

MGN-3/BIOBRAN ENHANCES GENERATION OF CYTOTOXIC CD8+ T CELLS VIA UPREGULATION OF DEC-205 EXPRESSION ON DENDRITIC CELLS

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Arabinoxylan rice bran (MGN-3/Biobran) has been shown to be a potent biological response modifier (BRM) that activates different arms of the immune system, including dendritic cells (DCs), which prime CD4+ helper T-cell responses. The present study explores the ability of MGN-3-activated DCs to prime CD8+ T cells and examines the mechanisms underlying its effect. Human monocyte-derived DCs were treated with MGN-3 (20 and 40 µg/ml). Results indicate that treatment with MGN-3 caused DCs to prime higher granzyme B-expressing CD8+ T cells. Tumor lysate-pulsed MGN-3 DC also increased tumor cell killing compared to DC-stimulated CD8+ T cells. This was associated with: i) increased expression of DEC-205 in MGN-3-activated DCs in a dose-dependent manner; and ii) MGN-3 induced significant production of Type III interferon, IL29, but not Type I IFNs α and β . These results suggest that MGN-3 is a potent natural adjuvant that efficiently activates DCs and may therefore be useful for mounting an efficient immune response against infections and cancer.

The anti-cancer effects of dietary rice bran derivatives have been examined by our laboratory and others. Arabinoxylan rice bran (MGN-3/Biobran) has been shown to exert tumor-inhibitory effects in animal models (1, 2) and in clinical trials in patients with hepatocellular carcinoma (3). Other rice bran derivatives have also been found to suppress the growth of tumor in animal models (4). The mechanisms by which rice bran-derived bioactive components exert anti-cancer effects include apoptosis induction (5, 6), anti-oxidant and radical-scavenging activity (7), inhibition of cell proliferation (8, 9), and alteration of the cell cycle (5). In addition, the immunomodulatory effects of rice bran derivatives have a profound effect on different immune cells, such as T and B cells (10, 11), NK cells (12-14), and dendritic cells (DC) (15, 16).

DCs are one of the major cells involved in generating anti-tumor immune response. Mature DCs are able to migrate to lymphoid organs, to present antigens to naive T cells, and to efficiently mount adaptive immune responses. Numerous studies in animal models and various clinical trials are underway for DCs based immunotherapy (17). Several DC-related receptors known as pattern recognition receptors (PRRs) have emerged as major sensors of pathogens (18-20). Exposure of DCs to ligands or agonists of all these pattern recognition receptors (PRRs) results in the activation and production of cytokines, which leads to activation of the type of CD4+ T cell. Upon recognition of the specific type of pathogen, DCs instruct CD4+ T cells to differentiate into one of several types of effector and regulatory cells. Due to these inherent

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properties, PRRs have been exploited as immune activating agents (17, 21).

Scientists are trying to find better immune-activating agents, such as biological response modifiers (BRMs), to fight against infection and cancer. Treatment with the BRM MGN-3 has been shown to cause activation and maturation of DCs, to produce several cytokines, and to activate DCs in priming CD4⁺ T cells to proliferate and secrete cytokines (15, 16). By contrast, the objective of the present study is to examine the role of MGN-3-stimulated DCs on the generation of CD8⁺ cytotoxic T lymphocytes (CTL) and the mechanisms underlying its effect. We find that MGN-3 activates DCs to prime higher granzyme B-expressing CD8⁺ cells. Furthermore, tumor lysate-pulsed MGN-3-activated DCs stimulate CD8⁺ T cells, demonstrated increased killing of tumor cells. These findings may be associated with an increased expression of DEC-205 and type III IFN production. This suggests 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.

MATERIALS AND METHODS

Antibodies and reagents

The following anti-human antibodies were used: DEC-205, Alexa647, 7-AAD, granzyme B, CD8⁺ PerCP, with respective isotype antibodies as negative control, all from BD Biosciences (San Jose, California, USA). *E. coli* LPS was purchased from InvivoGen (San Diego, California, USA). CFSE dye was purchased from Invitrogen (Carlsbad, California, USA), FACS analysis-flow cytometry was performed using FACScalibur (Becton-Dickenson, San Jose, California, USA) and analyzed using Flowjo software (Tree Star, Inc., Ashland, Oregon, USA). Human granulocyte macrophage/recombinant human IL-4 was purchased from PeproTech (Rocky Hill, New Jersey, USA). A negative selection kit was purchased from STEMCELL Technologies Inc. (Vancouver, Canada).

