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Norcantharidin Modulates Development of Dendritic Cells and Prolongs Skin Allograft Survival

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Background

To examine the effects of norcantharidin (NCTD) on development of human myeloid dendritic cells (DCs) in vitro and in skin allograft transplantation in vivo.

Methods

Human $CD14^+$ monocytes were isolated and triggered differentiation and maturation toward myeloid DCs with and without NCTD. The cell morphology, viability, cell death, expression of surface markers and co-stimulatory molecules, allostimulatory activity, and cytokine production were examined for characterization of DCs. The rejection of mice skin allograft model was used to translate the in vitro effect of cantharidin (CTD) and NCTD on DCs.

Results

DCs developed in the presence of NCTD showed decreased viability, cell death with necrosis, and lower expression of CD1a and CD83. DCs triggered in the presence of NCTD possessed a greater allostimulatory activity in naive $CD4^+CD45RA^+$ T cells. NCTD modulated DCs through calcineurin phospatase but not through mTOR or downstream molecule p70S6 kinase. In vivo, NCTD caused accumulation and co-localization of antigen-presenting cells (APCs) and regulatory T cells in the interfollicular area of the recipients' spleens. CTD and NCTD prolonged skin allograft survival along with less severe histopathological inflammatory reactions.

CTD, but not NCTD, treatment caused elevation of serum alanine aminotransferase and evident mortality of the recipients.

Conclusion

NCTD modulated the differentiation and maturation of human myeloid DCs and caused deviation of standard DC differentiation toward a tolerogenic phenotype through calcineurin phospatase inhibition. In vivo, both drugs effectively prolonged skin allograft survival. NCTD was less toxic than CTD, and thus, has potential for development as an immunosuppressant for transplant rejection.

Key Words: Cantharidin; Immunosuppressant; Norcantharidin; Dendritic Cells; Transplantation

Norcantharidin (NCTD, $C_8H_8O_4$), a water-soluble synthetic small molecule, is the demethylated form of cantharidin (CTD) (*1*). CTD ($C_{10}H_{12}O_4$) is a lipid-soluble bioactive compound isolated from the Chinese blister beetle (*Mylabris phalerata* Pallas) (*1*). CTD possesses activities that induce p53-dependent apoptosis and double strand breakage of DNA in various types of cancer cells (*1-3*). However, CTD causes mucosal erosion, the upper gastrointestinal tract bleeding, and renal dysfunction with its clinical application (*4*). This unfavorable toxicity profile limits the clinical use of CTD.

NCTD possesses anticancer activity as potent as CTD (*5*). Intriguingly, NCTD exhibits much less renal toxicity than CTD in the clinical setting (*1*). Thus, NCTD could be a feasible chemical derivative of CTD in terms of greater water solubility and less toxicity as a cancer treatment.

Besides the anti-cancer activity, both CTD and NCTD promote hematopoiesis while inhibiting cancer growth in vivo. This dual effect could benefit cancer patients and reduce or eliminate myelosuppression and possible life-threatening infection caused by immunosuppression. The hematopoiesis-promoting activity raises the possibility that CTD and NCTD could play a role in immunomodulation, especially for cell lineages originated from bone marrow, such as dendritic cells (DCs).

DCs are antigen-presenting cells (APCs) that induce innate and adaptive immune

responses (*6*). The precursors of DCs come from the bone marrow and migrate into the blood (*7*). Under different microenvironments or after various treatments, DCs differentiate into a less mature stage (*8-12*). This suggests that modulation of DC development could have therapeutic potential in the treatment or prevention of disorders involving unwanted immune responses, such as rejection of transplanted grafts (*13*).

In the present study, we evaluated the effect of NCTD on differentiation and maturation of DCs in vitro. A mouse skin allograft model was used to examine the immunomodulatory effect of NCTD in vivo.

Results

In vitro effect of NCTD on DCs

Morphological changes

The monocyte-derived DCs had typical morphological characteristics of mature DCs after stimulation with the cytokine combination, including being nonadherent and having multiple sharp cytoplasmic processes and abundant cytoplasm. The majority of NCTD-treated monocyte-derived DCs had thicker and blunter projections than those treated with the cytokine combination. (Fig. 1A) As the concentration of NCTD increased, the dendrites became fewer and shorter, and the cell contour became less intact. These morphological alterations in-NCTD-treated monocyte-derived DCs suggest an atypical maturation state during DC development.

Effect of NCTD on DC viability and death

As assessed by the trypan blue exclusion test, NCTD inhibited the viability of DCs in a dose-dependent manner (Fig 1B). The NCTD concentrations studied ranged from 1.25–20 μM and resulted in a 19%–59% decrease of cell viability, with an estimated concentration at which 50% of cells were killed (IC_{50}) of 10–20 μM. The cell death of NCTD-modulated DCs assessed by flow cytometry with Annexin V-FITC and PI double staining showed that the main mode of NCTD induced DC

death was necrosis (Fig. 1C).

NCTD modulation of DC surface marker expression

In comparison with the controls, NCTD inhibited the expression of CD1a, DC-SIGN, and CD83 in a dose-dependent manner (Fig. 2A). There were no significant differences in expression of the co-stimulatory molecules B7-2 (CD86) and B7.1 (CD80), CD40, and HLA-DR when treated with different doses of NCTD (Fig. 2B).

Effect of NCTD on secretion of IL-12 and IL-10

IL-12 levels produced by DCs in the presence of NCTD were similar to those of DCs incubated with the cytokine combination (data not shown). The secretion of IL-10 was inhibited by NCTD in a dose dependent manner (Fig. 2C).

Effect of NCTD on DC stimulation of allogeneic naive T cells

Both low- and high-concentration NCTD-treated DCs stimulated the proliferation of allogeneic $CD4^{+}CD45RA^{+}$ T cells (Fig. 2D). IFN-γ production by these allogeneic naive T cell had a similar profile to that of T cell proliferation for the NCTD-treated group (Fig. 2E). In addition, NCTD-treated DCs also stimulated the

proliferation of allogeneic $CD4^+$ $FOXp3^+$ Treg cells (12.0 ± 3.8) when compared with isotype control (0.5 ± 0.1)(Fig. 2F).

Expression of regulatory protein and activation of calcineurin phosphatase in NCTD-treated and CTD-treated DCs

To get insights into the mechanism of NCTD-induced immune silencing, we examined the expression of regulatory molecules involved in the immune tolerance. NCTD and CTD enhanced the expression of p70S6 kinaes and phosphorylation of p70S6 kinase at Thr-389 (Figure 3A). By contrast, the expression of phosphorylation of calcineurin (PP2B) phosphatase and the activity of cellular PP2B were inhibited by NCTD and CTD (Figure 3B and 3C). The results suggest that NCTD may induce immune tolerance through modulation of calcineurin, but not through down-regulating mTOR pathway.

In vivo effect of CTD and NCTD on skin transplantation

Gross observation

Acute skin graft rejection

Treatment with CTD and NCTD significantly prolonged skin graft survival in a dose-related manner. In the acute skin graft rejection experiment, CTD 1.0 mg/kg had

the best skin graft survival compared to the other groups. However, CTD caused greater mortality than the other treatment groups (4 of 6 mice treated with CTD 1.0 mg/kg died versus none in the NCTD-treated group). Treatment with NCTD 1 mg/kg resulted in similar skin survival as treatment with CTD 0.5mg/kg, which was better than treatment with NCTD 0.5 mg/kg.

Histological analysis

Acute skin graft rejection

By day 7, the skin allografts in the control and DMSO groups had severe epidermal necrosis and necrotizing vasculitis. NCTD 1.0 mg/kg and CTD 0.5 mg/kg treatment clearly reduced the inflammatory reaction with better integrity of the epidermis and less marked inflammatory cell infiltrates than the untreated and DMSO vehicle controls. NCTD 0.5 mg/kg had a moderate effect on maintaining epidermal integrity and reducing graft inflammation (Fig. 4A).

Prolonged skin graft rejection

To observe the longevity of the action of the CTD and NCTD, we extended the observation period beyond the subacute phase of skin graft healing. As shown in Figure 4B, treatment with CTD 0.5 mg/kg and NCTD 1 mg/kg markedly reduced the

rejection of the skin allografts and resulted in greater skin integrity. At the 21-day examination, CTD 0.5 mg/kg and NCTD 1 mg/kg both promoted significant prolongation of allograft survival (19.6 ± 1.9 and 18.2 ± 2.3 days, respectively) in comparison with the non-treated group and DMSO groups (11.6 ± 0.9 and 11.8 ± 1.1 days), with a statistically significant difference ($p < 0.01$) (Fig. 4C).

