

# Invariant natural killer T cells from children with versus without food allergy exhibit differential responsiveness to milk-derived sphingomyelin

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**Background:** A key immunologic feature of food allergy (FA) is the presence of a T<sub>H</sub>2-type cytokine bias. Ligation of the invariant natural killer T cell (iNKT) T-cell receptor (TCR) by sphingolipids presented via the CD1d molecule leads to copious secretion of T<sub>H</sub>2-type cytokines. Major food allergens (eg, milk, egg) are the richest dietary source of sphingolipids (food-derived sphingolipids [food-SLs]). Nonetheless, the role of iNKTs in FA is unknown.

**Objective:** To investigate the role of iNKTs in FA and to assess whether food-SL–CD1d complexes can engage the iNKT-TCR and induce iNKT functions.

**Methods:** PBMCs from 15 children with cow's milk allergy (MA), 12 children tolerant to cow's milk but with allergy to egg, and 13 healthy controls were incubated with  $\alpha$ -galactosylceramide ( $\alpha$ Gal), cow's milk–sphingomyelin, or hen's egg–ceramide. iNKTs were quantified, and their cytokine production and proliferation were assessed. Human CD1d tetramers loaded with milk-sphingomyelin or egg-ceramide were used to determine food-SL binding to the iNKT-TCR. **Results:** Milk-sphingomyelin, but not egg-ceramide, can engage the iNKT-TCR and induce iNKT proliferation and T<sub>H</sub>2-type cytokine secretion. Children with FA, especially those with MA, had significantly fewer peripheral blood iNKTs and their iNKTs exhibited a greater T<sub>H</sub>2 response to  $\alpha$ Gal and milk-sphingomyelin than iNKTs of healthy controls.

**Conclusion:** iNKTs from children with FA, especially those with MA, are reduced in number and exhibit a T<sub>H</sub>2 bias in response to  $\alpha$ Gal and milk-sphingomyelin. These data suggest a potential role for iNKTs in FA. (J Allergy Clin Immunol 2011;■■■:■■■–■■■.)

**Key words:** Food allergy, invariant natural killer T cells, sphingolipids

Food allergy (FA) is highly prevalent in children and on occasion can cause fatal allergic reactions.<sup>1</sup> It is postulated that in FA the presence of T<sub>H</sub>2 cytokines contributes to the breakdown of oral tolerance.<sup>2–4</sup> T<sub>H</sub>2-type cytokines can be produced by a variety of cells including invariant natural killer T cells (iNKTs).<sup>3,5</sup> Indeed, iNKTs, when appropriately stimulated, promote a T<sub>H</sub>2 response, IgE production, and subsequent sensitization to protein antigens.<sup>6,7</sup> Although iNKTs have pleiotropic roles in multiple diseases,<sup>8,9</sup> the role of iNKTs in FA has not been previously reported.

As opposed to conventional T cells that recognize peptides bound to highly polymorphic MHC molecules, iNKTs recognize sphingolipids presented by CD1d, a nonpolymorphic MHC class I–like molecule, via a semi-invariant V $\alpha$ 24–V $\beta$ 11 T-cell receptor (TCR). All iNKTs proliferate in response to  $\alpha$ -galactosylceramide ( $\alpha$ Gal).<sup>8</sup> Only a limited number of endogenous or bacterial sphingolipids are known to activate iNKTs.<sup>8,10</sup> After engagement of their invariant TCR by sphingolipids, iNKTs rapidly upregulate costimulatory molecules and secrete T<sub>H</sub>1-type and T<sub>H</sub>2-type cytokines. Thus, iNKTs may act similarly to innate immune cells to influence the activation and polarization of naive T cells during induction of adaptive responses.

The major food allergens in pediatric FA are egg and milk.<sup>1</sup> Interestingly, these foods are also the richest dietary source of sphingolipids, such as sphingomyelin, which have the potential to act as ligands of the iNKT-TCR.<sup>11</sup> It is estimated that the average individual consumes approximately 0.3 to 0.4 g sphingolipids every day.<sup>11</sup> For example, dairy products contain 70 to 400  $\mu$ g sphingomyelin per gram.<sup>12</sup> We undertook the current investigation to examine the role of iNKTs in the pathogenesis of FA. Here we show that a subset of iNKTs in normal individuals recognizes and responds to cow's milk–derived sphingomyelin (milk-SM), as demonstrated by increased proliferation and T<sub>H</sub>2-type cytokine production. Interestingly, the iNKT T<sub>H</sub>2-type cytokine response to milk-SM in children with FA, especially cow's milk allergy (FA-MA), is more pronounced, suggesting a role for iNKTs in the pathogenesis of FA. We hypothesize that milk-SM could promote a T<sub>H</sub>2-skewed environment in predisposed individuals by inducing iNKT cells to secrete T<sub>H</sub>2-type cytokines.

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**Abbreviations used**

|               |                                                    |
|---------------|----------------------------------------------------|
| $\alpha$ Gal: | $\alpha$ -Galactosylceramide                       |
| APC:          | Antigen-presenting cell                            |
| CFSE:         | 5,6-Carboxyfluoresceindiacetate succinimidyl ester |
| CHOP:         | The Children's Hospital of Philadelphia            |
| CM:           | Complete medium                                    |
| DMSO:         | Dimethyl sulfoxide                                 |
| Egg-CE:       | Hen's egg-ceramide                                 |
| FA:           | Food allergy                                       |
| FA-MA:        | Cow's milk allergy                                 |
| FA-NMA:       | Tolerance to milk and allergy to egg               |
| Food-SL:      | Food-derived sphingolipid                          |
| hCD1d:        | Unloaded human Cd1d tetramer                       |
| iNKT:         | Invariant natural killer T cell                    |
| Milk-SM:      | Cow's milk-derived sphingomyelin                   |
| ND:           | Normal adult donors                                |
| Non-FA:       | No history of FA, asthma, or allergic rhinitis     |
| PB:           | Peripheral blood                                   |
| PMA:          | Phorbol 12-myristate 13-acetate                    |
| rh:           | Recombinant human                                  |
| TCR:          | T-cell receptor                                    |

**METHODS****Study subjects**

Twenty-seven children with milk and/or egg allergy were recruited from the Allergy Clinic at The Children's Hospital of Philadelphia (CHOP), including 15 children with FA-MA (13 boys, 2 girls; average age  $\pm$  SD,  $5.4 \pm 2.5$  years; see this article's Table E1 in the Online Repository at [www.jacionline.org](http://www.jacionline.org)) and 12 children tolerant to milk but with allergy to egg (FA-NMA; 10 boys, 2 girls; average age  $\pm$  SD,  $4.1 \pm 1.8$  years; see this article's Table E2 in the Online Repository at [www.jacionline.org](http://www.jacionline.org)). To be eligible for study, patients needed to have all of the following characteristics: (1) positive skin prick test and/or presence of specific IgE in the serum to milk and/or egg; (2) positive food challenge test or recurrence of allergic reaction after accidental exposure to milk and/or egg; and (3) clinical stability on a diet excluding the offending food. Thirteen children without a history of FA, asthma, or allergic rhinitis (non-FA) were recruited from the primary care clinics at CHOP (10 boys, 3 girls; average age  $\pm$  SD,  $4.38 \pm 3.7$  years). All investigations were approved by the CHOP Internal Review Board. See additional Methods information in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org).

**Reagents**

Hen's egg-ceramide (egg-CE; 860051P-endotoxin-free) and milk-SM (860063P-endotoxin-free) were purchased from Avanti Polar Lipids (Alabaster, Ala).  $\alpha$ Gal was from Alexis Biochemicals (Farmingdale, NY). Human CD1d tetramers were unloaded (hCD1d) or loaded with the  $\alpha$ Gal analog PBS57 (PBS57-hCD1d), milk-SM (milk-SM-hCD1d) or egg-CE (egg-CE-hCD1d) and were provided by the MHC-Tetramer Core Facility, Emory University (Atlanta, Ga). Anti-V $\alpha$ 24 and anti-V $\beta$ 11 Abs were from Coulter-Immunotech (Marseille, France).

**Culture**

PBMCs were resuspended in complete medium (CM; AIM-V; 10% FCS; recombinant human [rh] IL-2 [40 U/mL]) in the presence of milk-SM (500 ng/mL), egg-CE (500 ng/mL),  $\alpha$ Gal (500 ng/mL), or dimethyl sulfoxide (DMSO). After 5 days, half of the CM was replaced with CM supplemented with rhIL-7 (5 ng/mL) and rhIL-15 (10 ng/mL).<sup>13,14</sup> iNKTs were also purified from human CD3<sup>+</sup> cells by cell sorting (95% to 99% purity; see additional Methods in the Online Repository).

**Flow cytometry**

iNKTs were defined as CD3<sup>+</sup>/V $\alpha$ 24<sup>+</sup>/V $\beta$ 11<sup>+</sup> or CD3<sup>+</sup>/PBS57-hCD1d<sup>+</sup> by flow cytometry (see Methods and this article's Fig E1 in the Online Repository at [www.jacionline.org](http://www.jacionline.org) for gating strategy used). 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. Stained cells were collected by using the FACScalibur Flow Cytometry System (Becton Dickinson, San Jose, Calif). Data were analyzed by using FlowJo software (Tree Star, Ashland, Ore).

**Measurement of iNKT proliferation**

See Methods in the Online Repository.

**RT-PCR analyses**

See Methods in the Online Repository.

**Statistical analysis**

See Methods in the Online Repository.

