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AQUEOUS EXTRACT OF *WITHANIA SOMNIFERA* (ASHWAGANDHA) ROOT AN INDIGENOUS MEDICINAL PLANT ENHANCES ANTIGEN SPECIFIC CELL-MEDIATED IMMUNE RESPONSE (CMIR) IN A T CELL LYMPHOMA

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Abstract:

Aims of the study: *Withania somnifera is an important Indian medicinal plant, widely used as a home remedy for several diseases in India. It is described as an herbal tonic and health food in Indian traditional medicine and considered as 'Indian Ginseng'. A series of animal studies show ashwagandha to have profound effects on the hematopoietic system and acts as an immunomodulator. However, there is inadequate evidence on their antitumor and immunostimulatory properties. Therefore, the present study is intended to investigate the effect of its aqueous root extract on Th1 immune response in a T cell lymphoma murine model.*

Materials and methods: *Groups of normal and DL-bearing BALB/c mice were fed with optimum dose of W. somnifera root extract for seven days, sensitized and challenged with OVA or tumor cell extract (TE). After 24 hrs of allergen or antigenic challenge, contact hypersensitivity (CHS), delayed type hypersensitivity (DTH) and production of IFN-γ and MIP-1α in footpad was measured.*

Results: *WSE treatment induces higher CHS and DTH immune response in both normal and DL-bearing mice as compared to normal and DL-bearing mice that received sterile PBS. It also increases thymocyte proliferation and production of IFN-γ and MIP-1α in DTH footpad of mice.*

Conclusion: *On the basis of result obtained, it can be concluded that WSE treatment significantly enhances antigen specific Th1 immune response in both normal healthy and DL-bearing mice and therefore, it can be used as immunostimulator and/or in combination therapy against the malignancies.*

Keywords: *W. somnifera; Herbal Medicine; Th1 immune response; Delayed type Hypersensitivity; IFN-γ; MIP-1α; T cell lymphoma.*

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1. INTRODUCTION

Withania somnifera L. Dunal (Family, Solanaceae) commonly known as Ashwagandha is an Ayurvedic Indian medicinal plant, which has been widely used as a home remedy for several disorders. *W. somnifera* is mentioned as herbal tonic and health food in Indian systems of traditional medicine. From ancient time, it is used as tonic to cure emaciation, debility, dyspepsia and other digestive problems, rheumatism, normalize physiological functions by working on the hypothalamic-pituitary-adrenal (HPA) axis and the neuroendocrine system. The plant extract isolated from its root is therapeutically most active part used in treating syphilis and asthma (Nadkarni et al., 1954; Tripathy et al., 1996). It possesses antisertogenic, anticancer, anabolic activity and has beneficial effects in the treatment of arthritis, geriatric problems and stress (Asthana et al., 1989). Like other adaptogenic herbs, it is a potent immunomodulatory agent and can serve to regulate immune response of a host by acting as a potent strengthening, immunomodulatory drug.

Recently, it has been reported that leaf and root extract of *W. somnifera* have profound hypoglycaemic effects and increases the insulin sensitivity in non-insulin dependent DM rats (Udayakumar et al., 2009; Anwer et al., 2008). Administration of an extract from the powdered root of the plant *W. somnifera* was found to stimulate immunological activity in BALB/c mice and found to counteract cyclophosphamide-induced immunosuppression by significantly increasing hemagglutinating antibody responses and hemolytic antibody responses towards sheep red blood cells (SRBC) (Davis and Kuttan, 2000; Agarwal et al., 1999; Davis and Kuttan, 1998). Furthermore, its extract has been found to enhance the phagocytic activity of macrophages in the host.

It has been demonstrated that *Withania somnifera* extract (WSE) have anti-inflammatory effect, suppress complement system, inhibits cell proliferation and show lysosomal membrane stabilizing effect on adjuvant-induced arthritis (Rasool and Varalakshmi, 2006; Rasool et al., 2006). Though, extract of *W. somnifera* reported to have no any significant effect on humoral immune response (Rasool and Varalakshmi, 2006). Various preclinical and clinical studies, on contrary, suggest that extract of *W. somnifera* have promising effect on immune responses (Diwanay et al., 2004b). It has been reported that *W. somnifera* extract when co-administered with lower immunogenic doses of DPT vaccine induces immune system against pertussis in experimental animals due to its immunomodulatory and

immunoprotective effects (Gautam et al., 2004a,b; Diwanay et al., 2004a). Sufficient dose of WSE has been found to increase the proliferation of T lymphocytes, complement system, humoral antibody response and Th1 immune response in normal and immunocompromised mice (Bani et al., 2006).

