

AWARD NUMBER: W81XWH-13-1-0497

TITLE: Induction of Food Allergy in Mice by Allergen Inhalation

PRINCIPAL INVESTIGATOR: Fred Finkelman, M.D.

CONTRACTING ORGANIZATION: Cincinnati Veterans Affairs Medical Center
Cincinnati, OH 45220

REPORT DATE: October 2015

TYPE OF REPORT: Annual Report

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE				<i>Form Approved</i> <i>OMB No. 0704-0188</i>	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.					
1. REPORT DATE October 2015		2. REPORT TYPE Annual		3. DATES COVERED 30 Sep 2014 - 29 Sep 2015	
4. TITLE AND SUBTITLE Induction of Food Allergy in Mice by Allergen Inhalation				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-13-1-0497	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Fred Finkelman, M.D. E-Mail: finkelfd@ucmail.uc.edu				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Cincinnati Veterans Affairs Medical Center 3200 Vine Street Cincinnati, OH 45220				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT The goal of our project is to use mouse models to determine whether and how inhaling egg white can sensitize for the development of egg white food allergy. Despite difficulties related to the complicated pregnancy and illness of a key researcher, considerable progress has been made: 1) We cannot induce an allergic response to aerosolized egg white, even if inflammation is induced by aspiration of saline. This suggests that additional stimuli are required to induce an allergic response by this route. 2) We have shown that egg yolk acts as an adjuvant for the development of allergic lung disease to egg white and induction of food allergy; 3) We have shown that egg yolk adjuvant activity is related to its induction of a cellular biochemical response called the unfolded protein response (UPR) and that the UPR induces epithelial cells to produce 3 cytokines that are essential for the induction of food allergy; and 4) we have shown that neutralization of any one of these 3 cytokines prevents food allergy induction, while neutralization of all 3 suppresses established food allergy. Additionally, preliminary data show that in vivo suppression of the UPR with an FDA-approved drug can suppress established food allergy.					
15. SUBJECT TERMS Food allergy, eggs, cytokines, unfolded protein response, allergic lung disease, mice					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT UU	18. NUMBER OF PAGES 10	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT Unclassified	b. ABSTRACT Unclassified	c. THIS PAGE Unclassified			19b. TELEPHONE NUMBER (include area code)

Table of Contents

	<u>Page</u>
1. Introduction.....	4
2. Keywords.....	4
3. Accomplishments.....	4
4. Impact.....	9
5. Changes/Problems.....	10
6. Products.....	10
7. Participants & Other Collaborating Organizations.....	10

INTRODUCTION: This proposal tests a hypothesis about the pathogenesis of food allergy (FA): the development of FA depends on a parenteral route of antigen (Ag) exposure, inflammation, Ag dose and Ag epitopes. Specifically, we hypothesized that: 1) the route of Ag exposure is critical for determining whether FA development is promoted or suppressed, with airway exposure being more likely than enteric exposure to promote FA; 2) inflammatory costimuli promote the induction of FA by inhaled Ags; 3) inhalation of sub-immunogenic quantities of Ag can induce tolerance instead of priming for FA; and 4) Ag inhalation can sensitize for the development of FA to subsequently ingested, cross-reactive Ags.

KEYWORDS: Mouse, food allergy, cytokines, eggs, antigen, allergen, inflammation, adjuvant, airways, sensitization

ACCOMPLISHMENTS:

What were the major goals of this project? The major goals of this project, were as stated in the approved SOW (please note that some extend into the third year of the project):

Aim 1: Determine the conditions under which inhalation of aerosolized egg white can prime for development of food allergy to egg white. Timeframe: months 1-20.

Task 1. Determine whether inflammation induced by aspiration of saline would allow exposure to aerosolized egg white to induce allergic airway disease and/or prime for food allergy

Task 2. Determine whether induction of allergic airway disease and/or priming for food allergy requires airway deposition of a higher dose of egg white than is accomplished by our aerosol protocol.

Task 3. Determine whether induction of allergic airway disease by inhalation of an unrelated allergen will allow exposure to aerosolized egg white (EW) to prime for food allergy (FA).

