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Antihelminthic niclosamide modulates dendritic cells activation and function



Chieh-Shan Wu^{a,b,c}, Yi-Rong Li^d, Jeremy J.W. Chen^{d,e}, Ying-Che Chen^d, Chiang-Liang Chu^f, I-Hong Pan^g, Yu-Shan Wu^h, Chi-Chen Lin^{d,e,i,j,*}

^a Department of Dermatology, Kaohsiung Veterans General Hospital, Taiwan, ROC

^b Department of Dermatology, Faculty of Medicine, College of Medicine, Kaohsiung Medical University, Taiwan, ROC

^c Graduate Institute of Medicine, College of Medicine, Kaohsiung Medical University, Taiwan, ROC

^d Institute of Biomedical Science, National Chung-Hsing University, Taichung, Taiwan, ROC

^e Rong Hsing Research Center for Translational Medicine, National Chung Hsing University, Taiwan, ROC

^f Graduate Institute of Immunology, College of Medicine, National Taiwan University, Taipei 100, Taiwan, ROC

^g Biomedical Technology and Device Research Laboratories, Industrial Technology Research Institute, Hsinchu, Taiwan, ROC

^h Department of Chemistry, Tunghai University, Taichung, Taiwan, ROC

ⁱ Department of Medical Research and Education, Taichung Veterans General Hospital, Taichung, Taiwan, ROC

^j Division of Chest Medicine, Department of Internal Medicine, Changhua Christian Hospital, Changhua, Taiwan, ROC

ARTICLE INFO

Article history:

Received 23 September 2013

Accepted 11 December 2013

Available online 28 December 2013

Keywords:

Dendritic cells

Niclosamide

Cytokine

T cell proliferation

Contact hypersensitivity (CHS)

ABSTRACT

Dendritic cells (DCs) link the sensing of the environment by the innate immune system to the initiation of adaptive immune responses. Accordingly, DCs are considered to be a major target in the development of immunomodulating compounds. In this study, the effect of niclosamide, a Food and Drug Administration-approved antihelminthic drug, on the activation of lipopolysaccharide (LPS)-stimulated murine bone marrow-derived DCs was examined. Our experimental results show that niclosamide reduced the pro-inflammatory cytokine and chemokine expression of LPS-activated DCs. In addition, niclosamide also affected the expression of MHC and costimulatory molecules and influenced the ability of the cells to take up antigens. Therefore, in mixed cell cultures composed of syngeneic OVA-specific T cells and DCs, niclosamide-treated DCs showed a decreased ability to stimulate T cell proliferation and IFN- γ production. Furthermore, intravenous injection of niclosamide also attenuated contact hypersensitivity (CHS) in mice during sensitization with 2,4-dinitro-1-fluorobenzene. Blocking the LPS-induced activation of MAPK-ERK, JNK and NF- κ B may contribute to the inhibitory effect of niclosamide on DC activation. Collectively, our findings suggest that niclosamide can manipulate the function of DCs. These results provide new insight into the immunopharmacological role of niclosamide and suggest that it may be useful for the treatment of chronic inflammatory disorders or DC-mediated autoimmune diseases.

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1. Introduction

Dendritic cells (DCs) are potent antigen presenting cells that are required for the initiation of T cell responses and function as a bridge between the innate and adaptive immune systems [1]. They are localized to and circulated within distinct compartments of the lymphoid and peripheral nonlymphoid organs at different stages of maturation. DCs normally reside in an immature state within the peripheral nonlymphoid tissues, where they serve as sentinels for incoming antigens, such as those associated with microbial

pathogens and tumors. Therefore, upon antigen capture and process in the peripheral nonlymphoid tissues, DCs migrate through the afferent lymphatic vessels to the secondary lymphoid organs, where they stimulate naïve T cells and undergo the morphological, phenotypic, and functional changes characteristic of maturation [2]. Mature DCs possess immunostimulatory properties such as reduced phagocytic activity, increased surface expression of the major histocompatibility complexes (MHC) that present Ag-peptides, increased expression of costimulatory molecules and increased secretion of cytokines and chemokines [3,4]. In contrast to the enhanced T cell immunity associated with mature DCs, immature DCs fail to stimulate T cell responses and are involved in the induction of peripheral T cell tolerance by generating functional Treg cells [5,6]. Thus, the potential to harness the power of DCs makes these cells attractive pharmacological targets [7–12].

* Corresponding author at: Institute of Biomedical Sciences, College of Life Science, National Chung Hsing University, Taichung, Taiwan, Republic of China. Fax: +886 4 23592705.

E-mail address: lincc@dragon.nchu.edu.tw (C.-C. Lin).

Recently, the development of other indications or rediscovery of the inherent values of FDA-approved drugs is a growing trend in the pharmaceutical industry. The repurposing or repositioning of an existing drug can accelerate the timeline and reduce the cost of bringing the drug to market because it eliminates the need for additional toxicological and pharmacokinetic assessments [13,14].

Niclosamide is a Food and Drug Administration-approved oral antihelminthic drug used to treat most tapeworms, including beef tapeworms and dwarf tapeworms. It has been used in humans for nearly 50 years. It is also used as a molluscicide for water treatment in schistosomiasis control programs [15]. The activity of niclosamide against these parasites is believed to be due to the inhibition of mitochondrial oxidative phosphorylation and anaerobic ATP production [16]. However, niclosamide is receiving renewed attention in light of new data showing that it has strong anti-neoplastic activity against a broad spectrum of cancer types. For example, several reports have indicated that niclosamide can suppress Wnt/ β -catenin signaling by targeting the Wnt co-receptor LRP6 on the cell surface, an activity that is closely associated with anti-proliferation and pro-apoptotic activities in prostate and breast cancer cells [17,18]. In addition, Jin et al. reported that niclosamide can inhibit the NF- κ B pathway and increase ROS levels in acute myelogenous leukemia stem cells; niclosamide also potently inhibited the growth of AML cells in vitro and in nude mice [19]. A study by Ren et al. demonstrated that niclosamide is a selective inhibitor of STAT3. Treatment with niclosamide inhibited the EGF-mediated STAT3 activity and inhibited the growth of several types of cancer cells with constitutive STAT3 activation (e.g., DU145, HeLa, A549) [20]. Moreover, using an automated cell-based screening assay, Balgi et al. observed that niclosamide can increase autophagy by inhibiting mammalian target of rapamycin complex 1 (mTORC1) signaling [21]. Independently, Fonseca et al. demonstrated that niclosamide suppresses mTORC1 signaling through the modulation of cytoplasmic pH in MCF-7 breast cancer cells [22]. Furthermore, Wang et al. reported that niclosamide potently suppresses the Notch-regulated gene C promoter-binding factor-1 (CBF-1) in K562 leukemia cells [23]. Taken together, these data suggest that the antitumor activity of niclosamide may be due to its ability to target multiple signaling pathways. However, until now, the cellular and molecular targets of this drug in the immune system have remained unknown, and the ability of niclosamide to modulate the functions of DCs, the most potent of the APCs, has not been defined.

