Signaling by the Epstein–Barr virus LMP1 protein induces potent cytotoxic CD4+ and CD8+ T cell responses

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The B-lymphotropic Epstein–Barr virus (EBV), pandemic in humans, is rapidly controlled on initial infection by T cell surveillance; thereafter, the virus establishes a lifelong latent infection in the host. If surveillance fails, fatal lymphoproliferation and lymphomagenesis ensue. The initial T cell response consists of predominantly CD8+ cytotoxic T cells and a smaller expansion of CD4+ cells. A major approach to treating EBV-associated lymphomas is adoptive transfer of autologous or allogeneic T cells that are stimulated/expanded on EBV-transformed B cells. Strikingly, the clinical response correlates with the frequency of CD4 cells in the infused T cells. Although in vitro studies suggested that EBV-specific CD4 cells develop cytotoxicity, they have not been comprehensively characterized and the molecular mechanism underlying their formation remains unknown. Our recent work, using a transgenic approach in mice, has revealed a central role for the EBV signaling molecule LMP1 in immune surveillance and transformation of EBV-infected B cells. The mouse model offers a unique tool for uncovering basic features of EBV immunity. Here, we show that LMP1 expression in B cells induces potent cytotoxic CD4 and CD8 T cell responses, by enhancing antigen presentation and costimulation by CD70, OX40 ligand, and 4-1BB ligand. Our data further suggest that cytotoxic CD4 cells hold superior therapeutic value for LMP1 (EBV)-driven lymphomas. These findings provide insights into EBV immunity, demonstrating that LMP1 signaling alone is sufficient to induce a prominent cytotoxic CD4 response, and suggest strategies for immunotherapy in EBV-related and other cancers.

Epstein–Barr virus | LMP1 | CD4+ cytotoxic T cells | costimulatory ligand

Epstein–Barr virus (EBV) is a potent tumorigenic virus with a narrow tropism primarily for B cells but also epithelial cells of primate origin. The virus infects and persists in >90% of humans worldwide (1). Primary EBV infection usually occurs during early childhood (beginning at ~6 mo after birth) and is asymptomatic (2–4); however, if the infection is delayed until adolescence, it may manifest as a self-limiting lymphoproliferative disorder known as infectious mononucleosis (5). EBV-infected B cells are rapidly eliminated by the host immune system, in which T cells play a major role (5). However, in a minute fraction of B cells, the virus acquires a latent state and persists for life (6). Under conditions of immunosuppression or immune deficiency, latent EBV can reactivate and spread, resulting in the rapid expansion of infected B cells and their malignant transformation, as seen in pathologies such as posttransplant lymphoproliferative disorder (PTLD) and AIDS-associated B cell lymphoma (5). EBV is also associated with other B cell malignancies that appear to have evaded immune surveillance via different strategies: Burkitt lymphoma cells express a severely limited number of EBV latent genes (8), while Hodgkin lymphoma cells produce immunosuppressive molecules that foster local immune privilege (9, 10). Apparently, host immune cells, particularly T cells, keep EBV-infected cells under constant surveillance, and EBV-driven malignancies only arise when surveillance fails.

T cell responses against EBV-infected or transformed B cells are believed to be directed at various latent gene products, and therefore a major approach for treating EBV-associated malignancies has been adoptive transfer of EBV-specific T cells, which are generated by stimulating/expanding autologous or allogeneic T cells on EBV-transformed B-lymphoblastoid cell lines (LCLs) (11). The therapeutic T cells mostly comprise predominantly CD8+ cytotoxic T cells (CTLs), with variable frequencies of CD4+ T cells. Strikingly, however, the results from a PTLD trial showed that clinical responses significantly correlated with the frequency of CD4 cells in the infused T cells (12). Although the precise function of the adoptive CD4 cells in these PTLD

Significance

Epstein–Barr virus (EBV) drives human B cell proliferation and transformation, but also potent T cell surveillance. When surveillance fails, EBV-driven malignancies arise. T cells can be stimulated/expanded on EBV-transformed B cells for adoptive therapy. Clinical data point to the therapeutic importance of CD4 T cells, perhaps through direct cytotoxicity; the mechanism underlying such an activity remains unknown. Previous studies show that signaling by the EBV oncoprotein LMP1 enhances antigen presentation. Here, we show that LMP1+ B cells provide costimulation through CD70 and OX40L to drive cytotoxic CD4 (and CD8) differentiation. In a mouse model of LMP1 (EBV)-driven lymphoma, cytotoxic CD4 cells have superior antitumor activity. These findings provide a mechanism for the EBV-mediated cytotoxic CD4 response and suggest strategies for immunotherapy in EBV-related and other cancers.

Author contributions: I.-K.C., Z.W., H.-J.K., K.R., and B.Z. designed research; I.-K.C., Z.W., O.K., M.H., Y.Q., and X.Z. performed research; I.-K.C., Y.L., K.R., and B.Z. analyzed data; I.-K.C., J.R., H.C., K.R., K.W.W., and B.Z. wrote the paper; and B.Z. supervised research.

Reviewers: A.B.R., University of Birmingham; and S.L.S., University of Massachusetts Medical School.

Conflict of interest statement: I.-K.C., Z.W., H.-J.K., K.R., and B.Z. have filed patent applications on aspects of this research.

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Data deposition: The microarray data have been deposited in the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) database, https://www.ncbi.nlm.nih.gov/geo (accession no. GSE108479).

