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An ancillary study of participants in a randomized, placebo-controlled trial suggests that ingestion of bovine lactoferrin promotes expression of interferon alpha in the human colon

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ABSTRACT

Studies using animal models have demonstrated that ingestion of bovine lactoferrin (bLF) is able to induce cytokine expression in the intestine and inhibit carcinogenesis in the colon and other organs of experimental animals. Consequently, a clinical trial was conducted in the National Cancer Center Hospital, Tokyo, Japan to determine whether ingestion of bLF affected the growth of colorectal polyps in humans. The Tokyo-trial found that ingestion of 3.0 g bLF suppressed the growth of colorectal polyps and increased the level of serum human lactoferrin in participants 63 years old or younger. The present study is a complementary study to the Tokyo-trial to determine if a change in the expression of one or more cytokines could be detected in the colon of the Tokyo-trial participants after ingesting bLF. We found that daily ingestion of 3.0 g bLF promoted the expression of interferon alpha in the colon of the Tokyo-trial participants.

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1. Introduction

Milk is a rich source of bioactive peptides (Korhonen, 2009). Lactoferrin is an approximately 80 kDa iron-binding glycoprotein present in the milk of most mammalian species. In 1995, McIntosh et al. reported that the whey fraction of bovine milk, which contains lactoferrin, protects against the development of intestinal cancers in rats (McIntosh, Regester, Le Leu, Royle, & Smithers, 1995). Since that time, several studies using animal models have demonstrated that ingestion of bovine lactoferrin (bLF) is able to induce cytokine expression in the intestine and inhibit carcinogenesis in the colon and other organs of experimental animals (Iigo et al., 2009; Tsuda et al., 2010).

As a result of these studies, a randomized, controlled clinical trial beginning in 2002 and ending in 2006 was conducted in the National Cancer Center Hospital, Tokyo, Japan to determine whether ingestion of bLF had an effect on the growth of colorectal polyps in humans; this trial is reported by Kozu et al. (2009). Briefly, trial participants ingested 0, 1.5, or 3.0 g bLF daily for 1 year. The size of adenomatous colorectal polyps, T-cell subpopulation numbers, natural killer cell activity and number, neutrophil number, and the serum levels of interleukin-18, interferon-gamma, and human lactoferrin (hLF) were measured. The Tokyo-trial reported that ingestion of 1.5 g bLF had no significant effect on any of the parameters measured; however, ingestion of 3.0 g bLF had two significant effects: (i) the growth of colorectal polyps was inhibited in trial participants 63 years old or younger and (ii) the level of hLF in the serum was increased in trial participants 63 years old or younger (Kozu et al., 2009). The authors of the Tokyo-trial concluded that ingestion of bLF inhibited the growth of adenomatous colon polyps and probably acted via modulation of immune system function.

The present study is a complementary study to the Tokyo-trial. The purpose of this ancillary study was to determine if a change in the expression of one or more cytokines could be detected in the colon of the Tokyo-trial participants who ingested bLF for 1 year. In the Tokyo-trial, normal tissue samples were collected from the trial participants before the trial began and after the trial ended. The present ancillary study examined the RNA extracted from these tissue samples. We found that ingestion of bLF promoted the expression of interferon alpha (IFNA) in the colon of the Tokyo-trial participants.

2. Methods

2.1. *The Tokyo-trial*

A blinded, randomized, controlled clinical trial beginning in 2002 and ending in 2006 was conducted in the National Cancer Center Hospital, Tokyo, Japan to determine if ingestion of bovine lactoferrin (bLF) would inhibit the growth of precancerous, adenomatous colorectal polyps in human patients. Trial participants took six tablets daily containing 0, 250, or 500 mg bLF. The bLF was approximately 10–20% iron saturated. The tablets also contained carbohydrate (D-sorbitol, maltitol, and corn starch) but not fat or dietary fiber and had a caloric value of 36 kcal. The tables were indistinguishable from each other in appearance, smell, and taste. There were no adverse effects associated with ingestion of bLF or the placebo. A description of the Tokyo-trail design, participants, interventions, outcomes, sample size, randomization, blinding, statistical methods, characteristics of the intent-to-treat population at the commencement of the trial, and the CONSORT flowchart are presented in Kozu et al. (2009).

2.2. *Ethics statement*

The Tokyo-trial was initiated after approval by the Ethical Committee of the National Cancer Center Hospital, Tokyo, Japan and is registered in the University Hospital Medical Information Network Clinical Trials Registry (UMIN-CTR; http://www.umin.ac.jp/ctr/index.htm) Tokyo, Japan, number C000000182. All trial participants provided written informed consent.

2.3. *Collection of RNA samples for analysis*

Prior to the start of the Tokyo-trial and at the end of the trial, a small tissue sample was collected from normal mucosa close to the target polyp; also at the end of the trial, the target polyps (and all other growths detected) were removed. Target polyps and tissue sample sites were located at the most proximal sites of the right colon (cecum to transverse colon) and the left colon (descending colon to rectum). Tissue and polyp samples were immediately transferred to the laboratory on ice and divided into halves; one half was used for histological analysis and RNA was extracted from the other half. RNA was extracted using the Isogen (Wako) extraction protocol according to the manufacturer's instructions. RNA was extracted immediately after obtaining the tissue specimens and then stored at –80 °C until use. RNA integrity was checked with an Agilent 2100 Bioanalyzer using an RNA Pico chip. RIN numbers were mostly 5.8 or greater: in these samples the TaqMan Real Time PCR cycle threshold of actin was 32 or less. In some samples, the RIN number was below 5.8: in these samples TaqMan Real Time PCR did not generate an actin signal. These samples are labeled NA (No Actin) in Tables 1–3, and Supplementary Table S2. In the present study we used only normal tissue samples, which were collected both at the beginning and at the end of the trial, for analysis of gene expression.

2.4. *RNA analysis*

RNA extracts were treated with DNase (Takara) and reverse transcribed using PrimeScript reverse transcriptase (Takara) with random hexamers (Invitrogen) and RNase OUT (Invitrogen) according to the manufacturers' instructions. Reverse transcriptase-negative controls were performed for all samples and cDNA-negative controls were performed for all primer pairs. All reverse transcriptase-negative controls and cDNA-negative controls were negative. A primary screening, using standard end-point PCR, was performed with samples from the 3.0 g bLF group. These samples were screened for alpha interferons (IFNAs), interferon beta (IFNB), interferon gamma (IFNG), IL-1A, IL-4, IL-7, IL-8, IL-12A, IL-12B, IL-15, IL-18, IL-22, IL-23A, GM-CSF, and TNF using the primers listed in Supplementary Table S1. IFNAs, IFNB, IFNG, IL-7, IL-12A, IL-12B, IL15, and IL-

