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Lycium barbarum polysaccharides regulate phenotypic and functional maturation of murine dendritic cells

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Abstract

Lycium barbarum polysaccharides (LBPs) have been known to have a variety of immunomodulatory functions including activation of T cells, B cells and NK cells. Dendritic cells (DC) are potent antigen-presenting cells that play pivotal roles in the initiation of the primary immune response. However, little is known about the immunomodulatory effects of LBPs on murine bone marrow derived dendritic cells (BMDC). In the present study, the effects of LBPs on the phenotypic and functional maturation of murine BMDC were investigated *in vitro*. Compared to the BMDC that were only subjected to treatment with RPMI1640, the co-expression of I-A/I-E, CD11c and secretion of IL-12 p40 by BMDC stimulated with LBPs (100 μg/ml) were increased. In addition, the endocytosis of FITC-dextran by LBPs-treated BMDC (100 μg/ml) was impaired, whereas the activation of proliferation of allogenic lymphocytes by BMDC was enhanced. Our results strongly suggest that LBPs are capable of promoting both the phenotypic and functional maturation of murine BMDC *in vitro*.
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Keywords: *Lycium barbarum* polysaccharides (LBPs); Dendritic cells (DC); Mixed lymphocyte reaction (MLR)

1. Introduction

Lycium barbarum, a well-known Chinese traditional medicine and also an edible food, plays multiple roles in pharmacological and biological functions including anti-aging activity, hypoglycemic and hypolipidemic effects, strengthening yang and improving eyesight. It has been reported that polysaccharides purified from *Lycium barbarum* are especially effective in modulating immune functions and inhibiting tumor growth (Gong et al., 2005; Gan et al., 2004; Cao et al., 1994). The immuno-modulation effects of LBPs are extensive, including increasing macrophage phagocytosis, the form of antibody secreted by spleen cells, spleen lymphocyte proliferation and CTL activity. Recently, the antitumor effects of LBPs have been investigated and are believed to be going through immune mechanisms (Gan et al., 2003, 2004).

DC, one of the most potent antigen-presenting cells (APC), are important for the initiation of primary immune response of both helper and cytotoxic T lymphocytes (Banchereua et al., 2000; Banchereua and Steinman, 1998; Lanzavecchia and Sallusto, 2001; Liu, 2001). After antigen capture, the DC precursors migrate to T cell regions of draining lymph nodes where they mature into functional DC. The functional DC further stimulate naive T cells by triggering the signaling pathway involving both major histocompatibility complex (MHC) molecules presenting antigen-peptides and co-stimulatory molecules (Austyn et al., 1988; Rock, 1996). The initial contact between DC and resting T cells may be mediated by a transient, high affinity interaction between DC-SIGN on the DC and the adhesion molecule ICAM-3 on the T cells (Geijtenbeek et al., 2000), followed by interactions through other adhesion molecules and their corresponding ligands (ICAM-1/LFA-1,

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LFA-1/CD2). Following TCR engagement, an intimate interaction often referred to as the immunological synapse evolves, where multiple interactions between co-stimulatory molecules on DC and their ligands on T cells result in final DC maturation and T cell activation (Banchereua et al., 2000; Lanzavecchia and Sallusto, 2000). There are no reports about LBPs inducing maturation of murine BMDC. Therefore, LBPs regulating phenotypic and functional maturation of murine BMDC were investigated in this study.

2. Materials and methods

2.1. Source of mice

Male or female C57BL/6J (H-2^b) and BALB/c (H-2^d) mice were purchased from the Department of Experimental Animal, College of Medicine, Zhejiang University, Hangzhou, China. Mice were used at 4–6 weeks of age.

2.2. Source of drugs

LBPs were purchased from Pharmagenesis Beijing Office. The percentage of polysaccharide of LBPs was about 84.32% according to the phenol-sulfuric acid colorimetric method. The molecular weights of LBPs were estimated to be 31,000 through high performance gel filtration chromatography. The LBPs mainly consist of mannose, glucose, galactose, arabinose, rhamnose and xylose.

