

## ACTIVATION OF HUMAN MONOCYTE-DERIVED DENDRITIC CELLS *IN VITRO* BY THE BIOLOGICAL RESPONSE MODIFIER ARABINOXYLAN RICE BRAN (MGN-3/BIOBRAN)

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Arabinoxylan rice bran (MGN-3/Biobran) is a potent biological response modifier (BRM) that activates natural killer (NK) cells, T cells and monocytes. Currently, little is known regarding the effects of MGN-3 on dendritic cells (DCs), the cell type that bridges innate and adaptive immunity. Therefore, we examined the stimulatory effects of MGN-3 on DCs. Human monocyte-derived DCs were treated with MGN-3 at different concentrations (5-20 µg/ml) for 24 hours *in vitro*. Activation of DCs was determined by assessing the expression of co-stimulatory and maturation markers (CD40, CD80, CD83, CD86 and HLA-DR) by flow cytometry, and production of cytokines by ELISA. DC function was determined by assessing their ability to activate naïve T cells. Activation of T cells was assessed by measuring cell proliferation and cytokine production. MGN-3 treatment, in a dose-dependent manner, resulted in: 1) up-regulation of the surface expression of CD83 and CD86, on DCs; 2) an increase in the production of pro-inflammatory and immuno-regulatory cytokines (IL-1β, IL-6, IL-10, TNF-α, IL-12p40 and low levels of IL-12p70 and IL-2) by DCs; and 3) MGN-3 stimulated DC induced CD4<sup>+</sup>T cell proliferation and their production of cytokines, IFN-γ, IL-10, IL-17. Results suggest that MGN-3 functions as a natural adjuvant for DC activation and thus may be used in DC-based vaccine strategies against infections and cancer.

Rice bran was obtained from milling rice. It is composed of pericarp and seed coat together as a major portion of the aleurone layer, a part of the endosperm, and some part of the embryo or germ. Accordingly, several health benefits have been identified with rice bran. It is a rich source of proteins, lipids, vitamins, and trace minerals (1), and is well-known as a great source of dietary fiber (2). Earlier studies have shown that rice bran lowers serum cholesterol, and additionally reduces

the postprandial lipemia in humans (3-4). It is of great interest to note that rice bran derivatives possess anti-cancer activity, *in vivo* and *in vitro*. Recent studies by our laboratory and that of others have demonstrated the anti-cancer activity of rice bran derivatives. Arabinoxylan rice bran (MGN-3/Biobran) exerts *in vivo* tumor inhibitory effects against Ehrlich carcinoma-bearing mice (5-6), and rice bran polyphenol, cycloartenyl ferulate, induces apoptosis in human colorectal adenocarcinoma *in*

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*vitro* (7).

Recently, the immune modulatory effects of rice bran have emerged as a focus of research. Our work, and that of others, has shown that MGN-3/Biobran and other rice bran-derived flavonoids exert anti-oxidant and radical-scavenging activities (5, 8-9). MGN-3 is obtained by reacting rice bran hemicellulose with multiple carbohydrate hydrolyzing enzymes from shiitake mushrooms. The main chemical structure of MGN-3 is arabinoxylan, with a xylose in its main chain and an arabinose polymer in its side chain (10). The anticancer activity of MGN-3 was attributed to its ability to activate natural killer (NK) cells (11-13) and T cells (10), and to inhibit T regulatory lymphocyte (T reg) generation in patients with locally limited or metastatic solid tumors (14). In addition, recent studies have indicated that MGN-3 could potentially activate dendritic cells (15).

