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Supplementary Materials for

Increased vaccine tolerability and protection via NF-κB modulation

B. A. Moser, R. C. Steinhardt, Y. Escalante-Buendia, D. A. Boltz, K. M. Barker, B. J. Cassaidy, M.G. Rosenberger, S. Yoo, B. G. McGonnigal, A. P. Esser-Kahn*

*Corresponding author. Email: aesserkahn@uchicago.edu

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This PDF file includes:

Figs. S1 to S14
References

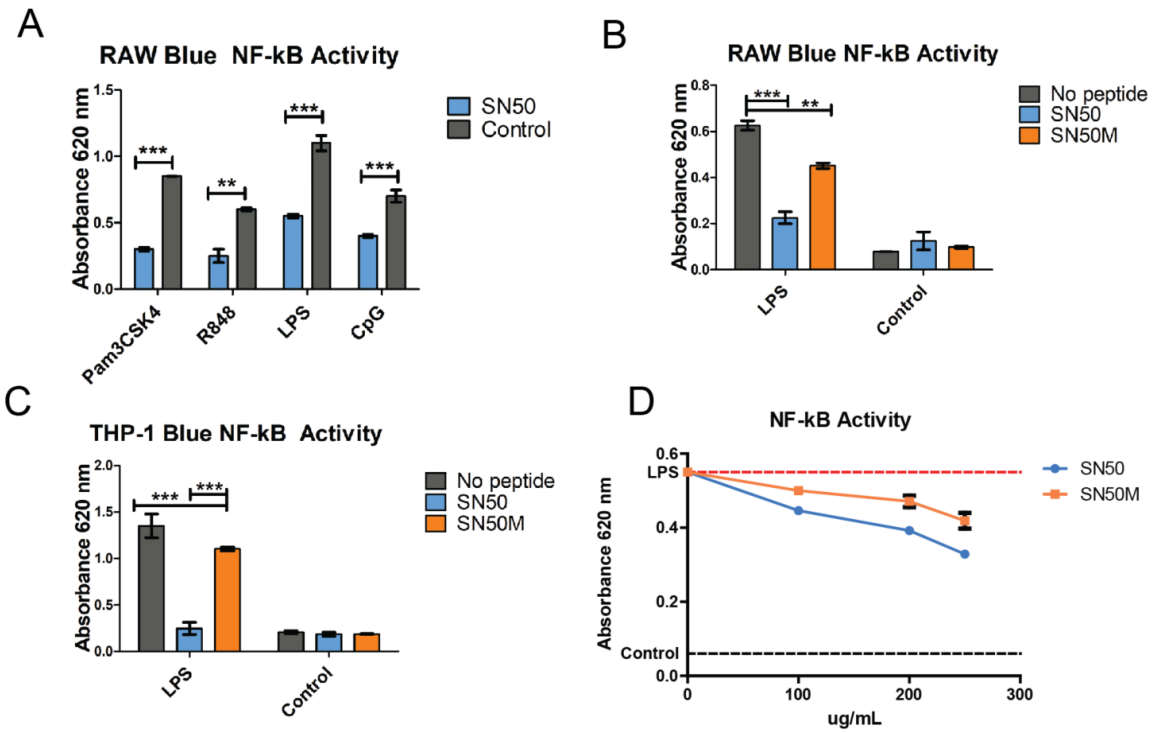

Figure S1. NF-kB activity in mouse and human cells. (A) NF-kB activity in RAW blue macrophages stimulated with various TLR agonists and SN50 (blue bars), TLR agonists alone (grey bars). Pam3CSK4 (100 ng/mL), R848 (1 µg/mL) LPS (10 ng/mL), CpG (5 µg/mL), SN50 (250 µg/mL). (B) RAW blue NF-kB activity of cells stimulated with SN50 (blue bars) and the control peptide SN50M (grey bars). SN50 and SN50M (250 µg/mL) (C) THP-1 NF-kB activity of cells stimulated with no peptide (grey bars), SN50 (blue bars) or SN50M (orange bars). LPS (1 µg /mL), SN50 and SN50M (150 µg/mL). (D) Concentration screen of 100 ng/ mL LPS and various concentrations of SN50 (blue line) and SN50M (orange line).