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1713607115/-/DCSupplemental.
patients was unclear, several in vitro studies reported that CD4 cells generated on LCLs exhibited direct cytotoxicity (13–16). The molecular mechanism underlying these “cytotoxic” CD4 responses remains a mystery.

The understanding of the basic immune responses to EBV had been limited by the paucity of small animal models, as the virus cannot infect nonprimate organisms. Our previous work, initially aimed at studying the EBV oncoprotein latent membrane protein 1 (LMP1) in B cell transformation in a transgenic mouse model, unexpectedly led to the discovery of a novel role of LMP1 in inducing immune surveillance relevant to EBV infection (17). Specifically, we coupled a conditional LMP1flSTOP allele with a CD19-cre allele to target the expression of LMP1 to the B lineage, starting at the pro-B stage; analysis of CD19-cre:LMP1flSTOP (CL) mice revealed that LMP1+ B cells were eliminated by T cells, as are EBV-infected B cells in humans. In the mice, T cell depletion resulted in rapid, fatal B cell proliferation and lymphomagenesis, resembling the EBV-driven malignancies seen in immunosuppressed patients. These findings revealed a central role for LMP1 in both the surveillance and transformation of EBV-infected B cells in vivo (17). Additional studies, from us and others, in which LMP1 was turned on initially in small populations of mature B cells in adult mice, corroborated the key role of LMP1 in inducing immune surveillance (18–20).

The LMP1-based mouse models offer a unique tool for uncovering basic features of EBV immunity. Previous studies have shown that LMP1 signaling-mediated B cell immunogenicity is a key to immune surveillance (17–20), and that the responding CD8 cells develop a typical antigen-specific cytotoxic response (17, 19). However, the identity of the T cell antigens in the mouse model remain elusive and may include a wide range of tumor-associated and self-antigens (17, 18, 21) (Discussion); in humans, the T cell antigens would include peptides derived from a variety of EBV proteins (6, 22). Other questions include the function/phenotype and formation of the antigen-specific CD4 cells, which are known to be capable of exerting potent antitumor activity in an IFN-γ– and TNF-α-independent manner, upon cotransfer with LMP1+ lymphomas into immunodeficient hosts (17). In this work, we focus on addressing these questions.

Results
Both CD4 and CD8 T Cells Develop Cytotoxic Responses to LMP1+ B Cells. We investigated the detailed time course and nature of immune surveillance in CL mice. Analysis of the dynamics of LMP1+ B cell and T cell responses revealed a peak T cell response against LMP1+ B cells on days 6–8 after birth, followed by rapid elimination of LMP1+ B cells (Fig. L4); T cell contracted thereafter, but some CD4 and CD8 effectors (CD69+ and CD44+CD62L−) persisted in the bone marrow (BM) (Fig. L4) and continued to eliminate newly arising LMP1+ B cells in the BM (the primary organ for B cell development). Accordingly, a small population of LMP1+ B cells was detected in the BM, but not in the spleen, of adult mice (Fig. L4).

Particularly striking was the high level of cytotoxic activity by CD4 cells, which had similar cytotoxic function as CD8 cells. CD4 and CD8 cells from the BM and spleen of day 6–8 CL mice displayed potent killing activity against LMP1+ lymphoma cells [derived from T cell-deficient CL mice (17)] ex vivo, but not against naive wild-type (WT) B cells (Fig. 1B). Remarkably, CD4 cells isolated from day 6–8 CL mice expressed perforin, granzyme B (GzmB), and CD107a, at levels similar to those of the CD8 cells (Fig. 1 C and D). In addition, these cells expressed Fas ligand (FasL) (Fig. 1C and D). The CD4 T cell killing of LMP1+ lymphoma targets could be slightly reduced by blocking the FasL–Fas apoptotic pathway, and more markedly suppressed by blocking MHC-II recognition; blocking both FasL and MHC-II resulted in an additive effect (Fig. 1E). These data suggest that CD4 cells kill LMP1+ B cells predominantly through the perforin–granzyme pathway, the main killing mechanism typically utilized by CD8 CTLs (23, 24), corroborating their similar cytotoxic function. This notion is in agreement with our previous findings that LMP1+ B cells remain well controlled in mice deficient for Fas (17) but not in mice deficient for perforin (19). Overall, our data demonstrate that LMP1 expression by B cells induces potent cytotoxic CD4 and CD8 T cell-mediated immunity.

Cytotoxic CD4 Cells Confer Potent Antitumor Immunity Against LMP1-Driven Lymphomas. Our previous work showed that, although ~50% of CD4 and CD8 cells in the BM of adult CL mice (referred to as “chronic stage” in this model system) maintain an activated phenotype (CD69+), the CD4 cells exhibited little cytotoxicity in an in vitro killing assay, in contrast to CD8 cells from the same mice (17) (Fig. 2A). Nevertheless, when the CD4 cells were cotransferred with LMP1+ lymphoma cells into immunodeficient hosts, they exhibited superior antitumor activity relative to that of the CD8 cells; their antitumor activity remained intact in the presence of antibodies blocking IFN-γ and TNF-α (17). We therefore tested the possibility that the CD4 cells controlled the tumors by regaining cytotoxicity in vivo. Indeed, CD4 cells that were recovered from the adoptive hosts at a point when actively engaging in tumor control (Materials and Methods) displayed potent killing activity ex vivo (Fig. 2A), associated with up-regulation of cytotoxic molecules perforin, GzmB, CD107a, and FasL, in contrast to the donor cells before transfer (Fig. 2B). These data indicate that CD4 cells control LMP1+ lymphomas through direct cytotoxicity.

