

ARTICLE

Glucocorticoids decrease the numbers and activation of mast cells by inducing the transactivation receptors of AGEs

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Abstract

Glucocorticoids (GCs) are potent anti-allergic compounds that function, at least in part, by inhibiting signaling pathways in mast cells. We hypothesized that the GC-induced mastocytopenia and suppression of mast cell activation are mediated by the advanced glycation end products (AGEs)/receptors of AGEs (RAGEs) signaling axis. We evaluated the role of AGEs in GC-mediated mastocytopenia and impaired mast cell degranulation in male Wistar rats and Swiss-Webster mice subcutaneously injected with dexamethasone or prednisolone (0.1 mg/kg) once a day for 21 consecutive days. The animals were treated with either the AGE inhibitor aminoguanidine (250 mg/kg), the RAGE antagonist FPS-ZM1 (1 mg/kg) or the galectin-3 antagonist GSC-100 (1 mg/kg) daily for 18 days, starting 3 days following GC treatment. Aminoguanidine inhibited GC-induced mast cell apoptosis and restored mast cell numbers in the pleural cavity of GC-treated rats. Aminoguanidine also reversed the GC-induced reduction in histamine release triggered by allergens or compound 48/80 in vitro. GC treatment induced RAGE and galectin expression in mast cells, and blocking these agents by FPS-ZM1 or GSC-100 significantly reversed mast cell numbers in the peritoneal cavity and mesenteric tissue of GC-treated mice. In addition, the combination of GC and AGE-induced mast cell apoptosis in vitro was inhibited by both FPS-ZM1 and GSC-100. We concluded that the GC-induced mastocytopenia and suppression of mast cell stimulation are associated with the gene transactivation of RAGE and galectin-3.

1 | INTRODUCTION

Mast cells are innate immune cells that play critical roles in homeostasis and the pathophysiology of various diseases, including allergies and anaphylaxis.¹ The activation of mast cells through high-affinity receptor for the Fc region of immunoglobulin E (IgE; FcεRI) following exposure to polyvalent Ags, is the major driver of allergic dysfunctions.² The allergen-induced activation of mast cells leads to the rapid release of a plethora of chemical mediators, including cytokines (TNF-α), biogenic amines (histamine), serglycin, proteoglycans, and mast cell-derived proteases (chymase and tryptase), stored in cytosolic granules.³

Glucocorticoids (GCs) are the most effective anti-allergic drugs currently used to improve the protracted symptoms or prevent the recurrence of episodes in allergic patients. In general, GCs are associated with the suppression of late-phase reactions, where the

major effector cells are T-lymphocytes and/or eosinophils.⁴ However, these drugs can also ameliorate the acute-phase reaction of allergic diseases, in which mast cells are highlighted as the master cells implicated in the pathogenesis of disease.¹ Notably, GCs can impact mast cell function and survival in several aspects, for example, by suppressing mast cell maturation, degranulation, and FcεRI expression,^{5–7} or by inducing apoptosis.⁸ However, GC therapy is often associated with undesired adverse effects, such as immunosuppression, loss of skeletal muscle mass, etc., particularly when used at high doses.⁹ GCs exert their effects primarily through the activation of genomic mechanisms, mediated by their intracellular receptors. During the activation of glucocorticoid receptor (GR) by GCs, the GC-GR complex forms and subsequently migrates to the cell nucleus to promote the transrepression of proinflammatory genes and transactivation of anti-inflammatory genes.¹⁰ GCs may also induce the transactivation and consequent expression of genes encoding receptors of advanced glycation end products (AGEs), such as receptors of AGE (RAGE) and galectin-3.^{11–14}

Abbreviations: Ag, antigen; AGEs, advanced glycation end products; FcεRI, high-affinity receptor for the Fc region of immunoglobulin E; GCs, glucocorticoids; GR, glucocorticoid receptor; β-hex, β-hexosaminidase, β-hex; IgE, Immunoglobulin E; RAGE, receptor of AGE

AGEs are products of the nonenzymatic glycation and oxidation of proteins and lipids, which appear up-regulated under conditions of hyperglycemia and oxidative stress, such as diabetes.¹⁵ AGEs also activate RAGE and galectin-3 present on the plasma membrane of several inflammatory cells. In mast cells, the activation of both RAGE and galectin-3 induces mast cell apoptosis.^{16,17} Because we previously showed that mast cells from diabetic animals are hyporesponsive to allergen stimulation^{8,18} and show high levels of apoptosis in a mechanism related to hypercorticism and increase of AGEs formation,^{19,20} we hypothesized that the GC-induced decrease in the numbers and activation of mast cells depends of the AGEs/RAGEs signaling pathway. Thus, the aim of the present study was to assess the contribution of the AGEs/RAGEs axis in GC-mediated mastocytopenia and impaired mast cell degranulation in mice and rats.

