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Cell-type specific regulation of galectin-3 expression by glucocorticoids in lung Clara cells and macrophages

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Summary. Bronchiolar Clara cells are integral components of lung homeostasis, predominantly distributed in distal airways. In addition to the 16 kDa Clara cell protein, a major secretory product with anti-inflammatory effects, rat Clara cells express the glycan-binding protein galectin-3 and secrete it into the airways. Given the essential role of galectin-3 in the control of inflammation and the well-established function of glucocorticoids (GCs) in lung physiology, here we investigated whether galectin-3 is a target of the regulatory effects of GCs. Adult male rats were subjected to bilateral adrenalectomy and the lungs were processed for light and transmission electron microscopy, immunoelectron microscopy and Western blot analysis. Profound changes in bronchiolar Clara cells and macrophage morphology could be observed by electron microscopy after adrenalectomy. While specific galectin-3 staining was detected in the nucleus and cytoplasm of Clara cells and macrophages from control animals, cytoplasmic galectin-3 expression was dramatically reduced after adrenalectomy in both cell types. This effect was cell-specific as it did not affect expression of this lectin in ciliated cells. After dexamethasone treatment, galectin-3 expression increased significantly in the nucleus and cytoplasm of macrophages and Clara cells. Western blot analysis showed a clear decrease in galectin-3 expression in ADX animals, which was recovered after a 7-day treatment with dexamethasone. In peritoneal macrophages, galectin-3 expression was also dependent on the effects of GCs both *in vivo* and *in vitro*. Our results identify a cell type-specific control of galectin-3 synthesis by GCs in lung bronchiolar Clara cells and interstitial macrophages, which may provide an alternative mechanism by which GCs contribute to modulate the inflammatory response.

Key words: Galectin-3, Glucocorticoids, Bronchiolar Clara cells, Macrophages

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Introduction

Galectins are a group of highly conserved lectins characterized by their ability to recognize multiple N-acetyl-lactosamine sequences, which can be displayed on both *N*- and *O*-glycans on cell surface glyco-conjugates (Gabius, 1997; Rabinovich and Toscano, 2009; Vasta, 2009). One of the best studied members of the family is galectin-3, a chimera-type lectin composed of two structurally distinct domains: a N-terminal non-lectin domain consisting of multiple repeats of a peptide sequence rich in proline, glycine, and tyrosine, and a C-terminal domain containing the carbohydrate-binding site (Dumic et al., 2006; Nieminen et al., 2008; Yang et al., 2008). First identified as the Mac-2 antigen in macrophages (Ho and Springer, 1982; Cherayil et al., 1989; Sato and Hughes, 1994), this ~31-kDa protein has been detected in different haematopoietic and non-haematopoietic tissues (Sundblad et al., 2011) and displays diverse biological roles ranging from immunomodulation to angiogenesis and wound healing (Dumic et al., 2006; Yang et al., 2008; Markowska et al.,

2010). Galectin-3 has been identified in a variety of cells involved in immune responses, including neutrophils, eosinophils, mast cells and dendritic cells, as well as monocytes and macrophages from different organs (Dumic et al., 2006; Toscano et al., 2007). It is also localized in highly specialized epithelia, including those covering the digestive tract (Nio et al., 2005) and lung airways (Kasper and Hughes, 1996), suggesting a protective mechanism to preserve mucosal integrity against a diversity of insults.

Through the control of a variety of biological processes, including cell adhesion, chemotaxis, cytokine secretion and apoptosis, galectin-3 can modulate innate and adaptive immune responses (Rabinovich and Toscano, 2009). *In vitro*, galectin-3 induces IL-1ß production by human monocytes (Liu et al., 1995) and stimulates L-selectin shedding, IL-8 secretion (Nieminen et al., 2005) and production of reactive oxygen species (Yamaoka et al., 1995; Almkvist et al., 2001) by neutrophils. Galectin-3 can also act as an adhesion molecule for neutrophils and eosinophils through cross-linking specific glyco-receptors (Kuwabara and Liu, 1996; Sato et al., 2002; Nieminen et al., 2007; Rao et al., 2007) and has been implicated in the recruitment of neutrophils into the alveoli during lung infection with *S. pneumoniae* (Sato et al., 2002). Moreover, galectin-3 not only promotes leukocyte recruitment (Nieminen et al., 2008), but also modulates T cell activation (Demetriou et al., 2001) and regulates macrophage chemotaxis and phagocytosis (Sano et al., 2000, 2003). Notably, this endogenous glycan-binding protein can also regulate T cell viability by either protecting cells from apoptosis or stimulating cell death depending on the intracellular or extracellular localization of the protein (Yang et al., 1996; Fukumori et al., 2003; Stillman et al., 2006). In addition, galectin-3 can down-regulate IL-5 synthesis when targeted to the inflammatory airways in a murine model of asthma (Cortegano et al., 1998; del Pozo et al., 2002; Lopez et al., 2006). However, galectin-3-deficient (*Lgals3*$^{-/-}$) mice displayed a lower Th2 response in the ovalbumin asthma model (Zuberi et al., 2004), suggesting distinct roles of endogenously-regulated versus exogenously-added galectin-3 in fine-tuning the Th1/Th2 cytokine balance. Collectively, these functions make galectin-3 an attractive target in the control of lung-associated inflammatory responses. However, despite accumulating evidence on the diversity of functions displayed by galectin-3, little is known about the stimuli and signaling pathways capable of regulating expression of this glycan-binding protein under physiological and pathological conditions.

Bronchiolar Clara cells are non-ciliated secretory cells abundant in the distal airway of the respiratory tract which play key roles in the maintenance of lung homeostasis. They are associated with important functions such as cellular proliferation, toxic metabolism and biosynthesis, as well as storage and release of secretory products (Massaro et al., 1994). Their main secretory product, the 16-kD Clara cell protein (CC16), has been implicated in the maintenance of lung homeostasis due to its considerable anti-inflammatory and immunosuppressive effects (Vasanthakumar et al., 1988; Dierynck et al., 1995; Jorens et al., 1995). Interestingly, Wasano and Yamamoto (1989) demonstrated that, in addition to CC16, rat lung bronchiolar Clara cells synthesize galectin-3 and secrete this lectin into the airways.

Glucocorticoids (GCs) are steroid hormones capable of modulating lung maturation and promoting pulmonary surfactant system development (Hitchcock, 1980). Particularly, GCs not only induce Clara cell differentiation (Nord et al., 1992), but also regulate CC16 synthesis (Hagen et al., 1990; Nord et al., 1992; Berg et al., 2002; Elia et al., 2003). In addition, a 14 kDa rat lung lectin has been shown to be regulated by dexamethasone treatment (Clerch et al., 1987, 1989). However, the effect of this steroid hormone on galectin-3 expression has not yet been examined.