18 were selected based on reports that these cytokines were induced in the intestine of mice or rats orally administered bLF (Iigo et al., 2004, 2009; Kuhara et al., 2000; Kuhara, Yamauchi, Tamura, & Okamura, 2006; Takakura, Wakabayashi, Yamauchi, & Takase, 2006; Wakabayashi, Takakura, Yamauchi, & Tamura, 2006; Wang et al., 2000). IL-4 was selected because it is produced by activated T cells, and ingestion of lactoferrin is reported to activate T cells in the intestine (Spadaro et al., 2007). IL-22 was selected because it is produced in response to pathogens in the gut (Zheng et al., 2008). IL-23A was selected because it dimerizes with IL-12B to form IL-23. IL-1A was selected because it is a pleiotropic cytokine involved in various immune responses. IL-8, GM-CSF and TNF were selected because they are key cytokines involved in immune cell function. All screening primers were designed using Primer Premier software (Premier Biosoft International). IFNA was detected in the majority of tissue samples while IFNG, IL-1A, IL-4, IL-7, IL-8, IL-12A, IL-12B, IL-15, IL-18, IL-22, IL-23A, GM-CSF, and TNF were either not detected or detected in only a small number of tissue samples. Therefore, all patient samples were screened for the presence of type I interferons (IFNAs and IFNB) using standard end-point PCR and the primers listed in Supplementary Table S1. All screening primers were designed using Primer Premier software (Premier Biosoft International). PCR-amplicons were purified using the Wizard SV Gel and PCR Clean-Up System (Promega: Tokyo, Japan) according to the manufacturer's instructions. Purified amplicons were sequenced using a 3130 Genetic Analyzer (Applied Biosystems) according to the manufacturer's instructions. Only IFNA2 and IFNB sequences were present in most of the samples. Therefore, using the primers and probes listed in Supplementary Table S1, TaqMan Real-Time PCR was used to analyze the relative expression of IFNA and IFNB with β-actin as an internal control. TaqMan Real-Time PCR was performed using an Applied Biosystems 7300 Real Time PCR system and PCR primers and TaqMan probes obtained from Applied Biosystems, according to the manufacturer's instructions.

2.5. *Statistics*

Differences in the mean values of IFNB and IFNA were analyzed using the two-tailed Student's T-test. The distribution of changes in IFNA gene expression, defined as decrease (≤–2.0 units), no-change (–2.0 to +2.0 units), and increase (≥2.0 units), was compared between treatment groups based on the Cochran–Mantel–Haenszel test. Statistical analyses were performed using JMP version 9.0 (SAS Institute Inc, Cary, NC). A p value of <0.05 was considered to be significant.

3. Results

3.1. *Identification of target cytokines*

A blinded, randomized, controlled clinical trial beginning in 2002 and ending in 2006 was conducted in the National Cancer Center Hospital, Tokyo, Japan to determine if ingestion of bovine lactoferrin (bLF) would inhibit the growth of precancerous, adenomatous colorectal polyps in human patients. Participants, randomized into one of three groups, ingested 0, 1.5, or 3.0 g bLF daily for 1 year. There were no adverse effects associated with ingestion of bLF or the placebo. The characteristics of the intent-to-treat population at the commencement of the Tokyo-trial and the CONSORT flowchart of the trial are presented in Kozu et al. (2009). There were 102 participants in the per-protocol set (PPS) population of the Tokyo-trial: see Supplementary Text S1 for a definition of PPS populations and the reason we used the PPS rather than the full analysis set (FAS) population samples for the present study. The placebo group contained 33 participants, the 1.5 g bLF group contained 37 participants, and the 3 g bLF group contained 32 participants. Normal tissue samples were obtained from the trial participants prior to the beginning of the trial and at the end of the trial, and RNA was extracted and stored at –80 °C. Five of the participants in the placebo group, six of the participants in the 1.5 g bLF group, and five of the participants in the 3.0 g bLF group had two target polyps. Therefore, the total number of samples was 38, 43, and 37 mucosa samples collected before the beginning of the trial and 38, 43, and 37 associated mucosa samples collected at the end of the trial from the placebo, 1.5 g bLF, and 3.0 g bLF participants, respectively. RNA was extracted from each of the samples.

Two sets of screening PCR were performed to identify target cytokines for further analysis. First, the samples collected from the 3.0 g bLF group were screened by standard end-point PCR using the primers indicated in Supplementary Table S1 for expression of IFNA-1,2,4,5,6,7,8,10,13,14,16,17,21, IFNB, IFNG, IL-4, IL-7, IL-8, IL-12A, IL-12B, IL-15, IL-18, IL-22, IL-23A, GM-CSF, and TNF. Expression of these cytokines, with the exception of IFNA and IFNB, was either not detected or detected in fewer than 20% of the samples. Therefore, only expression of type I interferons was examined further. The tissue samples were screened by standard PCR using the primers indicated in Supplementary Table S1 for the presence of the different type I interferons (IFNB and the 13 species of human IFNA (Pestka, 2007)). Because of IFNA redundancy, all of the amplicons generated by the second screening-PCR were sequenced. IFNA2 was the most widely detected type I interferon in the samples from the Tokyo-trial participants (data not shown). The other IFNA species sequences were either not detected or detected in a minority of samples. Therefore expression of IFNA2 was examined by TaqMan Real Time PCR using the primers listed in Supplementary Table S1. In addition, in this second screening, IFNB sequences were present in most of the samples, and therefore, expression of IFNB was also examined.

3.2. *Relative expression of IFNB and IFNA*

Once target interferon species were identified, we used TaqMan PCR to analyze the samples further. Each sample was tested in quintuplicate for the relative expression of IFNA using β-actin as an internal control. The relative expression of IFNB and IFNA is shown in Table 1 (placebo), Table 2 (1.5 g bLF), and Table 3 (3.0 g bLF); these data can be downloaded from Supplementary Table S2.

A potentially interesting observation, but one which is not part of this study, is that samples with relatively high IFNB expression also had relatively high IFNA expression. For example, samples in which the relative expression of IFNB was greater

Table 1 – The relative expression of IFNB and IFNA in tissue samples collected from the placebo group.

