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Invariant natural killer cells change after an oral allergy desensitization protocol for cow's milk

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Abstract

Background: Cow milk (CM) allergy (CMA) affects up to 3% of the pediatric population and recent data suggest that only about 50% will outgrow by age 8. Oral immunotherapy (OIT) is a type of immune-modulating treatment that is able to induce desensitization to food allergens, to increase tolerance threshold, to reduce the risk of anaphylaxis, and to improve the patient's quality of life. The examination of the immunological changes observed during the establishment of food allergy (FA) desensitization in FA patients is a window into the pathogenesis of food allergy and food tolerance development. In this pathway, we have previously found that invariant natural killer T cells (iNKTs) are involved in CM allergy sensitization and now examine their role in OIT.

Methods: In this study, 10 of the 11 children with CM induced anaphylaxis enrolled in a CMA OIT clinical trial and completed the protocol. Peripheral blood iNKTs were quantitatively and qualitatively via flow cytometry characterized ex-vivo and after culture with milk lipids before and after completing the OIT protocol.

Results: After completing OIT for CM, children were able to reintroduce CM in their diet. For the first time, we demonstrated that OIT induced a significant increase in the peripheral blood iNKT, as well as their switch from a T helper (Th-2; i.e. IL-4, IL-13) to Th-1 (i.e. IFN- γ) cytokine profile.

Conclusions and clinical relevance: This study confirms the efficacy and safety of CM-OIT as well as the role of iNKT cells in CM allergy.

Abbreviations: Invariant natural killer T cells (iNKT), α -galactosylceramide (α Gal), cow's milk (CM), double blind placebo controlled food challenge (DBPCFC), cow's milk allergy (CMA), dimethylsulfoxide (DMSO), interferon gamma (IFN- γ), interleukin 4 (IL-4), interleukin 13 (IL-13), food allergy (FA), , antibodies (Abs), human (h), human CD1d tetramers loaded with PBS57 (PBS57hCD1d), recombinant (r),

T helper cell type 1 (Th1), T helper cell type 2 (Th2), peripheral blood (PB), peripheral blood mononuclear cells (PBMCs), phorbol-12-myristate acetate (PMA), and Oral immunotherapy (OIT)

Key words: Food allergy, invariant natural killer T cells, cow's milk allergy

Introduction

Food allergy (FA) and FA induced anaphylaxis are increasing worldwide, especially in the Western world, and have become a public health issue in school age children and possibly in adults (1, 2). Cow's milk allergy (CMA) is the most common FA in children, with rates estimated at 2% to 3%(3).

Previously thought to be a benign FA with 80% of children outgrowing by age 5(4), more recent data suggest that only about 50% of children with CMA will outgrow by age 8 (5, 6). Significantly, for children and adolescents, FA, including CMA, is the major risk factor for anaphylaxis, a rare but life-threatening allergic event(7). Since CM is commonly found in the diet, accidental exposures are often seen and is the most common food induced reaction in schools; furthermore, it is a risk factor for triggering near fatal or fatal anaphylaxis (8, 9).

In the last 10 years, active research in the field of FA has shown that oral immunotherapy (OIT) is able to induce desensitization to food allergens, to increase tolerance threshold, to reduce the risk of anaphylaxis, and to improve the patient's quality of life (10-14). But, major impediments for OIT worldwide exist including 1) the possibility of acute reactions in any phase of the desensitization protocol, 2) uncertainty about long term efficacy, 3) the development of chronic non-IgE mediated food allergies such as eosinophilic esophagitis (EoE) once the food is reintroduced in the diet, and 4) compliance, as the lack thereof could be associated with severe life threatening reactions(15).

Nevertheless, food OIT offers a tremendous opportunity to gain insight into FA pathogenesis and food tolerance.

Although the pathogenic mechanisms leading to food sensitization and FA are not completely understood, various immunologic pathways including activation of effector cells through food-specific IgE antibodies, cell-mediated reactions, and combined pathways have been shown to be involved(16-18). Recent studies have shown that a subset of T cells responsive to CD1d presented lipid known as invariant Natural Killer T cells (iNKTs), may play a central role in regulating human immune responses (19-27). As opposed to conventional T cells that recognize peptides bound to highly polymorphic major histocompatibility complex (MHC)-molecules, iNKTs (also known as Type I NKT) recognize lipids presented by CD1d, a non-polymorphic MHC-class I-like molecule via a semi-invariant V α 24-V β 11 T cell receptor (TCR). A subgroup of NKT cells, that express a collection of rare but recurrent V α and V β chains and other TCRs are called Type II NKTs(28-30). The best known and more studied NKTs are the iNKTs or Type I NKTs and are the ones studied in the current paper (28-30) . All iNKTs proliferate in response to α -galactosylceramide (α Gal), which has been an important tool to study iNKTs. It is not a physiological ligand, as it is found only in marine sponges(31). Only a limited number of physiological stimuli, such as endogenous, food or bacterial lipids, have been found to activate iNKTs(31-34). Following the engagement of their invariant TCR by lipids, iNKTs rapidly upregulate costimulatory molecules and secrete Th1- and Th2-type cytokines. Thus, iNKTs may act similarly to innate immune cells to influence the activation and polarization of naïve T-cells during induction of adaptive responses.

We have previously demonstrated CM derived lipid, such as milk sphingomyelin (Milk-SM) can engage the iNKT-TCR and induce iNKT-proliferation and Th2-type cytokine secretion. Furthermore, we found that FA-children, especially those with CM allergy, have significantly fewer peripheral blood (PB)

iNKTs and their iNKTs exhibited a greater Th2-response to CM derived lipids compared to iNKTs of healthy controls, suggesting a potential role for iNKTs in CMA(34).

In the current study, we performed OIT to CM in 11 children with CM induced anaphylaxis. The primary objective was to study the safety and efficacy of CM OIT performed in highly allergic children with a history of anaphylaxis. Moreover, for the first time, we demonstrated that CM-OIT induces quantitatively and qualitatively differences in the iNKT population in children undergoing OIT, confirming that those cells may play a role in FA and specifically, in early onset FA such as CMA.

