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Regulation of eosinophilia and allergic airway inflammation by the glycan-binding protein galectin-1

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Galectin-1 (Gal-1), a glycan-binding protein with broad antiinflammatory activities, functions as a proresolving mediator in autoimmune and chronic inflammatory disorders. However, its role in allergic airway inflammation has not yet been elucidated. We evaluated the effects of Gal-1 on eosinophil function and its role in a mouse model of allergic asthma. Allergen exposure resulted in airway recruitment of Gal-1–expressing inflammatory cells, including eosinophils, as well as increased Gal-1 in extracellular spaces in the lungs. In vitro, extracellular Gal-1 exerted divergent effects on eosinophils that were N-glycan– and dose-dependent. At concentrations ≤0.25 μM, Gal-1 increased eosinophil adhesion to vascular cell adhesion molecule-1, caused redistribution of integrin CD49d to the periphery and cell clustering, but inhibited ERK(1/2) activation and eotaxin-1–induced migration. Exposure to concentrations ≥1 μM resulted in ERK(1/2)-dependent apoptosis and disruption of the F-actin cytoskeleton. At lower concentrations, Gal-1 did not alter expression of adhesion molecules (CD49d, CD18, CD11a, CD11b, L-selectin) or of the chemokine receptor CCR3, but decreased CD49d and CCR3 was observed in eosinophils treated with higher concentrations of this lectin. In vivo, allergen-challenged Gal-1–deficient mice exhibited increased recruitment of eosinophils and CD3$^+$ T lymphocytes in the airways as well as elevated peripheral blood and bone marrow eosinophils relative to corresponding WT mice. Further, these mice had an increased propensity to develop airway hyperresponsiveness and displayed significantly elevated levels of TNF-α in lung tissue. This study suggests that Gal-1 can limit eosinophil recruitment to allergic airways and suppresses airway inflammation by inhibiting cell migration and promoting eosinophil apoptosis.

galectin-1 | eosinophils | apoptosis | migration | allergic airway inflammation

Allergic asthma is an inflammatory disease of the airways that is associated with increased pulmonary recruitment of inflammatory cells, especially eosinophils, elevated levels of Th2 cytokines, proinflammatory chemokines, and growth factors that together contribute to the overall pathogenesis of the disease including the development of bronchoconstriction and airway hyperresponsiveness (AHR) (1). This disease is to a large extent driven by activation of Th2 cells and airway eosinophilia. Further, decreased number and/or function of regulatory T cells (Tregs) is also thought to contribute to atopic allergic disease and asthma in patients (2). Although various therapeutic approaches targeting T cells or cytokines and chemokines released by these cells have been developed, positive outcomes are either limited to small subpopulations (3) or vary significantly among patient groups, thus warranting the need for identification of novel or alternate therapeutic approaches.

Galectin-1 (Gal-1) is a glycan-binding protein that binds to *N*-acetyl-lactosamine (Gal-β1-3/4-*N*-acetyl-D-glucosamine or LacNAc) residues of complex N- and O-glycans on cell-surface glycoconjugates (4). Gal-1 has wide distribution in adult tissue including the lung, and is expressed by various cell types such as polymorphonuclear cells (PMNs), macrophages, dendritic cells (DCs), activated T cells, stromal cells, endothelial cells, and epithelial cells (5, 6). At a cellular level, Gal-1 may act either intracellularly or extracellularly, and is profoundly involved in resolving acute and chronic inflammation by affecting processes such as immune cell adhesion, migration, activation, signaling, proliferation, differentiation, and apoptosis (5, 7). Increasing evidence from multiple chronic inflammatory disease models supports the critical antiinflammatory role of exogenous and endogenous Gal-1 in limiting or resolving inflammation (6). Additionally, Gal-1 showed proresolving effects in models of acute inflammation where neutrophil recruitment (8) and extravasation (9) as well as mast cell degranulation were suppressed (9). The immunosuppressive role of Gal-1 was further supported by studies with Tregs where cells from Gal-1 null mice exhibited reduced regulatory activity (10). More recent studies emphasized the immunosuppressive effect of Gal-1 in macrophages and cells of the microglia compartment, which showed a shift toward an M2 phenotype and reduced secretion of proinflammatory cytokines upon exposure to this lectin (11).

Given the critical antiinflammatory and proresolving function of Gal-1 in innate and adaptive immune compartments and its broad expression in lung tissue, we hypothesized that this endogenous lectin might regulate eosinophil function and airway inflammation.

Significance

Allergic asthma is a chronic airway disease, and the number of individuals with asthma continues to grow. Eosinophils recruited to allergic airways contribute significantly to airway inflammation via release of proinflammatory mediators that cause epithelial tissue damage, bronchoconstriction, and airway remodeling. Here we show that galectin-1 (Gal-1), an endogenous immunoregulatory lectin, binds to eosinophil-expressed surface glycans to inhibit cell migration and induce apoptosis. Using a mouse model of allergic asthma, we show that mice lacking Gal-1 exhibit increased airway eosinophils and airway hyperresponsiveness compared with wild-type mice. Because Gal-1 plays an important role in regulating airway inflammation, identifying pathways to induce Gal-1 synthesis and/or favor its biological activity might enable exploitation of its proresolving function to suppress allergic asthma.

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Clinical studies have shown that sputum leukocytes (largely macrophages) from asthmatic patients have decreased intracellular Gal-1 relative to cells from healthy subjects, suggesting that decreased Gal-1 expression might favor exacerbation of asthma (12). In the present study, we examined the regulated expression and biological relevance of endogenous and exogenous/extracellular Gal-1 in eosinophil function in vitro and in a model of allergic asthma.

Results

Expression of Gal-1 Is Induced in Allergic Lungs. We examined the expression of Gal-1 in the lungs during allergic inflammation in a mouse model. In control mice, baseline Gal-1 expression was observed mostly in airway epithelial cells, smooth muscle cells, and endothelial cells by immunohistochemistry (IHC; Fig. 1*A*, *Upper*, *Left* and *Middle*). Acute or chronic ovalbumin (OVA) exposure resulted in increased Gal-1 expression in the lungs that was largely observed in the recruited inflammatory cells in addition to expression in airway epithelial cells, smooth muscle cells, and endothelial cells (Fig. 1*A*, *Lower*, *Left* and *Middle*). Further, increased Gal-1 expression was detected in extracellular spaces after allergen exposure (Fig. 1*A*, *Right*, *Upper* and *Lower*). Dual immunofluorescence (IF) staining of lung sections from allergen-challenged mice for Gal-1 and eosinophil peroxidase (EPX), an eosinophil-specific marker, revealed several cells that stained positively for Gal-1 (red) and EPX (green) in addition to cells that were positive for Gal-1 but negative for EPX (Fig. 1*B*, *Lower*). These findings indicate that eosinophils, the major inflammatory cells recruited to allergic airways, express Gal-1. Total lung Gal-1 expression was significantly higher in acute OVA-challenged mice compared with saline-exposed controls at the level of mRNA and protein (Fig. 1 *C* and *D*). Soluble Gal-1 was not detectable in the bronchoalveolar lavage fluid (BALF) of OVA-challenged mice, whereas Gal-3 was easily detected in these samples (Fig. S1*A*). Exposure to other allergens such as *Alternaria alternata* extract (Fig. S1*B*) or cockroach antigen (CRA; Fig. S1 *C* and *D*) also resulted in increased Gal-1 expression in the lungs of mice similar to OVA. Thus, allergic airway inflammation irrespective of the nature of allergen exposure results in up-regulated expression of Gal-1.

Gal-1 Binds to Eosinophils in a Carbohydrate-Dependent Manner. We examined Gal-1 expression at the cellular level using bone marrow (BM)-derived murine eosinophils and identified a protein band of ∼14.5 kDa corresponding to Gal-1 in its monomeric form (13) (Fig. 2*A*). Interestingly, flow cytometry studies revealed that Gal-1 expression by eosinophils (nonactivated) was detectable only when cells were permeabilized (Fig. 2*B*, *Right*), with no positive staining for this lectin under nonpermeabilized conditions (Fig. 2*B*, *Left*). This suggests that in resting eosinophils, Gal-1 is expressed intracellularly but is undetectable on the cell surface. Further, IF staining of permeabilized eosinophils indicated that Gal-1 was predominantly expressed in the cytosolic compartment (Fig. 2*C*).

Because allergen exposure results in increased Gal-1 expression in extracellular spaces (Fig. 1*A*), it is likely that eosinophils recruited to the airways are exposed to Gal-1 extracellularly. We found that soluble recombinant Gal-1 (rGal-1) binds to the surface of eosinophils in a dose-dependent fashion (Fig. 2*D*, *Left*). Exposure to rGal-1 in the presence of increasing concentrations of lactose, a specific disaccharide inhibitor, but not maltose (negative control), inhibited Gal-1 binding to the cell surface, indicating that Gal-1 binds to surface ligands on eosinophils in a carbohydrate-dependent manner (Fig. 2*D*, *Right*). Intracellular (protein and mRNA) and cell-surface expression of Gal-1 were not altered in eosinophils after stimulation/activation with TNF-α, eotaxin-1 (CCL11), or stem cell factor-1 (SCF-1) (Fig. S2 *A* and *B*). Further, Western blot analysis of culture supernatant concentrates (∼10-fold) from these activated eosinophils was negative for Gal-1 immunoreactivity, suggesting that Gal-1 may not be secreted in detectable levels by eosinophils.