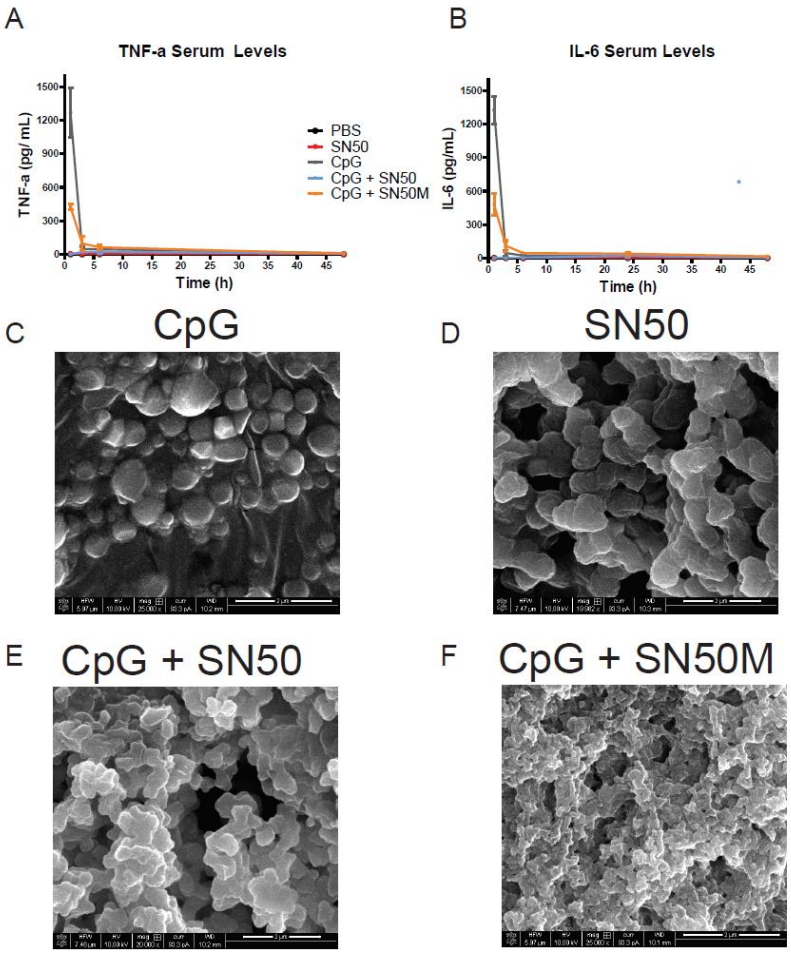

Figure S2. Proinflammatory Cytokine Analysis Time Course (A) Systemic TNF- α levels measured at 1h, 3h, 6h, 24, 48 h post vaccination. n = 3. (B) Systemic IL-6 levels. (C-F) SEM image of OVA vaccinations. (C) CpG + OVA (D) SN50 + OVA (E) CpG + SN50 + OVA (F) CpG + SN50M + OVA. Scale bar 2 um.

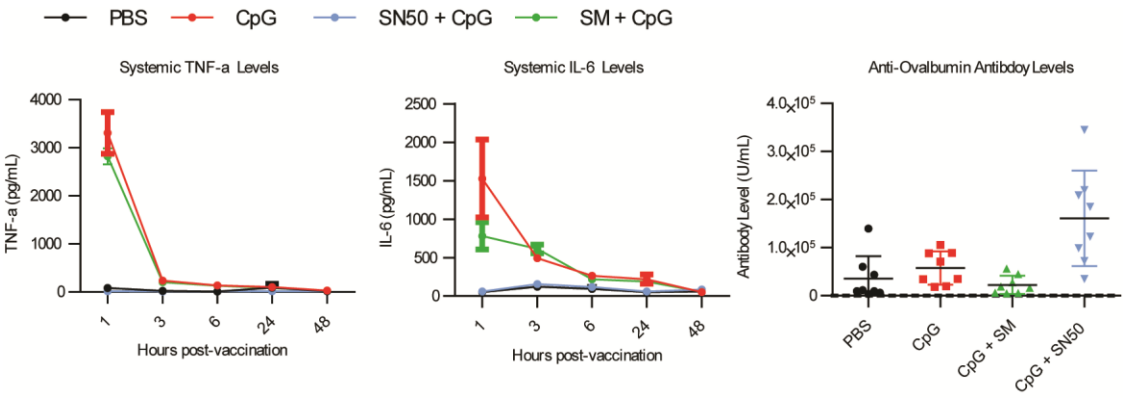

Figure S3. SM control peptide vaccination. Systemic TNF-a and IL-6 levels 1h post-vaccination and Anti-Ovalbumin antibody level on day 28 post-vaccination with: 50 ug CpG (red), CpG + 500 ug SN50 (blue), CpG + 500 ug SM (green) or PBS (black).

Gene expression fold change relative to OVA in draining lymph node

Gene	OVA	CpG	p value	CpG +SN50	p value
Gapdh	1.0	1.0		1.0	
Hprt1	1.0	1.4	0.820	1.5	0.396
Gusb	1.0	2.6	0.216	1.5	0.093
Agtr2	1.0	2.1	0.003	0.7	0.264
Bax	1.0	4.6	0.035	2.3	0.004
Bcl2	1.0	3.7	0.324	2.4	0.047
Bcl2l1	1.0	4.6	0.829	4.3	0.358
C3	1.0	1.4	0.165	2.7	0.404
Ccl19	1.0	1.4	0.584	1.1	0.067
Ccl2	1.0	19.4	0.067	52.7	0.007
Ccl3	1.0	17.9	0.527	23.0	0.002
Ccl5	1.0	2.0	0.333	1.6	0.155
Ccr2	1.0	0.6	0.076	1.0	0.027
Ccr4	1.0	0.7	0.238	1.0	0.423
Ccr7	1.0	2.8	0.281	2.2	0.152
Cd19	1.0	4.2	0.389	3.2	0.094
Cd28	1.0	1.2	0.308	0.9	0.199
Cd34	1.0	1.4	0.139	0.5	0.322
Cd38	1.0	6.0	0.028	2.6	0.004
Cd3e	1.0	2.8	0.110	1.5	0.043
Cd4	1.0	4.1	0.904	4.0	0.289
Cd40	1.0	8.1	0.436	6.5	0.015
Cd40lg	1.0	1.1	0.076	0.6	0.467
Cd68	1.0	1.4	0.062	2.4	0.153
Cd80	1.0	5.1	0.919	5.3	0.060
Cd86	1.0	4.5	0.154	7.6	0.004
Cd8a	1.0	1.9	0.720	1.7	0.042
Csf1	1.0	5.3	0.026	17.4	0.080
Csf2	1.0	1.9	0.888	2.0	0.337
Csf3	1.0	0.4	0.016	1.2	0.122
Ctla4	1.0	2.2	0.695	1.9	0.094
Cxcl10	1.0	25.7	0.027	88.8	0.020
Cxcl11	1.0	45.5	<0.001	425.7	0.005
Cxcr3	1.0	4.0	0.042	0.9	0.173
Cyp1a2	1.0	2.1	0.003	0.7	0.264
Cyp7a1	1.0	2.1	0.003	0.7	0.264
Edn1	1.0	3.3	0.005	33.3	0.013
Fas	1.0	2.6	0.780	2.8	0.003
Fasl	1.0	1.2	0.274	1.0	0.711
Fn1	1.0	1.1	0.005	3.7	0.822
Gzmb	1.0	109.1	0.217	157.9	0.038
H2-Ea	1.0	3.0	0.090	0.9	0.091
H2-Eb1	1.0	1.8	0.595	1.4	0.044
Hmox1	1.0	2.0	0.734	2.8	0.636
Icos	1.0	1.9	0.087	0.9	0.329
Ifng	1.0	9.6	0.012	55.2	0.028
Ikbkb	1.0	1.4	0.217	0.9	0.372