**RESULTS****Children with FA-MA have fewer peripheral blood iNKTs**

In many human disorders, iNKTs are reduced in number and/or function, suggesting a role for this lineage in disease pathogenesis.<sup>8,10</sup> To determine whether iNKTs are involved in FA, we measured the iNKT percentage and absolute number in 15 children with FA-MA (Table E1), 12 children with FA-NMA (Table E2), and 13 healthy controls (non-FA). In non-FA children, the percentage and absolute number of CD3<sup>+</sup>/V $\alpha$ 24<sup>+</sup>/V $\beta$ 11<sup>+</sup> iNKTs was consistent with those reported previously (mean %  $\pm$  SEM,  $0.29 \pm 0.25$ ; median %, 0.02; range, 0.001% to 3.6%; mean absolute number/mL  $\pm$  SEM,  $7982 \pm 6542$ ; median absolute number/mL, 1200; range, 0-86,400).<sup>13,15-17</sup> Similar results were obtained regardless of whether we identified iNKTs on the basis of V $\alpha$ 24/V $\beta$ 11 positivity or reactivity with PBS57-hCD1d tetramers (not shown). The percentage and absolute number/mL of iNKTs in children with FA-MA (mean %  $\pm$  SEM,  $0.01 \pm 0.003$ ; median %, 0.01; range, 0.001% to 0.04%; mean absolute number/mL  $\pm$  SEM,  $171.2 \pm 81.78$ ; median absolute number/mL, 57; range, 0-933) were significantly lower compared with children with FA-NMA (mean %  $\pm$  SEM,  $0.07 \pm 0.04$ ; median %, 0.017; range, 0.01% to 0.36%; mean absolute number/mL  $\pm$  SEM,  $735 \pm 2324$ ; median absolute number/mL, 735; range, 214-28,543) and non-FA controls (Fig 1, A and B; see this article's Fig E2 in the Online Repository at [www.jacionline.org](http://www.jacionline.org)).

To investigate whether the lower number of iNKTs observed in children with FA, particularly those with FA-MA, was a result of a reduced proliferative capacity, we examined iNKT responsiveness to a 10-day *in vitro* culture with the potent iNKT agonist  $\alpha$ Gal. iNKTs from children with FA-MA expanded in response to  $\alpha$ Gal, although the percentages of expanded iNKTs in  $\alpha$ Gal-stimulated PBMC cultures were lower in children with FA-MA and mirrored the low initial levels of iNKTs observed in fresh peripheral blood samples (Fig 1, C and D; see this article's Fig E3 in the Online Repository at [www.jacionline.org](http://www.jacionline.org)). The responsiveness of iNKTs from children with FA-MA to  $\alpha$ Gal was confirmed by measuring the expression of the activation marker CD25 on  $\alpha$ Gal-stimulated cells.  $\alpha$ Gal induced upregulation of

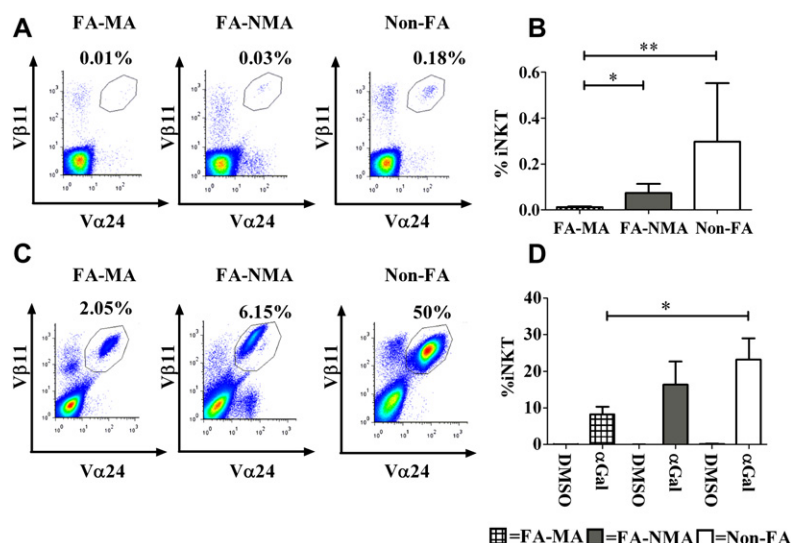


**FIG 1.** Children with FA-MA have significantly fewer PB-iNKTs that respond to  $\alpha$ Gal. Fresh PBMCs from 15 children with FA-MA, 12 children with FA-NMA, and 13 non-FA children were stained *ex vivo* for iNKTs ( $CD3^+V\alpha24^+V\beta11^+$ ; **A** and **B**) and then cultured for 10 days with  $\alpha$ Gal or DMSO (**C** and **D**). **A** and **C**, Dot plot of a representative experiment. **B** and **D**, Mean levels of iNKT cells expressed as  $CD3^+$  cells. \* $P < .02$ ; \*\* $P < .002$ .

CD25 equivalently in all study groups (see this article's Fig E4 in the Online Repository at [www.jacionline.org](http://www.jacionline.org)). Therefore, although iNKTs are reduced in number in patients with FA-MA, they do not appear to differ in their responsiveness to  $\alpha$ Gal.

### iNKTs from children with FA produce more $T_H2$ -type cytokines

iNKTs have the capacity to produce  $T_H1$ -type and  $T_H2$ -type cytokines, but in some diseases, they exhibit  $T_H2$  skewing.<sup>8,18,19</sup> We questioned whether iNKTs from children with FA might exhibit a  $T_H2$ -skewed cytokine profile, as has been observed for the T cells of patients with FA.<sup>1</sup> To test this hypothesis, we measured  $T_H1$ -type and  $T_H2$ -type cytokine production by  $\alpha$ Gal-expanded iNKTs after short-term stimulation with phorbol 12-myristate 13-acetate (PMA)/ionomycin. We observed that a greater percentage of iNKTs from children with FA, compared with controls, expressed IL-4 or IL-13 after  $\alpha$ Gal expansion and secondary stimulation, although such a difference reached statistical significance only in children with FA-NMA. iNKTs from children with FA-MA have a tendency even when unstimulated to produce more IL-4 compared with the other groups of children, although this did not reach statistical significance ( $P = .06$ ). Such a tendency was not enhanced by  $\alpha$ Gal expansion (Fig 2, A-C; see this article's Fig E5A-F, in the Online Repository at [www.jacionline.org](http://www.jacionline.org)). In contrast, production of IFN- $\gamma$  was similar in the iNKTs of all patient cohorts (Fig 2, A and D; Fig E5, G-I). Collectively, these data indicate that iNKTs from children with FA produce a  $T_H2$ -skewed cytokine profile in response to  $\alpha$ Gal.

### hCD1d tetramers loaded with milk-SM specifically bind to iNKTs

Sphingolipids are abundant in milk and egg.<sup>20</sup> Because the iNKTs from children with FA exhibited quantitative and

qualitative differences compared with the iNKTs of controls, we questioned whether milk-derived and/or egg-derived sphingolipids might drive iNKT functions. To address this possibility, we first examined whether food-derived sphingolipid (food-SL)-loaded hCD1d tetramers could bind to the invariant TCR present on normal iNKTs. PBMCs from 7 normal adult donors (NDs) were expanded with  $\alpha$ Gal and stained using hCD1d loaded with  $\alpha$ Gal, milk-SM, or egg-CE. We chose these 2 food-SLs because they differ in their head group (ie, substituents at the 1-hydroxyl [1-OH] position) of the sphingolipids.<sup>21,22</sup> Indeed, the iNKT TCR is capable of recognizing the head group of lipid antigens, ensuring specificity of antigen recognition and strength of signal. Hence, we hypothesized that milk-SM, which has a phosphocholine residue at the 1-OH position, but not egg-CE, which has only hydrogen in this position, might be able to activate iNKTs.<sup>22</sup> We observed that milk-SM-loaded but not egg-CE-loaded hCD1d tetramers stained a statistically higher number of peripheral blood (PB)-iNKTs previously expanded with  $\alpha$ Gal compared with unloaded hCD1d tetramers (Fig 3, A and B; see this article's Fig E6 in the Online Repository at [www.jacionline.org](http://www.jacionline.org)). Milk-SM-tetramer-TCR interactions were blocked by preincubation of cells with nonfluorescently conjugated PBS57-hCD1d tetramers, confirming that the milk-SM-tetramers were recognizing the iNKT invariant TCR and not binding nonspecifically to another structure on the surface of the iNKT cells. Milk-SM-loaded hCD1d recognized a mean percentage  $\pm$  SEM of iNKTs of  $0.02 \pm 0.005$  (median %, 0.027) and a mean absolute number/mL  $\pm$  SEM of  $4220 \pm 1170$  (median absolute number/mL, 3044). This is in contrast with unloaded tetramers, which recognized a mean percentage  $\pm$  SEM of iNKTs of  $0.0037 \pm 0.003$  (median %, 0.00044) and a mean absolute number/mL  $\pm$  SEM of  $432 \pm 304$  (median absolute number/mL, 82; Fig 3, A and B; Fig E6). This observation suggests that at least a small population of iNKTs from NDs has the capacity to bind milk-SM-loaded hCD1d tetramers.

