Blocking interleukin-4 enhances efficacy of vaccines for treatment of opioid abuse and prevention of opioid overdose

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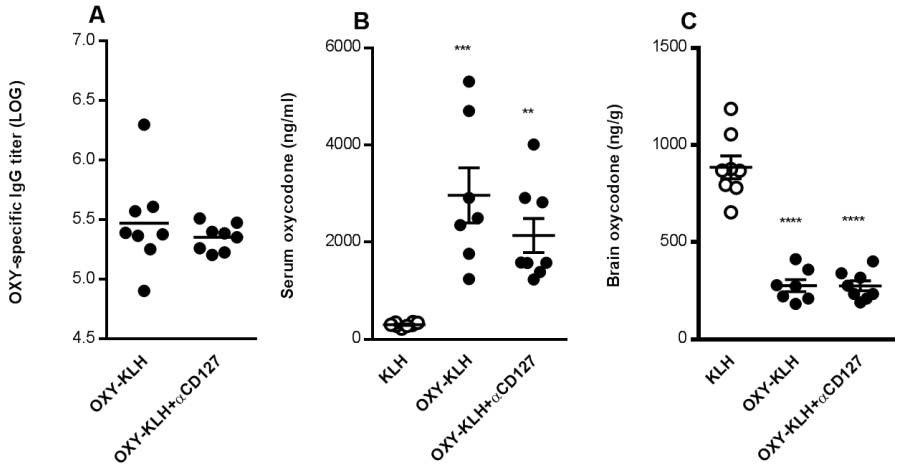
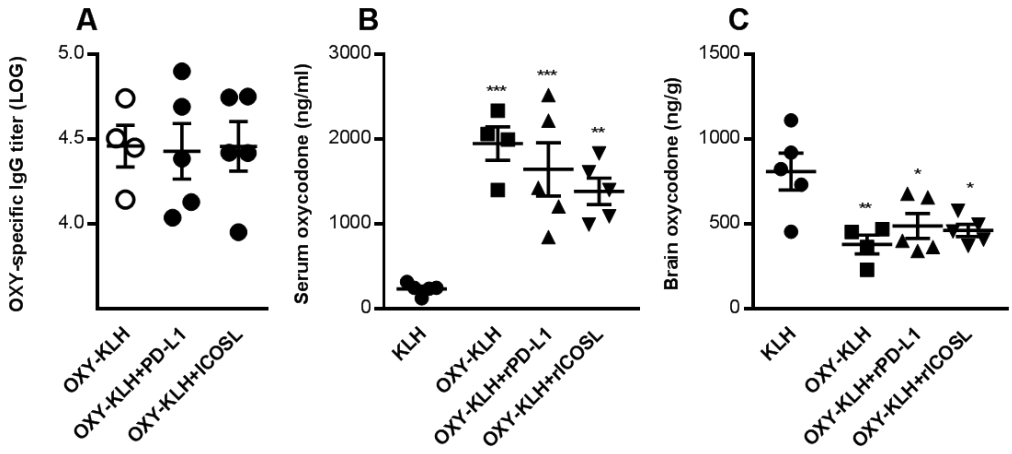
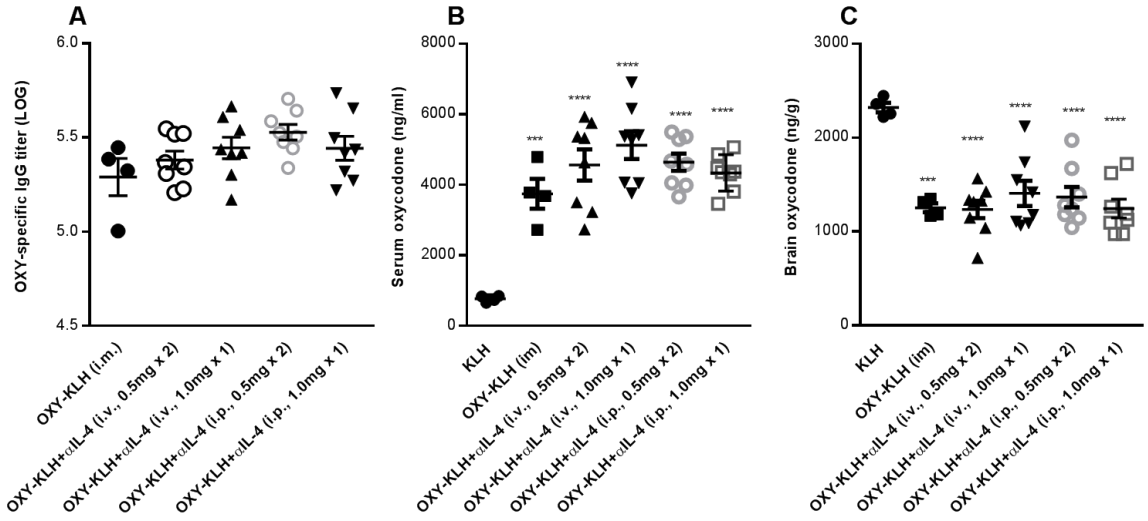
Supplementary Information



