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Effect of Dietary Ribonucleotides on Infant Immune Status. Part 2: Immune Cell Development

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ABSTRACT

The objective of this study was to determine whether dietary ribonucleotides alter immune cell phenotypes or function in the first year of life. Newborn term infants in a double-blind, 12-mo, multicenter trial were randomized to cow milk formula groups with (FN, $n = 138$) or without (F, $n = 147$) 72 mg/L supplemental ribonucleotides. A nonrandomized HMF cohort ($n = 192$) was concurrently enrolled. Eighty-eight immune blood cell types were characterized by flow cytometry. Data were analyzed by multivariate ANOVA (MANOVA), ANOVA, and repeated measures analysis (RMA), with adjustments made for multiple comparisons. Ribonucleotide feeding changed subpopulations of T and natural killer (NK) cells. FN had higher numbers and percentages of memory/effector (M/E) cytotoxic/suppressor ($CD45R0^+CD8^+$, RMA) T, Fas$^+$ M/E ($CD45R0^+CD95^+CD3^+$, 6 mo) T, and $CD56^+CD16^-$ NK cells ($CD56^+CD16^-CD3^-CD8^-$, 12 mo), and higher percentages of M/E helper ($CD45R0^+CD4^+$, RMA) T, Tc1 ($IFN\gamma^+CD4^-CD3^+$, RMA), total interferon (IFN)γT ($IFN\gamma^+CD4^{+/-}CD3^+$, RMA), Th2 ($IL\text{-}4^+CD4^+CD3^+$, 7 mo), and $CD57^+$ NK-T cells ($CD57^+CD56^-CD3^+$, 6 mo, 7 mo) compared with F. Percentages of naive helper T ($CD45RA^+CD4^+$, 12 mo) and numbers and percentages of $CD56^+$ NK-T cells ($CD56^+CD16^-CD3^+CD8^-$, 2 mo, 6 mo) were lower in FN than F. Percentages of M/E cytotoxic/suppressor, Th2, and $CD56^+CD16^-$ NK cells in FN were significantly higher than F but were not different from HMF, whereas F was significantly lower than HMF. Ribonucleotide supplementation of infant formula supported increased T-cell maturation and affected immunoregulatory NK cell subsets. These FN-associated immune cell profiles either did not differ from those infants fed HMF or tended to be more like those fed HMF than those fed F. **(*Pediatr Res* 56: 891–900, 2004)**

Abbreviations

APC, allophycocyanin
CD, cluster of differentiation
ExHMF, exclusive human milk-fed
F, formula without nucleotides
FBS, fetal bovine serum
FN, formula with supplemented nucleotides
FSC, forward light scatter
HBSS, Hanks' balanced salt solution
Hib PRP, Hib capsular polysaccharide (polyribosylribitol phosphate)
Hib-TITER, *Haemophilus influenzae* type b vaccine
HMF, human milk-fed
IFN, interferon
M/E cell, memory/effector cell
NK cell, natural killer cell
PerCP, peridinin chlorophyll protein
RMA, repeated measures analysis
SSC, side light scatter
Staph, *Staphylococcus aureus*
SWI, Similac with iron
Tc cell, cytotoxic T cell
Th cell, helper T cell
WBC, white blood cell

Immaturity of the infant immune system may place infants at risk for infectious disease–induced morbidity and mortality (1). Further, mode of feeding may affect susceptibility inasmuch as infants fed human milk appear to have fewer infections than formula-fed infants (1). Maternal secretory antibodies against environmental pathogens largely mediate the disease-preventive effect of human milk. However, other potentially immunoenhancing and anti-infective substances in human milk (growth factors, cytokines, oligosaccharides, complement, enzymes, lactoferrin) may also provide protective effects (2).

Ribonucleotide 5' monophosphates (nucleotides) exhibit immunopotentiating and gastrointestinal developmental effects (3–5). The nucleotide content of human milk is approximately 72 mg/L, comprising nucleosides, nucleotides, mono-, di-, and triphosphates, nucleotide adducts (nucleotide-containing com-

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pounds such as coenzymes), and free bases (6). In a prior study, cow milk–based infant formula supplemented with 72 mg/L of dietary nucleotides enhanced *Haemophilus influenzae* type b and diphtheria toxoid immunization responses compared with an unsupplemented control formula (7). In a recent study in Taiwan (8), nucleotide supplementation of formula increased serum IgA levels and reduced diarrhea incidence in healthy, term infants.

Dietary-induced changes in immune status may also be reflected in peripheral blood immune cell populations. Previous reports comparing immune cells from cow milk formula– or human milk–fed infants identified changes mainly among lymphocyte subsets (T, B, and NK cells), and lymphocyte-mediated functions, such as antibody production, lymphocyte proliferation, and cytokine production (2,9–11). Kuchan *et al.* (3) observed that nucleotide supplementation of cow milk formula tended to increase some lymphocyte populations compared with a control formula.

Immune development of infants fed unsupplemented or nucleotide-supplemented cow milk formula was further evaluated in this longitudinal, masked, 12-mo, parallel feeding trial. A nonrandomized, initially HMF group was concurrently enrolled. Changes in immune cell development may be reflected by differences in cell numbers and/or percentages, maturation, or functional state. Responses to childhood immunizations during the first year of life are reported elsewhere (12).

METHODS

Ethical considerations. Only infants whose mothers had decided not to breast-feed before enrollment were recruited for the formula-fed groups. Infants received cow milk–based formula, medical checks, and study vaccines during the trial. Each participating site's Institutional Review Board approved the study. Written informed consent was obtained from parent(s) or legal guardian(s).

Subjects, study design, and formula. This 12-mo, prospective, randomized, double-blind, parallel, longitudinal study of healthy, term, formula-fed infants was conducted at 18 sites (local hospitals and pediatric practices) in 10 states (AR, CA, FL, GA, IL, LA, NC, NY, OH, and PA) from fall 1996 to spring 1998. Formula-fed infants were enrolled before 8 d of age, and randomized to the control formula (F, $n = 147$) or the same formula supplemented with 72 mg/L ribonucleotide 5' monophosphates (FN, $n = 138$). Subjects were fed the assigned formula *ad libitum* from enrollment to 12 mo of age. Infants in the HMF reference group ($n = 192$) were enrolled before 8 d of age, fed human milk exclusively for at least 2 mo, and followed the study visit schedule for 12 mo. After 2 mo, infants whose parent(s) chose to wean were fed commercially available Similac with Iron (SWI, Ross Products Division, Abbott Laboratories, Columbus, OH), which at that time was not supplemented with ribonucleotides. Other foods were permitted for all infants after 4 mo of age. SWI was comparable to F except for a higher level of linoleic acid and a different protein blend (12). The proportion of HMF subjects whose milk source was exclusively human milk, or human milk in combination with formula, respectively, was 54% and 73% at 3 mo, 35% and 60% at 5 mo, 22% and 34% at 8 mo, and 15% and 30% at 12 mo.

