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Invariant Natural Killer T cells in children with Eosinophilic Esophagitis

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Abstract

Background—Eosinophilic esophagitis (EoE) is an atopic disease characterized by eosinophilic inflammation in which dietary antigens (in particular, milk) play a major role. EoE is most likely a mixed IgE and non-IgE food-mediated reaction in which over-expression of Th2 cytokines, particularly IL-13, play a major role; however, the cells responsible for IL-13 over-expression remain elusive. Th2-cytokines are secreted following the ligation of invariant natural killer T cell receptors to sphingolipids (SL). Sphingolipids (SL) are presented via the CD1d molecule on the iNKT cell surface. Cow's milk-derived SL has been shown to activate iNKTs from children with IgE-mediated food allergies to milk (FA-MA) to produce Th2 cytokines. The role of iNKTs and milk-SL in EoE pathogenesis is currently unknown.

Objective—To investigate the role of iNKTs and milk-SL in EoE.

Methods—Peripheral blood mononuclear cells (PBMCs) from 10 children with active EoE (EoE-A), 10 children with controlled EoE (EoE-C), and 16 healthy controls (Non-EoE) were measured ex-vivo and then incubated with α -galactosylceramide (α Gal) and milk-SL. iNKTs from peripheral blood (PB) and esophageal biopsies were studied.

Results—EoE-A-children had significantly fewer peripheral blood iNKTs with a greater Th2-response to α Gal and milk-SM compared to iNKTs of EoE-C and Non-EoE children. Additionally, EoE-A children had increased iNKT levels in esophageal biopsies compared to EoE-C children.

Conclusion—Milk-SLs are able to activate peripheral blood iNKTs in EoE-A children to produce Th2 cytokines. Additionally, iNKT levels are higher at the site of active esophageal eosinophilic inflammation.

Clinical Relevance—This study suggests that sphingolipids (SL) contained in milk may drive the development of EoE by promoting an iNKT cell-mediated Th2-type cytokine response that facilitates eosinophil-mediated allergic inflammation.

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Keywords

Eosinophilic esophagitis; invariant natural killer T cells; sphingolipids

INTRODUCTION

In recent years, eosinophilic esophagitis (EoE), an atopic disease characterized by esophageal eosinophilic inflammation, became increasingly prevalent (1). First described in 1993, its yearly incidence is now estimated to be equal to that of Crohn's disease (1, 2). Over the past several years, significant progress has been made in our understanding of EoE (3, 4). Dietary antigens, in particular, milk, play a major role in EoE pathogenesis (3, 5–7). EoE is most likely a mixed IgE and non-IgE food-mediated reaction (4, 8) in which T cell helper type 2 (Th2) cytokines, particularly Interleukin (IL)-13, thymic stromal lymphopoietin (TSLP), and eosinophilic chemokines such as eotaxin1-3/CCL-11-CCL24-CCL26 and RANTES/CCL5 are increasingly recognized as mediators, facilitating the establishment of a Th2 environment (9) (10). However, the cell-source and the initial stimuli responsible for IL-13 over-expression remain elusive.

Th2-type cytokines are produced by a variety of cells, one of which is the invariant natural killer T cell (iNKT), a subset of T cells responsive to sphingolipids rather than protein antigens (11, 12). iNKTs, when appropriately stimulated, promote a Th2-response leading to IgE production and subsequent sensitization to protein antigens (13, 14).

Recently we have shown that iNKTs may play a role in IgE mediated allergy to cow's milk (15). For the first time we demonstrated that cow's milk-derived sphingomyelin (milk-SM) could engage the iNKT-T Cell receptor (TCR) and induce iNKT proliferation and Th2-type cytokine secretion (15). Moreover, we demonstrated that iNKTs from children with IgE mediated food allergy (FA), especially those with milk allergy, are reduced in number and exhibit a Th2-bias in response to milk-SM. Furthermore, experimentation with gene-targeted mice revealed that peanut allergen-induced EoE was dependent on eotaxin and iNKTs, as CD1d and eotaxin-1/2 gene-deficient mice were protected from disease induction (16). The role of iNKTs in human EoE has not been previously reported.

