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EFFECT OF *BIFIDOBACTERIUM INFANTIS* ON INTERFERON- γ- INDUCED KERATINOCYTE APOPTOSIS: A POTENTIAL THERAPEUTIC APPROACH TO SKIN IMMUNE ABNORMALITIES

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Current management of atopic dermatitis is mainly directed to the reduction of cutaneous inflammation. Since patients with atopic dermatitis show abnormalities in immunoregulation, a therapy aimed to adjust their immune function could represent an alternative approach, particularly in the severe form of the disease. Indeed, T-lymphocytes constitute a large population of cellular infiltrate in atopic/allergic inflammation and a dysregulated T-cell induced keratinocyte apoptosis appears to be an important pathogenetic factor of the eczematous disease. In recent years, attention has been focused on the interaction between host and probiotics which may have anti-inflammatory properties and immunomodulatory activities. The aim of the present work is to investigate the effect of a selected probiotic extract, the *Bifidobacterium infantis* extract, on a human keratinocyte cell line (HaCaT) abnormal apoptosis induced by activated-T-lymphocyte. An *in vitro* model of atopic dermatitis was used to assess the ability of the probiotic extract to protect HaCaT from apoptosis induced by soluble factors (IFN-γ and CD95 ligand) released by human T-lymphocytes *in vitro* activated with anti-CD3/CD28 mAbs or Phytohemoagglutinin. Evidence is given that the bacterial extract treatment was able to totally prevent T lymphocyte-induced HaCaT cell apoptosis *in vitro*. The mechanism underlying this inhibitory effect has been suggested to depend on the ability of the bacterial extract to significantly reduce anti-CD3/CD28 mAbs and mitogen-induced T-cell proliferation, IFN-γ generation and CD95 ligand release. These preliminary results may represent an experimental basis for a potential therapeutic approach mainly targeting the skin disorders-associated immune abnormalities.

T-cells represent a large population of the cellular infiltrate and mediate a dysregulated cytokine response in cutaneous inflammatory processes, thus participating in the development of eczematous reactions associated with several skin diseases, including allergic contact dermatitis (ACD) and atopic dermatitis (AD) (1). A dendritic cell-dependent T cell-mediated immune response can be observed during contact hypersensitivity reactions and it is induced by epicutaneous sensitisation with hapten (2). Although AD is regarded as a Th2-type disease (3), IFN-γ, a Th1 type cytokine, has been

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reported to be expressed in the late stages of AD (4). Accordingly, a shift from Th2 in the acute phase to Th1 in the chronic phase in AD has been proposed (5-7). In particular, both type 2 IL-4 and IL-5 cytokines and type 1 IFN-γ cytokine have been suggested to play important roles in skin inflammation in a murine model of eczematous dermatitis (8). Furthermore, the injection of IFN-γ into the skin of human volunteers demonstrated that this cytokine can induce transient skin inflammation (9).

Homeostasis in epidermis, as tissue renewing, is mainly guaranteed by keratinocytes through their capacity to proliferate and differentiate into epithelial cells forming the skin barrier (10). Trautmann et al. reported that an altered keratinocyte apoptosis represents a major mechanism in the pathogenesis of eczematous disorders and could be one of the causes for the damage of epidermal barrier. IFN-γ release by skin-infiltrating T-cells was indeed shown to be capable of upregulating CD95 on keratinocytes, thus rendering them susceptible to apoptosis by CD95 ligand (CD95L) expressed on and/or released by the T-cell surface (1). Recently, several therapeutic approaches have been employed in correcting specific immune abnormalities in AD (11-12). Skin inflammatory diseases can benefit from immunomodulators and/or immunosuppressive agent-based treatments (i.e. cyclosporine A, topical tacrolimus/FK506, rapamycine) which are able to prevent the abnormal keratinocyte apoptosis mainly associated to these pathological conditions (13-15).

Many species of lactic acid bacteria (LAB) have been reported to be immunomodulatory both *in vitro* and *in vivo* (16-18), and to have beneficial effects on animal and human health [i.e., protection against enteric infections, use as an oral adjuvant, immunopotentiation in malnutrition and prevention of chemically induced tumors (19-20)], thus they are frequently used as probiotics. In particular, LAB-induced immune regulation is considered as one of the potential causes of their antitumoral activity (16, 21-22).

In a previous investigation, we analysed the effects of six LAB strains on human normal and tumoral lymphocytes *in vitro* (23). All examined strains, with regard to efficacy, were able to induce apoptosis of tumor cell lines, without affecting normal cell viability. This effect could be associated with the presence, on bacterial extracts, of arginine deiminase and/or neutral sphingomyelinase, which in turn were able, respectively, to prevent polyamine synthesis and to generate endogenous ceramide in tumor cells, thus leading to apoptotic cell death. Taken together, these findings led us to analyse, through an *in vitro* disease model of atopic dermatitis, the potential effect of one probiotic extract (*Bifidobacterium infantis* extract) on the abnormal T-cell activation which has been indicated to be mainly responsible for AD-associated apoptosis of keratinocytes. We therefore conducted a preliminary study on the effects of this bacterial extract on anti-CD3/CD28 mAbs or Phytohemoagglutinin-induced T-cell proliferation and IFN-γ generation, as well as on activated T-cell induced HaCaT apoptosis *in vitro*. Our results indicate that the *in vitro* treatment with a particular probiotic extract led to a significant decrease of activated T-cell proliferation, IFN-γ generation, CD95 ligand release and, consequently, of activated T cell-induced HaCaT apoptosis.