Aim 2. Determine whether airway-mediated induction of food allergy by one antigen increases the ability of a second, unrelated antigen to induce food allergy. Timeframe: months 1-12.

Aim 3. Determine whether ingestion of egg white will inhibit the ability of egg white inhalation to prime for development of egg white food allergy. Timeframe: months 13-24

Aim 4. Test the hypothesis that food regurgitation and aspiration may prime for food allergy. Timeframe: months 13-24.

Task 1: Determine the best time after feeding to recover partially digested egg white from the stomach: Duration: 4 weeks (month 13). Animal requirement: 72 mice.

Task 2: Perform a dose-response study that compares the abilities of fresh egg white vs. stomach-recovered egg white to induce allergic airway disease and initiate food allergy when inoculated intratracheally. Duration: 48 weeks (months 14-24).

Aim 5. Determine whether airway priming with birch pollen can induce murine food allergy to apple and celery. Timeframe: months 25-36.

Aim 6. Determine whether inhalation of aerosolized egg white can reverse

established egg white food allergy. Timeframe: months 1-36.

Task 1: Determine whether inhalation of low doses of aerosolized egg white can suppress established food allergy to this antigen.

Task 2: Histological evaluation of lungs from the same mice used in task 1 to determine effects of the aerosolized egg white on airway inflammation and fibrosis.

Task 3: Produce mAbs to IL-10R, TGF- β and CD25, which will be used in Aim 1, task 2, Aim 3 and Aim 6 task 3. Duration: 36 weeks (months 1-36).

Task 4: Determine whether mAbs to TGF- β , the IL-10R and/or CD25 will block the induction of tolerance by aerosolized egg white.

What was accomplished under these goals?

Aim 1, Task 1: Inducing airway inflammation by causing anesthetized mice to aspirate saline, in addition to having them breathe in aerosolized egg white (EW), still did not cause the development of severe allergic airway disease and sensitization for food allergy to egg white, unlike our original finding with aspiration of EW. The interpretation of this finding was complicated by a failure in many experiments to reproduce our original observation that aspiration of EW-sensitized mice to develop food allergy, although it always causes some degree of allergic airway disease. Our current interpretation is that two factors are involved: 1) Potentially most important, the presence of some egg yolk acts as an adjuvant for the development of allergic airway disease and food allergy to EW. This is relevant to human allergy, because egg yolk will generally be inhaled along with EW. Please see below for more detail about the adjuvant effect. 2) Our mouse suppliers and some details of the animal husbandry in our mouse colony have changed. This has resulted in reduced sensitivity to induction of allergic responses, possibly because of changes in bacterial flora.

Aim 1, Task 2: Increasing the dose of aerosolized EW did not induce allergic airway disease or prime for food allergy. The interpretation of this negative result is complicated by the issues discussed under Aim 1, Task 1 (failure of aspirated EW to prime for food allergy in many experiments).

Aim 1, Task 3: We have found that i.t. inoculation of house dust mite extract (HDM) along with EW for the initial 2 inoculations, followed by 17 inoculations (3/week) increases the severity of allergic airway disease that is induced (Figure 1). This is

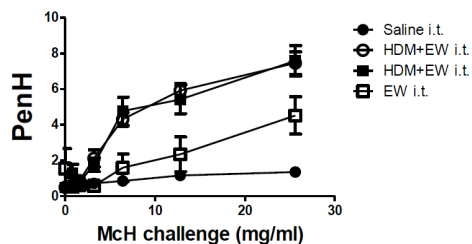


Figure 1. Inhalation of HDM with EW increases allergic airway disease beyond that induced by EW alone. BALB/c mice were inoculated i.t. with saline, EW, or HDM + EW for the first two inoculations, then with EW for 17 additional inoculations. Two separate groups of mice were inoculated with HDM + EW, to test reproducibility. Mice were tested by barometric plethysmography for responsiveness to mechacholine.