2.3. Generation of bone marrow-derived murine myeloid DC

DC were prepared as described previously with minor modifications (Inaba et al., 1992). Briefly, bone marrow cells were flushed from the femur and tibiae of C57BL/6J mice and depleted of RBC by hypotonic lysis using Tris-NH_4Cl. Cells with a starting number of 2×10^6 cells per ml were cultured in RPMI 1640 in six-well flat bottom plates (Orange Scientific) at 37 °C, 5% CO_2, supplemented with 10% fetal calf serum (FCS), 2 mmol/l L-glutamine, 100 U/ml penicillin G, 100 μg/ml streptomycin, 30 ng/ml rmGM-CSF (Peprotech) and 20 ng/ml rmIL-4 (Peprotech). On day 3, the old medium was replaced with fresh medium. On day 5, cells were purified by MACS columns (Miltenyi Biotec). CD11c$^+$ DC were acquired and divided into 3 groups. In parallel, 3 groups of DC were incubated at a concentration of 1×10^5/ml with either 100 μg/ml LBPs, serum-free RPMI media 1640 or 100 ng/ml LPS (serotype 0111:24; Sigma). The serum-free RPMI media 1640 and 100 ng/ml LPS groups were used as controls. On day 7, cells and culture supernatants were collected for further experiments and analysis.

2.4. Flow cytometric analysis

Cell surface expression of I-A/I-E or CD11c was determined by immunofluorescence staining. On day 7, cells were harvested, washed twice with PBS, and resuspended in washing buffer (PBS containing 2% FCS and 0.1% sodium azide). Cells were first blocked with 20% mixed mouse and rat serum for 15 min at 4 °C, and then stained with PE conjugated anti-mouse I-A/I-E antibody (BD Pharmingen) and FITC conjugated anti-mouse CD11c antibody (BD Pharmingen) for 30 min at 4 °C in the dark. Lastly the antibody-treated cells were washed twice with washing buffer. Cell surface co-expression of I-A/I-E and CD11c was detected by flow cytometry (Becton Dickinson). All the obtained data were analyzed by the CellQuest software package.

2.5. Cytokine assay

On day 7, DC culture supernatants were collected and the concentration of mouse IL-12 p40 unit was determined by a sandwich enzyme-linked immunosorbent assay (ELISA) kit (Biosource) according to the manufacturer's instructions. Cytokine concentrations were determined according to absorbance readings at 450 nm on a universal microplate reader (Bio-Tek Instruments).

2.6. Endocytos is assay

In order to analyze the endocytic capacity of DC on day 7, 1×10^5 cells were incubated at 37 °C for 1 h with 1 mg/ml FITC-dextran (Sigma). In the parallel experiment 1×10^5 cells were incubated at 4 °C for 1 h. After incubation, the cells were washed twice with cold HBSS. The cells were analyzed on a flow cytometer.

2.7. Mixed lymphocyte reaction (MLR) induced by DC

Responder mononuclear lymphocytes from H-2^d BALB/c's splenocytes were isolated by Ficoll-Urografin density gradient. On day 7, mature DC were harvested as inducers, treated with 25 μg/ml mitomycin C (AppliChem) for 45 min, then 5×10^3 cells were added to allogeneic lymphocytes (1×10^5 cells per well) in flat-bottom 96-well tissue culture plates for 120 h. Cell proliferation was estimated according to the cellular reduction of tetrazolium salt MTT (Sangon) by the mitochondrial dehydrogenase of viable cells into a blue formazan product that was measured spectrophotometrically.

2.8. Statistics

The results were expressed as means ± SD of the indicated number of experiments. The statistical significance was estimated using a Student's t-test for unpaired observations. A p value of <0.05 was considered to be significant.

3. Results

3.1. LBPs up-regulate the co-expression of I-A/I-E and CD11c on DC surface

LBPs (100 μg/ml) or LPS (100 ng/ml) treated DC showed an increased co-expression of I-A/I-E and CD11c on DC surface, and the double positive cells ratio were (41.42 ± 1.43)% or (54.13 ± 3.99)%, respectively, whereas that of RPMI 1640 was (35.68 ± 2.40)%. The difference was significant by paired t test analysis: $n = 4$, $P < 0.01$). One groups' figure of flow cytometric analysis is shown (Fig. 1).

