Progress Report Summary

A. Specific Aims

The specific aims are unchanged from the competing application. They are:

B. Studies and Results

In this year, we have continued to characterize the functional and transcriptional phenotype of human myeloid dendritic cells (mDCs) that are activated by peanut and other allergens. The functional assessment has been by co-culture with autologous naive CD4 T cells. Figure 1. shows that mDCs activated by peanut allergen are capable of providing co-stimulatory signals to naive T cells to induce proliferation. At the low concentrations (0.1 - 1 pg/ml) of the superantigen, staphylococcal enterotoxin B (SEB), used in these experiments, unstimulated DCs (treated with human albumin as a negative control) are incompetent for supporting T cell proliferation (top left panel), while mDCs stimulated with cholera toxin or LPS (bottom left and right, respectively) are competent. Peanut-stimulated DCs (top right) do induce naive T cell proliferation.

In addition, as we have shown previously using monocyte-derived DCs, directly isolated peripheral mDCs that have been activated with peanut allergen, induce Th2 differentiation of naive T cells (Figure 2).

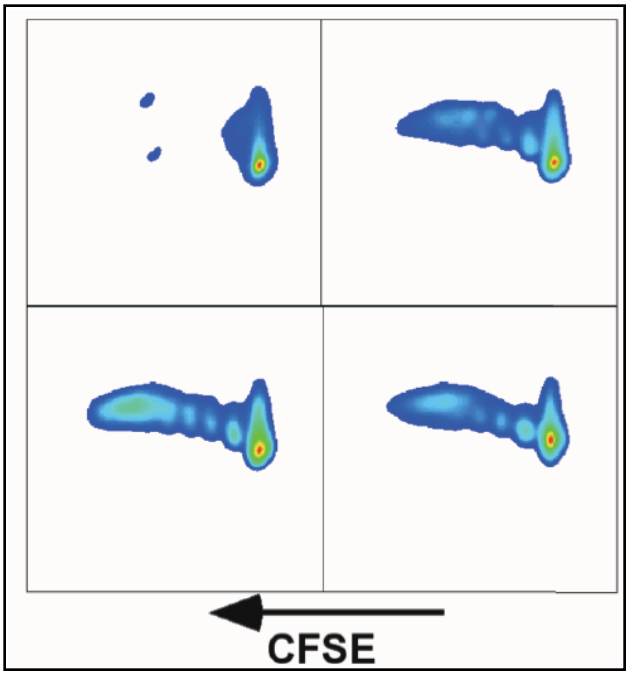

Figure 1. Proliferation of naive CD4 T cells induced by low dose super-antigen is dependent upon DC activation. Human myeloid DCs were isolated and incubated for 6 hours with medium alone (top left), peanut extract (top right), cholera toxin (bottom left), or LPS (bottom right) and then CFSE-labeled autologous CD4 T cells were added to the wells with 0.1 pg/ml SEB.

Expression profiling was not proposed in the competing proposal for this project, however, given that we have identified only modest changes in candidate pathways (Notch ligands, OX40L, CCL22, -- reviewed last year) downstream of peanut and Ara h 1 stimulation despite consistent functional effects on DCs stimulation of naive T cells, we reasoned that characterizing transcriptional changes in a less directed way, might inform new hypotheses.

In order to reduce the rate of false discovery, we pooled RNA from multiple DC stimulation experiments -- all from unique donors -- for use in microarray hybridization and then confirmed by quantitative RT-PCR the results on an individual donor basis. We first performed a pilot experiment comparing unstimulated mDCs to those stimulated with peanut allergen for 8 or 24 hours. Transcriptional changes observed in this pilot experiment can be viewed at http://openwetware.org/wiki/Shreffler:Notebook/Allergen_Adjuvant.

Based on the pilot data, we conducted a more extensive expression profiling experiment including several additional allergens (Ara h1, Ara h2, cow's milk, soy, *D. farinae*, cockroach, Birch pollen and cat dander) and controls (cholera toxin, LPS, human albumin) along with peanut extract. Plasma from anonymous donors was screened to identify those with low total IgE (<150 U/ml) and the absence of allergen-specific IgE to any of the allergens used in the experiment. Seven

donor mDCs of a consecutive 12 samples, met these criteria; these were stimulated for 24 hours and RNA was pooled for subsequent microarray hybridization without prior amplification using the Affymetrix HGU133 plus 2.0 chips. Additional RNA from each subject was also preserved for subsequent confirmation by RT-PCR.