MGN-3/Biobran

Arabinoxylan from rice bran (MGN-3/Biobran) 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 (11). MGN-3 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 (15, 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% CO₂ at 37°C in serum-free medium (AIM-V), human granulocyte macrophage/recombinant human IL-4. 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 100 ng/ml *E. coli* LPS as a positive control, or with MGN-3 (20 and 40 µg/ml) for 24 h.

DC phenotyping

The expression of cell surface markers was determined by flow cytometry. Briefly, gated CD11c+HLA-DR+DCs were analyzed for the expression of DEC-205 with the appropriate isotype antibody.

Cytokine production by DCs

Immature DCs were incubated with either LPS (100 ng/ml) or with MGN-3 (20, 40 µg/ml) for 24 h. The supernatants were collected and stored at -70°C until analyzed. The interferons Type III (IL29) from PBL and Type I IFNs α and β from RnDsystems were measured by ELISA kit as per manufacturer's protocol.

DC-CD8⁺ T cell cultures for granzyme B induction

Immature DCs were stimulated with LPS and MGN-3 (at concentration of 20µg/ml) as described above. Twenty-four hours later, cells were collected and washed. After washing, 2×10⁴ DCs were cultured with 1×10⁵ purified, allogeneic CD8⁺ T cells. Seven days later, cells were collected and surface-stained for CD8 PerCP. After fixing with 4% paraformaldehyde for 15 min at 37°C, the cells were washed and permeabilized (BD perm buffer). The cells were then stained with antibodies to granzyme B, and appropriate isotype controls (BD Biosciences, San Jose, California). Gated CD8⁺ T cells were analyzed for presence of granzyme B.

DC-CD8T⁺ cell cultures for tumor cell lysis

DCs (1×10⁶) were stimulated with MGN-3 and tumor lysate of PC3 (prostate cancer cells, American Type Culture Collection, Manassas, Virginia). After 24 h, cells were collected and washed. 2×10⁴ DCs were cultured with 1×10⁵ purified, allogeneic CD8⁺ T cells along with

tumor lysate for 7 days. The PC3 specific allogenic CD8+ T cells were generated by pulsing PMBCs with tumor lysate of PC3 for 10 days. Subsequently, CD8+ T cells were purified from the cultures by negative selection using magnetic beads. Purified CD8+ T cells (effectors) were incubated with CFSE-labeled PC3 cells (targets) at target:effector (T:E) ratio of 1:50 in a volume of 200 μ l. Labeling with cell tracking dye CFSE separates the targets from the effectors. Four hours later 5 μ l of 7-AAD was added to the cells to stain for dead cells. 7-AAD only enters the membrane of compromised cells and binds to DNA. Cells were acquired on FACS Calibur 10 min after adding 7-AAD. 10,000 CFSE-positive cells were collected per condition. Controls included CFSE-stained PC3 cells without effectors and 7-AAD- and CFSE-stained PC3 cells. Analysis was performed by gating on the target cells and measuring the 7-AAD-negative vs 7-AAD-positive cells. Cells positive for both 7-AAD and CFSE were considered lysed. Percentage of cytotoxicity was calculated by the following equation: (7-AAD-positive and CFSE-positive cells/total number of CFSE-positive cells) \times 100.

Statistics

All of the experiments were repeated with samples from 4-6 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

DEC-205 Expression

DCs were either treated with MGN-3 (20 and 40 μ g/ml) or LPS (100 ng/ml) or left untreated. The level of DC activation was examined under all three conditions. The data depicted in Fig. 1 displays the DEC-205 expression. Flow cytometry analysis revealed increased expression of DEC-205 in MGN-3-activated DCs in a dose-dependent manner.

MGN-3 activated DCs secrete Type I and III IFNs

Fig. 2 shows Type I and III IFN secretion by DCs post-treatment with MGN-3 at concentration of 20 μ g/ml. Fig. 2A shows that MGN-3 induces DCs, causing a significant increase in production of Type III interferon (IL29). The effect of MGN-3 was significant ($p < 0.05$) as compared to DCs alone. MGN-3 treatment also resulted in an increased production of the Type I IFNs α (Fig. 2B) and β (Fig. 2C). However, this increase was not statistically significant.

