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J Immunol (2023) 210 (9): 1272–1280.

<https://doi.org/10.4049/jimmunol.2200605>

IL-4 Predicts the Efficacy of a Candidate Antioxycodeone Vaccine and Alters Vaccine-Specific Antibody-Secreting Cell Proliferation in Mice

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Opioid use disorders (OUDs) are a public health concern in the United States and worldwide. Current medications for OUDs may trigger side effects and are often heavily regulated. A novel treatment strategy to be used alone or in combination with existing medications is active immunization with antiopioid vaccines, which stimulate production of opioid-specific Abs that bind to the target drug and prevent its distribution to the brain. Although antiopioid vaccines have shown promising preclinical efficacy, prior clinical evaluations of vaccines targeting stimulants indicate that efficacy is limited to a subset of subjects who achieve optimal Ab responses. We have previously reported that depletion of IL-4 with a mAb increased opioid-specific IgG_{2a} and total IgG, and it increased the number of germinal centers and germinal center T follicular helper cells in response to antiopioid vaccines via type I IL-4 signaling. The current study further investigates the mechanisms associated with IL-4-mediated increases in efficacy and whether IL-4 depletion affects specific processes involved in germinal center formation, including affinity maturation, class switching, and plasma cell differentiation in mice. Additionally, results demonstrate that preimmunization production of IL-4 after ex vivo whole blood stimulation predicted in vivo vaccine-induced Ab titers in outbred mice. Such mechanistic studies are critical for rational design of next-generation vaccine formulations, and they support the use of IL-4 as a predictive biomarker in ongoing OUD vaccine clinical studies. *The Journal of Immunology*, 2023, 210: 1272–1280.

Opioid use disorders (OUDs) affect >40 million individuals worldwide (1), which imposes an estimated annual economic burden of >1 trillion dollars in the United States alone (2). Current medications for OUD target the mu opioid receptor and include the mu opioid receptor agonist methadone, partial agonist buprenorphine, and antagonist naltrexone. Although effective, these medications are limited by poor accessibility and issues with patient compliance due to the potential for severe side effects. Active immunization with an antiopioid conjugate vaccine is a novel therapeutic strategy that may be useful alone or in combination with currently available pharmacotherapies. Antioxioid vaccines consist of an opioid-like hapten conjugated to a large immunogenic carrier protein such as subunit keyhole limpet hemocyanin (sKLH), tetanus toxoid, or cross-reactive material 197 and combined with adjuvants such as aluminum hydroxide (alum) to stimulate an immune response. Active immunization stimulates the production of opioid-specific polyclonal IgG Abs that can bind to the target drug and prevent its distribution to the brain, inhibiting centrally mediated opioid-induced effects (reviewed in Ref. 3). Proof of preclinical efficacy, selectivity, and safety has been shown for antiopioid vaccines against oxycodone/hydrocodone, heroin/morphine, fentanyl, and

fentanyl analogs (reviewed in Refs. 3–5), and an oxycodone vaccine (OXY-sKLH adsorbed on aluminum salts) is now under investigation in a first-in-human phase Ia/Ib clinical trial (NCT04458545).

To date, clinical trials for vaccines for substance use disorder (SUD) have achieved mixed success. Although some phase II trials for antinicotine vaccines have shown promising efficacy in increasing smoking cessation rates, phase III trials have failed to meet their primary efficacy endpoints (reviewed in Ref. 6). However, data from clinical trials for vaccine against either nicotine (7) or cocaine (8) provided proof of efficacy in increasing drug abstinence among individuals who produced high titers of high-affinity Abs. For example, anticocaine vaccine clinical trials have reported that individuals with >43 µg/ml serum drug-specific Abs had a >50% reduction in overall cocaine use and a greater incidence of cocaine-free urine, but only 38% of individuals attained this serum Ab concentration (8). These results highlight the importance of investigating strategies to increase OUD vaccine efficacy by increasing Ab production using novel adjuvants, vaccine delivery platforms, or identification of predictive biomarkers of vaccine efficacy.

Most first-generation SUD vaccine formulations, including most of those tested in clinical trials, used alum as an adjuvant (4, 6).

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Received for publication August 17, 2022. Accepted for publication February 27, 2023.

This work was supported by the National Institute on Drug Abuse Grants R01DA041730 (to M.P.), U01DA051658 (to M.P.), T32DA007097 (to B.C.), and F31DA054760 (to B.C.). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

B.C. designed and executed experiments, analyzed data, and wrote the manuscript; C.B. isolated and sequenced oxycodone-specific mAbs and edited the manuscript; D.H. developed the recombinant antioxioid mAbs with mutant Fc regions and edited the manuscript; and M.P. designed the study, analyzed data, and wrote the manuscript.

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The online version of this article contains supplemental material.

Abbreviations used in this article: alum, aluminum hydroxide; ASC, Ab-secreting cell; GC, germinal center; IRS2, insulin receptor substrate 2; K_{diss} , dissociation rate; LC-MS/MS, liquid chromatography–tandem mass spectrometry; ORF, open reading frame; OUD, opioid use disorder; SUD, substance use disorder; sKLH, subunit keyhole limpet hemocyanin; T_{fh}, T follicular helper; WT, wild-type.

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Alum is currently the most widely used adjuvant in Food and Drug Administration–approved vaccine formulations, and it is often chosen due to its known efficacy and safety profile (9). However, because current clinical studies suggest that alum may not be sufficient to produce an adequate Ab response in all subjects, new adjuvants are being investigated with OUD vaccines to increase vaccine efficacy. In preclinical studies, OUD vaccines have been tested with MF59 (10), LTA1 and dmLT (11), CpG oligodeoxynucleotide (12), dsRNA (13), MPLA (14), Advax (13), CFA (14), and ALF (15) with varying degrees of success, although many of these adjuvants may not be suitable for clinical translation due to cost, patent rights, or toxicity. As an alternative strategy to increase vaccine efficacy, and to provide mechanistic insights, our laboratory investigated whether the addition of immunomodulators, including mAbs to neutralize or deplete various cytokines, would increase the immune response to the candidate OXY-sKLH vaccine. It was found that a neutralizing mAb against IL-4 increased oxycodone-specific IgG titers, increased class switching from IgG₁ to IgG_{2a}, and increased efficacy of the vaccine after drug challenge (16).

