SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, six figures, and one table.

SUPPLEMENTAL FIGURE LEGENDS

Figure S1. Counter-Selection of LMP1$^+$ B Cells and Activation of T Cells in Adult *CD19-cre;LMP1^{flSTOP}* Mice, Related to Figure 2

(A) Numbers of CD19$^+$IgM$^+$ cells in spleens of 6-12 week old *CD19-cre* and *CD19-cre;LMP1^{flSTOP}* mice.

(B) Southern blot of EcoRI-digested genomic DNA to determine deletion of the STOP cassette within the *LMP1^{flSTOP}* allele. Flox, STOP cassette not deleted; del, STOP cassette deleted.

(C and D) FACS analysis (C) and frequency of various B-cell populations (D) in the BM of 6-12 week old *CD19-cre* and *CD19-cre;LMP1^{flSTOP}* mice. The cell populations were defined as follows: pro-B cells, B220$^+$IgM$^-$c-kit$^+$; pre-B cells, B220$^+$IgM$^-$CD25$^+$; immature B cells, B220^{int}IgM$^+$; and mature B cells, B220^{high}IgM$^+$.

(E) Frequency of Fas-expressing cells within B220$^+$IgM$^-$ and B220$^+$IgM$^+$ BM cells in these mice.

Bars in (D) and (E) show the respective mean values.

(F) FACS analysis of naive (CD62L$^+$) and effector/memory (CD62L$^-$CD44$^+$) CD4$^+$ and CD8$^+$ T cells in the BM of these mice.

Figure S2. Appearance of LMP1$^+$ B Cells and Activation of T Cells in Newborn *CD19-cre;LMP1^{flSTOP}* Mice, Related to Figure 2

(A) Frequency of CD19$^+$, CD19$^+$Fas$^+$, TCRβ^+CD8$^+$ and TCRβ^+CD4$^+$ cells in spleens of *CD19-cre* and *CD19-cre;LMP1^{flSTOP}* mice on day 3 and 8 after birth. Bars show the respective mean values; each dot indicates one mouse.

(B) Representative FACS analysis of B cells, CD8$^+$, CD4$^+$ T cells and their activation status (CD69 expression) in spleens of *CD19-cre* and *CD19-cre;LMP1^{flSTOP}* mice on day 8 after birth. Lowest panels: staining for the activation markers CD69 on TCRβ^+CD8$^+$ and TCRβ^+CD4$^+$ gated cells.

(C) Spleen sections of *CD19-cre* and *CD19-cre;LMP1^{flSTOP}* mice on day 8 after birth were stained with hematoxylin and eosin (H&E), anti-B220, anti-CD3 and terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL). In the TUNEL staining, apoptotic cells stain dark brown.

Figure S3, Antibody-Mediated Depletion of Immune Effectors in *CD19-cre;LMP1^{flSTOP}* Mice Leads to Outgrowth of LMP1$^+$ B Cells Active in Multiple Signaling Pathways, Related to Figure 3

(A) Frequency of CD19$^+$ (upper panel) and CD19$^+$Fas$^+$ cells (lower panel) in the BM of *CD19-cre* and *CD19-cre;LMP1^{flSTOP}* mice repetitively injected with PBS, or anti-CD4 and -CD8, or anti-CD4, -CD8, and -Thy1 antibodies. Mice were analyzed on day 13 after initiation of treatment. Bars represent median values; each dot indicates one mouse.

(B) Staining for intracellular cytokines in $CD4^+$ and $CD8^+$ T cells from the BM of the indicated mice. Data are representative of two independent experiments.

(C) Immunoblot analysis of various signaling pathways in B cells treated as indicated or isolated from *CD19-cre;LMP1^{flSTOP}* mice receiving the depleting anti-CD4, -CD8, and -Thy1 antibodies (Abs). Blotting of β-actin served as loading control; d3, day 3 of treatment.

(D) Cell cycle analysis of MACS-purified $CD19^+$ cells from *CD19-cre* and *CD19-cre;LMP1^{flSTOP}* mice receiving the depleting antibodies (Abs). The percentages of cells in the respective phases of the cell cycle are indicated.