MGN-3 induces granzyme-expressing cytotoxic CD8+ T cells

DCs also induce the generation of cytotoxic CD8+

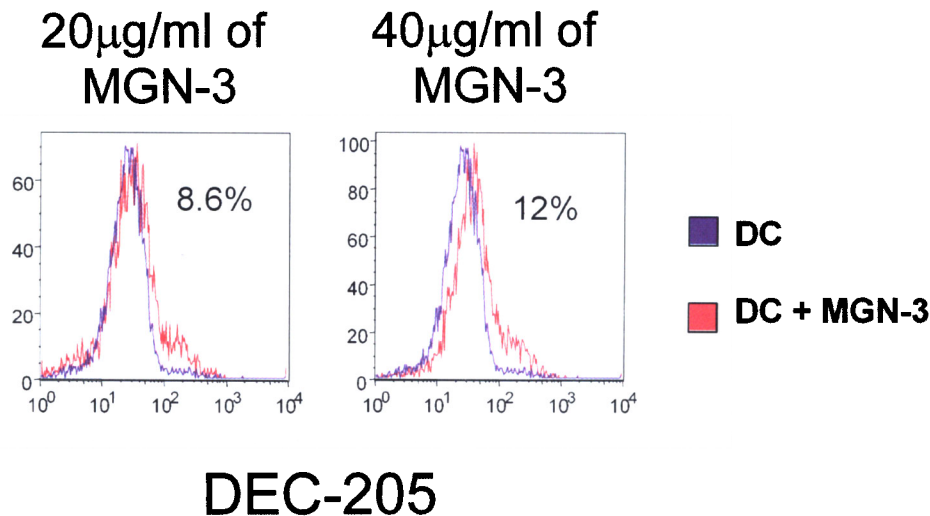


Fig. 1. MGN-3 activates DCs to induce DEC-205. Histogram depicts the percent positive DEC-205 expression in dose-dependent manner of MGN-3 (20 and 40 μ g/ml).

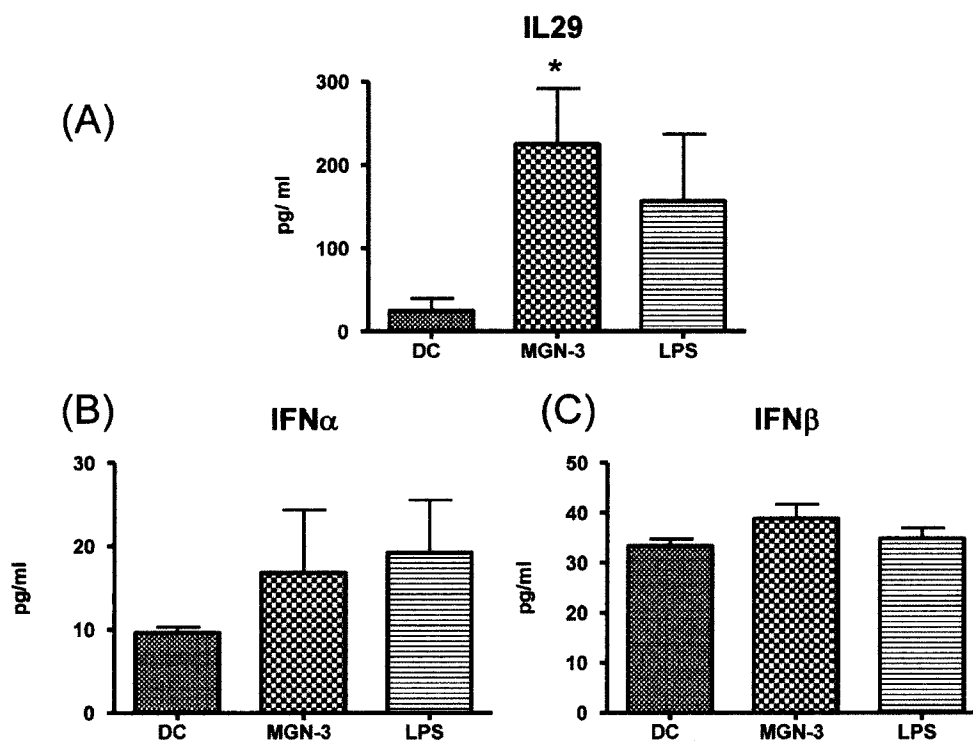


Fig. 2. MGN-3 (at concentration of 20 μ g/ml) activates DCs to induce a distinct profile of Type I and III IFN cytokine secretion. DCs were activated with MGN-3 and LPS for 48 h. Bar graphs depict the pg/ml level of (A) IL-29 (Type III IFNs), (B) IFN α , and (C) IFN β . Data represent the mean \pm S.E. of 6 experiments. *($p < 0.05$) as compared to DCs alone.