IL-4 is a pleiotropic cytokine that is produced by T cells, NKT cells, mast cells, innate lymphoid cells, eosinophils, and basophils (17). IL-4 can signal through either the type I or type II IL-4Rs. The type I receptor is a heterodimer of IL-4R α and IL-2R common γ -chain, and it is found on most lymphocytes and myeloid cells. Downstream of this receptor are STAT6 and insulin receptor substrate 2 (IRS2). The type II receptor consists of the IL-4R α and the IL-13R α 1, and it can signal in response to either IL-4 or IL-13. The type II receptor is found on most myeloid and nonhematopoietic cells, and it canonically signals downstream through STAT6 (reviewed in Ref. 18). IL-4 is extremely important for the protection against parasites and helminth worms (19–21) and has more recently been shown to be critical for germinal center (GC) formation in many contexts (22–30). Within the GC, IL-4 has been found to be secreted by NKT (25) and T follicular helper (T_{fh}) cells (31), and it can affect specific processes such as GC B cell differentiation (28), class switching to IgG₁ and IgE (27, 32), affinity maturation (28, 31) and the development of memory and plasma cells in activated B cells (22–24, 28). Immunization in IL-4–deficient mice (25) or in mice with STAT6-deficient B cells (26), Ab-based depletion of IL-4 (25, 30), or depletion of cells that produce IL-4 (25) led to smaller or misshapen GCs with fewer GC B cells and altered gene expression, which led to impaired Ab responses and decreased vaccine efficacy in a variety of viral infection models or after immunization with 4-hydroxy-3-nitrophenyl acetyl–OVA adsorbed on alum. In contrast, there is some evidence that depletion of IL-4 can positively affect GC formation after secondary immunization with OVA in CFA (33), and it leads to increased vaccine efficacy after administration of an HIV vaccine (34) or after challenge with respiratory syncytial virus (35). These contradicting reports suggest that the role of IL-4 in the GC may be dependent on Ag and/or adjuvant context. This is supported by evidence that Th₂-mediated responses are negatively affected by IL-4 depletion, whereas Th₁-mediated responses are not (28). The complex role of IL-4 in the GC was further highlighted by a recent study showing that IL-4R can activate signaling in some cell populations, while also acting as a decoy to sequester IL-4 away from the GC in other cell populations, such as follicular dendritic cells (24).

Previously, we found that immunization with OXY-sKLH or a model peptide-carrier conjugate vaccine in conjunction with IL-4 depletion led to an increase in GC-T_{fh} cells (16), changed the T cell transcription profile, and increased the number and size of GCs (36). These changes were mediated through type I IL-4 signaling, but not through STAT6. Therefore, we hypothesized that these changes may be mediated by a reduction in IRS2 signaling. NKT

cells, a major source of IL-4 during the GC response, were not necessary for vaccine efficacy (36). The current study sought to further elucidate the molecular signaling following IL-4 depletion in the context of antiopioid immunization, as well as its effect on GC processes such as affinity maturation, class switching, and plasma cell differentiation. Contrary to our original hypothesis, we found that the increase in efficacy after IL-4 depletion is not reproduced by ablation of IRS2 signaling. Additionally, IL-4 depletion did not change somatic hypermutation or affinity maturation in B cells after OXY-sKLH immunization. Although we have previously reported that IL-4 depletion increases class switching from IgG₁ to IgG_{2a}, we found that the Ab subclass itself did not affect the efficacy of an oxycodone-specific mAb in reducing oxycodone's effects after drug challenge. IL-4 depletion increased the number of oxycodone-specific Ab-secreting cells (ASCs) after three immunizations, but it did not lead to an increase in long-lived plasma cells in bone marrow. Finally, we investigated the potential of IL-4 as a predictive biomarker of vaccine efficacy and found that preimmunization IL-4 derived from ex vivo T cell stimulation negatively correlates with oxycodone-specific Ab titers and vaccine efficacy after immunization. These studies further uncover mechanisms of vaccine efficacy that can be used as a blueprint for rational design of next-generation vaccine formulations, and they support the exploration of IL-4 as a putative predictive biomarker in clinical studies.

Materials and Methods

Drugs and immunomodulators

Oxycodone HCl was obtained from Sigma-Aldrich (St. Louis, MO). Anti-IL-4 mAb (rat anti-mouse IgG₁, clone 11b11, catalog no. BE0045) was obtained from Bio X Cell (West Lebanon, NH). PHA-P was obtained from Invitrogen (catalog no. inh-phap), and LPS from *Escherichia coli* O55:B5 were obtained from Sigma-Aldrich (catalog no. L2880-10MG).

Ethics statement

Animal studies were performed according to the *Guide for the Care and Use of Laboratory Animals* and the National Institutes of Health. Protocols were approved by the University of Minnesota Institutional Animal Care and Use Committee. Animals were euthanized in American Association of Laboratory Animal Care–approved CO₂ chambers, and all efforts were made to minimize suffering.

Mice

Six- to 10-wk-old male wild-type BALB/c, C.129X1-*Il4ra*^{tm1Tch}/J (strain no. 007680), or J:DO diversity outbred mice (strain no. 009376) were obtained from The Jackson Laboratory (Bar Harbor, ME). Mice were group housed under a 14-h light/10-h dark cycle and had free access to food and water. All testing occurred during the light phase.

Hapten synthesis and conjugate vaccines

An oxycodone-based hapten containing a tetraglycine linker at the C6 position (OXY) was synthesized and conjugated to either sKLH (Biosyn, Carlsbad, CA) or chicken OVA as previously described (37, 38). Sixty micrograms of unconjugated sKLH or conjugate vaccine were adsorbed on 300 μ g of aluminum adjuvant (Alhydrogel 85, 2%, Brenntag Biosector, Frederikssund, Denmark) diluted in PBS for immunization at a total volume of 60 μ l.

Experimental design

For active immunization studies, mice were immunized on days 0, 14, and 28 with sKLH alone or OXY-sKLH i.m. in two sites (opposite legs) in the gastrocnemius muscle. For IL-4 depletion, 0.5 mg of anti-IL-4 was administered i.p. on days –2 and 1. Mice were bled via facial vein on day 34 for oxycodone-specific IgG analysis. J:DO mice were also bled on days –28 and –14 prior to study for in vitro stimulation of whole blood. On day 35, mice were challenged with 2.25 mg/kg oxycodone delivered s.c. Thirty minutes after injection, mice were euthanized and decapitated to collect brains and blood samples for LC-MS/MS analysis. For passive immunization studies, BALB/c mice were immunized i.p. with 40 mg/kg mAb. Twenty-

four hours later, mice were bled via facial vein to determine Ab concentration using biolayer interferometry. One hour later, mice were challenged with 2.25 mg/kg oxycodone s.c. After 30 min, mice were euthanized and blood and brain were collected to determine oxycodone concentration via LC-MS/MS analysis.