potentially important, because house dust in most of the US typically contains egg proteins as well as house dust mites; consequently, it is likely that this combination of antigens will be inhaled. Studies have not yet been performed to determine whether the initial HDM inoculation with EW makes mice susceptible to develop EW food allergy. This is because of the stronger effect that we later found of egg yolk plasma (EYP, the liquid part of egg yolk) on the development of both allergic airway disease and food

allergy to EW (Figure 2; food allergy is detected as diarrhea and anaphylactic shock,

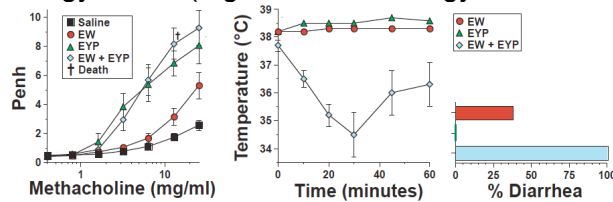


Figure 2. EYP enhances development of food allergy to EW. BALB/c mice were inoculated i.t. 3x/wk x 3 wks with saline, EW, EYP or EYP + EW, then tested for airway responsiveness to methacholine (left panel), after which they were inoculated o.g. 2x/wk x 4 wks with the same Ags and evaluated for development of shock (hypothermia) and diarrhea.

which is observed as diarrhea). We have not yet been able to test whether inhalation of an aerosol that contains both EW and egg yolk will induce severe allergic airway disease and prime for development of egg food allergy, because the high viscosity of egg yolk makes aerosol generation difficult with our equipment. We are trying to determine whether a low concentration of egg yolk will still have the adjuvant effect that we have observed with aspiration of EW plus egg yolk.

The strong effect of EYP on the development of airway hyperresponsiveness (AHR) and the strong synergy between EW and EYP on the development of food allergy had obvious human relevance, because EW and egg yolk (EY) are likely to be encountered together by humans, but was somewhat surprising, because the most clinically important egg allergens are present in EW, rather than EY. This, and the predominantly lipid constitution of EYP, made us wonder if EYP promoted airway and food allergy to eggs by virtue of possible adjuvant effects of its lipid components, rather than antigenic effects of its protein components. The former possibility seemed feasible, because we had recently found and reported that saturated fats, including those that are present in EYP, can induce epithelial cells to produce TSLP, IL-25 and IL-33, cytokines that have been shown to be important for the development of a food allergic response. To test this possibility, we evaluated whether EYP could induce epithelial cell expression of TSLP, IL-25 and/or IL-33 whether applied for 24 hours to mouse skin or inoculated i.t. 24 hours prior to harvesting mouse lungs. Results of this experiment (Figure 3) showed

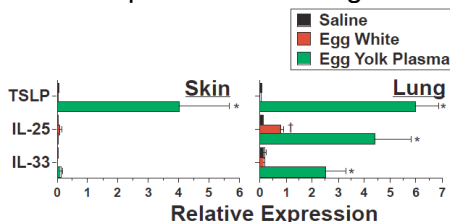


Figure 3. EYP induces pro-Th2 cytokine responses in vivo. BALB/c mice (6/gp) were inoculated i.t. with saline, EW, or EYP or had these applied to unabrased skin under an occlusive dressing. 24 hr later RNA was extracted from skin and lungs and relative expression of TSLP, IL-25 and IL-33 genes was determined by quantitative RT-PCR. * indicates a significant ($p < 0.05$) difference from saline and EW-treated mice; † indicates a significant difference from saline-treated mice.

that EYP strongly induced TSLP expression when applied to skin and all 3 of these cytokines when inoculated into the lungs. In contrast, EW has little ability to induce these cytokines. To determine whether induction of these cytokines was relevant, we first tested whether they are important for induction and maintenance of food allergy in another, more established model, in which food allergy is induced by inoculation with EW + purified saturated medium chain triglycerides by oral gavage (o.g.). Our results show that all three of these cytokines, which we call “pro-Th2 cytokines” because they promote a Th2 cytokine response, are essential for induction of food allergy in this model (Figure 4), while any one of these pro-Th2 cytokines can maintain established food allergy (Figure 5). These observations both explain how eggs (and other important nutrients that have a high saturated fat content, such as cow’s milk), can be such

common and important food allergens and provide an approach for the suppression of

