A Novel Function for Galectin-1 at the Crossroad of Innate and Adaptive Immunity: Galectin-1 Regulates Monocyte/Macrophage Physiology through a Nonapoptotic ERK-Dependent Pathway[1]

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Several environmental factors can differentially regulate monocyte and macrophage response patterns, resulting in the display of distinct functional phenotypes. Galectin-1, an endogenous lectin found at peripheral lymphoid organs and inflammatory sites, has shown immunoregulatory activity in vivo in experimental models of autoimmunity and cancer. Whereas compelling evidence has been accumulated regarding the effects of galectin-1 on T cell fate, limited information is available on how galectin-1 may impact other immune cell types. In the present study, we report a novel role for galectin-1 in the regulation of monocyte and macrophage physiology. Treatment with galectin-1 in vitro differentially regulates constitutive and inducible FcγRI expression on human monocytes and FcγRI-dependent phagocytosis. In addition, galectin-1 inhibits IFN-γ-induced MHC class II (MHC-II) expression and MHC-II-dependent Ag presentation in a dose-dependent manner. These regulatory effects were also evident in mouse macrophages recruited in response to inflammatory stimuli following treatment with recombinant galectin-1 and further confirmed in galectin-1-deficient mice. Investigation of the mechanisms involved in these functions showed that galectin-1 does not affect survival of human monocytes, but rather influences FcγRI- and MHC-II-dependent functions through active mechanisms involving modulation of an ERK1/2-dependent pathway. Our results provide evidence of a novel unrecognized role for galectin-1 in the control of monocyte/macrophage physiology with potential implications at the crossroad of innate and adaptive immunity. ***The Journal of Immunology,*** **2007, 178: 436–445.**

The ability to recognize a wide range of endogenous and exogenous ligands and to respond appropriately has placed monocytes and macrophages as central cells able to influence homeostasis as well as host defenses in innate and acquired immunity (1). Monocytes leave the unique environment of the bone marrow and enter the blood, where they are exposed to a plethora of environmental signals, including cytokines, chemokines, and soluble immunoregulatory factors, which are capable of impacting their phenotypic and functional characteristics (2). These cells selectively home to different tissues under the influence of chemokines and tissue-specific homing factors, where they can contribute, as activated macrophages, to innate and adaptive immune responses through expression of a variety of effector activities differentially regulated by the microenvironment of the different tissues (2). Of particular relevance are the receptors for Fc portion of IgG (FcγR), which mediate phagocytosis, respiratory burst, and secretion of proinflammatory cytokines (3). Among them, FcγRI, a high-affinity receptor, is expressed mainly in mononuclear phagocytes and is tightly regulated by IFN-γ (4). In contrast, monocytes and macrophages express other key molecules such as MHC class II (MHC-II),[4] which enables these cells to present Ags linking innate and adaptive immunity (5), and CD14, which is involved in the signaling pathway of bacterial LPS (1).

Galectins, a growing family of carbohydrate-binding proteins, have recently attracted the attention of immunologists as novel regulators of immune cell homeostasis (6–12). Galectin-1, a prototype member of this protein family, has the potential to regulate the inflammatory response (13–16) and confer immune privilege to tumor cells (17). How galectin-1 exerts its immunoregulatory activity is poorly understood, primarily because of its pleiotropic nature where it has been shown to affect T cell activation and proliferation (18, 19), T cell adhesion to extracellular matrix (20), and NO production (21). Foremost, however, has been the ability of galectin-1 to promote apoptosis of activated T cells through binding to specific carbohydrate ligands on cell surface glycoconjugates (22–24). Whereas compelling evidence has been accumulated regarding the effects of galectin-1 on T

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Received for publication November 29, 2005. Accepted for publication October 17, 2006.

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[1] This work was supported by grants from the Cancer Research Institute (USA), Mizutani Foundation for Glycoscience (Japan), Agencia de Promoción Científica y Tecnológica (PICT 2003-05-13787), Fundación Sales/Consejo Nacional de Investigaciones Científicas y Técnicas de Argentina Program, Wellcome Trust (U.K.), Fundación Florencio Fiorini, Fundación Bunge & Born, and University of Buenos Aires (UBACYT-M091; to G.A.R.), and by grants from Agencia Nacional de Promoción Científica y Tecnológica (PICT 05-14061; to M.A.I.). P.B., J.M.I., M.A.T., and G.A.B. thank Consejo Nacional de Investigaciones Científicas y Técnicas de Argentina for the fellowships granted.

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[4] Abbreviation used in this paper: MHC-II, MHC class II; MFI, median of fluorescence intensity; SRBC, sheep RBC; WT, wild type.

cell fate, scarce information is available on how galectin-1 may impact other immune cell types.

The present study was conducted to investigate the potential role of galectin-1 in the regulation of monocyte and macrophage physiology. We demonstrate here that galectin-1 can modulate constitutive and inducible FcγRI expression on human monocytes and FcγRI-dependent phagocytosis. In addition, galectin-1 regulates MHC-II expression and MHC-II-dependent Ag presentation in a dose-dependent manner. Interestingly, galectin-1 does not affect survival of human monocytes, but instead can modulate monocyte functions through an ERK1/2-dependent pathway. These effects were also observed in mouse inflammatory macrophages recruited to a glass cylinder following injection of recombinant galectin-1 and further confirmed in galectin-1-deficient mice. To the best of our knowledge, this is the first study demonstrating a critical role for galectin-1 in the regulation of monocyte and macrophage physiology with potential implications in the control of innate and adaptive immune responses.

Materials and Methods

Reagents and Abs

Tissue culture medium (RPMI 1640), Ficoll 400, human rIFN-γ, mouse rIFN-γ, lactose, sucrose, Nonidet P-40, and Tween 20 were purchased from Sigma-Aldrich. Percoll was obtained from Amersham Biosciences. FITC-labeled anti-human FcγRI Ab (clone 22), FITC-labeled anti-HLA-DR Ab (clone Immu-357), PE-labeled anti-human CD14 Ab (clone RM052), mouse IgG1 (clone 679.1Mc7), and mouse IgG2a (clone U7.27) isotype controls were from Immunotech. Rat anti-mouse FcγRII/III Ab (clone 2.4G2) and rat IgG2b isotype control were provided by Dr. M. Vermeulen (Academia Nacional de Medicina, Buenos Aires, Argentina). FITC-labeled goat anti-rat IgG (H+L) was obtained from Jackson ImmunoResearch Laboratories. FITC-labeled anti-mouse I-A^d (clone 39-10-8) mAb was obtained from BD Biosciences. Electrophoresis reagents were from Bio-Rad. MAPK inhibitors SB203580 and PD98059 were purchased from Calbiochem-Novabiochem. The anti-phospho-ERK1/2 mAb and anti-β-actin mAb were purchased from Santa Cruz Biotechnology.

Preparation of recombinant galectin-1 and the monomeric mutant N-*Gal-1*

Recombinant galectin-1 was produced and purified as described previously (25). In brief, *E. coli* BL21 (DE3) cells were transformed with expression plasmids constructed using pET expression systems (Novagen) and production of recombinant galectin-1 was induced by the addition of 1 mM isopropyl-β-D-thiogalactoside. Soluble fractions were obtained for subsequent purification by affinity chromatography on a lactosyl-Sepharose column (Sigma-Aldrich). The monomeric *N*-Gal-1 mutant described by Cho and Cummings (26) was produced from the *N*-Gal-1 expression vector provided by Dr. L. Baum (University of California Los Angeles School of Medicine, Los Angeles, CA). The purification procedure was essentially as previously described (26), except that a 3 × 7-cm lactosyl-Sepharose affinity column was used to isolate the recombinant monomeric protein. As galectin-1 has unpaired cysteines in the binding site that, in the absence of carbohydrate binding, can form intramolecular disulfide bonds and reduce its biological activity, we stored galectin-1 in a DTT buffer as has been indicated (27). LPS content of the purified samples (<60 ng/mg) was tested using a Gel Clot *Limulus* Test (Cape Cod).