Flow cytometry

Bone marrow collected from mouse femurs on day 100 postimmunization was analyzed for oxycodone-specific long-lived plasma cells. Bone marrow was processed to a single-cell suspension and stained extracellularly for allophycocyanin-eFluor 780 CD90.2 (eBioscience), allophycocyanin-eFluor 780 F4/80 (eBioscience), allophycocyanin-eFluor 780 Ly6G (eBioscience), and allophycocyanin-eFluor 780 CD11c (eBioscience) all as part of the dump gate, and with CD138 BV650 (eBioscience). Cells were permeabilized using a BD Cytotfix/Cytoperm kit and stained with PE-AF647 decoy, OXY-biotin-streptavidin-PE, and Pacific Orange F(ab')₂ (H+L) (Invitrogen). All fluorophores were diluted 1:100 for staining. Oxycodone-specific bait and decoy reagents were produced as previously described (16). Plasma cells were defined as dump⁺decoy⁺Ig⁺CD138⁺, and oxycodone-specific plasma cells were defined as plasma cells that were decoy⁺OXY⁺.

Ab analysis

Total IgG and IgG subclass-specific titers were measured via ELISA as previously described (16). Briefly, 96-well Costar plates were coated with 5 ng/well OXY-OVA in carbonate coating buffer overnight at 4°C. The following day, plates were blocked for 1 h with 1% porcine gelatin, and serum samples were serially diluted starting at 1:200 in PBS with Tween 20. Plates were incubated for 2 h, followed by overnight incubation with secondary Abs as follows: goat-anti-mouse IgG HRP (1:30,000; Jackson ImmunoResearch Laboratories, West Grove, PA), goat-anti-mouse IgG₁ (1:35,000; Alpha Diagnostic International, catalog no. 40126-GAF-BLK), or goat-anti-mouse IgG_{2a} (1:7500; Alpha Diagnostic International, catalog no. 40127-GAF-BLK). For IgE ELISAs, plates were coated with 5 ng/well OXY-OVA or IgE capture Ab (Bio-Techne, catalog no. MAB9935-100). Serum was diluted starting at 1:50, and primary Abs were incubated with 1:5000 anti-IgE Ab conjugated to HRP (VWR International, catalog no. 100242-786). Purified mouse IgE (BioLegend, catalog no. 401701) was used to obtain a concentration curve. The following day, plates were developed using SIGMAFAST OPD (*o*-phenylenediamine dihydrochloride) substrate (Sigma-Aldrich, St. Louis, MO).

BCR sequencing

BCR sequencing of previously characterized hybridomas generated from mice immunized with OXY-sKLH or OXY-sKLH+anti-IL-4 (39) was carried out as previously described (40). Briefly, 1–5 million cells were pelleted in a microfuge tube via centrifugation at 2000 rpm. Cells were washed with 1 ml of PBS, pelleted, and frozen at –80°C until processed. To extract RNA, pellets were thawed, and RNA was extracted using an RNeasy Mini kit (Qiagen, Hilden, Germany). RNA was reverse transcribed with a Maxima first-strand cDNA synthesis kit (Thermo Fisher Scientific, Waltham, MA). cDNA was amplified by PCR using Q5 high-fidelity DNA polymerase. The PCR product was then purified using a QIAquick PCR purification kit (Qiagen, Hilden, Germany). Amplified DNA was sent for Sanger sequencing at the University of Minnesota Genomics Center. H chain sequences were aligned to germline mouse H chain sequences using IgBLAST, and mutations in CDR1 and CDR2 regions were quantified.

Expression of oxycodone-specific mAbs

Oxycodone-binding mAb (clone HY1-3G8) (39) V_H and V_L sequences were cloned into pcDNA3.4 mammalian expression vectors (GenScript). The CMV promoter-driven pcDNA3.4 expression vector was modified to contain a Kozak consensus sequence preceding an open reading frame (ORF) with a murine IGHV signal peptide (MGWSCILFLVATATGVHS), or a murine IGKV signal peptide (METDTLLLVWLLWVPGSTG) for the Ab H and L chain expression vectors, respectively. The H chain ORF terminates with a murine IgG₁ or IgG_{2a} C region (accession nos. P01868.1 and P01863.1, <https://www.uniprot.org>), and the L chain vector ORF terminates with a murine IgK C region (accession no. P01837.2, <https://www.uniprot.org>). The IgG_{2a} sequence was mutated using site-directed mutagenesis to introduce L234A, L235A, and P329G mutations (IgG_{2a}-LALA-PG) to block FcγRI–IV binding as previously described (41). Oxycodone-specific mAbs were produced via transient expression in the Expi293 or ExpiCHO expression system (Thermo Fisher Scientific, catalog nos. A14635 and A29133) according to the manufacturer's instructions. Transfections were performed using a 2.5:1 ratio of LC vector/HC vector, with 1 µg of total vector DNA/ml of culture volume. Cell culture supernatant was harvested 7–10 d following transfection, and mAb was

purified from filtered cell culture supernatant via liquid chromatography on an ÄKTA pure system with a HiTrap protein G HP column (Cytiva, product no. 29048581), and buffer exchanged into PBS (pH 7.4).

ELISPOT

ELISPOT was performed using a Mabtech mouse IgG ELISPOT^{BASIC} kit (HRP). Polyvinylidene difluoride membrane ELISPOT plates were pretreated with 35% EtOH for 1 min and then washed five times with sterile water. Plates were then coated with 5 µg/ml OXY-OVA or 15 µg/ml IgG capture Ab overnight for detection of oxycodone-specific IgG-secreting cells or total IgG-secreting cells, respectively. The following day, plates were washed five times with PBS and blocked with DMEM+10% FBS. Meanwhile, spleens and lymph nodes from immunized mice were collected and processed to a single-cell suspension. Cells were washed three times with ClonaCell medium A (STEMCELL Technologies). Cells were counted and plated in triplicate in ClonaCell medium A at a density of 200,000 cells/well for OXY-OVA-coated wells and 50,000 cells/well for IgG-coated wells. Plates were incubated overnight at 37°C+5% CO₂ and spots were visualized using tetramethylbenzidine substrate according to the manufacturer's instructions. Images were acquired with an CTL BioSpot S5 core analyzer and analyzed using ImmunoSpot 7 software (Cellular Technology, Shaker Heights, OH).

Biolayer interferometry

Ab avidity assays were performed on an Octet RED96e instrument (Sartorius). Serum samples from immunized mice on day 35 (analyte) and biotinylated Ag, OXY-biotin (ligand), were diluted in PBS. Assays were performed by loading OXY-biotin onto prehydrated streptavidin sensors at 0.1 µg/ml (loading step 60 s) followed by 60 s baseline. Sensors were then moved into the analyte for 180 s for association, followed by a 300-s dissociation step. All steps were performed at room temperature with shaking at 1000 rpm. Serum samples were run at 1:200 dilution in PBS. Dissociation rate (*K*_{diss}) and response values were calculated using Sartorius HT analysis software version 11.1.3.50. All data were inspected for quality of fit to the calculated curve (*R*² > 0.95). Monoclonal serum Ab concentrations were calculated by fitting response values to a standard curve produced with an oxycodone-specific mAb.