Immunohistochemical study

We next examined the distribution profile of APCs and Treg cells in secondary lymphoid organs of the mice receiving skin allografts. In spleens of the controls, the APCs expressing MHC II molecules were found in the mantle zone of lymphoid follicles outside the germinal center (Fig. 5A). Intriguingly, the APCs in the NCTD (1 mg/kg) and CTD-treated (0.5 mg/kg) groups were found predominately in the interfollicular areas (IF) of lymphoid follicles (Fig. 5B and 5C). The number of APCs for control, NCTD-treated and CTD-treated group was 39.0 ± 3.0, 101.3 ± 34.8 and 130.7 ± 7.5, respectively. For the putative target of tolerogenic APCs, distribution of Foxp3-expressing Tregs in the recipient spleen was examined. In the control group, the amount of Treg was smaller and only a few Treg cells were found within the interfollicular areas of lymphoid follicles in the spleen (Fig. 5D). In contrast, in the NCTD (1 mg/kg) and CTD-treated (0.5 mg/kg) groups, abundant Foxp3-expressing

Treg cells accumulated in the interfollicular area, compatible with the location of APCs (Fig. 5E and 5F). The number of Treg for control, NCTD-treated and CTD-treated group was 12.3 ± 2.1, 31.0 ± 12.2 and 29.7 ± 14.0, respectively.

Serum cytokine levels

In the acute phase of rejection, the serum concentrations of IL-2, IL-4, IL-10, IL-12p70, TNF-α, and IFN-γ levels on day 7 were at basal levels, without significant differences between the drug (CTD and NCTD)-treated and control (untreated and DMSO) groups (data not show). However, IL-6 levels were elevated in the drug-treated groups for both CTD 0.5 mg/kg and NCTD 1 mg/kg in comparison with the control (untreated and DMSO) groups (p value < 0.01) (Fig. 6A).

Toxicity profile

In the acute phase of rejection, the serum concentrations of creatinine on day 7 were at the basal level, without significant difference between the drug-treated and control (untreated and DMSO) groups. However, alanine aminotransferase levels were elevated in the CTD-treated groups for both dosage regimens (0.5 mg/kg and 1 mg/kg) in comparison with the control groups ($p < 0.05$) (Fig. 6B). For bone marrow suppression, leukocyte counts were assessed and no significant change was noted in

any group.

Discussion

DCs are known to differentiate into various cell subsets, including immunogenic and tolerogenic populations, by different stimuli from microenvironments or after varying treatments. For example, tolerogenic DCs could be generated by immunosuppressive drugs such as vitamin D3 in combination with mycophenolate mofetil (*14*), platonin (*12*), or dexamethasone, resulting in differentiation of human DCs to a less mature stage (*11*). We previously reported that there was no significant cell death in peripheral blood mononuclear cells (PBMC) after treatment with 6.25 to100 μM NCTD for 3 days (*15*). The normal starting $CD14^+$ cells we used were sorted from PBMC, thus, NCTD at 10 – 20 μM may not toxic to the starting $CD14^+$ cells. In the current study, NCTD reduced the viability of DCs and causes cell death predominately due to necrosis (Fig. 1B and C). Nonetheless, the NCTD-treated DCs still possessed biological activity, including cytokine secretion (Fig. 2C), CD83 expression, and allostimulation of naïve T cell and Treg cell proliferation (Fig. 2D and 2F). This suggests that the immunomodulatory effect of NCTD-treated DCs may not solely be due to its cytotoxicity on DC precursors.

For allostimulation in vitro, NCTD-treated DCs resembled regulatory DCs in terms of surface phenotype and capacity to stimulate $CD4^+CD45RA^+$ naïve T cell, Treg cell proliferation and INF-γ production (Fig. 2D-F). Further characterization of

possible target cell lineage by DCs in vivo revealed that APCs, which included DCs and Foxp3$^+$ Treg cells, accumulated at the same interfollicular areas in secondary lymphoid organs of NCTD-treated recipient mice (Fig. 5A-F). This putative scenario for action of NCTD exists with some unclear intermediates and controversy, such as reduced secretion of IL-10 in vitro (Fig. 2C) and elevation of serum IL-6 level in vivo (Fig. 6A). These issues need further cellular and molecular investigation to clarify the interactions between the various cell lineages and cytokine network.

Various kinds of immunosuppressive agents acting through different mechanisms have been explored and used in clinical practice for preventing reject after transplantation. Early experimental data indicated that calcineurin inhibitors, through blocking T-cell activation, may induce the development of immunological tolerance (*15*). In contrast, mammalian target of rapamycin (mTOR) inhibitors allow T-cell activation, but not proliferation, could potentially promote immune tolerance. Moreover, recent publications indicate that expanding T-regulatory cells either through drug therapy or ex vivo cell therapy is being considered as novel transplant tolerance-induction strategies (*16*). In our study, we evaluated the effect of NCTD and CTD on expression of mTOR downstream molecule p70S6 kinase (p70S6K), and calcineurin (PP2B) phosphatase, respectively. We found that NCTD increased the expression of p70S6 k and phosphorylated p70S6K at Thr-389 (Figure 3A),

suggesting an mTOR activation-independent effect. Intriguingly, NCTD and CTD attenuated the expression of the phosphorylation of PP2B and decreased the enzyme activity of PP2B which indicates NCTD and CTD may induce immune silencing through calcineurin phosphatase inhibition in DCs (Fig. 3B and 3C).

IL-6 signaling increased the number of resting/immature DCs and decreased the number of activated/mature DCs in lymph nodes and in the spleen following LPS stimulation (*18*). In the current study, we found that the serum IL-6 levels were higher in the CTD-treated and NCTD-treated mice (Fig. 6A) whose skin grafts lasted much longer than the grafts of the controls (Fig. 3A-C). Whether this elevation correlates to the accumulation and co-localization of MHC II-expressing APCs and $Foxp3^+$ Treg cells remains to be elucidated.

Compared with CTD, NCTD possesses less toxicity to kidneys, gastrointestinal tract, bone marrow, and heart (*4*). The acute LD_{50} of NCTD is 11-fold higher than CTD (12.5 mg/kg) (*19*). The safety and efficacy of intra-arterial NCTD (0.5 mg/kg) applied in a rat solid liver tumor model are reported by Mack et al. (*20*). Because no pharmacokinetic data to estimate the transition, we determined the in vivo doses according to preliminary dose-finding experiments. Concerning the less toxicity than and similar potency to CTD (Fig. 6B), NCTD may have potential to be developed as a novel preventive and therapeutic agent against transplant rejection, based on its lower

toxicity and similar potency to CTD

To sum up, NCTD modulates the differentiation and maturation of human myeloid DCs toward a state of atypical maturation through calcineurin phosphatase inhibition. In the mouse skin allograft model, NCTD prolonged survival of skin grafts accompanied by accumulation and co-localization of APCs and Treg cells in the interfollicular area in the recipients' spleens. NCTD is less toxic than the naturally occurring compound CTD.

Methods

Generation of human dendritic cells

Human dendritic cells were generated as previously described (*12*). Briefly, human peripheral blood mononuclear cells were obtained from healthy donors. Subsequently, $CD14^+$ cells were purified using the miniMACS system. Immature DCs were generated from $CD14^+$ monocytes by culture in RPMI 1640 medium supplemented with 10% fetal calf serum, 100 ng/mL GM-CSF (Schering-Plough, Munich, Germany), 50 ng/mL of interleukin-4 (IL-4) (R&D Systems, Minneapolis, MN, USA), every 3 days for 6 days in a humidified 5% CO_2 incubator. To trigger maturation of DCs, immature DCs were incubated with a combination of pro-inflammatory cytokines including 5 ng/mL of tumor necrosis factor-α (TNF-α), 5 ng/mL of IL-1, 15 ng/mL of IL-6 (R&D Systems) and 1 μg/mL of prostaglandin E2 (PGE2) (Sigma-Aldrich, St. Louis, MO, USA) (*23, 24*). In some experiments, NCTD (0, 1.25, 2.5, 5, 10, 20 μM) was added at the beginning of $CD14^+$ cell culture to evaluate its effect on DC differentiation and maturation. NCTD was purchased from Sigma Company (SIGMA-ALDRICH, St. Louis, MO, USA) and dissolved in phosphate-buffered saline (PBS) as a stock solution (100 μM), respectively.