2 | MATERIAL AND METHODS

2.1 | Chemicals

Dexamethasone, prednisolone, compound 48/80, o-phthalaldehyde, aminoguanidine, L-NAME, glycated albumin, HBSS, Percoll, and toluidine blue dye were purchased from Sigma Chemical Co (St. Louis, MO, USA); FPS-ZM1, murine anti-DNP IgE mAb, and DNP-BSA were purchased from Calbiochem (San Diego, CA, USA); May-Grünwald dye and Giemsa dye were purchased from Merck (Rio de Janeiro, RJ, Brazil); and sodium heparin was purchased from Roche (São Paulo, SP, Brazil). GCS-100 was kindly supplied by the La Jolla Pharmaceutical Company (San Diego, CA, USA). All solutions were freshly prepared immediately prior to use.

2.2 | Animals

Male Wistar rats (180–200 g) and Swiss-Webster mice (20–25 g) were obtained from the Oswaldo Cruz Foundation breeding colony and used in accordance with the guidelines of the Committee on The Use of Laboratory Animals of the Oswaldo Cruz Foundation (CEUA-FIOCRUZ, license LW-23/11).

2.3 | Treatments

Male Wistar rats were randomly divided into 4 groups: control ($n = 5$), treated with dexamethasone or prednisolone ($n = 5$), treated with aminoguanidine (250 mg/kg; $n = 5$), and treated with dexamethasone or prednisolone plus aminoguanidine (250 mg/kg; $n = 5$). In another set of experiments, male Wistar rats were randomly divided into 4 groups: control ($n = 5$), treated with dexamethasone ($n = 5$), treated with aminoguanidine (50 mg/kg) or L-NAME ($n = 5$), and treated with dexamethasone plus aminoguanidine (50 mg/kg) or L-NAME ($n = 5$). In a third set of experiments, male Swiss-Webster mice were randomly divided into 6 experimental groups: control ($n = 5$), treated with dexamethasone ($n = 5$), treated with GCS-100 ($n = 5$), treated with FPS-ZM1 ($n = 5$), treated with dexamethasone plus GCS-100 ($n = 5$), and treated with dexamethasone plus FPS-ZM1 ($n = 5$).

The animals were treated with dexamethasone (0.1 mg/kg, s.c.)²¹ or prednisolone (0.1 mg/kg, s.c.) once a day for 21 consecutive days. Three days after initiating steroid injections, the animals were treated daily with either aminoguanidine (AGE inhibitor; 50 or 250 mg/kg, oral route),¹⁹ L-NAME (NOS inhibitor; 30 mg/kg, i.p.),²³ GCS-100 (galectin-3 blocker; 1 mg/kg, oral route) or FPS-ZM1 (RAGE blocker; 1 mg/kg, i.p.)²² for 18 consecutive days. Untreated rats received an equal volume of vehicle (saline 0.9% or DMSO 0.05%).

2.4 | Mast cell quantification in the pleural and peritoneal fluids

Twenty-four hours after the last injection of dexamethasone or prednisolone, the animals were sacrificed in a CO₂ chamber. The thoracic cavities of the rats were opened and rinsed with 3 mL of heparinized saline (10 U/mL). The pleural fluid volume was measured in a graduated syringe. The peritoneal cavities of the mice were washed with 3 mL of heparinized saline (10 U/mL); then, the cells were centrifuged at 150 × *g* for 10 min, the supernatant was discarded, and the pellet was resuspended in 300 μ L of heparinized saline (10 U/mL). After dilution of the cell samples (90 μ L) with toluidine blue dye solution (10 μ L), mast cells were counted in a Neubauer chamber under a light microscope (Olympus BX50, Shinjuku, Tokyo, Japan) at 200× magnification. The results are expressed as total mast cells × 10³ per cavity.

2.5 | Mast cell quantification in the mesentery

Twenty-four hours after the last injection of dexamethasone, the mice were sacrificed in a CO₂ chamber. The animals were then injected (i.p.) with 5 mL of paraformaldehyde (4%) diluted in sucrose solution (4%). After 15 min, the peritoneal cavity was opened, and the mesentery was recovered and placed on glass slides to dry at room temperature. The mast cell population was identified in the mesenteric membrane after staining with Giemsa dye. The number of mast cells was counted using an optical microscope (Olympus BX50) as previously described.²³

2.6 | Mast cell purification and stimulation

Mast cells were recovered from the pleural and peritoneal cavities and purified using a continuous Percoll gradient as previously described.²³ Isolated mast cells (10⁵ cells/mL) were sensitized with IgE anti-DNP Ab (0.5 mg/mL) at 37°C for 1 h and then stimulated with Ag (DNP-BSA, 0.5 mg/mL) for 1 h at 37°C in 95% air, 5% CO₂. Alternatively, mast cells were stimulated with compound 48/80 (0.05 μ g/mL) for 1 h at 37°C in 95% air, 5% CO₂. After centrifugation at 150 × *g* for 10 min, the supernatant was collected and stored at –20°C for histamine quantification according to Shore et al.²⁴

2.7 | Evaluation of mast cell apoptosis

Total cells were recovered from the peritoneal cavity and fixed with formaldehyde (4%) for apoptosis assessment using a horseradish peroxidase-labelled streptavidin (streptavidin-HRP) TUNEL assay (Roche Applied Science, Indianapolis, IN, USA), performed according to the manufacturer's instructions as previously described.²⁰ The number

of apoptotic mast cells is expressed as a percentage of the positive mast cell count for TUNEL.