Methods

Subjects underwent two double blind placebo controlled food challenges (DBPCFC) (within a one to two week span) with placebo (soy protein, Barry Farm Foods, Wapakoneta, Ohio) and CM lactose-free milk protein powder (Barry Farm Foods, Wapakoneta, Ohio). Doses were administered every 20 minutes (Supplemental Table S1). The food challenge was stopped in the event of an objective allergic reaction (sneezing, rhinorrhea, hives, angioedema, eczematous flares, nausea, vomiting, abdominal pain, cough, wheeze, and/or shortness of breath). Reactions were measured using the standardized Bock Scale(35). At the end of the study, subjects had an open challenge to eight ounces of CM to ascertain if they had been desensitized to CM.

Eleven children who had a reaction after the DBPCFC underwent desensitization to CM. This was a pilot study with an open desensitization protocol without a placebo-controlled desensitization. The initial build-up phase of the study occurred in the hospital with indicated doses (Supplemental Table 2S). Upon completion of the initial build-up day, subjects returned the following day to receive their first home dose of lactose-free CM. In the event that the subject was unable to build-up to the 50 mg dose, then the last tolerated dose was administered on the follow-up day. Dosing was adjusted and the duration of the desensitization was extended accordingly. Subjects were instructed to take the lactose-

free CM daily at approximately the same time of day with additional food. Patients were maintained on cetirizine daily while on the desensitization protocol (Supplemental Table S3). Each week, subjects returned for a follow-up visit to monitor adherence to dosage administration and to increase the dose to the next escalation amount (Supplemental Table S3). Subjects were built up (over 3-7 months) to a maintenance dose of eight grams of CM protein or eight ounces of CM. Home study journals were maintained by the family and were reviewed with the study physician on a weekly basis.

Twenty patients with suspected CM allergy consented to participate in the study. Only 10 were able to complete the study. Nine subjects were screen failures: 1 subject resulted to be allergic to Soy, 4 subjects passed CM challenge, 1 subject refused to do food challenge, 1 subject withdrew consent, 2 subjects refused to take daily antihistamine. Therefore, eleven subjects started the desensitization protocol. One subject had 2 episodes of anaphylaxis during the desensitization protocol and could not reach full dose of CM.

Dose adjustments:

In the event of an allergic reaction, the dose was repeated or reduced, depending on severity of reaction: the dose was reduced by one dose step if the reaction was purely cutaneous in nature. The dose was reduced by two steps if two organ systems were affected; if the subject had respiratory symptoms or had symptoms which required intramuscular epinephrine. After tolerating re-dosing, the dose was increased per protocol. If there were two separate allergic reactions that required epinephrine administration, the subject was dismissed from the study. For concurrent illness: if a subject was ill with gastroenteritis or with an upper respiratory tract infection, the subject did not advance his/her dosing. The subject continued another week at the last dose tolerated. Subjects were instructed to refrain from vigorous exercise for two hours after ingestion of the CM dose.

Allergy testing

Skin testing (SPT) to commercial CM extract (1:20 weight/volume) and commercial soy extract (1:40 weight/volume) (Greer Laboratories, Lenoir, NC, USA) were performed using standard methods (36). Blood samples were obtained (Supplemental Table S4). Milk-specific IgE using ImmunoCAP method was done at ARUP Laboratories, Salt Lake City, Utah. A level of CM specific IgE <0.35 kU/L was considered negative.

INKTs studies

Peripheral blood mononuclear cells (PBMCs) were separated via Ficoll gradient sedimentation according to standard protocols (Amersham Pharmacia, Uppsala, Sweden) and analyzed *ex vivo* or were resuspended in complete medium (AIM-V Life Technologies (Rockville, MD); 10% Fetal Calf serum (FCS); recombinant (r)hIL-2 [40 U/ml] BD-Biosciences (San Jose, CA)) in the presence of milk-SM (500ng/ml), or α Gal (500ng/ml) or dimethylsulfoxide (DMSO) (DMSO was a negative control). After 5 days, half the complete medium was replaced with complete medium-supplemented with rhIL-7 (5ng/ml- Peprotech, Rocky Hill, NJ) and rhIL-15 (10ng/ml- Sigma, Saint Louis, MO). Cells were cultured for a total of 10 days^{13,14}. CM-SM (860063P-endotoxin-free) was purchased from Avanti Polar Lipids (Alabaster, AL), it was stored per manufacturer recommendation in small aliquots at -80°C . For each experiment, 1 new aliquot was resuspended in DMSO used, and the remainder discarded. α Gal was purchased from Alexis Biochemicals (Farmingdale, NY), it was stored per manufacturer recommendation in small aliquots at -80°C . For each experiment 1 new aliquot was dissolved in DMSO, used, and the remainder was discarded.

Flow Cytometry

iNKTs were defined as CD3⁺/V α 24⁺/V β 11⁺ or CD3⁺/PBS57-hCD1d⁺ by flow cytometry as previously described (34, 37) . Briefly, human CD1d tetramers were unloaded (hCD1d) or loaded with the α Gal analogue PBS57 (PBS57-hCD1d provided by the MHC-Tetramer Core Facility, Emory University, Atlanta, GA). Anti-V α 24 and anti-V β 11 antibodies were from Coulter-Immunotech (Marseille, France).

The percentage of iNKTs was expressed as those cells staining positive and compared with cells stained similarly using isotype-matched control antibodies or unloaded hCD1d tetramers. For intracellular cytokine analysis, PBMCs were stimulated with phorbol-12-myristate acetate (PMA; 25ng/ml-Sigma, Saint Louis, MO) and ionomycin (500ng/ml, Sigma, Saint Louis, MO) for 4 hours in the presence of Brefeldin-A (BD-Biosciences, San Jose, CA). Cells were then fixed and permeabilized with Cytofix/Cytoperm (BD-Biosciences, San Jose, CA) following manufacturer instructions and stained with the indicated antibodies. Stained cells were collected using the FACScalibur Flow Cytometry System (Becton Dickinson, San Jose, CA). Data were analyzed using FlowJo software (Tree Star, Ashland, OR). Five hundred thousand to 1 million events were acquired in each condition for cytoflow examination). Gates were set according to both the DMSO-treated negative control and the isotype controls. Gates were placed consistently across samples. Full gating strategy is the same used in Jyonouchi et al(34)

Statistical Analysis

Invariant natural killer T cells were expressed as a percentage of peripheral blood mononuclear cells and were compared in different phases of the study (prior to desensitization and after desensitization, and 12 weeks after completing the desensitization study - Table 4S supplemental). Statistical significance between groups of data was determined using an unpaired 2-tail Welch T test or

Mann-Whitney U test or ANOVA and were calculated with a statistical package (GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego, California USA, www.graphpad.com). P values less than or equal to 0.05 were considered statistically significant(34, 37).