It is now well established that activation of cell-mediated immune response (CIMR) is necessary for the effective removal of tumor cells (Ganss et al., 2002; Hussain and Paterson, 2005). Delayed-type hypersensitivity (DTH) is an *in vivo* manifestation of cell-mediated immune response (CMIR), which provides protection against intracellular pathogens and tumor cells. It is characterized by activation and recruitment predominantly of T cells and macrophages and by resultant swelling at 24 to 48 hrs at the site of antigen entry in previously sensitized host (Black, 1999; Yang et al., 2001). It is well known that DTH is mediated by T helper 1 (Th1) cells secreting predominantly IFN-γ and IL-2. WSE selectively up regulate the production of Th1 cytokines like IFN-γ and IL-2, thereby increases DTH response in normal as well as immunosuppressed animal with no any significant effects on Th2 cytokines like IL-4 production (Bani et al., 2006; Bourgeols and Corinne, 2003). However, immunomodulatory actions of *W. somnifera* extract in various tumor systems including spontaneous transplantable T cell lymphoma, which mimics closely to human T cell lymphoma designated as Dalton's lymphoma that is highly deleterious and invasive killing the host within the very short period of life span with relatively no sign of host's control, is largely unknown.

Therefore, in the present study, we sought to investigate the effect of aqueous root extract of *W. somnifera* on the DTH response and T cell proliferation in Dalton's Lymphoma-bearing BALB/c mice. This study demonstrates that aqueous root extract of *W. somnifera* enhances effector phase of DTH response and production of Th1 cytokines and C-C chemokines at the site of antigen entry. It indicates that aqueous extract of *W. somnifera* has promising immunostimulatory function, and therefore can be used in the combination therapy against the cancer and other immunopathologies.

2. MATERIALS AND METHODS

2.1. Animals and tumor system

Healthy BALB/c mice of either sex at 8-12 weeks of age were used for the study. Mice were maintained in animal room facility of Department of Zoology, Banaras Hindu University and

were given food and water *ad libitum*. Mice were kept on utmost care under the guidelines of Animal Ethical Committee, BHU.

For tumor system, healthy BALB/c (H-2^d) mice of 8 to 10 weeks of age of either sex were injected intraperitoneally with 1.5×10^6 DL cells in 0.5 ml PBS. DL cells were maintained *in vivo* by serial transplantation and isolated from DL-bearing mice 18 days after transplantation, where yield of DL cells is higher. Alternatively, DL cells are cryopreserved for reference.

2.2. Reagents

Tissue culture medium RPMI-1640 was purchased from Hi-Media, Mumbai (India), IFN-γ and MIP-1α monoclonal anti-mice antibody was obtained by R&D Systems, Minneapolis, MN, USA. Roots of *W. somnifera* were obtained from Herbal Garden of Institute of Sciences, BHU, Varanasi. Fetal calf serum (FCS) was purchased from Invitrogen, Grands Island, NY, USA. Concanavalin-A (Con-A) obtained from Himedia, Mubai, India, and Cyclophosphammide and levamisole from Sigma-Aldrich, India. Unless otherwise specified, all other reagents required for the experiments were obtained either from Sigma Chemical Co., Bangalore, India or Super Religare Laboratories Ltd. (SRL), Mumabi, India

2.3. Preparation of Withania somnifera extract (WSE)

The extract of *W. somnifera* (L) Dunal was prepared by standard method as described earlier (Gautam et al., 2004b). Briefly, roots of *W. somnifera* were obtained from Herbal Garden of Institute of Medical Sciences, BHU, chopped and dried in oven. The chopped roots were crushed and boiled in water. The aqueous extract thus obtained was dried by heating and powder was prepared. Procedure resulted in 30 g of brownish hygroscopic extract obtained from 100 g powder of *W. somnifera* root.

2.4. Assessment of the contact hypersensitivity (CHS)

Mice were grouped into four control normal healthy mice, DL-bearing mice, and normal healthy and DL-bearing mice that received 100 mg/kg (2.5 mg/mouse) WSE orally previously described as standard doses (Gautam et al., 2004b) were shaved on their dorsum, and after 24 hrs they were sensitized with 0.5% DNFB (2,4-Dinitro-1-fluorobenzene) in vehicle (4:1 acetone and olive oil). Second exposure was given five days later with the same concentration of DNFB. After 24 hrs mice were challenged with 0.25% DNFB. Change in ear thickness was measured after 24 hrs post-challenge previously described as the time of

maximal response using engineer micrometer. Control group of mice received only sterile PBS and challenged with DNFB on their ears without prior sensitization.

2.5. Assessment of the delayed type hypersensitivity response (DTH)

Mice were grouped into four control normal healthy mice, DL-bearing mice, and normal healthy and DL-bearing mice that received 100 mg/kg (2.5 mg/mouse) WSE orally previously described as standard doses (Gautam et al., 2004b) were sensitized with 50 µl of 1 mg/ml OVA or tumor cell extract (TE) by an intra-dermal injection emulsified with complete Freund's adjuvant at two sites on the abdomen by Luer tip Hamilton syringe. Five days after immunization, mice were challenged with 25 µl of 1 mg/ml OVA or TE in PBS into left rear footpad, while the other rear footpad received a complete volume of sterile PBS as control and swelling in footpad was measured using engineer's micrometer.

