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Breastfeeding regulates development of immune system through TGF- β in mice pups

TGF- β suppresses innate immune activation

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Abstract

Background: Breast milk contains important nutrients and immunoregulatory factors that are essential for newborn infants. Recently, epidemiological studies suggested that breastfeeding prevents a wide range of infectious diseases and lowers the incidence of infant allergic diseases.

Methods: To examine the effects of breast milk in immunological development in infancy, we established an artificial rearing system for hand-feeding mice and compared mouse pups fed with either breast milk or milk substitute. All mice were sacrificed at 14 days of age and immune cell populations in the thymus, spleen, and small intestine were examined using flow cytometry.

Results: The number of thymocytes was higher whereas that of total immune cells of peripheral lymphoid tissues was lower in mice fed breast milk than in those fed milk substitute. In peripheral lymphoid tissues, the frequency of B cells was higher and the frequencies of CD8⁺ T cells, macrophages, dendritic cells, and granulocytes were significantly lower in breast milk-fed mice. The similar alteration in immune cells of thymus and peripheral lymphoid tissues as seen in milk substitute-fed mice was observed in pups reared by mother mice treated with anti-TGF- β mAb.

Conclusions: These results suggested that breast milk regulates the differentiation and expansion of innate and adaptive immune cells partly due to TGF- β . Our findings may provide the new concept that TGF- β in breast milk is involved in a new therapeutic strategy for innate immune system-mediated infant diseases.

Keywords:

artificial rearing system, breastfeeding, humoral immunity, innate immunity, TGF- β

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Introduction

Breast milk is the gold standard for feeding infants (1, 2) and contains critical nutrients and bioactive substances possessing protective and immunoregulatory effects (3-5). Breastfeeding prevents respiratory infections (6), diarrhea (7), urinary tract infections (8) otitis media (9), and necrotizing enterocolitis (10). Epidemiological studies suggest that breast milk reduces the risk of various immune-mediated childhood diseases, including type 1 diabetes (11), celiac disease (12), and inflammatory bowel disease (13). Recent studies revealed the relationship between breastfeeding and the development of allergic diseases, including atopic dermatitis (14), food allergies, and asthma.

It is well recognized that the major defense factor in breast milk is secretory IgA, which protects the intestinal mucosa by coating and binding to antigens in the mucus layer to regulate immune responses to dietary and bacterial antigens (15). Breast milk contains factors such as anti-inflammatory cytokines (TGF- β and IL-10) and pro-inflammatory cytokines (IL-1, IL-6, TNF- α , IFN- γ , IL-4, IL-5, and IL-13) (16). The balance of these cytokines modulates immune functions in infants. TGF- β in breast milk has been suggested to be associated with oral tolerance induction in childhood through IgA antibody production and the differentiation of regulatory T cells (17-21). However, the effect of TGF- β on the innate immune cells in infants remains unclear.

Although these findings indicate that breast milk contain beneficial factors for the infant's immune system, it is not easy to examine the immunological effects of breast milk in human infants. To examine the effects of breast milk in immunological development in infancy, we established an artificial rearing system for feeding pups of mice with our hands from postnatal (PN) day 2 (PN2),

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and assessed the population of intestinal immune cells together with thymic and splenic cells under the different nutritional environments, including breast milk and milk substitute. Furthermore, we demonstrated that breast milk controls differentiation and expansion of innate and acquired immune cells through TGF- β .

Materials and Methods

Mice

Pregnant-specific pathogen-free BALB/c mice were purchased from Charles River, Inc. (Yokohama, Japan), individually housed, and maintained according to institutional guidelines. They were given free access to distilled water, fed on pelleted diets, and allowed to spontaneously deliver. All animal experiments were approved by the Institutional Animal Care and Use Committee of Azabu University and Juntendo Animal Experimental Ethics Committee.

Composition and preparation of mice formula

The milk substitute formula was developed according to Yajima et al. (22), with slight modifications (Table 1). Casein and whey protein were used as protein sources; lactose was used as carbohydrate. Milk was homogenized twice under high pressure (800–1000 bar) using a high-pressure homogenizer (Panda PLUS 2000; Niro Soavi S.p.A., Parma, Italy), resulting in emulsified, sterilized, and smoothed milk, and stored at -80°C .