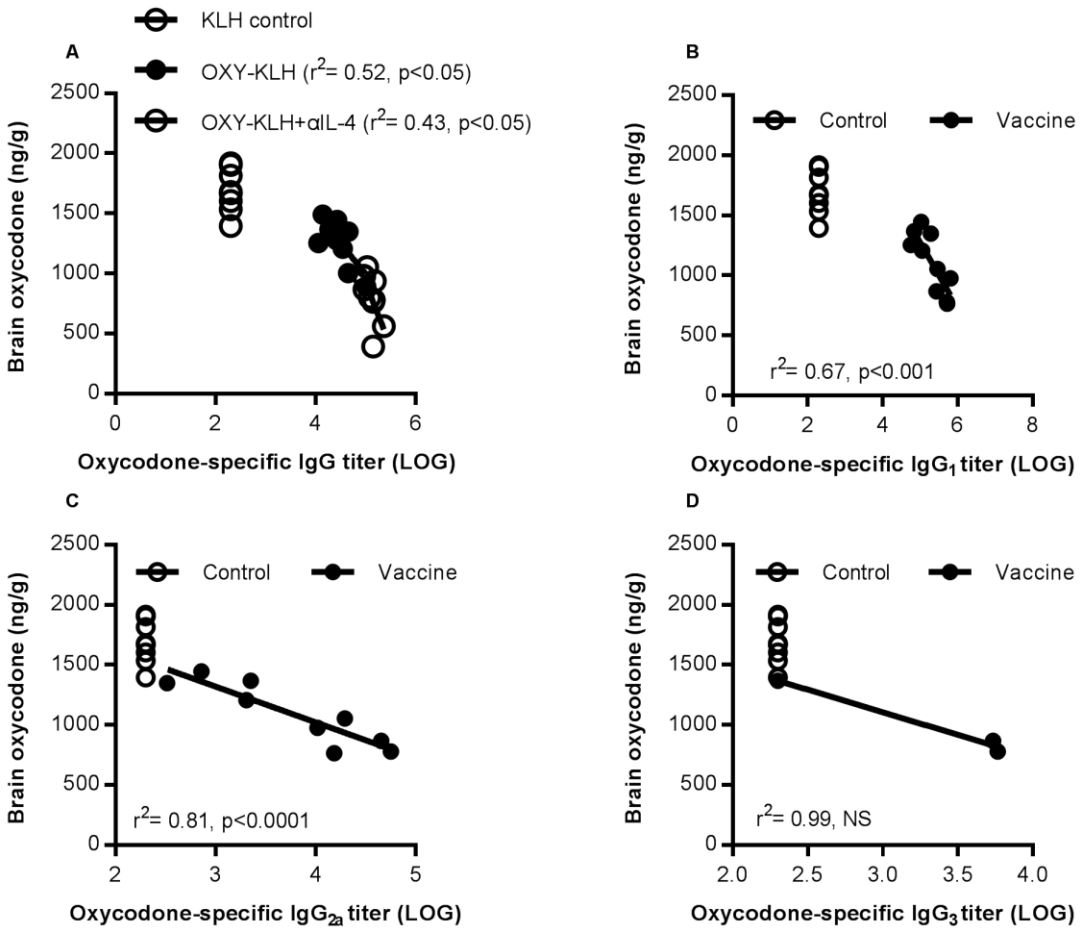
Supplemental Figure S1. IL-7 receptor blockage does not enhance OXY-KLH efficacy. Male BALB/c mice were immunized s.c. on days 0, 14, and 28, and challenged with 2.25 mg/kg s.c. oxycodone a week after the 3rd immunization. Mice received unconjugated KLH, OXY-KLH, or OXY-KLH plus an anti-IL-7α receptor mAb (αCD127, 1.0 mg per mouse, intraperitoneally). The OXY-KLH and KLH were adsorbed on alum adjuvant prior to administration. The mAb was administered 2 days prior and 1 day after the 1st immunization. A) oxycodone-specific IgG antibody titers, and effect of immunization on oxycodone distribution to B) serum and C) brain. Data are mean±SEM. Data shown are from one experiment (n=8). One-way ANOVA paired with Tukey's multiple comparisons. **p<0.01, ***p<0.001, ****p<0.0001 compared to KLH control.



