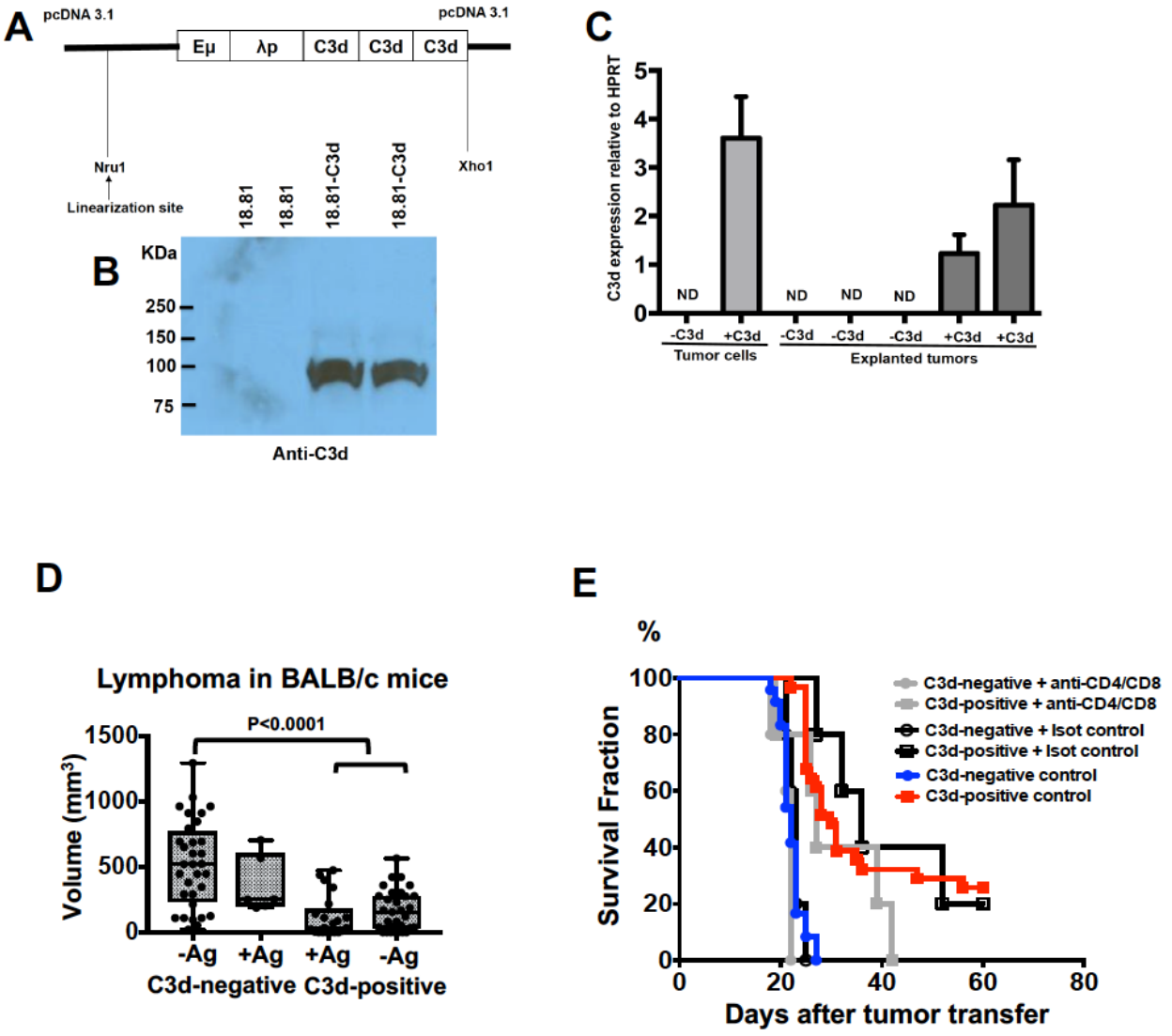
