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RESEARCH PAPER

TGF-β2, EGF and FGF21 influence the suckling rat intestinal maturation

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Abstract

Some of the growth factors present in breast milk, such as transforming growth factor-β (TGF-β), epidermal growth factor (EGF) and fibroblast growth factor 21 (FGF21), play important roles in the development of the intestinal tract. The aim of this study was to determine the effect of a supplementation with TGF-β2, EGF and FGF21 on suckling rats intestinal maturation. For this purpose, Wistar rats were supplemented daily with TGF-β2, EGF or FGF21 throughout the suckling period. We evaluated the functionality of the intestinal epithelial barrier through an *in vivo* dextran permeability assay, and by a histomorphometric and immunohistochemical study. In addition, the intestinal gene expression of tight junction-associated proteins, mucins, toll-like receptors, and maturation markers was analyzed. Moreover, the intraepithelial lymphocyte (IEL) phenotypical composition was established. During the suckling period, the supplementation with TGF-β2, EGF and FGF21 showed important signs of intestinal maturation. These results suggest that these molecules, present in breast milk, play a modulatory role in the maturation of the intestinal barrier function and the IEL composition during the suckling period.

Keywords: Growth factors; Breast milk; Intestinal barrier function; Intraepithelial lymphocytes; Neonatal rats; Intestinal immunity.

1. Introduction

The small intestine has important body functions beyond of those devoted to food digestion and nutrients absorption [1,2]. Among these, protective functions are outlined. In this sense, small intestine elements constitute the intestinal barrier that is crucial for gut and body homeostasis [3]. There is a mucus layer that covers intestinal epithelial cells [4]. Moreover, the gut microbiota is a key player that contributes to intestinal barrier maintenance [5]. The most predominant cells of the intestine, the intestinal epithelial cells (IECs) are primarily involved in absorption and also act as a barrier against pathogens [6,7]. IECs possess pattern-recognition receptors (PRRs) such as toll-like receptors (TLRs) and nucleotide-binding oligomerization domain receptors (NODs), which recognize antigenic molecules on intestinal microorganisms and play a role in innate immunity and inflammatory responses [6]. IECs function as a barrier due to their tight junctions (TJs), a group of proteins located on their apical-lateral membranes involved in joining IECs among them [8]. TJs are composed of transmembrane proteins (e.g. claudins and occludin) and cytosolic proteins (e.g. ZO-1), which regulate the paracellular passage of ions, solutes, and water [9]. Various extracellular stimuli, such as dietary factors, can regulate both TJ barrier function and paracellular permeability, and their correct function may prevent several diseases [8,9]. Intestinal epithelium also includes goblet cells and Paneth cells that secrete, primarily, mucins (MUC) and antimicrobial peptides, respectively [10–12].

Intraepithelial lymphocytes (IELs) are found along the full length of the intestinal tract, thus located both in the small and large intestines [3]. IELs are mainly composed of T cells and innate lymphoid cells (ILCs); however, they lack B cells as they are located mostly under the basal membrane in the lamina propria [6]. When exposed to antigens, IELs produce cytokines and growth factors to recruit effector cells, eliminate pathogens, and stimulate epithelial repair. Thus, IELs regulate both innate and adaptive immune responses to promote protection against infection and maintain barrier function [2,13–15].

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In the first days of life, luminal factors begin to stimulate the immune cells of the gut compartment [16]. In this context, breast milk is the main driving factor in the maturation, activation, and expansion of immune cells, especially at mucosal sites [17,18]. Among milk bioactive compounds, growth factors play a key role in directing immune protection and promoting the development of adaptive immune responses [15]. Some growth factors, such as transforming growth factor (TGF)-β, epidermal growth factor (EGF), and fibroblast growth factor 21 (FGF21) are present in breast milk from various mammals. In the case of rats, which are the focus of our study, the reported concentrations of these factors in the literature vary, but they are approximately as follows: TGF-β (~100–120 ng/mL), EGF (~4–10 pg/mL) and FGF21 (~0.6–0.8 ng/mL) [19–22]. These factors play important roles in the development of the gastrointestinal tract, among other vital functions in the developing infant [23,24]. They act throughout specific receptors located in the epithelium: TGFβRI/TGFβRII, EGFR/ErbB1, and FGFR1-4 for TGF-β, EGF and FGF21, respectively [25].The study of the gut-associated lymphoid tissue (GALT) is essential for understanding the mechanisms and interactions of nutritional supplementation with the immune system and for clarifying the phenotype and function of lymphocytes involved in immunological responses at the mucosal level. We hypothesized that these three growth factors might also play an important role in the development of the small intestine. Therefore, the objective of this study was to ascertain whether supplementation with TGF-β2, EGF or FGF21 in sucking rats could accelerate intestinal epithelial barrier maturation and IEL development.

2. Materials and methods

2.1. Animals

Pregnant Wistar rats (RjHan:WI) with 15 days of gestational age (G15) were obtained from Janvier Labs (Le Genest-Saint-Isle, France) and individually housed under controlled conditions of temperature and humidity in a 12:12 h light:dark cycle with free access to a commercial diet, corresponding to the American Institute of Nutrition 93G formulation [26] (Harlan Teklad, Madison, WI, USA) and water *ad libitum*. Pregnant rats were monitored daily and allowed to deliver naturally. The day after birth was identified as day 1 of life. Pups had free access to maternal milk and rat diet. The animals were weighed daily, and handling was done in the same time range on a scheduled basis, to avoid the influence of biological rhythms. The procedures of this study were performed in accordance with the Institutional Guidelines for the Care and Use of Laboratory Animals and were approved by the Ethical Committee for Animal Experimentation (CEEA) of the University of Barcelona (UB) and by the Catalonia Government (CEEA-UB Ref. 220/15 and DAAM 8521, respectively). Two experimental designs were carried out: a preliminary study aiming to establish the changes in the intestinal barrier functionality associated with age, and the main study aiming to assess the effects of the supplementation with growth factors on the intestinal barrier and on the IELs phenotype (Fig. S1).

2.2. Study of intestinal barrier function associated with age

In the preliminary study, an *in vivo* study was conducted to evaluate changes in intestinal epithelial barrier function during the suckling period. This approach should allow as to establish the adequate period at which the intestinal barrier closing is occurring in neonatal rats, and thus to define an appropriate period to evaluate the influence of bioactive factors on this function. To this end, Wistar rats aged 10 and 14 days (corresponding to the suckling period), 21 days (weaning day) and 28 days (1 week after weaning) were used (n=9 pups/age). Following euthanasia, blood samples for the permeability assay and small intestine tissues for gene expression analysis were collected.