The finding that, upon cotransfer with LMP1+ lymphoma cells, chronic-stage CD4 cells regain cytotoxicity and mediate superior antitumor activity relative to that of their CD8 counterparts, prompted us to test and compare these CD4 and CD8 cells for their therapeutic efficacy in a mouse model of PTLD, namely TCRβ−δ− CL mice bearing aggressive LMP1-driven primary lymphomas (17). Considering that the heavy tumor burden in these mice may establish an immunosuppressive environment and thereby impede the expansion and function of adoptive T cells, we pretreated the mice with radiation therapy (RT) to reduce the tumor burden and create a lymphopenic condition favorable for adoptive T cell expansion and function (25, 26), followed by transfer of a single dose (1 × 106 per recipient) of CD4 or CD8 cells. We found that RT alone moderately improved survival of tumor-bearing mice. The combination with adoptive CD8 cells further prolonged mice survival, and CD4 cells displayed even stronger antitumor activity than the CD8 cells (Fig. 2C). Thus, similar to the “T cell and LMP1+ lymphoma cotransfer system,” adoptive CD4 cells confer better tumor protection than CD8 cells in this primary LMP1+ lymphoma model, presumably also through direct cytotoxicity.
constructed an LMP1 mutant in which amino acids FWLY (38–41)
of transmembrane domain 1 (TM1) were changed to AALA (referred
to as LMP1TM1m): this abolishes LMP1 clustering and signaling (30).
We found that, after 7 d of coculture with LMP1+ B cells in vitro
(without addition of any exogenous cytokine), a sizable fraction
of naive CD4 cells was activated/expanded; this effect depended
on LMP1 signaling in B cells, as CD4 cells failed to expand on
LMP1TM1m-expressing B cells (Fig. 3B). Of note, the CD4 cells
expanded on LMP1+ B cells developed cytotoxicity toward their
specific targets—LMP1+ B cells, but not lipopolysaccharide (LPS)-
activated B cells (Fig. 3C). Thus, LMP1+ B cells function as APCs
to directly prime a cytotoxic CD4 response.

**LMP1+ B Cells Express Costimulatory Ligands Associated with the
Cytotoxic Differentiation of T Cells.** We next sought to uncover
the molecular mechanisms via which LMP1 signaling induces a
potent cytotoxic CD4 response. We focused on identifying cos-
stimulatory molecules that were expressed on LMP1+ B cells and
able to induce the cytotoxic differentiation of CD4 cells. Re-
cently, several studies described granzyme/perforin-featured cy-
totoxic CD4 T cells, whose differentiation is fully dependent on
the T-box transcription factor Eomesodermin (Eomes), but not
on the Th1 polarizing T-bet (29, 32–34). Furthermore, systemic
activation of 4-1BB and/or OX40 costimulatory pathways (by
agonist antibodies) induces high levels of Eomes in antigen-
primed CD4 cells, which then drives their cytotoxic differentia-
tion (32, 33, 35). Systemic CD27 activation also induces Eomes
expression in CD4 cells (33). Our data show that LMP1+ B cells
express greatly enhanced levels of 4-1BB ligand (4-1BBL),
OX40 ligand (OX40L), and CD70 (CD27 ligand), compared
with control B cells (Fig. 4A and B). Proinflammatory cytokines,
including IL27 and IL15, may also play a supportive role in cy-
totoxic CD4 cell generation (33). However, with the exception of