2.8 | Glucose and fructosamine quantification

Blood glycamia was determined using a glucose monitor (Johnson & Johnson, Los Angeles, CA, USA) in samples obtained from the tail vein. To evaluate plasma fructosamine levels, blood was collected from the abdominal aorta in heparinized (400 U/mL) saline and centrifuged for 10 min at $1000 \times g$, and the plasma was collected and stored at -20°C for fructosamine quantification using a commercial kit according to the manufacturer's instructions. The fructosamine kit was purchased from Labtest (Lagoa Santa, MG, Brazil).

2.9 | Determination of macro- and microscopic skeletal muscle atrophy indexes

Gastrocnemius muscles were dissected and placed in Millonig's fixative solution for 48 h. The tissues were dehydrated and cleared with xylene prior to paraffin embedding. Muscle sections were stained with hamatoxylin and eosin (H&E) and 5 different fields per animal were analyzed using a light microscope (Olympus BX50), coupled to a video camera (Olympus UC30) with a $200\times$ magnification. The mean muscle cell area was measured using Image-Pro-Plus 6.2 software (Media Cybernetics Inc., Rockville, MD, USA). To macroscopically assess muscle atrophy, the ratio between muscle weight and tibia length was determined.

2.10 | Blood leukocyte counts

Blood samples were obtained from the tail vein of rats or mice at 24 h after the last injection of dexamethasone. Total leukocyte number was determined using a Neubauer chamber and a light microscope after the dilution of the blood samples with 2% acetic acid solution, and the differential analysis was performed on blood smears stained with May-Grünwald-Giemsa dye.

2.11 | Western blot

Isolated mast cells (10^6 cells) and gastrocnemius muscles were obtained to evaluate the expression of Bax, galectin-3, and RAGE. All samples were homogenized in cold lysis buffer containing the Complete protease inhibitor cocktail (F. Hoffmann-La Roche Ltd., Basel, Switzerland) and 0.1% Triton X-100 in PBS. The lysate was centrifuged at $13,000 \times g$ for 10 min at 4°C . The supernatant was recovered and the proteins were quantified using a BCA assay according to the manufacturer's instructions (Sigma-Aldrich Corp., St. Louis, Missouri, USA). Equal amounts of protein ($30 \mu\text{g}/\text{lane}$) were loaded and separated on a 12.5% SDS-PAGE gel and transferred to nitrocellulose membranes (GE Healthcare, Little Chalfont, UK). After blocking in 5% (w/v) skimmed milk powder in TBS-T for 1 h, the membranes were exposed overnight at 4°C to either Bax, galectin-3- and RAGE-specific goat or rabbit polyclonal Abs (1:500; Santa Cruz Biotechnology, Dallas, Texas, USA), or a β -actin- or GAPDH-specific mouse or goat monoclonal Ab (1:1000; Santa Cruz Biotechnology), respectively, followed by 3 washes and

incubation with IRDye goat anti-rabbit secondary Ab or IRDye goat anti-mouse secondary Ab (both at 1:10 000; LI-COR Corporate) for 1 h at room temperature. The membranes were washed in TBS-T, and protein expression was detected using fluorescence using the Odyssey Image System (LI-COR Corporate, Lincoln, NE, USA). The band intensity was quantified through densitometry using Image-Pro Plus 6.2 software (Media Cybernetics Inc., Bethesda, MA, USA).

2.12 | Cell culture

We used RBL-2H3 cell line, which is a basophilic leukemia cell line isolated from Wistar rat basophilic cells maintained as tumors. Although these cells are derived from basophils, RBL-2H3 cell line is usually used in research articles as a mast cell line. Cells were used until the 20th passage. RBL-2H3 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 15% FBS and 2 mM L-glutamine and were grown at 37°C in a humidified 95% air, 5% CO_2 .

2.13 | RBL-2H3 cells degranulation assay in vitro

RBL-2H3 cells (1.25×10^5 cells/well) were pretreated with dexamethasone ($0.01 \mu\text{M}$) for 24 h and then stimulated with glycated albumin (0.03 mg/mL) for 1 h at 37°C in 95% air, 5% CO_2 . As positive control, RBL-2H3 cells were sensitized with IgE anti-DNP Ab ($1 \mu\text{g/mL}$) at 37°C for 20 h and then stimulated with Ag (DNP-BSA, 10 ng/mL) for 45 min at 37°C in 95% air, 5% CO_2 . After centrifugation at $150 \times g$ for 10 min, the supernatant was collected and stored at -20°C for β -hexosaminidase (β -hex) quantification according to Han et al.²⁵

2.14 | RBL-2H3 cells apoptosis assay in vitro

RBL-2H3 cells (1.5×10^5 cells/well) were pretreated with dexamethasone ($0.01 \mu\text{M}$) for 24 h and then stimulated with glycated albumin (0.03 mg/mL) for 24 h in the presence or absence of dexamethasone. In some experiments, FPS-ZM1 (0.1 ; 0.3 ; $1 \mu\text{M}$) or GSC-100 (0.1 ; 1 ; $10 \mu\text{g/mL}$) were coincubated with glycated albumin. Apoptosis was analyzed by permeabilizing and staining cells with propidium iodide as previously described.²⁶ Apoptosis values were determined by calculating the percentage of cells in phase Sub- G_0 of the cell cycle using CellQuest software (BD Bioscience Pharmingen, San Jose, CA, USA).

