The *in vivo* mechanism of action of CD20 monoclonal antibodies depends on local tumor burden

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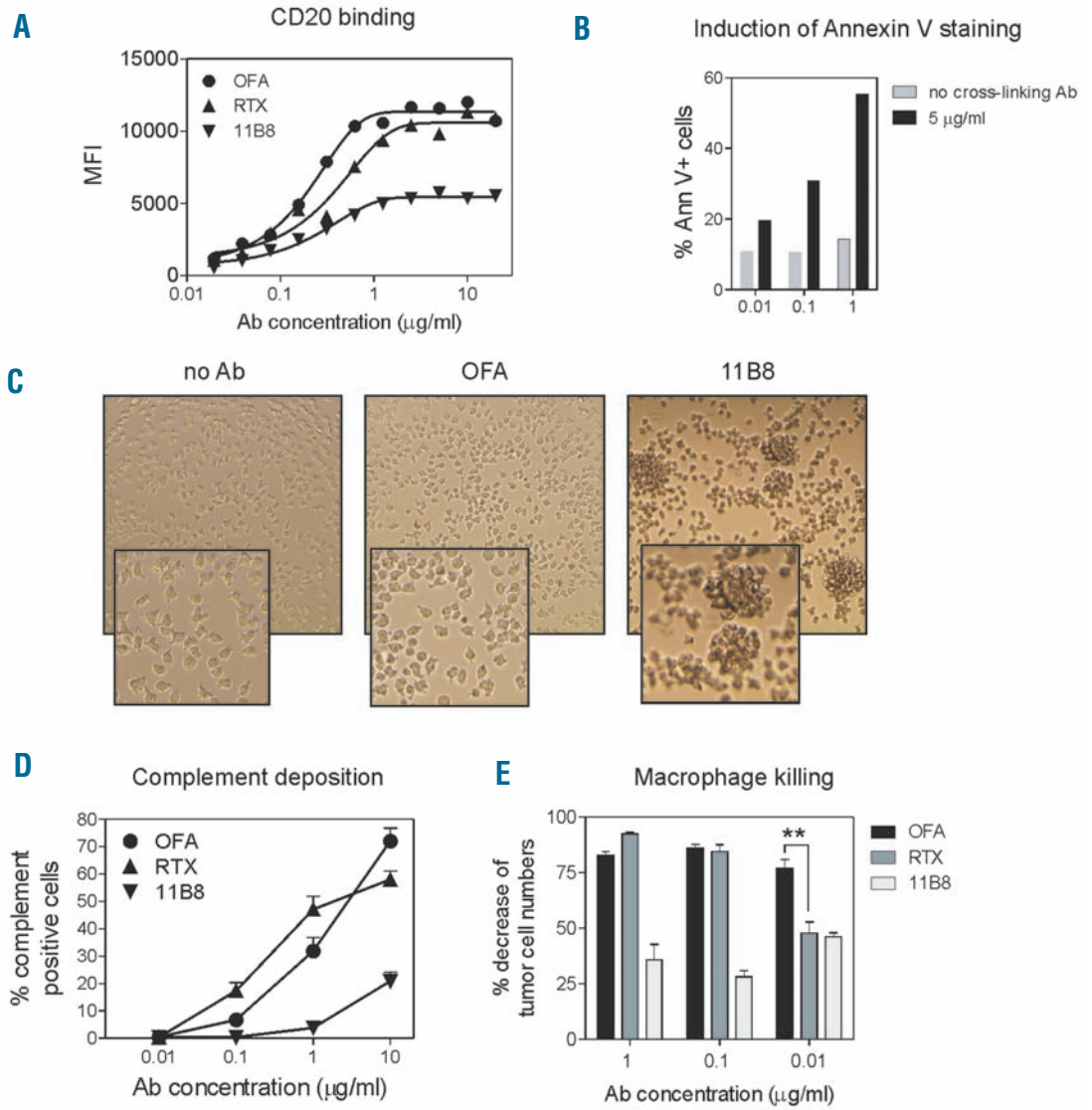
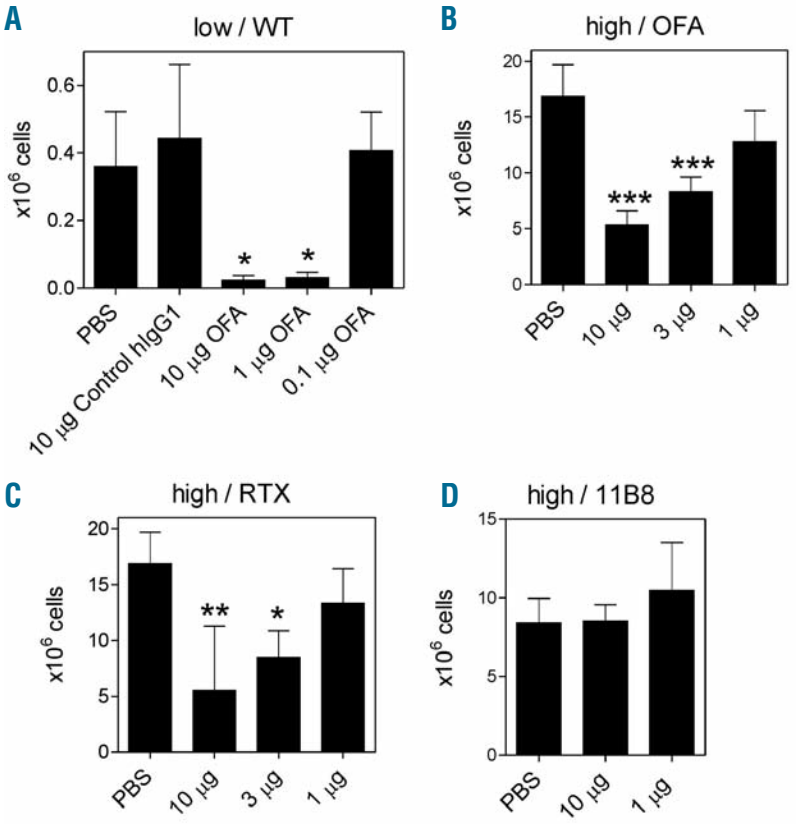
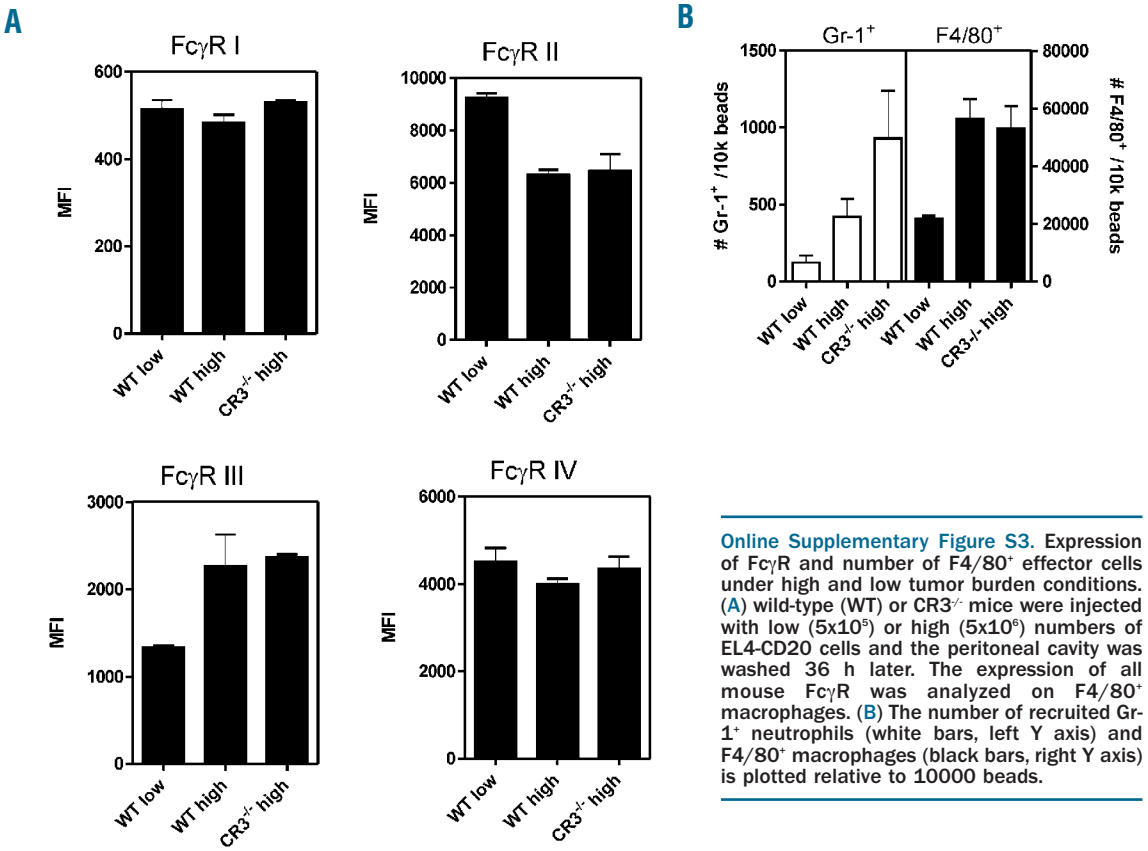