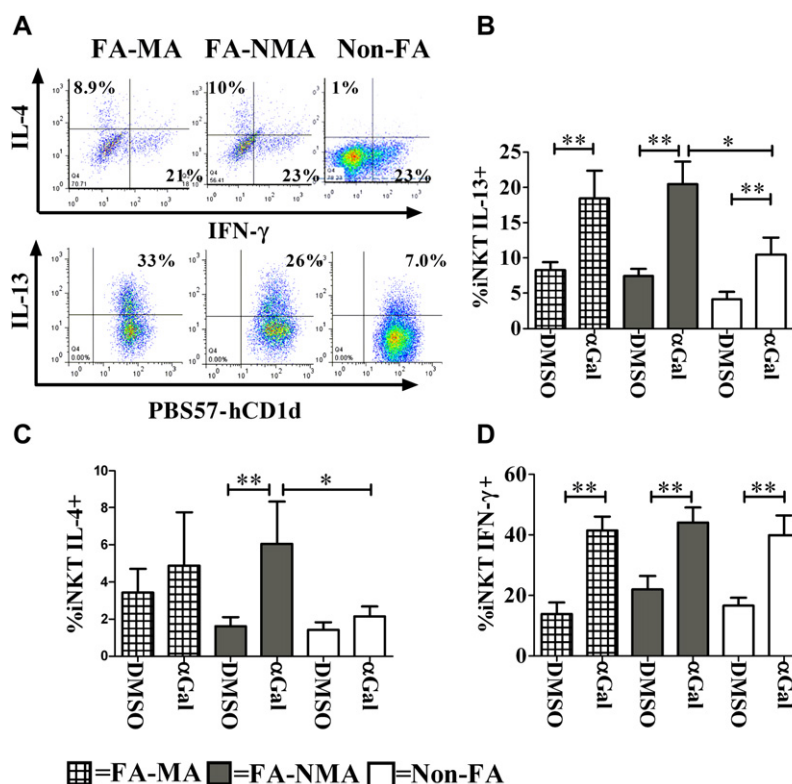


**FIG 2.** T<sub>H2</sub>-skewed cytokine responses of iNKTs from children with FA compared to with controls without allergy. PBMCs from 15 children with FA-MA, 12 children with FA-NMA, and 13 non-FA children were cultured for 10 days with αGal or DMSO, then stimulated for 4 hours with PMA and ionomycin and stained for intracellular cytokines. **A**, Dot plot of a typical experiment. **B-D**, Mean percentages of iNKTs staining for IL-13, IL-4 or IFN-γ. \**P* < .05; \*\**P* < .002.

### Milk-SM induces iNKT proliferation

To determine whether recognition of sphingolipids in the context of CD1d leads to TCR-mediated iNKT activation, we cultured PBMCs from 25 NDs in the presence of different lipids and assessed for iNKT expansion, CD25 expression, and iNKT proliferation. We observed that αGal or milk-SM, but not egg-CE or DMSO, induced an increase in the percentage and absolute number of iNKTs (Fig 4, A and B; see this article's Fig E7, A-G, in the Online Repository at [www.jacionline.org](http://www.jacionline.org)). Milk-SM, but not egg-CE, also induced CD25 expression on iNKTs, although less robustly than αGal (Fig 4, C; Fig E7, H). Finally, to confirm that the expansion of iNKTs after culture with αGal or food-SL was the result of iNKT proliferation, 5,6-carboxyfluoresceindiacetate succinimidyl ester (CFSE)-labeled iNKTs were stimulated with different lipids. αGal and milk-SM, but not egg-CE or DMSO, induced the proliferation of iNKTs, as determined by the dilution of CFSE (Fig 4, D). Collectively, these data reveal that naturally occurring milk-SM can engage the iNKT-TCR, presumably via its interaction with CD1d on antigen-presenting cells (APCs), and thereby lead to iNKT expansion and activation.

### More milk-SM-expanded iNKTs make IL-4 than αGal-expanded iNKTs

IFN-γ expression requires a longer and stronger iNKT-TCR stimulation than IL-4 expression.<sup>5</sup> The weaker proliferation of iNKTs after milk-SM stimulation (Fig 4) suggests that milk-SM

may bind only weakly to the iNKT-TCR. Therefore, we hypothesized that milk-SM would preferentially drive the production of IL-4 by iNKTs. To test this, we stimulated PBMCs that had been precultured with different lipids with PMA/ionomycin and measured the percentage of iNKTs producing IL-4 or IFN-γ. Consistent with our hypothesis, a greater percentage and number of milk-SM-expanded iNKTs expressed IL-4 (Fig 5, A; see this article's Fig E8, A-C, in the Online Repository at [www.jacionline.org](http://www.jacionline.org)) compared with αGal-expanded iNKTs, which preferentially produced IFN-γ (Fig 5, B; Fig E8, D-F).

To determine whether this cytokine response was a result of direct milk-SM-mediated iNKT activation as opposed to activation of other lineages within the PBMC cultures that then influenced iNKT cytokine responses, we cultured fluorescence-activated cell sorting-purified iNKTs from 5 NDs with DMSO-pulsed, milk-SM-pulsed, or αGal-pulsed APCs (Fig 5, C). After 14 days (the time necessary for iNKTs to expand sufficiently to provide enough cells for analysis), we measured the levels of mRNA encoding different cytokines in nonadherent cells. In these assays, we observed statistically higher levels of mRNA encoding IL-4 and reduced levels of mRNA encoding IFN-γ in iNKTs cultured in the presence of milk-SM compared with iNKTs cultured in the presence of αGal (Fig 5, D). We also observed a higher, but not statistically significant, increase in the level of IL-13-encoding and IL-5-encoding mRNA in the iNKTs from milk-SM-APC cultures. To assess the production of cytokines themselves, we sort-purified iNKTs from 5 NDs and cultured them for 72 hours with antigen-loaded APCs. Subsequently, we measured the levels

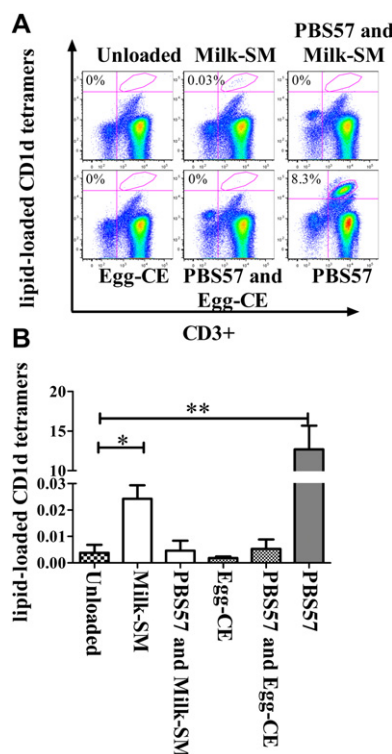


**FIG 3.** hCD1d tetramers loaded with milk-SM specifically bind to iNKTs.  $\alpha$ Gal-expanded PBMCs were stained with lipid-loaded hCD1d tetramers and anti-CD3 antibodies, or where indicated, were blocked by preincubation with nonfluorescently conjugated PBS57-hCD1d tetramers and then washed and stained with food-lipid-loaded hCD1d tetramers and anti-CD3 antibodies. **A**, Dot plot of a representative experiment. **B**, Mean percentage of CD3<sup>+</sup> cells stained with the indicated tetramers from 7 NDs. \* $P < .05$ ; \*\* $P < .005$ .

of cytokines secreted into the supernatants. Although we were not able to detect IL-4 under any of the conditions used, we observed lower levels of IFN- $\gamma$  and higher levels of IL-5 in the supernatants of iNKTs cultured with milk-SM-pulsed as opposed to  $\alpha$ Gal-pulsed APCs (Fig E8, G and H). These data suggest that iNKTs can indeed respond to the physiological presentation of milk-SM by APCs. Furthermore, milk-SM preferentially induced a T<sub>H</sub>2-skewed cytokine profile by human PB-iNKTs.

### Milk-SM-stimulated iNKTs expand more in children with FA-MA

Because milk-SMs activate ND-derived iNKTs to produce T<sub>H</sub>2 cytokines, we examined whether this effect would be greater in iNKTs from children with FA, who have a genetic predisposition to produce stronger T<sub>H</sub>2 responses.<sup>23</sup> Thus, we cultured PBMCs from children with FA and non-FA children for 10 days in the presence of milk-SM, egg-CE, or DMSO. We observed that milk-SM, but not egg-CE, induced a greater increase in iNKT percentage and absolute number in children with FA-MA compared with children with FA-NMA or non-FA children (Fig 6, A; see this article's Fig E9, A-C, in the Online Repository at [www.jacionline.org](http://www.jacionline.org)). Similarly, milk-SM upregulated CD25 expression more in iNKTs from children with FA-MA than the other 2 groups studied (Fig 6, B; Fig E9, D and E). Furthermore, when iNKTs from children with FA-MA were cultured in the presence of milk-SM and

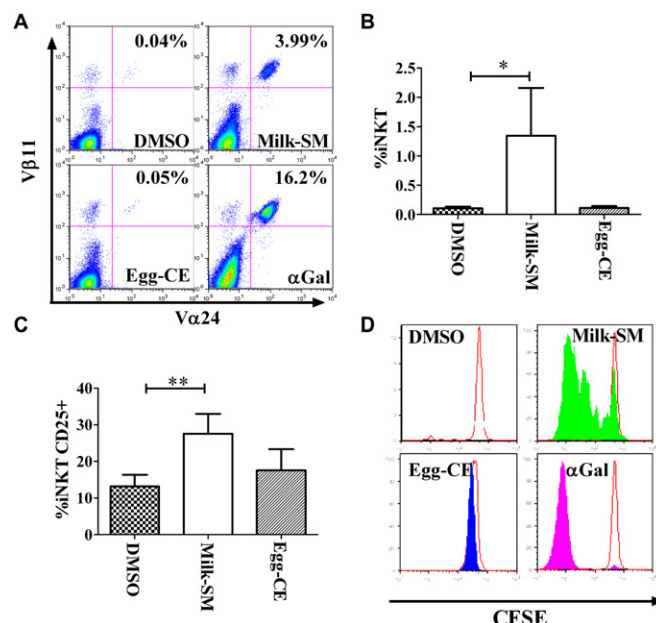


**FIG 4.** Milk-SM induces iNKT activation and expansion. PBMCs were cultured for 10 days with the indicated reagents. **A**, Dot plot of a representative experiment. **B**, Mean percentage of iNKTs after culture with lipids averaged from 25 NDs. **C**, Mean percentage of CD25<sup>+</sup> iNKTs averaged from 25 NDs. **D**, CFSE-labeled PBMCs were cultured with the indicated reagents. After 5 days, CFSE content in CD3<sup>+</sup>PBS57-hCD1d<sup>+</sup> cells was assessed by flow cytometry (n = 3 experiments). A representative experiment is shown. \* $P < .03$ ; \*\* $P < .001$ .

then stimulated with PMA/ionomycin, we observed more IL-13 and IL-4 and less IFN- $\gamma$  production, suggesting a stronger T<sub>H</sub>2 bias in cytokine production, compared with the other studied groups (Fig 6, C and D; see this article's Fig E10, A-D, in the Online Repository at [www.jacionline.org](http://www.jacionline.org)). Together, these observations demonstrate that iNKTs from children with FA-MA preferentially expand in response to milk-SM and that expanded iNKTs selectively produce T<sub>H</sub>2-type cytokines, suggesting that milk-SM might activate more iNKTs to produce T<sub>H</sub>2-type cytokines in certain individuals, such as those with FA to milk.

