production [8] and enhance mucosal immunity [9], NK cell activity [10,11], LAK activity [11], neutrophil activity [12,13], and macrophage cytotoxicity [14,15]. Previously we found that the pro-inflammatory cytokine interleukin-18 (IL-18) was produced by epithelial cells of the small intestine following administration of bLF [9,16]. IL-18 enhances Th1 type T and NK cell responses and generates $CD8^+$T cells [17].

Bezault et al. [18] report a protective effect of human-LF against the growth of solid tumors and the development of metastases in mice. bLF also inhibits lung metastasis of B16 melanoma and colon 26 tumor cells in mice [19,20] and is protective against tongue, esophagus, intestinal, lung, and bladder carcinogenesis in rats [21–24]. bLF administration results in a significant increase of $CD4^+$ and $CD8^+$T cells and asialo GM_1^+ (NK) cells in the blood [16] and in the lymphoid tissues and lamina propria of the small intestine [9]: $CD8^+$T and asialo $GM1^+$ cells exhibit marked cytotoxicity against Co26Lu cells *in vitro* [16]. The observed increase in T and NK cells may be due to the increased levels of mature IL-18 produced by epithelial cells of the small intestine after treatment with bLF [16].

In Co26Lu bearing-SCID mice, which are deficient in T and B cells, bLF also showed significant inhibition of tumor growth (Fig. 1) and lung metastatic colony formation [7]. However, the number of lung metastatic colonies was markedly increased when these mice were treated with anti-asialo GM_1 antibodies to weaken their NK cell activity. Bezault et al. [18] also report that LF-mediated antitumor effects were lost upon elimination of NK cell activity by pretreatment of mice with anti-asialo GM_1 antibodies. These results demonstrate the importance of activated NK cells for LF-mediated inhibition of tumor growth and metastasis.

The immunomodulatory and antitumor activities of bLF suggest that it may be a promising chemopreventer of carcinogenesis [25,26]. In this study we investigated pathways by which ingested bLF can exert its effects.

2. Materials and methods

2.1. bLF, bLFH, bLFcin and bTF

Bovine lactoferrin (bLF) (purity: greater than 96% of total protein by HPLC; endotoxin: less than 5.0 pg/mg of bLF; iron content: 143 ± 19 ng/mg of bLF, $n = 3$), a pepsin-generated hydrolysate of bLF (bLFH: endotoxin, 101.3 ± 25.7 pg/mg of bLFH; iron content, 100 ± 0 ng/mg of bLFH, $n = 3$), and lactoferricin (bLFcin: endotoxin, 101.3 ± 25.7 pg/mg of bLFcin, $n = 3$) were obtained from Morinaga Milk Industry Co., Ltd. (Zama, Japan). Bovine transferrin (bTF) (purity: greater than 98% of total protein by agarose electrophoresis; endotoxin, 16.4 ± 2.8 pg/mg of bTF; iron content, 13 ± 3 ng/mg of bTF, $n = 3$) was purchased from Sigma Co. Ltd. (St. Louis, MO). Endotoxin (by the modified limulus test) and iron (by the 2-nitroso-5-[*N*-*n*-propyl-*N*-(3-sulfopropyl)amino]phenol test) content were determined by SRL Inc., Hachioji, Japan.

2.2. Cytokines and antibodies

Anti-asialo GM_1 Ab was purchased from Wako Pure Chemical Industries (Osaka, Japan; caspase-1/ICE inhibitor (Acyl-Tyr-Val-Ala-Asp-chloromethylketone) was purchased from Medical & Biological Laboratories Co., Ltd. (Nagoya, Japan); recombinant murine IFNγ was purchased from Intergen Company (Purchase, NY); recombinant murine IL-7 was purchased from PeproTech House (London, England); recombinant murine IL-18 was purchased from Medical & Biological Laboratories Co., Ltd.; recombinant IFNαA/D (2.04×10^7 IU/ml) was obtained from Nippon Roche Research Center, (Kamakura, Japan); anti-mouse IFNα antibody (clone F18, rat IgG_1) was purchased from HyCult Biotechnology b.v.; goat anti-murine IL-1β (M-20, goat IgG) was purchased from Santa Cruz Biotechnology, Inc.; anti-murine IL-7 polyclonal antibody was purchased from Pepro Tech EC Ltd.; anti-mouse IL-12 antibody (p40/p70) was purchased from PharMingen, BD Biosciences; rabbit anti-mouse IL-15 antibody was purchased from Torrey Pines Biolabs, Inc.

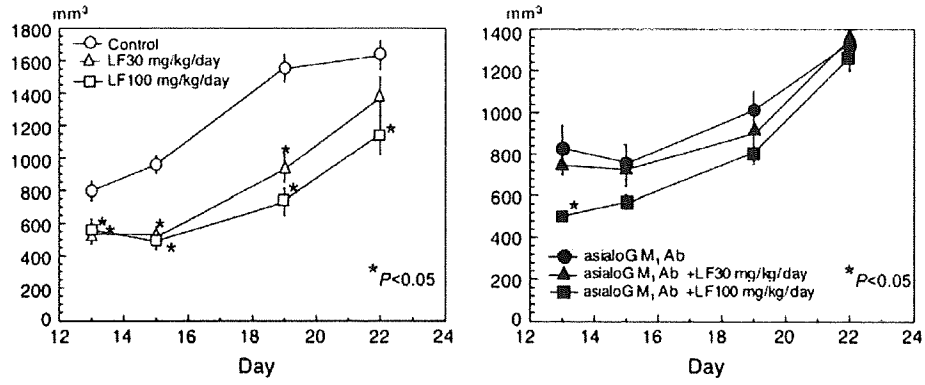

Fig. 1. Importance of activated NK cells for bLF-mediated inhibition of tumor growth (Co26Lu tumor-bearing SCID mice). 1×10^5 cells/mouse were subcutaneously implanted into the right thigh of SCID mice (Charles River Japan, female). bLF was administered at 30 or 300 mg/kg per day on days 5–9 and 12–16. Anti-asialo GM_1 Ab was injected (dilute 1/40, 0.2 ml/mouse i.v.) on days 5, 7, 12, and 14. The longest (a) and shortest (b) diameters of tumors at the Co26Lu cell injection site were measured twice a week using calipers, and the volume was calculated using the formula: $ab^2/2$ (mm^3). The experiment was terminated 22 days after implantation. The symbols represent the mean and standard error of tumor sizes ($n = 5–7$). *$p < 0.05$, Dunnett's multiple comparison *t*-test.

(Houston, TX); anti-rat IFNγ antibody (clone DB-1, mouse IgG_1) was purchased from BioLegend (San Diego, CA); and anti-TNFα (L-19) polyclonal antibody was purchased from Santa Cruz Biotechnology, Inc. Rabbit anti-human lactoferrin, specific for human lactoferrin, and rabbit anti-bovine lactoferrin antibodies, specific for bovine lactoferrin, were obtained from Morinaga Milk Industry Co. Ltd. (Zama, Japan). Anti-human IL-12/IL-23 p40 (clone: C8.3, mouse IgG_1) was purchased from BioLegend; anti-human IL-18 antibody (clone: 159-12B, rat IgG_{2a};detects both pro-IL-18 and mature IL-18) and anti-human IFNα antibody (clone: #14, mouse IgG_1) were purchased from Medical & Biological Laboratories Co., Ltd.; anti-human IFNγ antibody (clone: MD-1, mouse IgG_1) was purchased from BioLegend (San Diego, CA).

2.3. Mice

BALB/c mice (wild type mice), 5-week-old male and female, were obtained from Charles River Japan (Atsugi, Japan). IFNγ knockout mice (mice with a targeted disruption of the IFNγ gene, BALB/c-Ifng^{tm1Ts}, 4 ~ 5-weeks-old female, GKO mice) [27] were purchased from the Jackson Laboratory, Bar Harbor, ME. The animals were allowed free access to CE-2 pellet diet (CLEA Japan, Tokyo, Japan) and water, and were maintained in plastic cages on woodchip bedding under specific pathogen-free conditions in our animal facility with a controlled temperature of 24 ± 2 °C, humidity 60 ± 10%, and a 12 h light–dark cycle. All experiments were initiated when the mice became 6 weeks-old, and performed according to "Guide for the Care and Use of Laboratory Animals" of the Animal Study Committee of National Cancer Center Research Institute, Tokyo, Japan.

2.4. Organ culture of the small intestine

BALB/c mice were anesthetized with diethyl ether and then killed. The jejunum was excised (2 cm) and then washed with physiological saline. The jejunum was turned inside out and cultured in RPMI-1640 culture medium at 37 °C for 30 min with bLF, bTF, or bLFcin as shown in Fig. 2. After the 30 min culture period, the tissue specimens were cut perpendicularly with scissors and opened onto glass plates. The entire surface (epithelium, lamina propria, submucosa) of the jejunum was harvested by scraping with a slide glass. The sample was then placed on an aluminum plate cooled by acetone-dry ice and pressed flat using another plate. These samples were stored at −80 °C until use.

2.5. Peritoneal macrophage culture

Mice were injected intraperitoneally with 4 ml of thioglycollate broth (3 g/100 ml, Eiken Chemical Co. Ltd., Tokyo). Four days after injection, peritoneal exudate macrophages were obtained: using a plastic 10 ml syringe with a 22-gauge needle, mice were injected intraperitoneally with saline and massaged and the saline solution containing peritoneal exudate macrophages was recovered. Macrophages were washed once with Hanks' balanced salt solution. The harvested cells were centrifuged at 200 × *g* for 10 min, resuspended in culture medium (RPMI-1640, 10% FBS), and plated into a 24-well tissue culture plate (2×10^6 cells/2 ml/well). After incubation for 2 h, non-adherent cells were removed by vigorous washing, and fresh culture medium (RPMI-1640, 5% FBS) was added to the monolayer.

2.6. Spleen cell culture

Mice were anaesthetized and killed and the spleen harvested. Spleens were minced in petri dishes containing 5 ml media and pressed through a 120-stainless-steel mesh. Cells obtained were washed with Hanks' balanced salt solution, centrifuged at 200 × *g* for 10 min, resuspended in culture medium (RPMI-1640, 10% FBS), and plated into a 24-well tissue culture plate (2×10^6 cells/2 ml/well).

2.7. Enzyme-linked immunosorbent assay (ELISA) — frozen organ culture samples

ELISA was used to determine the levels of various cytokines. Frozen tissues were homogenized with a Sonifier 450 (Branson Ultrasonics Corp., Daburg, CT) in lysis buffer (0.067 M phosphate buffer, pH 7.2, 1% Nonidet P-40, 0.5% sodium deoxycholic acid, 0.1% sodium dodecyl sulfate, and 10 μg/ml phenylmethylsulfonyl fluoride, 2.5 μg/ml leupeptin and 20 U/ml aprotinin), homogenates were clarified by centrifugation at 15,000 rpm for 10 min at 4 °C, and the supernatants were collected and stored at −80 °C until testing. ELISA kits for murine IL-1β were purchased from Genzyme Techne (Minneapolis, MN). Mature IL-18 levels were determined using a mouse IL-18 ELISA kit (minimum detection limit: 25.0 pg/ml) purchased from Medical & Biological Laboratories Co., Ltd. (Nagoya, Japan).

2.8. Enzyme-linked immunosorbent assay (ELISA) — peritoneal macrophage, RAW 264.7 cells, and spleen cell cultures

Peritoneal macrophages and spleen cells were harvested as described above. RAW 264.7 mouse macrophages were obtained from Riken (Tsukuba, Japan). Cells were plated into 24-well tissue culture plates (2×10^6 cells/2 ml/well). After incubation for 24 h in 300 μg/ml bLF, cells were harvested and lysed in 100 μl of chilled lysis buffer (0.067 M phosphate buffer, pH 7.2, 1% Nonidet P-40, 0.5% sodium deoxycholic acid, 0.1% sodium dodecyl sulfate, and 10 μg/ml phenylmethylsulfonyl fluoride, 2.5 μg/ml leupeptin and 20 U/ml aprotinin), and then clarified by centrifugation at 15,000 rpm for 10 min. The supernatants were collected and stored at −80 °C until testing. An ELISA kit for mature mIL-18 (minimum detection limit: 25.0 pg/ml) (Medical & Biological Laboratories Co., Ltd., Nagoya, Japan) was used to measure IL-18 levels.

