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Lipoteichoic acid from *Lactobacillus rhamnosus GG* as an oral photoprotective agent against UV-induced carcinogenesis

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Abstract

Probiotics are live microorganisms that when administered in adequate amounts confer a health benefit on the host. Cell surface molecules of these micro-organisms are being studied in relation to their ability to interact with the host. The cell wall of *Lactobacilli* possesses lipoteichoic acids (LTA) which are molecules with immunomodulatory properties. UV radiation (UVR) has been proposed as the main cause of skin cancer because of its mutagenic and immunosuppressive effects. Photoprotection with some nutrition interventions including probiotics has recently been shown. The aim of the present study was to investigate whether the oral administration of purified LTA from *Lactobacillus rhamnosus GG* can modulate the immune-suppressive effect of UVR and skin tumour development in female Crl:SKH-1-hrBR mice. For this purpose, two irradiation models were studied: (1) a chronic irradiation scheme consisting of daily irradiations during twenty consecutive days and (2) a long-term irradiation schedule, irradiating the animals three times per week, during 34 weeks for tumour development. The results showed that T-cells in the inguinal lymph node of LTA-treated mice produced higher levels of (1) interferon-γ and (2) a number of total, helper and cytotoxic T-cells compared with non-treated mice. Moreover, a significant delay in tumour appearance was found in LTA-treated mice. An increased IgA^{+} cell number was found in the small intestine together with a higher number of activated dendritic cells in the mesenteric lymph nodes. The latter results might be indicative of a direct effect of LTA in the gut, affecting the cutaneous immune system and restoring homeostasis through the gut–skin axis.

Key words: UV radiation: Probiotics: Skin cancer: Lipoteichoic acid

At the beginning of the nineteenth century, Elie Metchnikoff wrote his work *The Prolongation of Life: Optimistic Studies*[1] where he stated that Bulgarians owed their longevity to the consumption of soured milk. Even if he did not use the term ‘probiotic’, which was first introduced in the 1960s[2], Metchnikoff had already identified yogurt as a functional food with important health benefits beyond nutrition.

Probiotics are defined as ‘live microorganisms that when administered in adequate amounts confer a health benefit on the host’[3]. These micro-organisms include different yeast and bacterial strains. The most studied bacterial genus within probiotics is *Lactobacillus*. *Lactobacilli* are lactic acid bacteria, associated with fermented foods mainly for their contribution to raw food preservation due to acidification and also because of their capacity to contribute to product characteristics such as flavour and texture[4]. Nutritional advantages of probiotics basically consist of preventive–curative effects against diseases including intestinal dysfunctions, gastrointestinal infections, inflammatory bowel disease and, possibly, colon cancer[5].

Industrial interest on health claims related to probiotics has been a great impulse to molecular research on the host–probiotic interaction. Cell surface molecules and extracellular components of these micro-organisms are being studied in relation to their ability to interact with the host.

The cell wall of *Lactobacilli* comprises peptidoglycan and teichoic acids. A great number of biological functions have been described for teichoic acid, e.g. surface protein binding, phage adsorption, cellular adhesion and interaction with the immune system. There are two types of teichoic acid described in *Lactobacilli*: wall teichoic acids, which are bound to *N*-acetyl muramic acid of the peptidoglycan, and lipoteichoic acids (LTA), which are anchored to the cytoplasmic membrane through a glycolipid.

In terms of structure, LTA from *Lactobacilli* are composed of poly-glycerol-phosphate (poly(Gro-P)), which are decorated

Abbreviations: ConA, concanavalin A; DC, dendritic cells; GALT, gut-associated lymphoid tissue; ILN, inguinal lymph node; LTA, lipoteichoic acid; MLN, mesenteric lymph nodes; RPMI, Roswell Park Memorial Institute; UVR, UV radiation.

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by D-alanyl esters. D-Alanylation in *Lactobacilli* is very important since it is involved in bacterial resistance to physicochemical conditions of the gastrointestinal tract and to defensins produced by the epithelial cells of the intestine[6–9].

Regarding its immunomodulatory effect, it is known that LTA can be recognised by Toll-like receptor type 2. After ligand binding, Toll-like receptor type 2 sequentially recruits the adaptor molecules MyD88, the IL-1 receptor-associated kinase and the TNF receptor-associated factor 6. In turn, these adaptor molecules activate the IκB kinase complex and the mitogen-activated protein kinases Jun N-terminal kinase, p38 and extracellular signal-regulated kinases 1 and 2, leading to the activation of NF-κB and activator protein 1, which results in the transcription of soluble mediators such as cytokines and chemokines[10,11].

LTA is one of the most important antigens in *Lactobacilli*, as it has a key role in the crosstalk between the host and bacteria in the intestine. The release of soluble mediators after the LTA interaction with the epithelial and immune cells present in the intestine generates an inflammatory microenvironment and the recruitment of certain cell types, such as T- and B-cells. LTA activates B-cells in the lamina propria, causing the Ig class switch of these cells with the consequent production of secretory IgA, the main Ig involved in intestinal immunity[12].

The effect of the oral administration of purified LTA has not been extensively studied. Literature data indicate its potential use in the prevention of group B streptococci infections in newborns. Cox *et al.* have evaluated LTA excretion and toxicity after oral administration to rabbits. In these studies, the amount of LTA administered was about 2–3 μg/g of animal weight, and they found LTA excretion in the urine and faeces until 4 d after the ingestion, with a peak of excretion at 24 h. These authors did not find any pathological alteration in the liver, spleen or kidneys of animals receiving LTA[13]. Nevertheless, no study regarding LTA bioavailability has been performed to date.