Gene	OVA	CpG	p value	CpG +SN50	p value
Il10	1.0	13.0	0.766	10.5	0.080
Il12a	1.0	4.2	0.255	1.7	0.095
Il12b	1.0	6.9	0.810	7.9	0.056
Il13	1.0	1.7	0.101	0.7	0.304
Il15	1.0	4.0	0.853	4.4	0.044
Il17a	1.0	1.4	0.076	0.5	0.672
Il18	1.0	2.7	0.236	1.7	0.057
Il1a	1.0	2.0	0.473	4.1	0.509
Il1b	1.0	13.2	0.765	15.4	0.013
Il2	1.0	0.9	0.236	0.4	0.619
Il2ra	1.0	2.0	0.726	1.6	0.372
Il3	1.0	2.1	0.003	0.7	0.264
Il4	1.0	2.2	0.173	0.5	0.412
Il5	1.0	2.1	0.003	0.7	0.264
Il6	1.0	278.1	0.425	146.6	0.003
Il7	1.0	0.7	0.003	0.2	0.647
Il9	1.0	2.1	0.003	0.7	0.264
Lrp2	1.0	0.4	0.541	0.6	0.047
Lta	1.0	2.1	0.003	0.7	0.264
Nfkb1	1.0	4.4	0.523	3.1	0.146
Nfkb2	1.0	1.1	0.991	1.1	0.884
Nos2	1.0	6.9	0.104	126.9	0.208
Prf1	1.0	4.4	0.119	2.4	0.104
Ptgs2	1.0	2.2	0.092	4.8	0.271
Ptprc	1.0	2.0	0.656	1.5	0.351
Sele	1.0	2.1	0.003	0.7	0.264
Selp	1.0	2.6	0.015	0.6	0.233
Ski	1.0	3.3	0.414	1.8	0.403
Smad3	1.0	3.7	0.407	2.1	0.414
Smad7	1.0	3.9	0.162	2.0	0.340
Socs1	1.0	1.0	0.786	1.1	0.970
Socs2	1.0	1.1	0.425	0.7	0.804
Stat1	1.0	7.4	0.726	8.8	0.014
Stat3	1.0	4.3	0.870	4.9	0.112
Stat4	1.0	2.5	0.345	1.5	0.100
Stat6	1.0	2.6	0.709	2.0	0.140
Tbx21	1.0	7.7	0.243	3.3	0.047
Tgfb1	1.0	1.8	0.186	0.9	0.215
Tnf	1.0	2.5	0.615	3.5	0.196
Tnfrsf1	1.0	2.4	0.287	1.6	0.039
Vcam1	1.0	1.1	0.768	0.8	0.903
Vegfa	1.0	5.1	0.338	2.8	0.106
Ace	1.0	2.9	0.575	3.8	0.134
Icam1	1.0	3.1	0.660	4.4	0.196
Lif	1.0	5.4	0.516	8.7	0.041
Ly96	1.0	4.2	0.138	2.0	0.015
Nfatc3	1.0	2.3	0.263	1.1	0.170
Nfatc4	1.0	0.8	0.291	0.2	0.546

Fig. S4. Gene expression in draining lymph node 24h post-vaccination (fold change relative to OVA vaccination) n = 3.