Dendritic cells (DCs) are the most influential and professional antigen presenting cells (APCs). Recent studies reported evidence of DC-based cancer immunotherapy (16). DCs are the bridge between innate and adaptive immunity because of their unique ability to sense pathogens and initiate immune response. These cells detect and respond to pathogens through Toll-like receptors (TLRs) and Nod-like receptors (17), in addition to the recently discovered C-type lectin receptor family that has also emerged as a major sensor of pathogens. In particular, C-type lectins recognize carbohydrate moieties on bacteria and fungi (18-19). Exposure of DCs to ligands or agonist of all these pattern recognition receptors (PRRs) results in production of cytokines, which modulate the type of T helper cell response and functions (16, 20). Upon sensing the specific type of pathogen, DCs instruct CD4<sup>+</sup>T cells to differentiate into a variety of effector and regulatory subsets, including classical Th1 cells and Th2 cells, follicular helper T cells, induced regulatory T cells, and the more recently defined Th17 cells (20). The type of the cytokines produced by DCs in response to various ligands dictates the type of T-helper cell responses (21-22). The present study was undertaken to examine the ability of MGN-3 to activate DCs in respect to the type of cytokines secreted and the type of T cell helper cells generated by MGN-3-activated DCs.

## MATERIALS AND METHODS

### *Antibodies and reagents*

The following anti-human antibodies were used: CD11c APC (Clone B-ly6), CD40 PE (clone 5C3), CD80 PE (Clone L307.4), CD83 PE (Clone HB15e), CD86 PE [Clone 2331 (FUN-1)], HLADR PerCP [Clone L243 (G46-6)], CD4 PerCP (Clone SK3) and CD45RA APC (Clone HI100), CD25 FITC (Clone M-A251) FoxP3 PE (Clone 259D/C7), CD127 Alexa647 (Clone hIL-7R-M21) all from BD Biosciences (San Jose, California). *E. coli* LPS was purchased from InvivoGen (San Diego, California). An isotype antibody was used as a negative control (BD Biosciences, San Jose, California). FACS analysis-flow cytometry was performed using FACScalibur (Becton-Dickenson, San Jose, California), and analyzed using Flowjo software (Tree Star, Inc., Ashland, Oregon, U.S.A.).

### *MGN-3/Biobran*

MGN-3/Biobran is an arabinoxylan extracted from rice bran that is treated enzymatically with an extract from Shiitake mushrooms. MGN-3/Biobran was kindly provided by Daiwa Pharmaceuticals Co. Ltd., Tokyo, Japan.

### *Isolation and culture of monocyte-derived DCs*

Monocyte-derived DCs were prepared essentially as described previously (21-22). Briefly, peripheral blood mononuclear cells (PBMC) from normal healthy donors (approved by the Institutional Review Board (IRB), Charles Drew University) were separated over Ficoll-hypaque density gradient centrifugation. The cells were allowed to adhere to culture plates for 2 h. Non-adherent cells were subsequently removed. The adherent monocytes were cultured for 6 days under a humidified atmosphere of 5% CO<sub>2</sub> at 37°C in RPMI 1640 supplemented with 10% FBS, 1 mM glutamine, 100 U/ml penicillin, 100 µg/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 DCs were collected after 6 days. The purity of the DCs obtained was >95%. DCs were subsequently pulsed with either 1 µg/ml *E. coli* LPS as a positive control, or with MGN-3 (5-20 µg/ml) for 24 h.

### *DC phenotyping*

The expression of cell surface markers was determined by flow cytometry. Briefly, gated CD11c<sup>+</sup>HLA-DR<sup>+</sup>DCs were analyzed for the expression of CD40, CD80, CD83, CD86 and HLADR with the appropriate isotype antibody



supplied by BD Pharmingen (San Diego, California, USA).

#### *Cytokine production by DCs*

Immature DCs were incubated with either LPS (1 µg/ml) or with MGN-3 (5, 10, or 20 µg/ml) for 24 h. The supernatants were collected and stored at -70°C until analyzed. The cytokines TNF-α, IL-1β, IL-6, IL-10, IL-12p40, IL-12p70 (BD Pharmingen) and IL-2 and IL-15 (ebiosciences, San Diego) in the supernatants were measured by specific ELISA kits as per the manufacturer's protocol.