Arbitrary patient number	Age	Gender	Sample location	Group	Relative IFN levels at the beginning of the trial				Relative IFN levels at the end of the trial			
					IFNB Mean	Std Dev	IFNA Mean	Std Dev	IFNB Mean	Std Dev	IFNA Mean	Std Dev
1	54	M	L	Placebo	0.01	0.03	1.99	0.65	12.50	2.54	84.49	4.42
2	59	M	L	Placebo	0.00	0.01	52.10	1.75	0.54	1.62	0.00	0.00
2	59	M	R	Placebo	0.00	0.00	0.08	0.01	2.30	0.64	0.00	0.00
3	58	M	R	Placebo	0.00	0.00	0.02	0.00	0.05	0.17	1.72	0.66
4	65	M	R	Placebo	0.00	0.00	91.27	1.42	0.35	0.40	4.59	0.73
5	57	M	R	Placebo	7.67	1.89	83.71	2.22	0.03	0.08	15.37	2.40
6	70	M	R	Placebo	0.93	0.11	1.96	0.94	0.00	0.00	96.52	2.47
7	67	M	L	Placebo	14.05	3.13	100.01	4.32	13.34	1.85	50.79	6.73
8	63	M	R	Placebo	6.38	0.86	33.77	4.63	1.04	0.41	62.11	1.71
9	73	M	L	Placebo	0.00	0.00	81.97	0.86	0.00	0.00	1.37	0.14
10	54	M	R	Placebo	0.05	0.01	0.07	0.03	0.24	0.47	54.21	4.41
11	63	M	R	Placebo	6.06	2.04	14.92	2.58	0.36	0.37	0.00	0.00
12	70	F	L	Placebo	7.05	1.61	74.58	4.35	0.12	0.05	12.90	2.53
13	54	F	R	Placebo	0.92	0.31	4.62	1.14	1.03	0.44	55.51	4.64
14	61	M	L	Placebo	1.12	0.46	0.00	0.00	0.04	0.02	0.20	0.16
15	71	M	R	Placebo	4.93	0.84	0.00	0.00	0.00	0.00	63.46	0.98
16	61	M	R	Placebo	1.34	0.59	19.77	3.47	68.99	0.28	1.77	0.72
17	60	M	R	Placebo	0.01	0.03	0.67	0.61	6.16	1.41	100.01	4.53
18	72	F	L	Placebo	29.27	3.01	100.11	7.78	0.25	0.74	62.25	1.01
18	72	F	R	Placebo	27.20	3.74	104.03	3.48	0.09	0.27	23.78	4.47
19	57	F	R	Placebo	15.85	3.55	69.34	1.03	0.02	0.03	8.48	1.68
20	46	M	L	Placebo	0.02	0.06	0.88	0.70	0.00	0.00	71.26	1.81
20	46	M	R	Placebo	0.13	0.39	55.58	3.42	0.45	0.15	69.24	0.80
21	68	M	R	Placebo	22.94	4.40	43.82	2.78	0.11	0.33	4.75	0.66
22	60	F	R	Placebo	99.00	0.24	0.03	0.01	13.81	3.04	99.85	0.23
23	56	M	R	Placebo	0.06	0.18	7.70	1.35	3.73	0.46	62.19	2.65
24	67	M	R	Placebo	0.13	0.39	30.24	4.73	27.47	2.38	100.02	7.09
25	66	M	L	Placebo	6.45	0.23	25.35	4.92	4.70	1.08	77.31	2.28
26	64	M	L	Placebo	6.93	1.59	6.48	0.91	94.63	0.79	33.40	3.67
27	59	M	L	Placebo	0.04	0.06	1.77	0.88	0.02	0.06	8.36	0.99
27	59	M	R	Placebo	0.03	0.01	0.33	0.02	40.29	1.60	100.01	3.14
28	66	M	R	Placebo	63.40	2.79	0.00	0.00	0.16	0.30	64.70	1.86
29	62	M	L	Placebo	7.07	1.62	0.98	0.47	15.06	2.59	0.05	0.02
30	75	M	L	Placebo	20.49	3.95	38.69	7.31	0.04	0.05	3.61	0.74
30	75	M	R	Placebo	14.37	3.17	93.96	4.28	0.00	0.00	65.40	3.49
31	59	F	R	Placebo	55.97	3.42	11.58	1.88	10.90	1.86	0.66	0.11
32	68	M	R	Placebo	0.13	0.03	6.08	0.61	0.46	0.10	0.11	0.02
33	63	M	L	Placebo	5.56	0.86	22.17	1.65	NA	NA	NA	NA

R: Right colon (cecum to transverse colon).
L: Left colon (descending colon to rectum).
NA: No actin.

Table 2 – The relative expression of IFNB and IFNA in tissue samples collected from the 1.5 g bLF group.

Arbitrary patient number	Age	Gender	Sample location	Group	Relative IFN levels at the beginning of the trial				Relative IFN levels at the end of the trial			
					IFNB Mean	Std Dev	IFNA Mean	Std Dev	IFNB Mean	Std Dev	IFNA Mean	Std Dev
34	67	M	R	1.5 g bLF	0.61	1.86	0.00	0.00	20.78	2.63	100.06	2.34
35	70	M	R	1.5 g bLF	0.00	0.00	0.00	0.00	0.00	0.00	0.08	0.00
36	60	F	R	1.5 g bLF	0.23	0.70	0.00	0.00	4.61	1.02	0.00	0.00
37	75	M	R	1.5 g bLF	10.26	0.64	104.87	2.34	0.03	0.05	31.92	1.94
38	55	M	L	1.5 g bLF	0.00	0.00	0.02	0.00	12.70	1.27	69.90	1.93
39	51	M	R	1.5 g bLF	11.50	0.99	0.00	0.00	46.17	0.29	120.31	4.85
40	70	M	L	1.5 g bLF	0.06	0.10	10.50	1.91	0.00	0.00	60.70	4.26
41	61	M	R	1.5 g bLF	0.03	0.01	0.05	0.01	6.04	1.34	66.69	4.93
42	74	M	R	1.5 g bLF	0.03	0.10	0.01	0.00	11.45	0.41	0.00	0.00
43	51	M	R	1.5 g bLF	0.00	0.00	0.00	0.00	2.71	0.62	0.01	0.02
44	63	M	R	1.5 g bLF	108.59	1.41	72.53	4.40	0.00	0.00	0.00	0.00
45	48	M	R	1.5 g bLF	0.00	0.00	62.91	4.95	10.60	1.18	101.05	4.84
46	61	M	L	1.5 g bLF	0.18	0.32	22.15	3.69	5.80	1.17	0.00	0.00
46	61	M	R	1.5 g bLF	58.12	1.55	0.00	0.00	0.05	0.09	0.00	0.00
47	56	M	R	1.5 g bLF	0.54	0.33	0.00	0.00	0.55	0.16	52.11	6.35
48	71	F	L	1.5 g bLF	12.70	1.04	1.54	0.35	98.18	2.31	100.01	3.85
48	71	F	R	1.5 g bLF	9.57	0.90	132.39	3.94	6.00	1.33	102.25	4.71
49	64	F	R	1.5 g bLF	12.91	2.49	100.76	2.53	0.12	0.36	81.14	6.02
50	55	F	L	1.5 g bLF	0.03	0.08	0.00	0.00	0.01	0.03	0.00	0.00
51	46	M	R	1.5 g bLF	62.36	1.34	0.01	0.00	0.14	0.10	100.03	0.75
52	63	M	R	1.5 g bLF	18.57	1.30	96.71	4.71	2.96	0.63	0.00	0.00
53	66	M	L	1.5 g bLF	0.89	0.33	0.00	0.00	127.43	1.00	85.69	11.47
53	66	M	R	1.5 g bLF	0.30	0.11	60.01	1.73	28.27	3.96	100.11	2.65
54	65	M	R	1.5 g bLF	0.50	0.03	14.65	0.90	16.92	1.38	103.09	15.49
55	57	M	R	1.5 g bLF	0.06	0.08	0.00	0.00	12.43	2.81	100.20	0.40
56	64	M	R	1.5 g bLF	0.02	0.05	0.00	0.00	12.49	2.37	91.79	6.14
57	61	M	R	1.5 g bLF	102.20	1.47	0.00	0.00	15.16	1.47	0.00	0.00
58	61	F	R	1.5 g bLF	102.17	1.37	77.79	2.49	3.95	0.79	0.00	0.00
59	64	M	L	1.5 g bLF	40.81	1.73	75.52	5.94	0.01	0.02	0.00	0.00
60	74	M	R	1.5 g bLF	0.00	0.00	0.00	0.00	4.24	0.94	1.19	0.24
61	61	M	L	1.5 g bLF	82.92	1.03	0.00	0.00	0.80	0.96	0.00	0.00
62	66	M	R	1.5 g bLF	5.49	1.24	0.00	0.00	5.29	1.17	0.00	0.00
63	64	F	R	1.5 g bLF	0.76	0.99	0.00	0.00	0.00	0.00	60.33	0.97
64	53	M	L	1.5 g bLF	69.77	0.30	64.16	2.09	0.09	0.16	53.95	5.53
64	53	M	R	1.5 g bLF	0.00	0.00	100.55	1.14	3.86	0.85	78.26	4.99
65	67	M	L	1.5 g bLF	96.42	0.81	96.86	6.64	NA	NA	NA	NA
66	50	M	L	1.5 g bLF	0.08	0.10	0.00	0.00	NA	NA	NA	NA
67	61	F	R	1.5 g bLF	129.20	2.03	80.56	1.92	NA	NA	NA	NA
44	63	M	L	1.5 g bLF	NA	NA	NA	NA	3.09	0.67	80.96	3.88
68	64	F	L	1.5 g bLF	NA	NA	NA	NA	2.00	0.46	0.00	0.00
69	57	M	R	1.5 g bLF	NA	NA	NA	NA	NA	NA	NA	NA
70	54	M	L	1.5 g bLF	NA	NA	NA	NA	NA	NA	NA	NA
70	54	M	R	1.5 g bLF	NA	NA	NA	NA	NA	NA	NA	NA