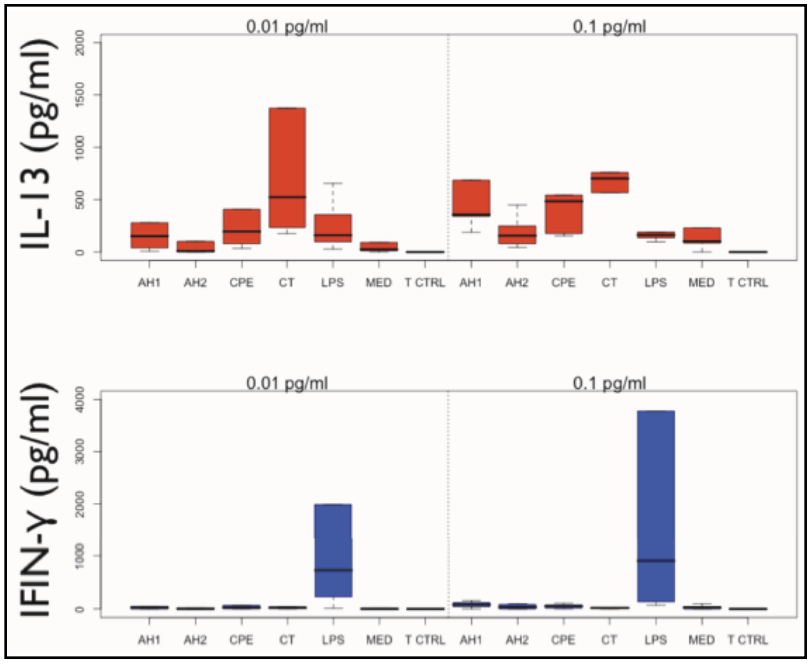

Figure 2. Cholera toxin, peanut extract and Ara h1 treated DCs induce Th2 differentiation of naive T cells.

Peanut allergen reproducibly induced transcriptional changes, while control human albumin did not (Figure 3). There was a very high degree of agreement between the pilot and subsequent experiment, as well as by qPCR (not shown).

Peanut allergen induced unique transcriptional changes in dendritic cells that were not shared with LPS or cholera toxin (Figure 4). We did not find evidence of pathways that were upregulated in common by allergens.

Because retinoic acid has recently been shown to be a potent regulator of naive T cell differentiation, including the promotion of gut-homing and Th2 or regulatory T cell phenotypes, we have decided to further characterize the ALDH1A2-inducing activity of peanut allergen. This gene expresses a retinal dehydrogenase, which has been shown to be the rate-limiting and inducible enzyme in the conversion of vitamin A to retinoic acid (RA). Recently, human basophils activated by IL-3 have been shown to induce the same gene, and by synthesis of RA, induce Th2 cell differentiation. Consistent with our findings (Figure 5), the same paper reported that LPS and a variety of other stimulants did not induce ALDH1A2 in human DCs. Murine DCs in the gastrointestinal-associated lymphoid tissue express this gene and produce RA, thereby promoting the differentiation of gut-homing T cells.