Artificial rearing using the milk substitute for mice

In the artificial rearing from PN2 (AR-2) group, newborn pups were separated from their mothers at PN2 and kept with parous, ovariectomized foster mothers. Pups were reared with milk substitute using the artificial rearing system comprising a custom-made nursing bottle, which had silicon nipples, milk inflow tube, milk overflow tube, and refill syringe (Meiji Dairies Corporation, Tokyo, Japan). The milk substitute was loaded into the nursing bottle using a sterilized syringe. Pups were fed every 3 h (five times daily). The bottles, nipples, and fresh milk were stored at 4°C after feeding to avoid bacterial growth. In the mother-reared (MR) group, pups were reared under normal conditions by their mothers throughout the study. The number of MR group pups was adjusted to 10 per mother to maintain similar growth rates as recorded for the AR-2 pups.

Study design

All pups were weighed daily; and all mice were sacrificed 14 days after birth. The internal organs were collected; the thymus was weighed. A small intestine segment (duodenum to the ileocecal valve) was isolated. The thymus, spleen, and small intestine were immersed in Hanks' balanced salt solution (HBSS; Thermo Fisher Scientific, Waltham, MA, USA) containing 5% fetal bovine serum (FBS; Thermo Fisher Scientific) and 15 mM HEPES buffer (HBSS-FBS-HEPES buffer).

Preparing thymocytes, splenic cells, and lamina propria cells

The thymus was triturated and washed with PBS containing 2% FBS. Collected cells were used as thymocytes. The spleen was placed in a 60-mm dish and injected with 1 mL of RPMI 1640 culture medium (Thermo Fisher Scientific) containing 100 U/mL collagenase (Wako, Osaka, Japan) using a 1-mL syringe with a 26G needle. Using a disposable scalpel, the tissue was finely cut up and incubated at 37°C for 30 min at 5% CO₂ in 5 mL of RPMI 1640 culture medium containing 400 U/mL collagenase. After adding 50 µL of 250 mM EDTA (Wako) to the dish, the spleen was pipetted and meshed through a filter. Collected cells were treated with ammonium-chloride-potassium lysis buffer to remove erythrocytes and used as splenic cells. Lamina propria (LP) cells from the small intestine were prepared for analysis. Visible Peyer's patches were excised; the intestine was longitudinally opened, washed with 10 mL HBSS-FBS-HEPES buffer in a 60-mm dish to remove fecal content, and then cut into 0.5–1.0 cm pieces. The pieces were washed with 45 mL HBSS-FBS-HEPES buffer and incubated in 30 mL HBSS buffer supplemented with 0.5 mM EDTA, 2 mM dithiothreitol (Wako), 5% FBS, and 15 mM HEPES in a shaking water bath at 165 strokes/min for 40 min at 37°C. Supernatant containing epithelial cells was removed; tissue pieces were washed with 40 mL of RPMI 1640 culture medium containing 10% FBS and 25 mM HEPES buffer. Then, the tissue pieces were incubated with 20 mL of RPMI 1640 that was supplemented with 50 µg/mL LiberaseTM (Roche, Basel, Switzerland), 15 µg/mL DNase1 (Roche), and 25 mM HEPES buffer at 37°C for 35 min in a shaking water bath at 165 strokes/min. Released cells were passed through 70- and 40-µm cell strainers, suspended with 40% Percoll (GE Healthcare, Uppsala, Sweden) solution, and centrifuged at 2800 rpm for 20 min. Cell pellets were washed and used as LP cells.

Flow cytometric analysis

Thymocytes, splenic cells, and LP cells were pretreated with unlabeled anti-CD16/32mAb (clone: 2.4G2) to avoid non-specific binding of antibodies to FcγR and stained with the following monoclonal antibodies (mAbs): CD8a-fluorescein isothiocyanate, CD25-Brilliant Violet 421, and F4/80-Brilliant Violet 421 (Biolegend, San Diego, CA, USA); CD3-Brilliant Violet 605, CD4-allophycocyanin (APC)-H7, CD19-PE-CF594, CD11b-Alexa Fluor 700, and CD45.2-Brilliant Violet 510 (BD Biosciences, San Jose, CA, USA); and CD11c-APC (TONBO Biosciences, San Diego, CA, USA). In most experiments, samples were fixed using Foxp-3 Staining Buffer Set (eBioscience, San Diego, CA, USA). Intracellular staining was performed using Foxp3-PerCP/Cy5.5 (eBioscience). Stained cells were analyzed using the BD LSRFortessa flow cytometer (BD Biosciences). Live cells were gated using Zombie Red (Biolegend) or 7-AAD (TONBO Biosciences) exclusion during acquisition on FACS. Flow cytometric analysis was performed using FlowJo Version 9.8.5 software (FlowJo, Ashland, OR, USA).