### DISCUSSION

In this study, we evaluated the response of iNKTs to food-SL in controls and children with FA. Our results indicate that there are quantitative and qualitative differences between the iNKT populations in controls and children with FA, especially in those with FA-MA, suggesting a possible role for iNKTs in the pathogenesis of FA. Here we demonstrate that children with FA-MA exhibit a 30-fold reduction in the percentage and a 15-fold reduction in absolute number of PB-iNKTs compared with non-FA children. The number of PB-iNKTs has been used as an indicator of iNKT involvement in human disease.<sup>14,24,25</sup> In conditions with low PB-iNKT numbers, there are conflicting reports regarding iNKT function.<sup>14,24-27</sup> To assess whether iNKTs in children with FA-MA have impaired function, iNKTs were stimulated with the potent iNKT agonist  $\alpha$ Gal *in vitro*. Despite lower starting percentages and absolute numbers, the iNKTs from children with FA-MA exhibited responsiveness to  $\alpha$ Gal that was similar to control iNKTs. From these experiments, we conclude that  $\alpha$ Gal-induced iNKT proliferation and activation are preserved in

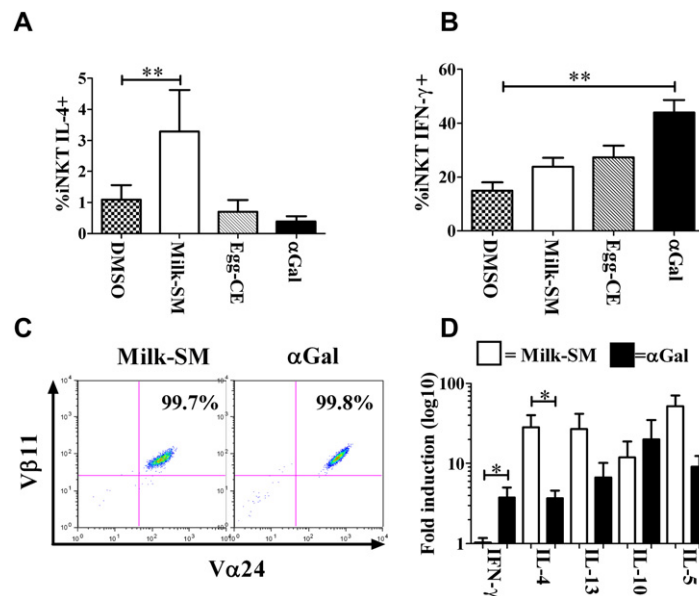


**FIG 5.** More milk-SM-activated iNKTs produce IL-4 compared with iNKTs activated by αGal. PBMCs from 25 NDs were cultured for 10 days with the indicated reagents and stimulated for 4 hours with PMA/ionomycin. **A** and **B**, Mean percentage of iNKTs expressing IL-4 or IFN-γ. **C**, Expansion of sort-enriched iNKTs cultured for 2 weeks with APCs prepulsed with the indicated reagents. **D**, Cytokine-mRNA expression of sorted iNKTs reported as relative expression of milk-SM or αGal versus DMSO-treated iNKTs (average from 5 different donors). \* $P < .05$ ; \*\* $P < .005$ .

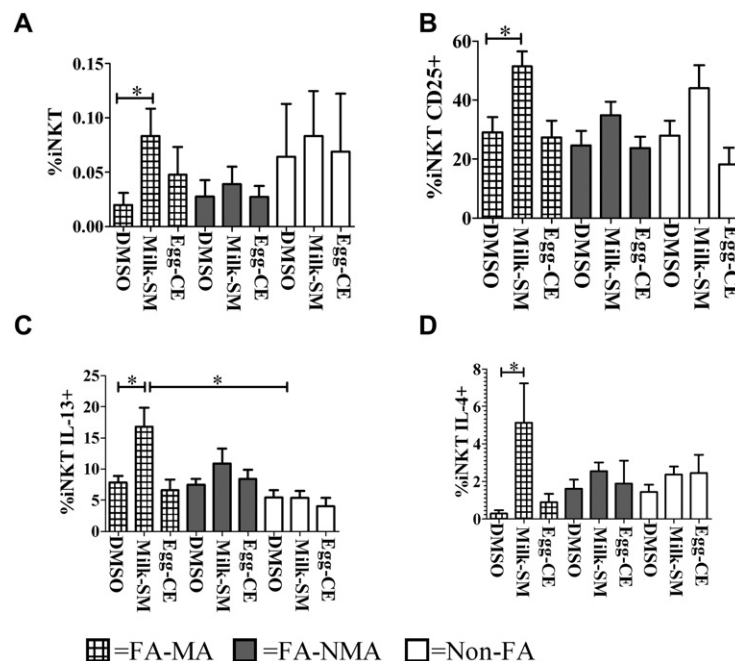


**FIG 6.** iNKTs from children with FA-MA expand more and have a stronger  $T_H2$  response to milk-SM than those from non-FA children. PBMCs from 15 children with FA-MA, 12 children with FA-NMA, and 13 non-FA children were cultured for 10 days with food-SL or DMSO. **A** and **B**, Mean percentage of total iNKTs and CD25<sup>+</sup> iNKTs when cultured with the indicated reagents. **C** and **D**, Mean percentage of iNKTs staining for IL-13 or IL-4. \* $P < .005$ .

children with FA-MA. We also observed that the iNKTs from children with FA, especially from those tolerant to milk, exhibited a skewed production of cytokines after expansion in the presence of αGal. Specifically, more iNKTs produced IL-4 and IL-13 in patients with FA compared with non-FA controls, although such a

difference reached statistical significance only in children with FA-NMA. These results mirror the findings previously reported in patients with asthma.<sup>28</sup> Thus, our results indicate a possible role for iNKTs in the development of FA through  $T_H2$ -type cytokine skewing.

The ligands that drive iNKT expansion and cytokine production in noninfectious diseases remain to be identified. Exposure of self-lipids because of lung inflammation or glycolipids from inhaled pollens has been postulated, but never demonstrated, to activate iNKTs in patients with asthma. All iNKTs proliferate in response to  $\alpha$ Gal, which is itself not believed to be a relevant physiological ligand in human diseases.<sup>8</sup> A small number of other ligands can activate iNKTs.<sup>8,29-31</sup> Interestingly, all these ligands are sphingolipids. Sphingolipids are widespread components found in eukaryotic cells, where they are sequestered (ie, not antigenic) in cell membranes.<sup>12,32</sup> Sphingolipids are common in many foods in our diet. Notably, the foods richest in sphingolipids (milk and egg) also represent the most common triggers of childhood FA.<sup>1</sup> Food-SLs inhibit colorectal cancer and in mice increase both  $T_H1$ -cytokine and  $T_H2$ -cytokine secretion in the small intestine by a mechanism that has not yet been clarified.<sup>20,33,34</sup> Thus, it remains possible that food-SLs could activate iNKTs.

To examine whether food-SLs specifically bind to the iNKT-TCR, hCD1d tetramers were loaded with milk-SM or egg-CE. Our experiments indicate that the iNKT-TCR was able to bind milk-SM on CD1d presentation, with a frequency of 300-fold fewer iNKTs compared with PBS57-loaded tetramers. When we cultured PBMCs from NDs in the presence of food-SL, we saw that milk-SM could expand iNKTs, albeit to a lesser extent than  $\alpha$ Gal. This finding mirrors the percentage of food sphingomyelin responsive iNKTs measured by tetramers and indicates that the iNKTs detected by tetramer analysis were not anergic.

Because we studied human iNKTs, which are relatively rare in the PB, in the current work we were not able to carry out an exhaustive specificity analysis by using many compounds related or unrelated to the ones tested. However, the fact that iNKTs failed to recognize egg-CE is indicative of some degree of specificity. We hypothesize that the difference in efficacy among different food-SLs in activating iNKTs may result from the diversity of the substituents at the 1-OH position of the sphingolipids.<sup>21,22</sup> Indeed, milk-SM, which has a phosphocholine residue at the 1-OH position, appears to be a stronger activator of iNKTs than egg-CE, which has only hydrogen in this position. Moreover, the length of the tail could explain why Fox et al<sup>35</sup> failed to find consistent activation of iNKTs using a synthetic sphingomyelin that contained a C18:1 acyl chain; our milk-SM is a mix of different sphingomyelin constituents with the main component C23:0 and the remainder C16:0, C18:0, C20:0, C20:4, C22:0, C24:0, and C24:1.

The molecular characteristics of sphingolipids influence the strength of signaling through the iNKT-TCR and determine distinct cytokine secretion patterns. Indeed, IFN- $\gamma$  expression requires stronger and longer iNKT stimulation than IL-4.<sup>5</sup> From our data, it appears that milk-SM is a weak ligand of the iNKT-TCR. Therefore, we hypothesized that it would drive an IL-4 response from iNKTs. To test this hypothesis, we measured iNKT  $T_H1$  (IFN- $\gamma$ ) and  $T_H2$  (IL-4, IL-13) cytokine expression after expansion with food-SL and stimulation with PMA/ionomycin. We recognize that this stimulation is not physiologic; however, the low numbers of iNKTs, even after milk-SM expansion, mandated the use of such an approach. With this method, we found that more ND milk-SM-responsive iNKTs secreted IL-4 compared with cells activated by  $\alpha$ Gal. To validate these data, we sorted iNKTs from 5 NDs and expanded them by using milk-SM-pulsed APCs. We subsequently assessed the levels of mRNA for  $T_H1/T_H2$ -type cytokines and confirmed that iNKTs

expanded with milk-SL exhibit more transcripts encoding  $T_H2$ -type cytokines even without PMA/ionomycin stimulation.