UV radiation (UVR) is indispensable for life on earth; however, prolonged exposures can be dangerous for human health. UVR has been proposed as the main cause of skin cancer such as basal and squamous cell carcinoma and cutaneous malignant melanoma[14]. UVR causes direct damage to cellular DNA, tissue inflammation, immune response suppression and free radical formation with the consequent oxidation of proteins, lipids and DNA[15,16]. Additionally, it has been demonstrated that chronic irradiation causes epidermal hyperplasia[17]. Hyperplasia is a key event in skin carcinogenesis, specifically in non-melanoma tumours (mainly basal and squamous cell carcinomas) where keratinocytes are the affected cell type. Hyperplasia is a result of both increased epidermal proliferation and apoptosis suppression[18]. Nevertheless, the loss in proliferation control is not the only event related to UVR-induced tumorigenesis. UV-mediated immunosuppression has been recognised as a condition for skin tumour development[19]. Furthermore, another consequence of chronic UVR is increased inguinal lymph node (ILN) cellularity in the absence of antigenic stimuli[20]. Even if the mechanism underlying this event is unclear, it has been postulated as a prerequisite for further immunosuppression. One possibility is that the increase in the number of cells is the result of cell migration from other organs[21,22].

Over the last 30 years, the immunosuppressive effect of UVR has been studied and described. There is growing evidence about the key role of UV-induced regulatory T-cells during photocarcinogenesis, since they are capable of inhibiting antitumoral effector functions. After UVR exposure, a cytokine cascade is initiated biasing the immune response towards a T helper 2 or T regulatory phenotype, which finally leads to the emergence of $CD4^{+}$-$CTLA4^{+}$ regulatory T-cells. IL-10 and IL-4 are the main cytokines involved in UV-induced immunosuppression. They are produced by T-cells, keratinocytes and other cell types in the skin after irradiation[23–25].

The relationship between the gut and the cutaneous immune systems is not clear; however, there are evidences about the existence of a crosstalk between them. The beneficial effect of probiotic consumption on atopic eczema[26], the development of specific IgA antibodies in gut-associated lymphoid tissue (GALT) after transcutaneous immunisation[27] and the re-establishment of skin homeostasis due to probiotic consumption after UV irradiation are evidences for the existence of a gut–skin axis susceptible to modulation with therapeutic ends[28].

Recently, photoprotection induced by specific nutrients has been demonstrated to be successful in preventing some of the harmful effects of UVR[29,30]. Over the last few years, probiotics have emerged as a new strategy in systemic photoprotection[30]. Gueniche *et al.*[27] showed that a 10 d supplementation with a specific probiotic (*Lactobacillus johnsonii*) was able to revert some of the immunosuppressive effects of UVR in female SKH:hr1 hairless mice.

Based on this bulk of knowledge, the aim of the present study was to investigate whether the oral administration of purified LTA from *Lactobacillus rhamnosus GG* (one of the most characterised probiotics)[31,32] can modulate the immune-suppressive effect of UVR and prevent skin tumour development in female SKH:hr1 hairless mice. In this sense, anti-inflammatory cytokines such as IL-10 and IL-4 were measured in cell-free culture supernatants of ILN and spleens from irradiated mice receiving LTA or PBS, or from a non-irradiated control group. Additionally, total T-cells ($CD3^{+}$) were determined in the epidermis and in the ILN, as well as helper ($CD3^{+}CD4^{+}CD8^{-}$) and cytotoxic T-cells ($CD3^{+}CD4^{-}CD8^{+}$). Furthermore, the effect of LTA ingestion on GALT was analysed to study the mechanisms underlying its immunomodulatory effect. Total IgA^{+} cells were determined in the lamina propria, and dendritic cells (DC) ($CD11c^{+}$), activated DC ($CD11c^{+}CD80^{+}$) and total activated antigen-presenting cells ($CD80^{+}$) were determined in the mesenteric lymph nodes (MLN).

For the present analysis, two irradiation models previously described by our group were used: a chronic irradiation scheme consisting of daily irradiations during twenty consecutive days, and a long-term irradiation schedule, irradiating the animals three times a week, during 34 weeks for tumour development[17,33].

Our hypothesis was that the oral administration of LTA would modulate the GALT and that through the gut–skin immune axis, this would restore skin homeostasis affected by UVR, reducing UVR-induced tumorigenesis.

Materials and methods

Lipoteichoic acid purification

LTA was isolated as described previously by Morath *et al.*[34]. Briefly, a previously established inoculum of *L. rhamnosus* GG (American Type Culture Collection 53 103) was cultured for 16 h in MRS broth (Britannia). Bacteria were harvested by centrifugation and washed three times with PBS. The pellet was mixed with an equal volume of *n*-butanol, under stirring for 30 min at room temperature. After centrifugation at 13 000 ***g*** for 20 min, the aqueous phase was lyophilised, resuspended with chromatography starting buffer (15 % *n*-propanol in 0·1 M-ammonium acetate, pH 4·7) and centrifuged at 45 000 ***g*** for 15 min. The supernatant was subjected to hydrophobic interaction chromatography in an octyl-sepharose matrix (GE Healthcare Life Sciences) eluting LTA with an increasing gradient of propanol. LTA-containing fractions were concentrated using vacuum centrifugation (Automatic Environmental Speedvac-Savant-Thermo) in order to eliminate *n*-propanol. LTA preparation was tested for purity by Western blot, as described previously by Dogi *et al.*[35], and by spectrophotometry, as described previously by Kim *et al.*[36].

Animal models and lipoteichoic acid administration

Female Crl:SKH-1-hrBR hairless mice between 8 and 12 weeks of age (20–25 g), purchased from Charles River Laboratories, were housed in quarters with a 12 h light–12 h dark cycle and maintained with water and food *ad libitum*.

The animals were irradiated on their back with UV light using an 8 W UVM-28 Mid-Range Wave (302 nm) lamp from Ultraviolet Products, which emits most of its energy within the UVB range (emission spectrum 280–370 nm) with a peak at 302 nm and including a 20–30 % amount of UVA. The lamp was calibrated with a UVX radiometer (Ultraviolet Products), and its power was determined as 1·2 mW/cm^2. Mice subjected to the chronic irradiation schedule (twelve animals) were exposed for 42 s to generate a dose of 50 mJ/cm^2 of UV corresponding to 0·25 minimal erythema dose. These animals were irradiated daily for twenty consecutive days. Mice in the long-term irradiation experiment (sixteen animals) were irradiated on their back with 50 mJ/cm^2, 0·25 minimal erythema dose every 2 d for a period of 34 weeks. These irradiation models have previously been established by our group[17,33]. In both irradiation schedules, half of the irradiated mice received 100 μl of LTA solution orally (1 mg/ml) in PBS before the irradiation, and the other half received 100 μl PBS. The solutions were orally administered by means of a feeding needle (Thomas Scientific).

