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Galectin-9 Induces Maturation of Human Monocyte-Derived Dendritic Cells

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Maturation of dendritic cells (DCs) is critical for initiation of immune responses and is regulated by various stimulatory signals. We assessed the role of galectin (Gal)-9 in DC maturation. Culture of immature DCs with exogenous Gal-9 markedly increased the surface expression of CD40, CD54, CD80, CD83, CD86, and HLA-DR in a dose-dependent manner, although Gal-9 had no or little effect on differentiation of human monocytes into immature DCs. Gal-9-treated DCs secreted IL-12 but not IL-10, and they elicited the production of Th1 cytokines (IFN-γ and IL-2) but not that of the Th2 cytokines (IL-4 and IL-5) by allogeneic CD4$^+$ T cells. These effects of Gal-9 on immature DCs were not essentially dependent on its lectin properties, given that they were inhibited only slightly by lactose. We further found that a Gal-9 mutant that lacks β-galactoside binding activity reproduced the above activities and that an anti-Gal-9 mAb suppressed them. Gal-9 induced phosphorylation of the MAPK p38 and ERK1/2 in DCs, and an inhibitor of p38 signaling, but not inhibitors of signaling by either ERK1/2 or PI3K, blocked Gal-9-induced up-regulation of costimulatory molecule expression and IL-12 production. These findings suggest that Gal-9 plays a role not only in innate immunity but also in acquired immunity by inducing DC maturation and promoting Th1 immune responses. ***The Journal of Immunology,*** **2005, 175: 2974–2981.**

Galectins (Gal)[2] are animal lectins that exhibit affinity for β-galactosides (1) and share certain conserved sequence elements (2). To date, 14 Gals have been cloned in mammals and shown to play modulatory roles in diverse biological processes such as cell adhesion and proliferation (3–6), T cell apoptosis (7, 8), and immunomodulation of inflammation (9–11).

Structurally, Gal-1 is classified as the prototype Gal, whereas Gal-3 is a chimera-type Gal, and Gal-8 and -9 belong to the tandem-repeat subfamily that is characterized by the presence of two distinct carbohydrate recognition domains (CRDs) joined by a linker peptide (12, 13). We have examined previously the roles of Gal in cells of the myeloid lineage, showing that Gal-3 preferentially acts on macrophages as an activator of chemoattraction (14), that Gal-8 induces adhesion activity and superoxide production in neutrophils (4), and that Gal-9 possesses eosinophil chemoattractant activity as well as induces superoxide production and prolongs cell survival in eosinophils (15). In addition, Gal-9 has been shown to induce apoptosis in a variety of cells, including activated T lymphocytes (8) and tumor cell lines (16).

Gal are also implicated in myeloid differentiation (17). We have thus shown that Gal-9 expression gradually decreases, whereas Gal-10 expression increases during eosinophil differentiation and that Gal-9 expression decreases, whereas Gal-3 expression increases during monocyte differentiation. However, we fail to detect changes in the levels of Gal-1 and Gal-8 expression during cell differentiation. Despite the importance of dendritic cell (DC) maturation in the initiation of immune responses and in DC-based vaccine therapies for various cancers, little is known of the possible roles of Gal in DC maturation. Gal-9 mRNAs were found to be present in both immature and mature DCs and to be increased 2-fold during DC maturation (18), indicating that Gal-9 may play a role in this process. We now investigate whether Gal-9 indeed contributes to DC maturation.

Materials and Methods

Expression and purification of recombinant proteins

Expression of recombinant human Gal-9 in *Escherichia coli* BL-21 cells was achieved as described previously (4, 19, 20). Given that it was difficult to obtain a stable preparation of a Gal-9 mutant in which both CRDs lack carbohydrate binding activity, we prepared a mutant (Gal-9 (R65D)) in which the N-terminal CRD lacks such activity; this mutant failed to exhibit detectable hemagglutination and eosinophil chemoattractant activities (19, 20). The mutation was confirmed by DNA sequencing.

Recombinant proteins were purified by affinity chromatography on a lactose-agarose column (Seikagaku Kogyo) or a glutathione-Sepharose column (Amersham Biosciences). Protein concentration was determined with bicinchoninic acid assay reagent (Pierce) and with BSA as a standard. The purity of the protein preparations was checked by SDS-PAGE as described previously (19, 20). All Gal preparations were checked for endotoxin contamination by Limulus assay (BioWhittaker); any contaminating LPS was removed with the use of polymer particles as described previously (21). The final endotoxin content of Gal used in this study was <0.008 endotoxin units (equivalent to 0.8 pg of LPS).

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[2] Abbreviations used in this paper: Gal, galectin; CRD, carbohydrate recognition domain; DC, dendritic cell.

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Generation and purification of a mAbs for human Gal-9

Murine mAbs against human Gal-9 were generated and purified from mice hyperimmunized with recombinant human Gal-9 by the procedures described previously (22). Anti-Gal-9 mAb (9S2-1) has been confirmed to be of neutralization effect on the functional study of Gal-9 (our unpublished data).

Generation of monocyte-derived DCs

Monocyte-derived DCs were generated as described previously (23). In brief, human PBMCs from healthy donors were isolated from the interface of a Ficoll-Paque density gradient (Amersham Biosciences). Monocytes were purified from the PBMCs by positive selection with a MACS column containing microbeads conjugated with Abs to CD14 (Miltenyi Biotec). The purity of the isolated $CD14^+$ monocytes was >90% as determined by flow cytometry.

