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Supplemental Information

Targeted *In Vivo* Delivery of NF-κB Decoy Inhibitor Augments Sensitivity of B Cell Lymphoma to Therapy

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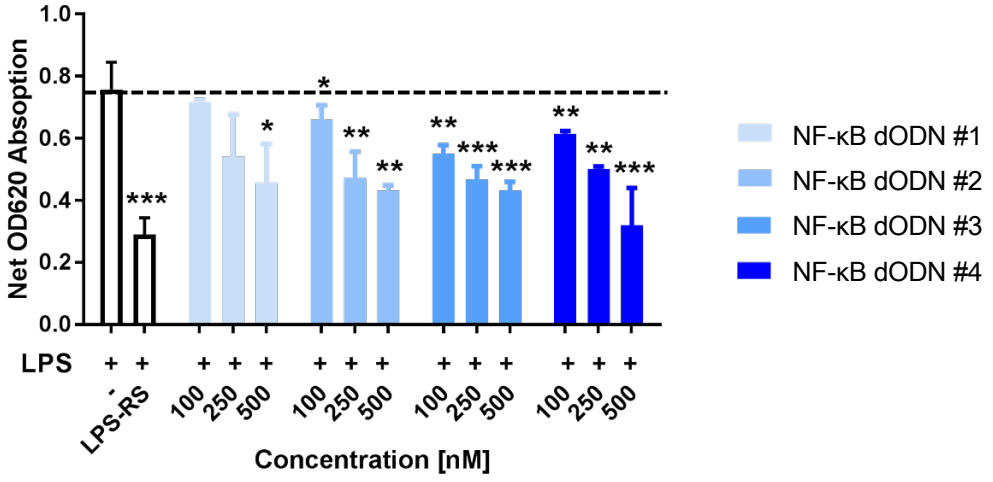
A NF-kBdODN sequences:

	Modified Sequences	References
#1	5’ - ooooo - TGGGGACTTTCCA - oooo - TGGAAAGTCCCCA - ooooo - 3’	(1)
#2	5’ - ooooo - TGGAAAGTCCCCA - oooo - TGGGGACTTTCCA - ooooo – 3’	(1)
#3	5’ - ooooo - G*A*T*CGAGGGGACTTTCCCTAGC - oooo - GCTAGGGAAAGTCCCCTCG*A*T*C - ooooo - 3'	(2)
#4	5’ - ooooo - C*C*T*TGAAGGGATTTCCCTCC - oooo – GGAGGGAAATCCCTTCA*A*G*G - ooooo - 3’	(3)