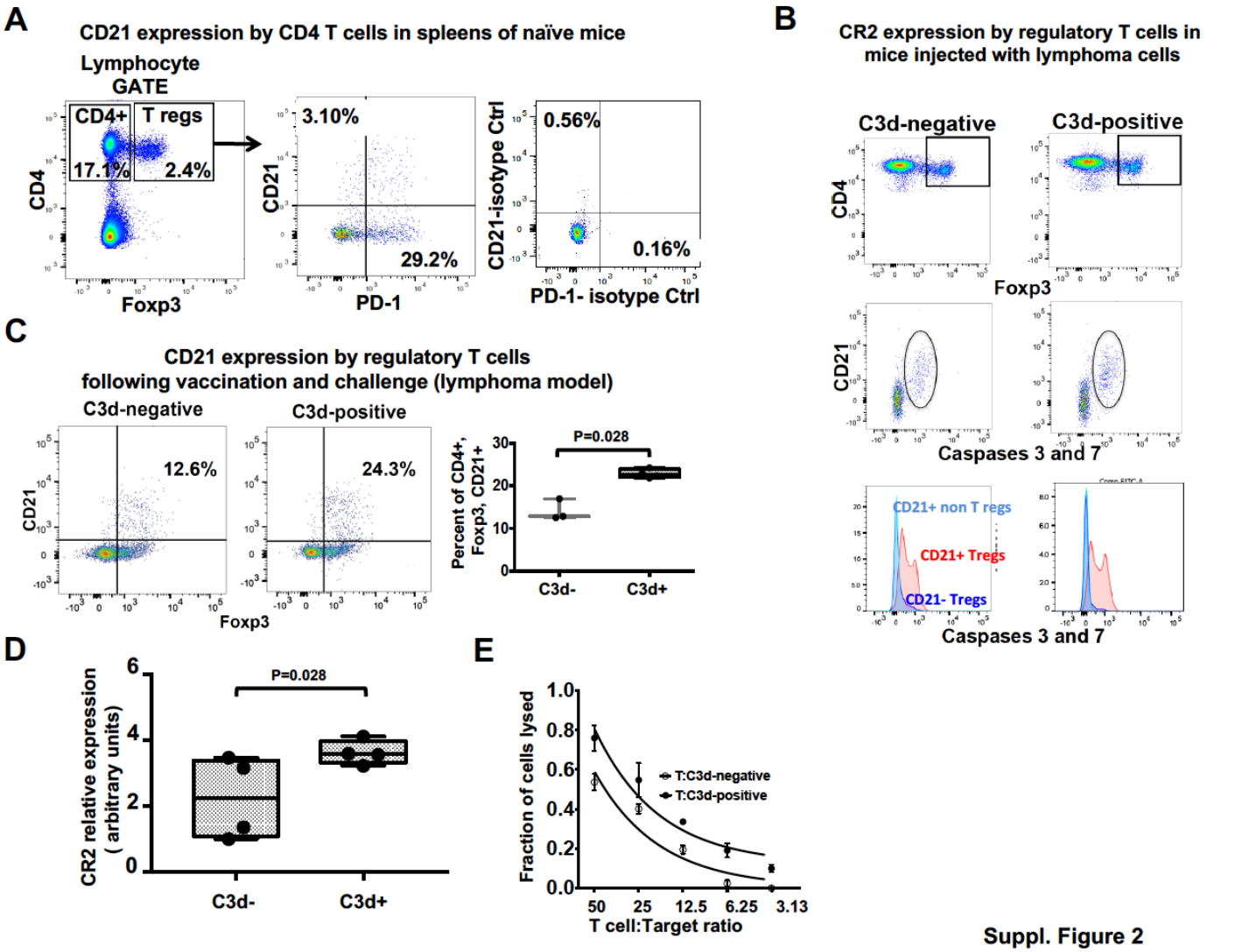
Supplemental Figures