2.6. Isolation and culture of macrophages

Macrophages were harvested from normal healthy and DL-bearing mice treated with 100 mg/ml of WSE aqueous extract by standard method. Briefly, after pain free cervical dislocation, peritoneal exudate cells (PECs) were isolated in chilled serum free RPMI 1640 culture medium from peritoneum. Cells were washed 3 times with PBS by centrifugation. Pellet was resuspended in incomplete culture medium and incubated for 2 hrs in round plastic Petri dishes (Tarson, Kolkata, India) at 37°C in humidified CO_2 incubator. Any nonadherent cells present in the cell suspension were discarded. Adherent cell population thus obtained were more than 95% cells macrophages as determined by nonspecific esterase staining.

2.7. Antigen pulsing of peritoneal exudate cells and immunization protocol

Purified macrophages were cultured and washed twice with RPMI-1640 culture medium supplemented with 2% FCS (v/v) and once with sterile PBS. 1×10^7 macrophages were incubated for 30 min in culture medium alone or culture medium containing OVA or TE at 37ºC in 5% CO_2 atmosphere and then washed as above. Viable macrophages were enumerated and 1×10^4 viable Ag-pulsed macrophages were injected subcutaneously (*s.c.*) into the flanks of each mouse. Viability before injections was always greater than 70% as confirmed by trypan blue staining.

2.8. MTT assay for thymocyte proliferation

MTT-assay was carried out to estimate thymocyte proliferation. Mice of 8-10 weeks old were injected intraperitoneally with 1.5×10^6 DL cells, and 18 days after DL transplantation, thymocytes were isolated. 2×10^4 thymocytes from both normal healthy mice and DL-bearing mice were co-incubated with 1.0×10^5 macrophages isolated from normal healthy mice (NH) or tumor-bearing host (TBH) previously treated with WSE, and stimulated with medium alone or medium containing concanavalin A in 96 well round bottom culture plate for 24 hrs. Thereafter, MTT-assay was carried out as described (Mossman, 1988). Each well was added with 50 µl MTT solution [3-(4,5-dimethylthiazol 2-yl)-2,5-diphenyltetrazolium bromide] dissolved in PBS at a concentration of 5.0 mg/ml and incubated at 37^0C for 4 hrs. Plate was centrifuged for 5 min at 100 × g at 4^0C and supernatant was carefully removed without disturbing the dark-blue formazan crystals. After that, 100 µl dimethyl sulfoxide (DMSO) was added in each well to dissolve the formazan crystals and absorbance were read on ELISA plate reader (Bio-Rad, Hercules, CA, USA) at 540 nm.

2.9. Quantification of IFN-γ and MIP-1α in DTH footpad

Production of IFN-γ and MIP-1α in DTH footpad of normal healthy and DL-bearing mice treated with WSE was determined by sandwich ELISA. Briefly, 96 well ELISA plates (Tarson, Kolkata, India) were coated overnight at room temperature with 0.5 pg/ml monoclonal antibody for IFN-γ and MIP-1α (R&D Systems, Minneapolis, MN, USA). After washing with PBS/0.05% Tween, nonspecific binding sites were blocked with PBS/0.05% Tween/2% BSA for 2 hrs at 37ºC. Samples were diluted in PBS/0.05%Tween/0.2% BSA. Blank values were obtained from wells incubated with medium alone. After washing with PBS/0.05% Tween 20 solution, wells were incubated with 0.5 µg/ml rabbit anti-mice IFN-γ and MIP-1α for 1 hr at 37ºC. The unbounded antibodies were removed by washing with washing buffer. Fifty microlitre (50 µl) alkaline phosphatase conjugated goat anti-rabbit IgG antibody (Bangalore Genei, Mumbai, India) diluted in PBS/0.05% Tween/0.2% BSA at a dilution of 1:5000 was added in each well and incubated for 60 min. Equal volume of p-nitro phenyl phosphate (NPP) at the concentration of 1.0 mg/ml was added in enzyme substrate buffer. Absorbance was read on ELISA Plate Reader (Bio-Rad, Hercules, CA, USA) at 405 nm after 10 min.

2.10. Statistical analysis

The unpaired and paired student's t-test was performed to test the significance of data obtained. The data were taken as significant at $P<0.05$. All statistical analysis was performed on Sigma plot Version 8.0 (Systat Software, San Jose, CA, USA).

3. RESULTS

3.1. Effect of WSE on contact hypersensitivity

Groups of normal and DL-bearing control mice or normal or DL-bearing mice that received 100 mg/kg WSE orally sensitized with DNFB on their shaved dorsum on days 0 and 1. Five days after first sensitization, left ear of mice was challenged with DNFB and right ear with sterile PBS and increase in ear thickness was measured at different time periods of 24, 48 and 72 hrs. Observation showed that DNFB sensitization significantly increases CHS response in normal healthy (Fig. 1B) and DL-bearing mice treated with WSE (Fig. 1D) as compared to the control group of normal healthy (Fig. 1A) and DL-bearing mice (Fig. 1C) that received sterile PBS only. Maximum ear swelling was observed only after 24 hrs of challenge with DNFB, which is reported to be optimum time for maximum hypersensitivity response