Simultaneously to each irradiation scheme, a group of mock-irradiated sex- and age-matched mice were used as controls, and handled in the same fashion as the irradiated animals. A total of six control female mice were included in the chronic model and eight in the long-term irradiation scheme. At 24 h after the last UV irradiation, both chronically and long-term irradiated mice were killed using a CO_2 gas chamber, and dorsal skin samples, ILN and spleens were removed. From chronically irradiated mice, four MLN and a small-intestinal section were obtained. The procedures involving animals were in compliance with the research animal use guidelines established by the Consejo Nacional de Investigaciones Científicas y Técnicas (Argentina) and were approved by the Review Board of Ethics of the Instituto de Estudios de la Inmunidad Humoral.

Histology and epidermal thickness determination

Specimens for histological examination were obtained from the skin of the irradiated area, fixed with 4 % neutral formalin and embedded in white paraffin. Serial paraffin sections, 4 μm thick, were prepared and stained with haematoxylin and eosin. At least three independent measurements were performed in two different slides per mouse. The observation and photography were performed using an Olympus BX-51 microscope (Olympus) with a Q color 3 Olympus digital camera. Epidermal thickness was measured with Image Pro 5.1.0.2 for Windows (Media Cybernetics).

Epidermal cell isolation

Skin samples of 1 cm^2 were taken from each mouse. The samples were incubated with 25 mg/ml of dispase (Invitrogen) in Roswell Park Memorial Institute (RPMI) medium for 2 h. After incubation, the epidermis was easily separated from the dermis. The epidermis was then manually dispersed with a tissue homogeniser (Thomas Scientific), passed through a 50 μm filter and cells were counted and prepared for flow cytometric analysis.

IgA$^+$ cell count in the lamina propria

The number of IgA$^+$ cells was determined on small-intestinal histological sections by a direct immunofluorescence assay. After deparaffinisation by immersion in xylene and rehydration in a graded ethanol series, paraffin sections (4 μm) were incubated with a 1:100 dilution of FITC-α-chain monospecific antibody (Bethyl) for 30 min and observed with an Olympus BX-51 fluorescence light microscope. The number of fluorescent cells was counted in forty fields at 1000×.

Measurement of apoptotic cells by the TUNEL

After deparaffinisation by immersion in xylene and rehydration in a graded ethanol series, the percentage of cells with DNA strand breaks in the assay epidermis was measured on paraffin sections (4 μm) using the TUNEL method, which detects digoxigenin-labelled 3′-OH ends of genomic DNA. Briefly, cells with DNA strand breaks were detected *in situ* using the ApopTag Plus Peroxidase *In Situ* Apoptosis Detection Kit (CHEMICON International) according to the manufacturer's instructions. Counterstain was performed with eosin. Total and apoptotic cells in the epidermis (combined basal and suprabasal layers) were counted in ten representative 400× magnification fields using an Olympus BX-51 microscope (Olympus).

In vitro proliferation

ILN and spleens from UV-irradiated or control mice were manually dispersed with a tissue homogeniser (Thomas Scientific)

and cells were counted and plated in ninety-six-well plates. Cells (4×10^5) were plated in replicates of three, together with 100 μl RPMI (Gibco) supplemented with 10 % fetal calf serum, streptomycin (100 μg/ml) and penicillin (100 U/ml), as described elsewhere[37]. Cells were incubated with the non-specific T-cell mitogen concanavalin A (ConA) (Sigma) at 4, 2, 1, 0·5 and 0·25 μg/ml. A basal proliferation control was also performed without the mitogen. After 72 h of incubation at 37°C with 5 % CO_2, supernatants were collected for cytokine determination.

Cytokine quantification in culture supernatants

IL-4, IL-10 and interferon-γ levels were measured by ELISA using the OPTEIA system (BD Biosciences) according to the manufacturer's instructions in cell-free culture supernatants of the cells treated with 4 μg/ml of ConA.

Flow cytometric analysis

The following anti-mouse antibodies were purchased from BD Biosciences: Alexa Fluor 647-anti-CD4, PE-anti-CD8, PE-anti-CD11c, FITC-anti-CD80 and FITC-anti-CD3ε with their corresponding isotype controls.

For staining of surface markers, lymph nodes and epidermal cells were incubated with antibodies diluted in staining buffer (PBS, 10 % fetal calf serum) for 30 min at 4°C, washed, and then fixed in 0·2 ml of 2 % formaldehyde (in PBS). Data were acquired on a PAS III cytometer (PARTEC) and analysed using Cyflogic software 1.2.1 (CyFlo Limited).

Tumour number and size

Mice were carefully examined once per week during the whole long-term irradiation model. The location and growth of each tumour exceeding 1 mm in diameter was determined and measured with a dermatoscope (MG13180).

Statistical analysis

All values are presented as means with their standard errors. Statistical significance was evaluated using one-way or two-way ANOVA, according to the experimental design. When variables had a normal distribution and showed homoscedasticity, a parametric ANOVA and Student–Newman–Keuls *post hoc* test was used. When samples did not have a normal distribution and did not show heteroscedasticity, a non-parametric ANOVA and Dunn *post hoc* test was used. Kaplan–Meier survival curve analysis was performed using log-rank and Wilcoxon–Gehan analysis. Graphical and statistical analyses were performed with GraphPad Prism 5.0 (GraphPad Software) and GraphPad Instat 2.0 (GraphPad Software), respectively. Values were considered significantly different at $P<0·05$.