B



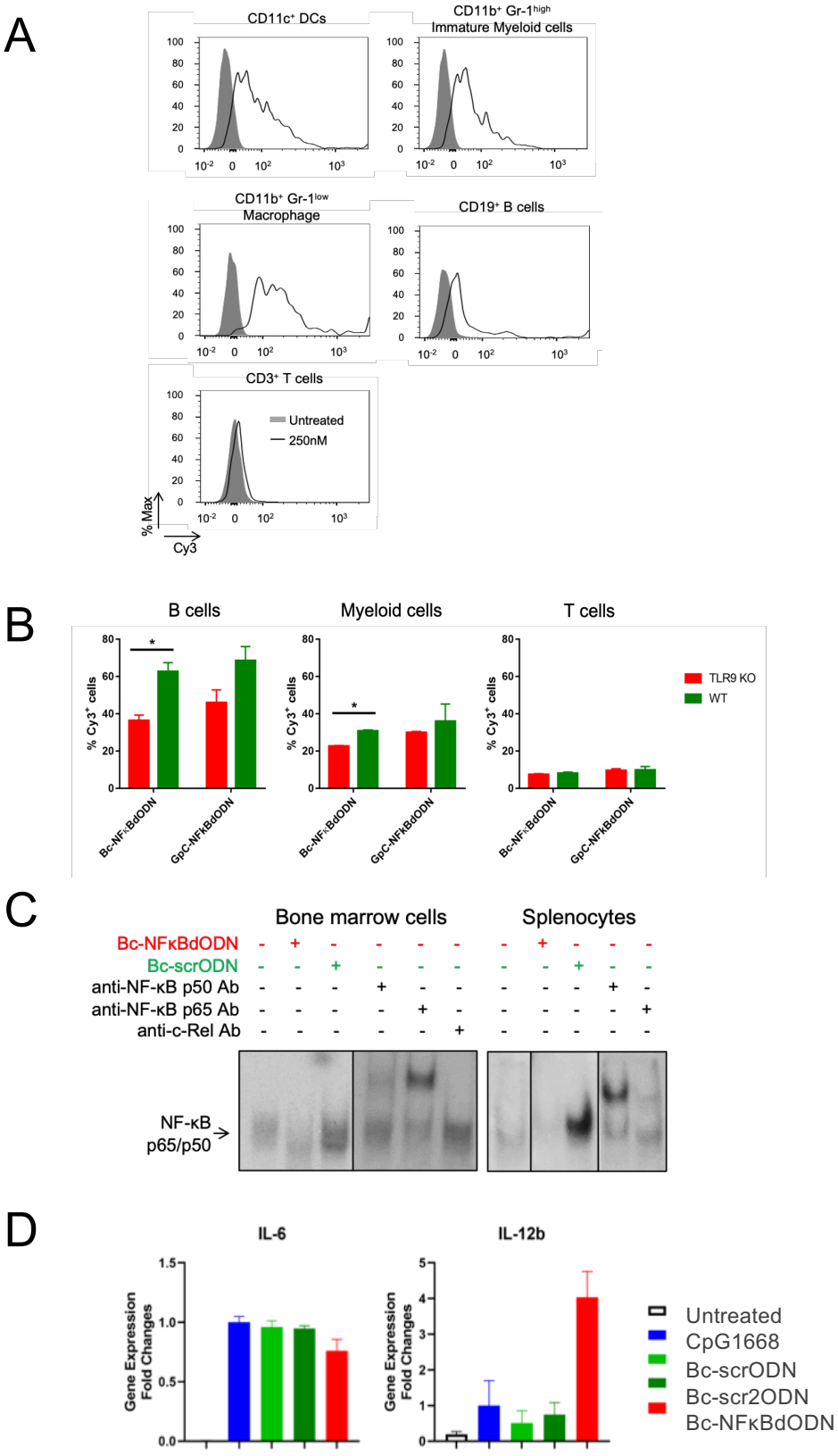
C

Bc-NFκBdOD$^{FITC/Alexa555}$:

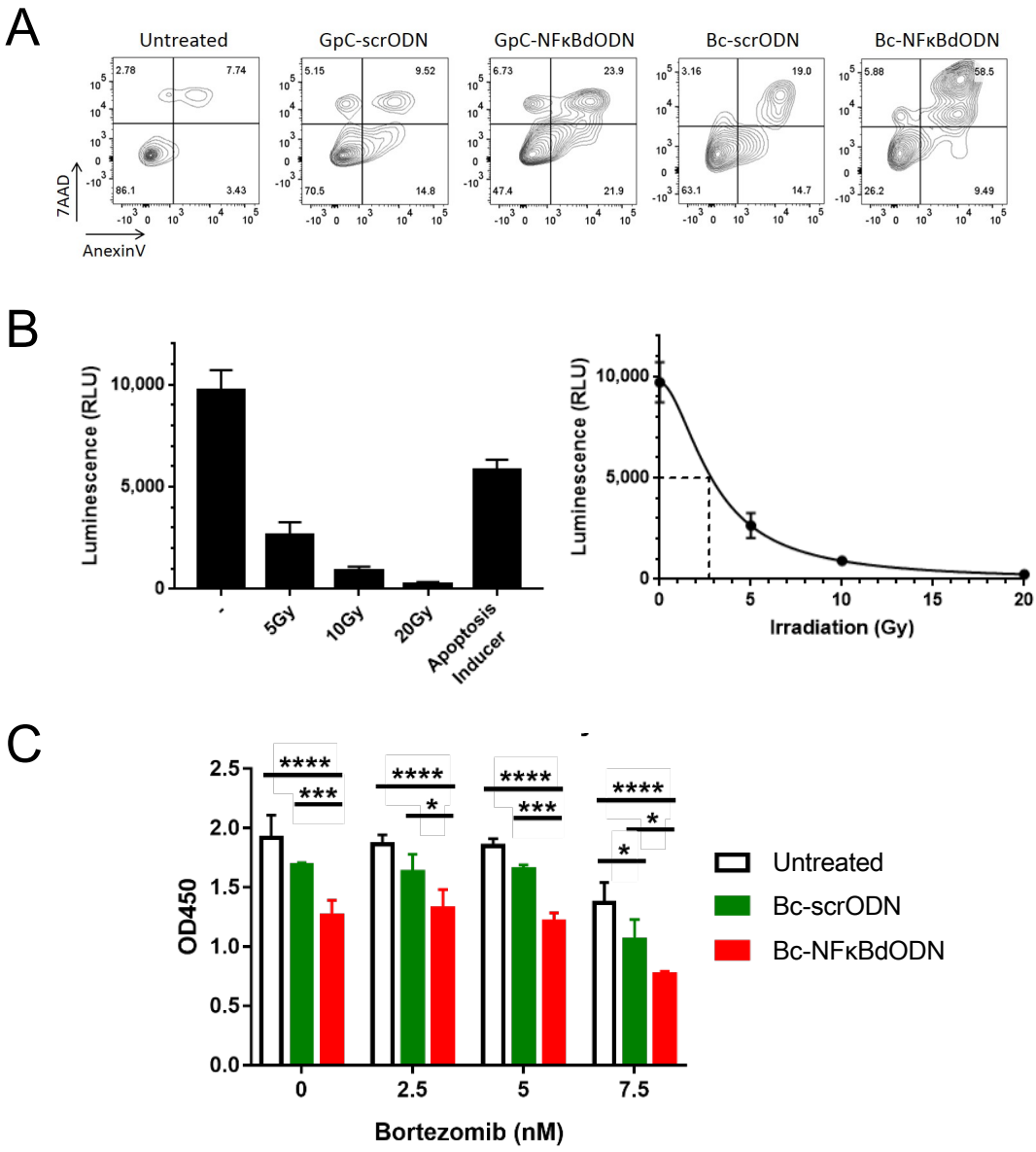
5’ T*C*C*A*T*G*A*C*G*T*T*C*C*T*G*A*T*G*C*T oo-**FITC**-oo C*C*T* TGA AGG GAT TTC CCT CC oo-**Alexa555**-oo GG AGG GAA ATC CCT TCA *A*G*G oooo 3’