In this initial study, we designed experiments to examine the potential therapeutic effects of niclosamide on the functional properties of DCs and to elucidate the molecular mechanisms involved. The present study demonstrates that niclosamide inhibits the ability of LPS-induced mouse bone marrow-derived DCs to secrete pro-inflammatory cytokines and affects the expression of costimulatory molecules, the ability of DCs to prime a T cell immune response in an syngeneic DC/T cell coculture and prevents 2,4-dinitro-1-fluorobenzene-induced contact hypersensitivity (CHS) in vivo. Consequently, blocking the LPS-induced NF- κ B, ERK and JNK activation in DCs may explain the inhibitory effect of niclosamide on DC activation. These results show for the first time that niclosamide can manipulate the immunostimulatory properties of DCs and may be useful in suppressing chronic inflammatory disorders or autoimmune diseases.

2. Methods

2.1. Mice and preparation of mouse bone marrow-derived DCs

Female C57BL/6 (H-2^b) mice (4–6 weeks old) were purchased from the National Laboratory Animal Center (Taipei, Taiwan). OT-I TCR transgenic mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA). OT-II TCR transgenic mice were provided by Dr. Clifford Lowell (UCSF, San Francisco, CA). The mice were housed

in a barrier facility at Taichung Veterans General Hospital (Taiwan) under the guidelines of the Institutional Animal Care and Use Committee, and all experiments were conducted in accordance with the institution's guidelines for animal experimentation. Mouse bone marrow-derived DCs were generated using a previously described method [24,25]. Briefly, bone marrow cells were flushed from the femurs and tibiae of C57BL/6 mice with RPMI 1640 using a syringe and a 25-gauge needle, and the erythrocytes were lysed with red blood cell lysis buffer. The bone marrow cells were suspended in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin G, 100 μ g/ml streptomycin, 20 ng/ml recombinant mouse granulocyte macrophage colony-stimulating factor (PeproTech) and 20 ng/ml recombinant mouse IL-4 and placed into a 24-well plate. The cells were incubated at 37 °C in a 5% CO₂ atmosphere. Fresh culture medium was added to the cells every 2 days. On day 7, the non-adherent or loosely adherent cells were harvested and classified as immature DCs. More than 80% of the cells expressed CD11c, as determined by flow cytometry (data not shown). CD11c⁺ DCs were further prepared by immunomagnetic selection from CD11c⁺-enriched BM cells by positive selection using anti-CD11-coated beads (Miltenyi Biotec, Auburn, CA, USA) according to the manufacturer's instructions; these cells were used for experiments. The purity of the CD11c⁺ cells was confirmed to be >90% by flow cytometry.

2.2. Flow cytometric analysis

DCs were cultured in the presence of 0.1% DMSO or 1.25 μ M niclosamide (Sigma, St. Louis, MO, USA; stock solution of 10 mM in DMSO) for 1 h followed by stimulation with 100 ng/ml of lipopolysaccharide (LPS) for 18 h. The control was untreated group. After incubation, the DCs were harvested and stained with specific antibodies. After mAb staining, the samples were analyzed for fluorescence on a FACSCalibur flow cytometer (BD Biosciences, Heidelberg, Germany). We used FITC- or phycoerythrin (PE)-conjugated monoclonal antibodies (mAbs) to stain for mouse CD11c, CD40, CD80, CD86, and MHC class II and class I expression (all from BD Biosciences, Mountain View, CA).

2.3. Cytokine release assays

Culture supernatants were collected from DCs propagated in the presence of 0.1% DMSO or indicated dose of niclosamide for 1 h before or after stimulation with LPS (100 ng/ml) or other TLR ligands, including peptidoglycan (1 μ g/ml, TLR1/TLR2), polyI:C (250 μ g/ml, TLR3), CpG ODN 1826 (200 nM, TLR-9) or imiquimod (5 μ g/ml, TLR-7) for 18 h or TNF- α for 4 h. All TLR ligands were purchased from InvivoGen, San Diego, CA. After incubation, the cytokine and chemokine levels in the supernatants of the DC cultures were determined using sandwich ELISA kits (R&D Systems, Minneapolis, MN). The viability of the cultured cells was assessed using the CCK-8 colorimetric assay kit according to the manufacturer's instructions (Sigma, St. Louis, MO). Cell viability of greater than 90% was observed in all of the experiments in this study.

2.4. OVA-specific T cell activation in vitro

The protocol for this assay was modified from our previous report [24]. The BMDCs generated from C57BL/6 were incubated with 2 μ g/ml OVA_{257–264} (OVAP₁) or OVA_{323–339} (OVAP₂) (synthesized by Echo Chemical Co., Taiwan) in the presence of 0.1% DMSO or niclosamide (1.25 μ M) at 37 °C for 1 h. Subsequently, DCs were stimulated with LPS (100 ng/ml) for 18 h. Control was untreated group. After incubation, the cells were harvested and washed with PBS. OVAP₁-specific CD8⁺ T cells or OVAP₂-specific CD4⁺ T cells were enriched from the splenocytes of OT-I and OT-II TCR transgenic mice (C57BL/6) by

MACS cell separation according to the manufacturer's protocol (Miltenyi Biotec). The enriched CD8⁺ and CD4⁺ T cells were cultured with stimulated DCs at various DC:T cell ratios for 96 h, and the proliferation of the cells was estimated based on [³H] thymidine uptake. In addition, the supernatants from the DC/T cell cultures were collected after 96 h, and the levels of IFN-gamma production were measured using an ELISA kit. The proportion of the CD8⁺ IFN-gamma⁺ or CD4⁺ IFN-gamma⁺ double-positive T cells were measured by intracellular cytokine staining using a FACSCalibur flow cytometry (BD Bioscience, MA, USA) as previous described [25].

2.5. OT-I/OT-II adoptive transfer

A total of 3×10^7 CFSE (10 μ M, Molecular Probes)-stained OT-I or OT-II spleen cells were injected intravenously (i.v.) into syngeneic C57BL/6 recipient mice on day 0. On day 1, BMDCs from C57BL/6 mice activated with with 2 μ g/ml OVA_{257–264} (OVAP₁) or OVA_{323–339} (OVAP₂) for 1 h at 37 °C, and 5×10^5 peptide-loaded BMDCs were i.v injected into the mice. At days 4, OT-I and OT-II cell proliferation were analyzed by dilution of CFSE labeling in the CD8⁺ or CD4⁺ population using flow cytometry.

2.6. Endocytosis assay

The endocytosis by DCs was assessed by dextran-FITC or OVA-FITC uptake, as described previously [26]. Briefly, enriched CD11c⁺ mouse bone marrow-derived DCs were treated with niclosamide (1.25 μ M), 0.1% DMSO plus LPS (100 ng/ml) or niclosamide

plus LPS for 18 h. The control was untreated group. The cells were then harvested and incubated at 37° for 1 h with 1 mg/ml dextran-FITC (molecular weight 42,000; Sigma) or 250 μ g/ml OVA-FITC (Invitrogen-Molecular Probes). The uptake of dextran-FITC or OVA-FITC by the DCs was analyzed using a FACSCalibur flow cytometer. In addition, parallel experiments were performed at 4 °C to demonstrate that the uptake of dextran by DCs is inhibited at low temperatures.