Results

Cow's Milk Oral Immunotherapy

Eleven subjects enrolled in the OIT trial. A majority were male (8/11) with multiple food allergies in 10/11 subjects (91%); all had seasonal allergies, 82% had asthma; and 55% had atopic dermatitis (Table 1). The subjects had elevated total IgE (mean 1353 kU/L, range 27-7730), elevated specific CM-IgE (mean 31 kU/L, range 1.71 to >100 kU/L) (Table 2). Skin prick test (SPT) wheal was on average 10 mm (range 7-20 mm). The reactive dose on the initial CM DBPCFC was an average of 0.3 gm (range 0.06-0.9 gm) with an average Bock scale score of 7.8 (range 3-13) (Table 2). During screening DBPCFC, seven subjects were treated with epinephrine.

On the first day of desensitization, subjects reached an average dose of 0.4 gm (range 0.02-0.5 gm) of CM protein (Table 3). Of the 11 subjects who started the protocol, 10 (90%) were able to finish the protocol and reach the 8 gm milk protein maintenance dose and continued for at least 12 weeks afterwards. During the desensitization protocol, all subjects were maintained on oral antihistamines (cetirizine or equivalent) (Table 3). One subject (Subject #10) had two reactions that required epinephrine at 36 mL and 56.25 mL for systemic reactions during an updose visit and a home dose, and was not able to complete desensitization. One other subject required epinephrine during a home study dose. One hundred percent (10/10 patients) stayed on the indicated dose until Oct 2016 (for 3 years since completion of OIT).

INKTs results

Peripheral Blood (PB)- iNKTs are lower in cow's milk allergy children

We have previously shown that PB-iNKTs are lower in CM allergy and in active eosinophilic esophagitis (EoE) suggesting a role for this lineage in disease pathogenesis(31, 34, 37). To determine whether iNKTs were increased during the oral desensitization protocol and therefore further implicating iNKTs' role in CM allergy pathogenesis, we measured the iNKT percentage (%) and absolute numbers (#) in ten children at three points: (1) before starting milk desensitization, (2) when subjects reached maintenance, and (3) after 12 weeks after being on maintenance dose (Supplemental Table S4). Before starting OIT, iNKT (CD3⁺/PBS57Tet⁺) were low as previously reported (mean % $\pm$ SEM: 0.01 $\pm$ 0.003, range 0.001-0.03%; mean #/mL of blood $\pm$ SEM 264 $\pm$ 75; range 19-746). Upon completion of OIT and being on maintenance dosing for 12 weeks, the percentage of iNKTs was significantly increased (Completion: mean % $\pm$ SEM 0.02 $\pm$ 0.006, range 0.001-0.05%; mean #/mL of blood $\pm$ SEM: 484 $\pm$ 153; range 23-1228 and Maintenance: mean % $\pm$ SEM: 0.03 $\pm$ 0.006, range 0.003-0.06%; mean #/mL of blood $\pm$ SEM 650 $\pm$ 158; range 19-1333; $p < 0.05$ Figure 1 A-B).

To investigate whether the increase in number of iNKTs observed in children undergoing OIT was due to a reduced proliferative capacity, we examined iNKT responsiveness to a 10-day *in vitro* culture with the potent iNKT cell agonist α Gal or a weak antigen Milk Sphingomyelin (Milk-SM). The percentages of expanded iNKTs in α Gal-stimulated and Milk-SM PBMC cultures were lower in children before undergoing OIT and mirrored the low initial levels of iNKTs observed in fresh peripheral blood samples (Figure 2A, B), and similarly the expansion of iNKT was significantly increased both upon α Gal and Milk-SM stimulation in children after 12 weeks of maintenance. Therefore, we can conclude that OIT increased the number (percentage and absolute) of PB iNKT cells and their responsiveness to lipids.

iNKTs from children who underwent OIT produce less Th2-type and more Th1 cytokines

iNKTs have the capacity to produce both Th1- and Th2-type cytokines; we have shown that they exhibit Th2-skewing in children with FA allergy and in EoE (34, 37). We questioned whether iNKTs from CM allergic children may switch their cytokine profile from Th2 to Th1 after undergoing CM- OIT. To test this hypothesis, we measured Th1- and Th2-type cytokine production by α Gal- or milk-SM expanded iNKTs following short-term stimulation with PMA/ionomycin. We observed that a greater percentage of iNKTs from CM allergic children before starting OIT expressed IL-4 or IL-13 after α Gal or Milk-SM expansion and secondary stimulation (Figure 4A-C) compared to iNKTs obtained from subjects who completed OIT. In contrast, production of IFN- γ was increased in the iNKTs in subjects who completed OIT compared to baseline (Figure 4A-C; Supplemental Figure 1S for representative experiment). Collectively, these data indicate that iNKTs from CM allergic children produce a Th2-skewed cytokine profile in response to α Gal and Milk-SM and OIT leads to a switch to a Th1 profile.

Discussion

Most of the current types of immunotherapy aimed at developing desensitization and eventual tolerance to an allergen are based on the well-known phenomenon described over 100 years ago, whereby administering progressively increasing doses of specific allergens over time stops the body from reacting to it(13, 15). Immunotherapy, both injectable and oral, is one of the mainstream treatments for asthma and allergic rhinitis (13, 15). In the past 10 years, OIT has been studied as a cure for food allergy as well, and accumulating evidence is showing that it may be an effective treatment(13, 15).

In this study, we used a protocol similar to many already published (38-41). In these protocols,

like ours, patients underwent an initial dose escalation day, performed in outpatient hospital setting, followed by a gradual dose build-up with weekly increase of the dose and a prolonged maintenance phase on high dose of allergen(38-41). Subjects had CM dose increments by approximately 25-30% under a study physician's supervision every week, followed by daily home ingestion of the tolerated doses in between, until the maintenance dose of 8 gm CM protein daily was achieved. Different from previous protocols, we kept patients on a daily antihistamine (Table 3) and reduced the dose in the event of illness in order to minimize discomfort and side effects, as specified in the Methods section. Our subjects experienced only minimal side effects (primarily oral pruritus, minimal sneezing, and mild abdominal pain). Compared to previously published studies, we had a higher rate of desensitization with 92% of subjects that were desensitized to reach a full dose of CM (≥ 8 gm CM protein per day). Other studies report no improvement in a small percentage of patients and partial tolerance achieved in 20% to 54%, all with significantly higher rates of acute reactions being reported(38-41). Although a small sample of subjects could play a role in this, our use of daily antihistamines and reduced dosing during illness may have increased our success rate and lowered the incidence of acute reactions.