#### *DC-T-cell cultures*

Allogenic CD4<sup>+</sup>T cells were purified by negative selection using a magnetic bead-based kit from Stem Cell Technology, Vancouver, BC, Canada. The purity of the naïve CD4<sup>+</sup>T-cells obtained ranged from 93 to 97% as determined by CD4 and CD45RA staining. CD4<sup>+</sup>T cells were labeled with CFSE dye. Allogenic CD4<sup>+</sup>T cells were cultured with DCs that were stimulated with LPS or MGN-3 (5-20 µg/ml) for 24 h as above. The DC-CD4<sup>+</sup>T were co-cultured for 5 days in U bottom 96 well plate. The ratio DC:CD4<sup>+</sup>T cells was 1:5 ( $2 \times 10^4$ : $1 \times 10^5$ ). Cell death due to CFSE dye was examined by Propidium Iodide (PI) staining and dead cells were excluded from analysis. The proliferation of T-cells was measured by dilution of dye. At the end of 5 days supernatant was collected and kept at -70°C. Cytokines IFN-γ, IL-10, IL-4 and IL-5 were detected using a specific ELISA kit from BD Pharmingen and IL-17 from eBioscience San Diego.

#### *Statistics*

All of the experiments were repeated with samples from 4-7 individual subjects. The probability of the mean values of two experimental groups was tested by the two-tailed *t*-test for paired samples. The level of significance was set at  $p < 0.05$ . For Bar graphs statistical analysis was performed using Graph Pad Prism software.

## RESULTS

#### *DC activation*

DCs were treated with MGN-3 (5, 10 and 20 µg/ml) or LPS (1 µg/ml) or untreated cultures. The level of DC activation was examined under all three conditions. The data depicted in Fig. 1 displays the levels of DC surface co-stimulatory and maturation markers, including CD40, CD80, CD83, CD86 and HLA-DR, post-treatment with MGN-3 or LPS. Flow cytometry analysis revealed that the MGN-3-treated DCs show a significant dose-dependent increase in

the expression of CD83 and CD86. DCs treated with LPS also show the up-regulation of these surface markers.

#### *Cytokine Production by DCs*

Data in Fig. 2 show that MGN-3 induced production of cytokines IL-1β, IL-6, IL-10, TNF-α, IL-12p40, IL-12p70, IL-2 and IL-15. The effect of MGN-3 was significant ( $p < 0.05$ ) as compared to DCs alone. DCs treated with LPS also produced significant levels of IL-6, IL-10, TNF-α, IL-12p40, and IL-12p70 cytokines. Unlike LPS, MGN-3 induced significant levels of IL-1 β in a dose-dependent manner.

#### *DC induced CD4<sup>+</sup>T cell proliferation*

To examine the capacity of the MGN-3 activated DCs to stimulate T cells, we co-cultured DC control or DCs activated with MGN-3 or LPS with alloreactive CD4<sup>+</sup>T cells. Co-culture with DC control resulted in proliferation of allogeneic CD4<sup>+</sup>T cells, whereas MGN-3 boosted DC-induced proliferation. Treatment with MGN-3 at a concentration of 10 µg/ml resulted in an increase in CD4<sup>+</sup>T cell proliferation to 64.6%, as compared with DC-CD4<sup>+</sup>T-cell proliferation alone 45.4%, thus representing a 1.4-fold (73.6%) increase in activity. The magnitude of T cell proliferation induced by MGN-3-activated DCs was similar to that of DC activated with LPS (Fig. 3).

