Supporting Information

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SI Text

Synthesis of Mouse and Human Cyclin B1 Recombinant Proteins. Mouse and human cyclin B1 were subcloned into pDEST-17 (Invitrogen). Bl21 codon+ BL21 RIPL bacteria cells (Invitrogen) were transformed and grown overnight in shaker flasks at 37 °C, in a constitutive expression state. Recombinant cyclin B1 was found in inclusion bodies and extracted with guanidine-HCL under reducing conditions. Solubilized inclusion bodies lysate was passed over a Q-Sepharose FF (Amersham Biosciences) packed column monitored by an AKTA prime chromatography system, and the protein was collected in flow through fractions. Fractions were run over Nickel-HP 5 mL HighTrap columns (Amersham Biosciences) in a modified refolding protocol. The protein was loaded onto the column, washed with guanidine, exchanged into a 6 M urea buffer, and then slowly exchanged into a 3 M Urea buffer. The protein was eluted in an imidazole gradient, and fractions were collected and run on a gel to determine which ones contained the protein. Full-length cyclin B1, both mouse and human, runs on Tris/glycine gels under reducing conditions at the predicted weight of 54 kDa, and only fractions containing full-length protein were collected. Protein was concentrated on 30 MWCO Amicon filters and assayed for endotoxin. Protein purity was analyzed by coomassie blue, Western blot, HPLC, and Limulus amebocyte lysate assay (LAL for endotoxin assessment).

Measurement of Human Anti-Cyclin B1 Antibody Responses. Wells of 96-well ELISA plates (Thermo) were each coated with 0.65 μg of recombinant human cyclin B1 protein in 50 μL PBS. Plates were sealed overnight at 4 °C and washed with PBS before use. Cyclin B1-coated wells and empty, background control wells were then blocked with 2.5% BSA in PBS (blocking buffer) for 1 h. Plasma samples were diluted 1:400 in blocking buffer in 96-well polypropylene plates (Nunc, Thermo Fisher) along with five control samples that represented the range of the assay. Fifty μL of each diluted sample was then transferred to the ELISA plates. Samples were incubated for 1 h and were subsequently washed with 1% PBS-Tween. Anti-human IgG (Sigma) was diluted in blocking buffer and incubated on the plates for 1 h. Plates were then washed as before and incubated with alkaline phosphatase substrate (SigmaFast Tabs) for 1 h in the dark. NaOH (3 M) was added to stop the reaction and plates were read immediately at 405 nm. After subtraction of background, samples run on separate days were normalized using the five sample controls. Briefly, the controls on all days were averaged and the difference between the overall mean and the mean on a given day was applied to all samples on that day.

Generation of Dendritic Cells. Peripheral blood mononuclear cells (PBMCs) from buffy coats were incubated in AIMV (Gibco, Invitrogen) at 37 °C for 1 h to obtain adherent monocytes. Nonadherent cells were removed for future use. Monocytes were then cultured in complete, serum-free AIMV media for six days in the presence of 400 U/mL GM-CSF (R&D) and 1,000 U/mL IL-4 (R&D). Additional media and GM-CSF/IL-4 were added again on day four. DCs were harvested for use on day six.

Cyclin B1 Peptide Library Synthesis. Cyclin B1 peptides were designed to span the entire cyclin B1 protein sequence and to represent the available T-cell epitopes. The peptides were synthesized in collaboration with the Proteomics Resource Center at the Rockefeller University using a published method (49). Peptides were optimized for synthesis with the epitope library fragment generation program PeptGen (available at http://www.hiv.lanl.gov). Integrity of each peptide was verified by matrix-assisted laser desorption/ionization (MALDI) mass spectrometry using a delayed extraction spectrometer system (Voyager; PerSeptive/Applied Biosystems).

LO2 Cell Line. LO2 cells were maintained in vitro in RPMI-1640 medium (Gibco, Invitrogen) supplemented with 10% heat-inactivated FBS (FBS, Cellgro; Media Tech), penicillin (100 U/mL), streptomycin (100 μg/mL), 0.3% glutamine (Gibco, Invitrogen), 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, and 10 μM β-mercaptoethanol (Gibco, Invitrogen). LO2 cells were s.c. inoculated into syngeneic C57BL/6 mice to establish a transplantable tumor model.

