Eur J Nutr (2013) 52:637–646
DOI 10.1007/s00394-012-0367-8

ORIGINAL CONTRIBUTION

Influence of breastfeeding versus formula feeding on lymphocyte subsets in infants at risk of coeliac disease: the PROFICEL study

Tamara Pozo-Rubio · Amalia Capilla · Jorge R. Mujico · Giada de Palma · Ascensión Marcos · Yolanda Sanz · Isabel Polanco · Maria Dolores García-Novo · Gemma Castillejo · Carmen Ribes-Koninckx · Vicente Varea · Francesc Palau · Luis Ortigosa · Luis Peña-Quintana · Esther Nova

Received: 16 January 2012 / Accepted: 24 April 2012 / Published online: 11 May 2012
© Springer-Verlag 2012

Abstract
Purpose In addition to genetic risk, environmental factors might influence coeliac disease (CD) development. We sought to assess the effect of the interaction between milk-feeding practices and the HLA-DQ genotype on peripheral lymphocyte subsets and their activation markers in infants at familial risk for CD.
Methods 170 newborns were classified in 3 different genetic risk groups (high risk, HR; intermediate risk, IR; and low risk, LR) after DQB1 and DQA1 typing. Lymphocyte subsets were studied at the age of 4 months by flow cytometry analysis.
Results 79 infants were receiving exclusive breastfeeding (BF) and 91 partial breastfeeding or formula feeding (FF). Regarding genetic risk, 40 infants were classified in HR group, 75 in IR group and 55 in LR group. Two-way ANOVA did not show significant interactions between the type of milk feeding and genetic risk group on the lymphocyte subsets analysed. One-way ANOVA for milk-feeding practice alone showed that the percentage of CD4 + CD25+ cells was significantly higher in BF group than in FF group (BF, 10.92 ± 2.71; FF, 9.94 ± 2.96; $p = 0.026$), and absolute counts of CD4 + CD38+ cells were significantly higher in FF group than in BF group (FF, 2,881.23 ± 973.48; BF, 2,557.95 ± 977.06; $p = 0.038$).

T. Pozo-Rubio · J. R. Mujico · A. Marcos · E. Nova (✉)
Immunonutrition Group, Department of Metabolism and Nutrition, Institute of Food Science, Technology and Nutrition (ICTAN), Spanish National Research Council (CSIC), C/Jose Antonio Novais 10, 28040 Madrid, Spain
e-mail: enova@ictan.csic.es

A. Capilla · F. Palau
Instituto de Biomedicina de Valencia (CSIC) and CIBER de Enfermedades Raras (CIBERER), Valencia, Spain

G. de Palma · Y. Sanz
Microbial Ecophysiology and Nutrition Group, Institute of Agrochemistry and Food Technology, Spanish National Research Council (IATA-CSIC), Valencia, Spain

I. Polanco
Gastroenterology and Paediatric Nutrition Service, Hospital Universitario La Paz, Madrid, Spain

M. D. García-Novo
Gastroenterology Unit, Hospital Universitario Infantil Niño Jesús, Madrid, Spain

G. Castillejo
Paediatric Gastroenterology Unit, Hospital Universitari Sant Joan de Reus, Universitat Rovira i Virgili, Tarragona, Spain

C. Ribes-Koninckx
Gastroenterology Unit, Hospital Infantil Universitario La Fe, Valencia, Spain

V. Varea
Paediatric Gastroenterology, Nutrition and Hepatology, Hospital Universitario Sant Joan de Deu Barcelona, Barcelona, Spain

L. Ortigosa
Gastroenterology, Hepatology and Nutrition Unit, Hospital Universitario Nuestra Señora de Candelaria, Santa Cruz de Tenerife, Canary Islands, Spain

L. Peña-Quintana
Nutrition Research Group, Department of Clinical Sciences, Centre for Health Sciences, University of Las Palmas de Gran Canaria, PO Box 550, 35080 Las Palmas, Spain

One-way ANOVA for genetic risk alone showed that absolute counts of NK cells were significantly higher in IR group than HR and LR groups (IR, 539.24 ± 340.63; HR, 405.01 ± 239.53; LR, 419.86 ± 262.85; $p = 0.028$).
Conclusion Lymphocyte subset profiles in the early stages of life could be modulated by milk-feeding practices and genetic risk separately. Breastfeeding might have a positive immunomodulatory effect on lymphocyte subsets in infants at risk of CD.

Keywords Lymphocyte subsets · Coeliac disease · Infants · HLA genotype · Breastfeeding · Formula feeding

Abbreviations

CD	Coeliac disease
PCR-SPP	Polymerase chain reaction-sequence-specific primers
HLA	Human leucocyte antigen
Treg	Regulatory T cells
NK	Natural killer
LR	Low genetic risk
IR	Intermediate genetic risk
HR	High genetic risk
BF	Breastfeeding
FF	Formula/mixture feeding

Introduction

Coeliac disease (CD) is an immune-mediated enteropathy triggered by the ingestion of gluten in genetically susceptible individuals. It is generally accepted that CD is a T-cell-mediated disease, in which gliadin-derived peptides activate lamina propria infiltrating T lymphocytes. The subsequent release of pro-inflammatory cytokines, in particular γ-interferon, leads to a profound tissue remodelling. However, an innate immune response also seems to be involved in the disease development [1]. This is a complex disorder, with environmental and genetic factors contributing to its aetiology. The major genetic risk factor in CD is represented by HLA-DQ genes. Several studies have documented that the HLA-DQA1*05 and DQB1*02 alleles, encoding for particular DQ2 molecules, confer high susceptibility to CD. This heterodimer can be encoded both in cis and in trans. The susceptibility to CD is increased in homozygous subjects with a cis haplotype or possessing a second HLA-DQB1*02 allele [2–4]. In Europe, approximately 90 % of patients have these genetic markers, whereas most of the remaining cases carry the HLA-DQA1*03 and DQB1*0302 alleles coding for DQ8 molecules [3, 5]. Gluten is the main environmental factor responsible for the signs and symptoms of the disease. Moreover, the interplay between other environmental elements and the genetic background is also thought to play a role in the disease risk, including the type of milk feeding, incidence of infections and intestinal dysbiosis [6].