Number of viable cells

DCs were harvested on day 8 and the numbers of viable cells were counted using the trypan blue dye exclusion test. The recovery rate of DC was estimated by dividing the number of harvested DCS by the total number of sorted $CD14^+$ monocytes.

Morphological observation

DCs were centrifuged onto microscope slides by using Cytospin[2] (Shandon Inc, Pittsburgh, PA). The glass slides were fixed with Liu's A solution for 45 seconds and Liu's B solution for 90 seconds. After washed out and air-dried, the DCs were observed under a light microscope (Olympus, Tokyo, Japan).

Apoptotic assay

Apoptosis of morphologic change was staining with Annexin-V fluorescein isothiocyanate (FITC) and propidium iodide (PI) double labeling kit purchased from BD Bioscience Pharmingen (San Diego, CA, USA). Annexin V-FITC and PI double-stain labeling were detected using a FACScan. AnnexinV(+)/PI(-) and AnnexinV(+)/PI(+) were defined as apoptosis and necrosis, respectively.

Flow cytometric analysis

Dual-color immunolabelling was performed using FITC- and phycoerythrin

(PE)-conjugated monoclonal antibodies (mAbs). The mouse anti-human mAbs IgG1:FITC/mouse IgG1:PE, and appropriate isotype controls were purchased from Serotec (Oxford, UK) and used for DC characterization as follows: anti-CD11c and anti-CD14 (for IgG-FITC), anti-CD1a-PE, anti-CD80-PE, anti-CD86-PE, anti-CD83-PE, anti-HLA-DR-PE, and anti-DC-SIGN-PE. 1×10^6 cells were applied to a FACS caliber flow cytometer (BD Biosciences, San Jose, CA). Data were collected and analyzed using CellQuest Software (BD Biosciences).

Allogeneic naive T cell proliferation, Tregs Identification and cytokine secretion

To purify $CD4^+CD45RA^+$ T cells, nonadherent cells from a culture of isolated mononuclear cells were used. Naive T cells were enriched with a $CD4^+CD45RA^+$ T cell isolation kit (Miltenyi Biotec) using the MiniMACS system. Monocyte-derived CTD-treated or NCTD-treated DCs were harvested and irradiated (30 Gy) with 6 MeV X-rays generated by a linear accelerator (Clinac® 1800, Varian Associates, Inc., CA) at a dose of 4.0 Gy/min in a single fraction. Thirty Gy-irradiated DCs were incubated with 1×10^6 allogeneic naive T cells at ratios of 1:10 or 1:30 for 5 days, after which 5 μM carboxyfluorescein succinimidyl ester (CFSE) was added to the T cell cultures for 18 h. The cells were then collected and the incorporated CFSE was detected using flow cytometry. To identify Tregs, allogeneic naive T cells were

stained with the following mAbs: anti-CD4 (PE or PerCP), anti-CD25 fluorescein isothiocyanate (FITC) (all from Becton Dickinson). Isotype controls were cells stained with IgG1 conjugated with the respective fluorochromes. Intracellular staining of FOXP3 was performed using the anti PE-FOXP3 staining set (eBioscience) according to the manufacturer` s instructions. In T cell population expressed CD4, CD25 or Foxp3, the percentage of $CD4^+$ $Foxp3^+$ T cells was analyzed by flow cytometry along with FACS Calibur using Cell Quest software.

Detection of cytokines produced by DC and stimulated allogeneic naive T cells

The levels of IL-12 (p40/70) in the DC supernatant and interferon- γ (IFN-γ) in the stimulated allogeneic T-cell supernatant were measured using enzyme-linked immunosorbent assay (ELISA) (R&D Systems) according to the manufacturer's instructions. The detection limits for IL-12 and IFN-γ were 5.0 and 8.0 pg/mL, respectively.

Western blotting

Western blot analysis was performed as previously described (*25*). Proteins were isolated from DCs after treatment with NCTD (10 and 20 μM) and CTD (1.25 and 2.5 μM) for 2 h. The protein concentration was determined by a bicinchoninic acid assay kit (Pierce, Rockford, Illinois). Equal amounts of proteins (50 μg in each lane) were

electrophoresed in 10% SDS-polyacrylamide gels. Proteins were transferred onto a nylon blotting membrane. The membrane was blocked with 5% de-fatted milk and immunoblotted with primary antibodies against various proteins (Transduction Laboratories, Lexington, Kentucky) at room temperature for 3 h. This was followed by incubation with horseradish peroxidase-labeled second antibodies (Transduction Laboratories) and development using the enhanced chemiluminescence system (Amersham Pharmacia, Piscataway, New Jersey).

Calcineurin Phosphatase Activity Assay.

A complete colorimetric nonradioactive calcineurin assay kit (BioMol, Plymouth Meeting, PA, Enzo Life Sciences International, Inc.) for measuring cellular calcineurin (PP2B) phosphatase activity was used. NCTD- and CTD- treated DCs were harvested and lysed in calcineurin assay buffer (50 mM Tris-HCl (pH 7.5), 0.5% Nonidet P-40, 1 mM EDTA, 1 mM CaCl2) (BioMol, Plymouth Meeting, PA), and clarified by centrifugation. The Quantizyme assay system AK-816 was performed by using 5000 cells per assay lysate according to the manufacturer's procedure (BIOMOL, Plymouth Meeting, PA). Calcineurin phosphatase activity was measured spectrophotometrically by detecting free-phosphate released from the calcineurin-specific RII phosphopeptide as described previously (*26*). Results are from three separate experiments.

Allograft transplantation model

Six- to eight-week-old male C57BL/6 (H-2^b) and BALB/c (H-2^d) mice were obtained from the Animal Resource Center of the National Science Council of Taiwan (Taipei, Taiwan). All experiments were approved by the animal ethics committee of Far Eastern Memorial Hospital, Taipei, Taiwan (study numbers FEMH-97-C-045 and FEMH-99-D-010).

Skin transplantation

Skin transplantation was conducted by a procedure modified from that described previously (*27*). Briefly, a 2 × 1-cm skin graft was removed from the flanks of C57BL/6 donor mice. A graft bed of right dorsal flanks of BALB/c recipients was removed an area of epidermis and dermis down to the level of the intrinsic muscle. The graft was fixed to the graft bed. No dressings or antibiotics were used. Rejection was defined as necrosis of more than 80% of the epidermal surface of the graft.

Drug preparation and treatment

The CTD and NCTD were dissolved in dimethyl sulfoxide (DMSO) and PBS at a stock concentration of 100 μM, respectively. In the first set of experiments, mice were sacrificed on day 7 after surgery and the skin grafts were harvested and

evaluated for severity of acute rejection. The mice were randomly assigned to six groups of six mice: 1) 0.5 mg/kg of CTD and NCTD intraperitoneal injection (i.p.) per day; 2) 1 mg/kg of CTD and NCTD i.p. per day; or 3) no drug treatment; 4) DMSO 200 μg/kg i.p. per day.

CTD 1 mg/kg i.p. per day was toxic for mice and the effect of NCTD 0.5 mg/kg i.p. per day was less than that of NCTD 1 mg/kg. Therefore, in the second set of experiments, 24 animals were randomly assigned to four groups receiving either 0.5 mg/kg CTD or 1 mg/kg NCTD, DMSO 200 μg/kg and no treatment for 21 days. Skin grafting was performed in the same manner as described above.

Histological evaluation

For histological analyses, skin grafts were fixed in formalin and embedded in paraffin. Five-μm sections were cut, deparaffinized, rehydrated, and stained with hematoxylin and eosin. A pathologist, blinded as to the experimental group the specimens were from, evaluated the slides to determine the presence of inflammation, rejection, infection, and other histological changes.

Immunohistochemical study

For immunohistochemical analyses, spleen, liver, and lymph nodes were fixed in

formalin and embedded in paraffin. According to the histological analysis of skin grafts, the groups treated with NCTD 1 mg/kg and CTD 0.5 mg/kg, and the control groups were enrolled. Antigen was retrieved using Target Retrieval Solution (pH 9.0) (Dako) in a decloaking chamber (Biocare Medical). Primary antibody major histocompatibility complex (MHC) class II (Abcam) was incubated and antibody (Ab) was detected using the MM-HRP-Polymer Kit (Biocare Medical) (*28*). For detection of forkhead/winged helix transcription factor 3 (Foxp3), a procedure was performed that described previously (*29*). A pathologist blinded to which experimental group the specimens were from evaluated the slides to determine the presence of DCs and other histological changes.