Eosinophils Express the Repertoire of Cell-Surface Glycans Required for Gal-1 Binding and Function. Gal-1 is well-known for its ability to recognize complex N- and O-glycans containing unmodified or terminally modified (α2,3-sialylated) LacNAc residues (5). To identify Gal-1–binding carbohydrate partners on eosinophils, we analyzed the glycophenotype of these cells using an array of labeled plant lectins with known carbohydrate-binding specificities by flow cytometry. Murine eosinophils express complex N-glycans [β1-6 branched N-linked carbohydrates, based on *Phaseolus vulgaris* leucoagglutinin (PHA)-L reactivity] as well as O-glycans [asialo core-1 O-glycans bearing terminal galactose residues (Galβ1-3GalNAcαSer-Thr), based on peanut agglutinin (PNA) reactivity] (Fig. 3*A*). Based on staining intensity, eosinophils expressed predominantly glycans containing poly-LacNAc [*Lycopersicon esculentum* (tomato) lectin (TL) reactivity] and to a lesser extent glycans containing α2,3-linked sialic acid residues [*Maackia amurensis* lectin-II (MAL-II) reactivity] and α2,6-linked sialic acid residues [*Sambucus nigra* lectin (SNA) reactivity], thus demonstrating the presence of many potential Gal-1–binding partners on their cell surface. When pretreated with these lectins,

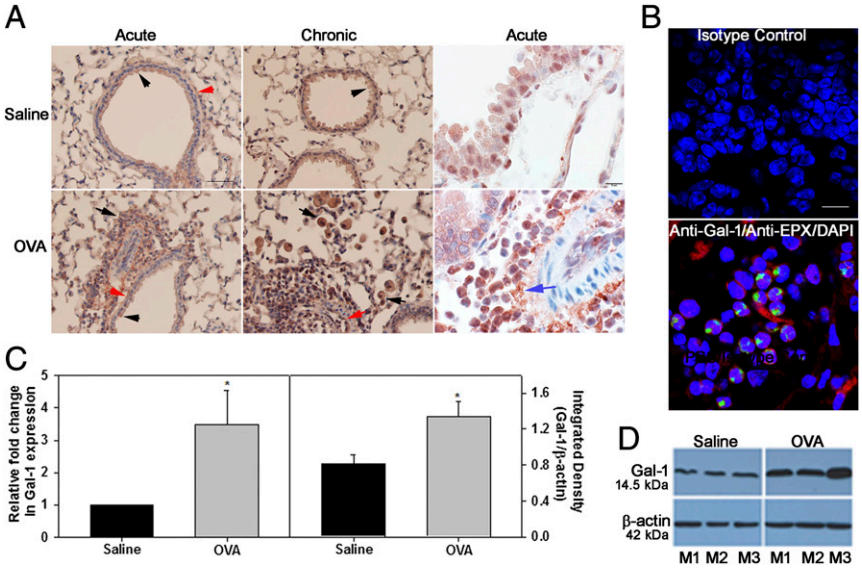

Fig. 1. Expression of Gal-1 is induced in allergic lungs. (*A*) Gal-1 expression in lungs of mice exposed to allergen challenge (or saline alone as control) by IHC. Gal-1 expression in airway epithelial cells (black arrowheads), endothelial cells (red arrow), smooth muscle cells (red arrowheads), inflammatory cells (black arrows), and extracellular spaces (blue arrow) is shown. [Scale bars, 50 μm (*Left*) and 10 μm (*Right*).] (*B*) Lung sections from OVA-exposed mice dual-stained with antibodies against Gal-1 (red) and eosinophil-specific mouse anti-human EPX (green) (*Bottom*) or rabbit and mouse IgG as controls (*Top*). (Scale bar, 10 μm.) (*C*) Gal-1 mRNA (*Left*) and protein (*Right*) expression in lungs of OVA (acute)-challenged or saline-exposed mice. (*D*) Gal-1 expression in lung tissue of three representative mice (M1–M3) for each group. Data are representative of $n = 6$ mice per group in *A* and $n = 3$ mice per group in *B*. Combined data (mean ± SEM) of $n = 5$–7 mice per group are shown in *C*. $^*P < 0.05$ (*Left*) and $^*P < 0.01$ (*Right*) in *C* for control versus allergen-challenged group.

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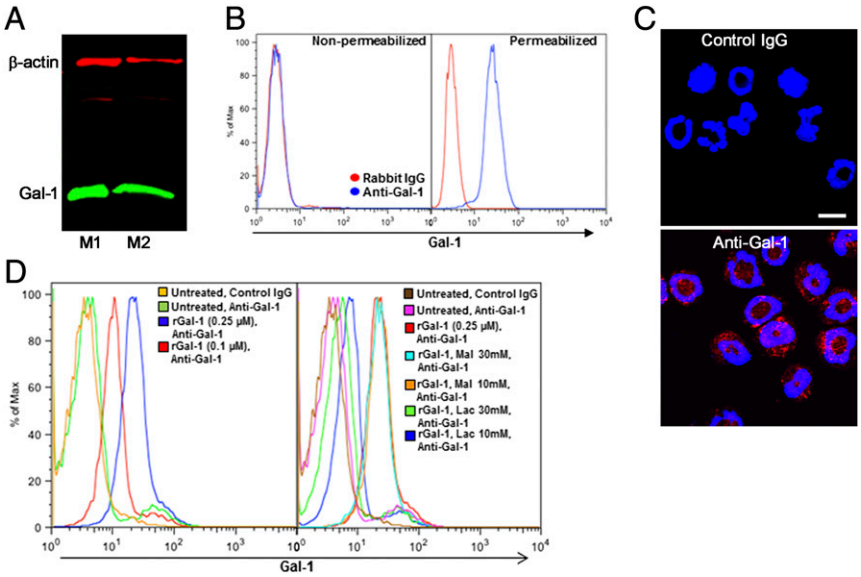

Fig. 2. Murine eosinophils express Gal-1 intracellularly and bind to Gal-1 on the cell surface. (*A*) Expression of Gal-1 in lysates of BM eosinophils from two representative mice (M1 and M2) by Western blot analysis. (*B*) Expression of Gal-1 in BM-derived eosinophils by flow cytometry. Note that Gal-1 expression is detected only in permeabilized cells. (*C*) IF staining of permeabilized eosinophils with anti–Gal-1 (*Bottom*) or rabbit IgG (*Top*). (Scale bar, 10 μm.) (*D*) Dose-dependent Gal-1 binding to nonpermeabilized eosinophils in the absence (*Left*) or presence (*Right*) of increasing concentrations of lactose (Lac) or maltose (Mal). Data are representative of three independent experiments with eosinophils from different mice.

only TL showed partial, albeit significant, reduction in binding of rGal-1 to the cell surface (Fig. 3*B*), substantiating the involvement of LacNAc residues in Gal-1 binding. Studies with eosinophils where O-linked glycosylation was specifically inhibited using benzyl-α-GalNAc (BG), an inhibitor of O-linked oligosaccharide chain elongation, demonstrated significantly increased Gal-1 binding (Fig. 3*C*). This suggests that, at least in the case of eosinophils, O-glycosylation may mask Gal-1 effects. However, binding of Gal-1 to eosinophils required complex branched N-glycans. Gal-1 binding to BM-derived eosinophils from mice deficient in UDP-*N*-acetylglucosamine:α-6-D-mannoside β1,6 *N*-acetylglucosaminyltransferase V (Mgat5, *Mgat5*$^{-/-}$), which lack tetraantennary N-glycans, was substantially lower than binding to their WT counterparts (Fig. 3*D*). These findings indicate that Gal-1 binding to eosinophils is mediated in part via interaction with complex N-linked glycans and does not require the presence of O-glycans.

Exogenous Gal-1 Induces Apoptosis in Eosinophils. Given the role of extracellular Gal-1 in promoting apoptosis of activated as well as fully differentiated T cells (14, 15) and modulating phosphatidylserine exposure (without engaging the full apoptotic program) in PMNs (16), we examined the direct effects of rGal-1 on eosinophil survival. Significant loss of cell viability (based on trypan blue dye staining) was observed when eosinophils were exposed to rGal-1 at concentrations of 1.0 μM or higher for 15 min, whereas no effect was noted at lower concentrations (Fig. 4*A*). Gal-1 induced phosphatidylserine exposure in eosinophils, an early apoptotic event, in a dose-dependent manner (Fig. 4*B*) at time points as early as 5 min at a concentration of 5 μM (Fig. 4*C*), as shown by annexin V staining. Under these conditions, rGal-1 also modulated later events in the apoptosis cascade, as shown by TUNEL assay. A substantial number of rGal-1–treated eosinophils were positive for TUNEL staining similar to cells treated with staurosporine, a well-known apoptotic stimulus, whereas control cells [PBS-treated (for Gal-1) and DMSO-treated (for staurosporine)] were negative for TUNEL staining (Fig. 4*D*). Treatment with rGal-1 also resulted in clustering of cells relative to control cells or cells treated with staurosporine, an effect commonly observed in the presence of exogenous Gal-1 (17). Further, rGal-1 exerted a similar effect on peripheral blood eosinophils. Treatment of peripheral blood leukocytes from allergen-challenged mice with 1.0 μM rGal-1 resulted in an approximately threefold increase in the number of Siglec-F–positive cells (eosinophils) that stained positive for annexin V (Fig. 4*E*). The glycan-binding requisite for Gal-1–induced eosinophil apoptosis was established by studies wherein treatment of cells with rGal-1 in the presence of lactose but not maltose completely inhibited apoptosis (Fig. 4*F*). In addition, a substantial number of eosinophils exposed to rGal-1 at 5 μM were found to undergo shape change, potentially cell shrinkage, based on the decrease in mean forward scatter (FSC) detected compared with untreated cells (Fig. 4*G*, *Left*). This decrease in FSC was inhibited by lactose but not maltose (Fig. 4*G*, *Right*).

To examine whether Gal-1–induced eosinophil apoptosis is mediated via cell-surface elongated O-glycans and/or complex branched N-glycans, cells treated with BG as described earlier or *Mgat5*$^{-/-}$ eosinophils were exposed to rGal-1. rGal-1–induced cell

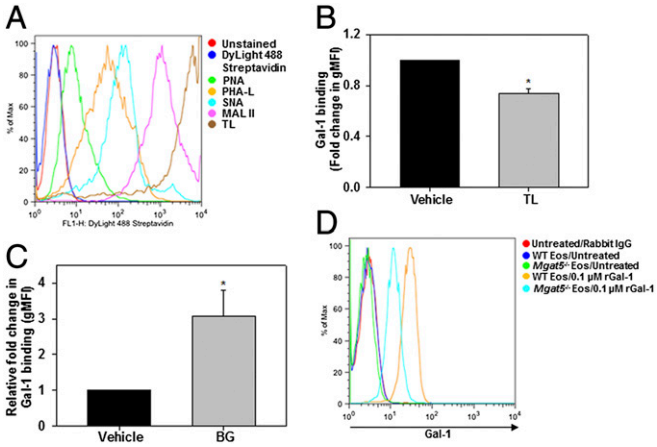

Fig. 3. Gal-1 binding to eosinophil-expressed glycans. (*A*) Glycophenotype analysis of BM eosinophils. Cells were incubated with specific plant lectins followed by streptavidin-conjugated DyLight 488 and then analyzed by flow cytometry. (*B*) Gal-1 binding to eosinophils pretreated with TL or vehicle (PBS) examined by flow cytometry with Gal-1 antibody. gMFI, geometric mean fluorescence intensity. (*C*) Role of O-glycans in Gal-1 binding. Eosinophils cultured in medium containing BG (2 mM) or vehicle (DMSO) were treated with rGal-1 and examined for Gal-1 binding. (*D*) Requirement of complex branched N-glycans for Gal-1 binding. WT and *Mgat5*$^{-/-}$ eosinophils (nonpermeabilized) were treated with rGal-1 and examined for Gal-1 binding. Data in *A* and *D* are representative of three independent experiments with eosinophils from different mice. Combined data (mean ± SEM) of four experiments are shown in *B* and *C*. *$P < 0.01$ in *B* and *$P < 0.03$ in *C* versus vehicle-treated cells.