For the induction of DC differentiation, the purified $CD14^+$ monocytes were cultured under a humidified atmosphere of 5% CO_2 at 37°C in RPMI 1640 supplemented with 10% FBS, 1 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 ng/ml human rGM-CSF (PeproTech), and 100 ng/ml human rIL-4 (R&D Systems). Half of the old medium was replaced every 2 days with fresh medium. After culture for 6 days, the cells became to exhibit morphology typical of immature DCs and a surface phenotype of $CD14^-$, $CD80^{low}$, $CD83^-$, and $CD86^{semi}$ as assessed by flow cytometry. To assess the influence of Gal-9 on differentiation of monocytes into immature DCs, the monocytes were cultured with different doses of Gal-9 in the same medium with or without GM-CSF and IL-4.

To induce further conversion of the cells into mature DCs, we added *E. coli* LPS (500 ng/ml) (Sigma-Aldrich) or 0.1 Klinische Einheit/ml OK432 (Chugai Pharmaceutical), a *Streptococcus*-derived anticancer immunotherapeutic agent and/or rGal-9 to the culture medium, and incubated the cells for an additional 2 days.

Various types of cells undergo apoptosis during the culture with 1 μM (~33 μg/ml) Gal-9 (8, 16). Indeed, after the culture of immature DCs for 2 days with Gal-9 at 33 μg/ml, the number of cells was <10% of that apparent for cultures incubated without Gal-9; in contrast, cell number was less affected by incubation with Gal-9 at 10 μg/ml (data not shown). Therefore, we used Gal-9 at a maximal concentration of 10 μg/ml for our experiments. When indicated, different concentrations of lactose were added to the culture medium, with sucrose at the same concentration serving as a control. In some cases, Gal-9 or LPS was heated (95°C, 20 min), or cells were preincubated for 1 h with 10 μg/ml polymixin B sulfate (Calbiochem). In other blocking experiments, cells were preincubated with anti-Gal-9 mAb (clone: 9S2-1, 10 μg/ml) or anti-TLR2 mAb (Alexis Biochemicals; clone: TL2.1, 10 μg/ml) or anti-TLR4 mAb (Alexis Biochemicals; clone: HTA125, 10 μg/ml) for 1 h before Gal-9 was added. In experiments with SB203580 (Calbiochem), PD98059 (Calbiochem), or wortmannin (Sigma-Aldrich), the cells were treated with the respective inhibitor at 37°C for 1 h before exposure to LPS or Gal-9; DMSO, at 0.1% (v/v), was used as a control given that the inhibitors were dissolved in this solvent.

Flow cytometric analysis

The expression of costimulatory molecules and HLA-DR on the surface of DCs was characterized by four-color flow cytometric analysis. FITC-conjugated mAbs to CD80 (mouse IgM, clone BB1), PE-conjugated mAbs to CD86 (mouse IgG2b, clone IT2.2), allophycocyanin-conjugated mAbs to CD83 (mouse IgG1, clone HB15e), PE-conjugated mAbs to CD14 (mouse IgG2a, clone M5E2), and allophycocyanin-conjugated mAbs to HLA-DR (mouse IgG2a, clone G46-6) were obtained from BD Biosciences. FITC-conjugated mAbs to CD54 (mouse IgG1, clone 84H10), PE-conjugated mAbs to CD40 (mouse IgG1, clone MAB89), and 7-aminoactinomycin D were obtained from Beckman Coulter. Before staining with Abs, DCs were incubated for 10 min on ice with human IgG (100 μg/ml) (Chemicon International) to block nonspecific binding of Abs to FcRs. Cells were analyzed with a FACSCalibur instrument (BD Biosciences); dead cells were excluded from analysis on the basis of 7-aminoactinomycin D staining. Data were processed with CellQuest software (BD Biosciences).

ELISA

The amounts of IL-12 (p70) and IL-10 released into the culture medium of DCs were measured with ELISA kits (Pierce). The detection limit for both IL-12 and IL-10 was 15.4 pg/ml.

Allogeneic MLR

For use as responder cells, T cells were isolated from PBMCs of healthy donors with a MACS column containing microbeads conjugated with Abs to CD4 (Miltenyi Biotec). DCs were treated for 30 min at 37°C with mitomycin C (50 μg/ml) (Kyowa Hakko Kogyo) and then washed three times for use as stimulator cells. Purified allogeneic T cells (>90% $CD4^+$ cells) were distributed at a density of 1×10^5 cells/well into U-bottom 96-well microplates. Various numbers of mitomycin C-treated DCs were then added to the wells, and the cell mixtures were cultured at 37°C under 5% CO_2. After culture for 3 days, 0.5 μCi of [^{3}H]thymidine (Amersham Biosciences) was added to each well, and the cells were incubated for an additional 18 h. The cells were then harvested on glass fiber filters, and the cell-associated radioactivity was assessed by scintillation spectroscopy with TopCount (Packard Instrument). Results were expressed in counts per minute and as the mean of triplicate cultures.

Production of Th1 and Th2 cytokines

After culture of DCs with T cells at a ratio of 1:10 for 48 h, the culture supernatants were collected and frozen at −30°C until analysis. The amounts of cytokines (IFN-γ, IL-2, IL-4, IL-5, IL-10, and TNF-α) in the culture supernatants were determined with a cytometric bead assay kit (BD Biosciences). In brief, the supernatants were mixed with human cytokine capture beads before the addition of PE detection reagent and incubation at room temperature for 3 h in the dark. After washing, the beads were analyzed with a FACSCalibur instrument and cytometric bead assay software (BD Biosciences).