Gene expression fold change relative to CpG in draining lymph node

Gene	OVA	p value	CpG	CpG + SN50	p value
Gapdh	1.0		1.0	1.0	
Hprt1	0.7	0.820	1.0	1.1	0.134
Gusb	0.4	0.216	1.0	0.6	0.473
Agtr2	0.5	0.003	1.0	0.3	0.471
Bax	0.2	0.035	1.0	0.5	0.020
Bcl2	0.3	0.324	1.0	0.6	0.154
Bcl2l1	0.2	0.829	1.0	0.9	0.379
C3	0.7	0.165	1.0	1.9	0.054
Ccl19	0.7	0.584	1.0	0.7	0.910
Ccl2	0.1	0.067	1.0	2.7	0.000
Ccl3	0.1	0.527	1.0	1.3	0.001
Ccl5	0.5	0.333	1.0	0.8	0.254
Ccr2	1.7	0.076	1.0	1.7	0.842
Ccr4	1.5	0.238	1.0	1.5	0.970
Ccr7	0.4	0.281	1.0	0.8	0.213
Cd19	0.2	0.389	1.0	0.8	0.142
Cd28	0.8	0.308	1.0	0.7	0.592
Cd34	0.7	0.139	1.0	0.4	0.273
Cd38	0.2	0.028	1.0	0.4	0.021
Cd3e	0.4	0.110	1.0	0.5	0.301
Cd4	0.2	0.904	1.0	1.0	0.296
Cd40	0.1	0.436	1.0	0.8	0.025
Cd40lg	0.9	0.076	1.0	0.6	0.130
Cd68	0.7	0.062	1.0	1.7	0.025
Cd80	0.2	0.919	1.0	1.0	0.044
Cd86	0.2	0.154	1.0	1.7	0.004
Cd8a	0.5	0.720	1.0	0.9	0.152
Csf1	0.2	0.026	1.0	3.3	0.018
Csf2	0.5	0.888	1.0	1.1	0.339
Csf3	2.5	0.016	1.0	3.0	0.663
Ctla4	0.4	0.695	1.0	0.9	0.129
Cxcl10	0.0	0.027	1.0	3.5	0.004
Cxcl11	0.0	0.000	1.0	9.4	0.002
Cxcr3	0.2	0.042	1.0	0.2	0.883
Cyp1a2	0.5	0.003	1.0	0.3	0.471
Cyp7a1	0.5	0.003	1.0	0.3	0.471
Edn1	0.3	0.005	1.0	10.1	0.014
Fas	0.4	0.780	1.0	1.1	0.023
Fasl	0.8	0.274	1.0	0.8	0.938
Fn1	0.9	0.005	1.0	3.4	0.021
Gzmb	0.0	0.217	1.0	1.4	0.028
H2-Ea	0.3	0.090	1.0	0.3	0.910
H2-Eb1	0.5	0.595	1.0	0.8	0.507
Hmox1	0.5	0.734	1.0	1.4	0.452
Icos	0.5	0.087	1.0	0.5	0.851
Ifng	0.1	0.012	1.0	5.8	0.003
Ikbkb	0.7	0.217	1.0	0.7	0.709

Gene	OVA	p value	CpG	CpG + SN50	p value
Il10	0.1	0.766	1.0	0.8	0.104
Il12a	0.2	0.255	1.0	0.4	0.341
Il12b	0.1	0.810	1.0	1.1	0.046
Il13	0.6	0.101	1.0	0.4	0.559
Il15	0.3	0.853	1.0	1.1	0.059
Il17a	0.7	0.076	1.0	0.4	0.437
Il18	0.4	0.236	1.0	0.6	0.243
Il1a	0.5	0.473	1.0	2.1	0.060
Il1b	0.1	0.765	1.0	1.2	0.002
Il2	1.1	0.236	1.0	0.5	0.210
Il2ra	0.5	0.726	1.0	0.8	0.467
Il3	0.5	0.003	1.0	0.3	0.471
Il4	0.4	0.173	1.0	0.2	0.584
Il5	0.5	0.003	1.0	0.3	0.471
Il6	0.0	0.425	1.0	0.5	0.003
Il7	1.4	0.003	1.0	0.3	0.123
Il9	0.5	0.003	1.0	0.3	0.471
Lrp2	2.3	0.541	1.0	1.4	0.437
Lta	0.5	0.003	1.0	0.3	0.471
Nfkb1	0.2	0.523	1.0	0.7	0.224
Nfkb2	0.9	0.991	1.0	1.0	0.829
Nos2	0.1	0.104	1.0	18.4	0.001
Prf1	0.2	0.119	1.0	0.6	0.245
Ptgs2	0.4	0.092	1.0	2.1	0.084
Ptprc	0.5	0.656	1.0	0.8	0.581
Sele	0.5	0.003	1.0	0.3	0.471
Selp	0.4	0.015	1.0	0.2	0.502
Ski	0.3	0.414	1.0	0.6	0.658
Smad3	0.3	0.407	1.0	0.6	0.610
Smad7	0.3	0.162	1.0	0.5	0.613
Socs1	1.0	0.786	1.0	1.1	0.804
Socs2	0.9	0.425	1.0	0.7	0.475
Stat1	0.1	0.726	1.0	1.2	0.018
Stat3	0.2	0.870	1.0	1.1	0.084
Stat4	0.4	0.345	1.0	0.6	0.425
Stat6	0.4	0.709	1.0	0.8	0.265
Tbx21	0.1	0.243	1.0	0.4	0.120
Tgfb1	0.6	0.186	1.0	0.5	0.754
Tnf	0.4	0.615	1.0	1.4	0.031
Tnfrsf18	0.4	0.287	1.0	0.6	0.270
Vcam1	0.9	0.768	1.0	0.8	0.821
Vegfa	0.2	0.338	1.0	0.6	0.236
Ace	0.3	0.575	1.0	1.3	0.091
Icam1	0.3	0.660	1.0	1.4	0.099
Lif	0.2	0.516	1.0	1.6	0.051
Ly96	0.2	0.138	1.0	0.5	0.141
Nfatc3	0.4	0.263	1.0	0.5	0.812
Nfatc4	1.3	0.291	1.0	0.3	0.219

Fig. S5. Gene expression in draining lymph node 24h post-vaccination (fold change relative to CpG vaccination) n = 3.

