Systems Biology of Inactivated Influenza Vaccine (IIV) in healthy adults with or without use of broad spectrum antibiotics.

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INVESTIGATOR SIGNATURE PAGE	
Protocol Systems Biology of Inactivated Influenza vaccine (IIV) in healthy adults with or without use of broad spectrum antibiotics**.**	**Version/Date:** 7.0/05/13/2016
	Principal Investigator: Nadine Rouphael, MD
Short Title*:* Responses to IIV in adults with or without antibiotics.	
INSTRUCTIONS: The Principal Investigator will print, sign, and date at the indicated location below. A copy should be kept in the investigator's records.	

I confirm that I have read the above protocol in the latest version. I understand it, and I will work according to principles of Good Clinical Practice (GCP) as described in the United States Code of Federal Regulations (CFR) 45 CFR part 46 and 21 CFR parts 50, 56, and 312 , and the International Conference on Harmonization (ICH) document "Guidance for Industry: E6 Good Clinical Practice: Consolidated Guidance" dated April 1996[1] and the spirit of the Declaration of Helsinki, Further, I will conduct the study in keeping with local, legal, and regulatory requirements.

As the Principal Investigator, I agree to conduct VAX-002: *Systems Biology of Inactivated Influenza vaccine (IIV) in healthy adults with or without use of broad spectrum antibiotics***.** I agree to carry out the study by the criteria written in the protocol and understand that no changes can be made to this protocol without written permission by the local IRB.

___NADINE ROUPHAEL, MD_____
Principal Investigator (Print)

_____ _____
Principal Investigator (Signature) Date

Synopsis

Title	Systems Biology of Inactivated Influenza Vaccine (IIV) in healthy adults with or without use of broad spectrum antibiotics
Short Title	Responses to IIV in adults with or without antibiotics
Rationale	Use of antibiotics can significantly impact the microbiota of patients. The status of the intestinal microbiome may impact the immune responses to vaccinations and alter functional protective immunity in humans. Here we hypothesize that the changes in the fecal microbiome induced by antibiotics translates into suboptimal Hemagglutination Inhibition Assay (HAI) titers.
Clinical Phase	N/A
Mechanistic Study	Yes
Principal Investigator	**Nadine Rouphael, MD (PI)**
Participating Site(s)	The Hope Clinic of the Emory Vaccine Center The Hope Clinic is a community-based vaccine research clinic and is the clinical arm of the Emory Vaccine Center at Emory University.
Accrual Objective	N=50 total (age 18-40)
Primary Study Objective	To compare HAI titers after vaccination with IIV in adults with or without use of antibiotics
Study Design	Single center, open mechanistic study in which subjects will be randomized to receive IIV per label at day 4 of a 5 day course of a specific antibiotic regimen (Group A) or IIV alone (Group B) Blood samples for immunologic testing will be collected at screening (from D -21 to D -1), on D0 (at vaccination), D1, D3, D7 (+/- 1 day), D30 (+/- 5 days), D90 (+/-14 days), D180 (+-/14 days), D365 (+/-28 days) post vaccination for both groups to study innate and/or adaptive immune responses. Stool samples will be collected in both groups at screening (from D -21 to D -1), on vaccination (D0), D1, D3, D7 (+/- 1 day) and D30 (+/- 5 days), D90 (+/-14 days), D180 (+/-14 days), D365 (+/- 28 days) to study the gut microbiome in both groups. For Group A, additional visit on D-6 to D-3 will occur with stool and blood samples collection. Study will be conducted outside the 2014-2015 and 2015-2016 influenza seasons.

	Antibiotics received by Group A will be started 3 days prior to vaccination (D-3) and continued on day of vaccination (D0) and for one day after vaccination (D1) for a total of 5 days • Flagyl® 500 mg po tid • Vancocin® 125 mg po qid • Neomycin sulfate ® 500mg po tid The dosage of each antibiotic is taken from their respective package inserts and does not exceed the maximum dose allowed for each antibiotic. The antibiotic regimen is a broad spectrum regimen covering all types of fecal flora (anaerobes, gram positive, and gram negative bacteria); has a good safety record; has poor systemic absorption for part of the regimen (Vancocin® and Neomycin sulfate®); and part of the regimen is used to treat *Clostridium difficile* infections (Vancocin® and Flagyl®). Subjects in Group A are asked to avoid all ethanol and any ethanol-containing drugs while taking antibiotics and for 48h before and after taking the antibiotics. Blood samples for safety laboratory testing (including CBC with differential, creatinine, potassium) will be collected at screening (from D-21 to D -1) and D 7 (+/- 1 day) for both groups. For Group A, stools will be screened for *Clostridium difficile* carriage by PCR at (from D-21 to D-1).
Study Duration	Approximately 12 months
Primary Endpoints	Comparison of HAI titers at D30 post vaccination in both groups
Secondary Endpoints	Frequency, severity, and causality of all adverse events in Group A subjects.
Exploratory Endpoints	• Analyses of microbiome at D0, D1, D3, D7, D30, D90, D180, D365 and comparison to baseline screening in both groups • Analysis of the repertoire and monoclonal antibodies from plasmablasts in a subset of vaccinees at D7 in both groups • Identification of innate immune signatures (traditional immune parameters measurements + array-based gene expression) at D1, D3 and D7 in both groups • Correlation of innate immune signatures at days D1, D3 and D7 with HAI titers at D30 in both groups • Comparison of HAI titers at D90, D180 and D365 post vaccination in both groups.

Inclusion Criteria	1. Healthy individuals aged 18-40 years 2. Able to understand and give informed consent 3. Women of child-bearing potential (not surgically sterile via tubal ligation, bilateral oophorectomy or hysterectomy or who are not postmenopausal for ≥1 year) must agree to practice adequate contraception that may include, but is not limited to, abstinence, monogamous relationship with vasectomized partner, barrier methods such as condoms, diaphragms, spermicides, intrauterine devices, and licensed hormonal methods* for 30 days before and 30 days after IIV vaccination. * Women of child-bearing potential using licensed hormonal methods must also use a second form of contraception. 4. Men must agree not get a sexual partner pregnant for 1 month after enrollment.
Exclusion Criteria	1. Receipt of the following: • Receipt of blood products 3 months prior to vaccination or expected receipt through 6 months after vaccination. • Receipt of any live virus vaccines within 4 weeks prior to vaccination or expected receipt within 4 weeks after vaccination.* • Receipt of any inactivated vaccine within 2 weeks or expected receipt within 2 weeks after vaccination.* • Receipt of any influenza vaccine in the past 3 seasons. • Receipt of any systemic antibiotic 6 months prior to vaccination or expected receipt 1 month after vaccination. • Receipt of probiotics and prebiotics 3 months prior to vaccination or expected receipt 1 month after vaccination. • Regular receipt of proton pump inhibitors, H2 receptor blockers, or antacids 3 months prior to vaccination or expected receipt 1 month after vaccination. 2. Documented influenza infection during the 2014-2015 or 2015-2016 influenza season. However, volunteers with prior upper respiratory infections during the 2014-2015 or 2015-2016 influenza illness will not be excluded from the study. 3. Presence of co-morbidities or immunosuppressive states such as: • Chronic medical problems including (but not limited to) insulin dependent diabetes, severe heart disease (including arrhythmias), severe lung disease, auto immune diseases, and grade 4 hypertension**.

	• Chronic neurologic conditions including seizure disorder, Parkinson's disease, myasthenia gravis, neuropathy, or history of encephalopathy, meningitis or ototoxicity. • Any history of gastrointestinal disease including (but not only): documented bacterial gastroenteritis or gastroenteritis associated with fever or associated with presence of blood/mucus in stools in the last 3 months; inflammatory bowel disease, and/or gastrointestinal surgery. • Any history of kidney or liver diseases. • Alcohol abuse, drug abuse, or psychiatric conditions that in the opinion of the investigator would preclude compliance with the trial or interpretation of safety or endpoint data. • Impaired immune function or known chronic infections including, but not limited to, known HIV, hepatitis B or C; organ transplantation; immunosuppression due to cancer; current and/or expected receipt of chemotherapy, radiation therapy, steroids* (i.e., more than 20 mg of prednisone given daily or on alternative days for 2 weeks or more in the past 90 days , or high dose inhaled corticosteroids***); and any other immunosuppressive therapies (including anti-TNF therapy), functional or anatomic asplenia, or congenital immunodeficiency. • Pregnancy or breast feeding 4. Conditions that could affect the safety of the volunteers, such as: • Severe reactions to prior vaccination with IIV, including anaphylaxis • History of Guillain Barré syndrome • History of bleeding disorders or current use of warfarin • Use of anticonvulsants • Use of digoxin or other forms of digitalis • Any allergy to any component of the vaccine including egg allergy • Allergy to vancomycin, metronidazole or neomycin as well as other aminoglycosides (gentamicin, tobramycin, amikacin, streptomycin) 5. Volunteers with any acute illness, including any fever ($\geq$ 100.4 F [$\geq$ 38.0C], regardless of the route) within 3 days prior to vaccination *. 6. Social, occupational, or any other condition that in the opinion of the investigator might interfere with compliance with the study and vaccine evaluation. 7. Positive *C difficile* testing by PCR at screening or history of *C difficile* infection. 8. Any grade 2 safety lab test results at screening.

	Note: *An individual who initially is excluded from study participation based on one or more of the time-limited exclusion criteria (e.g., acute illness, receipt or expected receipt of live or inactivated vaccines) may be reconsidered for enrollment once the condition has resolved as long as the subject continues to meet all other entry criteria. Subjects receiving ≥ 20 mg/day of prednisone or its equivalent daily or on alternate days for more than 2 weeks may enter the study after therapy has been discontinued for more than 3 months. ** Grade 4 hypertension per CTCAE criteria is defined as Life-threatening consequences (e.g., malignant hypertension, transient or permanent neurologic deficit, hypertensive. *** Subjects are excluded if on high dose intranasal steroids defined as > 960 mcg/day of beclomethasone dipropionate or equivalent.
Investigational Product(s)/ Intervention(s)	Licensed Inactivated Influenza vaccine (IIV): 2014-2015 and 2015-2016 Fluzone® (Sanofi Pasteur, Swiftwater, PA, USA). Antibitoics: Flagyl ®(Pfizer, New York, NY) Vancocin® (ViroPharma , Exton, PA) Neomycin sulfate® (X-Gen Pharmaceuticals, Big flats, NY)
Study Procedures	Antibiotic administration, vaccination, targeted physical examination, urine pregnancy testing, phlebotomy, stool collection, signs/symptom assessment (local and systemic) and vaccination site evaluation.
Statistical Considerations	Based on previous studies performed at the Hope Clinic, the rate of subjects that become ineligible during the study (i.e. do not meet exclusion/inclusion criteria anymore, lost to follow-ups, unable to have blood draws) is between 5 to 10%. Assuming a 10% attrition rate, 50 healthy adults will be recruited for the study, resulting in an expected 10 subjects per arm for data analysis.