The effect of environmental factors on future disease risk is relevant at the early stages of life when the immature neonate's gut undergoes the process of microbiota establishment and the immune system acquires full competence and tolerance to nonharmful antigens [7]. A particular feature of the neonatal immune system is the presence of a wide pool of naïve T cells waiting to participate in primary immune responses. Intestinal antigen exposure during neonatal life influences appropriate adult immune responses. T-cell differentiation occurs within the neonatal human intestine, and the T-cell receptor (TcR) repertoire of these developing immature T cells is likely to be influenced by luminal antigens [8]. Oral ingestion of food and environmental bacteria is a major route of antigenic exposure. After birth, lactation supports immunological defences through a number of molecules with antimicrobial activity and immunologically active components present in breast milk [9, 10]. Thus, breastfeeding is known to confer protection against infectious diseases in the short term and there is also some evidence of lower prevalence of inflammatory bowel diseases, childhood cancers and type I diabetes in breastfed infants [11].

As early events during extra-uterine life allow the maturation of the immune system, the peripheral lymphocyte subsets also exhibit certain changes. Blood cell analysis shows marked lymphocytosis at birth and at later stages of life compared to adulthood. An increase in T and B lymphocytes occurs during the first weeks of life, while NK cells decline sharply directly after birth [12, 13]. A high CD4/CD8 ratio in babies up to 2 years of age has been found compared to the values observed in normal adults, and this is mainly due to the large and expanding pool of naïve T CD4+ cells present during the first year of life [14, 15]. The ectoenzyme CD38 is expressed in almost all T and B cells [12] and decreases towards the adult life [15, 16]. Although breastfeeding is likely to contribute to these changes, only a limited number of studies have addressed the influence of milk-feeding practices on peripheral lymphocyte subsets or the immunocompetence of lactating children [17–20] and these findings are not consistent among them.

Taking in consideration how important the maturation of the immune system can be as a predisposing or protective factor in future disease development, we planned to assess the effect of milk-feeding practices on the levels of lymphocyte subsets and their activation markers in infants at risk of CD. Since genetic background is a key issue for disease development in this population, we also aimed to

find out possible interactions between type of milk feeding and the HLA genotype on the lymphocyte subsets of these infants.

Materials and methods

Subjects

This study was conducted in 4-month-old infants who are first-degree relatives of CD patients (at least one parent or sibling affected with CD). A total of 170 infants born between October 2006 and the end of 2010 were recruited from 6 hospitals geographically distributed throughout Spain. The 170 infants were divided according to milk-feeding practices into two groups: breastfed (BF) infants ($N = 79$), including those which had been exclusively breastfed until the age of 4 months, and formula-fed (FF) infants ($N = 91$), including babies receiving milk formula, either alone or in alternation with breast milk at the moment of analysis. Questions about delivery, infections, vaccinations, feeding practices, clinical symptoms and medical treatments were answered by the parents at the infant's age of 1 and 4 months in clinical visits with the paediatric gastroenterologist. To facilitate the collection of this information, a diary was given to the mothers upon recruitment (before delivery). The study was approved by the local ethic committees, and written informed consent was obtained from the parents of children included in the study.

This cohort of infants was analysed for the HLA class II DQA1 and DQB1 genotypes. The infants were classified into three main risk groups, according to their DQ haplotype, loosely based on the criterion of Bourgey et al. and considering the HLA distribution of the Eastern Spanish population [21]. We established the following risk groups: The first one included those individuals carrying the DQ2 haplotype, both in *cis* (DQA1*0501-DQB1*0201 in homozygosis) and in *trans* (DQA1*0201-DQB1*0202 with DQA1*0505-DQB1*0301 in heterozygosis) conformation. The second group included those subjects carrying the DQ2 along with any other haplotype, as well as subjects carrying the DQ8 haplotype (DQA1*0301-DQB1*0302) in homozygosis. The third group included those individuals with other common genotypes not associated with CD. Probabilities are approximated and have been estimated taking into account that in most cases, the genotype of the coeliac relative of each newborn was not considered. 40 infants were in the first risk group (high risk; HR) with the highest probabilities of developing CD (28–24 %) [4]. In the second group, with a probability between 7 and 8 %, 75 of the infants were grouped (intermediate risk; IR), and the remaining 55 were in the third group (low risk; LR), with less than 1 % risk of developing CD.

DNA isolation

DNA was extracted from buccal mucosa cells by scraping the inner side of the children's cheek with sterile swabs (Copan innovation, Sarstedt, Germany). The cotton was cut and incubated in DLB buffer (100 mM Tris–HCl pH 7.4, 10 mM EDTA pH 8, 10 mM NaCl per litre of distilled water), 10 % SDS and 10 µL proteinase K (20 mg/ml) at 65 °C for 1 h, and then, a standard phenol–chloroform method was carried out. Extracted DNA was stored in TE buffer (10 ml Tris–HCl pH 8, 200 µL EDTA 0.5 M pH 8 per litre of distilled water) at −20 °C after genotyping. The DNA concentration, around 50–100 µg/mL, was quantified using the NanoDrop© Spectrophotometer.

HLA-DQ genotyping

Low-resolution HLA-DQB1 typing was performed by PCR-SSP (polymerase chain reaction-sequence-specific primers) analysis. Each PCR was performed on about 60–90 ng of extracted DNA, 0.5 U of BIOTOOLS DNA polymerase (Biotools B&M S.A, Spain), 1 × PCR Master Mix (Dynal AllSet + TM SSP or Olerup SSPTM) containing nucleotides (200 µmol L^{-1} each), PCR buffer (50 mmol L^{-1} KCl, 1.5 mmol L^{-1} $MgCl_2$, 10 mmol L^{-1} Tris–HCl pH 8.3, 0.001 % w/v gelatine), 5 % glycerol and 100 µg mL^{-1} cresol red, 0.25 µmol L^{-1} of each allele- or group-specific primer pair and 0.1 µmol L^{-1} of internal positive control primer pair matching a segment of the human growth hormone gene in a final volume of 10 µL. Detailed PCR protocol: An initial denaturation step at 94 °C for 2 min was followed by 10 two-temperature cycles (94 °C for 10 s and 65 °C for 60 s) and 20 three-temperature cycles (94 °C for 10 s, 61 °C for 50 s and 72 °C for 30 s). Detection of amplified alleles was carried out by 2 % agarose gel electrophoresis and ethidium bromide staining. Although the allele DQB1*02 is a determinant of DQ2, HLA-DQA1 alleles were genotyped in a stepwise fashion for a high-resolution typing to sharpen the risk classification of each individual.

