

# Activation of Human Monocyte-derived Dendritic Cells *In Vitro* by Thymax, a Gross Thymic Extract

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**Abstract.** *Background:* We have recently demonstrated that Thymax, a gross thymic extract, induces an apoptotic effect against human breast cancer cells. In this study, the ability of Thymax to activate human dendritic cells (DCs) and the DC-directed T-cell response was examined in an *in vitro* culture model of peripheral blood mononuclear cells. *Materials and Methods:* The level of costimulatory molecules (CD40, CD80, CD83, CD86) and T-cell proliferation were analyzed by flow cytometry. Cytokine secretion was measured by ELISA. *Results:* Thymax activated DCs to secrete interleukin (IL)-12p40 and IL-6 cytokines and inhibited IL-10 production. Additionally, Thymax caused the up-regulation of CD80 and CD86 in DCs, leading to an increase in CD4<sup>+</sup> T-cells, which subsequently induced secretion of interferon-gamma (IFN- $\gamma$ ). *Conclusion:* Taken together, the data showed that Thymax activated DCs and, consequently, Th1 cells. Thus, Thymax is an immune-activating compound that needs to be evaluated extensively for its possible therapeutic properties.

Dendritic cells (DCs) are considered the most influential of the antigen-presenting cells (APCs) because of their unique role in initiating immunity against most types of antigens (Ags), and they are the bridge between innate and adaptive immunity. DCs are distributed in an immature state throughout the body and at the portal of entry for microbes. Immature DCs, which are highly phagocytic in nature, sense pathogens and capture Ags, leading to the activation of several arms of the immune system, including the secretion of cytokines and chemokines, which in turn activate the T and B lymphocytes. Therefore, DC activation is a key component in activating an efficient immune response for the eradication of infections.

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DCs have distinct stages of cell development, activation and maturation and have the potential to induce both immunity and tolerance (1). In the absence of inflammation, immature DCs located in peripheral tissues capture innocuous and cell-associated self-Ags and migrate to draining lymph nodes where they can induce tolerance (2). In the presence of inflammation and toll-like receptor (TLR) signals, they migrate to the lymphoid organs where, after maturation, they present captured Ag to naïve T-cells, inducing their differentiation into effector T-cells. Recent studies showed that the cytokines produced by DCs in response to a pathogen are critical in determining the type of T-cell response (3, 4).

Several agents demonstrate the ability to activate DCs to mount efficient immune responses, including a variety of cytokines (5), prostaglandin E<sub>2</sub> (6), poly(I:C) (7), galectin-9 (8), and pathogens (9-11). Thymax is a thymus gross extract that differs from other thymus extracts in that it is a complex of thymosin, thymomodulin and many other peptides. In addition, unlike other thymic factors, Thymax is introduced into the body by mouth. Attempts are currently being made to identify the active factors. We have recently demonstrated that Thymax has the ability to induce apoptosis in human breast cancer cells (12). The present study was undertaken to examine the ability of Thymax to activate human DCs and the DC-directed T-cell response in an *in vitro* culture model, since immunotherapy with DCs has been applied to various types of cancer (13, 14).

## Materials and Methods

*Thymax.* Thymax is a gross thymic extract obtained at acidic pH (through treatment with NaCl and L-ascorbic acid) as described in Ghoneum *et al.* (12). Thymax was provided by the YS Nature Company, Tokyo, Japan.

*Isolation and culture of monocyte-derived DCs.* Monocyte-derived dendritic cells (MDDCs) were prepared essentially as described previously (3, 4). Briefly, peripheral blood mononuclear cells (PBMCs) from normal healthy donors (approved by the Institutional Review Board [IRB], Charles Drew University) were separated over