2.3. Growth factors supplementation study

The main *in vivo* study was conducted to assess how growth factor supplementation affects the intestinal epithelial barrier function and to examine the alterations in the IEL phenotype throughout the suckling period. For this, on the first day of life, litters were culled to nine pups per lactating dam and were randomly distributed into four experimental groups: the reference (REF) group, the TGF-β2 group, the EGF group and the FGF21 group (n=3 litters of nine pups each/group). Sample size estimation was performed as described in previous studies [27,28].

Supplementation was daily carried out throughout the suckling period while allowing the pups to consume the breast milk of their mothers *ad libitum*. All pups were weighed daily and, 30 min before oral administration, were temporarily separated from their mothers to facilitate stomach emptying. They were then supplemented by oral gavage with a volume of 10 mL/kg/day, from day 1 to day 21 of age, as described in previous studies in the group [29,30]. Depending on the experimental group, pups received TGF-β2 at a dose of 35 μg/kg/day, EGF at 100 μg/kg/day or FGF21 at 5 μg/kg/day, as described in our preceding research [27,28]. The REF group received a matched volume of water. All animals started the study with a similar body weight (7.4±0.1 g/pup for all groups) and growth until day 21 of life was not affected due to the supplementations (61.8±1.6 g/pup for all groups). These findings were in line with a previous study [27]. In this main study, sample collection was performed at the age of 10, 14 and 21 days, with n=9 pups each time point/group (3 pups/litter).

2.4. Samples collection and processing

In both designs, the rats were anesthetized intramuscularly with ketamine (90 mg/kg) (Merial Laboratories S.A., Barcelona, Spain) and xylazine (10 mg/kg) (Bayer A.G., Leverkusen, Germany) to collect samples. In the four specified days of first experimental design (preliminary study) and on day 10 of second experimental design (main interventional study) blood samples were obtained by cardiac puncture in order to evaluate the intestinal permeability. Then, the abdomen was opened and the entire small intestine was carefully collected. After dissection, the small intestine was flushed with cold saline solution to remove the content. Intestine was weighed, measured and divided into three equal-length portions, designated as proximal, middle and distal. Samples of 0.5 cm of the middle small intestine of 10- and 21-day-old pups from both experimental designs were obtained and placed into tubes with RNAlater™ (Invitrogen, Thermo Fisher Scientific, Lithuania), and stored at -20°C until PCR analysis. In the second experimental design also distal small intestine portions of 10-day-old animals were collected and fixed overnight in 4% paraformaldehyde (Panreac, Barcelona, Spain) for histomorphometric and immunohistochemical assays. Then, the proximal small intestine portion was cut lengthwise along the mesenteric line and was sectioned into 2 cm piece and immersed into a tube with Hanks' balanced salt solution (HBSS) (Sigma-Aldrich) without calcium and magnesium and stored in ice until the IEL obtaining process.

2.5. Intestinal permeability assay

The permeability of the intestinal epithelial barrier was assessed by measuring the movement of 4-kDa-Dextran across the

paracellular pathway into the bloodstream, as in previous studies [31,32]. After 1 hour of separation of the pups from their mothers, rats were orally gavaged a 4 kDa-Dextran conjugated to fluorescein isothiocyanate (FITC) (Sigma-Aldrich) dissolved in PBS at a dosage of 500 mg/kg of body weight, using adapted low-capacity syringes. To account for any background fluorescence, a control sample pool was prepared from animals given an equivalent volume of PBS. Four hours post-Dextran administration, the animals were anesthetized and euthanized, and plasma was collected, diluted, and analyzed for fluorescence emission in triplicate using an excitation wavelength of 490 nm on the Modulus™ Microplate spectrophotometer.

2.6. Intestinal histomorphometric study

Fixed intestinal samples from 10-day-old pups were dehydrated in serial dilutions of 70%–100% ethanol followed by xylene (Honeywell Chemicals, Belgium), and then paraffin-embedded, microtome-sectioned and stained with periodic acid-Schiff (PAS)-hematoxylin, as previously described [31,33,34]. The architecture of intestine was analyzed by measuring the villi height (junction of the villi-crypts to the top of the villi), villi width (middle of the villi), villi area and epithelium perimeter of the villi (outer layer of the villi) [31]. For this, 4–10 villi per each section of distal small intestine from n=4 animals/group were evaluated. In addition, the number of goblet cells per villi was counted and was expressed as PAS-positive cells. Statistical analysis was performed using the average values of each animal.

2.7. Intestinal immunohistochemical analyses

Immunofluorescence staining for different TJs was performed as previously described [31]. Occludin, zonula occludens (ZO)-1, claudin-2 and claudin-4 staining was performed in paraffin embedded sections. For this purpose, paraffin was removed by xylene-ethanol gradient. For antigen retrieval, sections were boiled in Tris-EDTA solution (pH 9) and then washed twice with PBS solution. Then slides were permeabilized with PBS solution containing 0.2% Tween 20. To block nonspecific binding sites, slides were bathed in PBS-BSA 1% solution for 30 min at room temperature. For immunostaining, the primary antibodies (Ab) and dilutions used were antioccludin monoclonal (1:100), anti-ZO-1 polyclonal (1:50), anticlaudin-2 monoclonal (1:100), and anticlaudin-4 polyclonal (1:50) Ab, all from Thermo Fisher Scientific. The sections were incubated overnight at 4°C with primary Ab, and after incubation, the slides were rinsed three times with PBS with 0.05% Tween 20 for 10 min.

The tissue was then incubated with the secondary Ab, the Alexa Fluor® 555 donkey antirabbit IgG (H+L, Invitrogen), diluted 1:1,000 in blocking solution for 1 h at 5°C in a humidity chamber. Then, sections were washed three times with PBS with 0.05% Tween 20. Finally, for nuclei staining, sections were incubated for 10 min in a humidity chamber with 4′,6-diamidino-2-phenylindole (DAPI, 1:1,000, Invitrogen), then rinsed three times with PBS for 10 min and mounted with Fluoromount G™ (Invitrogen). Controls were incubated with secondary Ab only.

Images were taken and analyzed as previously reported [31].

2.8. Intestinal gene expression by real-time PCR

Reverse transcription and quantitative PCR were carried out as in previous studies [34–36] in small intestine samples from animals at days 10 and 21 in the preliminary study and from animals at day 21 in the interventional study. The specific PCR TaqMan® primers (AB) used to perform the PCR quantitative assay are shown in Table S1. The relative gene expression was normalized to the housekeeping gene *Gusb* (Rn00566655_m1, I) using the $2^{-\Delta\Delta Ct}$ method [37]. Results are expressed as percentage of values of each experimental group normalized to the mean value obtained for the REF group, which was set at 100%.

2.9. Isolation and purification of intraepithelial lymphocytes

IELs suspension was obtained from the proximal small intestine of 14- and 21-days-old pups of the main study following usual procedures used in our laboratory [38,39]. Cell number and viability were determined using a Countess™ Automated Cell Counter (Invitrogen) as previously described [38]. IELs were immediately immunophenotyped by multiple immunofluorescence staining technique.

