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Myosin IIa activation is crucial in breast cancer derived galectin-1 mediated tolerogenic dendritic cell differentiation

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ABSTRACT

Background: Tolerogenic dendritic cells (tDCs) play important roles in immune tolerance, autoimmune disease, tissue transplantation, and the tumor micro-environment. Factors that induce tDCs have been reported, however the intracellular mechanisms involved are rarely discussed.
Methods: Circulating $CD14^+CD16^+$ of breast cancer patients and induced $CD14^+CD16^+$ DCs were identified as tDCs by treating $CD14^+$ monocytes with galectin-1 and cancer cell-derived medium combined with IL-4 and GM-CSF. In addition, the 4T1 breast cancer syngeneic xenograft model was used to investigate the effect of galectin-1 in vivo.
Results: The $CD14^+CD16^+$ tDC population in the breast cancer patients was comparatively higher than that in the healthy donors, and both the MDA-MB-231 conditioned medium and galectin-1 could induce tDC differentiation. In a BALB/c animal model, the 4T1 breast cancer cell line enhanced IL-10 expression in $CD11c^+$ DCs which was down-regulated after knocking down the galectin-1 expression of 4T1 cells. Analysis of galectin-1 interacting proteins showed that myosin IIa was a major target of galectin-1 after internalization through a caveolin-dependent endocytosis. Myosin IIa specific inhibitor could diminish the effects of galectin-1 on monocyte-derived tDCs and also block the 4T1 cell induced $CD11c^+/Ly6G^+/IL\text{-}10^+$ in the BALB/c mice.
Conclusions: Galectin-1 can induce tDCs after internalizing into $CD14^+$ monocytes through the caveolae-dependent pathway and activating myosin IIa. For the breast cancer patients with a high galectin-1 expression, blebbistatin and genistein show potential in immune modulation and cancer immunotherapy.
General significance: Myosin IIa activation and galectin-1 endocytosis are important in tumor associated tDC development.

1. Introduction

Tolerogenic dendritic cells (tDCs) are very important in many biological functions and diseases. They secrete IL-10 but not IL-12, and drive the development of IL-10-producing regulatory T (Treg) cells [1]. One group of tDCs is also called DC-10 which may be found in the peripheral blood [2,3]. They are important for the regulation of hypersensitivity and autoimmune diseases [4,5].

Galectin-1 reportedly has immunosuppressive properties by inducing T cell apoptosis via binding to CD45RA/RO [6]. Previous research has demonstrated that it can enhance IL-10 expression in monocytes [7]. Galectin-1 belongs to a specialized family of lectins that contains one or more galactose carbohydrate recognition domain (CRD) and is widely expressed at inflammation and tumor growth sites and controls a diverse number of immune cell processes. High levels promote angiogenesis, cancer cell migration, and metastasis, and have been described in lung, breast, pancreatic, colorectal, and cervical cancers [7–13]. Many researches have also shown that it is a major effector in cancer progression and migration [14,15]. Recently, its importance has been noted in macrophage activation and DC migration [16,17]. Although its effects on myeloid lineage have been described, the mechanism is unclear.

Cytoskeleton molecules have recently been demonstrated to be involved in the biological function of myeloid linage, including CD36

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clustering and JNK pathway activation, and may be blocked by the myosin IIa specific inhibitor, blebbistatin [18]. Myosin IIa is composed of two regulatory light chains and two heavy chains encoded from the MYH9 gene. The major functions of myosin IIa include the actomyosin contraction of cells and the regulation of migration and morphology. Mutation of the MYH9 gene causes macro-thrombocytopenia and other diseases, including the loss of epithelial structures in the inner ear, lens, and kidneys that leads to hearing loss, cataracts, and nephritis [19–22]. The present study investigated the interaction of galectin-1 with myosin IIa and the effect of galectin-1 on tDC differentiation.

2. Materials and methods

2.1. Examination of tolerogenic DC

Nineteen breast cancer patients and 10 healthy donors were included in this study. Serum was separated by centrifugation and stored at −80 °C. The human peripheral blood from healthy adult volunteers was collected in syringes containing 1000 U/mL of preservative-free heparin. The peripheral blood monocytic cells (PBMCs) were isolated by density centrifugation using Ficoll–Hypaque and re-suspend in PBS and stained with FITC conjugated anti-CD16 monoclonal antibody and APC-Cy7 conjugated anti-CD14 monoclonal antibody (BD Pharmingen). The percentage of $CD14^+CD16^+$ was determined by FACS array flow cytometry (BD Pharmingen). The Institutional Review Board of Kaohsiung Medical University Hospital approved this study and all participants provided written informed consent in accordance with the Declaration of Helsinki.

2.2. Monocyte isolation and differentiation

Mononuclear cells were isolated from the blood by the Ficoll–Hypaque gradient (GE Healthcare Bio-Sciences, Little Chalfont, UK) from healthy donors. $CD14^+$ monocytes were purified using $CD14^+$ mAb-conjugated magnetic beads (MACS MicroBeads; Miltenyi Biotec) according to the manufacturer's protocol. The control group of MdDCs was generated by culturing $CD14^+$ monocytes in RPMI 1640 medium containing 10% FBS (Invitrogen, Carlsbad, CA) and 20 ng/mL GM-CSF and 10 ng/mL IL-4 (R&D Systems, Minneapolis, MN) for 5 days. In the galectin-1, MDA-MB-231 and MCF-7 groups, an extra 1 μg/mL of galectin-1 (ProSpec, East Brunswick, NJ) or 20% of cancer cell-conditioned medium was added. The medium was replaced with fresh medium containing GM-CSF and IL-4 on Day 3. After 5 days of incubation, CD14, CD16, CD1a, HLA-DR, CD40, CD80, CD86, CD163, and CD206 were determined by FACS array flow cytometry using the fluorochrome-conjugated monoclonal antibody (BD Pharmingen). The $CD16^+$ tDCs derived from monocytes or from the patients' PBMC were separated using $CD16^+$ mAb-conjugated magnetic beads (MACS MicroBeads; Miltenyi Biotec). For the stimulation of the MdDCs, immature MdDCs were stimulated with LPS (100 ng/mL) after priming with IFN-γ for 2 h. After 24 h of stimulation, the supernatants were collected and analyzed by BD™ Cytometric Bead Array.

2.3. Allogeneic mixed lymphocyte reaction

Human T cells were purified from PBMCs obtained from the healthy donors using immunomagnetic CD4 naive T cell isolation kit (MACS MicroBeads; Miltenyi Biotec) according to the manufacturer's protocol. Mixed lymphocyte reactions were carried out by culturing naive CD4 T cells (10^5 cells/well) for 7 days with activated MdDCs (10^4 cells/well) or $CD16^+$ myeloid cell isolated from the breast cancer patient PBMC in 96-well plates. After 5 days of incubation, the T cells were stained with FITC-conjugated anti-CD4 mAb, APC-conjugated anti-CD25 mAb, and the PE-conjugated ait-FoxP3 mAB using a FoxP3 staining kit (BD Pharmingen). The supernatants were then collected after centrifugation and analyzed by BD™ Cytometric Bead Array. $CD4^+$ T cells were labeled with 10 μM BrdU on Day 7 postculture (Millipore), and proliferation was then analyzed on Day 8 by the ELISA-based method.

