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Effect of *Lactobacillus brevis* KB290 on the cell-mediated cytotoxic activity of mouse splenocytes: a DNA microarray analysis

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Abstract

Lactic acid bacteria confer a variety of health benefits. Here, we investigate the mechanisms by which *Lactobacillus brevis* KB290 (KB290) enhances cell-mediated cytotoxic activity. Female BALB/c mice aged 9 weeks were fed a diet containing KB290 (3×10^9 colony-forming units/g) or starch for 1 d. The resulting cytotoxic activity of splenocytes against YAC-1 cells was measured using flow cytometry and analysed for gene expression using DNA microarray technology. KB290 enhanced the cell-mediated cytotoxic activity of splenocytes. DNA microarray analysis identified 327 up-regulated and 347 down-regulated genes that characterised the KB290 diet group. The up-regulated genes were significantly enriched in Gene Ontology terms related to immunity, and, especially, a positive regulation of T-cell-mediated cytotoxicity existed among these terms. Almost all the genes included in the term encoded major histocompatibility complex (MHC) class I molecules involved in the presentation of antigen to $CD8^+$ cytotoxic T cells. *Marco* and *Signr1* specific to marginal zone macrophages (MZM), antigen-presenting cells, were also up-regulated. Flow cytometric analysis confirmed that the proportion of MZM was significantly increased by KB290 ingestion. Additionally, the over-represented Kyoto Encyclopedia of Genes and Genomes pathways among the up-regulated genes were those for natural killer (NK) cell-mediated cytotoxicity and antigen processing and presentation. The results for the selected genes associated with NK cells and $CD8^+$ cytotoxic T cells were confirmed by quantitative RT-PCR. These results suggest that enhanced cytotoxic activity could be caused by the activation of NK cells and/or of $CD8^+$ cytotoxic T cells stimulated via MHC class I presentation.

Key words: ***Lactobacillus brevis*** **KB290: Cell-mediated cytotoxicity: DNA microarray: Marginal zone macrophages**

Lactic acid bacteria comprise a heterogeneous group of micro-organisms that produce lactic acid as a major end product of carbohydrate fermentation. Lactic acid bacterial strains are historically important because of their utility in food preservation and fermentation. Some strains are used as probiotics defined as live micro-organisms which, when administered in adequate amounts, confer a health benefit on the host[1,2]. Probiotic effects include modulation of the innate and adaptive immune systems, suppression of intestinal infections and alleviation of food allergies[3,4]. Since some probiotics enhance cell-mediated cytotoxic activity against tumour cells and virus-infected cells[5,6], it is possible that they could help protect consumers from the risk of tumour development and infectious diseases.

Lactobacillus brevis KB290 (KB290), which was isolated from the traditional Japanese pickle Suguki, is safe for humans, tolerates gastrointestinal juices and improves gut health[7], as well as being useful for an early intervention in the irritable bowel syndrome[8] – characteristics that meet the criteria for a probiotic strain. Humans who ingest KB290 show enhancement of interferon-α production capacity[9]. Oral administration of 1×10^9 colony-forming units (cfu)/d of KB290 for 14 d enhanced cell-mediated cytotoxic activity in mouse splenocytes (Y Fukui and K Katsura, unpublished results).

Natural killer (NK) cells act as cytolytic effector cells of the innate immune system. Activation of NK cells is induced by direct recognition of cells infected with viruses via NK

Abbreviations: cfu, colony-forming units; DC, dendritic cells; DEG, differentially expressed genes; GO, Gene Ontology; KB290, Lactobacillus brevis KB290; KEGG, Kyoto Encyclopedia of Genes and Genomes; MZM, marginal zone macrophages; NK, natural killer; PI3K, phosphatidylinositol 3-kinase; TCR, T-cell receptor.

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receptors as well as by cytokines produced by innate immune cells, such as dendritic cells (DC)[10,11]. $CD8^+$ cytotoxic T cells can also recognise and eradicate virally infected cells and tumours. DC and macrophages elicit $CD8^+$ cytotoxic T-cell activation by the interaction between MHC class I and the T-cell antigen receptor[12]. Lactic acid bacterial strains initiate NK–DC interactions via DC maturation and, as a consequence, NK cells increase their cytolytic potential[13]. However, the mechanism by which lactic acid bacteria modulate the function of effector cells *in vivo* is not fully understood because of a complex network of immune cells.

DNA microarray technology allows us to evaluate the expression of many genes at once and how they are up- and down-regulated in a particular type of cell or tissue. Nutrigenomics[14], an application of genomic technology to nutrition research, is also becoming important to the food industry, and many nutrigenomic studies have utilised new approaches such as bioinformatics to understand how nutrients influence gene expression. This technology has been used to study how probiotics modulate the immune system *in vivo* focused on intestinal mucosal immunity or the anti-inflammatory effect[15,16]. Here, we use the technology to investigate immune responses to ingested KB290 by focusing on cell-mediated cytotoxicity.

Materials and methods

Bacterial strain

KB290 was deposited as strain *L. brevis* JCM 17,312 in the Japan Collection of Microorganisms and has been maintained at Research Institute, Kagome Company Limited (Tochigi, Japan). Recognised as safe for human consumption[7], KB290 is used commercially in beverages and supplements.

Animal experiments

Specific pathogen-free female BALB/c mice, aged 9 weeks, were purchased from Charles River Laboratories Japan, Inc. They were housed at 20–24°C and 45–65% humidity in an animal laboratory with a 12 h light–12 h dark cycle timed from 07.30 hours. They were divided into two groups (n 6) with equal mean body weights and were fed a commercial normal diet (CE-2; CLEA Japan, Inc.) and sterile water during a 1-week acclimatisation period. Thereafter, lyophilised KB290 was added to the treatment group diet (3×10^9 cfu/g), while 5·8% (w/w) potato starch (Nippon Starch Chemical) was added to the control group diet. Daily intake of the KB290-containing diet per treated mouse was 3·63 g, or about 1×10^{10} cfu, as the combination of a high dose and the short duration of the treatment showed cell-mediated cytotoxicity reproducibly (Y Fukui and E Sasaki, unpublished results).

On day 1, mice were euthanised, their spleens were sampled and cell-mediated cytotoxic activity was measured in splenocytes before analysing gene expression using DNA microarrays.

The Animal Care and Use Committee of the Institute of Kagome Company Limited approved all protocols, which were in accordance with the guidelines established by the Japanese Society of Nutrition and Food Science (Law and Notification 6 of the Japanese Government).

YAC-1 target cells

Mouse lymphoma YAC-1 cells (Japanese Collection of Research Bioresources) were maintained in Roswell Park Memorial Institute-1640 (RPMI) medium (Sigma) supplemented with 10% (v/v) fetal bovine serum (Nippon Biotest), 5 mM-HEPES (Sigma), 1% penicillin–streptomycin solution (p4458; Sigma) and 100 μg streptomycin/ml (Sigma) (complete RPMI-1640 medium). NK cells[17], $CD8^+$ cytotoxic T cells[18] and macrophages[19] showed cytotoxicity against the cells.

Preparation of splenocytes

Individual mouse spleens were placed into 5 ml minimum essential medium Eagle (Sigma) containing 1% penicillin–streptomycin solution (p4458; Sigma), and crushed with frosted slide glass (Matsunami Trading) to obtain single-cell suspensions. The cells were passed through a sterile 70 μm nylon mesh filter (Becton Dickinson) and transferred to a 15 ml tube (Becton Dickinson) filled with complete RPMI-1640. They were centrifuged at 430 ***g*** for 3 min, collected, washed once in PBS (Invitrogen), counted with an automatic blood cell counter (Nihon Kohden) and adjusted the final concentration to 4×10^6 cells/ml in complete RPMI-1640 medium.