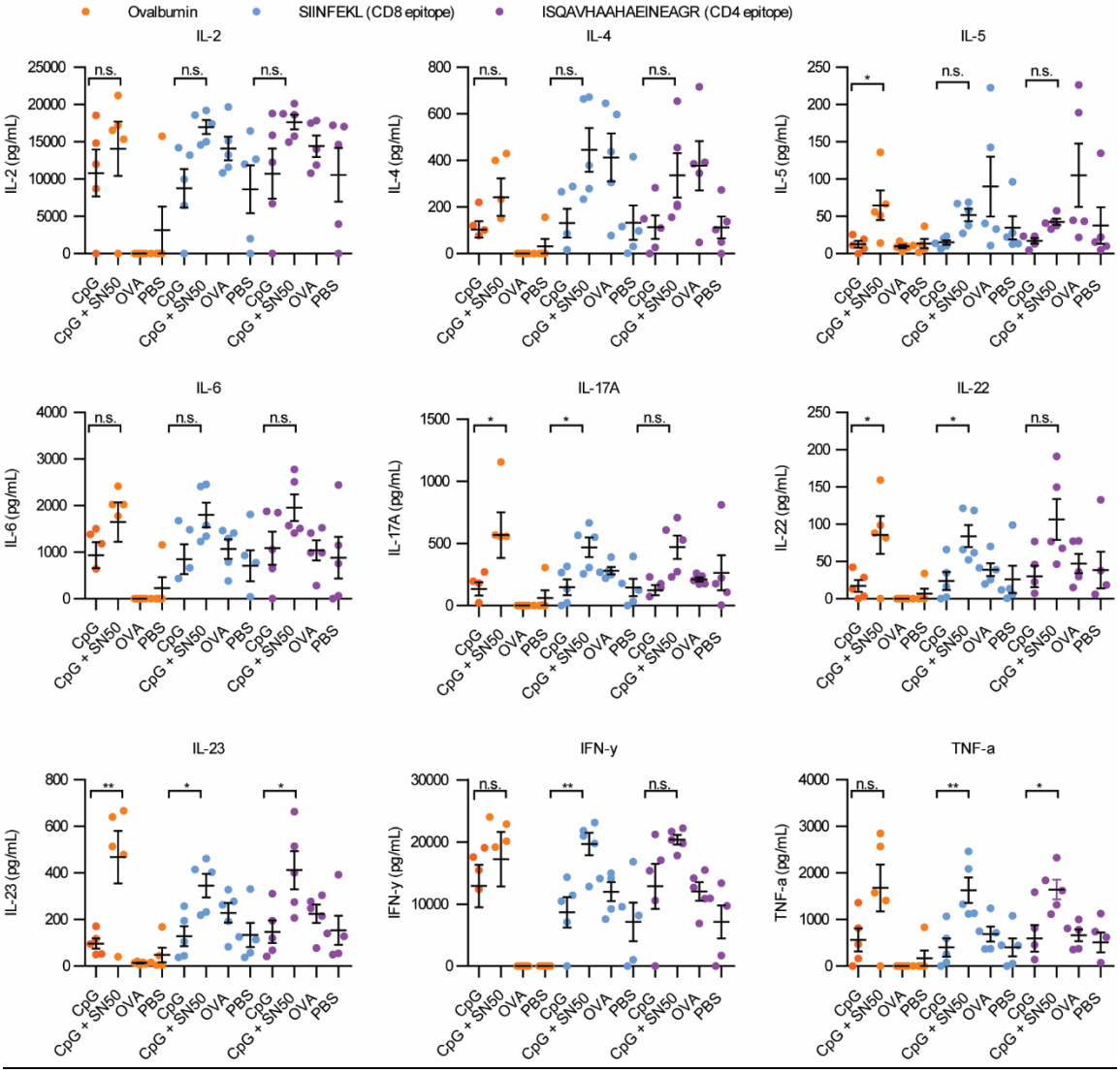

Figure S6. Splenocyte restimulation. Mice vaccinated with CpG + OVA (CpG), CpG + SN50 + OVA (CpG + SN50), OVA only (OVA), or PBS. Isolated splenocytes were stimulated with full ovalbumin protein (orange dots), SIINFEKL CD8 epitope (blue dots) or ISQAVHAAHAEINEAGR CD4 epitope (purple dots). n = 5.

