Protocol Title: Dietary Intervention in Milk Allergy and Tolerance Development

I. ABSTRACT

This 5-year prospective, randomized study will determine whether milk allergic children who tolerate low, medium or high doses of baked-milk and are subjected to frequent attempts at dose-escalation are more likely than children not subjected to frequent dose-escalation to achieve increases in their tolerated dose of milk at 36 months. This study will also attempt to delineate clinical phenotypes of milk-allergic patients based upon their response to various forms of milk proteins that vary in dose, extent of heat-denaturation and abundance of casein or whey proteins; to identify biomarkers differentiating the various subgroups; to elucidate immunologic changes that accompany acquisition of tolerance; and to determine at study end (36 months) whether participants in the heated milk dosing regimens are more likely (20% or greater difference in outcome) than non-participating comparison children to tolerate unheated milk.

II. BACKGROUND & SIGNIFICANCE

Milk is among the most common food allergens in infants and children. The majority of children outgrow their allergies with time, indeed, 80-85% of milk-allergic children become clinically tolerant to milk by 4 years of age, but the mechanisms by which food tolerance is achieved are unknown. Some milk allergic people are able to tolerate small amounts of milk in cooked, but not in the unprocessed form. During food processing, the native structure and conformational epitopes may be altered by heat and/or chemical treatments, eliminating IgE-binding conformational epitopes. A study by Sampson et al suggested that egg-allergic patients who developed significant amounts of IgE antibodies to "sequential" linear epitopes of ovomucoid (the major allergen in egg) were more likely to have persistent egg-allergy at an older age whereas the patients who predominantly developed IgE to "conformational epitopes" were more likely to outgrow their egg hypersensitivity. Furthermore, in the same study it was suggested that up to 50% of egg-allergic children may tolerate small amounts of extensively cooked egg products. Several milk proteins lack a fixed tertiary structure. Nevertheless, it was recently demonstrated that children with persistent IgE-medicated milk allergy recognized different αs1, αs2, κ-casein epitopes than the children who were likely to achieve clinical tolerance. Even though strict avoidance of the offending food is the current standard of care, there is no conclusive evidence that absolute dietary restriction is necessary for achieving clinical tolerance in all food-allergic children. Considering the imperfect compliance with strict food avoidance and the impact of dietary restriction on the life-style of the entire family of the food allergic person, we would like to determine whether milk allergic children who tolerate low, medium or high doses of baked-milk and are subjected to frequent attempts at dose-escalation are more likely than children not subjected to frequent dose-escalation to achieve increases in their tolerated dose at 36 months.

III. GOALS & METHODS (include sample size calculation, power analysis and statistician who helped develop plan)

The primary endpoint will be to determine if milk allergic children who tolerate low, medium or high doses of baked-milk and are subjected to frequent (every 6 months, "dose-escalation group")

attempts at dose-escalation are more likely than children not subjected to frequent dose-escalation (yearly attempt, "maintenance group") to achieve (75% dose-escalation group vs. 40% maintenance group) increases in their tolerated dose at 36 months. Secondary endpoints include:

1) To delineate clinical phenotypes of milk-allergic patients based upon their response to various forms of milk proteins that vary in dose, extent of heat-denaturation and abundance of casein or whey proteins.
2) To identify biomarkers differentiating the various subgroups.
3) To elucidate immunologic changes that accompany acquisition of tolerance including frequency of milk-specific regulatory T cells [Tregs], frequency of milk-specific Th2 cells, basophil reactivity to milk allergens, and qualitative differences in milk-specific antibodies such as the ratio of IgE to IgG and clonal diversity
4) To determine at study end (36 months) whether participants in the heated milk escalating dosing regimens are more likely (20% or greater difference in outcome) than non-participating comparison children to tolerate unheated milk.

We will enroll subjects between the ages of 4 and 10 years who have been diagnosed with milk allergy by either a positive prick skin test to milk and or/detectable serum milk-IgE and history of convincing reaction to milk within past 2 years OR serum milk-IgE of high predictive value >15 kIU/L or prick skin test to milk > 8mm wheal.

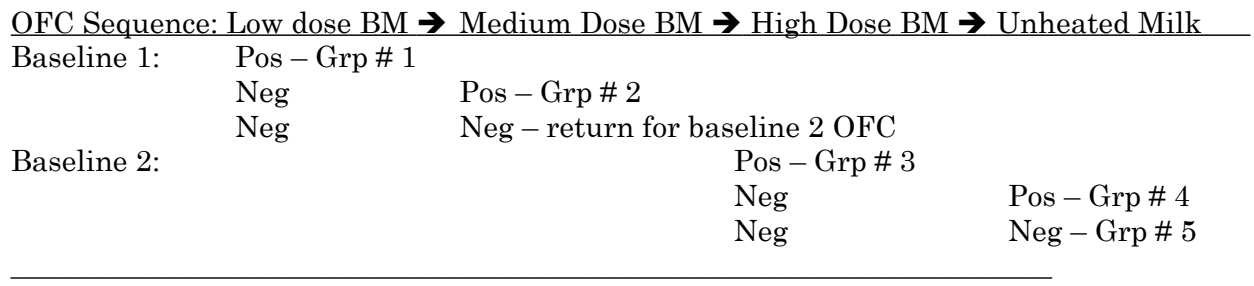
The sample size target is 180 individuals, with 60 subjects self-selected into the Comparison Arm and 120 subjects self selected into the Active Arm. Subjects within the Active Arm will be divided randomly and evenly into the Maintenance Arm (60 subjects) or Dose-Escalation Arm (60 subjects). Subjects enrolled into the comparison arm will be age-, gender- and milk IgE antibody serum level-matched with the subjects enrolled into the active arm. Subjects in the Maintenance and Dose-Escalation Arm will be stratified by age, gender and milk-specific IgE antibody serum level. There is 1 study site.