Apoptosis assay

The apoptosis of DCs were assayed as described previously (8). In brief, DCs were collected and resuspended in 300 μl of PBS and then 700 μl of 100% ethanol. After washing, the cells were incubated with 50 μg/ml RNase A (Sigma-Aldrich) for 30 min at 37°C, then with 50 μg/ml propidium iodide (Sigma-Aldrich) for 10 min. Stained cells were analyzed by flow cytometry.

Immunoblot analysis

Immature DCs were exposed to LPS (500 ng/ml) or Gal-9 (10 μg/ml), washed twice with ice-cold PBS, and subjected to ultrasonic treatment in lysis buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM PMSF, 10 mg/ml leupeptin, 1 mg/ml pepstatin A, 1 mg/ml aprotinin, 1 mM sodium orthovanadate, 50 mM NaF, 2 mM sodium pyrophosphate, and 1% Nonidet P40). The cell lysates were centrifuged for 15 min at 10,000 × *g*, the resulting supernatants were subjected to SDS-PAGE under reducing conditions, and the separated proteins were transferred to an Immobilon polyvinylidene difluoride membrane (Millipore). The membrane was incubated with 5% skim milk in a solution containing 50 mM Tris-HCl (pH 7.6), 150 mM NaCl, and 0.05% Tween 20 to block nonspecific binding before incubation overnight at 4°C with Abs specific for the phosphorylated forms of the MAPKs p38 or ERK1 or ERK2; it was then reprobed with Abs to total p38 or ERK1/2 (all Abs were purchased from Cell Signaling Technology). For reprobing, the membrane was incubated between Ab exposures in stripping buffer (62.5 mM Tris-HCl (pH 6.7), 100 mM 2-ME, and 2% SDS) for 30 min at 50°C with agitation. The blot was developed with a Phototope-HRP Western Blot Detection System (Cell Signaling Technology).

Statistical analysis

All data are expressed as means ± SD, and differences among groups were analyzed by the Mann-Whitney *U* test with the use of StatView software (Abacus Concepts). A value of $p < 0.05$ was considered statistically significant.

Results

Effects of Gal-9 on differentiation of monocytes into immature DCs and on DC maturation

We first examined the influence of Gal-9 on differentiation of monocytes into immature DCs. Purified human monocytes were cultured with Gal-9 alone (in medium without GM-CSF and IL-4 supplement) or Gal-9 in the medium supplemented with GM-CSF and IL-4 for 6 days. Then the cells were analyzed for surface markers by flow cytometry. As expected, the cells incubated with GM-CSF and IL-4 exhibited a typical phonotype of immature DCs with low expression of CD80 and CD86, whereas the cells without GM-CSF and IL-4 incubation did not (Fig. 1*A*). Exogenous Gal-9 exhibited no or little effect on GM-CSF and IL-4-mediated differentiation of monocytes into immature DCs (Fig. 1*A*).

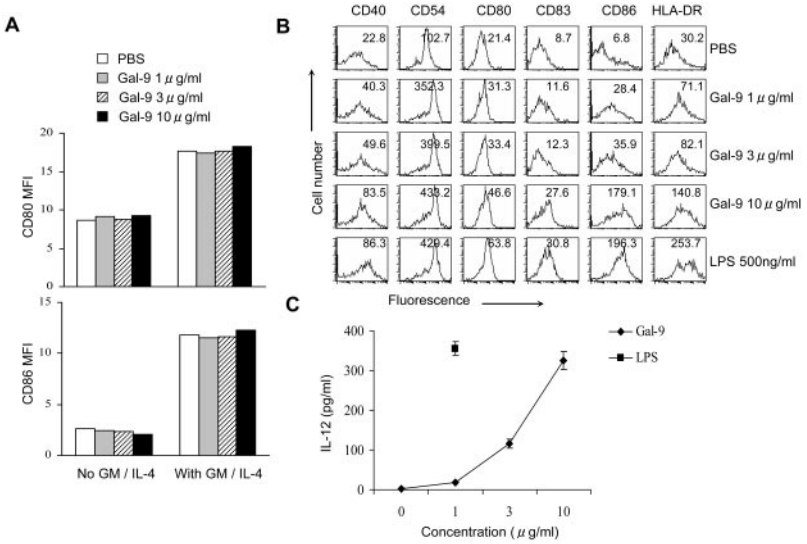

FIGURE 1. Effects of Gal-9 on DC differentiation and maturation. *A*, Purified human monocytes were cultured with Gal-9 alone (in medium without GM-CSF and IL-4 supplement) or Gal-9 in the medium supplemented with GM-CSF and IL-4 for 6 days at the indicated concentrations, and then cells were analyzed for surface markers by flow cytometry. Data are expressed as mean fluorescence intensity (MFI) for each molecule. *B*, Immature DCs were cultured for 2 days with Gal-9 or LPS at the indicated concentrations, after which the cells were analyzed for surface expression of the indicated molecules. Data are expressed as MFI for each molecule. *C*, The culture supernatants of DCs incubated as in *B* were collected and assayed for IL-12 by ELISA. Data are means ± SD of triplicates from one experiment. PBS was used as a negative control. Data in *A–C* are representative of five independent experiments.

