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Maintenance of granulocyte numbers during acute peritonitis is defective in galectin-3-null mutant mice

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SUMMARY

Galectin-3, also known as the macrophage marker Mac-2, is a member of a family of structurally related animal lectins that exhibit specificity for β-galactosides. In order to investigate the role of galectin-3 in acute inflammation, we have compared the number of leucocytes present in the peritoneal cavity of wild type and galectin-3 null mutant mice after intraperitoneal (i.p.) injection of thioglycolate broth. At day 1 after injection, we found no difference in the recruitment of mononuclear phagocytes and granulocytes to the peritoneal cavity. However, 4 days after thioglycolate injection, galectin-3 mutant mice exhibited a significantly reduced number of recoverable granulocytes compared to wild-type animals. As mutant granulocytes did not exhibit an accelerated rate of apoptosis and their uptake by macrophages appeared to be unaffected by the mutation, the phenotype described here suggests that galectin-3 participates in an additional level of control during the resolution of acute inflammation.

INTRODUCTION

Galectin-3 is a member of a family of structurally related animal lectins that exhibit specificity for β-galactosides which are bound in a Ca^{2+}-independent manner. These characteristics distinguish galectins from all other animal lectins.[1] The galectin family comprises 10 known members, many of which are expressed in a developmentally and tissue-restricted manner.[2] Galectins have been found in intracellular[3] and extracellular locations[4] but they lack a signal sequence and, where they have been studied, their mechanism of secretion is independent of the Golgi pathway.[5–7] In many cases galectins have been detected on cell surfaces but, because these proteins do not exhibit transmembrane sequences, their retention at the cell surface depends on the presence of appropriate β-galactoside-containing glycans.

Galectin-3 is a non-glycosylated, 30 000 MW protein which, in addition to the characteristic galectin carbohydrate recognition domain, also contains a unique amino-terminal domain that exhibits a collagenase-sensitive site[8] and can act as a substrate for transglutaminase.[9] The amino-terminal domain of galectin-3 is thought to be important for self-oligomerization and hence accounts for its functional multivalency.[10] Galectin-3 has been implicated in a variety of processes, such as RNA splicing[11] and apoptosis;[12] however, it is best documented as a cell–cell or cell–extracellular matrix interaction molecule, for example during embryonic development and tumour progression.[13]

We have recently generated galectin-3 null mutant mice and have shown that animals lacking this molecule are viable and have no overt abnormalities.[14] These results indicate that either galectin-3 is not required for survival under conventional animal house conditions, or that functional redundancy operates, possibly among members of the same gene family.

Within the immune system, galectin-3 was originally characterized in the mouse as the Mac-2 antigen expressed by inflammatory macrophages.[15] It was subsequently characterized as an immunoglobulin E (IgE) binding protein,[16] the major non-integrin laminin-binding protein of macrophages,[17] and was later found on the surface of human neutrophils,[18] eosinophils[19] and mast cells.[20] Addition of exogenous galectin-3 to neutrophils,[21] monocytes[22] and mast cells[23] led to their activation, suggesting a role in modification of the inflammatory response.

We have taken advantage of the availability of galectin-3 null mutant mice to investigate the role of this molecule in acute inflammation, by comparing the local response of wild type (wt) and mutant animals after intraperitoneal injection of thioglycolate broth. This is a well-defined model of acute inflammation that has been used extensively to dissect the molecular basis of inflammatory cell recruitment, activation and turnover.[24] Previous studies have shown that thioglycolate-stimulated macrophages retain galectin-3 at the cell surface and that this can be correlated with expression of appropriate

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Abbreviations: i.p., intraperitoneal; mAb, monoclonal antibody; wt, wild type.

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β-galactoside-containing glycans on the macrophage cell surface.[15,25] Using this model, we did not find significant differences in the day 1 recruitment of granulocytes and mononuclear phagocytes to the peritoneal cavity. However, 4 days after thioglycolate injection, galectin-3 null mutant mice exhibited a statistically significant reduction in the number of granulocytes present in the peritoneal cavity compared to wt mice. The present experiments suggest that this difference cannot be explained by accelerated apoptosis of mutant granulocytes or increased clearance of granulocytes by mutant inflammatory macrophages.

MATERIALS AND METHODS

Mice

All studies were performed using adult mice over 3 months old raised in a conventional animal facility. Galectin-3 null mutant mice were generated by standard gene targeting techniques.[14] Wt and mutant animals were caged together after weaning. All animals examined, whether wt or mutant, were from the same mixed genetic background (50% 129, 50% MF1).