T cell responses. To explore this, we determined the effect of MGN-3-stimulated DCs on CD8 $^+$ T cells. DCs cultured for 24 h with MGN-3 (at concentration of 20 mg/ml) were washed and cultured with purified CD8 $^+$ T cells. Seven days later the cells were collected and stained for intracellular granzyme B. DCs stimulated with MGN-3 induced significantly higher levels of granzyme B-positive CD8 $^+$ T cells ($p < 0.05$) as compared to unstimulated DC-CD8 $^+$ T cells (Fig. 3A and B). These data suggest that stimulation of DCs through MGN-3 is highly effective in priming cytotoxic T-cell responses.

MGN-3-stimulated DCs prime CD8 $^+$ T cells with higher cytolytic activity

Granzyme-expressing CD8 $^+$ T cells are able to kill tumor cells. PC3 cells were used as tumor target cells. PC3-specific CD8 $^+$ T cells were generated as described in methods. To determine the killing or lysis, purified CD8 $^+$ T cells (effectors) were co-

cultured with CFSE-labeled PC3 cells as targets. Target:effector (T:E) ratios were 1:25 and 1:50. Four hours later 7-AAD was added to the cells to stain dead cells. Controls included CFSE-stained PC3 cells without effectors and 7AAD- and CFSE-stained PC3 cells. Analysis was performed by gating on the target cells and measuring the 7AAD-negative vs 7AAD-positive cells. Cells positive for both 7-AAD and CFSE were considered lysed.

Fig. 4A shows flow cytometry studies revealing the percent specific lysis of PC3s from one representative experiment. MGN-3 (at concentration of 20 μ g/ml), as well as tumor lysate pulsed MGN-3 stimulated DCs, primed the CD8 $^+$ T cells with higher cytolytic activity (~38% lysis and ~41% lysis, respectively) as compared to unstimulated DCs primed CD8 $^+$ T cells (~35% lysis). A similar trend was also observed at the lower T:E ratio of 1:25, though to a lesser extent (data not shown).

Fig. 4B shows results (average with \pm S.E)