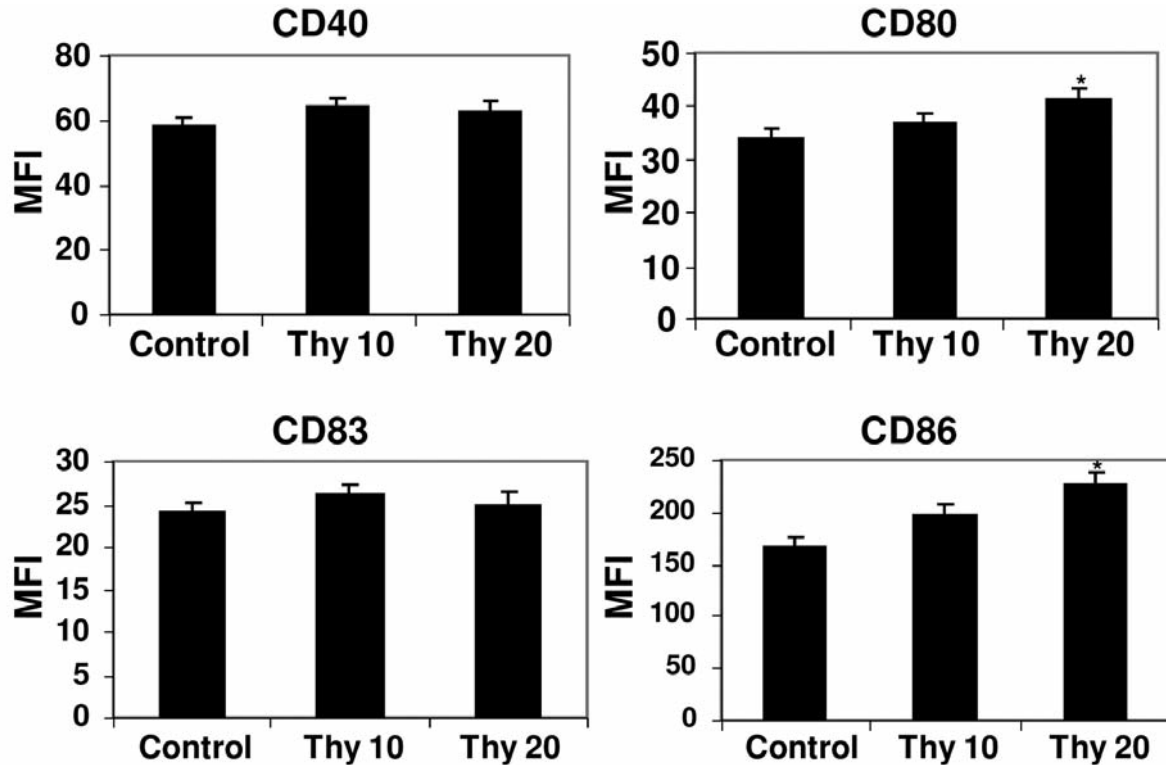


Figure 1. Effect of Thymax (Thy) on percent of DC activation: co-stimulatory and maturation markers CD40, CD80, CD83 and CD86. Monocyte-derived DCs were treated with Thymax (10 or 20  $\mu\text{g/ml}$ ) or LPS (1  $\mu\text{g/ml}$ , control) for 24 h. Expression of cell surface markers was determined by flow cytometry and is shown as the mean fluorescent intensity (MFI). One representative donor is shown, and the data show the mean $\pm$ SD from 3 individual experiments; \*  $p < 0.05$ ; as compared to control LPS-treated cells.

Ficoll-hypaque density gradient centrifugation. The cells were allowed to adhere to culture plates for 2 h. Non-adherent cells were removed. The resulting monocytes were cultured for 6 days under a humidified atmosphere of 5%  $\text{CO}_2$  at 37°C in RPMI 1640 supplemented with 10% FBS, 1 mM glutamine, 100 U/ml penicillin, 100  $\mu\text{g/ml}$  streptomycin, human granulocyte-macrophage colony-stimulating factor (GM-CSF) at 50 ng/ml (Peprotech, Rocky Hill, NJ, USA) and 10 ng/ml recombinant human IL-4 (Peprotech). Half of the medium was replaced every 2 days with fresh medium and MDDCs (CD1a<sup>+</sup>CD14<sup>-</sup>HLA<sup>-</sup>DR<sup>+</sup>CD11c<sup>+</sup>) were collected after 6 days. The purity of the MDDC obtained was >95%. After 48 h, the immature DCs were pulsed with 1  $\mu\text{g/ml}$  *E. coli* LPS as a positive control or Thymax.

**DC phenotyping.** The expression of cell surface markers was determined by flow cytometry. Briefly, gated CD14<sup>-</sup>CD11c<sup>+</sup>HLA<sup>-</sup>DR<sup>+</sup> DCs were analyzed for the expression of CD40, CD80, CD83 and CD86 with the appropriate antibodies supplied by BD Pharmingen (San Diego, CA, USA).