Given the essential role of GCs in lung Clara cell differentiation and their broad anti-inflammatory effects, the present study was conducted to investigate the effects of these steroid hormones on galectin-3 expression and subcellular distribution within lung Clara cells and macrophages. Understanding how galectins and their saccharide ligands are regulated in different tissues may contribute to the design of novel targeted anti-inflammatory and immunomodulatory strategies.

Materials and Methods

Animals

Studies were conducted on 8- to 12-week-old male Wistar rats (average weight 250 g). Animals were housed and cared for at the Animal Resource Facilities, Centro de Microscopía Electrónica, Universidad Nacional de Córdoba (Córdoba, Argentina) and the Instituto de Biología y Medicina Experimental in accordance with institutional guidelines.

Treatment

Rats were anesthetized with ketamine/xylazine (80 mg/kg weight) and subsequently submitted to bilateral trans-abdominal adrenalectomy or to sham operation (control group). Seven days after surgery, ADX rats were injected subcutaneously with either dexamethasone (2 mg/kg body weight) (DEX group) or saline solution (ADX group) once a day for 7 days. After surgery, animals were supplied with saline solution (0.9% wt/vol sodium chloride) and 1.5% (wt/vol) dextrose. Since the time that dexamethasone treatment was initiated, only saline solution was injected. Rat body weight and serum cortisol levels were registered to control efficiency of adrenalectomy. The protocols were approved by the Institutional Review Board of the Instituto de Biología y

Medicina Experimental (Buenos Aires) according to institutional and NIH guidelines.

Tissue processing

Rats were deeply anesthetized with chloral hydrate (300 mg/kg body weight) and exanguinated through a section at the inferior cava vein. Immediately, lungs were fixed by infusing a fixative solution through the tracheal duct at 30 cm water pressure. Diluted Karnovsky's mixture (1.5% (wt/vol) glutaraldehyde and 1.5% (wt/vol) formaldehyde in 0.1 M cacodylate buffer; pH 7.3) was used as fixative for electron and inmunoelectron microscopy, whereas 4% (wt/vol) formaldehyde was infused for immunohistochemistry. Fixed lungs were excised and placed overnight in fresh fixative. Blocks about 2-3 mm^3 were cut with razor blades.

Light microscope immunohistochemistry

For galectin-3 immunodetection, 5 μm thick lung paraffin sections were mounted on glass slides coated with 1% (wt/vol) polylysine, cleared with xylene, and rehydrated in decreasing concentration series of ethanol. Microwave pre-treatment (antigen retrieval method) was performed. To block endogenous peroxidase activity, slides were treated with H_2O_2 in methanol for 15 min. Sections were incubated for 30 min in 5% normal goat serum (Sigma) to block non-specific binding, followed by overnight incubation with a 1:500 dilution of a rabbit anti-galectin-3 antibody (kindly provided by Dr. Jun Hirabayashi, Ibaraki, Japan) at 4°C in a humidified chamber. Sections were then incubated with a biotinylated secondary antibody (Santa Cruz Biotechnology) and ABC complex (Vector Laboratories, Burlingame, CA). Diaminobenzidine (DAB; Sigma) was used as the chromogenic substrate for 10 min at RT and sections were rinsed in running water and counterstained with Harris hematoxylin.

Quantitative analysis of total galectin-3 was carried out in alveolar macrophages, using the Image Processing and Analysis in Java software (Image J). A total of 100 alveolar macrophages from each animal (n=3 per group) were analysed, and galectin-3 inmunolabeled area was expressed as positive area per macrophage total area ($\mu m^2/\mu m^2$).

Gold-complex preparation

Colloidal gold particles of 16 nm average diameter were prepared according to Frens (1973) using sodium citrate as a reducing agent. Gold particles were adsorbed to an IgG fraction purified from a goat antiserum raised against rabbit IgG (Sigma Chemical Co. St. Louis, MO, US). About 0.25 μg of protein was necessary to stabilize 1 μl of colloidal gold solution. Finally, the gold complex was centrifuged at 60,000xg for 2 h and the cell pellet was resuspended in PBS containing 0.01% (w/v) polyethylene glycol (PEG).

Electron microscopy

Blocks were treated with 1% osmium tetroxide for 2 h, dehydrated in graded concentrations of acetone and embedded in Araldite. Areas containing bronchioli were localized in semithin sections stained with 1% toluidine blue-1% borax solution. Thin sections were cut with a JEOL JUM-7 ultramicrotome and mounted onto copper grids, stained by uranyl acetate/ lead citrate technique and examined in a Siemens Elmiskop 101 or Zeiss 109 electron microscope.

For inmunoelectron microscopy (IMET), osmium fixation was omitted and lung blocks were dehydrated, embedded in LR White (London Resin Corporation, Berkshire, England) and polymerized at 50°C. Thin sections of ~60 nm thick were mounted on nickel grids and incubated overnight with a goat anti-galectin-3 antibody diluted 1:300 (kindly provided by Dr. F.T. Liu, University of California, Davis). Sections were further incubated with an anti-goat IgG-gold complex diluted 1:25 (25232, Electron Microscopy Sciences, Hatfield, USA) to detect specific galectin-3 staining. To assess the specificity of the primary antibody, additional slides were incubated with a normal goat IgG fraction (sc-2028 Santa Cruz, USA) diluted essentially as the primary antiserum. As an additional control, primary antibody was replaced with PBS-BSA in order to determine non-specific binding of the secondary antibody.

Quantitative analysis of immunoelectron microscopy was performed with Image J software on photographs of nucleus and cytoplasm of alveolar macrophages, ciliated cells and Clara cells obtained at a 10,000 x magnification. For each cell type, a total of 10 micrographs were taken from each compartment, from 3 animals per group. The number of gold particles (N) was expressed relative to μm^2 of nuclear or cytoplasmic area.

Purification of peritoneal macrophages and immunofluorescence microscopy

Peritoneal cells (PCs) from control, ADX and DEX rats were harvested using sterile PBS containing 5 U heparin and 20 μg/ml gentamicin (PBS-HG). Briefly, following sacrifice, rats were injected with 10 ml of ice-cold PBS-HG each and the anterior and lateral walls of the abdomen were gently massaged. The peritoneal cavity was opened and peritoneal washes were collected in ice-cold Petri dishes. Cells were centrifuged at 850xg for 10 min, and resuspended in RPMI 1640 with 20 μg/ml gentamicin, $5x10^{-5}$ M ß-mercaptoethanol and 10% heat-inactivated fetal calf serum (complete RPMI). The macrophage population was purified from PCs ($1x10^6$ cells) by adherent culture on coverslips. After a 3 h-incubation period at 37°C, non-adherent cells were removed. The resultant macrophage monolayer was 98% pure according to morphologic criteria. Coverslips were fixed in 4% (wt/vol) formaldehyde/sucrose in PBS for

15 min at room temperature. After three washes in PBS, coverslips were incubated for 60 min in 5% normal goat serum, 0.1% (wt/vol) Triton X-100 in PBS to block nonspecific binding, followed by overnight incubation with a 1:200 dilution of a mouse anti-galectin-3 antibody (kindly provided by Dr. F.T. Liu, University of California, Davis) in PBS 1% (wt/vol) BSA, 0.05% (vol/vol) Triton X-100 at 4°C in a humidified chamber. Cells were then incubated with a goat anti mouse-FITC-conjugated secondary antibody (BD Bioscience, California, USA) diluted 1:500 in PBS 1% BSA (wt/vol) for 2 h at room temperature in the dark. Cells were covered with Fluormount-G® (Southern Biotech, Birmingham, AL, USA) Antifade Reagent and coverslips were mounted on slides. Propidium iodide was used for staining nuclei. Specimens were examined in a Nikon E-800 confocal microscopy with Plan Apo 1.4 NA objective.