We next examined the effects of exogenous Gal-9 on DC maturation. Surprisingly, culture of immature DCs with Gal-9 for 2 days in the presence of GM-CSF and IL-4 resulted in a marked up-regulation of the surface expression of CD40, CD54, CD80, CD83, CD86, and HLA-DR dose dependently (Fig. 1*B*). In contrast to Gal-9, other Gal, such as Gal-1 and Gal-3, failed to up-regulate the surface expression of the above molecules (data not shown). In addition, Gal-9 greatly increased the production of IL-12 (p70) by DCs in a dose-dependent manner (Fig. 1*C*), whereas the amount of IL-10 was below the limit of detection (data not shown). LPS-treated DCs were used as a control.

Gal-9-induced enhancement of Ag-presenting activity and Th1 cytokine secretion in an allogeneic MLR

The ability to induce allogeneic T cell proliferation is a functional hallmark of DCs in vitro. We thus compared the allostimulatory activity of DCs before and after induction of maturation by LPS or treatment with Gal-9. Coculture of T cells with Gal-9-treated DCs resulted in enhancement of T cell proliferation (Fig. 2*A*), albeit to a lesser extent than that observed with LPS-treated DCs, indicating that Gal-9 potentiates the Ag-presenting activity of DCs in an allogeneic MLR. We also examined cytokine release into the culture supernatant during the MLR. Gal-9-treated DCs increased the secretion of the cytokines IFN-γ, TNF-α, and IL-2 from allogeneic $CD4^+$ T cells to an extent similar to that observed with LPS-treated DCs (Fig. 2*B*). Whereas the Gal-9-treated DCs had no effect on the production of IL-4 and IL-5, they also induced a significant increase of IL-10 secretion.

Gal-9-induced maturation of DCs is not caused by LPS contamination

To exclude the possibility that Gal-9-induced DC maturation is due to LPS contaminated, we pretreated Gal-9 and LPS with heating at 95°C or preincubated the cells for 1 h with polymixin B sulfate before induction. The activity of LPS was expectedly abrogated by polymixin B but not by heating. In contrast, the effect of Gal-9 on IL-12 production of DCs was completely blocked by heating but not by polymixin B (Fig. 3*A*), indicating that up-regulation of DC maturation by Gal-9 may not be the results of LPS contamination.

To confirm the above findings, we preincubated rGal-9 with blocking mAbs against Gal-9, TLR2, or TLR4. Among them, anti-Gal-9 mAb blocked the Gal-9-induced up-regulation of CD80, CD54, and CD40 expressions on the surfaces of Gal-9-treated DCs (Fig. 3*B*). In contrast, anti-TLR2 and TLR4 mAbs failed to block the Gal-9-induced up-regulation. Furthermore, we found that anti-Gal-9 mAb fails to block LPS-induced DC maturation (data not

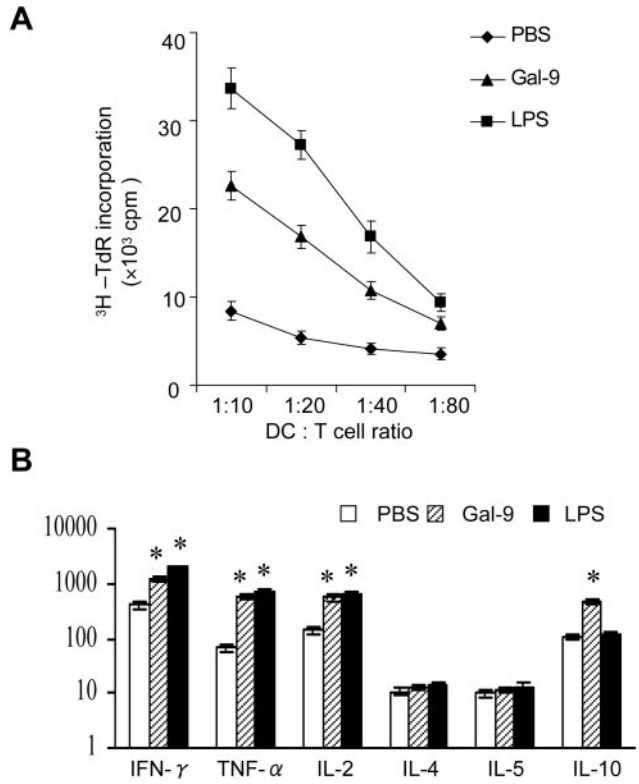

FIGURE 2. Gal-9-treated DCs elicited T cell proliferation and cytokine production in an allogeneic MLR. *A*, Allogeneic $CD4^+$ T cells were cultured for 4 days at the indicated ratios with DCs that had been treated for 2 days with Gal-9 (10 μg/ml), LPS (500 ng/ml), or PBS; [^{3}H]thymidine was added to the cultures for the final 18 h, and the proliferation of T cells was then determined by measurement of [^{3}H]thymidine uptake. *B*, Allogeneic $CD4^+$ T cells were cultured for 2 days with DCs that had been treated with Gal-9, LPS, or PBS as in *A*, after which the culture supernatants were assayed for the indicated cytokines. Data in *A* and *B* are means ± SD of triplicates from one experiment and are representative of four independent experiments.

