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Lung Cancer-Derived Galectin-1 Mediates Dendritic Cell Anergy through Inhibitor of DNA Binding 3/IL-10 Signaling Pathway

Po-Lin Kuo,†,‡*,‡‡ Jen-Yu Hung,§,¶,§‡ Shau-Ku Huang,‖ Shah-Hwa Chou,‡ Da-En Cheng,¶ Yuh-Jyh Jong,††,‡‡‡ Chih-Hsing Hung,§,¶¶ Chih-Jen Yang,‡,§ Ying-Ming Tsai,§ Ya-Ling Hsu,‖ and Ming-Shyan Huang‡,§

Lung cancer, one of the leading causes of death worldwide, is often associated with a state of immune suppression, but the molecular and functional basis remains enigmatic. Evidence is provided in this paper supporting the role of lung cancer-derived soluble lectin, galectin-1, as a culprit in dendritic cell (DC) anergy. We have shown that galectin-1 is highly expressed in lung cancer cell lines, together with the serum and surgical samples from lung cancer patients. Functionally, lung cancer-derived galectin-1 has been shown to alter the phenotypes of monocyte-derived DCs (MdDCs) and impair alloreactive T cell response, concomitant with the increase of CD4+CD25+FOXP3+ regulatory T cells. The regulatory effect of galectin-1 is mediated, in part, through its ability to induce, in an Id3 (inhibitor of DNA binding 3)-dependent manner, the expression of IL-10 in monocytes and MdDCs. This effect is inhibited by the addition of lactose, which normalizes the phenotypic and functional alterations seen in MdDCs. Of note, significant upregulation of IL-10 was seen in tumor-infiltrating CD11c+ DCs in human lung cancer samples. This was also noted in mice transplanted with lung cancer cells, but not in those receiving tumor cells with galectin-1 knockdown. Furthermore, a significant reduction was noted in lung cancer incidence and in the levels of IL-10–expressing, tumor-infiltrating DCs, in mice receiving galectin-1–silenced tumor cells. These results thus suggest that the galectin-1/IL-10 functional axis may be crucial in lung cancer-mediated immune suppression, and that galectin-1 may serve as a target in the development of lung cancer immunotherapy. The Journal of Immunology, 2011, 186: 1521–1530.

Lung cancer, one of the leading causes of death in the world, is associated with very poor prognoses even after surgical resection (1). Immunotherapy is regarded as a potentially effective treatment when conventional therapy strategies have attained their maximum advantage or are no longer effective in eliminating cancer. Dendritic cells (DCs) play pivotal roles in initiating innate and adaptive immune responses, which are responsible for the induction of anticancer immune responses (2). Accumulated evidence has also suggested that the emergence of tolerogenic DCs may be responsible for the development of tumor-mediated immune anergy (3, 4). DCs isolated from animals and human cancer patients have phenotypic aberrations and functional loss (3, 4). In addition, DCs located in the tumor’s microenvironment not only inhibit T cell-based anticancer immune response, but promote the tumor’s growth, and invasive and proangiogenic abilities (5–7). However, despite these consistent findings, the mechanisms by which cancer cells evade immune surveillance and mediate immune suppression have remained elusive, which has hindered progress in developing effective immunotherapy strategies.

Protein–glycan interactions play a crucial role in a variety of biological processes during cancer development, including cancer transformation, growth, metastasis, angiogenesis, and immune surveillance (8, 9). Galectins are glycan-binding proteins defined by their high affinity for N-acetylactosamine sequences displayed on both N- and O-glycans on cell surface glycoproteins or glycolipids. Galectin-1, the first discovered protein in the family, has been found to be upregulated in many cancers, including astrocytoma, melanoma, oral, colon, bladder, and ovarian carcinomas (10–12). Overexpression of galectin-1 in tumors has also been shown to correlate with the aggressiveness of these tumors and the development of metastasis (11–13). Moreover, recent studies have also suggested that tumors secrete galectin-1, which contributes to the immunosuppressive response and apoptosis of immune cells, particularly T cells (14–16). However, the mechanistic aspects of how galectin-1 causes DC anergy against tumors are...
currely unclear, and its potential involvement in lung cancer remains unexplored.