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GLOSSARY OF ABBREVIATIONS

ACIP	Advisory Committee on Immunization Practices
AADCRC	Asthma and Allergic Diseases Cooperative Research Center
AE	Adverse Event
CFR	Code of Federal Regulations
CRF	Case Report Form
CTCAE	Common Terminology Criteria for Adverse Events
DAIT	Division of Allergy, Immunology, and Transplantation
cGCP	Current Good Clinical Practice
GLP	Good Laboratory Practice
HAI	Hemagglutination Inhibition Assay
ICH	International Conference on Harmonization
IDE	Investigational Device Exemption
IFNg	Interferon gamma
IIV	Inactivated Influenza Vaccine
IL	InterLeukin
IM	IntraMuscular
ISM	Independent Safety Monitor
IND	Investigational New Drug
IRB	Institutional Review Board
MOP	Manual of Procedures
NCI	National Cancer Institute
NIAID	National Institute of Allergy and Infectious Diseases
NF kB	Nuclear Factor kappa-light-chain-enhancer of activated B cells
NSAIDs	NonSteroidal Anti-Inflammatory Drugs
PI	Principal Investigator

QID	Quater In Die (four times a day)
SAE	Serious Adverse Event
SAP	Statistical Analysis Plan
SOP	Standard Operating Procedure
Th17	T Helper 17 Cells
TID	Ter In Die (three times a day)
TLR	Toll-Like Receptor

1 BACKGROUND AND RATIONALE

1.1 Background

The human commensal flora is composed of diverse and complex communities of microorganisms, which outnumber host cells by a factor of ten (1). They inhabit barrier surfaces of major organs (e.g. skin) and tissues (e.g. gastrointestinal tract) and have evolved beneficial properties for the host that permits a symbiotic relationship to be maintained with the host (2). These include an array of functions ranging from enhancing digestion and metabolism to occupying a niche that competes against colonization with invasive pathogens (3-7). Moreover, associations between alterations or dysbiosis of the microbiota and susceptibility to metabolic and pro-inflammatory diseases such as obesity, diabetes, and inflammatory bowel diseases in humans have been widely reported (8-10). Thus, interactions between the host and microbiota are crucial factors supporting the balance of their symbiotic relationship.

Evidence emerging from the past decade highlights additional roles for the microbiota, most notably in influencing the development and homeostasis of the host immune system. Studies of germ-free animals, which are devoid of any detectable microorganisms, have revealed that commensal-derived signals are critical for maintaining a balance of pro-inflammatory and regulatory immune mechanisms (11-16). Germ-free mice exhibit reduced capacity of T cells to mount IL-17A and IFNg responses in both gut-associated tissues and the skin. Furthermore, these mice also exhibit increased frequency of regulatory CD4 T cells. Recent studies using animal model systems have also identified specific commensal species that can either promote pro-inflammatory or immunoregulatory pathways in the host. For example, colonization of mouse gut with segmented filamentous bacteria results in enhanced levels of Th17 cells in the gut (17), while Bacteroidetes fragilis or Clostridium subspecies results in the induction IL-10 responses and regulatory CD4+ T cells (18-20). Also, previous studies in mice have empirically demonstrated that the depletion of host microbiota – via orally administered broad-spectrum antibiotics can influence the development and homeostasis of the immune system (11-13, 17-19, 21-25). Together, these observations indicate that specific composition of microbiota is an important factor influencing the host immune system. Thus, it also suggests that any alterations to the gut microbiota can influence the capacity of the host immune system to mount or regulate responses against pathogens appropriately. Accordingly, several reports using mice suggest that in the absence of the microbiota, host susceptibility to infection is enhanced and the capacity to control the infection is compromised.

A major implication of these findings for global public health is the possibility that the microbiota also plays a role in immune responses to vaccines. If so, the status of the host microbiota may be a critical determinant of vaccine efficacy and alteration of mirobiota through antibiotic exposure could negatively impact vaccine efficacy. Currently, there is no published information in both humans and animal models whether the microbiota impacts the magnitude and/or quality of vaccine-induced immune responses. Examining the relationship between microbiota and vaccine immunogenicity is a novel concept, and understanding how such a phenomenon operates is a critical field to study. In this regard, the seasonal influenza vaccine would be an important area to focus upon given that it is one of the most widely and frequently administered vaccines worldwide. The goal of our study is to determine whether alteration of microbiota by antibiotic exposure can negatively impact the immunogenicity of IIV, and to assess the innate and adaptive immune mechanisms responsible for that phenomenon.

1.2 Immune Response to IIV Given With Antibiotics

Recent work from our group at the Emory Vaccine Center (Pulendran Laboratory) on the systems biology of the influenza vaccine has revealed a network of genes centered around TLR5 (26), which were found to be induced robustly in humans 3 days after vaccination with IIV. Intriguingly, this early expression of TLR5 exhibited a striking level of correlation with the magnitude of HAI titers at day 28 post vaccination. This was an unexpected observation given that TLR5 is not known to be a sensor of viral stimuli, but rather of bacterial flagellin. We addressed whether IIV was capable of signaling through TLR5 by utilizing a fibroblast cell line transfected with TLR5 and NF-Kb reporter gene, and, indeed, we found no indication that IIV was able to do so.

Thus, we subsequently determined whether TLR5 was essential for the induction of antibody responses to IIV vaccination. TLR5-deficient and wild-type littermate control mice were vaccinated with IIV and antigen specific antibody titers were measured at various time points following vaccination. We observed a striking reduction in the magnitude of antigen specific antibody response in TLR5-deficient mice. Given that we previously established that IIV itself does not signal through TLR5, we hypothesized that the microbiota-derived factors contribute to TLR5 mediated augmentation of the immunogenicity of IIV. To test this hypothesis, wild-type mice were administered a cocktail of broad-spectrum antibiotics (Neomycin, Ampicillin, Vancomycin,

Metronidazole) via their drinking water to eliminate the microbiota as previously described (12, 13, 20, 21, 25). Antibiotics were administered for 4 weeks prior to vaccination with IIV and continued without cessation for periods following vaccination at which antibody responses were measured. As early as Day 7 post vaccination, mice treated with antibiotics exhibited greater than 50% reduction in antigen-specific antibody titers in the serum when compared to untreated mice .We observed differences in antibody titers over periods exceeding 12 weeks following the primary vaccination. These observations were rigorously tested across 6 independent experiments with a total of 100 mice treated with antibiotics and 80 untreated control mice. Ongoing mechanistic studies indicate that boost/recall antibody responses are also reduced in antibiotic-treated mice.

In addition, we conducted longitudinal microbiome sampling in both untreated and antibiotic-treated animal groups to determine whether the impact of antibiotics on the humoral immune response can be attributed to compositional differences in the gut microbiota. The microbiome was analyzed using bacterial genomic DNA extracted from fecal samples and the 454 pyrosequencing platform to identify specific bacterial communities. A sequence clustering software originally developed by Robert Knight's group was used to determine the composition of the microbiota as previously described (27). The details of analysis methods are highlighted in 9.2.3.Analysis of exploratory endpoints. Our characterization of the microbiota in mice revealed that antibiotic treatment indeed reduced the bacterial load in the gut by a factor greater than ten and caused dramatic shifts in major bacterial communities of the gut. Specifically, greatest changes were observed in bacterial phyla, Fermicutes and Proteobacteria. The microbiota of untreated mice exhibited a greater presence of bacterial communities belonging to the Fermicutes phylum; whereas in treated mice, majority of these communities were eliminated and instead exhibited a predominance of species from the Proteobacteria phylum.

This indicated to us that the effects antibiotics and the status of the microbiota around the time of vaccination significantly impacted the antibody response. Collectively, our studies demonstrate that antibiotic treatment dramatically alters the composition of the host microbiota and results in dramatic reduction of antigen-specific antibody titers similar to the phenotype we previously observed in TLR5-deficient mice.

It is unclear how antibiotic exposure affects the human microbiota. Historical evidence on the effects of antibiotics in vivo is largely based on biologic assays that require culturing microbes. As the majority of microbial communities in the gut are anaerobes and, thus, difficult to culture, the

accuracy of our understanding of the microbiota remains limited (2). However, recent advances in pyrosequencing technologies are enabling a more detailed analysis of the human microbiome to be assessed (28-30). A few reports suggested that some antibiotics such as ciprofloxacin do alter the human microbiota, but revealed that only as little as a third of the bacterial taxa in the gut were affected by antibiotic treatment (31, 32). Moreover, analyses of the microbiome in these studies were limited to only an examination on the relative abundance of microbial communities and lacked quantifiable information such as overall bacterial load in the gut. Thus, given the relatively nascent stage of our capacity to characterize the human gut microbiome, our understanding of how it is influenced by antibiotics remains starkly unclear.

Using the data generated from our series of clinical flu vaccination trials (21), we found that the early induction of TLR5 expression (Day0 vs Day3 post vaccination) positively correlated to the levels of HAI titers measured on Day28 post vaccination. We have since demonstrated a causal link between TLR5 signaling and induction of antibody responses to IIV in mice. Determining whether a functional link can be established in humans is very challenging to address. However, one potential approach is to determine whether polymorphisms within the TLR5 gene contribute to responsiveness of a given individual to vaccination. We are specifically interested in the commonly-known TLR5 stop codon polymorphism (Hawn et al., 2003. J. Exp. Med.). This polymorphism refers to a SNP at nucleotide position 1174 (C -> T) resulting in a stop codon replacing arginine at amino acid position 392. This allele has been characterized as a dominant loss-of-function mutation as humans that are heterozygotes fail to respond to flagellin. Therefore, this is a highly intriguing and relevant SNP that will enable us to address a role of TLR5 expression in humans to vaccine-induced antibody responses.

In summary, the data from our experiments in mice suggest a novel role for commensal microbiota in enhancing vaccine-elicited immunity. Determining whether these effects are seen in humans after vaccination is vital to improve and optimize vaccine efficacy. Thus, we propose to apply our experimental data from mice to examine whether exposure to antibiotics given around vaccination in humans will negatively impact the ensuing immune response.

1.3 Rationale for Selection of Study Population

Twenty two healthy adults with no co-morbidities between the ages of 18-40 years will be recruited from the general population of metro Atlanta and enrolled at the Hope Clinic, located in downtown Decatur. In previous studies done at the Hope Clinic, 60% of subjects were female and 40% were male with good racial representation (55% Caucasian, 35% African American and 10% Asian). We chose the above age range to avoid the effect of immune senescence on vaccine efficacy since immune responses to IIV are known to decrease with age (33). Additionally, discharge rate for *Clostridium difficile* infection (CDI) from US short-stay hospitals is the lowest among adults between the ages of 18 to 44 and is less than 2.5 cases per 1000 discharges (42). This rate does not seem to be affected by the emergence of a hypervirulent strain of *Clostridium difficile* (NAP1/BI/027) resistant to quinolones (40). Therefore our study subjects will be healthy adults between the ages of 18-40 with no co-morbidities.