**Cytokine production by DCs.** Immature DCs were incubated with LPS (1  $\mu\text{g/ml}$ ) or 10 or 20  $\mu\text{g/ml}$  Thymax for 24 h. The supernatants were collected and stored at -70°C until analyzed. The cytokines TNF- $\alpha$ , interleukin (IL)-6, IL-10 and IL-12p40 in the supernatants were measured by specific ELISA kits (BD Pharmingen) as per the manufacturer's protocol.

**DC-T-cell cultures.** The ability of Thymax-treated DCs to stimulate T-cell functions was tested in an *in vitro* co-culture assay. The DCs were stimulated with LPS or Thymax for 24 h as above. After washing,  $2 \times 10^4$  MDDCs were co-cultured with carboxyfluorescein succinimidyl ester (CFSE) labeled  $1 \times 10^5$  purified T-cells (negatively selected using a magnetic bead-based kit from Stem Cell Technology, Vancouver, BC, Canada) for 5 days. T-cells cultured without DCs were also assessed. The purity of the T-cells obtained ranged from 93 to 97% as determined by CD3 staining. The proliferation of T-cells was measured by dilution of CFSE dye. The secretion of interferon-gamma (IFN- $\gamma$ ) in the supernatants was assessed using a specific ELISA (BD Pharmingen).

**Statistics.** All the experiments were repeated with samples from five individual subjects, which yielded similar results. The probability that the mean values of two experimental groups were identical was tested by the two-tailed *t*-test for paired samples. The level of significance was set at  $p < 0.05$ .

**Results**

**DC activation.** The data depicted in Figure 1 show the levels of DC surface co-stimulatory and maturation markers, including CD40, CD80, CD83 and CD86, post-treatment with Thymax or LPS. Flow cytometry analysis showed that the

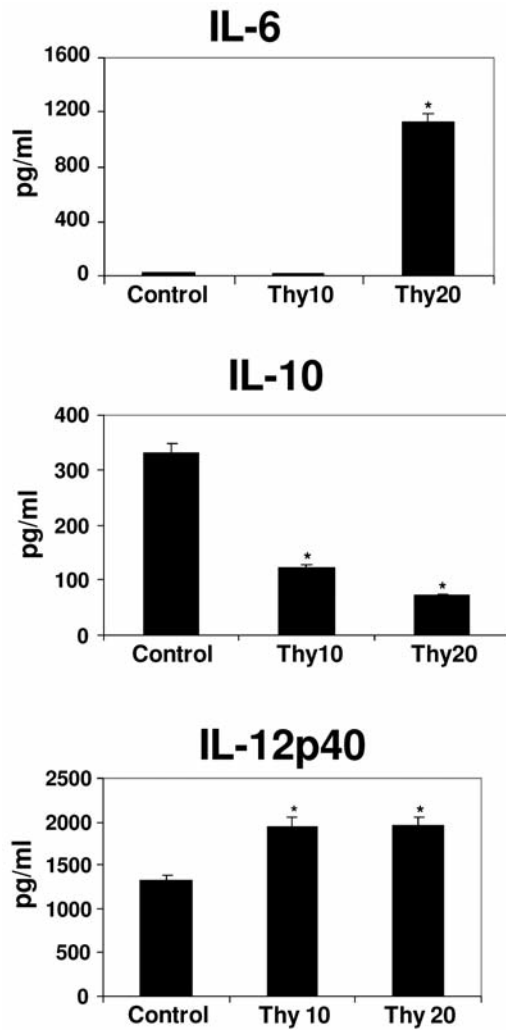


Figure 2. Effect of Thymax on cytokine production: the levels of IL-6, IL-10 and IL-12p40 secreted by the DCs. Immature DCs were incubated with LPS (1  $\mu$ g/ml) or 10 or 20  $\mu$ g/ml Thymax for 24 h. Supernatants were collected and IL-6, IL-10 and IL-12p40 were measured by specific ELISA kits. One representative donor is shown, and the data are the mean $\pm$ SD from 3 individual experiments; \* $p$ <0.05 as compared to control LPS-treated cells.

Thymax-treated DCs did not show increased expression of maturation markers CD40 and CD83. However, a significant dose-dependent increase in expression of CD80 and CD86 was observed (Figure 1) (Thymax 20  $\mu$ g/ml vs. LPS,  $p$ <0.05).