Animals

BALB/c male mice were obtained from the central Animal House of the Academia Nacional de Medicina. Galectin-1-deficient (*gal-1*$^{-/-}$) mice (129/Sv background) and age-matched male 129/Sv wild-type (WT) mice were provided by Dr. F. Poirier (Institut Jacques Monod, Paris, France) (28). All animals were housed and cared according to institutional guidelines for animal care and use at the Animal House of the Faculty of Exact and Natural Sciences (University of Buenos Aires). The ethics committee on the use and care for laboratory animals at the Faculty of Exact and Natural Sciences approved all the procedures used in this study.

Preparation of human monocytes and exposure to different treatments

PBMC were obtained by Ficoll-Hypaque gradient centrifugation from human blood collected from healthy adult volunteers who had taken no medication for at least 10 days before the day of sampling as described previously (29). Monocytes were obtained after centrifugation of PBMC on a Percoll gradient and resuspended in RPMI 1640 supplemented with 10% heat-inactivated FCS and 50 μg/ml gentamicin. Viability of human monocytes was >95% in all the experiments as measured by trypan blue exclusion test. The purified population of human monocytes was then subjected to phenotypic and functional assays following incubation with increasing concentrations of recombinant galectin-1 (0.4, 4, and 40 μg/ml) or the *N*-Gal-1 mutant (0.4, 4, 40, and 140 μg/ml) for different time periods in the presence or absence of IFN-γ. To evaluate whether individual galectin-1 effects were dependent on the carbohydrate-binding activity of this protein, human monocytes were exposed to different treatments in the presence of lactose or sucrose (10 or 30 mM). In addition, monocytes were also subjected to different treatments in the presence of the MAPK inhibitors SB203580 and PD98059 to disrupt p38 and ERK1/2 pathways.

Flow cytometry

Following different treatments, 0.5 × 10^6 human monocytes/ml were washed and incubated with appropriate dilutions of the following mAbs: FITC-labeled anti-human FcγRI (clone 22) or FITC-labeled anti-HLA-DR (clone Immu-357) or PE-labeled anti-human CD14 (clone RM052) from Immunotech. Purity of monocytes was determined as the percentage of CD14-positive cells and found to be >90% in all donors. Results were expressed as the percentage of median of fluorescence intensity (MFI) ± SEM. Nonspecific binding was determined using the appropriate mouse isotype-matched control IgG. In another set of experiments, 0.35 × 10^6 cells were obtained from glass cylinders introduced into different groups of mice, washed, and incubated with appropriate dilutions of FITC-labeled anti-mouse I-A^d mAb (clone 39-10-8; BD Biosciences). Cells were then washed, resuspended in Isoflow, and analyzed on a FACScan cytometer (BD Biosciences).

Phagocytosis assays

To evaluate phagocytosis, 0.5 ml of mononuclear cells (2 × 10^6 cells/ml) or inflammatory macrophages (1 × 10^6 cells/ml) were allowed to adhere to flat-bottom tissue culture chambers (Lab-Tek). Then, a suspension of 100 μl of 1% IgG-coated ^{51}Cr-labeled sheep RBC (SRBC) was added and incubated for 30 min at 37°C. After this period, cells were washed with RPMI 1640 to remove the nonphagocytosed SRBC. Then, cells were washed and removed from the chambers by adding 0.5% sodium deoxycholate and phagocytosis was evaluated by counting the radioactivity in the pellets. The obtained value was corrected by subtracting the percentage of uptake of unsensitized ^{51}Cr-SRBC by phagocytic cells (spontaneous phagocytosis).

Proliferation assays

PBMC at a concentration of 1 × 10^6 cells/ml were left to adhere in 96-well flat-bottom during 2 h at 37°C. Nonadherent cells were removed and the adherent cell population was incubated with galectin-1 (0.4, 4, or 40 μg/ml), IFN-γ (240 U/ml) or both at 37°C and 5% CO_2 for 24 h. After this period, cells were washed and incubated with nonadherent PBMC at 1 × 10^6 cells/ml in the absence (controls) or presence of *Mycobacterium tuberculosis* (1 × 10^6 bacteria/ml). Cells were incubated for 5 days at 37°C 5% CO_2 and pulsed with [^{3}H]thymidine for 18 h. Cells were then harvested and the index of [^{3}H]thymidine incorporation was calculated for each treatment as: counts per minute in the presence of *M. tuberculosis*/cpm in the absence of *M. tuberculosis*.

Western blot analysis

For each condition, monocytes at 2 × 10^6 cells/ml were used. Following different experimental treatments, cells were washed with PBS and lysed by incubation on ice for 20 min in 0.5 ml of 100 mM Tris-HCl (pH 8.0), 100 mM NaCl, 2 mM ethylenediamine tetraacetic acid, 1% Nonidet P-40, 1 mM Na_3VO_4, 50 mM NaF, 0.3 U/ml aprotinin, 2 mM PMSF, and 1 μg/ml each of leupeptin and pepstatin A. Lysates were then centrifuged for 15 min at 14,000 × *g*. Protein concentrations were determined using the microBCA protein assay (Pierce). Equal amounts of protein (60 μg) were then resolved on a 15% SDS-PAGE, and Western blot analysis was performed essentially as described (23). After electrophoresis, proteins were transferred to nitrocellulose membranes (Amersham Biosciences) for 1 h at 300 mA and blocked with PBS containing 3% nonfat dry milk for 1 h. Membranes were then probed with primary Ab in PBS 0.4% BSA (0.4 μg/ml anti-phospho-ERK1/2; Santa Cruz Biotechnology) overnight. After washing three times with PBS 0.2% Tween 20, blots were incubated for 1 h with a HRP-conjugated goat anti-mouse IgG (Bio-Rad). Immunoreactivity was detected using the ECL Western blot detection kit (Amersham

Biosciences). Films were then analyzed using the Scion Image Analysis software (Scion), and the intensity of the bands was recorded and expressed as arbitrary units. Equal loading was checked by Ponceau S staining and by incubation of the blots with an anti-β-actin Ab (Santa Cruz Biotechnology).

Apoptosis assays

Following treatment with increasing concentrations of recombinant galectin-1 for different periods, freshly isolated human monocytes were analyzed for their susceptibility to cell death using an annexin V binding assay (BD Biosciences) according to the manufacturer's recommended protocol and processed for flow cytometric analysis. Positive control of apoptosis included PHA-activated $CD4^+$ T cells cultured in the presence of recombinant galectin-1. Ten thousand events were acquired in a FACStar cytometer (BD Biosciences).

Recruitment of mouse macrophages following an inflammatory challenge

Glass cylinders of 2 cm long and 8 mm wide were introduced s.c. into 8- to 10-wk-old BALB/c as described previously (30). The volume of each cylinder is ~200 μl. After 20 days, the cylinders caused a chronic inflammatory process with the open ends of the cylinders closed by fibrotic tissue and predominantly infiltrated by macrophages. When the chronic inflammatory process was established, pyrogen-free vehicle control (PBS/DTT) or recombinant galectin-1 was injected at final concentrations of 0.4 and 40 μg/ml into the cylinder. Other groups of mice were injected with galectin-1 plus IFN-γ (750 U/ml) in the absence or presence of the ERK1/2 inhibitor PD98059. Then, cylinders were extracted from the mice and cells that had been recruited were stained with an anti-MHC-II FITC-labeled mAb ($I\text{-}A^d$ clone 39-10-8). Cells were then processed for flow cytometry and the macrophage population was analyzed using the macrophage-specific forward light scatter and side light scatter gates.