3.2. LBPs increase IL-12 p40 production of DC

LBPs (100 μg/ml) or LPS (100 ng/ml) treated DC showed an increased production of IL-12 p40 in culture supernatants of DC, and the production was 94.1 ± 12.2 pg/ml, 263.6 ± 13.8 pg/ml, respectively, compared with 57.3 ± 17.2 pg/ml of RPMI 1640. A significant difference was observed between RPMI 1640 control and 100 μg/ml LBPs group or LPS group (Fig. 2). The difference was significant by paired t test analysis: $n = 4$, $P < 0.01$).

3.3. LBPs inhibit the FITC-dextran uptake by DC

Mannose-receptor-mediated endocytosis was analyzed by the uptake of FITC-dextran. In DC treated by LBPs (100 μg/ml) or LPS (100 ng/ml), the percentage (30.96 ± 3.30 and 19.66 ± 2.01, respectively) of FITC-stained cells had a significantly lower uptake of FITC-dextran, compared with RPMI 1640 (68.52 ± 1.66)%. Parallel experiments were also

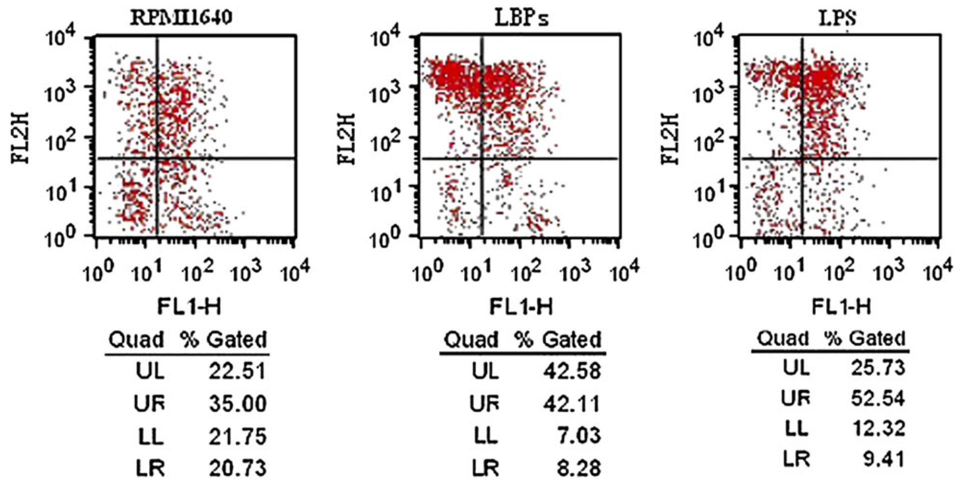

Fig. 1. Flow cytometric analysis of DC treated with RPMI 1640, LBPs or LPS. On day 5 of DC culture, cells were incubated at a concentration of 1×10^5 per well with 100 ng/ml LPS and 100 μg/ml LBPs. Serum-free RPMI 1640 was used as control. On day 7, cells were stained with R-PE conjugated anti-mouse I-A/I-E and FITC conjugated anti-mouse CD11c for 30 min at 4 °C in the dark. Fluorescence profiles were generated on a FACS flow cytometer. Histogram and density plots were produced by CellQuest software package. FL1-H: FITC conjugated anti-mouse CD11c; FL2-H: R-PE conjugated anti-mouse I-A/I-E.

performed at 4 °C (15.93 ± 1.02), FITC-dextran was internalized significantly less than DC at 37 °C. The difference was significant by paired t test analysis: $n = 4$, $P < 0.01$). One groups' figure of flow cytometric analysis is shown (Fig. 3).

3.4. LBPs facilitate the allostimulatory capacity of DC

The effects of LBPs on MLR induced by DC are illustrated in Fig. 4. LBPs (100 μg/ml)-treated DC or LPS (100 ng/ml)-treated DC stimulated proliferative responses more effectively than RPMI 1640-treated DC, and the proliferation ratio of the lymphocytes was (129.9 ± 12.48)% or (153.8 ± 14.15)%, respectively, compared with (100 ± 9.12)% of RPMI 1640 (Fig. 2). The difference was significant by paired t test analysis: $n = 5$, $P < 0.05$). (RPMI 1640 control served as 100%).