o = propandiol linker (C3 unit)

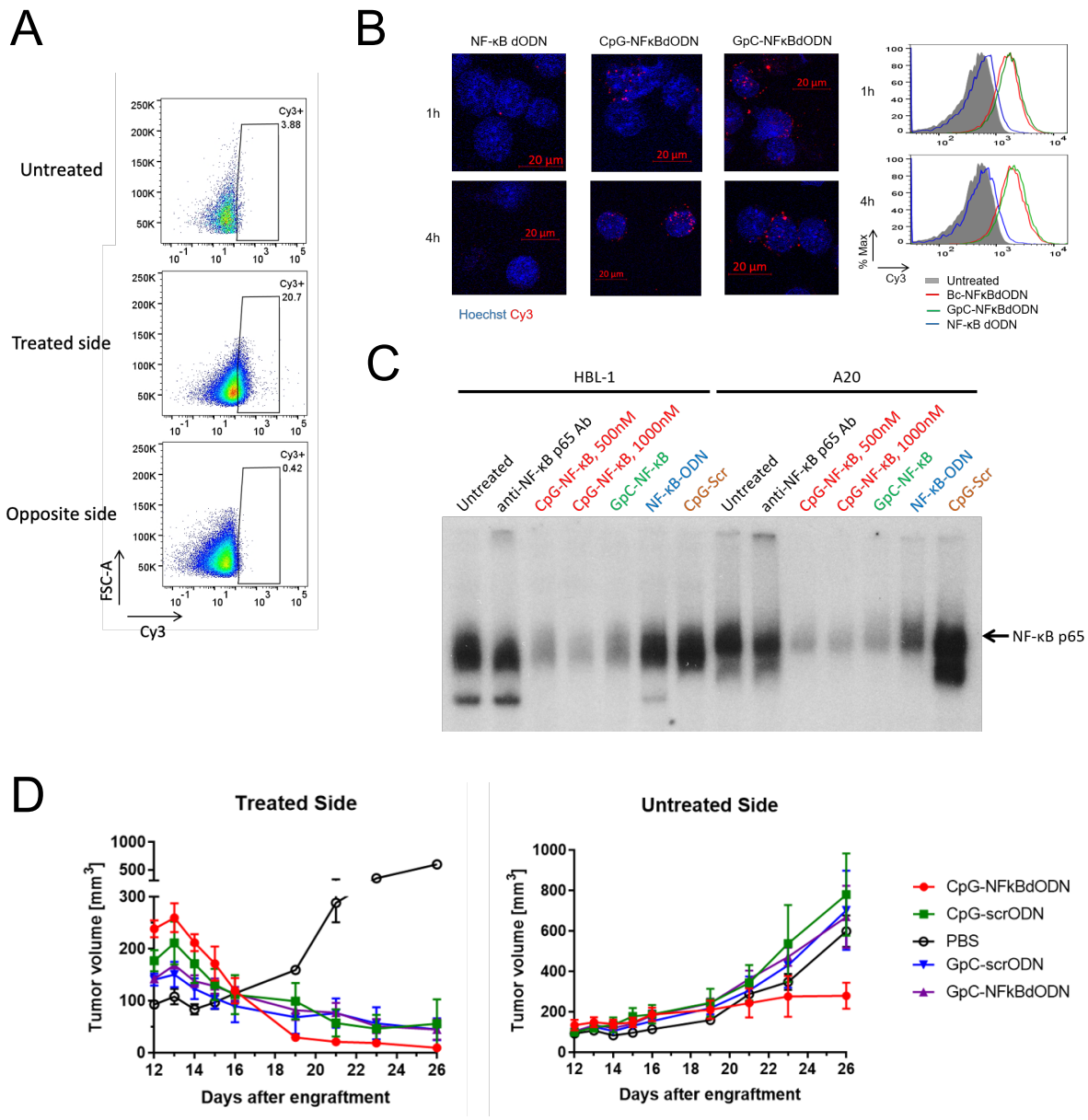
Supplemental Figure S1. Design of Bc(CpG)-NFκBdODN conjugates for selective targeting of myeloid immune cells and B cell lymphoma cells. (A) NFκB decoy ODN (dODN) sequences tested in this study. o means carbon linker, * indicates phosphorothioation. (**B)** RAW-Blue cells were treated three times every day using various NF-κB dODNs, 1 μg/mL LPS-RS as a negative control, then stimulated using 100 ng/mL LPS overnight. SEAP activity was measured colorimetrically. Shown are means±SEM (*n*=3/group). All statistical significance comparing with the untreated control, * p<0.05, ** p<0.01, *** p<0.001. **(C)** Sequence of an additional control conjugate used in the study, a dual-fluorescent Bc-NFκBdOD$^{FITC/Alexa555}$.