R: Right colon (cecum to transverse colon).
L: Left colon (descending colon to rectum).
NA: No actin.

Table 3 – The relative expression of IFNB and IFNA in tissue samples collected from the 3.0 g bLF group.

Arbitrary patient number	Age	Gender	Sample location	Group	Relative IFN levels at the beginning of the trial				Relative IFN levels at the end of the trial			
					IFNB Mean	Std Dev	IFNA Mean	Std Dev	IFNB Mean	Std Dev	IFNA Mean	Std Dev
71	69	M	R	3.0 g bLF	0.00	0.00	0.30	0.09	0.00	0.00	55.85	9.20
72	58	F	R	3.0 g bLF	0.00	0.00	1.27	0.24	0.13	0.25	42.65	6.52
73	60	M	R	3.0 g bLF	0.07	0.12	0.00	0.00	0.00	0.00	42.63	7.31
74	59	M	R	3.0 g bLF	0.23	0.70	40.22	5.48	0.00	0.00	103.59	5.19
75	61	M	R	3.0 g bLF	0.30	0.06	0.47	0.10	179.24	1.63	100.01	7.52
76	56	M	R	3.0 g bLF	0.14	0.43	0.01	0.00	114.75	1.33	116.55	9.73
77	64	M	L	3.0 g bLF	7.21	1.59	0.00	0.00	12.60	2.78	151.89	6.20
78	56	F	L	3.0 g bLF	2.52	0.57	23.60	4.57	0.00	0.00	61.92	1.97
79	69	M	L	3.0 g bLF	5.65	1.25	0.00	0.00	192.10	0.58	20.10	3.54
79	69	M	R	3.0 g bLF	7.69	0.80	6.24	1.16	177.28	0.54	173.96	8.99
80	66	M	R	3.0 g bLF	1.30	0.86	6.49	0.19	0.46	1.39	46.45	8.28
81	60	M	L	3.0 g bLF	10.37	2.38	100.08	7.02	0.00	0.00	13.10	2.38
82	73	M	R	3.0 g bLF	3.63	0.83	64.39	5.39	0.00	0.00	84.47	4.47
83	62	M	R	3.0 g bLF	1.11	1.27	0.17	0.04	0.25	0.56	65.94	11.10
84	62	M	L	3.0 g bLF	0.00	0.00	13.47	1.85	133.61	0.19	124.60	3.76
85	65	M	R	3.0 g bLF	0.00	0.00	120.80	7.59	0.00	0.00	132.71	5.70
86	48	M	L	3.0 g bLF	2.11	0.48	0.02	0.02	71.96	1.82	0.00	0.00
86	48	M	R	3.0 g bLF	1.22	1.40	0.00	0.00	0.00	0.00	47.65	8.15
87	70	F	R	3.0 g bLF	0.00	0.00	116.97	6.02	0.00	0.00	9.17	1.80
88	69	M	R	3.0 g bLF	0.03	0.09	12.24	3.45	0.35	1.07	123.61	4.21
89	70	M	R	3.0 g bLF	9.35	1.60	5.11	0.92	0.02	0.05	108.55	10.70
90	59	M	R	3.0 g bLF	1.84	1.94	0.00	0.00	0.08	0.25	30.48	6.11
91	70	M	L	3.0 g bLF	0.00	0.00	100.46	6.05	0.00	0.00	0.08	0.02
92	50	F	R	3.0 g bLF	0.26	0.78	6.47	1.18	0.00	0.00	23.72	3.80
93	67	F	R	3.0 g bLF	7.16	1.38	46.06	1.64	0.00	0.00	74.01	12.42
94	66	M	L	3.0 g bLF	0.04	0.13	0.00	0.00	1.16	1.32	116.88	6.68
95	64	M	L	3.0 g bLF	0.00	0.00	80.95	14.80	0.11	0.32	53.68	9.36
95	64	M	R	3.0 g bLF	20.40	4.52	0.00	0.00	0.00	0.00	1.16	0.27
88	69	M	L	3.0 g bLF	0.01	0.04	11.59	2.24	NA	NA	NA	NA
96	45	M	L	3.0 g bLF	0.03	0.09	2.76	0.10	NA	NA	NA	NA
96	45	M	R	3.0 g bLF	0.00	0.00	0.00	0.00	NA	NA	NA	NA
97	58	M	L	3.0 g bLF	0.03	0.09	12.86	2.34	NA	NA	NA	NA
98	66	M	R	3.0 g bLF	9.94	2.28	64.34	4.05	NA	NA	NA	NA
99	61	M	L	3.0 g bLF	NA	NA	NA	NA	0.00	0.00	123.32	9.33
100	72	M	L	3.0 g bLF	NA	NA	NA	NA	0.13	0.39	45.18	8.30
101	69	M	R	3.0 g bLF	NA	NA	NA	NA	0.20	0.60	37.57	5.50
102	72	M	L	3.0 g bLF	NA	NA	NA	NA	0.03	0.09	18.75	3.13

R: Right colon (cecum to transverse colon).
L: Left colon (descending colon to rectum).
NA: No actin.

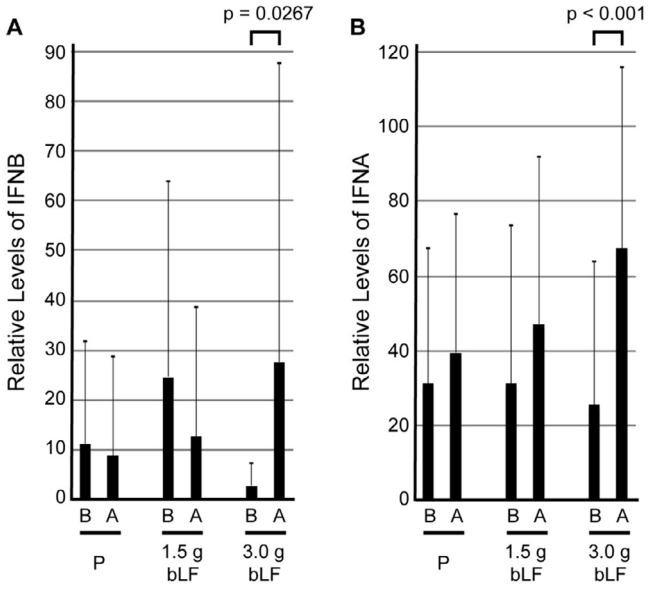


Fig. 1 – Relative levels of IFNB and IFNA at the beginning and end of the Tokyo-trial. (A) Relative levels of IFNB in tissue samples collected at the beginning, B, and after the end, A, of the Tokyo-trial from the placebo (P), 1.5 g bLF, and 3.0 g bLF group participants. Levels of IFNB were significantly higher in the 3.0 g bLF group after ingesting 3.0 g bLF daily for 1 year. (B) Relative levels of IFNA in tissue samples collected at the beginning, B, and after the end, A, of the Tokyo-trial from the placebo (P), 1.5 g bLF, and 3.0 g bLF group participants. Levels of IFNA were significantly higher in the 3.0 g bLF group after ingesting 3.0 g bLF daily for 1 year.

than 10 had significantly ($p < 0.001$) higher IFNA expression (64.8 ± 48.0) than samples in which the relative expression of IFNB was less than 10 (IFNA = 31.1 ± 37.7).