Purification and in vitro treatment of peritoneal macrophages

Untreated rats were injected intraperitoneally with 3 ml 3% (wt/vol) thioglycolate broth. One week later, peritoneal cells (PCs) were harvested as explained above. Macrophage population was purified from PCs ($1x10^6$ cells) by adherent culture in 100-mm-diameter tissue culture dishes. After a 2 h-incubation period at 37°C, nonadherent cells were removed, and monolayers of adherent cells were incubated overnight in the same medium. The resultant macrophage monolayer was 98% pure according to morphologic criteria. Macrophage populations were incubated for 24 h with increasing concentrations (10^{-9} M to 10^{-7} M) of dexamethasone (Sigma Aldrich) in complete RPMI medium.

Immunoblot analysis

Total cell lysates of the lungs and macrophages were prepared as described (Daroqui et al., 2007). Equal amounts of protein (15 μg/well) were resolved by 15% SDS-PAGE, blotted onto nitrocellulose membranes (Amersham) and probed with a goat anti-galectin-3 antibody as described (Acosta-Rodriguez et al., 2004). Briefly, blots were blocked for 30 min with PBS containing 0.05 % (vol/vol) Tween 20 (PBS-T) and 5 % (wt/vol) nonfat dry milk, and incubated for 1 h with a 1:3000 dilution of an anti-galectin-3 antibody in PBS-T and 1 % (wt/vol) nonfat dry milk. After three washes with PBS-T, blots were incubated for 1 h with peroxidase-labeled anti-goat antibody (Vector). Bound antibodies were detected after washing with PBS-T using enhanced chemoluminiscence (Amersham). Films were analyzed with Scion Image software.

Results

To evaluate the effects of endogenous GCs on galectin-3 expression in the lung, we first analyzed the morphological and ultrastructural changes that follow adrenalectomy in rats. Profound changes were observed by electron microscopy in the morphology of Clara cells and macrophages following adrenalectomy. The loss of secretory granules and the reduction of the apical cytoplasm (Fig. 1B) clearly contrasted with the classical morphology of Clara cells, which typically exhibit a dome-shaped apical cytoplasm that projects towards the bronchiolar space (Fig. 1A). Control alveolar macrophages are polyhedral cells with a central small nucleus and wide cytoplasm, and exhibit frequent projections of the plasma membrane (Fig. 1C). However, following corticosteroid deprivation, macrophages became round-shaped and exhibited scarce superficial projections (Fig. 1D).

In vivo expression of galectin-3 was evaluated by immunohistochemistry using light microscopy of paraffin-embedded lungs. Clara cells from control animals showed faint specific labeling at the apical cytoplasm and the plasma membrane. Ciliated cells which intermingled with non-ciliated Clara cells presented strong immunoreactivity (Fig. 2A). After adrenalectomy, specific galectin-3 immunostaining was scarcely observed in bronchiolar Clara cells, while expression of this glycan-binding protein was preserved in ciliated cells (Fig. 2B), suggesting cell-type specific regulation of galectin-3 by GCs. Remarkably, galectin-3 expression was recovered following daily treatment of adrenalectomised rats with dexamethasone (2 mg/kg body weight) for 7 days, and intense staining could be observed in the apical cytoplasm of some Clara cells (Fig. 2C). Control alveolar macrophages exhibited strong labeling delineating the plasma membrane, but modest and homogeneous immunolabeling within the cells (Fig. 3A). Similar to Clara cells, a reduction in cytoplasmic labeling, which contributed to emphasize nuclear labeling, could be observed after adrenalectomy (Fig 3B), which was confirmed by analysis of positive areas (Fig. 3D). Following treatment with dexamethasone, alveolar macrophages also recovered galectin-3 expression, which was mainly localized at the level of secretory granules (Fig. 3C,D).

The impact and specificity of adrenalectomy on galectin-3 levels were further analyzed by electron microscopy following immunogold labeling. In control animals, Clara and ciliated cells exhibited gold staining localized in the nucleus and spread in the cytoplasm (Fig. 4A,B). Notably, cilia were strongly labeled (Fig. 4B). After GCs deprivation, non-ciliated Clara cells showed a significant reduction in cytoplasmic labeling ($p<0.001$), while no changes were observed in the nucleus (Fig. 4C, Table 1). Meanwhile, both the cytoplasm (Fig. 4C) and nucleus (data not shown) of ciliated cells exhibited intense labeling, similar to that observed in controls (Table 1). Remarkably, upon dexamethasone treatment, a considerable increase in cytoplasmic and nuclear galectin-3 staining was observed in Clara cells ($p<0.05$) (Fig. 4D and Table 1).