For this experiment, we hypothesized that iNKTs play a role in EoE pathogenesis. To demonstrate such hypothesis, we measured the peripheral blood iNKT response to milk-SM in EoE-A, EoE-C, and healthy children (Non-EoE). Subsequently, we compared iNKT levels in esophageal biopsies and peripheral blood of EoE-A, EoE-C, and healthy children.

MATERIALS AND METHODS

Study Subjects

Twenty children with EoE were recruited from the Center for Pediatric Eosinophilic Disorders (CPED) at the Children's Hospital of Philadelphia (CHOP). Following most recent diagnostic criteria for EoE (17), children with typical EoE symptoms with a maximum eosinophil peak count of greater than 15/hpf in at least one of four esophageal pinch biopsies following eight weeks of proton pump inhibitor (PPI) therapy were defined as having active disease (EoE-A) (4, 18–20). Children who previously met criteria for EoE-A as defined above but now have less than 15 eosinophils/hpf in all four biopsies, no basal layer expansion, and no clinical symptoms were defined as EoE in remission (EoE-C). Our study population was 10 children with active EoE (EoE-A) (8 males, 2 females, avg age $\pm$ SD = 9.5 ± 5.6 years) and 10 children with controlled EoE (EoE-C) (4 males, 6 females, avg age $\pm$ SD = 8.8 ± 4.8 years). All children had been treated only with dietary intervention

(Table-E1). Sixteen children without a history of EoE, food allergy, or any other chronic disease (Non-EoE) were recruited from the primary care clinics and the Center for Eosinophilic disorders at CHOP (11 males and 5 females, with avg age $\pm$ SD = 8.7 ± 5.8 years). All investigations were approved by the CHOP Internal Review Board (See additional Methods information in the online repository).

Reagents

Cow's milk-SM (860063P) was purchased from Avanti Polar Lipids (Alabaster, AL). α -galactosylceramide (α Gal) was purchased from Alexis Biochemicals (Farmingdale, NY). Human CD1d tetramers (hCD1d), loaded or unloaded with the α Gal analogue PBS57 (PBS57-hCD1d), were provided by the MHC-Tetramer Core Facility at Emory University (Atlanta, GA). Anti-V α 24 and anti-V β 11 Abs were purchased from Coulter-Immunotech (Marseille, France). Anti-chemokine receptor (CCR) 3, 4, 5, and 7 were purchased from Becton Dickinson (San Jose, CA).

Esophageal Lymphocytes

Esophageal tissue from flash frozen biopsy was obtained from patients undergoing EGD for routine clinical care per IRB approved protocol. The esophageal biopsy was mashed through 70 μ m nylon mesh filters. Single cell suspensions were then analyzed via flow cytometry.

Culture

PBMCs were resuspended in complete medium (CM) (AIM-V; 10% FCS; rhIL-2 [40 U/ml]) in the presence of milk-SM (500ng/ml), α Gal (500ng/ml) or DMSO. After five days, half the CM was replaced with CM-supplemented with rhIL-2 (5ng/ml) and rhIL-15 (10ng/ml) (21) (22). (See additional Methods information in the online repository).

Flow Cytometry

INKTs were defined as CD3+/V α 24+/V β 11+ or CD3+/PBS57-hCD1d+ by flow cytometry (see Jyonouchi et al (15) for gating strategy used) and were expressed as those cells staining positive and compared to cells stained similarly using isotype-matched control antibodies or unloaded hCD1d tetramers. iNKs levels were reported as percentages of CD3+ lymphocytes and were considered undetectable if below <0.001%. Stained cells were collected using the FACScalibur Flow Cytometry System (Becton Dickinson, San Jose, CA). Data were analyzed using FlowJo8.3.3 software (Tree Star, Ashland, OR).

Stimulation of esophageal epithelial cells

Immortalized non-transformed human esophageal epithelial cells (EPC2-hTERT) were grown at 37° C in a humidified 5% CO₂ incubator, and maintained in keratinocyte serum free medium (KSFM, Invitrogen, city state) containing epidermal growth factor (1ng/mL), bovine pituitary extract (50ug/mL) (supplements provided by the manufacturer), and penicillin and streptomycin (100units/ml). Cells were seeded in triplicate and stimulated with poly (I:C) (103g/mL) [Invivogen (San Diego, CA)] for the indicated time points.