4. Discussion

It has been well-known for decades that LBPs are biologically active components of *Lycium barbarum* with potential pharmacological and biological functions. This is the first report that we are aware of showing the effects of LBPs on the generation and maturation of murine BMDC. In this study, we have shown that LBPs can induce maturation of murine BMDC and ready them for T cell-mediated immune responses. LBPs significantly increase the expression of membrane molecules I-A/I-E and CD11c. Meanwhile, LBPs markedly reduce the endocytic activity of DC and augment their capacity to promote the proliferation of naïve allogeneic T cells.

DC can differentiate from immature to mature stages by various stimulators, by cytokines such as TNF-α or pathogen products, or LPS (Banchereua et al., 2000). Immature DC have a high capacity for antigen capture and processing. Maturation of DC are characterized by a decreased antigen processing capacity, an increased cell surface expression of MHC and co-stimulatory molecules, and the secretion of IL-12, priming strong stimulation of $CD4^+$ and $CD8^+$T lymphocyte growth and differentiation (Banchereua et al., 2000; Banchereau and Steinman, 1998; Lanzavecchia and Sallusto, 2001). Mature DC can secrete IL-12 p70, the active protein, and then prime Th1 lymphocyte transformation. IL-12 p40 is a subunit of IL-12 p70, whose expression is inducible and correlated with the production of bioactive IL-12 p70 by DC; while another subunit, IL-12 p35, is encoded on separate genes and constitutively expressed by a variety of cell types (Schoenhaut et al., 1992; Trinchieri, 1995). Our data indicate that exposure to LBPs increase the production of bioactive IL-12 p70 in murine BMDC in the presence of GM-CSF/IL-4.

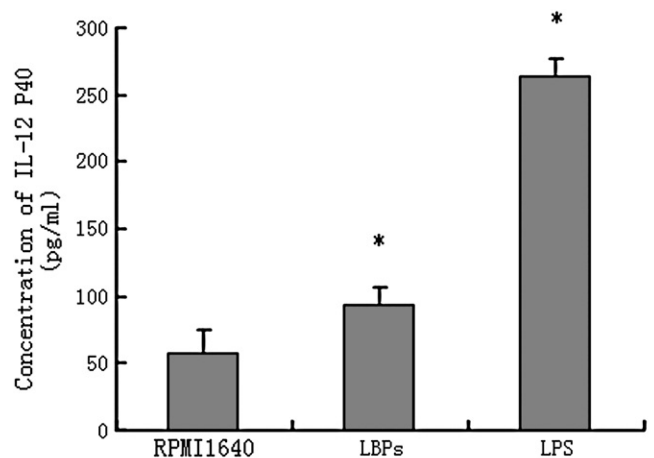

Fig. 2. Production of IL-12 p40 in culture supernatants of DC treated with RPMI 1640, LBPs or LPS. On day 5 of DC culture, cells were incubated at a concentration 1×10^5 per well with 100 ng/ml LPS and 100 μg/ml LBPs. RPMI 1640 was used as control. On day 7, culture supernatants were collected for detecting the level of mouse IL-12 P40 (by ELISA). Cytokine concentration was determined with a standard curve derived from known amounts of the IL-12 P40. $n = 4$, $^*P < 0.01$ vs. RPMI 1640.