Figure S4. Analysis of NF-κB activity, Clonality, and AID Expression in LMP1$^+$ Lymphoma Cells Arising in T-Cell Deficient *CD19-cre;LMP1^{flSTOP}* Mice, Related to Figure 5

(A) Top left panels: NF-κB DNA binding activity in nuclear extracts of $CD43^-$ splenic B cells stimulated overnight (O.N.) or not with anti-CD40 and of $LMP1^+$ tumor cells. I and II, NF-κB complexes. Bottom left panels: Identification of NF-κB family members in tumor cells by supershift using antibodies against RelA, RelB or p50. -, no addition of antibody; *, supershifted complexes. Right panels: Immunoblotting of p100, p52, α-Tubulin as well as β-Actin in cytoplasmic and nuclear extracts of the various samples. β-Actin, loading control; α-Tubulin, control for cytoplasmic contamination of nuclear extracts. Data are representative of two $LMP1^+$ tumors analyzed.

(B) Southern blot analysis of rearranged VDJ and DJ gene segments in various samples as indicated. B6 liver, genomic DNA from the liver of a C57BL/6 mouse serving as control for germline IgH configuration (dashed line); B6 $CD19^+$ B cells, normal B cells from the C57BL/6 mouse; wks, age of mice (weeks); RγT1, T2, tumor cells serially passaged in $Rag2^{-/-}\gamma c^{-/-}$ mice. Red arrows indicate clonal VDJ or DJ rearrangements in various tumors. The upper yellow box shows a shorter exposure for the corresponding samples.

(C) Immunoblotting of AID in various tumor cells or control B cells treated as indicated.

Figure S5, Conventional $CD4^+$ T, NKT, and $CD8^+$ T cells Control Non-Transformed and Transformed $LMP1^+$ B Cells, Related to Figure 6

(A) Both $CD4^+$ and $CD8^+$ T cells from *CD19-cre;LMP1^{flSTOP}* mice eliminated $LMP1^+$ B cells in transplanted animals. $CD19^+$ cells were sorted from the BM of *CD19-cre;LMP1^{flSTOP}* mice (C57BL/6xBALB/c F1) and transferred into $Rag2^{-/-}\gamma c^{-/-}$ mice ($2x10^5$ cells/mouse, i.v.) either alone or together with $TCR\beta^+CD4^+$ or $TCR\beta^+CD8^+$ T cells ($3x10^5$ cells/mouse) sorted from the BM of the same mice. Recipients were analyzed on day 39 after transplantation for the presence of $LMP1^+$ ($CD19^+Fas^+$) B cells. Data are representative of two recipients for each group.

(B) Intracellular IFNγ and TNFα staining in $TCR\beta^+CD4^+$ or $TCR\beta^+CD8^+$ T cells from the indicated mice cultured either alone or together with $LMP1^+$ tumor cells or normal B cells from WT mice. Data are representative of two experiments.

(C) Representative FACS analyses of LMP1$^+$ tumor cells (CD19$^+$Fas$^+$), conventional CD4$^+$ T cells (TCRβ^+CD1dTetramer$^-$CD4$^+$, denoted as CD4$^+$ T-NKT), and NKT cells (TCRβ^+CD1dTetramer$^+$CD4$^{+/-}$) recovered from the spleen of Rag2$^{-/-}\gamma$c$^{-/-}$ recipients. Splenic cells from a C57BL/6 mouse served as staining controls, in which the few CD19$^+$Fas$^+$ cells are probably germinal center B cells.

Figure S6. Immune Surveillance of LMP1$^+$ B Cells Does Not Depend on NKG2D, but LMP1$^+$ Tumors Can Be Therapeutically Targeted by NKG2D-Fc Fusion Protein, Related to Figure 7

(A) FACS analysis of CD19 and Fas expression on B cells, and NKG2D on NK cells (NK1.1$^+$CD3ε^-) from the spleen and bone marrow of the indicated mice. The gating information is shown on the left. CD19$^+$Fas$^+$ populations represent LMP1$^+$ B cells in mice carrying the LMP1^{flSTOP} allele, while in control mice (CD19-cre or NKG2D$^{-/-}$;CD19-cre), they are probably germinal center B cells. Data are representative of 3 mice of each genotype.

