Effect of *S. thermophilus* extracts on human T-cell induced keratinocyte apoptosis *in vitro:* Implications for a new therapeutic approach of atopic dermatitis-associated immune abnormalities.

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Abstract

Current management of atopic dermatitis is mainly directed to the reduction of cutaneous inflammation. Since patients with atopic dermatitis show abnormalities in immunoregulation, therapy directed to adjustment of 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 eczematous disease. The aim of the present work was to investigate the effect of *S. thermophilus* (a lactic acid bacterium) extracts on atopic dermatitis-associated T cell activation and abnormal keratinocyte apoptosis. An *in vitro* model of atopic dermatitis was used to assess the ability of bacterial extracts to protect keratinocytes from apoptosis induced by soluble factors (i.e. IFN-γ and CD95 ligand) derived from mitogen-activated human T-lymphocytes *in vitro*. Evidence is shown that *S. thermophilus* extracts *in vitro,* leading to a significant decrease of mitogen-induced T-cell proliferation, IFN-γ generation and CD95 ligand release, were indirectly able to totally prevent T cell-induced keratinocyte apoptosis. These results provide the experimental basis for a new therapeutical approach mainly targeting the atopic dermatitis-associated immune abnormalities.

Key words: T-cell activation, keratinocyte apoptosis, CD95 ligand, *S. thermophilus,* atopic dermatitis.

Introduction

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 reaction associated to several skin diseases including allergic contact dermatitis (ACD) and atopic dermatitis (AD) (1). A dendritic cell-dependent T cell-mediated immune response verifies 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 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 the type 2 cytokines IL-4 and IL-5, and the type 1 cytokine IFN-γ have been suggested to play important roles in the skin inflammation in a murine model of eczematous dermatitis (8). Furthermore, injection of IFN-γ into the skin of human volunteers demonstrated that this cytokine can induce transient skin inflammation (9).

Homeostasis in epidermis, as renewing tissue, is mainly garanteed by keratinocytes (KCs) through their capacity to proliferate and differentiate into epithelial cells forming the skin barrier (10). Trautmann et al. reported that an altered KC apoptosis represents a major mechanism in the pathogenesis of eczematous disorders and could be a cause for damaged epidermal barrier (1). IFN-γ, released by skin-infiltrating T-cells, indeed, was shown to be able of upregulating CD95 on KCs thus rendering them susceptible to apoptosis by CD95 ligand (CD95L) expressed on and/or released by the T-cell surface (1). Recently, several therapeutical approaches have been employed in correcting specific immune abnormalities in AD (11-12). Skin inflammatory diseases can benefit from immunosuppressive agent-based treatments (i.e. cyclosporine A, topical tacrolimus/FK506, rapamycine) able to prevent the abnormal KC apoptosis mainly associated to these pathological conditions (13-16).

Several studies have demonstrated that ceramides play an essential role in both the barrier and water-holding functions of healthy stratum corneum, (17, 18) suggesting that the dysfunction of the stratum corneum of

patients with AD could result from a ceramide deficiency (19, 20). In a previous study, our group demonstrated a significant increase in skin ceramide levels in healthy subjects, after a treatment *in vivo* with a cream containing a extract preparation of *S. salivarum* subspeciem *thermophilus,* a probiotic belonging to the lactic acid bacterium (LAB) group (21). The presence of high levels of neutral sphingomyelinase (nSMase) in this microrganism has been suggested to be responsible for the observed increase in stratum corneum ceramide levels, thus leading to an improvement in barrier function and maintenance of stratum corneum flexibility. Recently, we have also reported the beneficial effects on AD patients of a topical treatment with a cream containing *S. thermophilus,* which, also in this case, was shown to induce a relevant increase of skin ceramide levels which was associated to an improvement in the clinical signs and symptoms of AD (22). Taken together, these findings led us to analyse, through an *in vitro* disease model of atopic dermatitis, the potential effect of *S. thermophilus* extracts also on immune disfunction mainly responsible for AD-associated abnormal apoptosis of KCs (1). LAB are a group of bacteria belonging to a diverse genera used to bring about milk fermentation, and composed chiefly of bacteria whose primary metabolic end-product of carbohydrate metabolism is lactic acid. Many species of LAB have been reported to be immunomodulatory both *in vitro* and *in vivo* (23-25) and have beneficial effects on the health of animals and humans, i.e., protection against enteric infections, use as an oral adjuvant, immunopotentiation in malnutrition and prevention of chemically induced tumors (26-27). In particular, LAB-induced antimutagenic effects and immune regulation are considered the potential indirect causes of their antitumoral activity (28). In a previous investigation, we analysed the effects of six LAB strains on human normal and tumoral lymphocytes *in vitro* (28). All examined strains, regardless of efficacy, were able to induce apoptosis on human T leukaemia Jurkat cells, but not on normal human peripheral blood lymphocytes. The apoptotic death-inducing ability of bacterial extracts could be associated with the presence of arginine deiminase and/or nSMase, which in turn were able to prevent polyamine synthesis and generate endogenous ceramide in tumoral cells, respectively.