Reagents

Unless otherwise stated, all chemicals were purchased from Sigma (Poole, Dorset). Phosphate-buffered saline (PBS) was calcium and magnesium free. Thioglycolate broth was a gift from Dr Luisa Pomares (Oxford University, Oxford, UK). Rat anti-Mac-2 (galectin-3) monoclonal antibody (mAb) (M3/38 hybridoma supernatant) was a gift from Dr H. Leffler (University of California, San Francisco, CA, USA). FA11 mAb, specific for mouse macrophages[26] was kindly provided by Professor S. Gordon (Oxford University, Oxford, UK). Horseradish peroxidase-conjugated goat anti-rat immunoglobulin G (IgG) Fab′ fragments were purchased from Immunotech (Marseille, France).

Isolation and characterization of leucocytes

Peritoneal cells were harvested from the peritoneal cavity of mice by gentle lavage following injection of 5 ml of PBS. To induce an inflammatory reaction in the peritoneal cavity, mice were injected via the intraperitoneal (i.p.) route with 1 ml of thioglycolate broth and peritoneal cells were harvested on day 1 (18 h), day 2, day 3 or day 4 after injection. The proportion of granulocytes (neutrophils + eosinophils), macrophages and lymphocytes was determined on the basis of morphological criteria after Giemsa staining of cytospin preparations. The percentage of macrophages in each preparation was confirmed by immunocytochemical staining with the FA11 mAb.

Immunocytochemistry

Cells isolated from the peritoneal cavity were cytocentrifuged and endogenous peroxidase activity was inhibited with a solution of 0·6% hydrogen peroxide in methanol. The slides were washed in PBS containing 0·05% Triton-X-100 and 1% heat-inactivated horse serum, and stained overnight at 4° with a 1:20 dilution of the Mac-2 mAb or a 1:5 dilution of the FA11 mAb. After washing, the slides were incubated with a 1:500 dilution of the horseradish peroxidase-conjugated goat anti-rat IgG Fab′ for 1 h at room temperature. The enzymatic activity was detected with the chromogenic substrate diaminobenzidine.

Statistical analysis

Statistical analysis (Student's *t*-test, Mann–Whitney *U*-test) was performed using the program INSTAT from GraphPad Software (San Diego, CA).

Apoptosis assay

Neutrophils were isolated from thioglycolate-elicited peritoneal cell suspensions by depletion of adherent cells for 1 h at 37° in RPMI containing 10% fetal calf serum, as described previously.[27] Neutrophils were analysed either immediately or after culture for an additional 24 h in RPMI containing 10% fetal calf serum. In some experiments, recombinant murine G- and granulocyte–macrophage colony-stimulating factor (GM-CSF) (R&D Systems, Abingdon, Oxfordshire, UK) were added at 0·3 ng/ml and 1·5 ng/ml respectively to suppress apoptosis during the 24-h culture period. Apoptosis of neutrophils was assessed using the R&D Systems Apoptosis Detection Kit according to the manufacturer's instructions. Briefly, 10^6 cells were washed once in PBS, once in binding buffer, incubated with fluorescein isothiocyanate (FITC)-conjugated annexin-V and propidium iodide for 15 min at room temperature and analysed on a fluorescence-activated cell sorter (FACScan) flow cytometer.

Phagocytosis assay

The apoptotic neutrophil phagocytosis assay was carried out according to the procedure described by Lagasse *et al.*[27] Macrophages were harvested from mice injected 4 days previously with thioglycolate and 5×10^4 cells were plated onto each well of eight-chamber LabTek chamber slides (Nunc) and cultured for 24 h. Neutrophils were isolated as described above for the apoptosis assays and, after a 24-h cultivation period to induce apoptosis, 10^6 cells were seeded onto the preformed macrophage monolayers and incubated for 3·5 h, the cells being gently mixed after 2 h. The chamber slides were then rinsed, fixed and stained with benzidine to detect granulocytes. Under high-power light microscopy, phagocytosed neutrophils could be readily distinguished from the bound, non-phagocytosed cells. The number of phagocytosed neutrophils was determined from counts of 2000 macrophages for each well.

RESULTS

Mutant mice have reduced numbers of granulocytes in the peritoneal cavity 4 days after thioglycolate injection but no defect in their initial recruitment

Previous studies[15,25] showed that galectin-3 is expressed on the surface of inflammatory macrophages recruited to the peritoneal cavity following injection with thioglycolate. We therefore used this model system to determine whether galectin-3 could be implicated in the regulation of acute inflammation by comparing the inflammatory response in wt and galectin-3 null mutant mice.