2.7. Western blot analysis and NF- κ b activity assay

DCs were cultured in the presence of 0.1% DMSO or niclosamide (1.25 μ M) for 1 h and subsequently stimulated with LPS (100 ng/ml). The cells were harvested, and whole cell lysates were prepared at the 30 min after LPS stimulation. The control was untreated group. For protein detection, protein extracts (50 μ g/ml) were boiled, electrophoresed on 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were blocked for 1 h with 5% skim milk in TBS + 0.05% Tween 20. After blocking, the membranes were incubated with a primary antibody against phospho-p38, p38, phospho-p42/44, total p42/44, phospho JNK, β -Actin (Cell Signaling, Beverly, MA) or total JNK (SC-571; Santa Cruz Biotechnology, Santa Cruz, CA) overnight. The membranes were then washed and incubated with horseradish peroxidase-labeled secondary Abs (Jackson ImmunoResearch, West Grove, PA). The proteins were detected using a Western Lightning chemiluminescence reagent (Perkin Elmer Life Sciences, Boston, MA) and analyzed with an LAS3000 system (Fujifilm,

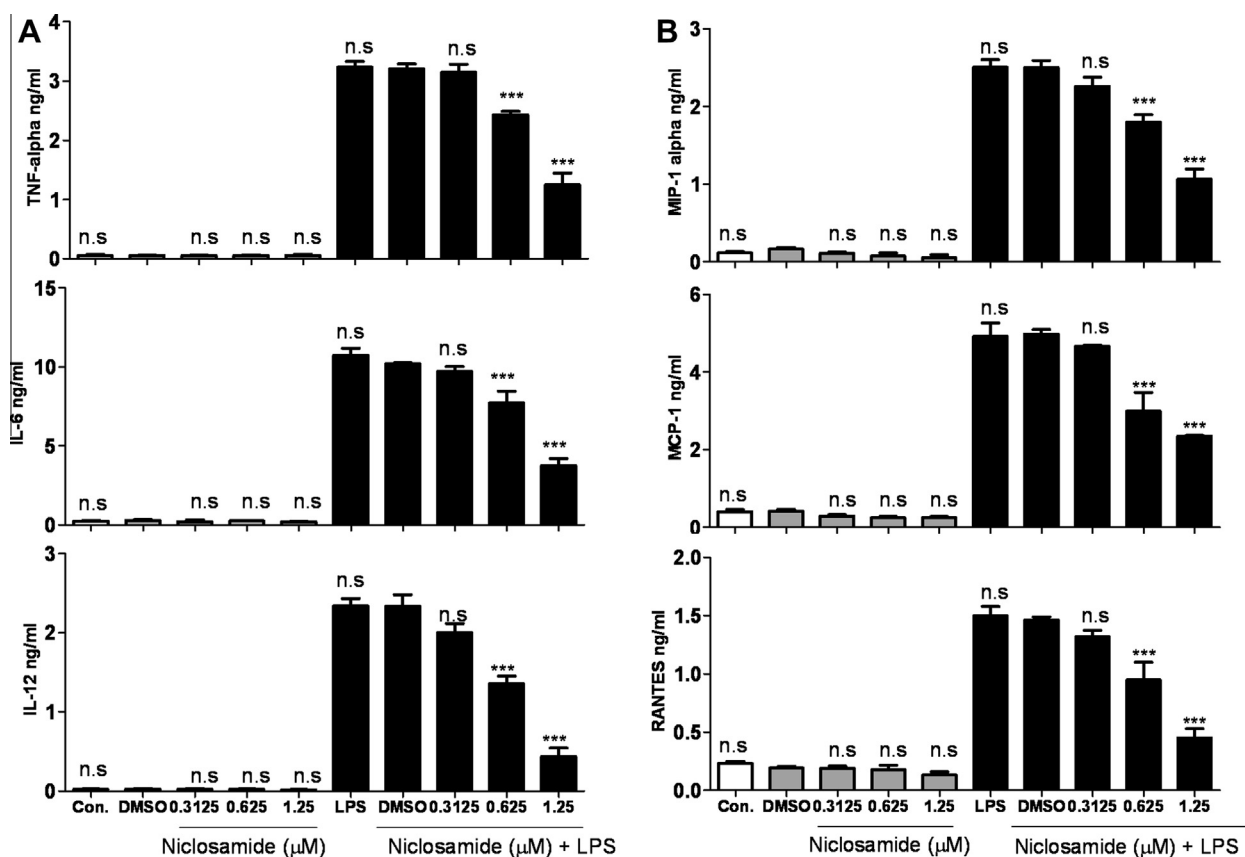


Fig. 1. Niclosamide inhibits the expression of cytokines and chemokines by LPS-stimulated mouse bone marrow-derived DCs. Mouse bone marrow-derived DCs were treated with 0.1% DMSO, indicated dose of niclosamide, LPS alone, 0.1% DMSO + 100 ng/ml LPS, indicated dose of niclosamide + 100 ng/ml LPS. The control group was untreated group (con.). (A) Supernatants were collected from cultures after 18 h (4 h for TNF-alpha), and the level of each cytokine and (B) chemokine was evaluated by ELISA. The cytokine and chemokine levels were compared between the niclosamide-treated and DMSO-treated DCs in the absence or presence of LPS (n.s. > 0.05, ***p < 0.001). All data are representative of three independent experiments showing similar results.

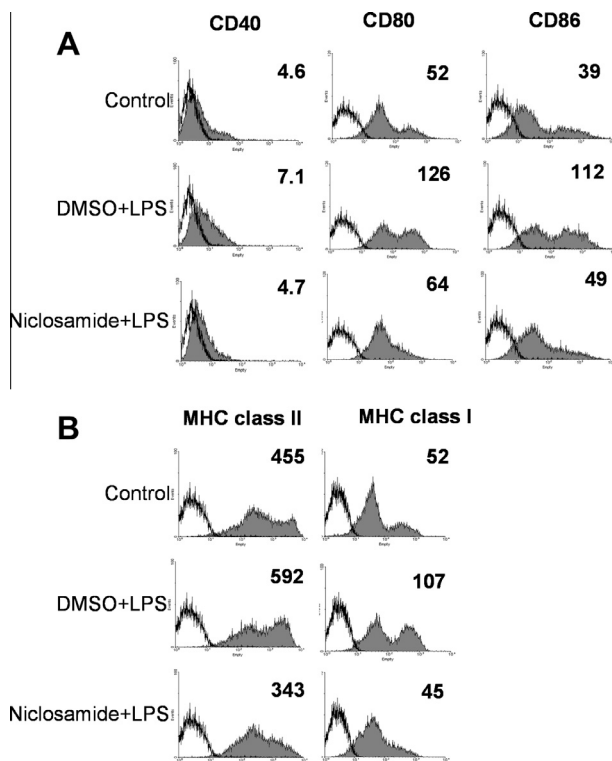


Fig. 2. Niclosamide affects the expression of immunomodulatory cell surface markers in LPS-induced DCs. DCs were treated with 0.1% DMSO + 100 ng/ml LPS or with 1.25 μ M niclosamide + 100 ng/ml LPS, as indicated. The control was untreated group. After an 18 h incubation, flow cytometry with fluorescently labeled Abs was used to assess the expression of the following surface markers: (A) CD40, CD80, CD86 and (B) MHC class II, MHC class I. The dark line indicates staining with an isotype-matched control Ab. The data represent the mean fluorescence intensity (MFI), and the results shown are representative of at least three independent experiments with similar results.

