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Functional characterization of an eosinophil-specific galectin, ovine galectin-14

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Abstract Across mammalian species, human galectin-10 and ovine galectin-14 are unique in their expression in eosinophils and their release into lung and gastrointestinal tissues following allergen or parasite challenge. Recombinant galectin-14 is active in carbohydrate binding assays and has been used in this study to unravel the function of this major eosinophil constituent. *In vitro* cultures revealed that galectin-14 is spontaneously released by eosinophils isolated from allergen-stimulated mammary gland lavage, but not by resting peripheral blood eosinophils. Galectin-14 secretion from peripheral blood eosinophils can be induced by the same stimuli that induce eosinophil degranulation. Flow cytometric analysis showed that recombinant galectin-14 can bind *in vitro* to eosinophils, neutrophils and activated lymphocytes. Glycan array screening indicated that galectin-14 recognizes terminal *N*-acetyllactosamine residues which can be modified with α1-2-fucosylation and, uniquely for a galectin, prefers α2- over α2-sialylation. Galectin-14 showed the greatest affinity for lacto-*N*-neotetraose, an immunomodulatory oligosaccharide expressed by helminths. Galectin-14 binds specifically to laminin *in vitro*, and to mucus and mucus producing cells on lung and intestinal tissue sections. *In vivo*, galectin-14 is abundantly present in mucus scrapings collected from either lungs or gastrointestinal tract following allergen or parasite challenge, respectively. These results suggest that *in vivo* secretion of eosinophil galectins may be specifically induced at epithelial surfaces after recruitment of eosinophils by allergic stimuli, and that eosinophil galectins may be involved in promoting adhesion and changing mucus properties during parasite infection and allergies.

Keywords Galectin · Eosinophil · Mucus · Glycan array · Allergy · Helminth

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Introduction

Galectins are a family of secreted animal lectins defined by their affinity for β-galactosides and significant sequence similarity in the carbohydrate-binding site [1]. Galectins are receiving increasing attention as possible regulators of inflammation, cell growth and tissue invasion [2–4], and as models for the study of divergent evolution [5–7]. Galectin-14 is a recently discovered member of the galectin family specifically expressed by ovine eosinophils [8]. The only other mammalian galactin with such a restricted expression is galectin-10 (previously known as the Charcot–Leyden crystal) present in human eosinophils and also basophils [9–11]. These cell types are typically associated with inflammation induced by allergies and helminth infection, and galectins are thought to play a central role in these processes [3, 12]. Both galectin-14 and galectin-10 are prototype galectins consisting of a monomer with a

single carbohydrate recognition domain (CRD) [8, 9]. The similar expression pattern of galectin-10 and galectin-14 in the cytoplasm and nucleus of human and ovine eosinophils respectively, and their release during the same pathological conditions [8, 10, 13], suggest that these two galectins may be functional orthologs in different species.

Purified native and recombinant galectin-10 proteins have unusual autocrystalisation properties that have made it difficult to assess the binding specificity and function of this galectin, and no specific ligands have been identified [9, 10, 13]. Purified native and cleaved recombinant galectin-14 also has a tendency to self-aggregate [8], however, the recombinant fusion protein has been shown to be active in a hemagglutination assay, with specific inhibition by lactose [8]. In the present study, the recombinant galectin-14 fusion protein was used to define the ligand specificity of galectin-14 using a glycan array assay and binding to inflammatory cells, extracellular matrix proteins and tissue sections. In addition, the *in vitro* and *in vivo* release patterns of native galectin-14 by eosinophils were determined. The results of these studies point to a possible novel function for eosinophil galectins in airway and gastrointestinal mucus.

Material and methods

All experimental animal procedures and collections of tissues and cells were approved by the Animal Experimental Ethics Committee of the University of Melbourne.

Reagents

Recombinant galectin-14 (rGST-galectin-14) and ovine galectin-11 (rGST-ovgal-11), are both glutathione S-transferase (GST) fusion proteins expressed in *Escherichia coli*, and galectin-14 monoclonal and polyclonal antibodies were prepared as previously described [8]. Recombinant GST from *Schistosoma japonicum* expressed in *E. coli* was purchased from Sigma. The eosinophil activators/degranulators calcium ionophore A23187 (CI), cytochalasin B (CB) and formyl-methionyl-leucyl-phenylalanine (FMLP) were purchased from Sigma. Recombinant human interleukin-5 (rhIL-5) was purchased from R&D Systems (Minneapolis, MN, USA).

Collection and purification of peripheral blood (PB) eosinophils

High blood eosinophilia was induced by infecting sheep with 100 metacercariae of the liver fluke, *Fasciola hepatica*. Approximately 1–2 months after infection, when circulating eosinophil levels were high, PB was collected and the percentage of eosinophils assessed on Giemsa-stained blood smears.

Two different methods were used to purify PB eosinophils. The first was to purify eosinophils to about 60% purity, in the absence of a red blood cell lysis step [14]. Eosinophils were collected from 45%/58.5% interface of the discontinuous Percoll gradients (GE Healthcare). Where PB eosinophils were required at higher levels of purity, eosinophils were purified according to the method of Woldehiwet [15], which included an isotonic lysis step. Eosinophils were obtained from the 65/70% interface of the discontinuous Percoll gradients and were >90% pure with no more than 8% contaminating neutrophils. This method was also used without the final percoll purification step to collect cells for flow cytometry analysis. Cells were washed three times in PBS/EDTA before use.