The pathophysiological mechanisms that cause allergen immunotherapy to work are not known, however OIT has been shown to induce regulatory T cells, which suppress T_H2 responses(42-44). Other immunological changes described in children who underwent OIT include increased levels of allergen-specific IgG₄, reductions in mast cell and basophil activation and specific IgE levels(42-44).

We have previously shown that children with CM allergy show quantitative and qualitative differences in their iNKT population compared to controls, suggesting a possible role for iNKTs in milk allergy pathogenesis(34). We also have previously demonstrated that milk-SM is able to bind iNKT-TCR upon CD1d presentation and stimulate iNKT to proliferate and produce Th2 cytokines with a strength about 300 times inferior to α Gal(34). More importantly, we observed that the iNKTs from CM allergic

children exhibit a Th2-skewed production of cytokines. These results mirror the findings previously reported in patients with asthma(26) and suggest a possible role for iNKTs in the development of FA through Th2-cytokine skewing(45).

In this paper, we hypothesized that if iNKTs were important in CM allergy pathogenesis, OIT to CM may induce quantitative and qualitative changes in iNKT population upon desensitization. We therefore measured iNKT levels and their Th1 (i.e. IFN- γ) and Th2 (IL-4; IL-13) cytokine expression *in vivo* and after culture with α Gal or milk-SM in ten children who underwent successful desensitization at three points (prior to OIT; completion of OIT at 8 g; and after 12 weeks of maintenance daily dosing). Similar to our previous studies(34, 37), milk-SM was used to show that iNKTs can be stimulated by a relevant allergen found in everyday life and present in CM, as opposed to α Gal, a pan-stimuli that is found only in marine sponges.(34).

Confirming our previous results, we observed very low levels of percentage and absolute numbers of iNKT in children with CM allergy (34). Upon reaching maintenance of OIT and after 12 weeks of daily maintenance dosage, we observed 4 key findings: (1) increased levels of percentage and absolute numbers of iNKT cells, (2) increased expansion upon antigen stimulation, (3) reduced production of Th2 cytokines, and (4) increase of Th1 cytokines. The levels are similar to levels we observed in children with food allergy and less than those in healthy children(34). This is not a surprise as subjects in our study have multiple food allergies and were only desensitized to one allergen (milk) with CM-OIT. The novel aspect of this study is that iNKT cells change during OIT and suggest a role of iNKTs in CM desensitization. Although we do not have any additional information about the precise immunological effects of the food-reactive iNKTs, we could postulate that iNKTs stimulated with milk-SM act as adjuvant cells that induce naïve T-cells to differentiate into Th2-cells and thereby promote IgE class switching during the humoral response to milk (46). This effect could be stronger early on in life

when the adaptive immune system is not well developed and could be reverted later in life when the T-regulatory population develops and children outgrow food allergy or if they undergo OIT.

The study has several limitations such as the small number and the lack of a population treated with placebo. Other CM OIT protocols, which included a placebo controlled group, have shown that outgrowing CM allergy in few months' time frame is very unlikely; however, we cannot exclude that a small percentage of our studied subjects could have acquired spontaneously the tolerance to CM. Even if the changes observed in iNKT cells with tolerance (either spontaneous or induced with OIT) could point to a mechanism in FA pathogenesis, as each subject functions as a control for him/herself, a larger double blind placebo controlled CM OIT study is needed to confirm the importance of iNKTs in CM allergy and their role in OIT.

In summary, we have demonstrated that lower PB-iNKTs and Th2-cytokine bias observed in children with CM-allergy is partially reversible upon OIT protocol completion and acquisition of CM desensitization status. The results, therefore, further suggest that iNKTs may play a role in CM sensitization and tolerance acquisition. If these results are confirmed in a larger DBPC CM desensitization trial, iNKT cells may present the opportunity for specific therapeutic modulation in CM allergy.

Table 1 Patient characteristics

Subject Number	Age (years)	Gender	Other Food Allergies/Sensitizations	Asthma	AR	AD
1	8	F	garlic, shellfish, peanuts, egg, tree nuts, sesame, pineapple	Y	Y	Y
2	8	F	peanuts, egg, tree nuts	Y	Y	Y
3	9	M	beef, egg, sesame, shellfish, peanut, tree nuts, green beans, peas	Y	Y	Y
4	9	M	peanuts, tree nuts, lentils, kidney beans	Y	Y	Y
5	6	F	egg, peanut	N	Y	N
6	8	M	egg, peanut, tree nuts, chicken, sesame OAS - bananas	Y	Y	Y
7	10	M	egg, peanut, tree nuts, coconut	Y	Y	N
8	12	M	Tree nuts, egg, peanut, shellfish	Y	Y	N
9	10	M	egg, tree nuts, peanut	Y	Y	Y
10	8	F	egg, tree nuts	Y	Y	N
11	13	M	None	N	Y	N

*F= female, M=Male, Y=yes, N=No, AR-Allergic Rhinitis, AD-atopic dermatitis, OAS-oral allergy syndrome

Table 2: Cow's Milk (CM) Allergy History

			Characteristics of patients at the time of First CM Challenge (Pre-therapy)				
Subject Number	Age (months) of initial reaction	Symptoms on initial reaction	CM Skin Test Size Wheal x flare (mm)	Total serum IgE(kU/L)	CM-specific serum IgE(kU/L)	Reactive of CM protein (g)	Bock Score
1	6	urticaria, emesis	20X22	2280	84.3	0.0556	10
2	6.5	emesis	12X30	841	9.73	0.889	5
3	2	eczema flare	11X28	7320	>100	0.0556	3
4	2	eczema flare	10X12	900	15	0.0556	5
5	6	urticaria, sneezing, watery eyes	8X30	550	3.24	0.444	6
6	12	lip & eye angioedema	7X18	420	22.4	0.0556	12
7	6	perioral erythema	6X22	123	3.68	0.0556	5
8	5	facial swelling, respiratory distress	10X15	787	29	0.889	11
9	7	emesis, urticaria	7X20	283	5.5	0.444	8
10	4	urticaria, abdominal pain	10X25	27	1.71	0.111	8
11	9	emesis	10X25	not done	48.8	0.222	13