Treatment with mAb for lactating mothers

Lactating mothers were intraperitoneally administered 1 mg of a mAb to TGF-β [clone: 1D11, American Type Culture Collection (ATCC)] or IL-10 (clone: JES5-2A5, ATCC), TGF-β control antibody (mouse IgG, Sigma-Aldrich, St. Louis, MO, USA), or IL-10 control antibody (Rat IgG, Sigma-Aldrich) twice a week from delivery until weaning. We sacrificed the offspring of mothers treated with the antibody at PN14 and collected the thymus, spleen, and small intestine from the pups. We prepared thymocytes, splenic cells, and LP cells as described in the flow cytometric analysis section.

Statistical analysis

All data were analyzed using GraphPad Prism (version 6, GraphPad, La Jolla, CA, USA). Differences between the groups were analyzed using Mann–Whitney’s U test. The significance level was defined at $p < 0.05$.

Results

Growth

First, we compared the growth rates of pups from PN2–PN14 between MR and AR-2 groups. The mean volume of milk substitute fed to AR-2 mice was 0.24–1.40 mL/day during the feeding period (data not shown). The growth rate was slightly slower in AR-2 mice than in MR mice from PN5–PN11. Thereafter, the body weight of AR-2 mice was not significantly different from that of MR mice (Figure 1a) (at PN14).

Immune cell numbers in the thymus, spleen, and intestine of pups

The thymus of MR mice was slightly larger than that of AR-2 mice, although body weight was similar between groups. The relative thymus weight at PN14 was significantly greater in MR mice than in AR-2 mice ($p = 0.0026$, Figure 1b).

To evaluate the effect of breast milk on lymphoid organs along with the thymus, we examined the number of immune cells in the thymus, spleen, and small intestine using flow cytometry. The number of CD45.2⁺ cells in the thymus was higher in MR mice than in AR-2 mice,

which was consistent with the thymus size ($p=0.0215$) (Figure 2). However, the numbers of CD45.2⁺ cells in the spleen and small intestine were significantly lower in MR mice than in AR-2 mice ($p=0.0014$ and $p<0.0001$, respectively) (Figure 2).

Comparison of immune cell populations in the thymus, spleen, and intestine

Because the total number of immune cells in the thymus, spleen, and small intestine was significantly different between MR and AR-2 groups, we examined the composition of immune cells in each organ. In the thymus, CD8⁺ T cell frequency was lower in MR mice ($p<0.0001$), whereas between-group CD4⁺ T cell frequency was not significantly different ($p=0.2340$). Conversely, CD4⁺CD8⁺ T cell frequency was higher in MR mice ($p=0.0002$) (Figure 3a). The proportion of Foxp3⁺ regulatory T cells among CD4⁺ T cells was significantly higher in MR mice ($p=0.0214$) (Figure 3a), but there were no significant differences observed in peripheral lymphoid organs, including the spleen and small intestine.

In the spleen and small intestine, frequencies of macrophages, dendritic cells, and granulocytes were lower in MR mice (spleen: $p<0.0001$, $p=0.0059$, and $p<0.0001$, respectively; intestine: $p=0.0418$, $p=0.0007$, and $p=0.0164$, respectively) (Figures 3b-c). Among T cells, the proportion of CD8⁺ T cells was smaller, whereas that of CD4⁺ T cells was larger in the MR group than in the AR-2 group (spleen: $p=0.0016$ and $p=0.0025$, respectively; intestine: $p=0.048$ and $p=0.0303$, respectively) (Figures 3b-c). As the number of total immune cells in the spleen and small intestine was significantly smaller in MR mice than in AR-2 mice, the median populations of CD4⁺ T cells was slightly lower in both the spleen and small intestine in MR mice (spleen: median 6.157×10^5 vs. median 5.145×10^5 , $p=0.5458$; intestine: median 2.234×10^5 vs. median 4.232×10^5 ,

p=0.0592). B cell frequency in the spleen and small intestine was significantly higher in MR mice than in AR-2 mice ($p<0.0001$ and $p=0.0002$, respectively) (Figures 3b-c). Therefore, CD8⁺ T cells and innate immune cells were less abundant and B cells were more abundant in MR mice than in AR-2 mice.