In a second set of experiments, glass cylinders were introduced into age-matched *gal-1*$^{-/-}$ and WT mice, and inflammatory macrophages were analyzed as described above. In a third set of experiments, recombinant galectin-1 (40 μg/ml) or vehicle control (PBS/DTT) was injected into the cylinder of *gal-1*$^{-/-}$ mice to evaluate whether galectin-1 was redundant or sufficient for the observed effects. Phenotypic analysis was performed essentially as described above. For functional assays, inflammatory macrophages were collected from the cylinders and evaluated for their ability to stimulate an allogeneic T cell response. Briefly, *gal-1*$^{-/-}$ and WT macrophages (1×10^6 cells/ml) were treated with mitomycin (25 μg/ml) and cultured with BALB/c responder spleen cells (1×10^6 cells/ml) for 4 days at 37°C and 5% CO_2. Cells were then pulsed with [^{3}H]thymidine for the final 18 h of culture and [^{3}H]thymidine incorporation was calculated.

Statistical analysis

Statistical analysis of the data was performed using the nonparametric paired Mann-Whitney *U* test. For the experiments in mice, the unpaired Student *t* test was used. Results are expressed as the mean ± SEM. Values of $p < 0.05$ were considered statistically significant.

Results

Galectin-1 differentially modulates constitutive and inducible FcγRI expression on human monocytes and influences FcγRI-dependent phagocytosis

The receptors for Fc portion of IgG (FcγR) have been considered a link between cellular and humoral immunity by serving as a bridge between Ab specificity and effector cell functions (3). One of them, FcγRI, is a high-affinity receptor capable of mediating phagocytosis, Ab-dependent cell-mediated cytotoxicity and secretion of cytokines (3). We first investigated whether galectin-1 could modulate constitutive expression of FcγRI on human monocytes. As shown in Fig. 1, galectin-1 at a low concentration of 0.4 μg/ml was able to significantly increase the levels of FcγRI expression (Fig. 1, *A* and *B*), while higher concentrations were not effective in modulating the constitutive expression of this immunoregulatory molecule (Fig. 1*B*). Furthermore, cell surface expression of FcγRII and FcγRIII was not altered by exposure to galectin-1 at any of the concentrations tested (data not shown). To determine whether this effect was mediated by galectin-carbohydrate interactions, we performed the assays in the presence of specific and nonspecific carbohydrate ligands. While addition of lactose, a galectin-specific sugar, completely inhibited galectin-1-induced up-regulation of FcγRI expression at a concentration of 30 mM (Fig. 1*B*), the disaccharide sucrose, which has very low affinity for galectin-1, was not capable of preventing this effect at any of the concentrations tested (Fig. 1*C*), indicating that galectin-1 binding to specific saccharide ligands on the surface of monocytes is necessary for the observed effects.

Galectin-1 exists as a noncovalent homodimer with a dimerization constant (K_d) of ~7 μM (26). The low concentrations of galectin-1 that were effective in modulating FcγRI expression suggested that this effect was mainly mediated by the monomeric form of this protein. To confirm this finding we used a monomeric mutant of galectin-1 (*N*-Gal-1) that exists as a monomer at concentrations even higher than 100 μg/ml, yet still retains carbohydrate-binding capacity (26). As shown in Fig. 1*D*, *N*-Gal-1 induced up-regulation of FcγRI expression even at very high concentrations (140 μg/ml), suggesting that the monomeric form of galectin-1 is mainly responsible for the observed effects.

It is well known that cytokines like IFN-γ augments the expression of FcγRI when added to human monocytes for 24 h (29). Therefore, we next investigated whether galectin-1 could modulate IFN-γ-induced FcγRI expression. Strikingly, when monocytes were simultaneously treated with IFN-γ and galectin-1 for 24 h, we could not find up-regulation of FcγRI. On the contrary, a significant dose-dependent inhibitory effect was observed (Fig. 1, *E* and *F*). Addition of lactose, but not sucrose, to the culture medium prevented the regulatory effects even at high concentrations of galectin-1 (Fig. 1, *F* and *G*). In addition, the inhibitory effect was still observed when monocytes were incubated with IFN-γ and the *N*-Gal-1 mutant (Fig. 1*H*), indicating that the monomeric form of galectin-1 is also relevant for this activity. In contrast, no significant changes were observed in the levels of FcγRI cell surface expression when human monocytes were pretreated with IFN-γ for 24 h and then exposed to galectin-1 (data not shown). Thus, galectin-1 differentially regulates constitutive and inducible FcγRI expression on the surface of human monocytes.

To determine whether differential FcγRI expression induced by galectin-1 may impact on monocyte functions linked to innate immunity, we evaluated the effect of galectin-1 on FcγRI-dependent phagocytosis of ^{51}Cr-labeled IgG-coated SRBC (Fig. 1*I*). In agreement with galectin-1-induced FcγRI up-regulation, we found increased levels of phagocytosis of ^{51}Cr-labeled SRBC when this protein was used at a concentration of 0.4 μg/ml. Furthermore, as expected by the dose-dependent inhibition of FcγRI expression on monocytes simultaneously exposed to IFN-γ and galectin-1, a dose-dependent inhibition of ^{51}Cr-labeled SRBC phagocytosis was clearly observed (Fig. 1*I*). These effects were also competed by lactose at a concentration of 30 mM and still observed when the monomeric mutant *N*-Gal-1 was used for the assays (data not shown). Thus, galectin-1 differentially modulates constitutive and inducible FcγRI expression on human monocytes and FcγRI-dependent functions such as phagocytosis through protein-carbohydrate interactions.

Galectin-1 inhibits constitutive and inducible MHC-II expression on human monocytes and interferes with MHC-II-dependent Ag presentation

MHC-II molecules are constitutively expressed on the surface of human monocytes, enabling them to present Ags and initiate the adaptive immune response (1, 5). To gain insights into the role of