Tokyo, Japan). Densitometric analysis was performed with ImageJ software (National Institutes of Health, Bethesda, MD, USA). To measure the NF- κ B transcriptional activity, purified BMDCs (1.5×10^6 cells/well) were stimulated with 0.1% DMSO or niclosamide (6.25, 12.5 μ M) for 1 h and subsequently stimulated with LPS (100 ng/ml) for 30 min in 6-well plates. The cells were then harvested, and nuclear extracts were prepared using the NE-PER Nuclear and Cytoplasmic Extraction system (Pierce) according to the manufacturer's instructions. A BCA protein assay kit (Pierce,

Rockford, IL, USA) was used to determine the protein concentration. For each assay, a total of 10 μ g nuclear extract was used in a TransAM NF- κ B p65 ELISA kit (Active Motif, Carlsbad, CA) according to the manufacturer's instructions.

2.8. 2,4-Dinitro-1-fluorobenzene-induced contact hypersensitivity assay

The 2,4-dinitro-1-fluorobenzene (DNFB; Sigma-Aldrich)-induced contact hypersensitivity (CHS) model was constructed using previously described methods, with some modifications [26]. The shaved belly of mice was painted daily with 20 μ l vehicle (acetone/olive oil at ratio of 1:4) or 0.5% (w/v) DNFB plus with i.v daily 50 μ l solvent solution (10% Cremophor EL; 0.9% NaCl) or 50 μ l niclosamide (2 mg/kg). Five days after sensitization, 20 μ l of DNFB 0.2% (w/v) were painted on both sides of the right ears of all of the mice. The CHS response was determined 16 h after exposure to DNFB through histological analysis using H&E staining and by evaluating the increased thickness of the ear (thickness of the right (challenged) ear minus the thickness of the left (unchallenged) ear) using an engineer's spring-loaded micrometer (Mitutoyo, Tokyo, Japan).

2.9. Statistical analysis

All statistical analyses were performed using GraphPad Prism software package version 4.0 (GraphPad Software; San Diego, CA, USA). Cytokine production, T cell proliferation in vitro and in vivo, IFN-gamma production, immunoblotting assay, and increased thickness of the ear were evaluated by one-way ANOVA followed by Tukey's post hoc test. When $p < 0.05$, the difference was considered to be statistically significant.

3. Results

3.1. Niclosamide decreases cytokine and chemokine production by LPS-stimulated mouse bone marrow-derived DCs

A major attribute of mature DCs is the synthesis and secretion of cytokines and chemokines that modulate inflammatory responses and T cell differentiation and are responsible for the adaptive immune response [2–4]. Therefore, in the first series of experiments, we investigated the effect of niclosamide on the production of pro-inflammatory cytokines and chemokines by

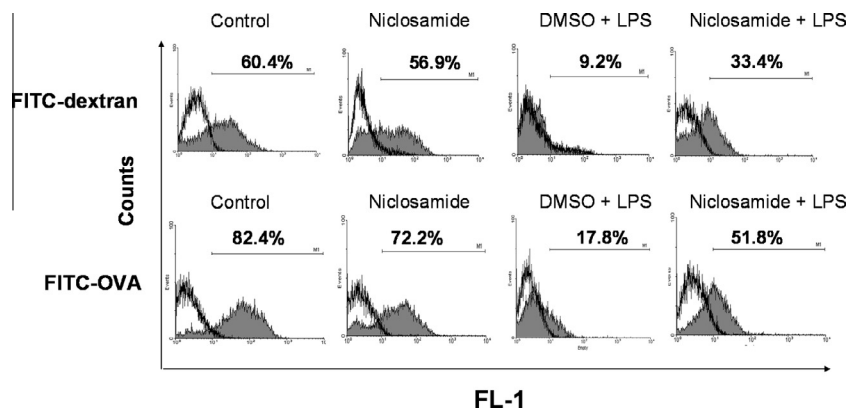


Fig. 3. Niclosamide affects endocytosis by DCs. DCs were treated with 0.1% DMSO, 1.25 μ M niclosamide, 0.1% DMSO + 100 ng/ml LPS or 12.5 μ M niclosamide + 100 ng/ml LPS for 18 h, followed by treatment with (A) dextran-FITC or (B) OVA-FITC for 1 h. The endocytic activity of the DCs was then determined by flow cytometry. The dark line represents the cells treated with dextran-FITC at 4 $^{\circ}$ C as a control for endocytosis. The numbers represent the percentages of dextran-FITC⁺ or OVA-FITC⁺ cells. All data are representative of three independent experiments showing similar results.