(B and D) Spleens and livers from Rag2$^{-/-}\gamma$c$^{-/-}$ animals transplanted with the indicated lymphoma lines derived from T cell-deficient *CD19-cre;LMP1^{flSTOP}* animals and repetitively treated with mNKG2D-Fc or isotype control (mouse IgG2a). Beginning one day after tumor transplantation, recipients received i.v. injections of 250 μg of mNKG2D-Fc or isotype control every 2 to 3 days for 7 times, and were analyzed on day 47 (815 tumor) and 34 (966 tumor) after tumor transplantation, respectively.

(C and E) Survival of mice transplanted with lymphoma cells and treated as in (B) and (D), respectively. Each dot indicates one mouse; * mice sacrificed at the termination of the experiment.

Table S1. Characterization of LMP1^{+} tumors, related to Figure 5

Mice	Genotype: CD19-cre; LMP1^{flSTOP}	Histology of primary tumors	Histology of secondary tumors	IRF4*	Surface markers of secondary tumors	Clonality
1485	TCR$\beta^{-/-}\delta^{+/-}$	DLBCL	DLBCL	+ (2)	CD19+, Fas+, AA4.1-, CD138-, CD43-, CD21+, CD23+, IgM+, Igλ+	Clonal
815	TCR$\beta^{-/-}\delta^{+/-}$	DLBCL	DLBCL	+ (2)	CD19+, Fas+, AA4.1-, CD138-, CD43-, CD21+, CD23+, IgM+, Igκ+	Clonal
966	TCR$\beta^{-/-}\delta^{-/-}$	DLBCL & plasmacytic	DLBCL	+ (1)	CD19+, Fas+, AA4.1-, CD138-, CD43-, CD21+, CD23+, IgG2+, Igκ+	Clonal
1019	TCR$\beta^{-/-}\delta^{-/-}$	plasmacytic	DLBCL	+ (1)	CD19+, Fas+, AA4.1-, CD138-, CD43-, CD21+, CD23+, IgG2+, Igκ+	Clonal
1115	TCR$\beta^{-/-}\delta^{-/-}$	plasmacytic	n.a	+ (1)	n.a	n.a

Secondary tumors, tumors propagated in immunodeficient host (Rag2$^{-/-}\gamma c^{-/-}$); n.a, not assayed.

*Immunohistochemistry was performed on primary tumors (1) or secondary tumors (2).

EXTENDED EXPERIMENTAL PROCEDURES

TAT-Cre Transduction

Splenic B cells were purified by depletion of $CD43^+$ cells by magnetic-activated cell sorting (MACS, Miltenyi Biotec) and treated with TAT-Cre as described previously (Koralov et al., 2008).

Cell Count and Viability

Cell numbers and viability were determined by the Guava ViaCount assay on a Guava PCA-96 system (Guava Technologies).

Flow Cytometry

Single cell suspensions prepared from various lymphoid organs were stained with the following antibodies: anti-B220 (RA3-6B2), -CD19 (1D3), -CD4 (L3T4), -CD8 (53-6.7), -CD5 (53-7.3), -CD138 (syndecan-1, 281-2), -CD49b (DX5), -Fas (Jo2), -CD43 (S7), -Igκ (187.1), -Igλ (R26-46), -IgG2a/2b (R2-40), and -TNFα (MP6-XT22), -H-$2K^b$ (AF6-88.5), -H-$2K^d$ (SF1-1.1), -I-A^b (AF6-120.1), -I-A^d (AMS-32.1), -CD86 (B7-2, GL1), -ICAM-1 (3E2) (all from BD); anti-NK1.1 (PK136), -CD3e (145-2C11), and -IL17 (TC11-18H10.1) (all from Biolegend); anti-CD69 (H1.2F3), -TCRβ (H57-597), -TCRδ (ebioGL3), -CD93 (AA4.1), -CD117 (c-kit, ACK2), -CD25 (PC61.5), -CD62L (MEL-14), -CD44 (IM7), -Thy1.2 (53-2.1), -IFNγ (XMG1.2), and -IL4 (11B11), -CD80 (B7-1, 16-10A1) (all from eBioscience); anti-IgM (Fab fragment, Jackson Immunoresearch); anti-mRae-1 (pan-specific) and

Rat IgG2a Isotype control (both from R&D Systems). Samples were acquired either on a FACSCalibur or FACSCanto II (BD Pharmingen), and analyzed using FlowJo software (Tree Star).