**Cytokine production.** The data indicated that Thymax was able to activate the DCs to secrete cytokines (Figure 2). Thymax at a concentration of 20  $\mu$ g/ml induced an increase in IL-6 secretion that was significantly higher than the control ( $p$ <0.05). Thymax also significantly induced the DCs to secrete IL-12p40, which was observed at both concentrations of 10 and 20  $\mu$ g/ml ( $p$ <0.05). On the other hand, exposure of

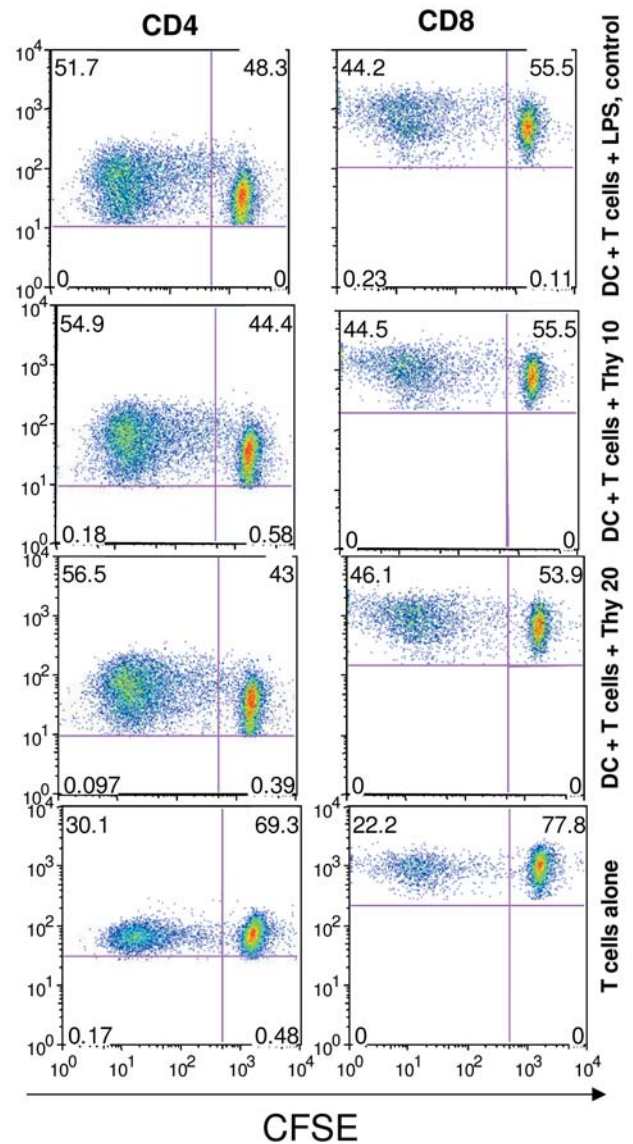


Figure 3. Effect of Thymax on the activation of T-cell proliferation: CD8<sup>+</sup> T-cells and CD4<sup>+</sup> T-cells. DCs were stimulated with LPS (1  $\mu$ g/ml) or Thymax (10 or 20  $\mu$ g/ml) for 24 h. After washing, DCs were cultured with CFSE-labeled T-cells for 5 days. Proliferation of T-cells was measured by dilution of CFSE dye. One representative donor is shown from 1 of 3 individual experiments.

the DCs to Thymax resulted in the down-regulation of IL-10 production, compared to LPS treatment ( $p$ <0.05).

**T-Cell proliferation.** The data in Figure 3 showed that CD8<sup>+</sup> T-cell proliferation did not change after DC treatment with Thymax as compared to LPS-treated cells. However, DC treatment with Thymax at a concentration of 20  $\mu$ g/ml caused an increase in CD4<sup>+</sup> T-cell proliferation to 56.5 %, as compared to 51.7% with the control DCs, treated with LPS.

*Thymax priming of DC-induced T-cells to secrete IFN- $\gamma$ .* The data in Figure 4 show that the LPS treatment of control DCs induced a 10-fold increase in IFN- $\gamma$ , as compared to T-cells alone. Treatment with Thymax primed DC-induced secretion of IFN- $\gamma$  from T-cells in a dose-dependent manner. Thymax at a concentration of 10  $\mu\text{g/ml}$  caused a 1.4-fold increase in IFN- $\gamma$  secretion that was further increased (1.5-fold) at a concentration of 20  $\mu\text{g/ml}$ , as compared to control DCs, treated with LPS ( $p < 0.05$ ).