1.4 Investigational Product(s)/Intervention (s)

The vaccine used in the study will be the seasonal 2014-2015 and 2015-2016 Fluzone® manufactured by Sanofi Pasteur (Swiftwater, PA USA)(34). Subjects in the study will receive the routine adult dose of 0.5 ml given by the intramuscular (IM) route. Fluzone® will be obtained from the Emory University pharmacy.

The antibiotic regimen will be given 3 days prior to vaccination, on the vaccination day, and one day after vaccination for a total for 5 days. The regimen will include the following antibiotics:

Flagyl ®(35) Pfizer, New York, NY) 500 mg po tid

Vancocin® (36) (ViroPharma , Exton, PA) 125 mg po qid

Neomycin sulfate® (37) (X-Gen Pharmaceuticals, Big flats, NY) 500 mg po tid

All antibiotics will be obtained from the Emory University pharmacy.

1.5 Rationale for Selection of Investigational Product(s)/Intervention(s) and Regimen

The majority of studies demonstrating that antibiotic-mediated depletion of host microbiota influence the development and homeostasis of the immune system employed a specific cocktail

of antibiotics consisting of Ampicillin (1 g/L), Neomycin (1 g/L), Vancomycin (0.5 g/L), and Metronidazole (0.5 g/L).

The basis for using this particular combination of antibiotics is to enable a wide spectrum of microbial communities to be targeted for depletion. Indeed, at the indicated doses, this cocktail has been shown to effectively deplete more than 90% of the existing flora in the gut and drastically alter the composition of the microbiota (22, 25). Specifically, the greatest changes were observed in bacterial phyla, Fermicutes and Proteobacteria as seen also in our independent experiments.

For this study we will use a broad spectrum antibiotic regimen affecting all bacteria in the gut flora based on our mice experiments that includes: Vancocin® (with activity against gram positive bacteria), Flagyl® (with activity against anaerobes) and Neomycin sulfate® (with activity against gram negative bacteria).

Additional characteristics of antibiotics selected in the regimen include:

- Poor systemic absorption for Vancocin® and Neomycin sulfate® (3%) when given orally which should result in less systemic side effects.

- Good safety profile for all 3 of these antibiotics used for more than half a century (Flagyl® was developed in 1960, Vancocin® in 1953 and Neomycin sulfate® in 1949).

- Although any antibiotic can cause *Clostridium difficile*-associated colitis, the current regimen includes Vancocin® and Flagyl®, both used at the same cumulative daily dosage to treat *Clostridium difficile* infection but administered in a shorter course of 5 days. For Neomycin sulfate®, the cumulative daily dosages are less than half of the minimum daily dosage used to treat adult hepatic encephalopathy and a 5-day course is same as that required for treating adult hepatic encephalopathy.

This antibiotic regimen (Vancocin®, Flagyl® and Neomycin sulfate®) will be administered three days before vaccination with IIV, on the day of vaccination and one day post-vaccination in subjects randomized to Group A (subjects in Group B will only receive IIV). This short course reduces the potential risk of bacterial and fungal superinfection, as well as preventing the development of antibiotic resistance.

1.6 Risks

1.6.1 Risks of Investigational Product(s)/Intervention(s)

IIV

Routine influenza vaccination is now recommended for all persons aged ≥6 months. Fluzonex® is an FDA approved IIV manufactured Sanofi Pasteur (Swiftwater, PA USA) and indicated for active immunization of persons 6 months of age and older against influenza disease caused by influenza virus subtypes A and type B contained in the vaccine. The most common (>10%) local adverse reactions and general adverse events observed with Fluzone® when given to adults were pain and redness at the injection site, and muscle aches, fatigue, and headache, respectively (34).

Acute and potentially life-threatening allergic reactions are also possible, especially in recipients of influenza vaccines with a history of severe egg allergy (34). As noted in the exclusion criteria, for safety considerations, individuals with an allergy to eggs will be excluded from this study. In addition, vaccinations will be administered per label and in clinical settings equipped to manage any potential allergic reactions that may occur following vaccine administration.

Associated with the 1976 flu vaccine, a few patients experienced temporary paralysis, a condition known as Guillain-Barré syndrome. However, this syndrome has not been observed with the more modern influenza vaccine preparations. Most individuals who develop Guillain-Barré Syndrome recover completely, although the recovery period may vary from weeks to a few years. Of those who develop Guillain-Barré Syndrome, approximately 30% continue to exhibit residual weakness after 3 years, and approximately 3% suffer a relapse of muscle weakness and tingling sensations years after the initial attack. Intensive surveillance of Guillain-Barré Syndrome following administration of inactivated influenza vaccines since 1976 has shown a slight increase in risk over background cases (more than one additional case of Guillain-Barré Syndrome per million persons) following vaccination, typically with onset within 6 weeks after vaccination (38). Interestingly, although vaccination rates have increased in the last 10 years, the numbers of reported cases of vaccine-associated Guillain-Barré Syndrome have declined (39).

Randomized, controlled clinical trials of the inactivated seasonal influenza vaccine in patients with asthma indicate that there is no significant increase in asthma exacerbations immediately after vaccination.

There may be other unknown side effects.

Antibiotics

The most common risk factor for *Clostridium difficile* infection (CDI) is antibiotic use. CDI typically results in mild illness with diarrhea, fever, and abdominal pain. Severe cases of CDI have resulted in ICU admissions, colectomy, and deaths, mostly occurring in elderly patients or patients with co-morbidities (41). However, hospital discharge rate for CDI from US short-stay hospitals is the lowest among adults between the ages of 18 to 44 and is less than 2.5 cases per 1000 discharges (42). This rate does not seem to be affected by the emergence of a hypervirulent strain of *Clostridium difficile* (NAP1/BI/027) resistant to quinolones (40). Therefore our study subjects will be healthy adults between the ages of 18-40 with no co-morbidities. Although CDI can occur with any antibiotic exposure, we specifically excluded quinolones from our antibiotic regimen as it has been the antibiotic most frequently associated with the epidemic strain (43).

Flagyl®

The most serious adverse reactions reported in patients treated with Flagyl® have been convulsive seizures, encephalopathy, aseptic meningitis, optic and peripheral neuropathy (35). Peripheral neuropathy is characterized mainly by numbness or paresthesia of an extremity (35). The most common adverse reactions reported have been referable to the gastrointestinal tract, particularly nausea, reported by approximately 12% of patients, and can be accompanied by headache, anorexia, and occasionally vomiting; diarrhea; epigastric distress; and abdominal cramping (35).

Vancocin®

Nephrotoxicity (renal failure, renal impairment, blood creatinine increased) has occurred following oral VANCOCIN therapy in randomized controlledclinical studies, and can occur either during or after completion of therapy. The risk of nephrotoxicity is increased in patients >65 years of age (36).

Ototoxicity has occurred in patients receiving vancomycin (36). It may be transient or permanent, but has been reported mainly in patients who have been given excessive intravenous doses, who have an underlying hearing loss, or who are receiving concomitant therapy with another ototoxic agent, such as an aminoglycoside (36).

The most common (>10%) adverse reactions associated with the use of Vancocin® in clinical trials included nausea (17%), abdominal pain (15%) and hypokalemia (13%). Other adverse events (>5%) included peripheral edema, fatigue, fever, diarrhea, vomiting, flatulence, urinary tract infection, back pain and headache. Also important/life-threatening side effects are rare (<1%) and include vasculitis, thrombocytopenia, nephrotoxicity, neurotoxicity and ototoxicity (36).

Neomycin sulfate®

Nephrotoxicity, ototoxicity, and neuromuscular blockage have been reported with the use of Neomycin sulfate® (37). Manifestations of neurotoxicity may include numbness, skin tingling, muscle twitching, and convulsions (37). The risk of hearing loss continues after drug withdrawal (37). Subjects concurrently receiving other neurotoxic and/or nephrotoxic drugs may have can have a possible enhancement of the nephrotoxicity and/or ototoxicity of neomycin (37). Concurrent or serial use of other aminoglycosides and polymyxins may enhance neomycin's nephrotoxicity and/or ototoxicity and potentiate neomycin sulfate's neuromuscular blocking effects (37).

The most common adverse reactions to oral neomycin sulfate are nausea, vomiting and diarrhea (37).

All three antibiotics in this regimen can lead to the overgrowth of non-susceptible bacteria.

1.6.2 Risk of Study Procedure

The discomforts of this study include having blood drawn, intramuscular (IM) injection, and possible allergic reactions to the components of IIV and the antibiotics.

Drawing blood causes transient discomfort and may cause fainting. Bruising at the blood draw site may occur but can be prevented or lessened by applying pressure to the draw site for several minutes. Intramuscular injection may also cause transient discomfort. The use of aseptic technique will make infection at the site where blood will be drawn or where the vaccination is given extremely unlikely. Vaccines used in the study will be a single dose syringe preventing the risk of over or under dosing and the potential risk of contamination.

1.6.3 Risk of Concomitant Medications, Prophylactic Medications and Rescue Medications

We do not anticipate the use of any other medication; however, should anaphylactic or hypersensitivity reactions occur, epinephrine (1:1000) and diphenhydramine injections are readily available at the Hope Clinic during vaccine use.

Epinephrine injection can be associated with high blood pressure, arrhythmia, lightheadedness, nervousness, restlessness, tremor, shortness of breath and diaphoresis. The frequency of these side effects is not defined. Diphenhydramine injection may be required to treat possible allergic reactions and its use can be associated with low blood pressure, arrhythmia, confusion, dizziness, sedation, restlessness, diarrhea, nausea and urinary retention. The frequency of these side effects is also not defined.

When facing a medical emergency, the clinic staff will follow the institutional SOP by calling 911 first. If needed, the subject will be transferred to Emory University Emergency Department for further care.

Subjects are allowed to use acetaminophen or NSAIDs if they experience a moderate to severe local or systemic side effects after vaccine administration.

1.7 Benefits

1.7.1 Benefits of Investigational Product(s)/Intervention(s)

None.

1.7.2 Benefits of Study Procedure(s)

None.

2 OBJECTIVES

Use of antibiotics can significantly impact the microbiota of patients. The status of the intestinal microbiome may impact the immune responses to vaccinations and alter functional protective immunity in humans. Here we hypothesize that the changes in the fecal microbiome induced by antibiotics translates into suboptimal Hemagglutination Inhibition Assay (HAI) titers.

2.1 Primary Objective(s)

Compare HAI titers to IIV at D30 post vaccination in the group receiving antibiotics to the group not receiving antibiotics.

2.2 Secondary Objectives

Evaluate the safety profile of the different antibiotics given in the study.

2.3 Exploratory Objectives

Analyze the microbiome in both groups.

Analyze the repertoire and monoclonal antibodies from plasmablasts in a subset of vaccinees in both groups.

Identify innate immune signatures (traditional immune parameters measurements + array-based gene expression) and correlate signatures with HAI titers at D30 in both groups.

Compare HAI titers to IIV at D90, D180 and D365 post vaccination in the group receiving antibiotics to the group not receiving antibiotics.