2.9. Enzyme-linked immunosorbent assay (ELISA) — peritoneal macrophage cultures

Peritoneal macrophages were harvested as described above. Cells were plated into 24-well tissue culture plates

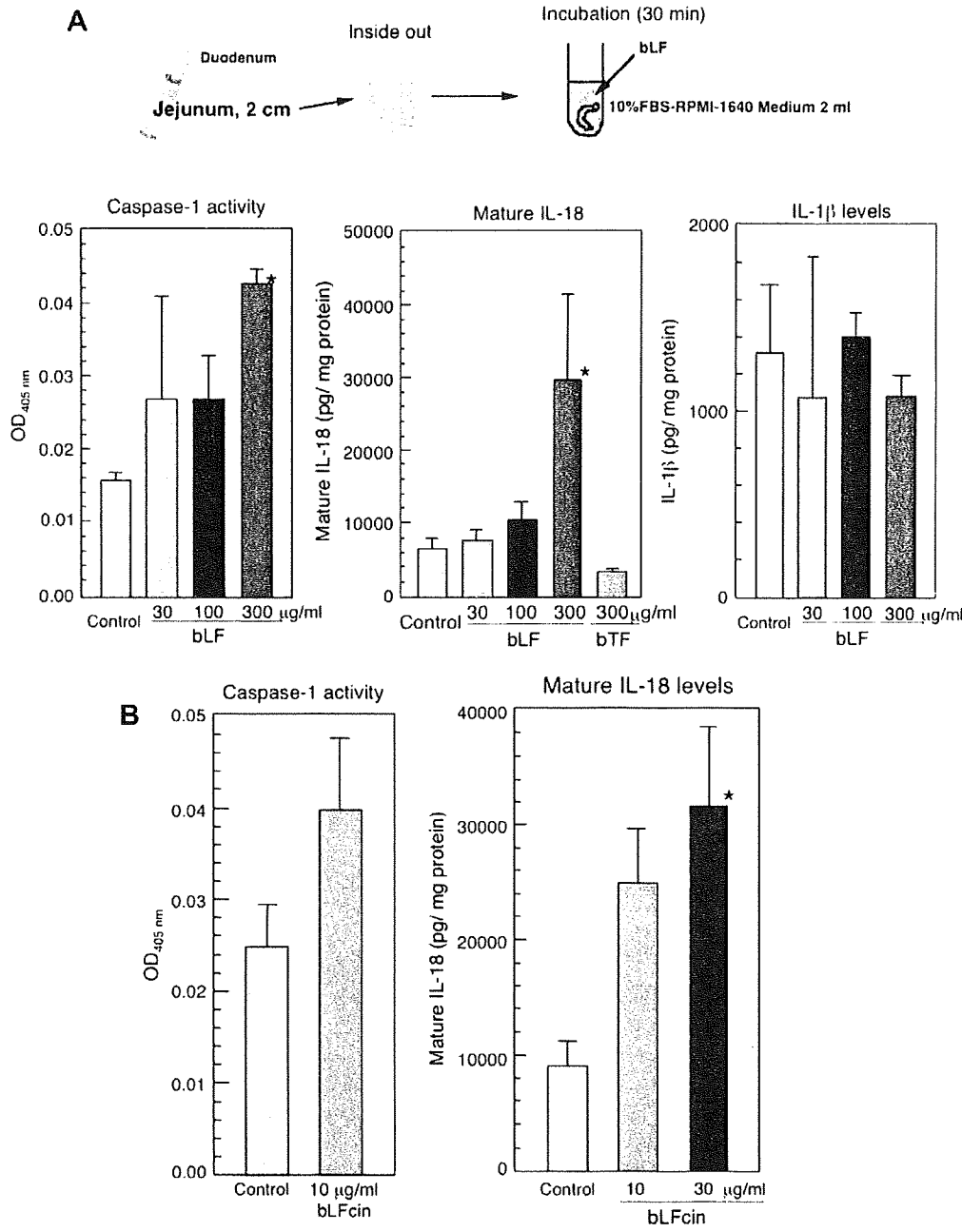

Fig. 2. Effects of bLF and bLFcin on caspase-1 activity and the level of mature IL-18 in the mucosa of organ cultured small intestine. Mouse jejunum in organ culture was incubated with lactoferrin (A) ($n = 7$) or lactoferricin (B) ($n = 3$) for 30 min, and caspase-1 activity and mature IL-18 or IL-1β levels were measured by ELISA. Error bars represent the standard error. *$p < 0.05$, Dunnett's multiple comparison t-test.

(2×10^6 cells/2 ml/well). After incubation for 24 h in bLF, rmIFNγ, anti-mIFNγ antibody, or caspase-1 inhibitor, cells were harvested and lysed in 100 μl of chilled lysis buffer (0.067 M phosphate buffer, pH 7.2, 1% Nonidet P-40, 0.5% sodium deoxycholic acid, 0.1% sodium dodecyl sulfate, and 10 μg/ml phenylmethylsulfonyl fluoride, 2.5 μg/ml leupeptin and 20 U/ml aprotinin), and then clarified by centrifugation at 15,000 rpm for 10 min. The supernatants were collected and stored at −80 °C until testing. An ELISA kit for mature mIL-18 (minimum detection limit: 25.0 pg/ml) (Medical & Biological Laboratories Co., Ltd., Nagoya, Japan) was used to measure IL-18 levels.

2.10. Caspase-1 activity

For assaying the activity of caspase-1, colorimetric protease assay kits were used (BioVision Research Products, Mountain View, CA). The samples were diluted to 200 μg protein in 50 μl Cell Lysis buffer and then 50 μl of Reaction buffer containing 10 mM DTT and 5 μl of 4 mM Tyr-Val-Ala-Asp-pNA

(chromophore *p*-nitroanilide) was added. The samples were incubated at 37 °C for 2 h. Caspase-1 activity levels were determined at 405 nm in a microtiter plate reader. In some experiments, Acyl-Tyr-Val-Ala-Asp-chloromethylketone (Bachem Biochemica GmbH, Heidelberg, Germany) at a concentration of 50 μM was added to inhibit caspase-1 activity; the inhibitor was applied 30 min before bLF addition.

2.11. Western blot analysis of cell cultures

Peritoneal macrophages were harvested as described above; Lewis Lung carcinoma cells and MCF7 human breast cancer epithelial cells were obtained from the National Cancer Center Research Institute (Tokyo, Japan); SW620 colon carcinoma cells were obtained from the American Type Culture Center (Rockville, MD); and SK-MEL 19 melanoma cells [28] were obtained from the Medical Research and Development Center, the Tokyo Metropolitan Institute of Medical Science (Tokyo, Japan). Cells were seeded in 24 well plates in RPMI-1640 medium with 10% FBS at a density of 1×10^6 cells/well and incubated for 24 h. The medium was then changed to serum-free medium containing various concentration of bLF. After a 24 h incubation, cells were washed twice with serum-free medium and lysed in 75 μl chilled lysis buffer containing 125 mM Tris–HCl (pH 6.8), 4.3% SDS, 30% glycerol, 10% 2-mercaptoethanol, 0.01% BPB, and protease inhibitor cocktail "Complete Mini" (Roche, Mannheim, Germany). Lysates were sonicated and clarified by centrifugation at 15,000 rpm for 45 min at 4 °C. Ten μg of each sample was separated by SDS-PAGE on 15/25% polyacrylamide gradient gels (Multigel II Mini, Daiichi Pure Chemicals Co. Ltd., Tokyo). Proteins were transferred to PVDF membrane (Immobilon-P, Millipore Corp., Bedford, MA) and detected by Western blot with appropriate antibodies. Immunoreactive bands were visualized with Amersham ECL Plus Western Blotting Detection System (GE Healthcare, Buckinghamshire, UK). Densitometric analysis of digitized blot images was carried out using NIH Image software.

2.12. Protein assay

Total protein content of the samples was assayed using Coomassie Dry Protein Assay Plates (Pierce, Rockford, IL) with bovine serum albumin as the protein standard (Fraction V, Sigma Chemical Co. St. Louis, MO).

2.13. Lung metastasis assay

Colon carcinoma 26 (obtained from the Cancer Institute of the Japanese Foundation for Cancer Research, Tokyo, Japan), are of BALB/c origin and have low metastatic potential. Metastatic tumor cells (Co26Lu) were obtained by sequential selection of tumor colonies in the lung: colon carcinoma 26 cells were subcutaneously implanted into the back of synergistic BALB/c mice and after tumor formation, colonies in the lung were recovered and cells from these lung metastases were subcutaneously implanted into the back of another mouse. After several rounds of sequential selection, highly metastatic Co26Lu cells were obtained.

Freshly excised Co26Lu cells from tumor-bearing mice were minced in Hanks' balanced salt solution (Life Technologies, Grand Island, NY) and strained through a 120-stainless-steel mesh. Viability was determined by trypan blue dye exclusion. Cells were diluted to the desired concentration and 1×10^5 cells/mouse were subcutaneously implanted into the right thigh of GKO and BALB/c mice ($n = 15$). bLF was administered at 30 or 300 mg/kg per day on days 3–7, 10–14, and 17–21 and the mice were killed on day 22. The lungs were excised and fixed in acetone and lung metastases were counted.

2.14. Treatment with bLF, bLFcin, and bTF and preparation of tissues

Normal BALB/c (WT) and GKO mice were used (five mice/group). bLF, bLFH, and bTF were dissolved in physiological saline (3 or 30 mg/ml) and administered at 30 mg/kg per day in consecutive treatments for 7 days or a single administration at 300 mg/kg. Three hours after the last oral administration of bLF, bLFH or bTF the mice were anesthetized with diethyl ether and killed. The small intestine, jejunum and ileum, was excised and washed with physiological saline. It was then cut perpendicularly with scissors and opened onto a glass plate. The entire surface (epithelium, lamina propria, submucosa) of the ileum was harvested by scraping with a slide glass. A portion of the harvested mucosa was used for RNA extraction; the remaining mucosa was placed on an aluminum plate cooled by acetone-dry ice and pressed flat using another plate. These samples were stored at −80 °C until use. The jejunum was fixed in acetone and used for immunohisochemistry.

2.15. Enzyme-linked immunosorbent assay (ELISA) — frozen tissue samples

ELISA was used to determine the levels of various cytokines. Frozen tissues were homogenized with a Sonifier 450 (Branson Ultrasonics Corp., Daburg, CT) in lysis buffer (0.067 M phosphate buffer, pH 7.2, 1% Nonidet P-40, 0.5% sodium deoxycholic acid, 0.1% sodium dodecyl sulfate, and 10 μg/ml phenylmethylsulfonyl fluoride, 2.5 μg/ml leupeptin and 20 U/ml aprotinin), homogenates were clarified by centrifugation at 15,000 rpm for 10 min at 4 °C, and the supernatants were collected and stored at −80 °C until testing. ELISA kits for murine IL-1β, IL-4, IL-6, IL-12, GM-CSF, and TNFα were purchased from Genzyme Techne (Minneapolis, MN). Mature IL-18 levels were determined using a mouse IL-18 ELISA kit (minimum detection limit: 25.0 pg/ml) purchased from Medical & Biological Laboratories Co., Ltd. (Nagoya, Japan).

2.16. PCR detection of IL-7, IL-15, IL-18, and caspase-1 mRNA expression

For investigation of IL-7, IL-15 IL-18, and caspase-1 mRNA levels, in the intestinal epithelium, bLF was administered orally to tumor-bearing mice on day 7 or days 1–7,

and the mice were killed under deep anesthesia with diethyl ether 3 h after the last oral administration and the small intestine was immediately removed. Total RNAs from mucosal tissues were isolated using ISOGEN (Nippon Gene, Toyama, Japan), followed by ethanol precipitation. Aliquots (5 μg) of total RNA were subjected to a DNase I digestion before reverse transcription (RT) to eliminate any remaining genomic DNA. The RT reaction was carried out for 1 h at 42 °C in a reaction mixture (20 μl) containing 50 ng of oligo(dT)12–18 primer, dNTP (0.5 mM each of dATP, dGTP, dCTP and dTTP), 200 units of Superscript™II (Life Technologies Inc., Gaithersburg, MD) and the first strand buffer, and terminated by incubation at 95 °C for 5 min.

PCR was performed in a reaction mixture (30 μl) containing cDNA, primers (10 pmol each), 0.2 mM dNTP and 1.5 units of Takara Ex Taq™ DNA polymerase (Takara Shuzo, Kyoto, Japan) in Ex Taq buffer. Primers (5′–3′) specific for murine IL-7, IL-15, IL-18, caspase-1 and β-actin sequences [29–32] were as follows:

IL-7, GCGGATCCATGTTCCATGTTTCTTTTAG and GCAAGCTTGTTTATATACTGCCCTTC, 483 bp;
IL-15, GCGGATCCAATGAAAATTTTGAAACC and GCGAATTCAGTCAGGACGTGTTGATG, 508 bp;
IL-18, GCGAATTCCAATGGCTGCCATGTCAG and GCAAGCTTACCTAACTTTGATGTAAG, 599 bp;
Caspase-1, GCCTGCAGATGGCTGACAAGATCCTGAGG and GCCTCGAGTTAATGTCCCGGGAAGAGGTAG, 1225 bp
β-actin, GCGGATCCATGGATGATGATATCGCCGC and GCAAGCTTCCTAGAAGCATTTGCGGTGG, 1144 bp.