Mice were analysed at day 0 (before injection) and on days 1 and 4 after i.p. injection of thioglycolate (Table 1, Fig. 1). We quantified the numbers of macrophages, granulocytes and lymphocytes at each time point. In five independent

Table 1. Kinetics of leucocyte recruitment in the peritoneal cavity of wild-type (wt) and galectin-3 mutant mice*

		Wild-type		Mutant		
	Day	*n*	Mean ± SEM $\times 10^5$	*n*	Mean ± SEM $\times 10^5$	*P*-value
Granulocytes	0	11	3·08 ± 1·00	10	1·32 ± 0·331	NS
	1	19	63·3 ± 11·3	20	49·0 ± 9·96	NS
	4	22	20·3 ± 3·13	21	5·09 ± 0·735	<0·0001
Macrophages	0	11	13·9 ± 3·55	10	13·1 ± 4·06	NS
	1	19	31·1 ± 6·24	20	19·0 ± 2·53	NS
	4	22	29·5 ± 5·88	21	39·0 ± 4·97	NS
Lymphocytes	0	11	84·8 ± 8·14	10	80·7 ± 19·2	NS
	1	19	62·2 ± 7·57	20	81·9 ± 9·12	NS
	4	22	60·6 ± 8·93	21	62·6 ± 10·4	NS

*At the indicated times after intraperitoneal injection of thioglycolate, cells were harvested from the peritoneal cavity by lavage and the numbers of macrophages (FA11+), granulocytes (eosinophils + neutrophils) and lymphocytes (remaining cells) were quantified for individual mice from cytospin preparations. Day 0 counts represent peritoneal cell populations from uninjected animals. The data were pooled from all experiments and analysed using the Mann–Whitney *U*-test.

n, total number of mice analysed for each group in three (day 0), five (day 1) and six (day 4) experiments.

NS, not significant.

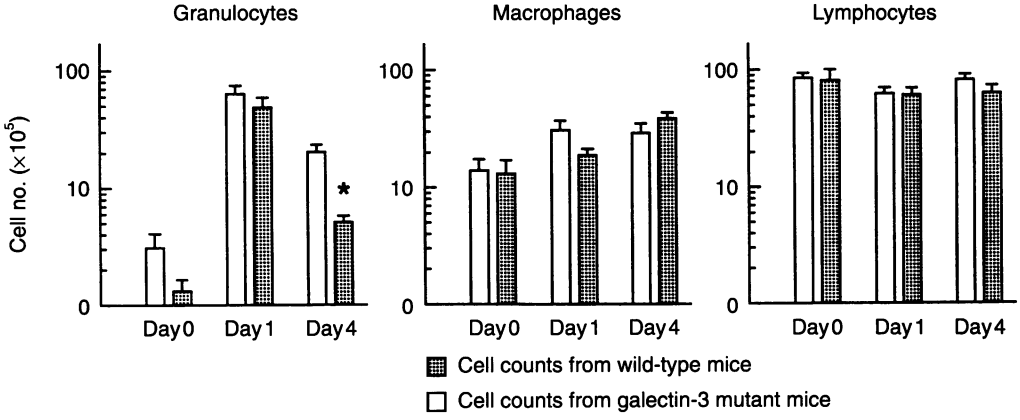

Figure 1. Kinetics of leucocyte recruitment in the peritoneal cavity of wild-type (wt) and galectin-3 mutant mice. Peritoneal cells were harvested at the indicated times after thioglycolate injection and the number of granulocytes, lymphocytes and macrophages were determined from cytospin preparations. The asterisk indicates $P<0{\cdot}0001$ (Mann–Whitney *U*-test).

experiments, no differences in either the percentage or absolute numbers of these cells were observed on day 1, but in six experiments carried out on day 4, there was a statistically significant, four-fold reduction in granulocyte numbers in mutant mice compared with wt mice (Table 1). In two separate experiments we also analysed granulocyte numbers on day 3 after thioglycolate injection, but no significant differences were observed (wt: $24\times10^5\pm5\times10^5$, $n=6$; mutant $22\times10^5\pm4\times10^5$, $n=7$). These results indicate that the absence of galectin-3 results in abnormal loss of granulocytes from the peritoneal cavity and that this occurs between days 3 and 4 after thioglycolate injection. This conclusion is based on a large number of independent experiments which were required because of the variable responses seen between individual mice. This variability is likely to be caused, in part, by the mixed genetic background of the wt and mutant mice used in these experiments.