Collection of eosinophil-enriched mammary lavage (MAL) inflammatory cells

It was previously established that large numbers of eosinophils are recruited into the mammary gland of sheep after infusion of parasites or parasite extracts [16]. Eosinophil-rich (~60% purity) MAL cells were collected from primed mature non-lactating Merino ewes 72 h post intramammary infusion of *Haemonchus contortus* L3 larvae as described previously [16, 17]. Cells were washed three times in PBS/EDTA before use.

In vitro cell culture

PB and MAL eosinophils (~60% purity) were set up at a density of 1×10^6 cells in a 1 ml volume of Dulbecco's modified Eagle's medium (DMEM, Gibco) in 24 well tissue culture plates with or without 5 ng/ml rhIL-5, cultured at 37°C for 0, 1, 2 and 3 h, and the cell free supernatants collected following centrifugation (350×*g* for 10 min) for analysis by Western blot.

PB eosinophils (>90% purity) were set up at a density of 1×10^6 cells in 1 ml volume of DMEM in 12 well tissue culture plates with or without activating agents 5 μM CI, 5 μg/ml CB, 10 μM FMLP and 0.01% dimethyl sulfoxide (DMSO; carrier solvent control). The cells were then cultured at 37°C for 1 h, and the supernatants collected following centrifugation at 350×*g* for 10 min to remove cells and again at 20,000×*g* for 5 min to remove other debris. Supernatants were immediately aliquoted on ice and stored frozen prior to analysis of eosinophil peroxidase (EPO) production and galectin-14 secretion by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot.

All experiments shown are representative of at least three separate experiments.

SDS-PAGE and Western blots

Cell free supernatants, cells and recombinant protein preparation were separated under reducing conditions by SDS-PAGE and transferred to 0.45 μm nitrocellulose membranes (MSI, Melbourne, Australia) by electroblotting at 100 V for 1 h. Membranes were incubated with either mAb supernatant or a 1/500 dilution of primary sera for 2 h at RT, followed by incubation with secondary antibody (HRP-conjugated anti-mouse Ig (Dako) or HRP-conjugated anti-rabbit Ig (Dako), respectively) for at least 1 h at RT. Signals were detected by enhanced chemiluminescence (GE Healthcare). Proteins separated by SDS-PAGE were visualized by silver staining [18].

EPO assay

EPO released by eosinophils activated *in vitro* was measured by microtitre plate assay. Thirty microliters of 20 mM potassium bromide and 100 μl of 3,3′,5,5′-tetramethyl benzidine (TMB) substrate solution (Pierce) was added to 70 μl of sample. Reactions were mixed, allowed to develop for 15–30 min at RT and stopped by addition of 100 μl of 2 M sulfuric acid. Absorbance was read on a dual wavelength mode plate reader (Bio-tek instruments) at 450 nm, ref 690 nm. EPO release was expressed as a percentage of the total peroxidase activity of 1×10^6 cells (collected prior to *in vitro* activation) as extrapolated from the linear part of a calibration curve prepared from dilutions of cells lysed with 0.1% Triton X-100.

Flow cytometry

Cells from PB and MAL (1×10^6/tube) were incubated with different concentrations of rGST-galectin-14 or rGST with or without lactose (300 mM) and incubated for 1 h at RT. Cells were washed in ice cold buffer (1% bovine serum albumin (BSA) and 0.05% sodium azide in PBS) and Fc receptors blocked for 15 min on ice with 5% normal sheep serum and 5% foetal calf serum in buffer. Anti-GST-phycoerythrin (PE; Martek, MD, USA) was added and the cells incubated for 25 min on ice. Following a wash step, 1×10^4 eosinophils were acquired using a FACSCalibur™ flow cytometer (Becton Dickinson, USA), gating on the high side scatter and highly autofluorescent population of cells. Dead cells were excluded using 7-amino-actinomycin (Sigma). Analysis was performed with FlowJo (Tree Star, USA).

Glycan array screening

The glycan binding specificity of galectin-14 was analyzed by the Consortium for Functional Glycomics using glycan plate array v3. Full details of protocols used and the ~200 glycans included in the array are presented on the Consortium's web site (http://www.functionalglycomics.org/static/consortium) and [19]. Briefly, purified rGST-galectin-14 was labeled with fluorescein isothiocyanate Alexa 488 and active labeled galectin purified on a lactosyl-sepharose column. Streptavidin-coated high-binding capacity black 384-well plates (Pierce) were coated with various biotinylated glycosides (30 pmol/well) in PBS, pH 7.4, overnight at 4°C. After washing, plates were incubated with the fluorescently labeled galectin-14 in replicates of $n=4$ wells for 1 h at RT in PBS, 0.05% Tween-20, 1% BSA. Plates were washed three times in the same buffer, without added albumin, before measuring fluorescence intensity in a fluorescent plate reader using excitation at 485 nm and emission at 535 nm. To analyze the results, all glycans were ranked according to their signal to noise (S/N) ratio by dividing their mean relative fluorescence units (from the four replicates) by the mean background generated in control wells lacking glycosides. This value was compared to the average S/N for all wells in the array, and the results were then ranked as high affinity (>3.5*avg. S/N), medium affinity (>2*avg. S/N), and low affinity (>1*avg. S/N).