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**Fig. 1.** LMP1-expressing B cells trigger cytotoxic CD4 and CD8 T cell responses in vivo. (A) Dynamics of LMP1-expressing B cells (CD19-Fas+; Fas is induced by
LMP1 signaling and is thus used as a reporter for LMP1 expression in B cells) and CD4+ and CD8+ T cells (Upper), and activation status (CD69+) of CD4
and CD8 cells (Lower), analyzed by FACS in the spleen (Spl) and BM of CL mice, compared with those in CD19-cre/+ littermate control (“C”) mice. The respective
mean values of at least three mice of each genotype are plotted at each time point. CD4+, TCRβ+CD1dTetramer-CD4+; CD8+, TCRβ+CD8+. (B) Cytotoxicity of
splenic CD4 and CD8 T cells from day 6–8 CL mice was assessed on LMP1+ lymphoma cells and naive wild-type (WT) control B cells. E:T ratio, effector-to-target
cell ratio. Data for LMP1+ lymphoma targets are representative of five independent experiments; for naive B cell controls are representative of two in-
dependent experiments. (C and D) FACS analysis of the indicated effector molecules in splenic total CD4 cells (C) and total CD8 cells (D) from day 6–8 CL mice,
compared with their counterparts from littermate control mice. For the CD4 analysis, Foxp3+ Tregs are excluded. Representative FACS plots are shown in the
Upper, and median fluorescence intensities (MFIs) in the Lower. Each symbol represents an individual mouse; bars show the respective mean values. (E) In
vitro killing assay was performed with CD4 T cells from day 6–8 CL mice on LMP1+ lymphoma cells, in the presence of MHC-II blocking antibody and/or Fas-Fc
(to block Fasl), or isotype control antibodies. Data are representative of two independent experiments using two different LMP1+ lymphoma cell lines. All
mice used in A–E are on a C57BL/6 × BALB/cF1 (CB6F1) background; the lymphoma cells are on a C57BL/6 × BALB/c mixed background; naive control B cells are
from WT CB6F1 mice.
B cells (– and A) are pooled from two independent experiments, in one B cells per recipient), or left
B cells through the CTCR "loses its ability
B Cells Drive Eomes-Programmed Cytotoxic CD4 and CD8
mice (Fig. 4 mice, compared with naive
mice (Fig. 4 mice). LMP1 signaling makes B cells suffice to induce cytotoxic CD4 response
B cells express ligands
mice (1 mice BM (chronic stage) and C purified CD4 T cells in triplicate wells of 12-well plates.
Cytotoxic CD4 cells confer potent antitumor immunity against LMP1-
and
Published online January 8, 2018
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mice revealed three populations of effector cells—Eomes T-bet, Eomes T-bet, and Eomes T-bet—in sharp
contrast to CD4 cells from control littermate mice (Fig. 4F). Furthermore, CD4 cells from CL mice expressed GzmB and/or
IFN-γ, in contrast to those from the control mice (Fig. 4F). GzmB expression depends on Eomes (32, 33), while IFN-γ is
mainly driven by T-bet (29); thus, our FACS analyses revealed three subtypes of effector CD4 cells in CL mice: (i) Eomes/GzmB-featured cytotoxic cells (similar to those described in ref. 33); (ii) T-bet/IFN-γ-expressing Th1 cells (29); and (iii) a population that displayed features of both the cells described in (i) and (ii) (these cells were similar to the "cytotoxic CD4 Th1 cells"; described in ref. 32). CD4 cells from CL mice exhibited no expression of GATA3 or RORγt (Fig. S2), indicating no commitment toward the Th2 or Th17 subsets (29). The costimulation pathways may similarly affect CD8 cells (32, 33), but in contrast to their CD4 counterparts, the CD8 cells in day 6–8 CL mice developed into a single, nearly uniform population, that was Eomes T-bet GzmB IFN-γ (Fig. 4G).
Overall, these findings indicate that the CD4 (and CD8) T cells skew toward an Eomes-programmed cytotoxic phenotype, perhaps due to costimulation by LMP1 B cells through the 4-1BB, OX40, and/or CD27 pathways.

LMP1 B Cells Drive Eomes-Programmed Cytotoxic CD4 and CD8 Responses via Costimulation by CD70, OX40L, and 4-1BBL. To address the roles of 4-1BL, OX40L, and CD70 in the LMP1 B cell-driven cytotoxic CD4 (and CD8) T cell generation, we employed a similar in vitro system as described in Fig. 3B, namely the coculture of T cells and LMP1 B cells. Our data show that, similar to ex vivo LMP1 B cells from CL mice (Fig. 4B),

the gene for the IL27 subunit β, the other cytokine genes were only marginally, if at all, induced in LMP1 B cells (Fig. S1).
We examined the levels of the corresponding costimulatory receptors on T cells as a sign of their functional interaction with the respective ligands on LMP1 B cells. In accord with their known patterns of expression following activation by their respective ligands, 4-1BB and OX40 were up-regulated, while CD27 was down-regulated on CD4 and CD8 effectors in day 6–8 CL mice, compared with naive CD4 and CD8 cells in littermate control mice (Fig. 4 C and D) (36).
Consistent with the plausible roles of 4-1BB and OX40 (and CD27) pathways in inducing the Eomes–Granzyme program in T cells, high levels of Eomes and GzmB were expressed in a major population of CD4 cells in day 6–8 CL mice (Fig. 4E). Systemic 4-1BB activation is known to result in selective expression of Eomes, without T-bet expression (33), while simultaneous activation of 4-1BB and OX40 induces both Eomes and T-bet in CD4 cells (32). Because LMP1 B cells express ligands for both pathways, we also examined T-bet expression in the CD4 cells: analysis of Eomes and T-bet expression by CD4 cells from CL mice revealed three populations of effector cells—Eomes T-bet, Eomes T-bet, and Eomes T-bet—in sharp contrast to CD4 cells from control littermate mice (Fig. 4F).
LMP1\(^+\) B cells generated in vitro (by retroviral transduction of mice and littermate controls. The frequencies (mean mice and littermate controls. Data in and MFI in the cells were sorted for array analysis. LMP1 CD4 cells (Fig. 5 CD4 cells from D and (CL B cells express high levels of costimulatory ligands associated with the cytotoxic differentiation of T cells. (CD8 cells (Fig. 5 CD4 cells (Fig. 5 SEM) of indicated populations are shown within the gates. (CL B cells from day 6 B cells drive the differentiation E of indicated populations on day 7 (Fig. S4 mice spleens, compared with splenic B cells from littermate control Tregs are excluded. (CL B cells compared with control B cells. Splenic B cells from YFP control B cells; 2 d posttreatment, reporter (YFP) cells and YFP control B cells; 2 d posttreatment, reporter (YFP) cells were sorted for array analysis.