The active arm of the trial will involve evaluation with oral food challenges [OFCs] to different forms of heat-denatured [baked] cow's milk protein and dietary modifications based on the outcomes of the OFCs. The comparison arm will involve children whose parents wish to continue strict avoidance of dietary milk protein but are willing to allow us to draw additional blood for mechanistic studies at 0 [baseline], 12, 24, and 36 months [along with routine, clinically indicated blood testing]. As described below, one-half of those children enrolled into the active arm, tolerating at least the low dose baked product, will be randomly assigned to the "dose-escalation" group and one-half to the "maintenance" group. Given the assumptions listed in the statistical section, 40 subjects per group will provide 80% power to detect differences between the two groups.
As noted in the Progress Report, milk-allergic children appear to tolerate different quantities and types of extensively heated [baked] milk proteins. In the proposed study protocol, subjects in the active arm will be challenged initially to a series of baked-milk products containing increasing quantities of milk proteins. Children tolerant to the high dose baked-milk who have milk-specific IgE and skin test results less than 95% predictive levels [i.e. milk-specific IgE < 15 kUA/L and mean wheal diameter < 8 mm, respectively] will also be challenged to 10 g of unheated [non-denatured] whole milk protein. Based upon the outcome of their baseline challenges, children will be classified in 5 groups: Group # 1 – do not tolerate milk in any form; Group # 2 – tolerate low dose baked-milk;

Group # 3 – tolerate low dose and medium dose baked-milk; Group # 4 – tolerate low, medium and high dose baked-milk but not regular unheated milk; and Group #5 – tolerate all forms of milk, i.e. "outgrown" milk allergy. Group # 1 will be instructed to continue strict avoidance of all forms of milk and to return for a repeat evaluation at 12 months. Group # 5 will be instructed to add regular milk/dairy products into the diet and will be discharged from the study. Groups # 2, 3, and 4 will be instructed to add heated milk into diet [at least 1 serving twice weekly] based upon the dose tolerated during a baseline OFC.

Subjects in Groups # 2, 3 and 4 will be randomly assigned to one of two cohorts: "dose-escalation" group consisting of subjects who will undergo graded challenges every 6 months or the "maintenance" group consisting of subjects who will undergo graded challenges yearly. During the follow up challenges, higher doses of baked-milk will be tested, starting from the dose that the subject reacted to at the previous visit, and continuing until the dose that elicits a reaction. Based upon the outcome of the follow up OFC, the subjects will be instructed to either continue their current diet [e.g. in case of a repeat failed OFC to the same dose of milk] or to expand their diet by adding other milk products. [Figure 1] For example, in a "dose-escalation" group, if a subject reacts to medium dose baked- milk during the baseline challenge, s/he will return in 6 months for another medium dose baked- milk challenge. If s/he fails the medium dose baked-milk challenge again, s/he will continue to eat low dose baked-milk for another 6 months whereas if s/he passes this challenge, s/he will be challenged to high dose baked-milk. If s/he fails the high dose baked-milk, s/he will be sent home and consume medium dose baked-milk products for 6 months before returning for another challenge to the high dose baked-milk. If s/he passes the high dose s/he will be sent home and consume high dose baked-milk products and will return in 6 months for a regular unheated milk challenge. In a "maintenance" group repeat OFC according to the same principles as outlined for a "dose-escalation" group will be done every 12 months.

Figure 1. Oral food challenge sequence in the active arm during the baseline evaluation



The study design is presented in the schematic:

Figure 2. Design of the clinical trial of baked-milk diet

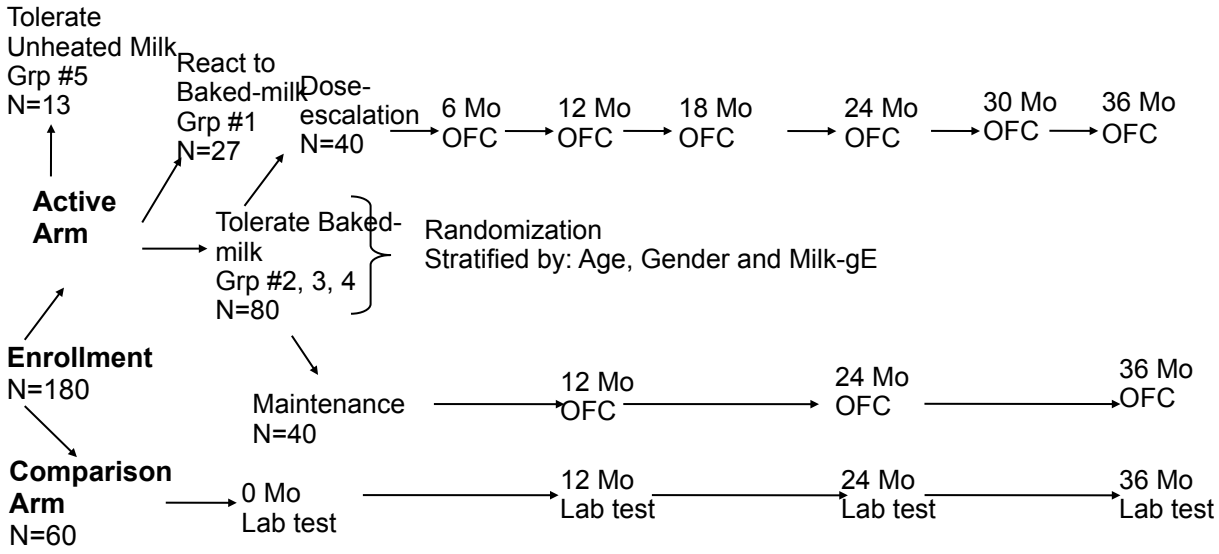
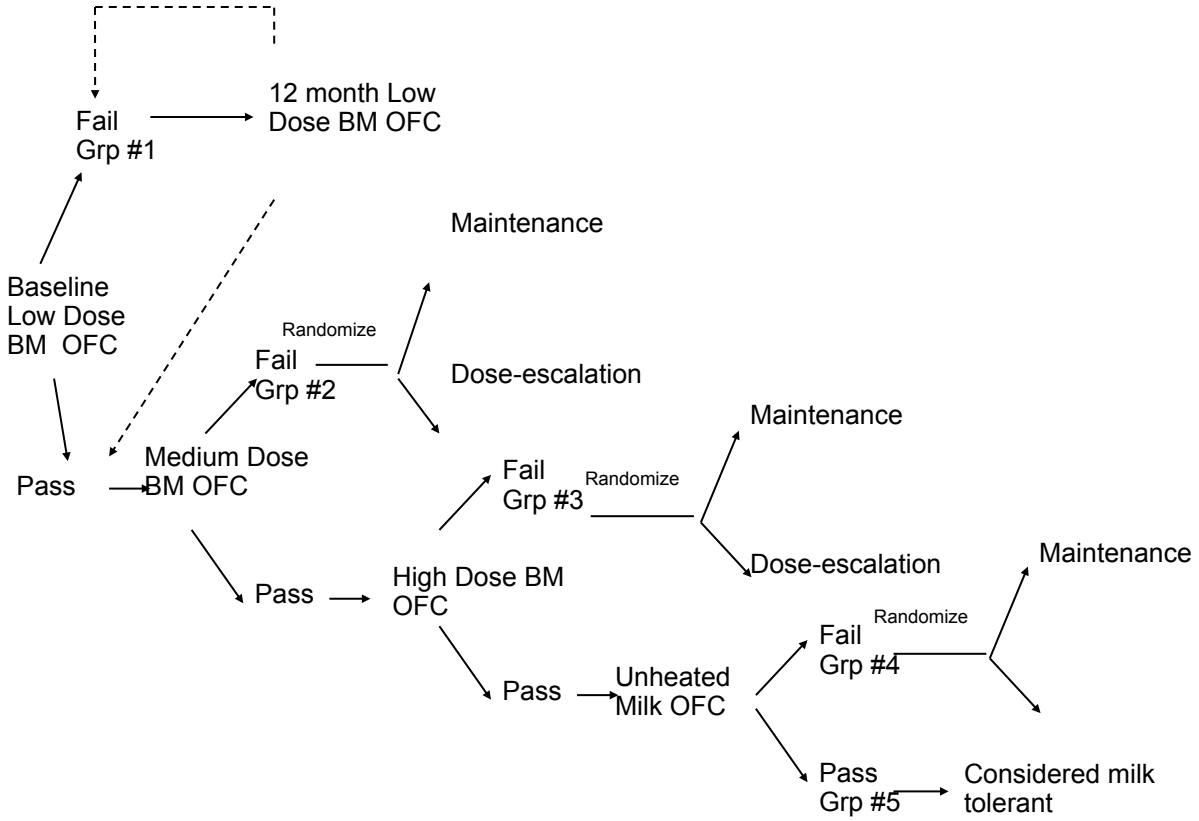


Figure 3. Scheme of randomization of subjects enrolled into the active arm of the study