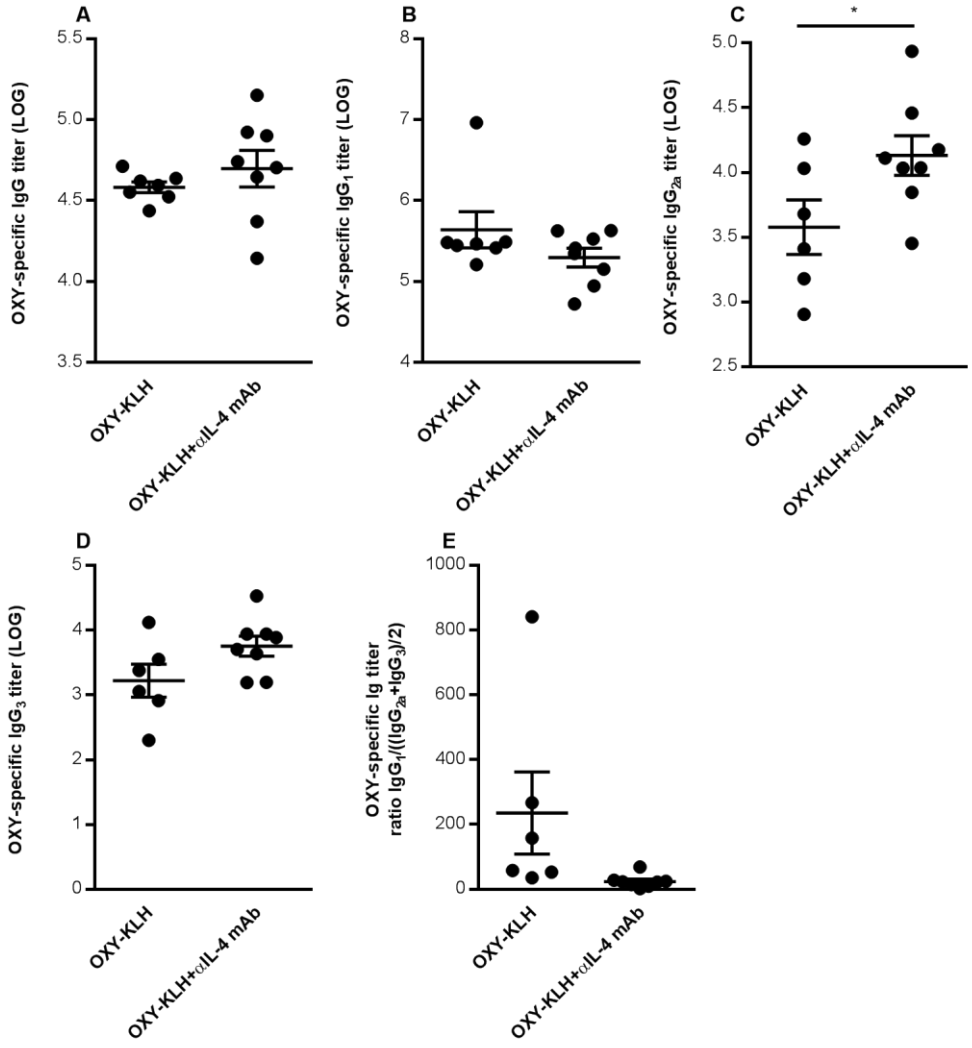
Supplemental Figure S2. **Co-stimulatory molecules do not enhance OXY-KLH efficacy.** Male BALB/c mice were immunized s.c. on days 0, 14, and 28, and challenged with 5 mg/kg s.c. oxycodone a week after the 3rd immunization. Mice received KLH, OXY-KLH, or OXY-KLH plus either recombinant Programmable Death Ligand 1 (rPD-L1) or recombinant ICOS ligand (rICOSL). The OXY-KLH and the unconjugated KLH were adsorbed on alum adjuvant prior to administration. PD-L1 and ICOSL were co-administered s.c. with vaccines. A) oxycodone-specific IgG antibody titers, effect of immunization on oxycodone distribution to B) serum and C) brain. Data are mean±SEM. Data shown are from one experiment (n=5). One-way ANOVA paired with Tukey's multiple comparisons. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 compared to KLH control.



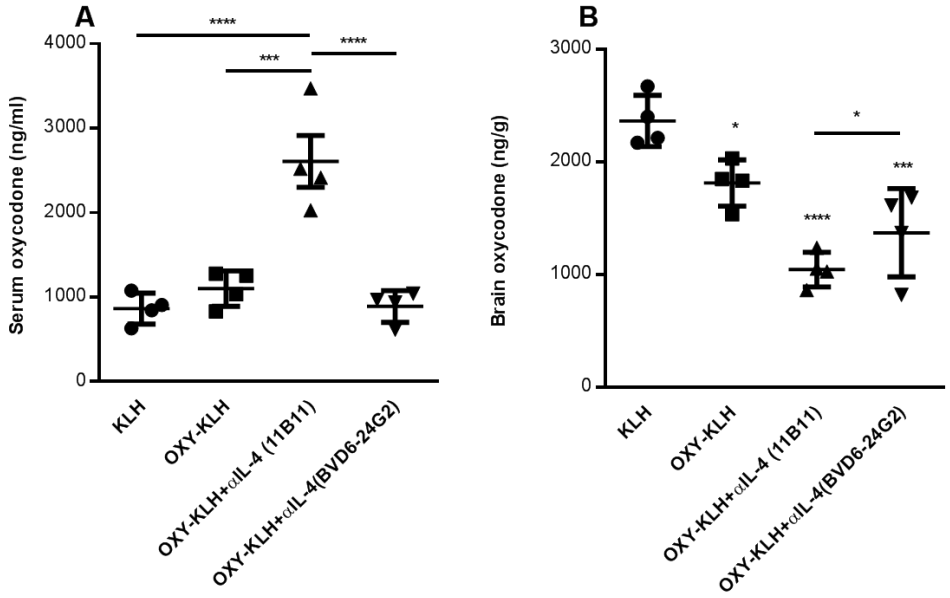
Supplemental Figure S3. Co-administration of OXY-KLH plus αIL-4 mAb is effective across clinically-relevant immunization regimens. Male BALB/c mice were immunized i.m. with OXY-KLH or KLH on days 0, 14 and 28, and challenged with 5.0 mg/kg oxycodone a week after the third immunization. Mice received KLH (n=4), OXY-KLH (n=4), or OXY-KLH plus the αIL-4 mAb (n=8). As labeled, αIL-4 mAb was administered either i.p. or i.v., either as 1 dose (1.0 mg) or 2 doses (0.5mg each). Data are from one experiment and expressed as mean±SEM. One-way ANOVA paired with Tukey's multiple comparisons. ***p<0.001, ****p<0.0001 compared to KLH.