Each formula batch (manufactured by Ross Products Division, Abbott Laboratories) met or exceeded nutrient levels recommended by the Committee on Nutrition of the American Academy for Pediatrics (13), as required by the Infant Formula Act of 1980 and subsequent amendments. The nucleotide formula was supplemented with 72 mg/L of yeast-derived, food-grade ribonucleotide 5' monophosphate monomers. Formula compositions and ribonucleotide concentrations for each of the formula batches are reported elsewhere (12).

Subjects were immunized as previously described (12) with Hib-TITER (*Haemophilus influenzae b*), Tripedia DTaP (diphtheria toxoid, tetanus toxoid, acellular pertussis) pediatric vaccine (Connaught, Swiftwater, PA), and Orimune (oral poliovirus, Orimune, Lederle, Pearl River, NY) at 2, 4, and 6 mo of age, consistent with the 1996 and 1998 recommendations of the American Academy of Pediatrics.

Blood collection and immune cell analysis. Sodium heparinized, coded blood samples were collected at 2, 6, 7, and 12 mo, packaged to maintain room temperature (RT), and transported to Ross Products Division where they were tested within 24 h of collection.

One milliliter of blood from each sample was used for granulocyte phagocytosis assays (see below), and 100 μL removed for a total WBC count. The remaining blood volume was marked on the collection tube and centrifuged for 10 min at 430 $\times$ *g*. The plasma fraction was then removed with a sterile transfer pipette and stored at -80°C for serological testing. The remaining cell fraction, referred to as plasma-free blood, was reconstituted to its original volume with RPMI 1640 culture medium (BioWhittaker, Walkersville, MD) plus 5% FBS (HyClone Laboratories, Logan, UT).

Immunophenotyping. Total WBC numbers were determined electronically using a Coulter Multisizer II (Beckman Coulter, Inc., Fullerton, CA). Cell processing, staining procedures, and flow cytometric analyses were performed using standard methods for flow cytometry (13). Plasma-free blood samples were stained using MAb labeled with FITC, phycoerythrin, PerCP, or APC fluorochromes [Becton Dickinson Immunocytometry Systems (BDIS), San Jose, CA] mixed in 3 or 4 fluor combinations. Appropriate isotype control antisera were used with each assay. Samples were evaluated using BDIS FACScan, BDIS FACSCalibur, or BDIS FACSort flow cytometers and BDIS Attractors software, as described previously (13). For the discrimination of lymphocytes, monocytes, neutrophils, eosinophils, and basophils, 10,000 leukocytes were counted, and results expressed as a percentage of the total WBC count. The absolute numbers of cells per microliter of blood were derived by multiplying percentages of leukocyte subset populations of interest by the WBC count per microliter. Approximately 10,000 lymphocytes, discriminated by forward (FSC) and side light scatter (SSC) characteristics, were counted from each of the immunophenotyping tubes. Lymphocyte subset results were expressed as percentages of total lymphocytes. Absolute lymphocyte cell numbers were derived

by multiplying the lymphocyte count per microliter by the percentage of the subset of interest.

Intracellular cytokine expression. T-cell-derived IFN-γ and IL-4 intracellular cytokines were induced using phorbol 12-myristate 13-acetate and ionomycin (14), and T-cell subsets were identified by surface staining with anti-CD4 PerCP and anti-CD3 APC MAb. Intracellular cytokine staining and flow cytometric analysis were performed as described previously (14).

CD40 ligand expression. Plasma-free blood was stimulated for 4 h at 37°C in a shaking waterbath with 0.02 μg/mL phorbol 12-myristate 13-acetate and 1.25 μg/mL ionomycin, or HBSS (unstimulated control). Cells were stained for 15 min at RT with anti-CD40L FITC, anti-CD69 PE, anti-CD3 PerCP, and anti-CD19 APC MAb. Erythrocytes were then lysed using 0.15M ammonium chloride, leukocytes were washed with PBS (BioWhittaker) plus 1% FBS, and fixed with 1% paraformaldehyde. Cells were analyzed by flow cytometry as above.

Granulocyte extravasation/migratory potential. Plasma-free blood was incubated for 10 min with 10^{-8} M N-formyl-met-leu-phe (Sigma Chemical Co., St. Louis, MO) or HBSS (unstimulated control) in a 37°C shaking waterbath. Cells were washed with PBS and stained with anti-CD11b FITC and anti-CD62L (L-selectin) phycoerythrin MAbs for 15 min at RT. Erythrocytes were lysed with BDIS FACS lysing solution and leukocytes were washed once with PBS containing 0.2% sodium azide, and then fixed in 1% paraformaldehyde. Cells were analyzed by flow cytometry. Approximately 10,000 granulocytes, determined by FSC and SSC characteristics, were analyzed for CD11b and CD62L expression.

Granulocyte phagocytosis. One milliliter of whole blood was treated with 0.15M ammonium chloride to lyse erythrocytes. Remaining leukocytes were washed twice with Dulbecco's PBS (BioWhittaker) plus 5% FBS. Cells were resuspended in HBSS and incubated for 10 min in a 37°C shaking waterbath with either heat-killed, plasma-opsonized Staph (Calbiochem-Novabiochem, La Jolla, CA), propidium iodide (PI)-stained Staph (100 μg/mL PI in HBSS; Molecular Probes, Inc., Eugene, OR), or HBSS alone. Approximately 10,000 granulocytes, determined by FSC and SSC, were analyzed by flow cytometry. The median fluorescence channel of granulocytes ingesting fluorescent PI-labeled bacteria determined phagocytosis; the greater the amount of PI-stained bacteria ingested, the higher the median fluorescence channel.

Statistical methods. Formula groups were randomized within each site stratified by gender. Random permuted blocks of size 2, designed to achieve balance in the feeding allocation after every four subjects, were used. Although the HMF group was not randomized at enrollment it was included as a reference group, as is commonly reported in infant feeding comparisons.