2.4. Reverse-transcription and real-time PCR

RNA isolation was performed using the TRIzol reagent (Invitrogen). cDNA was prepared using an oligo(dT) primer and reverse transcriptase (RT; Takara, Shiga, Japan) following standard protocols. Real-time PCR was performed using SYBR Green on an ABI 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA). Each PCR reaction mixture contained 200 nM of forward and reverse primer (5′-gcatcacagagg tgtgcatc-3′, 5′-aggccacgcacttaatcttg-3′), 10 μL 2× SYBR Green PCR Master Mix (Applied Biosystems), 5 μL cDNA, and RNase-free water for a total volume of 20 μl. The PCRs were conducted with a denaturation step at 95 °C for 10 min and then 40 cycles at 95 °C for 15 s and 60 °C for 1 min. All PCRs were performed in triplicate and normalized to an internal control GAPDH mRNA. The relative expression was presented using the $2^{(\text{Ct reference sample} - \text{Ct experimental sample})}$ method.

2.5. Analysis of galectin-1 interacting proteins

1 mg total cell lysate was applied to galectin-1 or BSA coupled affinity column generated using Micro Protein Coupling Kit (Thermo, San Jose, CA) and the eluate was analyzed by 2-D electrophoresis. Proteins were identified using silver staining. The stained gels were scanned using ImageScanner and LabScan 3.00 software (Amersham Biosciences). Image analysis was carried out using the ImageMaster 2D, version 2002.1 (Amersham Biosciences). The target protein spot was excised, trypsin-digested, and subjected to a MALDI-TOF MS for PMF using an in-house MASCOT software v2.2 (Matrix Science, London, UK).

2.6. Myosin light chain phosphorylation analysis

Cells were lysed on ice for 15 min using M-PER lysis reagent (Pierce, USA). The cell lysate was centrifuged at 14,000 ×*g* for 15 min and the supernatant fraction collected for immuno-blotting. Equivalent amounts of protein were resolved by SDS-PAGE (8–12%) and transferred to PVDF membranes. After blocking for 1 h in 5% non-fat dry milk in Tris-buffered saline, the membrane was incubated with anti-phosphorylated myosin regulatory light chain and beta-actin primary antibody for 16 h at 4 °C and then treated with the appropriate peroxidase-conjugated secondary antibody. Immuno-reactive proteins were detected using an enhanced chemiluminescence kit (Millipore, Bedford, MA) according to the manufacturer's instructions.

2.7. Biotin labeling and pull down assay

100 μg galectin-1 was dissolved in 200 μL amine-free PBS and labeled with biotin using an EasyLink biotin Conjugation Kit (Abcam Ltd., UK) according to the manufacturer's instructions. 1 μg/mL biotin conjugated galectin-1 was added into the culture medium, and the membrane and cytosol protein were extracted using a Transmembrane Protein Extract kit (Calbiochem) after 1 hour incubation. The same amount of extracted protein was incubated with 10 μg/mL anti-myosin antibody (Abcam) overnight and pulled down by protein G-agarose beads. The unbound protein was washed out and analyzed by SDS-PAGE.

2.8. FITC conjugated galectin-1 and immunofluorescence assay

100 μg galectin-1 was dissolved in 200 μL amine-free PBS and labeled with FITC using an EasyLink FITC Conjugation Kit (Abcam Ltd., UK) according to the manufacturer's instructions. 1 μg/mL FITC conjugated galectin-1 was added into the culture medium after different pre-treatment conditions. $CD14^+$ monocytes were incubated on a chamber slide for 16 h to allow them to become attached to the slides. The cells were pre-treated with different inhibitors for 1 h, and then

FITC conjugated galectin-1 was added and incubated for another 1 h. The slides were fixed with 4% paraformaldehyde and permeabilized with 0.5% Triton X-100, and then stained using an anti-myosin IIa antibody (Millipore). After washing with PBS containing 0.1% Tween 20, the slides were incubated with Dylight 549-conjugated secondary Ab (Rockland, Gilbertsville, PA), with DAPI for 1 h at room temperature.

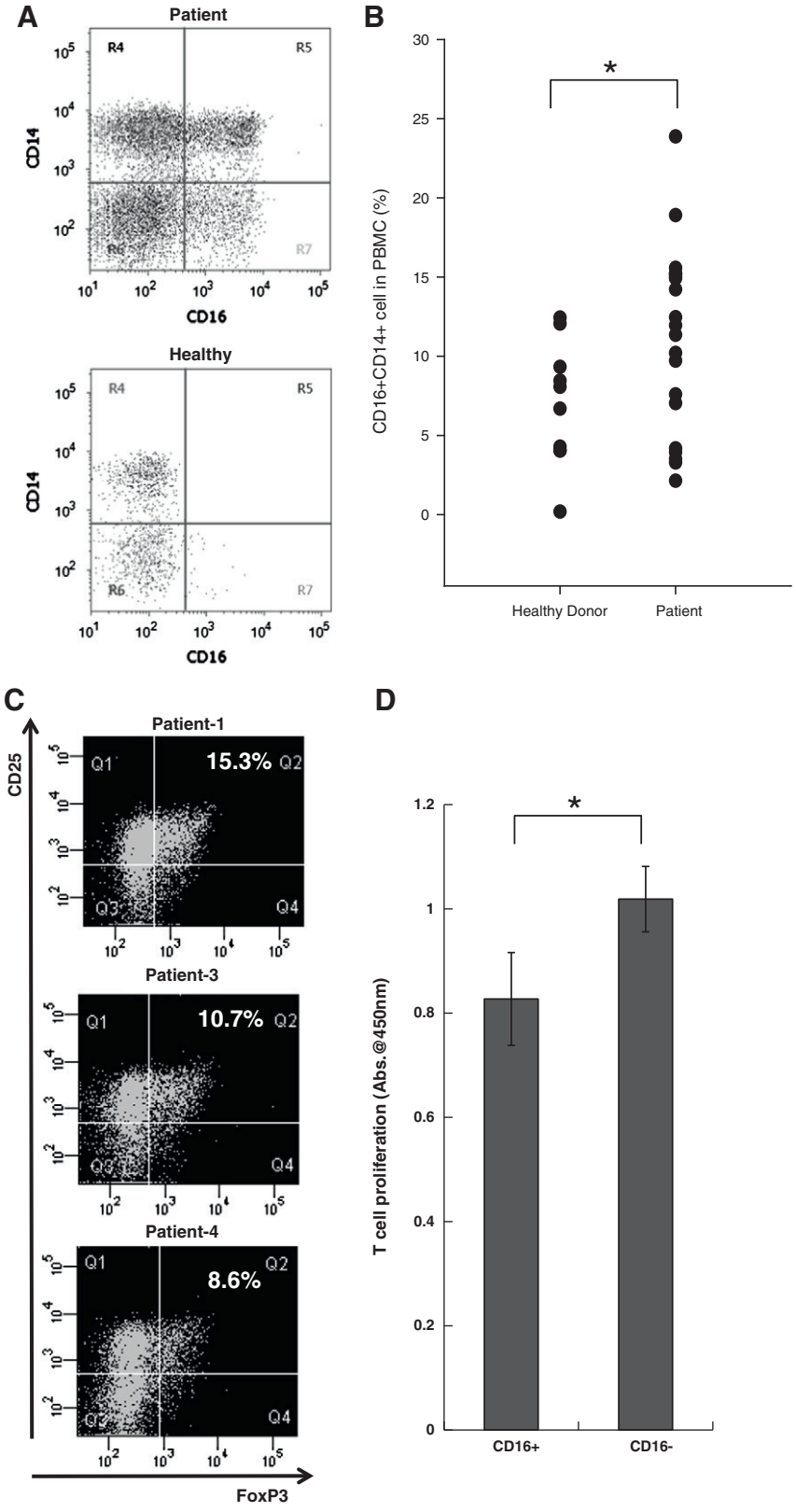


Fig. 1. Tolerogenic dendritic cells were increased in the breast cancer patients. (A and B) The PBMCs of 10 healthy donors and 19 breast cancer patients were stained with anti-CD14 and anti-CD16 antibodies. Myeloid cells were gated and the percentages of $CD14^+CD16^+$ cells are determined by FACS array flow cytometry. (C) The $CD16^+$ cell was isolated from the PBMCs of the breast cancer patients and co-cultured with naive $CD4^+$ T cells isolated from healthy donor for 7 days and stained using BD Pharmingen™ Human FoxP3 Buffer Set. The T cell proliferation was measured by BrdU incorporation assay. *Significant difference between the two test groups analyzed by ANOVA with the Student's *t* test ($p < 0.05$).

