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

Soma Jyonouchi, MD,[a,b]* Valsamma Abraham, PhD,[a,b]* Jordan S. Orange, MD, PhD,[a,b] Jonathan M. Spergel, MD, PhD,[a,b] Laura Gober, MD,[a,b] Emily Dudek, BS,[a,b] Rushani Saltzman, MD,[a,b] Kim E. Nichols, MD,[b,c] and Antonella Cianferoni, MD, PhD[a,b] *Philadelphia, Pa*

Background: A key immunologic feature of food allergy (FA) is the presence of a T_H2-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_H2-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 α-galactosylceramide (αGal), 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_H2-type cytokine secretion. Children with FA, especially those with MA, had significantly fewer peripheral blood iNKTs and their iNKTs exhibited a greater T_H2 response to αGal 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_H2 bias in response to αGal and milk-sphingomyelin. These data suggest a potential role for iNKTs in FA. (J Allergy Clin Immunol 2011;128:102-9.)

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.[1] It is postulated that in FA the presence of T_H2 cytokines contributes to the breakdown of oral tolerance.[2-4] T_H2-type cytokines can be produced by a variety of cells including invariant natural killer T cells (iNKTs).[3,5] Indeed, iNKTs, when appropriately stimulated, promote a T_H2 response, IgE production, and subsequent sensitization to protein antigens.[6,7] Although iNKTs have pleiotropic roles in multiple diseases,[8,9] 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 Vα24-Vβ11 T-cell receptor (TCR). All iNKTs proliferate in response to α-galactosylceramide (αGal).[8] Only a limited number of endogenous or bacterial sphingolipids are known to activate iNKTs.[8,10] After engagement of their invariant TCR by sphingolipids, iNKTs rapidly upregulate costimulatory molecules and secrete T_H1-type and T_H2-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.[1] 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.[11] It is estimated that the average individual consumes approximately 0.3 to 0.4 g sphingolipids every day.[11] For example, dairy products contain 70 to 400 μg sphingomyelin per gram.[12] 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_H2-type cytokine production. Interestingly, the iNKT T_H2-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_H2-skewed environment in predisposed individuals by inducing iNKT cells to secrete T_H2-type cytokines.

From [a]the Division of Allergy and Immunology, [b]the Department of Pediatrics, and [c]the Division of Oncology, The Children's Hospital of Philadelphia.

*These authors contributed equally to this work.

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Reprint requests: Antonella Cianferoni, MD, PhD, Division of Allergy and Immunology, The Children's Hospital of Philadelphia, ARC 1216H, 3615 Civic Center Blvd, Philadelphia, PA 19104. E-mail: cianferonia@email.chop.edu. Kim E. Nichols, MD, Division of Oncology, The Children's Hospital of Philadelphia, Colket Translational Research Bldg, Rm 3012, 3501 Civic Center Blvd, Philadelphia, PA 19104. E-mail: nicholsk@email.chop.edu.

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Abbreviations used

αGal: α-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 ± SD, 5.4 ± 2.5 years; see this article's Table E1 in the Online Repository at www.jacionline.org) and 12 children tolerant to milk but with allergy to egg (FA-NMA; 10 boys, 2 girls; average age ± SD, 4.1 ± 1.8 years; see this article's Table E2 in the Online Repository at 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 ± SD, 4.38 ± 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).

Reagents

Hen's egg–ceramide (egg-CE; 860051P-endotoxin-free) and milk-SM (860063P-endotoxin-free) were purchased from Avanti Polar Lipids (Alabaster, Ala). αGal was from Alexis Biochemicals (Farmingdale, NY). Human CD1d tetramers were unloaded (hCD1d) or loaded with the αGal 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-Vα24 and anti-Vβ11 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), αGal (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).[13,14] iNKTs were also purified from human $CD3^+$ cells by cell sorting (95% to 99% purity; see additional Methods in the Online Repository).

Flow cytometry

iNKTs were defined as $CD3^+/V\alpha24^+/V\beta11^+$ or $CD3^+$/PBS57-$hCD1d^+$ by flow cytometry (see Methods and this article's Fig E1 in the Online Repository at 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.[8,10] 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 $CD3^+/V\alpha24^+/V\beta11^+$ iNKTs was consistent with those reported previously (mean % ± SEM, 0.29 ± 0.25; median %, 0.02; range, 0.001% to 3.6%; mean absolute number/mL ± SEM, 7982 ± 6542; median absolute number/mL, 1200; range, 0-86,400).[13,15-17] Similar results were obtained regardless of whether we identified iNKTs on the basis of Vα24/Vβ11 positivity or reactivity with PBS57-hCD1d tetramers (not shown). The percentage and absolute number/mL of iNKTs in children with FA-MA (mean % ± SEM, 0.01 ± 0.003; median %, 0.01; range, 0.001% to 0.04%; mean absolute number/mL ± SEM, 171.2 ± 81.78; median absolute number/mL, 57; range, 0-933) were significantly lower compared with children with FA-NMA (mean % ± SEM, 0.07 ± 0.04; median %, 0.017; range, 0.01% to 0.36%; mean absolute number/mL ± SEM, 735 ± 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).

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 αGal. iNKTs from children with FA-MA expanded in response to αGal, although the percentages of expanded iNKTs in αGal-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). The responsiveness of iNKTs from children with FA-MA to αGal was confirmed by measuring the expression of the activation marker CD25 on αGal-stimulated cells. αGal induced upregulation of