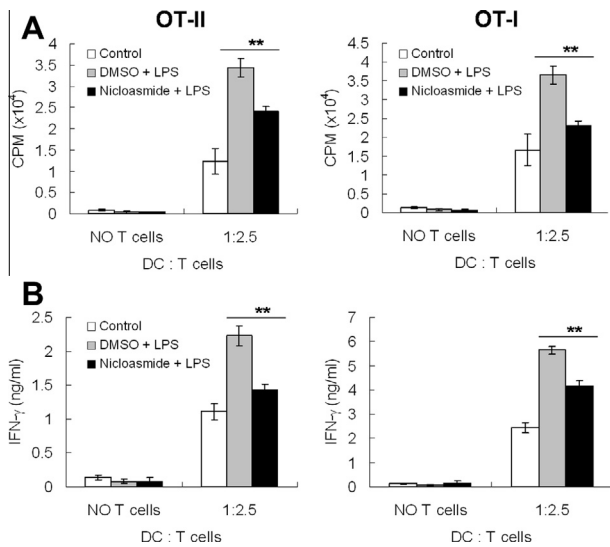


Fig. 4. Niclosamide inhibits Ag-specific T cell activation induced by LPS-stimulated DCs in vitro and in vivo. (A) DCs were incubated with 0.1% DMSO + 100 ng/ml LPS or 1.25 μ M niclosamide + 100 ng/ml LPS and then pulsed with OVA peptide for 24 h. The control group was untreated group. After incubation, the DCs were washed and co-cultured with OT-I CD8⁺ or OT-II CD4⁺ T cells at the indicated ratios of DC:T cells for 96 h. T cell proliferation was determined by exposure to [³H]-thymidine for 18 h prior to the end of the incubation, and cell-associated radioactivity was quantified. (B) After stimulation for 96 h, the supernatants were collected from the cultures. The IFN- γ level in the medium was determined by ELISA and (C) the percentage of IFN- γ -producing OVA-specific CD8⁺T and CD4⁺ T cells were determined using flow cytometry. The dot plot shows data from one representative sample. Bar graph mean \pm SD of samples from three wells. (D) C57BL/6 mice were adoptively transferred with CFSE-labeled OT-I or OT-II transgenic spleen cells. 24 h later, OVA_{257–264} (OVAP₁) or OVA_{323–339} (OVAP₂) peptide-loaded immature mouse DCs were pretreated in the presence or absence of niclosamide, stimulated with LPS were administered by the same route. Mice were evaluated for in vivo proliferation of OT-I and OT-II T cells 3 days after adoptive transfer. Histogram show CFSE-derived fluorescence for CD8⁺T cells (OT-I transfer) or CD4⁺T cells (OT-II transfer). Bar graph mean \pm SD of samples from three wells. * p < 0.05, ** p < 0.01 indicates a significant difference between the niclosamide-treated and 0.1% DMSO-treated LPS-activated DCs. All data are representative of three independent experiments with similar results.

LPS-stimulated mature mouse bone marrow-derived DCs. First, the supernatants were collected from LPS-activated mouse DCs propagated in the presence or absence of niclosamide, and the cytokine and chemokine levels in the supernatants were quantified by ELISA. Bacterial LPS is widely used as a strong inducer of DC activation. As shown in Fig. 1, DCs exposed to niclosamide exhibited a significant dose-dependent decrease in the levels of the LPS-induced pro-inflammatory cytokines (TNF- α , IL-6, IL-12) and chemokines (RANTES, MCP-1, MIP-1 α). In addition, the level of these cytokine or chemokine did not significant differ between the niclosamide-treated and DMSO-treated DCs in the absence of LPS. Moreover, the decrease in cytokine and chemokine levels was not due to an increase in the number of dead cells, as determined by CCK-8, because there were no marked differences in cell viability between 1.25 μ M niclosamide-treated cultures and DMSO-treated control cultures (Supplemental Fig. 1). Thus, these results suggest that niclosamide suppresses the inflammatory and immunostimulatory activity of DCs stimulated with LPS.

3.2. Niclosamide affects the expression of surface molecules and the endocytosis capacity of LPS-stimulated DCs

The activation of DCs is accompanied by the enhanced expression of surface molecules including the costimulatory molecules and major histocompatibility complex molecules (MHC) that mediate interactions with naïve T cells and contribute to T cell activation [4]. Thus, we determined whether treatment with

niclosamide affected the expression of surface molecules on mouse DCs. As shown in Fig. 2A, niclosamide treatment significantly altered the expression of the costimulatory molecules (CD80, CD86 and CD40) and the major histocompatibility complex molecules (MHC class II, MHC class I) (Fig. 2B) within 18 h compared to LPS-stimulated mature DCs that were not exposed to niclosamide. Thus, we suggest that niclosamide also impairs the LPS-induced phenotypic maturation of DCs. However, it is also important to note that immature DCs possess stronger endocytic activity than mature DCs [2,3]. Therefore, we examined the ability of niclosamide-treated DCs to endocytose dextran-FITC or OVA-FITC. As shown in Fig. 3A and B, the percentage of dextran-FITC- and OVA-FITC positive cells did not significant differ between the niclosamide-treated DCs and DMSO-treated DCs at 37 °C. Therefore, a lower percentage of LPS-stimulated DCs than DMSO-treated DCs were endocytic. Moreover, additional pretreatment with niclosamide also increased the endocytic capacity of LPS-stimulated DCs for dextran-FITC and OVA-FITC. These findings pertaining to the expression of surface molecules and endocytotic activity strongly suggest that niclosamide prevents the maturation of DCs.

3.3. Niclosamide decreases the ability of LPS-stimulated DCs to stimulate OVA-specific T cell proliferation

One critical function of activated DCs is to induce T cell proliferation and differentiation. Thus, we next studied the effects of niclosamide on the ability of DCs to induce OVA-specific T cell responses. OVA_{257–264} (OVAP₁) or OVA_{323–339} (OVAP₂) peptide-loaded immature mouse DCs were pretreated in the presence or absence of niclosamide, stimulated with LPS, and tested for their capacity to stimulate allogeneic OVA-specific CD8⁺ OT-I and CD4⁺ OT-II T cells. T cell proliferation was measured by [³H] thymidine incorporation. As shown in Fig. 4A, LPS-activated DCs promoted T cell proliferation, and this effect was abrogated by niclosamide. This suggests that niclosamide decreases the ability of LPS-treated DCs to stimulate CD4⁺ T cells. We also evaluated the effects of CD4⁺ T cell coculture with niclosamide-treated DCs on IFN- γ (Th1 cytokine) synthesis. As shown in Fig. 4B and C, a cytokine analysis by ELISA and intracellular cytokine staining revealed that CD4⁺ T and CD8⁺ T cells cocultured with niclosamide-treated DCs produced significantly lower amounts of IFN- γ in the medium (Fig. 4B) and percentage of IFN- γ producing CD4⁺ and CD8⁺ T cells (Fig. 4C) than the DMSO-treated DCs did in the presence of LPS. Next, we extended the in vitro results obtained for the proliferation of T cells to in vivo model. For this, CFSE-labeled spleen cells either from OT-I or OT-II mice were i.v injected into syngeneic C57BL/6 mice. 24 h later, OVA_{257–264} (OVAP₁) or OVA_{323–339} (OVAP₂) peptide-loaded immature mouse DCs were pretreated in the presence or absence of niclosamide, stimulated with LPS and administered by the same route. Three days after DCs injection, proliferation of CD4⁺ and CD8⁺ T cells was evaluated in spleens of recipient mice by dilution of the CFSE signal. As shown in Fig. 4D, proliferation of CD8⁺ or CD4⁺ was observed in mice injected with LPS-matured DCs, however this effect was attenuated by niclosamide. Overall, these data suggest that niclosamide impedes the ability of DCs to prime a T cells-biased immune response.