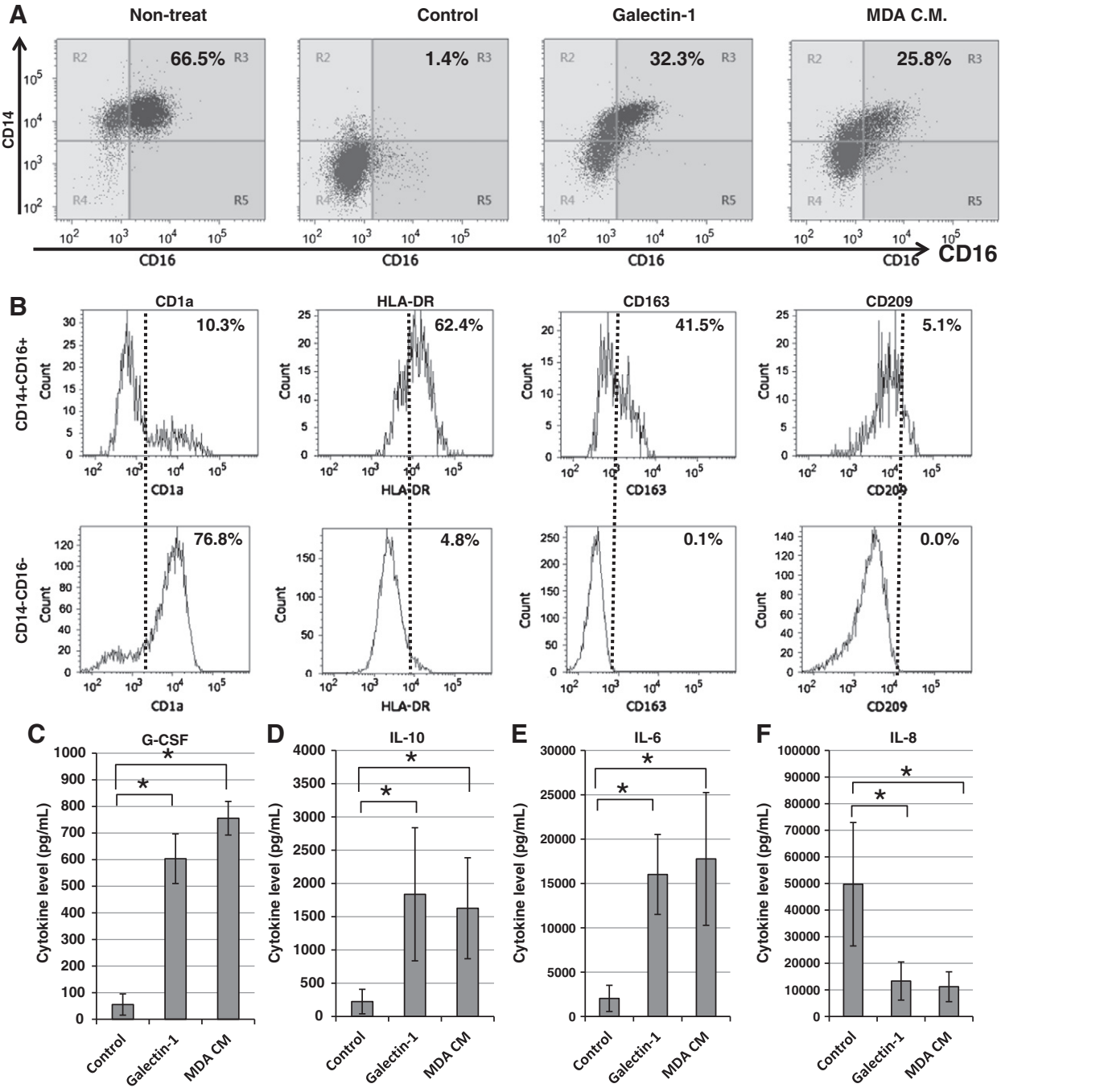


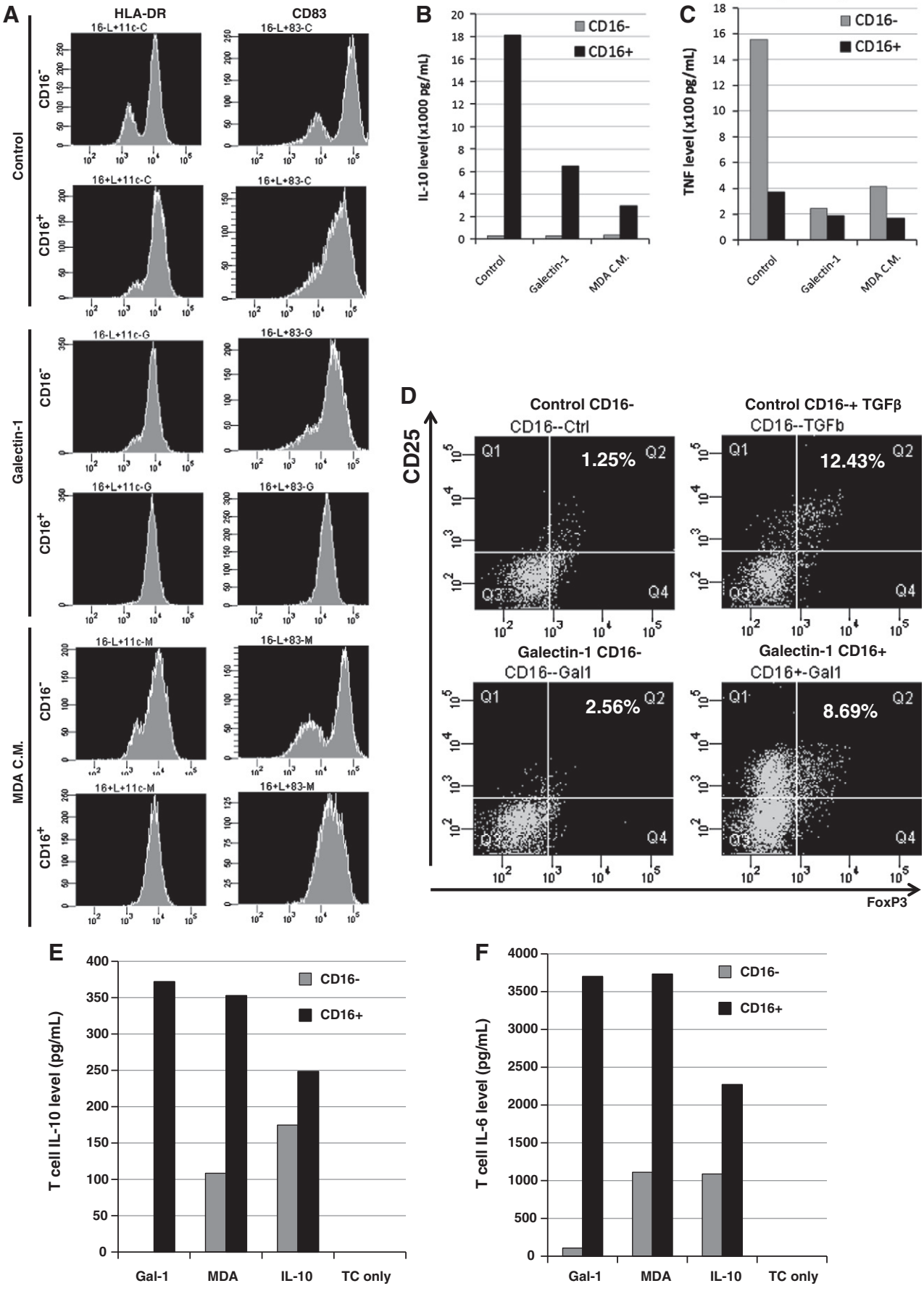
Fig. 2. Galectin-1 mimicked the ability of breast cancer to induce tDCs. (A) The CD14 and CD16 expressions of MdDC with different treatments. Non-treat: RPMI with 10% FBS only, control: RPMI with GM-CSF and IL-4, galectin-1: control plus 1 μg/mL galectin-1 and MDA CM: 20% MDA conditioned medium. (B) The surface marker expressions of $CD14^+/CD16^+$ and $CD14^-/CD16^-$ MdDC. (C–F) The cytokine levels secreted by MdDCs with different treatment were examined by BD™ Cytometric Bead Array. Data are representative of more than three independent experiments. *Significant difference between the two test groups analyzed by ANOVA with the Student's *t* test ($p < 0.05$).