Serum cytokine analysis

Serum obtained from the mouse blood samples were analyzed using Cytometric Bead Array kits according to the manufacturer's specifications (BD Biosciences). IL-6, IL-10, IL-12p70, and IFN-γ were detected simultaneously using the mouse inflammation kit. IL-2, IL-4, and TNF-α protein levels were measured using the mouse Th1/Th2 cytokine kit. For this assay, soluble cytokines were captured on microparticles and measured using flow cytometry as described previously (*30*).

Evaluation of leukocyte count, hepatic and renal functions

The white blood cell counts of the blood samples were analyzed by an automatic Coulter counter (Model Z1, Beckman Coulter Electronics, Fullerton, CA) and plasma levels of alanine aminotransferase and creatinine were measured by a standard colorimetric method and manufacturer-supplied reagents.

Statistical Analysis

The results are expressed as means ± standard errors of the means (SEMs). Comparison in each experiment was performed using an unpaired Student's *t*-test. For graft survival, repeated measurement one-way ANOVA followed by the Tukey post-test was performed using SPSS software, version 12.0 (SPSS, Chicago, IL, USA). A p value of less than 0.05 was considered statistically significant.

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Conflict of Interest: The authors declare that they have no conflict of interests.

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Figure Legends

Figure 1. (A) Morphology of monocyte-derived dendritic cells (DCs). DCs maturated in the presence of the cytokine combination and DCs differentiated in the presence of 1.25, 2.5, 5, 10, and 20 μM of norcantharidin (NCTD). Magnification for photograph is 1000X. (B) Effect of norcantharidin (NCTD) on dendritic cell (DC) viability and death. Viability of DCs treated with NCTD (1.25 to 20 μM). (C) Detection of necrosis and apoptotic cells by annexin V-propidium iodine (PI) double-stain labeling. Monocyte-derived DCs were treated with different concentrations of NCTD then stained with annexin V and PI, and then analyzed by flow cytometry. NCTD caused DC death by necrosis, especially in high-dose conditions. Similar results were obtained in three independent experiments. Data from three separate experiments are expressed as means ± standard errors of the means (SEMs). *$p < 0.05$in comparison to the control group (unpaired Student's *t*-test).

Figure 2. (A) Expression of surface molecules on monocyte-derived dendritic cells (DCs). Flow cytometric analysis of CD14, CD1a, DC-SIGN, and CD83. Norcantharidin (NCTD) inhibited the expression of CD1a, DC-SIGN, and CD83 in a dose dependent manner. CD14 was not expressed. NCTD did not de-differentiate DCs to monocytes; (B) Flow cytometric analysis of CD40, CD80, CD86 and HLA-DR.

There were no significant differences in expression of CD40, CD80, CD86, and HLA-DR under different doses of NCTD. (C) The secretion of IL-10 was inhibited by NCTD in a dose-dependent manner. (D) Proliferation and interferon (IFN)-γ secretion of allogeneic $CD4^+CD45RA^+$ naive T cells stimulated by dendritic cells (DCs) generated in various cultures. $CD4^+CD45RA^+$ T cell proliferation stimulated by NCTD-treated DCs as measured by carboxyfluorescein succinimidyl ester (CFSE) uptake. (F) IFN-γ production by NCTD-treated DC-stimulated $CD4^+CD45RA^+$ T cells measured by enzyme-linked immunosorbent assay. (F) The proliferation of allogeneic $CD4^+$ $Foxp3^+$ Treg cells. FACS analysis of $CD4^+$ $Foxp3^+$ T cells following mAb staining (12.0± 3.8) and isotpy control (0.5± 0.1), as described in the Materials and methods. Representative dot-blot figures were demonstrated. Data from 6 separate experiments are expressed as means ± standard errors of the means (SEMs). $*p < 0.05$ compared to the control group using unpaired Student's *t*-test.

Figure 3. Expression of p70S6K, calcineurin and calcineurin phosphatase activity in DCs treated with cantharidin (CTD) and norcantharidin (NCTD). DCs were treated with NCTD (0, 10, and 20 μM) and CTD (0, 1.25 and 2.5 μM) for 2 h and harvested for Western blot and calcineurin phosphatase activity analysis. (A) p70S6 kinaes and phosphorylation of p70S6 kinase at Thr-389; (B) PP2B and phosphorylated PP2B; (C)

The cellular calcineurin phosphatase activity.

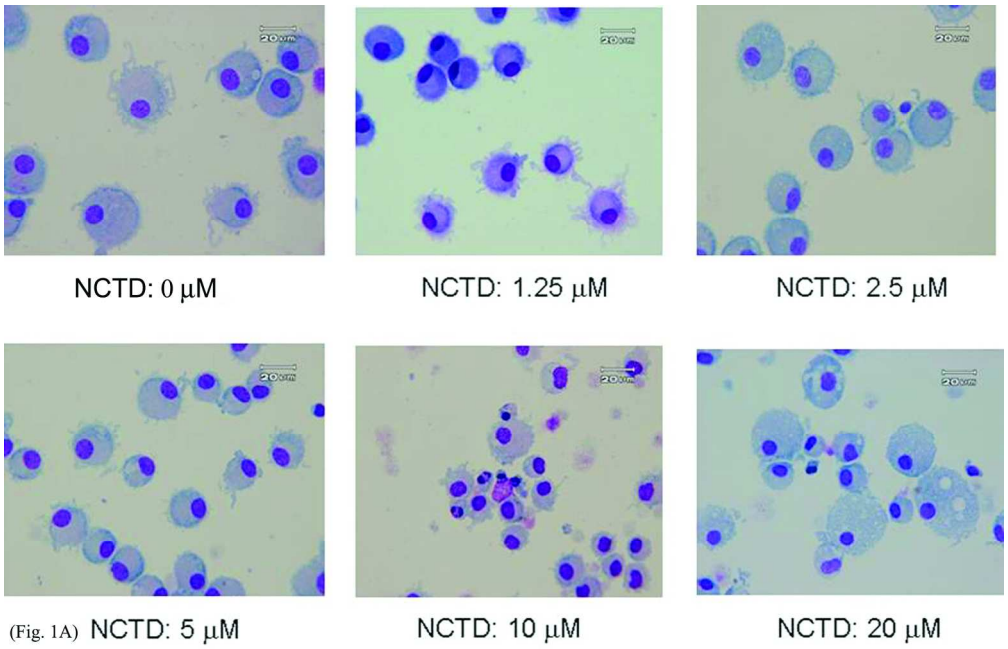
Figure 4. Comparison of gross and microscopic appearances of the skin grafts between the cantharidin (CTD)-treated, norcantharidin (NCTD)-treated, DMSO (dimethyl sulfoxide vehicle), and control mice. (A) Representative hematoxylin and eosin stained allografts harvested on day 7 after grafting (x 100 for original magnification; x 200 for *insets*). The extent of perivascular infiltration, epidermal degeneration, and dermal inflammation correlated with gross changes in the allografts. (B) Representative photographs of mouse skin allografts. Skin grafts were evaluated by visual and tactile inspection until necrosis. The necrotic graft was nonvascularized and had dried up or dropped off. (C) The 21-day of skin allograft (C57BL/6 to BALB/c) survival and median survival time of the graft (MST) in days (n = 6 in each group).

Figure 5. Comparison of spleen tissue histology. The spleen tissue sections were stained for major histocompatibility complex (MHC) class II and Foxp3 immunohistochemically after treatment with norcantharidin (NCTD) 1 mg/kg and cantharidin (CTD) 0.5 mg/kg and compared to the controls. (A) Antigen presenting cells (APCs) expressing MHC II antigens located in the mantle zone (MZ) of

lymphoid follicles (LF) in the control groups (Number of APCs: 39.0 ± 3.0). (B) APCs expressing MHC II antigens in the NCTD 1 mg/kg-treated group were predominately located in the interfollicular areas (IF) of the LF (Number of APCs: 101.3 ± 34.8). (C) APCs expressing MHC II antigens in the CTD 0.5 mg/kg-treated group were predominately located in the IF areas of the IL (Number of APCs: 130.7 ± 7.5). (x 200 for *original magnification*; x 600 for *insets*) (D) A few regulatory T (Treg) cells with Foxp3 expression were located in the IF area of the LF in the control groups (Number of Treg: 12.3 ± 2.1). (E) Regulatory T cells with Foxp3 expression in NCTD 1 mg/kg-treated groups were predominately located in the IF area of LF of spleen (Number of Treg: 31.0 ± 12.2). (F) Treg cells with Foxp3 expression in the CTD 0.5 mg/kg-treated groups were predominately located in the IF area of the LF (Number of Treg: 29.7 ± 14.0). (Red arrow) (x 200 for *original magnification*; x 600 for *insets*) The IHC stains represent results of one of six independent experiments, which showed similar patterns of expression.