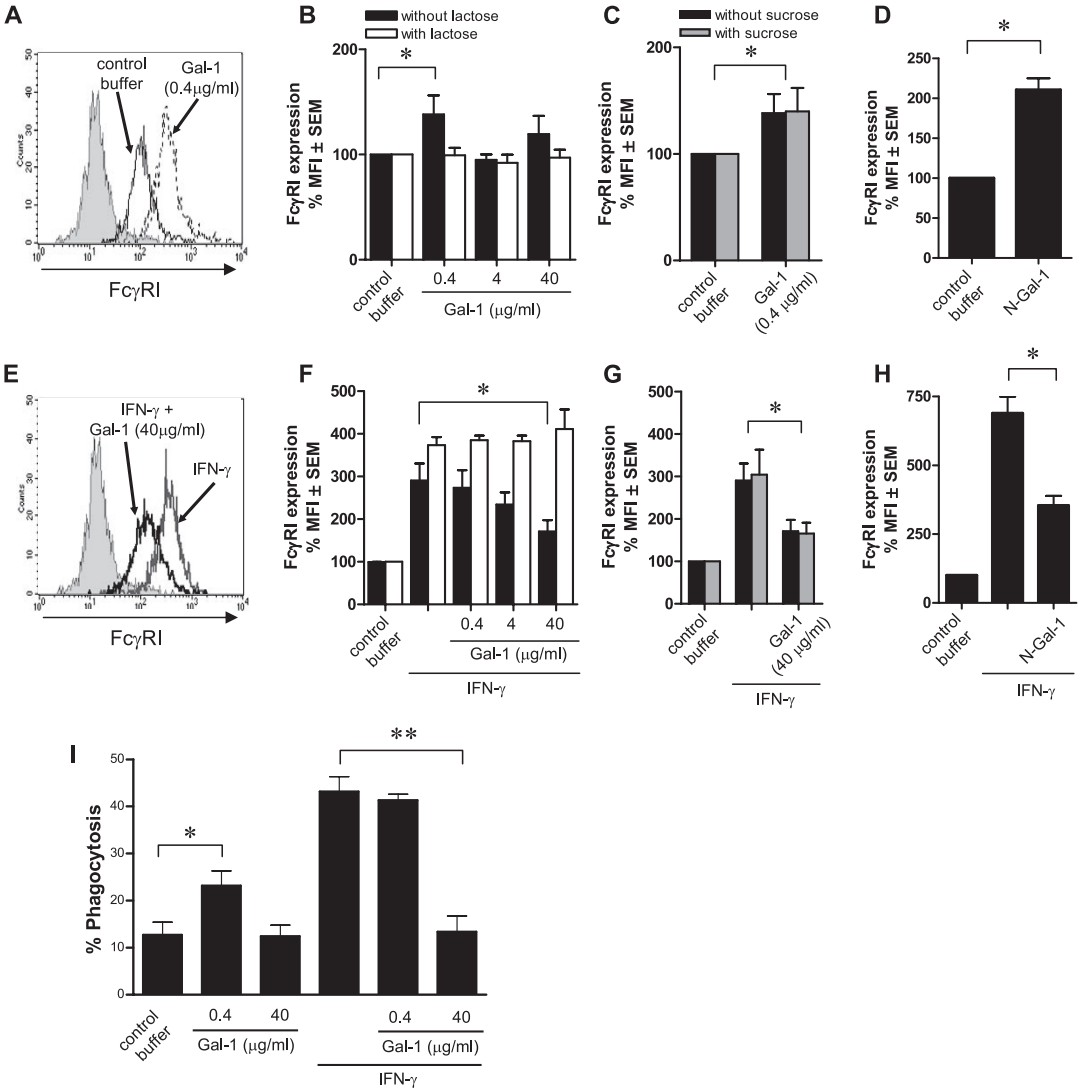

FIGURE 1. Galectin-1 differentially regulates constitute and inducible FcγRI expression on human monocytes and influences FcγRI-dependent phagocytosis. *A–C*, Human monocytes (0.5×10^6 cells/ml) were incubated with different concentrations of galectin-1 (0.4, 4, and 40 μg/ml) for 24 h in the absence or presence of 30 mM lactose (*B*) or sucrose (*C*). Cells were then stained with a FITC-conjugated anti-FcγRI mAb. Histogram (*A*) corresponds to 1 representative of 10 independent experiments, and bars (*B* and *C*) represent the percentage of MFI ± SEM of controls (*, $p < 0.05$ Gal-1 0.4 μg/ml vs control buffer). Nonspecific binding (filled histogram) was determined using a control isotype Ab. *D*, Human monocytes (0.5×10^6 cells/ml) were incubated with the monomeric *N*-Gal-1 mutant (140 μg/ml) for 24 h and stained as described above (*, $p < 0.05$ *N*-Gal-1 vs control buffer). *E–G*, Human monocytes (0.5×10^6 cells/ml) were incubated with IFN-γ (240 U/ml) and different concentrations of galectin-1 for 24 h in the absence or presence of lactose (*F*) or sucrose (*G*). Cells were then stained with a FITC-conjugated anti-FcγRI mAb. Histogram (*E*) corresponds to 1 representative of 10 independent experiments, and bars (*F* and *G*) represent the MFI ± SEM of controls (*, $p < 0.05$ Gal-1 plus IFN-γ vs IFN-γ alone). *H*, Human monocytes (0.5×10^6 cells/ml) were incubated with IFN-γ (240 U/ml) and the monomeric mutant *N*-Gal-1 for 24 h and stained as described above (*, $p < 0.05$ *N*-Gal-1 plus IFN-γ vs IFN-γ alone). *I*, Human monocytes (0.5×10^6 cells/ml) were incubated with or without IFN-γ (240 U/ml) in the absence or presence of increasing concentrations of galectin-1 (0.4, 4, and 40 μg/ml) for 24 h. Then, phagocytosis of ^{51}Cr-labeled IgG-coated SRBC was determined as described in *Materials and Methods.* Results obtained in five independent experiments are shown. Values represent percent phagocytosis ± SD (*, $p < 0.05$ Gal-1 vs buffer control; **, $p < 0.01$ Gal-1 plus IFN-γ vs IFN-γ alone).

galectin-1 in the modulation of monocyte functions linked to adaptive immunity, we examined the impact of this protein in constitutive and inducible MHC-II expression. As shown in Fig. 2, *A* and *B*, exposure of human monocytes to galectin-1 for 24 h significantly inhibited MHC-II expression in a dose-dependent manner. This effect was even more pronounced in human monocytes simultaneously exposed to IFN-γ and galectin-1 for 24 h (Fig. 2, *D* and *E*). Similarly to the effects described above, galectin-1-induced down-regulation of constitutive and inducible MHC-II expression was inhibited in the presence of lactose (Fig. 2, *B* and *E*), and this effect was still observed when the *N*-Gal-1 mutant was used in the assays (Fig. 2, *C* and *F*). Interestingly, no changes were detected in other relevant molecules such as CD14, MHC-I and CD11b following exposure of human monocytes to galectin-1 at different concentrations (data not shown).

To evaluate whether galectin-1-mediated regulation of MHC-II expression could impact on the APC activity of human monocytes, we explored the ability of galectin-1-treated or untreated APC to present mycobacterial Ags and stimulate the proliferation of nonadherent T cells. We found that exposure of adherent human monocytes to galectin-1 before the Ag presentation assay resulted in reduced ability of these cells to stimulate the proliferation of T cells in vitro (Fig. 2*G*). This dose-dependent effect was highly pronounced in the presence of IFN-γ (Fig. 2*G*) and was prevented