Immunological effect of anti-TGF- β mAb treatment in mothers during lactation on pups

TGF- β and IL-10 are important factors in breast milk for inducing immune tolerance in infants. To examine the effect of these cytokines on the composition of immune cells in pups, we treated lactating mothers with mAb against TGF- β or IL-10 twice a week from delivery until weaning (Figure 4a). Pups in both groups treated with anti-TGF- β or anti-IL-10 mAb had a similar weight as control group pups throughout the study ($p=0.7762$ and $p=0.3095$, respectively). The percent thymus weight relative to body weight was significantly smaller in pups of anti-TGF- β mAb-treated mice than in control pups ($p=0.0006$), whereas the relative thymus weight in pups of anti-IL-10 mAb-treated mice was not significantly different from that in the control group ($p=0.5476$) (Figure 4b).

To investigate the immunological impact of TGF- β and IL-10 on infants, we examined the number and composition of immune cells in the thymus, spleen, and small intestine. The number of CD45.2⁺ cells was significantly decreased in the thymus in the pups of anti-TGF- β mAb-treated mice ($p=0.0041$); however, no significant difference was noted between the anti-IL10 mAb treatment and control groups ($p=0.4365$) (Figure 4c). The numbers of CD45.2⁺ cells in the spleen and small intestine were not different in the anti-TGF- β and anti-IL10 mAb treatment groups relative to the control groups (spleen: $p=0.3636$ and $p=0.5476$, respectively; intestine: $p=0.5105$ and $p>0.9999$, respectively) (Figure 4c).

CD8⁺ and CD4⁺ T cell frequencies in the thymus increased in pups of anti-TGF- β mAb-treated mice ($p=0.0023$ and $p=0.0006$, respectively). In contrast, CD4⁺CD8⁺ T cell frequency was significantly decreased in pups of anti-TGF- β mAb-treated mice ($p=0.0070$), whereas these cells were slightly more abundant in pups of anti-IL-10 mAb-treated mice (Figure 4d).

In peripheral lymphoid tissues, CD8⁺ T cell counts increased and CD4⁺ T cell counts decreased in pups of anti-TGF- β mAb-treated mice in both the spleen and small intestine compared with control group pups (spleen: $p=0.0006$ and $p=0.0175$, respectively; intestine: $p=0.0262$ and $p=0.0262$, respectively), whereas both cell counts in pups of the anti-IL-10 mAb treatment group were not significantly different from controls. Frequencies of macrophages, dendritic cells, and granulocytes increased in the spleen and small intestine in pups of anti-TGF- β mAb-treated mice (spleen: $p=0.0006$, $p=0.0192$, and $p=0.0006$, respectively; intestine: $p=0.0111$, $p=0.3992$, and $p=0.2086$, respectively). Conversely, B cell frequency in the spleen and small intestine significantly decreased in the anti-TGF- β mAb group pups ($p=0.0006$ and $p=0.0012$, respectively) (Figure 5a-b). Thus, the change of immune cell compositions may be partly because of TGF- β in breast milk.

Discussion

We demonstrated the effect of breast milk on immune cells using mice reared with breast milk and milk substitute. The thymus was larger in MR mice than in AR-2 mice; the difference partly depended on TGF- β delivered from the mother. In peripheral lymphoid organs, including the spleen and small intestine, we observed the increased frequencies of myeloid cells and CD8⁺ T cells and decreased frequencies of B cells and CD4⁺ T cells in AR-2 mice than in MR mice. These differences were also confirmed in offspring of mother mice treated with anti-TGF- β mAb.