The spontaneous rate of apoptosis of galectin-3 mutant granulocytes is normal

To establish that galectin-3 is expressed on the recruited granulocytes, cytospins were prepared from peritoneal cells harvested 1 day after thioglycolate injection and stained with anti-galectin-3 mAb (Fig. 2). These cell populations comprised 60–65% granulocytes, the majority of which expressed galectin-3. As expected, galectin-3 was undetectable on mutant cells (Fig. 2). FACS analysis of live cells confirmed that galectin-3 was expressed on the surface of granulocytes (data not shown).

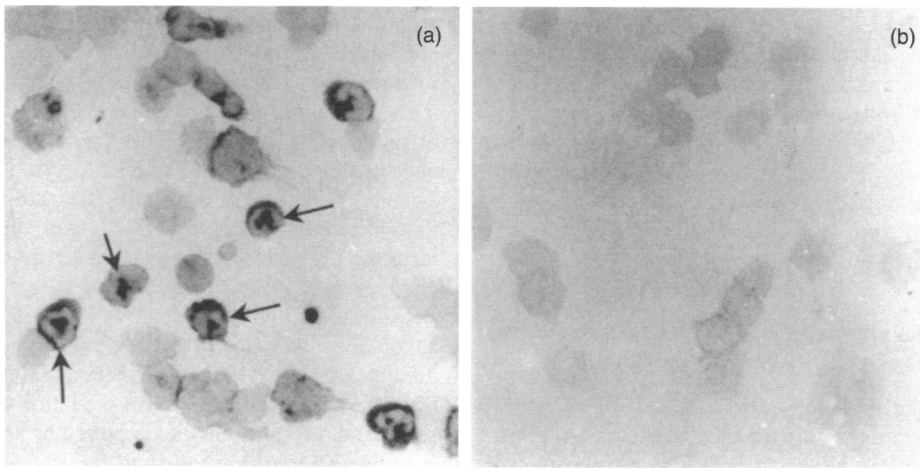

Figure 2. Thioglycolate-recruited granulocytes display galectin-3 at the cell surface. Day 1-recruited peritoneal cells were stained with anti-galectin-3 mAb. The mAb stained macrophages and granulocytes from wild-type (wt) animals (a) but not cells from mutant animals (b). Arrows indicate cells that are likely to be granulocytes.