In vitro whole-blood stimulation

In vitro stimulation of whole-blood samples was adapted from a previously described protocol (42). Facial blood samples were collected in EDTA-coated tubes (SAI Infusion Technologies, Lake Villa, IL). Blood was mixed with 200 µl of RPMI 1640 and plated in a 96-well plate. PHA-P was added to a final concentration of 10 µg/ml, and LPS was added to a final concentration of 1 ng/ml. Samples were incubated at 37°C with 5% CO₂ overnight. The following day, supernatants were collected and quantified with an IL-4 ELISA (BioLegend, San Diego, CA) using the manufacturer's instructions.

LC-MS/MS analysis of oxycodone concentrations

Blood and brain samples were processed and analyzed on an Agilent G6470A triple quadrupole LC-MS/MS system as previously described (43). Data acquisition and peak integration were analyzed using Mass Hunter software (Agilent Technologies, Tokyo, Japan).

Statistical analysis

Statistical analyses were performed using Prism version 9.1.2 (GraphPad Software, La Jolla, CA). Mean Ab titers and concentration, IgG_{2a}/IgG₁ ratios, and drug concentrations were analyzed by one-way ANOVA followed by a Tukey's multiple comparisons post hoc test. *K*_{diss} measurements and the number of H chain mutations were analyzed using a Mann–Whitney *U* test. Total IgG⁺ spots, the ratio of OXY⁺/IgG⁺ spots, total plasma cells, and OXY⁺ plasma cells were analyzed using a Student *t* test with or without Welch's correction, depending on whether the samples had equal variances as indicated by an *F* test. The relationship between oxycodone-specific Abs, serum and brain oxycodone concentrations, and LPS- or PHA-induced IL-4 concentrations were analyzed via a two-tailed Pearson correlation after determination of normality using the D'Agostino–Pearson's test.

Data availability

Data are available upon reasonable request by contacting the corresponding author. Oxycodone mAb binding sequences are confidential due to intellectual property rights.

Results

Ablation of IL-4R-mediated IRS2 signaling does not increase vaccine efficacy

We previously hypothesized that the increase in vaccine efficacy after IL-4 depletion was due to loss of IRS2 signaling downstream of the IL-4 receptor (36). To test this, wild-type (WT) BALB/c mice or mice with a Y500F mutation in the IL-4R that prevents IRS2 phosphorylation downstream of the IL-4R (IL-4R Y500F) were immunized with OXY-sKLH. Heterozygous littermates (IL-4R/IL-4R Y500F) were also immunized to test whether reduced IRS2 signaling would produce an equivalent increase in vaccine efficacy. A group of WT BALB/c mice was immunized with OXY-sKLH with Ab-based IL-4 depletion as a positive control. After three immunizations, IL-4R Y500F and IL-4R/IL-4R Y500F mice did not show increased oxycodone-specific IgG Ab titers (Fig. 1A) or an increased IgG_{2a}/IgG₁ ratio (Fig. 1B) compared with WT mice. At 30 min after oxycodone challenge, the serum/brain ratio of oxycodone in IL-4R Y500F and IL-4R/IL-4R Y500F mice was comparable to that of WT mice without IL-4 depletion (Fig. 1C). These data suggest that deletion of IRS2 signaling downstream of the IL-4R does not increase vaccine efficacy.

IL-4 depletion does not change somatic hypermutation or affinity maturation

IL-4 is involved in somatic hypermutation and affinity maturation of B cells in the GC to produce high-affinity Abs (28, 31). To determine whether depletion of IL-4 during antioioid immunization affects these processes, BCRs were sequenced from hybridomas previously isolated from mice that were immunized with OXY-sKLH with or without an IL-4-depleting Ab. BCR H chain sequences were compared with germline sequences using IgBLAST, and mutations in CDR1 and CDR2 were quantified. There was no significant difference in the number of mutations after immunization with or without IL-4 depletion, indicating that IL-4 depletion had no effect on somatic hypermutation (Fig. 2A). To further test this hypothesis, the K_{diss} of polyclonal Abs from WT mice immunized with or without IL-4 depletion from Fig. 1 was measured using biolayer interferometry as a correlate of Ab avidity. There were no differences in Ab avidity between the two groups (Fig. 2B),

suggesting that affinity maturation is unaffected by IL-4 depletion during antioioid immunization.

Oxycodone-specific IgE was not detected in mice immunized with OXY-sKLH

Because IL-4 is known to induce class switching to IgE after immunization (27, 32), we hypothesized that IL-4 produced after vaccination with OXY-sKLH may induce a subset of opioid-specific IgE Abs that is prevented by addition of an IL-4-depleting Ab. Without the influence of IL-4, these B cells would instead class switch to IgG, which would lead to the increased IgG titers seen in mice with depleted IL-4. Mice were immunized with OXY-sKLH with or without IL-4 depletion on days 0, 14, and 28, and then serum was collected on day 34 for analysis of oxycodone-specific and total IgE concentration. Although total IgE was detected and did decrease after IL-4 depletion, no oxycodone-specific IgE was detected in any groups (Supplemental Fig. 1), indicating that oxycodone-specific IgE is not produced in response to OXY-sKLH immunization, and that the reduction in IgE class switching is not the cause of the IL-4-mediated increase in vaccine efficacy.

IgG_{2a} Abs display equivalent efficacy to IgG₁ Abs against drug challenge in mice

We have reported that depletion of IL-4 increases class switching to IgG_{2a} after antioioid immunization and increases vaccine efficacy after drug challenge (16, 36). To directly assess whether the IgG_{2a} subclass itself is responsible for the increase in vaccine efficacy, an antioxycodone mAb (39) was expressed recombinantly as murine IgG₁, IgG_{2a}, or IgG_{2a} LALA-PG, the latter of which contains mutations to remove FcγRI-IV binding (41). Mice were passively immunized with recombinant mAb and then challenged with oxycodone 24 h later. Blood was collected immediately before challenge, and no differences in Ab concentration were observed between groups (Fig. 3A). Similarly, there were no differences in oxycodone concentration in the serum (Fig. 3B) or brain (Fig. 3C) between the passively immunized groups at 30 min after drug challenge. These data suggest that an increase in IgG_{2a} alone is not sufficient to explain the increase in efficacy seen after depletion of IL-4 during antioioid immunization. Because no differences were found between the WT IgG_{2a} and the IgG_{2a} LALA-PG displaying