Laminin binding

Laminin binding activity was measured according to the method of Mazurek *et al.* [20] with some modification. Briefly, the wells of a 96-well microtitre plate were coated with laminin from Engelbreth–Holm–Swarm murine sarcoma (Sigma) at 1 μg/100 μl of carbonate coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6) per well overnight at 4°C. Non-specific binding sites were blocked with 200 μl of 0.5% Tween 20 in PBS for 1 h. Plates were washed three times in 0.05% Tween 20 PBS and incubated with 50 μl serial dilutions of rGST-galectin-14 or rGST alone, diluted in 0.5% Tween 20 PBS, for 1.5 h at 37°C. Control wells contained 0.3 M lactose. After washing as before, 50 μl rabbit anti-GST HRP-conjugated antibody (Sigma) was added at 1/10,000 and the plates incubated for 37°C for 1 h. Washed plates were developed with 100 μl TMB substrate solution (Pierce) for 10 min in the dark at RT. The reaction was terminated with 100 μl 2 M sulfuric acid and the color change monitored using a dual wavelength mode plate reader (Bio-tek instruments) at 450 nm, ref 690 nm.

Binding of recombinant proteins to frozen tissue sections

Recombinant GST-galectin-14 and rGST were labeled with the thiol reactive probe 5-iodoacetamidofluorescein (Molecular probes, Invitrogen) according to the manufacturer's

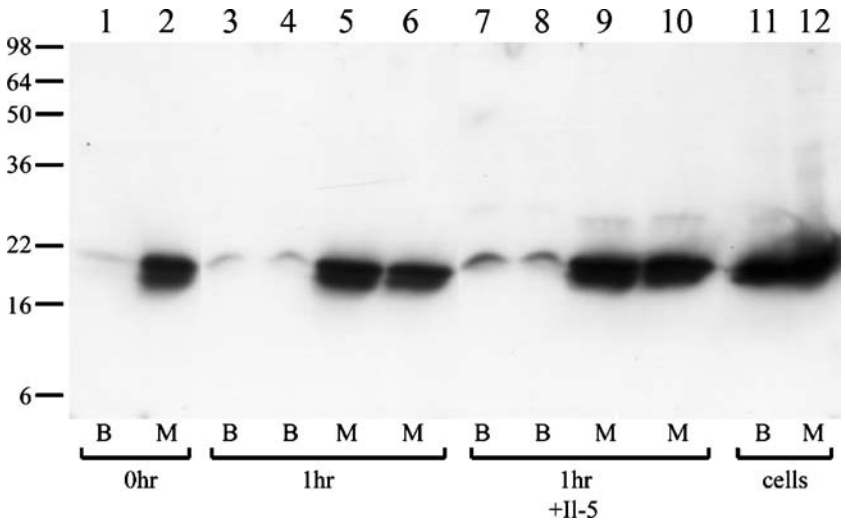


Fig. 1 Secretion of galectin-14 by blood and mammary lavage eosinophils. Eosinophil-rich (~60%) cell suspensions from peripheral blood (*B*) or mammary lavage (*M*) were cultured for up to 1 h, with or without rhIL-5. Both cell free supernatants (lanes 1-10) and whole cell suspensions (12 µl at a density of 1×10^6 cells in 1 ml (*lanes 11 and 12*) were analyzed by western blot for the presence of galectin-14 using galectin-14 mAb. *Lanes 1 and 2* Cell free supernatants collected at the time of setting up the culture from peripheral blood and mammary lavage cells, respectively. *Lanes 3–10* cell free supernatants collected after 1 h from duplicate cultures of peripheral blood in the absence (*lanes 3–4*) and presence of rhIL-5 (*lanes 7–8*), and mammary lavage cells in the absence (*lanes 5–6*) and presence of rhIL-5 (*lanes 9–10*). *Lanes 11 and 12* Whole cell suspensions from peripheral blood and mammary lavage cells, respectively, were analyzed prior to culture. kD sizes on left are based on prestained molecular weight markers

instructions. Labeling did not interfere with the activity of rGST-galectin-14 as assessed by hemagglutination assay [8].

Five-micrometer sections were cut from frozen tissue blocks, embedded in Optimal Cutting Temperature (O.C.T.) compound (Tissue-Tek). The sections were blocked with 3% BSA PBS for 30 min, incubated with fluorescein-labeled rGST-galectin-14 or rGST diluted to 10 µg/ml in blocking solution for 30 min, washed in PBS three times for 5 min each, and then examined under an epifluorescence Leica DM LB microscope (Leica microsystems, Inc.). Alternatively, slides were prepared as for immunohistochemistry but were incubated with rGST-galectin-14 or rGST-ovgal-11 at 10 µg/ml instead of the primary antibody and with anti-GST HRP-conjugated antibody (Sigma) as the secondary antibody.