Cell-mediated cytotoxicity assay

Flow cytometry measured the cell-mediated cytotoxic activity of splenocytes to calculate the percentage of the killed cells

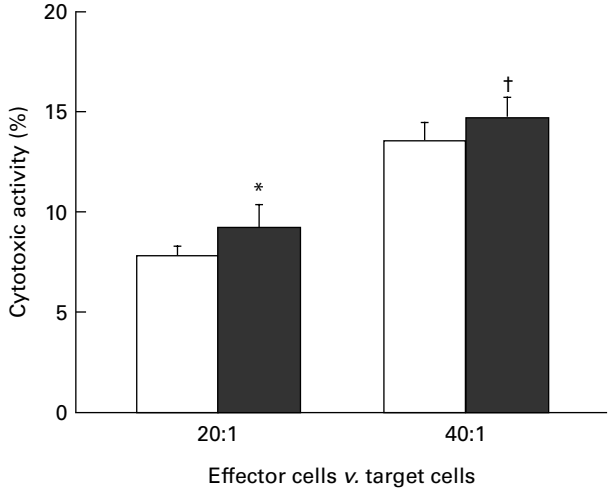

Fig. 1. Cell-mediated cytotoxicity associated with KB290 feeding. Splenocytes were collected 1 d after the commencement of feeding a diet containing *Lactobacillus brevis* KB290 (KB290) (3×10^9 colony-forming units/g; ■) or potato starch (□) and used as effector cells against YAC-1 target cells (1×10^4 cells/well). The effector:target cell ratios were 20:1 and 40:1. Values are means, with standard deviations represented by vertical bars (n 6). * Mean value was significantly different compared with the control group ($P<0·05$; Student's t test). † Mean value was marginally different from that of the control group ($P=0·067$).

GO ID	GO term	FDR-corrected *P* value
0001910	Cell killing*	
0031341	regulation of cell killing	$4{\cdot}85\times10^{-2}$
0001910	regulation of leucocyte-mediated cytotoxicity	$4{\cdot}85\times10^{-2}$
0001916	positive regulation of T-cell-mediated cytotoxicity	$1{\cdot}70\times10^{-2}$†
0002703	regulation of leucocyte-mediated immunity	$1{\cdot}15\times10^{-6}$
0002886	regulation of myeloid leucocyte-mediated immunity	$1{\cdot}86\times10^{-3}$†
0002682	regulation of immune system process	$8{\cdot}23\times10^{8}$
0002694	regulation of leucocyte activation	$4{\cdot}22\times10^{-5}$
0046635	positive regulation of α-β T-cell activation	$3{\cdot}32\times10^{-2}$†
0051249	regulation of lymphocyte activation	$1{\cdot}52\times10^{-3}$
0045577	regulation of β-cell differentiation	$6{\cdot}60\times10^{-3}$†
0050670	regulation of lymphocyte proliferation	$2{\cdot}16\times10^{-2}$
0046640	regulation of α-β T-cell proliferation	$4{\cdot}20\times10^{-2}$†
0002683	negative regulation of immune system process	$1{\cdot}51\times10^{-2}$
0002376	immune system process	$2{\cdot}24\times10^{-13}$
0019882	antigen processing and presentation	$8{\cdot}39\times10^{-3}$
0002474	antigen processing and presentation of peptide antigen via MHC class I	$6{\cdot}60\times10^{-3}$†
0045321	leucocyte activation	$4{\cdot}20\times10^{-3}$
0042110	T-cell activation	$6{\cdot}73\times10^{-3}$†
0002455	humoral immune response mediated by circulating Ig	$4{\cdot}85\times10^{-2}$†
0002250	adaptive immune response	$5{\cdot}62\times10^{-3}$
0002889	regulation of Ig-mediated immune response	$3{\cdot}32\times10^{-2}$†
0006955	immune response	$3{\cdot}36\times10^{-12}$
0002764	immune response-regulating signal transduction	$2{\cdot}65\times10^{-3}$
0002429	immune response-activating cell surface receptor signalling pathway	$2{\cdot}36\times10^{-2}$†
0050896	response to stimulus	$1{\cdot}75\times10^{-9}$
0006950	response to stress	$1{\cdot}55\times10^{-6}$
0006954	inflammatory response	$1{\cdot}87\times10^{-4}$
0002863	positive regulation of inflammatory response to antigenic stimulus	$8{\cdot}49\times10^{-4}$†
0034599	cellular response to oxidative stress	$3{\cdot}23\times10^{-3}$†
0042221	response to chemical stimulus	$1{\cdot}00\times10^{-3}$
0010033	response to organic substance	$1{\cdot}78\times10^{-2}$†
0009607	response to biotic stimulus	$2{\cdot}16\times10^{-2}$
0051707	response to other organism	$4{\cdot}36\times10^{-3}$†
0051704	multi-organism process	$5{\cdot}05\times10^{-3}$
0009987	cellular process	
0051014	actin filament severing	$2{\cdot}57\times10^{-2}$†
0030029	actin filament-based process	$9{\cdot}78\times10^{-3}$
0030036	actin cytoskeleton organisation	$1{\cdot}72\times10^{-2}$†
0071840	cellular component organisation or biogenesis	
0051289	protein homotetramerisation	$4{\cdot}20\times10^{-2}$†
0065007	biological regulation	
0050789	regulation of biological process	
0051239	regulation of multicellular organismal process	$3{\cdot}81\times10^{-3}$
0001817	regulation of cytokine production	$3{\cdot}45\times10^{-3}$†
0032844	regulation of homeostatic process	$1{\cdot}41\times10^{-3}$†
0032879	regulation of localisation	$1{\cdot}57\times10^{-2}$
0050766	positive regulation of phagocytosis	$1{\cdot}52\times10^{-2}$†
0051128	regulation of cellular component organisation	$2{\cdot}56\times10^{-2}$
0051693	actin filament capping	$1{\cdot}52\times10^{-2}$†
0043066	negative regulation of apoptosis	$1{\cdot}32\times10^{-2}$†
0010941	regulation of cell death	$1{\cdot}65\times10^{-3}$
0006917	induction of apoptosis	$4{\cdot}24\times10^{-2}$†
0050794	regulation of cellular process	
0065008	regulation of biological quality	$3{\cdot}18\times10^{-3}$
0048872	homeostasis of number of cells	$3{\cdot}76\times10^{-3}$†
0048878	chemical homeostasis	$5{\cdot}67\times10^{-3}$
0042632	cholesterol homeostasis	$3{\cdot}82\times10^{-2}$†
0030003	cellular cation homeostasis	$2{\cdot}61\times10^{-3}$†

Fig. 2. Significantly enriched Gene Ontology (GO) terms found in the up-regulated genes by *Lactobacillus brevis* KB290 (KB290) feeding ($P<0{\cdot}05$). * GO term with no *P* value means not significant. † False discovery rate (FDR)-corrected *P* values of the GO terms appearing in the deepest hierarchy.