Results

Histology and epidermal thickness determination

The effects of oral LTA administration on histological alterations produced in the epidermis by UV exposure were studied in skin sections, both in the chronic irradiation model and in the long-term irradiation scheme. An increase in epidermal thickness was observed in chronically irradiated mice receiving LTA and PBS (36·37 (se 7·20) μm and 44·69 (se 8·81) μm, respectively) compared with the control group (26·42 (se 2·82) μm) ($P<0·05$). No differences were found between the LTA and PBS treatments. Similar results were observed in the long-term irradiated mice. Those animals receiving LTA and PBS had an epidermal thickness of 68·05 (se 5·56) μm and 65·92 (se 6·38) μm, respectively, whereas the value for the control group was significantly lower (18·20 (se 1·42) μm) ($P<0·05$; Table 1).

Apoptotic cell percentage in the epidermis

In both irradiation models, UV exposure induced significant levels of apoptosis. Chronically irradiated mice receiving LTA had 22·43 (se 1·38) % of apoptotic cells, this value was not significantly different from the percentages obtained in mice treated with PBS which had 22·35 (se 0·89) % (Fig. 1(a)). The percentage of apoptotic cells in the control group was 12·55 (se 0·81) %, which was significantly lower ($P<0·05$) than that obtained in irradiated mice. Long-term irradiated mice receiving LTA had 31·26 (se 2·08) % of apoptotic cells, this percentage was not statistically different from that obtained in mice receiving PBS (28·76 (se 1·77) %). Percentages obtained in the control group were significantly lower than those obtained in irradiated mice (17·62 (se 1·66) %, $P<0·05$; Fig. 1(b)).

Epidermal T-cell number

UVR caused a decrease in epidermal T-cell percentage in both irradiation schemes. Chronically irradiated mice receiving

Table 1. Mean epidermal thickness, epidermal $CD3^+$ cells and inguinal lymph node (ILN) cell number in chronically and long-term irradiated mice (Mean values with their standard errors)

	Chronic irradiation						Long-term irradiation					
	Control		LTA		PBS		Control		LTA		PBS	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Epidermal thickness (μm)	26·42[b]	2·82	36·37[a]	7·20	44·69[a]	8·81	18·20[b]	1·42	68·05[a]	5·56	65·92[a]	6·38
Epidermal $CD3^+$ cells (%)	7·14[b]	0·49	0·91[a]	0·18	0·61[a]	0·05	5·99[b]	0·45	0·32[a]	0·08	0·87[a]	0·16
ILN cell number ($\times 10^7$)	1·14[a]	0·14	2·10[b]	0·16	1·50[a]	0·14	0·89[a]	0·15	3·35[b]	0·31	4·69[c]	0·27

* Mean values were significantly different ($P<0·05$).

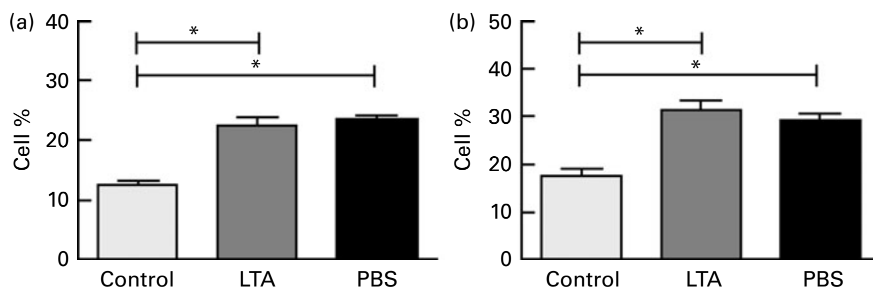


Fig. 1. Percentage of TUNEL-positive cells in the epidermis after (a) chronic and (b) long-term irradiation from control mice and from mice receiving lipoteichoic acid (LTA) or PBS. Values are means (n 6 for chronically irradiated and n 8 for long-term irradiated mice), with standard errors represented by vertical bars. * Mean values were significantly different ($P<0·05$).

LTA had 0·91 (SE 0·18) % of epidermal T-cells, this value was not different from that obtained in mice treated with PBS which had 0·61 (SE 0·05) % of T-cells. The percentage obtained in the control group was significantly higher than that observed in both irradiated groups (7·14 (SE 0·49) %, $P<0·05$). Long-term irradiated mice receiving LTA had 0·32 (SE 0·08) % of epidermal T-cells, whereas the animals administered with PBS had 0·87 (SE 0·16) %, these percentages were not statistically different from each other; however, the latter T-cell percentages were found to be lower than that obtained in the control group ($P<0·05$) which was 5·99 (SE 0·45) % (Table 1).

Cytokine production by inguinal lymph node and spleen T-cells

When analysing cytokine production in ConA-stimulated ILN cells, chronically irradiated mice receiving LTA and PBS showed an increase in IL-4 and IL-10 production compared with the control animals ($P<0·05$). IL-4 production in LTA-treated mice was 240·70 (SE 37·82) pg/ml, a value that was not statistically different from the production in PBS-treated mice which was 252·20 (SE 36·47) pg/ml. The production of IL-4 in the control group was 83·92 (SE 17·67) pg/ml (Fig. 2(a)). In the case of IL-10, the production in mice administered with LTA