Recombinant Protein Vaccination. C57BL/6 mice were immunized s.c. with 25 μg/100 μL per mouse recombinant human cyclin B1 (hCB1) protein, mouse cyclin B1 protein (mCB1), or 100 μL PBS as a control. At the time of immunization or PBS treatment, an immunostimulatory (IS) patch containing 20 μg heat-labile enterotoxin (LT) (provided by IOMAI Corporation) was applied to the immunization site. Repeat injections and LT/IS patch application were repeated twice in three-week intervals. Sera were collected to measure antibody response. Seventeen days after the last immunization, 3 mice per group were killed to study T-cell responses. The remaining mice from the human cyclin B1 and PBS groups, as well untreated, age-matched mice, were challenged with 1×10^6 LO2 cells s.c. Tumor growth and survival were monitored.

Construction of Mouse and Human Cyclin B1 pcDNA3.1 DNA Vectors. Human cyclin B1 cDNA derived from a HeLa cell line was a gift from Dr. Qimin Zhan (University of Pittsburgh). Mouse cyclin B1 cDNA was an RT-PCR product derived from the mouse $p53^{-/-}$ LO2 cell line. Briefly, RT-PCR was performed using primers ATGGCGCTCAGGGTCACTAG (forward) and CAGTCTATTGGAGTTATGCCTTTG (reverse). A band at $\approx$1.3 kb migrated on a 1.2% E-Gel Agarose gel (Invitrogen). The mouse cyclin B1 band was eluted using a MiniElute Kit (Qiagen) and subcloned into PCR2.1-TOPO vector (Invitrogen) and used to transform One-Shot TOP10 competent cells (Invitrogen) as described by the manufacturer. Colonies were picked for culture, and plasmids were isolated and identified positively by an *Eco*RI digest. Both cDNAs were then subcloned into the *Bam*HI-*Xho*I site of the pcDNA3.1 expression vector (Invitrogen). All inserts were verified by DNA sequencing.

ELISPOT Measurement of Mouse Anti-Cyclin B1 T-Cell Responses. Nitrocellulose plates (Millipore) were coated with anti-IFNγ capture antibody (BD Biosciences) overnight at 4 °C. DCs were loaded with mouse cyclin B1 protein for 2–6 h and mixed with autologous T cells at a DC/T cell ratio of 1:10 for 20 h at 37 °C. The cells were seeded at 10^5 cells per well. All assays were performed in serum-free AIMV medium (Gibco, Invitrogen). The plates were then washed with 0.1% Tween-20 in PBS and stained with anti-IFNγ mAb (BD PharMingen) for 2 h at 37 °C. The plates were washed, and either an avidin-peroxidase complex or an alkaline phosphatase-labeled avidin D antibody (Vector Laboratories) was added to the plates for 1 h. The plates were then developed using either AEC substrate (Sigma) or BCIP/NBT solution (KPL), and spots were quantified micro-

scopically with an inverted phase-contrast microscope (Carl Zeiss) along with a computer-assisted image analysis system (Immumoassay). Anti-CD4 or anti-CD8 blocking antibodies (BD Biosciences) were added to the cultures for blocking experiments.

Measurement of Mouse Anti-Cyclin B1 Antibody Responses. Cyclin B1 specific IgG levels were determined in sera with the use of 96-well ELISA plates (Immulon-2HB, Dynex Laboratories) were coated overnight with 0.1 μg per well cyclin B1. Plates were blocked with 0.5% casein/Tween-20 for 2 h and washed. Samples were serially diluted (2-fold) on ELISA plates and incubated overnight at 4 °C. IgG was detected with horseradish peroxidase conjugated goat anti-mouse IgG (Bio-Rad) and 2′2-azino-bis (3-ethylbenzthiazoline sulfonic acid) substrate (ABTS, KPL). The enzyme reaction was stopped using 1% sodium dodycyl sulfate. Antibody titers are reported as ELISA units, which correspond to the inverse dilution of the serum that yielded an OD_{405} of 1.0.

Cyclin B1 Protein Vaccination. In Fig. S3, we show that immunization with the mouse cyclin B1 protein elicits T cells specific for both mouse and human cyclin B1. In Fig. S3*B*, we show that immunization with human cyclin B1 protein elicits high titer antibodies reacting against both human (*Left*) and mouse (*Right*) cyclin B1. Fig. S3*C* shows that all of the cyclin B1-specific T-cell reactivity can be blocked with anti-CD4 antibody. When these mice were challenged with LO2 tumor s.c., neither vaccine was able to inhibit tumor growth. Thus, antibody and $CD4^+$ T-cell responses alone, which was the response induced with the recombinant protein immunization, were clearly not the desired tumor rejection response.