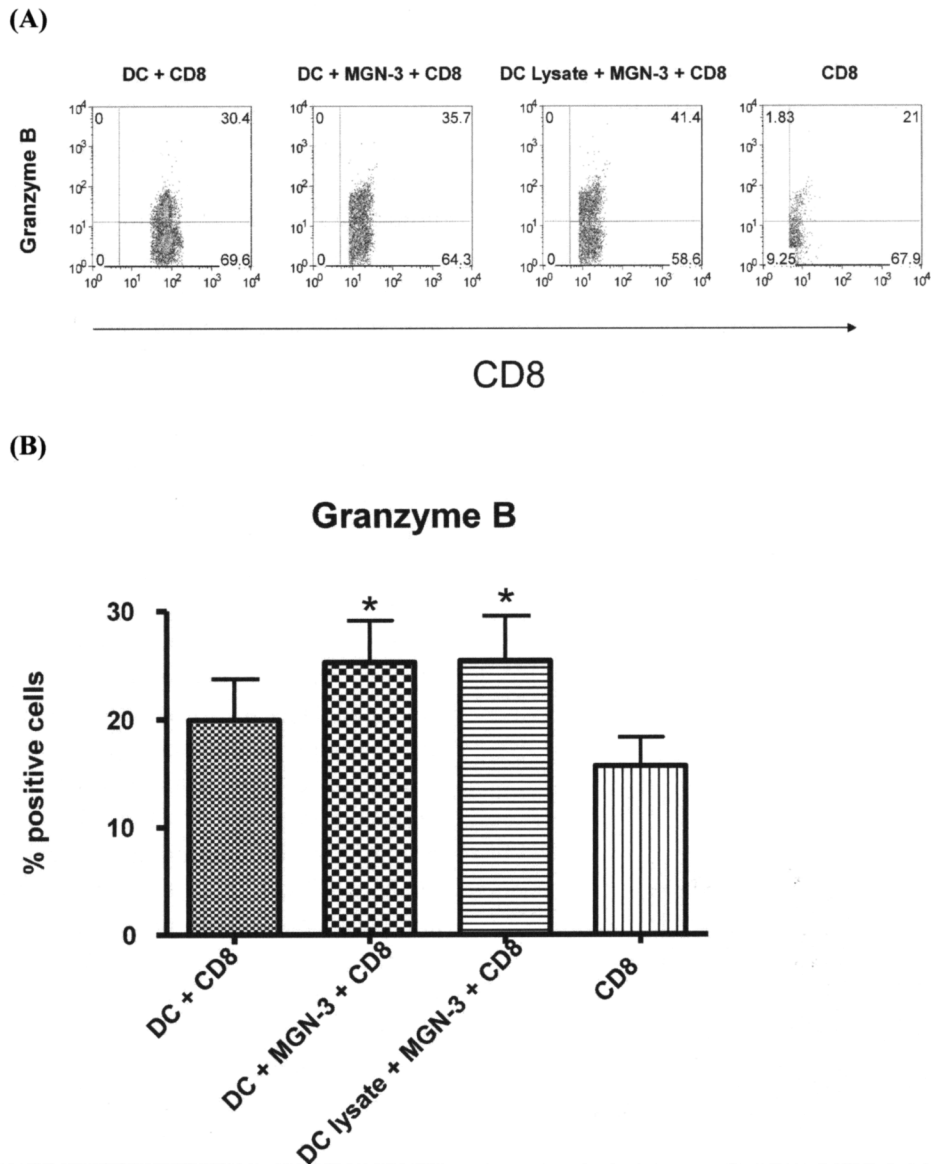


Fig. 3. MGN-3 (at concentration of 20 $\mu\text{g/ml}$) induces granzyme expressing cytotoxic CD8⁺ T cells. DCs activated with MGN-3 and tumor cell lysate and MGN-3 co-cultured with CD8⁺ T cells for 7 days. **A)** Granzyme B induction was determined by flow cytometry. **B)** Bar graphs depict the percentage of CD8⁺ T cells expressing granzyme B. Data represent the mean \pm S.E. of 5 experiments. *($p < 0.005$) as compared to unstimulated DC-CD8⁺ T cells.

from 5 experiments. Unstimulated DCs primed CD8⁺ T cells as well as CD8⁺ T cells alone show ~24% and ~18% lysis, while MGN-3 stimulated DCs with ~40% lysis and tumor lysate pulsed MGN-3 stimulated DCs with ~44% lysis ($p < 0.05$

as compared with unstimulated DC-CD8⁺ T cells). These data clearly suggest that stimulation of DCs through MGN-3 induces highly cytolytic CD8⁺ T cells. Tumor lysate-pulsed MGN-3-activated DCs stimulated CD8⁺ cell killing of tumor cells at an