3 STUDY DESIGN

This is a single center, mechanistic study in which 50 healthy subjects will be randomized to receive IIV with antibiotics (Group A) or IIV without antibiotics (Group B).

Study volunteers will be recruited from the general population of metropolitan Atlanta. The study will be conducted at the Hope Clinic of the Emory Vaccine Center (Decatur, GA). The expected duration of subject participation is one year.

3.1 Study Endpoints

3.1.1 Primary Endpoint(s)

- Comparison of HAI titers at D30 post vaccination in both groups.

3.1.2 Secondary Endpoint(s)

- Frequency, severity, and causality of all adverse events in Group A subjects.

3.1.3 Exploratory Endpoint(s)

- Analyses of microbiome at D0, D1, D3, D7 and D30, D90, D180, D365 and comparison to baseline screening in both groups.
- Analysis of the repertoire and monoclonal antibodies from plasmablasts in a subset of vaccinees at D7 in both groups.
- Identification of innate immune signatures (traditional immune parameters measurements + array-based gene expression) at D1, D3 andD7 in both groups.
- Correlation of innate immune signatures at days D1, D3 and D7 with HAI titers at D30 in both groups.
    - Comparison of HAI titers at D90, D180 and D365 post vaccination in both groups.

3.2 Study Completion

This study will be considered “completed” when the primary and secondary objectives have been met. After the study is completed, the Principal Investigator or Data Center will compile a final study report as per ICH E6 and 21CFR312. The study report will be submitted to the local IRB and ISM.

4 SELECTION OF STUDY SUBJECTS

4.1 Subject Inclusion Criteria

Subjects must meet all of the following inclusion criteria:

1. Healthy individuals between the ages of 18 and 40 years.
2. Able to understand and give informed consent.
3. Women of childbearing potential (not surgically sterile via tubal ligation, bilateral oophorectomy or hysterectomy or who are not postmenopausal for ≥ 1 year) must agree to practice adequate contraception that may include, but is not limited to, abstinence, monogamous relationship with vasectomized partner, barrier methods such as condoms, diaphragms, spermicides, intrauterine devices, and licensed hormonal methods* for 30 days before and 30 days after IIV vaccination.

   * Women of child-bearing potential using licensed hormonal methods must also use a second form of contraception.
4. Men must agree not get a sexual partner pregnant for 1 month after enrollment.

4.2 Subject Exclusion Criteria

Subjects who meet any of the following exclusion criteria will be excluded from study participation:

1. Receipt of the following:
   - Receipt of blood products 3 months prior to vaccination or expected receipt through 6 months after vaccination.
   - Receipt of any live virus vaccines within 4 weeks prior to vaccination or expected receipt within 4 weeks after vaccination.*
   - Receipt of any inactivated vaccine within 2 weeks or expected receipt within 2 weeks after vaccination.*
   - Receipt of any influenza vaccine in the past 3 seasons.
   - Receipt of any systemic antibiotic 6 months prior to vaccination or expected receipt 1 month after vaccination.

- Receipt of probiotics and prebiotics 3 months prior to vaccination or expected receipt 1 month after vaccination.
- Regular receipt of proton pump inhibitors, H2 receptor blockers, or antacids 3 months prior to vaccination or expected receipt 1 month after vaccination.

2. Documented influenza infection during the 2014-2015 or 2015-2016 influenza season. Not excluded from the study, volunteers with prior upper respiratory infections during the 2014-2015 or 2015-2016 influenza illness.
3. Presence of co-morbidities or immunosuppressive states such as:
   - Chronic medical problems including, but not limited to, insulin dependent diabetes, severe heart disease including arrhythmias, severe lung disease, auto immune diseases, and grade 4 hypertension**.
   - Chronic neurologic conditions including seizure disorder, Parkinson's disease, myasthenia gravis, neuropathy, or history of encephalopathy, meningitis or ototoxicity.
   - Any history of gastrointestinal disease including (but not only): documented bacterial gastroenteritis or gastroenteritis associated with fever or associated with presence of blood/mucus in stools in the last 3 months; inflammatory bowel disease, and/or gastrointestinal surgery.
   - Any history of kidney or liver diseases.
   - Alcohol or drug abuse and psychiatric conditions that in the opinion of the investigator would preclude compliance with the trial or interpretation of safety or endpoint data.
   - Impaired immune function or known chronic infections including, but not limited, to known HIV, hepatitis B or C; organ transplant; immunosuppression due to cancer; current and/or expected receipt of chemotherapy, radiation therapy, steroids* (i.e., more than 20 mg of prednisone given daily or on alternative days for 2 weeks or more in the past 90 days , or high dose inhaled corticosteroids*** or any other immunosuppressive therapies (including anti-TNF therapy), functional or anatomic asplenia and congenital immunodeficiency.
   - Pregnancy or breast feeding.

4. Conditions that could affect the safety of the volunteers, such as:
    - Severe reactions to prior vaccination with IIV, including anaphylaxis.
    - History of Guillain Barré syndrome.
    - History of bleeding disorders or current use of warfarin.
    - Use of anticonvulsants.
    - Use of digoxin or other forms of digitalis.
    - Any allergy to any component of the vaccine, including egg allergy.
    - Allergy to vancomycin, metronidazole or neomycin as well as other aminoglycosides (gentamicin, tobramycin, amikacin, streptomycin).
5. Volunteers with any acute illness, including any fever (≥ 100.4 F [≥ 38.0C], regardless of the route) within 3 days prior to vaccination *.
6. Social, occupational, or any other condition that in the opinion of the investigator might interfere with compliance with the study and vaccine evaluation.
7. Positive *C difficile* testing by PCR at screening or a history of *C difficile* infection.
8. Any grade 2 safety lab test results at screening.

Note:

*An individual who initially is excluded from study participation based on one or more of the time-limited exclusion criteria (e.g., acute illness, receipt or expected receipt of live or inactivated vaccines) may be reconsidered for enrollment once the condition has resolved as long as the subject continues to meet all other entry criteria.

Subjects receiving ≥ 20 mg/day of prednisone or its equivalent daily or on alternate days for more than 2 weeks may enter the study after therapy has been discontinued for more than 3 months.

** Grade 4 hypertension per CTCAE criteria is defined as Life-threatening consequences (e.g., malignant hypertension, transient or permanent neurologic deficit, hypertensive).

*** Subjects are excluded if on high dose intranasal steroids defined as > 960 mcg/day of beclomethasone dipropionate or equivalent.

4.3 Early Study Termination

Subjects may be terminated prior to study completion from the study for the following reasons:

A. The subject elects to withdraw consent from all future study activities, including follow-up.

B. The subject is considered by the PI to be “lost to follow-up” (i.e., no further follow-up is possible because attempts to reestablish contact with the subject have failed).

C. The subject dies.

D. The subject develops a medical condition or is started on new medication(s) not previously mentioned in the list of prohibited medications that, in the opinion of the investigator, may pose additional risks from participation in the study, may interfere with the subject’s ability to comply with study requirements, or may impact the quality of the data obtained from the study.

E. Blood is not able to be drawn (for technical or other reasons) or the subject does not tolerate multiple blood draw attempts.

F. As deemed necessary by the PI or her designee for noncompliance of any nature.

G. As deemed necessary by the PI after development of a related AE/SAE.

H. The subject meets one or more of the individual stopping rules delineated in section 8.

Subjects with early termination status are replaced as needed to preserve the statistical power needed to substantiate the primary endpoint (Refer to section 9.1).

Note:

Up to the discretion of the PI, subjects receiving concomitant medications may still continue with scheduled study blood draws. Subjects receiving concomitant medications along with subjects with early termination from this study for any reason may be replaced as needed to preserve the statistical power needed to substantiate the primary endpoint. (Refer to section 9.1)

5 INVESTIGATIONAL PRODUCT(S)/INTERVENTION MATERIAL(S), OTHER STUDY PRODUCTS (CONTROLS/PLACEBOS)

5.1 Investigational Product(s)/Intervention(s)

The Emory Investigational Drug Service will purchase Fluzone® manufactured by Sanofi Pasteur (Swiftwater, PA USA) from Emory Pharmacy. The Emory Investigational Drug Service will store the vaccines and will monitor temperatures of the refrigerator(s) containing the vaccines. All antibiotics (Flagyl ®(Pfizer, New York, NY); Vancocin® (ViroPharma , Exton, PA); Neomycin sulfate® (X-Gen Pharmaceuticals, Big flats, NY)) will be purchased from Emory Pharmacy as well. Refer to section 1.6, and applicable product labeling, for known and potential risks to human subjects associated with the investigational product(s) intervention(s).

5.2 Formulation, Packaging, and Labeling

IIV

Fluzone® is a suspension available in 5-mL multi-dose vials. Fluzone® is formulated to contain 45 micrograms (mcg) hemagglutinin (HA) per 0.5-mL dose, in the recommended ratio of 15 mcg HA of each of the following 3 strains: A/Christchurch/16/2010 NIB-74XP (H1N1) (an A/California/7/2009-like virus), A/Texas/50/2012 NYMC X-223A (H3N2) (an A/Victoria/361/2011-like virus), and 287 B/Massachusetts/2/2012 NYMC BX-51B.

Fluzone® is formulated without preservatives. Fluzone® does contain thimerosal. Each 5mL dose also contains sodium phosphate-buffered isotonic sodium chloride solution, gelatin, octylphenol ethoxylate, formaldehyde. Fluzone® does not contain natural rubber latex.

5.3 Preparation, Administration, and Dosage

IIV adult dosing is 0.5 ml and is given via the intramuscular route in the deltoid muscle at D0. It does not require any preparation and will come as a single dose in a prefilled syringe. IIV is stored between 2 to 8oC (36°F to 46°F) as per the manufacturers' instructions at the pharmacy. In the event of accidental deep-freezing or disruption of the cold chain, it will not be administered.

Antibiotics tablets are provided from IDS at room temperature (20º-25ºC (68º-77ºF)) to subjects in Group A, once they are deemed eligible to participate in the study at the screening visit (from D-21 to D-1). All antibiotics will be self-administered by the subject by mouth from D-3 until D1 (total of 5 days) as stated below:

- Flagyl® 500 mg po tid
- Vancocin® 125 mg po qid
- Neomycin sulfate ® 500mg po tid

5.4 Accountability of Investigational Product(s)/Intervention(s)

Under Title 21 of the Code of Federal Regulations (21CFR §312.62), the investigator will maintain adequate records of the disposition of the investigational product(s)/intervention material(s), including the date and quantity of the drug received, to whom the drug was dispensed (subject-by-subject accounting), and a detailed accounting of any investigational product(s)/intervention material(s) accidentally or deliberately destroyed.

Records for receipt, storage, use, and disposition will be maintained by the study site. A dispensing log will be kept current for each subject. This log will contain the identification of each subject, the name of the vaccine administered to the subject, the lot of vaccine received by the subject and the date and quantity of vaccine dispensed. A similar log is available for antibiotics dispensed for Group A subjects. A pill count is performed on D0, D1, and D7 to record compliance to the antibiotic regimen. All records regarding the disposition of the investigational product(s)/intervention material(s) will be available for inspection by the site monitor and the health authorities.