Before the first cycle, a denaturation step was performed at 95 °C for 7 min, PCR was carried out using a PCR thermal cycler sp (Takara Shuzo, Kyoto, Japan) with a regimen of 95 °C for 1 min, 58 °C for 1 min, and 72 °C for 1 min (30 cycles), and a final extension period of 7 min at 72 °C. The products were analyzed by electrophoresis through 1.5% agarose gels and stained with ethidium bromide, and densitometric analysis of digitized gel images was carried out using NIH Image software.

2.17. Immunohistochemical studies

For investigation of the effect of bLF on immune cells, 3 h after the final oral administration of bLF (30 mg/kg per day for 7 days; four mice/group) mice were anesthetized and killed, and the jejunum was removed. Jejuna obtained from mice were fixed in acetone at 4 °C and embedded in paraffin. After cutting and deparaffination, sections were incubated in 1% skim milk (Yukijirushi, Sapporo, Japan) – PBS at room temperature for 1 h to block non-specific antigens.

$CD4^+$ and $CD8a^+$ cells were detected with rat-anti-mouse CD4 mAb and CD8a mAb (Japan BD Biosciences Pharmingen, Tokyo, Japan). After washing blocked sections three times, sections were incubated in PBS containing 10% hydrogen peroxide at room temperature for 30 min to remove endogenous peroxidase activity before antibody was added. Sections were then incubated with antibody in a humidified chamber at room temperature for 2 h. After washing, the sections were incubated with biotin-conjugated anti-rat IgG antibody (Vectastain Elite ABC kit, Vector Laboratories, Burlingme, CA) for 3 h. Labeled cells were visualized with 0.03% 3,3-diaminobezidine tetrahydrochroride solution containing 0.1% hydrogen peroxide for 5 min.

IgM^+ and IgA^+ cells were detected with goat anti-mouse IgM Ab-FITC and goat anti-mouse IgA Ab-FITC (Sigma). After washing blocked sections three times, sections were incubated with antibody in a humidified chamber at 4 °C for 2 h. Labeled cells were visualized by fluorescent microscopy.

IL-7, IFNα, and caspase-1 (p-20) expressing cells were detected with rat anti-mouse IL-7 Ab (PeproTech EC. Ltd. London, UK), rat anti-mouse IFNα mAb (HyCult Biotechnology b.v., Uden, The Netherlands), and rabbit anti-mouse caspase-1 (p-20) antibody (Santa Cruz Biotechnology), respectively. After washing blocked sections three times, sections were incubated in PBS containing 10% hydrogen peroxide at room temperature for 30 min to remove endogenous peroxidase activity before antibody was added. Sections were then incubated with antibody in a humidified chamber at room temperature for 2 h. After washing, the sections were incubated with biotin-conjugated anti-rat IgG antibody or biotin-conjugated anti-rabbit IgG antibody (Vectastain Elite ABC kit, Vector Laboratories, Burlingme, CA) for 3 h. Labeled cells were visualized with 0.03% 3,3-diaminobezidine tetrahydrochroride solution containing 0.1% hydrogen peroxide for 5 min.

Results were expressed as the mean of the number of positive cells in the lamina propria per 10 light microscope fields (×200).

2.18. Western blot analysis of frozen tissue samples

Frozen tissue samples (100 mg) were homogenized in 1 ml chilled lysis buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, and protease inhibitor cocktail "Complete Mini™" (Roche, Mannheim, Germany). Lysates were centrifuged at 600 rpm for 20 min at 4 °C to remove nuclei and cell debris, and then clarified by centrifuged at 15,000 rpm for 45 min at 4 °C. Twenty μg of protein from each sample were separated by SDS-PAGE on 10% acrylamide, 0.2% bis gels. Proteins were transferred to Immobilon™ transfer membranes (Millipore Corp., Bedford, MA) and detected by Western blot with appropriate antibodies. Immunoreactive bands were visualized with ECL Plus Western Blotting Detection System (GE Healthcare, Buckinghamshire, UK). Densitometric analysis of digitized blot images was carried out using NIH Image software.

2.19. Treatment of GKO mice with rmIL-7, rmIL-18 or rIFNαA/D and metastasis assay

Co26Lu cells (1×10^5 cells/mouse) were subcutaneously implanted into the right thigh of GKO mice. Recombinant

murine IL-7 (*Escherichia coli*, PeproTech House, London, England), recombinant murine IL-18 (Medical & Biological Laboratories Co., Ltd.), and recombinant IFNαA/D (2.04×10^7 IU/ml, Nippon Roche Research Center, Kamakura, Japan) were administered intraperitoneally (i.p.) to GKO mice at 1 and 0.1 μg/mouse, 0.02 μg/mouse and 1×10^5 IU/mouse, respectively, on days 10, 13, 17, and 20. Endotoxin levels of rmIL-7 and IL-18 were less than 0.1 ng/μg protein by the LAL method. The longest (a) and shortest (b) diameters of tumors at the Co26Lu cell injection site were measured twice a week using calipers, and the volume was calculated using the formula: $ab^2/2$ (mm^3). The experiment was terminated 22 days after implantation, and the mice were killed. The lungs were removed, rinsed with saline, and fixed in acetone, and the lung metastatic colonies counted.

3. Results

3.1. Caspase-1 activity and generation of mature interleukin-18 levels in the mucosa of the small intestine in organ culture by bLF

In C57BL/6 or BALB/c mice, oral treatment with bLF or bLFcin increases mature IL-18 levels in the mucosa of the small and large intestines [7]. However, bLF is digested into numerous peptides by pepsin in the stomach [33]. We employed an organ culture system using mouse small intestine to investigate the ability of intact bLF and an N-terminal peptide fragment of bLF (bLFcin) to affect the activity of caspase-1 and the generation of mature IL-18: caspase-1 cleaves the proform of IL-18 to generate the 18 kDa bioactive molecule [34–36].

In organ culture experiments, caspase-1 activity and mature IL-18 levels in the mucosa of the small intestine were significantly increased following addition of bLF (Fig. 2A). Exposure to bLFcin also resulted in increases in caspase-1 activity and mature IL-18 levels (Fig. 2B). These results were specific since exposure to bTF did not result in elevated levels of mature IL-18, and exposure to bLF did not affect IL-1β levels. These results are similar to those obtained from previous *in vivo* experiments in which treatment with bLF or bLFcin caused an increase in IL-18 levels and caspase-1 activity in the mucosa of the small intestine [9]. Therefore, both intact bLF and an N-terminal peptide fragment of bLF, bLFcin, stimulate caspase-1 activity and the generation of mature IL-18.

3.2. bLF or bLFcin induction of cytokine production by peritoneal macrophages

Next, we investigated caspase-1 and cytokine production by peritoneal macrophages treated with bLF or bLFcin *in vitro*. Peritoneal macrophages, the macrophage cell line RAW264.7, and spleen cells were treated with bLF, and caspase-1 activity and mature IL-18 levels were measured. Treatment with 300 μg/ml bLF enhanced caspase-1 activity in peritoneal macrophages and possibly to some extent in spleen cells, but not in RAW264.7 cells (Fig. 3 left panel). bLF markedly enhanced production of mature IL-18 in peritoneal macrophages but not in Raw264.7 or spleen cells (Fig. 3 right panel). bLF-mediated induction of mature IL-18 by peritoneal macrophages was dose dependent and bovine transferrin (bTF) had no effect (Fig. 4). Recombinant murine interferon-gamma (rmIFNγ) also induced mature IL-18 production by peritoneal macrophages (Fig. 4). Importantly, bLF-mediated induction of mature IL-18 was abrogated by co-incubation of the cells with caspase-1 inhibitor (Acyl-Tyr-Val-Ala-Asp-chloromethylketone, Ac-YVAD-CMK) (Fig. 5). bLF-mediated induction of mature IL-18 was also inhibited by co-incubation with anti-mIFNγ antibody (Fig. 5). Finally, IFNγ induced production of mature IL-18 (Fig. 5), and this induction was inhibited by co-incubation of the cells with anti-mIFNγ antibody (data not shown), confirming the validity of this assay.

In Western blot analysis of macrophage lysates after addition of bLF or bLFcin at 1000 or 30 μg/ml, respectively, bLF induced expression of IFNγ and IL-15, and bLFcin induced expression of IL-15 (Fig. 6). Treatment with bLF decreased expression of IFNα while treatment with bLFcin decreased expression of TNFα (Fig. 6).

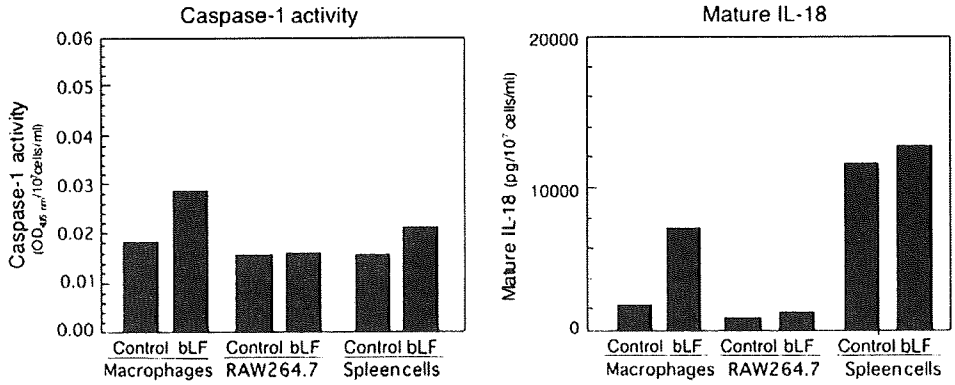

Fig. 3. Effects of bLF on caspase-1 activity and mature IL-18 production in peritoneal macrophages, RAW264.7 cells, and spleen (BALB/c mouse) cells. Cells were incubated for 24 h in bLF (300 μg/ml). Caspase-1 activity and mature IL-18 levels were determined by ELISA. Data are representative of two independent experiments.

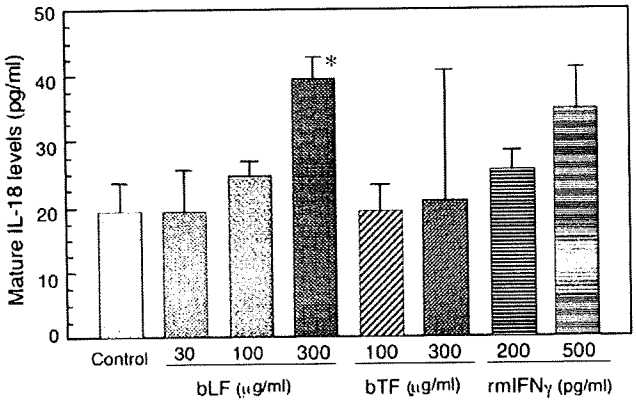

Fig. 4. Effect of bLF, bTF, and rmIFNγ on the production of mature IL-18 by macrophages. Macrophages were incubated for 24 h in bLF, bTF or rmIFNγ and mature IL-18 levels were measured by ELISA. Error bars represent the standard error ($n = 3$). *$p < 0.05$, Dunnett's multiple comparison t-test.

3.3. Cytokine induction in various tumor cell lines by bLF or bLFcin

bLF can induce caspase-1 activity and the generation of mature IL-18 in mouse intestinal tissue and macrophages and IFNγ and IL-15 expression in macrophages; bLFcin can induce caspase-1 activity and the generation of mature IL-18 in mouse intestinal tissue and IL-15 expression in macrophages. We next investigated the effect bLF or bLFcin had on cytokine expression in mouse and human tumor cell lines: Lewis lung carcinoma was the mouse tumor cell line used, and SW620 (colon), MCF-7 (breast), and SK-MEL-19 (melanoma) were the human cell lines used.

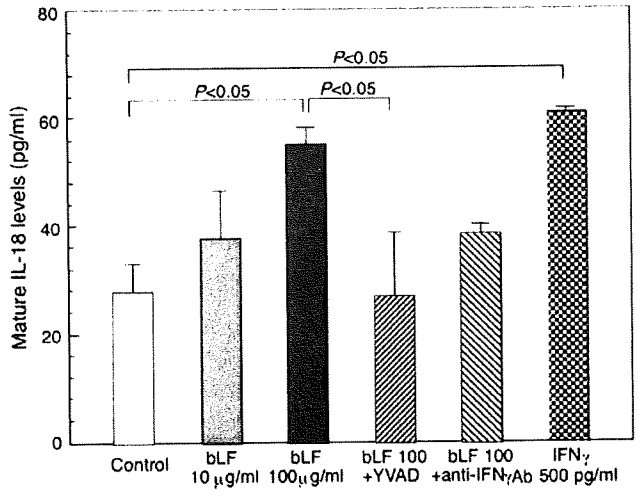

Fig. 5. Caspase-1 inhibitor (YVAD) and IFNγ antibodies block mature IL-18 production by macrophages treated with bLF. Peritoneal macrophages were treated with bLF, bLF + the caspase-1 inhibitor YVAD or bLF + IFNγ antibodies, and mature IL-18 levels were measured by ELISA. The error bars represent the standard error ($n = 3$), p values were calculated by Dunnett's multiple comparison t-test. YVAD = Acyl-Tyr-Val-Ala-Asp-chloromethylketone.