2.15 | Statistical analysis

Data are reported as the means $\pm$ SD. All data were evaluated to ensure normal distribution and statistically analyzed using one-way ANOVA, followed by a Student-Newman-Keuls post hoc test. Probability values (P) of 0.05 or less were considered significant.

3 | RESULTS

3.1 | GC-induced refractoriness and apoptosis of mast cells depend on AGEs

The treatment of Wistar rats with exogenous GCs, dexamethasone, or prednisolone (Fig. 1A and B, respectively) for 18 consecutive days

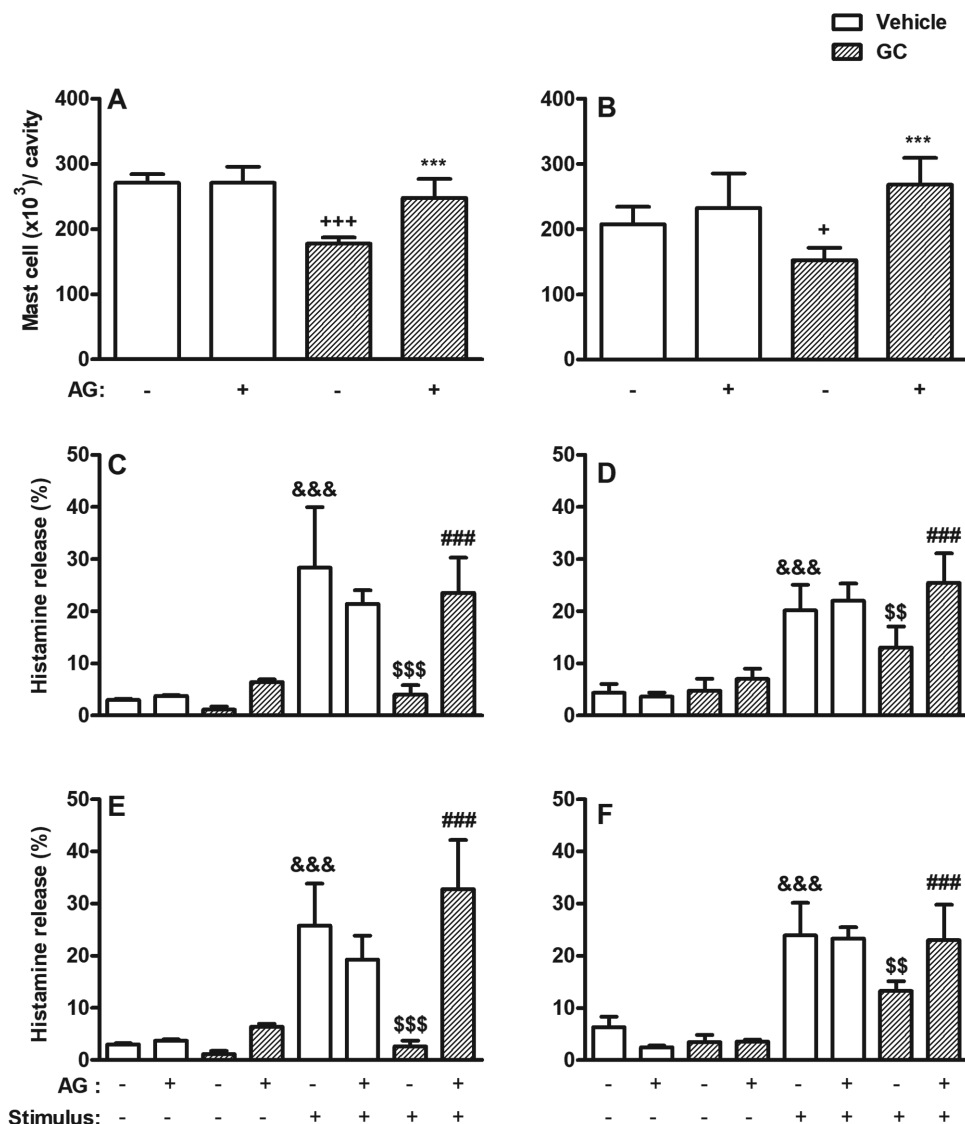


FIGURE 1 Aminoguanidine restores the reduction of mast cell numbers (A and B) and reactivity (C, D, E, and F) induced by GCs in Wistar rats. Dexamethasone (A, C, and E; 0.1 mg/kg) or prednisolone (B, D, and F; 0.1 mg/kg) was subcutaneously injected, once a day during 18 consecutive days. Treatment with aminoguanidine (250 mg/kg, oral route) was performed daily for 15 days, starting 3 days after initiating GC injections. A group of rats was treated daily with aminoguanidine for 15 consecutive days. Untreated rats received an equal amount of vehicle (saline 0.9%). Isolated peritoneal mast cells were collected from rats treated with GCs, aminoguanidine, GCs plus aminoguanidine, and vehicle. Mast cells were stimulated in vitro with Ag (C and D) or compound 48/80 (E and F). The data are expressed as the means $\pm$ SD of 5 and 4 rats to mast cell number and reactivity, respectively. $+P < 0.05$ compared to untreated rats. $+++P < 0.001$ compared to untreated rats. $***P < 0.001$ compared to rats treated with GC. $&&&P < 0.001$ compared to nonstimulated cells. $$$$P < 0.01$ compared to stimulated cells. $$$$P < 0.001$ compared to stimulated cells. $###P < 0.001$ compared to stimulated cells from rats treated with GC. AG, aminoguanidine

significantly reduced pleural mast cell numbers compared to control rats. Aminoguanidine (250 mg/kg) restored the number of mast cells in the pleural space of rats treated with either dexamethasone or prednisolone to values close to those in control animals (Fig. 1A and B, respectively). No change was observed in the number of mast cells present in the pleural space of rats treated with aminoguanidine alone (Fig. 1A and B).

As expected, purified peritoneal mast cells stimulated with Ag (Fig. 1C and D) or compound 48/80 (Fig. 1E and F) in vitro released a marked amount of histamine, an effect significantly inhibited in mast cells recovered from dexamethasone- (Fig. 1C and E) or prednisolone-treated rats (Fig. 1D and F). Peritoneal mast cells recovered from