GO ID	GO term	FDR-corrected *P* value
0071840	Cellular component organisation or biogenesis*	
0016043	Cellular component organisation	$1{\cdot}31\times10^{-2}$
0051276	chromosome organisation	$3{\cdot}18\times10^{-2}$
0006325	chromatin organisation	$4{\cdot}34\times10^{-2}$†
0008152	metabolic process	
0006730	One-carbon metabolic process	$3{\cdot}88\times10^{-2}$
0032259	methylation	$4{\cdot}23\times10^{-2}$
0043414	biopolymer methylation	$3{\cdot}61\times10^{-2}$†
0050896	response to stimulus	
0006950	response to stress	$3{\cdot}59\times10^{-2}$
0042060	wound healing	$3{\cdot}18\times10^{-2}$†
0032501	multicellular organismal process	
0050817	coagulation	$1{\cdot}89\times10^{-2}$
0007596	blood coagulation	$1{\cdot}89\times10^{-2}$†
0007599	haemostasis	$1{\cdot}42\times10^{-2}$
0050878	regulation of body fluid levels	$2{\cdot}98\times10^{-2}$
0065008	regulation of biological quality	$2{\cdot}89\times10^{-2}$
0065007	biological regulation	
0048519	negative regulation of biological process	$2{\cdot}32\times10^{-2}$
0048523	cellular cation homeostasis	$3{\cdot}50\times10^{-2}$†

Fig. 3. Significantly enriched Gene Ontology (GO) terms found in the down-regulated genes by *Lactobacillus brevis* KB290 (KB290) feeding ($P<0{\cdot}05$). * GO term with no *P* value means not significant. † False discovery rate (FDR)-corrected *P* values of the GO terms appearing in the deepest hierarchy.

stained with propidium iodide (PI; Sigma)[20]. YAC-1 cells (10^6 cells/ml) were labelled by incubation for 10 min with 20 μg/ml of 3,3′-dioctadecyloxacarbocyanine perchlorate (Dio; Sigma) at 37°C in a 5% CO_2 atmosphere. After washing them three times with PBS, the cells (10^5 cells/ml) were resuspended in complete RPMI-1640 medium and mixed 50 or 100 μl of the splenocyte suspension ($4{\cdot}0\times10^6$ cells/ml) with 100 μl of the YAC-1 cell suspension to achieve effector:target cell ratios of 20:1 and 40:1, and then added PI (25 μg/ml). To enhance cell contact, the samples were centrifuged for 5 min at 430 ***g*** and incubated for 2 h at 37°C in 5% CO_2. Target cell lysis was determined using a flow cytometer (FACSCalibur™; Becton Dickinson) and analysed with CELLQuest software (Becton Dickinson). The percentage of the dead target cells was calculated (%T_d) as (% Dio^+ PI^+ cells/% Dio^+ cells) × 100 and cell-mediated cytotoxic activity was estimated as %T_d (cultured with the effector cells) − %T_d (cultured without the effector cells). Each sample was assayed in triplicate and the means were recorded.

DNA microarray assay

Total RNA was isolated from each spleen sample with TRIzol reagent (Invitrogen), according to the manufacturer's instructions, and purified using the RNeasy Mini Kit (Qiagen) and checked the quality and quantity by agarose gel electrophoresis and spectrophotometry, respectively. Total RNA from individual samples was subjected to DNA microarray analysis as described previously[21]. Briefly, 100 ng of the purified total RNA were used to synthesise complementary DNA, and then biotinylated amplified RNA (aRNA) was transcribed with T7 RNA polymerase using the GeneChip 3' IVT Express Kit (Affymetrix). Agarose gel electrophoresis assayed aRNA quality, ascertaining its suitability for the experiments. aRNA was fragmented and then hybridised to an Affymetrix GeneChip mouse genome 430 2.0 array (Affymetrix) to determine the expression of over 45 000 probe sets fully covering the mouse genome, in accordance with the manufacturer's instructions. The array was hybridised at 45°C for 16 h, washed and stained with phycoerythrin. Fluorescence signals were scanned with the Affymetrix GeneChip System and Affymetrix GeneChip Command Console software was used to reduce the images to the intensity values for each probe (CEL files). All microarray data were submitted to the National Center for Biotechnology Information Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/; GEO Series ID GSE41127).

Quantitative RT-PCR

Single-strand complementary DNA was synthesised from 20 ng of total RNA using the PrimeScript RT reagent kit (Takara Bio). Quantitative RT-PCR analysis was performed on the 7000 Real-Time PCR System (Applied Biosystems) using SYBR Premix Ex Taq (Perfect Real Time; Takara Bio), according to the manufacturer's instructions. The reaction conditions were as follows: initial denaturation at 95°C for 30 s followed by forty cycles of denaturation at 95°C for 5 s and 60°C for 31 s. The primer sequences were as follows: IL-18 (*Il18*), forward 5′- AAGACTCTTGCGTCAACTTCAAGGA-3′ and reverse 5′-AGTCGGCCAAAGTTGTCTGATTC-3′; DNAX activation protein of 12 kDa (*Dap12*), forward 5′-CCGGAAACAACACATTGCTGAG-3′ and reverse 5′-GCCTCTGTGTGTTGAGGTCACTGTA-3′; lymphocyte-specific protein tyrosine kinase (*Lck*), forward 5′-TGGGACCTTCACCATCAAGTCA-3′ and reverse 5′-GTCAGGTCTCACCATGCGGTAG -3′; purine-nucleoside phosphorylase 1 (*Pnp*), forward 5′-CCAACTTTGAGACTGTGGCAGA-3′ and reverse 5′-CATGACAACCTTGTTCGTAATGAG-3′; glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*), forward 5′-AAATGGTGAAGGTCGGTGTG-3′ and reverse 5′-TGAAGGGGTCGTTGATGG-3′. Fold induction

Table 1. Significantly enriched Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway found in the differentially expressed genes (DEG) by *Lactobacillus brevis* KB290 (KB290) feeding ($P<0{\cdot}05$)

	KEGG ID	Pathway	FDR-corrected *P* value
Up-regulated	mmu04142	Lysosome	$1{\cdot}16\times10^{-3}$
	mmu05416	Viral myocarditis	$2{\cdot}09\times10^{-3}$
	mmu04670	Leukocyte transendothelial migration	$2{\cdot}10\times10^{-3}$
	mmu04612	Antigen processing and presentation	$4{\cdot}62\times10^{-3}$
	mmu04650	Natural killer cell-mediated cytotoxicity	$7{\cdot}57\times10^{-3}$
	mmu05330	Allograft rejection	$3{\cdot}14\times10^{-2}$
	mmu04662	B-cell receptor signalling pathway	$2{\cdot}98\times10^{-2}$
	mmu03010	Ribosome	$4{\cdot}77\times10^{-2}$
Down-regulated	mmu04512	ECM–receptor interaction	$1{\cdot}15\times10^{-3}$
	mmu04510	Focal adhesion	$6{\cdot}18\times10^{-3}$

FDR, false discovery rate; ECM, extracellular matrix.

values were calculated using the $2^{-\Delta\Delta Ct}$ method and target gene expression was normalised to *Gapdh*.

