

LF 15-0195 Generates Synergistic Tolerance by Promoting Formation of CD4⁺CD25⁺CTLA4⁺ T Cells

Xiaoping Xia,*‡ Xiao Zhang,†‡ Xuyan Huang,‡ Mu Li,§ Lina Zhao,* Donghua Tian,*
Robert Zhong,‡§^{||} and Wei-Ping Min,‡§^{||}

Summary: The purpose of the present study was to investigate the influence of 15-deoxyspergualin (LF) on the phenotypes and functions of dendritic cells (DCs) and T cells and to further illustrate the mechanism of LF-inducing immunologic tolerance. DCs from mice were cultured and treated with varying doses of LF at specific time-points. Fluorescence-activated cell sorting (FACS) was used to verify the changes of phenotypes in the cultured DCs labeled with fluorescent antibody. DCs were also used as stimulators in mixed leukocyte reaction to detect their ability to stimulate T-cell proliferation. DCs and T cells, treated with or without LF, were cultured together; phenotypes and cytokine profile of the T cells were identified and assayed by FACS and enzyme-linked immunosorbent assay. LF induced a dose- and time-dependent suppression of maturation of DCs and a dose-dependent suppression of T-cell proliferation in mixed leukocyte reaction when LF-treated DCs were used as stimulators. LF-treated DCs, cultured with naive T cells, could promote the formation of CD4⁺CD25⁺CTLA4⁺ T-cell subtypes and the production of higher levels of interleukin-10. It was suggested that the mechanism of LF-induced tolerance was inhibiting maturation and function of DC and inducing the formation of regulatory T-cell subtype by "suppressor DCs" to achieve a new immune balance.

Key Words: 15-deoxyspergualin, suppressive dendritic cells, regulatory T cell, immune tolerance

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15-Deoxyspergualin (DSG; LF 15-0195) is a novel immunosuppressive agent with low toxicity. In vivo it was able to induce immunologic tolerance in Balb/c mice (recipient) after heart transplantation from C57BL/6 mice

(donor). In vitro it could suppress T-cell activity via blocking nuclear factor- κ B signal and interleukin (IL) 2 production.¹ T-Cell activation is dependent on the existence of professional antigen-presenting cells (APCs), which present antigen to T cells. Dendritic cells (DCs) are the major type of APCs and the only ones that can stimulate premature T-cell response.^{2–4} Once APCs lose antigen recognition and presentation activities owing to external intervention, immunologic response may be terminated, leading to immunologic tolerance or reconstruction. This study was designed to identify the mechanism of immunologic toleration induced by LF though investigating whether LF could interfere with the phenotype, maturation, and function of DCs and T cells.

MATERIALS AND METHODS

Reagents and Animals

LF 15-0195, an analogue of DSG, was provided by Fournier Laboratory (Daix, France). Lipopolysaccharide (LPS) from *Escherichia coli* serotype 0127:B8 was purchased from Sigma (St. Louis, MO, USA). Male C57BL/6 and BALB/c mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA).

Generation of Bone Marrow-Derived DCs

Bone marrow cells were flushed from the femurs and tibias of C57BL/6 mice, washed, and cultured at a concentration of 2×10^6 cells/well in 24-well plates (Corning, Corning, NY, USA) in 2 mL of RPMI 1640 (Gibco Life Technologies, Burlington, Ontario, Canada) supplemented with 10% fetal calf serum (FCS) (Gibco), 100 U/mL penicillin, 100 μ g/mL streptomycin, 50 μ M 2-mercaptoethanol (Gibco), 10 ng/mL recombinant murine granulocyte-macrophage colony-stimulating factor (GM-CSF) (Peprotech, Rocky Hill, NJ, USA), and 10 ng/mL IL-4 (Peprotech). Nonadherent cells were removed after 48 hours of culture, and fresh medium was added every 48 hours. Different dosages of LF (0, 1, 5, 10 μ g/mL) were added into the culture medium at specific time-points (0, 2, 4, 6 days after culture). Treated DCs were used for further phenotype analysis, mixed leukocyte reaction, and enzyme-linked immunosorbent assay.

DC Line

An immature DC line, designed JAWS II, established from C57BL/6 mouse was obtained from American Type Culture Collection (ATCC). JAWS II cells were cultured in α -minimum essential medium (no. 12465-019; Gibco) without ribonucleosides and deoxyribonucleosides, containing

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From the *Department of Ophthalmology, 3rd Affiliated Hospital, Sun Yat-sen University, Guangzhou, China; †Department of Rheumatology, People's Hospital of Guangdong Province, Guangzhou, China; ‡Departments of Surgery, Pathology, Microbiology, and Immunology, University of Western Ontario, London, Canada; §Multi-Organ Transplant Program, London Health Science Centre, London, Canada; and ||Immunology and Transplantation, Lawson Health Research Institute, London, Canada.