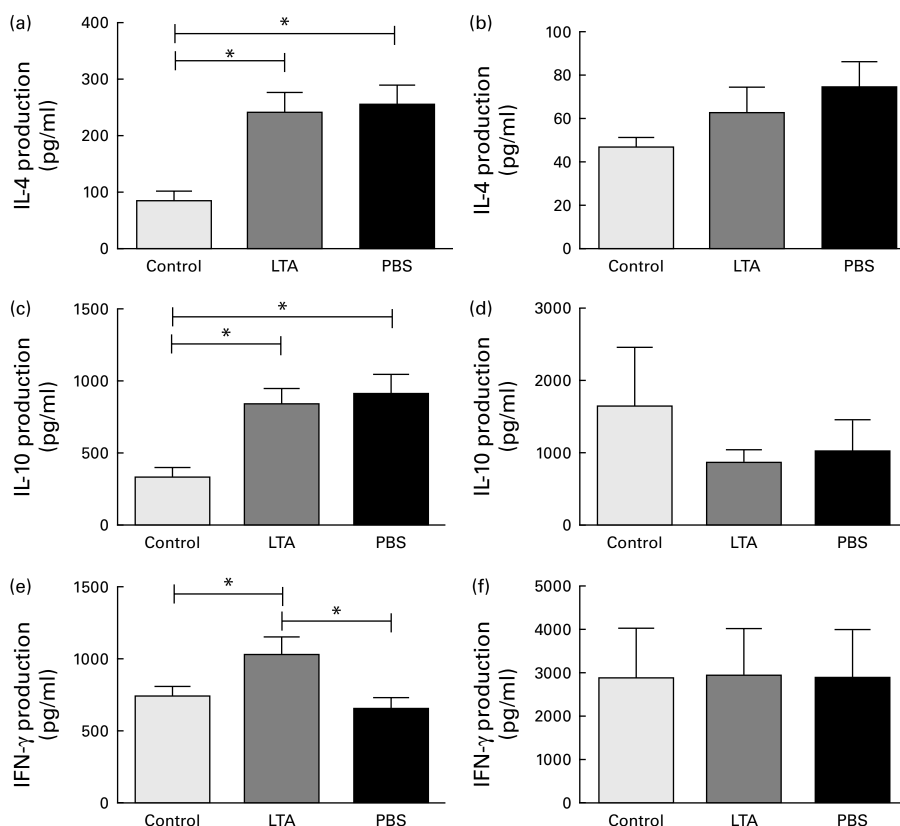


Fig. 2. Cytokine production in inguinal lymph node cells from (a, c, e) chronically and (b, d, f) long-term irradiated mice receiving lipoteichoic acid (LTA) or PBS and from the non-irradiated control group. Values are means (n 6 for chronically irradiated and n 8 for long-term irradiated mice), with standard errors represented by vertical bars. * Mean values were significantly different ($P<0·05$). IFN-γ, interferon-γ.

378 was 838·50 (SE 109·40) pg/ml, a value that was not different from
379 that in PBS-administered animals which was 921·00
380 (SE 121·50) pg/ml. The production of IL-10 in the control
381 group was 340·70 (SE 76·78) pg/ml (Fig. 2(c)). Interferon-γ
382 production was significantly increased in mice treated with
383 LTA (1033·10 (SE 126·15) pg/ml) compared with PBS-treated
384 (658·20 (SE 73·86) pg/ml) and non-irradiated mice (733·80
385 (SE 70·43) pg/ml) ($P<0·05$; Fig. 2(e)). In long-term irradiated
386 mice, no statistical differences were detected for any cytokine
387 (Fig. 2(b), (d) and (f)). No statistical differences were found
388 in cytokine production by ConA-stimulated spleen cells
389 from none of the groups in both irradiation models (data
390 not shown).

391 ### *Total cell count in the inguinal lymph node and spleen*

392 In the chronically irradiated animals, the number of ILN cells in
393 LTA-treated mice was 2·10 (SE 0·16) $\times 10^7$, a value that was sig-
394 nificantly higher than the number obtained in PBS-treated (1·50
395 (SE 0·14) $\times 10^7$) and control mice (1·14 (SE 0·14) $\times 10^7$). The
396 long-term irradiated animals showed an increase in ILN cell
397 number compared with control mice (0·89 (SE 0·15) $\times 10^7$,
398 $P<0·05$). The number of ILN cells in the PBS-treated group
399 (4·69 (SE 0·27) $\times 10^7$) was also significantly higher ($P<0·05$)
400 than those in LTA-treated mice (3·35 (SE 0·31) $\times 10^7$) (Table 1).
401 No differences were found in spleen cell number in either of the
402 irradiation schemes (data not shown).

403 ### *Phenotypic distribution of inguinal lymph node T-cell*
404 ### *populations*

405 In order to assess the effect of skin irradiation on ILN, helper
406 ($CD3^+CD4^+CD8^-$), cytotoxic ($CD3^+CD4^-CD8^+$) and total
407 T-cell ($CD3^+$) number were determined by flow cytometry.
408 After chronic irradiation, helper T-cell number was significantly
409 higher ($P<0·05$) in LTA-treated mice (19·98 (SE 0·83) $\times 10^6$ than
410 in the PBS-treated (13·01 (SE 2·50) $\times 10^6$) and control (11·69 (SE
411 1·35) $\times 10^6$) groups. As for cytotoxic T-cells, the results were
412 similar, with a significant increase detected in the LTA group
413 (13·23 (SE 0·73) $\times 10^6$) compared with PBS-treated (6·93 (SE
414 1·13) $\times 10^6$) and control (7·19 (SE 0·73) $\times 10^6$) mice ($P<0·05$).
415 Total T-cell number in LTA-treated mice was 35·00 (SE
416 1·61) $\times 10^6$, this number was significantly higher ($P<0·05$)
417 than the number obtained in PBS-treated (22·12 (SE
418 3·99) $\times 10^6$) and control (22·83 (SE 2·51) $\times 10^6$) mice
419 (Fig. 3(c), (e) and (g)). In the long-term irradiation model,
420 total T-cell number was significantly increased in PBS-treated
421 mice (37·33 (SE 1·98) $\times 10^6$) compared with LTA-treated
422 (26·50 (SE 2·68) $\times 10^6$) and control (26·13 (SE 3·16) $\times 10^6$)
423 mice ($P<0·05$). Helper T-cell number was also significantly
424 increased ($P<0·05$) in PBS-treated mice (16·13 (SE
425 0·98) $\times 10^6$) compared with LTA-treated (10·64 (SE
426 1·12) $\times 10^6$) and control mice (8·02 (SE 1·19) $\times 10^6$). The
427 number of cytotoxic T-cells was also significantly higher
428 ($P<0·05$) in PBS-treated mice (16·15 (SE 1·12) $\times 10^6$) than in
429 LTA-treated (8·26 (SE 0·88) $\times 10^6$) and control (8·54 (SE
430 1·26) $\times 10^6$) mice (Fig. 3(d), (f) and (h)).