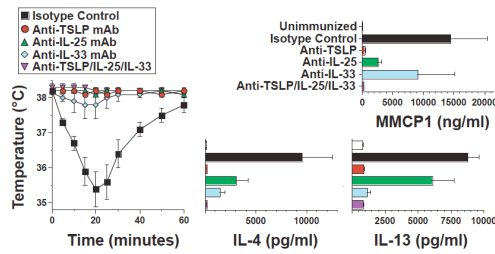


Figure 4. Anti-TSLP, anti-IL-25 and anti-IL-33 mAbs suppress food allergy induction by MCT + EW. BALB/c mice (6/group) were left untreated or were inoculated o.g. 3x a week for 3 weeks with 100 mg of EW + 100 µl of MCT. Mice were also injected i.p. 12 hr before each o.g. inoculation with mAbs to TSLP (500 µg), IL-25 (500 µg), or IL-33 (ST2L, 100 µg), with a combination of all 3 mAbs, or with isotype control mAbs. Mice were evaluated for development of hypothermia and MMCP1, IL-4 and IL-13 responses to their final o.g. challenge with 100 µl of MCT + 100 mg of EW.

established food allergy. Experiments are underway to try to repeat the observations illustrated in Figure 4 and Figure 5 using sensitization by the i.t. route with EW + EYP instead of o.g. sensitization with EW + MCT.

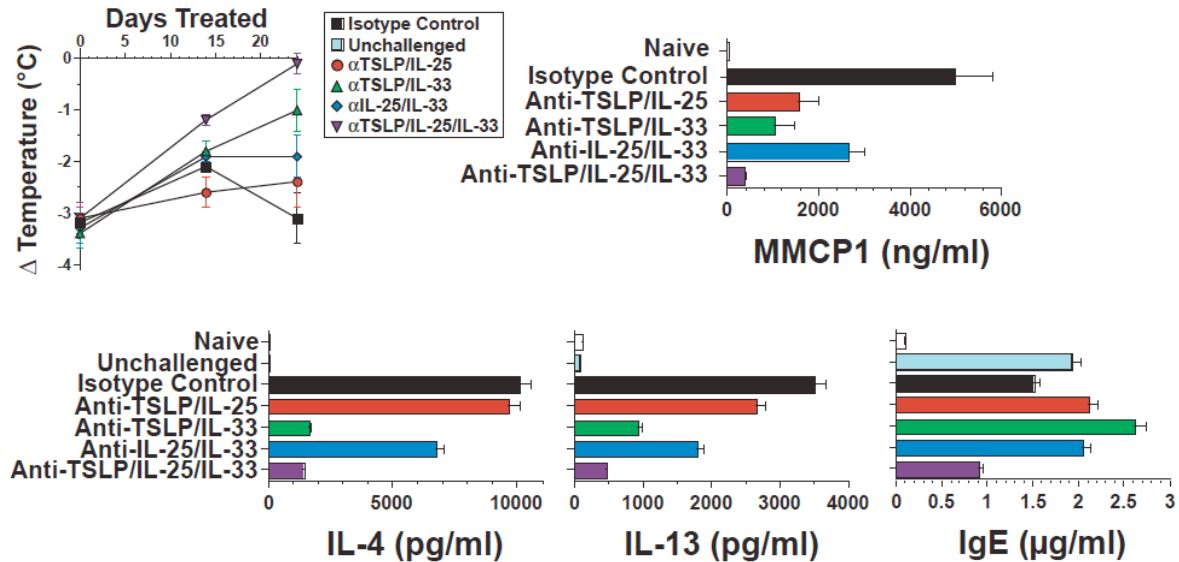


Figure 5. Suppression of established FA with anti-pro-Th2 mAbs. BALB/c mice were inoculated o.g. twice with MCT, then 2x/wk for 4 wk with MCT/EW. Mice with a temperature drop >3°C in response to o.g. challenge were then divided into groups of 5. These mice continued to be inoculated o.g. 2x/wk with MCT/EW for an additional 24 d, but also were injected i.p. with the mAbs shown 12 hr before each o.g. inoculation. Maximal decreases in rectal temperature were determined for the hr after o.g. inoculation on d 0, 14 and 24 after initiation of mAb treatment. Serum IgE and MMCP1 levels and IL-4 and IL-13 production were determined on d 24.