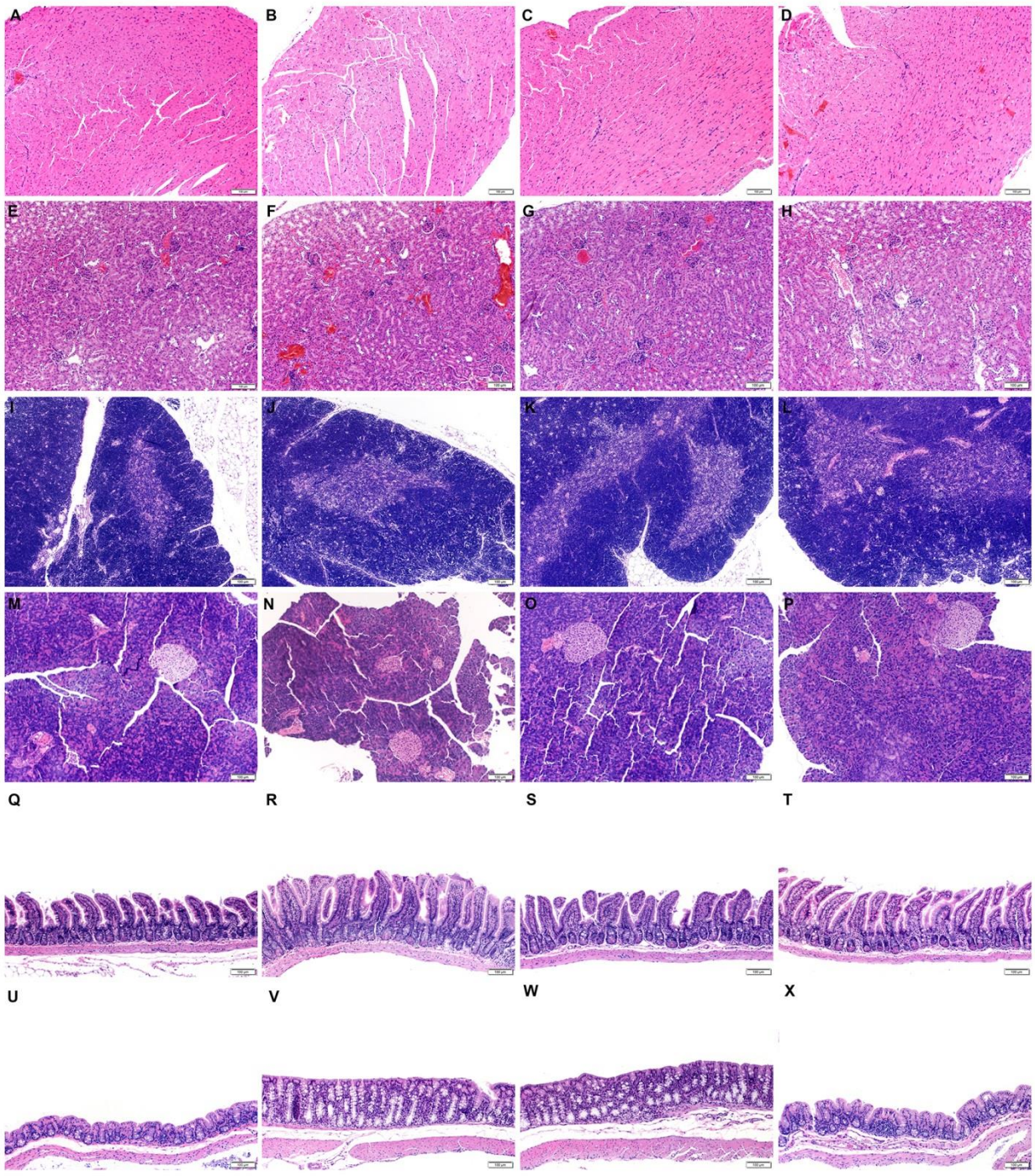
Supplemental Figure S4. Increased oxycodone-specific serum IgG, IgG_1, and IgG_{2a} antibody titers correlated with reduction in brain oxycodone. A-D) Analysis was performed on individual mice challenged with 5mg/kg oxycodone and reported in Figure 1E-G. A) Linear regression of oxycodone-specific serum IgG titers and brain concentration in mice treated with either OXY-KLH or OXY-KLH plus αIL-4 mAb (n=10 mice each group). The individual brain oxycodone concentrations in the KLH control group are provided as comparison (n=10). B-D) Linear regression of oxycodone-specific serum IgG_1, IgG_{2a}, and IgG_3 titers and brain concentration in immunized mice. The vaccine treatment group includes mice immunized with OXY-KLH (n=5) and OXY-KLH plus αIL-4 mAb (n=5). D) Most individual mice in this cohort had little to no detectable IgG_3 titers.