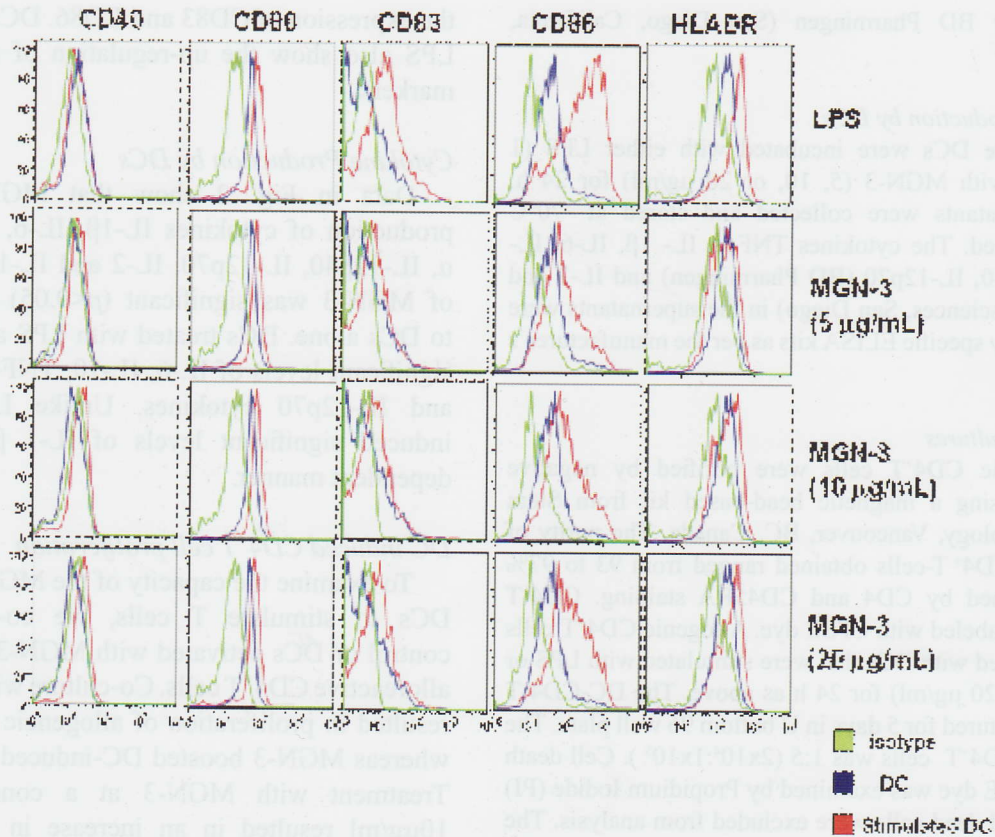
#### *Expression of activation marker, CD25 on CD4<sup>+</sup>T cells*

The data in Fig. 4 show that T cells co-cultured with MGN-3-activated DCs exhibited a significant increase in the expression of CD25 marker. MGN-3 at a concentration of 10 µg/ml caused a 1.3-fold increase in the expression of CD25 as compared to DC-CD4<sup>+</sup>T cells alone. In our current study, we examined T reg generation by MGN-3 stimulated by Intracellular FoxP3 and low CD127 but these CD4<sup>+</sup>T-cell do not exhibit T reg phenotype.

#### *MGN-3 activated DCs induce multiple cytokines (IFN-γ, IL-10 and IL-17) from T cells*

Cytokine secretion by T cells induced by DCs preactivated with or without MGN-3 or LPS was examined. Fig. 5 shows that, as expected, the LPS





**Fig. 1.** Effect of MGN-3 on DC co-stimulatory and maturation markers CD40, CD80, CD83, CD86, and HLADR. Monocyte-derived DCs were treated for 24 h with MGN-3 (5–20 µg/ml), LPS (1 µg/ml) as positive control. Isotype antibody was used as a negative control. Expression of cell surface markers was determined by flow cytometry. One representative experiment is shown from 4 individual experiments.