Intent-to-treat analyses were performed on cellular outcomes. The majority of cell populations were not normally distributed, therefore, data from these populations were $\log_{10}$-transformed to normalize the data before statistical evaluation. To reduce the burden of testing 88 individual cell populations, data were analyzed in 24 clusters of immune function/type related cells (*e.g.* type 1 T-cell cluster contained two components: Th1 and Tc1 cells; Table 1) using a per protocol two-group (FN *versus* F) multivariate ANOVA (MANOVA), and a three-group *posthoc* MANOVA (FN *versus* F *versus* HMF), using site as a blocking factor. Sites with low enrollment were combined in these assessments. The rationale for MANOVA was that, if clusters of related cell populations did not show differences among the treatment groups, then the differences between individual cluster components might not be statistically significant. In essence, time point MANOVA on clusters of immunologically related cells were performed followed by time point ANOVA on all cluster component cell types if a cluster was found to be significantly different. For simplicity, MANOVA-triggered ANOVA analyses are referred to throughout as being identified by MANOVA. Multiple pairwise comparisons of cell types by three-group MANOVA (FN *versus* F *versus* HMF) were implemented using Holm's step-down procedure to adjust significance levels. The smallest p value was tested at $0.05/3 = 0.0167$; if this was not found significant, then testing stopped and the groups were declared not different, otherwise the second lowest p value was tested at $0.05/2 = 0.025$, continuing only when significance was detected; the largest p value was tested at 0.05. In addition, two- and three-group RMA (to indicate change over time) of select cell types (selected by visual inspection of the data) were analyzed using SAS PROC MIXED (SAS Institute, Cary, NC). Multiple pairwise comparisons by three-group RMA were implemented using Holm's step-down procedure, as described above. The smallest p value was tested at $0.05/12 = 0.0042$,

Table 1. *Immunological/biological cell clusters for MANOVA analysis*

		No. of cluster component cell types
Leukocyte differential clusters		
Lymphocyte		4
Granulocyte		3
Granulocyte function cluster		4
Monocyte		1
T cell clusters	*CD markers*	
$\gamma\delta$ T cell	($\gamma\delta^+$ $CD3^+$)	3
T cell differentiation	($CD4^+CD8^+$)	3
Helper cell	($CD4^+$)	6
Cytotoxic/suppressor cell	($CD8^+$)	7
Memory/effector cell	($CD45R0^+$)	11
Naive cell	($CD45RA^+$)	7
Apoptosis capable cell	($CD95^+$)	3
Type 1 T cell	($IFN\gamma^+$)	2
Type 2 T cell	($IL\text{-}4^+$)	2
$CD40L^+$ T cell	($CD40L^+$ $CD3^+$)	3
$CD40L^-$ T cell	($CD40L^-CD3^+$)	3
B cell clusters		
Auto antibody B cell	($CD5^+$)	3
Primary B cell	($CD23^+$)	1
Secondary B cell	($CD23^-$)	2
NK/NK-T cell clusters		
$CD8^+$ NK		2
$CD16^+$ ADCC NK		4
$CD57^+$ NK		1
$CD56^+/CD57^+$ NK-T		7
$CD8^+$ NK & NK-T		4
$CD16^+$ADCC NK & NK-T		7

and the second lowest at $0.05/11 = 0.0045$, etc., to account for the three feeding groups and four time points. Finally, exploratory *post hoc* statistical analyses were performed to expand the MANOVA observations: two- and three-group time point ANOVA (unadjusted for site as a blocking factor), and two- and three-group RMA were performed on all cell populations.

Due to the large proportion of individuals who failed to generate cytokine positive Th0 cells, a categorical variable (responders/nonresponders) was defined and a Cochran-Mantel-Haenszel test was performed comparing the proportion of Th0 responders among the feeding groups.

Null hypothesis testing of feeding group differences for each cell population was two-sided and considered statistically significant at $\alpha = 0.05$. Because of the large number of cell comparisons, and because many comparisons should be considered not truly independent, a p value correction for multiplicity of endpoints (cell types) was not performed.

Percentage changes in the number or proportion of lymphocytes between FN and F were calculated as follows: ([FN − F] ÷ F) × 100%. Percentage changes in the number or proportion of lymphocytes between either formula and HMF were calculated as follows: ([F or FN] − [HMF] ÷ HMF) × 100%.

RESULTS

Significant cell type differences between FN and F formulas were judged at p values ≤0.05 (two-group analyses), whereas significant cell type differences among HMF and formula groups (three-group analyses) were judged with the lowest p value ≤ 0.0167 (0.05/3), the second lowest at $p \leq 0.025$ (0.05/2), and the largest at $p \leq 0.05$, using Holm's stepdown procedure, as described above, to account for multiple group comparisons. Consequently, differences between formula groups identified by two-group statistical analyses did not always reach significance by three-group analyses; in these instances p values > 0.0167 but ≤ 0.05 were regarded as suggestive of trends in the data. The most likely explanation for the loss of significance by three-group analysis is that the variability among the groups was increased by the addition of HMF, therefore, some differences between formulas were no longer statistically significant due to higher variability and multiple comparison adjustments.

With the exception of the leukocyte differential, granulocyte function, total T, B, and NK cell populations, and because of the large number of populations identified (88 cell types), only those populations that demonstrated differences between FN and F are reported.

Nucleotides did not affect WBC and leukocyte differential. There were no significant differences in WBC counts or in the number or percentage of lymphocytes, monocytes, neutrophils, basophils, or eosinophils between FN and F (two-group MANOVA or ANOVA) during the course of the study. Furthermore, there were no significant differences in these leukocyte populations between either formula and HMF (three-group MANOVA or ANOVA), except at 6 mo, where lymphocyte numbers were higher in F compared with HMF ($p = 0.01$).

Nucleotides did not affect granulocyte function. There were no significant differences in granulocyte phagocytosis or granulocyte extravasation potential between FN and F (two-group MANOVA or ANOVA). In addition, neither formula was significantly different from HMF (three-group MANOVA). However, by three-group ANOVA both formula groups at 2 mo (FN < HMF, $p = 0.003$; F < HMF, $p = 0.002$) had a slightly lower increase in the density of CD11b (a marker of extravasation affinity) on activated cells compared with HMF; no differences in CD62L levels were noted (data not shown). Upon activation, granulocytes typically shed CD62L and increase the expression of cell surface CD11b (15).

Nucleotides did not affect total T cells, B cells, or NK cells; MANOVA and RMA. Figure 1, *a–c*, depicts the profiles of total T ($CD3^+$), total B ($CD19\&20^+$), and total NK ($CD56^+CD57^{+/-}CD16^{+/-}CD3^-$) lymphocyte percentages and numbers over time. Statistical analyses comparing the change from time point to time point, either within or among the three feeding groups were not performed, however, these profiles appeared to be similar among the three groups. Figure 1, *a–c*, reveals no significant differences between FN and F for these cell populations, but does show significant differences between formula-fed and breast-fed infants, as described below.

There were no significant differences in T ($CD3^+$), Th ($CD3^+CD4^+$), and Tc ($CD3^+CD8^+$) cell numbers or percentages between FN and F groups (two- or three-group MANOVA). However, both formula groups had significantly higher (up to 10%) numbers of T cells at 2 and 6 mo, and higher proportions (up to 18%) of T cells at 2, 6, and 7 mo compared with HMF (Fig. 1-*a* and Fig. 3-group MANOVA). These T-cell differences were driven by naive Th cells, and not M/E T cells, as described below. At 7 mo, T cell numbers in FN were not different from HMF, whereas F was significantly higher than HMF ($p = 0.005$).

There were no significant differences in B cell ($CD19\&20^+$) numbers or percentages between formula groups. However, both formula groups had significantly lower proportions (up to 17%) of B cells at 2, 6, and 7 mo compared with HMF. FN had significantly lower numbers of B cells at 7 mo compared with HMF ($p = 0.001$), whereas F was not different from HMF (Fig. 1*b*).