Our finding that the thymus was larger in MR mice than in AR-2 mice is consistent with another report (23). Human breastfed infants have been also reported to possess considerably larger thymus than formula-fed infants (24). The thymic size of infants is important, because a positive correlation between the thymic size in the perinatal period and survival is shown in humans (25, 26). The smaller thymus size and similar change of thymic cell composition were observed in pups of mothers treated with anti-TGF- β mAb but not with anti-IL-10 mAb. Verhasselt et al. reported that the TGF- β 1 content in milk was reduced at least sixfold and the TGF- β 2 content was partially reduced after treatment with antibody to TGF- β (18). These results suggested that breast milk controls thymus growth and development partly through TGF- β , although it is unclear whether TGF- β directly affects thymic cells (27) or indirectly influences the entrance of precursor cells into the thymus or the emigration of mature T cells from the thymus.

In peripheral immune organs, frequencies of myeloid, CD8⁺ T cells, and B cells were altered in AR-2 mice. In humans, exclusive breastfeeding during the first month of life was associated with decreased whole-blood concentrations of monocytes and neutrophils compared with formula or mixed feeding (28), consistent with our findings. The suppression of humoral immunity in AR-2 mice was also consistent with previous findings (23), which identified by the decreased levels of serum immunoglobulins in milk substitute-fed mice. A similar alteration of these cells was observed in pups of mothers treated with anti-TGF- β mAb. Since TGF- β in breast milk can up-regulate the synthesis of IgA and Foxp3⁺ T cells, we expected to see the decrease of CD4⁺ T cells and the increase of CD8⁺ T cells after the treatment of anti-TGF- β mAb. Verhasselt et al. revealed that breastfeeding induced oral tolerance was mediated by CD4⁺CD25⁺ regulatory T cells and was depend on TGF- β signaling in T cells in the offspring (18). We also observed the decrease of Foxp3⁺ T cells in AR-2 mice. Since infants cannot produce TGF- β by themselves in early infancy (29) and orally administrated TGF- β

has been reported to retain and exert its activity in mice intestine (30), breast milk would be an important source of TGF- β for infants. However, the effect of TGF- β on CD8⁺ T cells in infants remains unclear. Further study is necessary to confirm the effect of CD8⁺ T cells increased in the intestinal mucosa.

Although the tolerogenic effect of TGF- β on specific food antigens has been reported, the relationship between innate immune cells and TGF- β was not investigated. Therefore, our findings that the higher frequencies of macrophages, dendritic cells, and granulocytes in pups of AR-2 mice or pups reared by mothers treated with anti-TGF- β mAb revealed a new role of TGF- β on innate immunity. We speculate that the innate immune system is involved in various allergic diseases at neonatal period, because the acquired immunity including the production of immunoglobulin is immature in infants. For example, food protein-induced enterocolitis syndrome (FPIES) that usually presents in infancy with vomiting, diarrhea, weight loss, and failure to thrive is poorly understood non-IgE-mediated food hypersensitivity. Even though there are few data regarding pathophysiology of FPIES, it is suggested that the involvement of local intestinal imbalance between TNF- α and TGF- β regulating the permeability of the intestinal barrier (31). Our findings support the idea that TGF- β and innate immune cells are strongly involved in FPIES. Experimental mouse models of non-IgE-mediated enterocolitis has not been established yet. Therefore, it is important to establish the non-IgE-mediated enterocolitis model to prove this hypothesis, which may provide a new therapeutic strategy for the disease.

There are 3 types of TGF- β ; such as β 1, β 2 and β 3 in mammals. Among them TGF- β 2 is dominant in human breast milk (32). TGF- β 1 is considered as a very important cytokine in developing infantile immune system since tolerance cannot be established in mice without the help of TGF- β 1

(33). Meanwhile TGF- β 1 and β 2 in breast milk are both necessary in IgA synthesis in the intestine.

Since anti-TGF- β mAb we used in this study neutralize both TGF- β 1 and - β 2, further study is necessary to analyze the effects of TGF- β 1 and - β 2 in the development of immune system.

Thus, our results indicate that TGF- β in breast milk directly affects the infant mucosal immune system. However, the increase of immune cell counts in peripheral lymphocyte organs was not obvious in the anti-TGF- β mAb treatment group. In addition to TGF- β , other factors such as secretory IgA, lactoferrin, and lysozyme in breast milk act on immunological cells. To confirm the effects of breast milk in relation to TGF- β , the number of IgA-bearing plasma cells among CD45.2⁺ cells in LP cells were also examined. However, we could not find any significant difference between the MR and AR-2 mice nor the pups of mothers treated with or without anti-TGF- β mAb (data not shown). Therefore, involvement of factors other than TGF- β in the alterations of immune cell numbers and the composition of immune cells remains to be examined.