rats treated with both GCs that received aminoguanidine showed reestablished responsiveness to stimulation with Ag (Fig. 1C and D) or compound 48/80 (Fig. 1E and F) in vitro. No change in the Ag- or compound 48/80-induced histamine release in vitro was observed in mast cells recovered from aminoguanidine-treated rats compared with control rats. Basal histamine release by mast cells showed no modification between all the groups studied (Fig. 1C, D, E, and F).

Notably, neither aminoguanidine, at a lower dose (50 mg/kg), which inhibits NOS but not AGEs, nor L-NAME (30 mg/kg), a classic nonselective NOS inhibitor, inhibited dexamethasone-induced pleural mastocytopenia (Fig. 2).

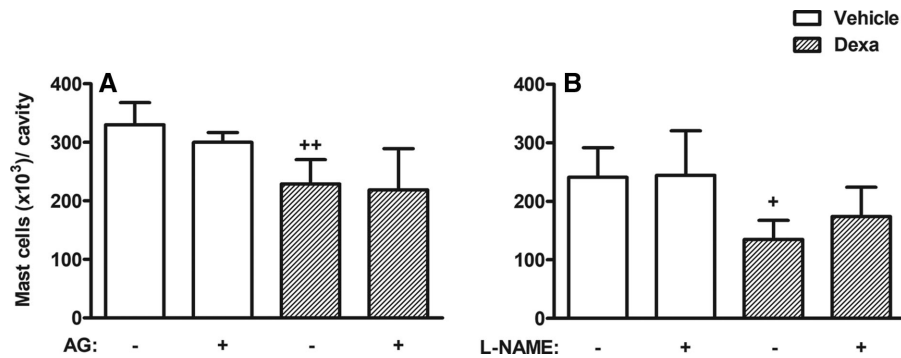


FIGURE 2 Inhibition of NOS does not restore the reduction of pleural mast cell number induced by dexamethasone in Wistar rats. Dexamethasone (0.1 mg/kg) was subcutaneously injected, once a day during 18 consecutive days. Treatment with aminoguanidine (50 mg/kg, oral route; A) or L-NAME (30 mg/kg, oral route; B) was performed daily for 15 days, starting 3 days after initiating dexamethasone injections. A group of rats was treated daily with aminoguanidine (A) or L-NAME (B) for 15 consecutive days. Untreated rats received an equal amount of vehicle (saline 0.9%). The data are expressed as the means $\pm$ SD of 5 rats. + P < 0.05 compared to untreated rats. ++ P < 0.01 compared to untreated rats. Dexa, dexamethasone; AG, aminoguanidine

As expected, mast cells recovered from dexamethasone-treated rats presented high levels of apoptotic cells compared to those from control rats, attested by TUNEL (Fig. 3A and C). Furthermore, dexamethasone induced an increased expression of the proapoptotic protein Bax in mast cells compared for those from control rats (Fig. 3F). Mast cells recovered from rats treated with dexamethasone plus aminoguanidine (250 mg/kg) showed a reduced rate of apoptotic mast cells and the expression of Bax in mast cells (Fig. 3D and F) compared to those treated with only dexamethasone (Fig. 3C and F). Aminoguanidine-treated rats did not show altered mast cell apoptosis (Fig. 3B), neither Bax expression (Fig. 3F). Quantitative data of TUNEL positive mast cells are shown in Fig. 3E.