IgA^+ cell count in the small-intestinal lamina propria 431

The number of IgA-producing B-cells in the lamina propria 432
(Fig. 4(b)) of the chronically irradiated animals treated with 433
LTA was 804·00 (SE 18·26). This value was significantly higher 434
($P<0·05$) than the number found in PBS-treated (427·70 435
(SE 62·76)) and control mice (528·80 (SE 67·12)) (Fig. 4(a)). 436

Antigen-presenting cell analysis in the mesenteric lymph 437
nodes 438

Total DC ($CD11c^+$), activated DC ($CD11c^+CD80^+$) and total 439
activated antigen-presenting cell ($CD80^+$) numbers in the 440
MLN were determined by flow cytometry in the chronic 441
irradiation model. A significant increase in activated DC 442
(Fig. 5(d)) was detected in LTA-treated mice (2410·00 443
(SE 427·20)) compared with PBS-treated (1451·00 (SE 192·80)) 444
and control (994·00 (SE 159·40)) mice ($P<0·05$). Total acti- 445
vated antigen-presenting cell number (Fig. 5(c)) was also sig- 446
nificantly increased ($P<0·05$) in LTA-treated mice (94 721·00 447
(SE 14 788·00)) compared with control (43 634·00 (SE 448
9555·00)) and PBS-treated (12 257·00 (SE 734·00)) mice. No sig- 449
nificant differences were found for the total DC number 450
(Fig. 5(b)) between the groups. 451

Tumour appearance kinetics 452

Tumour appearance was simultaneous in the LTA- and PBS- 453
treated animals and began around week 20. Nevertheless, 454
LTA-treated mice showed a significant slower progression in 455
tumour number, statistically compared with PBS-treated mice 456
(Fig. 6(a)). When the appearance of the fourth tumour was 457
taken as a death event for a death curve analysis, the difference 458
between LTA- and PBS-treated mice was significantly different. 459
Furthermore, the group of LTA-treated mice showed a 4-week 460
delay in the detection of the first animal with four tumours 461
(Fig. 6(b)). No tumour was detected in the control group 462
throughout the study. 463

Average tumour size was not different between the irradiated 464
groups along the study (Fig. 6(c)). 465

Discussion 466

The intake of some micro-organisms causes alterations in the 467
complex interactions between the immune system and intesti- 468
nal microbiota, and this does not only affect the GALT and the 469
other mucosal-associated lymphoid tissues, but it also affects, 470
in some way or another, the whole organism. 471

In the present study, LTA from *L. rhamnosus GG* was 472
employed. *L. rhamnosus GG is* one of the probiotic bacteria 473
with the most impressive scientific support as reviewed by 474
Goldin & Gorbach[32]. This antigen represents about 50 % of 475
the total weight of the *Lactobacilli* cell wall[4] and is one of the 476
strongest immunomodulators in this group of micro-organisms. 477

A model of chronic irradiation, which had already been set up 478
in our laboratory[33], was used to study, first, the effect of LTA 479
ingestion in an irradiation scheme applied for a shorter time 480
than that required to induce tumorigenesis. 481

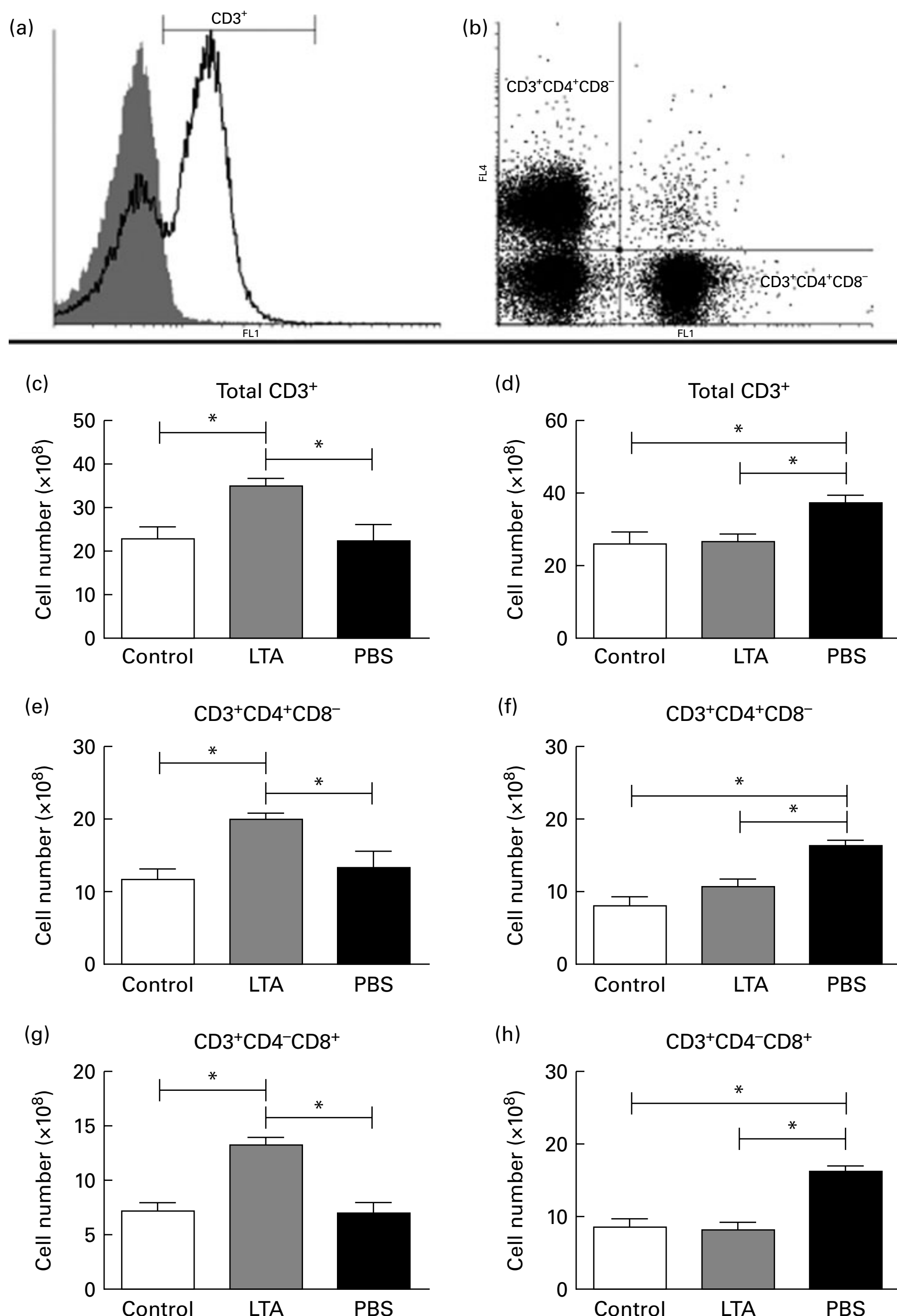
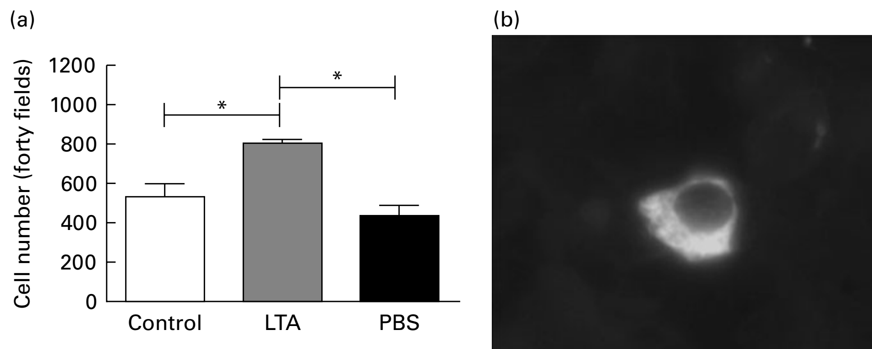