The slides were analyzed with a confocal laser-scanning microscope (Fluoview FV500; Olympus, Tokyo, Japan). Three-dimensional reconstructions of epidermal whole mounts were analyzed with Volocity software (Version 5.0, Perkin Elmer, Waltham, MA).

2.9. Knockdown galectin-1 in 4T1 cells

4T1 cells were purchased from ATCC and cultured in RPMI 1640 medium containing 10% FBS (Invitrogen, Carlsbad, CA). RNAi reagents

Fig. 3. Galectin-1-conditioned MdDCs induced regulatory T cells. The $CD16^+$ and $CD16^-$ MdDCs were separated by anti-CD16 micro-beads and treated with 10 ng/mL IFN-γ combined with 100 nM LPS for two days. (A) Surfaces CD83 and HLA-DR were analyzed by flow cytometry, and (B–C) cytokine levels were examined by Cytometric Bead Array. (D–F) Stimulated MdDCs of galectin-1 group were incubated with allogeneic naive $CD4^+$ T cells at a MdDCs/T cell ratio of 1:10. Intracellular FoxP3 expression was evaluated with a BD Pharmingen™ Human FoxP3 Buffer Set. Cytokine expressions were measured by Cytometric Bead Array. Data are representative of three independent experiments. *Significant difference between the two test groups analyzed by ANOVA with the Student's *t* test ($p < 0.05$).


A
HLA-DR
CD83
Control
Galectin-1
MDA C.M.
CD16-
CD16+
16-L+11c-C
16-L+83-C
16+L+11c-C
16+L+83-C
16-L+11c-G
16-L+83-G
16+L+11c-G
16+L+83-G
16-L+11c-M
16-L+83-M
16+L+11c-M
16+L+83-M
B
IL-10 level (x1000 pg/mL)
CD16-
CD16+
Control
Galectin-1
MDA C.M.
C
TNF level (x100 pg/mL)
CD16-
CD16+
Control
Galectin-1
MDA C.M.
D
CD25
FoxP3
Control CD16-
CD16--Ctrl
1.25%
Control CD16-+ TGFβ
CD16--TGFb
12.43%
Galectin-1 CD16-
CD16--Gal1
2.56%
Galectin-1 CD16+
CD16+-Gal1
8.69%
Q1
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E
T cell IL-10 level (pg/mL)
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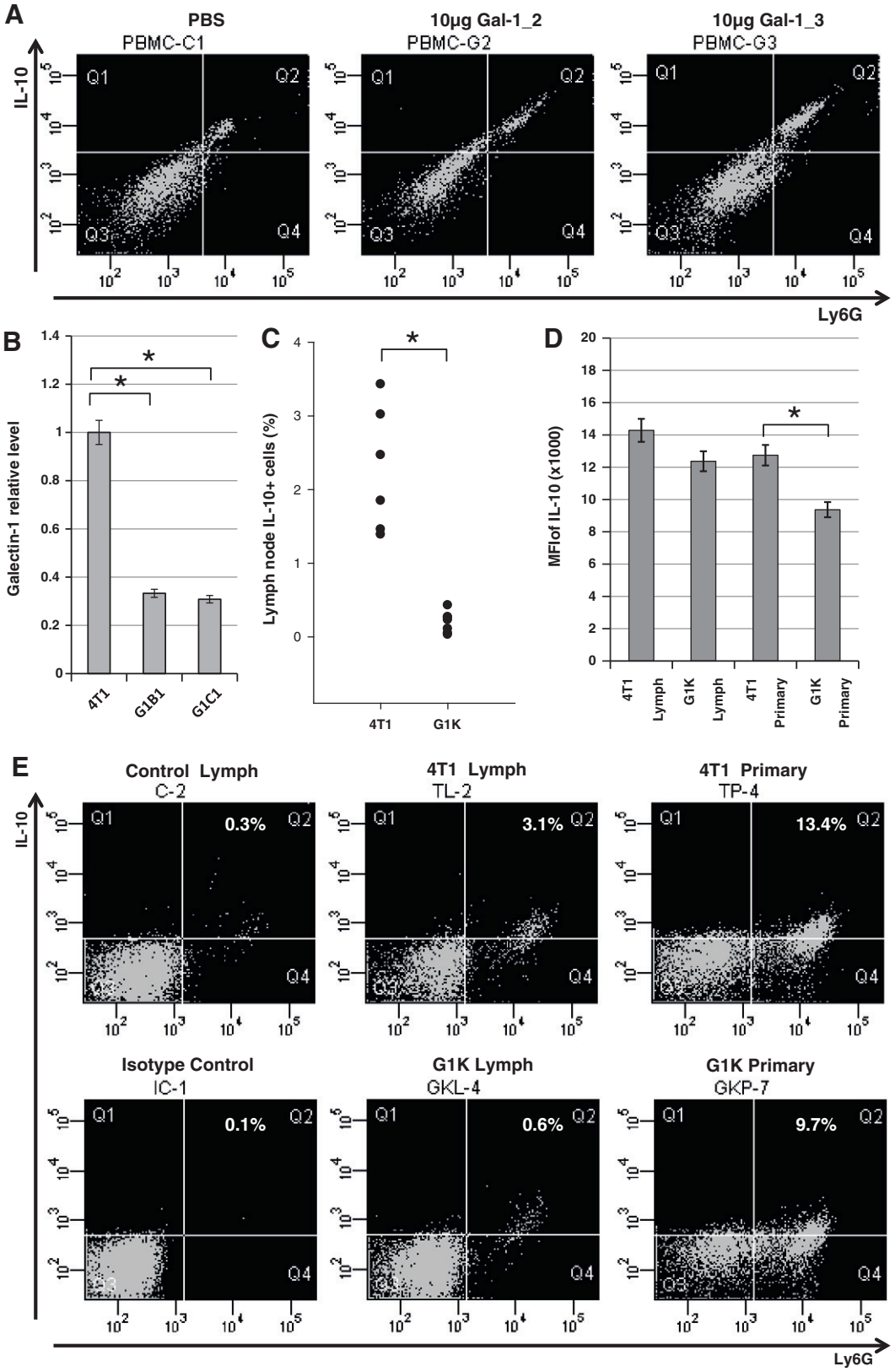