3.4. Niclosamide attenuated the MAPK and NF- κ B pathways in LPS-stimulated DCs

As shown previously, the activation of MAPKs and NF- κ B plays a pivotal role in the production of cytokines by DCs in response to inflammatory stimuli, including LPS, TNF- α , and interleukin-1 (IL-1) [27,28]. To gain insight into the mechanism of this inhibitory action of niclosamide, we investigated whether the activation of MAPKs and NF- κ B were altered by niclosamide in LPS-stimulated

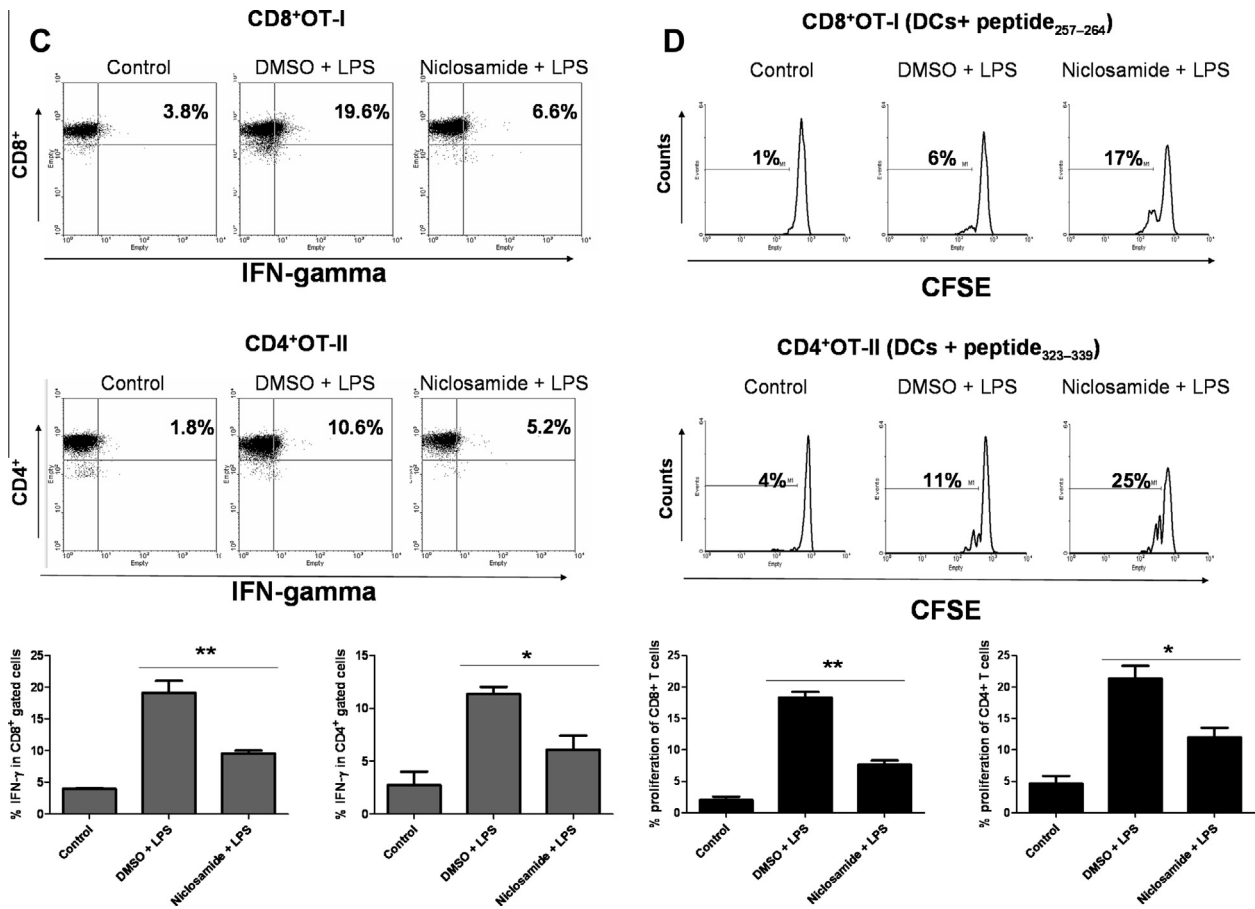


Fig. 4 (continued)

mouse DCs. Our results showed that pretreatment with niclosamide attenuated LPS-induced ERK and JNK-MAPK phosphorylation but did not affect p38 phosphorylation (Fig. 5A). To further determine whether niclosamide decreases the LPS-induced activation of the NF- κ B pathway, nuclear extracts were prepared, and NF- κ B binding activity was assessed using the TransAM NF- κ B transcription factor assay kit. As shown in Fig. 5B, LPS induced a significant level of p65 nuclear translocation and upregulated NF- κ B binding activity by 30 min after stimulation. However, the NF- κ B binding activity was dramatically reduced in niclosamide-treated BMDCs. Therefore, these results may partially explain the inhibitory effect of niclosamide on the maturation of LPS-induced DCs.

3.5. Niclosamide weakened the 2,4-dinitro-1-fluorobenzene-induced contact hypersensitivity (CHS) response in vivo

Contact hypersensitivity (CHS) is a typical DC mediated T cell-dependent, Ag-specific inflammatory response that is induced when the skin is exposed to haptens, such as DNFB [29]. This model was used to investigate the in vivo effect of niclosamide on the DC-mediated immune response. Our results showed that i.v injection of niclosamide (2 mg/kg) elicited less ear swelling than injection with vehicle control group (Fig. 6), suggests that niclosamide could potentially be used to prevent delayed-type hypersensitive diseases, such as allergic contact dermatitis.

4. Discussions

Niclosamide is a Food and Drug Administration-approved anti-helminthic agent of choice for the treatment of most tapeworm

infections. It was recently shown to have large spectrum of biological effects, including activity against the SARS virus and anti-toxin and anti-tumor activities in various systems [30,31,17–23]. Our present data show that niclosamide attenuates the LPS-induced pro-inflammatory cytokines and chemokines production, surface costimulatory and MHC molecules expression, the reduction in antigen uptake by DCs, and inhibits DC-triggered allogeneic T cell proliferation. To the best of our knowledge, this is the first study to report that niclosamide can modulate the phenotype and functional properties of DCs.

Both IL-6 and TNF- α are important mediators of a wide range of biologic activities and play numerous beneficial roles in the modulation of pro-inflammatory immune responses [32]. However, the dysregulation of the production of these two cytokines by DCs has been implicated in the pathogenesis of a variety of acute and chronic inflammatory diseases, as well as autoimmune diseases such as rheumatoid arthritis, ankylosing spondylitis, psoriasis and refractory asthma [33–35]. Therefore, small molecule inhibitors or monoclonal antibodies targeting these cytokines have been used in the clinical treatment of immune-related diseases. For example, multiple monoclonal antibodies against TNF, including infliximab, adalimumab and certolizumab pegol, have been used therapeutically, and the circulating receptor fusion protein etanercept (Enbrel) also targets TNF [36]. Other drugs, such as Tocilizumab, a humanized anti-IL-6R monoclonal antibody, target IL-6 [37]. Because our results indicate that niclosamide strongly inhibits the functions of the inflammatory cytokines TNF- α and IL-6 without toxicity, niclosamide should be further evaluated as a clinical immunosuppressive therapy or adjuvant immunotherapy drug.