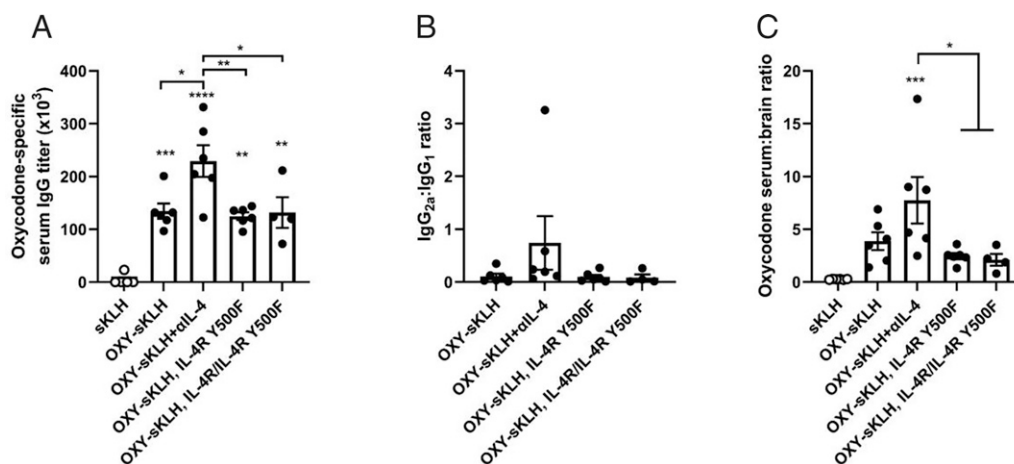


FIGURE 1. Ablation of IL-4-dependent IRS2 signaling does not increase vaccine efficacy after OXY-sKLH immunization. WT mice ($n = 6$ /group) were immunized on days 0, 14, and 28 with OXY-sKLH, with or without IL-4 depletion with an anti-IL-4 mAb and compared with IL-4R Y500F mice ($n = 6$) or their heterozygous littermates ($n = 4$). (A and B) Blood was collected via facial vein on day 34 to measure (A) oxycodone-specific serum IgG titers, and (B) ratio of IgG_{2a} versus IgG₁ subclass titers. On day 35, mice were challenged with 2.25 mg/kg oxycodone. (C) Thirty minutes later, blood and brain were collected to measure concentration of oxycodone in each, expressed as the serum/brain oxycodone ratio. Data are mean \pm SEM. Statistical analysis was performed via a one-way ANOVA with a Tukey's multiple comparison post hoc test. Data are from one independent experiment. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, compared with the control or as indicated by brackets.

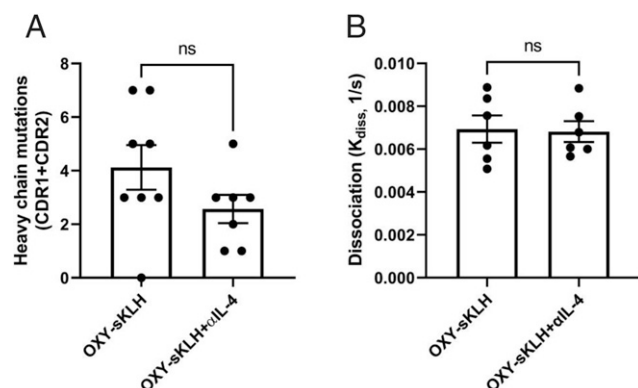


FIGURE 2. IL-4 depletion does not influence somatic hypermutation or affinity maturation. The H chain BCR sequence from hybridomas ($n = 7$ –8/group) generated by immunization with OXY-sKLH or OXY-sKLH with IL-4 depletion were analyzed against germline H chain sequences using IgBLAST. **(A)** Comparison of H chain CDR1 and CDR2 mutations in oxycodone-specific Abs after OXY-sKLH immunization with or without IL-4 depletion. **(B)** Sera from OXY-sKLH and OXY-sKLH+anti-IL-4 immunized mice from Fig. 1 ($n = 6$ /group) were analyzed for avidity via biolayer interferometry. Data are mean \pm SEM. Statistical analysis was performed via a Mann–Whitney U test. Data are from one independent experiment.

mutated Fc γ RI–IV binding sites, this study further supports previous reports that Ab-mediated effector functions do not play a role in opioid vaccine efficacy (44).

IL-4 depletion increases early Ab-secreting B cells but not long-term plasma cells

Previous literature has suggested that IL-4 is involved in the memory cell versus plasma cell choice in the GC (22–24, 28). To assess whether depletion of IL-4 increases the number of ASCs after antioioid vaccination, the same mice from Supplemental Fig. 1 were sacrificed on day 35, and then spleens and lymph nodes were harvested to perform Ag-specific ELISPOT. There were no differences in total IgG-secreting cells between mice immunized with OXY-sKLH and OXY-sKLH with an IL-4-depleting Ab (Fig. 4A). Conversely, there was a significant increase in oxycodone-specific IgG-secreting cells when normalized to the total IgG-secreting cells (Fig. 4B). A second cohort of mice was then immunized in a similar fashion and left until day 100 to assess whether this increase in early ASCs led to an

increase in oxycodone-specific long-lived plasma cells in the bone marrow (gating strategy provided in Supplemental Fig. 2). Analysis of bone marrow showed that there was no significant difference in total or oxycodone-specific long-lived plasma cells in mice immunized with or without IL-4 depletion (Fig. 4C, 4D). These data suggest that the depletion of IL-4 may increase plasmablast formation at day 35; however, these plasmablasts are short-lived and do not home to the bone marrow.

T cell–secreted IL-4 is a predictive biomarker of oxycodone vaccine efficacy in mice

Because depletion of IL-4 increases Ab titers and vaccine efficacy after OXY-sKLH immunization, we hypothesized that preimmunization production of IL-4 would predict postimmunization Ab titers and vaccine efficacy. To assess IL-4 as a predictive biomarker, blood was collected from genetically diverse outbred mice and stimulated ex vivo with either LPS (for innate immune cell stimulation) or PHA (for nonspecific T cell stimulation). After 24 h, supernatant was collected and analyzed for IL-4. Mice were then immunized with OXY-sKLH on days 0, 14, and 28, and blood was collected again on day 34 to assess oxycodone-specific Ab titers. The next day, mice were challenged with 2.25 mg/kg oxycodone, and blood and brain were collected at 30 min postchallenge to analyze the pharmacokinetics of oxycodone. Serum oxycodone-specific IgG concentration significantly correlated with serum and brain oxycodone concentration, as expected (Supplemental Fig. 3). Additionally, preimmunization concentration of IL-4 produced from T cells after ex vivo PHA stimulation showed a significant negative correlation with oxycodone-specific Ab concentration (Fig. 5A), a significant negative correlation with serum oxycodone concentration (Fig. 5B), and a significant positive correlation with brain oxycodone concentration after challenge (Fig. 5C). In contrast, these metrics did not correlate with preimmunization IL-4 produced by innate immune cells through stimulation with LPS (Fig. 5D–F). These data suggest that preimmunization T cell–derived IL-4 produced through nonspecific ex vivo stimulations can be used as a biomarker to predict anti-oxycodone vaccine efficacy.