Given the ability of milk-SM to activate iNKTs specifically to produce IL-4, we hypothesized that this effect would be greater in children with FA, because they may have a genetic predisposition to produce stronger  $T_H2$  responses.<sup>23</sup> In this study, we showed that sphingolipids contained in milk activate iNKTs to express  $T_H2$  cytokines preferentially in children with FA-MA. This effect was different from the one observed with  $\alpha$ Gal stimulation, which induced more  $T_H2$  cytokine production in FA-NMA, suggesting a more specific  $T_H2$  response to milk-SM in children with FA-MA. The iNKT response we observed with milk-SM is typical of a  $T_H2$ -biased response. Although we do not have any information about the downstream immunologic effects of the food-reactive iNKTs observed in this work, one could postulate that iNKTs stimulated with milk-SM favor a  $T_H2$ -biased environment and thereby promote IgE class-switching during the humoral response to milk.<sup>3</sup> This effect could be stronger early in life when the adaptive immune system is not well developed and could revert later in life when the regulatory T population develops and children outgrow FA. There is indeed evidence to suggest that iNKTs can provide T-cell help to B cells, resulting in enhanced immunoglobulin secretion.<sup>36</sup> In addition, primary sensitization to protein antigens is promoted by iNKTs when an iNKT agonist is coadministered with the allergen.<sup>6</sup>

In summary, we demonstrate here that iNKTs are reduced in number in FA-MA, and to a lesser extent in patients with FA-NMA, and that both subsets of iNKTs exhibit  $T_H2$ -skewed responses after activation with  $\alpha$ Gal. Of note, iNKTs from patients with FA-MA but not FA-NMA demonstrate a similar skewing of cytokine responses after exposure to milk-SM. Thus, iNKTs may play a role in FA, and more specifically in FA-MA pathogenesis, by recognizing specific food-SL and serving to create a  $T_H2$ -skewed environment. Further studies are needed to address why PB-iNKTs are lower in patients with FA-MA and whether the observed  $T_H2$ -cytokine bias is reversible on acquisition of food tolerance. Finally, we will need to investigate whether the observed characteristics in our study are specifically linked to FA-MA or to atopic individuals in general. The results, however, define a novel mechanism of promoting atopic inflammatory responses after exposure to allergen-derived physiological ligands. This may present the opportunity for specific therapeutic modulation of this interaction to interfere with the immunologic pathophysiology underlying the current FA pandemic.

We thank Drs Michael Brenner and Patrick Brennan from the Division of Rheumatology, Immunology and Allergy, Brigham and Women's Hospital, Boston, Mass, for their constructive comments. We also thank Dr Kathleen Sullivan and Kelly Maurer from the Children's Hospital of Philadelphia, Allergy and Immunology Division, for their support.

**Clinical implications: Milk-SMs activate PB-iNKTs to produce  $T_H2$  cytokines, and this effect is greater in children with FA-MA. Hence, sphingolipids contained in milk may promote an iNKT-mediated  $T_H2$ -type cytokine bias that facilitates sensitization to food allergens.**

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## METHODS

### Study subjects

Patients on immunosuppressive therapies or with acute illnesses were excluded. PB samples were drawn after informed consent was obtained. PBMCs from NDs were purchased from the Human Immunology Core (University of Pennsylvania, BRB, Philadelphia, Pa).

### Reagents

All other antibodies, rhIL-2, Brefeldin A, and Cytoperm/Cytofix were from BD Biosciences (San Jose, Calif). rhIL-15 was from Sigma (Saint Louis, Mo). rhIL-7 was from Peprotech (Rocky Hill, NJ). rhGM-CSF and rhIL-4 were from R&D Systems (Minneapolis, Minn). All lipids were prepared in DMSO. AIM-V medium was from Life Technologies (Rockville, Md).

### Culture

PBMCs were isolated by Ficoll gradient sedimentation according to standard protocols (Amersham Pharmacia, Uppsala, Sweden). Autologous APCs were generated from PBMCs by incubation with rhGM-CSF (50 ng/mL) and hIL-4 (100 ng/mL) for 5 days.<sup>E1,E2</sup> APCs were pulsed with lipids or DMSO overnight (37°C) and then irradiated (5000 rad). Irradiated-prepulsed APCs were cocultured with sorted iNKTs at a 1:1 ratio for 2 weeks.<sup>E1</sup>

### Flow cytometry

The gating strategy used to identify the iNKT population is shown in Fig E1. For intracellular cytokine analysis, PBMCs were stimulated with PMA (25 ng/mL) and ionomycin (500 ng/mL) for 4 hours in the presence of Brefeldin-A. Cells were then fixed and permeabilized with Cytofix/Cytoperm and stained with the indicated antibodies.

### Measurement of iNKT proliferation and cytokine release

Cell proliferation was measured by using CFSE dilution as described.<sup>E3</sup> Cytokines released into the supernatant were measured with the BD Cytometric Bead Array (San Jose, Calif).

### RT-PCR analyses

RNA was extracted and cDNA prepared as described.<sup>E4,E5</sup> Gene expression was analyzed by real-time PCR with 6-carboxyfluorescein (FAM)-labeled probes purchased from Applied Biosystems (Foster City, Calif). The relative expression of the gene of interest to the housekeeping gene  $\beta$ -actin was calculated as described.<sup>E4,E5</sup>

### Statistical analysis

Statistical analyses were performed by using PRISM software (GraphPad, San Diego, Calif). The Wilcoxon signed-rank test for matched pairs and the Mann-Whitney *U* test for unpaired samples were used. A *P* value <.05 was considered statistically significant.

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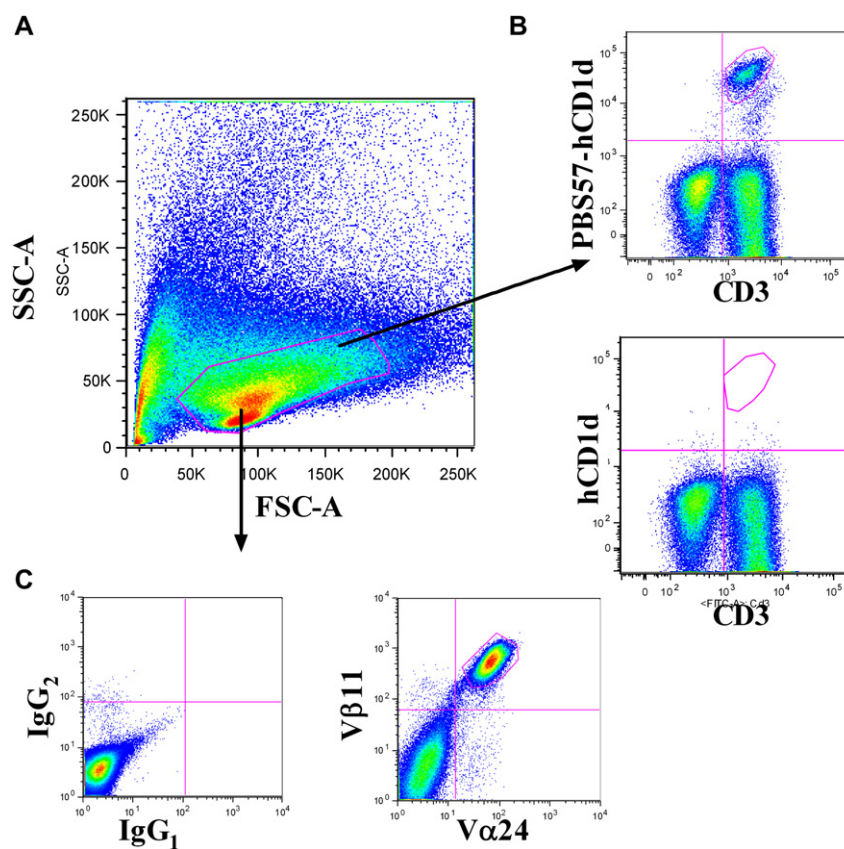


**FIG E1.** Gating strategy for iNKT detection. Representative profiles of iNKT frequencies detected in PBMCs (A) in a region made up of the lymphocytes by using (B) PBS57-loaded tetramer (*upper dot plot*) or unloaded human CD1d tetramer (*lower dot plot*), or (C) in combination with anti-CD3 or anti-Vα24 and anti-Vβ11 antibodies (*right dot plot*) or matched isotype control antibodies (*left dot plot*). FSC, Forward scatter; SSC, side scatter light.

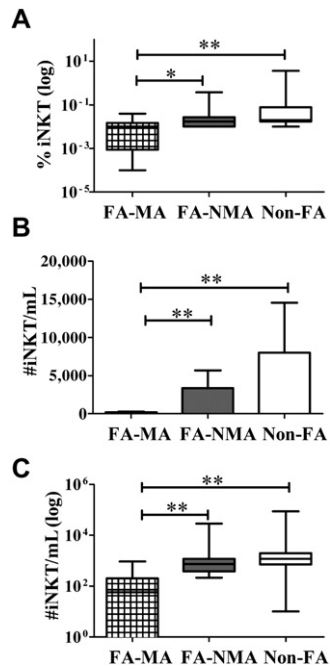


**FIG E2.** Children with FA-MA have fewer PB-iNKTs. Fresh PBMCs from 15 children with FA-MA, 12 children with FA-NMA, and 13 non-FA children were stained *ex vivo* for iNKTs (CD3<sup>+</sup>V $\alpha$ 24<sup>+</sup>V $\beta$ 11<sup>+</sup>). **A**, Median (*line in the box*), 25th and 75th percentiles (*lower and upper limit of the box*), and range (*upper and lower limits of whiskers*) of the percentages of iNKTs. **B**, Mean absolute number (#) of iNKTs per mL blood  $\pm$  SEM. **C**, Median, 25th and 75th percentiles, and range of absolute number of iNKTs per mL blood  $\pm$  SEM. \* $P < .02$ ; \*\* $P < .002$ .