Aim 2: We have induced allergic airway disease and subsequently, food allergy, to EW and then evaluated whether ingestion of an aqueous peanut extract would induce peanut food allergy. The results were negative, although we cannot exclude the possibility that more intense food allergy to EW or a different immunization schedule with peanut extract would have allowed induction of food allergy to peanut. We are continuing to try approaches to evaluate this question.

Aim 3: We have found that ingestion of EW suppresses the ability of aspirated EW to induce allergic airway disease. We are repeating this study to make certain that our observation is reproducible and to determine whether it is modified by ingestion of egg yolk with egg white. So far, we find that mice do not develop food allergy to EW if they

ingest EW + EYP without additional priming. This differs from our observations that use purified saturated fats, such as MCT, instead of EYP and may reflect the much lower amount of saturated fat in EYP than in the purified product.

Aim 4, Tasks 1 and 2: These tasks have not yet been completed. This is because our observations with mixtures of EW + EYP indicate that acidification/partial digestion is not necessary for airway sensitization to prime for food allergy. There is still an issue of how much EW + EYP is required to sensitize for food allergy development. This study is planned; if a relatively large quantity (>10 µg) is required, we will still determine whether acidification and partial digestion will decrease the quantity required to sensitize for food allergy development.

Aim 5: We inoculated mice i.t. with both crude birch pollen and a commercial birch pollen extract. Neither stimulated airway hyperresponsiveness to methacholine or sensitized mice to develop food allergy to celery or apple. This suggests that either the BALB/c mouse is not an appropriate species to use to model this or that stimuli in addition to airway inoculation with birch pollen are required.

Aim 6, Task 1: Mice were induced to develop allergic airway disease to egg white by airway inoculation with HDM + EW, followed by EW, as in Figure 1, or were inoculated i.t. with saline (negative control). Mice were then exposed to aerosolized EW or bovine serum albumin (BSA, negative control) 3x/week for 4 weeks, with the expectation that the relevant antigen (EW) might suppress airway hyperresponsiveness more than the irrelevant antigen (BSA). Instead, if anything, the EW aerosol acted to maintain airway responsiveness (Figure 6). This suggests that a low dose of an aerosolized

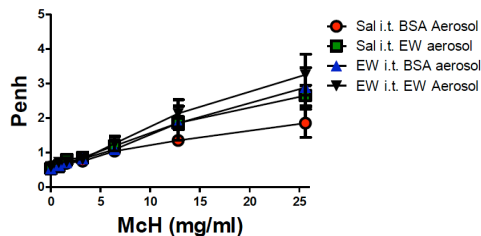


Figure 6. Inhalation of aerosolized EW fails to specifically suppress airway hyperresponsiveness in EW immunized mice. Mice were immunized i.t. to EW as in Figure 1, or were inoculated i.t. with saline, then were exposed 3x/wk for 4 wk to aerosolized EW or BSA. After this, they were evaluated for responsiveness to aerosolized methacholine.

antigen is not effective at suppressing established allergic airway disease to that antigen.

Aim 6, Task 2: This was not performed because of the negative results of Task 1.

Aim 6, Task 3: All of the mAbs have been prepared.

Aim 6, Task 4: The planned study cannot be performed because aerosolized EW failed to induce tolerance in EW-immune mice. Instead, with the permission of the Reviewers, we would like to follow up on the most exciting results of our study, those of Aim 1, Task 3, and determine the mechanism by which EYP promotes allergic airway disease and food allergy to EW; specifically, does it act as an additional antigen or as an adjuvant and, if it acts as an adjuvant by stimulating pro-Th2 cytokine production, how does it do this.