2.10. Immunofluorescence staining and flow cytometry analysis

IELs (2×10^5 cells) were stained with antirat monoclonal antibody (mAb) conjugated to FITC, phycoerythrin (PE), peridinin-chlorophyll-a-protein (PercP), allophycocyanin (APC) or APC-cyanine (Cy)7, as in previous studies [27,29]. The mAb used in this study are described in Table S2. A negative control staining using an isotype-matched mAb was included in each cell sample. For cell subset differentiation four different mAb panels were used (Fig. S2)

Analyses were performed as in previous studies [27,29] with the use of a Gallios™ Cytometer (Beckman Coulter, Miami, FL, USA) in the Flow Cytometry Unit of the Scientific and Technological Centers of the University of Barcelona (CCiTUB).

2.11. Statistical analysis

All statistical analyses were performed using the IBM Social Sciences Software Program (SPSS, version 22.0, Chicago, IL, USA). Levene's and Shapiro–Wilk tests were applied to assess homogeneity of variance and normality distribution, respectively. When the results demonstrated equality of variance and normal distribution, a one-way ANOVA test was carried out. Nonparametric tests were performed when equality of variance and/or normal distribution did not exist. Specifically, Kruskal–Wallis and Dunn's multiple comparison test was used to assess significance for independent samples. Significant differences were established at $P < .05$. All data in the text, tables and figures are expressed as the mean±standard error of the mean (S.E.M.).

3. Results

3.1. Intestinal barrier function during the suckling period

The *in vivo* intestinal permeability to FITC-Dextran (4 kDa) during the suckling period and 1 week after weaning (day 28) is summarized in Fig. 1A. Results showed a higher permeability on days 10 and 14 compared to days 21 and 28. Moreover, an acute decrease in the intestinal permeability was observed at day 21, which was similar to that in day 28, suggesting the achievement of intestinal maturity at the end of the sucking period. The gene expression of molecules related to intestinal barrier function was then evaluated at two time points: day 10, with still high permeability, and day 21, with low permeability, (Fig. 1B). It can be observed that at day 21, the gene expression of all molecules analyzed (occludin, ZO-1, claudin-4, MUC-2 and MUC-3) showed higher gene expression levels than at day 10 ($P < .05$). Thus, among the days studied here and assuming that earlier times were not explored, the time range between 10 and 14 days, when the

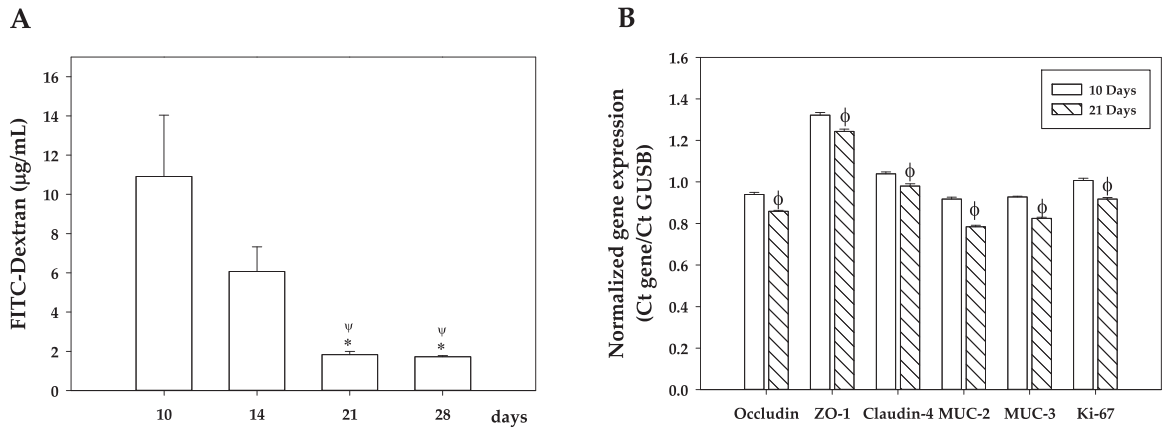

Fig. 1. Intestinal barrier function during the suckling period. (A) Intestinal permeability to FITC-Dextran (4 kDa) during the suckling period (10 and 14 days), weaning day (21 days) and one week after weaning (28 days). (B) Gene expression of tight junction molecules: occludin, zonula occludens-1 (ZO-1) and claudin-4, and mucins (MUC-2 and MUC-3) of small intestine samples at 10 and 21 days of life, results are normalized as ratio Ct gen/Ct GUSB; thus, lower bars mean higher gene expression than taller bars. Results are expressed as mean±S.E.M. (n=9 pups/group). Statistical differences: $^{*}P < .05$ vs. day 10; $^{\Psi}P < .05$ vs. day 14 and $^{\phi}p < .001$ vs. day 10.

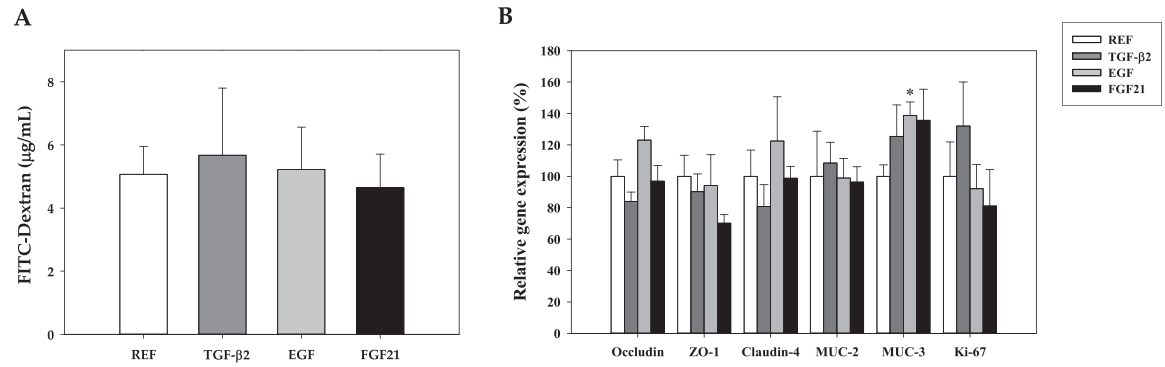

Fig. 2. Influence of growth factors supplementation in the intestinal barrier function at 10 days of life. (A) Intestinal permeability to FITC-Dextran (4kDa). (B) Relative gene expression of tight junction proteins occludin, zonula occludens-1 (ZO-1) and claudin-4, mucin genes (MUC-2 and MUC-3) and Ki-67 (marker of proliferation) were quantified by real-time PCR in small intestinal samples. Relative gene expression was calculated with respect to REF group, which corresponded to 100% of transcription. Results are expressed as mean±S.E.M. (n=9 pups/group). Statistical differences: $^{*}P < .05$ vs. REF.

epithelial barrier function was not yet fully mature, could be adequate for performing studies about the influence of the growth factors supplementation in suckling rats.