## Discussion

In this study, Thymax treatment caused an up-regulation of the B7 molecules, CD80 and CD86. These molecules provide co-stimulatory signals for optimal T-cell activation (15), which is mediated by CD28/cytotoxic T lymphocyte-associated antigen (CTLA)4-CD80/CD86 (CD28/B7) (16) and play an essential role in the proliferation and survival of CD4<sup>+</sup> T-cells (17-20). Recent analysis of the CD28/B7 pathway has revealed how this co-stimulatory signal induces the CD4<sup>+</sup> T-cell response with respect to cell proliferation and cytokine production. Two distinct patterns of T-cell cytokine production have been identified. The Th1, or Type 1, response is characterized by the production of IFN- $\gamma$  and targets intracellular antigens. The alternative response, Th2 or Type 2, is characterized by the production of IL-4 and IL-10 and is important in the allergy response and defense against extracellular antigens. In this study, Thymax activated the DCs to prime CD4<sup>+</sup> T-cells to proliferate and promoted a significant increase in the production of the Th1 cytokine IFN- $\gamma$ .

IL-12 is an important type 1 immune activation cytokine (21, 22). IL-12 is critical for the immune response to tumors (21), intracellular parasites, fungi, bacteria, and viruses (22, 23) and is produced by macrophages and DCs. These cells release IL-12p40 which is a major subunit of the biologically active IL-12p70. IL-12 primarily regulates Th1 cell differentiation while suppressing the expansion of Th2 cell clones. An increase in the levels of IL-12p40 secreted by the DCs was observed post-treatment with Thymax, which serves as a marker of DC maturity and Th1 induction (24). Thymax was able to activate the DCs to secrete another cytokine, IL-6, which has also been suggested to be a pivotal pro-inflammatory cytokine during acute infection (25).

It is of interest to note that Thymax treatment resulted in the down-regulation of IL-10, which is a regulatory cytokine that dampens the immune response in humans. IL-10 inhibits IFN- $\gamma$  production and Ag-specific proliferation of Th1 (26). IL-10 is also a potent inhibitor of IL-12 synthesis from activated human mononuclear cells (27) and it has been suggested that high IL-10 production levels in *Mycobacterium tuberculosis*-infected macrophages may cause the inhibition of IL-12 expression (9). Therefore, the up-regulation of IFN- $\gamma$  and IL-12 may be attributed to the ability of Thymax to cause down-regulation of IL-10.

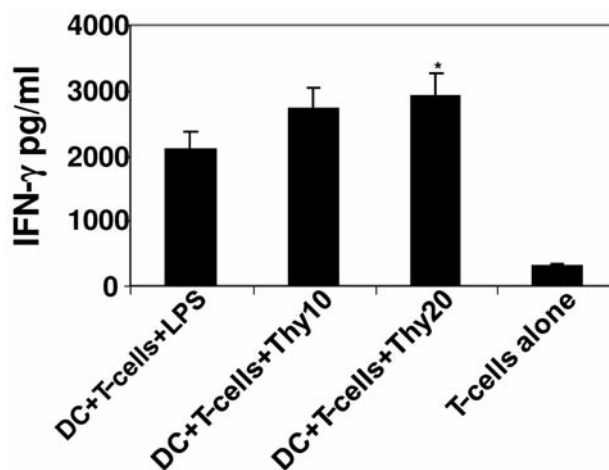


Figure 4. IFN- $\gamma$  secretion in Thymax primed DC-induced T-cells. DCs were stimulated with LPS (1  $\mu\text{g/ml}$ ) or Thymax (10 or 20  $\mu\text{g/ml}$ ) for 24 h. After washing, DCs were cultured with CFSE-labeled T-cells for 5 days. The secretion of IFN- $\gamma$  in the supernatants was assessed using specific ELISA. One representative donor is shown, and the data are the mean  $\pm$  SD from 3 individual experiments; \* $p < 0.05$ , as compared to control LPS treated cells.

Our recent study showed that Thymax induced apoptosis in human breast cancer cells *in vitro* (12). The induction of apoptosis was associated with disruption of the mitochondrial membrane potential (MMP), activation of caspase-9 and a decrease in the Bcl-2:Bax ratio (12). Furthermore, we recently found that Thymax treatment *in vivo* caused an activation of DC maturation (in press). Taken together with the present results showing that Thymax treatment *in vitro* is able to cause maturation of DCs and activation of T-cells, Thymax deserves further study as a potent antineoplastic agent.

## Conclusion

Thymax induces human DC maturation *in vitro*, as revealed by up-regulation of the surface expression of co-stimulatory molecules and IL-12 production. Thymax may thus represent a new class of adjuvants for the activation of human DCs.

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