Control rat alveolar macrophages also showed

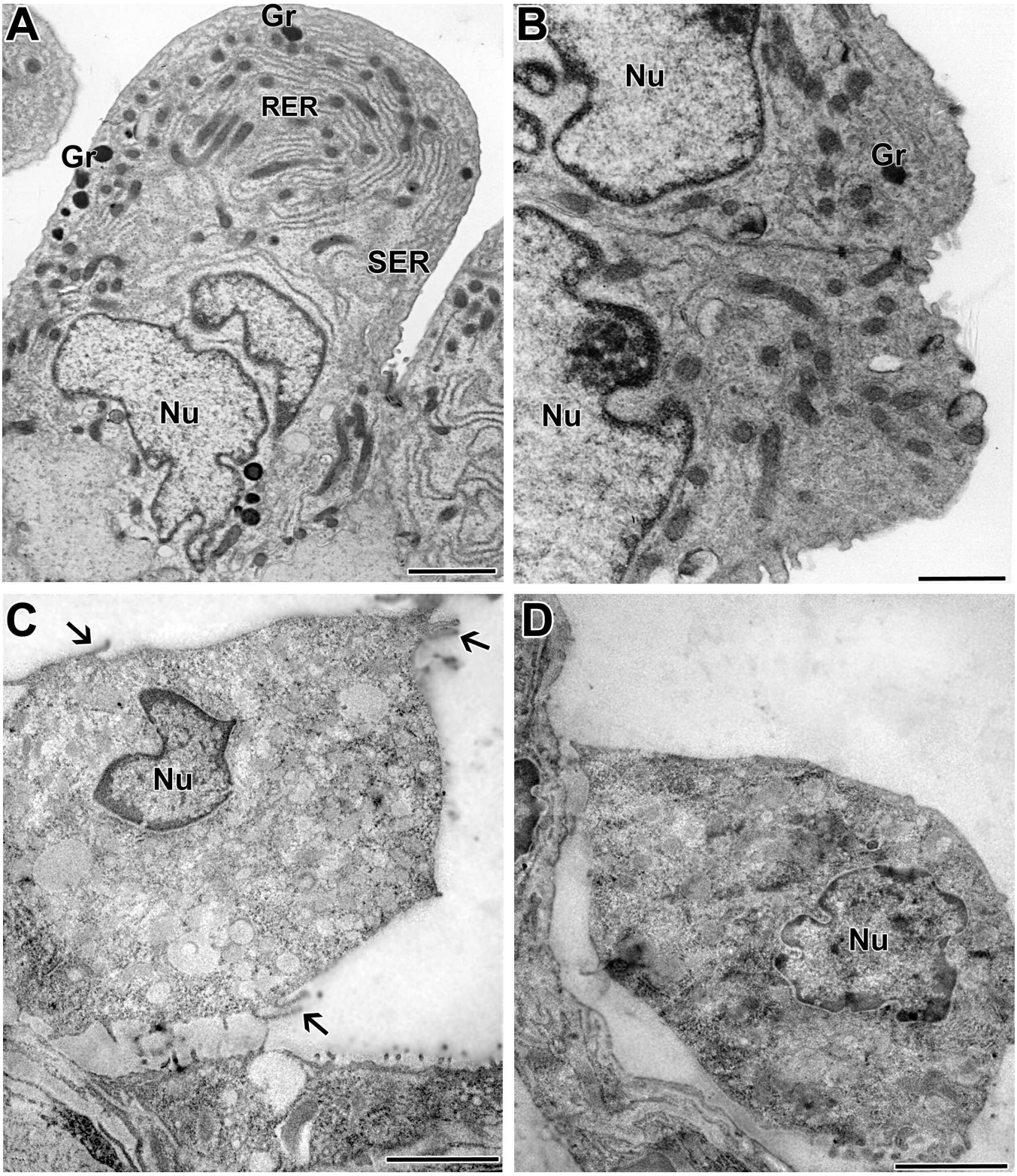

Fig. 1. Electron microscopy of ultrastructural alterations in lung bronchiolar Clara cells and alveolar macrophages following GC deprivation. **A.** An apical cupule with projections towards the bronchiolar space is observed in Clara cells from control animals. **B.** After adrenalectomy, Clara cells lose the smooth and rough endoplasmic reticulum (SER and RER) and secretory granules (Gr), and exhibit reduced apical cytoplasm. **C.** Control alveolar macrophages are poliedric cells and exhibit frequent projections at the plasma membrane. **D.** Following GC deprivation, macrophages present a higher nuclear/cytoplasm relationship, and lose their characteristic cytoplasmic projections at the plasma membrane (Arrows). Nu: nucleus. Bars: 2 μm.

galectin-3 positive staining, both in the nucleus and the cytoplasm (Fig. 5A). As shown by analysis of immunogold labeling, only cytoplasmic staining was affected by adrenalectomy (Fig. 5B and Table 1). After treatment with dexamethasone, a significant increase in galectin-3 immunoreactivity was observed in the cytoplasm and the nucleus of these cells ($p<0.001$) (Fig. 5C and Table 1) with considerable labeling at the levels of cytoplasmic projections and plasma membrane (Fig. 5D,E).

To confirm these findings, expression of galectin-3 in the lung of control and ADX rats was also examined by immunoblot analysis. A clear decrease in galectin-3 expression could be observed in total lung lysates from ADX animals. Moreover, following 7-day treatment with dexamethasone, galectin-3 expression was significantly restored (Fig. 6).

In addition, the impact of adrenalectomy on galectin-

Table 1. Gold particles number/area (N/μm^2) in lungs inmunolabelled for galectin-3 by immunoelectron microscopy.

	Macrophages		Clara cells		Ciliated cells	
	Nucleus	Citoplasm	Nucleus	Citoplasm	Nucleus	Citoplasm
Control	12.85±2.94	6.92±0.85	6.11±1.3	3.87±0.51	10.5±2.59	5.82±0.39
ADX	14.08±0.58	4.26±0.26**	5.83±0.62	1.94±0.19**	11.61±1.38	4.49±0.23
ADX-GLU	24.66±2.94**	9.36±0.62**	8.21±0.69*	5.07±0.20*	14.33±0.25	7.09±0.59*