In view of the suggested role of LAB in the regulation of the immune response, which in turn is needed also for the maintenance of skin integrity, we conducted a preliminary study on the effects of extracts of *Streptococcus salivarium subspeciem thermophilus* on mitogen-induced T-cell proliferation and IFN-γ generation, as well as on T-cell induced KC apoptosis *in vitro*, all events previously associated with atopic dermatitis. Our results indicate that *S. thermophilus* extracts *in vitro* led to a significant decrease of mitogen-induced T-cell proliferation, IFN-γ generation, CD95 ligand release, and consequently, of T cell-induced KC apoptosis.

Materials and Methods

Reagents. Media and culture reagents were purchased from EuroClone (Ltd. United Kingdom). Concanavalin A (ConA) was from Calbiochem (San Diego, CA, USA) and Phytohemoagglutinin-P (PHA) was from Sigma (St. Louis, MO, USA). [methyl-^{3}H]Thymidine 1 mCi/ml (specific activity 80 Ci/mmol) was purchased from Amersham (Buckinghamshire, UK).

Cell culture. The spontaneously immortalized human keratinocyte cell line HaCat was grown in plastic culture dishes (Nunc, Wiesbaden, Germany) containing Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 units penicillin per ml, 50 μg streptomycin per ml, and 50 μg gentamycin per ml. Confluent cells were subcultured every 3 days after detaching the cells with a 0.1% trypsin/0.02% ethylenediamine tetraacetic acid solution.

Preparation of bacterial extracts. S. thermophilus, obtained from VSL Pharmaceuticals (Gaithesburg, Maryland, USA) in a pure lyophilized form (10^8 colony-forming units [CFU]/gram), was routinely growth in MRS broth medium under aerobic conditions. For experiments *in vitro* on lymphocytes and keratinocytes, *S. thermophilus* cultures were washed and resuspended in 10 ml phosphate buffered solution (PBS), sonicated (10 cycles on-off altering 45 sec. on and 2 min. off) with a Vibracell sonicator (Sonic and Materials Inc., Danbury, CT), centrifuged at 7,500 g for 20 min at 4°C and the supernatants stored at –20°C until use (bacterial extract). The bacterial extract was added to cell cultures at 0.25 mg/ml, 1.25 mg/ml and 2.5 mg/ml (final concentration).

T lymphocyte isolation and culture. T lymphocytes from healthy donors were isolated from heparinized blood by density centrifugation over Ficoll-Hypaque, passage through nylon wool column, plastic adherence and Percoll fractionation. This procedure routinely yielded a >98% CD3 positive population, as analysed by immunofluorescence and flow cytometry. The cells were resuspended in culture medium (RPMI 1640 supplemented with 10% FCS, 2 g/ml sodium bicarbonate, 2 mM L-glutamine, 100 IU penicillin and 100 μg streptomycin/ml). The cells were counted and their viability, as assessed by Trypan blue dye exclusion, 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. Each culture comprised $2x10^5$ lymphocytes and PHA (20 μg/ml) and ConA (10 μg/ml) and/or bacteria (0.25 mg/ml, 1.25 mg/ml and 2.5 mg/ml) or nothing in a total volume of 200 μl of culture medium. Cell proliferation was evaluated by pulsing the cell cultures with 1 μCi of [^{3}H]thymidine during the last 6 hrs of a 72 hrs culture. Incorporation of [^{3}H]TdR was measured by standard liquid scintillation counting techniques after harvesting with a Skatron harvester.

Culture of human keratinocytes with T-cell conditioned medium. Conditioned medium was obtained from T-cells grown for 48 hrs in RPMI-1640 with or without PHA or ConA in the presence or absence of increasing concentrations of bacterial extracts. After centrifugation, the supernatants were added to keratinocytes and the culture was incubated for 72 hrs in 6-well plates ($3x10^5$ keratinocytes and $1x10^5$ T-cells).

KC apoptosis evaluation by propidium iodide solution. Apoptosis was measured by flow cytometry. After culturing, KCs were centrifuged and the pellets were gently resuspended in 1.5 ml hypotonic propidium iodide solution (PI, 50 μg/ml in 0.1% sodium citrate plus 0.1% Triton X-100, Sigma, St. Louis, MO, USA). The tubes were kept at 4°C in the dark overnight. The PI-fluorescence of individual nuclei was measured by flow cytometry with standard FACScan equipment (Becton Dickinson). The nuclei traversed the light beam of a 488 nm Argon laser. A 560 nm dichroid mirror (DM 570) and a 600 nm band pass filter (band width 35 nm) were used to collect the red fluorescence due to PI DNA staining, and the data were recorded in logarithmic scale in a Hewlett Packard (HP 9000, model 310) computer. The percentage of apoptotic cell nuclei (sub-diploid DNA peak in the DNA fluorescence histogram) was calculated with specific FACScan research software (Lysis II).