Discussion

Previous studies in genetic mouse models have indicated a key role for LMP1 in inducing immune surveillance of EBV-infected...
B cells, by showing that LMP1 expression makes B cells highly immunogenic, leading to their efficient elimination by T cells (17–20), similar to EBV-infected B cells in humans. LMP1 expression in mouse and human B cells is known to up-regulate MHC class I and class II molecules on the cell surface and enhance antigen presentation (17, 31, 37). In the present work, we show that LMP1 signaling in B cells up-regulates several costimulatory ligands—CD70, OX40L, and 4-1BBL—that drive Eomes-programmed cytotoxic CD4 and CD8 T cell differentiation (see schematic in Fig. 6); and that cytotoxic CD4 cells can confer potent antitumor immunity against LMP1-driven lymphomas (Fig. 2) (17). Understanding the key role of LMP1 in these processes could provide a powerful tool for developing cancer immunotherapies.

Fig. 5. LMP1+ B cells drive CD4 and CD8 CTLs through CD70, OX40 ligand, and 4-1BBL ligand. (A) FACS analysis of the indicated costimulatory ligands in the various B cells. Unstained vector-transduced B cells served as negative control. Representative FACS plots and MFI of three replicate wells from one of two independent experiments are shown. Bars show the respective mean values. (B) FACS analysis of Eomes expression in CD4 cells (excluding Foxp3+ Tregs), either freshly isolated from naive B6 mice (ex vivo), or after coculturing for 7 d with LMP1-B cells, in the presence of the indicated blocking antibodies, or corresponding isotype controls. Representative data from one of triplicate wells are shown, with the frequency of Eomes+ cells in the gate. Note that αOX40L and αCD70 share the same isotype control (Isotype2); for OX40L and CD70 double blocking, the “isotypes” means isotype2 used at a 2× concentration. (C) Numbers (mean ± SEM) of Eomes+ CD4 cells (excluding Foxp3+ Tregs) recovered from culture wells treated with the indicated blocking antibodies, relative to those recovered from corresponding isotype control-treated wells. (D) FACS analysis of Eomes expression in CD8 cells either freshly isolated from naive B6 mice, or after coculturing for 3 d with LMP1-B cells in the presence of the indicated blocking antibodies or corresponding isotype controls. Representative data from one of triplicate wells are shown, with the frequency of Eomes+ cells in the gate. For 4-1BBL, OX40L, and CD70 triple blocking (3 Abs), the isotypes means isotype1 used at a 1× concentration plus Isotype2 used at a 2× concentration. (E) Numbers (mean ± SEM) of Eomes+ CD8 cells recovered from culture wells treated with the indicated blocking antibodies, relative to those recovered from corresponding isotype control-treated wells. Data in A–E are representative of two independent experiments, using splenic B and T cells from 2- to 3-mo-old naive B6 mice.
LMP1 expression appears critical for the antigen-presenting function of EBV-transformed B cells. Ectopic expression of LMP1 in mouse and human B cells leads to highly up-regulated MHC class I and class II molecules on the cell surface and enhanced presentation of endogenously expressed antigens (17, 31, 37). LMP1 expression displays a cyclic pattern and varies over 100-fold in individual cells of a LCL culture (38, 39), and its expression level correlates with HLA-I expression and antigen-presenting function (38). Such variation is due to transcription regulation through as-yet-unknown mechanisms (39). In our mouse model and the retrovirus-mediated expression system, LMP1 is driven by constitutive promoters; although its expression leads to marked up-regulation of MHC-I (17), there appears no obvious correlation between the levels of LMP1 and MHC-I, in contrast to the clear correlation observed in LCLs (Fig. S5). Of note, the transgenic B cells (LMP1\textsuperscript{+} lymphoma cells) appear to express comparable levels of LMP1 as the HLA-I\textsuperscript{low} LCL cells, yet they are highly immunogenic (17), while the latter are presumably of low immunogenicity (38). Perhaps other EBV gene products interfere with antigen processing/presentation in LCLs (40), thereby dampening the potency of LMP1 (only LMP1\textsuperscript{h} LCL cells are highly immunogenic).

Whereas in humans the T cell antigens are known to comprise peptides derived from various EBV proteins, their identity remains elusive in the mouse model. Our previous work failed to reveal LMP1-derived T cell epitopes, but that does not exclude their existence (17). However, considering that (i) LMP1 expression up-regulates MHC class I and class II and enhances presentation of endogenously expressed antigens (17, 31, 37, 38); and (ii) CD4 and CD8 CTLs from CL mice lyse LMP1\textsuperscript{+} B cells, but not resting B cells (Fig. 1B) or WT B cells activated by LPS (through a pathway irrelevant to LMP1 signaling; Fig. 3C), we speculate that the T cell targets might include LMP1-induced cellular antigens. Because LMP1 is the key oncoprotein for EBV-driven tumorigenesis (41), the cellular antigens that are induced by LMP1 and recognized by T cells would be tumor-associated antigens (TAAs) belonging to the subgroup of “overexpression antigens” (21). In agreement with this notion, several recent studies show that stimulation by autologous EBV-LCLs can induce human cytotoxic CD4 cells that display HLA-II–restricted recognition of a broad range of EBV-negative B and T cell lymphomas (these clones do not recognize any known EBV latent antigens) (42–44), implying recognition of certain TAAs. An involvement of multiple LMP1-induced cellular antigens (TAAs) could perhaps explain the unusually rapid and potent T cell responses in the mouse model. The identification of such TAAs warrants further investigation.