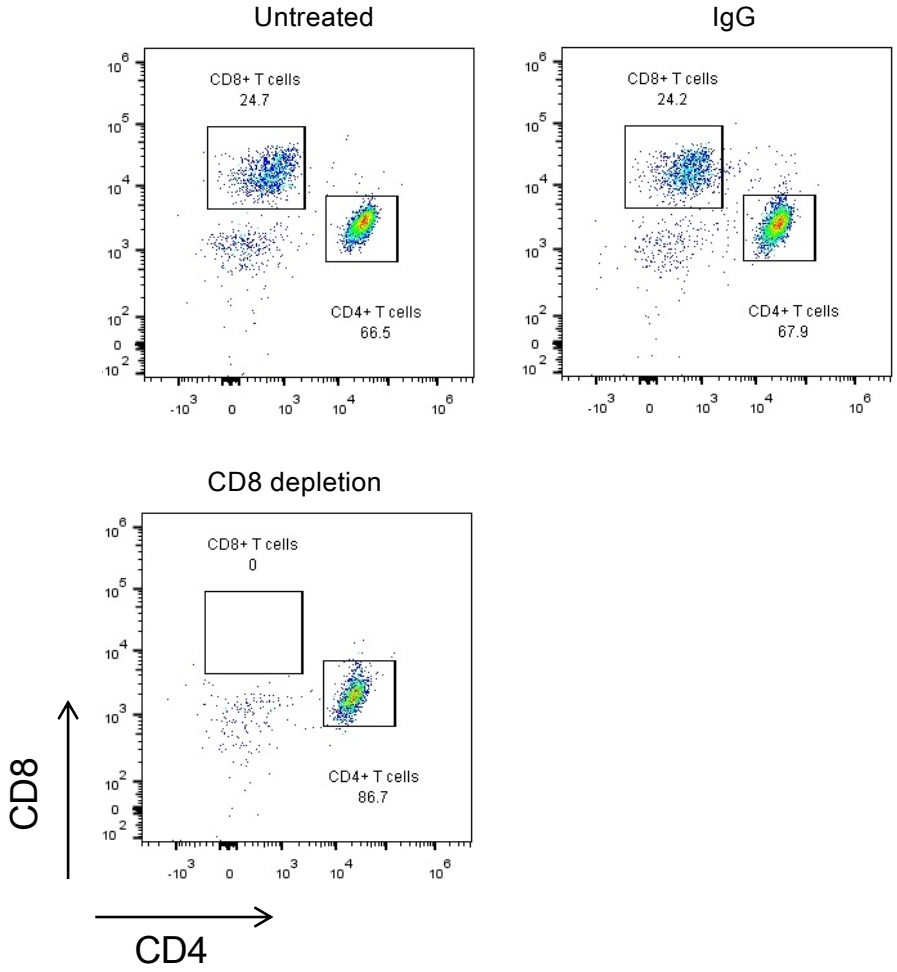
Supplemental Figure S2. Uptake and intracellular activity of Bc-NFκBdODN on mouse immune cells. (**A, B**) Single cell suspensions of freshly isolated mouse splenocytes from wild-type (A, B) or *Tlr9KO* (B) mice were incubated with 250 nM (A) or 100 nM (B) Cy3-labeled Bc-NFκBdODN overnight. Uptake of the constructs by specific immune cell populations were examined using flow cytometry. (**C**) Freshly isolated mouse bone marrow cells (top) or splenocytes (bottom) were stimulated with LPS (as above) and then treated using 1μM Bc-NFκBdODN, Bc-scrODN or NF-κB dODN. The NF-κB DNA-binding was assessed using gelshift (EMSA); antibodies specific to p65, p50 and c-Rel were used for supershift controls. Shown are representative images from one of three independent experiments. (**D**) Bc-NFκBdODN treatment altered expression of cytokine genes *in vitro*. RAW264.7 cells were treated for two days using 500 nM Bc-NFκBdODN, Bc-scrODN (two different sequences) or CpG1668 alone and the expression of *Il6* and *Il12B* genes was assessed using real-time qPCR (means ± SD).