RNA isolation and Quantitative RT-PCR

RNA was harvested from EPC2-hTERT cells using an RNeasy kit (Qiagen, Valencia, CA) according to manufacturer's recommendations. RNA samples (0.53g/sample) were reverse transcribed using a high capacity cDNA reverse transcriptase kit (Applied Biosystems, Foster City, CA). Preformulated Taqman® Gene Expression Assays were purchased from Applied Biosystems for RANTES/CCL5 and GAPDH. Quantitative RT-PCR was performed using the Taqman® Fast Universal PCR Master Mix kit (Applied Biosystems) and reactions were performed in triplicate using 96-well optical plates on a StepOnePlus Real-Time PCR

System (Applied Biosystems). GAPDH was used as an endogenous control to normalize the samples using the $\Delta\Delta CT$ method of relative quantitation, where CT is the threshold cycle.

Cytokine assay

24 hours after poly (I:C) stimulation, cell culture supernatants were collected and stored at -80°C until further use. RANTES/CCL5 secretion was quantified using a BioRad BioPlex™ (Hercules, CA) cytokine assay according to manufacturer's recommendations.

Statistical analysis

Results are shown as mean $\pm$ SEM. Statistical significance was determined by 2-tailed *students t-test* and non-parametric two-tailed Mann-Whitney test. Correlation analysis was performed using a non-parametric, two-tailed Spearman rank-order test with a 99% confidence interval, and a linear regression of the data with a 99% confidence interval is displayed. Results were considered significant at $P < 0.05$ (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). (See Methods information in the online repository).

RESULTS

iNKTs in the peripheral blood are lower in children with active EoE

In many human disorders, iNKTs are reduced in number and/or function, suggesting a role for this lineage in disease pathogenesis (23, 24). To determine whether iNKTs are involved in EoE pathogenesis, we first measured the iNKT percentage (%) in peripheral blood in 10 children with EoE-A, 10 children with EoE-C, and 16 healthy controls (non-EoE) (Table-1E). In non-EoE children, the peripheral blood percentage of CD3+/Va24+/Vb11+ iNKTs was consistent with those reported previously in normal controls (mean % $\pm$ SEM 0.22 ± 0.16 , range 0.01–3.6%; median 0.02) (21, 25–27). Similar results were obtained regardless of whether we identified iNKTs based on Va24/VB11 positivity or reactivity with PBS57-hCD1d tetramers (Figure 1A-and not shown). The percentage of iNKTs in the peripheral blood was significantly lower in EoE-A children (mean % $\pm$ SEM 0.01 ± 0.006 , range 0.001–0.07; median 0.01) compared to EoE-C children (mean % $\pm$ SEM 0.16 ± 0.08 , range 0.01–0.86%; median 0.05; $p=0.0034$) or to Non-EoE children (Figure 1-AB) ($p=0.0006$). This was similar to what was observed in children with milk-induced IgE mediated food allergy (15). No differences in the percentage of CD3+ T-cells were noticed among the groups studied (EoE mean percentages 72.8 ± 2.4 , Non-EoE 72.1 ± 3.2 , EoE-A 71 ± 3.9 , EoE-C 74 ± 1.6).

iNKTs from Children with EoE expand normally

To investigate whether the lower number of iNKTs observed in EoE-A children was due to a reduced proliferative capacity, we examined iNKT responsiveness to a 10 day *in vitro* culture with a weak or potent iNKT cell agonist Milk-SM or αGal , respectively. In contrast with children who have an IgE-mediated Food allergy to milk, iNKTs in both EoE (all 20 children with EoE regardless of their activity status) and non-EoE children expand when stimulated with milk-SM and αGal (Figure 2A and 2B). However, only iNKTs from children with EoE controlled, (EoE-C) but not EoE active (EoE-A), expand to milk-SM at a similar level as iNKTs in healthy (Non-EoE) children (Figure 2C)(15). This data suggests that children with active EoE may have fewer iNKTs responsive to milk-SM in the peripheral blood, possibly because they are recruited to the site of inflammation. Indeed iNKTs from children with active EoE don't appear to be anergic when stimulated with αGal (Figure 2D).

iNKTs levels are higher in esophageal biopsies of children with active EoE (EoE-A) compared to those with controlled EoE (EoE-C) or healthy controls (Non-EoE) and are inversely correlated with their PB iNKT levels