5.5 Assessment of Compliance with Investigational Product(s)/Intervention Material(s)

The number of used vaccine syringes will be tracked and reconciled by the nursing staff at the Hope Clinic prior to sending any vials of unused vaccine back to the Emory Investigational Drug Service.

5.6 Modification or Discontinuation of Investigational Product(s)/Intervention Material(s)

5.6.1 Modification of Investigational Product(s)/Intervention(s)

Unless IIV or any of the antibiotics are recalled by the manufacturers, there will be no discontinuation of administration of study vaccine and antibiotics.

5.6.2 Premature Discontinuation of Investigational Product(s)/Intervention(s)

Refer to sections 4.3 for possible causes of early study termination.

6 OTHER MEDICATIONS

6.1 Concomitant Medications

In accordance with exclusion criteria, subjects expected to receive prohibited medications (see section 6.4) will be considered ineligible for the study. All medications, therapies, or vaccines administered to study subjects after study entry will be documented at each visit.

6.2 Prophylactic Medications

Prophylactic medications will not be administered before vaccination or any other study procedures.

6.3 Rescue Medications

We do not anticipate the use of any rescue medication; however, should anaphylactic or hypersensitivity reactions occur, epinephrine (1:1000) and diphenhydramine injections are readily available at the Hope Clinic during vaccine use.

When facing a medical emergency, the clinic staff will follow the institutional SOP by calling 911 first. If needed, subject will be transferred to Emory University Emergency department for further care.

Subjects are allowed to use acetaminophen or NSAIDs if they experience a moderate to severe local or systemic side effect after vaccine administration.

6.4 Concomitant Study Medications

All medications and vaccines received by study subjects after administration of study vaccine should be reported to the study staff and recorded. This includes, but is not limited to, the following:

- blood products, chemotherapy, immunosuppressive therapy (including anti-INF therapy), and radiation therapy (administered at any time after study vaccination)
- inactivated vaccines (administered before the day 30 blood draw)
- live-attenuated vaccine (administered before the day 30 blood draw)

- any antibiotic aside from the study antibiotic (taken before the day 30 blood draw).
- any probiotics and prebiotics (taken before the day 30 blood draw).
- any proton pump inhibitors, H2 receptor blockers, or antacids (taken before the day 30 blood draw).

Any of the above medications could affect the innate and adaptive assay results and should not be used unless medically indicated.

Upon the discretion of the PI, subjects receiving concomitant medications may still continue with scheduled study blood draws. Subjects receiving concomitant medications and early terminated subjects may be replaced as needed to preserve the statistical power needed to substantiate the primary endpoint. (Refer to section 9.1.).

7 STUDY VISITS AND PROCEDURES

7.1 Enrollment and Randomization

This research study will be explained in lay terms to each potential research subject. The potential subject will sign an informed consent form before undergoing any screening study procedures. Subjects who are deemed eligible for the study (see sections 4.1 and 4.2) will be enrolled and assigned a unique subject number. Enrollment will occur over an approximate 6-month time span. The duration of participation for each subject is approximately 1 year

Twenty-two subjects will be randomized to Group A (receiving antibiotics) or to Group B (not receiving antibiotics) in a 1:1 ratio. All eligible subjects will receive IIV at D0.

7.2 Screening Visit(s) (From D-21 until D-1)

Subjects responding to study ads will call the Hope Clinic for a telephone screening where the eligibility criteria of the subject will be reviewed. If the subject is found to be eligible and continues to be interested in participating after reading the informed consent (emailed or mailed to him/her), a screening and enrollment appointment will be scheduled. Study staff will review the informed consent form with the subject and will answer all questions related to the study.

Once the subject signs the informed consent, a study participation number will be assigned. The volunteer will be asked to provide demographic information and information related to his/her medical history, including current medication use and vaccination history. The subject's vital signs will be recorded, and a targeted physical exam will be conducted as indicated, based on review of the subject's health status. For female volunteers who are of childbearing potential, a urine pregnancy test will be performed. Only females with a negative urine pregnancy test will be enrolled in the study. All subjects will have a stool sample collected from them. A stool kit will be mailed prior to the visit.

The subjects will be randomized to be in Group A or Group B in a 1:1 ratio and will have blood drawn (to measure CBC with differential, creatinine, and potassium) and immunologic assays.

This visit will last approximately 60 minutes.

7.3 BASELINE VISITS

Antibiotic initiation visit (Group A only) (From D-6 until D-3)

Subjects in Group A will also be given the three antibiotics and instructed on when and how to start taking the antibiotic pills (D-3 until D1). Subjects in Group A will provide stool and blood samples as well and should have a negative pregnancy test.

Subjects in Group A are asked to call the Hope Clinic for any symptom while on antibiotics-see below.

Vaccination (D0)

Study personnel will first review the subject's current health status, list of medications being taken, and note any change since the screening visit. The subject's vital signs will be recorded as well as height and weight and type of diet typically followed and a targeted physical exam will be conducted, as indicated by a review of the subject's health status. For female volunteers of childbearing potential, a urine pregnancy test will be performed. Only females with a negative urine pregnancy test will receive the vaccine. Inclusion/exclusion criteria will be also verified before vaccination. Subjects in Group A will be asked if they have had any symptoms since starting antibiotics, and a pill count is performed. All subjects will have a stool sample collected.

Blood for immunological assays will be drawn at this visit before administration of the vaccine. To ensure safety, each subject will be observed for a minimum of 15 minutes following vaccination to note the occurrence of any immediate hypersensitivity reactions. After 15 minutes, the injection site will be examined by either a RN or a MD and subject will be asked for systemic adverse reactions.

The subject will be provided with a written description of local and systemic vaccine reactions of mild, moderate and severe intensity (Refer to Appendix B and Appendix C). Subjects will be instructed to notify the study center by telephone if they develop any severe reactions within 1 week following vaccination.

At the end of the visit, the subject will also be instructed to promptly call the site if he/she develops any of the following:

- Illness or treatment from a physician or emergency department; and/or hospitalization due to any illness throughout the entire duration of the study;

- Development of any adverse event that limits self-care activities of daily living (e.g. bathing, dressing and undressing, feeding self, using the toilet, taking medications, and not bed ridden) even if he/she decides not to seek medical care;
- For Group A only: development of any of the following symptoms while on antibiotics and throughout D90:
    - diarrhea (≥ 3 unformed stools/24h) for more than 72h with either fever or abdominal pain*
    - tinnitus**
    - vertigo**
    - decrease hearing**
    - numbness/tingling**
- Beginning or discontinuing any medications/therapies during enrollment in the study.

Refer to section 8.2 for safety data that must be recorded and reported.

Note: Subjects calling the site for any of the above will receive further instructions on the proper course of action, including a return to the clinic for immediate evaluation, if appropriate.

This visit will last approximately 60 minutes.

7.4 Main Study Visits

All subjects will return for the study-related blood draws on Days 1, 7(+1), 21(+3), and 30 (+5) after immunization. All subjects will have stool collected at D1, D3, D7 D30, D90, D180 and D365. Additionally, for subjects, safety labs are obtained at D7.

On days 1, 3, 7, 30, 90, 180 and 365 study personnel will review current health status, including:

- Evaluation of local REs and systemic REs of grade 3 or higher severity developing after the previous visit (only until Day 7 Visit).

- Any solicited adverse event (AE) or serious adverse event (SAE) which may not have been reported by the subject by calling the site as directed at a prior visit.

- Development of any adverse event that limits self-care activities of daily living (e.g. bathing, dressing and undressing, feeding self, using the toilet, taking medications, and not bed ridden) even if he/she decides not to seek medical care.

- Any medications administered after vaccination.

  - Pill count for Group A (D1 and D3)

Subjects in Group A will also be instructed to call the site if he/she:

- Develops any symptoms while taking antibiotics

- Develops any of the following symptoms until D90:

  o diarrhea (≥ 3 unformed stools/24h) for more than 72h with either fever or abdominal pain*

  o tinnitus**

  o vertigo**

  o decrease hearing**

  o numbness/tingling**

A focused physical exam will be conducted (if indicated) based on results of the review of the subject's health status as outlined above.

Note:

* if *C difficile* infection is suspected, C diff. PCR testing will be performed. If positive, treatment and follow up for *C difficile* infection will be facilitated.

** if tinnitus, vertigo, decrease hearing, numbness/tingling are present, medical referral will be facilitated.

7.5 Visit Windows

Study visits should take place within the time limits below:

D0, D1,D3, D7 (+/-1), D30 (+/-5), D90 (+/-14), D180 (+/-14), D365 (+/-28).

7.6 Study Procedures

Refer to sections 7.1, 7.2, 7.3, and 7.4.

7.7 Study Arm Assignment Procedures

7.7.1 Blinding and Randomization

In this study 50 healthy subjects will be randomized to be in either Group A or Group B in a 1:1 ratio.

The randomization will be performed by the Emory Investigational Drug Service.

7.7.2 Securing Randomization Information

The information on randomization is kept at the Emory Investigational Drug Service.

7.7.3 Requirements for Unblinding

N/A.

7.7.4 Documentation of Unblinding

N/A.

8 SAFETY PROCEDURES

8.1 Stopping Rules

8.1.1 Study Stopping Rules

Study vaccination will be suspended pending expedited review of all pertinent data by the institutional review board and the ISM, if Fluzone® or antibiotics (Vancocin®, Flagyl®, and Neomycin sulfate®) were recalled by the manufacturer.

Also, vaccination will be suspended pending review of all pertinent data by the PI and the ISM after the occurrence of any of the following:

1 SAE

1 AE of Grade 4 severity.

2 AEs of Grade 3 severity of similar type other than expected events.

2 grade 2 AEs of abnormal laboratory values at D7.

2 cases of *C difficile* infections in Group A subjects.

1 case of ototoxicity.

1 case of neuropathy.

1 case of Guillain-Barré Syndrome.

8.1.2 Individual Subject Stopping Rules

Early study termination will occur in individual subjects due to any of the following circumstances:

- Development of a related serious adverse event.
- Development of a condition for which continued participation, in the opinion of the investigator, would pose a risk to the subject.
- Development of a condition for which continued participation, in the opinion of the investigator, would be likely to confound interpretation of the results of the study.

- Participation in the study may be terminated as deemed necessary by the PI or her designee for noncompliance with study procedures.
- Participation in the study may be terminated when blood is not able to be drawn (for technical or other reasons) at time points required for the primary endpoint, or the subject does not tolerate multiple blood draw attempts.
- Pregnancy

8.1.3 EARLY TERMINATION FROM STUDY VACCINES/ PROCEDURES WITH CONTINUED STUDY PARTICIPATION/FOLLOW-UP

Refer to section 4.3.

8.1.4 FOLLOW-UP AFTER EARLY STUDY TERMINATION

Subjects in Group A who are prematurely terminated from the study for reasons other than safety events will still be followed to monitor safety for 365 days after the last administered dose of antibiotics at the same intervals used for the Group A subjects who remain in the study.