Flow cytometric analysis

Flow cytometry determined the proportion of marginal zone macrophages (MZM) in the spleen. Incubating for 1 min at room temperature in 1 ml Red Blood Cell Lysing Buffer (Sigma) lysed erythrocytes from splenocyte suspension (10^6 cells/ml) before wash in ice-cold PBS (−) supplemented with 1 % (w/v) bovine serum albumin (Wako) and 0·1 % (w/v) sodium azide (staining buffer; Wako). The cells were treated with Rat Anti-Mouse CD16/CD32 (Clone 2.4G2; Becton Dickinson GmbH) for 5 min on ice to block the Fc receptor and then stained the cells with R-phycoerythrin (PE)-conjugated rat anti-mouse macrophage receptor with collagenous structure (MARCO, Clone ED31; AbD Serotec), Alexa Fluor® 647-conjugated rat anti-mouse CD209b antigen (SIGNR1, Clone ER-TR9; AbD Serotec) and fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse H-2 (Clone M1/42; BioLegend) on ice for 20 min. After washing the cells using staining buffer, the samples were analysed using the flow cytometer with CELLQuest software (Becton Dickinson).

Data analysis

Student's *t* test was applied to compare cell-mediated cytotoxic activity, quantitative RT-PCR and the proportion of MZM of the experimental *v.* the control group using SPSS 15.0 for Windows (SPSS Japan, Inc.).

The CEL files were quantified with the Factor Analysis for Robust Microarray Summarization (FARMS) algorithm[22] using statistical language R (version 2.12.1) and Bioconductor 2.7[23]. To detect the differentially expressed genes (DEG) between the control group and each of the KB290 groups, the rank products[24] method was used as a non-parametric statistic[25]. To identify functional classes of the DEG according to Biological Process in Gene Ontology (GO) and the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway, enrichment analyses were performed using the Expression Analysis Systematic Explorer (EASE) score, the modified Fisher's exact test *P* value[26] and Benjamini & Hochberg[27] false discovery rate corrections using the Database for Annotation, Visualization, and Integrated Discovery[28], a Web-accessible program, in accordance with the manuals available at the web site (http://david.abcc.ncifcrf.gov/home.jsp).

Results

Cell-mediated cytotoxicity

The effect of KB290 feeding for 1 d on cell-mediated cytotoxicity was investigated. Fig. 1 shows the cell-mediated cytotoxic activity of splenocytes from mice fed the KB290 or control diet. The activity was significantly ($P < 0{\cdot}05$) higher in the treated group at an effector:target cell ratio of 20:1, while the activity was marginal ($P = 0{\cdot}067$) at an effector:target cell ratio of 40:1.

Gene Ontology and the Kyoto Encyclopedia of Genes and Genomes analysis of DNA microarrays

To elucidate the mechanisms by which KB290 enhances cell-mediated cytotoxic activity, we used DNA microarrays and investigated gene expression changes in the spleen of mice ingested KB290. The rank products method combined with a qFARMS preprocessing algorithm nominated 395 up-regulated and 450 down-regulated probe sets in the group fed KB290 for 1 d (false discovery rate $<0{\cdot}05$). Of those, twelve probe sets were

Table 2. Up-regulated genes involved in the positive regulation of T-cell-mediated cytotoxicity

Gene name	Gene symbol	Description
β-2 Microglobulin	*B2m*	MHC class I protein complex
Histocompatibility 2, K1, K region	*H2-K1*	MHC class I protein complex
Histocompatibility 2, Q region locus 7	*H2-Q7*	MHC class I protein complex
Purine-nucleoside phosphorylase 1	*Pnp*	Transferase activity, transferring glycosyl groups

MHC, major histocompatibility complex.

Table 3. Up-regulated genes involved in the antigen processing and presentation of peptide antigen via major histocompatibility complex class I

Gene name	Gene symbol	Description
β-2 Microglobulin	*B2 m*	MHC class I protein complex
Fc receptor, IgE, high affinity I, gamma polypeptide	*Fcer1 g*	IgE receptor activity
Histocompatibility 2, K1, K region	*H2-K1*	MHC class I protein complex
Histocompatibility 2, D region; histocompatibility 2, D region locus 1	*H2-I*	MHC class I protein complex
Histocompatibility 2, Q region locus 7	*H2-Q7*	MHC class I protein complex

eliminated that were assigned as having both increased and decreased expression (this can be a property of the rank products method, which returns rank products statistics with false discovery rates for both up- and down-regulated probe sets independently). Finally, 383 up-regulated probe sets (327 unique genes) and 438 down-regulated probe sets (347 unique genes) were extracted. Full lists of the DEG are shown in Tables S1 and S2 (available online).

The GO and KEGG analysis identified functional classes of the DEG. The up- and down-regulated DEG fell into significantly (false discovery rate-corrected $P < 0{\cdot}05$) enriched GO terms (135 for the up-regulated genes summarised into fifty-three GO terms by removing redundant GO terms (Fig. 2) and fifteen for the down-regulated genes (Fig. 3)) and into significantly enriched KEGG pathways (eight for the up-regulated genes and two for the down-regulated genes (Table 1)). The over-represented GO terms and KEGG pathways among the up-regulated genes were involved in many immunologically relevant terms and pathways.