Blood analyses

At the age of 4 months, peripheral blood samples were drawn from the infants and collected in vacutainer tubes containing K_3EDTA. Immediately after, 1 mL of blood was mixed with an equal volume (1 mL) of preservative solution (Streck Cell Preservative™ CE. Streck, USA) and sent in cool temperature within 2–6 days to our laboratory for a centralised processing and flow cytometry analysis. Blood extractions were always performed previous to the standardised 4-month vaccination in the Spanish vaccination schedule. Complete blood counts and differential

counts were performed in situ at the corresponding enrolling centres by automated instrumentation. Blood samples were not taken if the infant presented an ongoing infection or had received a vaccination shot in the previous 6 weeks.

Flow cytometry analysis

Aliquots of blood mixed with the preservative solution (150 μL) were incubated for 30 min at room temperature and in the dark with 20 μL of fluorochrome-conjugated monoclonal antibodies specific for CD3+ (CD3-APC), CD4+ (CD4-PerCP-Cy5.5), CD8+ (CD8-PerCP-Cy5.5), CD45RA+ (CD45RA-FITC), CD45RO+ (CD45RO-PE), CD25+ (CD25-FITC), HLA-DR+ (HLA-DR-FITC), CD38+ (CD38-PE), in quadruple immunostainings, and 100 μL aliquots were incubated with the multitests CD3-FITC/CD16 + 56-PE/CD45-PerCP-Cy5.5/CD19-APC and CD3-FITC/CD8-PE/CD45-PerCP-Cy5.5/CD4-APC. Fluorochrome-conjugated isotype control immunoglobulins (IgG1 and IgG2a) from mouse were used for each monoclonal antibody to avoid any background fluorescence signal due to nonspecific binding. All the monoclonal antibodies were purchased from Becton–Dickinson (Sunnyvale, CA, USA). After incubation, samples were lysed with the BD FACS™ Lysing Solution (Becton–Dickinson) following the manufacturer's protocol. The samples were analysed with FACSCalibur Flow Cytometer (four-colour, dual-laser, Becton–Dickinson). The lympho gate was defined on the forward and side scatter patterns of lymphocytes. The analysis protocol gated on lymphocytes stained with PerCP and/or APC, and the selected population was then analysed with the two remaining colours (FITC and PE) to obtain cell percentages expressing the specific antigens. Cell subset counts were obtained by multiplying subset percentages times anchor marker counts, the later resulting by multiplying subset percentage times the absolute lymphocyte count.

Statistics

The normality of the distribution of variables was confirmed through normality graphs and Shapiro–Wilk tests. All variables fitted normal distribution. A univariant analysis of variance was employed with HLA risk and milk-feeding groups as fixed factors to assess the interaction between HLA status and type of milk feeding. When a significant interaction was found, differences between milk-feeding groups were analysed by t test comparison of means within each risk level, and differences between risk groups by one-way ANOVA within each feeding type level followed by post hoc analysis. When no interaction was obtained between the two factors, the analyses were carried out for each factor separately. A p value lower than 0.050 was considered statistically significant (Fig. 1).

Results

The demographic characteristics of the infants included in the study are presented in Table 1. All infants were full-term (39.02 ± 1.77 weeks of gestation), and the majority of them were vaginally delivered (118 out of 170). The size and the weight of the infants at the moment of the delivery were within standard ranges and did not differ significantly between the groups.

The percentage and cell counts of the main lymphocyte subsets and subsets expressing activation markers in the whole group of 4-month-old infants are shown in Table 2. Two-way ANOVA for type of milk feeding and genetic risk group did not show significant interactions between genetic risk and milk-feeding practices on the lymphocyte subsets analysed.

Two effects of the milk-feeding type alone were found (Table 3). Firstly, one-way ANOVA for milk-feeding practice alone showed that the percentage of CD4 + CD25+ cells was significantly higher in BF group than in FF group (BF, 10.92 ± 2.71; FF, 9.94 ± 2.96; $p = 0.026$), and secondly, absolute counts of CD4 + CD38 + cells were significantly higher in FF group than in BF group (FF, 2,881.23 ± 973.48; BF, 2,557.95 ± 977.06; $p = 0.038$). The effects of HLA genotype alone were also analysed (Table 4). One-way ANOVA for genetic risk alone showed that absolute counts of NK cells were significantly higher in IR group than HR and LR groups (IR, 539.24 ± 340.63; HR, 405.01 ± 239.53; LR, 419.86 ± 262.85; $p = 0.028$).

Discussion

In this study, the combined effect of HLA-DQ genotype and milk-feeding practices at 4 month of age on peripheral lymphocyte subsets was studied in a group of infants with at least one first-degree relative suffering from CD. The infants' HLA genotype was classified into genetic risk groups in concordance with the last results obtained from the Italian and Spanish population distribution [4, 21]. However, taking into account our sample size, the five-group initial classification was reduced to a three-group one. Our analysis did not reveal any interaction between type of feeding and genotype on lymphocyte subsets' profile, but both factors showed an independent effect on specific T-cell subsets.

The results obtained for the 20 different lymphocyte subsets measured (median and 10th and 90th percentiles) are in agreement with data published in the literature for