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Reprints: Xiao Zhang, Department of Rheumatology, People's Hospital of Guangdong Province, Guangzhou, China (e-mail: xiaozhang640@sohu.com).

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4 mM L-glutamine, 1 mM sodium pyruvate, 5 ng/mL murine GM-CSF, and 20% fetal bovine serum.

Generation of Spleen-Derived T Cells

T cells were prepared from Balb/c mice spleens, isolated by T-cell enrichment columns, and cultured in 5×10^5 cells/well for further experiments.

Phenotype Analysis of DC and T Cells (fluorescence-activated cell sorting)

Phenotypic analysis of cultured bone marrow-derived DCs (at the 8th day of culture) was performed on a FACScan (Becton Dickinson, San Jose, CA, USA). The fluorescein isothiocyanate phycoerythrin-conjugated anti-mouse monoclonal antibody were purchased from BD PharMingen (San Diego, CA, USA). I-A^d, CD11c, CD40, CD86, and IL-12 expression was assessed by intracellular staining using a cell permeabilization kit (Cedarlane Laboratories, Hornby, Ontario, Canada). All flow cytometric analyses were performed using appropriate isotype controls (Cedarlane).

C57BL/6 mice-derived DCs, treated with or without LF, were incubated with Balb/c mice-derived T cells for 3 days. After three-color staining, the phenotypic analysis of the T cells was performed on the above FACScan (gate CD4⁺ cells).

Mixed Leukocyte Reaction

Varying numbers of JAWS II DC, treated with or without LF (0, 1, 5, 10, 25 $\mu\text{g/mL}$) and LPS (0, 10 $\mu\text{g/mL}$) were seeded in triplicate in a flat-bottomed 96-well plate (Corning) for use as stimulator cells. T cells ($1-5 \times 10^5$ cells/well) were added to the DC cultures as reactor cells, with the final mixed leukocyte reaction taking place in 200 μL of RPMI 1640 (Gibco) supplemented with 10% FCS (Gibco), 100 U/mL of penicillin (Gibco), and 100 $\mu\text{g/mL}$ of streptomycin (Gibco). Cells were cultured for 3 days and pulsed with 1 μCi of [³H]thymidine (Amersham Pharmacia Biotech) for the last 16 hours of culture. Cells were harvested onto glass fiber

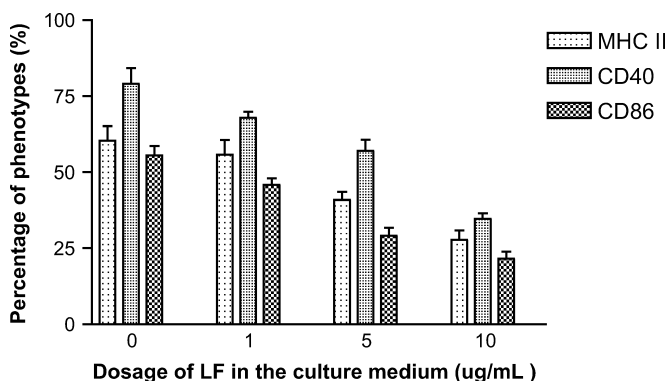


FIGURE 1. Effect of different dosage of LF on the maturity of DCs. Bone marrow derived DCs were cultured as described in Materials and Methods. LF were added into the culture at various concentrations as indicated. DCs were cultured for 8 days and activated by TNF α /LPS (10 ng/mL) in last 24 hrs. LF-treated and non-treated DCs were stained with FITC conjugated mAbs as indicated for cell surface molecules and analyzed by flow cytometry. Data shown are the mean \pm SD of three independent experiments.

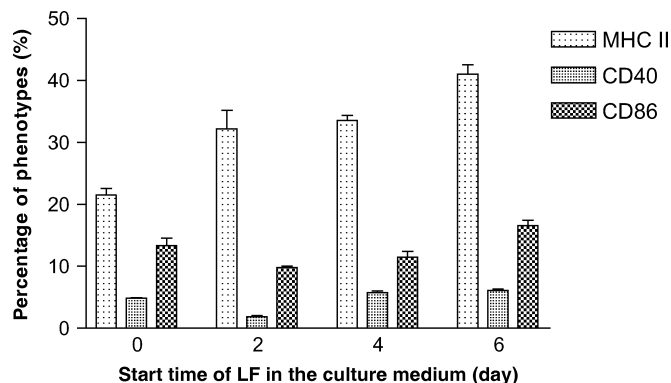


FIGURE 2. Effect of different start time-point of LF on the maturity of DCs. Bone marrow derived DCs were cultured as described in Materials and Methods. LF (10 $\mu\text{g/mL}$) were added into the culture at various time-points. DCs were cultured for 8 days and activated by TNF α /LPS (10 ng/mL) in last 24 hrs. LF-treated and non-treated DCs were stained with FITC conjugated mAbs as indicated for cell surface molecules and analyzed by flow cytometry. Data shown are the mean \pm SD of three independent experiments.

filters, and the radioactivity incorporated was quantitated using a Wallac Betaplate liquid scintillation counter (Beckman, Fullerton, CA, USA). Results were expressed as the mean \pm SEM counts per minute of triplicate cultures.