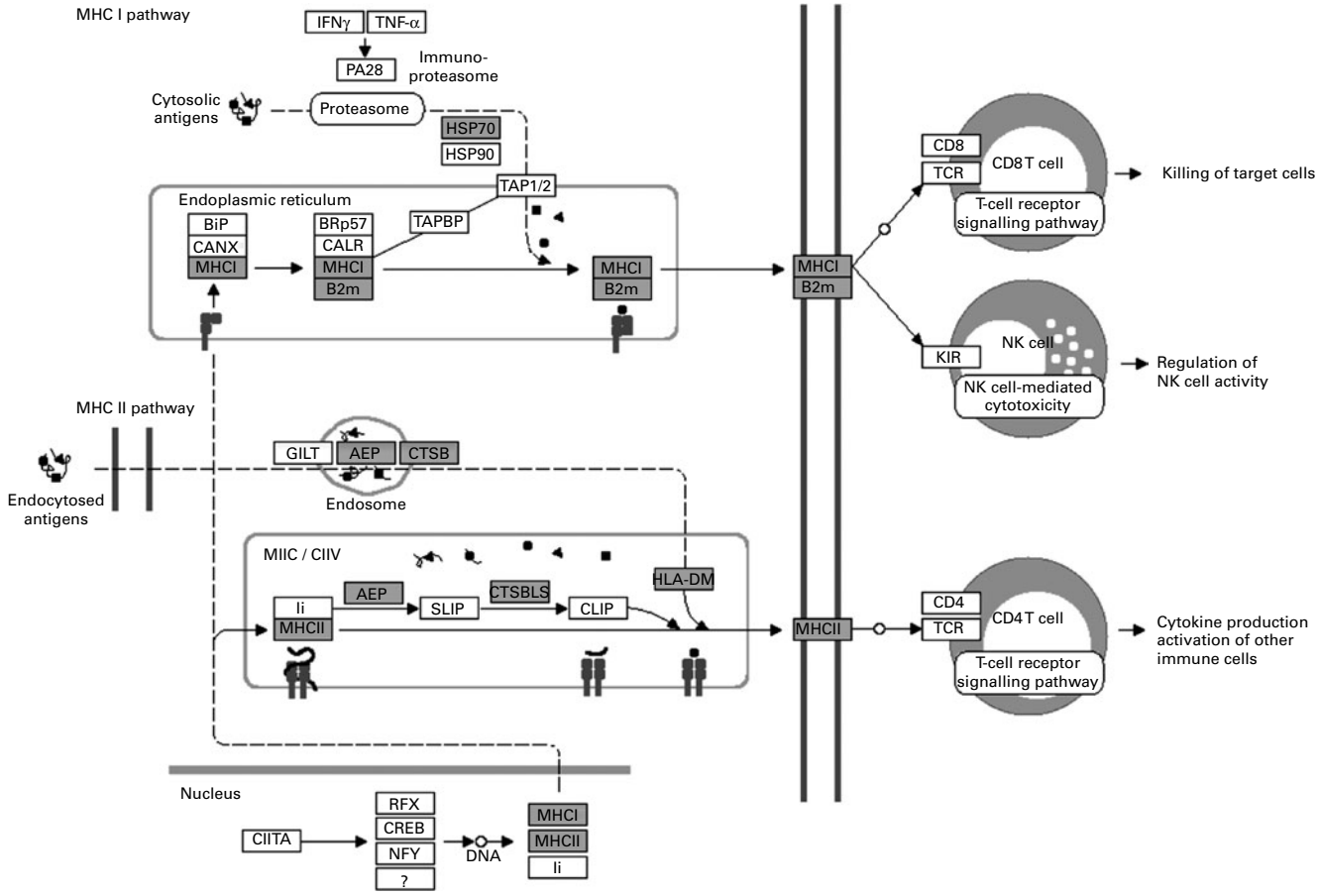

Fig. 4. Up-regulated genes involved in antigen processing and presentation (Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database; adaptation of the KEGG antigen processing and presentation pathway). Shaded boxes indicate genes significantly (false discovery rate $< 0{\cdot}05$) up-regulated by *Lactobacillus brevis* KB290 feeding as revealed by DNA microarray analysis. IFN, interferon; HSP, heat shock protein; MHC, major histocompatibility complex; B2M, β-2 microglobulin; TCR, T-cell receptor; NK, natural killer. PA28, proteasome (prosome, macropain) 28; BiP, heat shock protein 5; CANX, calnexin; BRp57, protein disulfide isomerase associated 3; CALR, calreticulin; TAPBP, TAP binding protein; TAP1/2, transporter 1/2, ATP-binding cassette; sub-family B (MDR/TAP); GILT, interferon gamma inducible protein 30; AEP, legumain; CTSB, cathepsin B; MIIC, MHC class II compartments; CIIV, class II vesicles; Ii, SLIP, and CLIP, CD74 antigen (invariant polypeptide of major histocompatibility complex, class II antigen-associated); CTSB/LB, cathepsin B; HLA-DM, histocompatibility 2, class II, locus DMa; CIITA, class II transactivator; RFX, regulatory factor X-associated protein; CREB, cAMP responsive element binding protein 1; NFY, nuclear transcription factor-Y; KIR, killer cell lectin-like receptor.

Gene expression profiles associated with cell-mediated cytotoxicity

GO terms appearing deeper in the hierarchical structure show more specificity, so they are more important. The deeper-level terms associated with cell-mediated cytotoxicity for each cluster of the hierarchy were searched, and the positive regulation of T-cell-mediated cytotoxicity and the antigen processing and presentation of peptide antigen via MHC class I in the immune system process were found. Almost all the up-regulated genes involved in both the GO terms encoded MHC class I molecules (β-2 microglobulin (*B2 m*), histocompatibility 2, K1, K region (*H2-k1*), histocompatibility 2, Q region locus 7 (*H2-q7*) and histocompatibility 2, D region locus 1 (*H2-l*); Tables 2 and 3).

The over-represented KEGG pathways associated with cell-mediated cytotoxicity were those of NK cell-mediated cytotoxicity and those of antigen processing and presentation (Table 1). Fig. 4 shows the significantly up-regulated genes involved in the latter. Heat shock protein 1B (*Hspa1b*), which has chaperone activity within MHC class I, was up-regulated other than genes directly encoding MHC class I molecules. The up-regulated genes were found in the MHC class I pathway as well as in the MHC class II pathway in antigen-presenting cells. However, there were no significantly up-regulated genes encoding CD4, CD8 and T-cell receptor (TCR) composing the TCR complex.

Since macrophages and DC stimulate $CD8^+$ cytotoxic T cells via MHC class I presentation as antigen-presenting cells, the relevant up-regulated genes were selected and categorised (Table 4). Glycoprotein 49A (*Gp49a*), *Signr1*, *Marco* and C-type lectin domain family 4, member n (*Clec4n*), which act as an antigen-capturing receptor, were up-regulated.

Many up-regulated genes were also implicated in NK cell-mediated cytotoxicity in the KEGG pathway and found in the NK cell activating signalling pathway, though cytotoxic molecules, such as perforin, granzyme and fibroblast-associated cell surface (FasL), were not up-regulated (Fig. 5). Moreover, the genes associated with the activation of NK cells were selected from the DEG, with the result that peroxiredoxin 1 (*Prdx1*), *Il18* and vimentin (*Vim*) were found to be up-regulated (Table 5).

Quantitative RT-PCR

To verify the changes in gene expression detected by DNA microarray analysis, quantitative RT-PCR was applied to four selected genes implicated in the positive regulation of T-cell-mediated cytotoxicity (*Pnp*), the NK cell activating signalling pathway (*Dap12*, *Lck*) and the cytotoxic activity of NK cells and T cells (*Il18*). The results from the quantitative RT-PCR of *Dap12*, *Il18*, *Lck* and *Pnp* argued for these from the DNA microarray (Fig. 6).

Proportion of marginal zone macrophages in the spleen

The genes encoding MHC class I molecules and the MZM-specific markers *Marco* and *Signr1* (Table 4) were up-regulated. Therefore, the control and the treatment groups were compared for the proportion of $MARCO^+$ $SIGNR1^+$ $H\text{-}2^+$ cells as MZM by flow cytometry. Typical figures for the flow cytometric analyses of splenocytes isolated from mice fed the control diet (upper panels) and the KB290 diet (lower panels) for 1 d are shown in Fig. 7(a). The proportion of $MARCO^+$ $SIGNR1^+$ $H\text{-}2^+$ cells in the group fed the KB290 diet was significantly ($P < 0{\cdot}05$) increased compared with the case of the control diet (Fig. 7(b)).

Discussion

It has been reported that immunomodulatory effects by probiotics are dose, administration time and strain

Table 4. Up-regulated genes related to macrophages by *Lactobacillus brevis* KB290 (KB290) ingestion

Gene name	Gene symbol	Description
Heat shock protein 1B	*Hspa1b* (*Hsp70*)	Chaperone activity within MHC class I
RNA-binding motif protein 3	*Rbm3*	Glycine-rich RNA-binding protein family production of Cox-2 after LPS stimulation
Schlafen 4	*Slfn4*	Proliferation-associated macrophages
Serum amyloid A3	*Saa3*	Adhesion and retention of monocytes
Ficolin A	*Fcna*	Act on complement activation in the lectin pathway
CD209b antigen	*Signr1* (*Cd209b*)	Recognise non-reductive end of LPS core region antigen capturing
Glycoprotein 49A	*Gp49a*	Antigen capturing and presentation to T cells
Leucocyte Ig-like receptor, subfamily B, member 4	*Lilrb4*	Suppression of T-cell proliferation induction of regulatory T cell
Macrophage receptor with collagenous structure	*Marco*	Capture blood bone apoptotic cells induce cytokine production expressed in marginal metalophillic macrophage and marginal zone macrophage
Predicted gene 10 883	*Gm10883*	Biological function is unknown
CDC-like kinase 1	*Clk1*	Response to antibiotic in macrophages
SAM domain and HD domain, 1	*Samhd1*	Anti-retroviral protein
DnaJ (Hsp40) homologue, subfamily B, member 1	*Dnajb1* (*hsp40*)	Biological function is unknown
Phospholipase A2, group VII (platelet-activating factor acetylhydrolase, plasma)	*Pla2g7*	Biological function is unknown
C-type lectin domain family 4, member n	*Clec4n* (*dectin2*)	C-type lectin-like receptor inflammatory response

MHC, major histocompatibility complex; LPS, lipopolysaccharide.