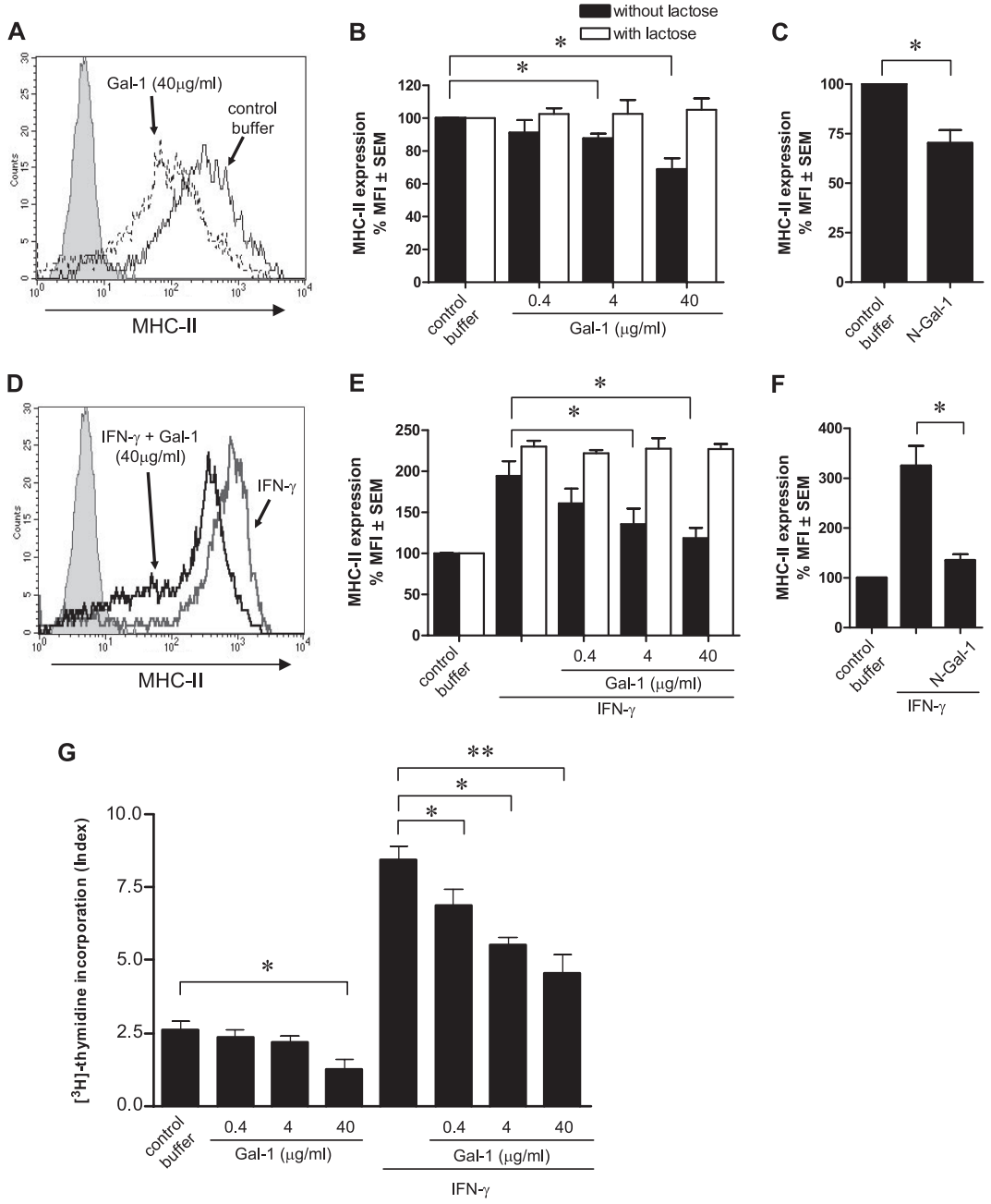

FIGURE 2. Galectin-1 regulates constitutive and inducible MHC-II expression on human monocytes and interferes with MHC-II-dependent Ag presentation. *A* and *B*, Human monocytes (0.5×10^6 cells/ml) were incubated with different concentrations of galectin-1 (0.4, 4, and 40 μg/ml) for 24 h in the absence or presence of lactose (30 mM). Cells were then stained with a FITC-conjugated anti-HLA-DR mAb. Histograms (*A*) correspond to 1 representative of 10 independent experiments, and bars (*B*) represent the MFI ± SEM of controls (∗, $p < 0.05$ Gal-1 vs control buffer). Nonspecific binding (filled histogram) was determined using a control isotype Ab. *C*, Human monocytes (0.5×10^6 cells/ml) were incubated with the monomeric mutant *N*-Gal-1 (140 μg/ml) for 24 h and stained as described above (∗, $p < 0.05$ *N*-Gal-1 vs buffer control). *D* and *E*, Human monocytes (0.5×10^6 cells/ml) were incubated with IFN-γ (240 U/ml) and different concentrations of galectin-1 (0.4, 4, and 40 μg/ml) for 24 h in the absence or presence of lactose (30 mM). Cells were then stained with a FITC-conjugated anti-HLA-DR mAb. Histograms (*D*) correspond to 1 representative of 10 independent experiments, and bars (*E*) represent the MFI ± SEM of controls (∗, $p < 0.05$ Gal-1 plus IFN-γ vs IFN-γ alone). *F*, Human monocytes (0.5×10^6 cells/ml) were incubated with IFN-γ (240 U/ml) and the monomeric mutant *N*-Gal-1 for 24 h and stained as described above (∗, $p < 0.05$ *N*-Gal-1 plus IFN-γ vs IFN-γ alone). *G*, Human monocytes (1×10^6 cells/ml) were incubated with or without IFN-γ (240 U/ml) in the absence or presence of increasing concentrations of galectin-1 (0.4, 4, and 40 μg/ml) for 24 h. Then, cells were washed and incubated with nonadherent PBMC (1×10^6 cells/ml) in the absence or presence of *Mycobacterium tuberculosis*. Values are calculated as the index of [^{3}H]thymidine incorporation ± SD as described in *Materials and Methods.* Results obtained in five independent experiments are shown (∗, $p < 0.05$ Gal-1 40 μg/ml vs control buffer; ∗, $p < 0.05$ Gal-1 (0.4 and 4 μg/ml) plus IFN-γ vs IFN-γ alone; ∗∗, $p < 0.01$ Gal-1 (40 μg/ml) plus IFN-γ vs IFN-γ alone).

by exposure of monocytes to galectin-1 in the presence of lactose (data not shown). Thus, inhibition of MHC-II expression by galectin-1 results in decreased ability of human monocytes to stimulate T cell proliferation. Our results suggest that galectin-1 found at sites of T cell activation could directly influence MHC-II expression and Ag presentation, suggesting another potential target for the immunoregulatory activity of this carbohydrate-binding protein.

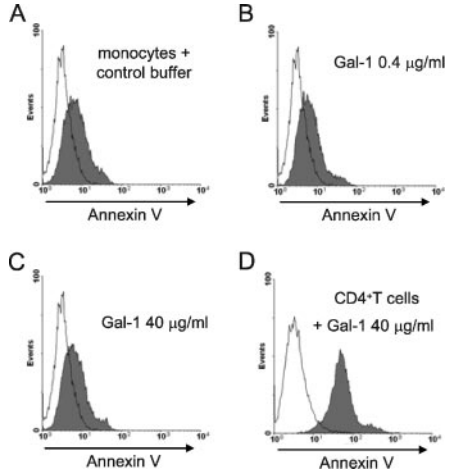

FIGURE 3. Galectin-1 does not affect survival of human monocytes. *A–C*, Human monocytes were cultured in the presence of control buffer (PBS/DTT) (*A*) or different concentrations of galectin-1 (*B* and *C*) for 24 h. Apoptosis was determined using the FITC-annexin V binding assay as described in *Materials and Methods*. A kinetic analysis revealed that galectin-1 does not affect the survival of human monocytes at any of the times tested (3, 6, and 18 h) (data not shown). *D*, In contrast, activated $CD4^+$ T cells were highly susceptible to the proapoptotic effects of galectin-1.

Galectin-1 does not affect the survival of human monocytes

Since galectin-1 has been implicated in the regulation of T cell apoptosis (22–24), we next examined whether this protein might control monocyte functions through modulation of cell survival. As shown in Fig. 3, no significant changes in the levels of annexin V binding were detected in human monocytes treated with control buffer (Fig. 3*A*) or increasing concentrations of galectin-1 (Fig. 3, *B* and *C*) for 18 h. Furthermore, a kinetic analysis revealed that recombinant galectin-1 does not affect the survival of human monocytes at any of the times tested (3, 6, and 18 h) (data not shown). In contrast, purified $CD4^+$ T cells activated for 72 h in the presence of PHA were highly susceptible to the proapoptotic effect of galectin-1 (Fig. 3*D*), as has been reported previously (24). These observations indicate that different immune cell types can display different phenotypes following exposure to galectin-1 and clearly demonstrates the differential susceptibility of monocytes and T cells to galectin-1-induced cell death.

Galectin-1 regulates FcγRI and MHC-II expression through an ERK1/2-dependent pathway

In monocytes and macrophages, several inflammatory stimuli can trigger MAPK phosphorylation (31, 32). The observation that activation of these kinases can play a pivotal role in regulating the effector function of these inflammatory cells prompted us to investigate the possible involvement of different MAPK-dependent signal transduction pathways in galectin-1-induced modulation of monocyte physiology. For this purpose, we examined the effects of disruption of different MAPK signaling pathways in galectin-1-induced regulation of FcγRI and MHC-II expression (Fig. 4). While treatment of human monocytes with SB203580 (a pharmacological inhibitor of p38 MAPK) did not alter the effects of galectin-1 in the regulation of constitutive and inducible FcγRI and MHC-II expression (Fig. 4, *B* and *D*), exposure of human monocytes to PD98059 (an inhibitor of MEK1 and MEK2 kinases that are responsible for ERK1/2 phosphorylation) significantly abrogated the regulatory effects of galectin-1 (Fig. 4, *A* and *C*). Interestingly, the ERK1/2 inhibitor was effective in preventing galectin-1 effects either when this protein was added alone or in combination with IFN-γ. However, this inhibitor was not capable of preventing the up-regulated expression of FcγRI and MHC-II induced by IFN-γ (Fig. 4, *A* and *C*).