Quantification of cytokines. Where indicated, IFN-γ and sCD95L were determinated by commercial ELISA kit (Human Interferon gamma ELISA Pierce Endogen; sFas Ligand ELISA MBL Co. Nagoya. Japan).

Statistical analysis. The Student's *t* test was performed by the STATPAC Computerized Program, and a P value <0.05 was used as the significance criterion.

RESULTS

Effect of S. thermophilus extracts on mitogen-induced proliferative response of human T-lymphocytes

In order to assess the effect of *S. thermophilus* on mitogen-induced proliferative response, human T-lymphocytes were treated for 72 hrs with three different concentrations (0.25, 1.25, 2.5 mg/ml) of bacterial extracts in the presence or absence of mitogen (PHA or ConA). A significant inhibition of both PHA- and ConA-induced proliferation was observed in the presence of *S. thermophilus* extracts ($P<0.001$) (figure 1). Toxic effects of bacterial extracts on T-lymphocytes could be excluded by cell viability test. The inhibitory effect was significantly and inversely correlated with bacterial extract concentration ($R=-0.909$, $P<0.001$ and $R=-0.875$, $P<0.001$, for cells treated with PHA and ConA, respectively). On the other hand, *S. thermophilus* extracts did significantly affect spontaneous [^{3}H]TdR incorporation from unstimulated lymphocytes only at higher concentration (2.5 mg/ml) ($P=0.005$).

Effects of S. thermophilus extracts on activated T lymphocyte-induced IFN-γ production and sCD95 ligand release

As expected, the incubation of T-lymphocytes with both PHA and ConA for 48 hrs was followed by a significant increase ($P<0.001$) of IFN-γ secretion in the culture medium (figure 2), as assessed by a specific enzyme-linked immunosorbent assay (ELISA). The addition in the culture medium of bacterial extracts at 0.25 mg/ml was able to strongly and significantly ($P<0.001$) inhibit either PHA- or ConA-induced IFN-γ generation. At higher concentrations (1.25 and 2.5 mg/ml), the presence of bacterial extracts totally abrogated mitogen-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 T-cells were cultured in the presence of bacterial extracts ($R=0.953$, $P<0.001$ for PHA-activated cells; $R=0.988$, $P<0.001$ for ConA-activated cells).

The effects *S. thermophilus* extracts on soluble CD95 ligand (sCD95L) release from mitogen-activated T lymphocytes *in vitro* were also investigated. The levels of sCD95L in the supernatants from T-lymphocytes,

incubated for 48 hrs with or without mitogens in the presence or absence of bacterial extracts, were measured by ELISA. The results of these experiments, as expected, showed the ability of the mitogens to significantly ($P<0.001$) increase the level of sCD95L (figure 3). Of note, the addition of *S.thermophilus* extracts to the cell cultures led to a relevant and significant ($P<0.001$) decrease of the levels of mitogen-induced sCD95L release by T-cells. On the other hand, bacterial extract treatment didn't influence the basal release of sCD95L. The extent of inhibition of sCD95L release 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 ConA-activated cells) or IFN-γ generation ($R=0.989$, $P<0.001$ for PHA-activated cells; $R=0.976$, $P<0.001$ for ConA-treated cells) observed when T-cells were cultured in the presence of bacterial extracts.

Effects of S. thermophilus extracts on T cell-mediated keratinocyte apoptosis

To assess the ability of mitogen-activated T-cells to induce KC apoptosis, we devised a KC-T cell coculture. To eliminate the disadvantage that apoptotic bodies or fragmented DNA could be derived from T-cells, the latter were incubated with the appropriate stimuli for 48 hrs and then centrifugated. The supernatants (conditioned media) were then added to KC culture for three days, afterwhich KCs were analysed for apoptosis by flow cytometry after nuclear staining with PI. This model allowed us to verify that KC apoptosis was induced by soluble factors secreted from mitogen-activated T-lymphocytes. Indeed, conditioned medium from both PHA- and ConA-activated T-cells, was able to induce relevant levels of KC apoptosis (range: 70-95%). In figure 4 is shown a representative experiment with KCs incubated for 3 days with Con-A-activated T-lymphocyte supernatant. Of note, supernatants from mitogen-activated T-lymphocytes cultured in the presence of *S. thermophilus* extracts were able to almost totally abrogate KC apoptosis. Interestingly, no effect on KC basal viability and apoptosis levels was revealed after incubation with supernatants from T-lymphocytes treated with bacterial extract alone. The effect on KC apoptosis could be attributed to the inhibitory effect of *S. thermophilus* extracts on T-cell mitogen activation and, consequently to IFN-γ production and sCD95L release.