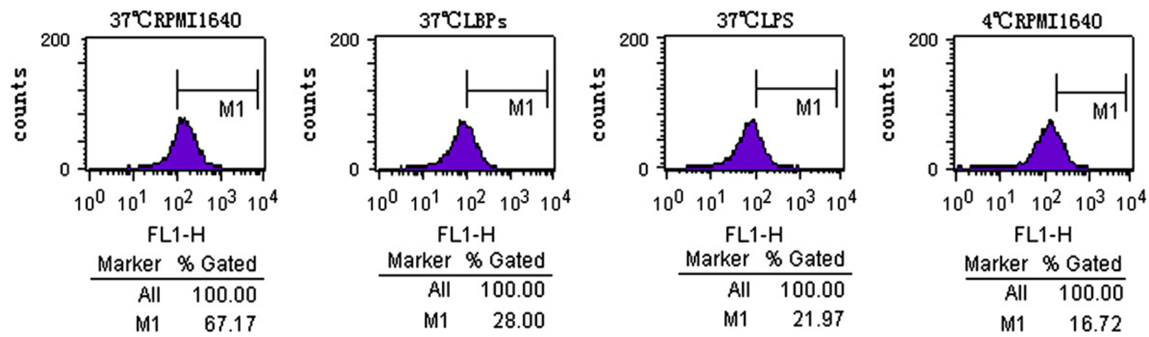

Fig. 3. Antigen uptake by DC stimulated by RPMI 1640, LBPs or LPS. On day 5 of DC culture, cells were incubated at a concentration of 1×10^5 per well with 100 ng/ml LPS and 100 μg/ml LBPs. On day 7, after cells were collected, the endocytic activity of the DC was determined by flow cytometry after treatment with FITC-dextran. Thereafter, the cells were washed twice with cold HBSS. The control endocytic activity was determined after FITC-dextran treatment at 4 °C. Data represents the percentages of cells.

Also, we investigated the capacity of LBPs-treated DC to internalize FITC-dextran via mannose receptor-mediated endocytosis. Both mechanisms are complex, energy-dependant processes that require the coordinated action of the actin cytoskeleton and are characteristic and distinctive properties of immature vs. mature DC (Sallusto et al., 1995; Garrett and Mellmann, 1999). These data further support the view that exposure to LBPs promotes the generation of functionally active, mature DC.

It is well-known that tumors are sources of biological substances and release certain immunosuppressive factors to evade the immune surveillance system of the host (Kanto et al., 2001). Through analysis of tumor specimens obtained from patients, a reduced number of DC infiltrated into tumors correlated with a poor prognosis for patients (Zeid and Muller, 1993; Tsuge et al., 2000). Also, the phenotypes and function of DC could be altered in tumors. The reduction of expression of co-stimulatory molecules, defective cytokine production, and full allostimulatory activity could be found in DC infiltrated into tumor tissue, which implies that tumor-derived factors can impede DC maturation (Gabrilovich et al., 1997; Troy et al., 1998). These effects appear to be maturation-dependent, acting only on DC precursors and not mature DC. Therefore, it may be better to use mature DC for clinical applications.

LBPs as a traditional Chinese herb have been extensively used in improving the immune activity of patients treated by chemical or radiation therapy. According to the results of this study, LBPs enhance the phenotypic and functional maturation of DC. Therefore, LBPs may increase the antitumor effects of DC-based vaccine therapy. Now we are investigating the antitumor effects of a combination therapy through an LBPs- and DC-based vaccine in a tumor-bearing mouse model.

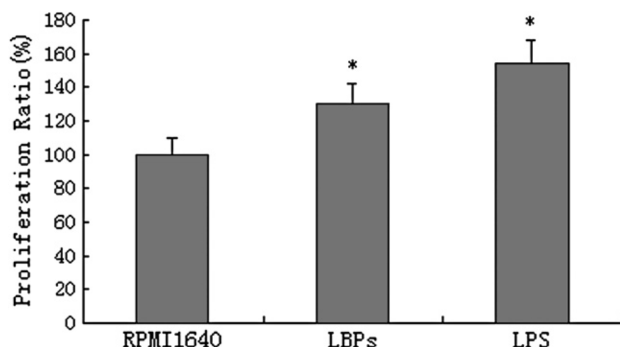

Fig. 4. Lymphocyte proliferation of MLR induced by DC. On day 5 of DC culture, cells were incubated with 100 ng/ml LPS and 100 μg/ml LBPs. RPMI 1640 was used as control. On day 7, mature DC was treated with 25 μg/ml mitomycin C and mononuclear lymphocytes from splenocytes were isolated by Ficoll-Urografin density gradient. H-2^d BABL/c responder spleen lymphocytes (1×10^5 cells per well) were cultured with H-2^b C57BL/6j inducer mature LBPs-treated DC, RPMI 1640-treated DC or LPS-treated DC (all at 5×10^3 cells per well). Cell proliferation was estimated by MTT method. $n = 5$, $^*P < 0.05$ vs. RPMI 1640.

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References

Austyn JM, Kupiec-Weglinski JW, Hankins DF, Morris PJ. Migration patterns of dendritic cells in the mouse. Homing to T cell-dependent areas of spleen, and bingding within marginal zone. J Exp Med 1988;167: 646−51.