Fig. 4. Increased amounts of IL-10 on the DCs in the tumors and lymph nodes of breast cancer-bearing mice. (A) The mice were administered with 10 μg in 100 μL PBS or PBS only every 2 days and the PBMCs were isolated from the tail vein blood. The Ly6G and IL-10 expression levels were determined after gating $CD11c^+$ cells. (B) The knockdown efficiency of two galectin-1 knockdown cell lines was determined by the ABI StepOne Real-Time PCR System. (C–E) The mice were injected with 4T1 or galectin-1 knockdown G1B1 (G1K) cells subcutaneously and sacrificed on Day 21. The intracellular IL-10 expression of cells in the lymph nodes (Lymph) and primary tumor mass (Primary) was detected by APC conjugated anti-IL-10 antibody after gating $CD11c^+$ cells. *Significant difference between the two test groups analyzed by ANOVA with the Student's *t* test ($p < 0.05$).

were obtained from the National RNAi Core Facility located at the Institute of Molecular Biology/Genomic Research Center, Academia Sinica, Taiwan, supported by a National Core Facility Program for Biotechnology grant from the National Science Council (NSC 100-2319-B-001-002), and two clones (TRCN0000011863, TRCN0000011865) were transfected into the 4T1 cells. Galectin-1 knockdown 4T1 cells were selected using 1 μg/mL puromycin. The knockdown efficiency was confirmed by real-time PCR.

2.10. Animal models and cell isolation

For galectin-1 induction in vivo, 10 μg recombinant mouse galectin-1 (R&D systems) in 100 μL PBS was administered intraperitoneally with the control group receiving 100 μL PBS every 2 days. After 14 days, leukocytes of the mice were obtained from the blood of tail veins and the red blood cells were lysed.

4T1 cells or galectin-1 knockdown cells were transplanted by subcutaneous injection into the fat pad of the BALB/c mice and treated with 1% DMSO in PBS or 250 μM blebbistatin 100 μL by intraperitoneal injection every 2 days. Lymph nodes and primary tumor mass were collected 14 and 21 days after the injection.

After sacrifice, the tissues were minced and incubated in RPMI 1640 medium with collagenase type 1 (200 U/mL; Worthington Biochemicals) for 1 h at 37 °C. The digested tissues were filtered through a 70-μm cell strainer and washed with RPMI 1640 medium. To analyze the number of myeloid suppressor cells, the surface expressions of CD11c and Ly6G were labeled by FITC-conjugated anti-CD11c and APC-Cy7 conjugated anti-Ly6G (BD Pharmingen), followed by intracellular staining of IL-10 by APC conjugated anti-IL-10 monoclonal antibody (BD Pharmingen). All of the procedures were in keeping with the IACUC guidelines at the National Laboratory Animal Center of the National Applied Research Laboratories.

2.11. Statistical analysis

Data were expressed as means ± SD. Analysis was performed using ANOVA and two-sided *t*-tests using Excel 2010 (Microsoft, Tulsa, USA). A p value < 0.05 was considered to be statistically significant.

3. Results

3.1. Galectin-1 mediated tolerogenic differentiation

The PBMCs were obtained from 10 healthy donors and 20 breast cancer patients. Tolerogenic DCs were identified in the PBMCs by CD14 and CD16 labeling in the myeloid cell region (Fig. 1A). The PBMCs of the breast cancer patients had a higher percentage of tDCs (7–11.5%) (Fig. 1B) (Table S3). The $CD16^+$ tDCs of 6 patients were further isolated and co-cultured with naive $CD4^+$ T cells isolated from the healthy donors. CD25/FoxP3 staining revealed the ability of $CD16^+$ tDCs to induce regulatory T cells and suppress T cell proliferation (Fig. 1C and D).

The pattern of surface markers of DCs derived from $CD14^+$ monocyte isolated from healthy donor in the presence of GM-CSF and IL-4 was examined after 5 days incubation. Compared to monocyte derived dendritic cells (MdDCs) with only GM-CSF and IL-4, the MdDCs cultured in galectin-1 containing medium had more $CD14^+CD16^+$ cells (Fig. 2A) and the MdDC cultured in cancer cell conditioned media containing medium also exhibited the same phenotype. The $CD14^+CD16^+$ population also showed higher CD163, CD209, and class II MHC expressions but lower CD1a expression (Fig. 2B). Expressions of IL-10, G-CSF, and IL-6 all increased after treatment with galectin-1 and MDA-MB-231 conditioned medium (Fig. 2C–E). However, IL-8 expressions decreased (Fig. 2F).

The $CD16^+$ and $CD16^-$ populations were later separated by microbeads and stimulated with IFN-γ and LPS. After LPS stimulation, the $CD16^-$ MdDCs showed activated DC features of higher HLA-DR and CD83 expressions (Fig. 3A). On the other hand, the $CD16^+$ galectin-1 derived DCs secreted more IL-10 than the $CD16^-$ DC, which was similar to the $CD16^+$ population induced by MDA C.M., but lower TNF secretion (Fig. 3B–C). The $CD14^+CD16^+$ DCs also had the ability to increase the regulatory T cell population from naïve $CD4^+$ T cells (Fig. 3D) and IL-6 and IL-10 secretions of T cells (Fig. 3E and F).

To induce tDCs in vivo, 10 μg galectin-1 in 100 μL PBS was administered intraperitoneally and the PBMCs of the mice were isolated after 14 days. The staining of CD11c, IL-10, and Ly6G showed that galectin-1 could induce IL-10 secreting DCs in vivo (Fig. 4A). Furthermore, 4T1 cells were administered subcutaneously into the fat pads of female mice. Immune cells in the lymph nodes were then extracted and analyzed. The $CD11c^+/Ly6G^+/IL\text{-}10^+$ cells were identified as immune suppressor cells in the tumors. Immune cells in the lymph nodes of the tumor-bearing mice also had higher IL-10 and Ly6G expressions (Fig. 4E).

In order to test the ability of galectin-1 to induce tDCs in the 4T1 bearing mice, two galectin-1 knockdown cell lines were generated (Fig. 4B). After knocking down the galectin-1 level in the 4T1 cells, the population of $Ly6G^+/IL\text{-}10^+$ cells in the lymph node $CD11c^+$ DCs is decreased (Fig. 4C and D) (Table S1). The IL-10 expression level of $Ly6G^+$ in primary tumors also decreased (Fig. 4D and E).