In order to determine if lower levels of iNKTs in the peripheral blood could be due to tissue sequestration we obtained biopsies from 6 EoE-A, 5 EoE -C, and 4 non-EoE children (patients characteristics are described in Table 2E), and analyzed their iNKT content via Flow cytometry. We observed that iNKTs in biopsies were significantly higher in children with EoE-A compared to those with EoE-C and non-EoE children ($p<0.005$, $p<0.05$ respectively) (Figure 3). In addition, we saw that peripheral blood iNKT levels were inversely correlated with iNKT levels in esophageal biopsies. (Spearman $r=-0.69$, $p=0.004$)

EoE-A children have PB-iNKT that express less CCR 3, 4 and 5

Chemokine receptor (CCR) expression has been used as a marker for tissue localization of immune cells (1, 28, 29). Therefore, we postulated that in children with EoE-A iNKTs express specific CCRs driving their tissue sequestration. To test this hypothesis, we expanded iNKTs with α Gal in 8 EoE subjects and 8 non-EoE subjects and measured the CCR3, CCR4 and CCR5 (important in eosinophilic inflammation) and CCR7 (involved in lymph node homing) as a control (29). CCR5, CCR3 and CCR4 expression on iNKTs was significantly decreased in EoE-A children compared to those with EoE-C (Figure 4A-B); however, only CCR4 and CCR5 levels in children with EoE-A were significantly lower than non-EoE ($p<0.02$) (Figure 4B). Additionally, CCR4 and CCR5 levels are significantly correlated with the levels of PB iNKTs (spearman $r=0.6$, $p<0.03$) in both EoE-A and EoE-C children.

Rothenberg et al (30) have previously shown that key cytokines involved in EoE pathogenesis are induced by TLR3 ligand stimulation of esophageal epithelial cells *in vitro*, which mimic a viral infection. We next determined whether innate immune responses to TLR3 stimulation of human esophageal epithelial cells could similarly induce the expression of CCR3, CCR5 and CCR4 binding chemokines. Using the immortalized non-transformed esophageal epithelial cell line EPC2-hTERT, we found that 24 hours of stimulation with the synthetic TLR3 ligand poly(I:C) led to robust mRNA expression of the chemokine CCL5/RANTES compared to unstimulated controls (Figure 4C). Maximal poly(I:C)-induced secretion of CCL5 protein by EPC2-hTERT cells at the 24 hour time point was also confirmed by a quantitative cytokine assay (Figure 4D).

Using primary esophageal cells isolated from non-EoE and EoE pediatric patients, we observed a significant higher induction of RANTES expression upon poly(I:C) stimulation in EoE children (Figure 4E). These data suggest that the esophageal epithelium is a source of chemokine expression. CCR3, CCR4 and CCR5 all bind to RANTES. Hence the lower levels of chemokine receptors we observed on iNKTs from EoE children may be due to local recruitment of cells expressing the receptor or may be due to CCR internalization after binding to their specific ligand (31).

iNKT cells from children with EoE-A produce more Th2 cytokines when pre-stimulated with milk-SM

iNKTs have the capacity to produce both Th1 and Th2-type cytokines, but in some diseases they exhibit Th2 skewing (23, 32, 33). We have previously demonstrated that in children with IgE mediated milk allergy, iNKTs preferentially produce Th2-type cytokines (15). We questioned whether iNKTs from EoE-children might also exhibit a Th2 skewed cytokine profile when stimulated with milk-derived lipids, as we have observed with iNKTs from FA patients (15). To test this hypothesis, we measured Th1 (e.g. $\text{IFN}\gamma$) and Th2-type cytokine (e.g. IL-13 and IL-4) production by milk SM and α Gal expanded iNKTs following short-

term stimulation with PMA/ionomycin. We observed that a greater percentage of iNKTs from both EoE-A and EoE-C children expressed IL-13 in all tested conditions compared to controls (Figure 5A,B). We found that only α Gal stimulation resulted in a statistically significant increase in iNKT IL-4 in both EoE-A and EoE-C children (Figure 5C). Milk-SM was able to induce iNKT IL-13 secretion in children with EoE-A and EoE-C but not in non-EoE children as previously described in children with milk FA (Figure 5A and Figure E1) (15). Children with EoE-A also expressed higher levels of IL-13 when stimulated with milk-SM compared to EoE-C (Figure 5E, $p=0.0047$) (Figure 5B). In children with EoE, iNKTs produced more IL-4 both when stimulated with α Gal and when stimulated with milk-SM compared to DMSO, in contrast to non-EoE children in whom only milk-SM was able to induce greater IL-4 than DMSO (Figure 1E). Children with EoE-A had higher levels of IL-4 expression in all tested conditions compared to EoE-C (Figure 5C). No differences were noticed in IFN- γ expression in any of the tested conditions (Figure 5D and Figure 1E). Taken together, these observations demonstrate that iNKTs in children with EoE, whether active or control, produce increased Th2 cytokines and that milk-SM is able to specifically activate iNKT IL-13, a key cytokine involved in EoE pathogenesis.