3.2. Effect of WSE on the induction of delayed type hypersensitivity

Control group of normal healthy and DL-bearing mice and WSE treated normal and DL-bearing mice were sensitized with 50 µl of sterile OVA or TE in the left footpad with Luer tip Hamilton syringe on days 0 and 1. Five days later, mice were challenged with 25 µl of sterile OVA or TE into left footpad and swelling in footpad was measured. Results indicate that mice received WSE showed 50% more swelling in the footpad as compared to the mice that received sterile PBS only. Further, to measure if the effect of DTH induction at one site has any contralateral effects at another site, sensitized group of mice were injected with 50 µl 10% WSE (w/v) in their right rear footpads and contralateral paws received equal volume of sterile PBS. After 24 hrs, both the footpads were challenged with 20 µl sterile OVA or TE and DTH responses were measured. It was observed that WSE treatment efficiently increases the DTH response in contralateral paws of both normal and DL-bearing mice (Fig. 2A & B) as compared to normal and DL-bearing mice that received sterile PBS only (Fig. 2C & D). However, the extent of footpad swelling was significantly low in contralateral paws of both normal healthy and DL-bearing mice. The augmentary effect of WSE on DTH reactivity was further confirmed by the treatment of cyclophosphamide, an immunosuppressant and levamisole, an immunostimulant which can be given by oral root. Cyclophophamide significantly suppress the DTH response, while WSE treatment significantly augments DTH reactivity in OVA or TE sensitized and challenged normal healthy and DL-bearing mice (Table 1).

3.3. Effect of adoptive transfer of macrophages on DTH response

Macrophages were harvested from normal healthy mice and single cell suspension was prepared. Cell suspension was treated with OVA and tumor extract (TE) constituted in sterile PBS and incubated in round plastic petri dishes at 37°C in 5% CO_2 atmosphere in CO_2 incubator for 2 hrs. The incubated peritoneal exudate cells enriched with macrophages (1×10^6 cells/mice) injected locally to control normal and DL-bearing and WSE treated normal and DL-bearing mice that had been sensitized with OVA or TE and DTH response was measured after 24 hrs. Macrophages treated with OVA significantly elicited higher DTH response in normal and DL-bearing mice treated with WSE (Fig. 3A & B) as compared to the normal and DL-bearing mice, where unpulsed macrophages were adoptively transferred (Fig. 3C & D). In any case, OVA sensitization induces higher DTH response in comparison to tumor cell extract (TE).

3.4. Effect of WSE on thymocyte proliferation

Effect of WSE on thymocyte proliferation was measured by MTT assay as described in Materials and methods. Result showed that when thymocytes were incubated with culture supernatants of NMO and TAMs that were incubated with medium alone had no significant effect on their proliferation efficiency while, culture supernatants of NMO and TAMs previously treated with WSE showed significantly higher thymocyte proliferation (Fig. 4), which indicates that WSE treatment may enhance the in vivo proliferation of T lymphocytes at the site of antigen delivery.

3.5. Production of IFN-γ and MIP-1α in DTH footpad

Th1 cytokine and chemokine are important mediators for elicitation of DTH responses. Therefore, to assess the production of IFN-γ and MIP-1α (Macrophage Inflammatory Protein-1α) in DTH footpads of normal and DL-bearing mice treated with WSE sensitized and challenged with OVA or TE were analyzed for IFN-γ and MIP-1α production by Sandwich ELISA. Higher production of IFN-γ and MIP-1α was observed in the DTH footpad of normal and DL-bearing mice sensitized with OVA as compared to the control group (Fig. 5A & B). Significantly low IFN-γ and MIP-1α production was observed in TE sensitized and challenged normal healthy and DL-bearing mice in comparison to OVA sensitized and challenged mice. IFN-γ and MIP-1α are important mediator molecules for the elicitation of DTH response.

Enhanced production of these important mediators at the site of antigen entry further strengthens the effector phase of DTH response.

4. DISCUSSION

In the present study, the immunomodulatory effect of root aqueous extract of *W. somnifera*, an important medicinal plant utilized from ancient time in Indian traditional medicine, was investigated. Experimental data reveals that oral administration of root aqueous extract WSE enhances contact hypersensitivity (CHS) and delayed type hypersensitivity (DTH) response in allergen/antigen challenged normal healthy and DL-bearing mice. However, the extent of allergen induced CHS response and antigen induced DTH responses were found low in DL-bearing host compared with normal healthy mice even after the treatment of aqueous root extract of *W. somnifera*. Comparatively, low CHS and DTH response in DL-bearing mice indicates that tumor cells release several immunosuppressive factors resulting into the suppression of immune response. It was observed that DL-bearing mice sensitized and challenged with OVA or tumor-cell extract (TE) treated with WSE induces higher DTH response as compared to control group of DL-bearing mice sensitized and challenged with OVA or TE. We observed that maximum DTH response occurred after 24 hrs of sensitization with sterile PBS and OVA as reported earlier (Back and Larsen, 1982).

The enhanced DTH response upon WSE treatment in DL-bearing mice suggests that oral administration of aqueous root extract of *W. somnifera* enhances the expression of those mediator molecules, which are able to enhance the immune response or neutralize the immunosuppressive agents produced by tumor cells. The observation on the augmentary effect of root extract of *W. somnifera* on DTH response has disagreement with the previous observation (Davis and Kuttan, 2000), however, this observation is in parallel with previous reports of Agarwal et al., 1999, which demonstrates the mild potentiation of DTH response upon WSE treatment. Further, sensitization with OVA induces higher DTH response as compared to tumor-cell extract (TE), which indicates that there may have some immunosuppressive factors in tumor-cell extract that results into suppressed DTH response in both normal healthy and DL-bearing mice.