Real-Time RT-PCR

Total RNA was isolated using the miRVana RNA isolation kit (Applied Biosystems, #AM1560) and cDNA was synthesized using the Superscript III cDNA Synthesis kit (Invitrogen, #18080-051). qRT-PCR was performed on the Step One Plus instrument (Applied Biosystems) using SYBR green PCR core reagents with AICDA specific primers: ATTCTGTCCGGCTAACC and CTATCCAGTCTCTTCAGAGAAATTTC. Samples were analyzed in duplicate and values normalized to HPRT (Calado et al., 2010) levels.

Immunoblot Analysis

Splenic B cells were purified by positive selection with anti-CD19 microbeads or by depletion of $CD43^+$ cells with anti-CD43 microbeads (Miltenyi Biotec). For some controls, purified (CD43-depleted) B cells were treated with anti-CD40 (1 μg/ml, HM40-3) with or without IL4 (25 ng/ml) for 3 days. The cells were lysed for 30 min on ice with lysis buffer (25 mM Hepes, pH7.9; 300 mM NaCl; 1.5 mM MgCl2; 1 mM EDTA, pH8.0; 1% Triton X-100) supplemented with 1 mM DTT, protease inhibitor cocktail (#11836170001, Roche Diagnostics), and phosphate inhibitors (1 mM sodium orthovanadate; 5 mM sodium fluoride). The following primary antibodies (all from Cell Signaling Technology unless indicated) were

used: Biotinylated anti-LMP1 (clone S12, gift from Dr. Fred Wang), anti-LMP1 (CS1-4, Dako), anti-NF-κB2 p100 (#4882), anti-phospho-IκBα (#9246), anti-IκBα (SC-371, Santa Cruz), anti-phospho-Erk (#9101), anti-Erk (#9102), anti-phospho-JNK (#9251), anti-JNK (#9258), anti-phospho-p38 (#9216), anti-p38 (#9212), anti-AID (polyclonal rabbit antibody, gift from Dr. FW Alt), anti-α-Tubulin (T5168, Sigma), and anti-β-Actin (A5316, Sigma).

Electromobility Shift Assay (EMSA)

Cytoplasmic and nuclear extracts were prepared using the NE-PER extraction kit according to the manufacturer's instruction (Thermo Scientific). Nuclear extracts were incubated with poly dI-dC competitor (GE Healthcare) and a ^{32}P-labelled κB probe (Promega). NF-κB-DNA complexes were subsequently separated by electrophoresis through a native polyacrylamide gel. For supershift assays, nuclear extracts were first incubated with anti-RelA (sc-109x), anti-RelB (sc-226x) or anti-p50 (sc-114x) antibodies from Santa Cruz Biotechnology.

Histology and Immunohistochemistry

Tissues were fixed with 10% formalin (Sigma), embedded in paraffin, sectioned at 5 μm and stained with hematoxylin and eosin (H&E). Immunohistochemistry was performed on OCT-embedded frozen sections with goat anti-IRF4 (MUM1) antibody (1:1000 dilution, SC-6059, Santa Cruz). Specimens of human PTLDs were fixed with B5-formalin (50:50 v/v) and stained on 5 μm sections with

hNKG2D-Fc to detect human NKG2D ligand expression. Mouse IgG2a was used as an isotype control.