Collection of mucus from gastrointestinal tract and lungs after parasite or allergen challenge

Sheep were immunized by seven weekly infections with 5,000–10,000 *Haemonchus contortus* L3 larvae, an important nematode parasite of sheep infecting the abomasum (third or true stomach). Three months after the last infection, sheep were challenged with 50,000 L3 and sacrificed 10 days later for mucus collection.

Allergen-challenged lungs were obtained by repeated lung challenge of sheep sensitized to house dust mite (HDM) as described in detail previously [8, 16, 21]. Briefly, sheep were primed by three subcutaneous injections of

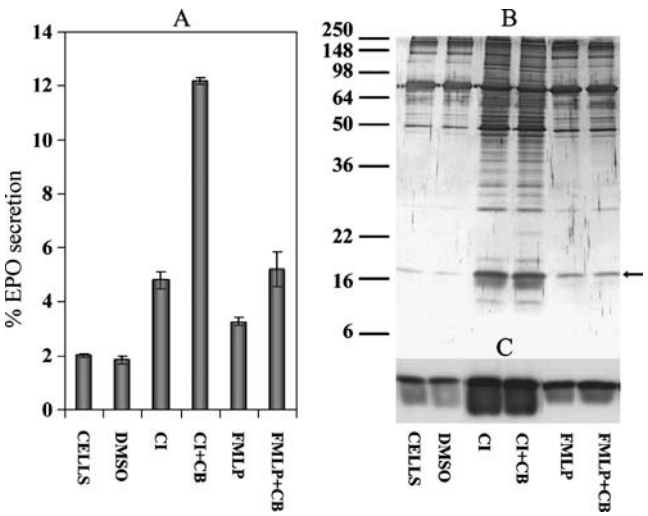


Fig. 2 Induction of galectin-14 secretion by blood eosinophils *in vitro*. Purified (>90%) peripheral blood eosinophils were cultured in either media alone (*CELLS*) or with media plus DMSO as a carrier solvent control (*DMSO*) or with media containing activating agents (*CI*, *CI+CB*, *FMLP* or *FMLP+CB*). The resulting cell free supernatants after 1 hr of culture was analyzed as follows: **a** eosinophil peroxidase (EPO) secretion was detected by microtitre plate assay using TMB as a substrate. **b** Total protein release was assessed by SDS-PAGE and silver staining. **c** Galectin-14 release was identified by western blotting with a galectin-14 mAb. Standard deviations are shown in panel **a**. Seventy microliters of each cell free supernatant was analyzed for EPO and 12 µl for silver staining and Western blotting

50 μg of house dust mite (HDM, *Dermatophagoides pteronyssinus*, CSL, Melbourne, Australia) in saline with aluminium hydroxide as adjuvant (1:1). A week later, they were challenged twice weekly for 6 weeks with 1 mg of solubilised HDM in saline in the lower lung lobe using a fibre optic bronchoscope (Pentax FG-16X, 5.5 mm OD). Sheep were sacrificed 72–96 h post-challenge for the collection of lung mucus.

Mucus was collected by gently scraping the surface of the lung and abomasal mucosa with a spatula, then diluted one third with phosphate buffered saline (PBS) and centrifuged to remove cellular debris.

Results

Galectin-14 is spontaneously released by tissue eosinophils, while its secretion by peripheral blood eosinophils can be induced after *in vitro* activation

Eosinophils purified to about 60% purity from blood (PB) or recruited into the mammary gland (MAL) were cultured *in vitro* with or without rhIL-5, a known potentiator of ovine eosinophils [22]. Cell free culture supernatants and eosinophils were analysed by western blot for the presence of galectin-14 (Fig. 1). High levels of galectin-14 were detected in the culture supernatants of MAL eosinophils at the time of setting up the cultures (Fig. 1, lane 2) and after 1 h in culture with (Fig. 1, lanes 9 and 10) or without rhIL-5 (Fig. 1, lanes 5 and 6). In contrast, very little galectin-14 could be detected in the culture supernatants from PB eosinophils, either at the time of setting up (Fig. 1, lane 1) or after 1 h in culture (Fig. 1, lanes 3 and 4). Some galectin-14 could be detected in the culture supernatants from PB eosinophils cultured in the presence of rhIL-5 (Fig. 1, lanes 7 and 8), but this was significantly less than that detected in MAL eosinophil culture supernatants at the same time point, with or without rhIL-5 (Fig. 1, lanes 5, 6, 9 and 10). Similar results were seen after 2 and 3 h in culture (data not shown). Comparable levels of galectin-14 were detected in whole PB and MAL eosinophils prior to cell culture (Fig. 1, lanes 11 and 12).