With regard to T cell costimulation, in agreement with our findings, Izawa et al. (45) recently reported that expression of CD70 is highly up-regulated in EBV-LCLs derived from healthy donors. Here, we have used a unique in vitro culture system, coupled with antibody-mediated blockade, to unravel the roles of the three costimulatory ligands, CD70, OX40L, and 4-1BBL, in the differentiation and expansion of LMP1\textsuperscript{+} B cell-driven cytotoxic T cells. In this effort, we monitored the Eomes–featured cytotoxic CD4 and CD8 responses; during the preparation of this manuscript, a new, Eomes-low/negative, subset of cytotoxic CD4 cells was described, which can be generated during certain virus infections in mice and phenotypically marked by expression of NKG2C/E (46); however, such NKG2C/E\textsuperscript{+} cells were barely seen in our in vitro system (Fig. S6). Our data document that CD70 and OX40L contribute to the generation of Eomes-programmed CD4 and CD8 CTLs, and that 4-1BBL contributes to the generation of CD8, but not CD4, CTLs. Among the three costimulatory ligands, CD70 apparently plays the dominant role in both CD4 and CD8 CTL generation. This is in line with the recent discovery of a critical role of the CD70–CD27 costimulatory pathway in EBV immunity: immune control of EBV infection is compromised in patients with defective CD70 or CD27, and culminates in EBV-associated lymphoproliferation and lymphomagenesis (45, 47–50). To our knowledge, there are no reports in the literature of OX40 or 4-1BB signaling deficiencies associated with EBV-related diseases. The human study highlights the key role of CD70 (on EBV-B cells) in the expansion of CD8 CTLs (45), while our data indicate that CD70 is also critical for priming CD8 CTLs and for generating CD4 CTLs. Overall, our findings explain why the CD4 T cells that are stimulated/expanded on autologous LCLs often skew toward a cytotoxic phenotype (13–16).

The observation that 4-1BBL is not involved in the generation of CD4 CTLs appears to contradict a report by Curran et al. (33), which showed that administration of 4-1BB agonist antibody in mice induces a CD4 CTL response. However, their findings were based on systemic activation of the 4-1BB pathway, raising the possibility that the observed cytotoxic CD4 differentiation was the result of an indirect effect of 4-1BB activation in myeloid APCs. Another factor that may explain the discrepancy between their results and ours is a different level of 4-1BB activation in the two systems.

EBV-LCLs have been widely used as APCs to generate EBV-specific T cells for adoptive therapy in EBV-associated lymphomas (11). Apparently, LMP1 signaling underlies the APC function of LCLs, by enhancing antigen presentation (17, 31, 37, 38) and costimulation for cytotoxic CD4 and CD8 responses (the present work). The T cells expanded on LCLs usually consist of predominantly CD8 CTLs, with variable frequencies of CD4 cells (presumably mostly CD4 CTLs), yet Haque et al. (12) reported that, in a PTLD trial, clinical responses strongly correlated with the frequency of CD4 cells in the infused T cells. Our results in T cell and LMP1\textsuperscript{+} lymphoma cotransfer experiments and in the mouse model of PTLD (Fig. 2) (17) further underscore the potency of cytotoxic CD4 cells. Whether other properties of CD4 cells, beyond the cytotoxic activity per se, make them superior to CD8 cells in tumor protection remains unclear. Nevertheless, our results suggest that increasing the abundance of CD4 CTLs in therapeutic T cells, or using solely CD4 CTLs, may lead to a better therapeutic efficacy in future trials. Toward this aim, CD4 and CD8 CTLs could be generated separately on LCLs and then mixed at a desired ratio for adoptive transfer.
Cytotoxic CD4 T cells also hold great potential for treating non–EBV-related cancers, as demonstrated in preclinical animal models (25, 26) and in patients (27, 28). However, these antigen-specific CD4 cells were either produced by a TCR transgene approach or spontaneously arose in cancer patients, and a general approach for rapid generation of tumor antigen-specific CD4 CTLs is so far unavailable. Although such CD4 CTLs may be generated using LCLs as an APC system (the desired antigens may be supplied through ectopic expression or peptide loading), their frequencies would be limited by competition from EBV-specific CD4 cells (6, 22). Perhaps for this reason, Hunder et al. (51) used LCLs only to stimulate/expand preselected tumor antigen-specific CD4 cells for melanoma therapy. In this context, LMP1-B cells may provide an urgently needed advance as an APC for generating CD4 CTLs (along with CD8 CTLs) against desired antigens for immunotherapy in EBV-unrelated cancers. Here, the competition from LMP1-specific T cells would be minor, as LMP1 protein is rarely targeted by human T cells (22). Another advantage in using LMP1-B cells as APCs lies in the brevity of the T cell production protocol (totaling about 11 d for preparation of LMP1-B cells and subsequent generation of antigen-specific T cells), in sharp contrast to the lengthy (2–3 mo) LCL-based protocol (11). Furthermore, one may speculate that LMP1 can be ectopically expressed in malignant B cells and thereby make them present endogenous antigens (such as TAAs and neoantigens) and simultaneously provide costimulatory signals for eliciting CD4 and CD8 CTL responses. This strategy may hold promise for T cell-based therapy in EBV-unrelated B cell lymphomas/leukemia.

Taken together, our findings provide insight into EBV immuno- munity, suggesting that EBV polarizes CD4 T cells toward a cytotoxic response via LMP1-induced costimulatory ligands, CD70 and OD40L. This suggests strategies for immunotherapy in EBV-related and other cancers.