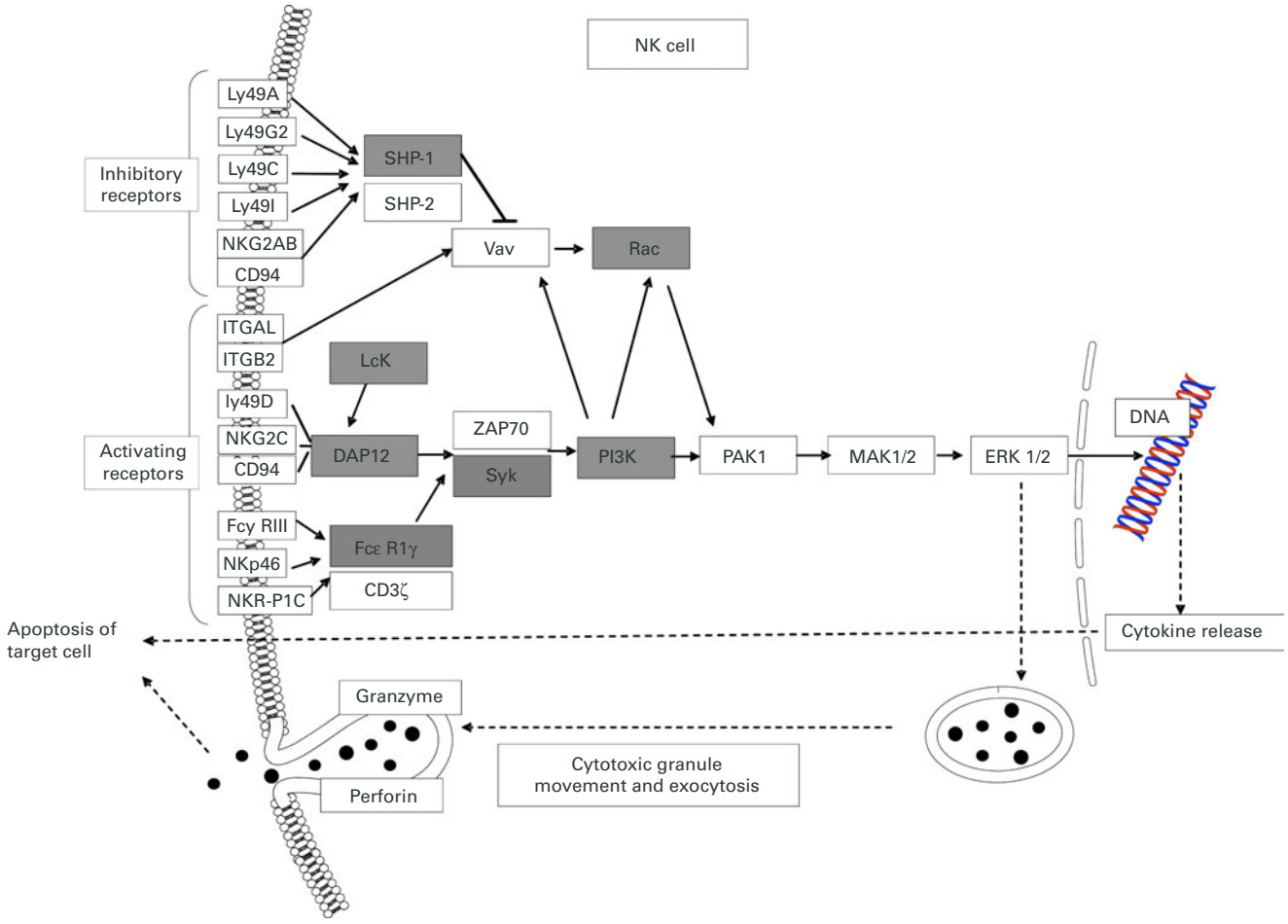

Fig. 5. Up-regulated genes involved in natural killer cell (NK)-mediated cytotoxicity (Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database; adaptation of the KEGG NK cell-mediated cytotoxicity pathway). Shaded boxes indicate genes significantly (false discovery rate $<0{\cdot}05$) up-regulated by KB290 feeding as revealed by DNA microarray analysis. Ly49a, killer cell lectin-like receptor, subfamily A, member 1; Ly49G2, killer cell lectin-like receptor, subfamily A, member 7; Ly49C, killer cell lectin-like receptor, subfamily A, member 3; Ly49I, killer cell lectin-like receptor subfamily A, member 9; NKG2AB, killer cell lectin-like receptor subfamily C, member 1; ITGAL, integrin alpha L; ITGB2, integrin beta 2; Ly49D, killer cell lectin-like receptor, subfamily A, member 4; NKG2C, killer cell lectin-like receptor subfamily C, member 2; FcyRIII, Fc receptor, IgG, low affinity IV; NKp46, natural cytotoxicity triggering receptor 1; NKR-P1C, killer cell lectin-like receptor subfamily B member 1C; SHP-1, Src homology region 2 domain-containing phosphatase 1; Vav, vav oncogene; Rac, ras-related Clck3 botulinum toxin substrate; Lck, lymphocyte-specific protein tyrosine kinase; DAP12, DNAX activation protein of 12 kDa; FcεRIγ, Fc receptor, IgE, high affinity I, gamma polypeptide; ZAP70, zeta-chain (TCR) associated protein kinase; Syk, spleen tyrosine kinase; PI3K, phosphatidylinositol 3-kinase; PAK1, p21 protein (Cdc42/Rac)-activated kinase 1; MAK1/2, mitogen-activated protein kinase kinase 1/2; ERK1/2, mitogen-activated protein kinase 1/2. (A colour version of this figure can be found online at http://www.journals.cambridge.org/bjn)

dependent[29,30]. Wang *et al.*[31] reported that probiotics VSL#3 ($11{\cdot}25 \times 10^9$ bacteria/(100 mg VSL#3 × mouse), suspended in 200 μl PBS) altered the distribution of DC subsets within the intestinal mucosa after 7 d. As 1 d feeding of KB290 (1×10^{10} cfu/d) was able to induce the cell-mediated cytotoxic activity of mouse splenocytes against YAC-1 cells reproducibly (Y Fukui and E Sasaki, unpublished results), we chose the same dose and duration to investigate the relationship between the cytotoxicity of splenocytes and gene expressions related to immune functions in the present study, and the combination successfully showed cytotoxicity also. Further investigation suggested that enhancement was caused by the activation of NK cells and/or of $CD8^+$ cytotoxic T cells stimulated via MHC class I up-regulation in antigen-presenting cells. As far as we know, the combination of a high dose and 1 d duration is a new trial to reveal the immune function of a probiotic.