Figure 6. (A) Interleukin (IL)-6 in cantharidin (CTD)- and norcantharidin (NCTD)-treated mice compared to that of the control and DMSO (dimethyl sulfoxide vehicle) groups. The levels of IL-6 were determined by cytometric bead array on day 7 after skin transplantation. *$p < 0.01$, **$p < 0.05$ versus the respective control (n = 6

in each group). (B) Plasma alanine aminotransferase (ALT) levels at different dosages of cantharidin (CTD) and norcantharidin (NCTD) treatment in mice compared to those of the control and DMSO (dimethyl sulfoxide vehicle) groups 7 days after skin transplantation. Elevated ALT occurred with treatment of CTD 0.5 mg/kg and CTD 1 mg/kg; however NCTD treatment did not increase ALT in the graft recipient mice (n = 6 in each group).



(Fig. 1A)

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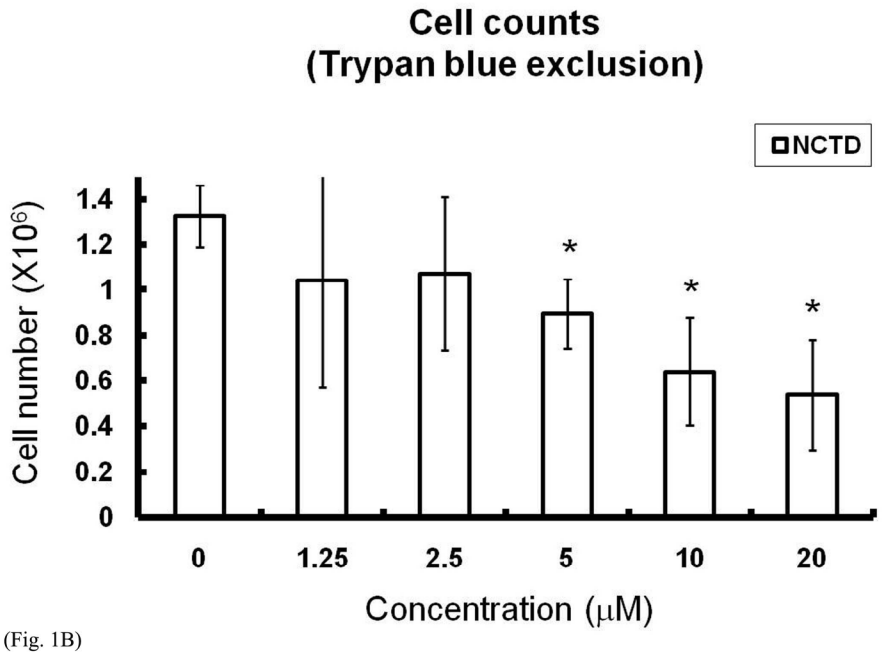

(Fig. 1B)

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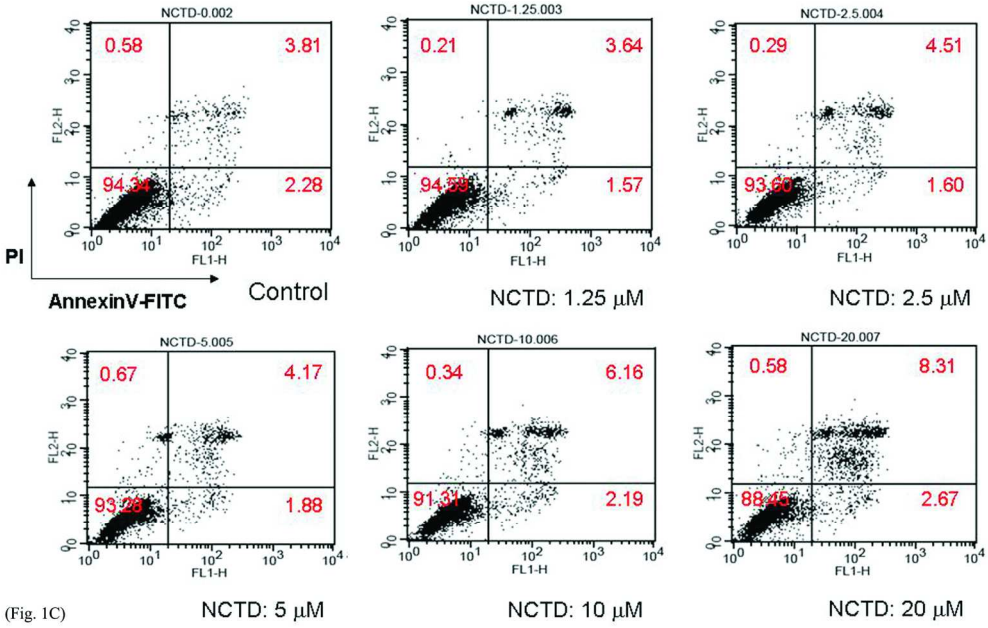

(Fig. 1C)

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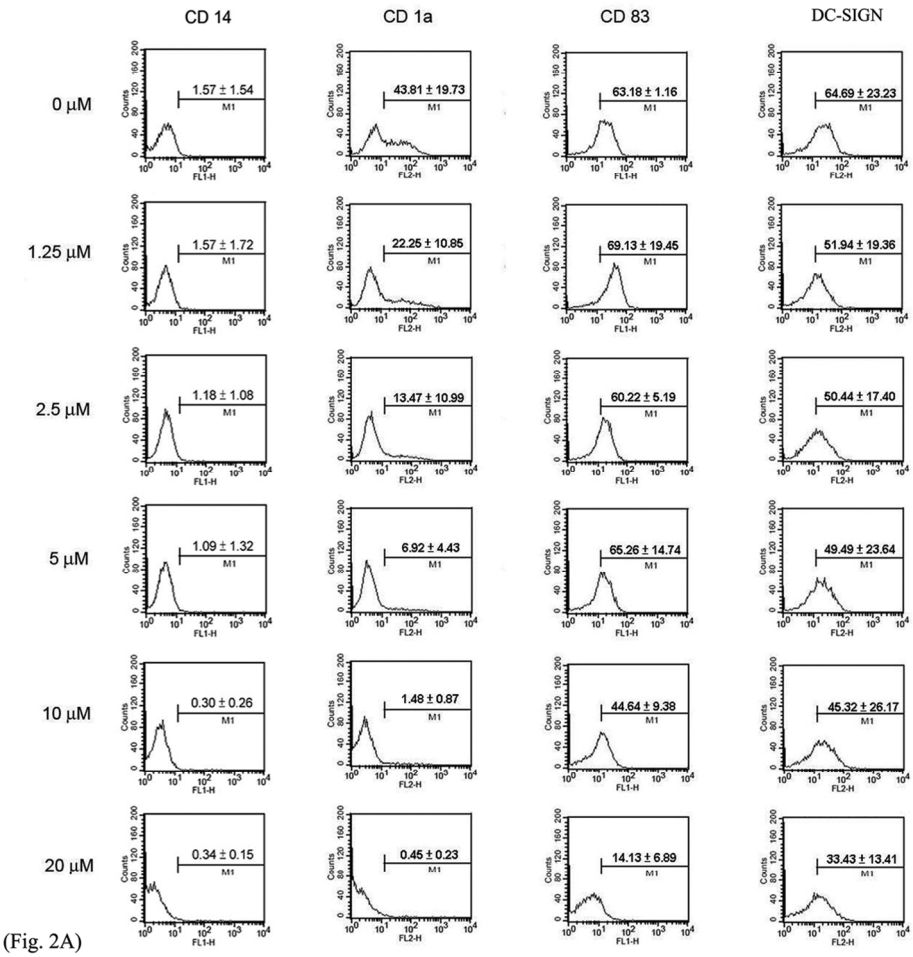

(Fig. 2A)