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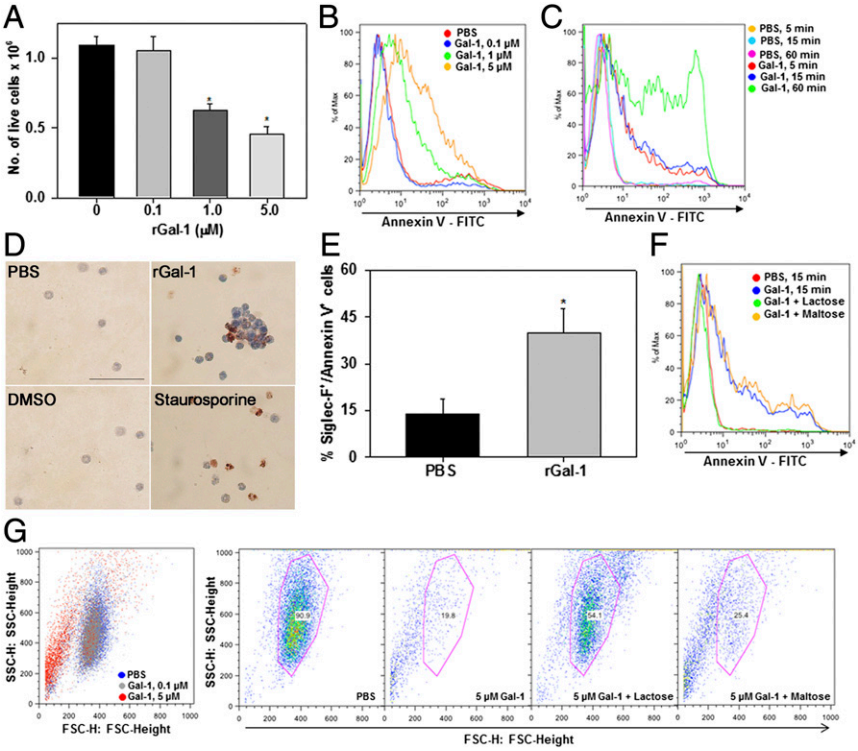

Fig. 4. Exogenous Gal-1 induces eosinophil apoptosis. (*A*) Effect of rGal-1 on the absolute number of live cells by trypan blue dye exclusion at the indicated doses. (*B* and *C*) Flow cytometric analysis after annexin V staining of eosinophils exposed to different concentrations of rGal-1 and eosinophils treated with 5.0 μM rGal-1 for different durations of time, respectively. (*D*) Analysis of cell death in eosinophils (stained brown) after rGal-1 or staurosporine (positive control) treatment by TUNEL assay. (Scale bar, 50 μM.) (*E*) Annexin V staining of peripheral blood leukocytes exposed to rGal-1. Annexin V positivity of cells gated for Siglec-F–positive staining (eosinophils) is shown. (*F*) Inhibition of Gal-1–induced apoptosis by lactose but not maltose (negative control). (*G*) Effect of rGal-1 on eosinophil shape change. FSC versus side scatter dot plots are shown. (*G*, *Left*) Dose-dependent cell shrinkage in Gal-1–treated eosinophils. (*G*, *Right*) Blockade of this effect by lactose but not maltose. Combined data (mean ± SEM) of three independent experiments with cells from $n = 3$–7 mice are shown in *A* and *E*. Data shown in *B*–*D*, *F*, and *G* are representative of two or three experiments with eosinophils from different mice. $^*P < 0.01$ in *A* and *E* for comparison with PBS-treated cells.

death was higher in BG-treated cells compared with corresponding vehicle-treated cells (Fig. 5*A*), confirming the fact that O-glycans may mask Gal-1 effects. On the other hand, *Mgat5*$^{-/-}$ eosinophils showed reduced Gal-1–induced apoptosis relative to WT eosinophils (Fig. 5*B*), suggesting that Gal-1–induced eosinophil apoptosis is partly mediated by complex β1-6 branched N-glycans. Exposure of cells to rGal-1 at proapoptotic concentrations (1–5 μM) resulted in a marked increase in phosphorylated ERK(1/2) relative to untreated cells (Fig. 5*C*, *Left* and *Right*). Surprisingly, this effect was associated with a significant decrease in the levels of total ERK(1/2). Interestingly, pretreatment of eosinophils with MG132, a proteasome inhibitor that inhibits degradation of ubiquitin-tagged proteins, before exposure to rGal-1 prevented the reduction in total ERK levels (Fig. 5*D*, *Left* and *Right*). These studies suggest that rGal-1–induced apoptosis is mediated via a rapid and strong activation of ERK(1/2) and is associated with degradation of ERK(1/2) by the proteasome.

To further investigate the signaling pathway(s) implicated in this effect, eosinophils were pretreated with inhibitors of signaling molecules such as Syk, Rho-associated protein kinase-1 (ROCK1), phosphoinositide 3-kinase (PI3K), mitogen-activated protein kinase kinase (MEK), and caspases before incubation with rGal-1 and then analyzed for annexin V binding. Significant dose-dependent inhibition of annexin V binding was noted only with the MEK inhibitor U0126, not with inhibitors of Syk, ROCK1, or PI3K (Fig. 5*E*). Because Gal-1–induced apoptosis in T cells is modulated via activation of caspases (18), we examined the involvement of caspases in Gal-1–induced apoptosis of eosinophils. Pretreatment with the pan-caspase inhibitor Z-VAD-FMK up to 100 μM did not inhibit Gal-1–induced eosinophil apoptosis. In contrast, this inhibitor substantially reduced staurosporine-induced apoptosis, which is known to be caspase-mediated at this concentration (Fig. 5*F*). These studies suggest that Gal-1–induced apoptosis of eosinophils involves activation of the ERK(1/2) signaling pathway and may be independent of caspase activation. This is further supported by the lack of caspase-3 activation in rGal-1–treated eosinophils. Unlike staurosporine, rGal-1 treatment did not result in an increase in expression of cleaved caspase-3 (~17 kDa) by Western blot analysis (Fig. 5*G*).

Exposure to Gal-1 Differentially Controls Eosinophil Adhesion and Migration. We examined the effect of rGal-1 on the ability of eosinophils to adhere to vascular cell adhesion molecule-1 (VCAM-1), which supports eosinophil trafficking and recruitment to sites of inflammation. Because rGal-1 induces apoptosis of eosinophils at concentrations of 1.0 μM or higher, these studies were carried out at lower doses of rGal-1. Cells exposed to lower concentrations of rGal-1 exhibited increased adhesion to recombinant murine VCAM-1 in a dose-dependent manner compared with untreated cells under static conditions (Fig. 6*A*). However, despite the increased adhesion, eosinophils treated with rGal-1 at these concentrations demonstrated significantly reduced migration toward eotaxin-1 in in vitro chemotaxis assays (Fig. 6*B*), an effect that was accompanied by inhibition of ERK(1/2) activation (Fig. 6*C*). This is in contrast to the increased activation of ERK(1/2) observed in eosinophils exposed to higher concentrations of rGal-1 in the apoptosis studies described above.

We investigated whether treatment with Gal-1 alters the expression of cell-surface adhesion molecules that mediate eosinophil adhesion and migration by flow cytometry. Exposure to Gal-1 up to 0.25 μM did not appear to alter the level of expression of α4 (CD49d), β2-, αL-, or αM-integrins, L-selectin, or the eotaxin receptor CCR3 on the cell surface (Fig. S3*A*). However, confocal microscopy following dual IF staining with antibodies against α4 and Gal-1 indicated that relative to the more dispersed α4 expression noted in untreated cells, treatment of eosinophils with rGal-1 not only induced cell clustering but also resulted in redistribution of α4 to the periphery/edge of individual cells in the cluster, where strong Gal-1 expression was also observed (Fig. 6*D*). Further, several cells exhibited colocalization of α4-integrin and Gal-1. This Gal-1–mediated effect was inhibited when cells were treated with lactose but not maltose. The ability of Gal-1 to interact with α4-integrins was investigated using human α4-transfected Chinese hamster ovary (α4β1-CHO-K1) cells that express functional α4β1 relative to control CHO-K1 cells that do not (Fig. S3*B*). α4-CHO-K1 and control CHO-K1 cells were found to inherently express Gal-1 on their cell surface. However, increased cell-surface binding of exogenous rGal-1 to α4-CHO-K1 cells was noted relative to nontransfected cells (Fig. S3*C*, *Upper*).

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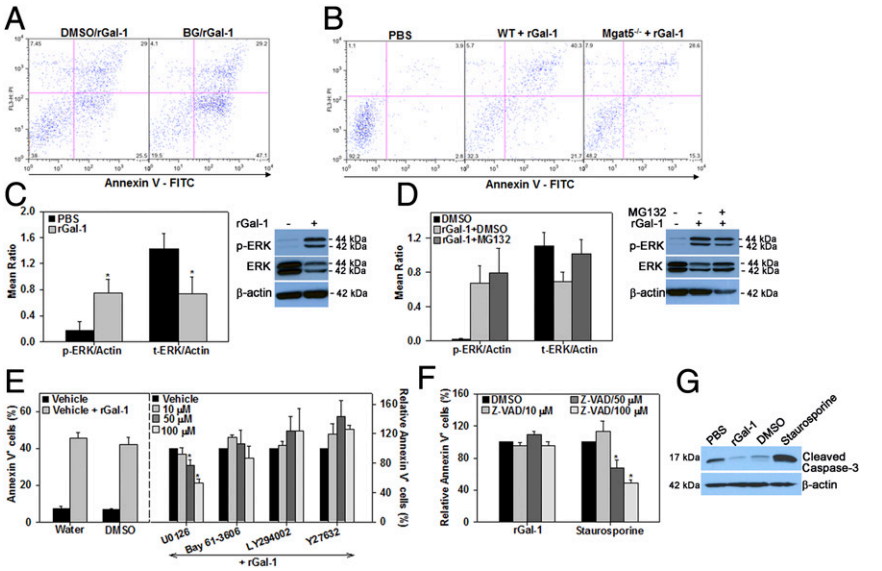