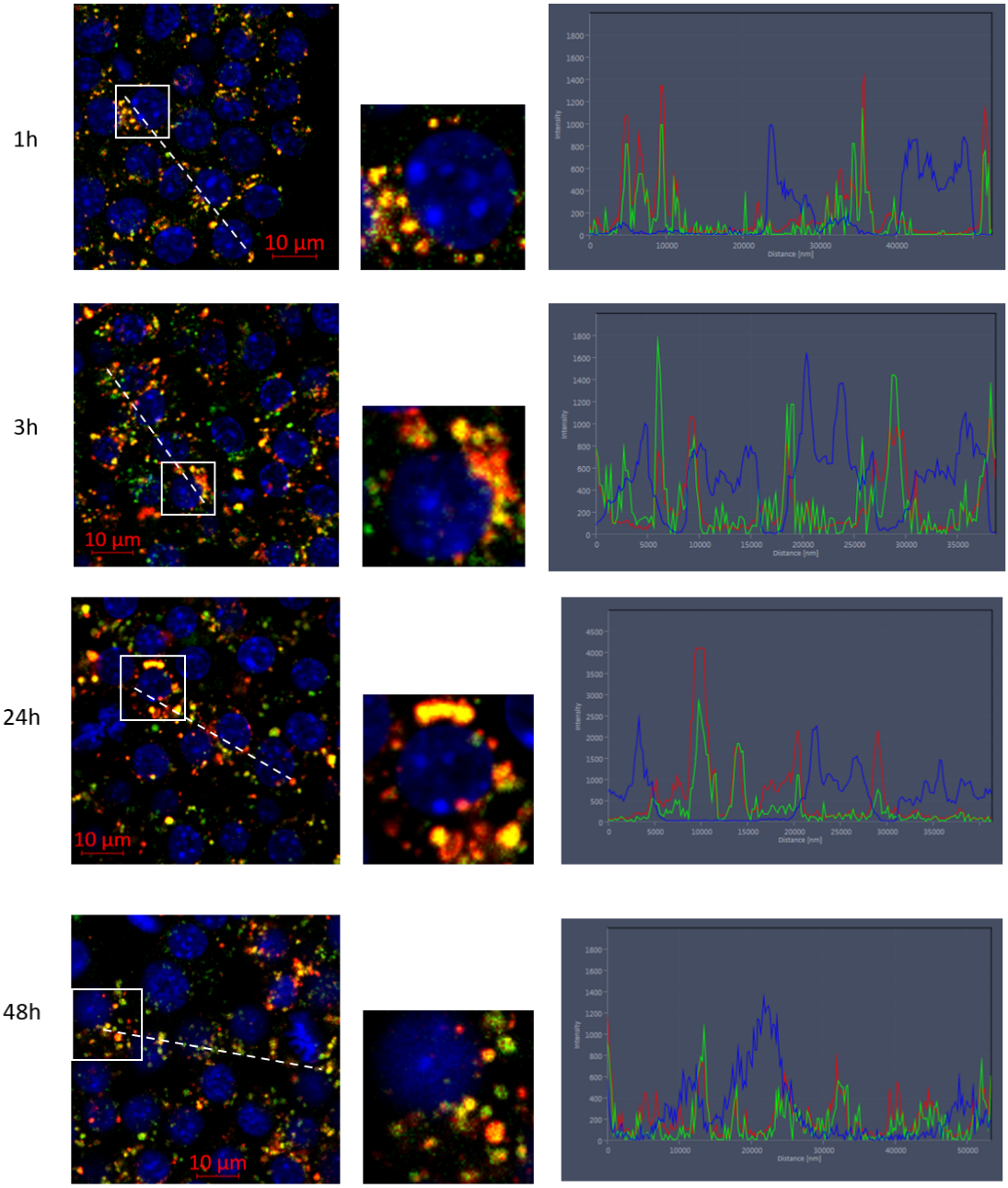
Supplemental Figure S3. Cytotoxic effects of various treatments types on human lymphoma cells in vitro. (**A**) Lack of TLR9 stimulation does not prevent cytotoxic effects resulting from the inhibition of NF-κB survival signaling in human lymphoma cells. U2932 cells were treated using 5 μM Bc-NFκBdODN, Bc-scrODN, GpC-NFκBdODN, GpC-scrODN or left untreated for 2 days. Cell viability has been measured using 7AAD and Annexin V staining using flow cytometry. **(B)** Dose-dependent effect of radiation on viability of human B cell lymphoma OCI-Ly3 cells were treated using series dosages of ionizing radiation. Cell death was measured by detection of the caspase 3 activation; shown is the LD_{50}. Apoptosis Inducer II (Thermo Fisher, MA) was used as positive control. (C**)** U2932 cells were treated with 500 nM Bc-NFκBdODN or Bc-scrODN and various concentrations of bortezomib daily for three days before testing cell viability using CCK-8 kit. Shown are means±SEM (n=3/group)