In agreement with these findings, Western blot analysis using a specific anti-phospho-ERK1/2 Ab showed a marked increase in

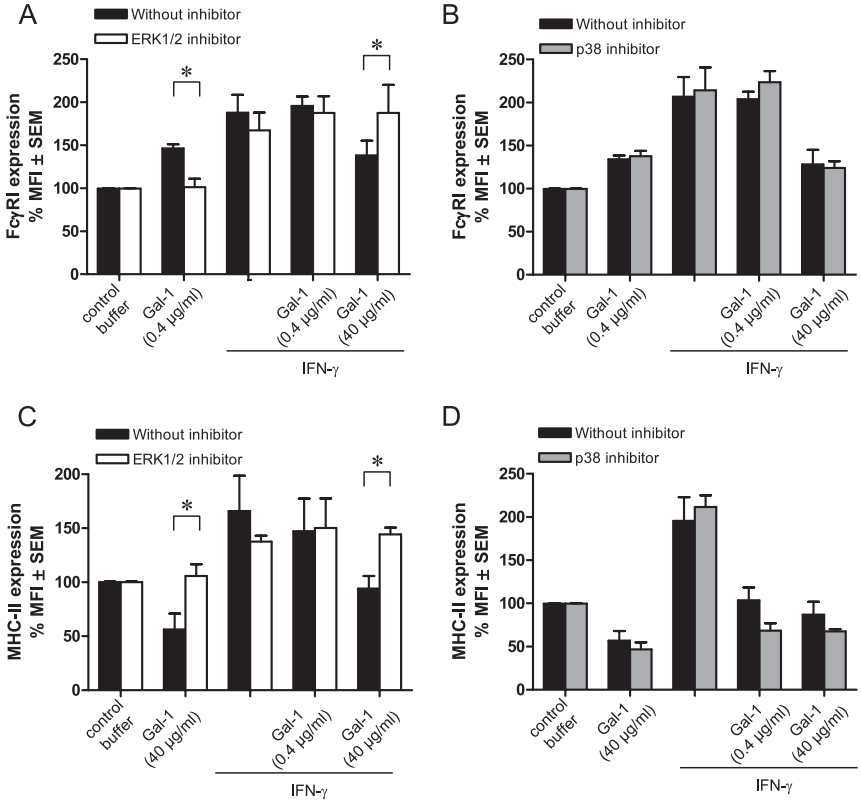

FIGURE 4. Galectin-1 controls FcγRI and MHC-II expression through modulation of an ERK1/2-dependent pathway. *A–D*, Human monocytes (0.5×10^6 cells/ml) were incubated with control buffer (PBS/DTT), galectin-1 alone, or IFN-γ (240 U/ml) plus galectin-1 (0.4 and 40 μg/ml) for 24 h in the absence or presence of the ERK1/2 inhibitor PD98059 (*A* and *C*) or the p38 inhibitor SB203580 (*B* and *D*). Cells were then stained with a FITC-labeled anti-FcγRI mAb (*A* and *B*) or a FITC-labeled anti-HLA-DR mAb (*C* and *D*). Results represent the MFI ± SEM of controls of five independent experiments (*, $p < 0.05$ Gal-1 plus PD98059 vs Gal-1 either in the absence or presence of IFN-γ).

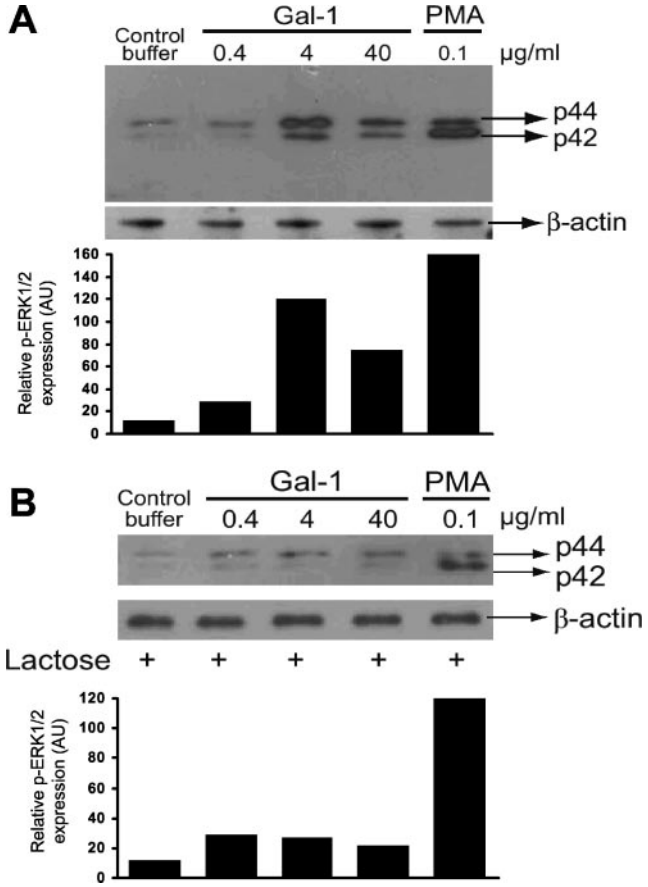

FIGURE 5. Galectin-1 induces ERK1/2 phosphorylation in freshly isolated human monocytes. *A* and *B*, Human monocytes (5×10^6 cells/ml) were incubated with galectin-1 at concentrations of 0.4, 4, and 40 μg/ml or PMA (0.1 μg/ml) for 5 min in the absence (*A*) or presence (*B*) of lactose (30 mM). ERK1/2 phosphorylation was assessed by Western blot using anti-phospho-ERK1/2 Ab. Equal protein loading (60 μg) was evaluated using an anti-β-actin polyclonal Ab. Immunoreactive protein bands were semi-quantified by densitometric analysis, and the sum of these bands (p42 + p44) was expressed as relative arbitrary units (AU). Representative of three independent experiments are shown.

ERK1/2 phosphorylation when monocytes were exposed to galectin-1 (4 and 40 μg/ml) for 15 min (Fig. 5, *lanes 3* and *4*). Human monocytes exposed to PMA (0.1 μg/ml) were used as positive controls (Fig. 5, *lane 5*). In addition, preincubation with PD98059 abolished phosphorylation of ERK1/2, confirming the specificity of the Ab and the effectiveness of the inhibitor (data not shown). Furthermore, exposure of human monocytes to galectin-1 in the presence of 30 mM lactose specifically prevented ERK1/2 phosphorylation induced by this lectin (Fig. 5*B*). Taken together, these data indicate that the ERK1/2-dependent pathway is critically involved in the regulatory effects of galectin-1 on human monocytes.

Galectin-1 negatively regulates MHC-II expression on macrophages recruited in response to inflammatory stimuli

To investigate the effects of galectin-1 in inflammatory macrophages, a glass cylinder was introduced s.c. into BALB/c mice as described previously (30, 33). After 20 days, the cylinders caused a chronic inflammatory process with open ends of the cylinders closed by fibrotic tissue and predominantly infiltrated by macrophages. Subsequently, galectin-1 and/or IFN-γ were injected into the cylinder, and 24 h later, inflammatory cells were isolated and the macrophage population was analyzed for MHC-II (I-A^d) expression by flow cytometry (Fig. 6). In accordance with the results obtained in vitro on human monocytes, injection of galectin-1 into the glass cylinder resulted in marked down-regulation of MHC-II on inflammatory macrophages either activated or not with IFN-γ (Fig. 6, *A* and *B*). Interestingly, this effect was prevented when galectin-1 was injected into the glass cylinder together with ERK1/2 inhibitors (Fig. 6*B*) but not in the presence of p38 inhibitors (data not shown). Thus, treatment with galectin-1 may trigger an ERK1/2-mediated pathway to regulate constitutive and inducible MHC-II expression on mouse macrophages recruited in response to inflammatory stimuli.