DISCUSSION

In this study, we evaluated the response of iNKTs to food-SL in controls and in EoE-children. Our results indicate that there are quantitative and qualitative differences between the iNKT cell populations in EoE vs. Non-EoE-children. These differences are particularly pronounced in those with EoE-A, suggesting a possible role for iNKTs in the pathogenesis of EoE. Here we demonstrate that EoE-A children exhibit a 15-fold reduction in the percentage of peripheral blood iNKTs compared to non-EoE or EoE-C children. The number of peripheral blood iNKTs has previously been used as an indicator of iNKT involvement in human disease (15, 22, 34, 35). In conditions with low peripheral blood iNKT numbers, there are conflicting reports regarding iNKT cell function (15, 22, 34–36). This lower level could be due to recruitment of iNKTs to the site of inflammation or inhibition of their proliferation.

To assess whether iNKTs in EoE children have impaired function, iNKTs were stimulated *in vitro* with a potent (α Gal) and a weak (milk-SM) iNKT cell agonist. Despite lower starting percentages, iNKTs from EoE-A children exhibited good responsiveness to α Gal and milk-SM which was similar to control iNKTs (Figure 2); these results match our previously reported findings in children with IgE mediated milk allergy (15).

Consequently, we postulated that low peripheral blood iNKT numbers could reflect recruitment to the site of active eosinophilic inflammation. Hence, we analyzed the iNKT levels via Flow cytometry in esophageal biopsies from children with EoE –A and EoE-C. For the first time we described that iNKTs were significantly higher in esophageal biopsies obtained from children with EoE-A compared to those with EoE-C ($p<0.05$) (Figure 3). Peripheral blood iNKT levels were inversely related to iNKT levels in tissue samples suggesting that iNKTs may play a role in the pathogenesis of EoE by being recruited at the level of eosinophilic inflammation.

As a result, we measured the CCR surface expression of peripheral blood iNKTs to provide a mechanism for iNKT localization (1, 28, 29). We found that iNKTs from children with EoE-A had lower levels of CCR5, CCR3 and CCR4 and that lower levels of CCR4 and CCR5 correlated with lower level of peripheral blood iNKTs. CCR3, CCR4 and CCR5 are ligands for important recruitment eosinophilic factors including to Eotaxin-3 and CCL5 (RANTES) which has been found to be up-regulated in EoE or other type of eosinophilic disorders and/or esophagitis (37, 38) (39) (40) (48). In the present study we also demonstrate

that mRNA expression of CCR3, CCR4 and CCR5 ligands was induced in primary esophageal epithelial cells after activation of the TLR3 pathway. We used TLR3 stimulation as it is known to stimulate other important cytokines for EoE inflammation such as TSLP (30) and because it mimics a viral infection. It is notable that viral gastroenteritis-like symptoms often precede the onset of EoE (30). The chemokines that was most strongly induced by TLR3 activation was RANTES/CCL5 in esophageal epithelial cells. CCL5 was also expressed at significantly higher levels in primary epithelial cells derived from EoE children compared to Non-EoE children. CCL5 binds to CCR3, CCR5 and CCR4 (41). Interestingly, even if CCR5 is considered the primary binding receptor for CCL5, in some cells subtypes, such as mast cells, it is responsible for CCL5 mediated chemotaxis. It is not known which receptor is important for RANTES-induced chemotaxis in iNKTs. The lower levels of chemokine receptors we observed on peripheral blood iNKTs may be due to esophageal recruitment of cells expressing the receptor or may be due to CCR internalization due to their activation when ligand is present (31). Taken together these results suggest that epithelial-derived RANTES may contribute to iNKT recruitment at the site of eosinophilic inflammation.