Materials and Methods

Mice. C57BL/6J (B6), CD19-cre, TCRγ−/−, TCRβ−/−, and YFpSTOP (all on a B6 background) and BALB/c were obtained from The Jackson Laboratory. Rag2−/− common γ chain−/− (Rag2−/−γc−/−) mice were bred in our mouse colony or purchased from Taconic. The LMP1−/− allele on a BALB/c background has been described (17). Homozygous CD19−/− mice were crossed with LMP1−/− heterozygous mice on BALB/c background to generate CD19−/−;LMP1−/− and CD19−/− control mice on a C57BL/6 × BALB/cF1 background. All mice were bred and maintained in the animal facilities at the Dana–Farber Cancer Institute (DFCI), under specific pathogen-free conditions. All animal experiments were conducted per protocols approved by the DFCI Institutional Animal Care and Use Committee.

Generation of EBV-LCLs. LCLs were established by infection of human primary B cells with the EBV strain B95.8, as previously described (44). The cells were maintained in RPMI medium 1640 (Gibico) supplemented with 10% FBS (Sigma) and 2 mM glutamine (Gibco). The human blood samples from (health donors) were obtained per DFCI IRB-approved protocol from the Brigham and Women’s Hospital Specimen Bank. These deidentified specimens were considered discarded tissues that did not require informed consent.

Flow Cytometry. Lymphoid single-cell suspensions were stained with the following monoclonal antibodies specific for mouse CD4 (L3T4), CD8 (53-6-7), CD19 (103), CD27 (LG.3A10), CD43 (57), C69 (91.23F), CD70 (FR70), 4-1BB (17B7), 4-1BBL (TKS-1), OX40 (OX-86), CD40L (RM134L), Fas (J02), TCRγ (H57-597), IFNγ (XMG1.2), GzmB (NGZB), Perforin (eBioOMAK-D), CD107a (1D4B), CD11a (1D4B), FasL (MFL1), TRAIL (N2B23), Fopo3 (Fik-165), Eome (Damos11mg), Tbet (4B10), GATA-3 (TWA3), RORγt (Q31-378), NK2GA/CE (20ds), NK2GA (16a11), H2-Kb (AF6-88.5), human CD19 (H1819), and HLA-ABC (W6/32) from BD Biosciences, or biotinylated or IFNγ (Invitrogen) or eFlour 560 (eBioscience) staining was used to exclude dead cells. Intracellular staining for GzmB, perforin, Fopo3, Eomes, Tbet, GATA-3, and RORγt was done with the Fixation/Permeabilization buffer (eBioscience). Intracellular staining for TNFa and IFNγ was conducted using the Intracellular Fixation/Permeabilization Buffer (eBioscience). All samples were acquired on a FACSAria II (BD Biosciences) and analyzed by FlowJo software (Tree Star).

Sorting (FACS) was performed using a FACSAria II (BD Biosciences). In all T cell-sorting experiments, the CD1d tetramer (NIH Tetramer Facility) was employed to exclude natural killer T cells.

Retroviral Constructs and Transduction. LMP1 cDNA was cloned into the MSCV-IRE- GFP or MSCV-Puro retroviral vector to generate MSCV-LMP1-IREs-GFP or MSCV-LMP1-Puro. To generate a retrovirus expressing the signaling-defective LMP1 mutant LMP1TM, amino acids FWLY (38–41) of the transmembrane domain 1 (TM1) of LMP1 were altered to AALA by QuiChang site-directed mutagenesis (Stratagene), and the resultant mutant was cloned into MSCV-IRE-GFP or MSCV-Puro retroviral vector. CD43-depleted (by using anti-CD43 microbeads from Miltenyi Biotec) splenic B cells were activated in vitro by 20 μg/ml LPS (Sigma) for 24 h, infected with retroviruses, and continually cultured in the presence of LPS. For B cells transduced with GFP-carrying retroviruses, at 48 or 72 h postinfection the cells were extensively washed and then used in downstream experiments (GFP+ indicates successfully transduced cells). For B cells transduced with Puro-carrying retroviruses, at 24 h postinfection the cells were selected with puromycin (6 μg/ml; Sigma) for 18 h, followed by extensive wash and recovery in fresh medium for 1 d before using in downstream experiments.

LMP1 Localization Analysis. cDNA for LMP1 or LMP1TM was each subcloned into the pcGAG-GFP vector (@11150; Addgene) to obtain C-terminally GFP-tagged constructs. The resultant plasmids (pcGAG-LMP1-GFP, pcGAG-LMP1TM-GFP, or vector control pcGAG-GFP) were then incorporated into mouse lymphoma B cells (line 775) (52). Twenty-four hours after electroporation, the cells were counterstained with the DNA-specific fluorescent dye Hoechst 33342 (blue; Sigma) and imaged with fluorescence microscopy.