MATERIALS AND METHODS

Media and chemicals

Media and culture reagents were purchased from EuroClone (Ltd. United Kingdom). Anti-CD3 (clone HIT3a; mouse anti-human monoclonal antibody) and anti-CD28 (clone CD28.2) mAbs were provided by Becton Dickinson (San Diego, CA, USA) and Phytohemoagglutinin-P (PHA) was from Sigma (St. Louis, MO, USA). Anti-CD95 mAb (clone ZB4) was purchased from Upstate (New York, USA). Neutralizing anti–human IFN-γ mAb (clone 25723) was from R&D Systems, Inc. (Minneapolis, USA). Fluorescein anti-mouse IgG1 was obtained from Vector Laboratories (Burlingame, CA, U.S.A.). [methyl-^{3}H]Thymidine 1 mCi mL^{-1} (specific activity 80 Ci mmol^{-1}) was purchased from Amersham (Buckinghamshire, UK).

Cell culture

The spontaneously immortalized human keratinocyte cell line HaCaT (24) was grown in plastic culture dishes (Nunc, Wiesbaden, Germany) containing Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, 100 U penicillin, 50 μg mL^{-1} streptomycin and 50 μg mL^{-1} gentamycin. Confluent cells were subcultured at day 3 after detaching the cells with a 0.1% trypsin/0.02% ethylenediamine tetraacetic acid solution.

Preparation of bacterial extracts

The bacterial strains, (*Streptococcus thermophilus*

(S244), Bifidobacterium longum, Lactobacillus acidophilus, Lactobacillus bulgaricus, Lactobacillus casei, Bifidobacterium infantis, Bifidobacterium breve), obtained from VSL Pharmaceuticals (Gaithesburg, Maryland, USA) in a pure lyophilized form (10^8 colony-forming units [CFU] g^{-1}), were routinely grown in MRS broth medium under aerobic conditions (25-26). Bacterial cultures were washed and re-suspended in 10 ml of phosphate buffered solution (PBS), sonicated (10 cycles on-off, alternating 45 sec. on and 2 min. off) with a Vibracell sonicator (Sonic and Materials Inc., Danbury, CT). Bacterial homogenates were centrifuged at 28,000 g for 20 min at 4°C in order to remove cell wall fragments and intact bacteria from cytosol, and the supernatant fraction (cytoplasm) was then filtered (0.45 μm). The filtrate was stored at –80°C until use. The protein concentration of bacterial extracts was determined using the Bio-Rad assay with bovine serum albumin standards (Sigma, St. Louis, MO, USA). The extracts were added to cell cultures to a final concentration of 25, 125, or 250 μg protein mL^{-1}.

T-lymphocyte isolation and culture

T-lymphocytes from healthy donors were isolated from heparinized blood by density centrifugation over Ficoll-Hypaque and MACS system (Pan T-Cell Isolation kit II cod 130-091-156), according to the instructions of the manufacturer (Miltenyi Biotec, Bergisch Gladbach, Germany). This procedure routinely yielded a >97% CD3 positive population, as analysed by flow cytometry. The cells were resuspended in culture medium (RPMI 1640 supplemented with 10% FCS, 2 mM L-glutamine, 100 IU mL^{-1} penicillin and 100 μg mL^{-1} streptomycin). The cells were counted and their viability, as assessed by Trypan blue dye exclusion and cytometric analysis, was routinely greater than 98%. The lymphocyte cultures were run in 96-well tissue culture plates and incubated in a humidified 5% CO_2 atmosphere at 37°C. The T-cells (10^6 mL^{-1}) were stimulated with PHA (20 μg mL^{-1}), anti-CD3 mAb (1 μg mL^{-1}) plus anti-CD28 mAb (1 μg mL^{-1}) in the presence or absence of bacterial extracts (final concentrations: 25, 125, or 250 μg protein mL^{-1}) in a total volume of 200 μl of culture medium. Experiments were performed according to the following schedule: a) untreated T-cell cultures; b) PHA- or anti-CD3/CD28 mAbs-stimulated T-cell cultures; c) bacterial extract treated T-lymphocytes; d) T-cells treated with bacterial extracts in the presence of either PHA or anti CD3/CD28 mAbs. Bacterial extracts and mAbs were added simultaneously. The viability and apoptosis of T-cells in the presence of different stimuli was tested by propidium iodide incorporation using flow cytometry (CELLquest software, Becton Dickinson). Cell proliferation was evaluated by pulsing the cell cultures with 1 μCi of [^{3}H]thymidine during the last 6 hrs of a 72 hr culture. Incorporation of [^{3}H]TdR was measured by standard liquid scintillation counting techniques after harvesting with a Skatron harvester.

Analysis by flow cytometry of anti-CD3 mAb binding to T-cells

In order to test whether each probiotic extract may interact with CD3-crosslinking, we measured anti-CD3 binding by indirect immune fluorescence. Briefly, T-cells were incubated for 30 minutes at 4°C with 250 μg mL^{-1} of each bacterial extract alone and with anti-CD3 mAb (1 μg mL^{-1}) in the presence or absence of the bacterial extract. Following a washout with PBS, the FITC-conjugated anti-mouse IgG was added for 30 min at 4°C after which the cells were analysed by cytofluorimetry. FITC-conjugated anti-mouse IgG was used as control isotype.

Culture of HaCaT with T-cell conditioned medium

Conditioned medium was obtained from T-cells grown for 48 hrs in RPMI-1640 with or without PHA, or anti-CD3 plus anti-CD28 in the presence or absence of increasing concentrations of each bacterial extract. After centrifugation, the T-cells supernatants were collected, diluted 1/5 and added to HaCaT ($1.2x10^5$ mL^{-1}) in 6-well plates. The culture was incubated for 72 hrs at 37°C in a humidified atmosphere containing 5% CO_2. Where indicated, the conditioned medium was preincubated for 30 minutes with neutralizing anti–human IFN-γ mAb (3 μg mL^{-1}) before addition to the HaCaT. After 72 hrs of incubation, pellets were collected and used to evaluate apoptosis level as described below.