3.2. Effect of supplementation with growth factors on the intestinal epithelial barrier function

To evaluate the influence of supplementation with growth factors on the intestinal barrier function, intestinal permeability, gene expression, histomorphometric and immunohistochemical studies were performed. In 10-days-old rats, no changes on intestinal permeability were observed after TGF-β2, EGF or FGF21 supplementation (Fig. 2A). In the study of the relative gene expression of proteins involved in TJ, mucins and proliferation at the same age (Fig. 2B), only MUC-3 levels were significantly increased due to EGF supplementation ($P < .05$ vs. REF group, Fig. 2B).

On day 21, none of the growth factor supplementations significantly modified the gene expression of occludin, ZO-1, claudin-4, MUC-2 or MUC-3 (Table S3). Most of the gene expression values in all experimental groups ranged between 80% and 120% of those in the REF group. However, although no statistically significant differences were observed, MUC-2 expression in the EGF-supplemented group tended to be reduced (~60%), while FGF21supplementation showed a trend towards higher expression of claudin-4 (~150%) and MUC-3 (~145%).

In addition, after 21 days of supplementation, the relative gene expression of several TLR such as TLR-2, -4, -5, -7, -9 was evaluated (Table S3). All TLR showed a similar Ct target gene/ Ct GUSB gene ratio with values between (~0.95 and ~1.12) being the TLR5 the one having the highest gene expression. Only the supplementation with EGF showed a trend to decrease the expression of TLR9 (~50%). The relative expression of genes encoding neonatal-Fc-receptor (FcRn) and B-lymphocyte-induced maturation-protein-1 (BLIMP-1) was also assessed on day 21 (Table S3). The FGF21 supplementation was able to significantly decrease the FcRn gene expression (~30%) compared to the REF group.

Histomorphometric analysis did not reveal significant effects of the growth factors supplementation on villi variables (Table 1). However, animals supplemented with TGF-β2 showed a decrease in muscle and submucosa layer thickness ($P < .05$ vs. REF group, Table 1). Moreover, the supplementation with FGF21 showed a tendency to increase villi width (~21%), villi height (~10%) and villi area (~35%) and a tendency to increase the number of goblet cells/villi (~20%). The goblet cell area in rats supplemented with EGF and FGF21 was larger than that in REF group (Table 1), although the number of goblet cells per villus remained similar.

Table 1
Histomorphometric variables of small intestine: villi width, height, area, perimeter and the number of goblet cells/villus at day 10 of the suckling period

	REF	TGF-β2	EGF	FGF21
Villi width (μm)	119.2±8.4	101.9±8.5	128.7±10.9	150.7±10.8
Villi height (μm)	415.9±24.4	431.9±19.0	402.3±42.4	455.0±25.1
Villi area (μm^2)	43638.0±2878.8	39854.8±2110.3	35620.5±4272.3	58704.0±7784.7
Villi perimeter (μm)	1042.7±79.1	941.8±33.6	1028.3±95.8	1042.2±54.9
Muscle layer (μm)	107.0±13.8	51.1±0.3*	74.8±5.4	98.1±19.0
Submucosa layer (μm)	121.5±13.95	78.2±5.3*	93.0±3.1	130.2±13.8
Goblet cells/villus	4.7±0.4	5.9±2.1	4.7±0.6	5.6±0.4
Goblet cells area (μm^2)	38.5±1.82	47.0±1.84	48.2±3.71*	51.7±1.13*

Table 2
Main lymphocyte subsets in intraepithelial lymphocytes

Table		REF	TGF-β2	EGF	FGF21
Day 14					
	TCR$\alpha\beta^+$ (%)	16.36±1.15	14.68±1.64	13.48±1.38	14.67±0.79
	TCR$\gamma\delta^+$ (%)	12.32±1.54	12.49±1.25	11.97±0.27	12.90±0.69
	NK (%)	38.80±0.82	41.71±3.52	41.70±1.90	33.92 ±2.92
	NKT (%)	28.90±2.88	29.45±1.96	28.32±1.47	34.05±2.31
	$CD4^+$ (%)	3.20±1.04	2.08±0.44	3.26±0.98	2.02±0.25
Day 21					
	TCR$\alpha\beta^+$ (%)	11.75±2.29	13.68±0.85	8.07±0.34$^{\Psi}$	9.79±0.23$^{\Psi}$
	TCR$\gamma\delta^+$ (%)	12.01±0.30	13.29±1.08$^{\Psi}$	11.14±0.49	13.23±0.55
	NK (%)	44.76±1.26	47.46±3.15	51.20±1.77$^{\Psi}$	49.37±1.24$^{\Psi}$
	NKT (%)	20.90±4.08	20.28±1.29$^{\Psi}$	20.13±0.75$^{\Psi}$	18.23±0.41$^{\Psi}$
	$CD4^+$ (%)	4.53±0.84	3.44±0.58	3.60±0.47	4.51±0.11$^{\Psi}$

Moreover, to analyze changes in the distribution of occludin, ZO-1, and claudins-2 and -4 due to supplementation, fluorescence immunohistochemical staining was performed (Figure S3). Supplementation with FGF21 resulted in an occludin staining pattern similar to that of the REF group, whereas supplementation with TGF-β2 and EGF showed images with lower fluorescence levels. Additionally, TGF-β2 supplementation led to higher fluorescence levels of ZO-1 compared to the REF group, while EGF and FGF21 exhibited staining profile similar to that of the REF group. The staining pattern of claudin-2 was consistent across all groups. Finally, claudin-4 was localized in the villus tip in the REF group but TGF-β2 and FGF21 supplementation showed higher fluorescence levels and a different staining pattern. In contrast, EGF supplementation resulted in lower fluorescence levels compared to the REF group (Figure S3).

Results are expressed as a mean±S.E.M. (n=9/group). Statistical differences: *P < .05 vs. REF. Four experimental groups: reference (REF), transforming growth factor-β2 (TGF-β2), epidermal growth factor (EGF) and fibroblast growth factor 21 (FGF21).

3.3. Intraepithelial lymphocytes composition

The main lymphocyte subsets in IELs were quantified at 14 and 21 days of life. At both ages, NK cell percentage was the highest (~34%–51%), followed by that of NKT cells (~18%–34%) and T cells (~19%–27%) (Table 2). None of the supplementations significantly modified the main composition of IELs compared to the REF group. However, differential effects between day 14 and day 21 due to the supplementations were observed. The proportion of NK cells, which did not change with age in the REF group, increased by about 23% and 46% after EGF and FGF21 supplementations, respectively (P < .05 day 21 vs. day 14), All the supplemented groups, but not REF one, exhibited a decrease with age in the proportion of NKT cells (~35% reduction, P < .05 vs. day 14) and TCR$\alpha\beta$+ cells (~20% decrease, P < .05 vs. day 14) in older animals.