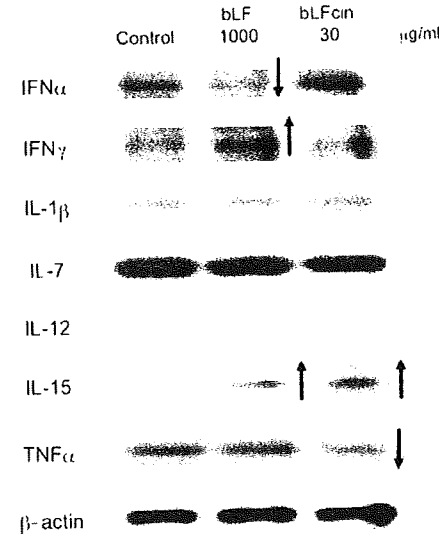

Fig. 6. Western blot analysis of cytokine production in peritoneal macrophages following addition of bLF or bLFcin (BALB/c mice). Macrophages were incubated for 24 h in bLF (1000 μg/ml) or bLFcin (30 μg/ml). Arrows indicate increases or decreases in expression compared to the control. Data are representative of two independent experiments.

Incubation of Lewis Lung carcinoma cells in bLFcin at 100 or 300 μg/ml for 48 h significantly decreased cell number, however, bLF at 1000 μg/ml or bLFcin at 30 μg/ml had no effect on cell number (Table 1). In Western blot analysis of Lewis lung carcinoma cells treated with bLF or bLFcin for 24 h, bLF induced increased expression of IFNγ (2-fold) and IL-1β (3-fold), and bLFcin (lower doses) induced increased expression of IL-1β (4-fold) (Fig. 7).

In Western blot analysis of human cells treated with bLF or bLFcin for 24 h, both bLF and bLFcin induced expression of human lactoferrin (hLF) (2-fold) and mature IL-18 (2 ~ 4-fold) by colon cancer SW620 cells (Fig. 8A); both bLF and bLFcin induced expression of hLF (7 ~ 29-fold), IFNα (4-fold), IL-12 (2 ~ 4-fold), and mature IL-18 (2 ~ 3-fold) by mammary cancer MCF-7 cells (Fig. 8B); and both bLF and bLFcin induced expression of IFNα (4 ~ 11-fold), IL-12 (2 ~ 5-fold) and mature IL-18 (3 ~ 4-fold) by melanoma SK-MEL19 cells (Fig. 8C). SK-MEL19 cells did not express hLF. In HT29 human colon cancer cells, hLF levels were unaffected by incubation in 1000 μg/ml bLF (87% of basal line), but were significantly decreased by incubation in 30 μg/ml bLFcin (46% of basal line) (data not shown).

Table 1
Effects of lactoferrin and lactoferricin on the growth of Lewis lung carcinoma cells 5% FCS in RPMI-1640 medium, 48 h incubation

	Concentration (μg/ml)	Growth (% of Control)
bLF	1000	96.2
bLFcin	30	100.0
	100	46.5
	300	13.2

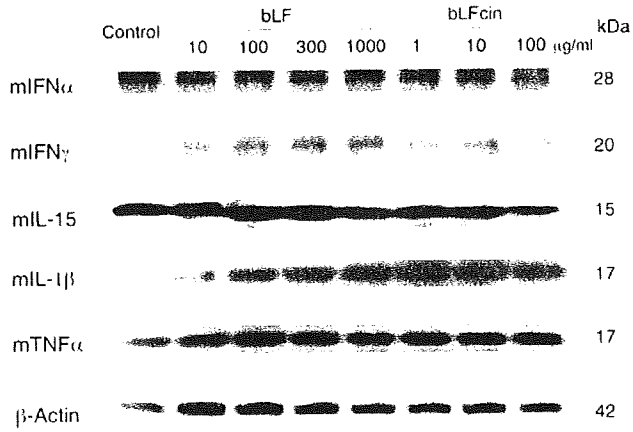

Fig. 7. Western blot analysis of cytokine production in Lewis Lung carcinoma cells following incubation with bLF or bLFcin for 24 h. Data are representative of two independent experiments.

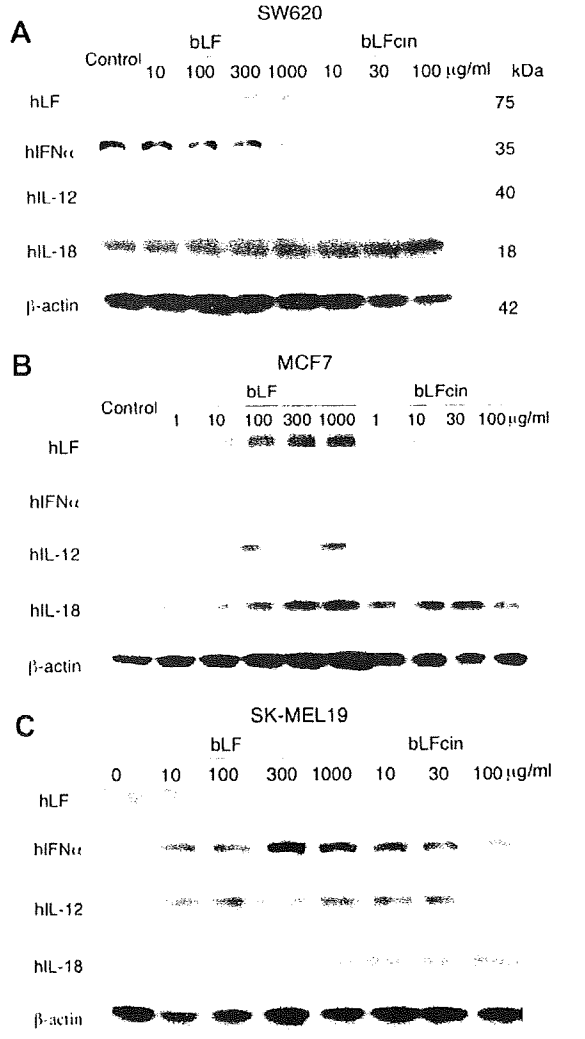

Fig. 8. Western blot analysis of cytokine production in SW620 (A) colon cancer cells, MCF7 (B) mammary carcinoma cells, and SK-MEL19 (C) melanoma cells following incubation with bLF or bLFcin for 24 h. Data are representative of three independent experiments.

In summary, exposure of cells to bLF or bLFcin tends to cause induction of a variety of cytokines. In addition, LF may activate an autocrine loop in some cells; notice the extremely high induction of hLF in the breast cancer cell line, MCF7. The specific effects of bLF and bLFcin, however, depend on the cell type.

3.4. Anti-metastatic activity following oral administration of bLF in GKO and BALB/c mice

We have shown that bLF and bLFcin can induce mouse small intestine to increase its production of mature IL-18. An important function of IL-18 is activation of T-cells and NK cells involved in cell mediated immune responses. Through its stimulation of immune cells, IL-18 has been shown to protect experimental animals against repeated challenges with tumor cells [37,38]. Therefore, we investigated the ability of bLF to inhibit lung metastasis by Co26Lu tumor cells. In addition, we used IFNγ KO (GKO) mice to assess the importance of IFNγ on the ability of bLF to inhibit lung metastasis of Co26Lu tumor cells.

The number of lung metastatic colonies in tumor-bearing GKO mice were markedly higher than in tumor-bearing BALB/c mice (Fig. 9), indicating that IFNγ inhibits the metastatic ability of Co26Lu cells. Oral treatment with bLF significantly inhibited lung metastatic colony formation in both GKO mice and BALB/c mice (Fig. 9), indicating that bLF can inhibit the metastatic ability of Co26Lu cells in the absence of IFNγ.

3.5. Cytokine levels in the small intestines in GKO and BALB/c mice following treatment with bLF

Since IFNγ is not required for bLF-mediated inhibition of Co26Lu metastasis to the lung, we compared bLF-mediated induction of cytokines in the small intestines of GKO and BALB/c mice. IL-1β, IL-6, and TNFα levels in the mucosa of the small intestine in both GKO and BALB/c mice were not markedly changed after treatment with bLF (Fig. 10). IL-12 levels showed a tendency to decrease in BALB/c mice. IL-4 and GM-CSF showed a tendency to increase in both GKO and BALB/c mice. Mature IL-18 levels in the small intestine of GKO mice were not affected by treatment with bLF, however, bLF did cause a significant increase in mature IL-18 in BALB/c mice (Fig. 11).

Expression of IL-7, IL-15, and IL-18 mRNA was examined in freshly isolated mucosal tissues of the small intestine of GKO and BALB/c mice following treatment with bLF at 30 or 300 mg/kg per day. IL-7 mRNA levels were significantly enhanced in GKO mice by administration of bLF (1.4- and 1.5-fold increase, $p < 0.05$ and $p < 0.01$, respectively), but bLF had no effect on IL-7 mRNA levels in BALB/c mice (0.9 ~ 1.3-fold increase $p > 0.05$); bLF had no significant effect on IL-15 mRNA levels in either GKO or BALB/c mice (Fig. 12); bLF had no significant effect on IL-18 mRNA levels

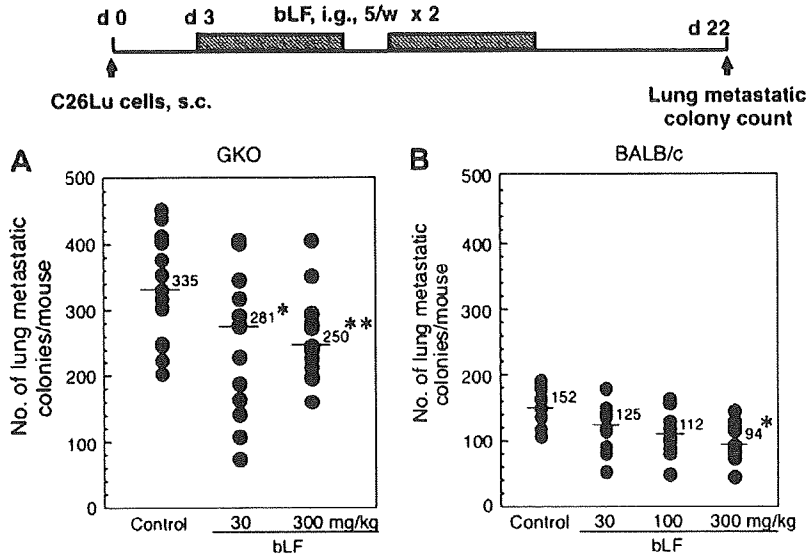

Fig. 9. Effects of oral administration of bLF on the number of lung metastatic colonies in Co26Lu-bearing GKO (A) and Co26Lu-bearing BALB/c (B) female mice. The numerical values represent the median of the number of lung metastatic colonies in 15 animals. *$p < 0.05$, **$p < 0.01$ vs Control, Mann–Whitney U-test.

in GKO mice (about 1.3-fold increase, $p > 0.05$), but bLF markedly elevated IL-18 mRNA levels in BALB/c mice (1.3 and 1.7-fold increase, $p > 0.05$ and $p < 0.01$, respectively) (Fig. 12).

Thus, bLF induced expression of IL-18 mRNA and generation of mature IL-18 in BALB/c mice. Since bLF had no effect on IL-18 expression in GKO mice, bLF induction of IL-18 is IFNγ dependent. In GKO mice, bLF induced expression of IL-7.

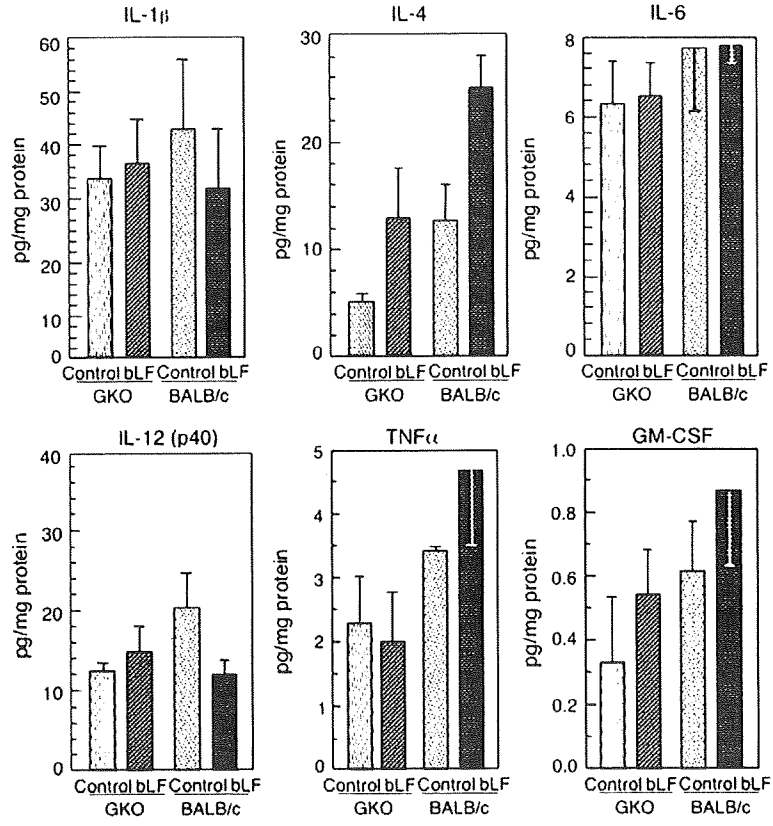

Fig. 10. Effect of bLF on various cytokine levels in the mucosa of the small intestines of GKO and BALB/c female mice. bLF was administered orally at 30 mg/kg per day for 7 days, and 3 h after the last administration the animals were anesthetized and killed, the jejunum removed, and cytokine levels measured. Error bars represent the standard error ($n = 5$).