Mouse spleen contains three subsets of DC[32,33] (myeloid ($CD11c^+$ MHC II^+ $CD11b^+$ cells), lymphoid ($CD11c^+$ MHC II^+ $CD8^+$ cells) and plasmacytoid ($CD11c^+$ MHC II^{low} $B220^+$ $Gr\text{-}1^+$ cells)) and three subsets of macrophages ($F4/80^+$ red pulp macrophages, $MARCO^+$ $SIGLEC1^+$ marginal metallophilic macrophages and $MARCO^+$ $SIGNR1^+$ MZM)[34]. Interestingly, the MZM-specific markers *Marco* and *Signr1* were up-regulated in the spleens of the treated mice. Furthermore, the proportion of MZM was significantly increased in the spleens of KB290-fed mice. MZM capture blood-borne bacterial antigens[35] and slightly induce an antigen-specific $CD8^+$ cytotoxic T-cell response[34]. Also, the up-regulated genes included those which contribute to the recognition of exogenous bacteria (*Clec4n*[36], *Marco*[37] and *Signr1*[38]), the suppression of intracellular micro-organisms (*Clk*[39], SAM domain and HD domain, 1 (*Samhd1*)[40]), the cross-priming of $CD8^+$ cytotoxic T cells (*Hspa1b*[41], *H2-k1* and

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Table 5. Up-regulated genes associated with the activation of natural killer (NK) cells by *Lactobacillus brevis* KB290 (KB290) ingestion

Gene name	Gene symbol	Description
Peroxiredoxin 1; predicted gene 7204	*Prdx1*	NK cell-mediated cytotoxicity hydrogen peroxide catabolic process
Interleukin 18	*Il18*	Augments NK cell activity
Vimentin	*Vim*	Ligand for activating NK receptor

H2-l), the activation of macrophages (schlafen 4 (*Slfn4*)[42]) and monocyte chemotaxis (human serum amyloid A 3 (*Saa3*)[43]). Transgenic overexpression of *Slfn4* in myeloid cells results in an increased number of macrophages in the liver and spleen[42]. SAA3 acts as part of a complex matrix that increases the adhesion and retention of monocytes[43]. Additionally, *Hspa1b*[44], *Slfn4*[42] and *Saa3*[45] were up-regulated in macrophages for bacterial stimulation. The gene encoding IL18 with potent capacity to augment the cytotoxic activity of NK cells[46] and T cells[47] was up-regulated. IL-18 is produced by macrophages when exposed to products such as lipopolysaccharide or oligodeoxynucleotides[47,48]. These results suggest that KB290 could activate antigen-presenting cells including macrophages. Other than the up-regulated genes associated with antigen-presenting cells, *Pnp* involved in the positive regulation of T-cell-mediated cytotoxicity was up-regulated. PNP increases the cytotoxicity of $CD8^+$ cytotoxic T cells[49]. LCK is an integral component of NK cell signalling as well as the TCR signalling complex, and important for the induction of cytotoxicity. Furthermore, the genes encoding MHC class I molecules were up-regulated. Although additional molecular and physiological studies are required to define which cells may contribute to augmenting cell-mediated cytotoxic activity, circumstantial evidence suggests that $CD8^+$ cytotoxic T cells might be possibly activated with antigen-presenting cells by KB290 feeding.

On the other hand, the responsible receptor genes for recognising antigens bound to MHC molecules, such as CD4, CD8 and TCR, were not up-regulated. The expression levels of the receptor are not always required for related cell activation. Paillard *et al.*[50] reported that *Tcr*, *Cd4* and *Cd8* mRNA levels were not up-regulated even when T cells were activated. Thus, the activation of these stimulants might be enough to increase cell-mediated cytotoxicity, even though genes encoding the TCR complex were not up-regulated. Although further studies are required to define which cells are responsible for augmentation of cell-mediated cytotoxic activity, circumstantial evidence suggests that $CD8^+$ cytotoxic T cells might be possibly activated with antigen-presenting cells by KB290 feeding.

KEGG analysis revealed that the up-regulated genes are enriched in NK cell-mediated cytotoxicity. NK cell function is regulated by a multitude of receptors, including the activating NK cell p46-related protein (NKp46) receptor, which also helps regulate NK cell interactions with other immune cells[51]. Vimentin, which was up-regulated in the spleen of treated mice, is a direct ligand of NKp46 and can induce the cytolytic activity of NK cells[52]. NKp46 non-covalently associates with the immunoreceptor tyrosine-based activation motif-containing adaptor proteins CD3ζ and/or the γ chain of fragment crystallisable receptors I (FcRI) for IgE (FcεRIγ)[53]. Although we failed to detect any apparent change in *Nkp46* expression, *Fcer1γ* and genes encoding other signal transduction molecules, such as spleen tyrosine kinase (SYK), phosphatidylinositol 3-kinase (PI3K) and RAC, were up-regulated by KB290 ingestion. Moreover, *Dap12* associated with multiple cell surface-activating receptors was also found to be up-regulated by the microarray and quantitative RT-PCR analyses. DAP12 contains an immunoreceptor tyrosine-based activation motif, which recruits SYK to trigger the cytotoxic cascade and cytokine release[54]. Genes encoding Src homology region 2 domain-containing phosphatase 1 (SHP-1) implicated in the inhibitory pathway were up-regulated at the same time as activating signal transduction genes. SHP-1 suppresses the vav oncogene-ras-related C3 botulinium toxin substrate (VAV–RAC) pathway implicated in NK cytotoxicity through an early dephosphorylation of Vav[55]. RAC is downstream of not only VAV but also PI3K in the signalling pathway that activates NK cytolytic function[56]. In considering that genes encoding PI3K and RAC were up-regulated, it is more likely that the pathway can be activated. Contrarily, perforin, granzyme B and FasL, which are cytotoxic molecules, were not up-regulated. However, it has been reported that up-regulated components of mitogen-activated protein kinase

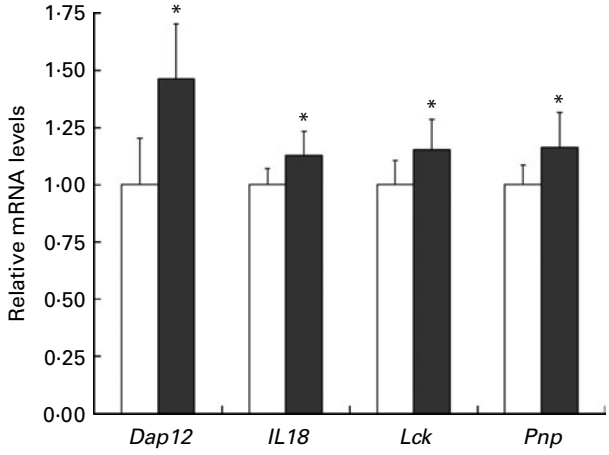

Fig. 6. Relative *Dap12* and *Il18* mRNA levels in the spleen of mice ingested *Lactobacillus brevis* KB290 (KB290). The relative expression ratio ($2^{-\Delta\Delta Ct}$) of each target gene in mice fed the KB290 (3×10^9 colony-forming units/g) diet (■) compared with those fed the control diet (□). The target gene expression was normalised to that of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*). Values are means, with standard deviations represented by vertical bars (*n* 6). * Mean values were significantly different compared with the control group ($P<0{\cdot}05$; Student's *t* test).