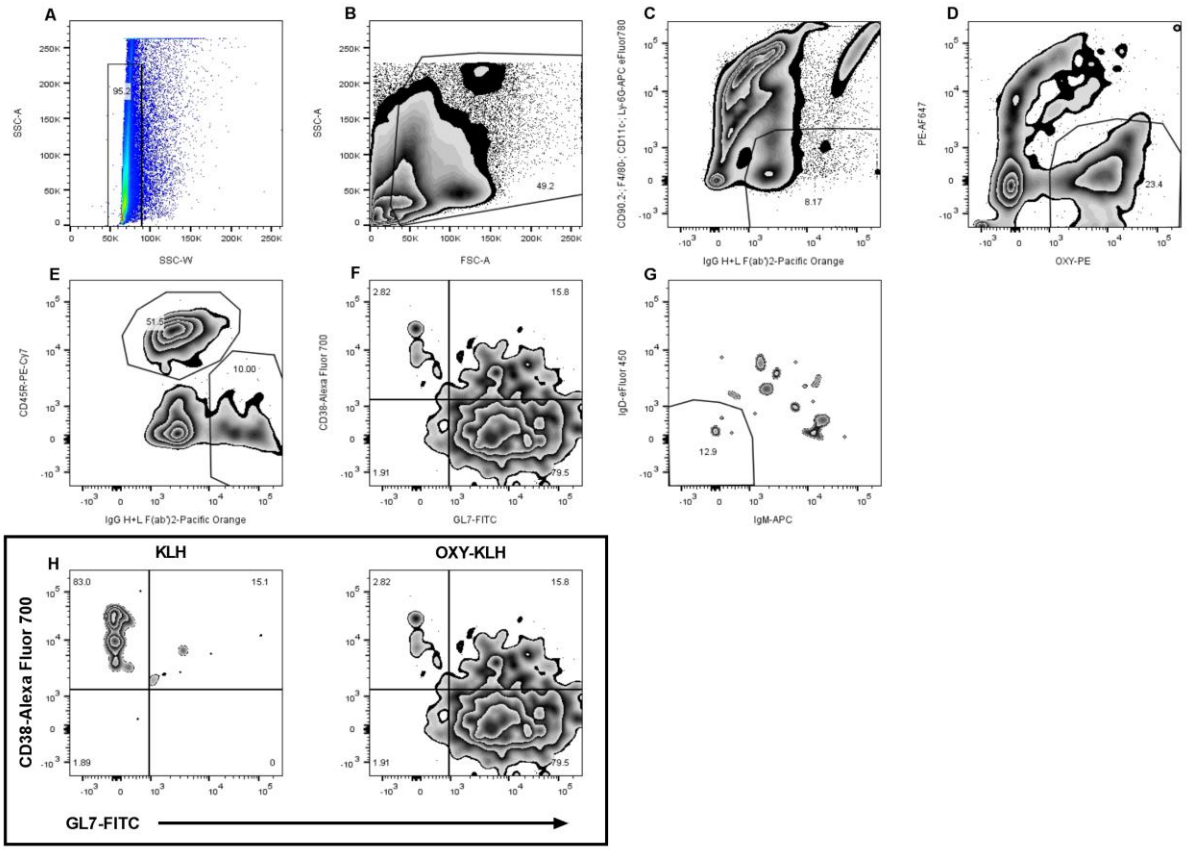
Supplemental Figure S5. Co-administration of OXY-KLH and αIL-4 mAb increases anti-oxycodone serum IgG_{2a} antibodies in C57Bl/6 mice. Male C57Bl/6 mice were immunized s.c. with OXY-KLH or OXY-KLH plus αIL-4 mAb i.p., and anti-oxycodone serum IgG antibodies were analyzed for IgG_1, IgG_{2a}, and IgG_3 subclass titers: A) total oxycodone-specific IgG titers, B) IgG_1, C) IgG_{2a}, D) IgG_3, and E) $IgG_1/((IgG_{2a} + IgG_3)/2)$. Data are from one experiment (n=8) and expressed as mean±SEM. Unpaired two-tailed t-test. *p<0.05.