Study Visit Schedule and Primary Evaluations, Including Laboratory Evaluations

I. Active Arm:

Baseline Visits: At the first baseline visit, the informed consent will be obtained, children will be prick skin tested and blood will be obtained for mechanistic studies. Stool sample will be obtained for measurement of fecal IgE/CD23. Weight and height will be measured and plotted on a growth chart. Food allergy quality of life questionnaire will be obtained. Children will undergo up to 2 food challenges during the day on the GCRC. The first challenge will take place in the morning and consist of low dose baked-milk product [e.g. muffin: 3.1 g baked-milk protein] given in a graded fashion every 15 minutes: 1 or 2 portions of a placebo food dependent upon the anxiety of the child, followed by 10%, 20%, 30% and 40% of the milk-containing food. If the child fails the low dose challenge [Group #1], i.e. develops objective allergic symptoms, s/he will be treated appropriately and will return in one year for a re-challenge to low dose milk. In the uncommon event of ambiguous food challenge outcomes (e.g., subjective symptoms) the food challenge format will be switched to a double-blind, placebo-controlled format. If the low dose is tolerated, the child will undergo a second challenge in the afternoon [at least 3 hrs following the completion of the first challenge] consisting of medium dose casein [e.g. cheese pizza: 4.5 g baked-milk protein] given in a graded fashion every 15 minutes: 5%, 10%, 25%, 30% and 30% [the dosing regimen is modified due to a higher total dose of milk administered]. If the child fails the medium dose challenge [Group # 2], s/he will be treated appropriately and randomly assigned to be in the "maintenance" or "dose-escalating" cohort. Subjects in Group # 2 will continue to eat low dose heated milk products at home until the next challenge visit. If the child passes the medium dose challenge, s/he will be discharged and return for a second set of baseline challenges within the following 2 – 3 week period. During this interim, the child will remain on a milk elimination diet. At the second baseline visit, the child will undergo a high dose milk challenge [e.g., rice pudding: 7.7 g baked-milk protein]; given in a graded fashion every 15 minutes: 5%, 10%, 25%, 30% and 30% [the dosing regimen is modified due to a higher total dose of milk administered]. If the child fails the high dose challenge [Group # 3], s/he will be treated appropriately and randomly assigned to be in the "maintenance" or "dose-escalation" cohort. Subjects in Group # 3 will continue to eat low and medium dose heated milk products at home until the next challenge visit. If the child passes the high dose milk challenge, s/he will be challenged 2 hours later to unheated milk protein. The challenge would consist of a single blind drink [e.g., 2 oz of chocolate soy milk + 6 oz regular 2% milk fortified with non-fat dry milk – 10 g milk protein] given in a graded fashion every 15 minutes: 5%, 10%, 15%, 20%, 25% and 25%. If the child fails the milk challenge [Group # 4], s/he will be treated appropriately and randomly assigned to be in the "maintenance" or "dose-escalation" cohort. Subjects in Group # 4 will continue to eat low, medium and high dose heated milk products at home until the next challenge visit. Children who tolerate the milk challenge [Group # 5] will be advised to add all milk products back to their diet, will be considered milk tolerant and discharged from the study. Following their baseline challenges, subjects in Groups # 2, 3, and 4 will be randomly assigned to the "dose-escalation" or "maintenance" cohorts. [Figure 3] Detailed dietary instructions will be provided for introduction of baked-milk products into diet at home, recommending at least one serving of baked-milk product twice weekly. Parents of the children will be required to notify study personnel about any unusual reactions/ concerns about worsening of atopic diseases [asthma, atopic dermatitis, allergic rhinitis].

Follow up visits: Subjects in the "maintenance" cohort will return on a yearly basis [12, 24, and 36 months] to restart the challenge sequence with the baked-milk product eliciting symptoms at the

previous challenge visit whereas the "dose-escalating" cohort will return every 6 months [6, 12, 18, 24, 30, and 36 months] to restart the challenge sequence. If they pass that challenge, they will be challenged to the next food in sequence until symptoms develop. [Figures 1 and 2] During each follow up visit, prick skin test, blood and stool sample for mechanistic studies will be obtained. Anthropometric measurements will be repeated. Food allergy quality of life questionnaire will be obtained. [Table 1]

II. Comparison Arm: Children fulfilling the eligibility criteria will be approached about participation in the trial. If parents of children prefer to continue strict dietary avoidance of milk protein, participation in comparison arm will be discussed. Following obtaining of a signed informed consent, a quality of life questionnaire will be administered and blood sample will be obtained for mechanistic studies. Children will return at yearly [12, 24 and 36 months] as indicated clinically for follow up of food allergy; during these visits, a quality of life questionnaire will be administered and an additional blood ample will be obtained for study tests [humoral, T cell and basophil]. [Table 1] Children will be considered persistently milk-allergic if they continue dietary milk avoidance and will be considered milk-tolerant if they successfully reintroduce milk protein/dairy into their diets. The parents of these children will be asked to call the investigators as soon as the child reintroduces milk into the diet.

Mechanistic studies will address the following hypotheses (See protocol for full explanation of methods, rationale, and analyses):

- Hypothesis 1: The development of clinical tolerance to milk is associated with an increase in the T regulatory phenotype of antigen-activated peripheral CD4+ T cells.

- Hypothesis 2: The development of clinical tolerance to milk is associated with the down-regulation of mast cells and basophils.

- Hypothesis 3: The persistence of milk allergy is associated with an epitope-specific IgE antibody profile that is distinct from persons who lose their clinical allergy to milk.

- Hypothesis 4: The persistence of clinical milk allergy is associated with the maintenance of a milk-specific, Th2- skewed T cell phenotype.

Statistical Analysis: In this project, subjects will be characterized at baseline as being in one of the five following ordinal categories: [1] tolerate no milk products, [2] tolerate low dose baked-milk, e.g. muffin, [3] tolerate medium dose baked-milk, e.g. pizza, [4] tolerate high dose baked-milk, e.g. rice pudding, [5] tolerate all milk products and milk. After the baseline assessment, subjects will be randomized to receive either a maintenance diet or a dose-escalation diet. Enrollment will occur over 18 months, and each subject will be followed for three years after enrollment. At each follow-up visit, as well as at the end of the three-year follow-up period, each subject will be classified on their degree of milk tolerance using the five ordered categories listed above.

The outcome variable is an ordered categorical variable, and it will be observed longitudinally on each subject from baseline until the end of the trial. The longitudinal data on each subject will be correlated, but the data from one subject to another will be independent. The model used for

analysis will be a logistic regression model with ordinal categorical outcomes. However, the longitudinal repeated observations taken on each subject require that the correlations in the data be properly modeled. All of these features are available in the SAS procedure PROC GLIMMIX. The independent variables in the longitudinal logistic regression model will be treatment group and baseline level of the milk tolerance variable. In particular, the interest will be on the population-averaged [marginal] model to compare the overall effect of the maintenance diet and the dose-escalation diet.