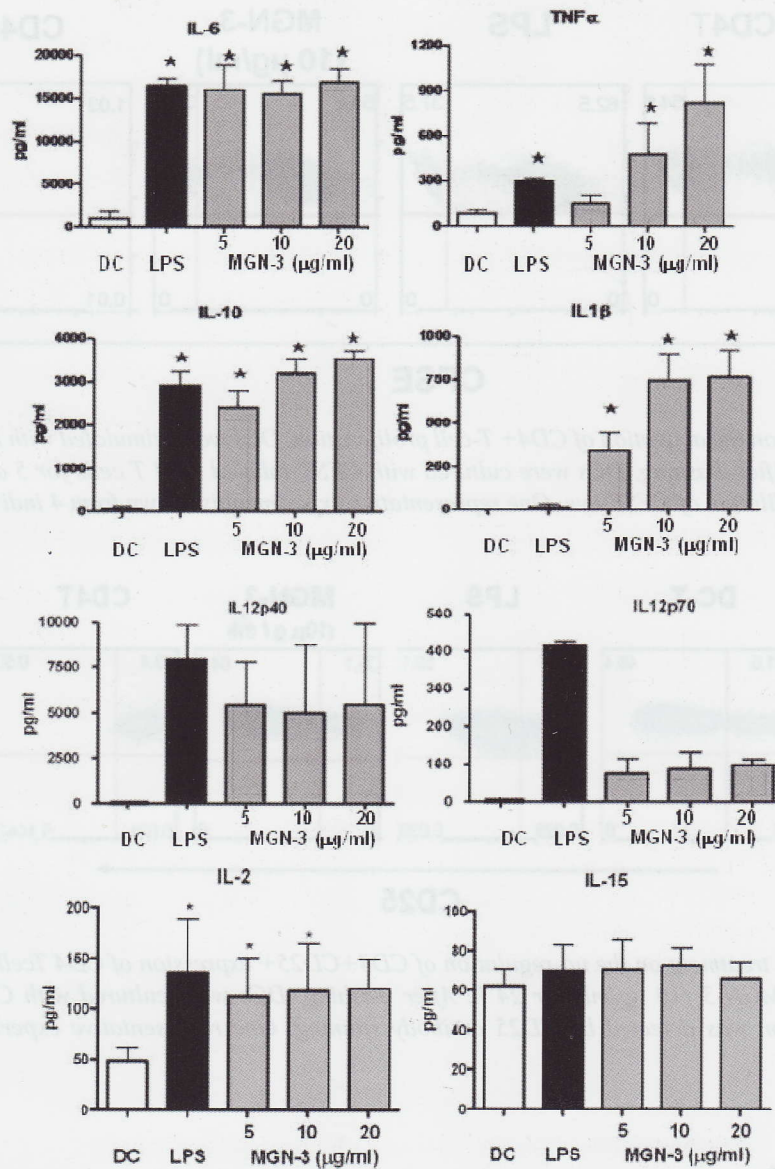
primed DCs displayed approximately a two-fold increase in IFN- $\gamma$ , as compared to unstimulated DC-T cells alone. MGN-3, at a concentration of 10 µg/ml, primed DC-induced secretion of IFN- $\gamma$  from T-cells, resulting in a 1.4-fold increase in IFN- $\gamma$  secretion as compared to control DC-CD4<sup>+</sup>T ( $p < 0.05$ ). Treatment with MGN-3 also resulted in a significant increase in IL-10 secretion (2 fold) over the control DC-CD4<sup>+</sup>T cells. IL-17 is detected in a dose-dependent manner in DC-T cells post treatment with MGN-3. TH2 cytokines IL-4 and IL-5 were not detected by MGN-3- and LPS-stimulated DC-CD4<sup>+</sup>T cells.

## DISCUSSION

The major function of DCs is to present antigens

to naïve T lymphocytes and induce effective immune responses. DC-based vaccines have emerged as a promising strategy for vaccination against infections and in the immunotherapy of cancer. In DC-based vaccines, the activation and maturation state of the DC is of importance for induction of appropriate immune responses. In this study we have established the fact MGN-3, an arabinoxylan rice bran, is a potent activator of DC maturation and function.