Indeed, a significant correlation was observed between the effect of bacterial extracts on i) ConA-induced T-cell proliferation and KC apoptosis ($R=0.972$, $P=0.028$), ii) INF-γ levels from ConA-activated T-cells and KC apoptosis ($R=0.992$, $P=0.008$) and iii) sCD95L release from ConA-activated T-cells and KC apoptosis ($R=0.954$, $P=0.046$). Similar results were obtained also with PHA-activated T-cells.

DISCUSSION

Several studies have suggested the role of 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 *S. thermophilus* extracts to affect the immune response of human T-lymphocytes in a disease model of AD *in vitro.*

Our results revealed that the treatment *in vitro* with *S. thermophilus* extract was able to strongly and dose-dependently influence the mitogen-induced proliferation of human T-lymphocytes, whereas no effect was observed when the cells were treated with *S. thermophilus* extracts alone. In considering that IFN-γ represents an important player in the skin inflammation (8, 9), the influence of *S. thermophilus* extracts treatment on the generation and release of this cytokine from mitogen-activated T-lymphocytes was also analysed. A clear direct correlation between the effects of bacterial extracts on mitogen-induced T-lymphocyte proliferation and IFN-γ production was observed. Indeed IFN-γ levels, generated by treatment with mitogens, was strongly and gradually reduced in the presence of bacterial extracts, even at the lower concentration of the latter.

Previous studies reported that AD-associated KC apoptosis is induced by activated-T-cell released IFN-γ through its ability to increase CD95L release from activated T-lymphocytes as well the expression of CD95 receptor on KCs (1, 29, 30). The experiments designed to assess the effect of *S. thermophilus* extracts on CD95L release from mitogen-activated T-lymphocytes have shown that bacterial extract treatment was able to strongly and dose-dependently reduce the levels of sCD95L released either from PHA or ConA treated T-cells.

In our experimental *in vitro* model, activated T cell-derived soluble factors were able to induce KC apoptosis. Of note, *S. thermophilus* extracts protected KC from T cell-induced cell death. This inhibitory effect appears to be indirect, being mediated by the impaired ability of activated-T cells to release adequate levels of sCD95L following treatment with bacterial

extracts. The proposed model summarising the observed effects of *S. thermophilus* on T-cell induced KC apoptosis is shown in figure 5.

Additional investigations are currently in progress in the attempt to identify the biomolecular mechanisms underlying the observed effects. Moreover, further research is also needed to assess the effect of a *S. thermophilus* extract-based treatment *in vivo* on AD-associated T-cell activation and KC apoptosis. In eczematous reactions, T-cells, indeed, represent 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 treatment of eczematous disorders (31-32). Considering that skin represents a functionally distinct immune compartment, and chronic inflammation of the skin is generally associated with tissue infiltration by T-cells (33), our investigation has a potential clinical significance, representing the experimental basis for a new therapeutical approach of atopic dermatitis-associated immune abnormalities.