In this study, we discovered that lung cancer cells secrete high amounts of galectin-1 and play a role in suppressing the response of DCs. Our data show that galectin-1 was able to cause a change in function of monocyte-derived DCs (MdDCs) by an IL-10 autocrine effect, which was regulated in an inhibitor of DNA binding 3 (Id3)-dependent manner. These findings also revealed that DCs infiltrating tumor tissues of mice and human patients express high levels of IL-10, suggesting both a novel role of galectin-1 in tumor-mediated immune suppression and its candidacy as a potential target for developing therapeutic strategies to strengthen the immune surveillance of the host in fighting lung cancer.

Materials and Methods

Lung cancer cells and conditioned media

Human lung cancer cells A549 and NCI-H460, and mouse Lewis lung carcinoma (LLC) cells were obtained from the Bioresource Collection and Research Center (Hsinchu City, Taiwan). Human bronchial epithelial cells were obtained from Lonza (Wokingham, UK). To obtain the conditioned medium (CM), we seeded cells at 2 × 10⁶ cells/100-mm dish and cultivated for 24 h. The medium was replaced and the supernatants were harvested after 48 h of incubation.

Serum samples from lung cancer patients

Preoperative blood samples were obtained from 40 lung cancer patients and 15 healthy donors admitted to the Division of Pulmonary and Critical Care Medicine, Kaohsiung Medical University Hospital (KMUH), Kaohsiung, Taiwan. Serum was separated by centrifugation and frozen at −80°C. Fifteen pairs of tumor and adjacent nontumor lung tissues were collected from all patients in accordance with the Declaration of Helsinki. Approval for these studies was obtained from the Institutional Review Board of KMUH, and informed consent was obtained from all patients in accordance with the Declaration of Helsinki.

Isolation of CD14⁺ monocytes and differentiation of MdDCs

Mononuclear cells were isolated from blood by Ficoll-Hypaque gradient (GE Healthcare Bio-Sciences, Little Chalfont, UK) from healthy consenting donors. CD14⁺ monocytes were purified using CD14⁺ mAb-conjugated magnetic beads (MACS MicroBeads; Miltenyi Biotech), according to the manufacturer’s protocol.

MdDCs were generated by culturing CD14⁺ monocytes in RPMI 1640 medium containing 10% FBS (Invitrogen, Carlsbad, CA) and 20 ng/ml IL-4 (R&D Systems, Minneapolis, MN) for 5 d.

Flow cytometry

MdDCs were characterized by the respective fluorochrome-conjugated mAb labels (CD1a-FITC or PE, CD80-PE, CD40-allophycocyanin, CD209-FITC, CD11b-PE, CD11c-allophycocyanin, CD1b-FITC, HLA-class II, HLA A chain-PE, CD14-allophycocyanin or FITC, CD83-FITC and CD86-allophycocyanin, all from BD Pharmingen, San Diego, CA), then analyzed using a BD FACSArray bioanalyzer. Data were collected by analyzing 10,000 events using CellQuest software (Becton Dickinson).

Allogeneic MLR

Human T cells were purified from PBMCs obtained from healthy consenting donors using immunomagnetic CD4 naïve T cell (MACS MicroBeads; Miltenyi Biotech) according to the manufacturer’s protocol. MLR was carried out by culturing naïve CD4 T cells (10⁶ cells/well) for a set number of days (4 d for cell proliferation assay, 1 d for cytokine assay, and 7 d for regulatory T cell [Treg] assay) with activated MdDCs (10⁵ cells/well) in 96-well plates. In some cases, CD4⁺ T cells were labeled with 10 μM BrdU on day 3 postculture (Millipore), and proliferation was then analyzed on day 4 by ELISA-based method.

Measurement of secreted factors

Supernatants from DCs or T cells were collected. Samples were tested for the presence of IL-2, IL-4, TNF-α, IL-10, IL-5, IL-12, IL-17, and IFN-γ by multiple cytokine analyses using cytokine bead array (Becton Dickinson). TGF-β and IL-27 levels were quantified using the Duoset ELISA Development System (R&D Systems) according to the manufacturer’s instructions. Galectin-1 levels were assessed by ELISA-based methods, as described previously.