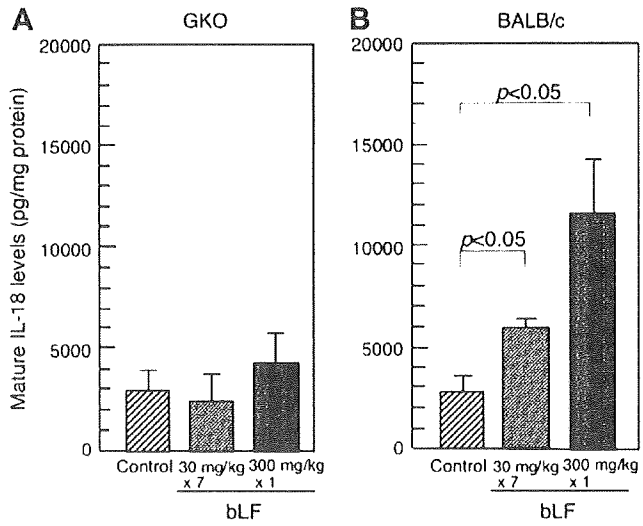

Fig. 11. Effect of bLF on mature IL-18 levels in the mucosa of the small intestine of GKO (A) and BALB/c (B) mice. bLF was administered orally at 30 mg/kg per day for 7 days or once at 300 mg/kg on day 7, and 3 h after last the administration the animals were anesthetized and killed, the jejunum removed, and mature IL-18 levels measured. Error bars represent the standard error ($n = 5$).

3.6. Analysis of caspase-1 expression and activity in the small intestine of GKO and BALB/c mice

Caspase-1 expression and activity in the small intestine of BALB/c mice is enhanced by treatment with bLF, and this effect is dependent on IFNγ. Therefore we investigated the role of IFNγ on bLF-mediated induction of caspase-1 expression and activity. In immunohistochemical and Western blot analysis, caspase-1 expression in epithelial cells of the small intestine was increased in both GKO and BALB/c mice after treatment with bLF (GKO: 1.6-fold; BALB/c: 1.9-fold). However, caspase-1 activity, as measured by generation of the cleaved-caspase-1 p-20 peptide, was markedly different between GKO and BALB/c mice (GKO: 0.8; BALB/c: 1.5-fold vs Control) (Fig. 13). Moreover, caspase-1 activity in the mucosa of the small intestine in GKO mice did not change following administration of bLF (120–123% of Control), but its activity in BALB/c mice was significantly enhanced (211–272% of Control, $p < 0.05$) (Table 2). Therefore, bLF induction of caspase-1 expression is not dependent on IFNγ, but generation of active caspase-1 is dependent on IFNγ.

3.7. Immunohistochemical analysis of various immune cells in the small intestine of GKO mice

bLF is thought to mediate its anti-metastasis activity by activation of immune cells in the intestine, and we have previously demonstrated that bLF increases the number of $CD4^+$ and $CD8^+$T and asialo GM_1^+ (NK) cells in BALB/c mice [9]. bLF treatment also inhibits Co26Lu metastasis to the lung in GKO mice, and this effect occurs in the absence of IFNγ, caspase-1, and IL-18. We therefore examined the effect

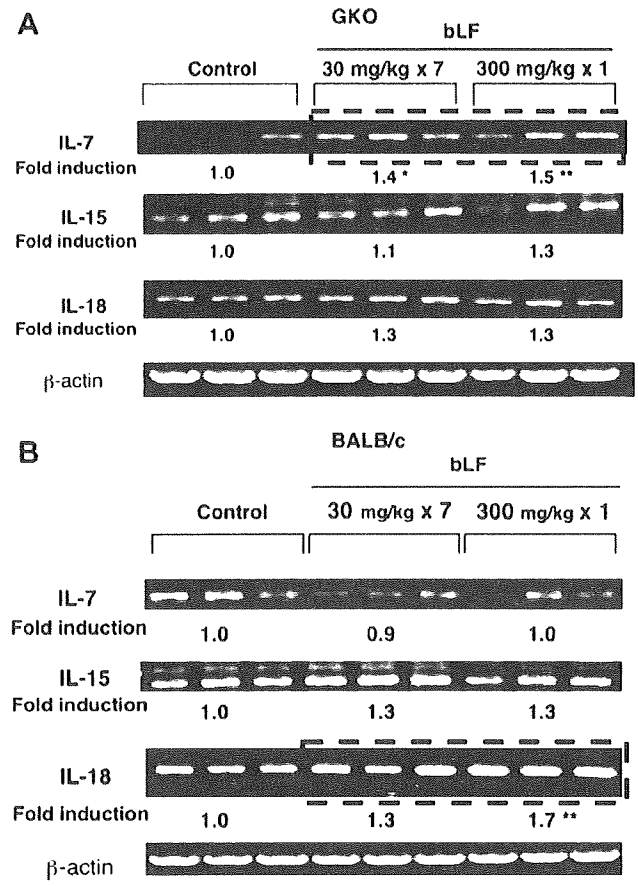

Fig. 12. Effect of bLF on the expression of IL-7, IL-15 and IL-18 mRNA in the mucosa of the small intestines of GKO (A) and BALB/c (B) female mice. bLF was administered orally at 30 mg/kg per day for 7 days or once at 300 mg/kg on day 7, and 3 h after the last administration of bLF the animals were anesthetized and killed and the jejunum removed. mRNA expression was detected by RT-PCR. Three mice were used for each condition. Expression of each transcript was normalized to β-actin and the mean expression values for each condition obtained. Numerical data represent the ratio of these values compared to the controls. Administration of bLF lead to an increase in IL-7 expression in GKO mice and to an increase in IL-18 expression in BALB/c mice. Data are representative of 2 independent experiments.

of bLF on immune cells in the small intestine of GKO mice. $CD4^+$, IgM^+ (data not shown), and IgA^+ cells were significantly increased by treatment with bLF, but $CD8^+$ cells were not (Fig. 14).

$IL\text{-}7^+$ cells in the small intestine are mainly intraepithelial lymphocytes (IEL), fibroblasts, and vascular endothelial cells. bLF also caused a marked increase in the number of $IL\text{-}7^+$ intraepithelial lymphocytes in GKO mice (Fig. 15B). In contrast, bLF did not induce expression of IL-7 in the small intestine of BALB/c mice or an increase in $IL\text{-}7^+$ intraepithelial lymphocytes (data not shown).

$IFN\alpha^+$ cells exist in the lamina propria of the small intestine and the number of these cells was also increased by treatment with bLF in GKO mice, but not in BALB/c mice (Fig. 16). IFNα protein expression was increased (1.8-fold) as shown by Western blot analysis (data not shown).

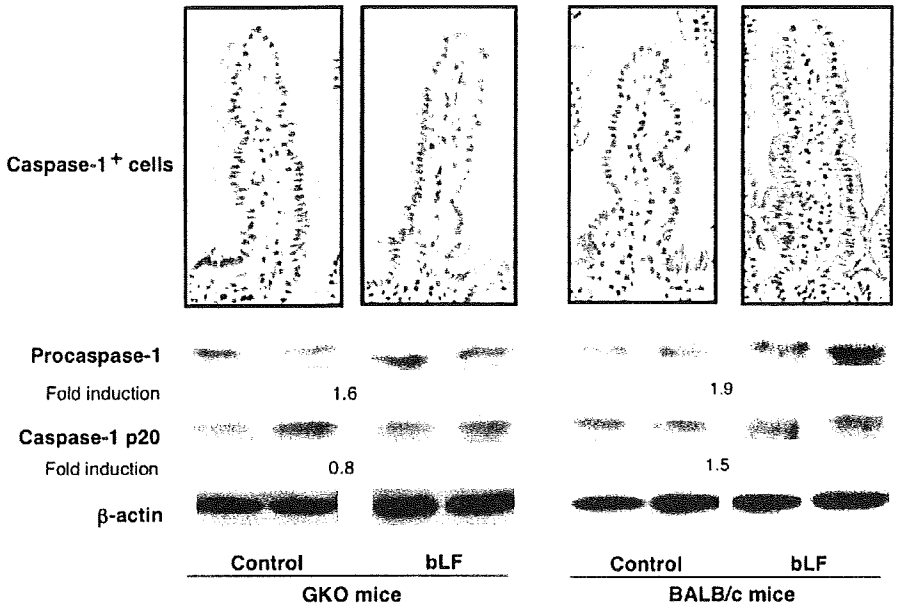

Fig. 13. Caspase-1$^+$ cells in the small intestine and Western blot analysis for procaspase-1 and active caspase-1 (caspase-1 p20). bLF was administered orally at 30 mg/kg per day for 7 days, and 3 h after the last administration of bLF the animals were anesthetized and killed, and the jejunum was removed. The results of two independent experiments are shown.

3.8. Anti-metastatic effects in tumor-bearing GKO mice by rmIL-18, rIFNαA/D, or rmIL-7 treatment

bLF inhibits Co26Lu metastasis to the lung in GKO mice in the absence of IFNγ, caspase-1, and IL-18. However, bLF induces IFNα and IL-7 in these mice. Therefore, we tested the effects of recombinant mature IL-18 (rmIL-18), recombinant IFNα (rIFNαA/D), and recombinant IL-7 (rIL-7) on Co26Lu metastasis to the lung in GKO mice. Intraperitoneal administration of IL-18 at 20 ng/mouse, IFNαA/D at 10^5 IU/mouse, or rmIL-7 at 0.1 μg/mouse caused significant retardation of tumor growth of subcutaneously implanted Co26Lu and also significant retardation of lung metastases (Table 3).

4. Discussion

LF is an important component of the innate immune system with potent antimicrobial activity and is found in a variety of exocrine secretions including gastrointestinal fluids. The small intestine has a well developed immune network that is involved in protection of the host from pathogens. For example, mucosa-associated bacteria (lactobacili and *E. coli*, etc.) in the small intestine can be potent stimulators of mononuclear cells of the intestinal lamina propria, inducing these cells to express IL-12 [39], which in turn activates intestinal immune cells and protects the small intestine against microbial infection. LF is part of the intestinal immune network [40–42]. It is an excellent marker of inflammatory bowel diseases such as ulcerative colitis and Crohn's disease [43], and fecal LF levels are also higher in patients with colorectal tumors than in healthy individuals [44]. Because ingested LF stimulates cytokine production [8] and enhances mucosal immunity [9], NK cell activity [10,11], LAK activity [11], neutrophil activity [12,13], and macrophage cytotoxicity [14,15], it is thought that orally ingested LF is also able to interact with epithelial cells and immune cells in the mucosa of the intestine.

Table 2
Effects of bLF on caspase-1 and -3 activities in the mucosa of the small intestine in GKO and BALB/c (WT) mice

Treatment (p.o.)	Caspase-1 activity	Caspase-3 activity (absorbance at 405 nm)
GKO mice ($n = 4$)		
Control (saline)	0.035 ± 0.002	0.214 ± 0.011
bLF (30 mg/kg × 7)	0.042 ± 0.011 (1.20)	
bLF (300 mg/kg × 1)	0.043 ± 0.012 (1.23)	0.221 ± 0.024 (1.03)
BALB/c (WT) mice ($n = 5$–7)		
Control (saline)	0.018 ± 0.003*	0.168 ± 0.014
bLF (30 mg/kg × 7)	0.049 ± 0.011* (2.72)	
bLF (300 mg/kg × 1)	0.038 ± 0.005 (2.11)	0.190 ± 0.026 (1.13)

Mucosal samples of the small intestine were diluted to 200 μg protein/50 μl. () = -fold. *$p < 0.05$.

There are several reports that administration of LF inhibits tumor growth and protects against carcinogenesis [18,45,46], reviewed in refs. [26,47,48]. In several rodent cancer models, it has been shown that this protective effect is associated with enhancement of the local intestinal mucosal immune response [9,16,47,49–52].