There were no significant differences in NK cell ($CD56^+CD57^{+/-}CD16^{+/-}CD3^-$) numbers or percentages between FN and F. However, NK cell proportions were significantly lower (14%) in both formula groups at 6 mo compared with HMF (Fig. 1*c*).

Nucleotides affected T-cell subsets; MANOVA and RMA. Figures 2–7 describe significant differences in T-cell subsets between FN and F, and compare each formula group to breast-fed infants. The proportion of naive helper ($CD45RA^+CD4^+$) T cells was significantly lower (5%) in FN compared with F at 12 mo (two-group MANOVA; Fig. 2). Both formula groups had significantly higher proportions (5–14%) of these cells at 2, 6, and 7 mo compared with HMF (three-group MANOVA). At 12 mo, naive helper T-cell percentages in FN were not significantly different from HMF, however, F was significantly

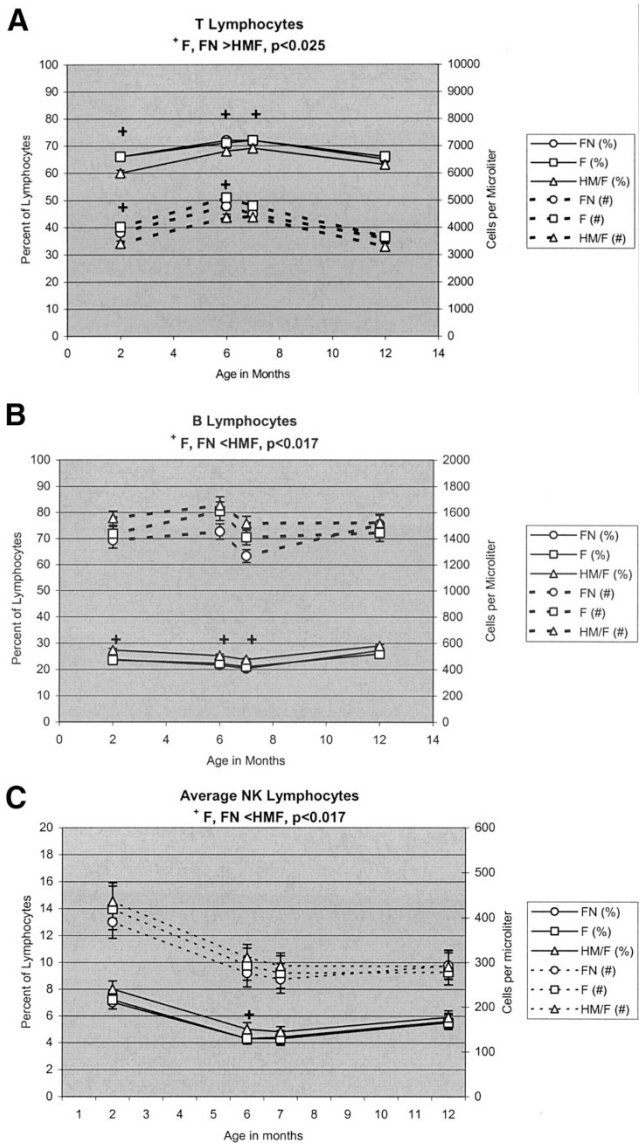

Figure 1. Panels *a–c* reveal no significant differences between FN and F but do show differences between formula-fed and breast-fed infants. (*a*) Both formula groups ($^+$) had significantly higher numbers of T cells ($CD3^+$) compared with HMF at 2 mo (FN > HMF, $p = 0.016$; F > HMF, $p = 0.0003$) and 6 mo (FN > HMF, $p = 0.022$; F > HMF, $p = 0.0001$), and higher percentages of T cells at 2, 6, and 7 mo (FN, F > HMF, $p = 0.0001$). FN numbers were not different from HMF at 7 mo, whereas F was significantly higher than HMF ($p = 0.005$). Data represent mean ± SEM. (*b*) Both formula groups (+) had significantly lower percentages of B cells ($CD19\&20^+$) compared with HMF at 2 mo (FN < HMF, $p = 0.0005$; F < HMF, $p = 0.0001$), 6 mo (FN < HMF, $p = 0.0001$; F < HMF, $p = 0.0005$), and 7 mo (FN < HMF, $p = 0.0001$; F < HMF, $p = 0.0004$). FN had significantly lower numbers of B cells at 7 mo compared with HMF ($p = 0.001$), whereas F was not different from HMF. Data represent mean ± SEM. (*c*) Both formula groups ($^+$) had lower percentages of NK cells ($CD56^+CD57^{+/-}CD16^{+/-}CD3^-$) at 6 mo (FN < HMF, $p = 0.006$, F < HMF, $p = 0.004$,) compared with HMF. Data represent geometric mean ± SEM.

higher than HMF, whereas FN was not different from F (three-group MANOVA).

Two-group RMA (indicating change across all time points) revealed higher percentages of M/E helper ($CD45R0^+CD4^+$) T cells in FN compared with F (Fig. 3). Neither formula group was significantly different from HMF (three-group RMA); however, the percentage differences in M/E helper T cells between FN and F were maintained. Figure 3 describes the MANOVA-identified time point differences between FN and F and comparisons with HMF.

Two-group RMA showed higher percentages and numbers of M/E cytotoxic/suppressor ($CD45R0^+CD8^+$) T cells in FN compared with F (Fig. 4). FN was not different from HMF in either percentages or numbers (three-group RMA), whereas F was significantly lower than HMF (percentages only) and FN (percentages and numbers). Figure 4 describes the MANOVA-identified time point differences between FN and F, and comparisons with HMF.

M/E Fas^+ (apoptosis competent, $CD45R0^+CD95^+CD3^+$) T-cell proportions (Fig. 5) were significantly higher (22%) in FN compared with F at 6 mo (two-group MANOVA). Neither formula group was different from HMF nor each other by three-group MANOVA.

Two-group RMA showed higher percentages of Tc1 ($IFN\gamma^+CD4^-CD3^+$) cells in FN compared with F (Fig. 6), and higher percentages of total $IFN\gamma^+$ (Th1 and Tc1) T cells in FN compared with F ($p = 0.026$, data not shown). Three-group RMA showed significantly higher percentages of Tc1 cells ($p = 0.007$) and total $IFN\gamma^+$ T cells ($p = 0.0002$, data not shown) in HMF compared with F. No pairwise differences were seen comparing FN to HMF or F. Tc1 cell proportions (Fig. 6) were significantly higher (33%) in FN compared with F at 12 mo (two-group MANOVA). F was lower than HMF at 7 mo by three-group MANOVA.

