independent animals per group.
k, Data are representative of two independent experiments, with $n = 3$ biologically independent animals per group.

m, $n = 5$ biologically independent animals per group, except the CD4 CTL + Isotype group ($n = 4$). Data are representative of two independent experiments. **$P = 0.0029$ by log-rank (Mantel-Cox) test.

Figure 4

a, Data are representative of three independent experiments each with a different patient.

b, Data are representative of three (OX40L), four (HLA-II) or six (CD80 and CD70) independent experiments each with 1–2 different patients.

c, Left panel, $n = 8$ patients. Data are pooled from six independent experiments. **$P = 0.0011$ by unpaired two-tailed Student’s $t$ test. Right panel, $n = 5$ patients. Data are pooled from three independent experiments. *$P = 0.0165$ by unpaired two-tailed Student’s $t$ test.

d, Data are pooled from three independent experiments each with a different patient.

e, Data are representative of two independent experiments. Right panel, $n = 3$ patients. **$P = 0.0072$ by unpaired two-tailed Student’s $t$ test.

f, Data are pooled from three independent experiments each with 1–2 different patients, and shown as mean of duplicate wells, except the single well for CTSH in Pt. 8.
Extended Data Figure 1
a, Data are representative of two independent experiments.
b, Data show mean of $n = 2$ technical replicates per sample group, except for both groups (WT CD4 and Eomes-null CD4) at the E:T ratio of 5:1 ($n = 1$). Data are representative of two independent experiments.
c, Data show mean of $n = 2$ technical replicates per sample group, except for the WT CD4 group at the E:T ratio of 50:1 ($n = 3$). Data are representative of two independent experiments.

Extended Data Figure 2
a, b, Numbers ($n$) of biologically independent animals are presented beside the respective group names. Data are pooled from two independent experiments.
c, Data show mean of $n = 2$ technical replicates per sample group. Representative data from one of two independent experiments using two different LMP1$^+$ lymphoma lines are shown.

Extended Data Figure 3
a, $n = 2$ (Day 0) or 4 (Days 7 and 28-32) biologically independent animals. Data are pooled from two (Day 28-32) or three (Day 7) independent experiments. The experiment for Day 0 was performed once. **$P = 0.0079$ by unpaired two-tailed Student’s $t$ test.
b, Data show mean of $n = 2$ biologically independent animals per group. Data are representative of two independent experiments.
c, Data are representative of three independent experiments, with $n = 4$ biologically independent animals per group.

Extended Data Figure 4
a, $n = 2$ biologically independent samples per group. This experiment was performed once.
b, $n = 2$ technical replicates per sample group. Data are representative of two independent experiments.
c, Data are representative of two independent experiments.

Extended Data Figure 5
a, Data are representative of two independent experiments, with $n = 4$ biologically independent animals per group.
b, This experiment was performed once, with $n = 2$ biologically independent animals per group.
c, Data are representative of two independent experiments, with $n = 4$ biologically independent animals per group, except for the EphA2-Tetrm in the ERT2-C group ($n = 3$).
d, Upper right panel, $n = 4$ (ERT2-C) or 5 (ERT2-CL) biologically independent animals. Data are pooled from three independent experiments. **$P = 0.0012$ by unpaired two-tailed Student’s $t$ test. Lower right panel, $n = 3$ biologically independent animals per group. Data are pooled from two independent experiments. ****$P < 0.0001$ by unpaired two-tailed Student’s $t$ test.

Extended Data Figure 7
Representative data from one of $n = 3$ biologically independent CL mice are shown along with a control mouse (C). This experiment was performed once.

Extended Data Figure 9
a, Data show mean of $n = 2$ technical replicates per sample group. Data are representative of two independent experiments.

b, Data are representative of two independent experiments, with $n = 4$ biologically independent animals per group, except for the Th0 CD4 control group where $n = 3$ from one experiment.

Extended Data Figure 10

a, Data are pooled from two independent experiments, each with a different patient.

b, Representative data from one of $n = 2$ patients are shown. This experiment was performed once.

c, d, Data are shown as mean of duplicate wells, except the single well in ‘PMA + Ionomycin’ group in Pt. 9. The experiments were performed once (c) or twice (each with a different patient) (d).

e, Data are pooled from three independent experiments each with 1–2 different patients.
Supplementary Fig. 1  |  Source images for western blot data.
Supplementary Fig. 2 | Representative gating strategies for cell analysis by flow cytometry. 

a, Gating strategy to determine active Caspase-3 in CellTrace-labeled target cells (Fig. 1a, 1c, 1d, 2j, 3d, 3f, 4d and Extended Data Fig. 1b, 1c, 2c, 9a). b, In vitro cultured cells were gated on the live fraction to analyse for levels of the indicated molecules in Fig. 1b, 2g, 3a, 3e, 4a, 4b. c, Gating strategy to analyse CellTrace dilution in CD4^+ T cells (Fig. 1e, 2f). d, Gating strategy to identify tetramer^CD8^+ T cell populations in CD8^+ enriched splenic cells (Fig. 2c, 2d and Extended Data Fig. 5). “Dump channel” includes antibodies specific for mouse CD4, CD11b, CD19 and CD49b. e, CD4^+ T cells co-cultured with B cell targets were gated for downstream analyses (Fig. 2h, 2i, 3c, 4e and Extended Data Fig. 1a, 4c, 10a, 10b). f, Gating strategy for analyses of B cells from spleens or mesenteric lymph nodes of mice (Fig. 2e, lower and Extended Data Fig. 3). g, Gating strategy for analyses of CD4^+ (excluding Foxp3^+ Tregs) or CD8^+ T cells from the spleen or bone marrow of mice (Extended Data Fig. 2a, 2b). h, Gating strategy for analyses of intra-tumoural CD45.1^+ adoptive CD4^+ T cells (excluding Foxp3^+ Tregs) (Fig. 3j, 3k and Extended Data Fig. 9b).
Supplementary Fig. 3 | Representative gating strategies for cell sorting. a, Gating strategy to sort mouse naive CD4 cells from splenocytes enriched by negative selection with an antibody cocktail (anti-CD11b, -CD19, -B220, -Gr-1 and -Ter119). “Dump channel” includes antibodies specific for mouse CD8 and CD25. b, c, Gating strategies to isolate human CD14+ monocytes and CD4+CD25^{low/-} T cells (b) or effector/memory CD4+ T cell subsets (excluding CD45RA+CCR7+ naive subset) (c) from peripheral blood mononuclear cells of chronic lymphocytic leukaemia patients after CD19 negative selection. “Dump channel” includes antibodies specific for human CD19, CD56 and TCRγδ.