One pathway which appears to be an effector of LF is generation of mature IL-18 and consequent activation of immune cells. Administration of bLF induces expression of IL-18 in the small intestine [9,16,46]. IL-18 stimulates both Th1 and Th2 type T helper cells, CD8$^+$ effector T cells, NK cells, and LAK cells [10,11,17,53–56]; and up-regulation and/or enhanced activity of CD4$^+$ and CD8$^+$ effector T-lymphocytes and NK and LAK cells is also observed upon LF

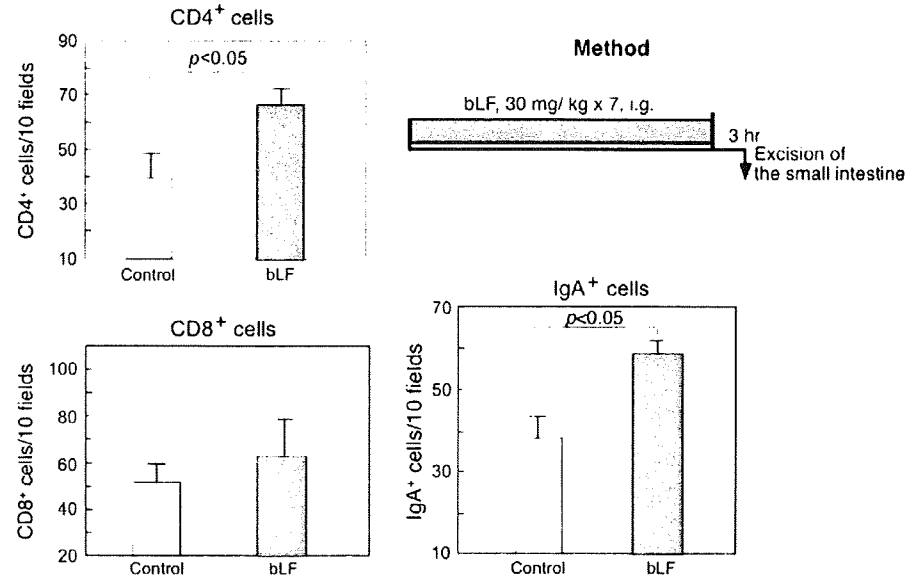

Fig. 14. Effects of bLF on the number of $CD4^+$, $CD8^+$, and IgA^+ cells in the lamina propria of the small intestine of GKO mice. bLF was administered orally at 30 mg/kg per day for 7 days, and 3 h after the last administration of bLF the animals were anesthetized and killed, and the jejunum was removed and the number of $CD4^+$, $CD8^+$, and IgA^+ cells counted. Error bars represent the standard error ($n = 5$).

administration [16,19,40,50,57]. LF also stimulates the induction and/or activation of B cells, macrophages, and neutrophils [54,58,59]. Wolf et al. report that hLF resulted in T cell-dependent tumor inhibition of head and neck squamous cell carcinoma *in vivo*, and that depletion of $CD3^+$ cells abolishes this effect [46]. Bezault et al. report that stimulation of NK cell activity is essential for LF-mediated prevention of metastasis in C57BL/6 mice: intraperitoneal administration of hLF to these mice inhibited the growth of solid tumors and prevented lung metastasis of murine melanoma cells, and this effect was lost upon deletion of NK cell function using antibody blocking experiments [18].

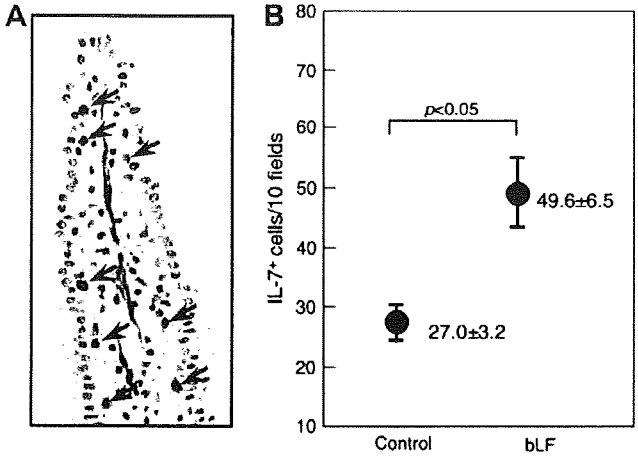

Fig. 15. Effects of bLF on the number of $IL\text{-}7^+$ cells in the small intestine of GKO mice. bLF was administered orally at 30 mg/kg per day for 7 days, and 3 h after the last administration of bLF the animals were anesthetized and killed, and the jejunum was removed. (A) $IL\text{-}7^+$ cells in the small intestine (arrows). (B) The number of $IL\text{-}7^+$ cells in the small intestine of GKO mice was significantly increased by treatment with bLF ($n = 5$).

In our study we confirmed the ability of bLF and digested bLF-peptides to induce expression of IL-18 and the generation of mature IL-18 in a variety of settings. Importantly, in IFNγ knockout (GKO) mice, bLF had no effect on IL-18 expression or maturation. Moreover, in settings in which bLF did induce generation of mature IL-18, maturation was abolished by caspase-1 inhibitors. Therefore, bLF-mediated induction of IL-18 expression is dependent upon IFNγ and bLF-mediated generation of mature IL-18 is dependent upon caspase-1.

Previously we showed that bLF can induce IFNγ in the intestine [50], and in this study we affirmed that bLF can induce expression of IFNγ by peritoneal macrophages and that both bLF and bLFcin can induce expression of IFNγ by Lewis Lung carcinoma cells. Moreover, bLF also induces expression

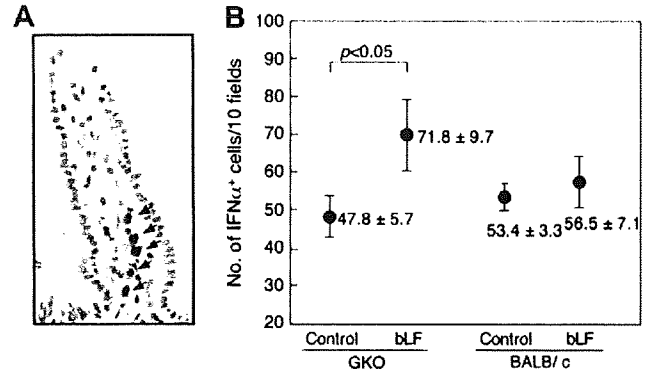

Fig. 16. Effects of bLF on the number of $IFN\alpha^+$ cells in the small intestine of GKO mice. bLF was administered orally at 30 mg/kg per day for 7 days, and 3 h after the last administration of bLF the animals were anesthetized and killed, and the jejunum was removed. (A) $IFN\alpha^+$ cells in the small intestine (arrows). (B) The number of $IFN\alpha^+$ cells in the small intestine of GKO mice was significantly increased by treatment with bLF ($n = 5$).

Table 3
Effect of IL-7, IL-18 and IFNαA/D on lung metastatic colony formation in Co26Lu-bearing GKO mice

Treatment (i.p.)	Dose (μg/mouse)	Tumor volume on day 20 (mm^3)	No. of metastatic colonies Median (range)	*p*-value
Control (saline)		1274 ± 254	233 (156–420)	
rmIL-7	0.1	1023 ± 68*	124 (53–165)	0.003
rmIL-7	1.0	1122 ± 172	143 (34–168)	0.007
rmIL-18	0.02	953 ± 55*	105 (51–169)	0.012
rIFNαA/D	1×10^5 IU	899 ± 59*	111 (91–168)	0.012

Cytokines were administered i.p. to Co26Lu-bearing GKO mice (5–7 mice/group) on days 10, 13, 17 and 20. *$p < 0.05$ vs Control.

of caspase-1 mRNA *in vivo*. However, while bLF administration results in generation of active caspase-1 in BALB/c mice, it has no effect on caspase-1 activity in GKO mice. Taken together, these results indicate that in BALB/c mice, bLF ingestion results in stimulation of IFNγ and caspase-1 expression; IFNγ in turn stimulates IL-18 expression and caspase-1 activity; and finally active caspase-1 cleaves pro-IL-18 to generate mature IL-18. The bLF stimulated generation of mature IL-18 subsequently acts on immune cells in the intestine which in turn effect the observed bLF anticarcinogenesis activity.

However, bLF also effects inhibition of tumor growth and metastasis in GKO mice, an animal model in which bLF administration does not result in induction of IFNγ expression or generation of caspase-1 activity or mature IL-18. This result is not inconsistent with some published reports. Kuhara et al. also report that bLF administration caused an increase in IFNγ and IL-18 and NK activity, however, bLF administration also caused increased NK activity in IL-18 KO mice [51]. In these mice, bLF induced increased expression of IFNα and IFNβ (type 1 IFNs) in Payer's patches and mesenteric lymph nodes, and blockade of induction of IFNα and IFNβ expression abolished bLF induction of NK activity. Using BALB/cByJ Jcl hypoglycemic mice, bLF administration does not result in elevated IFNγ or IL-18 expression [60]; however, bLF is host protective in this animal and does induce expression of IFNβ and IL-12. These cytokines are well characterized mediators of host defense against infections and cancer. bLF-mediated induction of IL-12 is also reported in other systems [61]. In the present study, we also show induction of type 1 IFNs and of interleukins other than IL-18 by bLF *in vitro* and in GKO mice *in vivo*. Therefore, administration of bLF can activate more than one effector pathway; which pathways are responsive to activation depend on the physiology of the gastrointestinal tract.

In the GKO mice, bLF did not induce IL-18 expression or maturation, but bLF did induce expression of IFNα and IL-7 and inhibited tumor growth and metastasis. In these mice, injection of recombinant murine IL-18 also inhibited tumor growth and metastasis. Thus, the IL-18 effector pathway is intact in GKO mice, but in the absence of IFNγ, bLF is unable to activate it. Importantly, injection of recombinant IFNαA/D or recombinant IL-7 also inhibited tumor growth and metastasis in these mice. Thus, in the absence of the IFNγ/IL-18 effector pathway, bLF is able to exert anticarcinogenesis activity by activation of a second effector pathway, an IFNα/IL-7 effector pathway.

Finally, it should be noted that bLF and bLFcin induced expression of IL-15 by peritoneal macrophages *in vitro*. That the induction of IL-15 was not observed when the entire intestinal mucosa was examined is not surprising since macrophages make up a very small proportion of this tissue. The specific role of IL-15, if any, in bLF-mediated immune modulation remains to be elucidated, however, IL-15 is a well known immune cell regulator [62–64] and could be a component of another bLF activated effector pathway.

5. Summary

Lactoferrin, originally isolated from milk, is an important component of the innate immune system. Several reports indicate that ingested lactoferrin can inhibit carcinogenesis. In this report we investigated effector pathways which link ingestion of lactoferrin to carcinogenesis. Our data support the concept that lactoferrin peptide fragments, especially lactoferricin, are able to stimulate cytokine production by cells in the mucosa of the small intestine. The subsequent activation of immune cells enhances immune surveillance, i.e., the ability of the immune system to recognize and remove malignant cells that arise during a person's lifetime, and lactoferrin's anticarcinogenesis effects are the result of this enhanced surveillance.

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The Mouse Lymphoma Assay Detects Recombination, Deletion, and Aneuploidy

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The mouse lymphoma assay (MLA) uses the thymidine kinase (*Tk*) gene of the L5178Y/*Tk*$^{+/-}$-3.7.2C mouse lymphoma cell line as a reporter gene to evaluate the mutagenicity of chemical and physical agents. The MLA is recommended by both the United States Food and Drug Administration and the United States Environmental Protection Agency as the preferred *in vitro* mammalian cell mutation assay for genetic toxicology screening because it detects a wide range of genetic alterations, including both point mutations and chromosomal mutations. However, the specific types of chromosomal mutations that can be detected by the MLA need further clarification. For this purpose, three chemicals, including two clastogens and an aneugen (3′-azido-3′-deoxythymidine, mitomycin C, and taxol), were used to induce *Tk* mutants. Loss of heterozygosity (LOH) analysis was used to select mutants that could be informative as to whether they resulted from deletion, mitotic recombination, or aneuploidy. A combination of additional methods, G-banding analysis, chromosome painting, and a real-time PCR method to detect the copy number (CN) of the *Tk* gene was then used to provide a detailed analysis. LOH involving at least 25% of chromosome 11, a normal karyotype, and a *Tk* CN of 2 would indicate that the mutant resulted from recombination, whereas LOH combined with a karyotypically visible deletion of chromosome 11 and a *Tk* CN of 1 would indicate a deletion. Aneuploidy was confirmed using G-banding combined with chromosome painting analysis for mutants showing LOH at every microsatellite marker on chromosome 11. From this analysis, it is clear that mouse lymphoma *Tk* mutants can result from recombination, deletion, and aneuploidy.

Key Words: mouse lymphoma assay; mutation type; loss of heterozygosity; cytogenetics; copy number; 3′-azido-3′-deoxythymidine; mitomycin C; taxol; thymidine kinase.