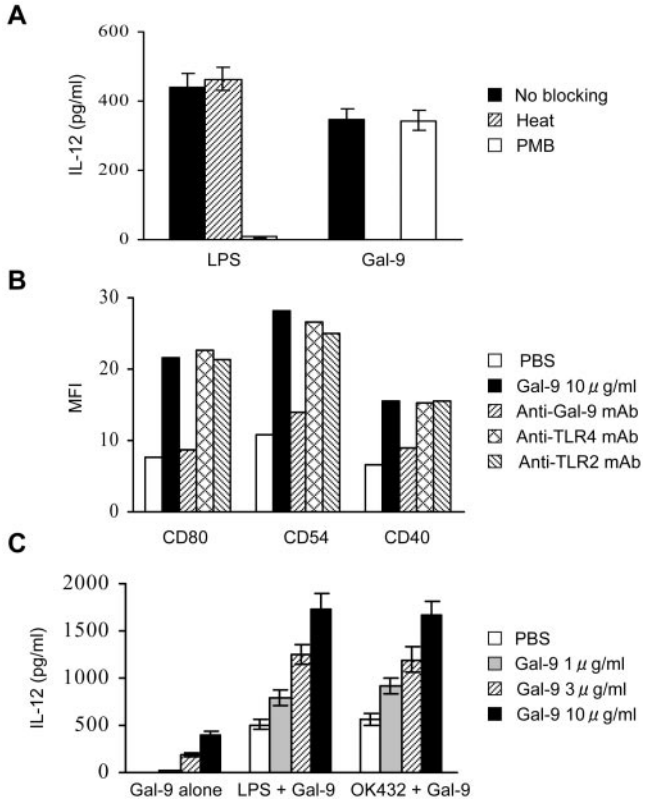

FIGURE 3. Gal-9-induced maturation of DCs is not caused by LPS contamination. *A*, Gal-9 and LPS were pretreated by heating (95°C, 20 min) or by preincubating the cells for 1 h with 10 μg/ml polymixin B sulfate before adding Gal-9 or LPS. After culture for 2 days, we collected the supernatant and assayed for IL-12 by ELISA. Data are means ± SD of triplicates from one experiment. *B*, Immature DCs were preincubated with anti-Gal-9 mAb (0.3 μM) or anti-TLR2 mAb (10 μg/ml) or anti-TLR4 mAb (10 μg/ml) for 1 h before Gal-9 was added, after which the cells were analyzed for surface expression of the indicated molecules by flow cytometry. Data are expressed as mean fluorescence intensity (MFI) for each molecule. *C*, Immature DCs were treated with Gal-9 alone at indicated concentration or in the presence of LPS or OK-432 for 2 days, after which the supernatant were collected and examined IL-12 by ELISA. Data are means ± SD of triplicates from one experiment. Data in *A–C* are representative of three independent experiments.

shown). Moreover, we found that Gal-9 dramatically synergizes with other maturation factors (LPS and OK-432) in IL-12 production of DCs in a dose-dependent manner (Fig. 3*C*). These results suggested that Gal-9-induced DC maturation is not due to LPS contaminated and is TLR2 and TLR4 independent.

Effect of lactose on Gal-9-induced DC maturation

Given that most biological effects of Gal are mediated by their carbohydrate-binding activity, we next examined whether this activity is required for Gal-9 to induce DC maturation. We first assessed the experiment whether lactose inhibits Gal-9-induced apoptosis of DCs. Consistent with our previously results (8), lactose, but not sucrose, could inhibit the Gal-9-induced apoptosis in a dose-dependent manner (Fig. 4*A*). In contrast, Gal-9-induced up-regulation of CD83 was not suppressed by lactose even at 30 mM lactose (Fig. 4*B*). Similarly, lactose slightly and not significantly inhibited Gal-9-induced up-regulation of the expression of other costimulatory molecules and HLA-DR on the surface of DCs (Table I).

To confirm that the ability of Gal-9 to induce DC maturation is independent of its lectin properties, we assessed the effect of a Gal-9 mutant that lacks the activities of β-galactoside binding, hemagglutination, eosinophil chemoattractant, and induction of apoptosis (19, 20) on this process. Treatment of DCs with the Gal-9 (R65D) mutant (10 μg/ml) expectedly increased the surface expression of costimulatory molecules and HLA-DR in DCs, although this effect was less pronounced than was that of wild-type Gal-9 (Table II). A higher concentration of Gal-9 (R65D) (30 μg/ml) induced an effect on the surface expression of these molecules similar to that observed with wild-type Gal-9 at 10 μg/ml. Moreover, Gal-9 (R65D) at 30 μg/ml increased IL-12 production by DCs to an extent similar to that apparent with the wild-type Gal at