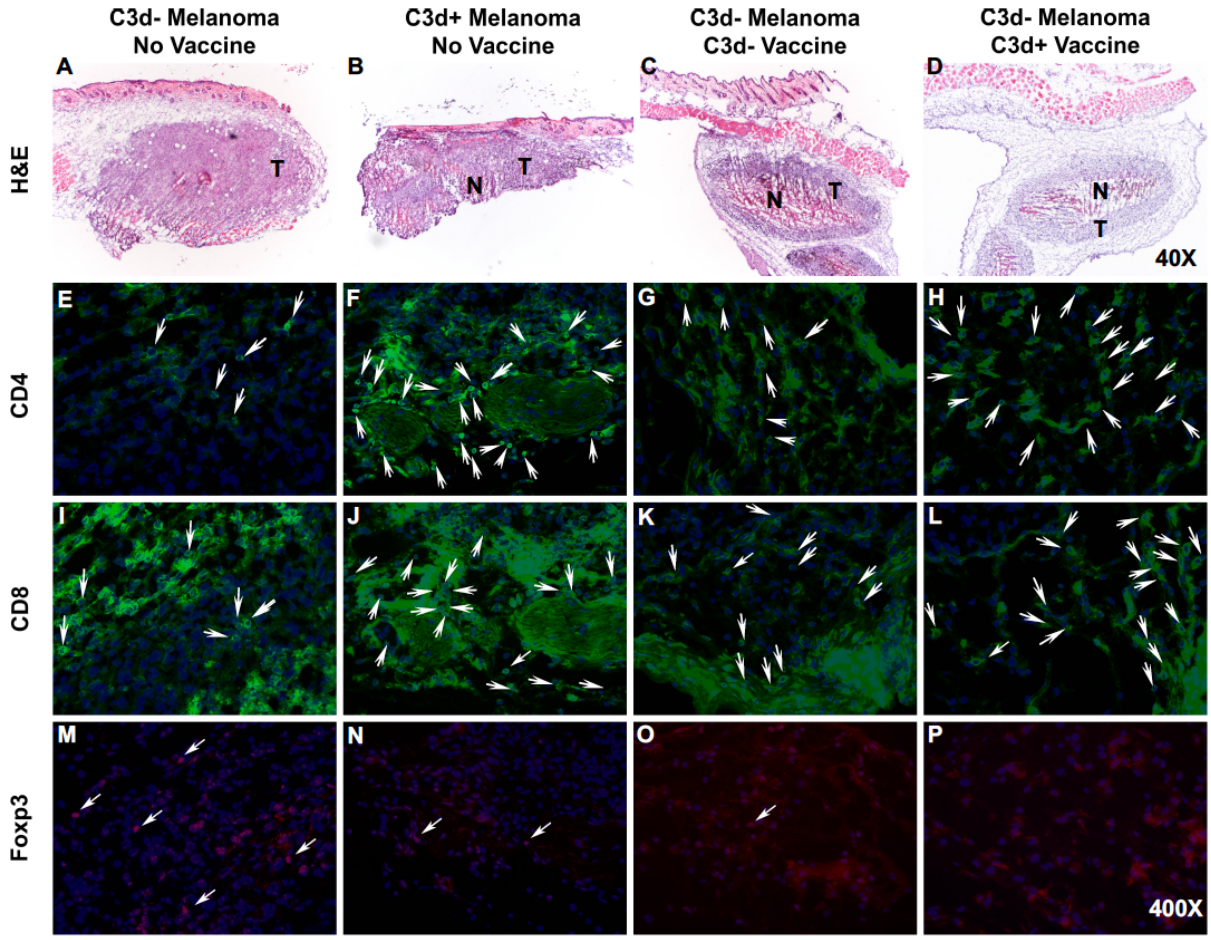


Suppl. Figure 1

Supplementary Figure 1: (A) Schematic of the C3d expression vector (for B cell lymphoma). C3d expression was driven by the immunoglobulin lambda 1 light chain promoter and by the immunoglobulin heavy chain major intronic enhancer, as shown. (B) Lymphoid cells stably transfected with this vector expressed the C3d protein. Shown is a Western Blot analysis of lysate obtained from 40-60x10^6 cells. Lysates were run on 7.5% SDS PAGE gel and C3d expression detected by rabbit anti-mouse C3d (1:1000). Three C3d copies had an apparent molecular mass of 100 KD. (C) Quantification of C3d mRNA measured by qPCR in cultured tumor cells or explanted tumors. (D) Lymphoma tumor volumes 10 days after injection of 10^7 HIV ENV(Ag)+C3d+, ENV(Ag)+C3d-, ENV(Ag)-C3d+ or ENV(Ag)-C3d- cells. On average Ag-C3d+ were 176 mm^3 and Ag-C3d- 523 mm^3, Ag+C3d+ were 119 mm^3 and Ag+C3d- were 361 mm^3. Boxes in graphs represent distribution of data between the 25 and the 75 percentiles. The mean is indicated by a horizontal line and whiskers represent maximum and minimum values. Statistical analysis was by the Mann-Whitney 2 tailed test. (E) Survival curves of mice injected with C3d+ or C3d- 5x10^3 tumor cells and either anti-CD4 and anti-CD8 monoclonal antibodies or with the respective isotype controls. Figure shows that depletion of CD4 and CD8 T cells accelerates tumor growth and decreases survival of mice injected with C3d+ tumor cells. Survival curves are Kaplan-Meier plots obtained with at least 5 mice per condition and differences between curves were analyzed by the Logrank Mantel-Cox test.