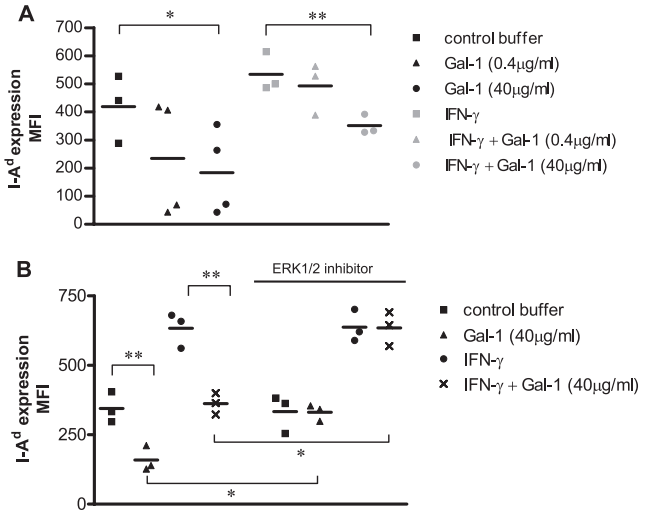

FIGURE 6. Galectin-1 regulates MHC-II surface expression on mouse macrophages recruited in response to inflammatory stimuli. A glass cylinder was introduced s.c. into BALB/c mice ($n = 3$–4 per group per experiment) as described in *Materials and Methods* inducing a chronic inflammatory process. After 20 days, galectin-1 was injected to the cylinder at concentrations of 0.4 and 40 μg/ml in the absence or presence of IFN-γ (3000 U/ml) (*A*). In another set of experiments, galectin-1 or galectin-1 plus IFN-γ were added to the glass cylinders in the presence of the ERK1/2 inhibitor PD98059 (*B*). After 24 h, inflammatory macrophages (0.35×10^5 cells/ml) were recovered from the cylinders and stained with an anti-mouse MHC-II (anti-I-A^d) mAb. Individual values are the MFI for each individual mouse tested of one of three independent experiments (*, $p < 0.05$ Gal-1 40 μg/ml vs control buffer; **, $p < 0.01$ Gal-1 40 μg/ml plus IFN-γ vs IFN-γ alone; *, $p < 0.05$ Gal-1 40 μg/ml plus PD98059 vs Gal-1 alone or Gal-1 plus IFN-γ).

Critical role for endogenous galectin-1 in the regulation of MHC-II expression and macrophage functions

To determine the impact of endogenous galectin-1 in the regulation of macrophage functions, a glass cylinder was introduced s.c. into *gal-1*$^{-/-}$ and WT mice essentially as described above. After 20 days, inflammatory cells were isolated and subjected to phenotypic and functional analysis. As shown in Fig. 7, *A* and *B*, inflammatory macrophages collected from *gal-1*$^{-/-}$ mice showed significantly higher levels of MHC-II (I-A^d) expression compared with WT mice. Accordingly, macrophages isolated from *gal-1*$^{-/-}$ mice demonstrated an increased ability to stimulate proliferation of allogeneic spleen cells from BALB/c mice (Fig. 7*C*). In addition, cells from *gal-1*$^{-/-}$ mice recruited in response to inflammatory stimuli demonstrated an increased capacity to phagocytose ^{51}Cr-labeled IgG coated-SRBC compared with macrophages isolated from WT mice (Fig. 7*D*).

To examine whether galectin-1 was sufficient or redundant for the effects observed in *gal-1*$^{-/-}$ mice, we examined whether recombinant galectin-1 injected into the glass cylinder was able to

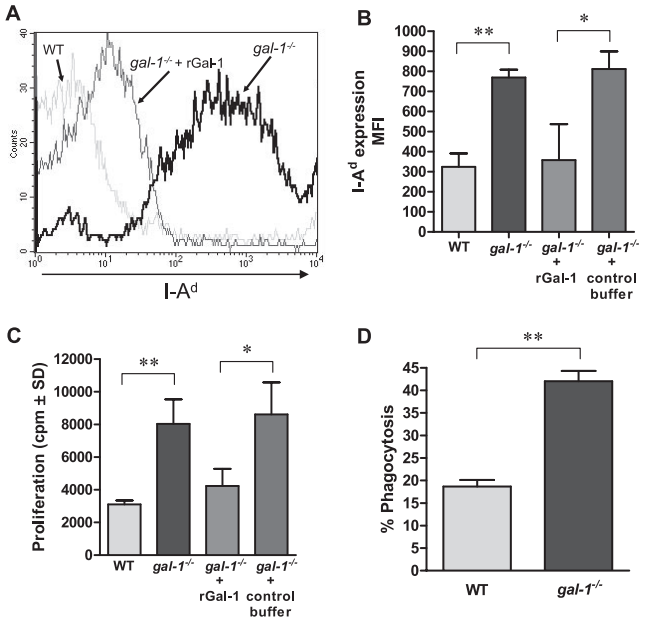

FIGURE 7. Critical role of endogenous galectin-1 in the regulation of MHC-II expression and macrophage functions. *A* and *B*, A glass cylinder was introduced s.c. into *gal-1*$^{-/-}$ or wild-type (WT) mice ($n = 4$ per group per experiment) as described in *Materials and Methods*, inducing a chronic inflammatory process. In other groups of mice, recombinant galectin-1 (rGal-1) or control buffer (PBS/DTT) was incorporated to the cylinders of *gal-1*$^{-/-}$ mice. After 20 days, inflammatory cells were recovered and stained with an anti-mouse MHC-II (anti-I-A^d) mAb (*A* and *B*). Individual values are the MFI for each individual mouse tested of one of two independent experiments (**, $p < 0.01$ *gal-1*$^{-/-}$ vs WT; *, $p < 0.05$ *gal-1*$^{-/-}$ plus rGal-1 vs *gal-1*$^{-/-}$ plus control buffer). *C*, Macrophages from *gal-1*$^{-/-}$ or WT mice exposed or not to recombinant galectin-1 or vehicle control were recovered from cylinders and cultured (1×10^6 cells/ml) with allogeneic spleen cells from BALB/c mice (1×10^6 cells/ml) for 4 days at 37°C and 5% CO_2. Cells were then pulsed with [^{3}H]thymidine for the final 18 h of culture and [^{3}H]thymidine incorporation was expressed as cpm ± SD (**, $p < 0.01$ *gal-1*$^{-/-}$ vs WT; *, $p < 0.05$ gal-1$^{-/-}$ plus rGal-1 vs *gal-1*$^{-/-}$ plus control buffer). *D*, Inflammatory macrophages from *gal-1*$^{-/-}$ or WT mice were recovered from cylinders and phagocytosis of ^{51}Cr-labeled IgG-coated SRBC was evaluated as described in *Materials and Methods* (**, $p < 0.01$ *gal-1*$^{-/-}$ vs WT). Results are representative of two or three independent experiments with $n = 4$ mice per experiment.

counteract up-regulated expression of MHC-II (I-A^d) in inflammatory macrophages from *gal-1*$^{-/-}$ mice. As shown in Fig. 7, *A* and *B*, injection of recombinant galectin-1 (40 μg/ml), but not control vehicle, into the glass cylinder significantly counteracted the increase in I-A^d levels observed in null mutant mice and almost completely abrogated the increased ability of *gal-1*$^{-/-}$ inflammatory macrophages to stimulate allogeneic T cell proliferation (Fig. 7*C*). However, we could not find differences in the levels of phagocytosis between *gal-1*$^{-/-}$ macrophages exposed to recombinant galectin-1 and *gal-1*$^{-/-}$ macrophages exposed to vehicle control (data not shown).

Since galectin-1 is abundant at inflammatory foci and sites of T cell activation, these results provide evidence that galectin-1 is not redundant and plays an essential role in the regulation of particular macrophage functions such as MHC-II-dependent APC activity with critical implications in the orchestration of effector immune responses.