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Legends

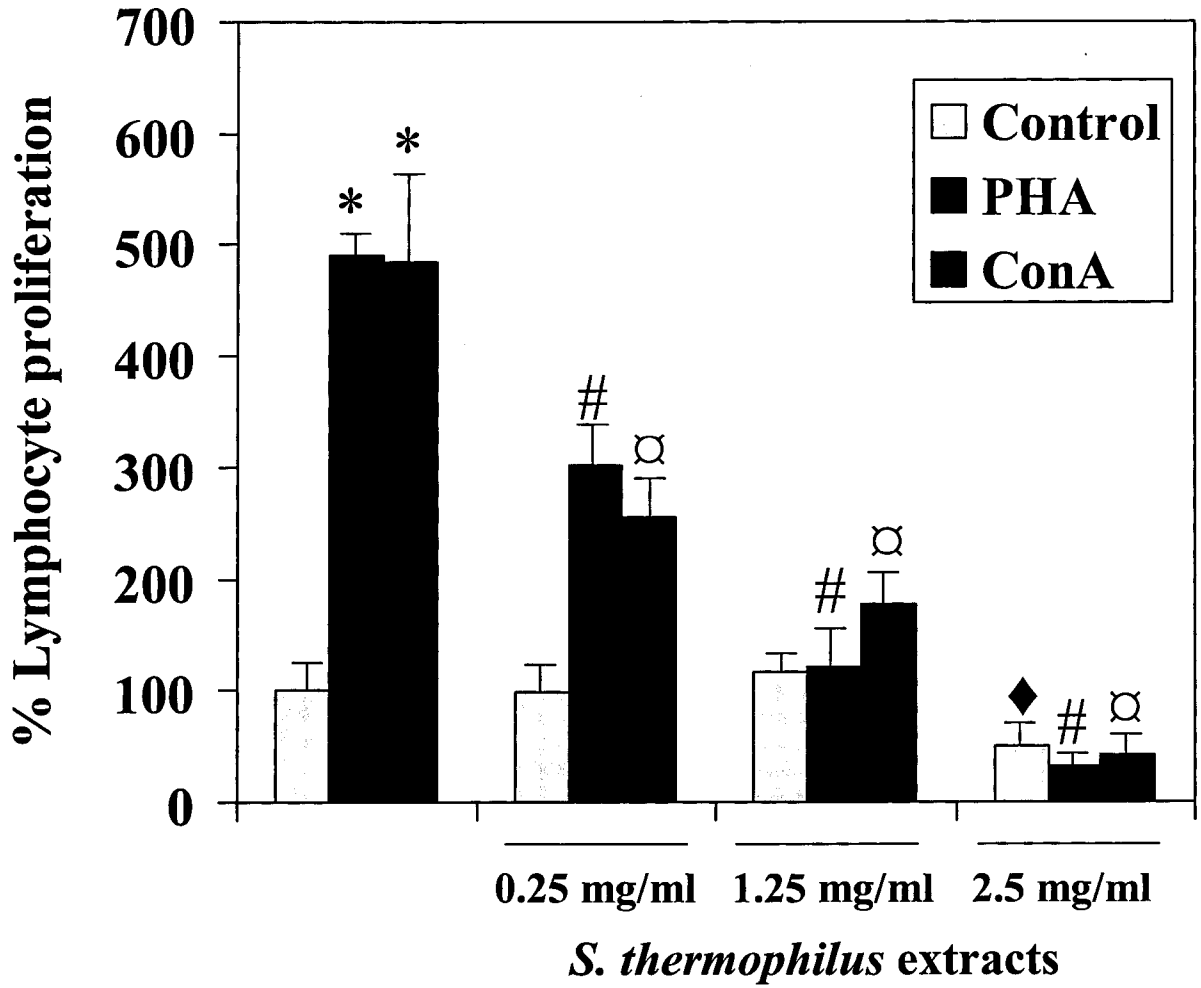
Figure 1. *Effect of S thermophilus extracts on mitogen-induced proliferation of human T-lymphocyte.* T- cells were incubated for 72 hrs with or without *S thermophilus* extracts at different amounts in the presence or absence of PHA (20 μg/ml) or ConA (10 μg/ml). The values, expressed as % cell proliferation respect to untreated cells meanly considered as 100%, represent the mean values of triplicate determinations ± SD. The results are representative of 1 from 3 experiments. T-cell proliferation was measured by [^{3}H]TdR incorporation. *P<0.001 and §P=0.005 when compared with untreated cells; $^{\#}$P<0.001 compared with PHA-activated T-cells; $^{\square}$P< 0.001 respect to ConA-activated T-cells.

Figure 2. *Effect of S thermophilus extracts on INF-γ production by mitogen-activated human T-cells* . The INF-γ levels were evaluated by a specific enzyme-linked immunosorbent assay (ELISA). Cytokine content was determined in supernatants of T-cells incubated for 48 hrs in the presence or absence of PHA (20 μg/ml) or ConA (10 μg/ml) and with or without *S thermophilus* extracts at different amounts. The results represent the mean values of duplicate determinations and are representative of 1 from 3 experiments. SD value were ever lower than 10% of mean value. *P<0.001 when compared with untreated cells; $^{\#}$P<0.001 compared with PHA-activated T-cells; $^{\square}$P< 0.001 respect to ConA-activated T-cells.

Figure 3. *Effect of S thermophilus extracts on sCD95 ligand release by mitogen-activated human T-cells.* The sCD95L levels were determined in conditioned medium of T-cells as measured by ELISA. T-cells were incubated for 48 hrs in the presence or absence of PHA (20 μg/ml) or ConA (10 μg/ml) and with or without *S thermophilus* extracts at different amounts. The results represent the mean values of triplicate determinations and are representative of 1 from 3 experiments. SD value were ever lower than 5% of mean value. *P<0.001 when compared with untreated cells. $^{\#}$P<0.001 compared with PHA-activated T-cells. $^{\square}$P< 0.001 respect to ConA-activated T-cells.

Figure 4. *Effect of S thermophilus extracts on T-cells-mediated keratinocyte apoptosis.* KC were incubated, for 3 days, with conditioned medium from (A) untreated T-cells, (B) T lymphocytes treated with bacterial extracts alone (2.5 mg/ml) (C), ConA-activated T-cells, (D) T-cells incubated with ConA in the presence of *S thermophilus* extracts (2.5 mg/ml). Nuclei were analysed with a FACScan cytofluorimeter. Data are representative of 1 from 3 experiments.