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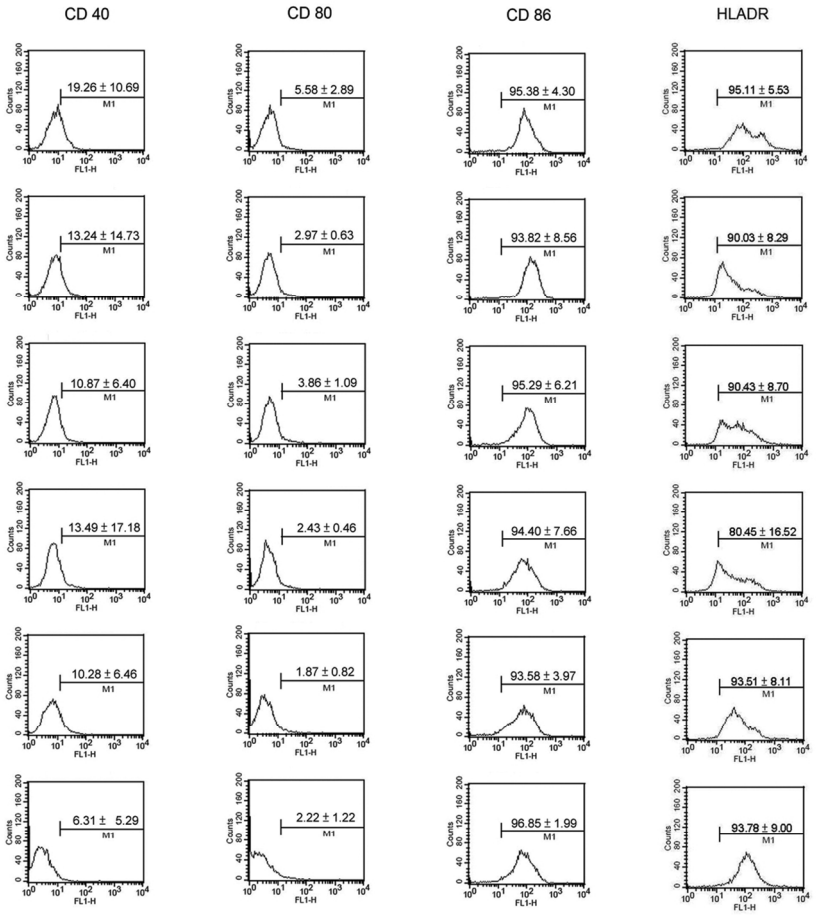

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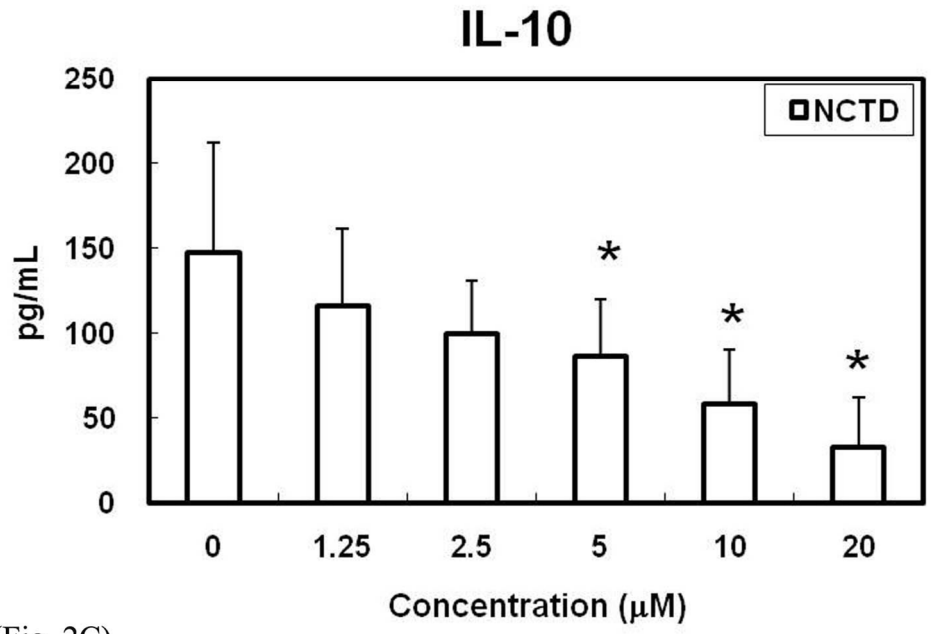

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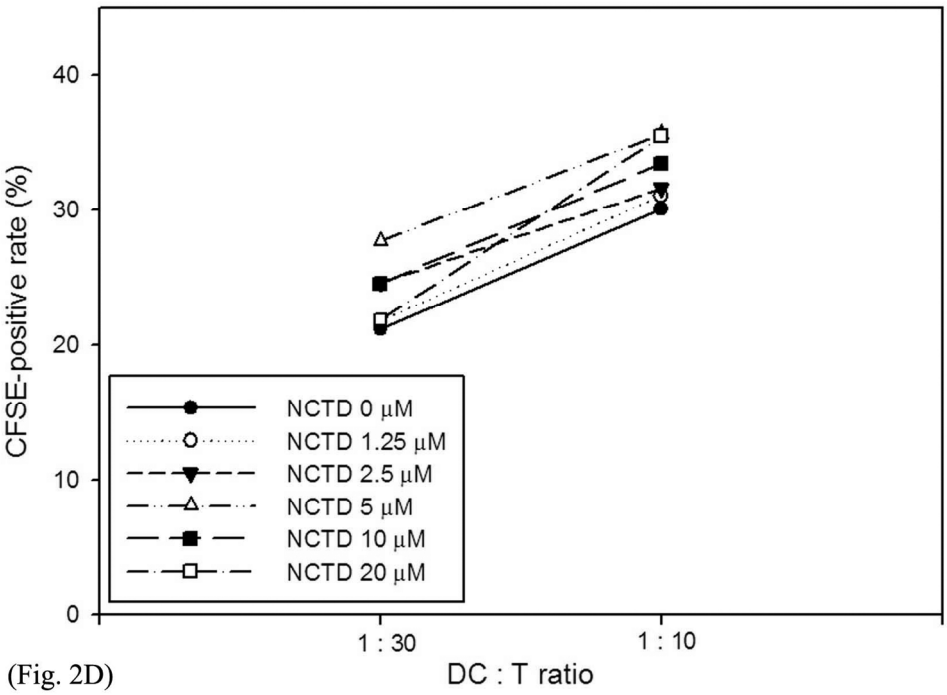

(Fig. 2D)

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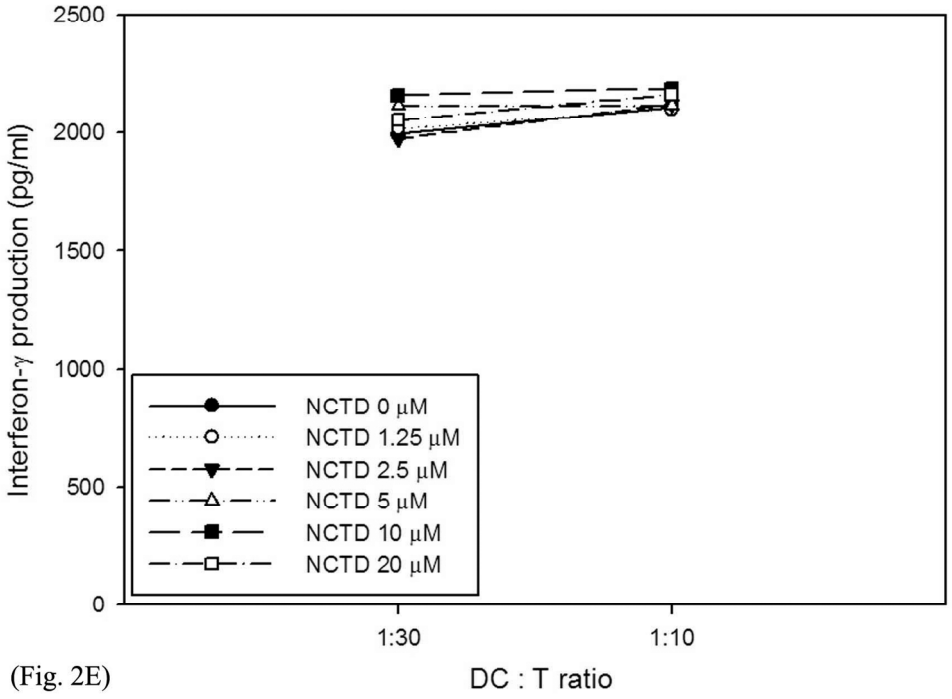