Fig. 5. Gal-1–induced apoptosis is mediated by complex branched N-glycans and involves activation of the ERK(1/2) signaling pathway. (*A*) Involvement of O-glycans in Gal-1–induced apoptosis. Eosinophils cultured in medium containing BG or vehicle (DMSO) were treated with rGal-1 (5 μM) and examined for annexin V positivity. (*B*) Requirement of complex branched N-glycans for Gal-1–induced apoptosis. WT and *Mgat5*$^{-/-}$ eosinophils were treated with rGal-1 and examined for annexin V positivity. (*C*) Induction of ERK(1/2) phosphorylation in Gal-1–treated eosinophils determined by densitometric analysis of Western blots. (*D*) Inhibition of ERK(1/2) degradation by proteasome inhibitor MG132 determined by densitometric analysis of Western blots. (*E*) Inhibition of Gal-1–induced apoptosis by MEK1/2 inhibitor. (*E*, *Right*) Annexin V staining of eosinophils pretreated with the indicated inhibitors of signaling molecules and then with rGal-1 relative to vehicle alone is shown. (*E*, *Left*) Annexin V positivity in vehicle-treated versus rGal-1–treated cells is shown. (*F*) Caspase-independent apoptosis of eosinophils by Gal-1. Cells were pretreated with caspase inhibitor (pan; Z-VAD-FMK) at the indicated doses (or vehicle) followed by rGal-1 or staurosporine and examined for annexin V positivity. (*G*) Western blot of Gal-1–treated eosinophils with antibodies against cleaved caspase-3. Data shown in *A* and *B* are representative of three experiments and in *G* of two experiments with eosinophils from different mice. Combined data (mean ± SEM) of three or four independent experiments in *C* and *D* and four to six independent experiments in *E* and *F* are shown. $*P < 0.05$ in *C*, $*P < 0.05$ for 50 μM and $*P < 0.01$ for 100 μM U0126 in *E*, and $*P < 0.01$ for 50 and 100 μM Z-VAD in *F* for comparison with PBS or vehicle-treated cells.

Further, this increased binding of exogenous rGal-1 to α4-CHO-K1 was glycan-dependent and inhibited by lactose (Fig. S3*C*, *Lower*). Because Gal-1 exerts differing effects on eosinophils depending on the concentration (alteration of adhesion and migration at lower concentrations versus apoptosis at higher concentrations), we next examined the effect of Gal-1 at a higher concentration (1.0 μM, proapoptotic) on cell-surface expression of these receptors. Interestingly, only expression of α4 and CCR3 was partially reduced at this concentration (Fig. 6*E*). Finally, because Gal-1 alters eosinophil motility and survival, its effect on the actin cytoskeleton was evaluated based on phalloidin staining (indicative of actin polymerization). Gal-1 at lower concentrations (0.25 μM) had no effect, whereas apoptosis-inducing concentrations resulted in a marked reduction in phalloidin binding relative to untreated cells by flow cytometry (Fig. 6*F*). Further, confocal microscopy revealed distinct phalloidin binding predominantly at the cell periphery/margin of control cells (Fig. 6*G*, *Left*), whereas Gal-1 treatment at proapoptotic concentrations caused disruption of this organized actin cytoskeleton in many cells, with an overall decrease in phalloidin binding (Fig. 6*G*, *Right*).

Gal-1 Deficiency Results in Increased Allergen-Induced Airway Inflammation. To understand the pathophysiological relevance of Gal-1 in allergic airway inflammation, WT and Gal-1–deficient (lectin galactoside-binding soluble 1 [*Lgals1*$^{-/-}$]) mice were sensitized and challenged with OVA. OVA challenge induced a marked influx of inflammatory leukocytes in the BALF of WT and *Lgals1*$^{-/-}$ mice. However, the total number of inflammatory cells and specifically of eosinophils and lymphocytes was significantly higher in the BALF of OVA-challenged *Lgals1*$^{-/-}$ mice (Fig. 7 *A* and *B*). Consistent with this finding, H&E staining showed evidence of greater lung tissue inflammation in OVA-challenged *Lgals1*$^{-/-}$ mice relative to WT mice (Fig. 7*C*) and a significantly increased number of lung tissue eosinophils and T cells based on IHC for major basic protein (MBP), an eosinophil-specific protein (Fig. 7 *D* and *E*), and CD3, a T cell-specific marker (Fig. 7 *F* and *G*), respectively. Along with increased airway eosinophils, peripheral blood and BM differential cell counts indicated a significantly higher percentage of eosinophils in OVA-challenged *Lgals1*$^{-/-}$ mice relative to WT counterparts (Fig. 7 *H* and *I*).

A larger number of blood vessels in the lungs of WT OVA-challenged mice exhibited eosinophils (MBP-positive cells) adherent along the vessel wall relative to lung blood vessels of *Lgals1*$^{-/-}$ mice (Fig. 8*A*, *Left*). Further, the number of adherent eosinophils per vessel was also significantly higher in WT OVA-challenged mice compared with *Lgals1*$^{-/-}$ counterparts (Fig. 8*A*, *Right*). AHR in control and OVA-challenged WT and *Lgals1*$^{-/-}$ mice exposed to increasing concentrations of aerosolized methacholine was evaluated (Fig. 8*B*). Control mice of both genotypes displayed only a marginal increase in pulmonary resistance (R_L) with increasing doses of methacholine. In OVA-challenged *Lgals1*$^{-/-}$ mice, airway resistance was significantly higher compared with corresponding control mice even at low doses of methacholine challenge (i.e., 6 mg/mL), and remained elevated at higher doses. OVA-challenged WT mice, on the other hand, exhibited increased airway resistance relative to WT control mice only at higher doses of methacholine (>25 mg/mL). More importantly, airway resistance in OVA-challenged *Lgals1*$^{-/-}$ mice was significantly higher than in WT counterparts at 6, 12, and 25 mg/mL methacholine. These findings are indicative of a higher tendency to develop AHR in OVA-challenged *Lgals1*$^{-/-}$ and are consistent with the increased cellular inflammation observed in these mice relative to WT allergen-challenged mice. In addition to cellular inflammation, airway mucus accumulation and increased airway smooth muscle mass are important factors that contribute to increased airway resistance. OVA challenge increased airway mucus secretion and airway smooth muscle mass in WT and *Lgals1*$^{-/-}$ mice compared with corresponding control mice (Fig. 8 *C*–*F*). Whereas there was no significant difference between the OVA-challenged groups with respect to airway mucus secretion (Fig. 8 *C* and *D*), smooth muscle mass in OVA-challenged *Lgals1*$^{-/-}$ mice was lower than in WT mice (Fig. 8 *E* and *F*).

Modulation of Cytokines and Chemokines in Airways of Allergen-Challenged WT and *Lgals1*$^{-/-}$ Mice. Consistent with the asthma phenotype, IL-5, TNF-α, TGF-β, eotaxin-1, and eotaxin-2 (CCL24) were all significantly elevated in the BALF of OVA-challenged mice of both genotypes compared with corresponding control mice. No significant difference in the levels of any of these mediators was noted between OVA-challenged *Lgals1*$^{-/-}$ and WT mice (Fig. 9*A* and Fig. S4*A*). However, TNF-α levels were significantly higher in lung tissue from OVA-challenged *Lgals1*$^{-/-}$ mice than in WT counterparts, and IL-5 and IL-13 also tended to be higher (Fig. 9*B*).

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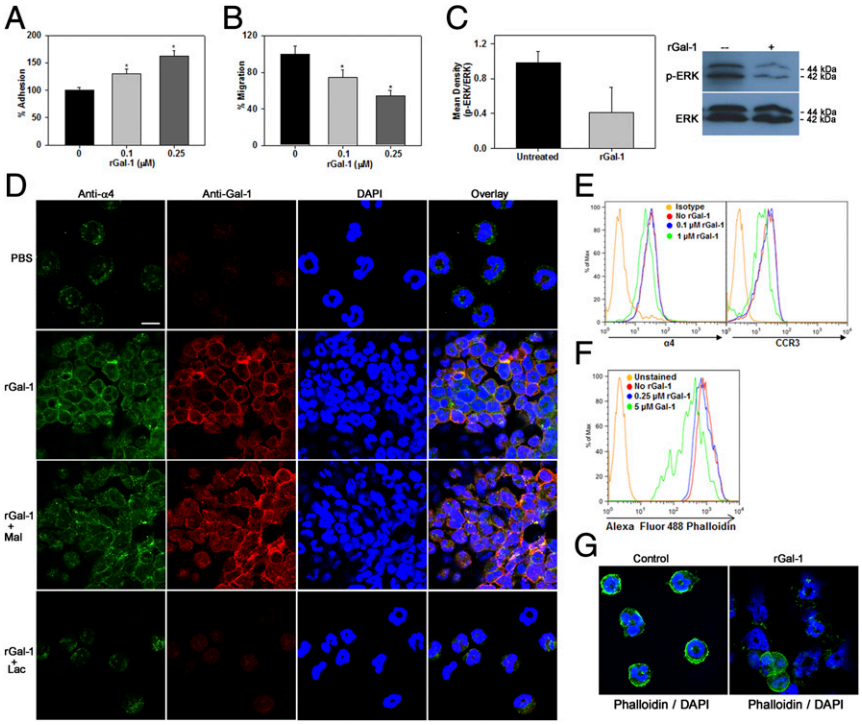

Fig. 6. Extracellular Gal-1 differentially regulates eosinophil adhesion and migration. (*A* and *B*) Effect of rGal-1 at subapoptotic concentrations (<0.25 μM) on eosinophil adhesion to VCAM-1–coated coverslips (*A*) and eotaxin-1–induced migration (*B*). (*C*) Inhibition of ERK activation by Gal-1 at subapoptotic concentrations by Western blot analysis. (*D*) Clustering of eosinophils, redistribution of CD49d (α4), and colocalized expression of rGal-1 with α4-integrin by dual IF staining with anti-α4 (green) and anti–Gal-1 (red). (Scale bar, 10 μm.) (*E*) Effect of rGal-1 at proapoptotic concentrations on expression of α4 and CCR3 by flow cytometry. (*F* and *G*) Total F-actin and actin cytoskeleton in eosinophils treated with rGal-1 at proapoptotic concentrations. Combined data (mean ± SEM) of three experiments in duplicate in *A*, four experiments in triplicate in *B*, and three experiments in *C* (*Left*) are shown. Data shown in *D*–*G* are representative of three or four independent experiments with eosinophils from different mice. $^*P < 0.05$ for 0.1 μM and $^*P < 0.01$ for 0.25 μM rGal-1–treated cells compared with untreated in *A* and *B*.