Fig. 3. T-cell populations in (c, e, g) chronically and (d, f, h) long-term irradiated mice receiving lipoteichoic acid (LTA) or PBS and in the non-irradiated control group. Values are means of total cell number (*n* 6 for chronically irradiated and *n* 8 for long-term irradiated mice), with standard errors represented by vertical bars. (a) Histogram showing the $CD3^+$ population and (b) dot plot showing the $CD3^+CD4^+CD8^-$ and $CD3^+CD4^-CD8^+$ cells. * Mean values were significantly different ($P < 0.05$).

Q13 Q14

482 The results demonstrated that LTA ingestion did not improve 483 UVR-induced epidermal cell alterations, since it did not 484 modify the increase in epidermal thickness, the number of 485 apoptotic cells and the decrease in intraepithelial T-cells. LTA 486 consumption did not affect IL-10 and IL-4 production by 487 ConA-stimulated ILN T-cells, which were increased after UVR 488 skin exposure. Nevertheless, T-cells from the LTA-treated ani- 489 mals showed a significant increase in interferon-γ levels, compared with the controls and mice treated with PBS. This 490 result would indicate a partial restoration of homeostasis in 491 the draining lymph nodes, since interferon-γ is a cytokine 492 classically associated with inflammatory processes which 493 would be opposing the effect of IL-10 and IL-4[38]. 494

495 Flow cytometric analysis of ILN T-cell populations showed an 496 increase in total, helper and cytotoxic T-cells in mice treated 497 with LTA. This phenomenon could be associated with the



Q15 **Fig. 4.** (a) IgA-positive cells in the lamina propria of chronically irradiated mice. (b) Small-intestinal samples were stained with FITC-anti-IgA antibodies and positive cells were counted in forty fields per animal. Values are means, with standard errors represented by vertical bars. * Mean values were significantly different ($P<0·05$).

498 increased number of activated DC, which is also observed in the MLN of these mice. Indeed, activated DC activate T-cells that express α4β7 integrin, which binds mucosal addressin cell adhesion molecule-1, a molecule constitutively expressed in the mucosal-associated lymphoid tissues[39]. Recently, Ohmatsu *et al.*[40] have shown that this integrin mediates lymphocyte migration to the skin under inflammatory conditions. Therefore, it is possible that after UVR-induced tissue damage, T-cells already activated in the MLN may migrate to the cutaneous immune system. This phenomenon, in association with the higher number of activated DC in the MLN of LTA-treated mice, might be responsible for the increased number of T-cells in the skin-draining lymph nodes of these animals. 510

511 In order to assess the effect of LTA on the intestinal immune cells, lamina propria IgA^+ cells were counted. After LTA consumption, a significant increase in the number of these cells was observed. B-cell activation by LTA can occur by its binding to Toll-like receptor type 2 or by crosslinking B-cell receptors. LTA can reach lamina propia B-cells directly by diffusion through the intestinal epithelial cells or by internalisation (non-degradative endocytosis) into DC which afterwards present LTA to B-cells in the lamina propria. Once the lamina propria B-cells are activated, they produce IgA with multiple specificities and low affinity. These Ig mediate commensal bacteria exclusion and protection from some pathogens such as rotaviruses and *Salmonella typhimurium*[12]. These results indicate that the oral administration of LTA had a direct effect on the GALT, in particular on B-cells. 525

526 Considering the photoprotective effects of LTA administration on the chronic irradiation model, we decided to evaluate its use in a tumorigenic irradiation model, which we had previously characterised studying non-steroidal anti-inflammatory 529