Discussion

As testing of antioioid vaccines in clinical settings begins, it is imperative to optimize vaccine efficacy to maximize their chance of success. The data from previous clinical trials of SUD vaccines

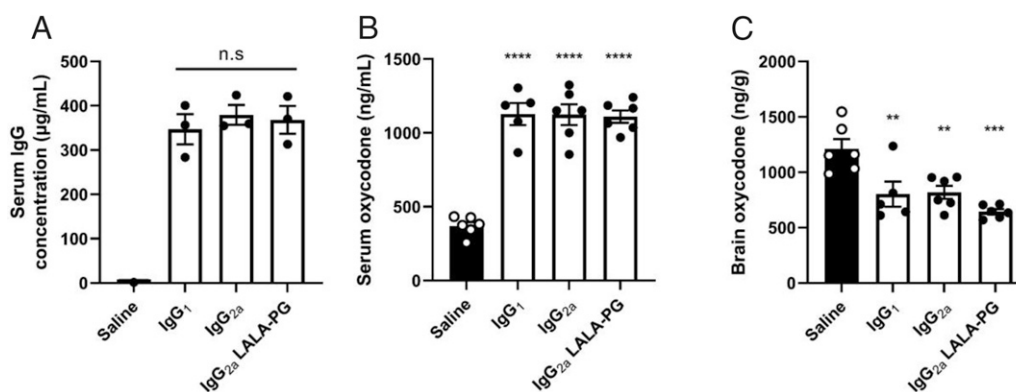


FIGURE 3. IgG subclass and Ab mediated effector functions do not influence vaccine efficacy. Mice ($n = 6$ /group) were passively immunized with recombinant mAb expressed as IgG₁, IgG_{2a}, or IgG_{2a} LALA-PG (mutation which prevents binding to Fc γ RI–IV). **(A)** One day later, serum was collected to measure Ab concentration via biolayer interferometry. **(B and C)** Mice were then challenged with 2.25 mg/kg oxycodone and (B) blood and (C) brain were collected 30 min later to determine opioid concentration by LC–MS/MS. Data are mean \pm SEM. Statistical analysis performed via one-way ANOVA with a Tukey's multiple comparison post hoc test. Two independent experiments were performed. Data shown are representative from one experiment. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, compared with control.

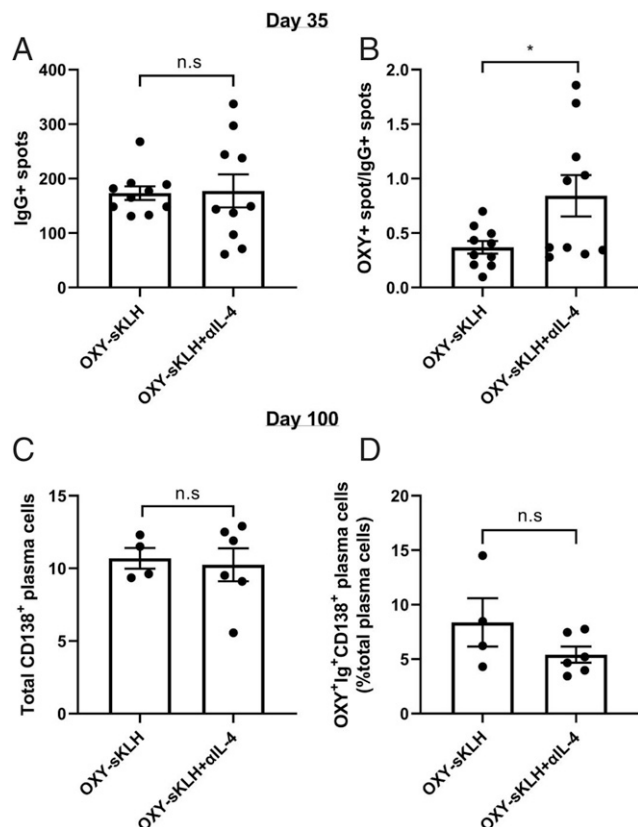


FIGURE 4. IL-4 depletion increases early oxycodone-specific Ab-secreting B cells. Mice ($n = 10$ /group, combined from two independent experiments) were immunized with OXY-sKLH with or without IL-4 depletion. After three immunizations, spleens and lymph nodes were collected and processed into a single-cell suspension. Total and oxycodone-specific Ab-secreting cells (ASCs) were measured by ELISPOT. **(A)** Number of total IgG ASCs and **(B)** oxycodone-specific ASCs normalized to total IgG ASCs. A second cohort of mice ($n = 4$ –6/group, from one independent experiment) were similarly immunized and bone marrow was collected on day 100. **(C)** Total plasma cells in the bone marrow and **(D)** oxycodone-specific plasma cells as a percent of total plasma cells were identified via flow cytometry. Data are mean \pm SEM. Statistical analysis was performed via a Student t test (A, C, and D) or a Welch's t test (B). * $p < 0.05$.

indicate that generation of high concentrations of high-affinity Abs is important to achieve significant vaccine efficacy (7, 8); however, the immunological mechanisms underlying optimal Ab generation are not well characterized. Our previous studies indicate that depletion of IL-4 increased the efficacy of OUD vaccines for oxycodone and fentanyl by increasing total IgG titers and increasing class switching from IgG₁ to IgG_{2a} (16, 36). The role of IL-4 in GC formation appears to be specific to Ag/adjuvant context, with it being critical for GC formation in some studies, whereas in other studies GCs are enhanced in its absence (16, 24–26, 28, 33–36). We have previously shown that in the context of an OXY-sKLH vaccine, IL-4 depletion increases GC formation (36), increases Ag-specific GC-T_H cells (16), and modulates T cell transcription via signaling through the type I IL-4 receptor, but not through STAT6 (36). Additionally, we have found that depletion of IL-4 increases the efficacy of other antioioid vaccines, including a lead fentanyl vaccine (F-CRM) (36), suggesting that these findings may be applicable to additional OUD or SUD conjugate vaccines. To extend our previous findings, we tested whether this increase in efficacy could be recapitulated by ablation of IRS2 signaling downstream of the IL-4 receptor. Furthermore, we tested the effect of IL-4 depletion on

specific GC processes that IL-4 has been shown to regulate, including somatic hypermutation and affinity maturation, class switching to IgG₁ and IgE, and plasma cell generation. Finally, we tested whether the relative inherent ability of T cells or innate immune cells to produce IL-4 could be used as a predictive biomarker of vaccine efficacy.