We studied iNKT intracellular cytokine expression after milk-SM or α Gal expansion and PMA/Iono stimulation to understand if iNKTs contribute to local inflammation by secreting Th2 cytokines. Although we recognize that our iNKT stimulation is not physiologic, we believed that low numbers of iNKTs, even after milk-SM expansion, mandated the use of such an approach to study cytokine release from iNKTs (15). In the present study we show that the expression of prototypical Th2 cytokines (IL-13 and IL-4), but not the prototypical Th1 cytokine (IFN γ), was more pronounced in children with EoE regardless of their disease's activity (Figure 1E). Interestingly, IL-13 was only specifically induced by milk-SM in children with EoE regardless of their disease's activity, and EoE-A children had specifically increased levels of IL-13 after milk-SM stimulation compared to EoE-C children (Figure 5B). These results mirror the findings previously reported in patients with IgE mediated milk FA and suggest that iNKTs may contribute to Th2 inflammation by secreting Th2 cytokines if appropriately activated in a Th2 skewing environment such as the one present in a diseases characterized by high levels of TSLP (24, 42) (15, 43).

Our results indicate a possible role for iNKTs in the development of EoE through Th2-type cytokine skewing, possibly stimulated by milk-derived lipids. Milk is the most common food allergen trigger in EoE indicating a potential pathway for milk inducing EoE. The ligands that drive iNKT expansion and cytokine production in non-infectious diseases remain to be identified. All iNKTs proliferate in response to α Gal, which is not believed to be a relevant physiological ligand in human diseases(23). A small number of other sphingolipid (SL) ligands able to activate iNKTs have been identified(23, 44–46). Sphingolipids are widespread membrane components found in eukaryotic cells, where they are sequestered (i.e. not antigenic) in cell membranes (47, 48). However they are also common in many foods in our diet. Notably, the foods richest in SL (*i.e.* milk, egg) also represent the most common triggers of childhood FA and EoE(49). Our results also indicate that iNKTs stimulated by milk-derived lipids could be an important source of IL-13, a key cytokine in EoE pathogenesis, providing an alternative mechanism for milk-induced inflammation that differs from classical protein antigen mediated T-cell activation and IgE sensitization.

In summary, iNKTs are reduced in number in children with EoE-A compared to children with EoE-C and normal children. iNKTs in esophageal biopsies are elevated in children with EoE-A and may be recruited at the site of inflammation via CCR3, 4 and 5 ligation. iNKTs from children with EoE exhibit Th2-skewed responses with increased production of IL-13 following milk-SM exposure. Children with EoE-A appear to produce the greatest amounts

of IL-13 following stimulation with milk-SM. Thus, iNKTs may play a role in EoE pathogenesis by recognizing specific milk-derived lipids and secreting cytokines that help establish a Th2-skewed microenvironment. Milk is the most common food allergen trigger in EoE (50) and a milk-free diet often induces remission in children with EoE even if no objective signs of sensitization (serum IgE, skin prick and patch tests results) (8) are found, suggesting no involvement of IgE-mediated immune mediated mechanisms. The mechanism described above could explain the clinical symptoms induced by milk in children with EoE.

Our results, for the first time, define a possible role of iNKT and milk-derived lipids in the pathogenesis of this complex inflammatory disease.

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Abbreviations

Abs	antibodies
αGal	α-galactosylceramide
milk-SM	cow's milk sphingomyelin
CCL	chemokine ligand
CCR	chemokines receptor
EoE	Eosinophilic Esophagitis
EoE-A	children with active eosinophilic esophagitis
EoE-C	children with controlled eosinophilic esophagitis
Non-FA-children	children without FA
FA-MA	children with allergy to milk
FA	food allergy
food-SL	food-derived sphingolipids
PBS57hCD1d	human CD1d tetramers loaded with PBS57
IFN-γ	interferon gamma
IL-4	interleukin 4
IL-13	interleukin 13
iGb3	isoglobotrihexosylceramide
iNKTs	invariant natural killer T cells
PB	peripheral blood
PBMCs	peripheral blood mononuclear cells
PIM4	phosphaatidylinositolmannoside

PE	phosphatidylethanolamine
SL	sphingolipids
Th1	T helper cell type 1
Th2	T helper cell type 2
hCD1d	unloaded human Cd1d tetramers
TSLP	Thymic stromal lymphopoietin