Real-time RT-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 the ABI 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA). Each PCR reaction mixture contained 200 nM of each primer, 10 μL 2× SYBR Green PCR Master Mix (Applied Biosystems), 5 μL cDNA, and RNase-free water with a total volume of 20 μL. The PCR reaction was carried out with a denaturation step at 95°C for 10 min and then for 40 cycles at 95°C for 15 s and 60°C for 1 min. All PCRs were performed in triplicate and normalized to internal control GAPDH mRNA. Relative expression was presented using the 2⁻¹ΔCT reference sample – Ct experimental sample method.

Immunoblot/Immunoprecipitation

Cells were lysed on ice for 15 min by M-PER lysis reagent (Pierce, Rockford, IL). Cell lysate was centrifuged at 14,000 × g for 15 min, and the supernatant fraction collected for immunoblot. Equivalent amounts of protein were resolved by SDS-PAGE (8–12%) and transferred to polyvinylidene difluoride membranes. After blocking for 1 h in 5% nonfat dry milk in TBS, the membrane was incubated with the desired primary Ab for 1–16 h. The membrane was then treated with appropriate peroxidase-conjugated secondary Ab, and the immunoreactive proteins detected using an ECL kit (Millipore), according to the manufacturer’s instructions. Immunoprecipitation was performed by ProFound IP (Pierce) kit according to the manufacturer’s instructions.

IL-10 promoter assay

Plasmid pLL-10-Luc was constructed by inserting the proximal promoter sequence (−895 ± 121 bp; relative to the transcription initiation site) of human IL-10 gene into a transfected luciferase reporter vector (Panomics, Fremont, CA), which was confirmed by DNA sequencing. The Renilla luciferase vector (Promega, Madison, WI) was also cotransfected with the pLL-10-Luc as an internal control by electroporation (Amaxa Nucleofector II system). Promoter activity was measured in the cellular extracts using a dual-luciferase reporter assay system (Promega) and expressed as the luciferase activity (luciferase activity/Renilla activity) relative to that of the control group.

Gene knockdown by small interfering RNA

Monocytes were transfected with 1 μmol/l nontarget, Id3, or IL-10 Accell small interfering RNA (siRNA) pool (Dharmacon) in Accell delivery media (B-005000), according to the manufacturer’s instructions. Positive control Accell GAPDH siRNA and scrambled Accell siRNA pool were used in the experiments. After 72-h transfection, the medium was changed to whole media. The changes of Id3 and IL-10 were measured by real-time PCR, as described earlier. Knockdown of galectin-1 in A549 was performed by using a lentiviral expression system provided by National RNAi Core Facility (Taipei, Taiwan). Lentiviruses were produced by cotransfecting HEK293T with pLKO-AS2 or pLKO-AS2-LGALS1 short hairpin RNA (shRNA) and two packaging plasmids (pCMVVDR8.91 and pMD.G). Knockdown of galectin-1 in LLC cells was carried out using galectin-1 shRNA (m) lentiviral particles (Santa Cruz Biotechnology, Santa Cruz, CA) according to manufacturer’s instructions. Monocytes were nucleofected (Amaxa) with either 0.5 μg of the Id3-expressing plasmid or GFP-expressing plasmid. At 24 h posttransfection, monocytes were seeded in 24-well plates, and the culture medium was collected for IL-10 ELISA assay.

Animal models and isolation of CD11c⁺ cells from lungs

LLC cells were transplanted by the tail vein into C57BL/6 mice. Lung tissue was collected 14 d after injection, then minced and incubated in RPMI 1640 medium with collagenase type I (300 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. CD11c⁺ DCs were isolated from the cell suspension by CD11c magnetic beads (Miltenyi Biotech). Mouse bone marrow cells were harvested from the long bones of

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the limbs. Bone marrow cells were placed in RPMI 1640 containing murine GM-CSF (20 ng/ml) and murine IL-4 (20 ng/ml; R&D Systems), with or without murine galectin-1 or LLC-CM, for 48 h. The expression of various mRNAs was assayed by real-time PCR.

Immunofluorescence

Noncancerous and cancerous lung tissue obtained from both human and mouse were embedded in OCT and frozen in liquid nitrogen. Sections (3–5 μm) were fixed with acetone at −20°C and then stained using anti–galectin-1 Ab (R&D Systems) or costained by anti-CD11c Ab and anti–IL-10 Ab (1:10; Abcam, Cambridge, U.K.). After washing with PBS containing 0.1% Tween 20, slides were incubated with Dylight 488- or Dylight 549-conjugated secondary Abs (Rockland, Gilbertsville, PA), with or without DAPI, for 1 h at room temperature. The data were analyzed with a confocal laser-scanning microscope (Fluoview FV500; Olympus, Tokyo, Japan).