Q16

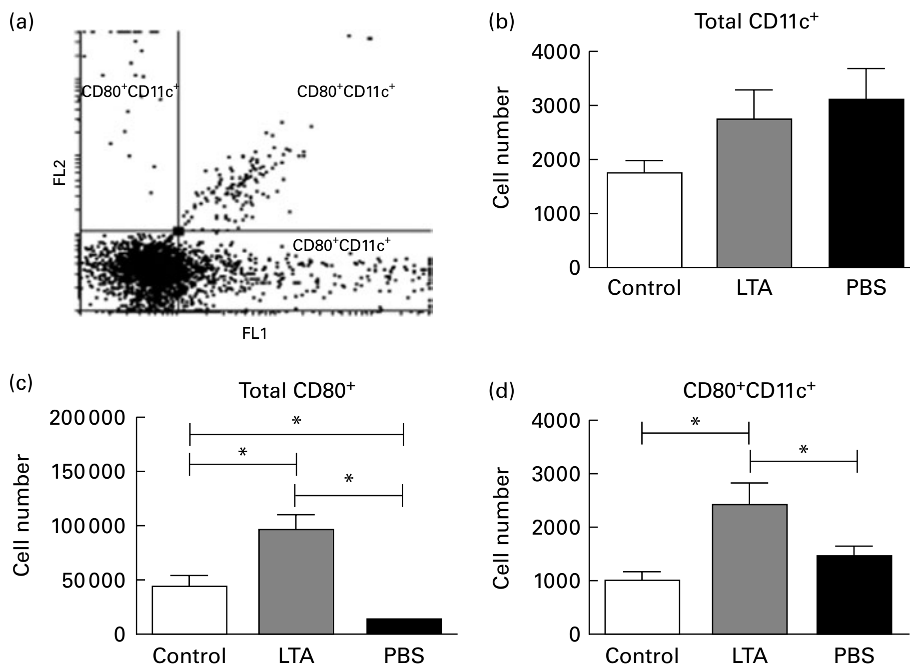


Fig. 5. Antigen-presenting cells (APC) in the mesenteric lymph nodes of chronically irradiated mice were analysed. (b) Total dendritic cells (DC), (c) total activated Q15 APC and (d) total activated DC number were obtained from (a) the dot plot of double staining with anti-CD80 and anti-CD11c. Values are means, with standard errors represented by vertical bars. * Mean values were significantly different ($P<0·05$).

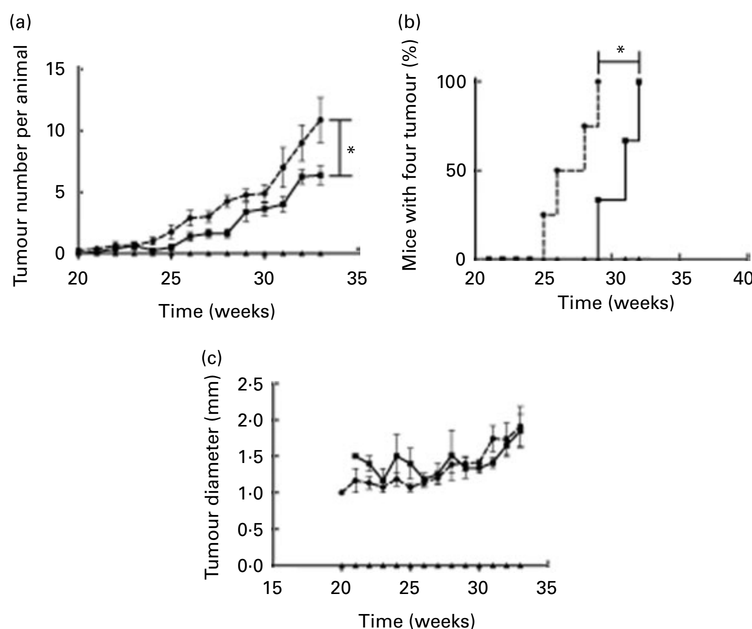


Fig. 6. (a) Tumour appearance kinetics, (b) death curve considering the appearance of the fourth tumour as a death event and (c) tumour size kinetics. Values Q15 are means, with standard errors represented by vertical bars. * Mean values were significantly different ($P< 0·05$). ·■·, PBS; ■, lipoteichoic acid; ▲, control.

530 drugs[17]. The results found in this model showed that LTA 531 consumption delays tumour appearance. The kinetics in the 532 appearance of tumours was significantly slower in the LTA-533 treated animals, since there was a 4-week difference between 534 the appearance of the first animal with four tumours in the 535 PBS-treated group *v.* the LTA-treated group. This period of 536 time is clinically relevant considering life expectancy in hairless 537 mice, which is about 2 years. The effect on tumorigenesis was 538 exclusively associated with the delay in tumour appearance 539 since, once installed, LTA consumption did not affect tumour 540 size progression. The increased cellularity found in the skin-541 draining lymph nodes of PBS-treated mice, associated with 542 the rise in the number of total, helper and cytotoxic T-cells, is 543 probably the consequence of the higher tumour number in 544 this group of animals compared with the LTA-treated or the 545 control group.

546 The amount of LTA given to mice in the present study was 547 based on toxicity assays done by Cox *et al.*[13], who safely admi-548 nistered about 2–3 μg LTA/g of animal weight. In mice weigh-549 ing about 30–35 g, the amount of LTA represents 550 approximately 100 μg. The animals receiving the LTA solution 551 behave exactly in the same usual way after oral administration 552 as did PBS-treated mice. Since LTA was obtained from a probio-553 tic bacteria and was administered in an isotonic solution, no 554 acute responses from mice were expected. Nevertheless, further 555 studies considering different doses should be performed.

556 Finally, in the present study, we found a photoprotective 557 effect of the consumption of an immunostimulant antigen 558 from *L. rhamnosus GG*, a micro-organism with recognised 559 probiotic characteristics, the consumption of which is safe and 560 massive nowadays. This effect is reflected in tumour develop-561 ment delay, and it could be mediated by a transitory increase 562 in cytotoxic and helper T-cells in the draining lymph nodes, 563 as observed in the chronic irradiation model. Even though it is clear that the probiotic effect of a bacterium cannot be just 564 limited to one of its antigens, it is interesting to consider the 565 beneficial effect of the administration of subcellular fractions 566 of these micro-organisms on the host's health. Further studies 567 on the molecular and biochemical mechanisms underlying the 568 effects observed are needed. Moreover, it is also very important 569 to explore new applications and, from the industrial point of 570 view, to develop technologies that allow improving the pro- 571 duction and the incorporation of these molecules to food 572 matrices. 573

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596 of the manuscript and approved the final manuscript. The
597 authors declare that they have no conflicts of interest.

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