Every effort will be made to collect complete data from each enrolled subject. However, it is recognized that in a longitudinal study over three years there will inevitably be missing data points. The strategy for dealing with missing data in the statistical analysis will be to use direct likelihood maximization [Beunckens C, et al, 2005]. This method does not depend on data deletion [such as complete case analysis] or on imputation [such as last observation carried forward]. In this particular trial, the primary outcome variable is the categorical variable of degree of milk tolerance. In such a situation, the direct likelihood method is implemented using PROC GLIMMIX in SAS to estimate the mean profile from the generalized linear mixed model for observed longitudinal data. [Beunckens C, et al, 2005; Lachin JM, 1977]

IV. PROGRESS REPORT/PRELIMINARY STUDIES

In our ongoing clinical trial 01-1209, eighty four children with suspected CMA were challenged to extensively heated cow's milk baked into muffins and waffles [3 g milk protein]. Preliminary results were presented at the AAAAI 2007 Meeting in San Diego, CA. [Bloom K, et al. recognition of Clinical Tolerance to Baked Milk Products in Milk-Allergic Children. J Allergy Clin Immunol, 2007, 119-S118] Children tolerant to baked-milk were subsequently challenged to regular unheated CM. Children who tolerated the muffin and waffle were advised to add comparable baked product to their diets, and were monitored with anthropometric measurements, serum milk-specific IgE and IgG4 levels, and prick skin test to cow's milk at each visit. Intestinal permeability was re-evaluated at 3 months. Nine (11%) study subjects tolerated both the baked and unheated (regular) milk challenge and were considered to have "outgrown" their milk allergy [clinical tolerance]. Nineteen (23%) children reacted to baked milk products and were placed on a strict milk-avoidance diet. Fifty-six (66%) children tolerated baked-milk; of those, 37 children had CMA confirmed with an unheated milk challenge and 19 were considered milk-allergic because of a recent reaction and/or milk-specific IgE levels and /or prick skin test mean wheal diameters >95% predictive of clinical reactivity. Characteristics of the different groups of CM-allergic children are presented in the Table 1. The 56 children tolerating the baked-milk challenges were instructed to add baked-milk products into their diets. Children returned for follow up at 3 months (data currently available for 43 children), 6 months (31 children), and 12 months (23 children). In an effort to retain study subjects, children tolerating muffins were challenged to less extensively heated cow's milk in the form of pizza [6 g casein] and / or macaroni and cheese [6 g casein; 2 g whey].

Table 1. Characteristics of cow's milk-allergic children at baseline

N=84	Outgrown N=19	P*	Heated cow's milk tolerant N=56	P**	Allergic to heated cow's milk N=19	P***

Age, years	7.3 (3-15)	0.9	7.6 (2.8-6.7)	0.4	8.5 (1.6-16.4)	0.9
CM PST wheal [mm]	6.0 (0.0-8.0)	0.007	8.0 (4.0-19.0)	0.2	9.5 (3.0-24.0)	0.014
CM-IgE [kUa/L]	0.8 (0.01-3.3)	0.2	2.6 (0.01-79.1)	0.02	6.8 (0.48-101)	0.03
Casein-IgE [kUa/L]	2.9 (0.48-56.0)	0.2	1.1 (0.01-101)	0.3	2.8 (0.42-207)	0.6
Casein-IgG4 [kUa/L]	1.4 (0.09-31)	0.1	0.6 (0.01-23.8)	0.3	0.8 (0.04-6.7)	0.5
Beta-lactoglobulin-IgE [kUa/L]	1.7 (0.01-13.0)	0.7	0.5 (0.01-63.7)	0.6	0.97 (0.01-171.0)	0.9
Beta-lactoglobulin-IgG4 [kUa/L]	1.3 (0.06-31)	0.02	0.3 (0.001-11.3)	<0.001	1.9 (0.08-7.4)	0.5
IP# (LacMan ratio)	0.023 (0.01-0.07)	0.7	0.023 (0.01-0.07)	0.07	0.018 (0.01-0.03)	0.5

Results expressed as median (range); #IP-intestinal permeability expressed as a urinary ratio of lactulose and mannitol; P value less than 0.05 was considered statistically significant; P* passed all versus passed baked; P** passed baked versus failed baked; P***passed all versus failed baked; calculated with Sigma Stat 2.03, T-test or Mann-Whitney U test.

The 56 children tolerating the baked-milk challenges were instructed to add baked-milk products into their diets. Children returned for follow up at 3 months (data currently available for 43 children), 6 months (31 children), and 12 months (23 children). In an effort to retain study subjects, children tolerating muffins were challenged to less extensively heated cow's milk in the form of pizza [6 g casein] and / or macaroni and cheese [6 g casein; 1 g whey].

Twenty one (80%) tolerated and 5 (20%) reacted to pizza, whereas 15 (71%) tolerated and 6 (39%) reacted to macaroni and cheese. At the 12 month follow-up, 15 children underwent an oral challenge to unheated, regular milk; 9 passed and 6 reacted. There were no significant changes in growth pattern or intestinal permeability in children ingesting baked-milk compared to normal controls. The comparisons of immunologic parameters at different time points in children ingesting baked CM are presented in the Table 2.

Table 2. Comparison of immunologic parameters and intestinal permeability (IP) in children ingesting baked cow's milk in their diet (baked [heated] cow's milk–tolerant children).