The increased recruitment of eosinophils to the airways and peripheral blood of allergen-challenged *Lgals1*$^{-/-}$ mice relative to WT mice (Fig. 7) is unlikely to be entirely due to elevated eotaxin-1 and -2 because levels of these chemokines were not found to be substantially different between the two allergen-challenged groups. Thus, we examined inherent differences between WT and *Lgals1*$^{-/-}$ eosinophils with respect to migration in vivo. BM eosinophils from WT and *Lgals1*$^{-/-}$ mice were carboxyfluorescein succinimidyl ester (CFSE)-labeled and infused into WT mice with thioglycollate (TG)-induced peritoneal inflammation. There was a twofold increase in the number of CFSE-labeled *Lgals1*$^{-/-}$ eosinophils recruited to the inflamed peritoneum relative to WT eosinophils based on flow cytometry analysis of the peritoneal fluid (Fig. 9*C* and Fig. S4*B*). This was reflected in a larger number of total eosinophils (endogenous eosinophils plus infused eosinophils) in the peritoneum of WT mice that received *Lgals1*$^{-/-}$ eosinophils compared with WT mice that were infused with WT eosinophils (Fig. S4*C*). We also compared survival of WT versus *Lgals1*$^{-/-}$ eosinophils in culture medium containing low serum up to 24 h but found no inherent difference in constitutive apoptosis based on annexin V staining (Fig. S4*D*), suggesting that endogenous Gal-1 did not regulate eosinophil viability. Collectively, these findings suggest that Gal-1–deficient eosinophils exhibit increased ability to recruit to sites of inflammation compared with WT eosinophils, which could also contribute to the increased eosinophilia observed in allergen-challenged *Lgals1*$^{-/-}$ mice.

Discussion

Eosinophils recruited to allergic airways serve as a reservoir for proinflammatory cytokines, chemokines, growth factors, and cytotoxic granule proteins (MBP, EPX, and eosinophil cationic protein) that cause significant epithelial tissue damage and contribute to bronchoconstriction, mucus production, vascular permeability, and airway remodeling (19). Given the homeostatic/antiinflammatory role of Gal-1 in multiple chronic disease models as well as acute inflammation models (5, 6), we examined the role played by Gal-1 in allergic airway inflammation and asthma. Exposure to various allergens (OVA, *Alternaria*, and CRA) resulted in increased Gal-1 expression in the lungs due to the recruitment of Gal-1–expressing inflammatory cells, including eosinophils, as well as increased expression of Gal-1 in the basement membrane of the airway epithelium and extracellular spaces beneath the epithelium and blood vessel wall. Expression of Gal-1 has been demonstrated in the nucleus, cytosol, and intracellular side of cell membranes as well as extracellularly in culture supernatants and in the ECM of tissues despite the absence of a secretion signal sequence (5, 20). Based on techniques used in the current study, soluble Gal-1 was not detectable in the BALF of OVA-challenged mice. Gal-1 could be secreted at concentrations below the detection limit of our assay or the secreted Gal-1 may rapidly bind to cell-surface glycans or the ECM and thus not remain in a soluble form at concentrations high enough to be detected.

Studies with BM-derived eosinophils confirmed that eosinophils (nonactivated) express Gal-1 intracellularly but not on the cell surface. These findings are consistent with Gal-1 expression in activated T cells where only intracellular expression was noted, with no detectable cell-surface expression or secretion (15). Further, intracellular Gal-1 expression or expression on the cell surface was not induced after stimulation/activation with asthma-inducing mediators such as TNF-α, eotaxin-1, or SCF-1. Despite the lack of endogenous cell-surface Gal-1 expression, murine eosinophils were found to rapidly bind to exogenous/extracellular Gal-1 on the cell surface in a carbohydrate-dependent manner. Gal-1 is known to bind preferentially to complex N- and O-glycans containing terminal modified or unmodified LacNAc residues (5), and can therefore bind to a host of cell-surface glycoproteins that contain these glycan components. Glycophenotyping of murine eosinophils confirmed the expression of complex N- and O-glycans bearing LacNAc residues on the cell surface that could potentially bind to Gal-1. Further, lectin inhibition studies demonstrated the involvement of LacNAc residues of eosinophil-expressed cell-surface glycans in binding to Gal-1. Studies to determine the relative requirement of N- versus O-glycans for Gal-1 binding suggest that eosinophil-expressed complex N-glycans are involved in this interaction. Although O-glycans do not appear to be required for Gal-1 binding, based on increased binding of exogenous Gal-1 in the absence of complex O-glycans, it is possible that O-glycans may play a role in regulating Gal-1

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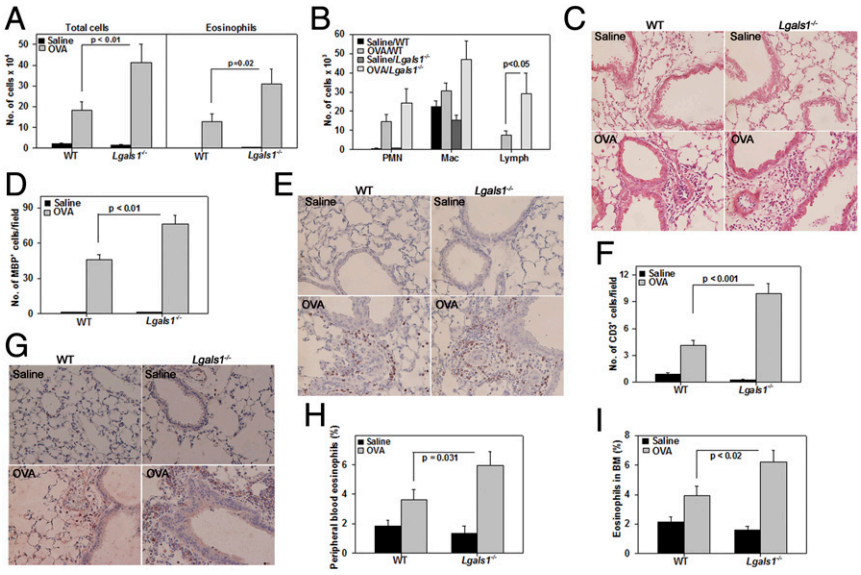

Fig. 7. Gal-1 deficiency leads to increased airway inflammation after allergen exposure. (*A* and *B*) Number of total cells (*A*, *Left*) and eosinophils (*A*, *Right*) and number of PMNs, macrophages (Mac), and lymphocytes (Lymph) (*B*) in the BALF from saline- and OVA-challenged WT and *Lgals1*$^{-/-}$ mice. (*C*) Cellular infiltration in lung tissue after H&E staining. Representative images are shown. (Scale bar, 50 μm.) (*D* and *E*) Quantitation of lung tissue eosinophils and representative images after MBP IHC (stained reddish brown). (Scale bar, 50 μm.) (*F* and *G*) Quantitation of lung tissue T cells and representative images after CD3ε IHC (stained brown). (Scale bar, 50 μm.) (*H* and *I*) Quantitation of eosinophils in peripheral blood and BM, respectively, after Hema 3 staining. Combined data (mean ± SEM) of mice from three independent experiments (n = 8–9 mice for OVA groups and n = 6 mice for saline groups in *A*, *B*, *D*, and *F*; n = 7–8 mice for OVA groups and n = 7 mice for saline groups in *H* and *I*) are shown.

binding to N-glycans by masking N-glycans that participate in binding to Gal-1. This is in contrast to activated T cells, where both N- and O-glycans participate in Gal-1 binding (14).

Although eosinophils did not appear to secrete Gal-1 in response to stimulation with TNF-α, eotaxin-1, or SCF-1 in vitro, these mediators may play a different role in vivo and induce Gal-1 secretion, which could potentially exert autocrine effects. Further, in vivo, eosinophils are likely to be exposed to varying concentrations of soluble Gal-1 released by other cells such as endothelial cells (20) or to Gal-1 presented by the ECM (21) during inflammation. Our in vitro studies demonstrate that Gal-1 rapidly induces apoptosis of eosinophils at high concentrations, typical of inflammatory reactions. The proapoptotic effect of Gal-1 has previously been demonstrated in other cell types such as T cells (14), B cells (22), epithelial cells (23), and endothelial cells (24) but not macrophages, DCs, or mast cells (25). Gal-1–induced apoptosis of both BM-derived as well as peripheral blood eosinophils from allergen-challenged mice occurred at lower concentrations and more rapidly (≥1 μM by 15 min) than in T cells (14), B cells (22), and endothelial cells (24). Further, Gal-1 treatment caused cell shrinkage in eosinophils, a hallmark feature of apoptosis (26). These Gal-1–mediated effects were significantly inhibited in the presence of lactose, establishing their carbohydrate-dependent nature. Gal-1 at higher concentrations also caused a marked reduction in total F-actin levels (indicative of F-actin depolymerization) and disruption of the cytoskeleton, consistent with cell disintegration into apoptotic bodies (26). Previous studies investigating the effect of immobilized Gal-1 (coated on plastic supports) on adhesion and migration of human eosinophils reported altered actin polymerization/depolymerization dynamics with a prevalence of glomerular actin (27), suggesting that the actin cytoskeleton is a target of Gal-1–mediated signaling. Eosinophils exposed to proapoptotic concentrations of Gal-1 exhibited reduced cell-surface expression of α4 and CCR3. It is possible that these receptors are rapidly recycled or shed, a phenomenon previously described in other cell types during apoptosis (28) that may serve as a mechanism to suppress eosinophil activation and recruitment.