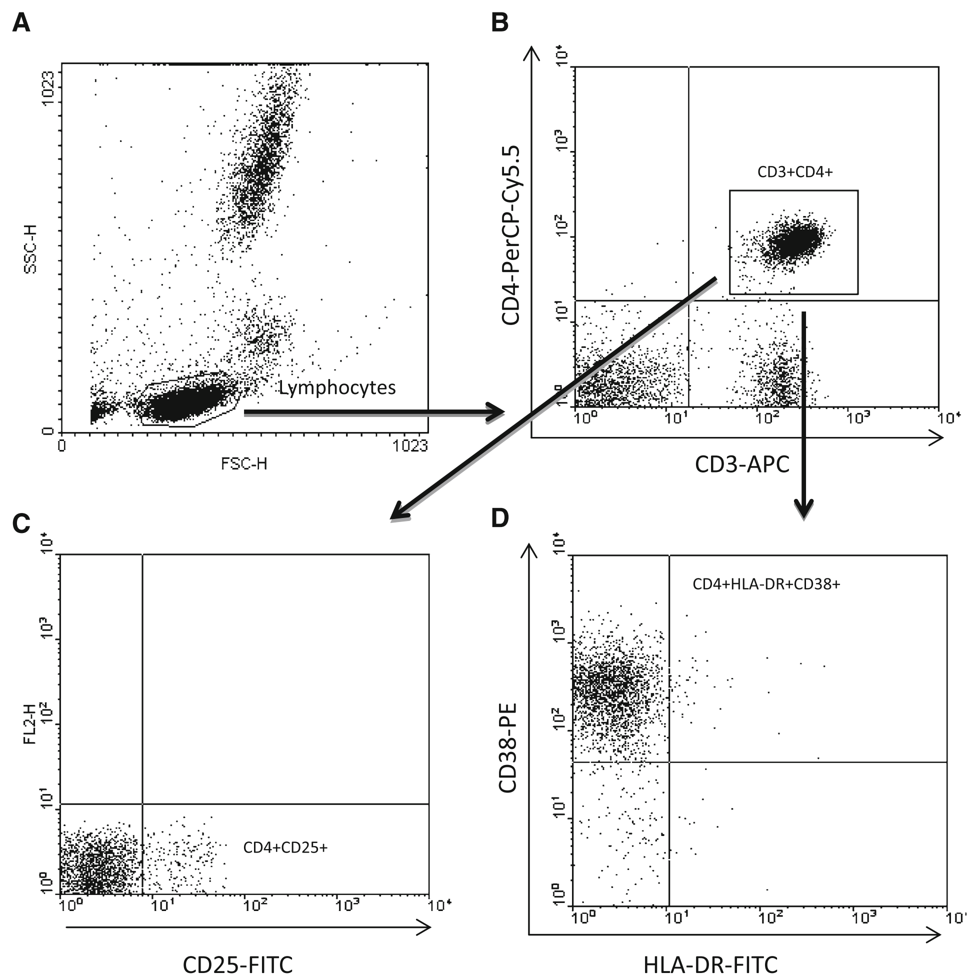

Fig. 1 Four-colour flow cytometry analysis of total blood from a 4-month-old infant. **a** Lymphocytes were gated in the forward scatter (FSC) and side scatter (SSC) histogram according to the size and complexity of the total white blood cells. **b** Helper T cells (CD3 + CD4+) were gated and used as the anchor marker to analyse CD4 + CD25+ **c** and CD4 + CD38+ and CD4 + HLA-DR + CD38+ (**d**)

Table 1 Demographic variables in coeliac disease risk infants

	Total subjects (n = 170)
Delivery	
Vaginal	118/170
Caesarean	52/170
Size (cm)	49.73 ± 2.32
Weight (g)	3,270.57 ± 458.64
Weeks of gestation	39.02 ± 1.77
Infants at 4 months of age	
Breastfeeding	Subjects (n)
Exclusive at 4 months	79
Formula feeding	Subjects (n)
Formula before 4 months	91

3- to 6-month-old healthy infants [15, 22]. However, no reference data have been published so far for this age range in the Spanish population.

A role for breastfeeding as an environmental factor with the capacity to produce immune modulation that might perhaps delay or reduce the risk of developing CD has been suggested by several authors [23–25]. In our population of infants at risk, we have only found a slight modulation of the lymphocyte subset counts by breastfeeding, since most of the subsets did not show differences between breastfed and formula-fed infants. In the literature, the effects of breastfeeding *versus* formula feeding on lymphocyte subsets are controversial, but two different studies have found an increased percentage of CD8+ cells in breastfed infants at 6 and 8 months, compared to formula-fed infants [19, 20]. In our infants, no differences were found in the CD4+ and CD8+ lymphocytes; however, we found lower CD4 + CD38+ counts and higher CD4 + CD25+ percentage in breastfed infants compared to FF infants. CD38+ has a role in T-cell activation and differentiation [26] and is constitutively expressed in newborns decreasing towards the adult life. The fact that lower CD4 + CD38+ counts are found in breastfed children supports the enhancing effect of breastfeeding on lymphocyte maturation.

Regarding the lower CD4 + CD25+ percentage with formula feeding, this might reflect a lower number of regulatory T cells in these infants. However, since we did not use a specific Treg marker, a major drawback of our

Table 2 Cell subset percentages and counts of peripheral blood lymphocytes in 4-month-old infants at risk of coeliac disease

Subsets	Relative frequencies (%) $N = 170$	Absolute counts (cells/μL) $N = 163$
CD45 + CD3+	65 (55–74)	3,706 (2,563–5,780)
Mature T cells		
CD45 + CD4+	46 (38–56)	2,719 (1,816–4,255)
Helper T cells		
CD45 + CD8+	14 (10–22)	831 (520–1,439)
Cytotoxic T cells		
CD45 + CD3-(CD56 + 16)+	6 (4–12)	373 (189–949)
NK cells		
CD45 + CD19+	23 (15–34)	1,291 (755–2,206)
Mature B cells		
CD4 + CD45RA+	87 (77–92)	2,264 (1,453–3,684)
Naïve T4 cells		
CD8 + CD45RA+	90 (77–96)	722 (447–1,217)
Naïve T8 cells		
CD3 + CD45RO+	12 (7–23)	446 (264–918)
Pan Memory T cells		
CD3 + CD4 + CD45RO+	9 (5–14)	321 (190–516)
Memory T4 cells		
CD3 + CD8 + CD45RO+	2 (1–7)	81 (36–277)
Memory T8 cells		
CD4 + CD25+	10 (7–14)	271 (184–439)
Activated T4 cells		
CD8 + CD25+	1 (0–4)	11 (4–35)
Activated T8 cells		
CD4 + HLA-DR	3 (2–6)	79 (42–179)
Activated T4 cells		
CD4 + CD38+	96 (93–98)	2,538 (1,645–4,112)
Activated T4 cells		
CD4 + HLA-DR + CD38+	2 (1–5)	65 (31–165)
Activated T4 cells		
CD8 + HLA-DR+	4 (2–16)	40 (14–183)
Activated T8 cells		
CD8 + CD38+	95 (89–98)	786 (481–1,404)
Activated T8 cells		
CD8 + HLA-DR + CD38+	4 (2–16)	37 (11–166)
Activated T8 cells		
CD4 + CD28+	100 (99–100)	2,719 (1,787–4,245)
Activation primed T4 cells		
CD8 + CD28+	95 (72–98)	747 (469–1,219)
Activation primed T8 cells		
Ratio CD4 + /CD8+	3 (2–5)	
Lymphocytes		5,805 (3,930–8,500)