N=52*	Month 0	Month 3	P	Month 6	P	Month 12	P
CM PST wheal [mm]+	7.5 (4.0-19.0)	7.0 (2.0-10.0)	<0.001	7.0 (3.0-13.0)	0.04	6.2 (3.0-7.0)	<0.001
CM-IgE	2.6 (0.01-79.1)	1.7 (0.01-63.7)	0.35	0.9 (0.01-17.7)	0.021	1.3 (0.01-8.1)	0.027
Casein-IgE	1.1 (0.03-101)	ND	NA	ND	NA	ND	NA
Casein-IgG4	0.6 (0.01-23.8)	0.9 (0.05-14.7)	0.003	1.7 (0.01-4.4)	<0.001	0.8 (0.01-3.4)	0.15
Beta-lactoglobulin-IgE	0.5 (0.01-63.7)	ND	NA	ND	NA	ND	NA
Beta-lactoglobulin-IgG4	0.3 (0.01-31.0)	0.6 (0.01-31.0)	0.76	0.7 (0.01-4.4)	0.95	1.4 (0.01-4.9)	0.4
IP (LacMan ratio)	0.023 (0.01-0.07)	0.03 (0.01-0.06)	0.49	NA	NA	NA	NA

* Four children withdrew from the study following the baseline evaluation due to reported inconveniences; P values for comparisons between baseline (month 0) and follow up visits (month 3, 6, and 12); + data expressed as median (range); NA-not applicable; ND-not done; For the analysis of the UNICAP measurements of specific IgE and IgG4 antibody levels, a value of 0.01[kUa/L] was arbitrarily assigned for any undetectable level; P value less than 0.05 was considered statistically significant; calculated with Sigma Stat 2.03, T-test or Mann-Whitney U test.

Sera from 60 subjects enrolled were examined by peptide microarray analysis: 7 "outgrew" their milk allergy (tolerated a regular milk challenge), 15 had persistent milk allergy (failed extensively-heated [baked] milk challenge [muffin containing 3 g milk protein]) and 38 tolerated limited amounts of extensively heated milk, but not natural milk. IgE antibodies from subjects who outgrew their milk allergy tended to identify fewer milk peptides (for both IgE and IgG4) as compared to sera from those with persistent milk allergy. [Wang J et al. J Allergy Clin Immunol 2006] Individuals who were tolerant to extensively processed milk had binding patterns intermediate between those with transient and persistent milk allergy. With the limited number of subjects investigated to date,

we have not been able to identify specific milk epitopes that uniformly distinguish between the three groups. The IgE to IgG4 ratio was calculated for each milk peptide for each patient. Individuals with persistent milk allergy had significantly more peptides with elevated IgE/IgG4 ratios (ratio > 2) than those with transient milk allergy. The location of these peptides with high IgE/IgG4 ratios did not cluster in specific milk proteins nor did they correlate with specific epitopes previously identified using SPOTs membrane technology. Our peptide microarray immunoassay findings suggest that patients with persistent milk allergy bind to more milk peptides with higher IgE to IgG4 ratios of mean binding intensities as compared to those with transient milk allergy. These differences are not associated with serum milk-specific IgE concentrations or correlated with specific IgG4 levels to casein and beta-lactoglobulin. Additional modifications to the peptide microarray protocol are underway to further enhance the sensitivity, reproducibility, and efficiency of this assay.

We investigated the relationship between the frequency of casein-specific Tregs and the resolution of CMA in children enrolled in our baked-milk study. [Shreffler WG, et al. Onset of Clinical Tolerance to Milk Proteins is Associated with Increased CD25+CD27+ Casein-Specific T Cells. J Allergy Clin Immunol, 2007, 119-S160] Subjects were defined by strict clinical criteria as either “Control” (no history of milk allergy), “Outgrown” (previously allergic, but tolerated regular milk challenge), baked-cow’s milk product [HCM] tolerant (tolerated 3 g milk protein in baked muffin but reacted to regular milk, or “Allergic” (react to baked-milk in muffin). Serially obtained patient PBMCs were labeled with CFSE and cultured for 7 days with media alone, 50 μg/ml purified α-, β-, and κ-caseins, or anti-CD3/ -CD28 beads. Cultures were supplemented with IL-2 and expanded as necessary. The phenotype and proliferation were characterized by flow cytometry using mAbs for CD3, CD4, CD25 and CD27. Either propidium iodide or ethidium monoazide was added to better exclude dead cells. Casein-induced proliferation was restricted to CD4+ T cells and proliferating cells could be clearly phenotyped with respect to CD25 and CD27 expression.

The frequency of proliferating CD25+ CD27+ T cells from the PBMCs of children HCM tolerant, while variable, was significantly greater than that of other patient groups (Figure 2A). This difference was greatest for HCM group compared with either Allergic or Control subjects ($p<0.02$ for both), while in comparison to the group “outgrowing” their CMA, the difference was less ($p<0.05$). There was no apparent difference in Treg frequency between the other groups. This was not simply a reflection of overall differences in general casein-induced proliferation, since there was no significant difference between groups in CD4+ cell proliferation, except for the CD25+ CD27+ subset (Figure 2B). In addition, there was no correlation between the proliferation of CD25+ CD27+ T cells and non-CD25+ CD27+ T cells ($r=0.02$, $p=0.9$). No significant difference in the frequency of CD25+ CD27+ T cells proliferating to polyclonal stimulation was seen between the clinical groups (Figure 2C). Proliferation from IL-2 media alone [control] was negligible (less than 1% CFSElo CD3+ CD4+ CD25+ CD27+ of total CD3+ CD4+ for all subjects) and there were no differences between groups. While we had hypothesized that there would be a higher frequency of CD25+ CD27+ T cells in the children tolerating baked-milk compared to those reactive to baked-milk [Allergic group], we were surprised that there was no difference between Allergic and Control groups and that children who had outgrown their milk allergy had a frequency of casein-induced proliferating CD25+ CD27+ cells only slightly greater than Controls. Interestingly, 1 of 7 Controls, who was an outlier (>3 fold higher frequency of CD25+ CD27+ than any other in that group), was a child who did not eat milk-containing foods by parental preference. These results support the role of Tregs during the development of clinical tolerance to a major food allergen in humans.