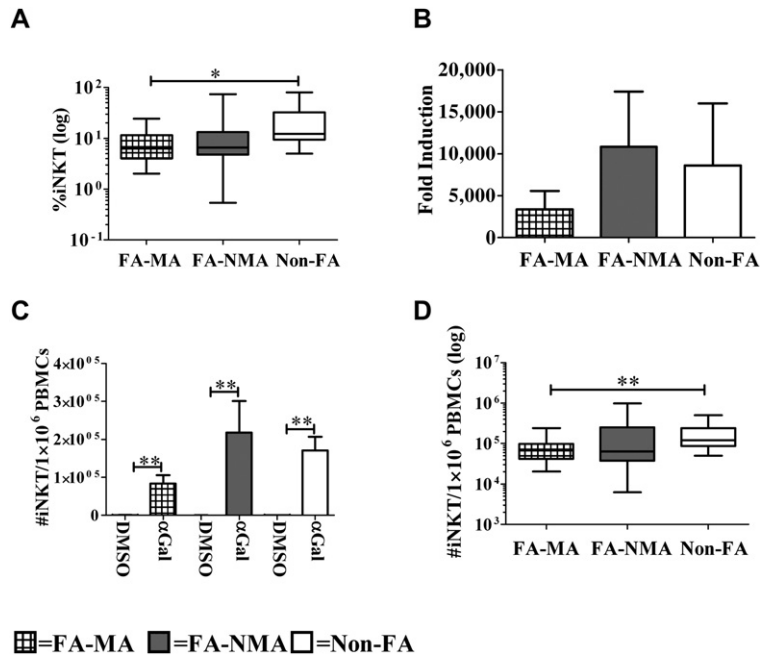


**FIG E3.** iNKTs from children with FA children expand to  $\alpha$ Gal. PBMCs were cultured for 10 days with  $\alpha$ Gal or DMSO. **A**, Median, 25th and 75th percentiles, and range of the percentages of iNKTs. **B**, Mean fold induction of the percentages of iNKTs treated with  $\alpha$ Gal compared with those treated with DMSO. **C**, Mean absolute number (#) of iNKTs in PBMCs pretreated with  $\alpha$ Gal. **D**, Median, 25th and 75th percentiles, and range of the absolute number of iNKTs per  $1 \times 10^6$  PBMCs. \* $P < .02$ ; \*\* $P < .002$ .

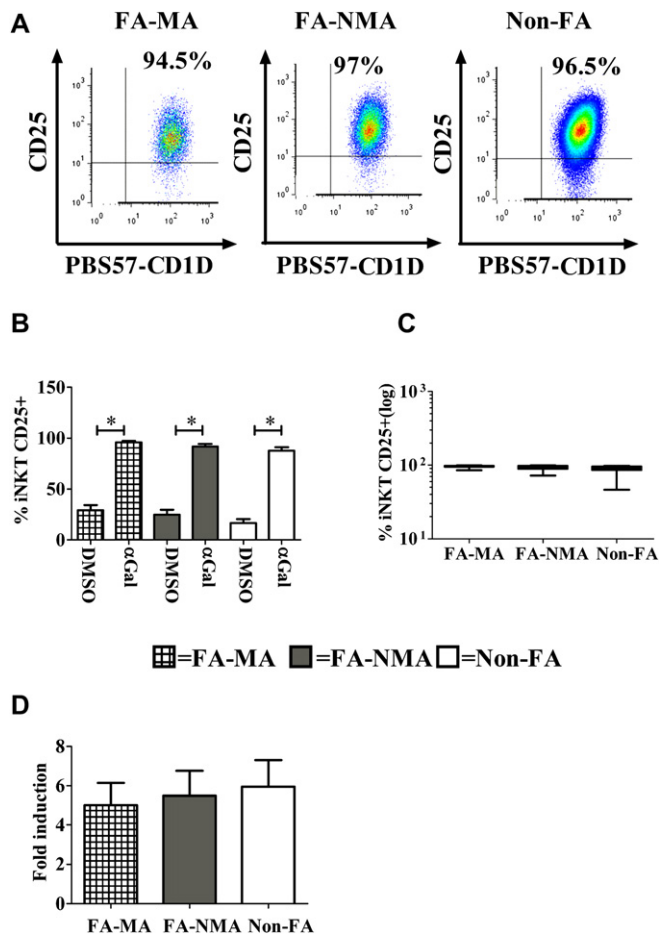


**FIG E4.** iNKTs from children with FA are activated by  $\alpha$ Gal. PBMCs were cultured for 10 days with  $\alpha$ Gal or DMSO. **A**, Typical experiment showing percentage of iNKTs expressing CD25. **B**, Mean percentage of iNKTs expressing CD25. **C**, Median, 25th and 75th percentiles, and range of the percentages iNKTs expressing CD25. **D**, Mean fold induction of the percentage of CD25<sup>+</sup> iNKTs after culture with  $\alpha$ Gal compared with those cultured with DMSO. \* $P < .02$ .

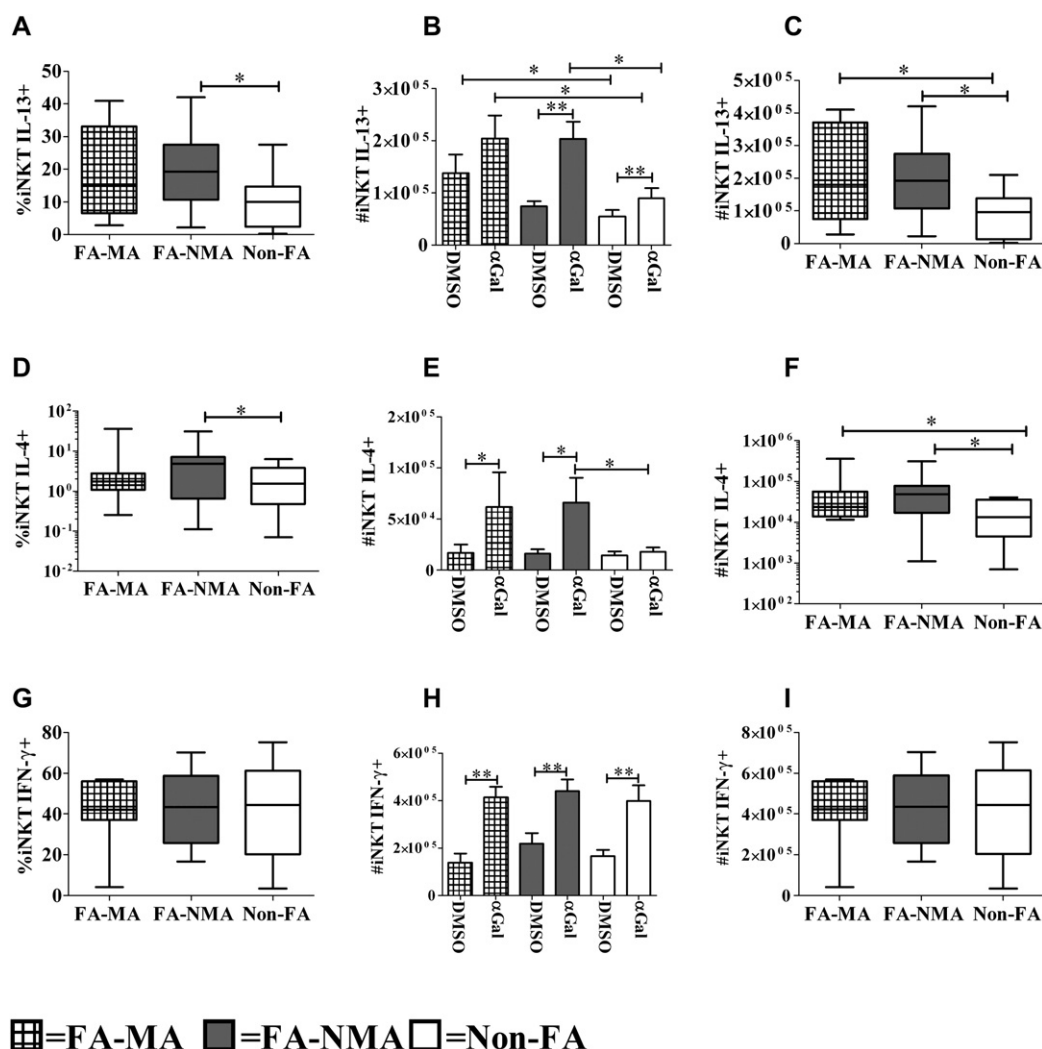


**FIG E5.** More iNKTs from children with FA express IL-4 and IL-13 than iNKTs from non-FA patients. After 10 days in culture with  $\alpha$ Gal or DMSO, PBMCs were stimulated with PMA and ionomycin and stained for intracellular cytokines. **A, D, G,** Median, 25th and 75th percentiles, and range of percentage of iNKTs expressing IL-13, IL-4, and IFN- $\gamma$  in  $\alpha$ Gal-stimulated cultures. **B, E, H,** Absolute number (#)  $\pm$  SEM of iNKTs per  $1 \times 10^6$  PBMCs expressing IL-4, IL-13, and IFN- $\gamma$ , respectively. **C, F, I,** Median, 25th and 75th percentiles, and range of the absolute number of iNKTs per  $1 \times 10^6$  PBMCs expressing IL-13, IL-4, and IFN- $\gamma$  in  $\alpha$ Gal-stimulated cultures. \* $P < .02$ ; \*\* $P < .002$ .