3.2. Galectin-1 interacted with myosin IIa

To identify the interacting protein of galectin-1, a galectin-1 coupled affinity column was generated, and a BSA coupled column was used as a negative control. The total protein extract of monocytes was applied

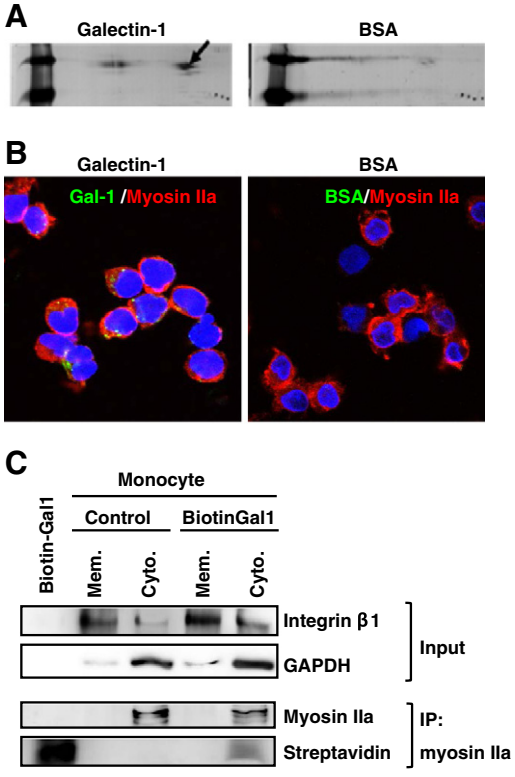

Fig. 5. The interaction of galectin-1 and myosin IIa. (A) 1 mg of $CD14^+$ monocyte lysate in 100 μL was applied to the galectin-1 or BSA affinity column. The binding proteins were washed out by high salt buffer and analyzed by two-dimensional electrophoresis. Arrow: the spot of myosin IIa. (B) $CD14^+$ monocytes were cultured on a cover slide chamber for 16 h and treated with FITC-galectin-1 (1 μg/mL). After incubation for 1 h, the cells were washed by PBS and fixed. Cells were later permeabilized and stained with an anti-myosin IIa antibody. Z-stack images were taken by a confocal microscope. (C) The $CD14^+$ monocytes were mixed with or without biotin-labeled galectin-1 for 1 h and the protein in different proportions was extracted and pulled down by an anti-myosin IIa antibody. Mem: membrane proteins, Cyto: cytosol proteins. Data are representative of three independent experiments.

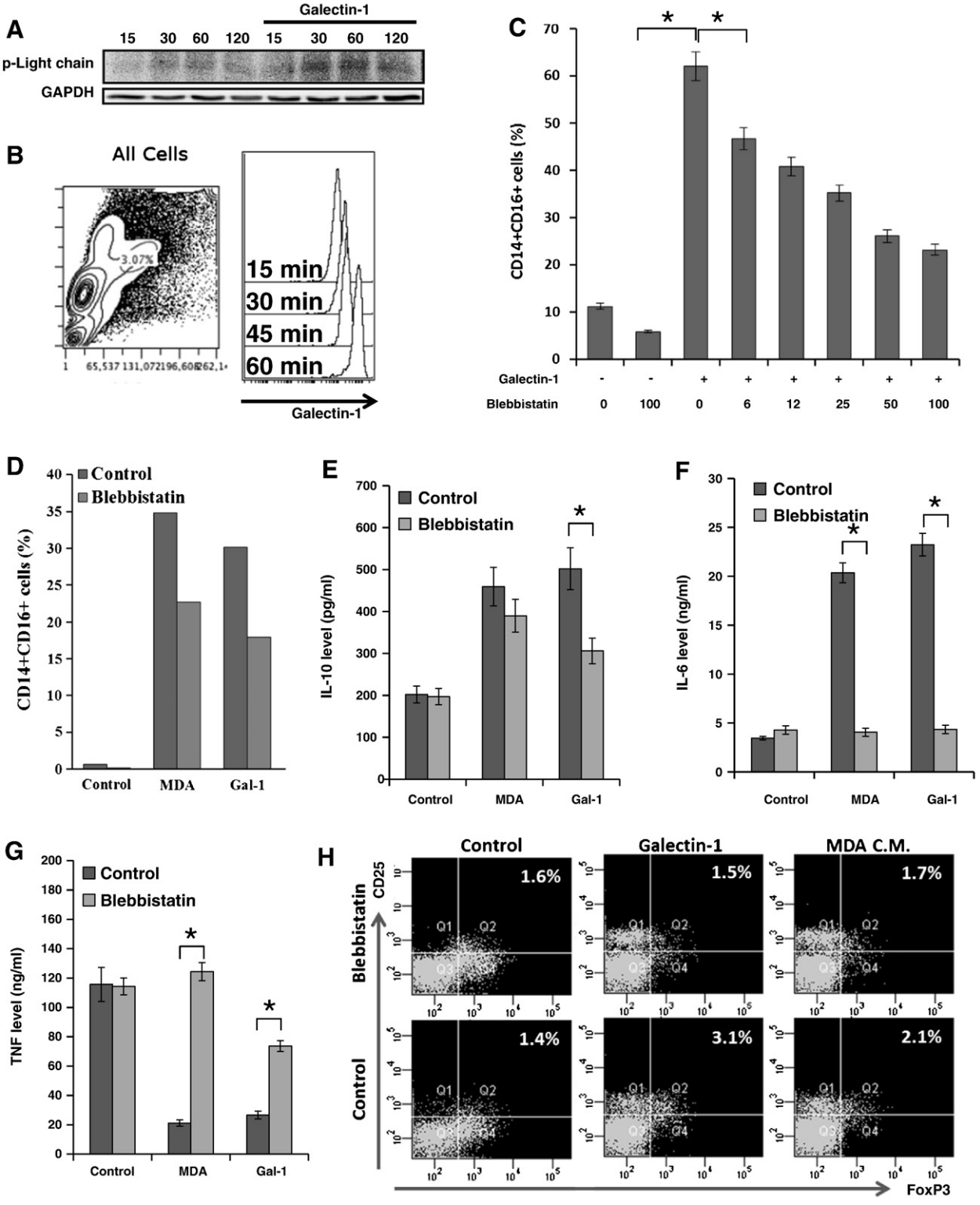

Fig. 6. Blebbistatin blocked galectin-1-induced tolerogenic DC differentiation. (A) $CD14^+$ monocytes isolated from human PBMCs were incubated with 20 ng/mL GM-CSF and IL-4 only, or combined with 1 μg/mL galectin-1 and lysed at different time points. (B) The human PBMCs were mixed with FITC conjugated galectin-1 and washed with acid wash buffer at each time point. (C–F) $CD14^+$ monocytes were incubated with galectin-1 and different concentrations of blebbistatin for 5 days, and the percentages of $CD14^+/CD16^+$ cells were assayed by flow cytometry. Cytokine expressions were measured by Cytometric Bead Array. (G) The MdDCs were stimulated by LPS for two days and the expression of TNFs was measured by Cytometric Bead Array. The stimulated MdDCs were later incubated with allogeneic naive $CD4^+$ T cells at a MdDCs/T cell ratio of 1:10. (H) Regulatory T cells were assayed with a BD Pharmingen™ Human FoxP3 Buffer Set. Data are representative of two independent experiments. The mice were injected with 1×10^6 4T1 cells subcutaneously near the fat pad on Day 0, and then administered with 1% DMSO or 250 nM blebbistatin 100 μL every two days. The mice were sacrificed on Day 21 and cells from the lymph nodes or primary tumor mass were isolated and stained with CD11c, Ly6G and IL-10 antibodies (I). The percentages of $Ly6G^+/IL\text{-}10^+$ cells in the $CD11c^+$ cells are indicated (J). Data are representative of seven mice per group. *Significant difference between the two test groups analyzed by ANOVA with the Student's *t* test ($p < 0.05$).