PB eosinophils purified to >90% purity were cultured with activation/degranulation agents and degranulation was measured by the release of EPO. After 1 h of culture, the cell free supernatants were analysed for EPO activity (Fig. 2a), total protein content by SDS-PAGE and silver staining (Fig. 2b), and for galectin-14 by Western blot (Fig. 2c). Cells cultured in the absence of a stimulus or with

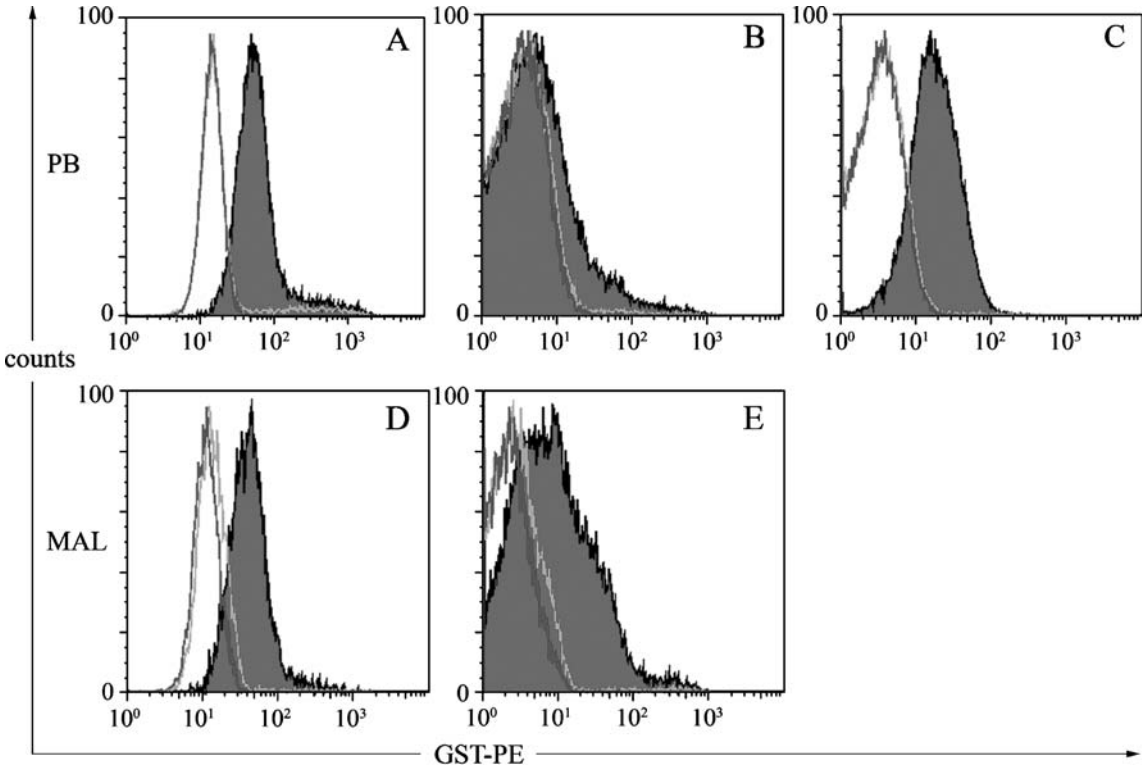


Fig. 3 Flow cytometric analysis of galectin-14 binding to PB or MAL leukocytes. PB eosinophils (**a**), PB lymphocytes (**b**), PB neutrophils (**c**), MAL eosinophils (**d**) or MAL lymphocytes (**e**) were gated using their distinctive forward and side scatter profiles as described previously [16]. The *solid histogram* in each plot represents binding with 0.6 μM rGST-galectin-14. The controls 1 μM rGST alone and 0.6 μM rGST-galectin-14 plus 300 mM lactose are shown by the *open histograms*, which overlap in each case. Binding was detected with an anti-GST PE-conjugated antibody. Profiles shown are representative of three separate experiments

the carrier solvent DMSO alone, released low levels of both EPO and the approximately 16 kDa protein (Fig. 2b, arrowed) identified by western blotting as galectin-14 (Fig. 2c). All the chemical stimuli used, CI, CI+CB, FMLP, FMLP+CB, led to varying degrees of eosinophil degranulation and all stimulated the concurrent release of galectin-14 when compared to the unstimulated controls.

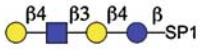
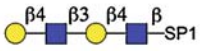
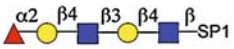
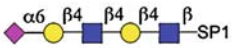
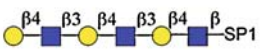
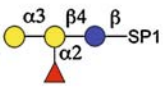
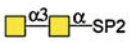
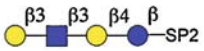
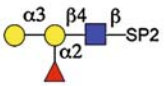
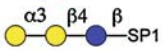
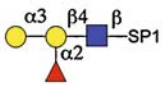
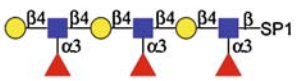
Galectin-14 binds to both blood and luminal granulocytes, but only to luminal lymphocytes

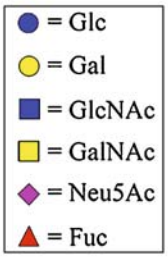
Fluorescence activated cell sorting analysis detected the binding of rGST-galectin-14 to the surface of PB eosinophils and neutrophils, and to MAL eosinophils and lymphocytes (Fig. 3). No rGST-galectin-14 binding was observed on PB lymphocytes. Galectin-14 binding to immune cells was found to be concentration dependent (data not shown) and was not detected in the presence of 300 mM lactose, suggesting that galectin-14 was binding in a carbohydrate dependent manner to cell surface glycoconjugates. No binding was observed after incubation of leukocytes with rGST, or with the anti-GST-PE antibody alone (data not shown).