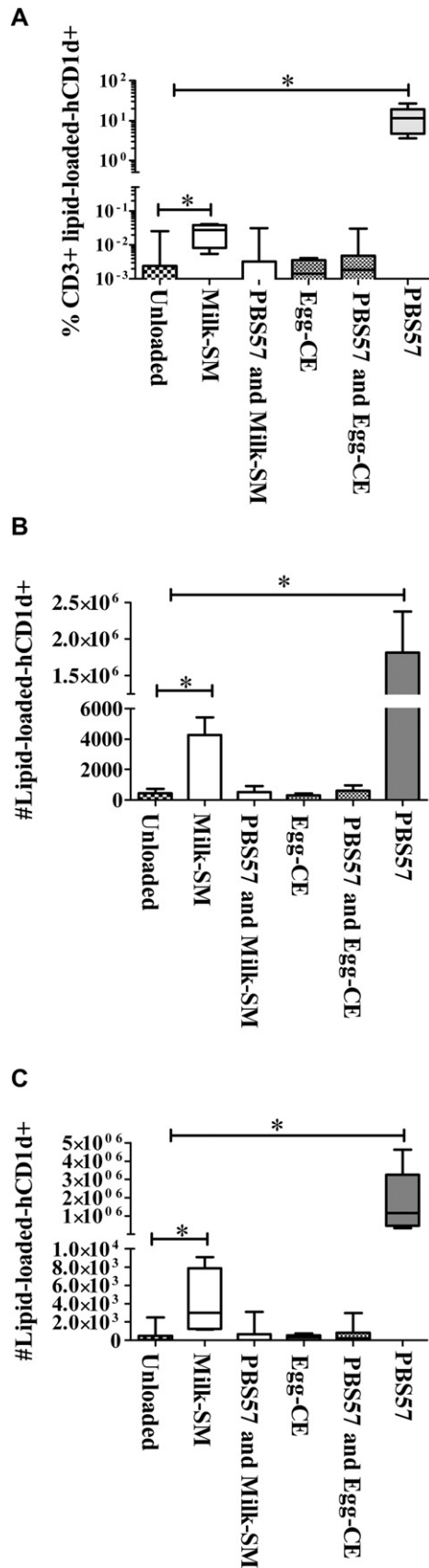


**FIG E6.** Human CD1d tetramers loaded with milk-SM specifically bind iNKTs.  $\alpha$ Gal-expanded PBMCs from 7 NDs were stained with lipid-loaded hCD1d tetramers and anti-CD3 antibodies, or where indicated, were blocked with PBS57-hCD1d and stained with food-lipid-loaded hCD1d tetramers and anti-CD3 antibodies. **A**, Median, 25th and 75th percentiles, and range of percentage iNKTs stained with the indicated tetramers. **B**, Mean absolute number (#) of iNKTs per  $10^6$  PBMCs that stained with the indicated tetramers. **C**, Median, 25th and 75th percentiles, and range of the absolute number of iNKTs per  $10^6$  PBMCs that stained with the indicated tetramers. \* $P < .05$ .

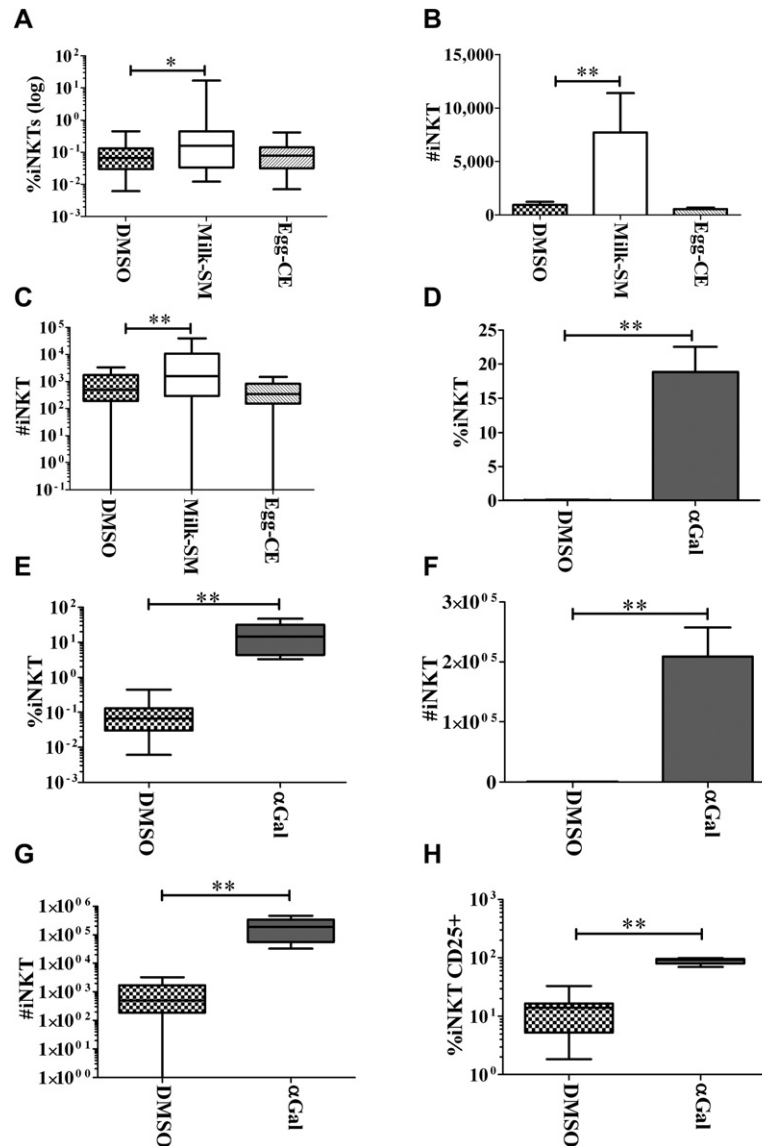


**FIG E7.** Milk-SM induces iNKT activation and expansion. PMBCs from 25 NDs were cultured for 10 days with indicated reagents and examined for expansion and CD25 expression. **A** and **E**, Median, 25th and 75th percentiles, and range of percentage iNKTs. **B** and **F**, Mean absolute number (#) of iNKTs (per  $1 \times 10^6$  PBMCs). **C** and **G**, Median, 25th and 75th percentiles, and range of the absolute number of iNKTs (per  $1 \times 10^6$  PBMCs). **D**, Mean percentage of iNKTs. **H**, Median, 25th and 75th percentiles, and range of percentage of CD25<sup>+</sup> iNKTs. \* $P < .05$ ; \*\* $P < .005$ .

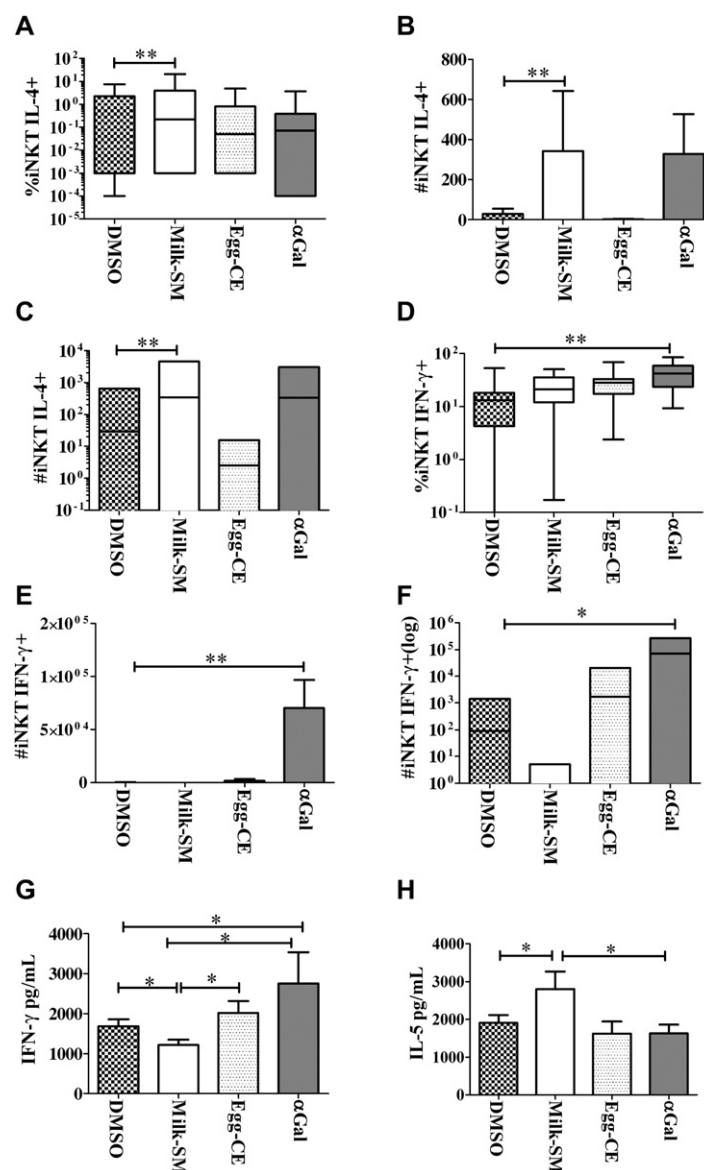


**FIG E8.** More milk-SM-expanded iNKTs produce IL-4 compared with iNKTs expanded by αGal. PBMCs from 25 NDs were cultured with the indicated reagents for 10 days, stimulated with PMA and ionomycin, and examined for cytokine production. **A** and **D**, Median, 25th and 75th percentiles, and range of the percentage of iNKTs. **B** and **E**, Mean absolute number (#) of iNKTs. **C** and **F**, Median and range of the absolute number of iNKTs. Fluorescence-activated cell sorting-sorted iNKTs from 5 different NDs were cultured with lipid-pulsed APCs for 72 hours, and release of IFN-γ (**G**) and IL-5 (**H**) was measured. \**P* < .05; \*\**P* < .003.