Th2 ($IL\text{-}4^+CD4^+CD3^+$) cell proportions (Fig. 7) were significantly higher (27%) in FN compared with F at 7 mo (two-group MANOVA). At 7 mo, Th2 cell percentages in FN were not significantly different from HMF (three-group MANOVA), whereas F was significantly lower than HMF and FN.

Subjects were defined as Th0 cell ($IFN\gamma^+IL\text{-}4^+CD4^+CD3^+$) responders or Th0 cell nonresponders based on the sensitivity of the intracellular cytokine assay (16).

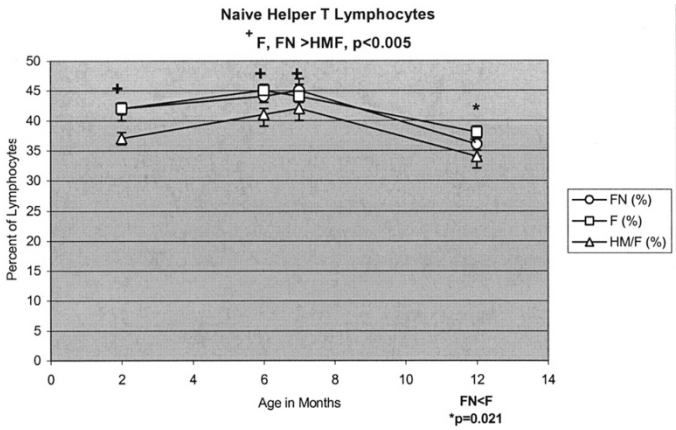

Figure 2. Naive helper ($CD45RA^+CD4^+$) T-cell percentages (*) were lower in FN *vs* F at 12 mo (two-group MANOVA). Both formula groups ($^+$) had higher percentages of these cells at 2, 6, and 7 mo *vs* HMF ($p < 0.005$). At 12 mo, naive helper T-cell percentages (three-group MANOVA) in F were significantly higher than HMF ($p = 0.0001$), whereas FN was not different from HMF ($p = 0.028$) or F ($p = 0.066$). Data represent geometric mean ± SEM.

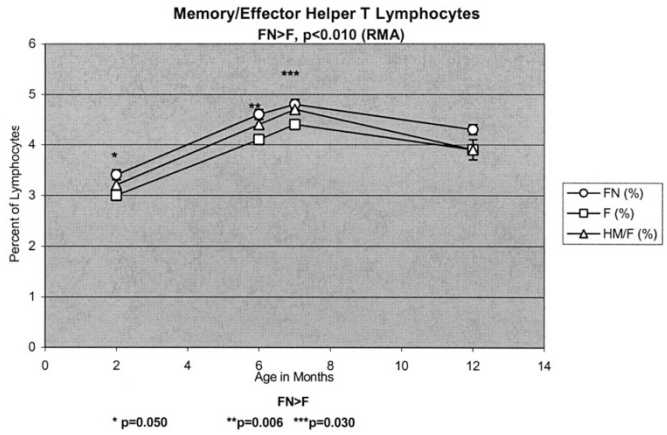

Figure 3. Two-group ($p = 0.006$) and three-group ($p = 0.009$) RMA revealed higher percentages of M/E helper T cells ($CD45R0^+CD4^+$) in FN *vs* F; neither formula group differed from HMF. M/E helper T-cell percentages were significantly higher in FN compared with F at ^{*2} (two-group MANOVA), $^{**}6$ (two-group MANOVA), and $^{***}7$ mo (two-group ANOVA). Neither formula group differed from HMF by three-group MANOVA, and the percentage differences between formulas at 2 ($p = 0.045$) and 7 mo ($p > 0.05$) did not reach significance, whereas at 6 mo ($p = 0.01$) percentages of these cells in FN were greater compared with F. Data represent mean ± SEM.

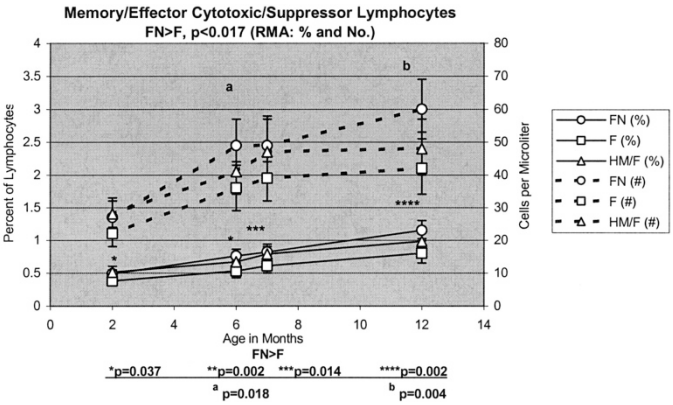

Figure 4. Two- and three-group RMA showed higher percentages and numbers of M/E cytotoxic/suppressor ($CD45R0^+CD8^+$) T cells in FN *vs* F (two-group, $p = 0.002$ and 0.006, respectively; three-group, $p = 0.006$ and $p = 0.012$, respectively). FN was not different from HMF in either percentages or numbers (three-group RMA), whereas F was significantly lower than HMF (F < HMF, $p = 0.009$, percentages only) and FN (F<FN $p = 0.002$ percentages; $p = 0.006$ numbers). Percentages of M/E cytotoxic/suppressor T (Tc) cells were significantly higher in FN *vs* F (two-group MANOVA) at ^{*2}, $^{**}6$, $^{***}7$, and $^{***}12$ mo. Percentages of M/E Tc cells in FN were not significantly different from HMF (three-group MANOVA) at 2 or 7 mo, although F was significantly different from HMF at 2 mo (F < HMF, $p = 0.007$) and tended to be different from HMF at 7 mo ($p = 0.037$). Neither formula significantly differed from HMF at 6 and 12 mo. Three-group MANOVA supported the formula differences in M/E Tc cells at 6 (FN > F, $p = 0.006$) and 12 mo (FN > F, $p = 0.005$), although the differences in percentages between FN and F at 2 and 7 mo did not reach significance ($p = 0.048$ and 0.026, respectively). Numbers of M/E Tc cells were higher at ^{a}6 mo and ^{b}12 mo in FN compared with F (two-group ANOVA); neither formula group differed from HMF. Data represent geometric mean ± SEM.

Although Th0 cells represent an infrequent T-cell cytokine population, two-group Cochran-Mantel-Haenszel analysis revealed significantly more Th0-positive subjects in FN (54%) compared with F (36%) at 6 mo ($p = 0.005$). No differences were seen among the feeding groups in a three-group analysis.