Figure 1 shows the mean level of expression of IFNB and IFNA in the placebo, 1.5 g bLF, and 3.0 g bLF groups before and at the end of the trial. The mean and standard deviation of the relative expression of IFNB is 11.2 ± 20.6 (placebo before), 8.6 ± 19.9 (placebo after), 24.7 ± 39.3 (1.5 g bLF before), 12.6 ± 26.3 (1.5 g bLF after), 2.8 ± 4.6 (3.0 g bLF before), and 27.6 ± 60.2 (3.0 g bLF after). The relative expression of IFNB after ingesting 3.0 g bLF for 1 year is significantly increased compared to its expression before the beginning of the trial ($p = 0.027$). The mean and standard deviation of the relative expression of IFNA is 31.1 ± 36.3 (placebo before), 39.5 ± 37.1 (placebo after), 30.9 ± 42.6 (1.5 g bLF before), 47.1 ± 44.8 (1.5 g bLF after), 25.4 ± 38.3 (3.0 g bLF before), and 67.2 ± 48.5 (3.0 g bLF after). The relative expression of IFNA after ingesting 3.0 g bLF for 1 year is significantly increased compared to its expression before the beginning of the trial ($p < 0.001$).

For each tissue sample-couplet (i.e., the sample collected from a patient before the beginning of the trial and the sample from that patient at the end of the trial) the relative expression of IFNB and IFNA in the sample collected at the end of the trial period was compared with the relative expression of IFNB and IFNA in the sample collected prior to the beginning of the trial. Only sample-couplets from which β-actin was successfully amplified in all TaqMan PCRs from the sample collected prior to the beginning of the trial and the associated sample collected at the end of the trial were used for this analysis: β-actin was successfully amplified in 37 of the 38 placebo group sample-couplets, in 35 of the 43 1.5 g bLF group sample-couplets, and in 28 of the 37 3.0 g bLF group sample-couplets. Due to the detection limit of the Applied Biosystems 7300 Real Time System, a two-fold change in IFN expression was used as the limit of detection for a change in IFN expression, i.e., if IFN expression at the end of the trial period changed by less than two-fold when compared to its expression prior to the beginning of the trial period, then IFN expression was judged to be unchanged. Also, when associated samples both had relative values below 0.1, interferon expression was judged to be unchanged.

In the placebo group, expression of IFNB was increased by two-fold or more in 13 (35%) of the sample-couplets, decreased by two-fold or more in 17 (49%) of the sample-couplets, and unchanged in seven (19%) of the sample couplets. In the 1.5 g bLF group, expression of IFNB was increased by more than two-fold in 16 (46%) of the sample-couplets, decreased by two-fold or more in 13 (37%) of the sample-couplets, and unchanged in six (17%) of the sample couplets. In the 3.0 g bLF group, expression of IFNB was increased by more than two-fold in 10 (36%) of the sample-couplets, decreased by two-fold or more in 10 (36%) of the sample-couplets, and unchanged in eight (29%) of the sample couplets. There was no significant difference in the distribution of IFNB expression between any of the trial groups.

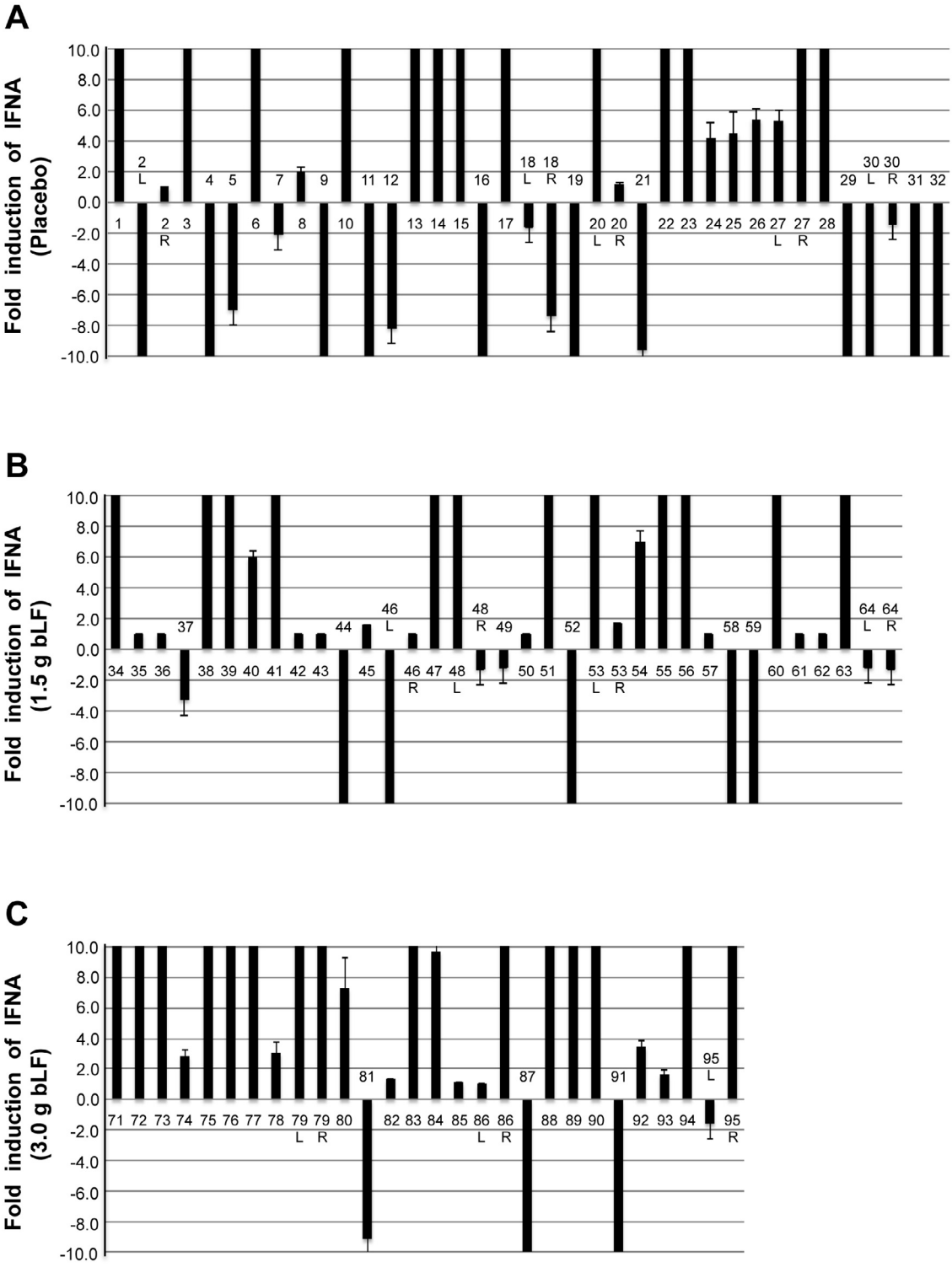
In contrast to IFNB, there was a significant difference in the distribution of IFNA expression between the 3.0 g bLF group and the placebo and 1.5 g bLF groups. Figure 2 is a graphical representation of the fold-induction of IFNA expression at the end of the trial period in the placebo group (1A), 1.5 g bLF group (1B), and 3.0 g bLF group (1C). In the placebo group, expression of IFNA was increased by two-fold or more in 18 (49%) of the sample-couplets, decreased by two-fold or more in 15 (41%) of the sample-couplets, and unchanged in four (11%) of the sample couplets. In the 1.5 g bLF group, expression of IFNA was increased by more than two-fold in 14 (40%) of the sample-couplets, decreased by two-fold or more in six (17%) of the sample-couplets, and unchanged in 15 (43%) of the sample couplets. In the 3.0 g bLF group, expression of IFNA was increased by more than two-fold in 20 (71%) of the sample-couplets, decreased by two-fold or more in three (11%) of the sample-couplets, and unchanged in five (19%) of the sample couplets. There was no significant difference in the distribution of IFNA expression between the 1.5 g bLF and the placebo groups ($p = 0.4619$); however, there was a significant difference in the distribution of IFNA expression in the 3.0 g bLF group compared to the 1.5 g bLF group ($p = 0.0409$) and the placebo group ($p = 0.0173$).