HaCaT apoptosis evaluation by propidium iodide solution

DNA flow cytometry was performed as described elsewhere (27). After the treatments the cells were harvested, washed twice with PBS and centrifuged. The pellets (10^6) were gently resuspended in 1 ml of hypotonic propidium iodide (PI) solution (50 μg mL^{-1} in 0,1% sodium citrate + 0,1 % Triton X-100; Sigma, St Louis, MO). The tubes were kept overnight at 4°C in the dark. The PI fluorescence of individual nuclei and the percentage of apoptotic cell nuclei (subdiploid DNA peak in the DNA fluorescence histogram) were measured by flow cytometry with standard fluorescein-activated cell scan equipment (FACScan Becton Dickinson, San Josè, CA). Where indicated, the HaCaT were pretreated with anti-CD95 mAb (250 ng mL^{-1}), known to block induction of apoptosis via CD95 receptor in Jurkat T cells.

Quantification of cytokines

Cell cultures were prepared as above-described for the T-lymphocyte isolation and culture. The plates were incubated

at 37°C in 5% CO_2 for 24, 48 and 72 hrs. Cell cultures were then centrifuged and supernatants were collected and stored at -70°C. IFN-γ and sCD95L levels in supernatants were determined by commercial ELISA kit (Human Interferon gamma ELISA Pierce Endogen; sCD95 Ligand ELISA MBL Co. Nagoya. Japan), as recommended by the manufacturer. The 48-h incubation was chosen since at this time the higher production for both cytokines was observed.

Statistical analysis

Statistical analysis of data was done by using one-way ANOVA followed by Student's t test or by the correlation analysis (Prism 3.0 GraphPad Sofwtware, San Diego). Data were expressed as mean ± SD and P value <0.05 was used as the significance criterion.

RESULTS

Effect of bacterial extracts on anti-CD3/CD28 mAbs or PHA-induced proliferative response of human T-lymphocytes

In order to assess the possible effect of probiotics on anti-CD3/CD28 mAbs and PHA-induced proliferative response, we firstly treated for 72 hrs human T-lymphocytes with seven different strains (250 μg protein mL^{-1}) of LAB (*Streptococcus thermophilus, Bifidobacterium longum, Lactobacillus acidophilus, Lactobacillus bulgaricus, Lactobacillus casei, Bifidobacterium infantis* and *Bifidobacterium breve*) extracts in the presence or absence of PHA or anti-CD3/CD28 mAbs. All tested bacterial extracts significantly (P<0.001) suppressed T-cells proliferation (Fig. 1A), even if an intergroup difference in terms of the proliferation decrease obtained with the different bacterial extracts could be observed (range % inhibition: about 50% to almost 100%), *Bifidobacterium infantis* being the most efficient. Based on these results, all subsequent experiments designed to analyse the underlying mechanisms of the above reported effect were performed with *Bifidobacterium infantis* extracts.

To further delineate the effect of *Bifidobacterium infantis* extracts on anti-CD3/CD28 mAbs and PHA-induced proliferative response, human T-lymphocytes were treated with three different concentrations (25, 125, 250 μg protein mL^{-1}) of bacterial extract in the presence or absence of PHA or anti-CD3/CD28 mAbs. Fig. 1B shows that exposure to the bacterial extract caused a significant and concentration-dependent decrease in activated T-cells [^{3}H]thymidine incorporation (% inhibition range: 60-95%). Toxic effects potentially due to the bacterial extract based treatment on T-lymphocytes could be excluded since no significant influence on cell viability was observed, as analysed by both Trypan blue exclusion and propidium iodide incorporation using flow cytometry. A significant inverse correlation between the inhibitory effect and bacterial extract concentration was observed (R=-0.908, P<0.001 and R=-0.7, P<0.03 for cells treated with PHA and anti-CD3/CD28 mAbs, respectively). On the other hand, bacterial extract did not significantly affect spontaneous [^{3}H]TdR incorporation from lymphocytes at basal conditions (without PHA or anti-CD3/CD28 mAbs).

Effect of Bifidobacterium infantis on anti-CD3 mAb binding to human T-lymphocytes

To exclude the possibility that the above reported inhibitory effect could be due to the interference of bacterial extract with the mAb induced-cross-linking of surface receptors, the binding of anti-CD3 mAb in the presence of the bacterial extract was evaluated. As shown in Fig.2, the anti-CD3 mAb binding, as analysed by cytofluorimetry, was not influenced in the presence of the bacterial extract at higher concentration (250 μg mL^{-1}).

Effects of Bifidobacterium infantis on activated T lymphocyte-induced IFN-γ production and sCD95 ligand release

The incubation of T-lymphocytes with both PHA and anti-CD3/CD28 mAbs for 48 hrs, as expected, was followed by a significant increase (P<0.001) of IFN-γ secretion in the culture medium (Fig. 3A), as assessed by a specific enzyme-linked immunosorbent assay (ELISA). The addition of bacterial extract (25 μg mL^{-1}) in the culture medium was able to strongly and significantly (P<0.001) inhibit either PHA- or anti-CD3/CD28 mAbs-induced IFN-γ generation. At higher concentrations (125 and 250 μg mL^{-1}), the presence of the bacterial extract totally abrogated PHA- or anti-CD3/CD28 mAbs-induced IFN-γ release which returned to basal levels. The extent of inhibition of IFN-γ secretion well correlated with the impairment of cell proliferation observed when activated T-cells were cultured in the presence of the bacterial extract (R=0.953, P<0.001 for PHA-

activated cells; R=0.988, P<0.001 for anti-CD3/CD28 mAbs-activated cells).