CM= Cow's milk

Table 3: Patients desensitization

Subject Number	Highest Tolerated dose on Initial CM Desensitization(g)	Bock Score Initial CM Desensitization	Starting dose (g/ml of CM) for Desensitization	Antihistamine used
1	0.5	4	0.32 (9.6)	Cetirizine 7.5 mg
2	0.5	1	0.5 (15)	Cetirizine 7.5 mg
3	0.08	17	0.01 (0.3)	Cetirizine 10 mg
4	0.5	1	0.5(15)	Fexofenadine 30 mg
5	0.5	6	0.16 (4.8)	Cetirizine 5 mg
6	0.5	10	0.16 (4.8)	Cetirizine 5 mg
7	0.32	3	0.16 (4.8)	Cetirizine 10 mg
8	0.5	0	0.5 (15)	Cetirizine 10 mg
9	0.5	2	0.5 (15)	Cetirizine 10 mg
10	0.5	0	0.39 (11.7)	Cetirizine 5 mg
11	0.02	5	0.01 (0.3)	Cetirizine 10 mg

CM= cow's milk

Figure 1: iNKTs in children with IgE mediated cow's milk (CM) allergy increase upon completion of oral immunotherapy (OIT) to CM.

Fresh PBMCs from 10 CM allergy subjects were stained *ex vivo* for iNKTs (CD3⁺PBS57CD1dtet⁺) before (Pre), upon completion (End) and after 12 weeks (After) of OIT to CM. (A) show mean levels of iNKT cells expressed as percentage of live lymphocytes (B) show absolute number of iNKTs per ml. *p<0.02, **p<0.002. PBMC=peripheral blood monocytes, CD1dtet =CD1d tetramers, OIT=oral immunotherapy, CM= Cow's milk, iNKT= invariant natural killer T cells, αGal=alpha galactosylceramide, milk-SM= milk sphingomyelin

Figure 2: iNKTs expand upon completion of OIT when stimulated with αGal or Milk-SM.

10 CM allergy subjects were studied at three points: before (Pre), upon completion (End) and after 12 weeks (After) of OIT to CM. The PBMCs were cultured for 10 days with αGal (A-B), milk-SM (B-C) and were stained for iNKTs (CD3⁺PBS57CD1dtet⁺). (A, C) show mean levels of iNKT cells expressed as percentage of live lymphocytes (B,D) show absolute number of iNKTs. *p<0.02, **p<0.002.

PBMC=peripheral blood monocytes, CD1dtet =CD1d tetramers, OIT=oral immunotherapy, CM= Cow's milk, iNKT= invariant natural killer T cells, αGal=alpha galactosylceramide, milk-SM= milk sphingomyelin

Figure 3: iNKTs express lower levels of IL-4 and IL-13 and higher levels of IFNγ when maintenance dose of OIT was established for 12 weeks.

PBMCs were pretreated 10 days with αGal, Milk-SM or DMSO, stimulated with PMA and ionomycin and stained for intracellular cytokines. Percentage of iNKTs staining for IL-4 (A), IL13 (B), or IFNγ (C) pretreated with indicated reagents are shown. *p <0.05, **<0.02, ***p<0.003.

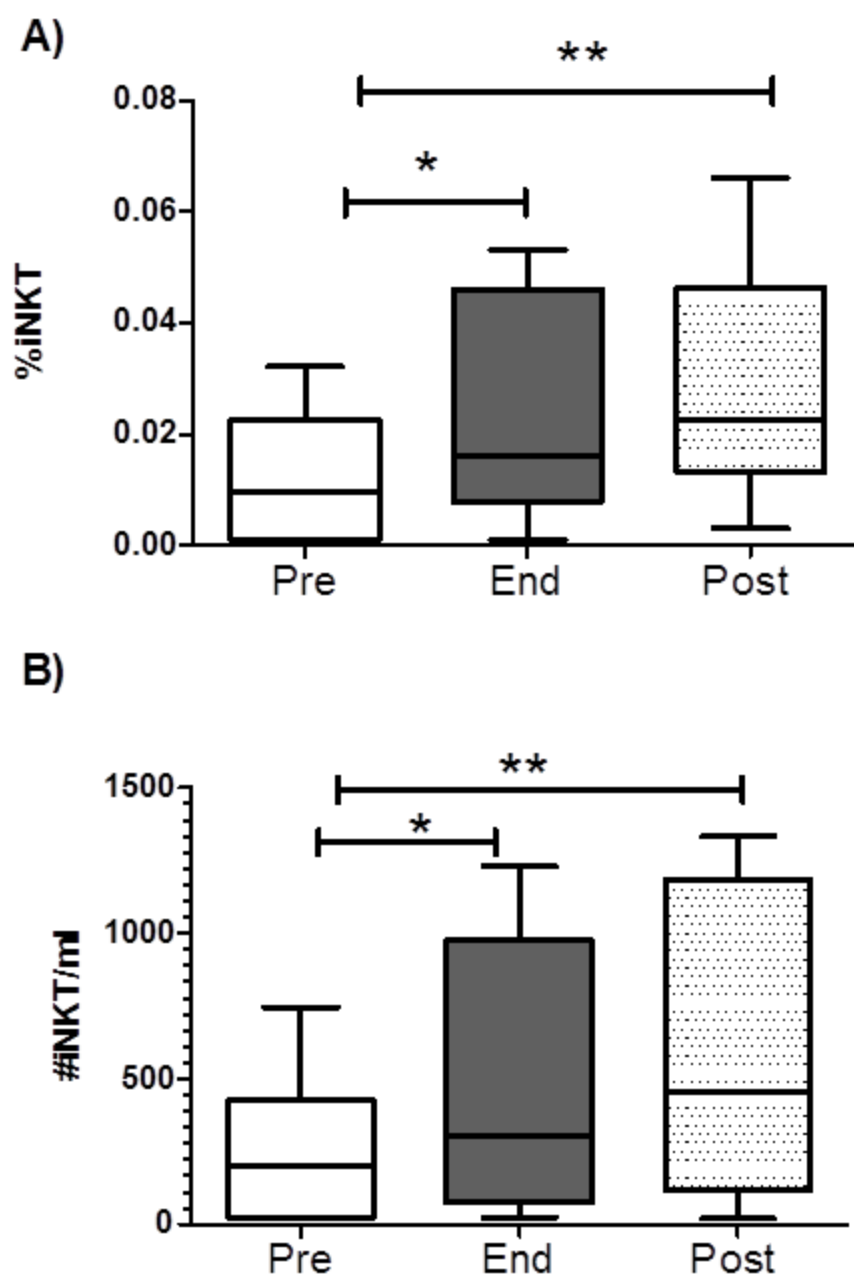
PBMC=peripheral blood monocytes, CD1dtet =CD1d tetramers, OIT=oral immunotherapy, CM= Cow's milk, iNKT= invariant natural killer T cells, αGal=alpha galactosylceramide, milk-SM= milk sphingomyelin