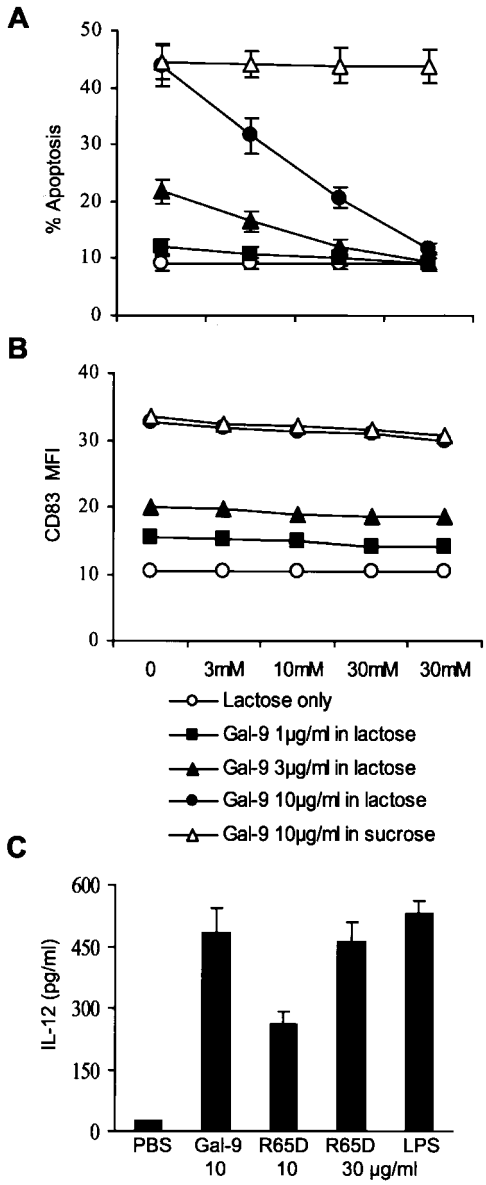

FIGURE 4. Effect of lactose on Gal-9-induced maturation of DCs. *A*, Immature DCs were cultured for 2 days with Gal-9 in the presence of lactose or sucrose at indicated concentrations, after which the cells were assayed for apoptosis by propidium iodide staining. Data are means ± SD of triplicates from one experiment. *B*, Cells in *A* were analyzed surface expression of the indicated molecules by flow cytometry. Data are expressed as mean fluorescence intensity (MFI). *C*, Immature DCs were incubated for 2 days with Gal-9 (10 μg/ml), the Gal-9(R65D) mutant (10 or 30 μg/ml), LPS (500 ng/ml), or PBS, after which culture supernatants were assayed for IL-12. Data are means ± SD of triplicates from one experiment. Data in *A–C* are representative of three independent experiments.

Table I. *Effect of lactose on the Gal-9-induced up-regulation of costimulatory molecules and HLA-DR on the surface of DCs*[a]

Cell Surface Marker	Treatment	MFI	
		Sucrose	Lactose
CD80	PBS	30.5	29.1
	Gal-9	61.5	54.7
	LPS	62.6	84.3
CD86	PBS	18.9	18.9
	Gal-9	196.3	167.1
	LPS	191.9	207.9
CD40	PBS	58.8	59.4
	Gal-9	187.7	137.7
	LPS	224.6	239.9
HLA-DR	PBS	46.6	47.5
	Gal-9	162.5	147.7
	LPS	253.7	254.9

[a] Immature DCs were cultured for 2 days with Gal-9 (10 μg/ml), LPS (500 ng/ml), or PBS in the presence of 20 mM lactose or 20 mM sucrose, after which the cells were analyzed for the surface expression of the indicated molecules by flow cytometry. Data are representative of three independent experiments. MFI, Mean fluorescence intensity.

10 μg/ml (Fig. 4*C*), confirming that the β-galactoside-binding activity of Gal-9 is not essentially required for the ability of this Gal to induce DC maturation.

Phosphorylation of p38 MAPK and ERK1/2 during Gal-9-induced DC maturation

Agents that induce DC maturation, including LPS and TNF-α, also elicit the phosphorylation of the MAPKs p38 and ERK1/2 (24). Therefore, we examined the effect of Gal-9 on the phosphorylation of these signaling molecules in immature DCs by immunoblot analysis. Treatment of immature DCs with Gal-9 induced the phosphorylation of both p38 MAPK and ERK1/2 within 10 min (Fig. 5).

Effects of signaling inhibitors on Gal-9-induced DC maturation

To evaluate further the contribution of MAPK-signaling pathways to Gal-9-induced DC maturation, we examined the effects of pretreatment of immature DCs with PD98059 (ERK1/2 inhibitor) (25), SB203580 (p38 MAPK inhibitor) (26), or wortmannin (PI3K inhibitor) on this process. The up-regulation of CD40, CD80, CD83, CD86, and HLA-DR induced by Gal-9 was prevented by pretreatment of immature DCs with SB203580 but not by that with PD98059 (Fig. 6, *A* and *B*) or wortmannin (data not shown). Moreover, IL-12 production induced by Gal-9 in DCs was also almost completely inhibited by SB203580 but was not affected by PD98059 (Fig. 6*C*) or wortmannin (data not shown).

Discussion

We have shown that Gal-9 induces DC maturation in vitro, as revealed by up-regulation of the surface expression of costimulatory and HLA molecules and of IL-12 production in a dose-dependent manner, and that this Gal-9-mediated induction may not depend on its lectin properties and TLR2 or TLR4. Moreover, DCs treated with Gal-9 promote both T cell proliferation and production of Th1 cytokines from those cells in an allogeneic MLR. In contrast to LPS-treated DCs, Gal-9-treated DCs also enhance the production of IL-10, a Th2 cytokine, by T cells, suggesting that DCs that matured in response to Gal-9, like those treated with glucocorticoids or PGE_2 (27, 28), also stimulate T cells responsible for IL-10 synthesis.

Gal-9 is released from T cells in response to stimulation with specific Ags (29), Con A (30), or PMA (31), and its release is probably associated with the activity of an unidentified matrix metalloproteinase (31). Moreover, various types of cells, including endothelial cells, fibroblasts, tissue macrophages, mast cells, and eosinophils, express Gal-9 both in the cytoplasm and on the cell surface, and numerous stimuli, have been found to regulate Gal-9 expression (32, 33). Taken together, these observations suggest that Gal-9 is a candidate for a natural inducer of DC maturation in vivo.