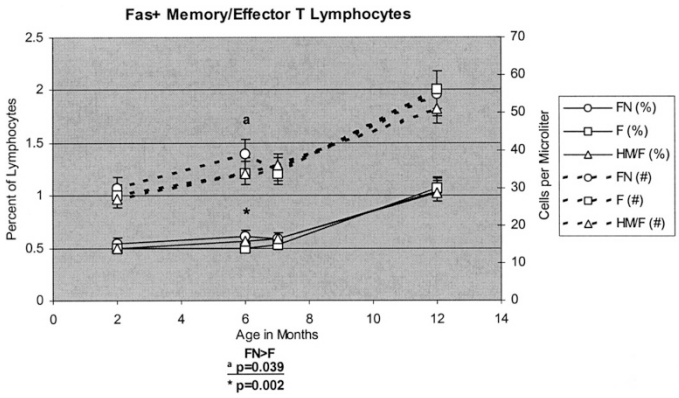

Figure 5. *Percentages (two-group MANOVA) and ^{a}numbers (two-group ANOVA) of M/E Fas^+ (apoptosis competent; $CD45R0^+CD95^+CD3^+$) T cells were higher in FN *vs* F at 6 mo. By three-group analyses, neither formula group was different from HMF. Data represent geometric mean ± SEM.

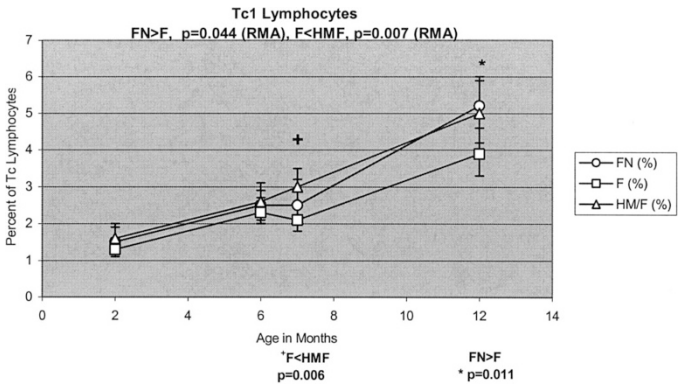

Figure 6. Two-group RMA showed higher percentages of Tc1 ($IFN\gamma^+CD4^-CD3^+$) cells in FN *vs* F ($p = 0.044$). Three-group RMA showed lower percentages of Tc1 cells ($p = 0.007$) in F compared with HMF. No pairwise differences were seen comparing FN to HMF or F. Percentages of Tc1 cells were higher in FN *vs* F ($p = 0.011$, two-group MANOVA) at 12 mo (*). Tc1 percentages in F were lower than HMF at 7 mo ($^+$) by three-group MANOVA ($p = 0.006$). Data represent geometric mean ± SEM.

Proportions of naive $CD4^+CD8^+$ T cells were significantly lower (12.5%) in FN compared with F at 12 mo (two-group MANOVA). However, there were no significant feeding differences by three-group MANOVA (data not shown). Proportions of M/E $CD4^+CD8^+$ T cells were significantly higher (20%) in FN compared with F at 6 mo (two-group MANOVA). However, there were no significant feeding differences by three-group MANOVA (data not shown).

Exploratory RMA on all cell populations supported the initial RMA results and did not reveal additional nucleotide effects.

Nucleotides affected NK and NK-T cells: exploratory two-group and three-group ANOVA. Two- and three-group ANOVA time point analyses for each cell type supported the results of the two- and three-group MANOVA. However, several lymphocyte populations were not seen by MANOVA cluster analysis but were identified by time point ANOVA of individual cell types, generally because the MANOVA cluster was not significant at a given time point. ANOVA revealed additional changes in M/E T cells and NK subsets. FN had up

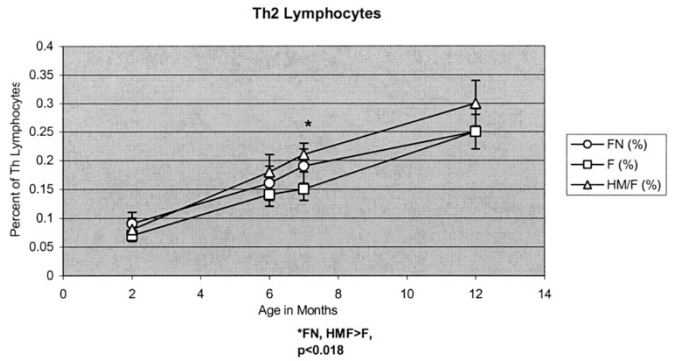

Figure 7. Percentages of Th2 (IL-4$^+$CD4$^+$CD3$^+$) cells were higher in FN *vs* F ($p = 0.017$, two-group MANOVA) at 7 mo (*). By three-group MANOVA, Th2 cell percentages in FN were not significantly different from HMF at 7 mo, whereas F was significantly lower than HMF and FN (F < HMF, $p = 0.001$; F < FN, $p = 0.018$). Data represent geometric mean ± SEM.

to 43% higher numbers of M/E Tc cells (6 mo, 12 mo), 9% higher proportions of M/E Th cells (7 mo), 15% higher numbers of M/E Fas$^+$ cells (6 mo), 11% higher proportions of M/E Fas$^-$ T cells (12 mo), and 22% higher proportions of $\gamma^+\delta^+$ Th cells (6 mo) compared with F. With respect to NK subsets, FN had 17–18% higher numbers and proportions of CD56$^+$CD16$^-$ NK cells, 30–42% higher proportions of CD57$^+$ NK-T cells, and 20–29% lower numbers and proportions of CD56$^+$ NK-T cells compared with F (Table 2).

Exclusive breast-feeding (ExHMF)* versus *mixed breast-feeding (HMF) and nucleotide effects. After 2 mo of exclusive breast-feeding, infants weaned to HMF were compared with infants whose milk source was exclusively human milk (ExHMF) at 6 mo and 7 mo (*n* value insufficient at 12 mo). Because ribonucleotide feeding affected eight lymphocyte populations (M/E Th, M/E Tc, IFNγ, Tc1, Th2, CD56$^+$NK, CD56$^+$NK-T, CD57$^+$NK-T) at these time points, only those cell types were analyzed comparing HMF or ExHMF. There were no significant differences in these cell populations at 6 mo and 7 mo between ExHMF and HMF. Three-group analyses using ExHMF, FN, and F supported the nucleotide effects seen using HMF as a reference group. Similar to the results with HMF, FN immune cell types either did not differ from those infants fed ExHMF, or tended to be more like those fed ExHMF than those fed F (except for CD56$^+$ NK-T cells at 6 mo, FN<ExHMF, although no significant differences by RMA).

DISCUSSION

This large study of 477 infants represents an extensive, longitudinal assessment of infant immune development during the first year of life. Several statistical methods were applied to elucidate the possible effects of nucleotides on immune cell development. These techniques included multivariate time point ANOVA (MANOVA) of clusters of functionally related cell types, and exploratory time point ANOVA of all cell types, which led to similar but not identical conclusions. ANOVA analysis of all cell types supported the nucleotide effects identified by MANOVA. However, ANOVA also revealed nucleotide effects not identified by MANOVA, including changes in M/E Tc cell numbers and NK cell subsets (Table 2). MANOVA analysis did reduce the burden of testing individual cell types but it did not identify all nucleotide-associated cell changes. Some of the nucleotide-associated changes affected cell percentages but not absolute cell numbers. In these instances, nucleotide effects on cell percentages may have been driven by changes in the target cell of interest (*i.e.* numerator) or the parent lymphocyte population (*i.e.* denominator), or both. Although the absolute cell numbers may not have been significantly different, relationships among cells (expressed as a percentage) were significantly different.