Our study suggested that breast milk regulates the differentiation and expansion of innate and adaptive immune cells through TGF- β . Meanwhile, Turfkruyer et al. also investigated the immunological effects of breast milk and suggesting that physiological vitamin A and IFN- γ , which were both contained in breast milk, can stimulate Th1 differentiation and induction of oral intolerance in early infancy (34). Further study is necessary to confirm the development of infantile immune system and oral intolerance in relation to breast feeding.

In summary, breast milk controls the differentiation and expansion of infant innate and acquired immune cells, partly because of TGF- β . Breastfeeding offers an immunoglobulin-rich condition, including both in the serum and intestine, by suppling milk-derived IgA and TGF- β to

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increase B cells. In addition, breast milk suppresses the excess reaction of CD8⁺ T cells and innate immune cells directly by TGF- β and indirectly by other factors. Our findings shed light on the effects of breast milk on the infant intestinal immune system and may leads to a new therapeutic strategy for innate immune system-mediated infant diseases.

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Authorship contribution

K.S., T.S. and S.M. designed the study; K.S., A.K., F.K., A.H., and T.M. performed experiments; K.S., A.K., F.K., A.H., and S.M. collected and assembled data; K.S., A.K., F.K., A.C., K.H., and S.M. analyzed and interpreted data; K.S., A.K., A.H., A.C., T.M., and S.M. provided reagents and mice; K.S., A.C., and S.M. drafted the article; A.K., T.M., T.S., and S.M. gave technical support and conceptual advice. All authors read and approved the final manuscript.

Disclosure

The authors declare no conflict of interest.

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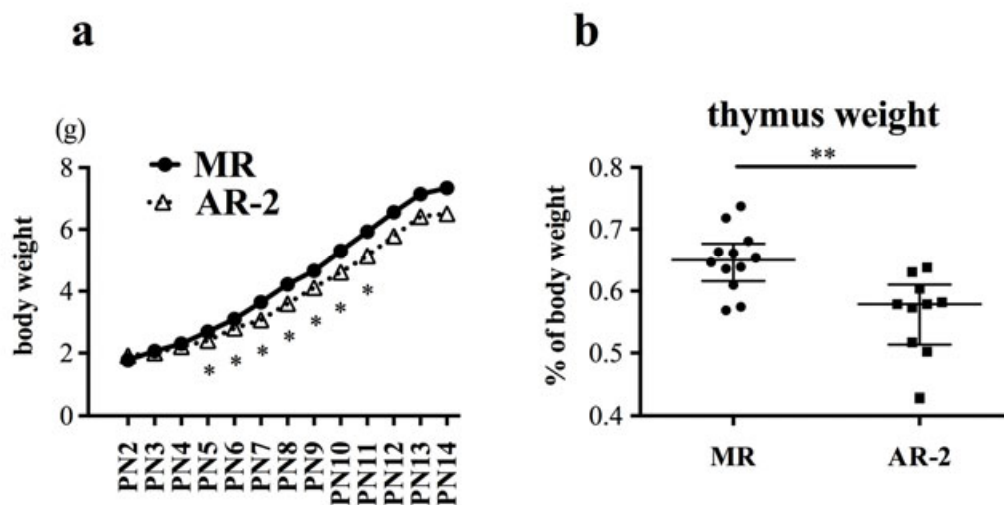
Table 1 Composition of artificial milk ^{a)}