Figure 3 shows the relative expression of IFNB (2A) and IFNA (2B) in trial participants who did not ingest bLF (placebo-before, placebo-after, 1.5 g bLF-before, 3.0 g bLF before) and the relative expression of IFNB and IFNA in the 3.0 g bLF group at the end of the trial. As can be seen, the proportion of trial participants with low expression of IFNA (for example, relative expression less than 10) in their colon tissue samples is markedly less in the group that ingested bLF.

4. Discussion

In the clinical trial referred to in this report (the Tokyo-trial), participants ingested 0, 1.5, or 3.0 g bLF for 1 year to determine whether ingestion of bLF had an effect on the growth of colorectal polyps in humans (this trial is reported by Kozu et al., 2009). The present study used normal tissue samples collected from the Tokyo-trial participants before the trial began and after the trial ended to determine if a change in the expression of one or more cytokines could be detected in the colon of the Tokyo-trial participants who ingested bLF.

To identify cytokines for analysis, we initially screened samples from the 3.0 g bLF group, the group that had a response to ingestion of bLF. In this preliminary screening IFNG, IL-4, IL-7, IL-8, IL-12A, IL-12B, IL-15, IL-18, IL-22, IL-23A, GM-CSF, and TNF were either not detected or detected in a small number of samples. Lack of detection of these cytokines was



not unexpected: An important point is that patients diagnosed as having diseased or infected colons were excluded from the Tokyo-trial. Consequently, the colons of the trial participants would not have excessive numbers of activated immune cells secreting cytokines. A second point is that the intestine is a dynamic and heterogeneous environment, with the mucosa constantly interacting with food antigens and the intestinal flora, and therefore, cytokine expression in the intestine is expected to differ at different sites and at different times. A third point is that in a human trial, unlike animal studies in which the entire intestine can be examined, only very small tissue samples are available for examination. The tissue samples examined in this study had a colon mucosal surface area of less than 10 mm^2. In order for a cytokine to be detected in a majority of samples, it would have to be transcribed in a majority of 10 mm^2 sections of the colon. This is unlikely in the normal human colon for most cytokines. Lack of detection of expression of these cytokines made it impossible to determine whether or not ingestion of bLF affected their expression in the colon of the Tokyo-trial participants; therefore, these cytokines were not examined further.

In contrast to the other cytokines, expression of type I IFN mRNA was detected in most of the samples from the 3.0 g bLF group. Type I interferons are widespread immune system modulators, and, unlike other cytokines, type I interferons can be expressed by all known nucleated cells (Hervas-Stubbs et al., 2011; Mancuso et al., 2007; Pichlmair & Reis e Sousa, 2007; Theofilopoulos, Baccala, Beutler, & Kono, 2005; Trinchieri, 2010). The samples were subsequently screened by standard endpoint PCR for the presence of the different type I interferons (IFNB and the 13 species of human IFNA (Pestka, 2007)), followed by sequencing of the PCR-generated amplicons to confirm the identification of the interferon. IFNA2 sequences were present in most of the tissue samples. IFNB sequences, while less widespread than IFNA, were present in more than 50% of the samples. The other IFNA species were either not identified by amplicon sequencing or their sequences were present in only a minority of samples. Therefore, we examined the relative expression of IFNA2 and IFNB by TaqMan Real-Time PCR using the primers listed in Supplementary Table S1.

Patients ingesting 3.0 g bLF for 1 year had a significant increase in IFNB expression in the colon. However, this mathematically significant increase does not appear to reflect a physiologically significant increase. As can be seen from Tables 1–3, detection of IFNB was sporadic. Relative expression values greater than 10 for IFNB are observed in less than 25% of the samples. In addition, the mean expression level in the colons of the 3.0 g bLF group before the trial began (mean relative expression = 2.8) is significantly lower than in the 1.5 g bLF group (mean relative expression = 24.7; $p = 0.0016$) and the placebo group (mean relative expression = 11.2; $p = 0.0192$). This suggests that the increase seen in IFNB expression after 1 year in the 3.0 g bLF group may be due to the low level of IFNB expression in the samples collected prior to the beginning of the trial. Notably, when the relative expression of IFNB in all patients which had not ingested bLF (placebo-before, placebo-after, 1.5 g bLF-before, 3.0 g bLF-before) are combined (mean relative expression = 12.2), there is only a non-significant increase in the expression of IFNB in the 3.0 group at the end of the trial (mean relative expression = 27.6; $p = 0.1632$).

Patients ingesting 3.0 g bLF for 1 year also had a significant increase in IFNA expression in the colon. In contrast to IFNB expression, relative expression values greater than 10 for IFNA2 are observed in more than 50% of the samples. In addition, the mean expression level in the colons of the 3.0 g bLF group before the trial began (mean relative expression = 25.4) is not significantly different from the 1.5 g bLF group (mean relative expression = 30.9; $p = 0.5665$) or the placebo group (mean relative expression = 31.1; $p = 0.5245$). Moreover, when the relative expression of IFNA in all patients who had not ingested bLF (placebo-before, placebo-after, 1.5 g bLF-before, 3.0 g bLF-before) are combined (mean relative expression = 31.9), there is a significant increase in the expression of IFNA in the 3.0 group at the end of the trial (mean relative expression = 67.2; $p = 0.0004$).