Analysis of immune cell populations revealed significant differences between the two formula feeding groups for several lymphocyte types associated with immune maturation. Immune maturation of T cells is associated with a decrease in naive T lymphocytes and an increase in M/E T lymphocytes, both of which may be identified by the presence or absence of cell surface markers, and/or the production of various cytokines. Naive T cells (CD45RA$^+$), which predominate in the infant immune system, often have not previously encountered antigen and mediate primary immune responses *via* IL-2 cytokine production. M/E T cells (CD45R0$^+$) are produced as a result of primary antigen exposure, and their numbers expand after subsequent antigen challenges (booster immunization, environmental pathogens). During this process, most T cells differentiate into effector cells to facilitate antibody production or act as cytotoxic cells, and then undergo apoptosis. Remaining T cells become memory cells capable of mediating a more potent secondary, or memory immune response (17). These cells produce type 1 cytokines (*e.g.* IL-2, IFN-γ), and/or type 2 cytokines (*e.g.* IL-4, IL-5).

The relative proportion of several M/E T lymphocyte populations increased up to 44% in the nucleotide group compared with the control group [M/E Th cells, M/E Tc cells, apoptosis competent (Fas$^+$) M/E T cells, Fas$^-$ M/E T cells, and M/E T cells with both helper (CD4) and cytotoxic/suppressor (CD8) markers]. The number of M/E Tc cells and Fas$^+$ T cells also increased up to 43% in the nucleotide group compared with the control group. M/E T-cell populations in the nucleotide group were comparable to those in the HMF group, whereas M/E Tc cells (RMA) in F were lower than HMF. The HMF group represents an appropriate reference group for breast-fed infants inasmuch as there were no differences in M/E T cell-populations between exclusively breast-fed and HMF infants. Consistent with changes in M/E T cells, percentages of T lymphocytes positive for intracellular IFN-γ (Tc1) and for IL-4 (Th2) were also higher in the nucleotide group compared with the control group; Tc1 and Th2 cells represent subsets of M/E T cells. These cytokine-positive T cells in the nucleotide group were comparable to those in the HMF group, whereas total IFNγ (RMA), Tc1 (RMA), and Th2 (7 mo) cells in the control group were lower than HMF. The increase in M/E T cells was associated with a concomitant decrease in the proportion of naive T lymphocytes (naive helper T cells and naive T cells with both helper and cytotoxic/suppressor markers) in the nucleotide group compared with the control group.

Decreases in naive T-cell populations and increases in memory T-cell populations characterize normal immune matura-

Table 2. *Effect of nucleotides on NK-T cells and NK cells (exploratory ANOVA)*

Cell population	Feeding	Age			
		2 mo	6 mo	7 mo	12 mo
NK-T cells					
$CD57^+$ NK-T (% of lymphocytes) ($CD57^+$ $CD56^-CD3^+$)	FN	0.05 (0.04–0.06)	**0.13 (0.11–0.16)**	**0.17 (0.14–0.19)**	0.33 (0.27–0.41)
	F	0.05 (0.04–0.06)	**0.10 (0.08–0.12)**	**0.12 (0.10–0.15)**	0.28 (0.23–0.33)
	HMF	0.06 (0.05–0.07)	0.14 (0.11–0.17)	0.18 (0.14–0.21)	0.42 (0.35–0.50)
$CD56^+$ NK-T (% of lymphocytes) ($CD56^+CD16^-CD8^-CD3^+$)	FN	**0.04 (0.04–0.05)**	0.06 (0.05–0.07)	0.08 (0.07–0.10)	0.14 (0.12–0.16)
	F	**0.05 (0.05–0.07)**	0.08 (0.06–0.09)	0.08 (0.07–0.10)	0.12 (0.11–0.14)
	HMF	0.04 (0.04–0.05)	0.08 (0.07–0.10)	0.07 (0.06–0.08)	0.15 (0.13–0.17)
$CD56^+$ NK-T (#) ($CD56^+CD16^-CD8^-CD3^+$)	FN	**2.2 (1.8–2.8)**	**3.8 (3.1–4.6)**	4.6 (3.7–5.8)	7.3 (6.4–8.3)
	F	**3.1 (2.5–3.8)**	**5.1 (4.3–6.1)**	5.3 (4.4–6.5)	6.3 (5.5–7.3)
	HMF	2.1 (1.7–2.5)	5.1 (4.4–5.9)	4.1 (3.5–4.8)	7.4 (6.6–8.3)
NK cells					
$CD56^+$ NK (% of lymphocytes) ($CD56^+CD16^-CD8^-CD3^-$)	FN	0.44 (0.41–0.48)	0.44 (0.40–0.47)	0.45 (0.42–0.48)	**0.49 (0.45–0.54)**
	F	0.43 (0.39–0.46)	0.41 (0.38–0.45)	0.44 (0.40–0.47)	**0.42 (0.39–0.47)**
	HMF	0.59 (0.56–0.64)	0.52 (0.49–0.56)	0.48 (0.45–0.51)	0.52 (0.49–0.56)
$CD56^+$ NK (#) ($CD56^+CD16^-CD8^-CD3^-$)	FN	24 (22–27)	28 (25–31)	27 (24–29)	**26 (23–28)**
	F	25 (23–27)	28 (25–31)	28 (26–31)	**22 (20–24)**
	HMF	32 (30–35)	32 (30–35)	29 (27–31)	26 (24–28)

Data represent geometric means with 95% confidence intervals in parentheses. Bold items reflect significant differences between formulas (two-group analyses, $p < 0.05$). NK-T and NK subset populations are expressed as proportions (%) or numbers (#).

tion/development and normal responses to vaccinations and infections (18,19). Increases in memory cells in nucleotide-fed infants compared with control-fed infants may be beneficial, as increases in memory cells correlate with higher vaccine antibody titers and improved cell-mediated immune responses. The inability to generate adequate levels of memory cells is associated with some types of immunodeficiency and less robust cell-mediated responses (20–22). Increased levels of Tc1 and total IFNγ-producing cells in nucleotide-fed infants compared with control-fed infants may also be beneficial, as increases in IFNγ correlate with proliferative responses, an indicator of cell-mediated immunity, and inversely correlate with the severity of respiratory syncytial disease; that is, the greater the amount of IFNγ, the less severe the disease (23, 24). The increased proportion of Th2 cells in the nucleotide group may enhance mucosal and systemic antibody responses. Finally, increased proportions of Th0 responders in the nucleotide group compared with the control group may also support T-cell maturation, because naive Th cells transition to Th0 after encountering antigen and subsequently differentiate into Th1 or Th2 cells (25).