Previous studies have shown that depletion of IL-13 during active immunization against oxycodone does not produce an increase in vaccine efficacy equivalent to IL-4 depletion, indicating that the increase in efficacy is mediated by a reduction in type I signaling (36). Furthermore, whereas genetic deletion of IL-4 in mice recapitulates the increase in vaccine efficacy seen after Ab-based depletion, deletion of IL-4R or STAT6 does not (36). Similarly, in the current study, mice bearing a Y500F mutation in the IL-4Ra chain to eliminate downstream signaling through phosphorylation of IRS2 did not show an increase in vaccine efficacy compared with WT mice. Taken together, these results were not able to implicate IL-13, IL-4R, STAT6, or IRS2, which are the main components of the canonical IL-4 signaling pathway, in the mechanism of the increase in vaccine efficacy following IL-4 depletion. Some literature suggests that IL-4 may signal through STAT5 in certain contexts (45), making this a possible contributor to the increase in efficacy seen after IL-4 depletion; however, testing this hypothesis may be challenging due to the lack of highly specific STAT5 pharmacological inhibitors and STAT5's role downstream of other signaling pathways, including IL-2 family receptors (IL-2, IL-7, IL-9, and IL-15), GM-CSF, erythropoietin, thrombopoietin, epidermal growth factor, and platelet-derived growth factor (46). Another potential hypothesis is that the effect is mediated through inhibition of IL-4 signaling through a non-IL-4R-based pathway, although there is no evidence to date of IL-4 signaling through a noncanonical receptor. One caveat to these studies is the use of full genetic deletions to test the functions of IL-4 signaling. Not only can genetic manipulations lead to uncharacterized immune system deficiencies, but the elucidation of the increasingly complex and often opposing role of IL-4 signaling in different cell types (24, 28) makes one speculate that deletion of IL-4 signaling in all cell types may be hiding phenotypes specific to certain cell populations that would require more nuanced approaches to assess, such as conditional knockouts or adoptive transfer.

IL-4 has been shown to be important for somatic hypermutation and affinity maturation in some contexts (28, 31). Studies in IL-4-deficient animals have shown a significant reduction in activation-induced cytidine deaminase (28) and a reduction in affinity of Abs to the target Ag. These changes were more pronounced in IgG₁-secreting B cells compared with IgG_{2a}-secreting B cells (31). In the current study, no differences were seen in the number of mutations in H chain CDR1 and CDR2 in antioxycodone mAbs isolated from vaccination with or without IL-4 depletion. A limitation of this method is that mAb selection using hybridoma technology may be biased toward high-affinity clones, and a limited number of sequences were obtained to compare between groups. Therefore, as a follow-up experiment, Ab avidity in polyclonal sera was measured by biolayer interferometry, and no difference was found in Ab avidity between groups with or without IL-4 depletion. Because the polyclonal Ab response consisted of both IgG₁ and IgG_{2a} Abs, it is possible that there were differences in affinity maturation in the IgG₁ subset that were not detected due to compensation in affinity from the IgG_{2a} subset. Regardless, any changes in affinity that may be present in a subset of cells did not prevent an increase in efficacy after drug challenge, indicating that Ab affinity is not necessary for the increased efficacy in this specific context.

We have long reported that increases in IgG_{2a}, along with increases in IgG₁, have correlated with increases in vaccine efficacy in a variety of adjuvant contexts (16, 36). In this study, we directly

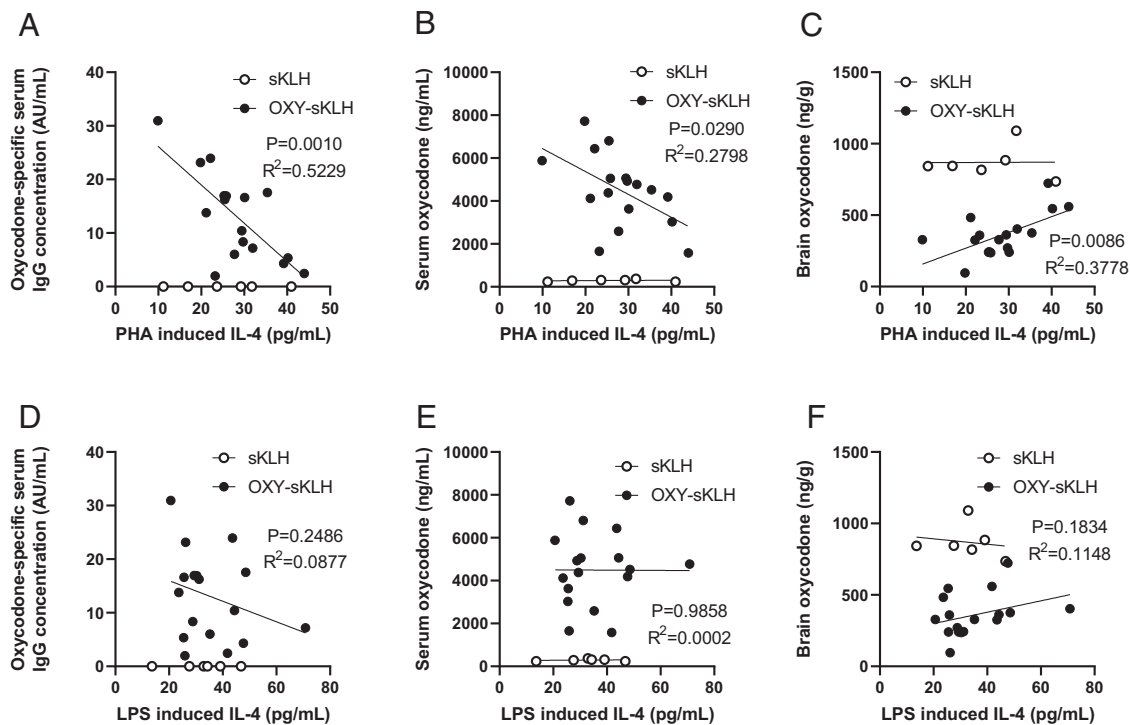


FIGURE 5. Preimmunization IL-4 concentration correlates with vaccine efficacy in mice. Outbred diversity mice (J:DO; The Jackson Laboratory) mice were bled preimmunization, and whole blood was plated for *in vitro* stimulation with either PHA (to stimulate T cells) or LPS (to stimulate innate immune cells). After 24 h, supernatant was collected and IL-4 was measured via ELISA. Mice ($n = 18$ active immunization, $n = 6$ control immunization) were then immunized three times and blood was collected 7 d after the final immunization. Oxydodone-specific IgG concentration was measured by ELISA. Mice were subsequently challenged with oxydodone (2.25 mg/kg, s.c.), and blood and brain were collected 30 min postchallenge to measure concentration of oxydodone via LC-MS/MS. **(A–C)** Linear correlation between (A) oxydodone-specific IgG concentration and PHA-induced IL-4, (B) postchallenge serum oxydodone concentration and PHA-induced IL-4, and (C) postchallenge brain oxydodone concentration and PHA-induced IL-4. **(D–F)** There was no linear correlation between (D) oxydodone-specific IgG concentration and LPS-induced IL-4, (E) postchallenge serum oxydodone concentration and LPS-induced IL-4, or (F) postchallenge brain oxydodone concentration and LPS-induced IL-4. Linear associations were determined via a Pearson correlation. Data are from one independent experiment.