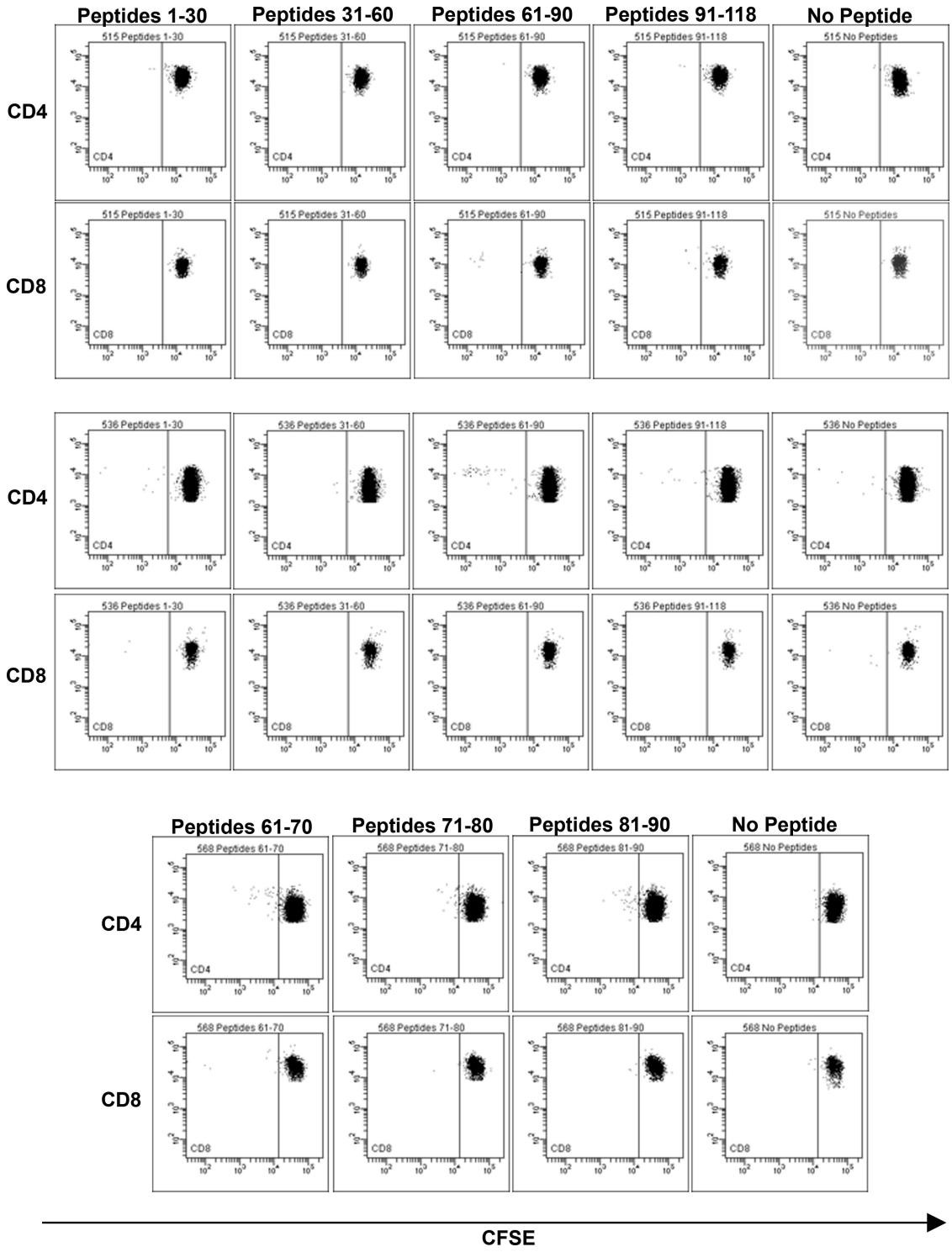

Fig. S1. T-cell proliferation in response to peptides pools of the cyclin B1 peptide library. PBMC from heavy smokers who were negative for lung cancer by computed tomography (CT) scan were labeled with CFSE and stimulated with ≈2 μg/mL/peptide recombinant cyclin B1 peptides. After six days of culture, PBMC were stained with cell surface markers and proliferation was assessed by flow cytometry. (*Top* and *Middle*) 118 peptides of the cyclin B1 library were initially divided into three pools of 30 and one pool of 28 peptides; data from two individuals are shown. (*Lower*) Pool 61–90 was then broken into three pools of 10 peptides each; data from one individual shown.