Results are expressed as a mean±S.E.M. (n=9 per group). Statistical differences: $^{\Psi}P$ < .05 vs. same group at day 14. Four experimental groups: reference (REF), transforming growth factor-β2 (TGF-β2), epidermal growth factor (EGF) and fibroblast growth factor 21 (FGF21).

The CD8 co-expression was also studied in each of the main IEL subsets (Fig. 3). Overall, no changes were observed in the proportion of $CD8^+$ cells within the TCR$\alpha\beta^+$ subset due to growth factors supplementation or age, with the exception of a reduction by the age in FGF21 group (25% reduction, P < .05 day 21 vs. day 14, Fig. 3A). In contrast, all studied groups showed lower levels of $CD8^+$ cells in the TCR$\gamma\delta^+$ population with age (P < .05, Fig. 3B). Additionally, TGF-β2 supplementation increased the proportion of $CD8^+$ TCR$\gamma\delta^+$ cells in this population at 14 days of life compared to the REF group (P < .05, Fig. 3B).

Moreover, the supplementation with EGF and FGF21 decreased the percentage of NKT cells co-expressing CD8 at 21 days due to both the supplementation and the age (P < .05, Fig. 3C). In the case of NK $CD8^+$ cells, the EGF supplementation increased this proportion at day 21 compared to day 14 (25% increase, Fig. 3D).

When analyzing the overall presence of $CD8^+$ cells in IELs, it can be observed that the high proportion found at the age of 14 days (60%–70%) in all groups decreased with age, falling to 50% by 21 days (P < .05, Fig. 4A). Only FGF21 supplementation was able to accelerate such age-associated reduction compared with that of the REF group (P < .05). Moreover, FGF21 supplementation doubled the proportion of $CD4^+$ cells (Table 2) at 21 days of life (P < .05 vs. day 14).

With regard to the results from the CD8$\alpha\alpha$/CD8$\alpha\beta$ analysis, the REF group showed a relative reduction in the proportion of CD8$\alpha\beta^+$ cells (Fig. 4B) and an increase in CD8$\alpha\alpha^+$ cells (Fig. 4C).

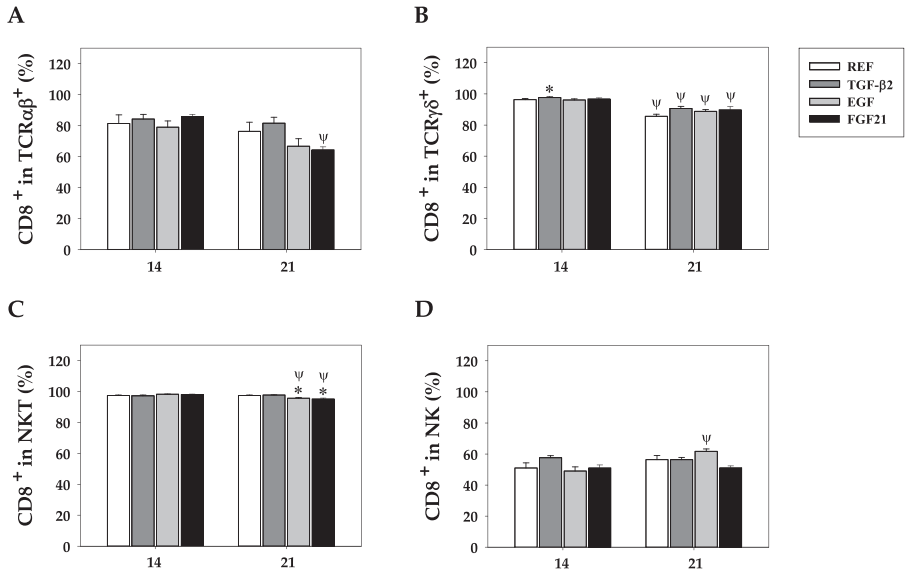

Fig. 3. Surface expression of CD8 molecule in the main IEL subsets at 14 and 21 days of life. Percentage of $CD8^+$ cells in (A) $TCR\alpha\beta^+$; (B) $TCR\gamma\delta^+$; (C) NKT; and (D) NK IEL. Results are expressed as mean±S.E.M. (n=9 pups/group). Statistical differences: $^*P < .05$ vs. REF group at same age; $^{\Psi}P < .05$ vs. same group at day 14.

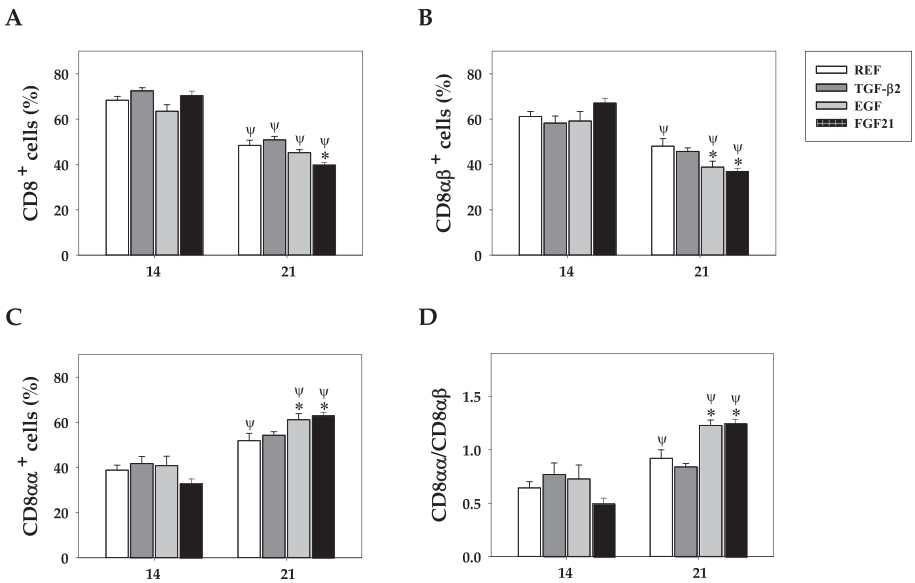

Fig. 4. Proportion of $CD8^+$ cells, $CD8\alpha\beta^+$ cells, $CD8\alpha\alpha^+$ cells, and ratio $CD8\alpha\alpha/CD8\alpha\beta$ in IEL at 14 and 21 days of life. Percentage of (A) $CD8^+$ cells; (B) $CD8\alpha\beta^+$ cells; (C) $CD8\alpha\alpha^+$ cells and (D) $CD8\alpha\alpha/CD8\alpha\beta$ ratio in IEL. Results are expressed as mean±S.E.M. (n=9 pups/group). Statistical differences: $^*P < .05$ vs. REF group at same age; $^{\Psi}P < .05$ vs. same group at day 14.