Statistical analysis

Data were expressed as means ± SD. Statistical comparisons of the results were made using ANOVA. Significant differences (p < 0.05) between the means of the two test groups were analyzed by Student t test.

Results

Galectin-1 is expressed differently in lung cancer cell lines and in patients with lung cancer

Gene expression profiling of lung cancer cells was initially performed to begin assessing the effect of lung cancer on immune regulation. The results showed that, when compared with normal bronchial epithelial cells, significantly increased expression of genes encoding several known immune modulators was found. Among them, galectin-1 was found to be highly expressed in the culture media of two lung cancer cell lines, A549 and NCI-H460 (Supplemental Fig. 1A). The expression of galectin-1 mRNA was significantly greater in tumor sections than in nontumor sections by real-time PCR analysis (n = 15; p = 0.00026; Fig. 1A). In addition, galectin-1 levels were also increased in the sera of the 40 lung cancer patients, compared with that obtained from the 15 healthy donors (Supplemental Fig. 1B).

Phenotypic changes of MdDCs in the presence of galectin-1 and lung cancer cell-CM

To examine whether galectin-1 influences the function of DCs, the effect of lung cancer-CM and galectin-1 on the expression of surface markers on MdDCs was examined. As expected, monocytes cultured in the presence of IL-4 and GM-CSF differentiated into immature MdDCs with characteristic marker expression, including CD209 and CD11c, whereas the expression of CD14, a monocyte marker, was lost (Fig. 1B). However, significant modification was noted for the expression pattern of CD1a and CD14 in cells cultured in media containing lung cancer-CM or galectin-1. Compared with control MdDCs, CD1a expression was lower in MdDCs treated with A549-CM, NCI-H460-CM, and galectin-1, and a subpopulation of these cells still showed significant expression of CD14 (Fig. 1B). The relative expression levels of CD80 and CD1b were lower in A549-CM−, NCI-H460-CM−, and galectin-1−treated MdDCs (Fig. 1B). Importantly, the addition of a galectin-1 blocker, disaccharide lactose, known to prevent binding of galectin-1 to glycosylated proteins, reversed the mod-

![FIGURE 1. Lung cancers change MdDC phenotype by secreting galectin-1. A. The expression of galectin-1 on normal and cancerous lung sections. The expression of galectin-1 in normal and cancer section was determined by real-time PCR (n = 15). B. The effect of lung cancer-CM and galectin-1 on the surface markers of MdDCs. C. Lactose reverses the effect of lung cancer-CM and galectin-1 on the phenotypes of MdDCs. CD14^+ monocytes were cultured with RPMI 1640, A549-CM (20%), NCI-H460-CM (20%), and galectin-1 (2 μg/ml) presented in GM-CSF (20 ng/ml) and IL-4 (20 ng/ml) with or without lactose (50 mg/ml) for 5 d. The expression of various surface markers was analyzed by flow cytometry. The percentage of positive cells and mean fluorescence intensity (within parentheses) is indicated. D. Sera from lung cancer patients changed the phenotypes of MdDCs. CD14^+ monocytes were cultured for 5 d in the presence of GM-CSF and IL-4, with sera (15%) from healthy donors and lung cancer patients. Results are representative of at least four independent experiments. Horizontal bars represent means. The asterisk indicates a significant difference between the two test groups, as analyzed by ANOVA with Student t test (*p < 0.05).]
ulatory activity of A549-CM, NCI-H460-CM, and galectin-1 on alterations of MdDCs (Fig. 1C, Supplemental Fig. 1C). For comparison, cytokines that have been found to be overexpressed in lung cancer cell lines, including CXCL1, CXCL5, and IL-8 (17, 18), showed no apparent regulatory effects on the phenotype of MdDC (Supplemental Fig. 1D).

Moreover, when monocytes were cultured in the presence of 15% serum from each of the 20 lung cancer patients, similar alterations in the patterns of CD1a and CD14 expression were found, comparable with those seen in MdDCs cultured in the presence of healthy donor sera. A reduction of CD1a, CD1b, CD40, and CD80 expression was also observed when the cells were cultured in the presence of patient’s sera. Again, the expression of CD14 remained high in a subset of MdDCs when lung cancer patient sera were present in the culture media (Fig. 1D, Supplemental Fig. 1E).