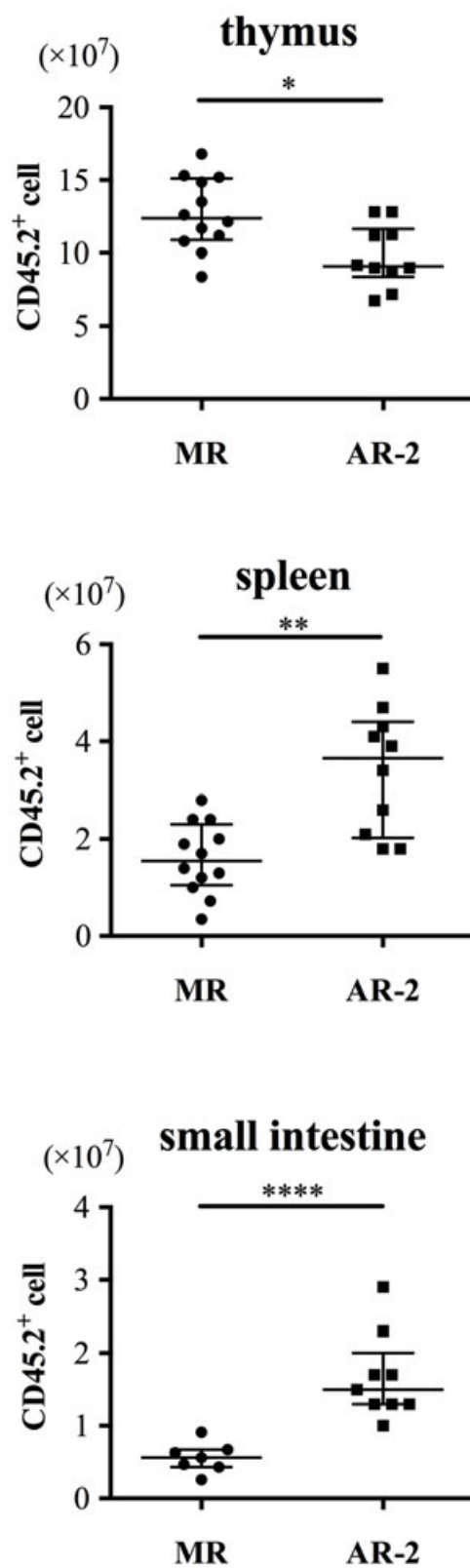
	Ingredient	Amount (weight /100 mL milk)
Protein (g)	Whey protein isolate	4.0
	Whey protein hydrolyzed	5.0
	Casein	4.0
	Serine	0.02875
	Cystine	0.3
	Tryptophan	0.027
	Methionine	0.0045
Carbohydrate (g)	Lactose	1.89
Fat (g)	MCT	1.25
	Palm oil	8.25
	Coconut oil	2.5
	Corn oil	0.5
	Soybean oil	2.75
	Linseed oil	0.75
	Cholesterol	0.04
Minerals (mg)	NaOH	2
	KOH	150
	GlyCaPO ₄	800
	MgCl ₂ 6H ₂ O	190
	CaCl ₂ 2H ₂ O	170