**: P<0.001 vs Control; *: P<0.05 vs Control

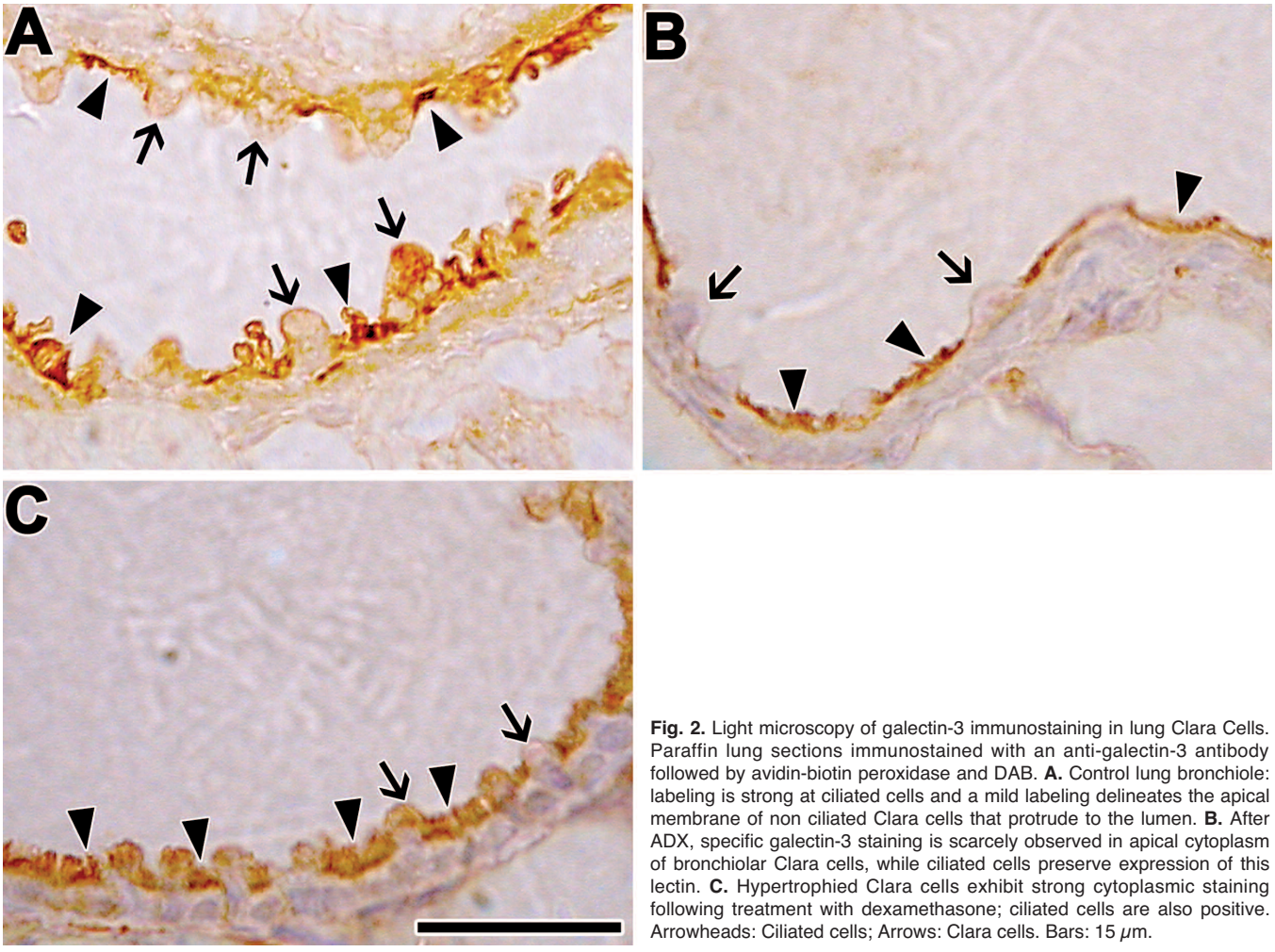

Fig. 2. Light microscopy of galectin-3 immunostaining in lung Clara Cells. Paraffin lung sections immunostained with an anti-galectin-3 antibody followed by avidin-biotin peroxidase and DAB. **A.** Control lung bronchiole: labeling is strong at ciliated cells and a mild labeling delineates the apical membrane of non ciliated Clara cells that protrude to the lumen. **B.** After ADX, specific galectin-3 staining is scarcely observed in apical cytoplasm of bronchiolar Clara cells, while ciliated cells preserve expression of this lectin. **C.** Hypertrophied Clara cells exhibit strong cytoplasmic staining following treatment with dexamethasone; ciliated cells are also positive. Arrowheads: Ciliated cells; Arrows: Clara cells. Bars: 15 μm.

3 expression was also analyzed in peritoneal macrophages. By immunofluorescence staining, rat peritoneal macrophages from control animals showed intense galectin-3 expression, mostly localized in the cytoplasmic compartment (Fig. 7A), which was significantly reduced after GC deprivation (Fig. 7B). GC-mediated regulation of galectin-3 expression was further confirmed by immunoblot analysis of peritoneal macrophages treated *in vitro* with dexamethasone. Constitutive expression of galectin-3 was detected in peritoneal macrophages, which increased in a dose-dependent manner following exposure *in vitro* to different concentrations of dexamethasone (Fig. 8). Collectively, these data demonstrate that GCs, either endogenously-regulated or exogenously-added, can control galectin-3 expression in lung Clara cells and macrophages.

Discussion

Galectin-3 is one of the most abundant lectins expressed in highly specialized epithelia, including lung airway epithelium, where it may play potential roles in preserving mucosal integrity from environmental antigens (Kasper and Hughes, 1996; Kim et al, 2007; Yang et al., 2008). First described in Clara cells by Wasano and Yamamoto (1989) in normal rat lung, galectin-3 is also expressed in alveolar macrophages and bronchial epithelial cells (Kasper and Hughes, 1996). In spite of the established roles of GCs in lung maturation and Clara cell differentiation, the impact of these steroid hormones on the expression of galectins, which are critical regulators of the inflammatory response, has not yet been investigated.

In the present work we showed that galectin-3 synthesis is strongly down-regulated in bronchiolar Clara cells and macrophages following GC deprivation. Although at a glance the effects on macrophages might be less evident than in Clara cells, quantitative analysis of immunolabeled galectin-3 either at light or electron microscopy confirmed these findings. Interestingly, this effect was cell-type specific, as expression of this glycan-binding protein was preserved in ciliated cells even after adrenalectomy. In addition, as shown by