Lung cancer-CM and galectin-1 modify the function and T cell response of MdDCs

We assessed the effect of lung cancer-CM and galectin-1 on the function of MdDCs. Exposure of MdDCs to LPS increased all mature markers of MdDCs, including CD1a, CD83, and CD86. In contrast, A549-CM, NCI-H460-CM, and galectin-1 suppressed the upregulation of CD1a, CD83, and CD86 in LPS-stimulated MdDCs (Fig. 2A). A549-CM, NCI-H460-CM, and galectin-1 also decreased the ability of MdDCs to produce proinflammatory cytokines IL-12 and TNF-α, whereas they increased secretion of immunosuppressive IL-10 (Fig. 2B).

The major function of DCs is to present Ags to the T cells and induce T cell proliferation and activation (19). The functional consequences of lung cancer-CM– and galectin-1–conditioned MdDCs on the adaptive T cell responses were then examined. In comparison with those cultured in normal conditions, lung cancer-CM– or galectin-1–conditioned MdDCs showed impaired ability to induce naive CD4+ T cell proliferation (Fig. 2C). Significantly, coculture of naive CD4+ T cells with A549-CM–, NCI-H460-CM–, and galectin-1–conditioned MdDCs led to secretion of significantly lower amounts of Th1 cytokines (IFN-γ and TNF-α) and increased IL-10 production, when compared with CD4+ T cells stimulated by unconditioned MdDCs. In addition, the expression of Th2 cytokines, IL-4 and IL-13, as well as IL-17A, was also enhanced in CD4+ T cells after coculture with lung cancer-CM– or galectin-1–conditioned MdDCs (Supplemental Table II). Furthermore, significantly increased frequency of the naturally oc-
curring CD4⁺CD25⁺FOXP3⁺ Tregs was noted when naive CD4⁺ T cells were cocultured with lung cancer-CM– and galectin-1–conditioned MdDCs (Fig. 2D). Interestingly, the impact of conditioned MdDCs on the generation of Tregs was corroborated by the finding that A549-CM, NCI-H460-CM, and galectin-1 all increased MdDCs’ production of IL-27 and TGF-β (Supplemental Fig. 2A, 2B), both of which have been reported to induce CD4⁺CD25⁺FOXP3⁺ and generate Tregs from naive T cells (20).

*Lung cancer-CM and galectin-1 increased the expression of IL-10, which changes the phenotypes of MdDCs by autocrine effect*

Previous studies have reported that tumors can inhibit the differentiation of MdDCs from monocytes through their ability to produce IL-10 (21, 22). However, neither A549-CM nor NCI-H460-CM expressed detectable levels of IL-10 protein (data not shown). In contrast, A549-CM, NCI-H460-CM, and galectin-1 increased the production of IL-10 in mRNA and protein levels in CD14⁺ monocytes (Fig. 3A, Supplemental Fig. 3A). Addition of recombinant human IL-10 (rhIL-10) attenuated the stimulation of CD1a, CD80, and CD1b, and reversed the decrease of CD14 caused by GM-CSF and IL-4 (Fig. 3B). This inhibitory pattern coincided with the inhibitory effect of lung cancer cell-CM and galectin-1 on MdDCs. The addition of lactose decreased the effect of A549-CM, NCI-H460-CM, and galectin-1 on the upregulation of IL-10 (Fig. 3C). Moreover, galectin-1 knockdown A549-CM lost its activity in upregulating IL-10 (Supplemental Fig. 3B). To evaluate the contribution of IL-10 to the inhibitory effect of lung cancer-CM and galectin-1 on MdDCs, CD14⁺ monocytes were treated with RPMI 1640, A549-CM (20%), NCI-H460-CM (20%), and galectin-1 (2 μg/ml) or IL-10 (20 ng/ml) presented in GM-CSF and IL-4 with or without lactose (50 mg/ml), for the indicated times (5 d for surface marker assay, 1 d for lactose blocking assay). mRNA levels and various cell surface markers were measured. D. IL-10 knockdown reversed the alteration of lung cancer-CM and galectin-1 on MdDC phenotype. siRNA transfection CD14⁺ monocytes were treated with RPMI 1640, lung cancer-CM (20%), and galectin-1 (2 μg/ml) presented in GM-CSF and IL-4 for 5 d. Surface markers were assessed by flow cytometry. Results are representative of at least three independent experiments. Asterisk indicates a significant difference between the two test groups, as analyzed by ANOVA with Student t test post hoc (*p < 0.05; **p < 0.01).
cancer-CM and galectin-1 on MdDC differentiation, we interfered with the effects of IL-10 by siRNA transfection. Compared with nontarget siRNA-transfected CD14+ monocytes, IL-10 siRNA effectively decreased IL-10 expression in CD14+ monocytes by 85% (Supplemental Fig. 3C). Knockdown of IL-10 by siRNA from CD14+ monocytes was sufficient to reverse the effect of lung cancer-CM and galectin-1 on the differentiation of DCs (Fig. 3D).