Interestingly, other nutritional factors have been shown to modulate T-cell immune maturation. Specifically, supplementation of preterm formula with docosahexanoic acid and arachidonic acid increased immune maturation at 42 d of age compared with unsupplemented formula, as indicated by a ~25% increase in the proportion of circulating M/E helper T cells, and a decrease in the proportion of naive helper T cells to levels consistent with those in human milk (26).

Nucleotide feeding was associated with a significantly elevated neutralizing antibody response to poliovirus type 1, a transient increase in Hib protection levels at 6 mo (>1 μg/mL), and trends favoring nucleotide supplementation for antibody responses to Hib and diphtheria antigens (12). In a previous study (7), nucleotide supplementation was associated with significantly higher Hib and diphtheria antibody responses (FN > F, FN > HMF). Although M/E T cells were not measured by Pickering *et al.* (7), Cordle *et al.* (16) observed a 29% increase in the number of M/E Th cells in infants fed Isomil (a soy-based formula with inherent nucleotide levels ~300 mg/L) supplemented with nucleotides (~74 mg/L) compared with those fed HMF. The same group (27) also reported an increased antibody response to the PRP component of HibTITER vaccine in the nucleotide-fed group compared with HMF. Enhanced levels of M/E T cells may be responsible for driving the significantly improved vaccine antibody responses seen in these studies (7,12,27).

With regard to nucleotides and animal research, Jyonouchi *et al*. (28) reported nucleotide-mediated increases in type 1–associated antibodies (anti-keyhole limpet hemocyanin IgG2a and IgG2b) in type 1– dominant mice, indicating that dietary nucleotides shift a type 1–predominant immune system further in the same direction. Consistent with this observation, Jyonouchi *et al.* (29) also showed dietary nucleotides result in a type 1 shift in anti-ovalbumin antibody responses in type 2–predominant mice. In addition, lymphocytes isolated from mice fed nucleotides expressed more IFNγ mRNA, and produced more IFNγ and more IFNγ^+ T cells after restimulation with antigen in culture (28–30). These changes indicate that nucleotide supplementation results in shifts toward type 1 responses. Thus, the current clinical finding that nucleotides enhance the proportion of type 1 T cells (IFNγ^+) supports Jyonouchi's observations in mice fed nucleotides. The current study also demonstrated nucleotides support a type 1/type 2 cytokine balance consistent with breast-fed infants.

Nucleotide-associated changes were not restricted to T cells. Nucleotide supplementation enhanced the numbers and proportions of $CD56^+CD16^-$ NK cells, enhanced the proportions of $CD57^+$ NK-T cells (NK-T cells express CD3 and CD56 and/or CD57), and lowered the numbers and proportions of $CD56^+$ NK-T cells compared with the control-fed infants. The changes in NK and NK-T cells were consistent with the HMF group, whereas the control group significantly differed from HMF. HMF represents an appropriate reference group for breast-fed infants because there were no differences in these NK or NK-T-cell populations between exclusively breast-fed and HMF infants. $CD56^+CD16^-$ NK cells represent ~10% of $CD56^+$ NK cells (supported by the current study), they express high levels of CD56 (the majority of NK cells are $CD56^{dim}$), have lower natural cytotoxicity compared with $CD56^{dim}$ cells but have similar levels of cytotoxicity after activation with IL-2 or IL-12, proliferate more vigorously than $CD56^{dim}$ NK cells, and are the primary source of NK-derived immunoregulatory cytokines (IFNγ, tumor necrosis factor-β, IL-10, IL-13, granulocyte macrophage colony-stimulating factor) (31). The natural ligand of NK-T cells is thought to be hydrophobic (probably lipids and glycolipids), and is presented by major histocompatibility complex class I–like molecules (CD1d proteins) on antigen-presenting cells (32). NK-T cells produce high levels of immunoregulatory cytokines, including IFN-γ and IL-4 (32). They also exhibit potent cytolytic activity (31) and have been shown to increase during infancy (16) and with chronologic aging (33). The majority of $CD57^+$ NK-T cells express CD8 ($\alpha\beta$ heterodimer) but lack the CD28 costimulatory molecule. These cells increase during human cytomegalovirus, or human immunodeficiency virus infections, and after bone marrow and kidney transplantation (34,35). $CD56^+$ NK-T cells are thought to be liver-derived (36), whereas $CD57^+$ NK-T cells are bone marrow–derived (37). Evidently, nucleotides influence some lymphocytes of the innate immune system, which may lead to an increased ability to eliminate intracellular pathogens and/or tumor cells.

With regard to clinical outcomes, there were no significant differences between HMF and formula-fed infants in physician visits due to infectious [otitis media, respiratory tract infections (independent of otitis media), other infections] or noninfectious causes (12). Nucleotide effects might be clinically apparent in infant populations different from those currently studied.

In summary, dietary nucleotides affected T lymphocyte subsets (memory/effector T cells and naive T cells), and NK lymphocyte subsets ($CD56^+CD16^-$ NK cells, and NK-T cells) in infants. Collectively, these results suggest an increased maturation of the cellular immune system in the nucleotide group compared with the control group. Furthermore, the nucleotide-associated changes were consistent with those found in HMF infants. These changes represent shifts in lymphocyte populations capable of responding to a plethora of antigens. Consistent with previous data (16), the addition of nucleotides did not result in any shifts in the overall distribution of the major classes of immune cells. The five major WBC categories studied included neutrophils, eosinophils, basophils, monocytes, and lymphocytes; there were no differences between formula groups, or between either formula and HMF groups (except at 6 mo, where lymphocyte numbers were higher in F *versus* HMF). Furthermore, there were no differences in the major lymphocyte types (total T, B, or NK cells) between the formula groups. However, also consistent with previous reports, infants fed either formula had significantly increased numbers and percentages of T cells resulting in decreased percentages of B cells and NK cells compared with HMF infants (10,11).

The present data provided additional observations, namely, granulocyte phagocytic ability and granulocyte extravasation (migration) potential were not affected by supplemental nucleotides. Similarly, supplemental nucleotides did not affect T-B lymphocyte cooperation or lymphocyte activation as mediated, respectively, by CD40 ligand or CD69 expression on T cells (data not shown).

CONCLUSIONS

Nucleotide-associated increases in memory/effector T-cell populations and changes in NK cell subtypes provide evidence that infant formula supplemented with levels of nucleotides similar to levels in human milk (72 mg/L) may facilitate maturation and immunoregulatory shifts in some lymphocyte populations consistent with HMF infants. These shifts might support increased antibody responses and immune cell protection. The nucleotide-associated changes in immunoregulatory NK cell subsets may also enhance innate immune responses against tumors and/or intracellular pathogens. Finally, this study established the important role that certain key nutrients may play when included in infant formula in helping to provide some of the benefits associated with breast-feeding.

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