tested whether IgG_{2a} Abs have inherently greater capacity than IgG₁ to protect against oxydodone after challenge by passively immunizing mice with an antioxydodone mAb expressed as IgG₁, IgG_{2a}, and the FcγR-silent IgG_{2a}-LALA-PG variant. This study showed no difference between the mAbs in their efficacy in preventing oxydodone distribution to the brain, indicating that an individual IgG subclass is not inherently sufficient or responsible for increasing vaccine efficacy at the time of drug challenge. We hypothesize that activation of multiple IgG subclasses is required for achieving higher overall IgG Ab titers (i.e., IgG₁+IgG_{2a}+IgG_{2b}) and a more effective response against opioids. The lack of noticeable effects of the FcγR-silent IgG_{2a}-LALA-PG variant further reinforces previous reports that Ab-mediated effector functions are not necessary for vaccine efficacy after drug challenge (44). One alternative hypothesis for the correlation between IgG_{2a} production and increased vaccine efficacy is that an early IgG_{2a} response during immunization increases Fc-mediated Ag presentation of immune complexes, which in turn increases overall Ab production. Another plausible explanation is that rather than needing a Th₁-polarized response, adjuvants that can stimulate both Th₁ and Th₂ responses can engage nonoverlapping populations of B and T cells, leading to an overall increase in Ab production. These results will be important for future vaccine design and adjuvant selection to pair Th₁- and Th₂-inducing adjuvants.

During GC formation, IL-4 has been implicated in the differentiation of Ab-secreting plasma cells and in the plasma versus memory cell choice (24, 28). Because IL-4 depletion increases GC-T_{fh} cells in the GC, which results in increased Ab titers, we hypothesized

that the increased number of GC-T_{fh} cells leads to increased interactions with GC B cells, increasing survival and differentiation into plasma cells. We found that immunization with OXY-sKLH and a depleting IL-4 mAb increased the number of oxydodone-specific ASCs at day 35, visualized via ELISPOT. When determining whether the increase in plasmablast formation translated into an increase in long-lived plasma cells in the bone marrow, we found no significant difference in oxydodone-specific plasma cells at day 100. This suggests that the increase in plasmablasts caused by IL-4 depletion is transient, and these cells likely die during the contraction of the immune response, or alternatively they may become tissue-resident plasma cells in other organs. Transient increases in ASCs may be beneficial for OUD/SUD vaccines, as recent arguments have suggested that long-lived and/or permanent immunity to drugs of abuse raise ethical issues of bodily autonomy. Although long-lasting treatment may be beneficial in some ways for sustained abstinence due to ease of patient burden to receive frequent treatment, some have suggested that permanent blockade of opioids through immunization could lead to ethical considerations relating to patient choice to continue treatment, or even the possibility of coerced treatment within vulnerable populations (47). As vaccines for OUDs and other SUDs undergo clinical evaluation, these ethical issues will have to be considered carefully. The current results do suggest that further investigation is needed into the mechanisms of IL-4 before considering it to be used as a molecular adjuvant; however, given the current results, we hypothesized that IL-4 may hold additional promise as a predictive biomarker of vaccine efficacy.

As OXY-sKLH is currently undergoing clinical evaluation, it is necessary to test putative biomarkers to identify correlates of protection, which may be critical to identifying individuals who would most benefit from OUD vaccines. Previous studies have identified that the preimmunization frequency of oxycodone-specific naive B cells correlates positively with vaccine response (48), providing the first putative biomarker for efficacy of the OXY-sKLH vaccine. Owing to our findings that IL-4 depletion increases vaccine efficacy, we hypothesized that IL-4 may also have utility as a predictive biomarker, with those who inherently produced lower levels of IL-4 after immunization (Th₁-biased immune response) responding better to antioioid immunization than those who have an innately Th₂-biased immune response. Therefore, we tested whether the concentration of preimmunization IL-4 after nonspecific stimulation of either T cells or innate immune cells in vitro would correlate with postimmunization vaccine response in vivo. We found that in genetically diverse outbred mice, higher levels of preimmunization IL-4 produced by T cells stimulated with PHA negatively correlates with oxycodone-specific IgG titers. These data are consistent with our previous findings that IL-4 depletion affects GC-T_h cells and the T cell transcription program (16, 36). A similar personalized medicine approach could be implemented as a screening strategy for patients by analyzing the potential of their T cells to produce IL-4 or other cytokines prevaccination through in vitro or ex vivo assays to determine which individuals would respond favorably to antioioid immunization.

In this final experiment, we did not see a correlation between innate immune cell-derived IL-4 through stimulation with LPS and vaccine response. Note that TLR4, the receptor to which LPS binds, has been shown to be necessary for vaccine efficacy after OXY-sKLH immunization (14), yet TLR4 agonists have not shown consistent success in increasing the efficacy of vaccines for OUD (14, 15). In the human population, IL-4 and its downstream signaling components (IL-4R, STAT6) are highly polymorphic loci with high minor allele frequencies for individual single-nucleotide polymorphisms (49–57), making it an attractive target for a predictive biomarker in clinical studies. In fact, IL-4 has been shown to correlate with efficacy in vaccines against hepatitis B (49, 58), Japanese encephalitis virus (51), measles (52), tetanus and diphtheria (59), and *Streptococcus pneumoniae* (60). The current results support the use of IL-4 as a putative predictive biomarker for patient stratification in future clinical studies for OUD vaccines, including an ongoing phase I trial for OXY-sKLH, and ultimately for individualized or personalized treatment of patients with OUD.

Acknowledgments

We thank the University of Minnesota Flow Cytometry Resource for technical and research support, and Dr. Steve Jameson (University of Minnesota) for the gift of IL-4R Y500F mice.

Disclosures

M.P. is the inventor of “Cytokine signaling immunomodulators and methods.” M.P., C.B., and D.H. are inventors on other pending patents related to vaccines or Abs against opioids. B.C. has no financial conflicts of interest.

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