Glycan array screening

Analysis of the glycan binding specificity of galectin-14 by glycan array analysis (Table 1) demonstrated that galectin-14 recognises classic galectin binding structures containing a

Table 1 Binding of fluorescein-labelled rGST-galectin-14 to glycan array

Glycan No.	Glycan structure-spacer	Name	S/N ratio
High Affinity			
32		LNnT	5.65
34		LN2	4.38
105		2'F-Di-LN	4.02
109		6'SiaDi-LN	3.90
35		LN3	3.84
Medium Affinity			
200		B-tetra Lac	3.21
73		Fs-2	3.20
169		LNT	2.93
168		B (type 2)	2.87
193		Galili-tri	2.87
201		B-tetra type 2	2.45
33		LNT-2	2.36
102		Tri-Lex	2.33



The array consisted of ~200 biotin-labelled glycosides attached to streptavidin-coated wells on microtitre plates. See "Experimental procedures" for binding conditions. Binding is classed as high or medium affinity depending on the S/N ratio

terminal *N*-acetyllactosamine (Galβ1-4GlcNAc). Overall, galectin-14 prefers linear type 2 polylactosamine glycans (Galβ1-4GlcNAc-R as found in LNnT) to the type 1 (Galβ1-3GlcNAc-R as found in LNT) and blood group B to A and O glycans. The degree of binding decreased with increasing number of lactosamine repeats (LNnT>LN2> LN3), however binding to single *N*-acetyllactosamine structures such as LN was low. Galectin-14 also recognises α1-2 fucosylated and α2-sialylated poly-*N*-acetyllactosamine glycans with nearly the same affinity as the corresponding non-modified structures.

Galectin-14 binding to laminin

Laminin is a large glycoprotein and an integral constituent of basement membranes underlying the epithelia. Laminin contains glycans that show positive binding on the glycan array and the potential of galectin-14 binding to laminin was therefore examined. *In vitro*, rGST-galectin-14 bound to laminin in a dose dependent manner (Fig. 4). This binding was reduced to the level of the GST control, when lactose was added; implying that binding of rGST-galectin-14 to laminin is mediated by the carbohydrate recognition domain of galectin-14.

No significant levels of *in vitro* eosinophil binding to laminin could be observed either for blood or luminal eosinophils, in the presence or absence of rhIL-5 or activation/degranulation agents and this was not altered by the presence of various concentrations of galectin-14 (data not shown).

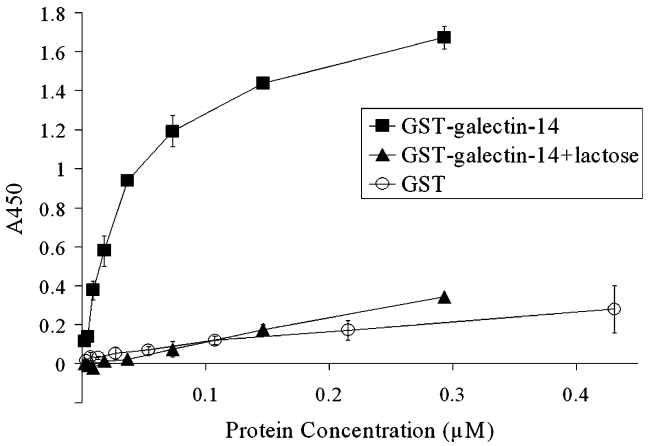


Fig. 4 Saturation binding of rGST-galectin-14 protein to immobilized laminin. Laminin binding of rGST-galectin-14 was analyzed using a solid-phase binding assay in a 96-well microtitre plate. Serial dilutions of rGST-galectin-14 (*filled square*) and rGST (*filled triangle*) were added to microtitre wells containing laminin. 0.3 M lactose was added to rGST-galectin-14 to inhibit binding (*empty circle*). The level of bound protein was monitored by the binding of rabbit anti-GST HRP antibody developed with TMB substrate and detected by a change in absorbance at 450 nm

Binding of galectin-14 to lung and gut tissue sections

Galectin-14 release has been observed in the lung after allergen challenge and in the gastrointestinal tract after parasite infection [8]. To examine the binding of galectin-14 to relevant tissues in the absence of endogenous galectin-14 release *i.e.* in the absence of tissue eosinophilia, rGST-galectin-14 was used as a probe to detect galectin-14 ligands in both unstimulated lung and gastrointestinal tissue sections (Fig. 5). Fluorescein labeled rGST-galectin-14 (Fig. 5a), but not rGST (Fig. 5c), strongly bound to the surface mucus layer of the lung and also appeared to bind the underlying mucus-secreting cells within the epithelial layer. A similar pattern of binding was also observed with rGST-galectin-14 in the gastrointestinal tract using indirect immunoperoxidase staining with anti-GST antibodies (Fig. 5b). Little binding was observed when the same tissue was probed with a different galectin, rGST-ovgal-11 (Fig. 5d). rGST-galectin-14 was also able to bind to intestinal mucus absorbed on ELISA plates, and this binding was inhibited by lactose (not shown). Taken together these results suggest that mucins are likely glycoprotein ligands for galectin-14 in both the lung and the gastrointestinal tract.