Furthermore, macrophages isolated from normal mice when injected intraperitoneally into the normal and DL-bearing mice pre-treated with WSE induces higher DTH immune

response as compared to the DTH immune response in control group of mice that received sterile PBS only. Macrophages are thought to play a central role in DTH response (Gajewski et al., 1991). It was observed that adoptive transfer of macrophages that are pulsed with OVA upon transfer to WSE treated or control group of mice, induces higher DTH response as compared to the macrophages pulsed with sterile PBS or TE. Augmentation in DTH response upon adoptive transfer of macrophages into WSE treated mice suggests that the DTH inducing capacity of WSE is possibly due to its ability to upregulate inflammatory cytokine production in macrophages. It has been reported that WSE treatment selectively upregulate the expression of Th1 cytokines in antigen challenged mice (Bani et al., 2006). It might also possible that WSE treatment may enhance antigen presentation by macrophages to $CD4^+$ T cells which resulted into enhanced Th1 response leading to significantly higher DTH response in the normal healthy and DL-bearing mice where macrophages were adoptively transferred. However, the adoptive transfer of TE-pulsed macrophages induces strong DTH reactivity but significantly lower than OVA peptide in WSE treated normal and DL-bearing mice.

The enhanced production of IFN-γ in DTH foot pad in normal and DL-bearing mice treated with WSE further supports the assumption that WSE treatment selectively augments Th1 cytokine production. Correspondingly, enhanced production of MIP-1α in DTH footpad was observed in WSE treated normal healthy and DL-bearing mice. Production of both IFN-γ and MIP-1α was higher in normal healthy mice and DL-bearing mice treated with WSE as compared to the control group of normal healthy and DL-bearing mice. IFN-γ is a Th1 cytokine, which stimulates macrophages and T cells to produce proinflammatory cytokines and chemokines in the internal environment, on the other hand, MIP-1α is a strong chemoattractant, which mediates the infiltration of macrophages and other immune cells at the site of inflammation or antigen entry. Enhanced production of these mediator molecules upon WSE treatment indicates that aqueous root extract of *W. somnifera* enhances DTH response in recipient mice by enhancing the expression of IFN-γ and MIP-1α, which further amplifies Th1 immune response at the site of antigen delivery resulting into concomitant footpad swelling. Moreover, WSE treatment enhanced thymocyte proliferation in both normal healthy and DL-bearing mice. It is probably due to the production of IL-1β by

macrophages in their culture supernatant, responsible for T cell proliferation in adequate amount.

Previously, it was reported that highly suppressed immune system in DL-bearing host can be modulated in their early stage of DL cell progression by giving the treatment of effective immunostimulating agent (Loeffler et al., 1992; Mizoguchi et al., 1992). In the present study, the augmentation of DTH response in DL-bearing mice upon treatment with WSE indicates that WSE treatment enhances production and proliferation of Th1 cells resulting into enhanced DTH reactivity in recipient mice. It may also be possible that WSE treatment may suppress the production of Th2 cytokines in treated mice, which need to be investigated in detail to show the elaborated view of immunomodulatory effect of *W. somnifera* aqueous root extract in both normal healthy and tumor-bearing mice. Further, it will be beneficial to investigate the active ingredients of *W. somnifera* aqueous root extract. Study is suggestive of its importance in combinational therapy against the malignancies or as an adjuvant.

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Table 1

Effect of WSE on delayed type hypersensitivity. Normal healthy and DL-bearing mice were sensitized and challenged with OVA or TE and after 24 hrs footpad thickness was measured.

Group	Treatment	PBS	Cyclophosphamide	Levamisole	WSE
Normal healthy mice	**OVA**	0.503 ± 0.037	0.578 ± 0.0218	0.820 ± 0.042	0.654 ± 0.0394
	TE	0.450 ± 0.036	0.526 ± 0.0183	0.752 ± 0.0112	0.602 ± 0.0407
DL-bearing mice	**OVA**	0.344 ± 0.033	0.634 ± 0.0471	1.129 ± 0.0836	0.903 ± 0.0703
	TE	0.307 ± 0.029	0.568 ± 0.0519	1.035 ± 0.1052	0.828 ± 0.0665

Normal healthy mice and DL-bearing mice were treated with cyclophosphamide as negative control, levamisole as a positive control or aqueous root extract of W. somnifera (WSE). Cyclophosphamide acts as immunosuppressant, while levamisole found to be immunostimulatory and both the drugs can be

administered through oral root. Data are significant at P<0.5. Data represent mean footpad thickness ± SEM of thee independent experimental settings.