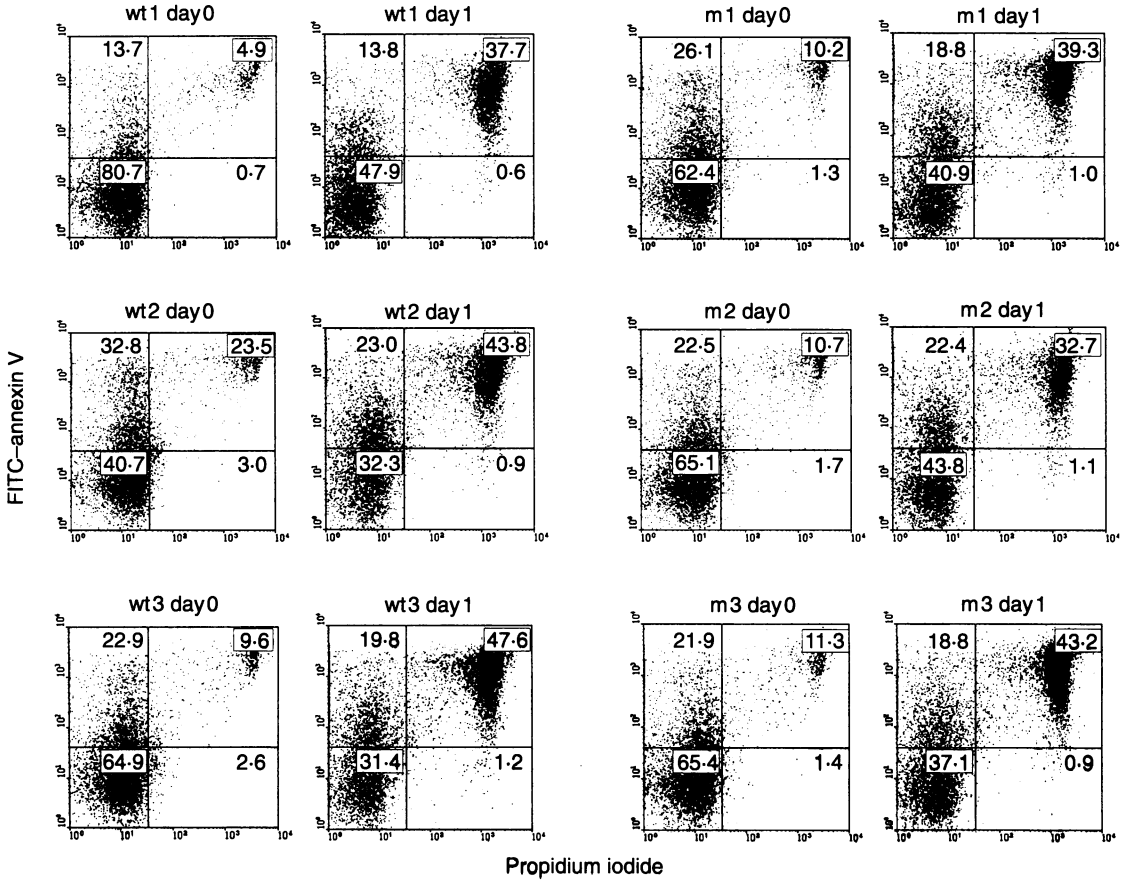

Figure 3. *In vitro* apoptosis of recruited granulocytes. Granulocytes were isolated from day 1-recruited peritoneal cells harvested from wild-type (wt) and mutant (m) mice and stained with FITC–annexin-V and propidium iodide (PI), either immediately after isolation (day 0) or after 24 h of culture in medium containing 10% fetal calf serum (day 1). The numbers in each quadrant represent the percentage values of the total number of cells analysed. Viable cells are FITC−/PI−; cells undergoing apoptosis are FITC+/PI−; post-apoptotic cells are FITC+/PI+. Data from three individual animals of each genotype are presented.

One possible explanation for the reduced numbers of granulocytes in mutant mice is that the absence of galectin-3 increases their rate of spontaneous apoptosis. This possibility was examined using FITC–annexin-V staining of thioglycolate-recruited granulocytes either immediately after harvest or after 24 h of culture (Fig. 3). The binding of annexin-V to the cell surface is established as one of the earliest indicators of programmed cell death.[28] Using annexin-V in conjunction

with propidium iodide to detect post-apoptotic (permeable) cells, there were no detectable differences in rates of apoptosis between wt and mutant granulocytes at both time-points analysed (Fig. 3). In both cases, the viability of granulocytes decreased from ~63% to ~39% during the culture period. Furthermore, addition of 1·5 ng/ml GM-CSF and 0·3 ng/ml G-CSF, which maintained the viability of the cells at ~69% during the culture period, did not reveal differences in the rates of apoptosis (data not shown).

Apoptotic granulocytes are phagocytosed at the same rate by wt and mutant macrophages

We next explored the possibility that the lack of galectin-3 leads to increased granulocyte clearance by macrophages at the inflammatory site. The phagocytic activity of macrophages for apoptotic granulocytes was determined using an *in vitro* assay, as previously described.[27] We found that there was no significant difference in the phagocytic activity between wt and mutant macrophages (~7% of macrophages exhibiting phagocytosis) (Table 2). This reflected specific phagocytosis of apoptotic granulocytes because fewer than 1% of the macrophages were phagocytic when exposed to freshly harvested granulocytes (data not shown).

DISCUSSION

Because galectin-3 null mutant mice are viable, it is possible to initiate studies on the role of this lectin in acute inflammatory processes. In previous studies, galectin-3 has been extensively examined as a surface molecule, expressed on macrophages, elicited by i.p. injection of thioglycolate.[15,25] Therefore, this model was chosen in the present study to test the possible functional significance of galectin-3 in the context of acute inflammation. The most striking observation was that the mutant mice had greatly reduced numbers of granulocytes on day 4 after thioglycolate injection, but no differences were observed at earlier time-points, including day 3. This finding indicates that galectin-3 plays a role in the maintenance of granulocyte numbers in the inflamed peritoneal cavity. This phenotype is very robust as it was easily detected despite considerable variability in the leucocyte numbers seen between individual animals. We cannot exclude the possibility that other, more subtle, differences exist between wt and mutant mice in this experimental system.