We hypothesized that inclusion of dietary milk protein would lead to an increased frequency of casein-specific CD25+ CD27+ T cells in the peripheral blood. We compared subjects from the HCM tolerant group who were either excluding or including milk in their diet. Interestingly, the frequency of CD25+ CD27+ T cells was significantly greater in the HCM group at baseline, before inclusion of milk into their diet ($p<0.01$). The difference between the HCM tolerant and Allergic groups was more significant ($p<0.001$) when only subjects excluding milk were compared (7 of 15 HCM baked-milk tolerant and 7 of 7 milk allergic).

CD25+ CD27+ T cells have a Treg phenotype.

We sought to further characterize the phenotype of the CD25+ CD27+ cells from a subset of HCM subjects utilizing other reported markers of the CD4+ CD25+ Treg cells, including FoxP3 and genes that have been shown to be directly regulated by FoxP3 including CTLA-4, GITR, and CD127. With in vitro culture of PBMCs in the presence of IL-2, there was expansion of FoxP3+ CD25+ CD127- cells. Cells proliferating in response to casein were largely FoxP3 positive, however, a subset of these proliferating cells had higher levels of FoxP3 expression and this correlated with the highest CD25 and the lowest CD127 expression.

Proliferating CD25+ CD27+ T cells include the FoxP3 bright population and have higher overall expression of FoxP3 and CTLA-4 expression than proliferating CD25+ CD27- T cells. However, neither the CD25+ CD27+ nor the CD25+ CD127- phenotype is restricted to the CD25bright FoxP3bright subset and CD127 expression was low on all proliferating CD25+, not only the CD25+ CD27+ subset. We were unable to detect differences in expression of GITR.

We compared the responsiveness of patient's basophils to milk allergen as well as control stimulants. [Wanich N, et al. Utility of the Direct Basophil Activation Test in Predicting Tolerance to Dietary Milk Protein in Children with a History of Cow's Milk Allergy. J Allergy Clin Immunol, 2007, 119-S122] Patients who were HCM tolerant on challenge had significantly lower response to milk allergen than allergic patients ($p<0.001$ for Allergic vs. HCM tolerant). However, responses to anti-IgE and fMLP were not significantly different across groups. This finding was consistent with a trend for difference in skin testing between these groups. The median milk skin test wheal for this allergic group was 17 mm with a range of 5-24 versus 8 [3-13] for the HCM tolerant subjects. We hypothesized that the milk-specific hyporesponsiveness may be due to competition of specific IgG for antigen binding by sensitized basophils and to test this we compared basophil responsiveness to milk allergen or anti-IgE before and after washing the cells to remove plasma proteins. We found that 8 of 14 individuals had an enhanced basophil response (9.5 [9.15-13.2] versus 25.1 [22.4-32.2] median [25-75%]; $p<0.01$). There was no significant change in anti-IgE response.

Overall, this study demonstrated that the majority of children with CMA can safely ingest baked-milk products without experiencing allergic symptoms or having any adverse effects on gastrointestinal permeability or growth. Traditional allergy tests, i.e. prick skin test and milk-specific IgE levels, do not distinguish which children will tolerate baked-milk products and which will not. However, those children tolerating baked-milk products possess a greater frequency of milk-specific T regulatory cells cultured from their peripheral blood and decreased milk-induced activation of peripheral blood basophils compared to children unable to ingest any form of milk product. Preliminary data suggests that the children ingesting baked-milk products over time tend to have an increase in casein-specific IgG4 with no change in IgG4 response to whey proteins, and a decrease

in milk-specific T regulatory cell activity when tolerance to regular, unheated milk is achieved. While our current study was not designed to address this question, it appears that children tolerant to baked-milk and ingesting baked-milk products may be "outgrowing" their milk allergy more quickly than those children who can tolerate baked-milk products but elected not to add them to their diet. In summary, we believe that these findings will change the current paradigm of managing the vast majority of milk-allergic patients and dramatically simplify their lives and those of their families and care-givers.

V. FUTURE PLANS

The results of this study may provide a basis for approaching other food allergies (e.g., peanut, egg) using these techniques. Mechanistic studies may elucidate immunologic mechanisms of oral tolerance.

VI. HUMAN SUBJECTS

Subjects of Protocol

Children and adolescents ages 4 to 10 will be enrolled.

Inclusion Criteria

Subjects who meet *all* of the following criteria are eligible for enrollment as study participants:

- Age 4 to 10 years, either sex, any race, any ethnicity
- Either:
  - A positive prick skin test to milk and or/detectable serum milk-IgE and history of convincing reaction to milk within past 2 years OR
  - Serum milk-IgE of high predictive value >15 kIU/L or prick skin test to milk > 8mm wheal
- Written informed consent from parent/guardian
- All females of child-bearing age must be using appropriate birth control

Exclusion Criteria

Subjects who meet *any* of these criteria are *not* eligible for enrollment as study participants:

- Serum cow's milk-specific IgE antibody level > 35 kIU/L
- Recent (within the past 12 months) anaphylactic reaction to milk
- No evidence of IgE antibody to milk by prick skin test and/or RAST within 6 months of study enrollment.
- Unstable atopic disease such as asthma, atopic dermatitis, or allergic rhinitis within 7 days of the baseline visit.
- Allergic eosinophilic gastroenteritis caused by milk
- Use of short-acting antihistamines (diphenhydramine, etc.) more than one time within 3 days of challenge.