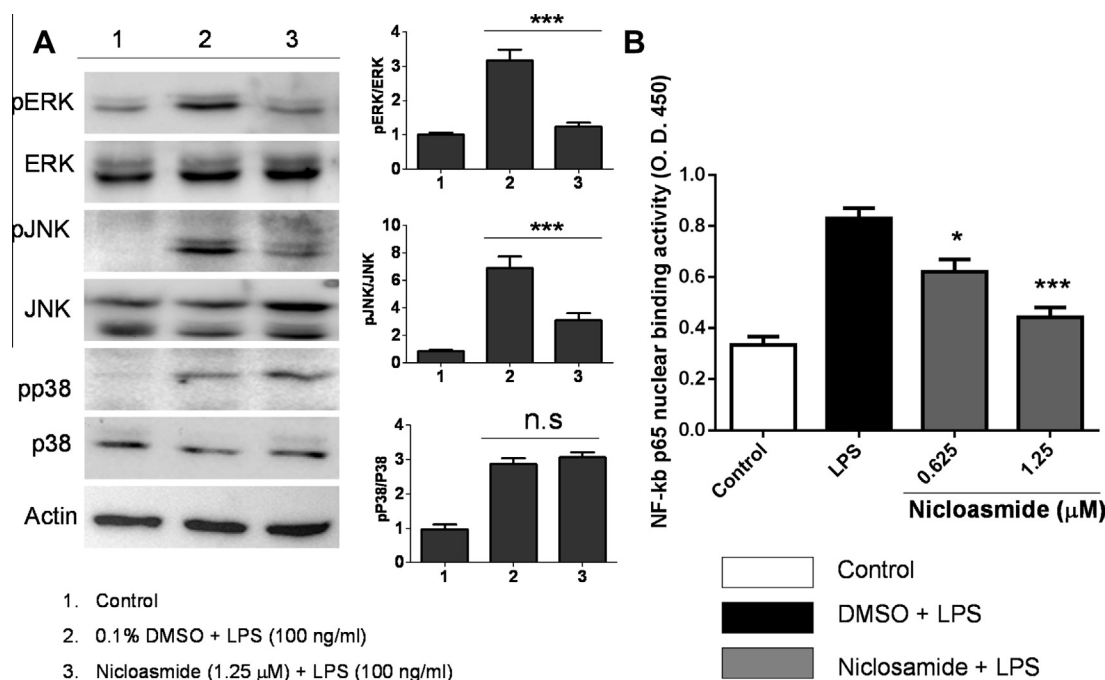


Fig. 5. Niclosamide inhibits MAPK-ERK and JNK phosphorylation and NF- κ B activity in DCs. DCs were treated with 0.1% DMSO + 100 ng/ml LPS or 12.5 μ M niclosamide + 100 ng/ml LPS. The control was untreated group. Whole cell lysates or nuclear fractions of the lysates were collected at the 30 min after LPS treatment. (A) The level of MAPK phosphorylation was examined by western blot analysis with antibodies against ERK, JNK, p38 and MAPK (both phosphorylated and unphosphorylated). Bands were analyzed by ImageJ and normalized to actin. (B) The NF- κ B assay was performed as described in the Materials and Methods. The OD was measured at 450 nm. The data shown are the mean \pm SD of samples from three wells. n.s. $p > 0.05$, * $p < 0.05$, *** $p < 0.001$ indicates a significant difference between the niclosamide-treated and 0.1% DMSO-treated LPS-activated DCs. All data are representative of three independent experiments with similar results.

IL-12 has been identified as a cytokine that strongly influences the differentiation of naïve CD4 cells into T helper 1 (Th1) cells that produce IFN- γ and aid in cell-mediated immunity [38]. The present study demonstrated that niclosamide can significantly reduce LPS-induced IL-12 production in BMDCs and can also down regulate the capacity of LPS-stimulated DCs to induce IFN- γ expression by allogeneic CD4⁺ T and CD8⁺ T cells (Fig. 3B). These observations suggest that niclosamide may be effective in several chronic inflammatory diseases described as Th1-dominant diseases. However, we cannot exclude the possibility that niclosamide may modulate the responses of other types of T lymphocytes. Specifically, the TLR-4 ligand LPS was used in our study to stimulate DC maturation. LPS has been shown to induce strong Th1-like responses instead of Th2 immune responses [39]. Our data did not indicate that IL-4 was expressed in DCs after LPS stimulation (data not shown). Therefore, the utilization of agents capable of stimulating a Th2 response, such as dust mite allergens [40], to further determine the effects of niclosamide on DC-mediated Th2 polarization should be examined in the future.

To determine the mechanism by which niclosamide inhibits DC function, we examined TLR4-related downstream signaling pathways. NF- κ B has been demonstrated to regulate the expression of pro-inflammatory mediators, including costimulatory molecules, cytokines and adhesion molecules, in DCs, and it is upregulated as DCs mature [41]. In addition, experiments in NF- κ B subunit knockout mice further indicated that c-Rel and p50 control the expression of costimulatory molecules (CD40) and cytokines (IL-12, IL-18) during the T cell responses induced by lipopolysaccharide (LPS)-stimulated DCs. [42]. A previous study demonstrated that the TNF- α -induced NF- κ B activity in acute myelogenous leukemia stem cells was significantly suppressed by niclosamide [19]. Our present study showed that niclosamide inhibited NF- κ B activity in LPS-treated DCs (Fig. 5B), suggesting that the inhibition of NF- κ B activation might be an important mechanism of the

inhibition of DC maturation by niclosamide. In addition, the mitogen-activated protein kinase (MAPK) pathways: c-Jun N-terminal (JNK), extracellular signal-regulated kinase (ERK) and p38, which direct the expression of various genes related to DC maturation and plays a major role in DCs function [27,28]. In this study, we also observed that niclosamide moderately decreased ERK and JNK but did not change p38 activation in LPS-treated BMDCs, suggesting that niclosamide inhibits TLR-related signaling, perhaps including the NF- κ B and MAPK-ERK, JNK signaling pathways. However, niclosamide has been demonstrated to regulate multiple signaling pathways, and some of these pathways are involved in immune responses, such as AKT [19] and STAT3 [20]. Thus, additional studies are needed to determine whether other pathways are involved in this effect and to fully elucidate the signaling pathway(s) responsible for the inhibition of DC maturation by niclosamide.

It is worth noting that although our current study demonstrates for the first time that niclosamide can inhibit the LPS-induced MAPK-ERK signaling pathway in BMDCs, it was recently reported that niclosamide dose not affect the induction of the MEK/ERK/MNK pathway by serum starvation in MCF-7 cells [22]. Our preliminary inferences suggest at least two possible causes for the differences in the results. First, the difference in the effect of ERK activation by niclosamide may be due to species-specific or cell-type differences. Second, niclosamide may be not directly target ERK but may instead regulate the pathway upstream of ERK. Thus, niclosamide has different effects on ERK activation in serum-starved MCF-7 cells and LPS-treated BMDCs. However, the identification of possible mechanisms responsible for these different effects may lead to future applications for niclosamide in the treatment of clinical diseases.