Figure 5. *Proposed scheme about the effect of S thermophilus extracts on an in vitro disease model of atopic dermatitis.*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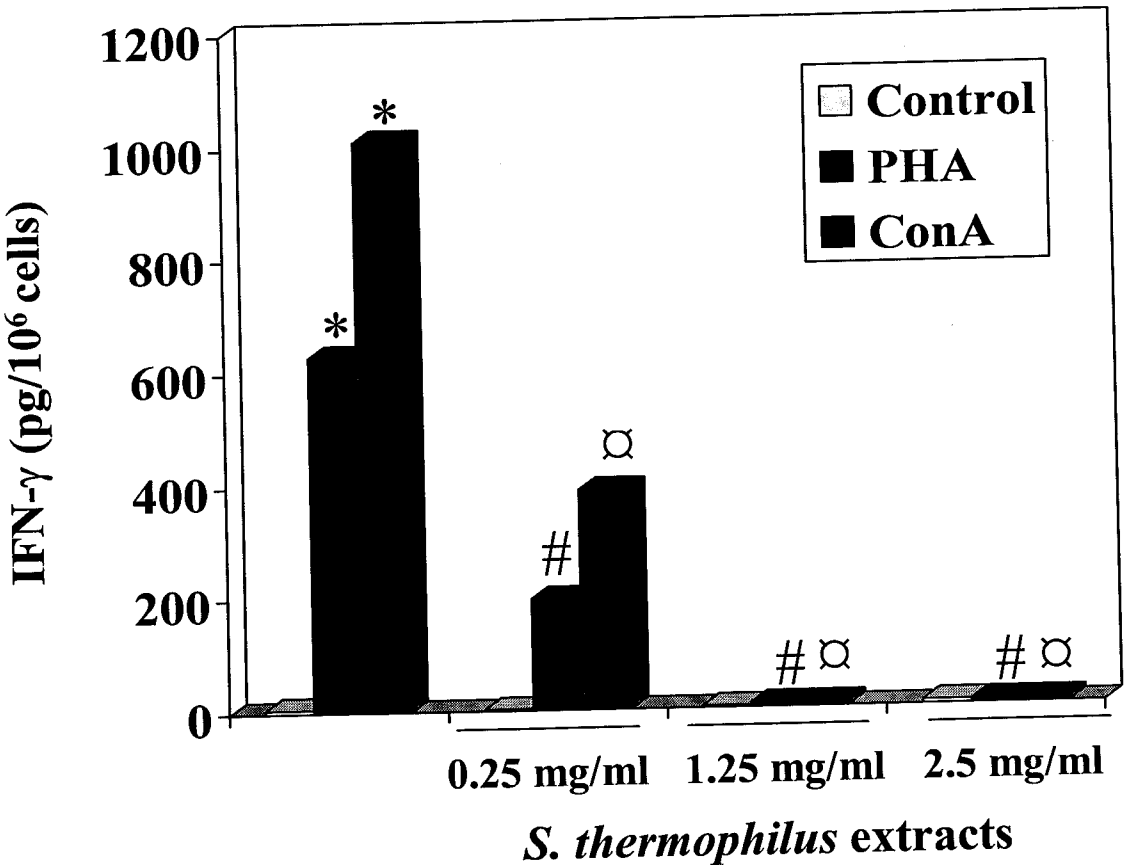