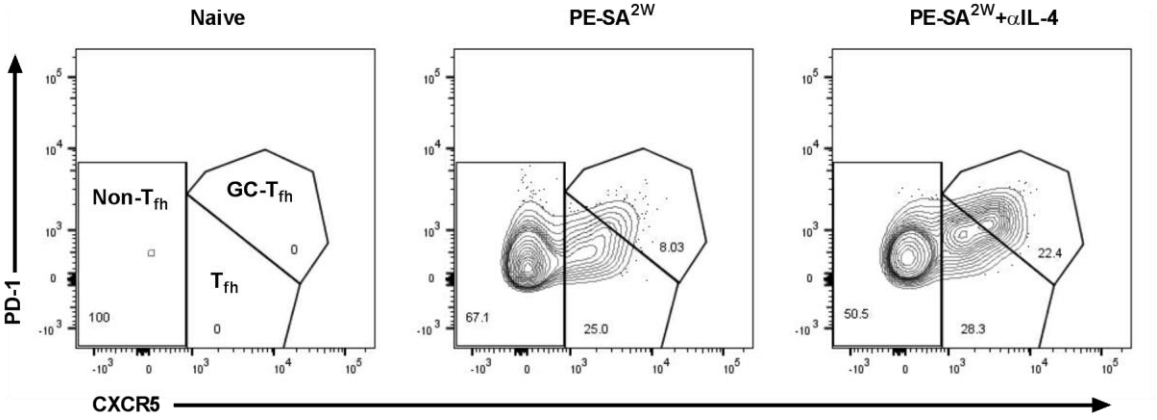
Supplemental Figure S6. The OXY-KLH efficacy is enhanced by neutralizing αIL-4 mAb but not by non-neutralizing αIL-4 mAb. Male BALB/c mice were immunized i.m. with KLH or OXY-KLH on days 0, 14 and 28, and challenged with 5.0 mg/kg oxycodone a week after the third immunization. Mice received KLH (n=4), OXY-KLH (n=4), and OXY-KLH plus either neutralizing αIL-4 mAb (11B11, n=4) or non-neutralizing αIL-4 mAb (BVD6-24G2, n=4). The αIL-4 mAb was administered i.p. as 2 doses (0.5mg each). Data are from one experiment and expressed as mean±SEM. One-way ANOVA paired with Tukey's multiple comparisons. *p<0.05, ***p<0.001, and ****p<0.0001 compared to KLH or brackets to indicate significance between groups.



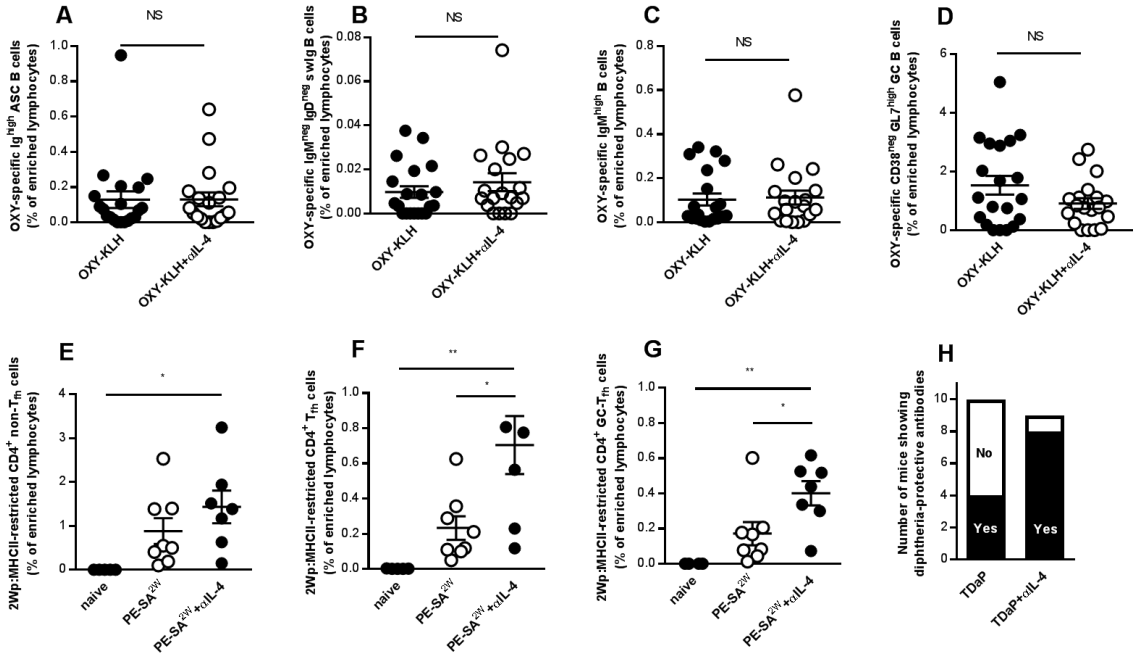
Supplemental Figure S7. Co-administration of OXY-KLH plus αIL-4 mAb is safe. No significant pathology was identified in A-D) heart, E-H) kidney, I-L) thymus, M-P) pancreas, Q-T) small intestine, and U-X) large intestine in mice immunized with KLH (s.c.), OXY-KLH (s.c.), αIL-4 (i.p.), or OXY-KLH (s.c.) plus αIL-4 (i.p.).