Enzyme-Linked Immunosorbent Assay

JAWS II DC (1×10^5), treated with or without LF, were cultured with Balb/c mice-derived T cells (5×10^6) for 48 hours. The supernatants were harvested and assessed for IL-10 and IL-2 by enzyme-linked immunosorbent assay (ELISA). Cytokine-specific ELISA (Endogen, Rockford, IL, USA) was

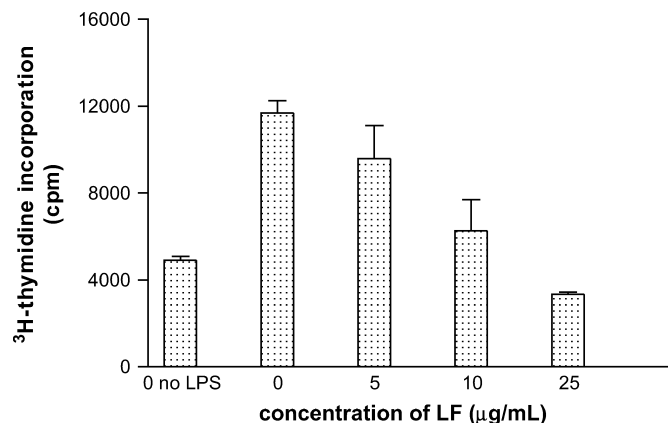
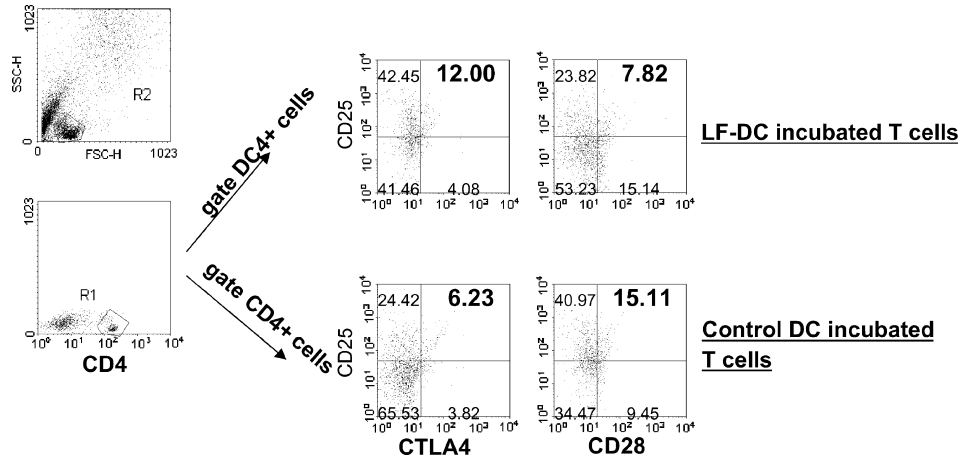


FIGURE 3. LF inhibits DC allostimulatory capacity in MLR. Stimulators comprised 0.5×10^5 JAWS II cells seeded into a 96-well plate. Cells were pretreated with LF at the indicated concentrations for 12 h and subsequently stimulated with 10 ng/mL of TNF α /LPS for 24 h. Responders consisted of 5×10^5 of BALB/c T cells. Stimulators and responders were co-cultured and proliferation was assessed as described in "Materials and Methods". Data shown are the mean \pm SD of three independent experiments.

FIGURE 4. LF treated DC increased CD4⁺CD25⁺CTLA4⁺ T cells and decreased CD4⁺CD25⁺28⁺ T cells. Allogeneic T cells were isolated from BALB/c spleens and incubated with JAWS II cells that were pretreated with 10 μg/mL LF for 12 h. After 7 days incubation, cells were triple stained with FITC-CD4, Cy5-CD25, PE-CTLA4 and PE-CD28 mAbs, and analyzed by flow cytometry. Data shown here is a representative of 3 experiments.



used for detecting protein concentrations in culture supernatants according to the manufacturer's instructions using a Benchmark Microplate Reader (Bio-Rad Laboratories, Hercules, CA, USA). The amounts of IL-10 and IL-2 were determined from standard curves generated with recombinant murine IL-10 and IL-2 (Endogen).