The effects of lung cancer cell-CM and galectin-1 are mediated by their ability to modify the expression and interaction of Id3 and E2A protein

The basic helix-loop-helix (bHLH) proteins and HLH inhibitor Id family proteins have been reported to be involved in many types of cell differentiation, including DCs (23, 24). We therefore assessed the effects of lung cancer-CM and galectin-1 on the expression of Ids and E protein. The results showed that although lung cancer-CM or galectin-1 did not modify the expression of Id1, Id2, and E2-2 (data not shown), they increased Id3 expression in CD14+ monocytes. In addition, A549-CM, NCI-H460-CM, and galectin-1 also decreased the levels of E2A (Fig. 4A). Furthermore, two lung cancer-CMs and galectin-1 also increased the association of Id3 and E2A in CD14+ monocytes in a time-dependent manner (Fig. 4B).

Knockdown of Id3 was carried out using the siRNA-based method to better understand the role of Id3. The results showed that, in comparison with control siRNA transfection, Id3 siRNA reduced Id3 expression in CD14+ monocytes (Supplemental Fig. 4A). Specific knockdown of Id3 expression reversed the inhibitory effect of A549-CM and NCI-H460-CM on the differentiation of MdDCs from CD14+ monocytes (Fig. 4C). Interestingly, galectin-1 decreased the cell viability of CD14+ monocytes after a 48-h treatment if Id3 was knocked down by siRNA. However, the effect of galectin-1 on CD14 level was reversed in cells with Id3 knockdown after a 24-h treatment (Fig. 4C). Moreover, the addition of lactose inhibited lung cancer-CM and galectin-1–mediated upregulation of Id3, and galectin-1 knockdown A549-CM lost its activity in upregulating Id3, suggesting that galectin-1 is involved in lung cancer-mediated Id3 upregulation (Fig. 4D, Supplemental Fig. 4B).

To determine whether IL-10 upregulation is controlled by Id3, we assessed the levels of IL-10 in monocytes that had undergone Id3 knockdown or Id3 overexpression. Id3 knockdown abrogated A549-CM–, NCI-H460-CM–, and galectin-1–mediated upregulation of IL-10 (Fig. 5A), whereas Id3 overexpression enhanced IL-10 production in CD14+ monocytes (Supplemental Fig. 5A). In

![FIGURE 4](http://www.jimmunol.org/)