Banchereau J, Steinman RM. Dendritic cells and the control of immunity. Nature 1998;392:245−52.

Banchereua J, Briere F, Caux C, Davoust J, Lebecque S, Liu YJ, et al. Immunobiology of dendritic cells. Annu Rev Immunol 2000;18:767−811.

Cao GW, Yang WG, Du P. Observation of the effects of LAK/IL-2 therapy combining with *Lycium barbarum* polysaccharides in the treatment of 75 cancer patients. Zhonghua Zhong Liu Za Zhi 1994;16:428−31.

Gabrilovich DI, Corak J, Ciernik IF, Kavanaugh D, Carbone DP. Decreased antigen presentation by dendritic cells in patients with breast cancer. Clin Cancer Res 1997;3:483−90.

Gan L, Zhang SH, Liu Q, Xu HB. A polysaccharide-protein complex from *Lycium barbarum* upregulates cytokine expression in human peripheral blood mononuclear cells. Eur J Pharmacol 2003;3:217−22.

Gan L, Hua ZS, Liang YX, Bi XH. Immunomodulation and antitumor activity by a polysaccharide-protein complex from *Lycium barbarum*. Int Immunopharmacol 2004;4:563−9.

Garrett WS, Mellmann I. Studies of endocytosis. In: Lotze MT, Thomson AW, editors. Dendritic Cells. San Diego: Academic Press; 1999. p. 693.

Geijtenbeek TH, Torensma R, van Vliet SJ, van Duijnhoven GC, Adema GJ, van Kooyk Y, et al. Identification of DC-SIGN, a novel dendritic cell-specific ICAM-3 receptor that supports primary immune responses. Cell 2000;100:575–85.

Gong H, Shen P, Jin L, Xing C, Tang F. Therapeutic effects of *Lycium barbarum* polysaccharide (LBP) on irradiation or chemotherapy-induced myelosuppressive mice. Cancer Biother Radiopharm 2005;20:155–62.

Inaba K, Inaba M, Romani N, Aya H, Deguchi M, Ikehara S, et al. Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. J Exp Med 1992;176:1693–702.

Kanto T, Kalinski P, Hunter OC, Lotze MT, Amoscato AA. Ceramide mediates tumor-induced dendritic cell apoptosis. J Immunol 2001;167:3773–84.

Lanzavecchia A, Sallusto F. From synapses to immunological memory: the role of sustained T cell stimulation. Curr Opin Immunol 2000;12: 92–8.

Lanzavecchia A, Sallusto F. Regulation of T cell immunity by dendritic cells. Cell 2001;106:263–6.

Liu YJ. Dendritic cell subsets and lineages, and their functions in innate and adaptive immunity. Cell 2001;106:259–62.

Rock KL. A new foreign policy: MHC class molecules monitor the outside world. Immunol Today 1996;17:131–7.

Sallusto F, Cella M, Danieli C, Lanzavecchia A. Dendritic cells used macropinocytosis and the mannose receptor to concentrate macromolecules in the major histocompatibility complex class II compartment: downregulation by cytokines and bacterial products. J Exp Med 1995;182:389–400.

Schoenhaut DS, Chua AO, Wolitzky AG, Quinn PM, Dwyer CM, McComas W, et al. Cloning and expression of murine IL-12. J Immunol 1992;148:3433–40.

Trinchieri G. Interleukin-12: a proinflammatory cytokine with immunoregulatory functions that bridge innate resistance and antigen-specific adaptive immunity. Annu Rev Immunol 1995;13:251–76.

Troy AJ, Summers KL, Davidson PJ, Atkinson CH, Hart DN. Minimal recruitment and activation of dendritic cells within renal cell carcinoma. Clin Cancer Res 1998;4:585–93.

Tsuge T, Yamakawa M, Tsukamoto M. Infiltrating dendritic/Landerhans cells in primary breast cancer. Breast Cancer Res Treat 2000;59:141–52.

Zeid NA, Muller HK. S100 positive dendritic cells in human lung tumors associated with cell differentiation and enhanced survival. Pathology 1993; 25:338–43.