Subjects in Group A and B who are prematurely terminated from the study due to an AE will be followed until resolution of the AE or until 30 days after a subject terminates from the study, whichever comes later. (Note: subjects in Group A terminate study participation 90 days +/-14 days after the last dose of antibiotics is administered; subjects in Group B terminate study participation at Day 30 +/- 5 days)Resolution of an AE is defined as the return to baseline status or as stabilization of the condition with the expectation that it will remain chronic.

After assessing terminated subjects for safety under the provisions stated above, the subject will be seen in clinic, if necessary.

8.1.5 SUBJECT REPLACEMENT

In the case that premature termination causes the attrition rate to be higher than 10%, we will recruit extra patients to maintain the target sample size of 10 subjects/group and the statistical power to substantiate primary endpoint. Please refer to section 9 - Statistical Analysis.

8.2 Adverse Events

This section defines the types of adverse events that may occur, and outlines the procedures for appropriately adverse event collecting, grading, recording, and reporting.

Information in this section complies with 21CFR 312; ICH Guideline E2A: Clinical Safety Data Management: Definitions and Standards for Expedited Reporting and ICH E6: Guideline for Good Clinical Practice; and applies the standards set forth in the National Cancer Institute (NCI), Common Terminology Criteria for Adverse Events Version 4.0 [Published: May 28, 2009; revised version 4.03; June 14. 2010, http://evs.nci.nih.gov/ftp1/CTCAE/About.html]

These criteria have been reviewed by the study investigators and have been determined to be appropriate for this study population.

8.2.1 Safety Reporting

8.2.1.1 Adverse Events (AE)

Adverse event means any untoward medical occurrence associated with the use of a drug in humans, whether or not considered drug related.

An adverse event (also referred to as an adverse experience) can be any unfavorable and unintended sign (e.g., an abnormal laboratory finding), symptom, or disease temporally associated with the use of a drug, without any judgment about causality. An adverse event can arise from any use of the drug (e.g., off-label use, use in combination with another drug) and from any route of administration, formulation, or dose, including an overdose.

8.2.1.2 Suspected Adverse Reaction (SAR)

Suspected adverse reaction means any adverse event for which there is a reasonable possibility that the drug caused the adverse event.

Suspected adverse reaction implies a lesser degree of certainty about causality than adverse reaction, which means any adverse event caused by a drug.

SARs after vaccine administration may include:

- Local reactions: hardening of the skin at the injection site, injection site redness, injection site swelling, pain at the site of injection, bruising of the skin at the injection site.

- Systemic reactions: aching muscles, headache, joint pain, fatigue, fever, and shivering.

8.2.1.3 SERIOUS ADVERSE EVENT (SAE) OR SERIOUS SUSPECTED ADVERSE REACTION

An adverse event or suspected adverse reaction is considered ‘‘serious’’ if, in the view of the investigator, it results in any of the following outcomes:

- Death

- A life-threatening adverse event

- Inpatient hospitalization or prolongation of existing hospitalization

- A persistent or significant incapacity or substantial disruption of the ability to conduct normal life functions

- A congenital anomaly/birth defect.

Important medical events that may not result in death, be life-threatening, or require hospitalization may be considered serious when, based upon appropriate medical judgment, they may jeopardize the subject and may require medical or surgical intervention to prevent one of the outcomes listed in this definition.

Examples of such medical events include allergic bronchospasm requiring intensive treatment in an emergency room or at home, blood dyscrasias, or convulsions that do not result in inpatient hospitalization, or the development of drug dependency or drug abuse.

8.2.1.4 Life-Threatening Adverse Event or Life-Threatening Suspected Adverse Reaction

An adverse event or suspected adverse reaction is considered ‘‘life-threatening’’ if, in the view of the investigator, its occurrence places the patient or subject at immediate risk of death.

It does not include an adverse event or suspected adverse reaction that, had it occurred in a more severe form, might have caused death.

8.2.1.5 Unexpected Adverse Event or Unexpected Suspected Adverse Reaction

An adverse event or suspected adverse reaction is considered ‘‘unexpected’’ if it is not listed in the Summary of Product Characteristics or is not listed at the specificity or severity that has been observed; or, if the Summary of Product Characteristics is not required or available, is not consistent with the risk information described in the general investigational plan or elsewhere in the current application, as amended.

For example, under this definition, hepatic necrosis would be unexpected (by virtue of greater severity) if the investigator brochure referred only to elevated hepatic enzymes or hepatitis. Similarly, cerebral thromboembolism and cerebral vasculitis would be unexpected (by virtue of greater specificity) if the investigator brochure listed only cerebral vascular accidents.

‘‘Unexpected,’’ as used in this definition, also refers to adverse events or suspected adverse reactions that are mentioned in the Summary of Product Characteristics as occurring with a class of drugs or as anticipated from the pharmacological properties of the drug, but are not specifically mentioned as occurring with the particular drug under investigation.

8.2.1.6 Independent Safety Monitoring

The ISM is a physician with relevant expertise in vaccine trials whose primary responsibility will be to provide independent safety monitoring in a timely fashion and to provide recommendations regarding the safe continuation of this study.

The ISM will evaluate safety data generated from study subjects including all SAEs against the known safety profile of the study vaccine to assess for possible changes to the overall risk of the study.

Contact information for the ISM is listed on page 3 of this protocol.

The ISM will communicate with the PI as needed to discuss any safety events of special interest developing during the study and when conducting the review of the monthly reports of cumulative safety data. The study has provisions for a back-up ISM to ensure that independent safety

monitoring happens at all times during the study. Additional roles and responsibilities of the ISM are described in section 8.2.4 below.

8.2.2 Collecting and Recording Adverse Events and Pregnancy

Adverse events may be identified during this study through any of these methods:

1. Examination of the subject during study visits.
2. Questioning the subject during study visits.
3. Receiving a safety report from the subject at any time during the study

Note: subjects will be asked to call the site if they develop any of the following:

- Illness or treatment from a physician or emergency department the entire duration of the study;
- Any adverse event that limits self-care activities of daily living (e.g. bathing, dressing and undressing, feeding self, using the toilet, taking medications, and not bed ridden) even if he/she decides not to seek medical care;
- Local REs and/or systemic REs of grade 3 or higher severity after the previous visit (only until Day 7 Visit).

4. Receiving a safety report from subject in Group A if they:

- Develop any symptoms while on antibiotics
- Develop any of the following until D90:
  - diarrhea (≥ 3 unformed stools/24h) for more than 72h with either fever or abdominal pain
  - tinnitus
  - vertigo*
  - decrease hearing
  - numbness/tingling

A complete recording of safety events in the CRF will include event term, date(s) of onset and

resolution/stabilization, assessment of severity, relationship to study vaccine or procedures/intervention(s) such as phlebotomy, expectedness, determination of whether the AE qualifies as serious or non-serious, treatment required, action taken with study participation, and outcome. AEs qualifying as serious also require a narrative of the event. Updates in safety events will be recorded as additional information becomes available.

Adverse events will be followed until they are resolved. Resolution of an AE is defined as the return to baseline status or as stabilization of the condition with the expectation that it will remain chronic.

Information on pregnancies will be collected from the time a subject signs the consent until the subject completes study participation. Cumulatively monthly reports of safety data will capture any pregnancies and pregnancy outcomes at least on a quarterly basis.

If a subject becomes pregnant after study entry, the investigator will discuss with the subject and/or the treating physician the known possible risks to the fetus.

Subjects becoming pregnant after study entry will be withdrawn from the study and followed until the end of the pregnancy. A pregnancy resulting in congenital anomaly/birth defect will be considered a SAE. Any premature termination of the pregnancy will also be reported and assessed as an SAE as needed.

8.2.3 Grading and Attribution of Adverse Events

8.2.3.1 Grading Criteria

Adverse events will be graded according to the criteria set forth in the National Cancer Institute's Common Terminology Criteria for Adverse Events Version 4.0 [May 28, 2009;revised version 4.03; June 14. 2010, http://evs.nci.nih.gov/ftp1/CTCAE/About.html]

This document (referred to herein as the NCI-CTCAE manual) provides a common language to describe levels of severity, to analyze and interpret data, and to articulate the clinical significance of all adverse events.

All adverse events whether or not listed in the NCI-CTCAE will be graded on a scale from 1 to 5 according to the following standards in the NCI-CTCAE manual (A semi-colon indicates 'or' within the description of the grade):

Grade 1 Mild; asymptomatic or mild symptoms; clinical or diagnostic observations only; intervention not indicated.

Grade 2 Moderate; minimal, local or noninvasive intervention indicated; limiting age-appropriate instrumental ADL*.

Grade 3 Severe or medically significant but not immediately life-threatening; hospitalization or prolongation of hospitalization indicated; disabling; limiting self-care ADL**.

Grade 4 Life-threatening consequences; urgent intervention indicated.

Grade 5 Death related to AE.

*Instrumental Activities of Daily Living (ADL) refer to preparing meals, shopping for groceries or clothes, using the telephone, managing money, etc.

**Self-care Activities of Daily Living (ADL) refer to bathing, dressing and undressing, feeding self, using the toilet, taking medications, and not bedridden.

Not all Grades are appropriate for all AEs; therefore, some AEs are listed with fewer than five options for Grade selection.

Anaphylaxis is a disorder characterized by an acute inflammatory reaction resulting from the release of histamine and histamine-like substances from mast cells, causing a hypersensitivity immune response. Clinically, it presents with breathing difficulty, dizziness, hypotension, cyanosis and loss of consciousness and may lead to death.

Severity grading of anaphylaxis as per the NCI-CTCAE manual is as follows:

Grade 1= not applicable

Grade 2= not applicable

Grade 3= Symptomatic bronchospasm, with or without urticaria; parenteral intervention indicated; allergy-related edema/angioedema; hypotension

Grade 4= Life-threatening consequences; urgent intervention indicated

Grade 5= Death

Severity grading of AEs of laboratory abnormalities will be assessed as per the NCI-CTCAE manual.

8.2.3.2 Definition of Attribution

The site investigator will initially determine the relationship of an adverse event to the study vaccine or study procedures (blood draw). The investigator's determination of causality will be used for FDA reporting purposes.

The relationship of an AE to study participation will be determined using definitions in the table below:

Code	Descriptor	Definition (guidelines)
UNRELATED CATEGORY		
1	Unrelated	The adverse event is clearly not related to study. The event is completely related to an etiology other than the study product or study intervention (the alternative etiology must be documented in the study subject's medical record)
2	Unlikely	The adverse event is doubtfully related to study and likely to be related to factors other than study product or study intervention.
RELATED CATEGORIES		
3	Possible	The adverse event may be related to study. There is an association between the event and the administration of study product and there is a plausible mechanism for the event to be related to the study product; there may be also an alternative etiology, such as characteristics of the subject's clinical status and/or underlying disease
4	Probable	The adverse event is likely related to study. There is (1) an association between the event and the administration of study product or study intervention, (2) a plausible mechanism for the event to be related to the study product, and (3) the event

		could not be reasonably explained by known characteristics of the subject's clinical status and or an alternative etiology is not apparent
5	Definite	The adverse event is clearly related to study. There is (1) an association between the event and the administration of the study product or study intervention, (2) a plausible mechanism for the event to be related to the related to the study product, and (3) causes other than the study product have been ruled out and/or the event re-appeared on re-exposure to the study product

8.2.4 Reporting Serious Adverse Events to the Independent Safety Monitor

The Principal Investigator will notify the ISM by email of any SAE within 24 hours of becoming aware of the event. The initial SAE CRF should be signed by the PI or co-PI and include as much information as possible.