In Vitro Killing Assay

$CD4^+$ or $CD8^+$ T cells (containing 50% activated, $CD69^+$ cells) were sorted by Fluorescence-activated cell sorting (FACS) from the BM of *CD19-cre;LMP1^{flSTOP}* or the spleen of OT-II and OT-I mice bred to BALB/c (all on a (C57BL/6xBALB/c) F1 background). The T cells were co-cultured with $4x10^3$ tumor cells (line 966, derived from a T cell-deficient *CD19-cre;LMP1^{flSTOP}* animal on a C57BL/6xBALB/c mixed background, expressing MHC-I and -II of haplotypes b and d) at various effector:target ratios for 4 hours in 96-well plates, followed by active Caspase-3 staining (BD) (He et al., 2005). NK cells ($NK1.1^+DX5^+CD3^-$) were purified from the BM of *CD19-cre;LMP1^{flSTOP}* mice by MACS using anti-DX5 microbeads and subsequent FACS. Purified NK cells were expanded for 5 days with recombinant murine IL-2 (2000U/ml, Peprotech), washed and incubated for 5 min at 37^oC with the following antibodies: NKG2D blocking antibodies CX5 and MI6, isotype control rat IgG (all at 30 µg/ml; eBioscience), Fas-ligand neutralizing fusion protein rmFas-Fc, and isotype control human IgG1 (both at 10 µg/ml; R&D Systems), either alone or in combinations. The NK cells were then co-cultured with $4x10^3$ tumor cells (line 966) at various effector:target ratios for 4 hours before staining for active Caspase-3. In all killing assays, effector-target mixtures in U-bottom 96-well plates were spun at 200 rpm for 2 min before moving to incubator, and cultures were stained with anti-CD19, anti-CD4, anti-CD8, and

anti-NK1.1 to identify tumor cells and effector cells. Active Caspase-3 positive $CD19^+$ cells represent apoptotic tumor cells. % specific killing = % apoptotic target cells of cultures with both effectors and targets - % apoptotic target cells of cultures with targets alone.

Intracellular Cytokine Staining

In one set of experiments, BM cells from *CD19-cre* or *CD19-cre;LMP1^{flSTOP}* mice were cultured in the presence of 0.1 μM PMA, 1 μM Ionomycin, and 10 μg/ml Brefeldin A (BFA) for 4.5 hrs at 37°C. In another set of experiments, $TCR\beta^+CD4^+$ or $TCR\beta^+CD8^+$ cells were FACS sorted from the BM of *CD19-cre;LMP1^{flSTOP}* or the spleen of WT mice (all on a (C57BL/6xBALB/c) F1 background), and co-cultured with $LMP1^+$ tumor B cells ($H\text{-}2K^{b/d}$ and $I\text{-}A/E^{b/d}$) or B cells from the WT mice. $2.5x10^5$ T cells were cultured with $4x10^5$ B cells for 51 hrs in 96-well U-bottom plate and supplemented with 10 μg/ml of BFA in the last 8 hrs. After the surface staining for TCRβ, CD4, CD8, and CD19, cells were fixed with 2% paraformaldehyde, permeabilized with 0.5% Saponin, and stained for various intracellular cytokines. Samples were acquired on FACSCanto II and analyzed using FlowJo software.

Anti-TNF-α and Anti-IFN-γ Treatment

Primary mice or $Rag2^{-/-}\gamma c^{-/-}$ recipients engrafted with $1x10^4$ $LMP1^+$ tumor cells ± $20x10^4$ $TCR\beta^+CD4^+$ cells from the BM of *CD19-cre;LMP1^{flSTOP}* donors were injected (i.p.) with anti-TNF-α (TN3-19.12) and/or anti-IFN-γ (H22) blocking

antibodies (250 μg of each) in a 7-day interval, and analyzed 21 or 28 days after the beginning of the treatment, respectively (Koebel et al., 2007).

LMP1 Epitope Screening

To identify T cell epitopes, we synthesized 20-mer peptides overlapping in 10 amino acids, covering the full-length LMP1 protein (JPT Peptide Technologies) and dissolved them into dimethyl sulfoxide (DMSO). B cells isolated from the spleen of C57BL/6 mice were pulsed with 5 μM of each individual peptide for 24 hrs in the presence of 20 μg/ml of LPS to activate B cells. After intensive washing, $1x10^5$ B cells were co-cultured with $3x10^4$ of CFSE-labeled $TCR\beta^+CD4^+$ or $TCR\beta^+CD8^+$ cells sorted from the BM of *CD19-cre;LMP1^{flSTOP}* mice (on a C57BL/6 background) in a 96-well U-bottom plate for 4 days, and the cells were stained for TCRβ, CD4, CD8, and CD19, and analyzed for CFSE levels.

Analysis of Tumor Clonality by Southern Blotting has been described previously (Calado et al., 2010).

Cell Cycle Analysis was done as described previously (Xiao et al., 2008).

SUPPLEMENTAL REFERENCES

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