3.2 | GC-induced loss of body weight, skeletal muscle atrophy, and circulating lymphopenia are not associated with AGEs

Treatment with dexamethasone decreased the body weight of rats, which was not modified by aminoguanidine (250 mg/kg) (Fig. 4A). Dexamethasone also induced gastrocnemius muscle atrophy, evidenced as a reduction in the ratio between muscle weight and tibia length (Fig. 4B). This muscle atrophy was associated with a decrease in the mean muscle cell area (Fig. 4E and G) compared to control rats (Fig. 4C and G). Neither the macroscopic nor microscopic gastrocnemius muscle atrophy induced by dexamethasone was altered in rats after treatment with aminoguanidine (250 mg/kg; Fig. 4B, F, and G). The baseline levels remained unaltered in aminoguanidine-treated rats, such as gastrocnemius muscle weight/tibia length ratio (Fig. 4B) and mean muscle cell area (Fig. 4D and G).

We also assessed whether aminoguanidine (250 mg/kg) interfered with the immunosuppressive effect of GCs. Compared with control rats, dexamethasone caused a significant reduction in total leukocyte numbers detected in peripheral blood, evidenced as a decrease in the numbers of lymphocytes, but not neutrophils and mononuclear cells. This dexamethasone-induced lymphopenia was also insensitive to aminoguanidine treatment, also failed to alter the levels of total leuko-

cytes, mononuclear cells, and neutrophils in both dexamethasone-treated and untreated animals (Fig. 4H).

3.3 | GC induces transactivation of RAGE and galectin-3 in mast cells but not in skeletal muscle cells

We investigated whether GCs interfered with the AGEs-RAGEs axis in the experimental protocol employed. First, neither dexamethasone nor prednisolone changed glycemia conditions or plasma fructosamine levels in rats (Table 1). Furthermore, mast cells from dexamethasone-treated rats showed the overexpression of both receptors for AGEs, RAGE and galectin-3, compared to the mast cells from control rats (Fig. 5A and B, respectively). The dexamethasone-induced transactivation of RAGE and galectin-3 in mast cells was reversed by aminoguanidine under conditions where no alterations in RAGE and galectin-3 expression in the mast cells of control rats were noted (Fig. 5A and B, respectively). Nevertheless, treatment with dexamethasone did not modify the expression of either RAGE or galectin-3 in the gastrocnemius muscle (Fig. 5C and D, respectively). Aminoguanidine (250 mg/kg) also failed to alter the expression of either RAGE or galectin-3 in the gastrocnemius muscle in both dexamethasone-treated and untreated rats (Fig. 5C and D, respectively).

3.4 | Blockade of RAGE and galectin-3 inhibits the mastocytopenia induced by GCs

Dexamethasone significantly reduced peritoneal and mesenteric mast cell numbers compared to control mice (Fig. 6A and B, respectively). Both RAGE (FPS-ZM1, 1 mg/kg) and galectin-3 (GCS-100, 1 mg/kg) antagonists restored the number of mast cells to almost to baseline levels in the peritoneal cavity and mesenteric tissue of mice treated with dexamethasone (Fig. 6A and B, respectively). No change was observed in the number of mast cells present in the peritoneal cavity and mesenteric tissue of mice treated with FPS-ZM1 or GCS-100 compared to control mice (Fig. 6A and B, respectively).