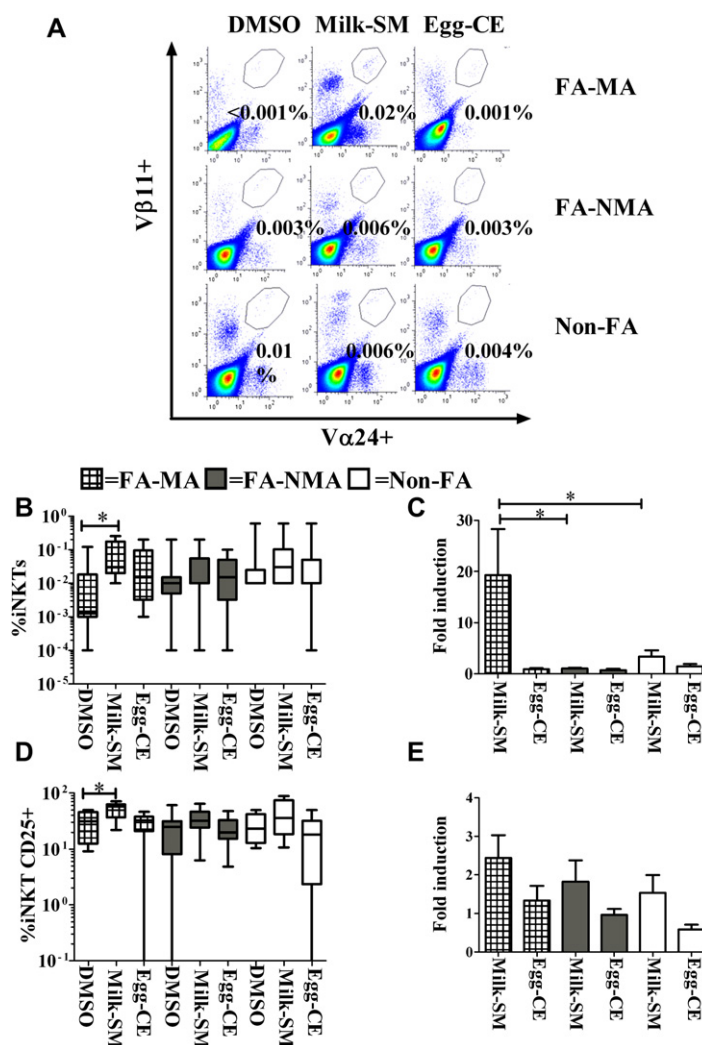


**FIG E9.** iNKTs from children with FA-MA expand more in response to milk-SM compared with iNKTs from non-FA children. PMBCs were cultured for 10 days with/without food-SL and then examined for expansion and CD25 expression. **A**, A typical experiment. **B**, Median, 25th and 75th percentiles, and range of the percentage of iNKTs. **C**, Mean fold induction of the percentage  $\pm$  SEM of iNKTs pretreated with indicated reagents compared with those pretreated with DMSO. **D**, Median, 25th and 75th percentiles, and range of the percentage of CD25<sup>+</sup> iNKTs. **E**, Mean fold induction of iNKTs expressing CD25 after culture with the indicated reagents compared with culture with DMSO. \* $P < .05$ .

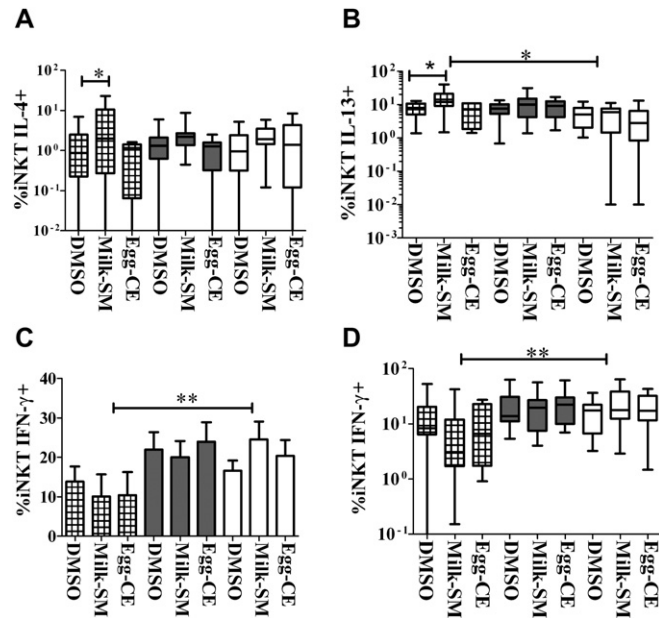


**FIG E10.** iNKTs from children with FA-MA exhibit a stronger T<sub>H</sub>2 response to milk-SM than those from non-FA children. PMBCs were cultured for 10 days with/without food-SL and then stimulated with PMA and ionomycin. **A, B, D,** Median, 25th and 75th percentiles, and range of the percentages of IL-4<sup>+</sup>, IL-13<sup>+</sup> or IFN-γ<sup>+</sup> iNKTs, respectively. **C,** Mean percentage of iNKTs staining for IFN-γ. \**P* < .05; \*\**P* < .005.

**TABLE E1.** Clinical characteristics of 15 children with cow's milk allergy

| Patient no. | Age (mo) | Skin prick test<br>(mm wheal × mm flare) | ImmunoCAP (kU/L) | Clinical reaction |
|-------------|----------|------------------------------------------|------------------|-------------------|
| 1           | 22       | NA                                       | Milk (1.3)       | Hives, vomiting   |
|             |          | NA                                       | Egg (3.0)        | Hives             |
|             |          | NA                                       | Peanut (0.9)     | Hives             |
| 2           | 72       | Milk (7 × 15)                            | NA               | Cough, wheeze     |
| 3           | 24       | Milk (5 × 25)                            | Milk (68.5)      | Hives, vomiting   |
| 4           | 72       | Milk (8 × 20)                            | Milk (>100)      | Hives, vomiting   |
| 5           | 96       | Milk (5 × 20)                            | Milk (13.8)      | Hives             |
|             |          | Egg (5 × 20)                             | Egg (2.5)        | Vomiting          |
| 6           | 24       | Milk (6 × 30)                            | Milk (71.9)      | Hives             |
| 7           | 96       | Milk (7 × 30)                            | Milk (4.3)       | Angioedema        |
| 8           | 48       | Milk (8 × 20)                            | Milk (>100)      | Hives             |
| 9           | 60       |                                          | Milk (>100)      | Anaphylaxis       |
|             |          | Peanut (15 × 30)                         | Peanut (>100)    | Anaphylaxis       |
| 10          | 96       | Milk (20 × 22)                           | Milk (84.4)      | Anaphylaxis       |
|             |          | Egg (4 × 15)                             | Egg (12.6)       | Anaphylaxis       |
|             |          | Peanuts (10 × 20)                        | Peanut (50)      | Anaphylaxis       |
| 11          | 96       | Milk (12 × 30)                           | Milk (9.3)       | Anaphylaxis       |
|             |          | Egg (10 × 20)                            | Egg (9.3)        | Anaphylaxis       |
|             |          | Peanuts (10 × 16)                        | Peanut (19)      | Anaphylaxis       |
|             |          |                                          |                  |                   |
| 12          | 108      | Milk (11 × 28)                           | Milk (>100)      | Anaphylaxis       |
|             |          | Egg (10 × 20)                            | NA               | Anaphylaxis       |
| 13          | 108      | Milk (10 × 12)                           | NA               | Anaphylaxis       |
| 14          | 72       | Milk (8 × 30)                            | Milk (3.2)       | Anaphylaxis       |
| 15          | 24       | Milk (4 × 25)                            | Milk (>100)      | Anaphylaxis       |

NA, Not available.

Some patients listed have multiple skin prick test or ImmunoCAP–positive foods. The foods with proven clinical reactions are listed. Positive tests without history of ingestion are not listed.

**TABLE E2.** Clinical characteristics of 12 children with FA-NMA

| Patient no. | Age (mo) | Skin prick test (mm wheal × mm flare) | ImmunoCAP (kU/L) | Clinical reaction |
|-------------|----------|---------------------------------------|------------------|-------------------|
| 1           | 36       | Egg (9 × 30)                          | Egg (31)         | Hives, vomiting   |
| 2           | 36       | Egg (12 × 15)                         | Egg (15.8)       | Angioedema        |
| 3           | 60       | Egg (5 × 25)                          | Egg (0.8)        | Hives             |
| 4           | 108      | Egg (15 × 18)                         | Egg (130)        | Hives             |
|             |          | Peanut (15 × 30)                      | Peanut (123)     | Angioedema        |
| 5           | 84       | Egg (4 × 10)                          | Egg (11.6)       | Hives, vomiting   |
|             |          | Peanuts (9 × 30)                      | Peanuts (>100)   | Hives, vomiting   |
| 6           | 24       | Egg (4 × 12)                          | NA               | Hives, eczema     |
| 7           | 72       | Egg (15 × 30)                         | Egg (>100)       | Hives, wheeze     |
| 8           | 24       | Egg (7 × 40)                          | Egg (7.7)        | Angioedema, hives |
| 9           | 48       | Egg (7 × 40)                          | Egg (7)          | Angioedema, hives |
|             |          | Peas (4 × 30)                         | NA               | Hives             |
| 10          | 48       | Egg (6 × 10)                          | Egg (5)          | Rash, vomiting    |
| 11          | 36       | Egg (7 × 13)                          | Egg (2.0)        | Anaphylaxis       |
| 12          | 72       | Egg (4 × 7)                           | Egg (0.5)        | Anaphylaxis       |

NA, Not available.

Some patients listed have multiple skin prick test or ImmunoCAP–positive foods. The foods with proven clinical reactions are listed. Positive tests without history of ingestion are not listed.