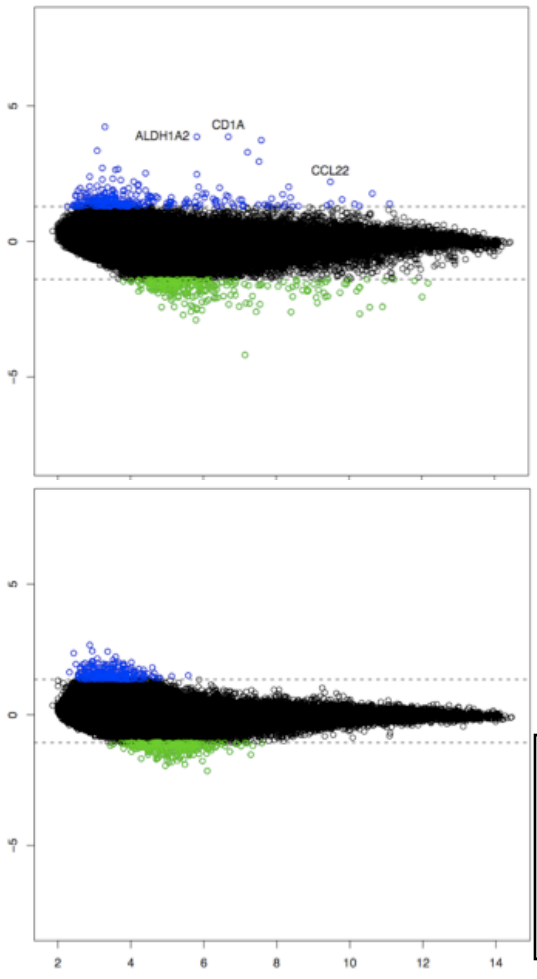

Figure 3. Peanut allergen induces transcriptional changes in human mDCs. The ratio of gene expression following stimulation with either peanut (top) or human albumin (bottom) to medium alone was plotted against the baseline expression. Broken lines mark the upper and lower 0.5%. Examples of peanut allergen-induced genes are labeled: ALDH1A2 (gene ID: 8854); CD1A (909); CCL22 (6367)

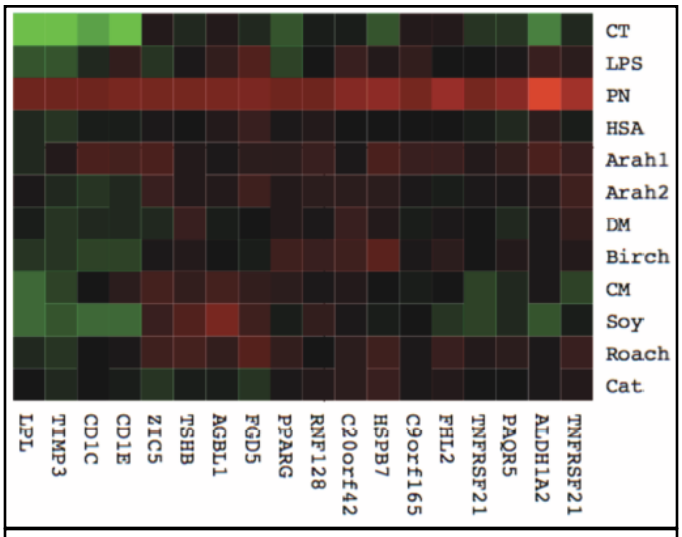

Figure 4. Example of genes uniquely upregulated (indicated by red color) by peanut allergen (PN).

Induction of ALDH1A2 is 4-5 times stronger when DCs are stimulated with plate-bound peanut allergen (not shown), suggesting a receptor-mediated mechanism and the activity has been isolated to an HPLC fraction of peanut extract (not shown) that is currently being analyzed for identification of candidate ligands. Notably, this activity is distinct from both Ara h 1 and Ara h 2.

We have also shown that retinal dehydrogenase is induced on the protein level by peanut allergen, by a flow cytometry based assay detecting the conversion of a fluorescent substrate of the enzyme (Aldefluor, Molecular

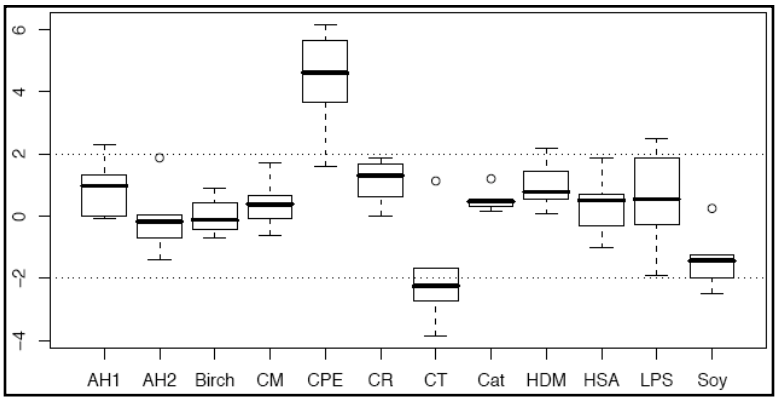

Figure 5. Quantitative RT-PCR data for fold expression of ALDH1A2 following stimulation with relative to unstimulated (log_2 scale). Sample size is 10 donors. AH1, AH2 -- Ara h 1 and 2; CM -- cow's milk; CPE -- crude peanut extract (i.e. peanut allergen); CR -- cockroach; CT -- cholera toxin; HDM -- dust mite; HSA -- human albumin. Horizontal lines mark four-fold expression change.