In activated T cells, Gal-1–induced cell death has been shown to involve the participation of AP-1 (4), NF-κB (29), and Bcl-2 (4) as well as activation of caspases (18), although caspase-independent T-cell apoptosis has also been shown to occur (30). In the current study, Gal-1–induced apoptosis of eosinophils did not appear to involve activation of caspases, similar to the effects observed in endothelial cells (24). However, a role for ERK(1/2) was identified. Exposure to rGal-1 at concentrations >1 μM caused a marked increase in phosphorylation of ERK(1/2), and eosinophil apoptosis was inhibited by the MEK inhibitor U0126. Whereas activation of the ERK(1/2) pathway is typically known to govern cell proliferation and differentiation and is protective against apoptosis (31), recent studies indicate that activation of ERK by certain stimuli can indeed act as a cell-death signal and

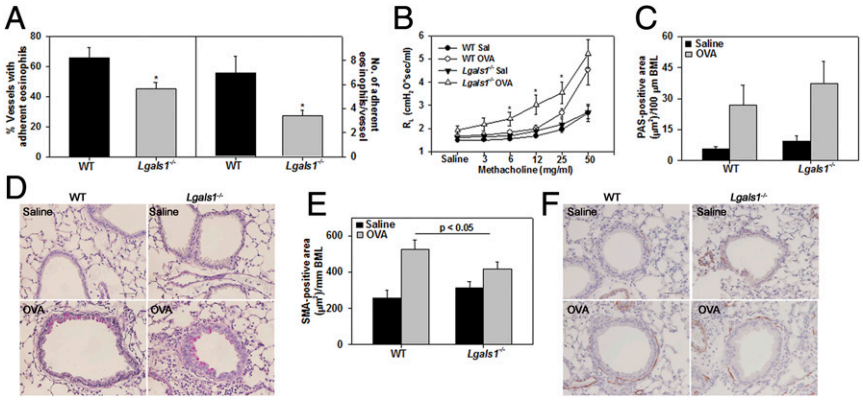

Fig. 8. Increased airway reactivity in allergen-challenged Gal-1–deficient mice. (*A*) Quantitation of the number of lung blood vessels with eosinophils (MBP-positive cells) and the number of adherent eosinophils in the blood vessels of OVA-challenged WT and *Lgals1*$^{-/-}$ mice. (*B*) Pulmonary resistance in saline- and OVA-challenged WT and *Lgals1*$^{-/-}$ mice following exposure to aerosolized methacholine. (*C* and *D*) Quantitation of mucus secretion (stained pink) in airways of saline- and OVA-challenged WT and *Lgals1*$^{-/-}$ mice along with representative images. BML, basement membrane length; PAS, periodic acid–Schiff reagent. (Scale bar, 50 μm.) (*E* and *F*) Quantitation of airway smooth muscle mass based on α-SMA IHC (stained brown). Representative images are shown in *F*. (Scale bar, 50 μm.) Combined data (mean ± SEM) of mice from two independent experiments (n = 7–8 mice per group) in *A* and three independent experiments (n = 7–8 mice for OVA groups and n = 6–7 mice for saline groups) in *B*, *C*, and *E* are shown. *P < 0.025 in *A* and *P < 0.05 at 6 and 25 mg/mL and *P < 0.03 at 12 mg/mL methacholine in *B* for comparison of OVA-challenged groups.

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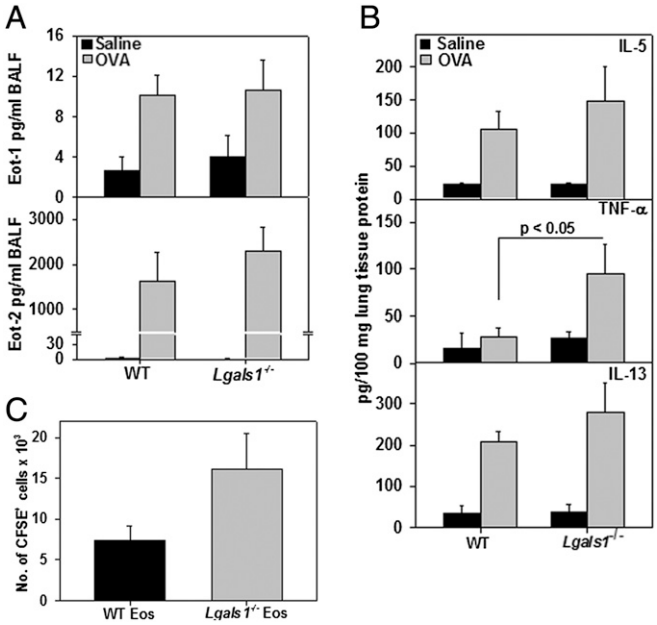

Fig. 9. Inflammatory cytokines and chemokines in airways of allergen-challenged WT and Gal-1–deficient mice. (*A*) Eotaxin-1 and -2 (Eot-1, Eot-2) levels in the BALF of saline- and OVA-challenged WT and *Lgals1*$^{-/-}$ mice. (*B*) IL-5, TNF-α, and IL-13 levels in lung lysates from the above groups of mice. Combined data (mean ± SEM) of mice from three independent experiments (n = 7–9 mice for OVA groups and n = 6–7 mice for saline groups) are shown in *A* and *B*. (*C*) Recruitment of i.v. infused CFSE-labeled BM eosinophils from WT or *Lgals1*$^{-/-}$ mice to the peritoneum of mice with TG-induced inflammation. Combined data (mean ± SD) of two out of three experiments in duplicate mice with similar results are shown.

facilitate engagement of apoptotic programs (32). Interestingly, in the current study, increased ERK(1/2) activation was associated with a drastic reduction/degradation of ERK(1/2) by the proteasome. Previous studies have demonstrated that, in addition to functioning as an upstream activator of ERK(1/2), MEKK1 has E3 ubiquitin ligase activity and can thus mediate ubiquitination and degradation of ERK(1/2), which may serve as a negative regulatory mechanism for decreasing ERK(1/2) activity in stress-induced cells (33).

Apart from its ability to induce apoptosis, Gal-1 has been shown to affect immune cell motility. Whereas PMNs were less migratory toward IL-8 after treatment with Gal-1 (34), DCs demonstrated increased chemokine-induced migration (35) in vitro. Further, at lower concentrations (subapoptotic), Gal-1 has been shown to inhibit T-cell adhesion to ECM proteins in a dose-dependent manner (36). Our studies show that Gal-1 treatment at subapoptotic concentrations (up to 0.25 μM) causes eosinophils to form clusters, increases their adhesion to VCAM-1, and alters distribution of α4-integrin to the cell periphery but does not affect the expression level of adhesion receptors (α4- or β2-integrins and L-selectin). In this regard, Gal-1 has been reported to directly bind to β1-integrins on vascular smooth muscle cells, transiently increasing the availability of its active form on the cell surface and thus modulating adhesion (37). Treatment of eosinophils with Gal-1 at the same concentrations that increased adhesion resulted in decreased eotaxin-1–induced migration, with the expression level of CCR3 remaining unaltered. Exogenous Gal-1–induced clustering of cells along with increased adhesion to VCAM-1 and inhibition of ERK(1/2) activation [a signaling molecule required for eotaxin-1–induced eosinophil migration (38)] may result in the cells being less motile to undergo directed movement toward eotaxin-1. These in vitro findings are further supported by observations in our model of allergic airway inflammation where eosinophils adherent in lung blood vessels were more commonly noted in allergen-challenged WT mice than in *Lgals1*$^{-/-}$ mice, potentially due to exposure to Gal-1 secreted by or presented on the surface of endothelial cells. Indeed, endothelial cells in allergen-challenged mice express Gal-1 based on our IHC data. Additionally, allergen-challenged *Lgals1*$^{-/-}$ mice exhibited increased lung tissue eosinophils relative to WT counterparts despite similar levels of the eosinophil-specific chemokines eotaxin-1 and -2, which could be due to the lack of the inhibitory/limiting effects of Gal-1 on eosinophil migration that was noted in vitro.

Consistent with the inhibitory effect of exogenous Gal-1 on eosinophil migration in vitro and the increased number of blood vessels with adherent eosinophils in allergen-challenged WT mice relative to Gal-1–deficient mice, eosinophil recruitment in the BALF and lung tissue after allergen challenge was significantly lower in WT mice than in Gal-1–deficient counterparts. Further, these mice had lower peripheral blood and BM eosinophilia. In a WT setting, depending on concentrations in the local milieu, extracellular Gal-1 may regulate eosinophilia by inducing apoptosis of differentiated eosinophils in the BM or inhibit their migration/mobilization from the BM to sites of inflammation. Indeed, previous studies have shown that administration of Gal-1 can prevent cyclophosphamide- and G-CSF–induced migration of progenitor cells from the BM in vivo (39). Along with the increased eosinophilia, allergen-challenged Gal-1–deficient mice exhibited significantly elevated T cells and AHR. However, airway smooth muscle mass was somewhat lower in allergen-challenged Gal-1–deficient mice despite more inflammation. Gal-1 is secreted by vascular smooth muscle cells and is known to induce vascular smooth muscle proliferation, attachment, spreading, and migration (37). It is conceivable that it may have a similar effect on airway smooth muscle cells, which might explain the decrease in α-smooth muscle actin (α-SMA)–positive cells in allergen-challenged Gal-1–deficient mice relative to WT counterparts. Finally, TNF-α in the lung tissue of allergen-challenged Gal-1–deficient mice was significantly higher relative to WT counterparts. TNF-α is secreted by eosinophils and T cells, both of which are elevated in allergen-challenged Gal-1–deficient mice. It is known to promote migration of inflammatory cells as well as induction of AHR (40) and inhibit the suppressive function of both naturally occurring and induced Tregs in asthmatic patients (41). In this regard, studies have shown that Gal-1 is required for the regulatory activity of $CD4^+CD25^+$ Tregs (10). The inherently increased ability of Gal-1–deficient eosinophils to recruit to sites of inflammation versus WT cells as noted in the peritonitis model, suggestive of a role for intracellular Gal-1 in regulating eosinophil migration independent of the extracellular effect of Gal-1, could also be an important player responsible for the increased eosinophilia in allergen-challenged Gal-1–deficient mice. In a recent study, administration of rGal-1 to mice with IgE-mediated allergic conjunctivitis resolved clinical signs as well as reduction in Th2 cytokines, eotaxin, RANTES, and MAPK signaling. However, this was associated with increased Gal-1 expression and eosinophilia in the conjunctiva relative to untreated mice (42).

Overall, our studies demonstrate that Gal-1, which is induced in the lung in response to allergen challenge, is important in regulating airway inflammation. This is supported by our in vitro findings that extracellular Gal-1 can bind to eosinophil-expressed cell-surface glycans in a carbohydrate-dependent manner to inhibit cell migration and induce apoptosis as well as our in vivo studies demonstrating increased eosinophilia and airway inflammation in the absence of the endogenous lectin. Targeting eosinophils is an important strategy for alleviating not only allergic asthma but potentially even other eosinophil-driven disorders.

Materials and Methods

Mice. Mice with a deletion of the gene encoding Gal-1 (*Lgals1*$^{-/-}$), originally provided by F. Poirier, Institut Jacques Monod, Paris, France, or Mgat5 (*Mgat5*$^{-/-}$; obtained from the Consortium for Functional Glycomics) (43), both on C57BL/6 background, were bred in-house. Age- and gender-matched C57BL/6 mice (8–12 wk old) were used as WT controls. All studies involving

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mice were performed following standards and procedures approved by the Institutional Animal Care and Use Committee at the University of Minnesota.