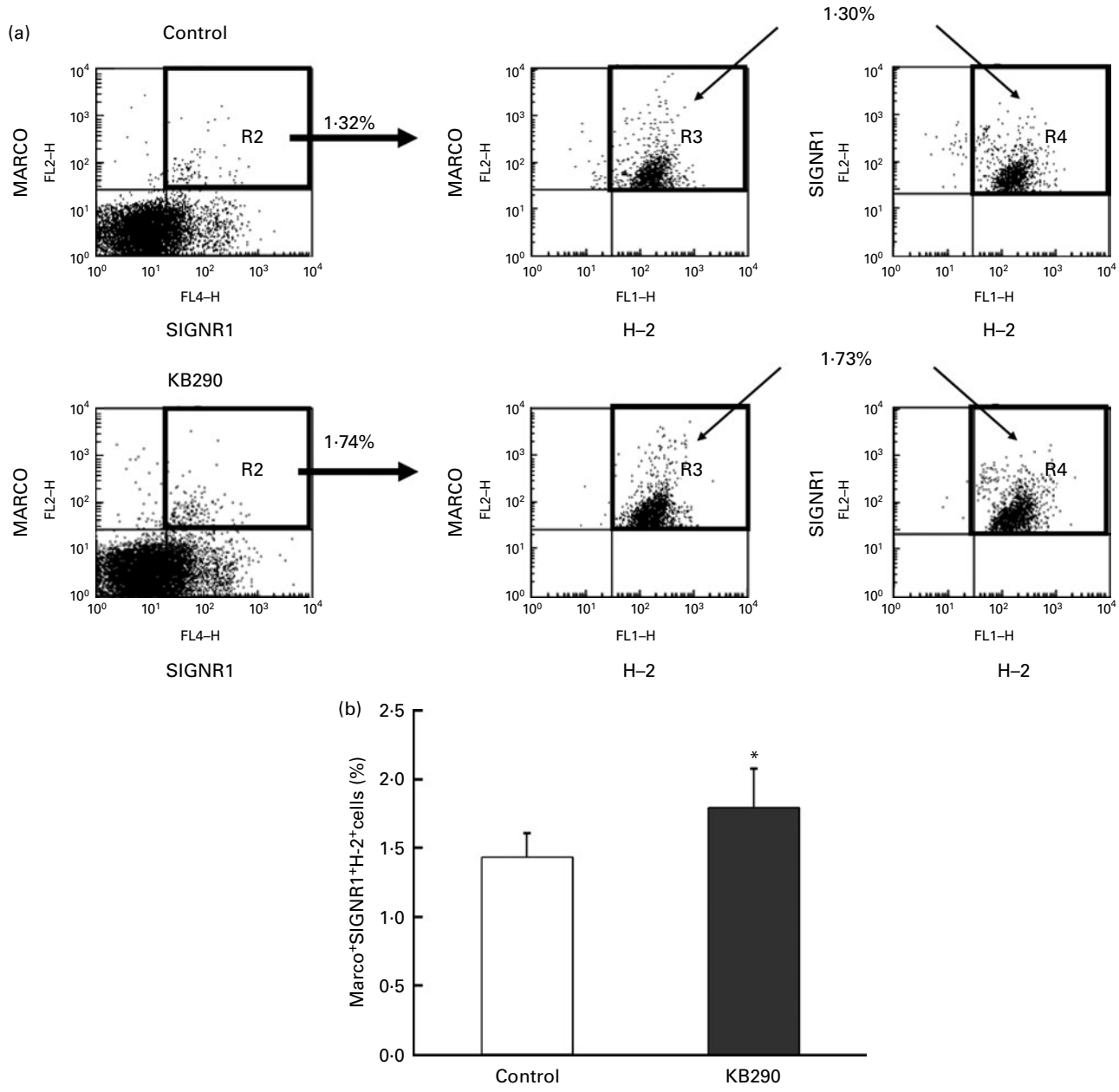

Fig. 7. Effect of *Lactobacillus brevis* KB290 (KB290) on the proportion of marginal zone macrophages in splenocytes. Splenocytes were collected from mice fed the KB290 or control diet for 1 d and analysed for MARCO, SIGNR1 and histocompatability-2 (H-2) expression. (a) Staining with anti-MARCO and anti-H-2 is shown in the right panels for selected $MARCO^+$ $SIGNR1^+$ cells (as shown in the left panels). Percentage of positive cells is indicated in each quadrant. One representative sample for each group is shown. (b) The percentage of $MARCO^+$ $SIGNR1^+$ $H\text{-}2^+$ cells among the total cells is shown. ■, Control group; □, KB290 group. Data from one of two experiments with similar results are shown. Values are means, with standard deviations represented by vertical bars (n 6). * Mean value was significantly different compared with the control group ($P<0{\cdot}05$; Student's t test). FL2-H, fluorescence channel 2 height; FL4-H, fluorescene channel 4 height; FL1-H, fluorescence channel 1 height. Fluorescein isothiocyanate was detected in FL1, R-phycoerythrin was measured in FL2-H and Alexa Fluor 647 was detected in FL4.

(MAPK) and cytotoxicity pathways are possibly associated with effective cytotoxicity function[57]. For these results, there is a possibility that the cytotoxic activity of NK cells was enhanced in the spleen of mice fed KB290. However, the up-regulated genes in the NK cell activating signalling pathway were involved in signalling in other cell types. LCK, PI3K and SHP-1 are components of the TCR signalling pathway (KEGG pathway). PI3K, RAC, SHP-1 and SKY are included in the B-cell receptor signalling pathway (KEGG pathway). Further studies are required to define which cells are responsible for the enhancement of cell-mediated cytotoxic activity by KB290.

DC and macrophages reside in Peyer's patches, capturing antigen shuttled through M-cells. In the lamina propria, DC extend their dendrite in the intestinal lumen, and directly sample intestinal bacteria[58]. Therefore, these cells are important in responding to commensal bacteria in the intestine. Probiotics can induce IL-12 in lymph node DC, activating

NK cells to produce interferon-γ and to increase their cytolytic potential[13]. On the other hand, the concentration of bacterial peptidoglycan in serum correlates with the concentration in faeces[59]. Peptidoglycan stimulates innate immunity, inducing macrophages to produce cytokines. The administration of lipopolysaccharide, zymosan or *Listeria monocytogenes* to mice results in the up-regulation of MARCO[60]. These observations suggest that treatment with bacterial components may not only cause an increase in the percentage of MARCO-positive cells capable of participating in bacterial phagocytosis, but they also induce an increase in the amount of bacterial uptake by individual macrophages. Actually, specific markers of MZM, which capture blood-borne particles, were up-regulated in the spleens of KB290-fed mice in the present study. Some genes that contribute to phagocytosis and macrophage antigen presentation were also up-regulated on 1 d. Furthermore, the proportion of MZM by flow cytometric analysis was significantly increased by KB290 ingestion. These finding shed light on the new aspect of the immunomodulatory effect of probiotics. Further studies in gut-associated lymphoid and cardiovascular tissue are required to define whether bacterial components reach the spleen via the circulatory system or activated DC migrate to the spleen through the mesenteric lymph.

In summary, high-dose KB290 (about 1×10^{10} cfu) feeding for 1 d enhanced the cell-mediated cytotoxic activity of splenocytes. The response could be due to the activation of NK cells following stimulation, with natural cytotoxicity receptor ligand and/or $CD8^+$ cytotoxic T cells being stimulated via MHC class I presentation. To our knowledge, the present study is the first to define the effect of ingested probiotics on changes in mouse spleen gene expression using DNA microarray analysis associated with the enhancement of cytotoxic activity. However, the present investigation covered only one tissue on day 1; gene expression changes need to be studied over time and in other tissues to uncover the mechanisms underlying the enhanced cytotoxic effect seen in animals fed KB290.

Supplementary material

To view supplementary material for this article, please visit http://dx.doi.org/10.1017/S0007114513000767

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