This effect led to an age-associated pattern of increasing the $CD8\alpha\alpha/CD8\alpha\beta$ ratio (Fig. 4D). The supplementations with EGF and FGF21 enhanced the age maturation process of this phenotypical markers observed in the REF group.

3.4. Intraepithelial lymphocytes homing and activation patterns

The surface αE/CD62L molecular pattern was evaluated in IELs at 14 (Table S4) and 21 days of life (Fig. 5). As it can be observed, the proportion of αE^+CD62L^- cells is the highest at 21 day (around 80% in REF group). This cell phenotype was also predominant at 14 days; however, its proportion decreased with age in all groups, independently of the supplementation given, thus, all following the same physiological maturation process. However, some changes compared to the REF group were found due to TGF-β2 supplementation at 14 days ($P < .05$, Table S4) and to FGF21 at 21 days ($P < .05$ vs. REF group, Fig. 5D). Regarding the other αE/CD62L subsets, the supplementation with TGF-β2, EGF and FGF21 induced higher proportions of αE^-CD62L^- and αE^-CD62L^+ subsets at 21 days of life compared to day 14 ($P < .05$), however these changes were not observed in the REF group. On the other hand, FGF21 group had higher values of αE^+CD62L^+ cells ($P < .05$, day 21 vs. day 14).

The proportion of integrin αE^+ cells and selectin $CD62L^+$ cells in $CD8^+$, $CD4^+$ and $CD8^+CD4^+$ were further studied (Table S5). All studied groups also showed age-associated changes in αE and CD62L expressions in the $CD8^+$ IEL subset ($P < .05$ day 21 vs. day 14). However, only the EGF supplementation was able to induce

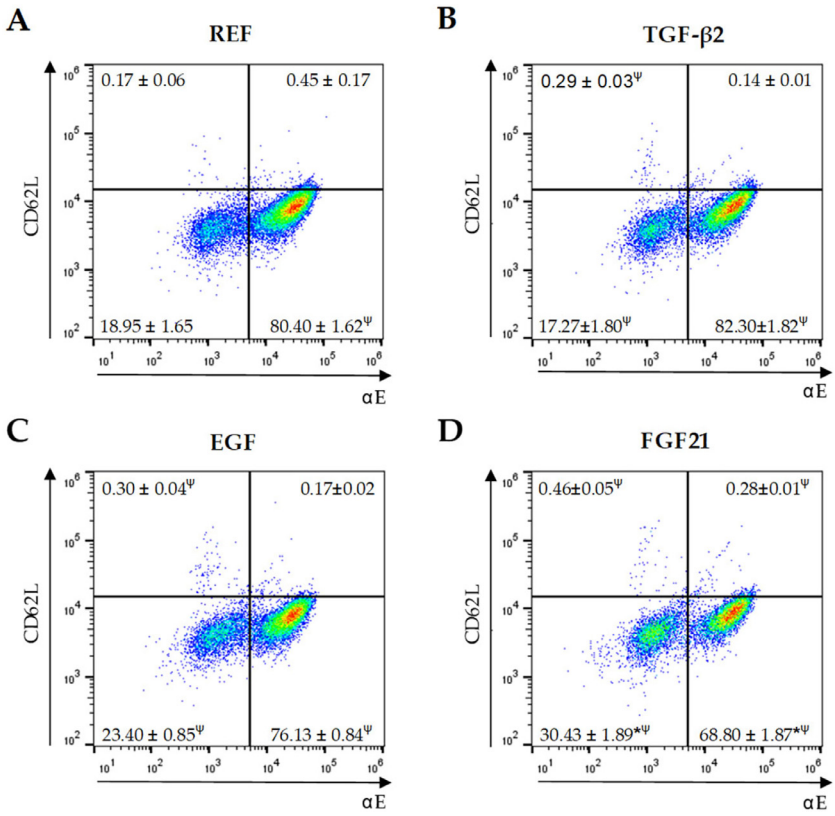

Fig. 5. Surface expression of αE integrin and CD62L selectin in IELs at 21 days of life. Representative histograms of CD62L/αE integrin surface expression and mean±S.E.M. (n=9 pups/group) in the four experimental groups: (A) REF; (B) TGF-β2; (C) EGF; and (D) FGF21. Statistical differences: $^{\Psi}P < .05$ vs. same group at day 14.

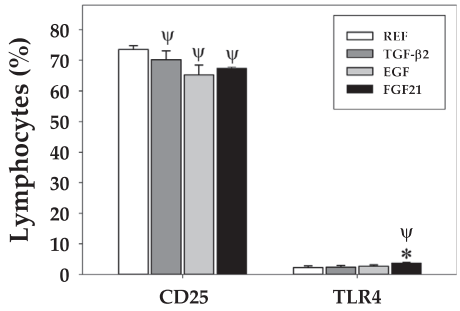

Fig. 6. Percentage of CD25 and TLR4 in IELs at 21 days of life. Results are expressed as mean±S.E.M. (n=9 pups/group). Statistical differences: $^{*}P < .05$ vs. REF group at same age, $^{\Psi}P < .05$ vs. same group at day 14.

changes with respect to the REF group, promoting, overall, a decrease of αE molecule expression in CD8$^+$ (~4%) at day 21 with respect to the REF group.

On the other hand, no changes were observed in the αE and CD62L expressions in CD4$^+$ cells with any group. With regard to the αE/CD62L pattern in CD8$^+$CD4$^+$ cells, the FGF21 supplementation increased the proportion of αE$^+$CD62L$^+$ (~36%) whereas decreased that of αE$^+$CD62L$^-$ (48%) compared to the REF group at 21 days (Table S6, $P < .05$).

Finally, the overall CD25 and TLR4 surface expression of the isolated IELs at 21 days of life was also determined (Fig. 6). All the supplementations reduced the activation state (CD25 proportion) from day 14 to day 21, whereas no changes due to age were observed in the REF group. With regard to TLR4$^+$ cells in IEL, FGF21 supplementation induced a slight but significant increase in its proportion ($P < .05$ vs. REF group at 21 days, Fig. 6).

4. Discussion

Newborns have an immature intestine with underdeveloped barrier function. In the early days of life, a large number of macromolecules can pass through the epithelium into systemic circulation, increasing susceptibility to infectious agents and potentially leading to the development of necrotizing enterocolitis (NEC) [17,40]. This early immaturity and its effects are more pronounced in preterm newborns [41]. For that reason, it is important that the intestinal barrier and immune system of the newborn mature rapidly and properly. Recent reviews have highlighted the importance of the programmed developmental steps and the response to exogenous stimuli in establishing the proper immune homeostasis [42]. In addition, weaning has been identified as a crucial period in the immune system ontogeny due to the “window exposure” to the microbiota [43]. However, despite early microbial colonization, proper immune system maturation is necessary for the correct establishment of intestinal microbe-host interaction [44].