Figure 1. Effect of WSE on CHS response in normal healthy. Mice were treated with WSE, sensitized with 0.5% DNFB in vehicle and 5 days after, challenged with 0.25% DNFB in vehicle on left ear. After challenge, the increase in ear thickness was measured in control group (A) and treated (B) normal healthy mice, control group (C) and treated (D) DL-bearing mice at different time intervals of 24, 48 and 72 hrs. Control group of mice received only sterile PBS. Data are presented as mean of difference in ear thickness ± SEM. *Significant differences at P < 0.05 between DNFB-painted mice and DNFB-painted mice treated with WSE after 24 hrs of time interval.

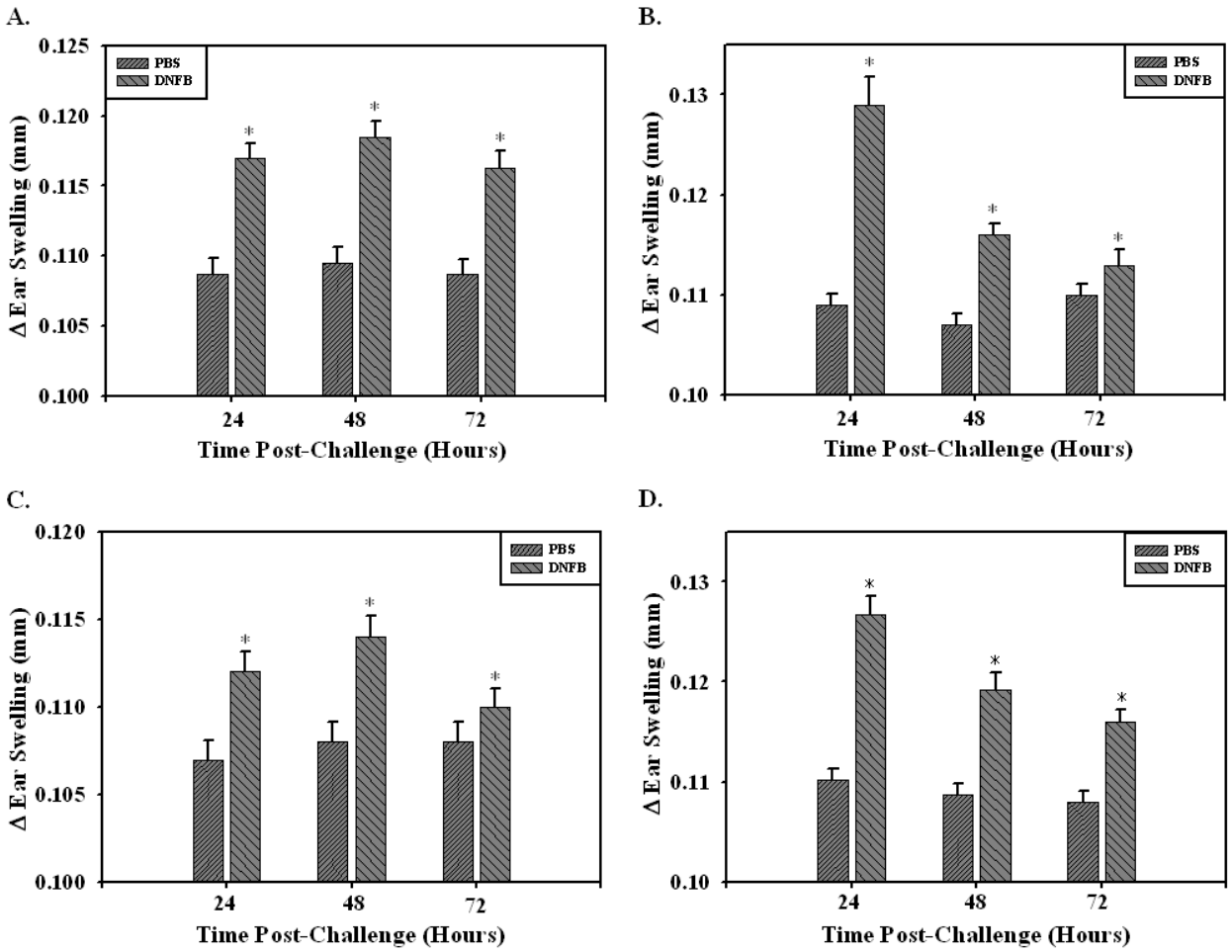

Figure 2. Effect of WSE on delayed type hypersensitivity reaction in normal healthy and DL-bearing mice. Both control and WSE treated normal healthy and DL-bearing mice were sensitized with OVA or TE into left rear footpad by luer tip Hamilton syringe and 5 days later, challenged with OVA or TE. The increase in thickness of footpad of WSE treated normal

healthy mice (A), and DL-bearing mice (B); and control group of normal healthy mice (C) and DL-bearing mice (D) that received sterile PBS only were measured at different time intervals of 24, 48 and 72 hrs. Data are presented as mean of difference of footpad thickness ± SEM of three independent readings and symbols * indicate that data is significant at $p<0.05$.