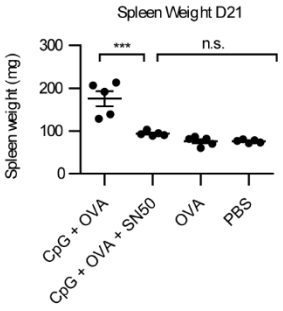

Figure S7. Spleen weight 21 days post-vaccination. n = 5

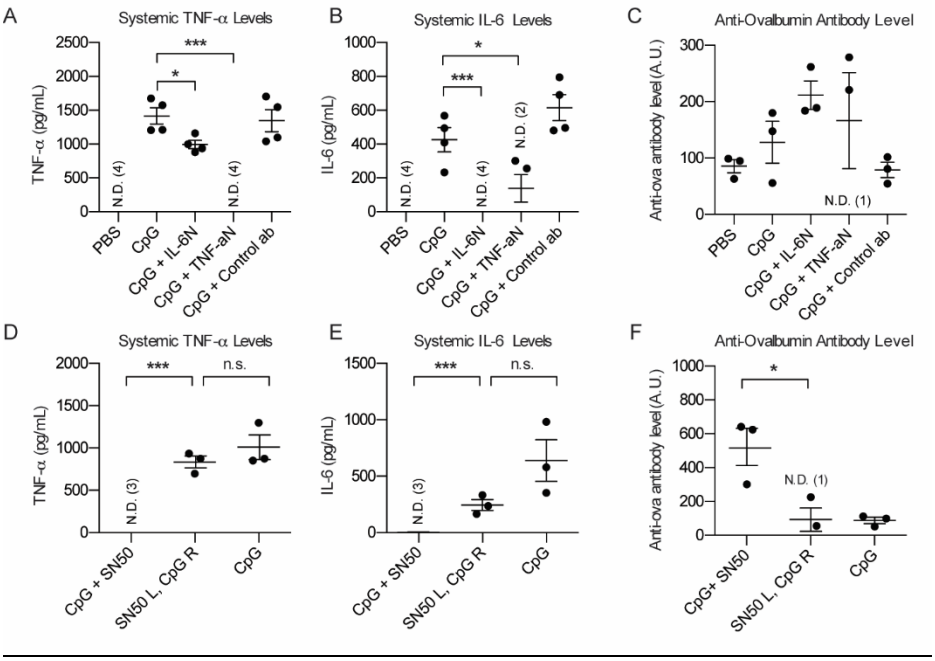

Figure S8. Exploration of SN50 mechanism of action. (A) Systemic TNF- α levels 1h post-vaccination with CpG, CpG + IL-6N, CpG + TNF- αN or CpG + Control ab, n = 4. (B) Systemic IL-6 levels 1h post-vaccination with CpG, CpG + IL-6N, CpG + TNF- αN or CpG + Control ab. (C) Anti-ovalbumin antibody level, day 28. (D) Systemic TNF-α levels in mice vaccinated with mixed CpG and SN50 (CpG + SN50), SN50 in left limb and CpG + OVA in right limb (SN50L, CpG R, or CpG alone, n =3. (E) Systemic IL-6 levels. (F) Anti-OVA antibody level, day 28.

To more directly examine how early systemic expression of TNF-α and IL-6 impact the immediate inflammatory response and downstream adaptive immune response. We vaccinated mice with CpG and either TNF- α neutralizing antibody (TNF- αN) or IL-6 neutralizing antibody (IL-6N) and measured the systemic cytokines (**Fig. S8**). The CpG + IL-6N group demonstrated a 1.4 -fold decrease in TNF- α expression and a complete reduction of systemic IL-6 expression. The CpG + TNF- α N group demonstrated complete elimination of systemic TNF- α and a 3-fold reduction of IL-6 expression. This result was confirmed by a control isotype antibody to rule out any nonspecific interactions. Although both IL-6N and TNF-αN groups demonstrated higher average antibody levels, these differences were not statistically significant (**Fig. S8**). This indicates that reducing TNF-α and IL-6 from CpG with the initial vaccination is not detrimental to antibody production, but also does not enhance it.

Upon observing this in vivo modulation, we sought to determine whether SN50 acts locally or systemically. To examine this mechanism, we injected SN50 i.m. in the left hind limb and immediately injected CpG + OVA (SN50 L + CpG R) in the right hind limb. There was no significant difference in systemic cytokine levels tested between CpG and the SN50 L + CpG R group (**Fig. S8**), whereas SN50 + CpG + OVA (SN50 + CpG) injected simultaneously

demonstrated reduction of TNF- α and IL-6. On day 28, we analyzed serum antibody concentrations, revealing a 5.5 fold difference between the SN50 + CpG and the SN50 L +CpG R group (**Fig. S8**). This demonstrates the importance of coadministration of the components and therefore indicates that SN50 is acting locally to both increase safety and protection.

From these experiments, we conclude that SN50 acts locally at the injection site to inhibit immediate cytokine production, containing inflammation before it is distributed systemically. Based on our in vitro data, we believe CpG + SN50 enables TNF- α and IL-6 production locally at reduced levels. Our in vitro data suggests that immune cells exposed to CpG + SN50 express higher levels of cell surface receptors important for antigen presentation and effective T cell activation.

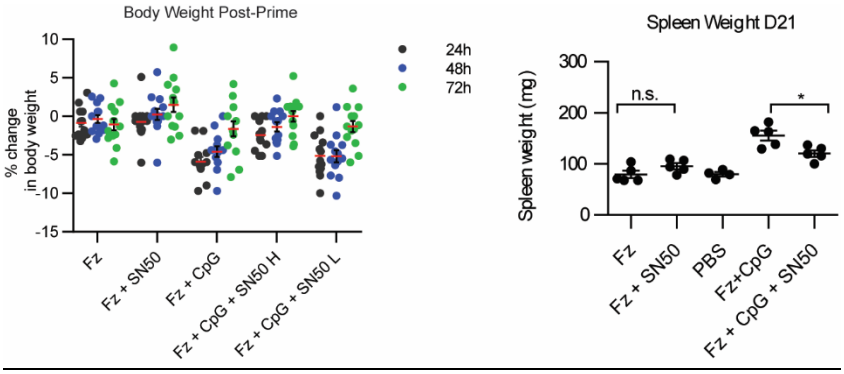

Figure S9. Weight Loss Post-Vaccination. (A) Weight loss 24h (black dot), 48h (blue dot) and 72h (green dot) post prime. Fz = Fluzone 2017-2018 flu vaccine. n = 13. (B) Spleen weight d21 post vaccination n = 5.