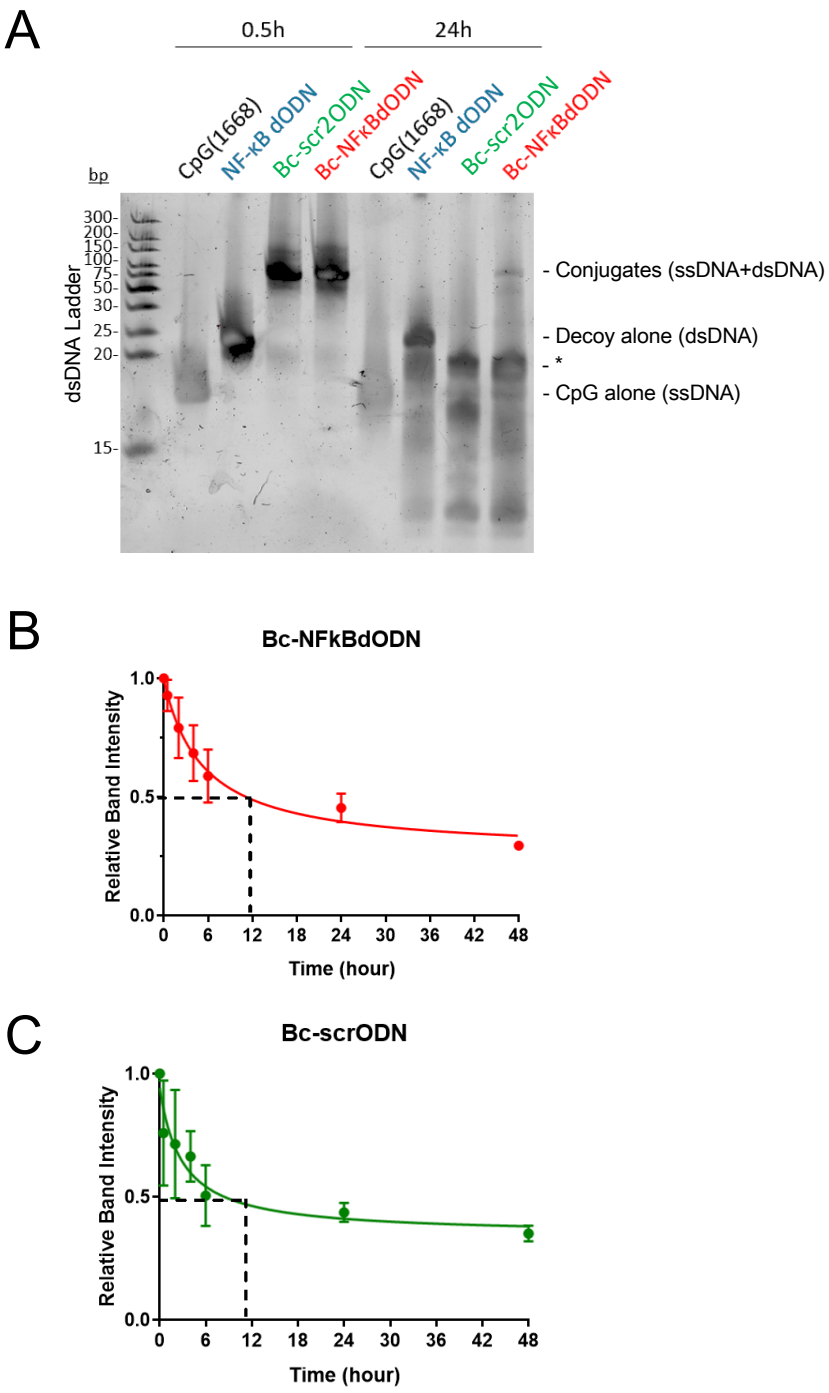
Supplemental Figure S4. TLR9 activation supports biological activity of Bc-NFκBdODN *in vivo*. (A) The biodistribution of intratumorally injected Bc-NFκBdODN to a distant tumor site. A20 cells were engrafted subcutaneously at both flanks of BALB/c mice, tumors on one side were injected using 1mg/kg Cy3-labeled Bc-NFκBdODN. Tumors from both sides were collected 3h post injection to detect oligonucleotide internalization using flow cytometry. **(B)** A20 cells were incubated in the presence of 100 nM Cy3-labeled Bc(CpG)-NFκBdODN or GpC-NFκBdODN for 1 or 4h then examined using confocal microscopy (left panels) or flow cytometry (right panels). **(C)** A20 or HBL-1 lymohoma cells were incubated in the presence of 500 or 1,000 nM Bc-NFκBdODN, GpC-NFκBdODN, NF-κB dODN or Bc-scrODN. Next, the cells were treated using LPS (100 ng/mL) for 30 min to activate NF-κB. The NF-κB DNA-binding activity was measured in nuclear extracts using specific, radiolabeled DNA binding probe and gel shift (EMSA) assay. **(D)** A20 cells were engrafted subcutaneously in both flanks of BALB/c mice. Tumors on one side were treated with 1mg/kg Bc-NFκBdODN or Bc-scrODN or 5mg/kg GpC-NFκBdODN or GpC-scrODN every day intratumorally for 2 weeks while tumors on the other side were left untreated; n=5/each group, shown are means ± SEM.