Galectin-14 is secreted into the mucus after parasite and allergen challenge

To explore the relevance of the *in vitro* galectin14 binding to lung and gastrointestinal tissue sections, mucus was collected from the lung and stomach of sheep with induced tissue eosinophilia at these sites from either allergen exposure (HDM) or parasite infection (*H. contortus*). Mucus collected from both challenged lung and stomach were found to contain large quantities of a protein of ~17 kDa that reacts with polyclonal anti-galectin-14 antibodies by western blot (Fig. 6). A similar result was seen using anti-galectin-14 mAbs (data not shown). This demonstrates that galectin-14 is released by eosinophils during allergen challenge or parasite infection and becomes associated with mucus.

Discussion

The results of the present study indicate that, while galectin-14 is constitutively expressed by ovine eosinophils, it is only secreted by eosinophils, which have migrated into the tissues in response to allergen or parasitic stimuli. Previous studies have observed abundant extracellular galectin-14 in the dermis of sheep after eosinophilia was induced by scab mite infection [23] and in the bronchoalveolar fluid of ovine lungs after allergen chal-

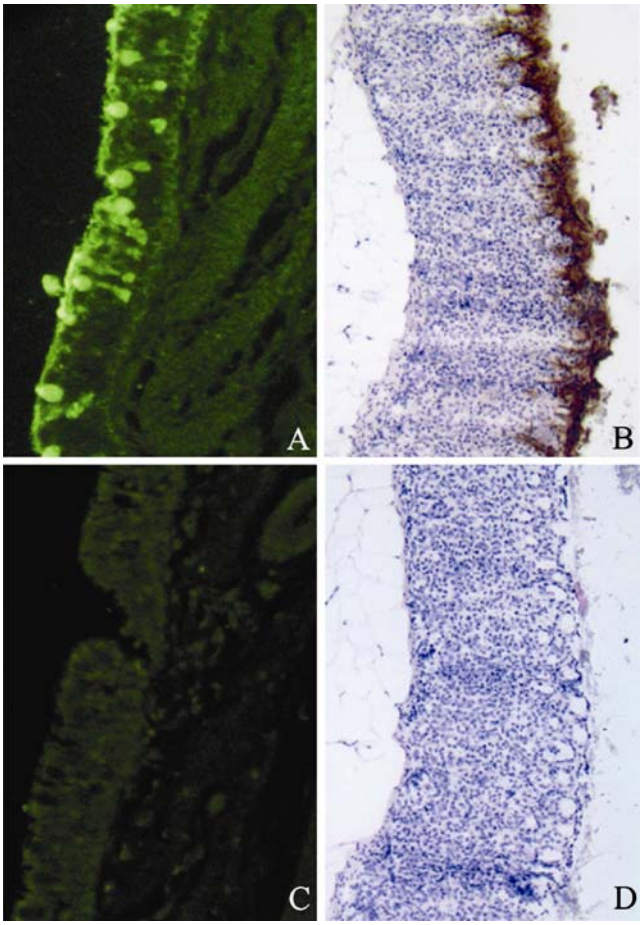


Fig. 5 Binding of galectin-14 on lung and intestinal tissue sections. Frozen tissue sections from healthy sheep were incubated with recombinant proteins; lung tissue with rGST-galectin-14-fluorescein (**a**) or rGST-fluorescein (**c**) and intestinal tract tissue with rGST-galectin-14 (**b**) or rGST-ovgal-11 (**d**). Protein binding was monitored directly by fluorescence microscopy (**a**, **c**) or with an anti-GST HRP-conjugated antibody (**b**, **d**). The tissues (**b**, **d**) were counter-stained with hematoxylin. Magnification: **a** and **c** ×200; **b** and **d**, ×100

lenge [8]. However, it was unclear whether an activation signal is required for the release of galectin-14. The present studies demonstrate that galectin-14 was spontaneously secreted by eosinophils isolated from mammary lavage following parasite infusion, while little galectin-14 was released by resting peripheral blood eosinophils. This is in agreement with previous studies, which have shown that galectin expression and/or secretion is regulated by the activation state of cells which produce them (reviewed in [24]). *In vitro* studies also indicate that the same stimuli that induce eosinophil degranulation, as measured by peroxidase release, will also induce galectin-14 secretion. In particular, it appears that galectin-14, like galectin-10 [25], is rapidly released during calcium ionophore-induced degranulation of eosinophils.

The localisation of galectin-14 to eosinophils and its secretion in response to appropriate stimuli, suggests that it has an important extracellular role in allergic-type inflammation. Binding studies showed that galectin-14 not only binds to eosinophils, but also to other inflammatory cells in the local tissue environment, such as neutrophils and lymphocytes. This binding was specific, as galectin-14 did not bind to peripheral blood lymphocytes, but only those from the mammary lavage. Previous studies have shown that most mammary lavage lymphocytes display an activated phenotype compared to blood lymphocytes [16] suggesting that binding to lymphocytes only occurs when they are activated. Other galectins have been shown to induce apoptosis after binding to inflammatory cells in the local area [24, 26]. However, incubation of blood leukocytes and Con-A activated lymphocytes with recombinant galectin-14 failed to induce apoptosis after 1-4 hrs, as determined by Annexin V staining (data not shown).