Opportunities for training and professional development: One post-doctoral fellow, Durga Krishnamurthy, was hired under this contract. I have met with her at least weekly to discuss results and plan additional experiments throughout the period of this

contract, with the exception of her annual leave time and the 4 months following her complicated delivery.

Dissemination of results: Abstracts are being presented at the American Association of Immunology Annual meeting this year by Dr. Marat Khodoun and Dr. Unni Samavedam that describe our results. The former has been chosen for both oral and poster presentation; the latter for poster presentation. The latter has received an award for exceptional merit.

Plans for the next reporting period:

1. Determine whether induction of allergic airway disease by HDM + EW primes for EW food allergy.
2. Determine dose of EW + EYP required to induce allergic airway disease and sensitize for development of food allergy. If $> 10 \mu\text{g}$ (total dose), determine if dose requirement decreases with acidification and partial digestion.
3. Compare the abilities of ingested EW vs. EW + EYP to prevent the development of allergic airway disease in response to i.t. EW or EW + EYP.
4. Determine whether IL-25, IL-33 and TSLP are required for induction and/or maintenance of allergic airway disease and food allergy when these are induced by inhalation of EW + EYP, followed by oral gavage with EW + EYP.
5. With permission, determine whether EYP acts primarily as an antigen or an adjuvant and, if the latter, how it induced epithelial cell expression of pro-Th2 cytokine genes.

IMPACT:

Impact on the principal discipline: The principal impact was 3-fold:

1. We demonstrated that inhalation of egg white plus egg yolk can sensitize to allow the development of food allergy in response to ingested EW + EY. This demonstrates an alternative pathway, aside from skin sensitization, that can allow the development of egg allergy, one of the most common food allergies.
2. We demonstrated synergy between egg white and egg yolk in the induction of allergic airway disease and food allergy. This suggests that the two component of eggs have distinct roles in the induction of egg allergy and that the use of egg white as a nutrient, without egg yolk (or with an unsaturated fat substituting for egg yolk) might prevent the development of egg allergy.
3. We demonstrated that egg yolk, but not egg white, induces lung and skin epithelial cells to express three cytokines (hormones of the immune system) that promote the development of food allergy. We showed that all three of these cytokines are required to induce food allergy in our model, while any one will maintain established food allergy. This suggests that an approach that neutralizes all 3 of these cytokines or inhibits their production may be necessary to suppress established food allergy.

Impact on other disciplines: The discovery that egg yolk plasma, and other saturated fats, may act as Th2 adjuvants may provide a relatively safe and useful adjuvant to use for vaccination.

Impact on technology transfer: Nothing to report

Impact on society beyond science and technology: Nothing to report

CHANGES/PROBLEMS:

Changes in Approach: Nothing to report

Delays: A significant delay has been caused by pregnancy/delivery-related problems, followed by child-care problems, of the post-doctoral fellow who is doing a great deal of the work on this project. She is now back at work full-time and I am hopeful that we will be able to catch up.

Changes in expenditures: Nothing to report

Changes in human subjects, vertebrate animals, biohazards/select agents: Nothing to report.

PRODUCTS:**Publications:**

1. Marat Khodoun, Durga Krishnamurthy, Richard Strait, Joel Tocker, and Fred D. Finkelman. Suppression of established food allergy by a combination of anti-TSLP, anti-IL-33, and anti-IL-25 monoclonal antibodies. (Abstract). *J. Immunol.* In press.
2. Unni K. Samavedam, Marat Khodoun M, David Wu, Simon P .Hogan, and Fred D. Finkelman. Saturated fatty acids promote allergic (Th2) cytokine responses by activation of unfolded protein response (UPR) and ER stress. Abstract). *J. Immunol.* In press.

Other products: None

PARTICIPANTS AND OTHER COLLABORATING ORGANIZATIONS:

Fred D. Finkelman: No change
Marat Khodoun: No change
Durga Krishnamurthy: No change
Charles Perkins: No change
Crystal Potter: No change