The SAE case report form will be re-submitted and signed by the PI or co-PI to the ISM with updated relevant medical information as needed until the event is considered closed by the ISM.

8.2.5 Reporting Serious Adverse Events to the FDA

N/A.

8.2.6 Notifying Institutional Review Board

The Principal Investigator will ensure the timely dissemination of SAE information, including SAEs requiring expedited review by the ISM and resulting ISM memorandums following evaluations of safety data, to the IRB in accordance with IRB regulations and guidelines.

8.3 Protocol Deviations

Deviations occur when the Investigator, site study staff, or subjects fail to adhere to protocol requirements or when there is non-adherence to (GCP) as delineated in ICH E6 (R1) guidelines.

Upon determination that a protocol deviation has occurred, the study staff will a) notify the Principal Investigator, and b) will complete the Protocol Deviation form. There could be a discussion between the Principal Investigator and the ISM to determine the effect of the protocol deviation on the study subject and his/her further study participation, the effect of the protocol deviation on the overall study, and corrective actions.

The Principal Investigator will complete and sign the Protocol Deviation form and submit it to the ISM Monitor and to the site IRB, per IRB regulations.

Identification of protocol deviations will be done through the quality manager at the study site. Reviewing and reporting of minor protocol deviations will be discussed at the Hope Clinic monthly meetings. Major protocol deviations will be discussed with the PI and the ISM.

All study deviations will be captured in monthly reports along with safety data and forwarded to the ISM.

9 SAMPLE SIZE CALCULATIONS AND STATISTICAL PLAN

9.1 Sample Size and Power Calculations

Based on previous studies performed at the Hope Clinic, the rate of subjects that become ineligible during the study (i.e. do not meet exclusion/inclusion criteria anymore, lost to follow-ups, unable to have blood draws) is between 5 to 10%. Assuming a 10% attrition rate, 50 healthy adults will be recruited for the study, resulting in an expected 10 subjects per arm for data analysis. In the case that the sample size of subjects missing any of the 2 draws required for the primary endpoint [meaning draws at days 0 and day 30] is higher than 10% of the 50 subjects to be recruited for the study, we will recruit extra patients to maintain the target sample size of 10/group and the statistical power to prove primary endpoint.

9.1.1 Primary and Secondary Endpoints

The comparison of HAI titer will be conducted in three ways: direct comparison of HAI titers, comparing seroprotection rate, and comparing seroconversion rate.

For the comparison of the mean log2 HAI titers between the two groups, with 10 subjects in each group, at the alpha level of 0.05, we can reject the null hypothesis of equal means with 80% power if the true effect size ((mean0-mean1)/sigma, assuming equal variance) is 1.3 using an unpaired one-sided t-test.

Based on a previous study of Fluzone® in a large cohort, we expect 98% of subjects without antibiotics treatment will reach seroprotection, i.e. HAI titers of ≥1:40. Our null hypothesis is the two groups have equal proportions of subjects who will reach seroprotection, and the alternative hypothesis is the antibiotic treatment lowers the proportion of subjects reaching seroprotection. The sample size of 10 subjects per treatment group will allow us 80% power to detect a difference in proportions of 0.41 or higher in a one-sided test of two proportions using a significance level of 0.05.

Based on a previous study of Fluzone® in a large cohort, we expect 65% of subjects without antibiotics treatment will achieve seroconversion, i.e. ≥four-fold increase in HAI titers over baseline. The sample size of 10 subjects per treatment group will allow us 80% power to detect

a difference in proportions of 0.50 or higher in a one-sided test of two proportions using a significance level of 0.05.

9.2 Data Analysis

Data from the study will be analyzed using the statistical software R . High-throughput data will be analyzed using appropriate methods, either by stand-alone software or modules in leading statistical softwares, e.g. the Bioconductor in the R framework (44). For cytokine data, missing values will be dealt with using multiple imputations. For microarray data, nearest neighbor method and local least squares may be used to fill missing values. Unsupervised learning techniques such as PCA, PLSDA, clustering, and factor analysis will be used for the visualization and identification of global patterns (45). For descriptive endpoints, we will generate summary statistics, and visualize the data using histograms and boxplots when applicable. For the identification of innate signatures, the two treatment groups will be analyzed separately.

9.2.1 Analyses of Primary Endpoint

Comparison of HAI Titers at D30 Post-Vaccination in Both Groups

Data from the study will be analyzed using the statistical software R. We will take three alternative routes for data analysis. The first is directly comparing HAI titers between the treatment groups; the second is comparing the proportions of subjects achieving seroprotection from each group; and the third is comparing the proportions of subjects achieving seroconversion from each group.

The first data analysis route is directly comparing the means/medians of the HAI titers between the two groups at D30. We will first log2 – transform the HAI titers for each subject. The log2 HAI titers of each group will be summarized and presented using histograms and strip charts for visual comparison. We will use the t-test to compare the mean log2 HAI titers between the two groups. The Wilcoxon test may also be used depending on the distribution of the data.

In addition, we will compare the two groups based on the proportion of achieving seroprotection and seroconversion. For seroprotection, we will dichotomize the outcome into D30 HAI titer ≥1:40 or D30 HAI titer <1:40. For seroconvertion, we will dichotomize the outcome into D30 HAI titer ≥four-fold or <four-fold rise relative to baseline titer. If a subject has negative titer at D0, we will consider the subject a seroconverter if his/her HAI reaches 1:40 at D30. We will then compare the two treatment groups by conducting a Fisher's exact test on the 2×2 contingency table of

treatment versus dichotomized HAI titers. We will also report the estimated proportion of seroprotection/seroconversion and its 95% confidence interval for each group.

9.2.2 Analyses of Secondary Endpoint

9.2.3 Analysis of Exploratory Endpoints

Exploratory Endpoint 1: Analyses of microbiome at D0, D7, and D30 in Group A and comparison to baseline Screening

Sequence results will be analyzed using the open source software package called Quantitative Insights into Microbial Ecology (QIIME) (46). Primary objective of our analyses will be to characterize the composition of microbiota samples and to identify specific microbial communities significantly associated with an experimental group.

Prior to analysis, sequences will undergo quality filtering using QIIME and the sequence quality scores annotated to each raw sequence data. The 16s gene sequences will be aligned and clustered using UCLUST, which is based on a pair-wise identity threshold of 97% (47). Clustered sequences will be assigned to operational taxonomic units (OTUs) (48). A single representative sequence for each OTU will be aligned using PyNAST to enable taxonomic classification and generation of phylogenetic trees for subsequent analyses (46, 49, 50).

OTUs with taxonomic assignments will allow QIIME to assemble a matrix of OTU abundance in each sample. This matrix will then be used to summarize communities by taxonomic composition and abundance, which can be visualized together as area/bar graphs or heatmaps. Information from phylogenetic trees will be used to compute distances or levels of dissimilarity between microbial communities using Unifrac (51). This tool will enable compositional comparisons between samples (beta diversity) to be conducted. For this purpose, we will use the OTU matrix to calculate distance or dissimilarity of samples between experimental groups using weighted and unweighted UniFrac metrics. QIIME will then generate 2 and 3 dimensional Principal Coordinate Analysis plots to represent the distance matrix data. Furthermore, QIIME codes a tool based on G test of Independence to identify OTUs that are differentially represented across experimental groups. This tool will also be conducted and applied to the beta diversity Principal Coordinate Analyses to help explain which OTUs are significantly associated with a particular experimental group.

For longitudinal analyses of samples within each group, semivariogram plots will be generated using dissimilarity metrics over time (Euclidean) plotted against community dissimilarity (UniFrac) (46,52).

Exploratory endpoint 2: Analysis of the repertoire and monoclonal antibodies from plasmablasts in a subset of vaccinees at D7 in both groups

The repertoire and monoclonal antibody levels will be presented as descriptions of the actual values. Summary statistics, boxplots and histograms will be used to summarize the data.

Exploratory endpoint 3: Identification of innate immune signatures (traditional immune parameters measurements + array-based gene expression) at D1, D3 and D7 in both groups

We will take the measurements on the day of vaccination as baseline. For traditional immune parameters, we will test if an immune parameter shows significant change over baseline at day 1, 3, and 7 after each vaccination, using a two-sided paired t-test. Fold-change (ratio between the mean values of the two groups) will be combined with the test p-value in a selection criterion as appropriate. Based on previous studies similar in nature, the tentative selection criterion is p-value ≤ 0.01 and fold change ≥ 3.

For the gene expression data, we will use the method Significance Analysis of Microarrays (SAM) with paired design to find differentially expressed genes (53). False discovery rate (FDR) will be used as selection criterion (54). The tentative selection criterion is FDR ≤ 0.1.

Exploratory endpoint 4: Correlation of innate immune signatures at days D1, D3 and D7 with HAI titers at D30 in both groups

For traditional immune parameters, we will calculate the correlation coefficient between the immune parameter and the adaptive immune response (HAI titers at D30) he p-values associated with the correlation coefficients will be used to select traditional immune parameters that are associated with the respective adaptive immune responses. The tentative selection criterion is p-value ≤ 0.01.

For gene expression data, we will use the method Significance Analysis of Microarrays (SAM) with quantitative outcome to identify genes that are significantly associated with the respective

adaptive immune responses (HAI titers at D30). False discovery rate (FDR) will be used as selection criterion. The tentative selection criterion is FDR ≤ 0.1.

Exploratory endpoint 5: Comparison of HAI Titers at D30 Post-Vaccination in Both Groups

Analysis similar to 9.2.1.

9.2.4 Patient Populations

We will use all subjects who are randomized, receive the vaccine, complete at least one day of antibiotic treatment, and have measurements at days 0 and 21 in the data analysis.

9.2.5 Study Subject Baseline Characteristics and Demographics

A summary of descriptive statistics for baseline and demographic characteristics will be provided for all enrolled subjects. Demographic data will include age, race, sex, and medical history, including current medication use and vaccination history.

9.2.6 Status of Study Subjects

Status of study subjects (continue in the study vs. left the study) will be incorporated into the periodic safety reports.

9.3 Interim Analyses

No interim analysis will be conducted.

9.4 Deviations from Statistical Plan

The principal features of the study design and of the plan for statistical analysis of the data are outlined in this protocol. Any changes in these principal features will require a protocol amendment and will be described in the final report. These changes will be subject to review by the IRB, ISM.