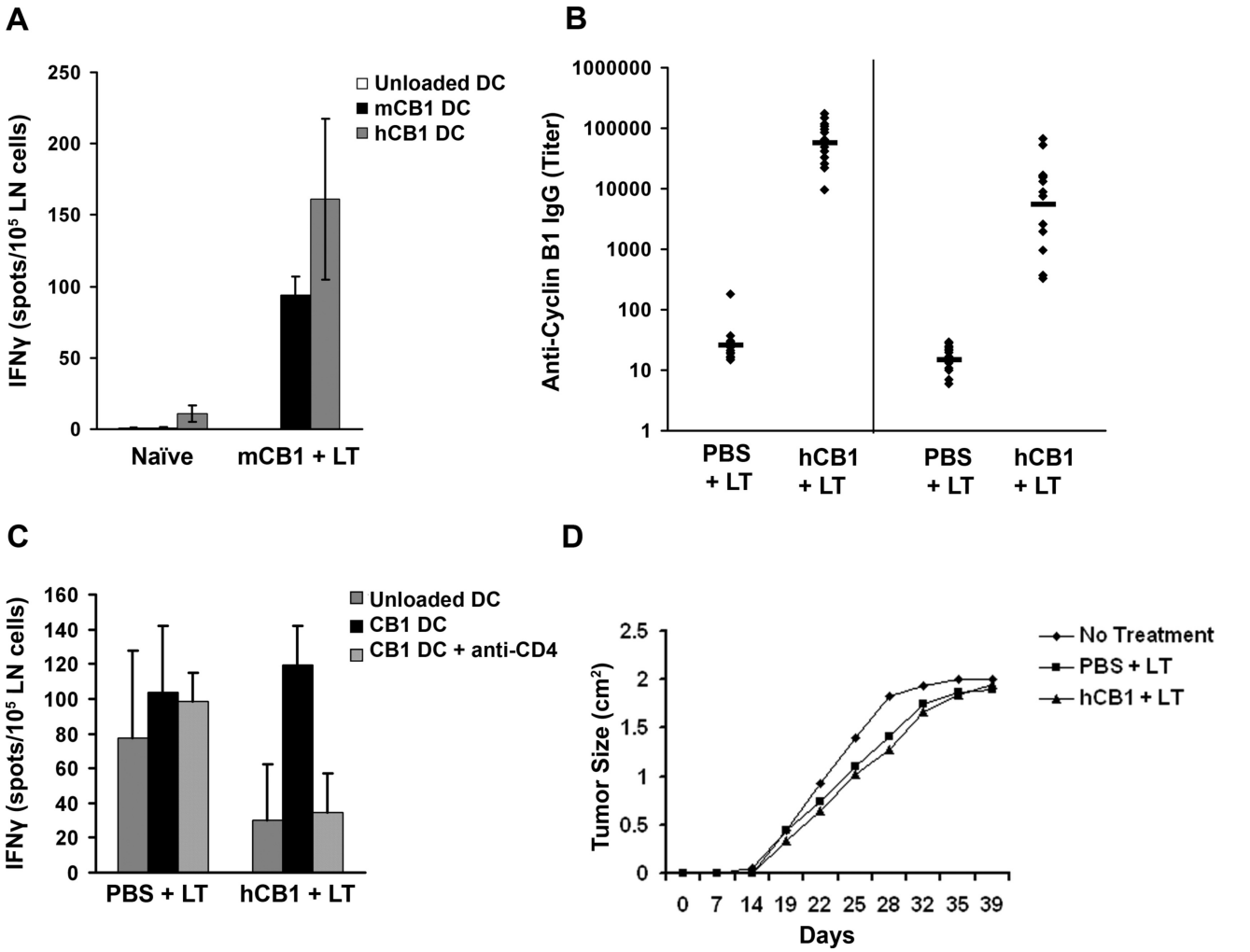

Fig. S2. Cyclin B1 immunity elicited by immunization with recombinant cyclin B1 protein. C57BL/6 mice were immunized s.c. with either mouse (*A*, mCB1) or human (*B–D*, hCB1) recombinant cyclin B1 protein and boosted with the protein + LT/IS patch twice in three-week intervals. Mice immunized with PBS (*A–D*) or not treated (*D*) were used as controls. Error bars indicate SD. (*A*) Mice immunized with mouse cyclin B1 protein generate IFNγ producing T cells specific for both mouse and human cyclin B1. (*B*) Both anti-human (*Left*) and anti-mouse (*Right*) cyclin B1 antibody responses were elicited by human cyclin B1 protein immunization. Bars indicate geometric mean. (*C*) Cyclin B1-specific T-cell responses were elicited by priming and boosting with human cyclin B1 protein and the LT/IS patch. The response can be blocked with anti-CD4 antibody. LT patch alone induced nonspecific T-cell activation. (*D*) Compared with untreated mice, neither LT alone nor human cyclin B1 protein + LT was able to prevent or delay tumor growth.