The mouse lymphoma assay (MLA), using the thymidine kinase (*Tk*) gene of the L5178Y/*Tk*$^{+/-}$-3.7.2C mouse lymphoma cell line as a reporter gene of mutation, is preferred by a number of international regulatory agencies, including the United States Food and Drug Administration and the United States Environmental Protection Agency, as the *in vitro* mammalian mutation assay in the genetic toxicology screening battery. The decision to prefer the MLA was based on previous research demonstrating that the assay detects most of the mutational events known to be associated with the etiology of cancer and other human diseases, including point mutations and a number of different types of chromosomal mutations (Applegate *et al.*, Blazak *et al.*, 1989; Chen *et al.*, 2002; Clive *et al.*, 1990; 1990; Honma *et al.*, 2001; Hozier *et al.*, 1981, 1992; Liechty *et al.*, 1998; Moore *et al.*, 1985; Zhang *et al.*, 1996).

It is important to determine the various mutation types that can be detected by this assay, so that MLA data can be properly interpreted. Since the development of this assay, quite a number of studies have been conducted to understand the types of mutations that can be detected by the MLA. Several cytogenetic studies have been conducted to analyze *Tk* mutants induced by various mutagens (Blazak *et al.*, 1986, 1989; Hozier *et al.*, 1981; Moore *et al.*, 1985; Zhang *et al.*, 1996). It is clear that many small colony (SC) mutants have recognizable chromosome rearrangements involving chromosome 11, which contains the *Tk* gene. The *Tk*$^{-}$ and *Tk*$^{+}$ chromosomes can be distinguished by a centromeric heteromorphism: the *Tk*$^{+}$ chromosome has a bigger centromere (Hozier *et al.*, 1982; Sawyer *et al.*, 1985). This finding greatly helps the cytogenetic characterization of chromosome aberrations in *Tk* mutants. However, without the help of molecular genetics, small chromosome changes cannot be detected using cytogenetic methods alone; mitotic recombination cannot be identified because the karyotype of the mutant would be normal. Applegate *et al.* (1990) identified a *Nco I* restriction fragment length polymorphism that distinguishes the *Tk*$^{+}$ and *Tk*$^{-}$ alleles. Several studies using Southern blot analysis to determine the status of the *Tk*$^{+}$ allele were subsequently conducted (Applegate *et al.*, 1990; Clive *et al.*, 1990). The

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presence of the Tk^+ allele suggests an intragenic mutation, while the loss of the Tk^+ allele indicates a chromosome mutation. Most large colony (LC) mutants induced by point mutagens, such as ethyl methanesulfonate, retain the Tk^+ allele; while most SC mutants induced by clastogens, such as bleomycin, lose the Tk^+ allele. An allele-specific PCR technique was developed to identify the presence or absence of the Tk^+ allele. Furthermore, based on microsatellite polymorphisms, the loss of heterozygosity (LOH) pattern of the entire chromosome 11 can be investigated (Liechty *et al.*, 1994, 1996, 1998). This labor-saving approach allows many mutants to be analyzed; however, without the detection of *Tk* gene copy number (CN), one cannot distinguish between a deletion and a recombination event. Southern blot analysis can be used to detect *Tk* gene CN (Applegate *et al.*, 1990), but it is resource intensive, requires a relatively large amount of DNA, and the measurement is not precise (Joseph *et al.*, 1993). Therefore, we developed a real-time PCR method to detect the CN of the *Tk* gene (Wang *et al.*, 2007). By combining LOH analysis of chromosome 11 and cytogenetic analysis, we were able to distinguish between deletion and recombination events.

Another important issue for the MLA is whether the assay can detect aneuploidy. Aneuploidy plays a significant role in many adverse human health conditions, including spontaneous abortions, birth defects, and cancer (Aardema *et al.*, 1998; Duesberg *et al.*, 1999; Oshimura and Barrett, 1986; Sen, 2000). Aneugens can interact with the spindle apparatus or impair its function, thereby inducing aneuploidy due to nondisjunction mechanisms. Several aneugens are carcinogens (Cimino *et al.*, 1986; Oshimura and Barrett, 1986). Although the MLA has been shown to detect aneugens, its ability to adequately detect aneuploidy is unclear. Applegate *et al.* (1990) reported that at least some *Tk* mutants show aneuploidy. Honma *et al.* (2001) evaluated two aneugens, colchicine, and vinblastine, using the MLA. The two chemicals did not induce a significant mutant frequency (MF) increase after the regular 3-h treatment. Although they did show positive responses after the long-term (24 h) treatment, the increase of MF was not high. Therefore, many of the mutants that were isolated and analyzed from the chemical-treated cultures would be spontaneous rather than induced mutants.

Overall, the specific types of chromosomal mutations that are detected by the MLA need further clarification. In this study, *Tk* mutants from cultures treated with different chemicals, including two clastogens and an aneugen (3′-azido-3′-deoxythymidine [AZT], mitomycin C and taxol), were used. A combined strategy of both molecular genetic and cytogenetic methods was used to analyze the mutants. LOH analysis of chromosome 11 was conducted first, and then the *Tk* mutants showing LOH involving a large portion of chromosome 11 were selected for further cytogenetic analysis and *Tk* CN detection. It should be noted that it was not our intent to provide a complete analysis of the types of mutational events induced by these three chemicals; rather, we used these chemicals to give us mutants for our analysis that would be expected to include deletions, mitotic recombination, and aneuploidy.

MATERIALS AND METHODS

Cell culture. The L5178Y/$Tk^{+/-}$ -3.7.2C mouse lymphoma cells were cultured in suspension using Fischer's medium for leukemic cells of mice (Quality Biologicals, Gaithersburg, MD) supplemented with 10% heat-inactivated horse serum, 200 μg/ml sodium pyruvate, 100 unit/ml penicillin, 100 μg/ml streptomycin, and 0.05% (vol/vol) pluronic F68 (Invitrogen, Carlsbad, CA). The cultures were incubated at 37°C in an atmosphere of 5% CO_2 and saturated humidity and maintained in logarithmic growth.

Tk mutant induction. *Tk* mutants isolated from AZT-treated and taxol-treated cultures were from previous studies using the microwell version of the assay (Moore *et al.*, 2005; Wang *et al.*, 2007). For the induction of *Tk* mutants using mitomycin C (obtained from Sigma, St Louis, MO), the protocol for the microwell version of the mouse lymphoma assay described by Chen and Moore (2004) was followed. Briefly, cells were centrifuged and resuspended at a concentration of 0.2×10^6 cells/ml in 50 ml of medium in 75-cm^2 polystyrene flasks. Mitomycin C dissolved in dimethyl sulfoxide (DMSO) was added from a stock solution to the cell cultures. Additional DMSO was added to give a final volume of 100 μl. The flasks were incubated for 4 h, and then the cells were centrifuged, washed twice, and resuspended in fresh medium. The cells were transferred to new 75-cm^2 flasks and cultured for 2 days for mutant expression with cell counts and cell density adjustment made after 1 day. Then the cells were cloned in 96-well plates in medium containing 3 μg/ml trifluorothymidine (TFT) for selection and medium without TFT for the measurement of cloning efficiency. After 12 days, the 96-well plates were evaluated by eye using a Quebec dark field colony counter to determine the presence or absence of colonies in each well and to enumerate the number of small and LC mutants. Total MF, SC MF, and LC MF were determined, and the relative total growth values that measures cytotoxicity were calculated according to the published protocol (Chen and Moore, 2004). Mutant colonies were randomly selected and isolated from the cultures treated with the highest test dose of AZT (1 mg/ml), mitomycin C (0.4 μg/ml), or taxol (1 μg/ml), respectively.

DNA extraction. The isolated mutant colonies were cultured in fresh medium for several days to obtain sufficient cells for subsequent analysis. Genomic DNA was extracted from 3×10^6 cells of each *Tk* mutant clone using the Qiagen DNeasy tissue kit (Valencia, CA) and stored at − 20°C.

Tk gene LOH analysis. *Tk* gene LOH analysis was conducted using the allele-specific PCR described by Liechty *et al.* (1996), with some modifications (Wang *et al.*, 2007). Basically, the microsatellite locus *D11Agl2* that resides in the *Tk* gene was amplified using a touchdown PCR method. The PCR was performed in 96-well plates using a PCR System 9700 (Applied Biosystems, Foster City, CA). The reaction products were separated by 2% agarose gel electrophoresis, stained with 1 μg/ml ethidium bromide, and visualized with a UV light box.

Chromosome 11 LOH analysis. In addition to microsatellite marker *D11Agl2*, eight other microsatellite loci on mouse chromosome 11 (*D11Mit 42, 59, 36, 29, 22, 20, 19*, and *74*) were used (Wang *et al.*, 2007). The nine microsatellite loci are almost evenly distributed along the length of the chromosome (with locations at 78.0, 72.0, 58.5, 47.6, 40.0, 25.0, 20.0, 13.0, and 0.0cM, respectively). LOH analysis was performed at each microsatellite locus using allele-specific PCR as previously described (Wang *et al.*, 2007). The PCR products were separated and visualized as above.

Tk gene dosage analysis. For those mutants showing LOH involving a large portion of chromosome 11 (at least including microsatellite markers *D11Agl2* and *D11Mit 42*), *Tk* gene CN was evaluated using the real-time PCR $2^{-\Delta\Delta Ct}$ method described previously (Wang *et al.*, 2007). Briefly, a fragment of the *Tk* gene and a fragment of an unrelated gene (*H-2K*) on chromosome

17 were amplified simultaneously. The *H-2K* gene fragment was used as an endogenous reference for PCR relative quantitation.

According to the criteria set by Honma *et al.* (2001), the CN of *Tk* mutants was classified as <1.2, 1.2–1.8, and >1.8, which sets the ranges for the hemizygous, mosaic, and homozygous states of the *Tk* gene, respectively.

G-banding analysis. For the mutants showing LOH involving a large portion of chromosome 11 (at least including microsatellite markers *D11Agl2* and *D11Mit 42*), G-banding analysis was performed to examine the alteration of chromosome 11. The protocol established by Sawyer *et al.* (1985) was followed with some revision. Briefly, a 12-ml cell culture (approximately 8–10 × 10^6 cells) was centrifuged at 200 × g for 10 min. The supernatant was discarded, and the cell pellet was resuspended with gentle agitation in a hypotonic solution (10 ml 75mM KCl). Then 120 µl of 10 µg/ml colchicine (Sigma) was added. The cell suspension was then incubated at 37°C for 20 min. Five drops of fixative (5:2, methanol:acetic acid) were added at the end of incubation with gentle agitation to avoid cell clumping. Then the cells were centrifuged, and the supernatant was discarded leaving approximately 0.5 ml of solution over the pellet. The pellet was then gently agitated, and 10 ml of fixative was added. After gentle mixing, the cell suspension was incubated at room temperature for 20 min. The cell suspension was centrifuged, and the fixative was changed twice to eliminate cell debris and to ensure good spreading and staining of chromosomes. The cell pellet was resuspended in about 1 ml fixative to make the slides. For karyotypic analysis, cells were dropped onto precleaned glass slides (soaked in 95% ethanol overnight) and air dried. Trypsin treatment before Giemsa staining was used to sharpen bands and increase contrast. At least 10 cells were examined per clone.

Chromosome painting analysis. Chromosome 11 painting analysis was performed as described by Zhang *et al.* (1996) with some modification. Briefly, colchicine was added to a 12-ml cell culture (approximately 8–10 × 10^6 cells) at a final concentration of 0.5 µg/ml. Cells were incubated for 1 h at 37°C in an atmosphere of 5% CO_2 and saturated humidity. Then the cell culture was centrifuged at 200 × g for 10 min and resuspended in hypotonic solution (75mM KCl), followed by incubation in a water bath at 37°C for 20 min. After that, five drops of fixative (3:1, methanol:acetic acid) were added with gentle agitation. The rest of the cell fixation was accomplished using the procedure described in the G-banding analysis section above.

A whole-chromosome painting probe specific for mouse chromosome 11 labeled with biotin was purchased from Cambio (Cambridge, UK). The chromosome painting procedure described in the manufacturer's instructions was followed. Briefly, chromosomal DNA on slides was denatured with 2× sodium chloride-sodium citrate buffer (SSC)-70% formamide solution (1× SSC is 0.15M NaCl + 0.015M sodium citrate) at 70°C for 2 min followed by dehydration through an ethanol series (70%, 85%, and 100%). Probes were added to the metaphase preparations. After overnight hybridization at 37°C, the slides were washed three times with 2× SSC-50% formamide solution and three times with 2× SSC at 45°C. The slides were then incubated with fluorescent avidin-DCS (Vector Laboratories, Burlingame, CA) solution at 37°C for 30 min and washed with 2× SSC containing 0.1% Tween 20. The slides were then incubated with biotinylated goat anti-avidin DCS (Vector Laboratories) at 37°C for 30 min followed by a second fluorescent avidin-DCS treatment. The slides were washed, counterstained with propidium iodide solution, and mounted. At least 10 metaphases from each slide were examined with fluorescence microscopy.