The effect of the bacterial extract on soluble CD95 ligand (sCD95L) release from PHA- or anti-CD3/CD28 mAbs-activated T-lymphocytes *in vitro* was also investigated. The levels of sCD95L in the supernatants from T-lymphocytes, incubated for 48 hrs with or without stimuli in the presence or absence of bacterial extract, were measured by ELISA. The results of these experiments showed the ability of the stimuli to significantly (P<0.001) increase the level of sCD95L (Fig. 3B). It is worthy of note that the presence of *Bifidobacterium* extract in the cell cultures led to a relevant and significant (P<0.001) decrease of the sCD95L release by PHA- or anti-CD3/CD28 mAbs-stimulated T-cells, without affecting the basal release of sCD95L. The extent of inhibition of sCD95L release was concentration-dependent and well correlated either with the impairment of cell proliferation (R=0.981, P<0.001 for PHA-activated cells; R=0.942, P<0.001 for anti-CD3/CD28 mAbs-activated cells) or IFN-γ generation (R=0.989, P<0.001 for PHA-activated cells; R=0.976, P<0.001 for anti-CD3/CD28 mAbs-treated cells) observed when T-cells were cultured in the presence of the bacterial extract.

Effect of Bifidobacterium infantis on T cell-mediated HaCaT apoptosis

To confirm the ability of activated T-cells to induce HaCaT apoptosis, we investigated the effect of T-cell supernatants on HaCaT culture. To eliminate the disadvantage of apoptotic bodies or fragmented DNA being derived from T-cells, the latter were incubated with the appropriate stimuli for 48 hrs and then centrifuged. The conditioned media were then added to HaCaT culture for three days and the cells were subsequently analysed for apoptosis by flow cytometry. This model allowed us to verify that HaCaT apoptosis was induced by soluble factors secreted from PHA- or anti-CD3/CD28 mAbs-activated T-lymphocytes. In Fig. 4 the mean values (±SD) of data from 3 experiments in duplicate are shown. As evident, the apoptosis levels in HaCaT treated with supernatants from PHA or anti-CD3/CD28 mAbs-activated T-lymphocytes appeared to be comparable. The presence of neutralizing anti–human IFN-γ in the conditioned media or the preincubation of HaCaT with blocking anti-CD95 mAb dramatically reduced the apoptosis (approximately 65-80% and 70-80% reduction, respectively), thus confirming previous observations showing that this event could be totally due to the IFN-γ released from activated T-cells and CD95 system (1).

According to these results and as reported by other authors (1), keratinocyte apoptosis being induced by IFN-γ and sCD95 ligand, to evaluate the effect of the bacterial extract we used it at concentration of 250 $\mu g\ mL^{-1}$ since this concentration, in respect to the other concentrations, completely abrogated PHA- or anti-CD3/CD28 mAbs-induced IFN-γ production and sCD95 ligand release. Supernatants from both anti-CD3/CD28 mAb- and PHA-stimulated T-lymphocytes, cultured in the presence of the bacterial extract, were able to almost totally abrogate HaCaT apoptosis. It was noted that when the bacterial extract was added directly to HaCaT cultures in the presence of the conditioned medium derived from activated-T cells, the apoptosis level did not change, thus suggesting that the inhibitory effect of bacterial extract on HaCaT apoptosis was not directed on HaCaT, being mainly attributed to its suppressive action on T-cell activation and, consequently, to IFN-γ generation and sCD95L release. Fig. 5 displays a representative experiment showing that, as expected, while no effect could be observed when HaCaT were incubated with supernatant from untreated and unstimulated T-cells (panel A), the conditioned medium from anti-CD3/CD28 mAb- and PHA-stimulated T-cells was able to induce relevant levels of HaCaT apoptosis (range: 80-100%) (panel B and C respectively). Similar results were obtained when the bacterial extract was added directly to HaCaT culture (panel D, E and F). It is of interest that the medium conditioned from activated T-cells in the presence of the bacterial extract was able to reduce the percentage number of hypodiploid nuclei (panel H and I). Altogether, these findings show the ability of probiotic extract to strongly inhibit and prevent the HaCaT apoptosis induced by soluble factors from activated-T-cells.