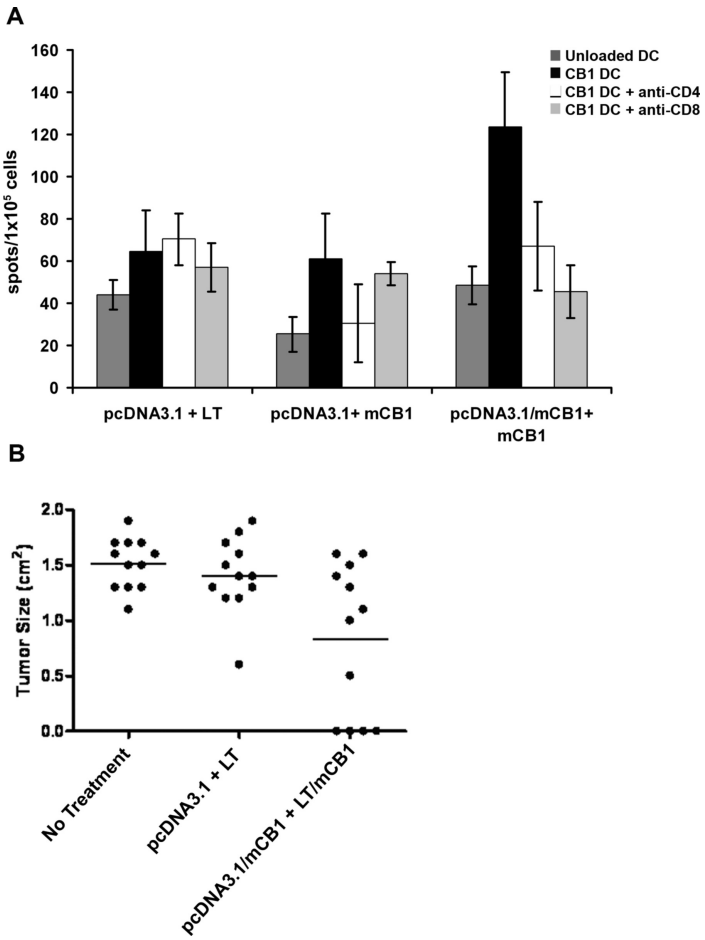

Fig. S3. Mouse Cyclin B1 DNA prime-protein boost vaccination elicits $CD8^+$ T cells and protects from tumor challenge. C57BL/6 mice were primed as four experimental groups and boosted twice in three-week intervals. pcDNA3.1 + LT: mice were primed with an empty DNA vector and boosted with heat labile enterotoxin (LT) via an immunostimulatory patch (IS); pcDNA3.1 + mCB1: mice were primed with an empty DNA vector and boosted with mouse cyclin B1 protein (mCB1) in the presence of the LT/IS patch; pcDNA3.1/mCB1 + mCB1: mice were primed with the pcDNA3.1 vector containing mCB1 cDNA and boosted with the LT/IS patch + mCB1 protein. (*A*) Blocking CD8 activity significantly reduces the production of IFNγ only in mice vaccinated with mCB1 DNA prime-protein boost. Error bars, SE. (*B*) On day 25 after vaccination, mice vaccinated with mCB1 DNA prime-protein boost had significantly lower mean tumor volume than adjuvant only ($P = 0.0162$) and no treatment ($P = 0.0034$) controls. Bars, mean.