Online Supplementary Figure S1. *In vitro* characterization of CD20 monoclonal antibody (mAb) using EL4-CD20 target cells. (A) CD20 expression on EL4-CD20 cells *in vitro*. Bound CD20 mAb was detected by FITC-labeled F(ab')2 fragments of goat anti-human IgG antibody (Ab). Data are representative of two independent experiments. (B) Induction of annexin V positivity after *in vitro* cross-linking of ofatumumab (OFA). Bound OFA was cross-linked by 5 μg/mL rabbit F(ab')2-anti-human IgG Ab for 24 h. The percentage of annexin V$^+$ cells was assessed by cytofluorimetry. Data are representative of six independent experiments. (C) Induction of homotypic aggregates by CD20 mAb binding *in vitro*. EL4-CD20 cells were incubated for 24 h with 1 μg/mL OFA or 11B8 (20x magnification). (D) Complement deposition on EL4-CD20 cells *in vitro*. Pooled (n=3) normal mouse serum (0.5%) was incubated with CD20 mAb opsonized EL4-CD20 cells for 45 min at 37°C. Deposited complement fragments were detected using a mAb against C3b/iC3b/C3c. (E) *In vitro* macrophage killing assay. EL4-CD20 cells were labeled with CFSE and opsonized with CD20 mAb (1, 0.1, 0.01 μg/mL) for 30 min. Bone marrow derived macrophages (BMDM) were incubated with opsonized EL4-CD20 cells for 16 h at a E:T ratio of 10:1. The number of CFSE+ EL4-CD20 cells was determined by using a constant amount of beads. The condition without CD20 mAb was used to set the 0% level; the condition without tumor cells was used to set the 100% level and killing is expressed as percentage decrease in cell numbers compared to control (**P<0.01, ANOVA). Data are representative of five independent experiments.



Online Supplementary Figure S2. Titration of CD20 monoclonal antibody (mAb) in the EL4-CD20 model using high and low tumor burden conditions. EL4-CD20 intraperitoneal model: mice were injected i.p. with CFSE-labeled EL4-CD20 cells and 16 h later were given CD20 mAb or phosphate-buffered saline (PBS). After 24 h the number of tumor cells in a peritoneal wash was determined using TrueCount tubes (4-8 mice / group; ***P<0.001, **P<0.01, *P<0.05; ANOVA). (A) Wild-type (WT) mice were injected with 5x10^5 EL4-CD20 cells and 10 µg non-specific human IgG or various concentrations of ofatumumab (OFA). WT mice were injected with 5x10^6 EL4-CD20 cells and various amounts of OFA (B), rituximab (RTX) (C) or 11B8 (D).



Online Supplementary Figure S3. Expression of FcγR and number of F4/80$^+$ effector cells under high and low tumor burden conditions. (A) wild-type (WT) or CR3$^{-/-}$ mice were injected with low (5x10^5) or high (5x10^6) numbers of EL4-CD20 cells and the peritoneal cavity was washed 36 h later. The expression of all mouse FcγR was analyzed on F4/80$^+$ macrophages. (B) The number of recruited Gr-1$^+$ neutrophils (white bars, left Y axis) and F4/80$^+$ macrophages (black bars, right Y axis) is plotted relative to 10000 beads.

Online Supplementary Table S1. Average total numbers of EL4-CD20 cells in all experiments recovered from the peritoneum 24 h after CD20 monoclonal antiboby administration. Average fold reduction is calculated compared to numbers from mice treated with phosphate-buffered saline.

		Median	Minimum	Maximum	Average reduction
Low tumor burden ($5x10^5$)	PBS	$3.9x10^5$	$1.2x10^5$	$14.2x10^5$	
	OFA	$0.17x10^5$	$0.01x10^5$	$0.46x10^5$	95.63%
	RTX	$0.18x10^5$	$0.02x10^5$	$0.56x10^5$	95.37%
	11B8	$1.16x10^5$	$0.001x10^5$	$3.5x10^5$	70.23%
High tumor burden ($5x10^6$)	PBS	$17.76x10^6$	$1.75x10^6$	$34.98x10^6$	
	OFA	$5.32x10^6$	$0.77x10^6$	$21.15x10^6$	69.96%
	RTX	$6.07x10^6$	$0.09x10^6$	$16.7x10^6$	65.75%
	11B8	$14.13x10^6$	$4.95x10^6$	$21.16x10^6$	20%