Gal play an important role in innate immunity by modulating effector cell function. For example, Gal-3 exhibits macrophage chemoattractant activity (14), and Gal-9 was originally identified as an eosinophil chemoattractant (29). A variety of eosinophil functions are also activated by Gal-9, and Gal-9 expressed on the surface of fibroblasts and endothelial cells mediates adhesion to eosinophils (3). The effects of Gal-9 on eosinophils are dependent on its lectin properties (19). In contrast, the ability of Gal-9 to induce DC maturation has been found to be independent of these properties: thus, lactose shows a minimal inhibitory effect on Gal-9-induced DC maturation, and the CRD mutant Gal-9 (R65D), which lacks β-galactoside-binding activity, mimics this effect of the wild-type protein, albeit at slightly higher concentrations. Thus, the β-galactoside-binding activity of Gal-9 is not essentially required to induce DC maturation. It has been shown that only the reduced form of Gal-1 possesses lectin activity, whereas the oxidized form of Gal-1 exhibits a different biological activity though it has no lectin activity (34, 35). Of course, it remains to be determined whether the ability of Gal-9 to induce DC maturation is related to its redox status or not. Moreover, Suk et al. (36) have demonstrated that natural autoantibodies against Gal-9 are detected in normal human sera. We have found in the present experiments that anti-Gal-9 mAb significantly inhibits the Gal-9-induced DC maturation. Therefore, it should be clarified whether

Table II. *Effect of a CRD mutant of Gal-9 on the surface phenotype of DCs*[a]

Cell Surface Marker	MFI				
	PBS	Gal-9 (10 μg/ml)	Gal-9 (R65D) (10 μg/ml)	Gal-9 (R65D) (30 μg/ml)	LPS
CD80	5.8	38.5	24.3	39.1	39.6
CD83	3.5	24.7	15.6	20.4	28.8
CD86	4.6	69.2	39.9	72.4	79.8
CD40	40.3	124.1	84.7	116.5	155.4
HLA-DR	25.9	85.4	58.7	84.6	109.5

[a] Immature DCs were incubated for 2 days with Gal-9 (10 μg/ml), the Gal-9 (R65D) mutant (10 or 30 μg/ml), LPS (500 ng/ml), or PBS, after which the cells were analyzed for surface expression of the indicated proteins by flow cytometry. Data are representative of four independent experiments. MFI, mean fluorescence intensity.

autoantibodies against Gal-9 in serum could regulate Gal-9-induced DC maturation.

DC maturation induced by Gal-9 probably depends on signaling by p38 MAPK but not on that by ERK1/2, similar to maturation induced by LPS or CD40L (23). Gal-4 stimulates intestinal $CD4^+$ T cells in mice to produce IL-6 through binding at the immunologic synapse (37). Gal-4 is a tandem repeat-type Gal, containing two CRDs, as is Gal-9 (12, 38). Thus, it is possible that Gal-9 induces DC maturation by binding to DCs at the immunologic synapse, initiating this effect through activation of a p38 MAPK-signaling pathway. However, the Gal-4-induced production of IL-6 by $CD4^+$ T cells appears to be largely dependent on signaling by protein kinase C-θ, suggesting that the signaling molecules activated by Gal-9 and Gal-4 differ. Although synapse formation and intracellular signaling by T cells are relatively well characterized, those by DCs are poorly understood (38).

DC maturation is induced not only by LPS and Gal-9 but by a variety of cytokines (such as IFN-γ, IL-1β, and TNF-α), PGs, poly(I:C), and bacteria (23, 28, 39–42). We have previously shown that many of these factors also up-regulate Gal-9 expression in various cell types (3, 43, 44). Therefore, it is possible that the up-regulation of Gal-9 expression in DCs or other cells in response to such factors is responsible for the induction of DC maturation. Recently, Vray et al. (45) have shown that *Trypanosoma cruzi* infection up-regulated the expression of Gal-3 and of its ligands in DCs with functional consequences on their capacities of adhesion and migration, indicating Gal may play important roles in modulation of DCs. Our results suggest that Gal-9 plays an important role not only in innate immunity by modulating granulocyte functions but also in acquired immunity by inducing DC maturation. Thus, this Gal might contribute to cross-talk between innate and acquired immunity.

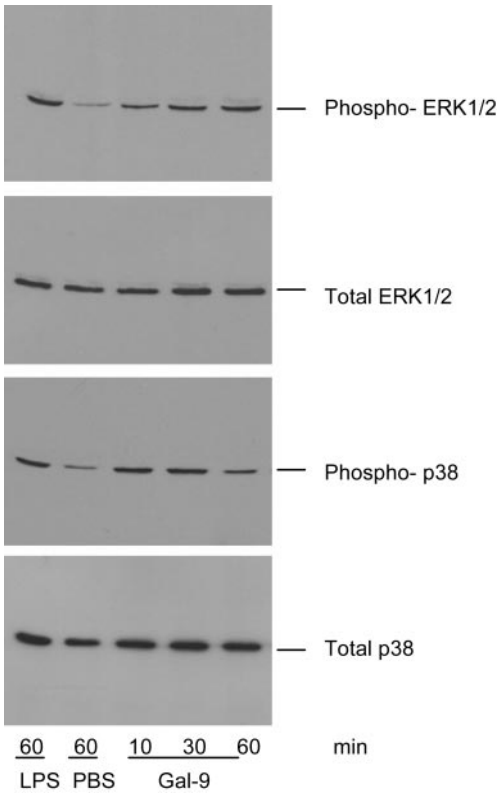

FIGURE 5. Effect of Gal-9 on the phosphorylation of p38 MAPK and ERK1/2 in DCs. Immature DCs were incubated for the indicated times with Gal-9 (10 μg/ml), LPS (500 ng/ml), or PBS, after which cell lysates were subjected to immunoblot analysis with Abs specific for phosphorylated forms of p38 MAPK or ERK1/2. The blot was reprobed with Abs to total p38 MAPK or total ERK1/2. Data are representative of three independent experiments.