Recent work by Cholujoa et al. has shown the ability of MGN-3 to induce maturation of DCs as determined by upregulation of DC maturation marker CD83 and CD86 (15). Our data confirm the upregulation of these maturation markers and further show that MGN-3 triggers production of pro-inflammatory and immuno-regulatory cytokines (IL-1 $\beta$ , IL-6, IL-10, TNF- $\alpha$ , IL-12p40 and low levels



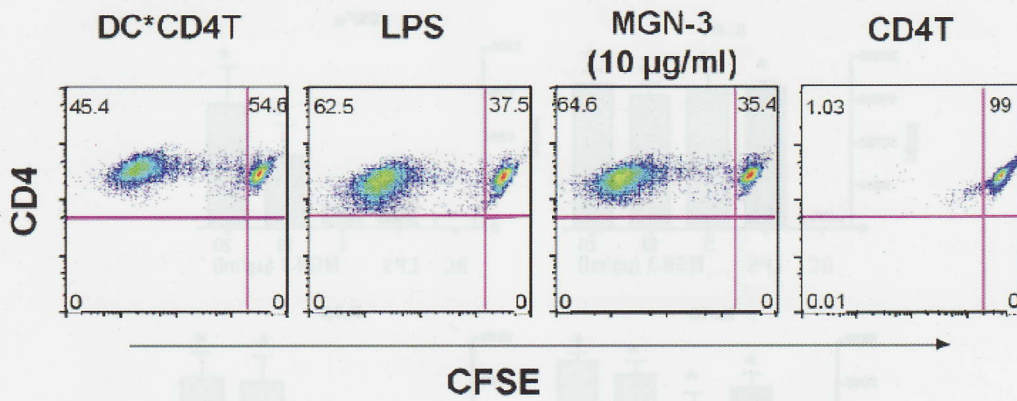
**Fig. 2.** Effect of MGN-3 on cytokine production. Immature DCs were incubated with LPS (1 µg/ml) or MGN-3 (5-20 µg/ml) for 24 h. Supernatants were collected and the levels of IL-6, TNF-α, IL-10, IL-1β, IL-12p40, IL-12p70, IL-2, IL-15 were measured by specific ELISA kits. The data are the mean ± SD from 7 individual experiments; ★*p*<0.05 as compared to DCs alone.

of IL-12p70, IL-2) by DCs. In addition, we show that MGN-3-stimulated DCs are able to expand CD4<sup>+</sup>T-cells to produce IFN-γ, IL-10, and IL-17. Taken together, the results of this study suggest that MGN-3 can be used as an adjuvant in DC-based vaccination strategies.

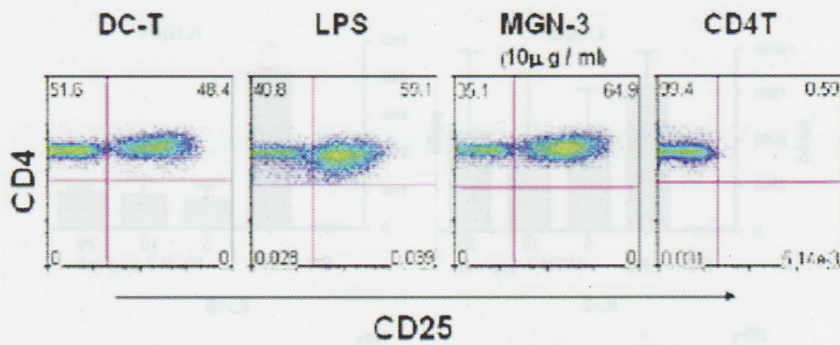
MGN-3-treated DCs elicited the production of

multifunctional cytokines. IL-1β, IL-6, and TNF-α are known as pro-inflammatory cytokines and play a role in phagocytosis of invading pathogens (18-19, 23). Cytokine IL-10 possesses anti-cancer activity and can inhibit the growth of a squamous cell carcinoma (SCC-13) cell line. In addition, it can also decrease tumorigenicity and increase