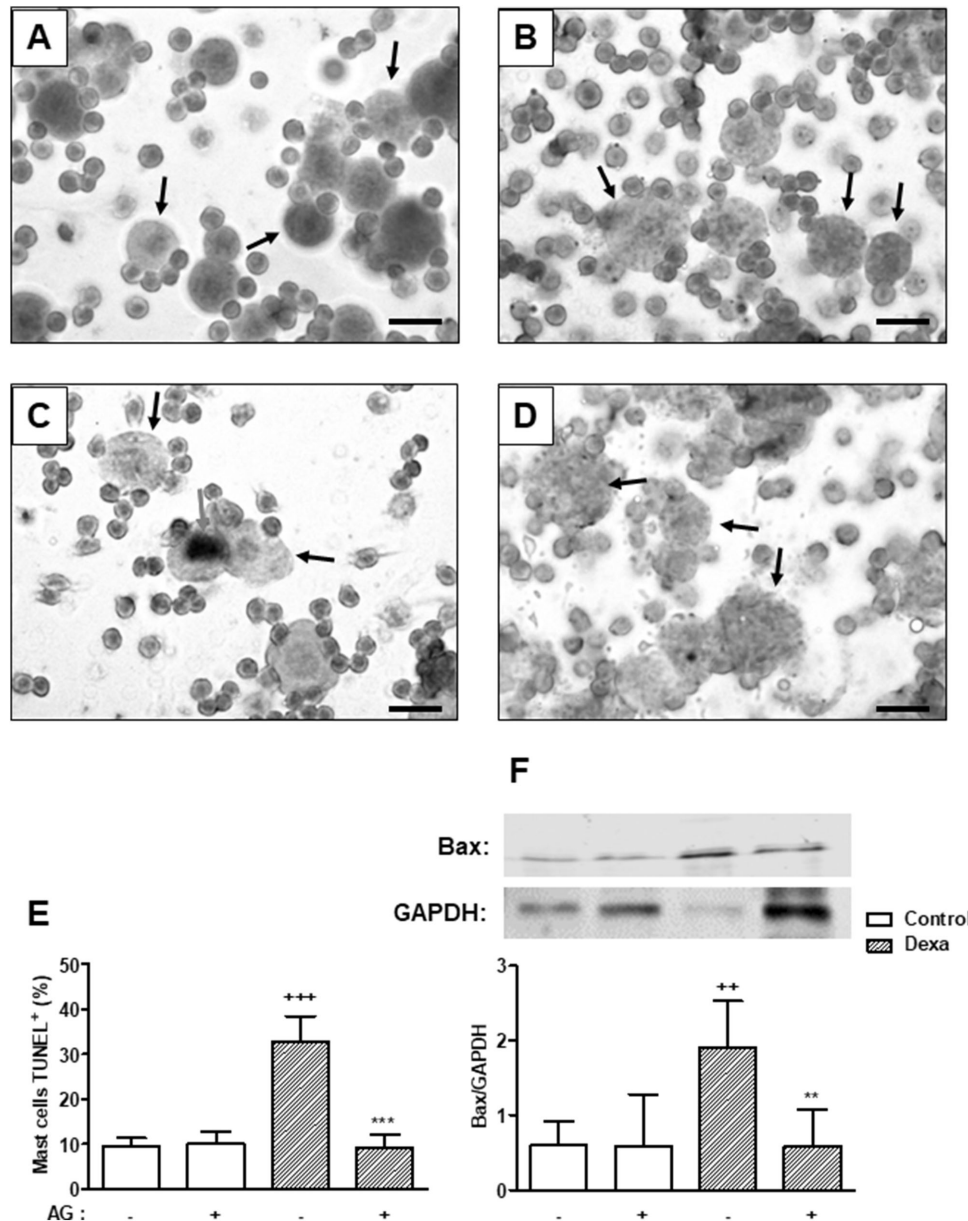


FIGURE 3 Aminoguanidine restores apoptosis of mast cells induced by dexamethasone in Wistar rats. Dexamethasone (0.1 mg/kg) was subcutaneously injected, once a day during 18 consecutive days. Treatment with aminoguanidine (250 mg/kg, oral route) was performed daily for 15 days, starting 3 days after initiating dexamethasone injections. A group of rats was treated daily with aminoguanidine for 15 consecutive days. Untreated rats received an equal amount of vehicle (saline 0.9%). Panels show photomicrographs of toluidine blue-stained mast cells (black arrows) plus TUNEL marking (grey arrow), from the peritoneal cavity of untreated (A), aminoguanidine-treated (B), and dexamethasone-treated (C), and dexamethasone plus aminoguanidine-treated (D) rats. Panel E shows that aminoguanidine abolished the increased apoptosis in mast cells from dexamethasone-treated rats. Panel F shows representative samples of Bax expression in isolated mast cells. The expression of Bax was determined by western blot. The data are normalized to GAPDH. The data are expressed as the means $\pm$ SD of 4 or 5 rats for TUNEL and Bax expression analysis, respectively. $++P < 0.01$ compared to untreated rats. $+++P < 0.001$ compared to untreated rats. $**P < 0.01$ compared to dexamethasone-treated rats. $***P < 0.001$ compared to dexamethasone-treated rats. Dexa, dexamethasone; AG, aminoguanidine

3.5 | Blockade of RAGEs and galectin-3 does not alter the loss of body weight and circulating lymphopenia induced by GC

Dexamethasone decreased total leukocyte numbers in the peripheral blood compared to control mice, reflected by reduced numbers of lymphocytes, but not neutrophils and mononuclear cells, a phenomenon

demonstrated as insensitive to treatment with either RAGE (FPS-ZM1, 1 mg/kg) or galectin-3 (GCS-100, 1 mg/kg) antagonists. Treatment with FPS-ZM1 or GCS-100 did not alter the basal numbers of total leukocytes, mononuclear cells, and neutrophils in dexamethasone and control groups (Fig. 7A). Treatment with dexamethasone reduced the body weight of rats; however, neither FPS-ZM1 nor GCS-100 restored the body weight of treated or control mice (Fig. 7B).