**FIGURE 4.** Lung cancer-CM and galectin-1 change the expressions and interaction of Id3 and E2A protein. A, The amount of Id3 and E2A proteins. B, Interaction of Id3 and E2A. CD14+ monocytes were treated with RPMI 1640, A549-CM (20%), NCI-H460-CM (20%), and galectin-1 (2 μg/ml) presented in GM-CSF/IL-4 for the indicated times. Protein levels and Id3/E2A interaction were determined by immunoblot and immunoprecipitation, respectively. C, Id3 knockdown reversed the alteration of lung cancer-CM and galectin-1 on MdDCs. Id3 knockdown was performed by Acell scramble or Id3 siRNA (1 μM). After 72-h transfection, siRNA-transfected CD14+ monocytes were treated with RPMI 1640, A549-CM (20%), NCI-H460-CM (20%), and galectin-1 (2 μg/ml) for 5 (lung cancer-CM) or 1 d (galectin-1), and the surface markers were assessed by flow cytometry. D, Lactose inhibited lung cancer-CM and galectin-1–mediated Id3 upregulation. Results are representative of at least three independent experiments. Asterisk indicates a significant difference between the two test groups, as analyzed by ANOVA with Student t test post hoc (*p < 0.05; **p < 0.01).
addition, significantly increased IL-10 promoter activity was noted in CD14+ monocytes in the presence of A459-CM, NCI-H460-CM, or galectin-1 (Fig. 5B). Increased IL-10 promoter activity was also found in cells overexpressing Id3 (Supplemental Fig. 5B). Interestingly, IL-10 knockdown also decreased the upregulation of Id3 by lung cancer-CM and galectin-1 in CD14+ monocytes (Fig. 5C), whereas the addition of rhIL-10 in the culture media also increased Id3 expression in CD14+ monocytes after 12 and 24 h of exposure (Supplemental Fig. 5C), suggesting the presence of positive feedback between the regulation of Id3 and IL-10. We have further assessed the molecular mechanism of IL-10 on the regulation of Id3 by using various kinase inhibitors (MEK1/2, p38, JNK, PI3K/AKT, pan-protein kinase C (PKC) and PKCδ, and Jak2 inhibitors). The results showed that only PI3K/AKT inhibitor decreased the upregulation of IL-10 on Id3 expression. In contrast, Jak2 and pan-PKC inhibitors increased the enhancement of Id3 by IL-10 (Fig. 5D). Immunoblot assay also revealed that rhIL-10 increased the phosphorylation of AKT (Thr308 and Ser473; Supplemental Fig. 5D). These data suggested that activation of AKT may be involved in IL-10–mediated Id3 upregulation.

High amounts of IL-10 in CD11c+ DCs are found in the tumor sections of lung cancer-bearing mice and human lung cancer patients

To confirm the role of galectin-1 on lung cancer-mediated immune suppression in humans, we characterized the amount of IL-10 in tumor-infiltrating CD11c+ DCs. Immunofluorescence staining of the marginal region of patient’s lung cancers revealed the presence of many CD11c+ DCs that had infiltrated the area around the tumor, and those areas expressed greater levels of IL-10 in comparison with tissue sections of nontumor regions (Fig. 6A). In contrast with CD11c+ DCs from nontumor regions, CD11c+ DCs isolated from fresh lung cancers also showed a stronger ability to produce IL-10 within 24 h in culture (Fig. 6B). However, no statistical differences could be found between sera of lung cancer patients and healthy donors (Supplemental Fig. 6), suggesting the changes of IL-10 are found only in the affected lung.

Finally, we used animal experiments to determine whether lung cancer increased IL-10 expression in DCs in vivo, and whether galectin-1 acts as a major regulator on upregulation of IL-10 in CD11c+ DCs.
immune cells. We injected mouse lung cancer cell line LLC into mice and allowed the tumor to develop for 14 d. Compared with normal lung tissues of mice, LLC expresses high levels of galectin-1 (Supplemental Fig. 7A). LLC-CM and murine galectin-1 (mgalectin-1) also increased the production of IL-10, in an autocrine fashion, via a mechanism involving Id3. Furthermore, galectin-1-modified MDSCs were shown to influence the expression of IFN-γ whereas increasing the secretion of IL-10 from T cells. In the same culture conditions, significantly increased frequency of CD4+CD25+FOXP3+ Tregs was also noted. These findings suggest that lung cancer-derived galectin-1 may be a novel candidate in conferring the ability of lung cancer to evade immune surveillance.

Galectin-1 has been reported to regulate immune responses by controlling DC differentiation and T cell survival, cytokine secretion, and transendothelial migration (27–29). Galectin-1 is also believed to be involved in immunosuppression by increasing inhibitory activity of CD4+CD25+FOXP3+ Tregs (16). Recent evidence indicates that galectin-1 increases MDSCs to produce IL-27, which, in turn, results in IL-10–mediated T cell tolerance, thereby resulting in immunosuppression (29). However, whether galectin-1 is involved in generating similar immunosuppressive mechanisms in lung cancer has not been determined. Our data demonstrate that galectin-1 is involved in lung cancer-mediated immune suppression by virtue of its ability to modify DC phenotype and induce IL-10 production, which not only decreases T cell activation but also increases the CD4+CD25+FOXP3+ Treg population. Several lines of evidence support this view. First, galectin-1–containing lung cancer cell medium and patient sera selectively alter the expression of CD1a and CD14 in MDSCs and the upregulation of IL-10. Second, this effect is mediated through, at least in part, its recognition of glycosylated cellular proteins, as the blockade of galectin-1 binding by lactose significantly decreased the effect of galectin-1.