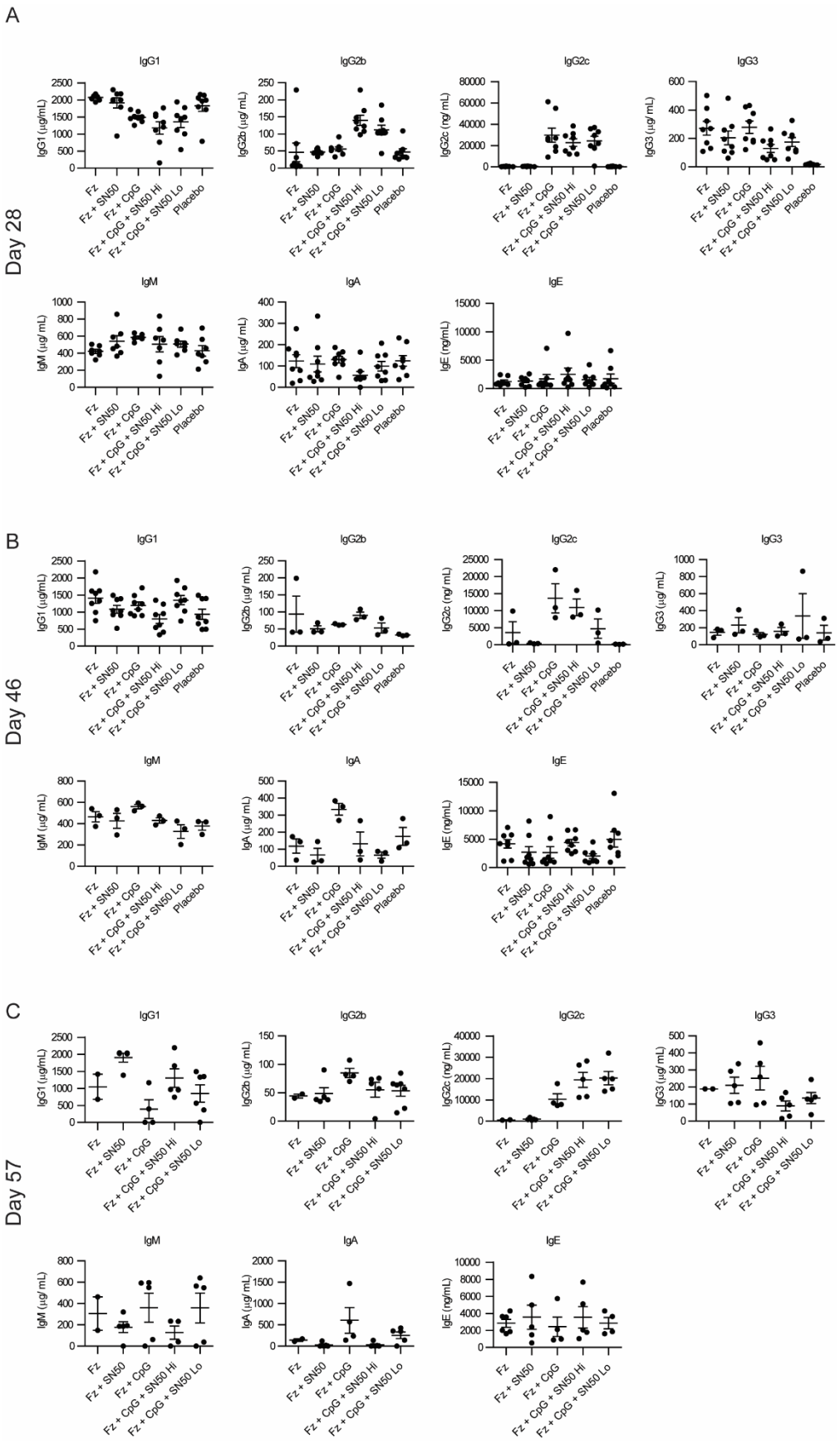
A
Day 28
IgG1
IgG1 (µg/mL)
IgG2b
IgG2b (µg/mL)
IgG2c
IgG2c (ng/mL)
IgG3
IgG3 (µg/mL)
IgM
IgM (µg/mL)
IgA
IgA (µg/mL)
IgE
IgE (ng/mL)
B
Day 46
IgG1
IgG1 (µg/mL)
IgG2b
IgG2b (µg/mL)
IgG2c
IgG2c (ng/mL)
IgG3
IgG3 (µg/mL)
IgM
IgM (µg/mL)
IgA
IgA (µg/mL)
IgE
IgE (ng/mL)
C
Day 57
IgG1
IgG1 (µg/mL)
IgG2b
IgG2b (µg/mL)
IgG2c
IgG2c (ng/mL)
IgG3
IgG3 (µg/mL)
IgM
IgM (µg/mL)
IgA
IgA (µg/mL)
IgE
IgE (ng/mL)
Fz
Fz + SN50
Fz + CpG
Fz + CpG + SN50 Hi
Fz + CpG + SN50 Lo
Placebo

Figure S10. Serum IgG1, IgG2b, IgG2c, IgG3, IgM, IgA and IgE Antibody concentrations at (A) day 28 post-vaccination, n=8. (B) Day 46 (d3 post infection) n=3. (C) Day 57 (d14 post infection) Fz (n= 2), Fz + SN50 (n=5), Fz + CpG (n=5), Fz + CpG + SN50 Hi (n=5), Fz + CpG + SN50 Lo (n=5). Fz = Fluzone 2017-2018 flu vaccine.

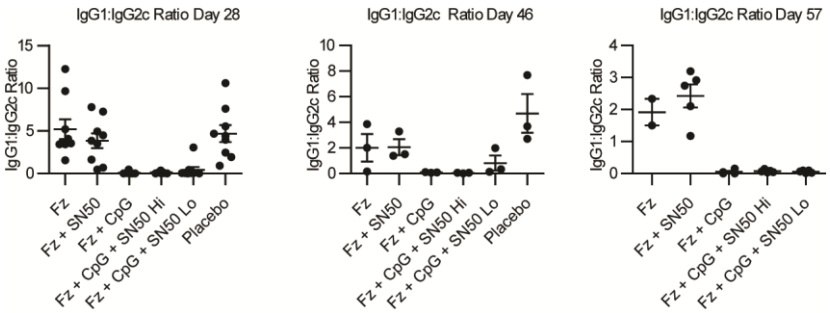

Figure S11. Serum IgG1:IgG2 Antibody concentration ratios at (A) day 28 post-vaccination, n = 8. (B) Day 46 (d3 post infection) n = 3. (C) Day 57 (d14 post infection) Fz (n = 2), Fz + SN50 (n = 5), Fz + CpG (n = 5), Fz + CpG + SN50 Hi (n = 5), Fz + CpG + SN50 Lo (n = 5). Fz = Fluzone 2017-2018 flu vaccine.