In general, it is known that certain bioactive compounds present in breast milk play a crucial role in the maturation of the gastrointestinal tract [17,23,24,45]. EGF is a molecule clearly involved in the intestinal growth, proliferation of crypt enterocyte and mucosal repair [46,47]. TGF-β2 is a key cytokine regulating enterocyte differentiation and immune tolerance [47,48]. FGF21 is a factor recently described to be present in breast milk with metabolic functions in the early life [21]. Thus, all together, may participate in this maturation process. The presence of specific receptors (EGFR/ErbB1, TGFβRI/TGFβRII and FGFR1-4, respectively)

found in the newborn intestinal epithelium reinforces a local role [25]. In this context, the present study was designed to ascertain the effect of TGF-β2, EGF and FGF21 on the maturity of barrier function of the small intestine, includingthe IELs evaluation, during and at the end of the suckling period in rats.

As a general view, none of the interventions with the bioactive factors assayed were able to impact the intestinal growth in terms of weight and length or histological analysis. Although the literature is scarce about the effect of TGF-β2 or FGF21 on intestinal development, it is generally recognized that EGF has a crucial function in the early development of the intestinal architecture by increasing villi height, crypt depth, etc., in different animal models (pigs, rabbits, mice and rats) [46,49]. In our study, EGF did not induce such changes; however, it has to be taken into account that some of these effects have been described after receiving EGF by a nonphysiological route, such as the intraperitoneal one [50]; or using oral administration of EGF in concentrations that exceed the physiological concentration in milk [51], which may explain such differences reported in the literature compared to our results.

With regard to the development of the intestinal functionality, in the present study although it was not studied at earlier ages, shows that the intestinal barrier function is still in development in 2-weeks-old rats but acquire permeability characteristics on day 21, the weaning day. These results agree with previous studies demonstrating that, at the end of the suckling period, rats have gut closure followed by a marked permeability decrease [38,52–54]. Moreover, our results reinforce the existence of a functional immaturity in the middle of the suckling period (14 days) which is associated with lower gene expression of TJ proteins (occludin, ZO-1 and claudin-4) and secretory mucin genes (MUC-2 and MUC-3) with respect to weanling rats. These results are in line with previous studies in rodents, and in humans [55]. Thus, this particular period of life might be adequate for studying the influence of external factors, such as dietary components, on the intestinal barrier function. We cannot discard that this time period of opportunity to modulate the barrier function could happen even earlier, but it has not been assessed here. Even though, the interventional study with the growth factors could have been performed also at later times, from 14 to 21 days, in order to have more impact derived from their supplementations.

Thus, the first aim of this nutritional approach using growth factors present in breast milk was to determine whether the supplementation had an impact on the maturation of the neonatal intestinal barrier. It has been shown, in various neonatal animal models, that breast milk has an important role in enhancing intestinal growth and function [51], a process in which the formation of TJ is a key step for the enhancement of the epithelial barrier [56]. However, we observed that the supplementations with the growth factors did not have a significant effect on intestinal permeability or TJ gene expression or localization in the present approach. These results do not match with other studies showing that TGF-α and EGF are able to decrease gut permeability by increasing the expression of TJ proteins [46,57,58]. However, we observed that EGF supplementation, but not other factors, enhanced the acquisition of the capacity of mucin production, specifically MUC-3, after 10 days of supplementation. In line with this result, a positive association has been described between TNF-α and MUC-3 mRNA levels both at preclinical [59] and clinical levels [60]. Thus, according to these data, it could be inferred that the MUC-3 increase might be caused by higher TNF-α levels in the GALT. In this case, as stated in a previous study, EGF supplementation can increase TNF-α production by mesenteric lymph nodes cells in suckling rats [27], which could explain the rise in the MUC-3 gene expression observed in the present study. For the other breast milk factors, the lack of effect of TGF-β2 and FGF21 supplementations on intestinal permeability, TJ gene expression and localization cannot be compared to others due to the absence of literature in this topic, thus further investigation should be performed to corroborate our findings.

In addition to the developmental epithelial barrier function study, the expression of FcRn and BLIMP-1 was determined, two genes that are present in the early postnatal intestinal epithelium, the expression of which is used for intestinal maturation assessment [61,58]. In rodents, their expression decreases throughout the suckling period being the lowest at weaning, suggesting that these molecules may not be functional in the adult rodent gut and therefore are specific to this particular period of life [61,62]. FcRn acts as the intestinal IgG receptor and allows its transfer from the suckling gut lumen to the systemic [63], whereas BLIMP-1 is a regulating element in gut maturation [61]. Globally, their expressions were not affected by the supplementations except for the group receiving FGF21, in which a significant decrease in the expression of FcRn was observed. Thus, it can be suggested that this factor would accelerate the gut maturation process, which is accompanied with a reduction in the expression of this IgG receptor. However, parallel studies evaluating the impact of FGF21 on systemic immune response using this model did not show an impact on the overall plasma IgG levels at the end of suckling [28].

For an effective gate-keeper function development of the intestinal epithelium an acquisition of adult-like TLR signaling is also necessary [64]. To evaluate that, the relative gene expression of TLR2, 4, 5, 7, and 9 was studied. The supplementation with EGF was able to downmodulate the TLR9 gene expression at 21 days of life. It has been demonstrated that the gene expression of TLR9 in Swiss Webster mice small intestine was increased with age [65], thus promoting more intense immune responses. In that model, the activation of TLR9 with CpG-DNA limited TLR4 signaling in enterocytes and reduced the severity and the extent of an experimental NEC [66]. Taking this information into account, the present results concerning the effect of EGF supplementation on TLR9 suggest that when the intestinal mucosa is still developing, EGF could be important in the prevention of inflammatory disorders in early life such as NEC [23,41,45,47].

Regarding the histomorphometric study, a decrease in the thickness of the musculature and submucosa layer in TGF-β2-supplemented suckling rats was found. Little is known about the role this growth factor on musculature layer, but Moore-Olufemi *et al.*[66] showed that TGF-β3 increased the cellular contraction. So, it could be similar in TGF-β2 and thus would explain the lower musculature layer thickness. With regard to goblet cell area, the supplementation with EGF and FGF21 increased their size. This result agrees with an increase in the goblet cells area after the administration of this growth factor during the first 17 days of suckling in a model of rat prematurity [31]. This result reinforces the positive effect of EGF on the mucus production and on the enhancement of the intestinal barrier. In case of FGF21, this result was not in line with literature [67]. Overall, further research is required in this regard.