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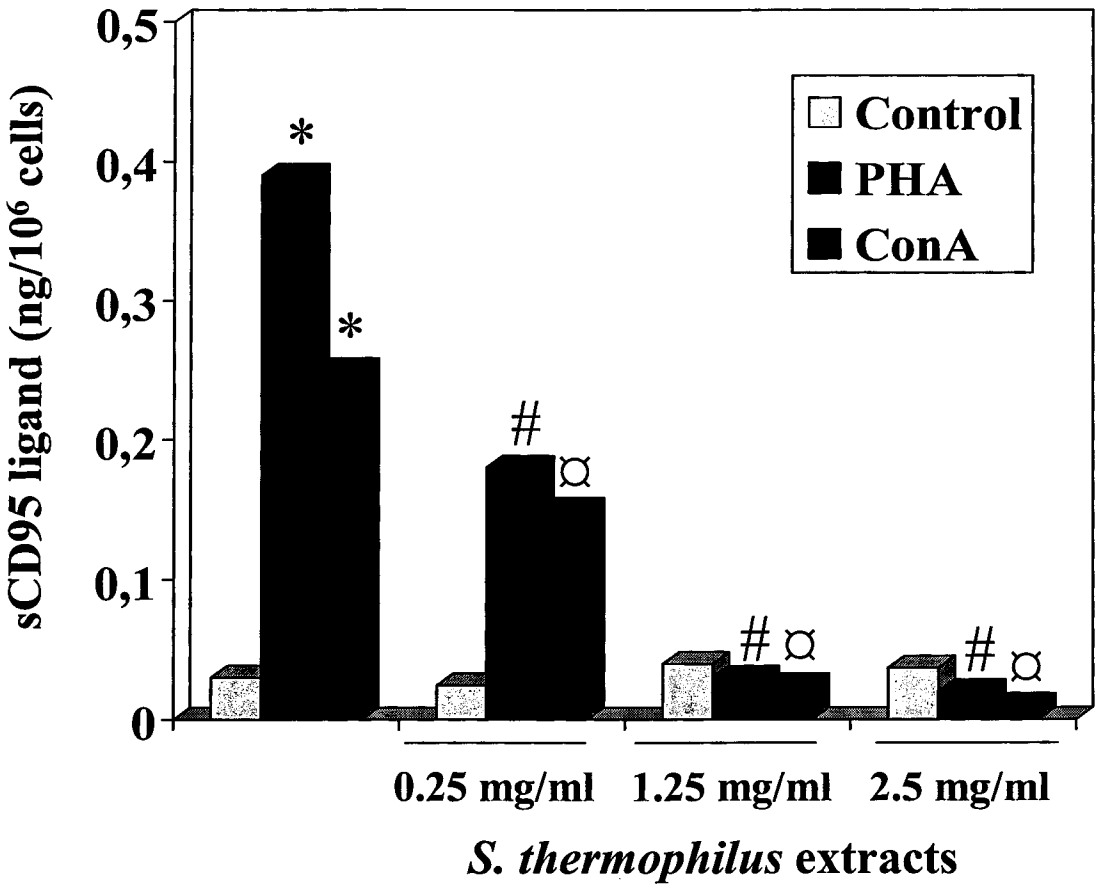
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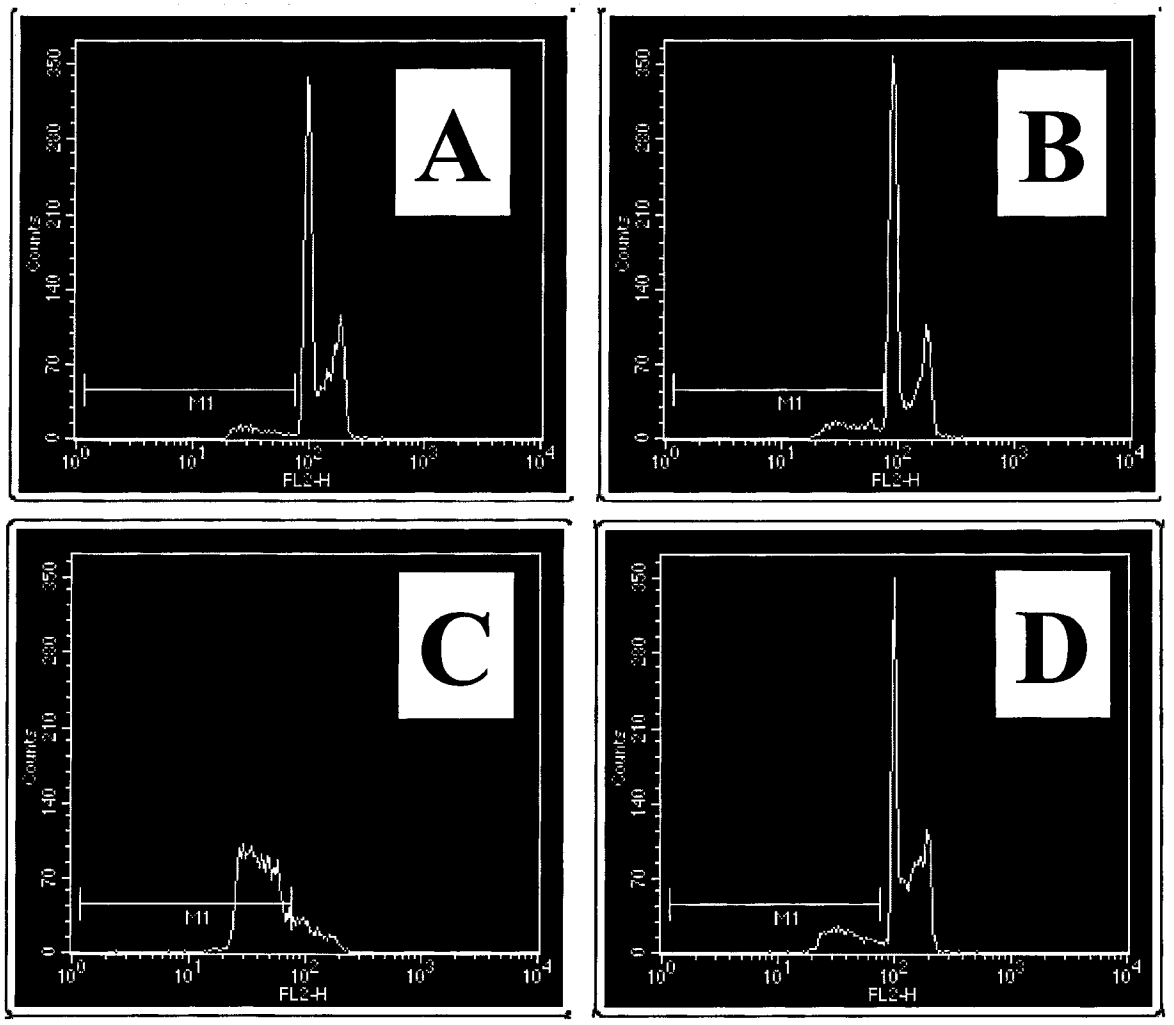
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FL2-H