Values are medians (10th and 90th percentiles) of the relative frequencies or absolute counts of cells expressing the indicated markers referred to the lymphocyte population defined by the anchor marker, which appears in first place of the subset name

study is the impossibility to differentiate within the CD4 + CD25+ population what proportion is activated to produce Th1 cytokines and what proportion is Treg to produce IL-10 and TGF-β. The importance of the Treg cells in the susceptibility of atopic disease in children with a familial history of the disease has been pointed out recently [27], even though their role is still not well defined. The modulation exerted by breastfeeding on this population seems relevant, since different animal and human studies have revealed that breast milk induces oral

Table 3 Lymphocyte subset percentages and counts in coeliac disease risk infants classified into groups according to milk-feeding practice

Lymphocyte subsets		Breastfeeding ($N = 79$)	Formula feeding ($N = 91$)	p
CD45 + CD3+	%	64 ± 8	65 ± 7	0.626
Mature T cells	cel./µL	3,824 ± 1,386	4,055 ± 1,224	0.260
CD45 + CD4+	%	46 ± 7	47 ± 7	0.264
Helper T cells	cel./µL	2,726 ± 945	2,991 ± 989	0.086
CD45 + CD8 +	%	16 ± 5	15 ± 5	0.262
Cytotoxic T cells	cel./µL	956 ± 513	925 ± 388	0.670
CD45 + CD3-(CD56 + 16)+	%	8 ± 4	7 ± 3	0.085
NK cells	cel./µL	494 ± 346	447 ± 253	0.317
CD45 + CD19+	%	23 ± 8	24 ± 7	0.767
Mature B cells	cel./µL	1,386 ± 649	1,489 ± 666	0.320
CD3 + CD45RO+	%	14 ± 7	14 ± 8	0.747
Pan memory T cells	cel./µL	518 ± 359	583 ± 431	0.307
CD4 + CD45RA+	%	86 ± 6	85 ± 9	0.594
Naïve T4 cells	cel./µL	2,352 ± 887	2,568 ± 908	0.136
CD3 + CD4 + CD45RO+	%	9 ± 4	10 ± 5	0.423
Memory T4 cells	cel./µL	331 ± 202	392 ± 263	0.109
CD8 + CD45RA+	%	88 ± 9	87 ± 9	0.711
Naïve T8 cells	cel./µL	832 ± 456	789 ± 277	0.468
CD3 + CD8 + CD45RO+	%	3 ± 3	3 ± 4	0.493
Memory T8 cells	cel./µL	125 ± 151	149 ± 209	0.417
CD4 + CD25+	%	11 ± 3	10 ± 3	**0.026**
Activated T4 cells	cel./µL	287 ± 91	289 ± 106	0.889
CD8 + CD25+	%	2 ± 1	2 ± 2	0.321
Activated T8 cells	cel./µL	16 ± 16	18 ± 21	0.461
CD4 + HLA-DR	%	4 ± 2	4 ± 2	0.919
Activated T4 cells	cel./µL	93 ± 54	103 ± 61	0.315
CD4 + CD38+	%	94 ± 11	96 ± 2	0.065
Activated T4 cells	cel./µL	2,558 ± 977	2,881 ± 973	**0.038**
CD4 + HLA-DR + CD38+	%	3 ± 2	3 ± 2	0.619
Activated T4 cells	cel./µL	76 ± 52	88 ± 56	0.173
CD8 + HLA-DR+	%	8 ± 10	7 ± 7	0.637
Activated T8 cells	cel./µL	93 ± 181	72 ± 110	0.375
CD8 + CD38+	%	93 ± 11	94 ± 5	0.196
Activated T8 cells	cel./µL	893 ± 503	869 ± 341	0.723
CD8 + HLA-DR + CD38+	%	7 ± 10	7 ± 6	0.663
Activated T8 cells	cel./µL	88 ± 175	67 ± 99	0.346
CD4 + CD28+	%	100 ± 1	100 ± 1	0.900
Activation primed T4 cells	cel./µL	2,718 ± 944	2,985 ± 992	0.083
CD8 + CD28+	%	90 ± 13	90 ± 13	0.794
Activation primed T8 cells	cel./µL	840 ± 437	815 ± 293	0.672
Ratio CD4 + /CD8+	%	3 ± 1	3 ± 1	0.295
Lymphocytes	cel./µL	5,985 ± 2,036	6,308 ± 1,876	0.294

Data are mean ± SD. One-way ANOVA ($p < 0.05$)

Significant differences are highlighted in bold

tolerance and prevents asthma and other autoimmune disorders development through an increase in CD4 + CD25 + Foxp3 + regulatory T cells [28–30]. Several in vitro studies showed that specific milk components, like exosomes [31], *lactobacilli* [32] and lactadherine milk protein [33], activate or increase the number of regulatory T cells (CD4 + CD25 + Foxp3+). Lactadherine, in addition, has been involved in protection against rotavirus

Table 4 Lymphocyte subset percentages and counts in coeliac disease risk infants classified into groups according to genetic risk