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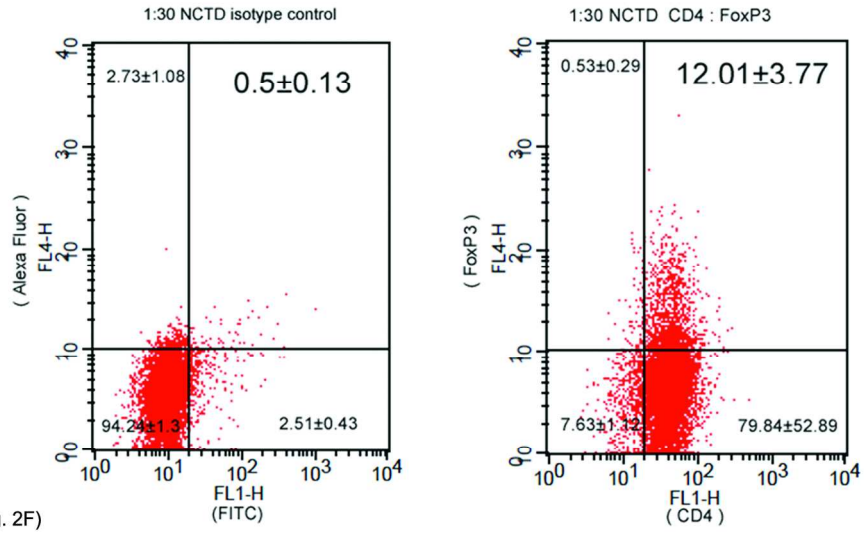

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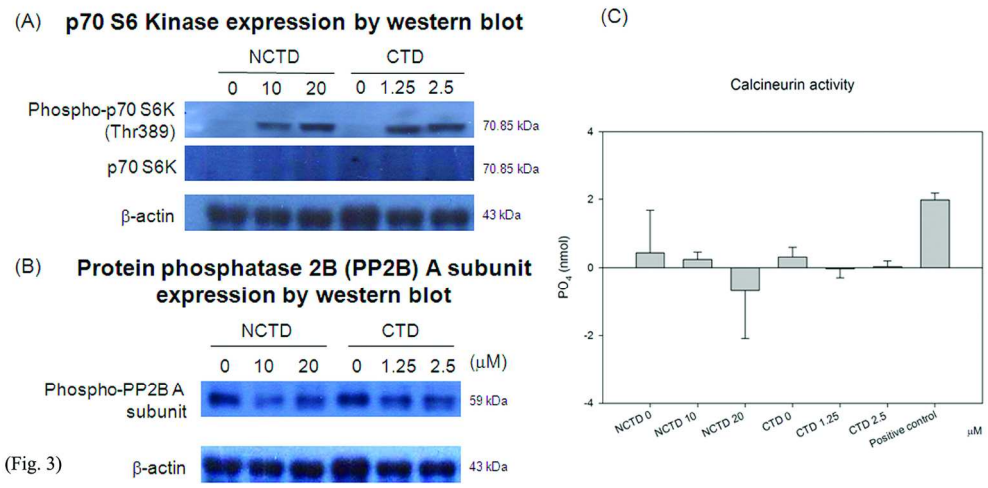

(Fig. 3)

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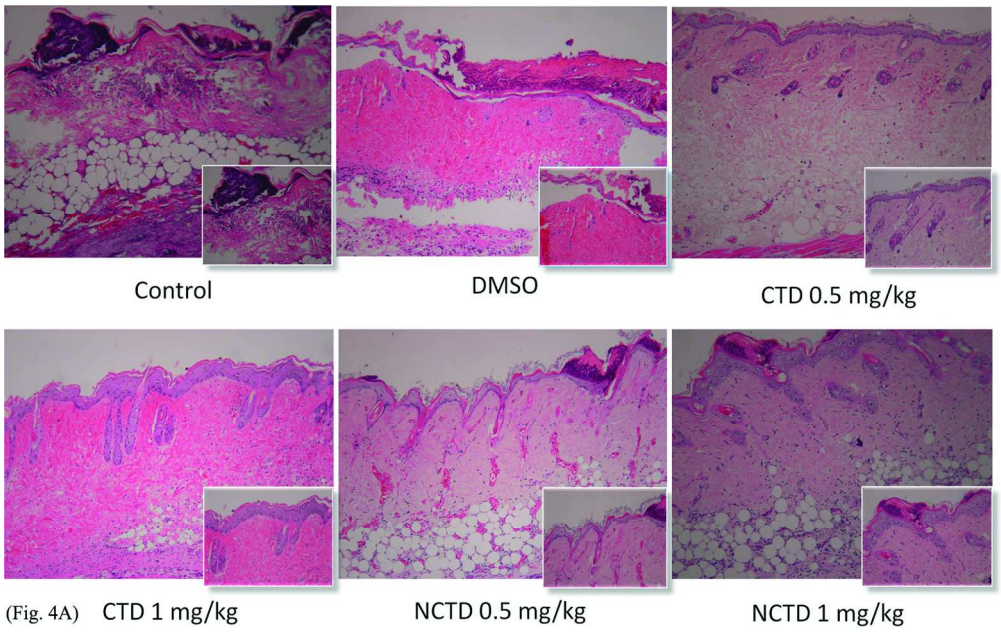

(Fig. 4A)

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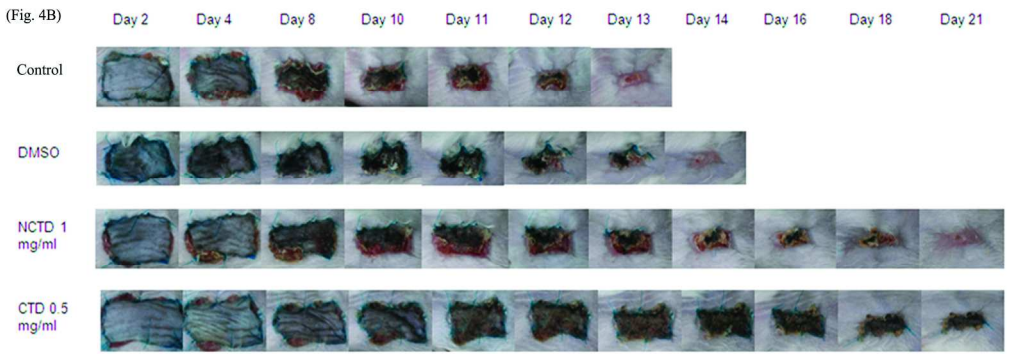