DISCUSSION

Several studies have suggested the role of T cells

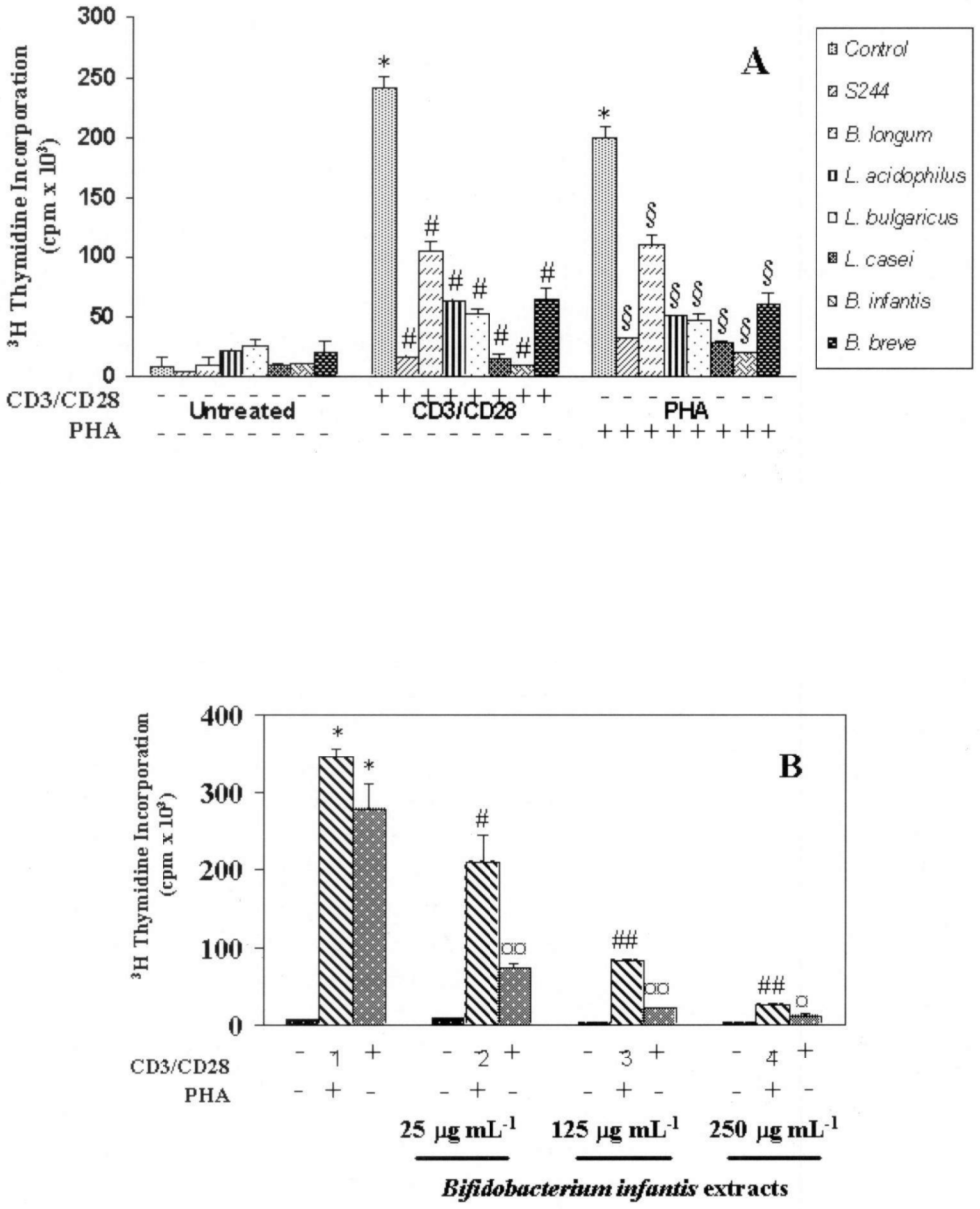

Fig. 1. *Effect of bacterial extracts on anti-CD3/CD28 mAbs and PHA-induced proliferation of human T-lymphocytes. T-cell proliferation, measured by [3H]TdR incorporation, is expressed as counts per minute, values represent the mean±SD of three determinations from one experiment representative of three. (Panel A): proliferation was assessed in cultures containing isolated lymphocytes treated with different bacterial extracts used at the same protein concentration (250 μg mL^{-1}), with anti-CD3/CD28 mAbs (1 μg mL^{-1}), or PHA (20 μg mL^{-1}) in the presence or absence of bacterial extracts. * = P<0.001 when compared to untreated T cells; # = P<0.001 compared to anti-CD3/CD28-stimulated T cells; § = P<0.001 compared to PHA-stimulated T cells. (Panel B): T-cells were incubated for 72 hrs with or without Bifidobacterium infantis (protein concentration of 25, 125, 250 μg mL^{-1}) in presence or absence of PHA (20 μg mL^{-1}) or anti-CD3/CD28 mAbs (1 μg mL^{-1}). * = P<0.001 when compared to untreated cells; ## = P<0.001 and # = P=0.003 compared to PHA-stimulated T cells; ¤¤ = P<0.001 and ¤ = P=0.007 compared to anti-CD3/CD28-stimulated T-cells.*