The change in the distribution of IFNB and IFNA expression (decreased, no change, or increased) supports the proposal that ingestion of bLF promotes the expression if IFNA, but does not support the proposal that ingestion of bLF promotes the expression of IFNB. The intestinal environment is constantly changing due to the substances we eat and drink and because of the interaction of the intestinal flora with the intestinal mucosa (Artis, 2008; O'Hara & Shanahan, 2006). Intestinal epithelial cells (IECs) and immune cells express a wide range of pattern recognition receptors (PRRs) that can recognize microbial factors such as lipopolysaccharide,

Fig. 2 – Fold-induction of IFNA expression after daily ingestion of placebo, 1.5 g bLF, or 3 g bLF for 1 year. The relative expression of IFNA in the tissue sample collected at the end of the trial was compared with its relative expression in the associated sample collected at the beginning of the trial. Reduction of IFN expression is calculated as the negative inverse of the change in the expression of IFN [–1/(IFN-after/IFN-before)]; thus, if the relative expression of IFN at the end of the trial was 25% of its expression at the start of the trial, the fold-induction is –4. Because the expression of IFNA in several of the samples was very low or non-detectable, extremely large induction/reduction values were obtained in these sample-couplets; therefore, a 10-fold increase or decrease in expression is used as a cut-off for the fold-induction of IFNA in this figure. When associated samples both had relative values below 0.1, expression of IFNA was judged to be unchanged. (A) Fold-induction of IFNA expression at the end of the trial period in the placebo group. (B) Fold-induction in IFNA expression at the end of the trial period in the 1.5 g bLF group. (C) Fold-induction in IFNA expression at the end of the trial period in the 3 g bLF group. There was a significant difference in the distribution of IFNA expression in the 3.0 g bLF group compared to the 1.5 g bLF group ($p = 0.0409$) and the placebo group ($p = 0.0173$). The distribution of IFNA expression in the 1.5 g bLF and the placebo groups was not significantly different ($p = 0.4619$). Patient numbers correspond to those in Tables 1–3, and Supplementary Table S2.

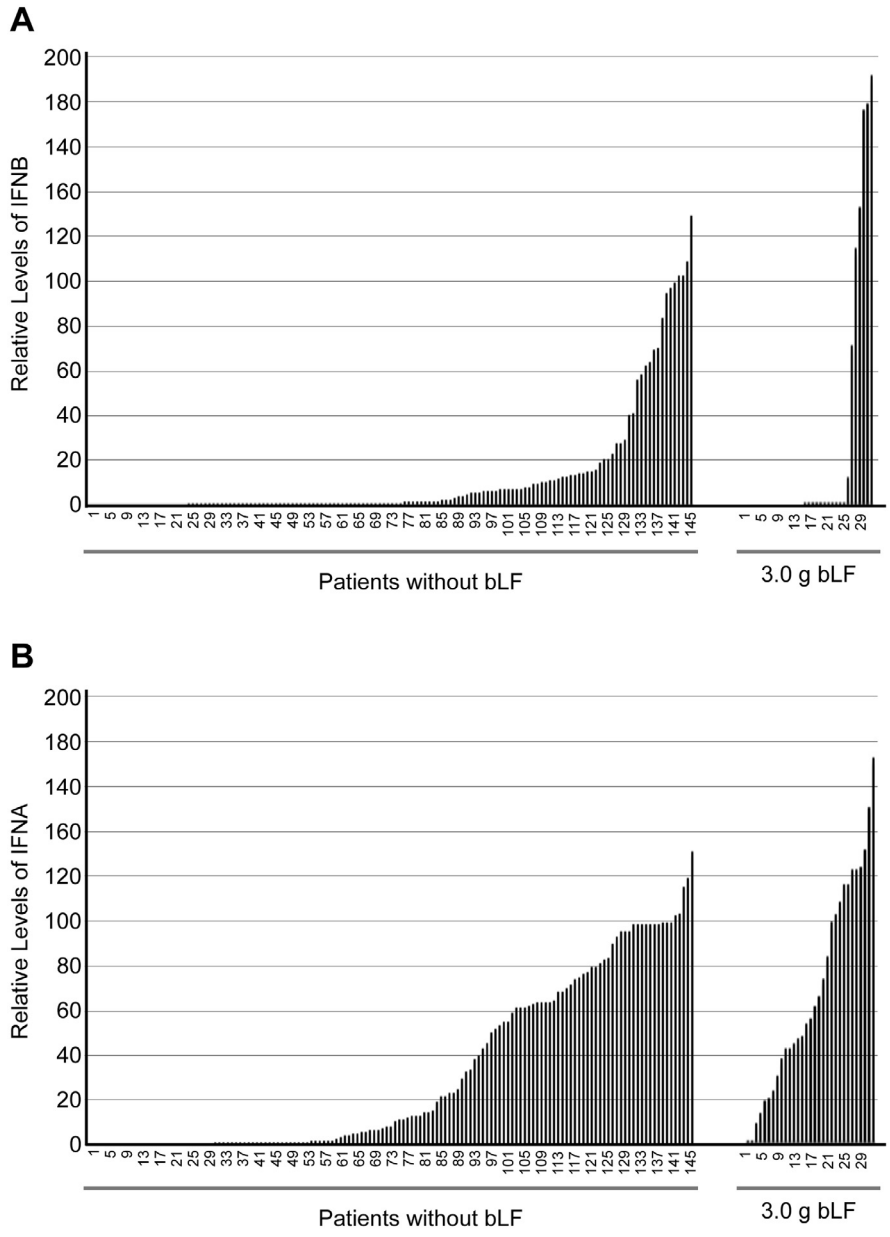


Fig. 3 – Relative levels of IFNB and IFNA in Tokyo-trial participants who did not ingest bLF and Tokyo-trial participants who ingested 3.0 g bLF daily for 1 year. (A) Relative levels of IFNB in trial participants who did not ingest bLF (placebo-before, placebo-after, 1.5 g bLF-before, 3.0 g bLF before) and trial participants who ingested 3.0 g bLF daily for 1 year. (B) Relative levels of IFNA in trial participants who did not ingest bLF (placebo-before, placebo-after, 1.5 g bLF-before, 3.0 g bLF before) and trial participants who ingested 3.0 g bLF daily for 1 year.

lipoproteins, flagellin, and unmethylated CpG-containing DNA (Abreu, Fukata, & Arditi, 2005; Artis, 2008; Hall et al., 2008; O'Hara & Shanahan, 2006; Yrlid et al., 2006). Therefore, signaling through these receptors will change in accordance with the intestinal environment. Consequently, the expression levels of genes which are expressed by IECs and immune cells and which respond to PRR signaling, such as type I interferons (Kawai & Akira, 2010; Kawai et al., 2004; Paun et al., 2008; Takeuchi & Akira, 2010; Yrlid et al., 2006), will fluctuate. In a population composed of a random grouping of individuals, this fluctuation is expected to appear random, resulting in an increase in expression in about half of the population with detectable cytokine expression and a decrease in expression in about half of the population with detectable cytokine expression. This is the result seen in the placebo group for both IFNA and IFNB. In the 3 g bLF group, in contrast to the placebo group, IFNA expression was increased by more than two-fold in most of the participants and only a few participants exhibited decreased IFNA expression. The distribution of IFNA expression in the 3.0 g bLF group was significantly different from the placebo group ($p = 0.0173$). This is the result expected if ingestion of bLF promoted expression of IFNA. In contrast to IFNA expression, there

was no significant difference in the distribution of IFNB expression between the 3.0 g bLF group and the placebo (or 1.5 g bLF) group. Therefore, while it is possible that ingestion of bLF promoted IFNB expression in the colon, our overall results can not be used to support this conclusion.