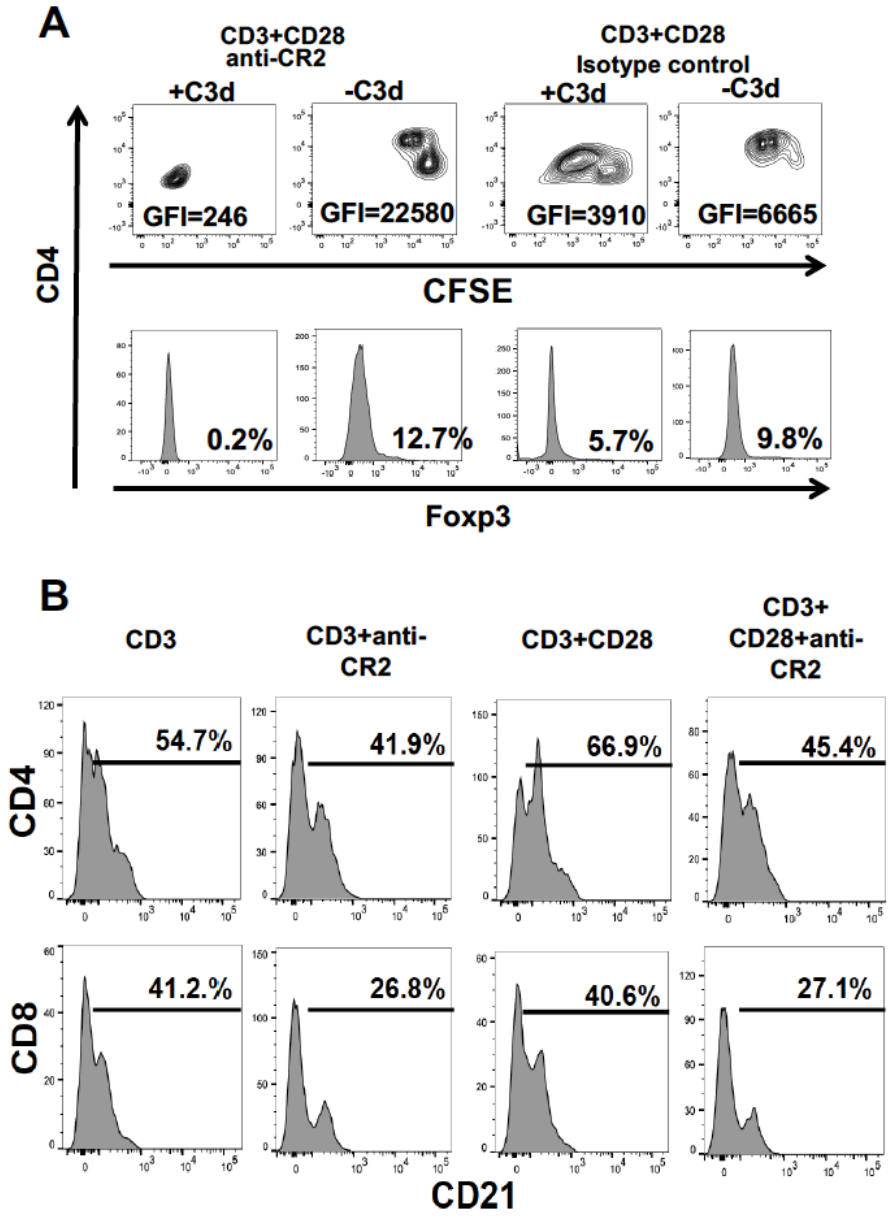


Supplementary Figure 2: (A) Flow cytometry analysis of CD21 surface expression by Treg in spleens of naïve mice detected with rat anti-mouse CD21/35 antibody (7G6). The percent of CD4+, CD21+ T cells is indicated. Shown in the right diagram is the isotype control plot for CD21 and PD-1 antibodies. (B) Depiction of the gating strategy for the analysis of apoptotic Treg (CD4+, Foxp3+) in spleens of mice challenged with the cognate tumors, 10 days prior. (C) Flow cytometry analysis of CD21 expression by Treg in spleens of mice vaccinated and challenged with the cognate tumors, 10 days prior. Graph depicts the frequency of CD21+ Treg in mice after challenge. Flow cytometry plots shown are representative of 5 or more experiments. (D) CR2 relative expression in Treg isolated from C3d+ or C3d- lymphomas 8-10 days after transfer. Delta CTs were calculated by subtracting the CR2 average CT from the GAPDH average CT. (E) Cytotoxicity of splenic T cells isolated from lymphoma bearing mice were cultured with irradiated tumor cells, derived from the same clone that originated the tumors, in the presence of IL-2 for 6 days. Cytotoxicity against C3d+ or C3d- targets at various E:T ratios was measured by assaying release of LDH. Cytokine expression and cytotoxicity assays were repeated (3x) and performed at 3 different times, 3, 5 and 6 days. Controls to measure background lysis of responder or effector cells alone (by using an irrelevant target control) were subtracted from results. Comparison of the averages was done by 2 tailed unpaired T test



Suppl. Figure 3