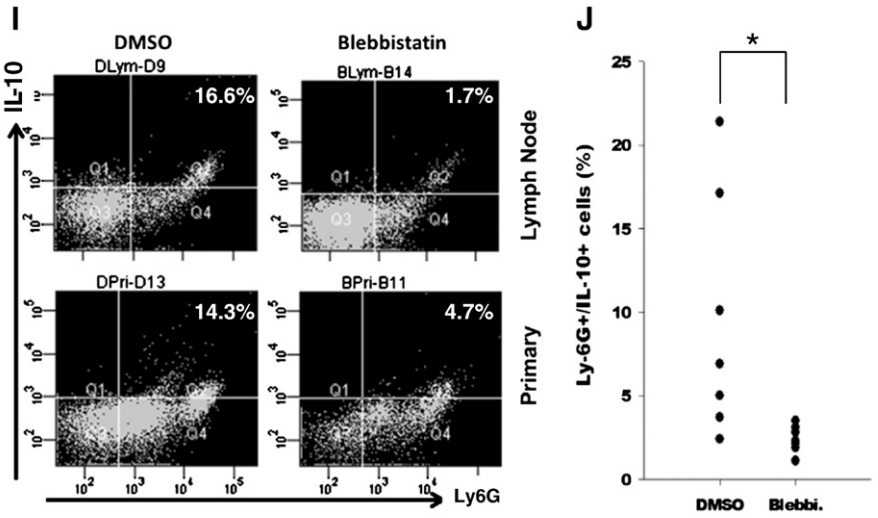

Fig. 6 (*continued*).

and the eluents of three repeats were analyzed by 2-D electrophoresis and LC–MS-MS. After comparing differences in the results from the galectin-1 and BSA coupled columns, MYH9, a myosin IIa component, was found to be a major target of galectin-1 (Fig. 5A). Minor changes were also analyzed by directly applying the eluent onto the LC–MS-MS to obtain myosin IIa interacting proteins (Table S2).

To investigate the binding of myosin and galectin-1 in PBMCs, FITC conjugated galectin-1 was administrated to the monocyte and extra galectin-1 was washed out after 1 h. Then the monocytes were fixed and stained with an anti-myosin IIa antibody. Confocal microscopy was later used to examine the location of galectin-1 and myosin IIa which revealed that galectin-1 could partially co-localize with myosin IIa inside the monocytes (Fig. 5B). For further confirmation, myosin IIa pull down of membrane and cytosol was performed. Integrin beta1 was used for the membrane protein control and GAPDH for the cytosol protein. After pulling down by myosin IIa antibody, both myosin IIa and exogenous galectin-1 could be detected in the cytosol protein but not in the membrane protein (Fig. 5C).

3.3. Myosin IIa activation was important in galectin-1 induced tolerogenic DCs

Myosin light chain ser-19 phosphorylation was an important marker in myosin IIa activation. Galectin-1 caused a time-related increase in myosin IIa activation (Fig. 6A). The exogenous galectin-1 endocytosis of monocytes is determined by the wash out surface bound FITC conjugated galectin-1 at each time point (Fig. 6B). To further investigate the importance of myosin IIa in galectin-1 mediated tDC differentiation, myosin IIa specific inhibitor, blebbistatin has been used which had dose-related inhibitory effects on galectin-1-induced $CD14^+CD16^+$ DC differentiation (Fig. 6C). Blebbistatin could also decrease the MDA C.M.-induced $CD14^+CD16^+$ population increment (Fig. 6D) and inhibit the secretion of IL-6 and IL-10 (Fig. 6E and F) of MdDCs but had no effect on IL-8 and G-CSF (data not shown). The secretion of TNFs of MdDC induced by LPS was restored after the cells were treated with blebbistatin during the differentiation period (Fig. 6G). The ability of tolerogenic DC induced by galectin-1 and cancer cell derived medium to induce regulatory T cells was also inhibited by blebbistatin (Fig. 6H).

The 4T1 breast cancer cells were used for an in vivo model to examine the importance of myosin IIa in the tumor micro-environment. BALB/c mice were injected with 4T1 cells subcutaneously, followed by treatment with 100 μL of 250 μM blebbistatin once every two days. After 21 days of treatment, the CD11c positive cells in the lymph nodes and primary tumor masses of blebbistatin-treated mice showed lower Ly6G and IL-10 expressions (Fig. 6I and J).

3.4. Galectin-1 internalization was required to induce tolerogenic DC differentiation

To further investigate whether the entry of galectin-1 into the cytoplasm of monocytes was necessary to induce tDC differentiation, different endocytosis inhibitors were used, including phenylarsine oxide and chlorpromazine for clathrin-dependent endocytosis, genistein for caveolae-dependent endocytosis, and rottlerin for fluid phase endocytosis. The cells were treated with different endocytosis inhibitors before the addition of galectin-1. Genistein and phenylarsine oxide inhibited the galectin-1-induced tolerogenic DC differentiation (Fig. 7A and B). Genistein, but not phenylarsine oxide, decreased the internalization of galectin-1, as shown by confocal microscopy (Fig. 7C). Genistein also blocked the galectin-1-induced myosin light chain phosphorylation (Fig. 7E) and decreased the monocyte IL-10 secretion (Fig. 7F).

4. Discussion

Galectin-1 is important in cancer progression and is an important marker in cancer prognosis, including colorectal, oral, lung, ovarian, thyroid, and breast cancers [9,14,23–25]. Previous studies have mainly focused on galectin-1-induced T cell apoptosis [26–28]. A previous study has demonstrated that galectin-1 not only controls the immune system by inducing T cell apoptosis, but also enhances the IL-10 secretion of monocytes. IL-10 has been identified as the most important cytokine that induces tolerance of auto-antigen and cancer cells. Generally, it is considered that tumor-associated tDCs are up-regulated by cancer cell mediated IL-6, IL-10, and M-CSF. The present study demonstrates that galectin-1 can be an important factor for increasing IL-10 and IL-6 concentration in the tumor micro-environment.

This is the first report to describe the galectin-1 mediated tDCs as the $CD1a^-CD11c^+CD14^+CD16^+CD40^{med}CD83^{med}CD86^{med}CD163^+$ $CD206^+CD209^+HLA\text{-}DR^+$ DCs which are similar to DC-10 population found in human PBMCs. These cells also had higher IL-6, IL-10, and G-CSF secretions, and lower IL-8 expression. Galectin-1 induced tDCs expressed lower IL-12 and TNF levels compared to normal DCs when stimulated by LPS. The micro-array data also demonstrated that galectin-1 can up-regulate oncostatin M, VEGF, CXCL1, and CCL5 mRNA expressions in monocytes (data not shown). Such secretion factors have also been reported to be important factors in the tumor micro-environment. These results indicate that galectin-1 is an important target in cancer immune therapy rather than IL-6, IL-10, and M-CSF.