Probes; data not shown).

C. Significance

Allergy to peanut is persistent and severe and is increasing in prevalence. The capacity of peanut allergen to directly activate dendritic cells is one mechanism that may help explain why peanut is associated with strong immune responses and that understanding may lead to novel interventions.

The finding that a component of peanut allergen specifically and uniquely induces a pathway (retinoic acid synthesis) that has been implicated in the differentiation of gut-homing T cells that have either a Th2 or regulatory phenotype is particularly significant in this context.

D. Plans

Aim 1. We plan to use the transcriptional induction of ALDH1A2 to purify the active component of peanut allergen. We have already isolated a single HPLC fraction, containing a prominent ~38 kD candidate protein ligand. We are also testing whether peanut activated DCs are capable of producing RA that can then influence naive T cell differentiation in trans in two ways: using the DC/ T cell co-culture system with and without allergen activation with the addition of exogenous ligands of ALDH1A2 (retinal) in comparison to RA, and co-culture of DCs with transfected TCR+ Jurkat cells containing a RA response element (RARE) driving luciferase expression.

We will also perform RA receptor (RAR) and ALDH1A2 blocking experiments using the DC/ T cell co-culture to determine whether Th2 differentiation is dependent upon or enhanced by RA production.

Aim 2. The priority for Aim 2 remains to determine whether Arah1-indcued and peanut-induced DC transcriptional responses are dependent on DC-SIGN. We have performed antibody blocking experiments, not discussed above due to space contraints, in immature BDCA1+ mDCs directly isolated from peripheral blood with positive results; however, we are still trying siRNA-mediated knockdown experiments in monocyte-derived DCs to further test this hypothesis. We will also now explore comparative transcriptional changes induced by Ara h 1 and peanut allergen to recently deposited microarray data for DCs stimulated with anti-DC-SIGN.

Aim 3. Preliminary in vitro data supports a role for Arah1 as an adjuvant in the murine system. We will continue with the proposed studies in vivo using naive DO11.10 cells transfered to syngeneic Balb/c recipients followed by s.c. and i.p. immunization with native purified Ara h 1.

E. Publications

There have been no publications in this past year directly related to this project. There is a manuscript in progress describing the data briefly summarized above. I have had nine papers published in the last grant period, two of which were primary reports of which I was the first or senior author.

F. Project-Generated Resources

As reported last year, we have generated several novel DC-SIGN constructs and stable cell lines, which are available upon request. We have also now generated a significant expression profiling data set, which will be deposited to the public database.

G. Research Development

My research development has been further advanced in the second year funding period. I have received a competitive score (12th percentile) on an A1 R01 application to NIAID and am preparing to submit an R01 (A0) application on this peanut adjuvant project for the third cycle this fall. I presented an abstract at the 2009 Joint Keystone Symposium on Dendritic Cells and Pattern Recognition Molecules and also presented or mentored abstracts and invited talks at the 2009 AAAAI annual meeting.

H. Other Activities

I have continued to give 20% effort in clinical and other academic activities. I was elected to Fellow status at the AAAAI. I attend 1-1.5 sessions per week in the faculty practice in the Division of Pediatric Allergy. I was a group teacher for the first-year medical student immunology course. I continue to teach the Allergy/ Immunology fellows formally and informally and mentor students and a post-doc in the lab. I serve 4 weeks/ year on the Allergy consult service.

I. Research Development and Activities Planned for the Next Year

The focus of my career and research development for the coming year will be on submitting a competitive R01 application to provide ongoing support for this project. I have also submitted together with my post-doctoral fellow, Dr. Bert Ruiter, a grant application on comparative DC responses to allergen between atopic and non-atopic individuals.

J. Mentor's Report (Sampson)

Dr. Shreffler has made substantial progress towards a successful independent research career in the field of allergy/ immunology during this first year of K08 funding. With continued protected time, I anticipate Dr. Shreffler continuing to make outstanding progress on his project. The Department of Pediatrics and the Division of Allergy and Immunology fully support this development and are committed to protecting required time for research and career development activities.

Hugh A. Sampson, M.D.
Professor of Pediatrics
Dean for Translational Research