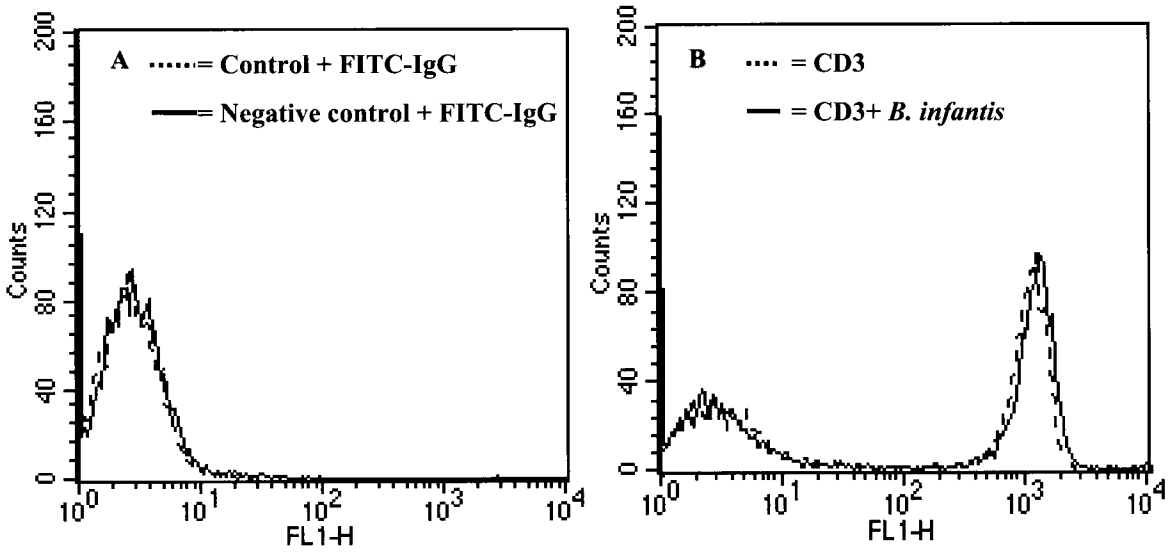

Fig. 2. *Effect of Bifidobacterium infantis extracts on anti-CD3 mAb binding to human T-lymphocytes. T-cells were cultured with bacterial extracts alone (250 μg mL^{-1}) or stimulated with anti-CD3 mAb (1 μg mL^{-1}) in presence or absence of 250 μg mL^{-1} of bacterial extract. (A). Control isotype stained (FITC-conjugated anti-mouse IgG) in presence (solid line) or absence (dotted line) of bacterial extracts. (B). Anti-CD3 mAb binding in presence (solid line) or absence (dotted line) of bacterial extracts. The data shown are from one representative of three independent experiments.*

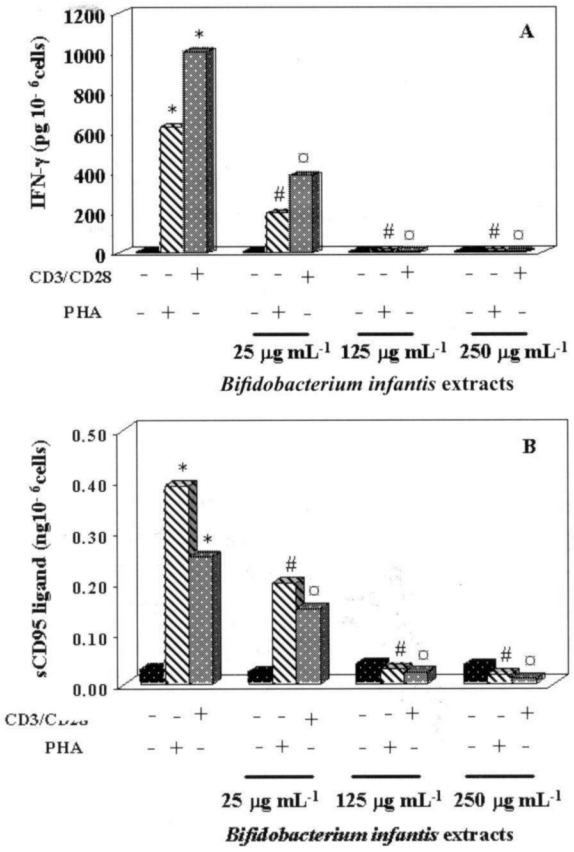

Fig. 3. *Effect of Bifidobacterium infantis extracts on IFN-γ and sCD95 ligand production by activated human T-cells. The IFN-γ (A) and sCD95 ligand (B) levels were evaluated by a specific ELISA. The values represent the mean values of duplicate determinations and are representative of 1 of 3 experiments. SD values were ever lower than 5% of mean values. *P<0.001 compared to untreated T-cells. #P<0.001 compared to PHA-stimulated T-cells. ¤P< 0.001 compared to anti-CD3/CD28-stimulated T-cells.*

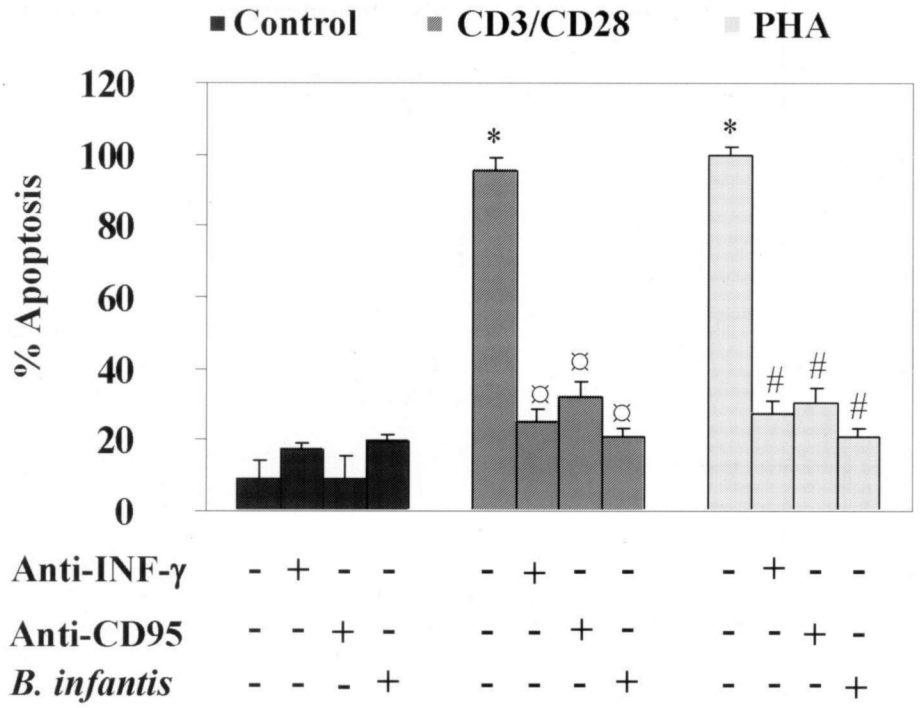

Fig. 4. *HaCaT cell apoptosis levels in presence of conditioned medium from unstimulated or stimulated T cells cultured in presence or absence of Bifidobacterium infantis extracts. The Y axis represents the % of HaCaT cells apoptosis induced by the supernatants of unstimulated or stimulated (with PHA or anti-CD3/CD28 mAbs) T-cells. Histograms show the difference between treatments with bacterial extracts (250 μg mL^{-1}), neutralizing anti–human IFN-γ mAb (3 μg mL^{-1}), anti-CD95 mAb (clone ZB4; 250 μg mL^{-1}) on the apoptotic behaviour. Values are expressed as the mean ±SD of 2 determinations. Shown data represent one out of three independent experiments. *P<0.001 when compared to untreated, ¤P<0.001 compared to anti-CD3/CD28 mAbs-stimulated T-cells, #P<0.001 compared to PHA-stimulated T-cells.*