It is of interest to note that IL-10–producing CD11c+ DCs were found to infiltrate cancerous lung tissue of LLC-bearing mice, whereas this phenomenon is significantly decreased in the lung sections of galectin-1 knockdown LLC-bearing mice. Furthermore, CD11c+ DCs isolated from fresh galectin-1–high-expressing human tumor tissue also produced high amounts of IL-10 compared with CD11c+ DCs purified from galectin-1–low-expressing nontumor regions. These results from experimental cell studies, mouse models, and clinical patient sections strongly suggest that galectin-1 is an important effector molecule through which lung cancer cells evade the host’s immune surveillance.

Although some studies have reported that exposure of MDSCs to high doses (20 μM) of galectin-1 increased DC activation and migration ability (30), our study showed that lower concentrations (2 μg/ml), which are close to natural physiological levels, can switch DCs from their immunostimulatory characteristics to a
Tolerogenic phenotype in human and mouse DCs when added during differentiation. This difference may be caused by the concentration and the timing of DC exposure to galectin-1.

IL-10 in the tumor microenvironment can be produced from hematopoietic and nonhematopoietic sources (25, 31). IL-10 has been reported to be expressed in a variety of human malignancies (25, 32, 33). In addition to tumor cells, tumor-associated macrophages have also been shown to express an increased production of IL-10 and low levels of IL-12 and TNF-α (34, 35). The presence of IL-10–producing DCs within tumors is associated with increased Treg populations, which have been implicated in playing a crucial role in the occurrence of tumor-mediated immune escape (36, 37). Indeed, increase of IL-10 production in human cancers is correlated with poor prognosis (38, 39). We have observed that although lung cancer expresses low levels of IL-10, the main source of this cytokine in the tumor microenvironment is the tumor-infiltrating CD11c+ DCs.

bHLH family transcription factors play an important role in determining the differentiation of cell lineages (23). The bHLH protein has been identified as being required for the development of plasmacytoid DCs (pDCs), as seen in E-box protein-deficient mice, which have a lower percentage of pDCs (23). bHLH transcription factors are antagonized by Id proteins, of which four (Id1–4) have been identified. Heterodimerization of Id with E-box protein results in the loss of E2A protein’s DNA binding ability (23). Id3 has negative functions in the immune system, including blocking of B and T cell development and immunity, and CD123high pDC2 differentiation (40, 41). To our knowledge, our data demonstrate that lung cancer-CM and galectin-1 increased the expression of Id3, which, in turn, interacts with E2A protein. Knockdown Id3 by specific siRNA in monocytes reversed effects of lung cancer-CM and galectin-1 on the changes of MdDC phenotype and the increase of IL-10 levels. Overexpression of Id3 significantly enhanced IL-10 production in monocytes. Indeed, genetic blockade of IL-10 not only abrogated lung cancer-CM and galectin-1–mediated MdDC phenotypic alteration, but also attenuated the upregulation of Id3 by AKT activation, suggesting the presence of positive feedback between Id3 and IL-10. Id3 has been reported as being involved in TGF-β–activated alternative activation of macrophages, which express increased levels of IL-10 (42). In our studies, those signaling events known to be associated with IL-10 expression were examined, but neither cAMP nor any members of the MAPK family were found to be activated in galectin-1–treated cells (data not shown). Therefore, further studies are required to investigate the signaling pathway of Id3 on the regulation of IL-10 expression.

Taken together, our findings suggest a new mechanism of immune surveillance against lung cancer, in which cancer-derived galectin-1 not only modifies DC phenotype and impairs DC function, but even causes an increase of immunosuppressive Tregs.
Galectin-1 induced monocytes to produce IL-10, which, in turn, results in DC anergy by autocrine effects. We also detected IL-10–expressing CD11c+ DCs infiltrating mouse tumors and human lung cancers. Knockdown of galectin-1 decreased the level of infiltrating IL-10–expressing DCs in lung cancer and significantly decreased the incidence of cancer development in mice. In light of these findings, inhibition of galectin-1 is an attractive therapeutic target for lung cancer-mediated immunosuppression.

Disclosures

The authors have no financial conflicts of interest.

References