Lymphocyte subsets		High risk (*N* = 40)	Intermediate risk (*N* = 75)	Low risk (*N* = 55)	*p*
CD45 + CD3+	%	66 ± 7	64 ± 8	64 ± 8	0.406
Mature T cells	cel./μL	4,008 ± 1,419	4,149 ± 1,391	3,621 ± 1,017	0.079
CD45 + CD4+	%	48 ± 8	46 ± 7	47 ± 7	0.491
Helper T cells	cel./μL	2,880 ± 1,058	3,002 ± 1,038	2,678 ± 799	0.188
CD45 + CD8+	%	15 ± 5	15 ± 5	15 ± 5	0.872
Cytotoxic T cells	cel./μL	942 ± 470	1,014 ± 511	834 ± 302	0.086
CD45 + CD3-(CD56+16)+	%	6 ± 2	8 ± 4	7 ± 4	0.090
NK cells	cel./μL	405 ± 240	539 ± 341	420 ± 263	**0.028**
CD45 + CD19+	%	23 ± 7	23 ± 8	24 ± 7	0.666
Mature B cells	cel./μL	1,365 ± 687	1,490 ± 633	1,429 ± 678	0.633
CD3 + CD45RO+	%	15 ± 9	14 ± 8	13 ± 5	0.462
Pan memory T cells	cel./μL	603 ± 461	587 ± 430	468 ± 284	0.183
CD4 + CD45RA+	%	85 ± 10	85 ± 7	87 ± 5	0.477
Naïve T4 cells	cel./μL	2,475 ± 1,032	2,572 ± 960	2,325 ± 697	0.333
CD3 + CD4 + CD45RO+	%	10 ± 7	9 ± 4	9 ± 4	0.510
Memory T4 cells	cel./μL	395 ± 327	379 ± 202	320 ± 203	0.263
CD8 + CD45RA+	%	86 ± 10	87 ± 10	88 ± 7	0.427
Naïve T8 cells	cel./μL	806 ± 393	860 ± 412	738 ± 272	0.200
CD3 + CD8 + CD45RO+	%	4 ± 3	3 ± 4	3 ± 2	0.418
Memory T8 cells	cel./μL	160 ± 163	155 ± 239	98 ± 79	0.174
CD4 + CD25+	%	10 ± 3	11 ± 3	10 ± 3	0.167
Activated T4 cells	cel./μL	269 ± 94	307 ± 92	275 ± 110	0.083
CD8 + CD25+	%	2 ± 2	2 ± 1	2 ± 2	0.362
Activated T8 cells	cel./μL	15 ± 23	20 ± 20	13 ± 11	0.084
CD4 + HLA-DR	%	3 ± 2	4 ± 2	4 ± 2	0.532
Activated T4 cells	cel./μL	90 ± 58	104 ± 62	96 ± 53	0.486
CD4 + CD38+	%	96 ± 2	94 ± 11	96 ± 2	0.332
Activated T4 cells	cel./μL	2,777 ± 1,049	2,829 ± 1,080	2,567 ± 777	0.328
CD4 + HLA-DR + CD38+	%	3 ± 2	3 ± 2	3 ± 1	0.642
Activated T4 cells	cel./μL	77 ± 56	88 ± 59	79 ± 46	0.479
CD8 + HLA-DR+	%	7 ± 9	7 ± 9	7 ± 7	0.973
Activated T8 cells	cel./μL	93 ± 195	90 ± 156	62 ± 76	0.488
CD8 + CD38+	%	94 ± 5	93 ± 12	95 ± 3	0.376
Activated T8 cells	cel./μL	888 ± 454	941 ± 477	791 ± 289	0.146
CD8 + HLA-DR + CD38+	%	7 ± 9	7 ± 9	7 ± 7	0.966
Activated T8 cells	cel./μL	89 ± 1,888	84 ± 146	57 ± 72	0.481
CD4 + CD28+	%	100 ± 1	100 ± 1	100 ± 1	0.969
Activation primed T4 cells	cel./μL	2,873 ± 1,058	2,996 ± 1,038	2,671 ± 799	0.187
CD8 + CD28+	%	89 ± 13	89 ± 15	92 ± 9	0.349
Activation primed T8 cells	cel./μL	807 ± 310	883 ± 434	762 ± 283	0.177
Ratio CD4 + /CD8+	%	3 ± 1	3 ± 1	3 ± 1	0.709
Lymphocytes	cel./μL	6,107 ± 2,075	6,489 ± 2,020	5,745 ± 1,702	0.110

Data are mean ± SD. One-way ANOVA ($p < 0.05$)

Significant differences are highlighted in bold

infection [34–37]; some investigations have linked this pathogen to coeliac disease development [38].

Overall, our results showing a percentage of T CD4 + CD25+ cells significantly higher in BF group than in FF group, and absolute counts of T CD4 + CD38+ cells significantly higher in FF group than in BF group, suggest that breastfed infants could have a more mature immune system that formula-fed infants due to the beneficial

properties of human milk. This would be an argument in favour of the recommendation to introduce gluten in predisposed infants while they are still breastfed as a means to possibly lessen the toxicity of gliadin peptides [39]. However, since at the time of our lymphocyte subset analysis, gluten introduction had not occurred yet, it will be interesting to find out whether this apparently increased number of regulatory T-cell clones is associated with a better response to gliadin after gluten introduction. In addition, there are studies that demonstrate that breast milk contains gliadin peptides [40] and gliadin-specific IgA antibodies [40–42] that could be involved in the modulation of the immune response in neonates [40], possibly with a preventive role [42].

Regarding higher absolute counts of NK cells in IR group than HR and LR groups, the possibility exists that this is only a spurious finding, with no biological relevance. However, the association of specific genotypes with a certain marker, a function or a disease, is usually difficult to explain. In this sense, the concept that genetic factors influence the regulation of lymphocyte subpopulations has been supported by several published studies [43–46]. Therefore, more research is needed to clarify the true meaning of the association between NK cells and HLA-DQ genotype in the population studied.

In conclusion, according to our results, the effect of breast milk on lymphocyte subsets could be beneficial in infants at risk of CD and further studies are necessary to assess the combined effect of milk-feeding practices and gluten introduction practices on that risk.

Acknowledgments We gratefully acknowledge the assistance of the statistician Dr. Laura Barrios in the statistical analysis. Supported by grants AGL2007-66126-C03-01/ALI, AGL2007-66126-C03-02/ALI and AGL2007-66126-C03-03/ALI, from the Spanish Ministry of Science and Innovation and grants 200570F0091 and 200570F0093 from CSIC. T. Pozo and G. de Palma were recipients of a personal grant from the JAE/I3P Program of CSIC (Spain).

Conflict of interest The authors declare that they have no conflict of interest.