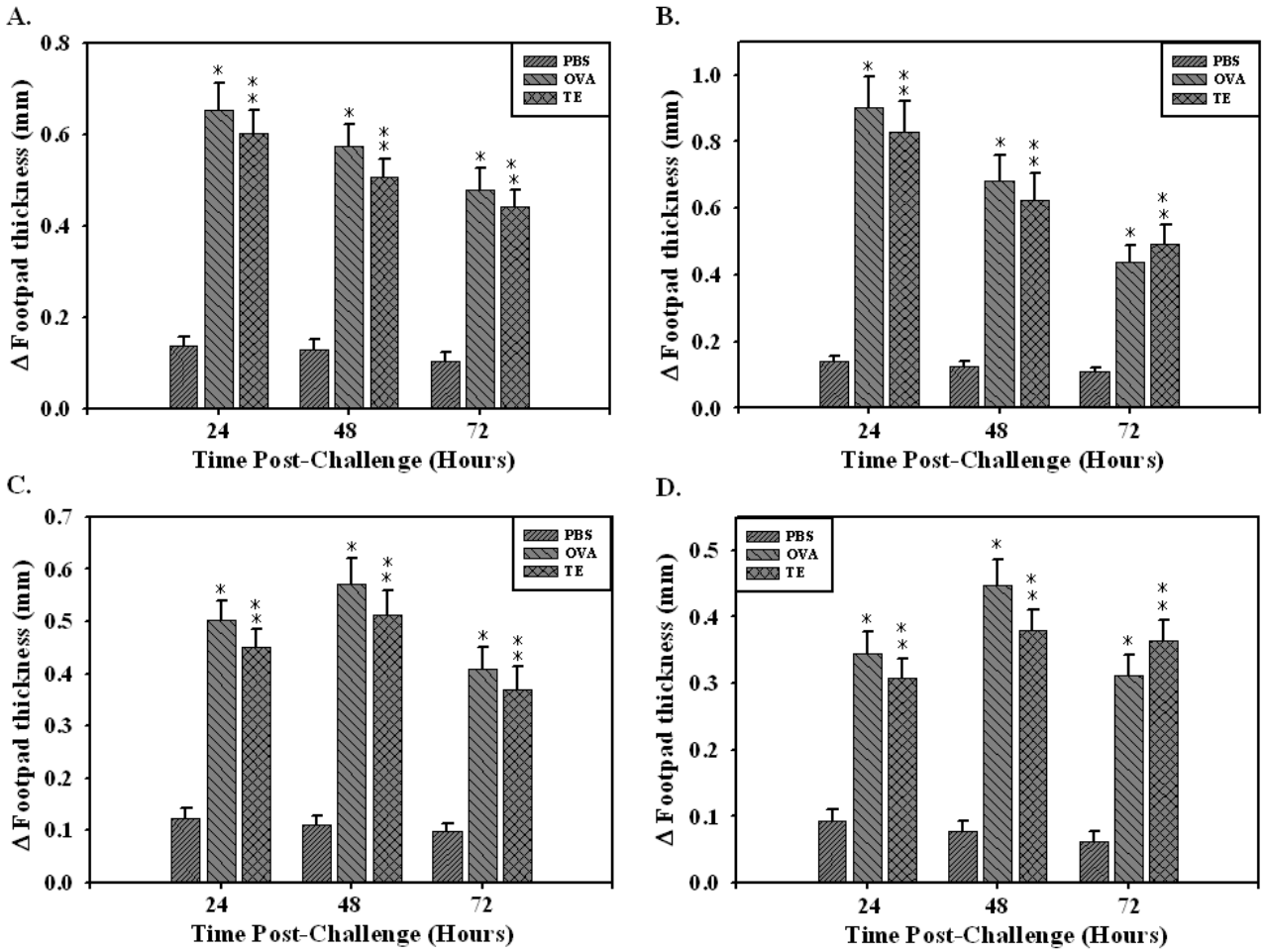

Figure 3. Effect of adoptive transfer of macrophage on DTH response. Macrophages were harvested from normal healthy mice incubated for 24 hrs with culture medium alone or culture medium containing OVA or TE for 24 hrs and 1×10^4 macrophages were transferred to WSE treated normal healthy mice (A) and DL-bearing mice (B); and control group of normal healthy mice (C) and DL-bearing mice (D) that received sterile PBS only and increase in footpad thickness was measured after 24, 48 and 72 hrs of time intervals. The results shown are representative of three independent experiments. Data are presented as mean of difference of footpad thickness ± SEM of three independent reading and symbols * indicate that data is significant at $p<0.05$.