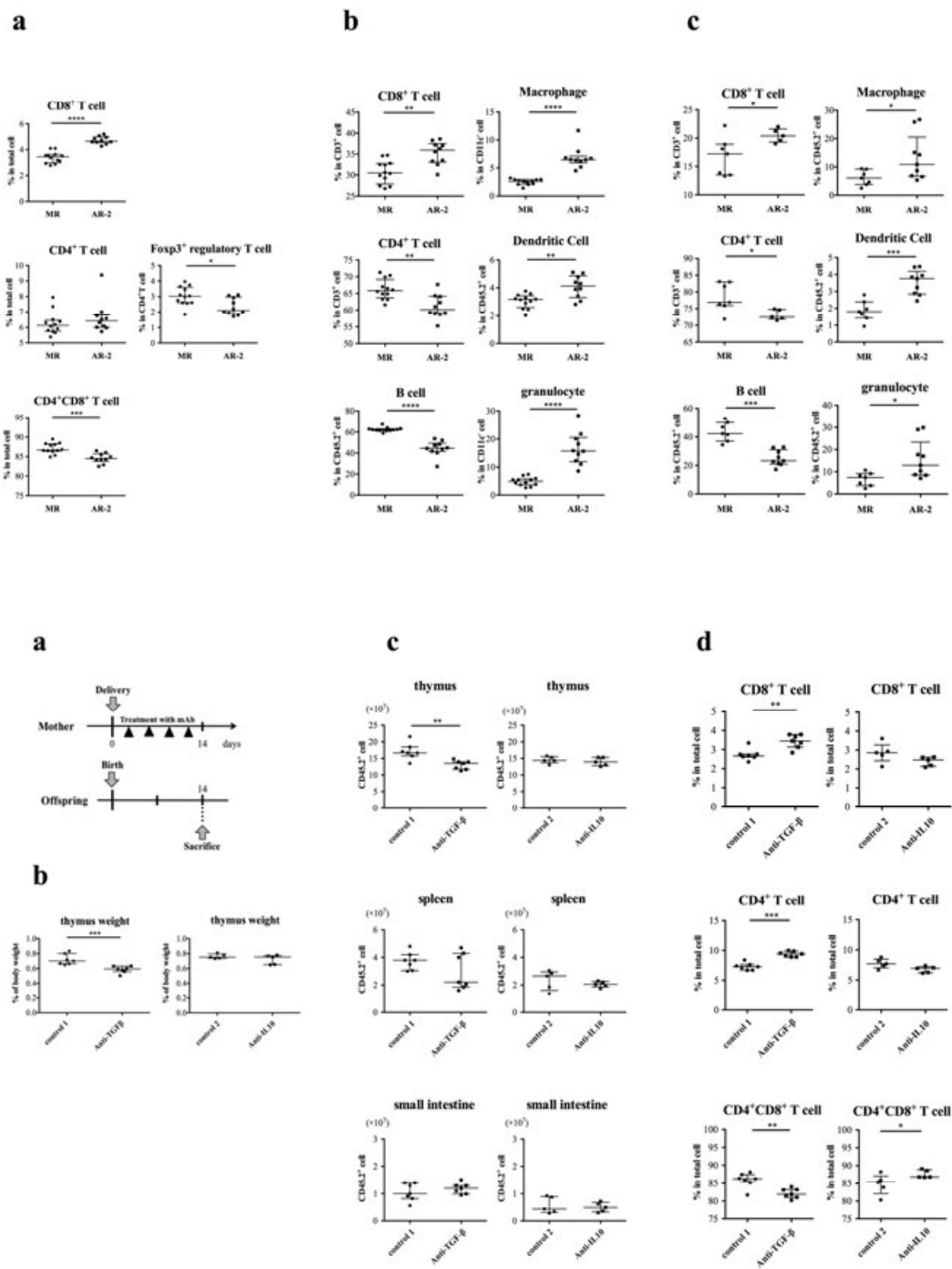
	CaCO ₃	184
	Ca-Citrate	120
	Na ₂ HPO ₄	80
	KH ₂ PO ₄	8
	FeSO ₄	24
	Citrate H ₂ O	0.5
	ZnSO ₄	6
	CuSO ₄	1.5
	MnSO ₄	0.25
	NaF	0.155
	KI	0.25
	K ₂ SO ₄	163.5
	Na ₂ SiO ₃ 9H ₂ O	5.075
	Na ₂ O ₄ Se	0.035
	H ₈ MoN ₂ O ₄ 4H ₂ O	0.0275
	CrH ₂₄ KO ₂₀ S ₂	0.9625
	Li ₂ CO ₃	0.06
	H ₃ BO ₃	0.285
	NiCO ₃	0.1125
	NH ₄ VO ₃	0.0225
Vitamins (mg)	Vitamin mix	400
	Vitamin C	200
	Vitamin K3	1.9825

	Vitamin A	0.1284
	Vitamin D	23.46
	Vitamin E	0.0025
Others (mg)	Carnitine	4.0
	Picolinate	2.0
	Ethanolamine	3.5
	Taurine	15.0
	Tricholine Citrate	147.0

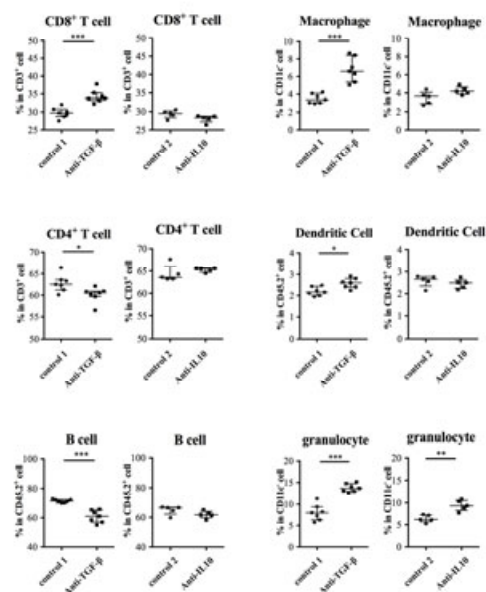
a) The artificial milk formula was made according to the method of Yajima et al. (26)







a



b