Although our statistical methods and time-points for measurements will adhere to what is proposed herein, our experience with analyses of similar studies in the past has underscored the need for flexibility and adaptability in trying different statistical approaches to arrive at the most informative results, especially for the high-throughput data.

As such, we may use alternative approaches such as the gene set enrichment analyses, or other approaches, and run additional statistical analyses of data generated by use of assays at time points other than those stated in the primary, secondary and exploratory endpoints, on an ad hoc basis (55).

10 IDENTIFICATION AND ACCESS TO SOURCE DATA

10.1 Identifying Source Data

The investigator will keep accurate records to ensure that the conduct of the study is fully documented. Data forms are either considered source or protocol-specific CRFs as detailed in the MOPs.

10.2 Updating Source Documentation

Documents describing the safety profile of investigational products, such as the investigator's brochure and the package insert, will be amended as needed by the investigational products manufacturer to ensure that the description of safety information adequately reflects any new clinical findings.

The Principal Investigator (or co-PI) will provide the ISM and the IRB with the most up-to-date versions of the above documents as soon as the Principal Investigator (or co-PI) becomes aware of any changes. For purchased investigational products, the Principal Investigator (or co-PI) will confirm that there are no changes to the package insert every 3 months. In case of package insert changes, the Principal Investigator (or co-PI) will notify the ISM and the IRB.

10.3 Permitting Access to Source Data

The investigational site participating in this study will maintain the highest degree of confidentiality for the clinical and research information obtained from the subjects in this study. Medical and research records will be maintained at the study site in the strictest confidence. However, as a part of the quality assurance and legal responsibilities of an investigation, the investigational site will permit authorized representatives of regulatory authorities to examine (and when required by applicable law, to copy) clinical records for the purpose of quality assurance reviews, audits, and evaluations of the study safety and progress. Unless required by the laws that permit copying of records, only the coded identity associated with documents or with other subject data may be copied (and all personally identifying information will be removed). Authorized representatives

described above are bound to maintain the strict confidentiality of medical and research information that is linked to identify individuals.

11 QUALITY CONTROL AND QUALITY ASSURANCE

The Principal Investigator (or co-PI) will keep accurate records to ensure that the conduct of the study is fully documented. The investigator will ensure that all CRFs and subject study files are legible and complete for every subject.

When the CRFs are complete, they will be reviewed and signed by the Principal Investigator or co-PI. All discrepancies identified will be reviewed, and any resulting queries will be resolved with the Principal Investigator (or co-PI) and the CRFs will be amended as needed.

The Principal Investigator (or co-PI), through the use of an internal Quality Management Plan, appropriate site quality control, and quality assurance monitoring staff, will be responsible for the regular review of the conduct of the trial, for verifying adherence to the protocol, and for confirming the completeness, consistency, and accuracy of all documented data and accuracy of source documentation verification. The reports of the internal site monitor will be submitted to the Principal Investigator (or co-PI).

As per the clinical monitoring plan, site monitoring will be conducted by the independent site monitor in accordance with established Good Clinical Practices (ICH GCP 5.1.1, 5.2, 5.18.1) and the Code of Federal Regulations, as applicable. The overall objectives of site monitoring visits are to ensure:

1. Site compliance with the current version of the approved protocol, consent, documents and local Institutional Review Board requirements.

2. Accuracy and completeness of data entry.

3. Required regulatory documents are current and maintained in the protocol-specific regulatory binder.

4. A designated percent of signed consent forms, inclusion/exclusion criteria, primary, secondary, and tertiary endpoints, safety monitoring parameters, deviations, and serious adverse events are documented.

5. Procedures are in place to administer and monitor study drug / product accountability and documentation of destruction policies.

6. The research staff is adequately trained with respect to GCP and GLP.

7. All observed anomalies or protocol deviations are reported and identify an action plan to minimize study dropouts and non-compliance with defined study procedures.

The results of the site monitoring report will be discussed with the PI, the staff to ensure compliance with the monitor's findings.

12 ETHICAL CONSIDERATIONS AND COMPLIANCE WITH GOOD CLINICAL PRACTICE

12.1 Statement of Compliance

This study was designed to ensure the protection of subjects according to the ethical principles of the Declaration of Helsinki and amendments concerning medical research in human subjects. This clinical study will be conducted using current good clinical practice (cGCP), as delineated in Guidance for Industry: E6 Good Clinical Practice Consolidated Guidance [1], and according to the criteria specified in this study protocol. Before study initiation, the protocol the informed consent documents will be reviewed and approved by IRB, as well as any other appropriate health authorities. Any amendments to the protocol or to the consent materials will also be approved by the appropriate bodies listed above prior to implementation.

12.2 Informed Consent Process

The informed consent form will provide information about the study to a prospective subject or subject's legal representative to allow for an informed decision about participation in the study. Prospective subject or subject's legal representative must be given ample opportunity to review the informed consent and inquire about the results of the study. All subjects (or their legally acceptable representative) must read, sign, and date a consent form prior to study participation. Consent materials for subjects who do not speak or read English will be translated into the subjects' appropriate language.

The informed consent form will be revised and receive IRB approval whenever important new safety information is available, whenever the protocol is amended, and/or whenever any new information becomes available that may affect participation in the trial.

A copy of the informed consent form will be given to a prospective subject for review prior to any study procedure. The Principal Investigator or an approved designee will discuss the consent with the prospective subject and answer questions. The prospective subject will be told that being in the trial is voluntary and that he or she may withdraw from the study at any time, for any reason.

13 PUBLICATIONS

Publication of any data from this study must be carried out in agreement of sponsor and investigators.

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15 APPENDICES

15.1 Appendix A: Schedule of Events

Day	D-21 D-1	D-6 D-3[f]	D0	D1	D3	D7 +/-1	D30 +/-5	D90 +/-14	D180 +/-14	
Visit	1		2	3	4	5	6	7		D +
Informed consent & HIPPA	X									
Demographic and Medical History (including medication and vaccine history).	X									
Verify eligibility	X		X							
PID assignment	X									
Vital signs, height and weight	X		X							
Focused physical exam (only if indicated based on review of health status)	X	X	X	X	X	X	X	X	X	
Urine pregnancy test (for female volunteers of childbearing potential)	X	X	X							
Stool sample collection	X	X	X	X	X	X	X	X	X	
Randomization	X									
Assessment of health status while on antibiotics (Group A only)[a]			X	X						
Distribute assigned antibiotics and instruct subject when and how to take (Group A only)		X								
Vaccination			X							
Study drug accountability (Group A only)			X	X	X					

Day	D-21 D-1	D-6 D-3[f]	D0	D1	D3	D7 +/-1	D30 +/-5	D90 +/-14	D180 +/-14	
Visit	1		2	3	4	5	6	7		D +
Instruction given to study subjects on safety events and concomitant medications.[b]	X		X	X	X	X	X			
Assessment of health status [c] and concomitant medications after administration of study vaccine			X	X	X	X	X	X	X	
Blood draw for Innate Assays and Adaptive Assays [d]	X	X	X	X	X	X	X	X	X	
Blood draw for safety labs[e]	X					X				

Footnotes:

[a] Subjects in Group A are asked to report any symptoms experienced while on antibiotics (from D-3 until D1).
[b] Any Adverse Event including – but not limited to- vaccine reactions and local or systemic Reactogenicity Events of grade 3 or higher severity or serious adverse event (SAE) occurring after vaccination while the subjects is still at the clinical site will be recorded and reported.
[c] Instruction includes the following:

1. Subjects will be provided (on Day 0 only) with a written description of local and systemic vaccine reactions of mild, moderate and severe intensity and instructed to call the site to report reactogencity events of grade 3 (severe) or higher severity within 1 week following vaccination.
2. Subjects will also be instructed to promptly call the site if he/she develops any of the following:
   - Illness or treatment from a physician or emergency department; and/or hospitalization due to any illness throughout the entire duration of the study;
   - Any adverse event that limits self-care activities of daily living (e.g. bathing, dressing and undressing, feeding self, using the toilet, taking medications, and not bed ridden) even if he/she decides not to seek medical care; or
   - Any of the following symptoms until D90 (Group A subject only):
     - diarrhea (≥ 3 unformed stools/24h) for more than 72h with either fever or abdominal pain*
     - tinnitus**
     - vertigo**
     - decrease hearing**
     - numbness/tingling**
   - he/she starts/stops medications during enrollment in the study.

[d] Innate assays will not be conducted at day 30. Adaptive assays will not be conducted at day 1.
[e] Safety labs include CBC with differential, creatinine, and potassium.
[F] Only for Group A

15.2 Appendix B. Severity Scale for Local Vaccine Reactions (local reactogenicity events)

	INJECTION SITE REACTIONS		
	Grade		
	1	2	3
Swelling/ Induration/	Mild induration, able to move skin parallel to plane (sliding) and perpendicular to skin (pinching up)	Moderate induration, able to slide skin, unable to pinch skin; limiting instrumental activities of daily living	Severe induration, unable to slide or pinch skin; limiting arm movement limiting self-care activities of daily living
Redness/ erythema	Asymptomatic or mild symptoms; intervention not indicated	Moderate; minimal, local; limiting age-appropriate instrumental activities of daily living	Severe but not immediately life-threatening; hospitalization or prolongation of existing hospitalization indicated; disabling; limiting self-care activities of daily living
Pain/tenderness	Mild	Moderate pain; limiting instrumental activities of daily living	Severe pain; limiting self-care activities of daily living

Note:
Instrumental Activities of Daily Living refer to preparing meals, shopping for groceries or clothes, using the telephone, managing money, etc.
Self-care Activities of Daily Living refer to bathing, dressing and undressing, feeding self, using the toilet, taking medications, and not bedridden.

15.3 Appendix C. Severity Scale for Generalized Vaccine Reactions (systemic reactogenicity events)

	GENERAL ADVERSE REACTIONS		
	Grade		
	1	2	3
Fatigue/asthenia	Fatigue relieved by rest	Fatigue not relieved by rest; limiting instrumental activities of daily living	Fatigue not relieved by rest, limiting self-care activities of daily living
Body ache/myalgia	Mild pain	Moderate pain; limiting instrumental activities of daily living	Severe pain; limiting self-care ADL
Headache	Mild pain	Moderate pain; limiting instrumental activities of daily living	Severe pain; limiting self-care activities of daily living
Joint pain	Mild pain	Moderate pain; limiting instrumental activities of daily living	Severe pain; limiting self-care activities of daily living
Chills	Mild sensation of cold; shivering; chattering of teeth	Moderate tremor of the entire body; narcotics indicated	Severe or prolonged, not responsive to narcotics
Fever	38.0 - 39.0 degrees C (100.4 - 102.2 degrees F)	>39.0 - 40.0 degrees C (102.3 - 104.0 degrees F)	>40.0 degrees C (>104.0 degrees F) for <=24 hrs

Note:
Instrumental Activities of Daily Living refer to preparing meals, shopping for groceries or clothes, using the telephone, managing money, etc.
Self-care Activities of Daily Living refer to bathing, dressing and undressing, feeding self, using the toilet, taking medications, and not bedridden