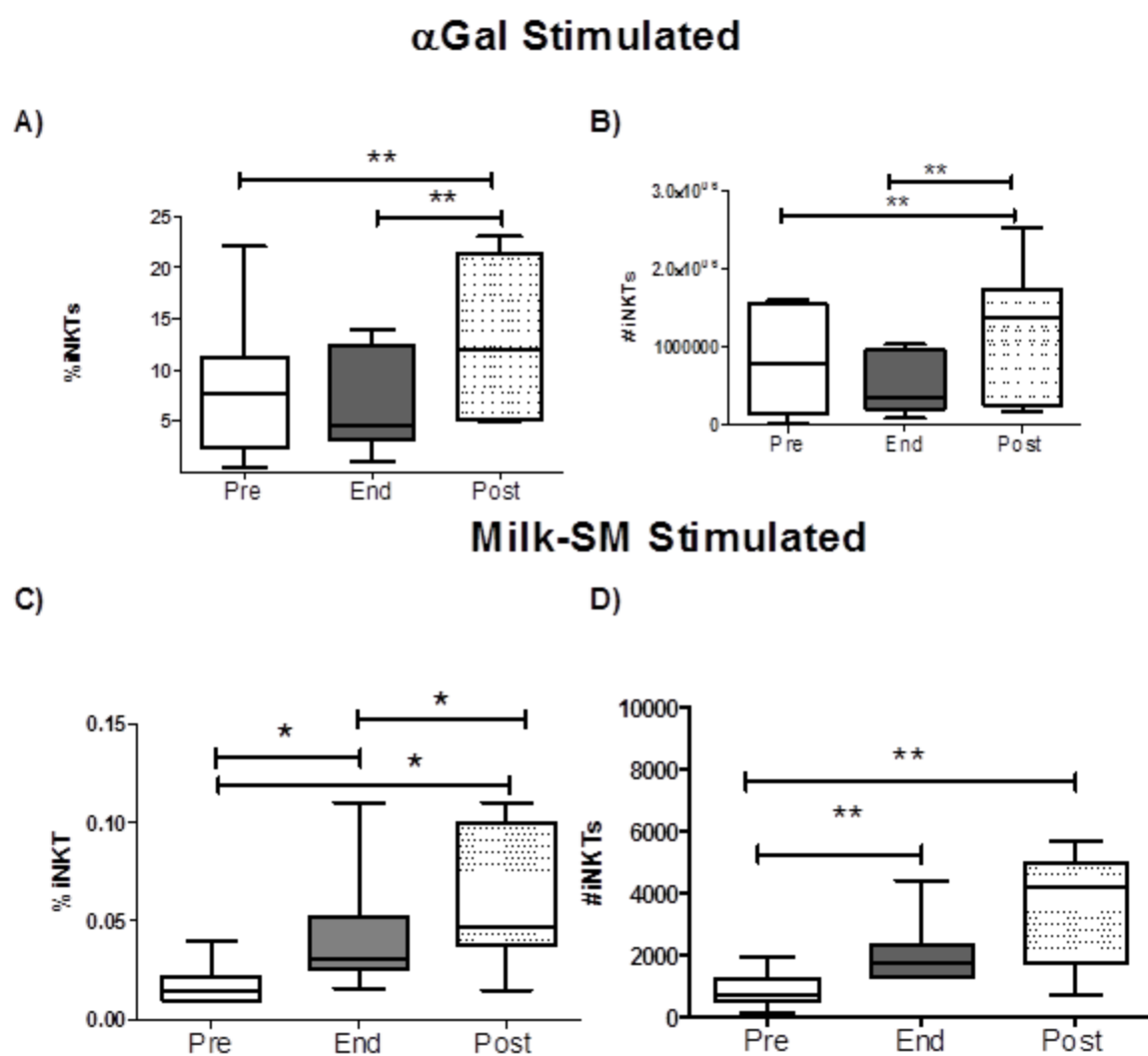
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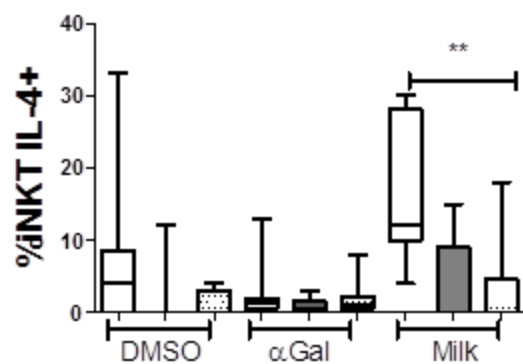
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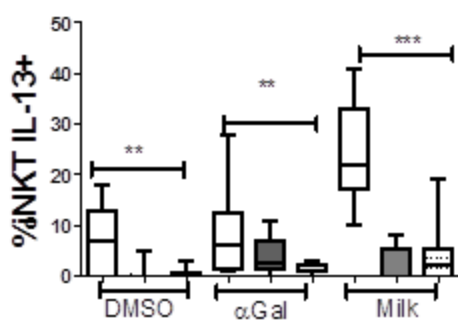




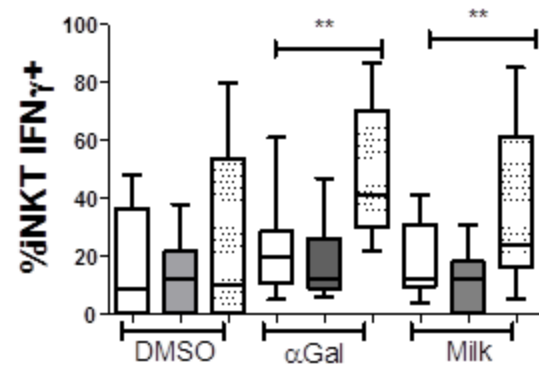
A)