References

1. Gianfrani C, Auricchio S, Troncone R (2005) Adaptive and innate immune responses in celiac disease. Immunol Lett 99(2): 141–145. doi:10.1016/j.imlet.2005.02.017
2. Dubois PC, van Heel DA (2008) Translational mini-review series on the immunogenetics of gut disease: immunogenetics of coeliac disease. Clin Exp Immunol 153(2):162–173. doi:10.1111/j.1365-2249.2008.03704.x
3. Kagnoff MF (2007) Celiac disease: pathogenesis of a model immunogenetic disease. J Clin Invest 117(1):41–49. doi:10.1172/JCI30253
4. Bourgey M, Calcagno G, Tinto N, Gennarelli D, Margaritte-Jeannin P, Greco L, Limongelli MG, Esposito O, Marano C, Troncone R, Spampanato A, Clerget-Darpoux F, Sacchetti L (2007) HLA related genetic risk for coeliac disease. Gut 56(8): 1054–1059. doi:10.1136/gut.2006.108530
5. Karell K, Louka AS, Moodie SJ, Ascher H, Clot F, Greco L, Ciclitira PJ, Sollid LM, Partanen J (2003) HLA types in celiac disease patients not carrying the DQA1*05-DQB1*02 (DQ2) heterodimer: results from the European genetics cluster on celiac disease. Hum Immunol 64(4):469–477
6. Nova E, Pozo T, Sanz Y, Marcos A (2010) Dietary strategies of immunomodulation in infants at risk for celiac disease. Proc Nutr Soc 69(3):347–353. doi:10.1017/S0029665110001825
7. Westerbeek EA, van den Berg A, Lafeber HN, Knol J, Fetter WP, van Elburg RM (2006) The intestinal bacterial colonisation in preterm infants: a review of the literature. Clin Nutr 25(3):361–368. doi:10.1016/j.clnu.2006.03.002
8. Williams AM, Bland PW, Phillips AC, Turner S, Brooklyn T, Shaya G, Spicer RD, Probert CS (2004) Intestinal alpha beta T cells differentiate and rearrange antigen receptor genes in situ in the human infant. J Immunol 173(12):7190–7199
9. Niers L, Stasse-Wolthuis M, Rombouts FM, Rijkers GT (2007) Nutritional support for the infant's immune system. Nutr Rev 65(8 Pt 1):347–360
10. Martin R, Langa S, Reviriego C, Jiminez E, Marin ML, Xaus J, Fernandez L, Rodriguez JM (2003) Human milk is a source of lactic acid bacteria for the infant gut. J Pediatr 143(6):754–758. doi:10.1016/j.jpeds.2003.09.028
11. Schack-Nielsen L, Michaelsen KF (2007) Advances in our understanding of the biology of human milk and its effects on the offspring. J Nutr 137(2):503S–510S
12. de Vries E, de Bruin-Versteeg S, Comans-Bitter WM, de Groot R, Hop WC, Boerma GJ, Lotgering FK, van Dongen JJ (2000) Longitudinal survey of lymphocyte subpopulations in the first year of life. Pediatr Res 47(4 Pt 1):528–537
13. Comans-Bitter WM, de Groot R, van den Beemd R, Neijens HJ, Hop WC, Groeneveld K, Hooijkaas H, van Dongen JJ (1997) Immunophenotyping of blood lymphocytes in childhood. Reference values for lymphocyte subpopulations. J Pediatr 130(3):388–393
14. Panaro A, Amati A, di Loreto M, Felle R, Ferrante M, Papadia AM, Porfido N, Gambatesa V, Dell'Osso A, Lucivero G (1991) Lymphocyte subpopulations in pediatric age. Definition of reference values by flow cytometry. Allergol Immunopathol Madr 19(3):109–112
15. Shearer WT, Rosenblatt HM, Gelman RS, Oyomopito R, Plaeger S, Stiehm ER, Wara DW, Douglas SD, Luzuriaga K, McFarland EJ, Yogev R, Rathore MH, Levy W, Graham BL, Spector SA (2003) Lymphocyte subsets in healthy children from birth through 18 years of age: the Pediatric AIDS Clinical Trials Group P1009 study. J Allergy Clin Immunol 112(5):973–980. doi:10.1016/j.jaci.2003.07.003
16. Jackson DG, Bell JI (1990) Isolation of a cDNA encoding the human CD38 (T10) molecule, a cell surface glycoprotein with an unusual discontinuous pattern of expression during lymphocyte differentiation. J Immunol 144(7):2811–2815
17. Carver JD, Pimentel B, Wiener DA, Lowell NE, Barness LA (1991) Infant feeding effects on flow cytometric analysis of blood. J Clin Lab Anal 5(1):54–56
18. Cummins AG, Eglinton BA, Gonzalez A, Roberton DM (1994) Immune activation during infancy in healthy humans. J Clin Immunol 14(2):107–115
19. Hawkes JS, Neumann MA, Gibson RA (1999) The effect of breast feeding on lymphocyte subpopulations in healthy term infants at 6 months of age. Pediatr Res 45(5 Pt 1):648–651
20. Jeppesen DL, Hasselbalch H, Lisse IM, Ersboll AK, Engelmann MD (2004) T-lymphocyte subsets, thymic size and breastfeeding in infancy. Pediatr Allergy Immunol 15(2):127–132. doi:10.1111/j.1399-3038.2004.00032.xPAI032