- Use of medium-acting antihistamines (hydroxyzine, loratadine, etc.) more than one time within 7 days of challenge.
- Maintenance therapy or use of beta-blockers and ACE inhibitors within 12-24 hours of challenge.
- Participation in any other trials of therapeutic interventions for food allergy.
- Therapy with anti-IgE for asthma [within 1 year of enrollment]

Sources of Research Materials:

Blood samples, stool samples, interview, physical examinations, results of oral food challenges done during the study as part of study procedures. All data collected will be specifically for research purposes.

Risks to the Subjects

To date, the PI has performed over 3,000 oral food challenges without a serious life-threatening anaphylactic reaction. Currently no known reliable alternative test procedure is available to substitute for the oral food challenge in documenting clinical food hypersensitivity. Several precautions are taken to minimize the risk of a major anaphylactic reaction secondary to food challenges. The decision to insert an intravenous catheter prior to the initiation of the challenge will be made by the study physician on a case-by-case basis. Food antigens are introduced in very small quantities and increased in a graded fashion, appropriate individualized doses of epinephrine for intravenous and subcutaneous use, nebulae Albuterol with oxygen, corticosteroid for intravenous use, and diphenhydramine for intravenous and oral use are available at the bedside, and nursing staff, Clinical Research Nurse, Study Physician, monitoring equipment [pulse oximeter and automatic blood pressure cuff], and complete resuscitation equipment are available during the challenge. The challenges are terminated when objective signs of a reaction have occurred. Protocols are maintained for the treatment of reactions. Risks attendant to blood drawing will be minimized by using sterile disposable equipment and by adhering strictly to blood drawing allowances indicated above. Allergy skin testing is expected to cause a local wheal/flare response, but the testing is routine and standard for the diagnosis of allergy. Systemic reactions including anaphylaxis to skin testing are extraordinarily rare, but emergency medications are at hand for treatment of allergic reactions as discussed above. Lastly, as outlined in the protocol, subjects will be in generally good health.

2. Adequacy of Protection Against Risks

Recruitment and Consent Procedures:

Consent will be obtained at the time of initial evaluation prior to performing any screening procedures. A private location will be provided in which the potential parent/guardian will have the opportunity to review the consent document, discuss it with whomever they like, digest the information that has been presented and make an informed decision, without any element of coercion or being in an anxiety provoking situation, as to whether they wish to participate. In addition, the study will be explained to the potential participants in language that they understand so that they may provide assent to participate.

3. Potential Benefits of the Proposed Research to the Subjects and Others

Subjects will derive two major benefits from the study. Accurate diagnosis of milk hypersensitivity will lead to appropriate dietary modifications and prevention of accidental exposures and reactions. Characterization of tolerance to baked milk will enable a group of patients to expand their diets to include a variety of products of high nutritional value.

4. Importance of Knowledge to Be Gained

There are currently no treatments for food allergy, other than avoidance. In addition to the benefit for the participant, an effective protocol could be targeted for treatment of others in the future (societal benefit) for milk or other food allergies. An effective therapy would reduce patient anxiety and increase safety. Additionally, data obtained from mechanistic studies may help us gain a better understanding of oral tolerance and may present opportunities to address other means to alter immune responses.

VII. INCLUSION OF WOMEN AND MINORITIES

Children meeting inclusion criteria will be enrolled regardless of gender or race.

VIII. INCLUSION OF CHILDREN

This is a study of children and adolescents.

IX. DATA SAFETY AND MONITORING PLAN (provide complete details, see http://www.mssm.edu/gcrc/forms/DSMP_template.pdf for guidance

Data Quality

The quality of the data will be monitored by the PI and Coinvestigators. The following elements will be monitored: recruitment proceeding as expected; cohort characteristics match the inclusion/ exclusion criteria; deviations from protocol; timeliness, accuracy and confidentiality of all information both in study documents and database.

Safety Monitoring

Safety monitoring for adverse events (AEs) will be conducted in real time by the PI and the Research Coordinator. All adverse events will be indicated on the source documentation for the study, and on specific adverse event report form. The following information about adverse events will be collected:

1) the onset and resolution of the AE
2) an assessment of the severity or intensity
3) an assessment of the relationship of the event to the study
4) action taken

The PI will determine the severity of the event, will assign attribution to the event, and will monitor the event until its resolution.

All serious adverse events (SAEs) will be reported to the IRB and the GCRC Research Subject Advocate (RSA) within 24 hours of the investigative team learning about them. The initial

report will be conducted by phone, email or fax. This initial contact will later be followed by a written report to the IRB (using the SAE reporting form), copied to the GCRC RSA.

Unexpected AEs and expected AEs that occur at a greater frequency or intensity than expected will be reported to the IRB and GCRC RSA within 15 business days.

Adverse Events

Anticipated adverse events include: pain or bruising at the puncture site for blood draws, itching and redness at the site of prick skin tests, allergic reaction experienced during the milk oral food challenges.

Data Safety and Monitoring Board

A data safety monitoring board has been convened by the NIAID/NIH. Information about this DSMB is attached.