in cutaneous inflammatory processes (i.e. atopic dermatitis), where they represent a large population of the cellular infiltrate and mediate a dysregulated cytokine response which, in turn, could be responsible for abnormal keratinocyte apoptosis (1-7). Our study was conducted to assess the ability of a *Bifidobacteria* extract to affect the immune response of human T-lymphocytes in a disease model of AD *in vitro*.

Our results demonstrate that all the analysed probiotic extracts were able to significantly inhibit human T-lymphocyte proliferation induced by anti-CD3 plus anti-CD28 mAbs *in vitro*, even if at different extents (range 50-100%), the *Bifidobacterium infantis* being the most efficient in this effect. No effect was observed when cells were treated with bacterial extracts only. The inhibitory effect on activated T-cell proliferation was significant even at the lowest concentration of *Bifidobacterium infantis* extract and could not be attributed either to toxic effects or to any interference of the bacterial extract with membrane receptor crosslinking. Indeed, the treatment with bacterial extract did not influence cell viability analysed by Trypan blue exclusion as well anti-CD3 binding, as assayed by cytofluorimetry.

The activation of T cells with both PHA and anti-CD3/CD28 mAbs, as expected, led to the induction of IFN-γ generation (28). Taking into account that IFN-γ plays an important role in skin inflammation (8-9), the influence of the bacterial extract treatment on the generation and release of this cytokine from stimulated T-lymphocytes was also analysed. IFN-γ levels generated by treatment with PHA or anti-CD3/CD28 mAbs appeared to be significantly inhibited in the presence of the bacterial extract even at the lowest concentration of the latter, and totally abrogated at 125 and 250 μg mL^{-1}. Taken