In a separate study, we found that niclosamide was able to repress the expression of pro-inflammatory cytokines regardless of whether it was administered before or after LPS stimulation

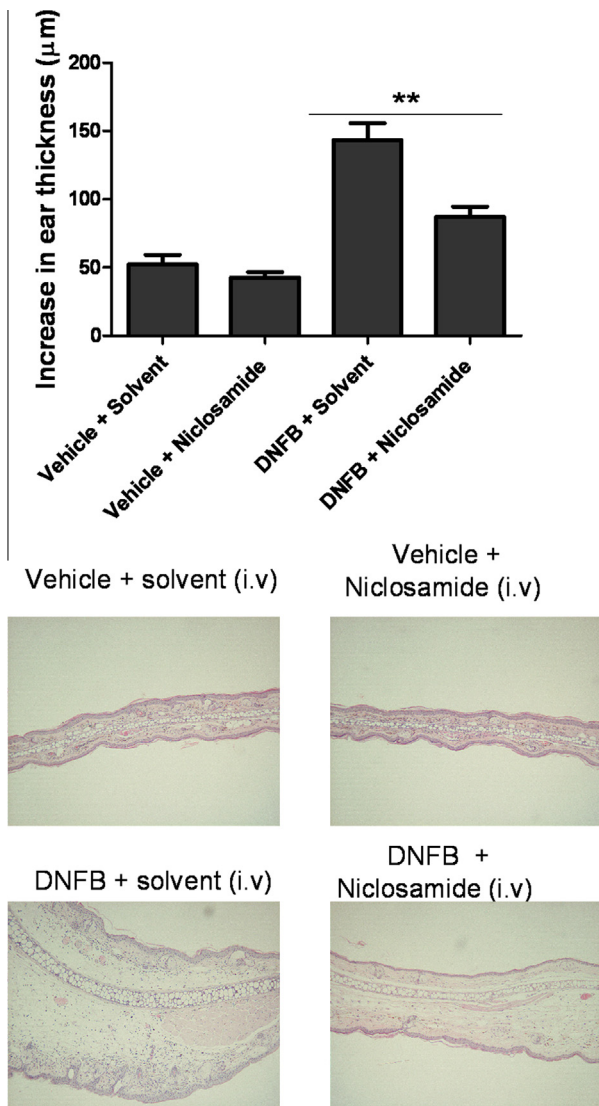


Fig. 6. Intravenous administration of niclosamide reduced the DNFB-induced contact hypersensitivity (CHS) response in mice. The DNFB-induced CHS experiments were performed as described in the Materials and methods. Briefly, (The shaved belly of mice was painted daily with 20 µl vehicle (acetone/olive oil at ratio of 1:4) or 0.5% (w/v) DNFB plus with i.v daily 50 µl solvent solution (10% Cremophor EL, 0.9% NaCl) or 50 µl niclosamide (2 mg/kg). Five days after sensitization, 20 µl of DNFB 0.2% (w/v) were painted on both sides of the right ears of all of the mice. (A) The increase in ear thickness was measured after 16 h. The data are shown as mean ± SD from six mice and representative of two independent experiments yielding similar result, ** $p < 0.01$ indicates a significant difference between the DNFB + Solvent (i.v) and DNFB + (Niclosamide). (B). Representative histopathology from the ear tissues. Cryosection of ears from different treated mice prepared at 24 h after hapten challenge were stained with hematoxylin and eosin (H&E), original magnification × 100.

(Supplemental Fig. 2). This suggests that the anti-inflammatory and immunomodulatory effects of niclosamide could be clinically effective for the prevention or treatment of various diseases. In addition, we also tested whether niclosamide could modulate the activation of immature DCs by other TLR ligands, namely peptidoglycan (TLR1/TLR2), polyI:C (TLR3), CpG ODN 1826 (TLR-9) and imiquimod (TLR-7). Each TLR ligand increased the release of pro-inflammatory IL-6 and IL-12 into the culture medium, and this release was reduced by treatment with 1.25 µM niclosamide (Supplemental Fig. 3). Although the mechanism through which niclosamide interferes with DC activation upon stimulation with different TLR ligands is largely unknown, we suggest that it may be related to the effect of niclosamide via the suppression of the MAPK and

NF-κB signaling pathway. In this pathway, NF-κB is believed to play an important role in various TLR ligand-stimulated selective cytokine and chemokine secretions from dendritic cells [43].

Previous studies showed that niclosamide has very low toxicity in mammals (oral LD50 in rats >5000 mg/kg) [44,45]. In addition, a single oral administration of 5 mg/kg niclosamide can generate a maximal plasma concentration of 1.08 µM in rat [46]. Another possibility to achieve higher plasma concentrations of the drug may be intravenous administration. For instance, a single intravenous administration of 2 mg/kg niclosamide to rats gave rise to a peak plasma concentration of 25 µM [46], which is sufficient to modulate DC function, as extrapolated from our data.

Naïve dendritic cells when activated using TLR ligands or pro-inflammatory cytokines, shift their metabolism from oxidative phosphorylation to aerobic glycolysis (i.e., the Warburg effect). Kraczyk et al. described this phenomenon in DCs and also demonstrated how TLR stimulation is essential for dendritic cell maturation [47]. Consequently, according to previous studies, uncoupling of mitochondrial oxidative phosphorylation was also believed to be its antihelminthic mechanism of action [48]. Thus, we hypothesized that immunomodulatory effects of niclosamides on dendritic cells could, in part, be due to its disruption of metabolism.

Niclosamide has been FDA approved for human use against parasitic helminthic infestations for decades. In recent years, niclosamide was shown to have antiviral effects. For example, Jurgeit et al. found that niclosamide is an entry inhibitor for a number of pH-dependent respiratory viruses, including influenza virus and human rhinoviruses [49]. Imperi et al. showed that at micromolar concentrations, niclosamide has high inhibitory activity against *Pseudomonas aeruginosa* QS and virulence both in vitro and in vivo [31]. Moreover, Wu et al. demonstrated that niclosamide was able to inhibit replication of SARS-CoV; viral antigen synthesis was totally abolished at a niclosamide concentration of 1.56 µM, suggesting that this drug is a possible candidate for the treatment of SARS-CoV infection [30]. This is the first study demonstrating the ability of niclosamide in modulating the activation of immature DCs by several types of TLR ligands. Since DCs are crucial for the elimination of pathogens and tumors [50,51], further studies also should examine the risks associated with long-term niclosamide use.

In summary, our results demonstrate for the first time that the FDA-approved drug niclosamide inhibits LPS-induced DC maturation and cytokine, costimulatory molecule, and MHC molecule expression. In addition, niclosamide-treated DCs inhibited antigen specific T cell responses, prompting us to speculate that this might be an important function of niclosamide. However, we also believe that additional issues warrant future investigation including the molecular mechanisms underlying the ability of niclosamide to suppress DC function.

Authors' contributions

Chieh-Shan Wu, Yi-Rong Li and Jeremy J. W. Chen contributed equally to this work.

Conflict of interests

The authors declare that they have no conflict of interests.

Acknowledgments

This study was supported by grants from the VGHKS – 101-061, 102-053 and Ministry of Education, Taiwan under the ATU plan

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.cellimm.2013.12.006>.

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