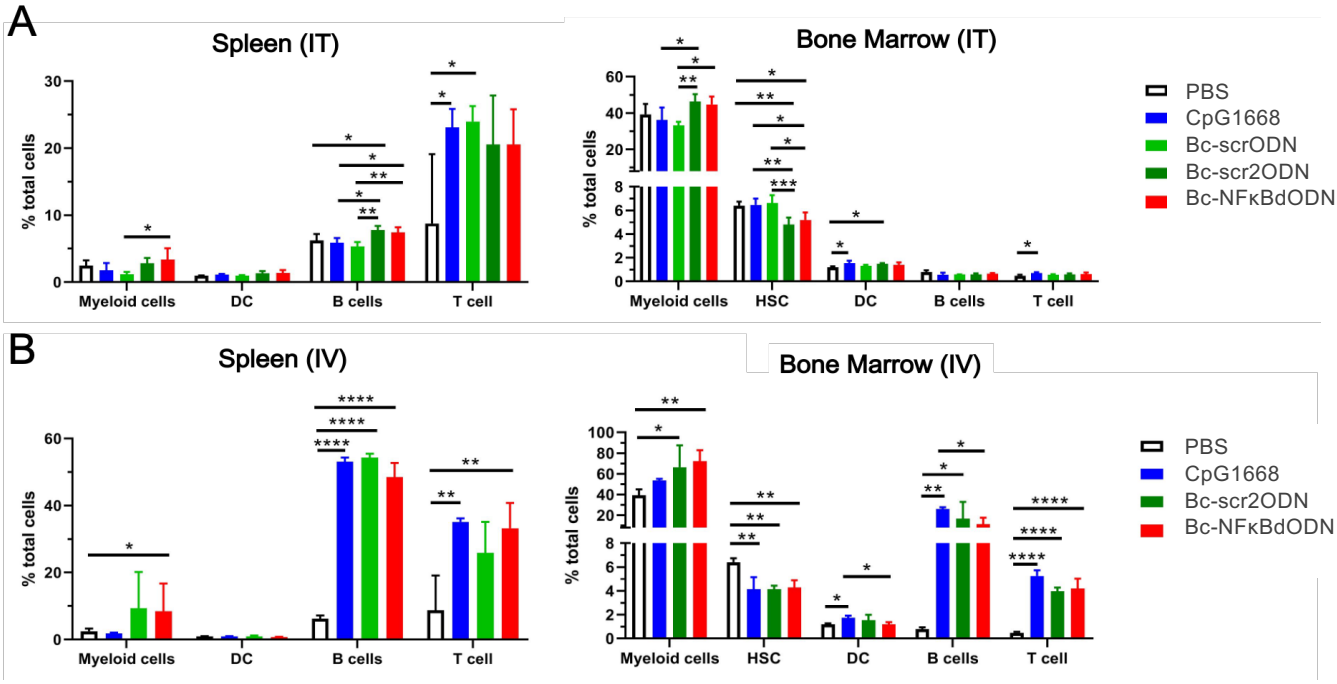
Supplemental Figure S5. Verification of the CD8 T cell depletion in vivo. Mice were injected using 0.2 µg of CD8-specific antibodies 6 days after tumor engraftment, the percentages of circulating CD8 and CD4 T cells were verified using flow cytometry two days late. Shown are representative results (n=5/group).



Supplemental Figure S6. Bc-NFκBdODN conjugate undergoes partial intracellular cleavage separating both parts of the molecule. Mouse RAW264.7 macrophages were incubated in the presence of Bc-NFκBdODN$^{FITC/Alexa555}$ for 1, 3, 24 or 48 h. The colocalization of FITC and Cy3 signals, indicating Bc(CpG) and decoy part of the molecule was examined using confocal microscopy and analyzed using LSM ImageBrowser (version 4.2.0.121; Zeiss). DAPI was used for staining of nuclei.



Supplemental Figure S7. Serum stability of various tested oligonucleotides in human serum. (**A**) Bc-NFκBdODN, Bc-scrODN, NF-κB dODN or CpG ODN were incubated in 50% human serum for the indicated times and analyzed using 15% SDS/Urea PAGE gels. *, indicates position of a putative CpG-linker fragment generated as a result of decoy cleavage (**B, C**) Stability of Bc-NFκBdODN (**B**) and the Bc-scr2ODN (**C**) in 50% human serum. Quantification of bands using triplicate samples; means ± SEM.



Supplemental Figure S8. Local or systemic administration of Bc-NFκBdODN does not result in myeloablation or lymphodepletion. Mice-bearing A20 engrafted subcutaneously were treated every other day using 1mg/kg Bc-NFκBdODN, Bc-scrODN (old and/or new scrambled sequence), equivalent molar amount of CpG ODN injected intratumorally (IT) for 2 weeks (**A**) or intravenously (IV) for 4 weeks (**B**). Mice were then euthanized to harvest spleens and bone marrow for the analysis of immune cell profiles using flow cytometry; shown are means ± SEM (A: *n*=5/group; B: *n*=5/PBS group, *n*=4/Bc-NFκBdODN group, *n*=2/Bc-scrODN group, *n*=3/CpG ODN group).

Supplemental References

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