Supplemental Figure S8. Gating strategy for flow cytometric analysis of hapten-specific B cells and characterization of hapten-specific germinal center (GC) B cells from mouse lymphoid organs 7 d post-vaccination with OXY-KLH or unconjugated KLH control. A) Singlet gating to remove any aggregated cells. B) Total lymphocytes. C) Total B cells expressing gamma immunoglobulin G heavy and light chain (IgG+ [H+L]) and excluding the following non-B cell markers: CD90.2 (T cells), Gr-1 (neutrophils), CD11.c (dendritic cells), and F4/80 (macrophages). D) OXY-specific B cells bound to OXY-Streptavidin-PE were differentiated from B cells bound to a Streptavidin-PE^{AF647} decoy (1,2). E) OXY-specific B cells were identified as either non antibody-secreting cells (non-ASC) expressing CD45R^{high} or antibody-secreting cells (ASC) expressing high levels of gamma immunoglobulin (IgG^{high}). F) OXY-specific CD45R^{high} non-ASC B cells were identified as GL7^{+} GC, CD38^{+} naïve/memory (N/M), or GL7^{+}/CD38^{+} precursor memory B cells. G) N/M B cells were identified as IgM^{high} or IgM^{low}/IgD^{low} switched immunoglobulin (swIg) cells. H) Representative comparison of CD38 and GL7 expression in mice immunized with either KLH or OXY-KLH adsorbed on alum adjuvant.



Supplemental Figure S9. Gating strategy for analysis of 2W1S:MHCII-restricted T cells in C57Bl/6 mice immunized with PE-SA^{2W} with or without αIL-4 mAb, or naïve mice as control. Analysis of 2W-specific *CD4*$^{+}$ T cells was performed as described (1, 3, 4). Lymph nodes and spleens are processed to single-cell suspensions. Samples are incubated with 2W1S:I-A^{b} tetramers conjugated to PE and APC, and BV421 anti-CXCR5 (3). Samples are then enriched with anti-PE and anti-APC magnetic beads and magnetic columns (3). After magnetic enrichment, samples are labeled with PE-Cy7 labeled CD45R, CD11b/c, CD11c, F4/80 antibodies to identify non-T cell populations, and then with T cell markers: PE anti-CD4, BV510 anti-CD8a, APC-eFluor780 anti-CD4, PerCP-eFluor710 anti-CD90.2, FITC antiCD279 (PD-1), and AF700 anti-CD44. Flow cytometric analysis: a) singlets through standard FSC-W and FSC-A, b) T cells are CD90.2^{high} not expressing non-T cell markers, which are then classified as CD8^{high} T cells or CD4^{high} T cells, c) Within the CD4^{high} T cell subset, 2W1S-specific CD4^{+} T cells are identified as PE^{high} and APC^{high}, and d) 2W1S-specific CD4^{+} T cells are classified as CXCR5^{neg} non-Tfh, CXCR5^{mid} PD-1^{neg} Tfh, and CXCR5^{high} PD-1^{+} GC-Tfh (4).



Supplemental Figure S10. A-G) Data from Figure 5A-F expressed as percentages of lymphocytes in enriched samples from individual mice. Analysis of the OXY-specific B cell population: A) plasma cells or antibody-secreting cells (ACS), B) switched immunoglobulin (swIg), C) IgM^{high} naïve and memory, and D) germinal center (GC). Analysis of the 2Wp:MHCII-restricted $CD4^+$ T cell population: E) non-Tfh, F) Tfh and G) GC-Tfh. *p<0.05, **p<0.01. H) Number of mice that showed detectable (IC_{50}>10) protective antibody titers against diphtheria toxin. Chi-square analysis, $\chi 2$, df= 4.866, 1, z=2.206, p<0.05, two tailed.

Supplemental table S1. Effect of combining immunomodulators with vaccines on baseline nociception.

Formulation	Sample size	Baseline nociception (s)	Body weight (g)
KLH	n=49	17.04±1.03	27.20±0.26
OXY-KLH	n=48	13.93±0.57*	26.80±0.29
OXY-KLH + αIL-4	n=38	15.22±0.73	27.13±0.22
OXY-KLH + αCD25	n=20	12.38±1.03**	26.02±0.25*

Data are from mice immunized with KLH (s.c.), OXY-KLH (s.c.), OXY-KLH (s.c.) plus either αIL-4 or αCD25 mAb (i.p.) from experiments presented in Figures 1 and 2. Nociception is expressed as delay to hind paw lifts on a hotplate set at 54^{o}C. Data are expressed as mean±SEM. One-way ANOVA paired with Tukey's multiple comparisons test. *$p<0.05$ and **$p<0.01$ from KLH control.

Supplemental table S2. Combining immunomodulators with vaccines: blood biochemistry.