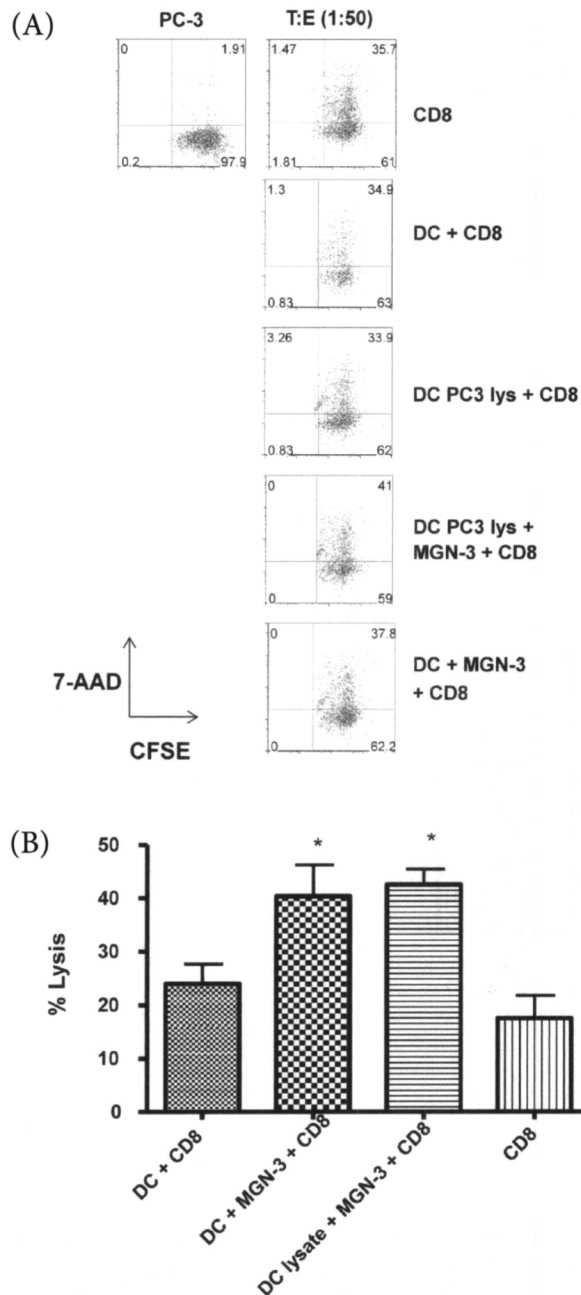


Fig. 4. MGN-3 stimulation primes higher cytotoxic CD8+ T cells. DCs activated with MGN-3 (at concentration of 20 $\mu\text{g/ml}$) and tumor cell lysate plus MGN-3 co-cultured with CD8+ T cells for 7 days. Purified CD8+ T cells (effectors) were co-cultured with CFSE labeled PC3 cells (target) at target:effector ratio of 1:50. Cells positive for both 7-AAD and CFSE were considered lysed. Flow cytometry studies show the percent specific lysis of PC3. Data from one representative experiment is shown in (A) and \pm S.E. of 5 experiments in (B). * $(p < 0.05)$ as compared with unstimulated DC-CD8+ T cells.

even higher rate.

DISCUSSION

Earlier studies by our group and others have demonstrated the ability of arabinoxylan rice bran, MGN-3/Biobran, to induce maturation and activation of DCs. Initial studies showed that MGN-3 induced upregulation of DC costimulatory markers and production of pro-inflammatory and immunoregulatory cytokines (15, 16). MGN-3-stimulated DCs induce the proliferation of CD4+ T cells and the production of cytokines IFN- γ , IL-10, and IL-17 (15). However, there is limited information regarding the pattern recognition receptors involved in MGN-3 recognition. The present study was designed to examine the role of MGN-3-stimulated DCs on CD8+ cells and CTLs generation.

The results of the current study revealed an increase in the expression of DEC-205 in MGN-3-activated DCs in a dose-dependent manner. DEC-205 belongs to the macrophage mannose receptor family of C-type lectin endocytic receptors (23). DEC-205 is a type I cell surface protein expressed primarily by DCs upregulated during the maturation of DCs (22, 24, 25). It has endocytic capacity (23) and facilitates antigen presentation by DCs (26).

In this study, we also showed increased Type III IFN secretion by DCs. Type III IFNs, or IFN- λ s, also known as IL-28/29, are important regulators of the skin's defense system. They play an important role in clearing viral and microbial infections and in fighting against tumors (27). They are primarily expressed on epithelium-like tissues, in contrast with Type I IFN receptors, which are expressed on most cell types. Both Type I and Type III IFNs share similar expression and biological activities, but they may play distinct roles in antiviral and anti-cancer response (28, 29). For example, Agrawal showed that cell signaling by Type III IFNs may be a more effective antiviral responses due to enhanced production and prolonged action in the mucosal compartments that are exposed to the outside (30). Type III IFN has been shown to be a potent effector of the immune system in stimulating the cytotoxic effects of CD8+ T cells to kill cancer cells (31, 32).

Results of this study showed that MGN-3-activated DCs primed CD8+ T cells to express a

greater granzyme B level, and tumor lysate-pulsed MGN-3-activated DCs stimulated CD8⁺ cells to exert higher tumor-cell-killing activity. These results were associated with an increased level of DEC-205 expression. Several reports indicate the critical roles of DEC-205 and CD8⁺ T cell responses against cancer and viruses (33). DEC-205 is important to the uptake of extracellular proteins. After binding to DEC-205, proteins are internalized, processed, and presented in a complex with MHC II. Cognate T cells are stimulated to proliferate (34). The expression of DEC-205 is positively correlated with that of CD8⁺ T cell responses (33).

In conclusion, data of the current study suggest that MGN-3 functions as a natural adjuvant for DC activation. Future research might investigate the potential use of MGN-3 as a useful antiviral agent that enhances mucosal responses via IL-29 secretion, DEC-205 activation, and CTL generation, and thus may be used in DC-based vaccine strategies against infections and cancer.

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