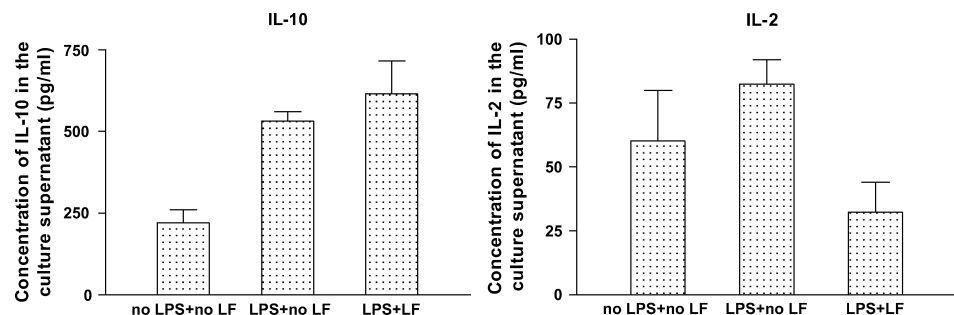
RESULTS

The higher the LF dosage in culture medium, the more suppressive the mature phenotype (major histocompatibility complex [MHC] II, CD40, CD86, and DEC205) of DCs. However, CD11c was not affected. The earlier the LF was added into the medium, the stronger the suppression of DC maturity that was observed (Figs. 1 and 2).

A dosage-dependent suppression of T-cell proliferation was observed in a mixed reaction. In this experiment, DCs were treated with different dosages of LF, which was used as stimulator. Higher dosage of LF treatment of DCs resulted in stronger suppression of T-cell proliferation (Fig. 3).

LF-treated DCs could induce the differentiation of untreated T cells into the regulatory CD4⁺CD25⁺CTLA4⁺ subtype. The percentage of this subtype increased with the elevated dosage of LF. It was also found that the content of IL-10 in the supernatant of this group was significantly higher than that of other groups (Figs. 4 and 5). This specific subtype could not be differentiated by either untreated DCs or T cells without the stimulation of DCs.

FIGURE 5. LF treated DCs increased IL-10 but inhibited IL-2 in T cells. Allogeneic T cells were isolated from BALB/c spleens and incubated with JAWS II cells that were pretreated with 10 μg/mL LF for 12 h. After 48 h incubation, the supernatants were harvested and the IL-2 and IL-10 were detected by ELISA as described in "Materials and Methods". Data shown are the mean ± SD of three independent experiments.



DISCUSSION

DCs have high expression of MHC and compound-stimulating molecules. Their ability to induce T-cell activation and tolerance was 100 times greater than that of macrophages. DC maturity and functional activity were represented by the expression of the surface molecules, because these molecules respectively represent the first and second signals in the antigen-presenting pathway. When LF was added to DC culture medium, it was observed that DCs appeared to have a decreased expression of MHC, CD86, and CD40 and became more premature as the dosage of LF increased. In addition, it was also observed that the earlier the intervention with LF occurred, the stronger the suppression appeared. This suggests that early treatment with LF may result in a more potent suppression of DC maturity. Because lacking of co-stimulating factor in these premature DCs, the T cell would become anergy or apoptosis.^{5,6} Upon an examination of anti-heart transplantation rejection in vivo, it was also shown that the usage of LF 2 weeks after surgery resulted in a prolonged survival of donor, whereas monotherapy for T-cell suppressor or DC suppressor could not prevent the rejection.⁷ This demonstrated that both T cells and DCs must be suppressed to establish a long-lasting immunologic tolerance in the body.

To determine what effect LF-induced premature DCs have on T-cell activation and immunologic tolerance, we looked at T-cell proliferation and differentiation. This study showed that deficiency in the maturity degree of DCs and their ability to induce T-cell proliferation was correlated with the dosage of LF treatment, suggesting that premature DCs have

deficiency in presenting antigen and signal, which are essential for activation and proliferation of primitive T cells. To look at any other mechanism, LF-treated or untreated DCs and T cells were arranged in four culture groups to observe their differentiation and secretion. It was found that the mixed cultivation of LF-treated immature DCs and LF-treated T cells could induce a unique T-cell subtype ($CD4^+CD25^+CTLA4^+$ T cell). This subtype is thought to be a modulating T cell with immunologic anergy or inhibitive potential. Meanwhile, the expression of IL-10 in the supernatant of this group was observed to be much higher than that in the other groups. Therefore, LF may induce a “suppressor DC,” which could induce production of modulating T cell (processing the surface molecule $CD4^+CD25^+CTLA4^+$, mainly secreting IL-10 and having low proliferation ability) in vitro. This modulating T cell in turn could induce antigen-specific immunologic tolerance either in vitro or in vivo.^{8,9} To sum up, it is believed that the immunologic suppression induced by LF is due to (a) the inhibition of DC maturation and functioning and (b) interference with T-cell differentiation directly or via the “suppressor DC,” which increased the number of $CD4^+CD25^+CTLA4^+$ T cell and ultimately achieved immunologic tolerance.

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