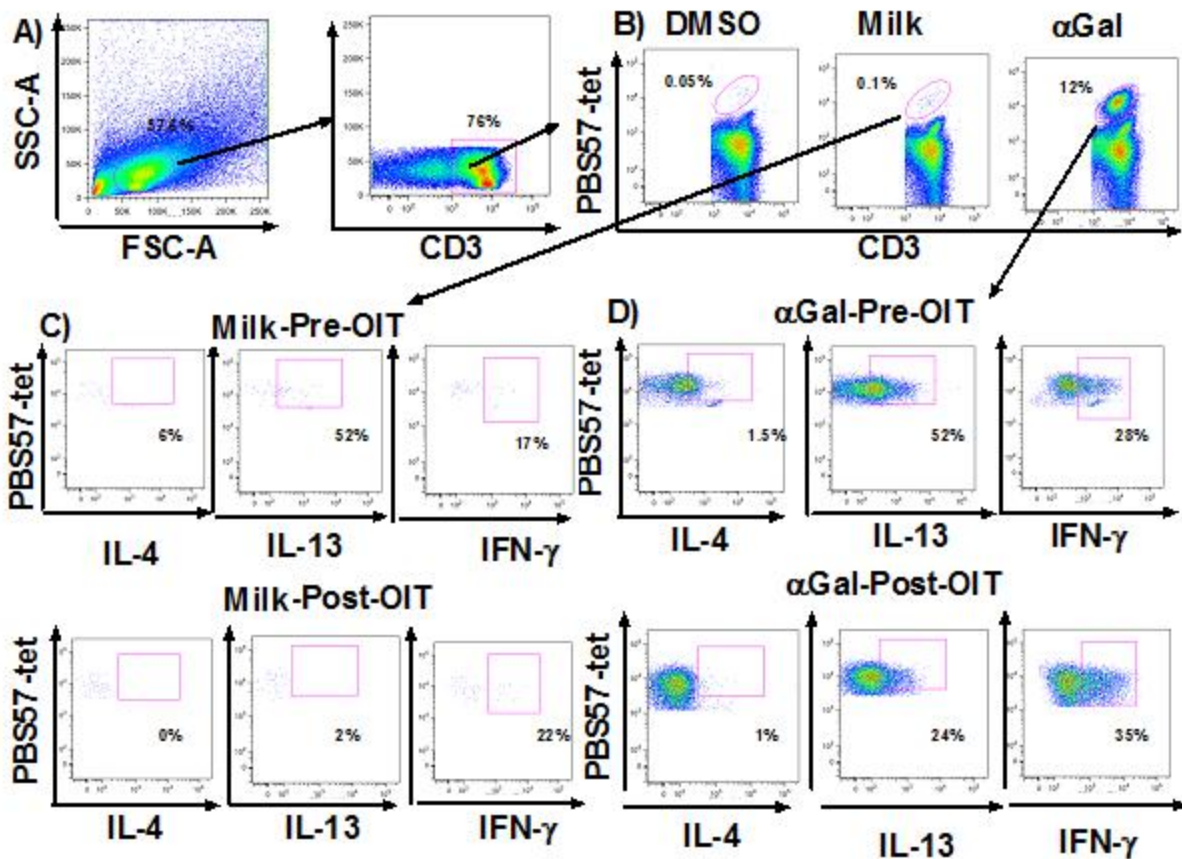


B)



C)





Supplemental methods

Supplemental methods

Subjects who underwent DBPCFC satisfied all inclusion criteria. Then inclusion criteria were: 1) six to 18 years of age with a history of cow's milk allergy; 2) had a history (within the past 6 months) of a positive skin prick test to cow's milk extract and an immunocap IgE level greater than 0.35 kU/L; 3) positive allergic reaction history to cow's milk within the past 12 months; 4) were in good health, as determined by medical history and physical examination performed by a study physician, with no history of PICU admission for asthma or FA induced anaphylaxis; 5) females of childbearing potential were using an effective method of contraception and agreed to continue to practice an acceptable method of contraception for the duration of their participation in the study; 6) Informed consent of parent or legal guardian was obtained prior to study enrollment. The study was approved by The Children's Hospital of Philadelphia IRB, Clinical Trial #7320. The exclusion criteria include: 1) Tolerating 8 grams of cow's milk protein; 2) Pregnancy 3) Soy allergy 4) Food protein induced enterocolitis syndrome to milk; 5) Anaphylaxis to milk requiring overnight hospitalization; 6) Intubation related to asthma and/or a history of an intensive care unit admission for asthma management; 7) Current diagnosis of severe persistent asthma [FEV1 (forced expiratory volume in 1 second) < 60% of predicted, as defined by NHLBI (National Heart, Lung, and Blood Institute) guidelines, despite current therapy]; 8) Current diagnosis of severe atopic dermatitis; 9) Serious chronic medical condition, including neurologic, hepatic, renal, endocrine, cardiovascular, gastrointestinal, pulmonary, or metabolic disease; 10) Use of oral or injection steroids within one month of protocol initial visit; 11) Acute illness within one week prior to the first dose of oral immunotherapy; 12) Use of antihistamines within seven days prior to DBPCFC; 13) Use of

chronic immunomodulatory therapy; 14) Participating in another experimental therapy study; and 15) Inability to discontinue antihistamines for skin testing and food challenges.

DPCFC dosing (Table S1) and initial build-up day dosing (Table S2) was dosed every 20 minutes and stopped for objective symptoms based on the consensus PRACTALL guidelines (1). Patients were sent home on a daily dose for a week and returned for increase in dosing as per Table S3. Dosing was reduced for acute illness or systemic reaction by 50% and subjects continued on that dose until well. When well, patients with acute illness resumed their original dose. For patients with acute reactions, they were maintained on the lower dose until the next scheduled visit and then resumed increase by standard methods (Table S3).