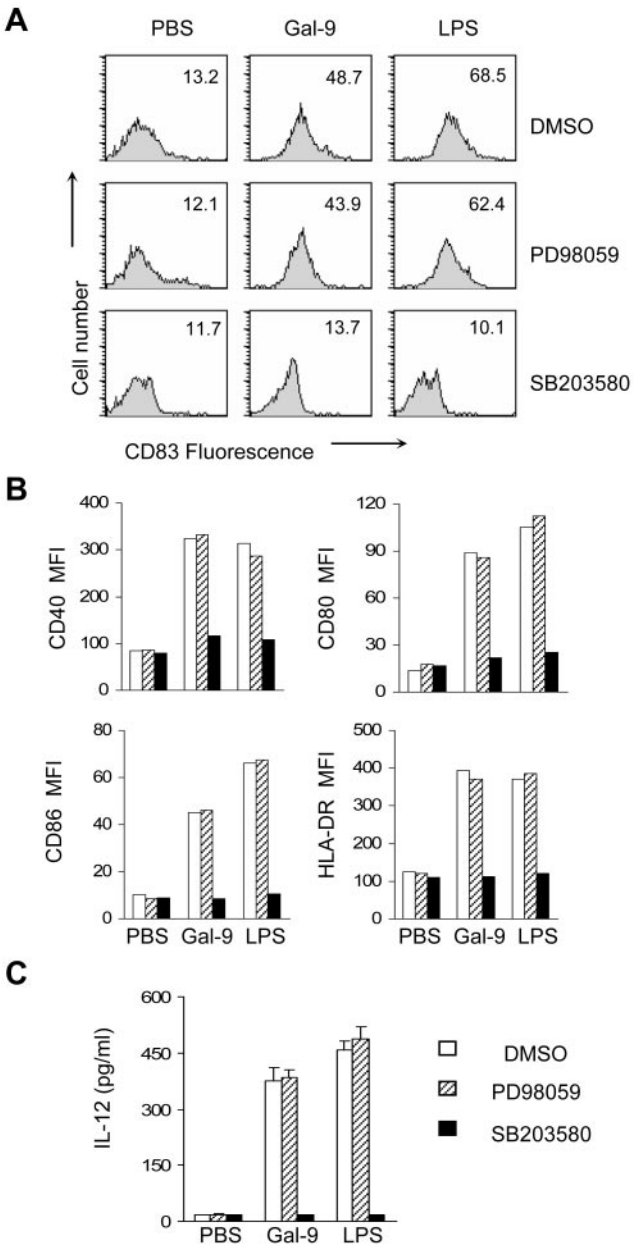

FIGURE 6. Effects of SB203580 and PD98059 on Gal-9-induced DCs maturation. Immature DCs were incubated for 1 h with 50 μM PD98059, 50 μM SB203580, or DMSO (vehicle) and then cultured for 2 days with Gal-9 (10 μg/ml), LPS (500 ng/ml), or PBS in the continued presence of these agents, as indicated. The cells were then analyzed for surface expression of the indicated molecules by flow cytometry. *A*, Histograms of CD83 expression on the cells. *B*, Surface expression of the indicated molecules by flow cytometry analysis. Data are expressed as mean fluorescence intensity (MFI). *C*, The amounts of IL-12 in the culture supernatant were determined by ELISA. Data means ± SD of triplicates from one experiment. Data in *A–C* are representative of four independent experiments.

DCs are professional APCs that are required for the initiation of immune responses (46). Thus they are essential for induction of a primary CTL response to tumor cells (47). Immunotherapy with

DCs has been applied to various types of cancer, including malignant melanoma, non-Hodgkin's lymphoma, multiple melanoma, prostate cancer, colorectal cancer, and non-small cell lung cancer (48, 49). Moreover, DCs constitutively secrete exosomes, which themselves are regarded as a potential mediator of tumor therapy (50–52). Proteomics analysis has revealed that exosomal proteins include Gal-3, thioredoxin peroxidase II, and Alix (53). It remains to be determined whether Gal-9 is also present in exosomes. However, Gal-9 has been shown to induce apoptosis not only in activated $CD4^+$ T cells but also in various types of malignant tumor cells, including malignant melanoma and lymphoma cells (8, 16). The proapoptotic activity of Gal-9 is greater than that of Gal-1 (54), and Gal-9 fails to induce apoptosis in resting T cells or normal cells at concentrations at which it does so in activated T cells and cancer cells (8). We have further described that Gal-9 induces aggregation of breast cancer cells and that Gal-9 can be a prognostic factor with antimetastatic activity in breast cancer (55). Thus, Gal-9 is a potential candidate, together with DCs, for use in antitumor immune therapy.

Disclosures

The authors have no financial conflict of interest.

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