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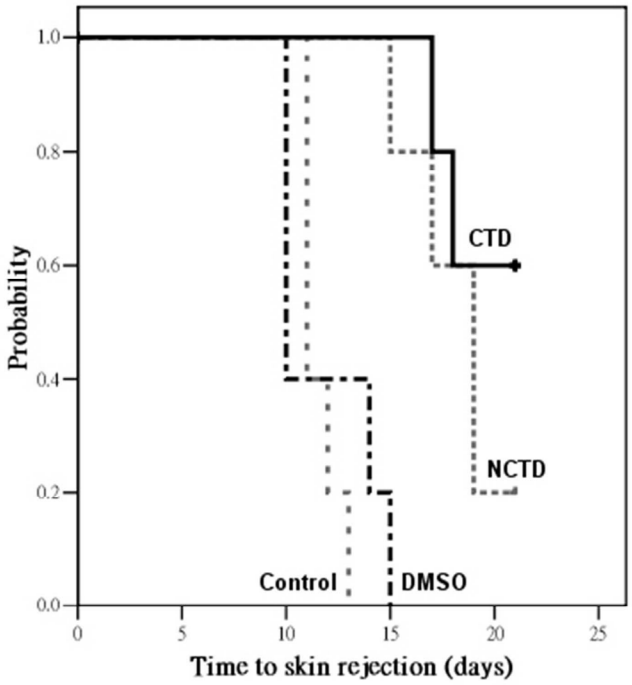
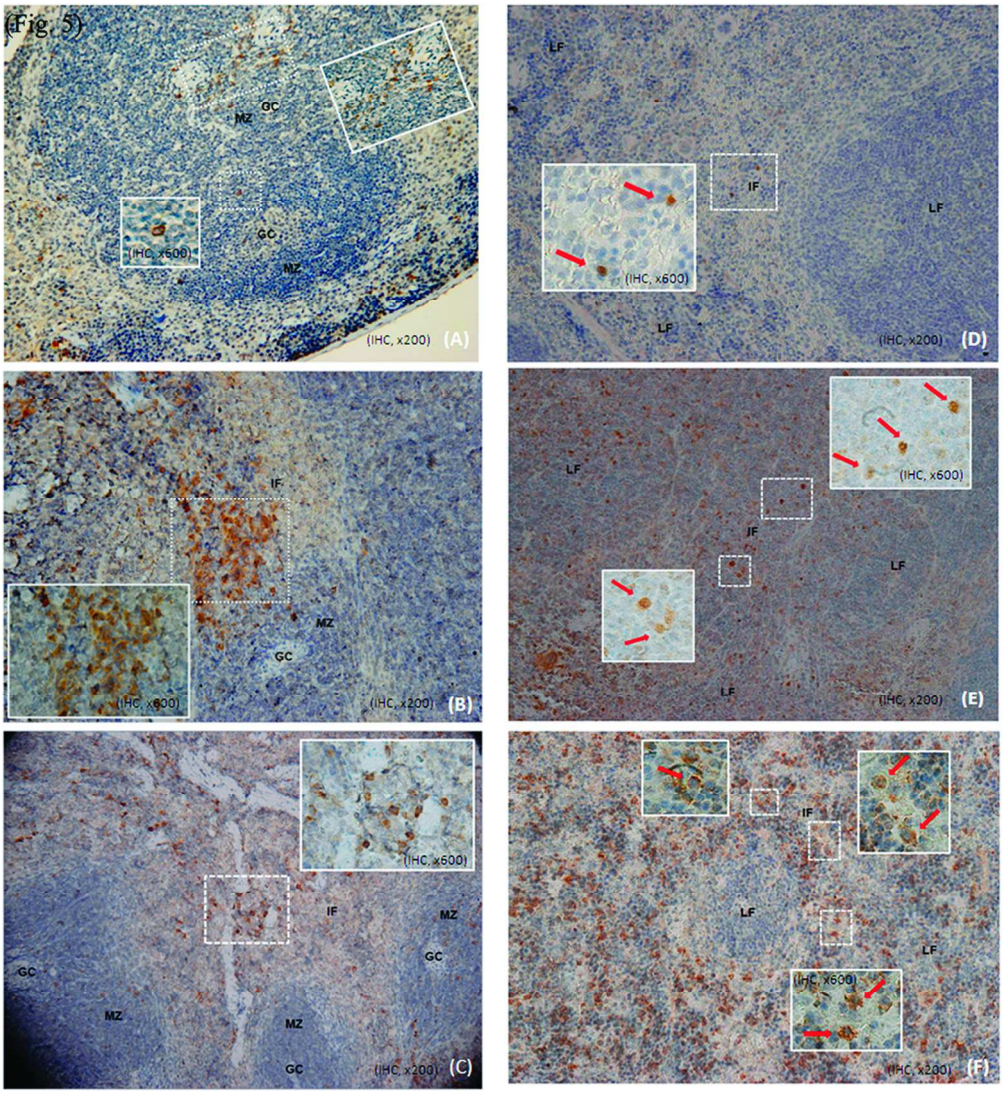
(Fig. 4C)



	Groups			
	Control	DMSO	NCTD 1 mg/kg	CTD 0.5 mg/kg
MST ± SD (days)	11.6 ± 0.9	11.8 ± 1.1	18.2 ± 2.3	19.6 ± 1.9

180x223mm (300 x 300 DPI)

(Fig. 5)



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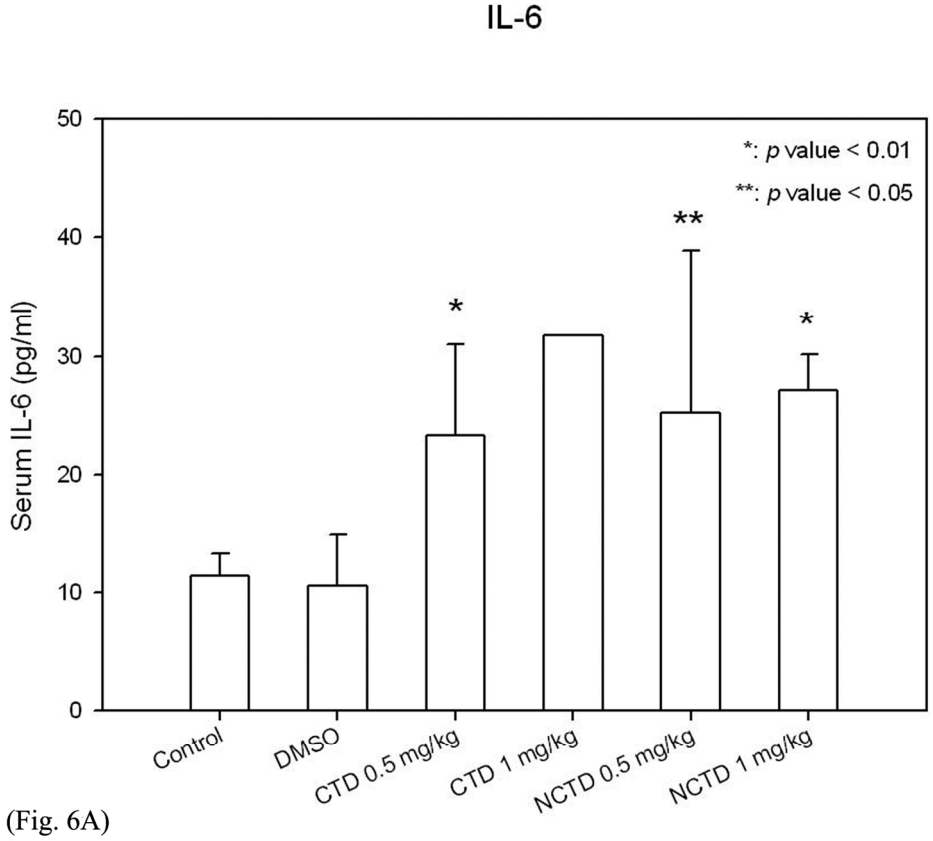

(Fig. 6A)

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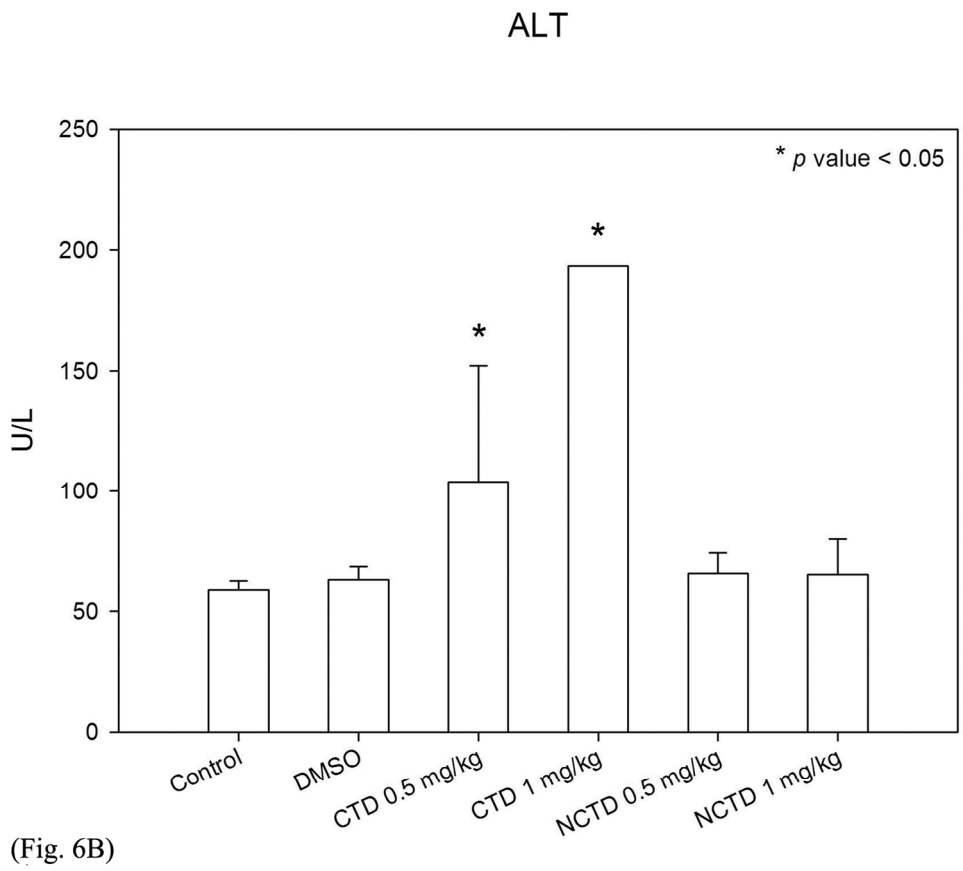

(Fig. 6B)

150x130mm (300 x 300 DPI)