Another interesting phenomenon demonstrated in this study is that exogenous factors can activate intracellular responses only after

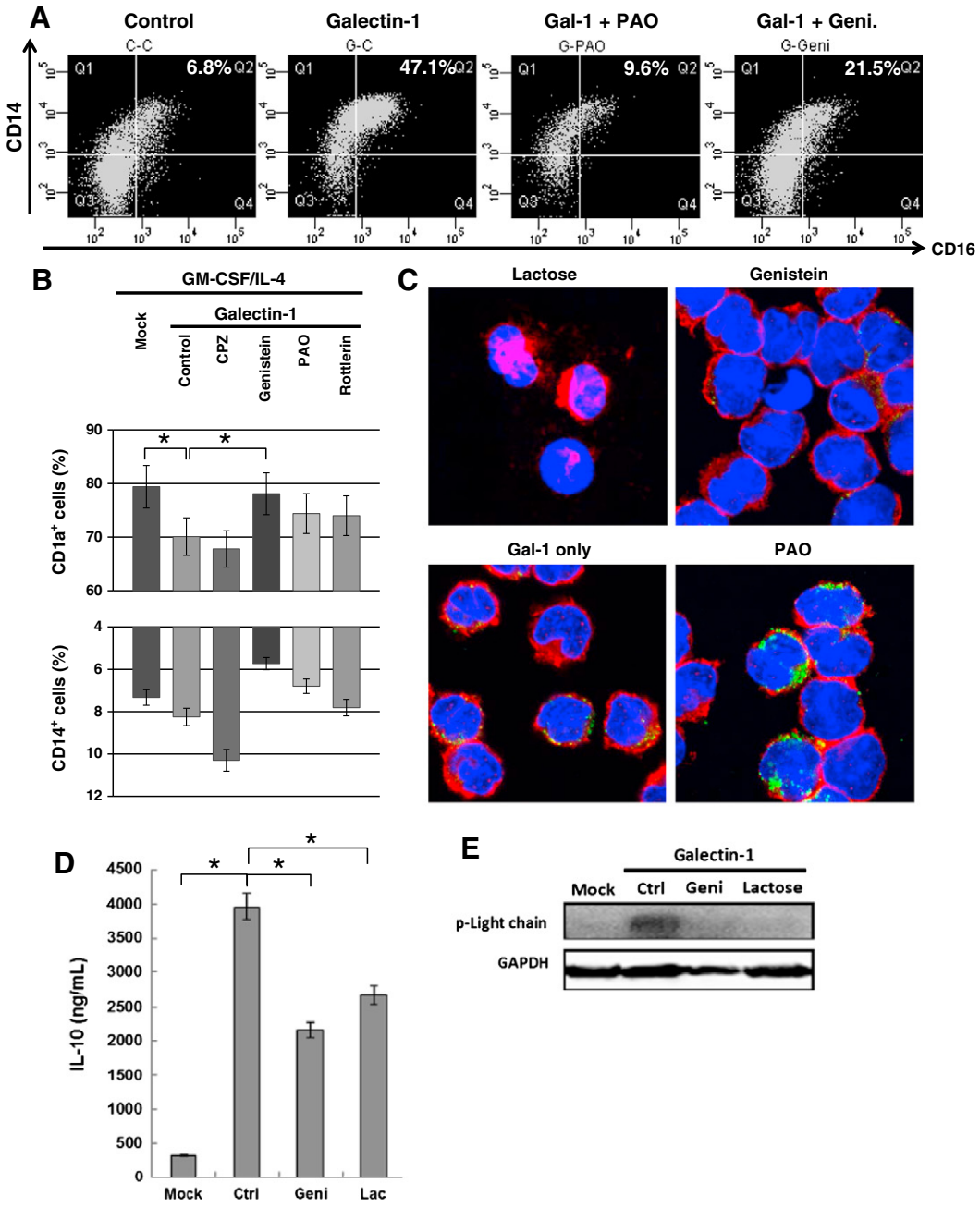

Fig. 7. Genistein inhibited galectin-1 internalization and tDC differentiation. The monocytes were incubated with RPMI, genistein (25 μM), chlorpromazine (CPZ, 200 μM), phenylarsine oxide (PAO, 100 μM), rottlerin (25 μM), or lactose (25 mM), and then treated with galectin-1. (A, B and D) After 5 days of incubation, surface marker expressions and cytokine levels were analyzed by flow cytometry and Cytometric Bead Array. (E) $CD14^+$ monocytes were pre-treated with genistein or lactose for 1 h and treated with galectin-1 for another hour. The cells were lysed and the phosphorylation of regulatory myosin light chain was examined by western blotting. (C) The distribution of galectin-1 was assayed by confocal microscopy after incubation with FITC conjugated galectin-1 for 30 min. The red fluorescence refers to myosin IIa. Data are representative of two independent experiments.

internalization. Fajka-Boja et al. reported that galectin-1 can be internalized in T cells with GM1 ganglioside [29]. However, these findings showed that the monocytes had a higher ability in galectin-1 internalization (Fig. S1). This also suggests that galectin-1 endocytosis in monocytes may be through a caveolae-dependent pathway, which is different from endocytosis in T cells.

Myosin IIa has been reported to be a receptor for several extracellular molecules, including herpes simplex virus and soluble CD163 [30–32]. A previous report also showed the same result for an interaction between galectin-1 and myosin IIa [33]. The mass spectrum data in this study shows that the galectin-1 column captured many molecules, including actins, ezrin, vimentin, and S100 proteins which have previously been reported to interact with myosin IIa [34–37]. This suggests that galectin-1 can interact with these molecules via myosin IIa. In this study, galectin-1 internalization was mediated by a caveolae-dependent pathway, which is a subtype of lipid raft. Previous studies have indicated that endogenous galectin-1 may stabilize Ras anchorage and mediate signal transduction [38,39] and also that the myosin light chain kinase is one of the downstream factors of Ras [40]. This implies that the interaction of Ras, galectin-1 and myosin IIa may participate in myosin IIa activation.

Blebbistatin is a myosin IIa specific inhibitor that inhibits ATPase on the myosin heavy chain [41–43]. Blebbistatin has been used as an inhibitor for actomyosin contraction and cell migration, and previous reports

have shown that it can inhibit cancer cell migration and invasion [44,45]. Recent reports have demonstrated that myosin IIa also plays an important role in intracellular signaling, including the JAK1/STAT3 and JNK/c-Jun pathways. Treatment with blebbistatin has been reported to down-regulate the expressions of oncostatin M and gp130 in fibroblasts and the CD36-mediated JNK pathway [18,46]. The JAK1/STAT3 pathway is very important for IL-6, IL-10, and oncostatin M secretions and auto-enhancement [47–49]. This is consistent with the present findings that galectin-1 can increase IL-6 and IL-10 secretions and that blebbistatin can inhibit the function of galectin-1. Blebbistatin can also inhibit the differentiation of tDCs and reduce the amount of $CD4^+CD25^+Foxp3^+$ regulatory T cells. Taken together, blebbistatin can inhibit tumor metastasis and enhance immunity in the tumor micro-environment. This study is also the first to use blebbistatin as in vivo treatment. The results showed that blebbistatin did not cause significant differences in body weight or activity of the mice by local injections of 0.25 mg/kg.

Fulcher et al. reported that galectin-1 treatment may induce myosin regulatory light chain (MRLC) phosphorylation [50]. In this study, galectin-1 increased MRLC phosphorylation which can activate the myosin IIa pathway. This is the first report to show that myosin IIa plays an important role in myeloid linage differentiation by regulating cell surface makers and cytokine secretion. This study further shows that blebbistatin can down-regulate the tolerogenic ability of DC-10. Compared to mature DCs, galectin-1 induced more Foxp3 expression and more $CD4^+CD25^+Foxp3^+$ regulatory T cells rather than $CD25^+Foxp3^-$ effector T cells [51].

The results of this study revealed a novel mechanism for tDC differentiation by the interaction of myosin IIa and galectin-1 through caveolae-dependent endocytosis. For the cancers with a high galectin-1 expression, blebbistatin or genistein may be a promising adjuvant in cancer immunotherapy. Combined with other immunotherapy methods, this may eliminate the tumors that escape from the immune system due to immune suppression and tolerance.

Conflict of interest statement

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbagen.2014.01.026.

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