Table 2. *In vitro* phagocytosis of apoptotic granulocytes by macrophages*

	Phagocytosis†	
Mouse	Wild-type macrophages + Wild-type granulocytes	Mutant macrophages + Mutant granulocytes
1	140	117
2	133	166
3	121	150

*Pools of granulocytes were isolated from day 1-recruited peritoneal cells and cultured for 24 hr in medium containing 10% fetal calf serum to induce apoptosis. Apoptotic granulocytes were added to monolayers of day 4 thioglycolate-elicited macrophages, from three different animals, which had been cultured for 24 hr prior to addition of granulocytes. Phagocytosis was allowed to proceed for 3·5 hr. Phagocytosed granulocytes were visualized after staining with benzidine as a peroxidase substrate.

†The data show the number of macrophages that have phagocytosed at least one granulocyte from 2000 macrophages analysed.

With a notable exception,[29] it is now generally accepted that granulocytes are cleared at sites of inflammation as a result of apoptosis followed by macrophage recognition and phagocytosis, via specific receptor–ligand interactions.[30–32] Because the granulocyte counts on days 1 and 3 after thioglycolate injection showed that the phenotype was not caused by differences in the initial recruitment of granulocytes, we initially hypothesized that the clear-cut reduction in granulocytes seen between days 3 and 4 could be caused by accelerated apoptosis. A previous report suggested that galectin-3 might function as an anti-apoptotic factor; this was based on experimental observations and also its postulated homology with Bcl-2.[12] Furthermore, in the case of Mac-1-deficient mice, it was proposed that the dramatically increased number of granulocytes present in the inflamed peritoneal cavity was a result of reduced apoptosis, a suggestion that was at least partially supported by the enhanced rates of apoptosis observed for mutant granulocytes *in vitro*.[33] However, in the case of the galectin-3 mutants, annexin-V staining of day 1 thioglycolate-elicited granulocytes revealed that there were no differences in the *in vitro* rates of apoptosis between wt and mutant cells. This indicates that enhanced apoptosis of galectin-3 deficient granulocytes is unlikely to explain the observed phenotype.

A second possible explanation that we considered was that in the absence of galectin-3, apoptotic granulocytes are more efficiently cleared by macrophages. For example, ligands on apoptotic granulocytes that could potentially be recognized by macrophage receptors might normally be masked by galectin-3. In the absence of the lectin, these sites could be unmasked and lead to enhanced recognition and clearance. Alternatively, if galectin-3 binds to oligosaccharide ligands carried by macrophage receptors involved in recognition of apoptotic granulocytes, like CD36[34] or scavenger receptors,[35] the absence of galectin-3 could affect macrophage clearance functions. It is noteworthy in this respect that one of the prominent potential macrophage counter-receptors for galectin-3 was found to be Mac-1,[36] a promiscuous receptor with multiple ligand specificities.[37] However, when we compared the phagocytic activity of wt and galectin-3 mutant macrophages, there were no detectable differences in *in vitro* uptake of wt and mutant apoptotic granulocytes, arguing against a role of the lectin in modulating clearance functions of macrophages.

Our results thus indicate that the decline in granulocyte numbers observed between days 3 and 4 of the inflammatory response is unlikely to be the result of a role of galectin-3 in either granulocyte apoptosis or phagocytic uptake by macrophages. As these are considered to be the two major mechanisms leading to the resolution of acute inflammation,[38] the present report provides evidence for an additional regulatory pathway. An attractive model to explain our findings is that granulocytes are normally retained in the inflamed peritoneal cavity through a galectin-3-dependent mechanism. One way in which this might occur is through a bridging mechanism, whereby galectin-3 normally impedes neutrophil exit by cross-linking ligands on neutrophils with ligands present on extra-

cellular components. For example, it has been demonstrated previously that galectin-3 is able to promote attachment of granulocytes to laminin and activate divalent cation-dependent adhesion to other extracellular components like fibronectin.[39] It will be interesting in the future to determine whether the granulocytes recruited in mutant mice migrate from the peritoneal cavity to the draining parathymic lymph nodes. This is a process that has recently been shown to occur in the case of inflammatory macrophages, but there is no evidence that it is a normal pathway for granulocytes.[38]

In conclusion, we report the first phenotypic defect associated with the galectin-3 mutation. This carbohydrate-binding protein appears to be involved in the control of an acute inflammatory process, specifically at the level of granulocyte maintenance, through a hitherto unrecognized mechanism. Regardless of the molecular basis for this phenotype, it will be of interest to explore the consequences of this mutation in the context of host defence to pathogens. Indeed, it has been previously suggested that galectins might be involved in host–pathogen interactions.[40,41]

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