Supplementary Figure 3: Representative images of melanoma tumors expressing or not C3d (10 days after implantation), or of C3d- tumors (14 days after implantation) following C3d+ or C3d- vaccination 35 days prior to tumor implantation. A-D. Tumor nodules (T) in subcutaneous tissue. C3d+ and vaccine treated tumors have central necrosis (N) and an edematous stroma with brisk inflammation, (Hematoxilin and Eosin, 40x). E-H. Membrane staining for CD4 (green fluorescence) highlights T_H cells in tumor and adjacent stroma. Examples of positive cells given by white arrows. (400x). I-L Membrane staining for CD8 (green fluorescence) highlights cytotoxic lymphocytes in tumor and adjacent stroma. Examples given by white arrows. Confluent staining is necrosis or keratin (400x). M-P Solid nuclear staining for Foxp3 (green fluorescence) highlights Treg cells in tumor and adjacent stroma. All positive cells highlighted by white arrows. Punctate staining is background (400x).



Suppl. Figure 4

Supplementary Figure 4: Impact of free C3d on T cell proliferation stimulated with anti-CD3, anti-CD28 and IL2. T cells were magnetically isolated from spleens of naïve mice. (A) Figure shows flow cytometry analysis of CFSE or Foxp3 expression on gated CD4-positive cells after 5 days in culture, as indicated. The geometric fluorescence intensities (GFI) and the percentages of Foxp3+ cells are indicated. The figure is representative of 5 independent experiments. (B) Figure shows flow cytometry analysis of CD21 expression gated CD4+ or CD8+ T cells, following stimulation with anti CD3 and or anti CD28 +/- anti CR2 for 5 days. Figure shows that anti CR2 decreases detection of expression of CD21 on the surface of lymphocytes.