The Tokyo-trial reported that ingestion of 3 g bLF inhibited the growth of colorectal polyps and increased the levels of hLF in the serum of trial participants 63 years old or younger (Kozu et al., 2009). Induction of IFNA in the human colon and consequent IFNA-mediated suppression of cell growth (Bekisz, Baron, Balinsky, Morrow, & Zoon, 2010) and IFNA-mediated priming of neutrophils (Brassard, Grace, & Bordens, 2002; Hervas-Stubbs et al., 2011; Theofilopoulos et al., 2005) is a plausible mechanism by which ingestion of bLF mediated these effects in the Tokyo-trial participants. For the interested reader, this hypothetical model, the results of the Tokyo-trial, and why the effects were age-dependent is discussed in detail in Supplementary Text S2. Also discussed in Supplementary Text S2 is the evidence that neither bLF itself nor bLF-derived peptides exerted direct toxic effects against colorectal polyps in the Tokyo-trial.

In a related study by Iigo et al. (2014), using Tokyo-trial samples prepared for histological analysis, we found that participants ingesting 3.0 g bLF had increased numbers of CD4+ cells and NK cells in their colorectal polyps. The increase in polyp-associated CD4+ cells is consistent with the increase of peritumoral and intratumoral CD4+ cells seen 15 days after intralesional injection of basal cell carcinomas with IFNA (Mozzanica et al., 1990) and suggest that induction of IFNA in the colons of participants ingesting 3.0 g bLF resulted in an increase of CD4+ cells in the colon mucosa and, consequently, in the colorectal polyps of these trial participants. The increase in polyp-associated NK cells is consistent with IFNA-mediated induction of the expression of CXCL10 (Lande et al., 2003), which is chemotactic for NK cells (Lande et al., 2003; Megjugorac, Young, Amrute, Olshalsky, & Fitzgerald-Bocarsly, 2004). While the target polyps in the Tokyo-trial were precancerous, and consequently were not targeted by the immune system, the presence of increased numbers of CD4+ cells and NK cells will enhance the ability of the immune system to target and remove cells when they become transformed into cells with increased tumorigenic potential. Taken together, our results combined with the data discussed above suggest that promotion of IFNA expression increases the responsiveness of the immune system.

The ability of bLF to prime immune effector cells means that the health benefits of ingesting bLF go beyond its protective effects against cancer. By priming the immune system, ingestion of bLF is able to enhance the immune response against the myriad diseases and infections to which humans are subject. In support of this possibility, a recent study reports that daily ingestion of 600 mg of a bLF/whey protein Ig-rich fraction combination significantly decreased the occurrence of colds reported and decreased the number of cold-related symptoms (Vitetta et al., 2013). This effect may be of particular importance in the elderly whose immune function is declining. It should be noted, however, that the model we propose in Supplementary Text S2 suggests that for people with reduced gastric digestive capability, predigested bLF may be a more beneficial dietary supplement than bLF.

Consumption of LF by adult mammals has effects distinct from those of endogenous LF. Endogenous LF is present at moderate to high levels in tear film, upper airway fluids, seminal fluid, and in the specific granules of neutrophils and is involved in mucosal and neutrophil-mediated immunity (reviewed in Alexander, Iigo, Yamauchi, Suzui, & Tsuda, 2012). The primary function of endogenous LF in mucosal fluids is to promote the non-lethal, non-inflammatory removal of microbial pathogens away from cells and tissues. Our model, as discussed in Supplementary Text S2, predicts that the differences in the activities of ingested and endogenous LF in the adult are due to the differences in the activities of the lactoferrin protein and the antimicrobial peptides derived from gastric digestion of lactoferrin. This paradigm is consistent with the proposed activities of other bioactive peptides liberated by gastrointestinal digestion of milk proteins (Korhonen, 2009). It should be noted that the effects of the consumption of LF by infants and adults are also distinct: Because lactoferrin is highly resistant to digestion in the infant GI tract, LF in the milk ingested by infants will have little microbicidal activity and will function primarily as a microbiostatic agent promoting the non-lethal, non-inflammatory removal of microbial pathogens away from the intestinal epithelium (see Alexander et al., 2012).

A major limitation of our study is the small number of samples analyzed. Consequently, while significance was obtained, follow up studies with a larger number of participants is needed to confirm our results. In addition, this study used samples obtained from human volunteers; consequently: (i) Tissue samples were not obtained from the small intestine; therefore, the effect of bLF on cytokine production in the small intestine and in Peyer's Patches was not assessed. (ii) While we show that ingestion of bLF promoted expression of IFNA2 RNA in the colon of the Tokyo-trial participants, the size of the tissue samples and the number of samples was too small to rigorously assess the effect of ingestion of bLF on the expression of other cytokines. (iii) Tissue samples for protein extraction were not available; therefore, expression of IFNA protein in the colon could not be analyzed.

Importantly, in all of the animal and human studies conducted to date, which include chronic administration to animals and daily intake for 1 year by human patients, no adverse events related to ingestion of lactoferrin have been reported. In trials with patients with chronic hepatitis C, 15 patients were given 3.6 g bLF for 6 months (Iwasa et al., 2002), 15 patients were given 7.2 g bLF daily for 8 weeks (Okada et al., 2002), and 97 patients were given 1.8 g bLF daily for 12 weeks (Ueno et al., 2006). No adverse events related to bLF ingestion were reported by any of these trials. In a clinical trial to determine whether ingestion of bLF had an effect on colorectal polyp growth, ingestion of 3.0 g bLF daily for 1 year had no adverse effects on the trial participants (Kozu et al., 2009). Four long term cancer survivors with metastatic renal cell carcinoma receiving recombinant human lactoferrin at a dose of 9 g per day given in cycles of 2 weeks on/2 weeks off for 30 months or longer showed no adverse events related to lactoferrin (Lewis & Hayes, 2011). Finally, the European Food Safety Authority (EFSA) Panel on Dietetic Products, Nutrition and Allergies (NDA) has concluded that intake of bLF up to 3.4 g per day by an adult is safe (EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA),

2012). Taken together, these data indicate that ingestion of bLF is safe. However, the effects of consuming higher amounts of lactoferrin than noted here are unknown and should be avoided.

5. Conclusions

Trial participants ingested 0, 1.5, or 3.0 g bLF for 1 year. Our ancillary study found a significant increase in IFNB expression in the colons of the participants ingesting 3.0 g bLF; however, the overall expression of IFNB in the trial participants and the distribution of IFNB expression (decreased, no change, or increased) in the 3.0 g bLF group preclude concluding that this mathematically significant increase is physiologically significant. Our study also found a significant increase in IFNA expression in the colons of the 3.0 bLF group, and the overall expression of IFNA in the trial participants and the distribution of IFNA expression (decreased, no change, or increased) in the 3.0 g bLF group supports the conclusion that ingestion of 3.0 g bLF for 1 year resulted in increased expression of IFNA in the human colon. Our study validates the results obtained from animal studies: ingestion of bLF promotes expression of immune modulating cytokines in the intestine.

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Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.jff.2014.06.028.

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