Culture of Murine Eosinophils and Preparation of Recombinant Gal-1. Eosinophils were cultured from naïve BM of WT, *Lgals1*$^{-/-}$, and *Mgat5*$^{-/-}$ mice as described previously (43, 44). Expression and purification of recombinant Gal-1 were accomplished as outlined previously (45). LPS was removed by Detoxi-Gel endotoxin removing gel (Pierce), and samples were tested for LPS content using the gel-clot *Limulus* test (<0.5 IU/mg).

Cell Lysis and Western Blot Analysis. Detailed methods for cell lysis and Western blotting are described in *SI Materials and Methods*.

Gal-1 Expression and Binding to Eosinophils. Expression of Gal-1 by murine eosinophils was evaluated by flow cytometry and confocal microscopy after IF staining as detailed in *SI Materials and Methods* using polyclonal antibodies against human Gal-1 prepared as described previously (46).

To determine whether Gal-1 binds to eosinophils, cells were treated with rGal-1 at 0.1 or 0.25 μM in PBS or vehicle alone for 15 min at 37 °C. After washing (once with PBS), Gal-1 expression on the cell surface (nonpermeabilized) was examined by flow cytometry using antibodies against Gal-1 (*SI Materials and Methods*). In some experiments, cells were incubated with rGal-1 in the presence of lactose (10 or 30 mM), a Gal-1–specific disaccharide, or maltose as a control before analysis.

Glycophenotyping and Gal-1–Binding Partners Expressed in Eosinophils. Biotinylated plant lectins with known specificities were used to identify glycan epitopes on the cell surface of BM-derived eosinophils. Cells were incubated with TL, MAL-II, SNA, PHA-L, or PNA (all at 20 μg/mL; Vector Laboratories) or vehicle (PBS) for 30 min followed by DyLight 488 conjugated to streptavidin (Vector Laboratories). In some studies, eosinophils were preincubated with the above lectins or vehicle alone for 20 min at room temperature and then treated with rGal-1 (0.1 μM). Gal-1 binding to the cell surface was examined by flow cytometry as described earlier. Results were expressed as fold change in geometric mean fluorescence intensity relative to vehicle-treated cells.

To examine the requirement of O-glycans, eosinophils were cultured in medium containing BG (2 mM) or vehicle (DMSO) for 65 h as described (14) and then examined for rGal-1 binding by flow cytometry. To examine the requirement of complex branched N-glycans, binding of rGal-1 to BM eosinophils from *Mgat5*$^{-/-}$ mice relative to WT mice was evaluated.

Apoptosis Assays. Eosinophils were treated with 0.1–5 μM rGal-1 in PBS (or PBS only) at 37 °C for up to 60 min and examined for cell viability by trypan blue exclusion. In another set of experiments, eosinophils were treated with rGal-1, washed, and analyzed by flow cytometry using the Annexin V-FITC Apoptosis Detection Kit (eBioscience). Briefly, eosinophils were collected after treatment with Gal-1, resuspended in binding buffer, and stained with FITC-conjugated annexin V for 15 min followed by propidium iodide (PI). To additionally examine the effect of Gal-1 on peripheral blood eosinophils, total leukocytes (after lysis of red blood cells) obtained from allergen-challenged mice were exposed to PBS or rGal-1 (1 μM for 15 min at 37 °C). Cells were dual-stained with annexin V-FITC and phycoerythrin (PE)-conjugated rat anti-mouse Siglec-F (BD Biosciences). PE-conjugated rat IgG was used as isotype control. Changes in annexin V positivity in the Siglec-F–positive population (eosinophils) were determined by flow cytometry. Involvement of O- versus complex N-glycans in Gal-1–mediated apoptosis was examined by exposing eosinophils cultured in medium containing BG or BM eosinophils from *Mgat5*$^{-/-}$ mice to rGal-1 (5 μM) and analyzed for annexin V staining. To examine the role of specific signaling molecules in Gal-1–mediated eosinophil apoptosis, cells were incubated with Y-27632 (inhibitor of ROCK1; Cayman Chemical), U0126 (inhibitor of MEK; Cayman Chemical), LY294002 (inhibitor of PI3K; Sigma-Aldrich), Bay 61-3606 (synonym Syk inhibitor IV, inhibitor of Syk tyrosine kinase; EMD Millipore), or pan-caspase inhibitor [synonym Z-VAD (OMe)-FMK; Santa Cruz Biotechnology] at 10, 50, or 100 μM for 20 min in DMEM at 37 °C and then exposed to rGal-1 (1–5 μM) or PBS alone as described above. Cells treated with staurosporine (20 μM; Santa Cruz Biotechnology), a known inducer of caspase-mediated apoptosis (47), served as a positive control. Cells preincubated with DMSO (Sigma-Aldrich) or water alone in media served as the vehicle control. Additionally, cells treated with rGal-1 or staurosporine (20 μM) for 60 min were applied to poly-L-lysine–coated slides and evaluated by TUNEL assay (DeadEnd Colorimetric TUNEL System; Promega).

Adhesion and Migration Assays. The effects of Gal-1 on eosinophil adhesion and migration were evaluated as described previously (48, 49) and are detailed in *SI Materials and Methods*.

Cell-Surface Receptor Expression and Actin Polymerization. Changes in the expression of cell-surface adhesion molecules and the actin cytoskeleton (based on phalloidin binding) after treatment with rGal-1 were examined by flow cytometry and confocal microscopy as detailed in *SI Materials and Methods*.

Evaluation of Gal-1–α4-Integrin Interaction. BM eosinophils were treated with rGal-1 (0.25 μM) in the presence or absence of 30 mM lactose (or maltose) or with buffer alone in suspension and then cytocentrifuged (75 × *g*) onto glass slides. Cells were dual-stained with anti–Gal-1 polyclonal antibodies (30 μg/mL) and anti-CD49d mAb (clone R1-2, 10 μg/mL; BD Biosciences) followed by Rhodamine Red-X–conjugated goat anti-rabbit IgG and FITC-conjugated goat anti-rat IgG, respectively (both from Jackson ImmunoResearch Laboratories), and examined by confocal microscopy. Rabbit IgG (for Gal-1) and rat IgG (for CD49d) were used as negative controls. In some studies, α4-transfected CHO-K1, kindly provided by Yoshikazu Takada, Scripps Research Institute, La Jolla, CA (50), or nontransfected control CHO-K1 cells cultured to 70% confluence were treated with PBS or rGal-1 and examined for Gal-1 binding by flow cytometry with antibodies against Gal-1 (see *SI Materials and Methods* for details).

Models of Allergic Airway Inflammation. For acute allergen-induced airway inflammation, WT or *Lgals1*$^{-/-}$ mice (C57BL/6; 8–12 wk old) were sensitized and challenged with OVA (grade V; Sigma-Aldrich) as described (51). Control mice were administered saline instead of OVA for sensitization and challenge. Additionally, lungs from C57BL/6 WT mice exposed to chronic challenge with OVA (52), acute challenge with CRA (Hollister-Stier Laboratories) (48), or an extract of *A. alternata* (Greer) (53) were harvested. Mice were killed 24 h after the last allergen challenge in all models.

Measurement of Airway Responsiveness. Pulmonary function in control and acute OVA-challenged WT and *Lgals1*$^{-/-}$ mice was assessed by invasive plethysmography (FinePointe RC System; Buxco) (51). Changes in pulmonary resistance were monitored continuously in response to saline followed by increasing concentrations of inhaled methacholine (3–50 mg/mL) nebulized for 18–20 s and expressed as mean R_L values following each dose of methacholine.

Sample Collection and Analysis. Total cell counts in the BALF were determined using a hemocytometer. Differential cell counts in the BALF as well as blood and BM were determined based on morphologic and histologic criteria after staining cytocentrifuged samples with the Hema 3 System (Thermo Fisher Scientific). BALF supernatants were stored at −80 °C for later analysis. Right lungs were snap-frozen and left lungs were perfused and fixed in 4% (vol/vol) paraformaldehyde before paraffin embedding. *Lgals1* expression in lung tissue was examined by quantitative PCR as described in *SI Materials and Methods*.

Lung Histology. Paraffin-embedded tissue sections from OVA-challenged WT and *Lgals1*$^{-/-}$ mice were evaluated for cellular infiltration [total cells, eosinophils (MBP-positive), and lymphocytes (CD3-positive)], Gal-1 expression, smooth muscle hyperplasia, and airway mucus secretion by histology as described in *SI Materials and Methods*. To examine Gal-1 expression by lung tissue eosinophils, sections were dual-stained with antibodies against Gal-1 (4 μg/mL) and mAb against EPX (10 μg/mL) (54) and examined by confocal microscopy.

Measurement of Lung Cytokines and Chemokines. Th1 (IL-2, IFN-γ)/Th2 (IL-4, IL-5, IL-13) cytokine and TNF-α levels in supernatants of lung tissue lysates and/or BALF were determined by flow cytometry using CBA Flex Set Kits (BD Biosciences) and expressed as pg cytokine per 100 mg lung tissue protein or pg/mL BALF. Eotaxin-1, eotaxin-2, and TGF-β1 in the BALF were measured using ELISA kits (R&D Systems).

In Vivo Migration Assay. Migration/recruitment of infused CFSE-labeled BM-derived eosinophils from WT versus *Lgals1*$^{-/-}$ mice was evaluated in a model of TG-induced peritoneal inflammation (43). Total and differential cell counts in the peritoneal fluid were determined and cells were analyzed by flow cytometry to determine the number of CFSE-labeled eosinophils.

Statistical Analysis. Results are expressed as mean ± SEM (or mean ± SD where noted) of combined data from multiple experiments. Statistical

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comparison between two groups was determined by an unpaired Student's *t* test. Comparisons between control and multiple treatment conditions were carried out by ANOVA followed by Dunnett's posttest. A P value < 0.05 was considered to be significant.