In Vitro Killing Assay. Various target cells were labeled with CellTrace Violet (Invitrogen) before use. CD4 and CD8 T cells were purified from the BM or spleen of mice by FACS. The T cells were then cocultured with 2 or 4 × 106 target cells at different effector/target ratios for 4 h in 96-well round-bottomed plates, followed by active Caspase-3 staining (BD Biosciences) (17, 53). For the blocking assay, target cells were preincubated with anti-HMC1 (IA/IE) blocking antibody (MS/114.15.2) or with isotype control rat lgG2b (both at 10 μg/ml; Biologend) for 20 min at 37 °C, whereas the CD4 T cells were preincubated with the Fas-ligand neutralizing fusion protein rmFas-Fc or with isotype control human IgG1 (both at 10 μg/ml; R&D Sys- tems) for 20 min at 37 °C. In all killing assays, effector/target mixtures in 96-well plates were spun down at 8 × g for 2 min before the incubation at 37 °C; cultures were stained for CD4 or CD8 (to exclude effector cells) and analyzed for active Caspase-3 levels in CellTrace-labeled target cells. Active Caspase-3−CellTrace− cells represent apoptotic target cells. Percentage (% specific killing = % apoptotic target cells in cultures with both effectors and targets − % apoptotic target cells in cultures with targets alone.

Adoptive Cell Transfer. In one set of experiments, Rag2−/−;γc−/− mice lacking B, T, and natural killer cells were transferred (i.v.) with 2 × 106 LMP1 lymphoma cells (line 966) (17), and 2 d later with 1 × 106 CD4 T cells sorted from the BM of adult (day 42–44) CL mice. Splenic cells were isolated from recipients 10 d after the tumor transfer (our preliminary histological observation suggested CD4 cells actively controlling LMP1 tumor cells at this point) and processed for CD4 cell sorting for in vitro killing assay and FACS analysis of cytotoxic molecules in the CD4 cells. In another set of experiments to test the therapeutic effect of adoptive T cells, CD4 or CD8 T cells were sorted from the BM of adult (day 42–44) CL mice, and 1 × 106 cells of each were transferred (i.v.) into TCRγ−/−;γc−/− CL mice at 8 wk of age [at the time point they usually have clonal LMP1+ lymphomas (17)]. One day before T cell transfer, the CD4 and CD8 T cell treatment groups and a control group received 500 rad of total body irradiation (RT).

Gene Expression Profiling. B cells were isolated from the spleens of YFP-positive and LMP1−/−YFP mice, by CD43 depletion using magnetic-activated cell sorting (Miltenyi Biotec); the cells were treated with TAT-Cre as previously described (54). Two days posttreatment, reporter (YFP)+ cells were sorted, and total RNA was extracted with TRIzol reagent (Invitrogen) according to manufacturer’s specifications and analyzed on GeneChip Mouse Gene 2.0 ST arrays (Affymetrix) at the Molecular Biology Core Facility at DFCI.

Generation of Cytotoxic CD4 T Cells on LMP1-Expressing B Cells in Vitro. Sorted CD4 T cells from the spleens of naive B6 mice were plated in 12-well plates at 1.5 × 105 per well with irradiated (500 rad) LMP1+ or LMP1TM+B cells at a
Blockade of Costimulatory Ligands During LMP1+ B Cell-Driven Cytotoxic T Cell Production. Irradiated LMP1-expressing B cells were preincubated with blocking antibodies against CD70 (FR70, rat IgG2b), OK40L (RM134L, rat IgG2b), and/or 4-1BBL (T-1, rat IgG2a), or the corresponding isotype controls (all at 10 μg/mL, Biologend), for 50 min at 37°C. Splenic CD4 (1 × 10⁶) or CD8 cells (0.5 × 10⁶) sorted from naive B6 mice were subsequently cocultured with the pretreated LMP1+ B cells at a 1:1 ratio in 24-well plates. The CD4 T cells were harvested for FACS analysis after 3 d of coculture, whereas the CD8 T cells were restimulated at day 5 with 0.5 × 10⁶ of the respective B cells for an additional 2 d, followed by FACS analysis.

Immunoblot Analysis. Protein extraction and immunoblotting were performed as previously described (17), with the following primary antibodies: anti-LMP1 (clone 512; gift from Dr. Fred Wang, Brigham and Women’s Hospital, Boston) and anti–β-Actin (clone 4C4; Invitrogen Biosystems).

Statistical Analysis. Statistical significance was determined by unpaired two-tailed Student’s t test, except where indicated; a value of P < 0.05 was considered significant (ns, not significant; *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001). Survival curves were compared using the log-rank test.

ACKNOWLEDGMENTS. We thank D. W. Paul for technical assistance, the DFCI Hematologic Neoplasia Flow Cytometry Core for excellent assistance with the flow cytometry studies and cell sorting, Dr. B. E. Gewurz for the EBV (B95.8) virus, D. P. Leahy for administrative assistance, and A. M. Sing for critical reading of the manuscript. We are grateful to the NIH Tetramer Facility at Emory University for providing the PE-conjugated mCD1d-PBS57 tetramer. This work was supported by DFCI Faculty Start-up Funds (to B.Z.).
51. Hunder NN, et al. (2008) Treatment of metastatic melanoma with autologous CD4+ T cells against NY-ESO-1. *N Engl J Med* 358:2698–2703.

52. Zhang B, et al. (2015) An oncogenic role for alternative NF-κB signaling in DLBCL revealed upon deregulated BCL6 expression. *Cell Rep* 11:715–726.

53. He L, et al. (2005) A sensitive flow cytometry-based cytotoxic T-lymphocyte assay through detection of cleaved caspase 3 in target cells. *J Immunol Methods* 304:43–59.

54. Koralov SB, et al. (2008) Dicer ablation affects antibody diversity and cell survival in the B lymphocyte lineage. *Cell* 132:860–874.