Discussion

In the present study, we provide evidence of a novel role for galectin-1 in the regulation of monocyte and macrophage physiology. By using different experimental approaches, we demonstrate that galectin-1 can differentially regulate the expression and function of critical regulatory molecules involved in innate and adaptive immunity, such as FcγRI and MHC-II. Interestingly, galectin-1 does not affect survival of human monocytes, but instead can modulate monocyte activity through ERK1/2-dependent pathways. These effects were also observed in macrophages recruited in response to inflammatory stimuli following treatment with galectin-1 and further confirmed in *gal-1*$^{-/-}$ mice. To the best of our knowledge, this is the first evidence demonstrating an essential role for galectin-1 in the regulation of monocyte and macrophage physiology with critical implications in the development of adaptive immune responses.

Although cells of the mononuclear phagocyte system (monocytes and macrophages) share similar functional properties, they have marked phenotypic heterogeneity as a result of cellular differentiation, tissue distribution, and responsiveness to a variety of endogenous and exogenous stimuli (1, 34). Interaction of these stimuli with specific cell surface receptors may induce five different states of macrophage activation, which may determine the quality and magnitude of adaptive immune responses: 1) "innate activation," which is induced by microbial products that are recognized by pattern recognition receptors and is responsible for the production of proinflammatory cytokines; 2) "humoral activation," which is triggered by cross-linking of Fc and complement receptors and is critical for functions such as phagocytosis and secretion of pro- and anti-inflammatory cytokines; 3) "classical activation," which is typically mediated by the priming stimulus IFN-γ and is critical for the microbicidal activity of macrophages and DTH responses; 4) "alternative activation," which is triggered by IL-4 and IL-13, induces arginase expression, and determines the generation of Th2 responses; and 5) "deactivation," which is triggered by anti-inflammatory cytokines and uptake of apoptotic cells and results in MHC-II down-regulation and secretion of high levels of IL-10, TGF-β, and PGE_2 (34). The present study together with our previous observations that galectin-1 favors arginase activity (21) and promotes PGE_2 production (35) suggests that this endogenous lectin might promote a state of "alternative activation" or "deactivation" in elicited macrophages.

Expression and function of other members of the galectin family, in particular galectin-3, have been well studied within the monocyte and macrophage populations (8). It has been demonstrated that galectin-3 stimulates different macrophage functions, such as LPS-induced IL-1 production (36), chemotaxis of human monocytes (37), and phagocytosis (38). Furthermore, peritoneal macrophages from galectin-3-deficient mice displayed higher levels of apoptosis compared with cells from wild-type mice, suggesting that this carbohydrate-binding protein may also protect macrophages from cell death (39). In addition, recent findings indicate that galectin-3 is a critical regulator of chronic inflammation, myofibroblast activation, and hepatic fibrosis (40). Taking these findings into consideration, it has been proposed that different members of the galectin family may act in concert to regulate the initiation of the inflammatory response (41–45).

Strikingly, galectin-1 at a concentration of 0.4 μg/ml (~0.03 μM) could up-regulate FcγRI cell surface expression, while this effect was not observed at higher concentrations of this protein. Since the dimerization constant of galectin-1 is ~7 μM, we speculated that this effect was mainly mediated by the monomeric form of this protein. To confirm this finding, we used a monomeric mutant of galectin-1 (*N*-Gal-1) that exists as a monomer at concentrations even higher than 100 μg/ml, yet still retains carbohydrate-binding capacity (26). Our results showed that *N*-Gal-1 induced up-regulation of FcγRI expression even at higher

concentrations, indicating that the monomeric form of galectin-1 is mainly responsible for the observed effects.

However, galectin-1 was able to suppress inducible FcγRI and MHC-II expression in a dose-dependent manner, and this effect was paralleled by a dose-dependent inhibition of FcγRI-dependent phagocytosis and Ag-presenting ability of these cells. This double-edge effect of galectin-1 is not totally surprising. In fact, we have previously demonstrated, in a model of *Trypanosoma cruzi* infection, that exogenously added galectin-1 induces a biphasic modulation of parasite replication in peritoneal macrophages isolated from infected mice or in macrophage cell lines infected in vitro with living trypomastigotes (46). While low concentrations of galectin-1 increased parasite replication, high doses of galectin-1 decreased the number of intracellular amastigotes and extracellular trypomastigotes by inducing apoptosis of highly activated macrophages (45).

Recently, Fulcher et al. (44) demonstrated that galectin-1 can influence the initiation of an adaptive immune response by inducing dendritic cell maturation and activating a genetic program involved in dendritic cell migration through the extracellular matrix (44). Furthermore, dendritic cells engineered to overexpress galectin-1 synthesized increased levels of proinflammatory cytokines and showed enhanced ability to stimulate naive T cells (45). In addition, Dias-Baruffi et al. (47) demonstrated that addition of galectin-1 induces cell surface exposure of phosphatidylserine, thus favoring recognition by phagocytic leukocytes. Similar to our findings, these effects were dependent on protein-carbohydrate interactions because incubation of the specific disaccharide lactose was able to prevent galectin-1 effects either when this protein was added at low or high concentrations. However, in contrast to the effects described above, we observed that regulation of monocyte and macrophage functions by galectin-1 does not require dimerization of this protein. Thus, it seems apparent that galectin-1 may play different roles in the modulation of cells of the myelomonocytic lineage, and these effects may depend on the activation or differentiation state of these cells, the magnitude of protein-glycan or protein-protein interactions, and the relative abundance of the monomeric or dimeric forms of this protein.

The MAPK cascade is known to participate in multiple cellular functions, such as locomotion, proliferation, differentiation, and survival of monocytes and macrophages (31, 32). The MAPKs are phosphorylated on threonine-tyrosine residues by distinct MAPK kinases. ERK1/2 are activated by a variety of growth factors and play a critical role in the regulation of monocyte physiology (31). In contrast, JNK and p38 are typically activated by cellular stress or proinflammatory cytokines that are known to induce cell death (31). It is intriguing that, despite similar carbohydrate specificities, galectins 1 and 3 can trigger different signal transduction pathways on different cell types. In this sense, we have recently demonstrated that galectin-3 induces p38 but not ERK1/2 phosphorylation in human primed neutrophils (48). However, in the present study, we demonstrate that galectin-1 may trigger an ERK1/2- but not p38-dependent signal transduction pathway in human monocytes. In agreement with our findings, Vespa et al. (49) demonstrated that galectin-1 synergizes with TCR engagement to specifically enhance ERK activation. In addition, Levy et al. (50) reported sustained induction of ERK1/2 in the regulation of tumor cell spreading upon ligation of integrins by galectin-8, and Elad-Sfadia et al. (51) showed that galectin-3 triggers a Ras signal that attenuates ERK activation in tumor cells. Thus, it seems that different members of the galectin family may trigger different signal transduction pathways and modulate a great diversity of biological functions in different cell types. Since galectin-1 expression is upregulated during macrophage activation (23, 52), we might speculate that galectin-1 could exert an autocrine negative regulatory effect to control macrophage effector functions during the development of innate and adaptive immune responses.

Although *gal-1*$^{-/-}$ mice do not have apparent phenotypic abnormalities (53), recent evidence indicates that these mice can have subtle but critical alterations in some cells and tissues, including sensory and motoneurons (54, 55). Our results indicate that targeted disruption of the *gal-1* gene can also have important consequences in immune physiology. Whether these alterations can result in increased susceptibility to immune-related pathology still remains to be elucidated.

In summary, the results presented herein provide evidence of a novel role for galectin-1 in the regulation of monocyte and macrophage physiology. This carbohydrate-binding protein can modulate critical immunoregulatory molecules and their associated functions through a nonapoptotic ERK1/2-dependent pathway. Our findings provide alternative explanatory mechanisms for the immunoregulatory effects of galectin-1 in experimental models of acute and chronic inflammation at the crossroad of innate and adaptive immunity.

Acknowledgments

We thank Dr. J. Hirabayashi and Dr. L. Baum for providing the plasmids for preparation of recombinant galectin-1 and the monomeric *N*-Gal-1 mutant. We are also indebted to Dr. F. Poirier for generating and sharing the galectin-1-deficient mice.

Disclosures

The authors have no financial conflict of interest.

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