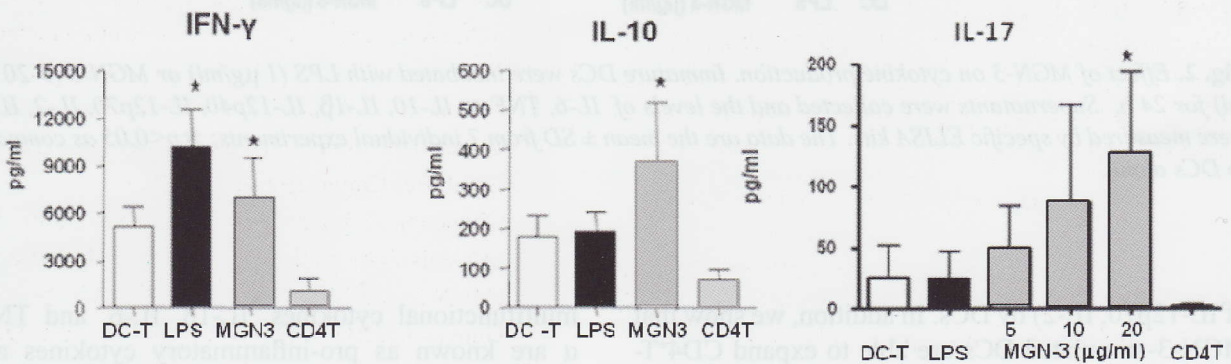




**Fig. 3.** Effect of MGN-3 on the activation of CD4<sup>+</sup> T-cell proliferation. DCs were stimulated with LPS (1 µg/ml) or MGN-3 (10 µg/ml) for 24 h. After washing, DCs were cultured with CFSE-labeled CD4 T cells for 5 days. Proliferation of T cells was measured by dilution of CFSE dye. One representative experiment is shown from 4 individual experiments.



**Fig. 4.** Effect of MGN-3 treatment on the up-regulation of CD4<sup>+</sup>CD25<sup>+</sup> expression of CD4 T cells. DCs were stimulated with LPS (1 µg/ml) or MGN-3 (10 µg/ml) for 24 h. After washing, DCs were cultured with CD4 T cells for 5 days. CD4<sup>+</sup>CD25<sup>+</sup> expression was detected by CD25 antibody staining. One representative experiment is shown from 4 individual experiments.



**Fig. 5.** Cytokine secretion in MGN-3 primed DC-induced T cells. DCs were stimulated with LPS (1 µg/ml) or MGN-3 (10 µg/ml) for 24 h. After washing, DCs were cultured with CFSE-labeled T cells for 5 days. The secretion of IFN-γ, IL-10, and IL-17 in the supernatants was assessed using specific ELISA kits. The data are the mean ± SD from 4 individual experiments; \*p < 0.05, as compared to control DCs.



antigenicity of tumor cells (24-27). The significantly increased production of these cytokines by DC post treatment with MGN-3 suggests its potential for use as an adjuvant in anticancer and infectious disease vaccines. Furthermore, adjuvants that expand T cells in sufficient quantity and quality are of value in inducing protective immunity. In this study we showed an increased production of cytokines IL-2, IL-12p40 and IL-12p70 post treatment with MGN-3. DC-derived IL-2 is primarily involved in survival and expansion of T cells, whereas IL-12p40 and IL-12p70 are mainly involved in driving IFN- $\gamma$  secreting CD4<sup>+</sup>T cells.

IFN- $\gamma$  has been found to exert antitumor activity (28-30). We have previously shown that MGN-3 treatment caused a significant reduction in tumor volume in mice that was associated with increased levels of IFN- $\gamma$  (5). In cancer patients, MGN-3 inhibits T regulatory cell formation and promotes T helper (TH) production with a statistically significant increase in the ratio of TH/T reg cells (14). In our current study, we examined whether or not MGN-3 stimulated DC to induce T reg (FoxP3<sup>+</sup> and low CD127<sup>+</sup>) cells. We did not detect T regs (data not shown).

Two alternative mechanisms can be proposed to account for the observed stimulatory effects of MGN-3 on DCs. First, it is possible that MGN-3 binds to the cell surface via pattern recognition receptors (TLRs and/or C type lectins) and triggers signaling pathways involved in cell activation and cytokine production. Alternatively, MGN-3 could bind intracellular receptors (NLRP3 inflammasome) and signal cell activation pathways. These two possibilities are not mutually exclusive. Although the exact mechanism has not been established, our results suggest that enhancing DC function with MGN-3 should be beneficial for DC-based vaccine improvement.

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