Treatment	Protein, total (g/dL)	Bilirubin, total (mg/dL)	ALT (U/L)	AST (U/L)	Urea nitrogen (mg/dL)	Cholesterol (mg/dL)	Body weight (g)
KLH	5.7 ± 0.32	0.2 ± 0.10	71.6 ± 3.2	626 ± 24.7	20 ± 1.5	161 ± 10.4	26.5 ± 0.39
OXY-KLH	5.4 ± 0.17	0.3 ± 0.11	74 ± 4.7	709 ± 51.6	20 ± 1.0	154 ± 11.0	27.4 ± 0.75
αIL-4 mAb	5.8 ± 0.06	0.46 ± 0.03	104 ± 9.5	605 ± 253	27 ± 2.8	163 ± 0.3	27.7 ± 0.72
OXY-KLH + αIL-4 mAb	5.5 ± 0.26	0.27 ± 0.09	101 ± 32.3	840 ± 286	24 ± 1.0	158 ± 13.0	27.1 ± 0.24
Reference Values*	4.2 - 5.8	0 - 0.5	41 - 131	55 - 32	7 - 26	111 - 246	

Blood biochemistry analysis in BALB/c mice immunized with KLH (s.c.), OXY-KLH (s.c.), αIL-4 mAb (i.p.), and OXY-KLH (s.c.) plus αIL-4 mAb (i.p.). Reported values for protein, bilirubin, ALT (alanine aminotransferase), AST (aspartate aminotransferase), urea nitrogen and total cholesterol. Data are from one experiment and reported as mean±SEM (n=3/group). One way ANOVA paired with Tukey's multiple comparisons test. *Reference values for BALB/c mice as reported from the Jackson Laboratory.

Supplemental table S3. List of reagents and antibodies for flow cytometry analysis.

Reagent	Catalog #	Manufacturer
B cell analysis		
$OXY(Gly)_4$-peptide-biotin-streptavidin-PE	--	Custom, Pravetoni
NH_2-peptide-biotin-streptavidin-PE^{AF647}	--	Custom, Pravetoni
FITC rat-anti-mouse T and B cell Activation Antigen (clone GL7)	55366	BD Pharmingen
CD45R (B220) PE-Cyanine7 (Clone: RA3-6B2) (formerly eBioscience)	25-0452-82	ThermoFisher Scientific
CD38 Alexa Fluor® 700 (Clone: 90)	56-0381-82	ThermoFisher Scientific
IgM APC (Clone: II/41)	17-5790-82	ThermoFisher Scientific
CD11c APC-eFluor® 780 (Clone: N418)	47-0114-82	ThermoFisher Scientific
CD90.2 (Thy-1.2) APC-eFluor® 780 (Clone: 53-2.1)	47-0902-82	ThermoFisher Scientific
F4/80 Antigen APC-eFluor® 780 (Clone: BM8)	47-4801-82	ThermoFisher Scientific
Ly-6G (Gr-1) APC-eFluor® 780 (Clone: RB6-8C5)	47-5931-82	ThermoFisher Scientific
IgD eFluor® 450 (Clone: 11-26c)	48-5993-82	ThermoFisher Scientific
IgG H+L F(ab')2 Pacific Orange	P31585	Invitrogen (Life Technologies)
B cell compensation staining		
CD4 FITC (Clone: GK1.5)	11-0041-85	ThermoFisher Scientific
CD4 PE (Clone: GK1.5)	12-0041-83	ThermoFisher Scientific
CD4 PE-Cyanine5 (Clone: GK1.5)	15-0041-83	ThermoFisher Scientific
T cell analysis		
2Wp:MCHII-PE and –APC	--	Custom, Marc Jenkins
CD44 AlexaFluor700 clone IM7	56-0441-82	ThermoFisher Scientific
CD45R (B220) PE-Cy7 clone RA3-6B2	25-0452-82	ThermoFisher Scientific
CD11b PE-Cy7 clone M1/70	552850	BD biosciences
CD11c PE-Cy7 clone N418	25-0114-82	ThermoFisher Scientific
CD279 (PD-1) FITC clone J43	11-9985-82	ThermoFisher Scientific
CD4 APC clone GK1.5	17-0041-82	ThermoFisher Scientific
CD4 APC-eFluor780 clone RM4-5	47-0042-82	ThermoFisher Scientific
CD8a BV510 clone 53-6.7	563068	BD biosciences
CD90.2 (Thy-1.2) PerCP-eFluor710 clone 30-H12	46-0903-82	ThermoFisher Scientific
CXCR5 BV421 clone 2G8	562889	BD biosciences
F4/80 PE-Cy7 clone BM8	25-4801-82	ThermoFisher Scientific

Supplemental figure references

1. Laudenbach M, Baruffaldi F, Vervacke JS et al. The frequency of naive and early-activated hapten-specific B cell subsets dictates the efficacy of a therapeutic vaccine against prescription opioid abuse. J Immunol 2015;194:5926-36.
2. Taylor JJ, Laudenbach M, Tucker AM, Jenkins MK, Pravetoni M. Hapten-specific naive B cells are biomarkers of vaccine efficacy against drugs of abuse. J Immunol Methods 2014;405:74-86.
3. Tubo NJ, Pagan AJ, Taylor JJ et al. Single Naive CD4(+) T Cells from a Diverse Repertoire Produce Different Effector Cell Types during Infection. Cell 2013;153:785-96.
4. Yang JA, Tubo NJ, Gearhart MD, Bardwell VJ, Jenkins MK. Cutting edge: Bcl6-interacting corepressor contributes to germinal center T follicular helper cell formation and B cell helper function. J Immunol. 2015 Jun 15;194(12):5604-8.