RESULTS

The *Tk* MFs for the AZT-, mitomycin C–, and taxol-treated cultures used for mutant analysis are shown in Table 1. The complete MF data for AZT can be found in Wang *et al.* (2007), for taxol in Moore *et al.*, (2005), and for mitomycin C in Figure 1. At the highest dose tested, all three chemicals induced high *Tk* MFs (4.8-, 16.6-, and 7.3-fold over the concurrent negative control for AZT, mitomycin C, and taxol, respectively). This indicated that most of the analyzed *Tk* mutants were chemical-induced mutants rather than spontaneous background mutants.

TABLE 1
Tk Gene MFs in Mouse Lymphoma Cells Treated with AZT, Mitomycin C, and Taxol (at the Highest dose Tested)

			Tk MF (× 10^{-6})	
Chemical	Concentration	SC[a] (%)	Control	Treated culture
AZT[b]	1 mg/ml	65%	84	407
Mitomycin C	0.4 µg/ml	44%	56	928
Taxol[c]	1 µg/ml	41%	50	366

[a]SC mutants, as a percent of all *Tk* mutant colonies obtained.
[b]Data from Wang *et al.* (2007).
[c]Data from Moore et al. (2005).

For our detailed analysis to determine if mutants can result from deletion, mitotic recombination, and aneuploidy, we needed mutants with large chromosomal alterations. Therefore, a number of mutants were first screened using nine microsatellite markers spanning chromosome 11. Mutants showing partial chromosome 11 LOH including at least microsatellite markers *Agl2* and *Mit42* were selected for further analysis using G-banding. In addition, *Tk* gene CN of these mutants was evaluated. Chromosome painting analysis was performed for some of the mutants showing LOH at every chromosome 11 microsatellite marker.

Fifteen mutants from the 1-mg/ml AZT–treated culture (Wang *et al.*, 2007) showing LOH including at least microsatellite markers *Agl2* and *Mit42* were analyzed using G-banding analysis. Nineteen mutants from the 0.4-µg/ml mitomycin C–treated culture were analyzed using chromosome 11 LOH analysis; then four mutants showing partial chromosome 11 LOH were analyzed using *Tk* gene dosage analysis and G-banding analysis, and two mutants showing LOH at

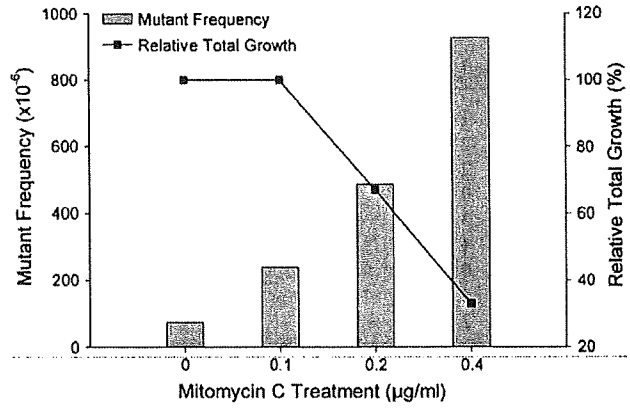


FIG. 1. *Tk* gene MFs and relative total growth values of mouse lymphoma cells treated with mitomycin C.

TABLE 2
The Number of Mutants that were Analyzed Using G-banding Analysis or Chromosome Painting Analysis

Treatment	LOH pattern	Number of mutants analyzed using G-banding analysis	Number of mutants analyzed using chromosome painting analysis
1 mg/ml AZT	Partial LOH including at least microsatellite markers *Agl2* and *Mit42*	15	
0.4 μg/ml mitomycin C	Partial LOH including at least microsatellite markers *Agl2* and *Mit42*	4	
	Complete LOH		2
1 μg/ml taxol	Complete LOH		7

every microsatellite marker on chromosome 11 were analyzed using chromosome painting analysis. Twenty mutants from the 1-μg/ml taxol–treated culture were isolated for chromosome 11 LOH analysis, and seven mutants showing LOH at every microsatellite marker on chromosome 11 were analyzed using chromosome painting analysis. The number of mutants that were analyzed using the combined strategy is summarized in Table 2.

The results of the G-banding analysis can be classified as normal chromosome 11, visible deletion, or complex chromosome alterations. The number of chromosome 11 revealed by the chromosome painting analysis can be classified as one (indicating chromosome loss), two (indicating chromosome duplication after loss), or more than two (including either aneuploidy or polyploidy). The numbers of metaphase spreads showing different numbers of chromosome 11 as revealed by chromosome painting are shown in Table 3. The combined results of the chromosome 11 LOH analysis, *Tk* CN detection, and G-banding/chromosome painting analysis are shown in Table 4.

TABLE 3
The Number of Metaphase Spreads Showing Different Numbers of Chromosome 11 in the *Tk* Mutants Isolated from Mitomycin C– or Taxol-Treated Cultures as Revealed by Chromosome Painting

	Number of chromosome 11				
Mutant[a]	1	2	3	4	>4
ML7	1	9	0	0	0
MS3	3	4	2	1	0
TL2	2	8	0	0	0
TL4	0	9	0	1	0
TL8	3	7	0	0	0
TS2	1	7	0	1	1
TS4	0	8	1	1	0
TS6	0	2	5	0	3
TS7	2	8	0	0	0

[a]The first letter of the mutant name indicates the chemical exposure (M, mitomycin C; T, taxol); the second letter indicates colony size of the mutant (L, large; S, small). All the mutants showed LOH at every microsatellite marker on chromosome 11. Ten metaphase spreads were counted for each *Tk* mutant.

In total, 28 mutants showing partial or complete chromosome 11 LOH were analyzed using this combined strategy. Among them, nine mutants (A1B4, A1B5, A2D4, A3C2, A5B3, A6A2, ML4, ML10, and MS6) retained a normal karyotype, while at least three microsatellite markers showed LOH. With a *Tk* CN of 2, the mutation type of these mutants can be identified as recombination. A representative metaphase cell is shown in Figure 2. Five mutants (A1C6, A3A4, A5C2, A5D6, and A6C2) were identified to be deletions. They all have partial chromosome 11 LOH patterns, one copy of the *Tk* gene, and chromosome 11 showing visible deletions or deletions combined with translocation (representative metaphase cell shown in Fig. 2). Five mutants (A5C6, A5D2, A7B5, A7C1, and MS8) showed complex chromosome alterations. They all have partial chromosome 11 LOH patterns, with more than one copy of the *Tk* gene. G-banding analysis showed that the Tk^- chromosome appears to be normal, while the Tk^+ chromosome is abnormally long (Fig. 3). It is speculated that this complex alteration was the result of multiple events: deletion, duplication (aneuploidy), and translocation. First, the Tk^+ chromosome was partially deleted, which resulted in the LOH pattern; then the Tk^- chromosome was duplicated and translocations occurred. The duplicated Tk^- chromosome was translocated directly to the damaged Tk^+ chromosome or other chromosomes. Mutants A5D2 and MS8 have *Tk* CNs between 1 and 2, indicating that they are mosaic mutants. They may be a mixture of cells with duplicated Tk^- chromosome after the partial deletion of the Tk^+ chromosome or the Tk^- chromosome was not duplicated. For those mutants that showed complete LOH of chromosome 11 (ML7, MS3, TL2, TL4, TL8, TS2, TS4, TS6, and TS7), chromosome painting analysis indicated that they were all mosaic mutants, and overall the major mechanism appears to be the Tk^- chromosome duplication after the loss of the Tk^+ chromosome. Representative photos for chromosome loss and chromosome duplication after loss are shown in Figure 4.

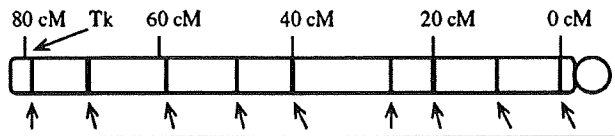
DISCUSSION

A complete evaluation of genetic toxicology data includes an analysis of the mode of action by which the test chemical induced mutation (Dearfield and Moore, 2005). Because there are a number of genotoxicity tests and different tests detect different types of genotoxic damage, they may give a mixture of both positive and negative results. Therefore, it is important to identify the types of mutations detected by the different assays in order to evaluate the data properly.

As mentioned before, it has been established that the MLA can detect both point mutations and chromosomal mutations

TABLE 4

Combined Results of the LOH Analysis, *Tk* Gene CN Detection, and G-Banding/Chromosome Painting Analysis for the *Tk* (Mutants Isolated from AZT-, Mitomycin C–, or Taxol-Treated Cultures

		LOH pattern[c]										
												
		80 cM Tk		60 cM		40 cM		20 cM		0 cM		
Mutants[a]	L/S[b]	*Agl2*	*42*	*59*	*36*	*29*	*22*	*20*	*19*	*74*	*Tk* CN[d]	G-banding/chromosome painting[e]
A1B4	L	○	○	○	○	○	●	●	●	●	>1.8	Recombination
A1B5	L	○	○	○	○	●	●	●	●	●	>1.8	Recombination
A1C6	L	○	○	●	●	●	●	●	●	●	<1.2	Deletion
A2D4	L	○	○	○	●	●	●	●	●	●	>1.8	Recombination
A3A4	S	○	○	●	●	●	●	●	●	●	<1.2	Deletion
A3C2	S	○	○	○	○	○	●	●	●	●	>1.8	Recombination
A5B3	S	○	○	○	○	○	●	●	●	●	>1.8	Recombination
A5C2	S	○	○	●	●	●	●	●	●	●	<1.2	Deletion
A5C6	S	○	○	○	●	●	●	●	●	●	>1.8	Deletion with aneuploidy
A5D2	S	○	○	○	●	●	●	●	●	●	1.2–1.8	Deletion with/without aneuploidy
A5D6	S	○	○	○	○	●	●	●	●	●	<1.2	Deletion with translocation
A6A2	S	○	○	○	●	●	●	●	●	●	>1.8	Recombination
A6C2	S	○	○	○	●	●	●	●	●	●	<1.2	Deletion
A7B5	S	○	○	○	●	●	●	●	●	●	>1.8	Deletion with aneuploidy
A7C1	S	○	○	○	●	●	●	●	●	●	>1.8	Deletion with aneuploidy
ML4	L	○	○	○	○	●	●	●	●	●	>1.8	Recombination
ML10	L	○	○	○	●	●	●	●	●	●	>1.8	Recombination
MS6	S	○	○	○	●	●	●	●	●	●	>1.8	Recombination
MS8	S	○	○	●	●	●	●	●	●	●	1.2–1.8	Deletion with/without aneuploidy
ML7	L	○	○	○	○	○	○	○	○	○	NA[f]	Chromosome loss/duplication
MS3	S	○	○	○	○	○	○	○	○	○	NA	Chromosome loss/duplication
TL2	L	○	○	○	○	○	○	○	○	○	NA	Chromosome loss/duplication
TL4	L	○	○	○	○	○	○	○	○	○	NA	Chromosome duplication
TL8	L	○	○	○	○	○	○	○	○	○	NA	Chromosome loss/duplication
TS2	S	○	○	○	○	○	○	○	○	○	NA	Chromosome loss/duplication
TS4	S	○	○	○	○	○	○	○	○	○	NA	Chromosome duplication
TS6	S	○	○	○	○	○	○	○	○	○	NA	Chromosome duplication
TS7	S	○	○	○	○	○	○	○	○	○	NA	Chromosome loss/duplication

●, retain heterozygosity; ○, LOH.

[a]The first letter of the mutant name indicates the chemical exposure. A, AZT; M, mitomycin C; T, taxol. For mutants from AZT-treated culture, LOH analysis and *Tk* gene CN detection was done in a previous study (Wang *et al.*, 2007).

[b]L, large colony mutant; S, small colony mutant.

[c]*D11Agl2*, *D11Mit 42*, *59*, *36*, *29*, *22*, *20*, *19*, and *74* are nine microsatellite markers that are almost evenly distributed along the length of chromosome 11.

[d]The *Tk* gene CN analysis was performed for mutants showing microsatellite LOH for at least markers *Agl2* and *Mit 42* but also retaining microsatellite heterozygosity for at least marker *Mit 74* (partial LOH of chromosome 11).

[e]G-banding analysis was performed for the mutants showing LOH at least at microsatellite markers *Agl2* and *Mit 42* but also retaining heterozygosity at least at microsatellite marker *Mit 74* (partial LOH of chromosome 11). Chromosome painting analysis was performed for the mutants showing LOH at every microsatellite marker on chromosome 11 (complete LOH of chromosome 11).

[f]Not analyzed.

(Applegate *et al.*, 1990; Chen *et al.*, 2002; Honma *et al.*, 2001; Hozier *et al.*, 1981; Liechty *et al.*, 1998; Moore *et al.*, 1985). A large experimental trial including 45 labs (Honma *et al.*, 1999) suggested that the MLA and the *in vitro* chromosome aberration test were basically "equivalent" as to their ability to give positive responses for clastogens. It is important to recognize that the MLA detects small-scale alterations that are too small to be detected by cytogenetic assays. In addition, the MLA detects recombination, which may lead to recessive mutations and plays an important role in the deactivation of