Many of the biological properties of galectins are thought to depend on their ability to bind β-galactosides. The elucidation of the unique glycan-binding specificity of galectin-14 should therefore lead to a better understanding of its biological role. Screening on a glycan array revealed that galectin-14 bound to type 2 polylactosamine glycans, Galβ1-4GlcNAc, with a preference for low numbers of *N*-acetyllactosamine repeats (must be >1). Galectin-14 showed no preference for branched glycans, but was tolerant of α1-fucosylated terminal substitutions. Galectin-14 however, seems unusual among galectins in its preference for α2-6- but not α-sialylation. Recently, it has been demonstrated that receptor sialylation can significantly and uniquely alter cellular sensitivity towards galectins-1, 2 and

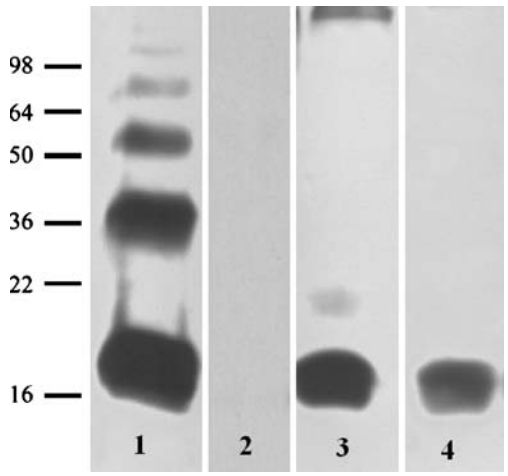


Fig. 6 Galectin-14 in mucus scrapings after allergen or parasite challenge. Mucus scrapings were collected from the lung of HDM sensitized and lung challenged sheep 72 h post-challenge (*lane 3*). Stomach mucus scrapings were collected from two separate sheep before (*lane 2*) and 10 days post *H. contortus* challenge infection (*lane 4*). Twelve microliters of mucus scrapings diluted one third with PBS were analyzed by western blotting. The membrane was stained with anti-galectin-14 polyclonal serum. Recombinant galectin-14 was included as a positive control (*lane 1*)

3 [27]. Specifically, any sialylation blocked glycan recognition by galectin-2, galectin-1 was tolerant of α2-3- but not α2-sialylation, while galectin-3 was more tolerant of either α2-3- or α2-sialylation but its binding did not appear to be regulated by it. It remains to be seen whether glycan sialylation will form the basis of the modulation of galectin-14 activity, or merely represents a species adaptation for ligand binding.

The highest binding of galectin-14 on the glycan array was observed with lacto-*N*-neotetraose (LNnT). This oligosaccharide is expressed by helminth parasites and has been shown to skew the immune response into a type 2 or regulatory phenotype, in a ligand-specific manner [28]. The nature of the LNnT ligand is unknown, however it is interesting to speculate on the role of galectin-14 in this process. Low numbers of eosinophils are present in most resting tissues, particularly the lung and gastrointestinal tract, and galectin-14 release by tissue eosinophils would be able to interact immediately with helminth glycans such as LNnT upon infection. Specific binding of galectin-14 to the surface of liver fluke has recently been demonstrated (Young *et al.*, submitted).

The glycan array data also suggest a number of potential tissue-based ligands for galectin-14 including the extracellular matrix protein laminin. Laminin carries polylactosamine side chains containing Galβ1-4GlcNAcβ1-3 repeating units [29] and has been identified as a ligand for galectins-1 and 3 which is thought to regulate cell migration [30]. However, although galectin-14 was able to bind laminin, we were unable to show that galectin-14 had any influence on the binding of eosinophils to laminin *in vitro* (data not shown). In addition, no binding to extracellular matrix was apparent in tissue sections with fluorescently-labeled recombinant galectin-14. These results suggest that galectin-14 is not involved in tissue cell migration. However, it can not be excluded that the recombinant galectin-14 used in these studies is not fully active in these assays.

Recombinant galectin-14 was shown to strongly bind to the epithelial mucus layer and mucus-secreting cells in the respiratory and gastrointestinal tracts. The diversity of carbohydrate epitopes presented by mucins, which include polylactosamine chains, would indicate mucin to be an ideal ligand for galectin-14. The functional relevance of this interaction is significantly strengthened by the presence of large amounts of native galectin-14 in mucus scrapings from the respiratory tract following allergen challenge and from the gastrointestinal tract during parasite infection. Several galectins have been detected in the epithelial layers of the gastrointestinal or respiratory tracts [31], but only galectin-1 has been shown to bind to epithelial mucin [32]. However, unlike resident gastrointestinal tract galectins, secretion of galectin-14 is dependent on eosinophil recruitment and activation, suggesting a targeted rather than housekeeping function. Once released into the mucus layer, galectin-14 may cross-link mucins thereby changing the rheological properties of the mucus or increasing its adhesiveness. Changes in mucus properties are an integral part of the host defence against gastrointestinal parasites [33] and are also involved in eosinophil-induced pathologies such as allergic asthma [34, 35]. The similar expression and release profiles of ovine galectin-14 and human galectin-10 and the functional and binding studies reported here for galectin-14, indicate a possible role for eosinophil galectins in these important disease processes.

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