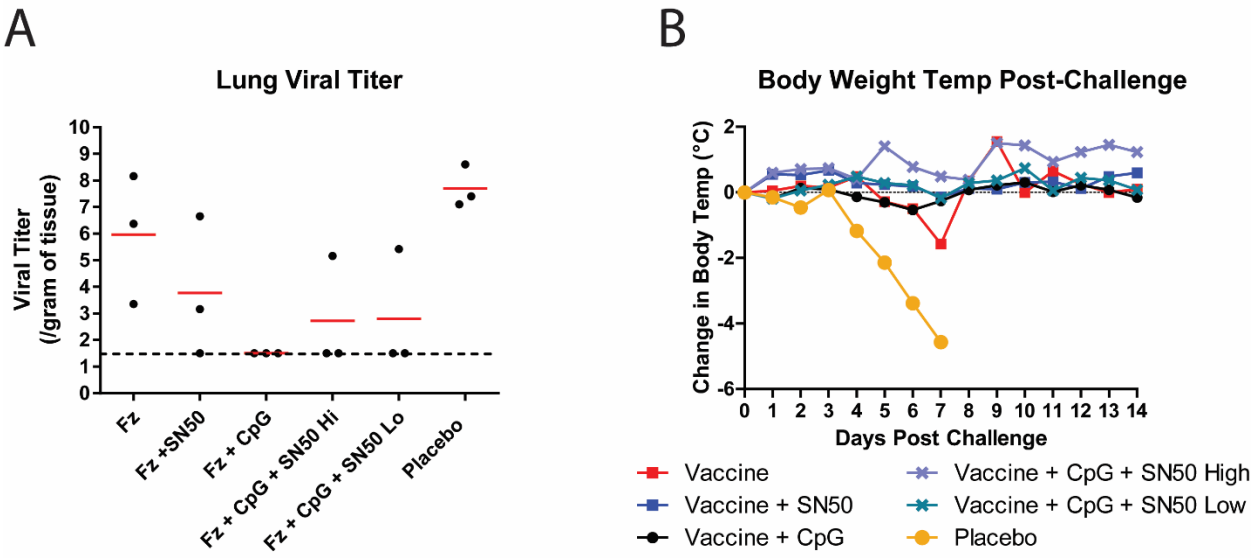

Figure S12. (A) Lung viral titer d3 post-infection. (B) Full temperature curve for 14 days post-challenge. Fz (black line), Fz + SN50 (blue line), Fz + CpG (grey line), Fz + CpG + SN50 Hi (red line), Fz + CpG + SN50 Lo (purple line), Placebo (yellow line).

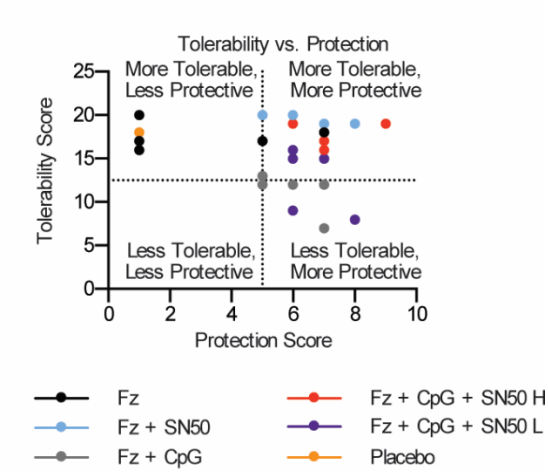

Figure S13. Tolerability vs. Protection score

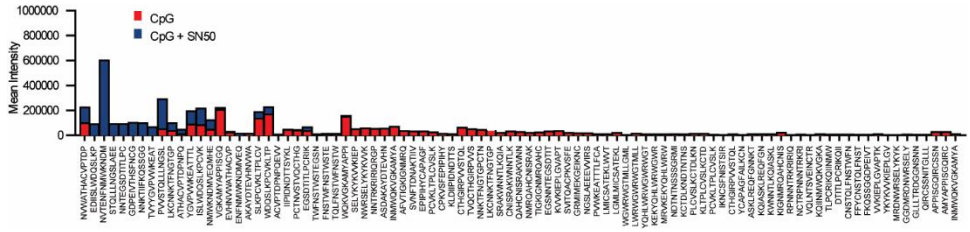

Figure S14. Mean intensity of each recognized epitope by CpG (red bars) or CpG + SN50 (blue bars).

With some diseases, including HIV, high titers of non-neutralizing antibodies can lead to antibody-dependent enhancement and increased infection risk (70–73). In other situations, including with HIV, non-neutralizing antibodies can be beneficial (73–75). The key is to induce the correct types of antibodies at the right time. In general, increased abundance of broadly neutralizing antibodies, correlate with improved disease outcomes (76–78). The most highly recognized epitope in the CpG + SN50 group corresponds to the epitope recognized by the recently isolated 35O22 monoclonal antibody (79). Antibodies isolated from mice vaccinated with CpG + SN50 also recognize the CD4 binding site recognized by several potent, broadly neutralizing antibodies (VRC01, VRC03, b12) (80–82). Based on the epitopes recognized by the serum samples, we hypothesize that these antibodies may be beneficial to protection. It is possible that altering the epitopes recognized could enhance certain vaccinations where specific epitopes are difficult to target with standard immunization strategies. Additionally, varying levels of antigen, adjuvant or SN50 may shift epitope recognition and we are currently examining the functional biological significance of such a shift.

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