Table S1. DBPCFC Dosing

<i>Dose of milk protein powder</i>
1/16 teaspoon = 0.056 gm
1/8 teaspoon = 0.111 gm
¼ teaspoon = 0.222 gm
½ teaspoon = 0.444 gm
1 teaspoon = 0.889 gm
2 teaspoons = 1.778 gm
Cumulative 9 teaspoons (equivalent to 8 grams cow's milk protein or 8 ounces cow's milk)

Table S2: Initial Build-up Day Dosing of Protein

#	<i>Dose</i>	<i>% Increase</i>
1	1 mg = 0.3 mL of 1:10 dilution of cow's milk	50
2	2 mg = 0.6 mL of 1:10 dilution of cow's milk	50
3	4 mg = 1.2 mL of 1:10 dilution of cow's milk	50
4	8 mg = 2.4 mL of 1:10 dilution of cow's milk	50
5	10 mg = 0.3 mL of cow's milk	25
6	20 mg = 0.6 mL	50
7	40 mg = 1.2 mL	50
8	80 mg = 2.4 mL	50
9	160 mg = 4.8 mL	50
10	320 mg = 9.6 mL	50
11	500 mg = 15 mL	36

Table S3: Desensitization Protocol for Build-up to Maintenance Dose

<i>Week</i>	<i>Dose</i> <i>(milligrams of cow's milk protein)</i>	<i>% Increase</i>
4	625 mg	25%
5	780 mg	25%
6	975 mg	25%
7	1200 mg	23%
8	1500 mg = 45 mL of cow's milk	25%
9	1875 mg	25%
10	2300 mg	23%
11	2875 mg	25%
12	3500 mg	22%
13	4375 mg	25%
14	5500 mg	26%
15	6875 mg	25%
16	8000 mg = 8 ounces of cow's milk	16%

Table S4: Timeline for Blood Work-up

<i>Timepoint</i>	<i>Studies</i>
Initial visit	-Immunocap testing for milk (if not within prior 6 months) -Baseline IgE
Prior to initial food challenges	Blood for immune cell analysis
Once on maintenance	Blood for immune cell analysis
After 12 weeks on maintenance	Blood for immune cell analysis

Figure 1 S: representative experiment of data presented in Figure 4

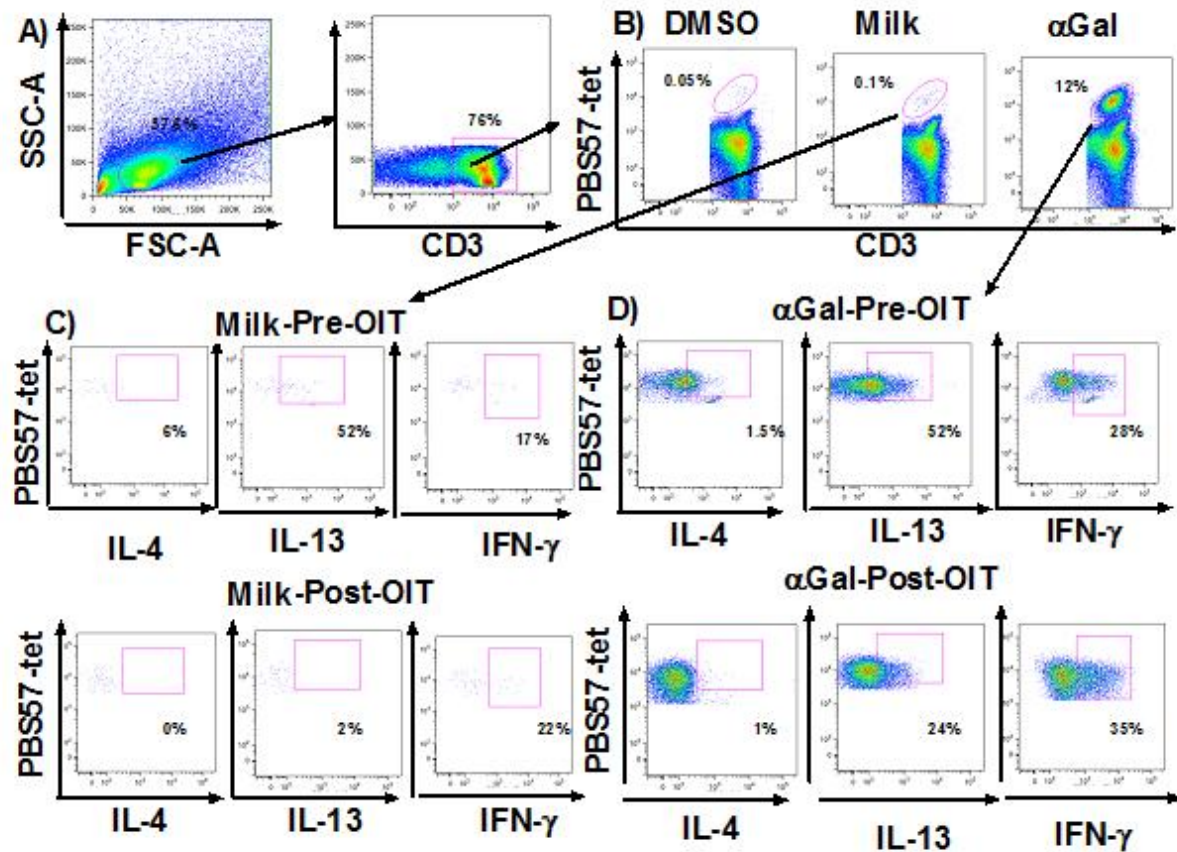


Figure 1 S: iNKTs express lower IL-4 and IL-13 and higher level of IFN γ when maintenance dose of OIT was established for 12 weeks.

PBMCs from patient #6 were obtained before starting CM-OIT (Pre-OIT) or 12 week after finishing OIT (Post-OIT), they were pretreated 10 days with DMSO, Milk-SM or α Gal and stimulated with PMA and ionomycin and stained for intracellular cytokines as indicated (IL-4, IL13, INF γ). Representative experiment. Panel A shows gating strategy for lymphocytes and CD3⁺ cells. Panel B show iNKT population in PBMCs cultured for 10 days with either DMSO, Milk-SM or α Gal. (Numbers in graphic are percentage of gated CD3⁺ lymphocytes). Panel C shows cytokine expression in iNKTs cells stimulated for 10 days with Milk-SM (Numbers in graphic are percentage of gated iNKTs) and Panel D shows C shows cytokine expression in iNKTs cells stimulated for 10 days with α Gal (Numbers in graphic are percentage of gated iNKTs). Gates were set according to both the DMSO-treated negative control () and the isotype controls. Gates were placed consistently across samples. Full gating strategy is the same used in Jyonouchi et al (1, 2).

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