The GALT development process during suckling is well documented in humans [68] and even more exhaustively in rodents [69]. Specifically, the percentage of IEL in rats increases after birth and the proportion of IEL subsets shows an age-dependent maturation process [69]. It is suggested that IEL, besides participating in effector and regulatory immune responses, can participate in tissue surveillance and maintenance of barrier function [2,13,70]. Thus, the present study also focuses on this element of the intestinal defense.

In agreement with previous findings [38,39,69,71,72], it is showed that the main IEL subsets are NK and NKT cells. This is characteristic in rat early life, much more than in adults, and

it can be related to a protective role during the suckling period [38,69,73]. With regard to NK cells, here it is shown that their proportion is high and invariable with age over suckling, which corroborated previous findings [38,73]. However, EGF and FGF21 supplementations increased its proportion at the end of the suckling period, suggesting a potentiation of the innate immunity at this stage. This effect was not always accompanied with changes in the co-expression of CD8 in their surface – a marker mostly present during suckling, but not in older NK cells – which has been ascribed to a characteristic intestinal phenotype [38]. The supplementation with EGF and FGF21 affect NKT development by down-regulating the CD8 surface molecule. These overall findings on NK and NKT lineages may suggest that the supplementation with these bioactive factors, and specifically with EGF and FGF21, may reflect a higher immune maturation of IEL in the small intestine.

In addition to the cells involved in the innate immune responses in early life, T cells are also a major population in the IEL population during this period, without changes in proportion during the last week of suckling, as observed in previous studies [38,39,71,72]. Regarding the interventions, and similar to the effect of EGF and FGF21 on the NKT cells, these active factors also modulated their development. Nevertheless, it is known that intestinal T cells are highly heterogeneous and are distinguished based on T-cell surface receptors TCR$\gamma\delta$ or $\alpha\beta$, based on the $\alpha\alpha$ or $\alpha\beta$ chains on the CD8 molecule or the CD4 membrane co-receptor [7,74–77]. In fact, the T-cell age-decreasing pattern found here after EGF and FGF21 supplementation during suckling is associated with a reduction in TCR$\alpha\beta$ and CD8 co-expression. Overall, it seems that both EGF and FGF21 have an influence on NKT and T TCR$\alpha\beta$ IEL maturation.

Another important obtained result is that the supplementation with TGF-β2 was able to increase the proportion of T TCR$\gamma\delta$+ CD8+ cells at 14 days compared to REF animals. It has been previously described that this T-cell subset colonizes the epithelium in the first days of life and has an important immunoprotective and immunoregulatory role in this period [78]. To date, preterm infants with NEC have lower levels of T TCR$\gamma\delta$+ CD8+ cells compared to term infants [79] and the loss of this subset could represent a disproportional lack of immune regulatory function in the IEL population [80]. Taken all together, it can be hypothesized that the action of this important subset in early life may be driven by TGF-β2 pathways.

Focusing on CD8 expression in IEL, a clear developmental pattern of the CD8$\alpha\alpha$/CD8$\alpha\beta$ ratio was found, in partial agreement with previous studies [38]. This developmental change is potentiated in those animals receiving EGF and FGF21 supplementations, but not in those administrated with TGF-β2. Thus, the first two bioactive breast milk factors seem to be acting on the maturation of IEL, overall promoting a CD8 $\alpha\alpha$ phenotype, that is more typical from a mature intestine.

One of the molecules mediating the selective localization or intestinal retention of IEL is the integrin αE (CD103) [81]. The αE integrin mediates T-cell adhesion to epithelial cells through its binding to E-cadherin (CD62L), a member of the cadherin family of adhesion molecules that is expressed selectively on epithelial cells [81]. It is known that αE integrin is widely expressed on intestinal IEL in both humans and rodents [72,81,82]. The supplementation with TGF-β2 over 2 weeks induced higher levels of $\alpha E^{+}CD62L^{-}$ cell phenotype, which is predominant during life. This effect is in line with previous studies suggesting that TGF-β2 can be involved in αE expression on the surface of IEL [8,83]. On the other hand, FGF21 seems to down-regulate the proportion of $\alpha E^{+}CD62L^{-}$ cells after 21 days of supplementation, achieving the acquisition of a more mature phenotype. In a study conducted in adult αE-deficient mice, the results showed that this molecule is a key homing factor for T cells, particularly for T TCR$\alpha\beta^{+}$ cells compared to T TCR$\gamma\delta$+ [81]. In line with this, our results showed a lower $\alpha E^{+}CD62L^{-}$ cell proportion in the FGF21 group at day 21, which may be linked to the lower T TCR$\alpha\beta^{+}$ cell proportion also found that day.

In the current study, the TLR4 surface expression on IEL was also studied, and a very low proportion of IEL were positive for this marker. The low presence of this molecule allows to support previous findings reported in suckling rats at birth [84] and in the IELs' surface at the end of suckling [72]. With regard to the interventions performed, the supplementation with FGF21 for 21 days was related with an increase in the percentage of $TLR4^{+}$ IEL. This effect could be explained by the fact that TLR4 expression in both enterocytes and immune cells might increase in response to stressors, such as formula feeding and asphyxia stress [85]. In this context, FGF21 has been considered as a stress hormone in several pathological conditions [86,87], thus the plausible explanation for the effect of FGF21 on TLR4 may be that the FGF21 is acting as a stress inductor in this context to potentiate immune responses, which are still not fully orchestrated in early life. However, the phenotypical variability between the different developing animals, and the low proportion of some surface biomarkers (i.e. CD62L) in IEL could have had an impact in the overall results of the study.

Finally, it must be considered that this work has an experimental limitation, due to the difficulty to determine if the effects seen in this study are directly caused by the supplemented components or, indirectly, by the effect that these growth factors might have had on some other mediators involved in the intestinal maturation. In addition, it must be considered that the rats kept their physiological process of suckling, thus, they are not deprived from these components during the study.

5. Conclusions

To sum up, our study demonstrates the capacity of TGF-β2, EGF and FGF21 to differentially modulate the neonatal maturation of the intestinal barrier function and IEL composition. The intestinal immunomodulatory action of FGF21 has been described for the first time here, thus further studies should target this growth factor to better understand its role in the immune system. On the other hand, these results reinforce the role of the bioactive factors present in breast milk in the development of the intestine and immunity. Further studies are needed to fully understand the implications of these factors during suckling, and mainly in the first days of life.

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Declaration of competing interest

The authors declare that there are no conflicts of interest.

CRediT authorship contribution statement

Blanca Grases-Pintó: Writing – original draft, Investigation. **Paulina Torres-Castro:** Investigation. **Mar Abril-Gil:** Investigation.

Margarida Castell: Supervision, Data curation. **María J. Rodríguez-Lagunas:** Supervision, Methodology, Investigation. **Francisco J. Pérez-Cano:** Writing – review & editing, Writing – original draft, Supervision, Formal analysis, Conceptualization. **Àngels Franch:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jnutbio.2024.109778.

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