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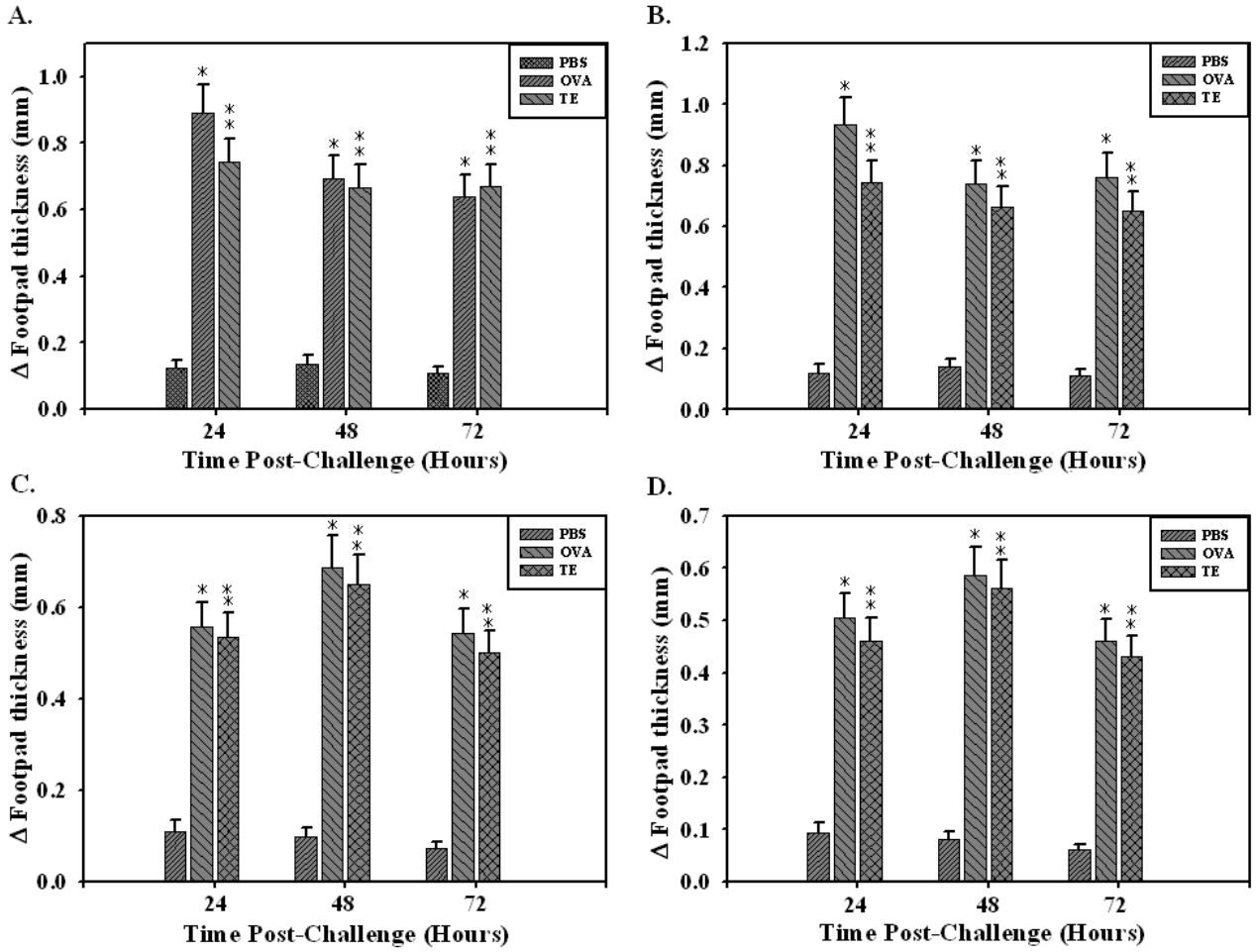

Figure 4. Thymocyte Proliferation. 1.0 × 10^5 NMO and TAMs were treated with WSE for 24 hours and 2 × 10^4 thymocytes were added in each well and MTT assay was performed as described in Materials and methods. The absorbance was read at 540 nm and data are presented as the mean absorbance ± SEM of three independent experiments in triplicate. The symbols * indicate that data are significant at $p<0.05$.

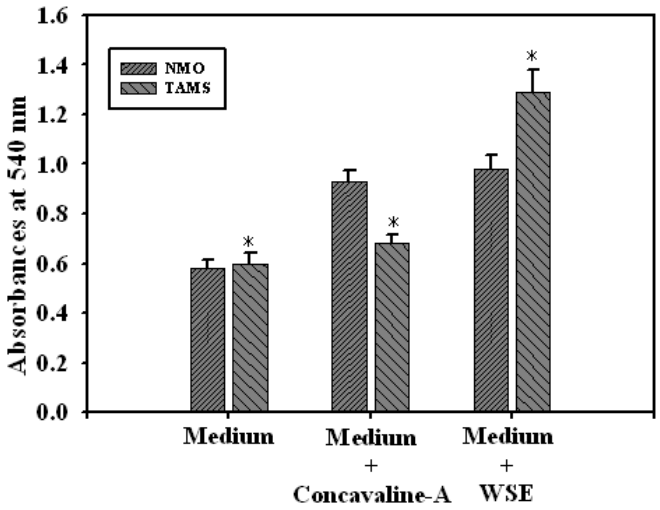

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Figure 5. Effect of WSE on the production of IFN-γ and MIP-1α in DTH footpad. WSE treated normal healthy mice and DL-bearing mice were sensitized and challenged with OVA or TE. The left footpad of challenged mice was analyzed for MIP-1α (A) and IFN-γ (B) production at different time intervals of 24, 48 and 72 hrs, and MIP-1α and IFN-γ level were measured in both normal healthy and DL-bearing mice. Data represent the mean concentration ± SEM of three independent experiments in triplicate and symbols * indicate that data is significant at $p<0.05$.