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Figure 1. EoE-A children have significantly fewer PB- iNKTs

Fresh PBMCs from 20 EoE children, and 16 Non-EoE-children were stained *ex vivo* for iNKTs (CD3+Vα24+Vβ11+) or (CD3+ Cd1D PBS57) mean levels of iNKT cells are reported expressed as percentage of CD3+ T cells. A) Shows a typical experiment B) Shows mean levels of iNKT % (CD3+Vα24+Vβ11+). Mann-Whitney test *p<0.05, **p<0.005. EoE = all children with EoE regardless of their activity status, EoE-A = children with active EoE, EoE-C= children with controlled EoE, Non-EoE = non-allergic healthy controls.

Figure 2. iNKT derived from children with EoE-C expand more upon milk SM stimulation PBMCs from 20 EoE (10 EoE-A and 10 EoE-C), 16 non-EoE children were cultured for 10 days with DMSO, milk-SM (See Panel B–C) or αGal (see panel D). Panel A) Shows a typical experiment Panel B show iNKT expansion to milk-SM in 20 children with EoE regardless of their activity status vs Non-EoE Panel-C- show iNKT expansion to milk-SM in 10 children with EoE-A vs 10 children with EoE-C Panel D show iNKT expansion after αGal stimulation in EoE, EoE-A, EoE-C and Non-EoE children. B,C and D) Show mean levels of iNKT % (CD3+Vα24+Vβ11 Mann-Whitney test *p < 0.05 EoE = all children with EoE regardless of their activity status, EoE-A = children with active EoE, EoE-C= children with controlled EoE, Non-EoE = non-allergic healthy controls.

Figure 3. iNKTs levels are higher in children in Esophageal biopsies of children with EoE-A compared to those with EoE-C and inversely correlate with their PB iNKT levels
Flash frozen biopsies of 11 children with EoE (5 EoE-A and 6EoE-C) and 4 healthy children (Non-EoE) were stained *ex vivo* for iNKTs (CD3+V α 24+V β 11+) and mean levels of iNKT cells are reported expressed as percentage of CD3+ T cells. Levels of iNKT % (CD3+V α 24+V β 11+) in biopsies and PB are reported. Mann-Whitney test * $p < 0.05$ ** $p < 0.005$. EoE-A = children with active EoE, EoE-C= children with controlled EoE, Non-EoE = non-allergic healthy controls.

Figure 4. EoE children have significantly fewer peripheral iNKTs expressing CCR3, CCR4 and CCR5, while Epithelial esophageal cells of children with EoE express significantly higher levels of RANTES

In panel A and B Fresh PBMCs from 8 EoE-A, 8 EoE-C and 8 non-EoE children were cultured for 10 days with α Gal and then analyzed for their CCR expression. (A) shows a typical experiment B) shows the mean percentage of iNKT expressing the indicated CCR. Mann-Whitney test $*p < 0.05$. **In panel C and D esophageal cell line EPC2-hTERT** were used In Panel C CCL5 RNA expression from EPC2-hTERT stimulated with Poly (I:C) for the indicated time was analyzed (N=3) **In panel D is shown CCL5 protein expression** from EPC2-hTERT stimulated with Poly (I:C) for 24 hours t-test $p < 0.03$ In panel E are primary

cells line obtained from 3 EoE patients (EoE) and from 3 healthy controls (Non-EoE) were stimulated with Poly (I:C) for 24 hrs, and CCL5 mRNA Levels were measured (N=3) T-test * $p < 0.04$,

Figure 5. iNKTs from EoE children are Th2 skewed and produce more IL-13 upon milk-SM stimulation, especially if they have EoE-A
 PMBCs from 20 EoE children (10 with EoE-A and 10 with EoE-C) and 16 Non-EoE - children were cultured for 10 days with food-SL or DMSO and then stimulated with PMA and Ionomycin. A) Shows a typical experiment B show percentage of iNKT expressing IL-13, percentage of iNKT expressing IL-4, Dpercentage of iNKT expressing IFN- γ . Mann-Whitney test **p <0.005 * <0.05 EoE = all children with EoE regardless of their activity status, EoE-A = children with active EoE, EoE-C= children with controlled EoE, Non-EoE = non-allergic healthy controls.