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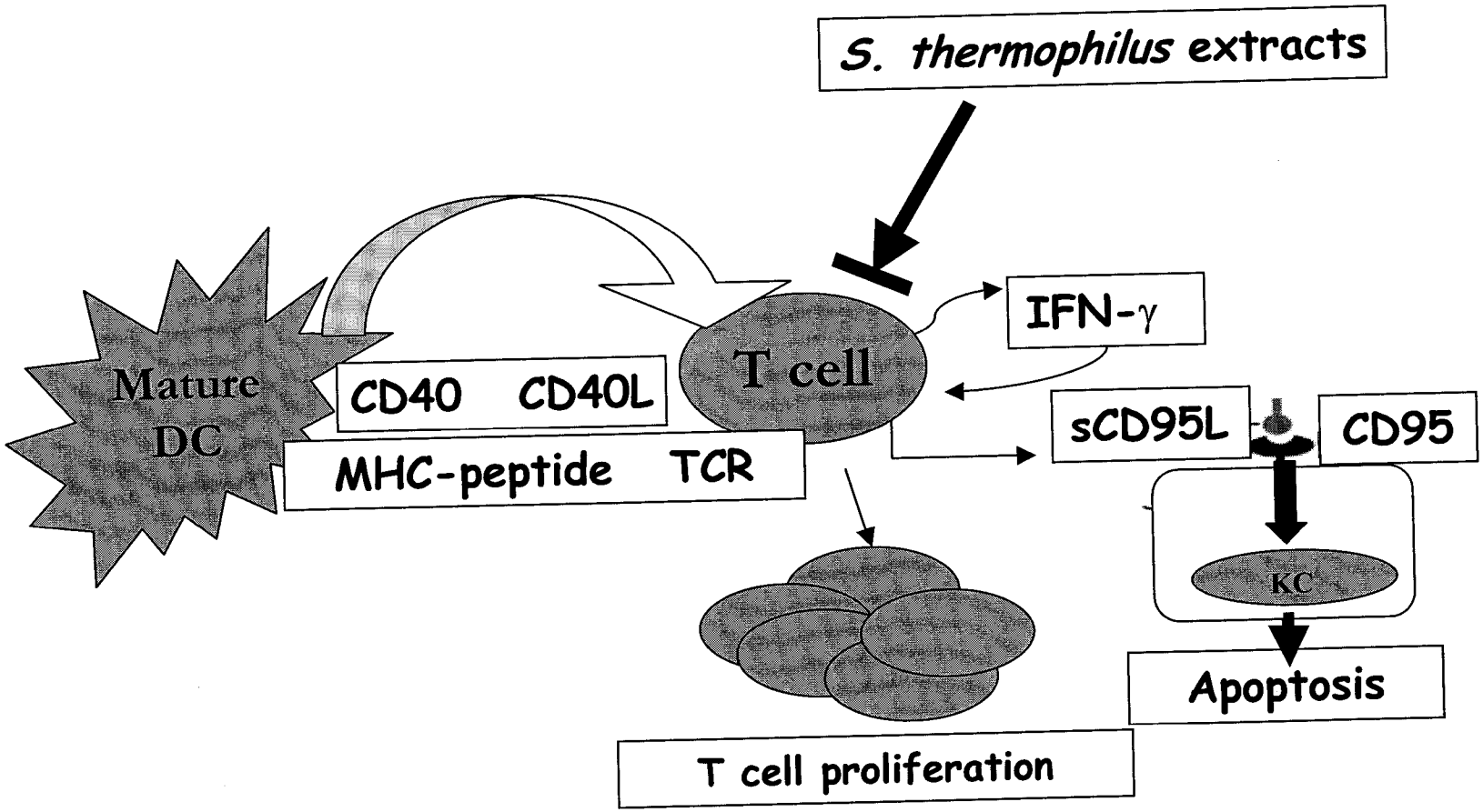
S. thermophilus extracts
Mature DC
CD40 CD40L
MHC-peptide TCR
T cell
IFN-γ
sCD95L
CD95
KC
Apoptosis
T cell proliferation