21. Donat E, Planelles D, Capilla-Villanueva A, Montoro JA, Palau F, Ribes-Koninckx C (2009) Allelic distribution and the effect of haplotype combination for HLA type II loci in the celiac disease population of the Valencian community (Spain). Tissue Antigens 73(3):255–261. doi:10.1111/j.1399-0039.2008.01191.x
22. Berrington JE, Barge D, Fenton AC, Cant AJ, Spickett GP (2005) Lymphocyte subsets in term and significantly preterm UK infants in the first year of life analysed by single platform flow cytometry. Clin Exp Immunol 140(2):289–292. doi:10.1111/j.1365-2249.2005.02767.x
23. Peters U, Schneeweiss S, Trautwein EA, Erbersdobler HF (2001) A case-control study of the effect of infant feeding on celiac disease. Ann Nutr Metab 45(4):135–142
24. Ivarsson A, Hernell O, Stenlund H, Persson LA (2002) Breast-feeding protects against celiac disease. Am J Clin Nutr 75(5): 914–921
25. Branski D, Fasano A, Troncone R (2006) Latest developments in the pathogenesis and treatment of celiac disease. J Pediatr 149(3):295–300. doi:10.1016/j.jpeds.2006.06.003
26. Kimpimaki T, Erkkola M, Korhonen S, Kupila A, Virtanen SM, Ilonen J, Simell O, Knip M (2001) Short-term exclusive breast-feeding predisposes young children with increased genetic risk of Type I diabetes to progressive beta-cell autoimmunity. Diabetologia 44(1):63–69
27. McLoughlin RM, Calatroni A, Visness CM, Wallace PK, Cruikshank WW, Tuzova M, Ly NP, Ruiz-Perez B, Kattan M, Bloomberg GR, Lederman H, Gern JE, Gold DR (2011) Longitudinal relationship of early life immunomodulatory T cell phenotype and function to development of allergic sensitization in an urban cohort. Clin Exp Allergy J Br Soc Allergy Clin Immunol. doi:10.1111/j.1365-2222.2011.03882.x
28. Brugman S, Visser JT, Hillebrands JL, Bos NA, Rozing J (2009) Prolonged exclusive breastfeeding reduces autoimmune diabetes incidence and increases regulatory T-cell frequency in bio-breeding diabetes-prone rats. Diabetes Metab Res Rev 25(4):380–387. doi:10.1002/dmrr.953
29. Mosconi E, Rekima A, Seitz-Polski B, Kanda A, Fleury S, Tissandie E, Monteiro R, Dombrowicz DD, Julia V, Glaichenhaus N, Verhasselt V (2010) Breast milk immune complexes are potent inducers of oral tolerance in neonates and prevent asthma development. Mucosal Immunol 3(5):461–474. doi:10.1038/mi.2010.23
30. Lan RY, Ansari AA, Lian ZX, Gershwin ME (2005) Regulatory T cells: development, function and role in autoimmunity. Autoimmun Rev 4(6):351–363. doi:10.1016/j.autrev.2005.01.007
31. Admyre C, Johansson SM, Qazi KR, Filen JJ, Lahesmaa R, Norman M, Neve EP, Scheynius A, Gabrielsson S (2007) Exosomes with immune modulatory features are present in human breast milk. J Immunol 179(3):1969–1978
32. Perez-Cano FJ, Dong H, Yaqoob P (2010) In vitro immunomodulatory activity of Lactobacillus fermentum CECT5716 and Lactobacillus salivarius CECT5713: two probiotic strains isolated from human breast milk. Immunobiology 215(12):996–1004. doi:10.1016/j.imbio.2010.01.004
33. Zhou YJ, Gao J, Yang HM, Yuan XL, Chen TX, He ZJ (2010) The role of the lactadherin in promoting intestinal DCs development in vivo and vitro. Clin Dev Immunol 2010:357541. doi:10.1155/2010/357541
34. Newburg DS, Peterson JA, Ruiz-Palacios GM, Matson DO, Morrow AL, Shults J, Guerrero ML, Chaturvedi P, Newburg SO, Scallan CD, Taylor MR, Ceriani RL, Pickering LK (1998) Role of human-milk lactadherin in protection against symptomatic rotavirus infection. Lancet 351(9110):1160–1164
35. Newburg DS (1999) Human milk glycoconjugates that inhibit pathogens. Curr Med Chem 6(2):117–127
36. Peterson JA, Scallan CD, Ceriani RL, Hamosh M (2001) Structural and functional aspects of three major glycoproteins of the human milk fat globule membrane. Adv Exp Med Biol 501:179–187
37. Kvistgaard AS, Pallesen LT, Arias CF, Lopez S, Petersen TE, Heegaard CW, Rasmussen JT (2004) Inhibitory effects of human and bovine milk constituents on rotavirus infections. J Dairy Sci 87(12):4088–4096. doi:10.3168/jds.S0022-0302(04)73551-1
38. Stene LC, Honeyman MC, Hoffenberg EJ, Haas JE, Sokol RJ, Emery L, Taki I, Norris JM, Erlich HA, Eisenbarth GS, Rewers M (2006) Rotavirus infection frequency and risk of celiac disease autoimmunity in early childhood: a longitudinal study. Am J Gastroenterol 101(10):2333–2340. doi:10.1111/j.1572-0241.2006.00741.x
39. Guandalini S (2007) The influence of gluten: weaning recommendations for healthy children and children at risk for celiac disease. Nestle Nutr Workshop Ser Pediatr Program 60:139–151. doi:10.1159/0000106366 (Discussion 151–135)
40. Chirdo FG, Rumbo M, Anon MC, Fossati CA (1998) Presence of high levels of non-degraded gliadin in breast milk from healthy mothers. Scandinavian J Gastroenterol 33(11):1186–1192
41. Rumbo M, Chirdo FG, Anon MC, Fossati CA (1998) Detection and characterization of antibodies specific to food antigens (gliadin, ovalbumin and beta-lactoglobulin) in human serum, saliva, colostrum and milk. Clin Exp Immunol 112(3):453–458
42. Ozkan T, Ozeke T, Meral A (2000) Gliadin-specific IgA antibodies in breast milk. J Int Med Res 28(5):234–240
43. Hall MA, Norman PJ, Thiel B, Tiwari H, Peiffer A, Vaughan RW, Prescott S, Leppert M, Schork NJ, Lanchbury JS (2002) Quantitative trait loci on chromosomes 1, 2, 3, 4, 8, 9, 11, 12, and 18 control variation in levels of T and B lymphocyte subpopulations. Am J Hum Genet 70(5):1172–1182. doi:10.1086/340090
44. Amadori A, Zamarchi R, De Silvestro G, Forza G, Cavatton G, Danieli GA, Clementi M, Chieco-Bianchi L (1995) Genetic control of the CD4/CD8 T-cell ratio in humans. Nat Med 1(12): 1279–1283
45. Clementi M, Forabosco P, Amadori A, Zamarchi R, De Silvestro G, Di Gianantonio E, Chieco-Bianchi L, Tenconi R (1999) CD4 and CD8 T lymphocyte inheritance. Evidence for major autosomal recessive genes. Hum Genet 105(4):337–342
46. Puglisi F, Capuano P, Simone M, Verzillo F, Laurentaci C (1999) T-cell activity in HLA-associated autoimmune diseases. Panminerva Med 41(4):315–317