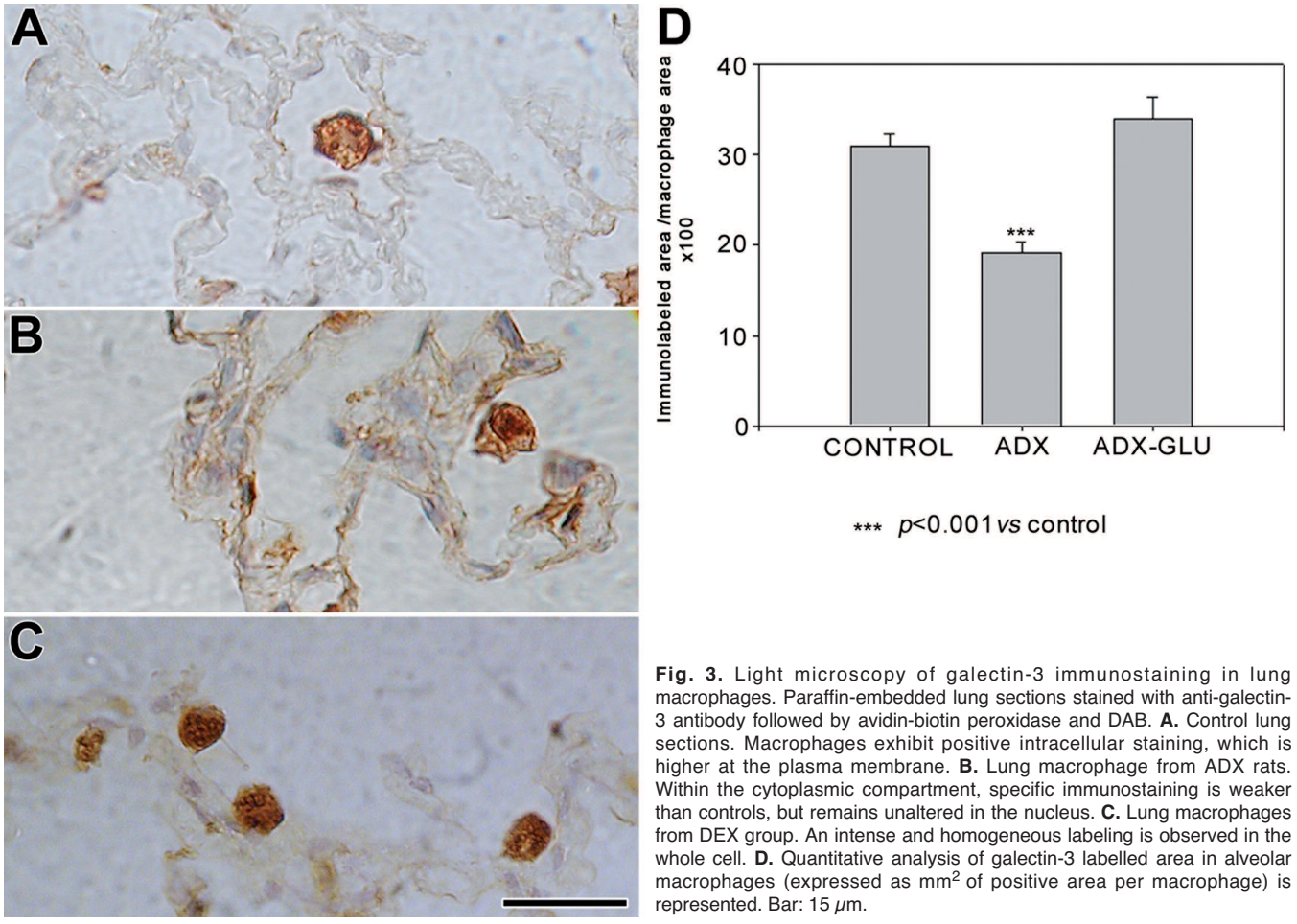

Fig. 3. Light microscopy of galectin-3 immunostaining in lung macrophages. Paraffin-embedded lung sections stained with anti-galectin-3 antibody followed by avidin-biotin peroxidase and DAB. **A.** Control lung sections. Macrophages exhibit positive intracellular staining, which is higher at the plasma membrane. **B.** Lung macrophage from ADX rats. Within the cytoplasmic compartment, specific immunostaining is weaker than controls, but remains unaltered in the nucleus. **C.** Lung macrophages from DEX group. An intense and homogeneous labeling is observed in the whole cell. **D.** Quantitative analysis of galectin-3 labelled area in alveolar macrophages (expressed as mm^2 of positive area per macrophage) is represented. Bar: 15 μm.

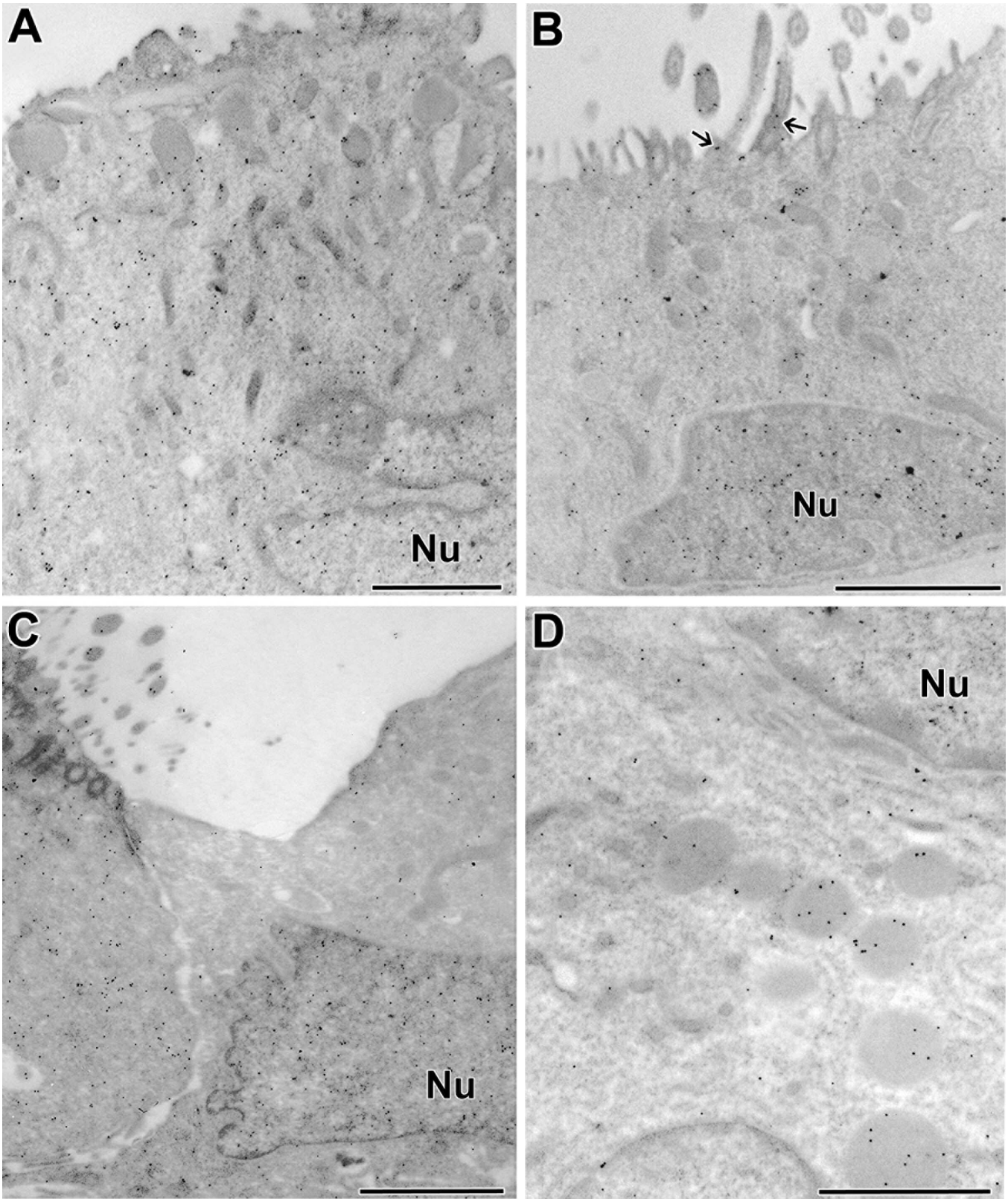

Fig. 4. Electron microscopy of galectin-3 immunogold labeling in bronchiolar epithelium. LR White embedded lung sections stained with anti-galectin-3 antibody followed by IgG-gold complex. **A.** In control Clara cells, galectin-3 is detected free in the cytoplasm, and associated with eucromatin structures in the nucleus. **B.** The numerous cilias (arrows) at the apical face of control ciliated cells exhibit intense gold labeling, in addition to the cytoplasm and nucleus. **C.** Non-ciliated Clara cells of ADX animals show gold labeling mainly restricted to the nucleus (Nu), specifically localized in eucromatin structures, whereas their cytoplasmic labelling is very faint. In contrast, both cytoplasm and cilias of ciliated cells conserve intense labeling. **D.** After dexamethasone treatment, galectin-3 is detected in the cytoplasm, either free or associated with numerous secretory granules (Gr), as well as in the nucleus of Clara cells. Bars: 2 μm.