1. Hamid Q, Tulic M (2009) Immunobiology of asthma. *Annu Rev Physiol* 71:489–507.
2. Robinson DS (2009) Regulatory T cells and asthma. *Clin Exp Allergy* 39(9):1314–1323.
3. Holgate ST (2011) Pathophysiology of asthma: What has our current understanding taught us about new therapeutic approaches? *J Allergy Clin Immunol* 128(3):495–505.
4. Rabinovich GA, Toscano MA (2009) Turning 'sweet' on immunity: Galectin-glycan interactions in immune tolerance and inflammation. *Nat Rev Immunol* 9(5):338–352.
5. Camby I, Le Mercier M, Lefranc F, Kiss R (2006) Galectin-1: A small protein with major functions. *Glycobiology* 16(11):137R–157R.
6. Rabinovich GA, Croci DO (2012) Regulatory circuits mediated by lectin-glycan interactions in autoimmunity and cancer. *Immunity* 36(3):322–335.
7. Liu F-T, Yang R-Y, Hsu DK (2012) Galectins in acute and chronic inflammation. *Ann N Y Acad Sci* 1253:80–91.
8. Cooper D, Norling LV, Perretti M (2008) Novel insights into the inhibitory effects of galectin-1 on neutrophil recruitment under flow. *J Leukoc Biol* 83(6):1459–1466.
9. Rabinovich GA, Sotomayor CE, Riera CM, Bianco I, Correa SG (2000) Evidence of a role for galectin-1 in acute inflammation. *Eur J Immunol* 30(5):1331–1339.
10. Garín MI, et al. (2007) Galectin-1: A key effector of regulation mediated by $CD4^+CD25^+$ T cells. *Blood* 109(5):2058–2065.
11. Rostoker R, et al. (2013) Galectin-1 induces 12/15-lipoxygenase expression in murine macrophages and favors their conversion toward a pro-resolving phenotype. *Prostaglandins Other Lipid Mediat* 107:85–94.
12. Sanchez-Cuellar S, et al. (2012) Reduced expression of galectin-1 and galectin-9 by leucocytes in asthma patients. *Clin Exp Immunol* 170(3):365–374.
13. Rabinovich GA, et al. (1999) Recombinant galectin-1 and its genetic delivery suppress collagen-induced arthritis via T cell apoptosis. *J Exp Med* 190(3):385–398.
14. Perillo NL, Pace KE, Seilhamer JJ, Baum LG (1995) Apoptosis of T cells mediated by galectin-1. *Nature* 378(6558):736–739.
15. Deák M, et al. (2015) Novel role for galectin-1 in T-cells under physiological and pathological conditions. *Immunobiology* 220(4):483–489.
16. Stowell SR, et al. (2007) Human galectin-1, -2, and -4 induce surface exposure of phosphatidylserine in activated human neutrophils but not in activated T cells. *Blood* 109(1):219–227.
17. Rabinovich GA, et al. (1997) Specific inhibition of lymphocyte proliferation and induction of apoptosis by CLL-I, a β-galactoside-binding lectin. *J Biochem* 122(2): 365–373.
18. Matarrese P, et al. (2005) Galectin-1 sensitizes resting human T lymphocytes to Fas (CD95)-mediated cell death via mitochondrial hyperpolarization, budding, and fission. *J Biol Chem* 280(8):6969–6985.
19. Kita H (2011) Eosinophils: Multifaceted biological properties and roles in health and disease. *Immunol Rev* 242(1):161–177.
20. He J, Baum LG (2006) Endothelial cell expression of galectin-1 induced by prostate cancer cells inhibits T-cell transendothelial migration. *Lab Invest* 86(6):578–590.
21. He J, Baum LG (2006) Galectin interactions with extracellular matrix and effects on cellular function. *Methods Enzymol* 417:247–256.
22. Fouillit M, et al. (2000) Regulation of CD45-induced signaling by galectin-1 in Burkitt lymphoma B cells. *Glycobiology* 10(4):413–419.
23. Muglia C, et al. (2011) The glycan-binding protein galectin-1 controls survival of epithelial cells along the crypt-villus axis of small intestine. *Cell Death Dis* 2:e163.
24. Jouve N, et al. (2013) The involvement of CD146 and its novel ligand galectin-1 in apoptotic regulation of endothelial cells. *J Biol Chem* 288(4):2571–2579.
25. Liu FT, Rabinovich GA (2010) Galectins: Regulators of acute and chronic inflammation. *Ann N Y Acad Sci* 1183:158–182.
26. Elmore S (2007) Apoptosis: A review of programmed cell death. *Toxicol Pathol* 35(4): 495–516.
27. Delbrouck C, et al. (2002) Galectin-1 is overexpressed in nasal polyps under budesonide and inhibits eosinophil migration. *Lab Invest* 82(2):147–158.
28. Ilan N, Mohsenin A, Cheung L, Madri JA (2001) PECAM-1 shedding during apoptosis generates a membrane-anchored truncated molecule with unique signaling characteristics. *FASEB J* 15(2):362–372.
29. Koh HS, et al. (2008) CD7 expression and galectin-1-induced apoptosis of immature thymocytes are directly regulated by NF-kappaB upon T-cell activation. *Biochem Biophys Res Commun* 370(1):149–153.
30. Cedeno-Laurent F, Dimitroff CJ (2012) Galectin-1 research in T cell immunity: Past, present and future. *Clin Immunol* 142(2):107–116.
31. Zhang W, Liu HT (2002) MAPK signal pathways in the regulation of cell proliferation in mammalian cells. *Cell Res* 12(1):9–18.
32. Cagnol S, Chambard J-C (2010) ERK and cell death: Mechanisms of ERK-induced cell death—Apoptosis, autophagy and senescence. *FEBS J* 277(1):2–21.
33. Lu Z, Xu S, Joazeiro C, Cobb MH, Hunter T (2002) The PHD domain of MEKK1 acts as an E3 ubiquitin ligase and mediates ubiquitination and degradation of ERK1/2. *Mol Cell* 9(5):945–956.
34. La M, et al. (2003) A novel biological activity for galectin-1: Inhibition of leukocyte-endothelial cell interactions in experimental inflammation. *Am J Pathol* 163(4): 1505–1515.
35. Fulcher JA, et al. (2009) Galectin-1 co-clusters CD43/CD45 on dendritic cells and induces cell activation and migration through Syk and protein kinase C signaling. *J Biol Chem* 284(39):26860–26870.
36. Rabinovich GA, et al. (1999) Specific inhibition of T-cell adhesion to extracellular matrix and proinflammatory cytokine secretion by human recombinant galectin-1. *Immunology* 97(1):100–106.
37. Moiseeva EP, Williams B, Goodall AH, Samani NJ (2003) Galectin-1 interacts with β-1 subunit of integrin. *Biochem Biophys Res Commun* 310(3):1010–1016.
38. Boehme SA, et al. (1999) Activation of mitogen-activated protein kinase regulates eotaxin-induced eosinophil migration. *J Immunol* 163(3):1611–1618.
39. Kiss J, et al. (2007) A novel anti-inflammatory function of human galectin-1: Inhibition of hematopoietic progenitor cell mobilization. *Exp Hematol* 35(2):305–313.
40. Choi I-W, et al. (2005) TNF-α induces the late-phase airway hyperresponsiveness and airway inflammation through cytosolic phospholipase A(2) activation. *J Allergy Clin Immunol* 116(3):537–543.
41. Lin YL, Shieh CC, Wang JY (2008) The functional insufficiency of human $CD4^+CD25^{high}$ T-regulatory cells in allergic asthma is subjected to TNF-α modulation. *Allergy* 63(1): 67–74.
42. Mello CB, et al. (2015) Immunomodulatory effects of galectin-1 on an IgE-mediated allergic conjunctivitis model. *Invest Ophthalmol Vis Sci* 56(2):693–704.
43. Bahaie NS, et al. (2011) N-glycans differentially regulate eosinophil and neutrophil recruitment during allergic airway inflammation. *J Biol Chem* 286(44):38231–38241.
44. Dyer KD, et al. (2008) Functionally competent eosinophils differentiated ex vivo in high purity from normal mouse bone marrow. *J Immunol* 181(6):4004–4009.
45. Barrionuevo P, et al. (2007) 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. *J Immunol* 178(1):436–445.
46. Rabinovich GA, et al. (1998) Activated rat macrophages produce a galectin-1-like protein that induces apoptosis of T cells: Biochemical and functional characterization. *J Immunol* 160(10):4831–4840.
47. Bertrand R, Solary E, O'Connor P, Kohn KW, Pommier Y (1994) Induction of a common pathway of apoptosis by staurosporine. *Exp Cell Res* 211(2):314–321.
48. Kang BN, et al. (2012) The p110δ subunit of PI3K regulates bone marrow-derived eosinophil trafficking and airway eosinophilia in allergen-challenged mice. *Am J Physiol Lung Cell Mol Physiol* 302(11):L1179–L1191.
49. Ge XN, Ha SG, Liu FT, Rao SP, Sriramarao P (2013) Eosinophil-expressed galectin-3 regulates cell trafficking and migration. *Front Pharmacol* 4:37.
50. Matsuura N, et al. (1996) Induction of experimental bone metastasis in mice by transfection of integrin alpha 4 beta 1 into tumor cells. *Am J Pathol* 148(1):55–61.
51. Bahaie NS, et al. (2012) Regulation of eosinophil trafficking by SWAP-70 and its role in allergic airway inflammation. *J Immunol* 188(3):1479–1490.
52. Ge XN, et al. (2010) Allergen-induced airway remodeling is impaired in galectin-3-deficient mice. *J Immunol* 185(2):1205–1214.
53. Ha SG, et al. (2013) ORMDL3 promotes eosinophil trafficking and activation via regulation of integrins and CD48. *Nat Commun* 4:2479.
54. Ochkur SI, et al. (2012) The development of a sensitive and specific ELISA for mouse eosinophil peroxidase: Assessment of eosinophil degranulation ex vivo and in models of human disease. *J Immunol Methods* 375(1–2):138–147.
55. Hirabayashi J, Ayaki H, Soma G, Kasai K (1989) Production and purification of a recombinant human 14 kDa β-galactoside-binding lectin. *FEBS Lett* 250(2):161–165.
56. Abramoff MD, Magalhaes PJ, Ram SJ (2004) Image processing with ImageJ. *Biophotonics Int* 11(7):36–42.
57. Zuberi RI, et al. (2009) Deficiency of endothelial heparan sulfates attenuates allergic airway inflammation. *J Immunol* 183(6):3971–3979.
58. Pirot N, et al. (2014) Lung endothelial barrier disruption in Lyl1-deficient mice. *Am J Physiol Lung Cell Mol Physiol* 306(8):L775–L785.
59. Chung L-Y, et al. (2012) Galectin-1 promotes lung cancer progression and chemoresistance by upregulating p38 MAPK, ERK, and cyclooxygenase-2. *Clin Cancer Res* 18(15):4037–4047.
60. Schmittgen TD, Livak KJ (2008) Analyzing real-time PCR data by the comparative C(T) method. *Nat Protoc* 3(6):1101–1108.

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