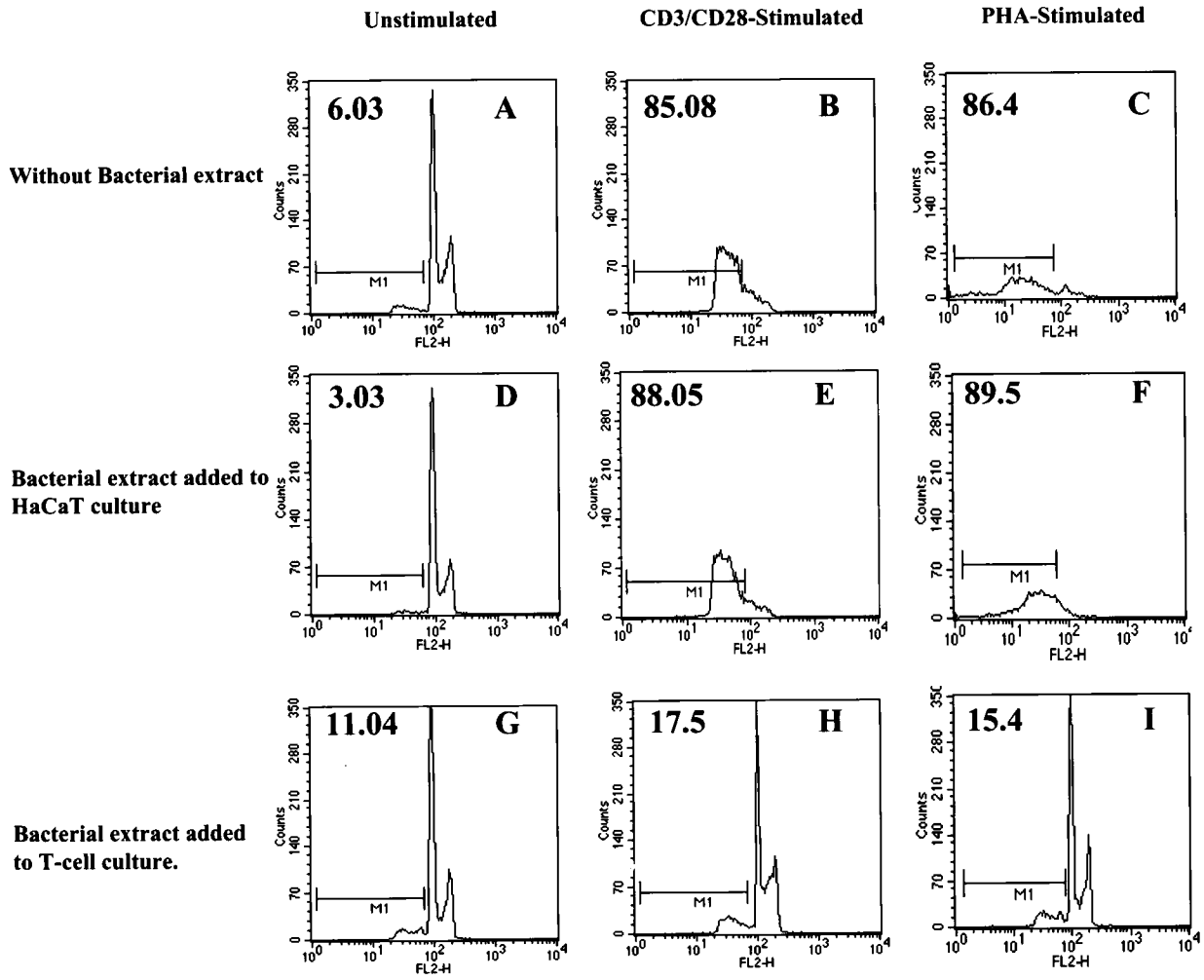

Fig. 5. *Effect of Bifidobacterium infantis extracts on HaCaT apoptosis induced by conditioned medium from unstimulated or stimulated T cells, as determined through flow cytometry. Data represent the results of one out of 3 independent experiments. HaCaT cells were incubated for 3 days with conditioned medium from unstimulated T-cells (A, D and G), anti-CD3/CD28 stimulated T-cells (B, E and H) and PHA-stimulated T-cells (C, F and I). D, E and F show HaCaT apoptosis when the bacterial extracts (250 μg mL^{-1}) were directly added to cell culture; G, H and I show HaCaT apoptosis when the bacterial extracts (250 μg mL^{-1}) were incubated with stimulated T-cells. M1 indicates the apoptotic peak representing the cells with a sub-diploid DNA content.*

together, these results suggest that the bacterial extract treatment *in vitro* was able to significantly or totally prevent either T-cell proliferation or IFN-γ generation. Previous studies reported that AD skin-infiltrating activated-T-cells upregulate the expression of CD95 receptor on keratinocytes through IFN-γ and induce apoptosis by CD95 ligand which could be expressed on T-cell surface or released into the extracellular microenvironment (1, 29-30). Experiments designed to assess the effect of the bacterial extract on CD95L released from activated T-lymphocytes, showed that the treatment was able to strongly and concentration-dependently inhibit the levels of sCD95L released either from PHA or anti-CD3/CD28 mAb stimulated T-cells.

In our experimental *in vitro* model, activated T cell-derived soluble factors were able to induce HaCaT apoptosis. Of note, when stimulated, T-cells were cultured in the presence of the bacterial extract, the conditioned medium was unable to induce HaCaT apoptotic death, the effect being comparable to that observed either when blocking anti-CD95

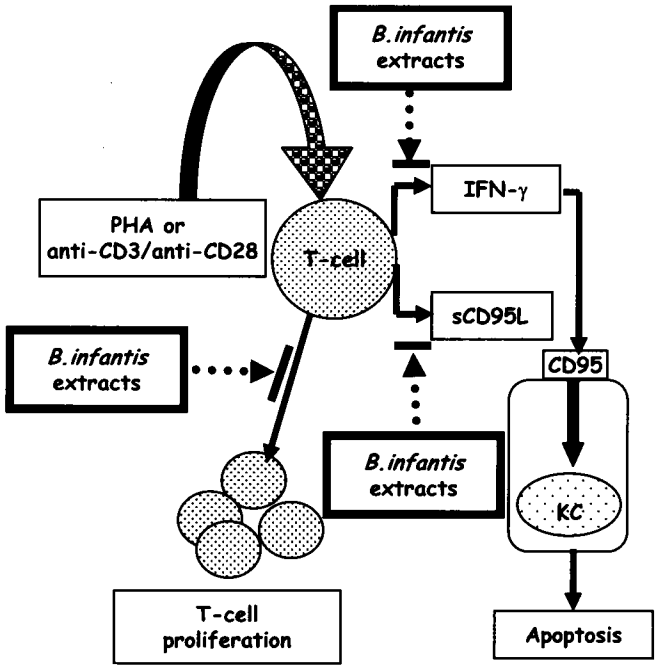

Fig. 6. *Proposed model of the effect of Bifidobacterium infantis extracts on prevention of keratinocyte apoptosis mediated by activated T-cells.*

mAb (about 70-80%) or neutralizing anti-IFN-γ mAb (about 65-80%) were used. Overall, these results suggest that keratinocyte apoptosis could be mediated by T cell released IFN-γ, and almost totally attributed to CD95 system, confirming previously reported results (1). The bacterial extract-induced inhibitory effect on HaCaT apoptosis appeared to be indirect, being mediated by the impaired ability of activated-T cells to release adequate levels of IFN-γ and sCD95L. This hypothesis was further supported by the results from experiments indicating that no inhibitory effect could be observed on keratinocyte apoptosis when the bacterial extract was directly added to HaCaT cell cultures in the presence of conditioned medium from activated-T cells. The proposed model summarising the observed effects of *Bifidobacterium* extract on T-cell induced keratinocyte apoptosis is shown in Fig. 6. Taken together, these results could represent the experimental basis for a new therapeutic approach to immune abnormalities-associated atopic dermatitis.

Judging from our findings, the components of *Bifidobacterium infantis*, considered to be responsible for the observed immunosuppression, could be located in the cytoplasm and not in cell wall fractions. However, additional investigations are needed in order to identify the immunomodulator components and the biomolecular mechanisms by which these factors operate. Moreover, further research is also needed to assess the effect of an experimental *Bifidobacteria* extract *in vivo* treatment on the AD-associated T-cell activation and keratinocyte apoptosis. In the eczematous reactions, T-cells act as important mediators of the inflammatory processes, as supported by the observation that immunosuppressive drugs such as FK506 and glucocorticoids, which block T-cells activation, are effective in the treatment of eczematous disorders (1). Skin represents a functionally distinct immune compartment, and chronic inflammation of the skin is generally associated with tissue infiltration by T-cells (31).

Finally, since a reduced ratio of *Bifidobacteria* to *Clostridia* has been observed to precede the development of atopy, and patients with allergic diseases resulted in being less often colonized by *Enterococci* and *Bifidobacteria* but more often by *Clostridia* and *Staphylococci* compared to not allergic patients (32-34), our investigation could have

a potential clinical significance also in this context.

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