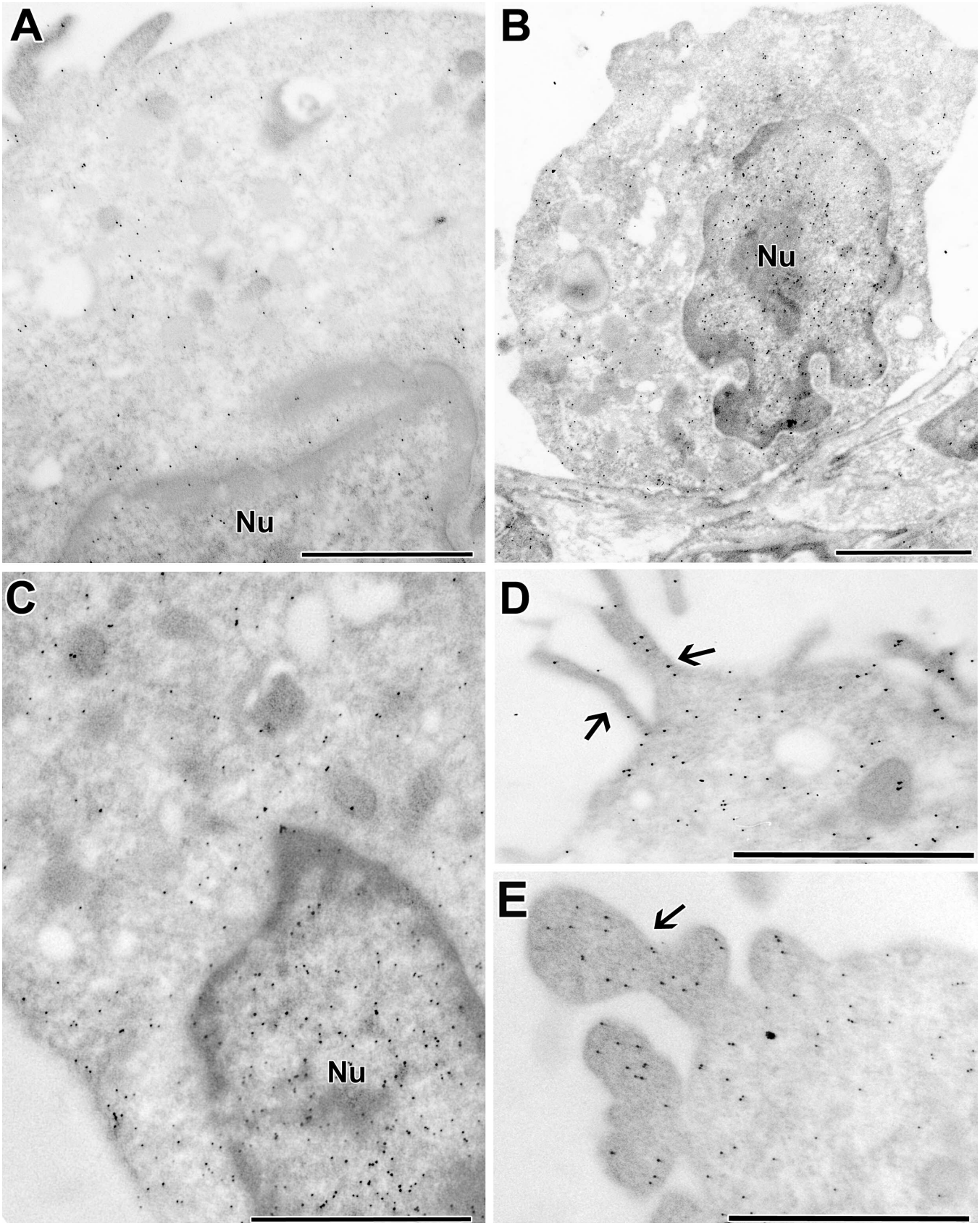

Fig. 5. Electron microscopy of galectin-3 staining in alveolar macrophages. **A.** In control alveolar macrophages, galectin-3 positivity is detected both in the nucleus and the cytoplasm. **B.** Following adrenalectomy, cytoplasmic labelling is almost undetectable while nuclear staining is intense and localized within the euchromatin. **C.** Dexamethasone treatment intensifies cytoplasmic and nuclear localization of galectin-3. **D and E.** Numerous gold particles are observed at the cytoplasmatic proyections (arrows) of macrophage surface. Nu: nucleus. Bars: 2 μm.

immunoelectron microscopy and its quantitative analysis, specific cytoplasmic staining was markedly reduced in Clara cells and macrophages, while their nuclear labeling was mostly preserved, suggesting that GC effects are not only cell-type specific, but also target particular subcellular compartments. Galectin-3 has been proposed to shuttle between the cytoplasm and nucleus (Wang et al, 2004). Depending on the cell type, specific experimental conditions or tissue distribution, galectin-3 was reported to be exclusively cytoplasmic, predominantly nuclear, or distributed between the two compartments (Haudek et al., 2009). This intracellular mechanism of nucleo-cytoplasmic shuttling may contribute to the differences observed in Clara cells and macrophages upon GC treatment.

Remarkably, specific galectin-3 staining was restored following dexamethasone treatment and this effect was confirmed *in vitro* upon culture of purified macrophages in the presence of this anti-inflammatory agent. Although other neuroendocrine signals might also contribute to regulation of galectin-3 expression, recovery of its expression following dexamethasone treatment clearly implies that GCs, the major effectors of hypothalamic-pituitary-adrenal (HPA) axis, are critical mediators of the observed effect. On the other hand, as confirmed by quantitative analysis of gold particles, while adrenalectomy affected almost exclusively the cytoplasmic content of Clara cells and macrophages, dexamethasone treatment led to an increase of galectin-3 staining in both compartments. This observation may be associated with the frequently described supra-physiological effects of dexamethasone, a synthetic GC, which are much more pronounced than those observed for its endogenous counterpart. These differences could also account for the weak increase in cytoplasmic galectin-3 following dexamethasone treatment in ciliated cells, which were not affected by GC deprivation.

Interestingly, other proteins essential for Clara cell biology have also been demonstrated to be a target of GCs modulation. In the rat lung, expression of CC16 mRNA is regulated by these steroid hormones (Hagen et al, 1990) and GC deprivation due to adrenalectomy induces a strong reduction in CC16 synthesis (Elia et al, 2003). Remarkably, stimulation of the pulmonary surfactant system and expression of the surfactant-associated proteins also depend on the action of GCs (Ballard et al., 1996; Grier and Halliday., 2004).

The profound morphological and ultrastructural changes observed in Clara cell and macrophage morphology following adrenalectomy may suggest that constitutive expression of galectin-3 sustained by GCs

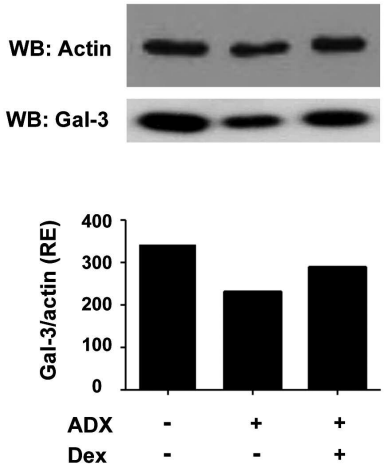

Fig. 6. Western blot analysis of galectin-3 (Gal-3) expression in rat lung homogenates. The expression of Gal-3 (lower blot) compared to ß-actin expression (upper blot) in total lung homogenates obtained from control or ADX rats before and after a 7-day treatment with dexamethasone (DEX). A densitometric analysis is shown. RE: relative expression. Results are representative of three independent experiments with n=3 rats per group.

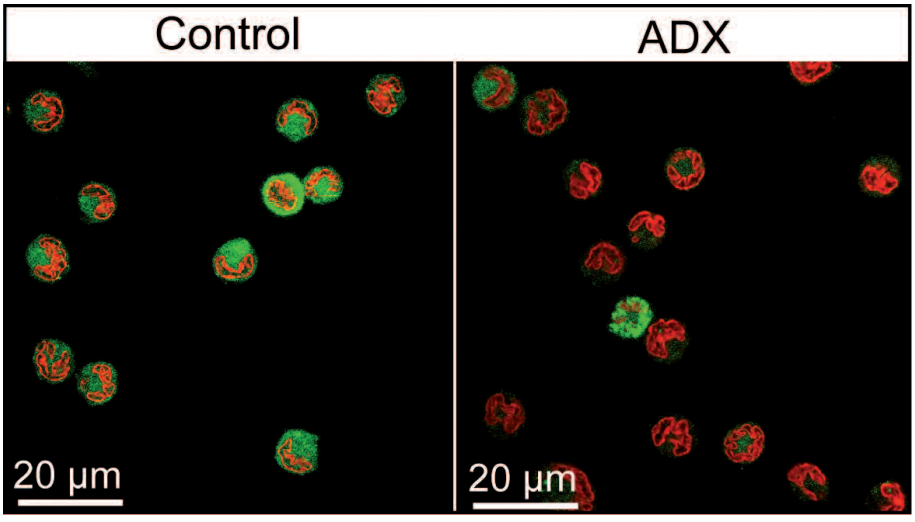

Fig. 7. Impact of GC deprivation on galectin-3 expression in peritoneal rat macrophages. Immunofluorescence and laser confocal microscopy of peritoneal macrophages stained with anti-galectin-3 antibody and propidium iodide. **A.** Rat peritoneal macrophages from control animals show intense galectin-3 positive staining, mostly in the cytoplasm. **B.** Following ADX, galectin-3 positive staining is significantly reduced. Results are representative of three independent experiments with n=3 rats per group.

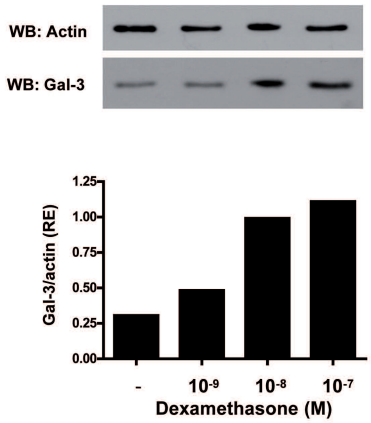

Fig. 8. Western blot analysis of galectin-3 (Gal-3) expression in rat peritoneal macrophages. The expression of Gal-3 (lower blot) compared to ß-actin expression (upper blot) in total cell lysates of peritoneal macrophages from control rats following *in vitro* treatment with increasing concentrations of dexamethasone (DEX). RE: relative expression. Results are representative of three independent experiments with n=3 rats per group.

could be associated with cell differentiation programs. In the present study not only bronchiolar, but also extra-pulmonary macrophages, were shown to be responsive to GCs. Indeed, GC-dependent expression of galectin-3 was also evident in peritoneal macrophages, which showed a dose-dependent up-regulation of galectin-3 expression following exposure to dexamethasone *in vitro*. Our findings are in accordance with those reported by Dabelic and colleagues (2006), who demonstrated *in vitro* that macrophage-like cells exposed to clinically relevant doses of immunomodulatory drugs modulate galectin-3 synthesis both at mRNA and protein levels.

As mentioned above, galectin-3 modulates a variety of cellular processes critical to immunological homeostasis. In different animal models of lung inflammation, a significantly increased secretion of galectin-3 was detected in the alveolar space, mainly in resident alveolar macrophages, but also in alveolar parenchyma cells (Kasper and Hughes., 1996; Sato et al., 2002; Zuberi et al., 2004; Andre et al., 2006) and in bronchoalveolar lavage fluid (Sato et al., 2002; Zuberi et al., 2004). Interestingly, galectin-3 expression is also increased in distal airways of patients with severe chronic obstructive pulmonary disease (Pilette et al., 2007).

Clara cells have been identified as central players in protecting the airway from environmental insults. Their diverse functions in lung homeostasis include roles in xenobiotic metabolism, immune system regulation and progenitor cell activity (Reynolds and Malkinson, 2010). Although localized early in Clara cells (Wasano and Yamamoto, 1989), galectin-3 has never been specifically associated with the immunomodulatory functions of these cells. Our results suggest that galectin-3 might contribute to the immunoregulatory activity of Clara cells during lung inflammation, through its capacity to modulate immune cell survival, chemotaxis, migration and cytokine synthesis. Moreover, considering the role of Clara cells as progenitors for the repair of bronchial epithelium after injury (Evans et al., 1978; Massaro et al., 1994), and given the anti-apoptotic effects of intracellular galectin-3 (Yang et al., 1996; Akahani et al, 1997), it is possible to speculate that galectin-3 might also contribute to GC-dependent mechanisms of epithelial cell repair through modulation of cell growth, survival, angiogenesis and proliferation (Yang et al, 2008). Moreover, the differential regulation of cytoplasmic and nuclear galectin-3 described here might be in accordance with the different functions displayed by this multifunctional protein, which depend on its subcellular distribution. While nuclear galectin-3 has been implicated in spliceosome assembly and regulation of gene transcription, cytosolic galectin-3 has been involved in the regulation of cell proliferation, differentiation and survival (Dumic et al., 2006).

Given the critical role of galectin-3 in phagocytic clearance of microorganisms and apoptotic cells (Sano et al., 2003), GC-dependent regulation of this lectin in cells of the monocyte/ macrophage lineage may contribute to control phagocytosis, thereby influencing the resolution of inflammation and suggesting an essential role of galectin-3-glycan lattices in the anti-inflammatory effects of GCs (Sternberg, 2006). Thus, galectin-3 may represent an alternative mediator by which GCs regulate early and late inflammatory responses, as well as the differentiation of Clara cell phenotype, which are key events in lung homeostasis. In this sense, a tightly regulated equilibrium of anti-inflammatory and pro-inflammatory mediators may control the response of Clara cells to pathogen invasion, tissue injury and toxic exposure.

It is well-established that interruption of the HPA axis, through surgical or pharmacological intervention, renders otherwise resistant animal models highly susceptible to inflammation, thus indicating the critical role of GCs in modulating innate and acquired immune function. Regulation of galectin-3 in the lungs may represent one of the multiple potential mechanisms by which GCs contribute to modulate innate and adaptive immune responses.

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