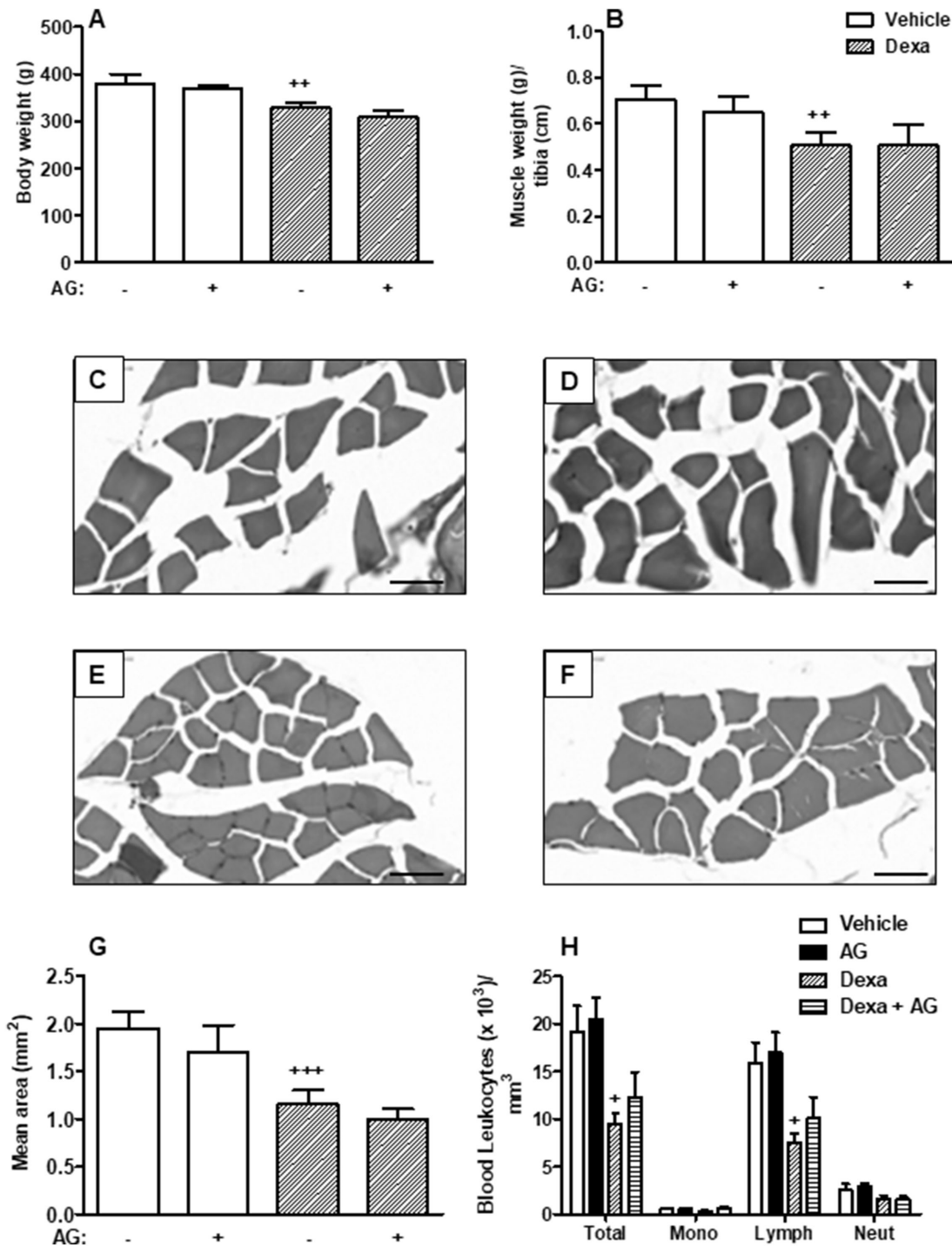


FIGURE 4 Aminoguanidine does not alter the reduction of body weight, neither skeletal muscle atrophy nor peripheral lymphopenia induced by dexamethasone in Wistar rats. Dexamethasone (0.1 mg/kg) was subcutaneously injected, once a day during 18 consecutive days. Treatment with aminoguanidine (250 mg/kg, oral route) was performed daily for 15 days, starting 3 days after initiating dexamethasone injections. A group of rats was treated daily with aminoguanidine for 15 consecutive days. Untreated rats received an equal amount of vehicle (saline 0.9%). Panels A and B show body weight and the relation between gastrocnemius muscle weight and tibia length, respectively. Panels show photomicrographs of HE-stained gastrocnemius muscle of untreated (C), aminoguanidine-treated (D), dexamethasone-treated (E), and dexamethasone plus aminoguanidine-treated rats (F), respectively. Quantification of the mean muscle cell area (G). Analysis of total and differential leukocyte numbers in the peripheral blood. The data are expressed as the means $\pm$ SD of 5 rats. $+P < 0.05$ compared to untreated rats. $++P < 0.01$ compared to untreated rats. $+++P < 0.001$ compared to untreated rats. Dexa, dexamethasone; AG, aminoguanidine; Total, total leukocytes; Mono, monocytes; Lymph, lymphocytes; Neut, neutrophils

TABLE 1 GCs do not alter blood glucose and plasma fructosamine levels in Wistar rats

Group	Glycaemia (mmol/L)	Fructosamine (μ mol/L)
Control	6 $\pm$ 0.54	347 $\pm$ 96.9
Dexamethasone	5 $\pm$ 0.55	304 $\pm$ 97.2
Prednisolone	5 $\pm$ 0.46	390 $\pm$ 80.9

Dexamethasone or prednisolone (0.1 mg/kg) were injected s.c. once a day during 18 consecutive days. Values represent mean $\pm$ SD of 5 rats.

3.6 | Blockade of RAGE and galectin-3 inhibits the GC-induced apoptosis of mast cells in vitro

Initially, we treated mast cells in vitro for 24 h with dexamethasone and then stimulated the cells with glycated albumin for 24 h. We observed that glycated albumin induced mast cell apoptosis in vitro when the cells were pretreated with dexamethasone at concentrations in which neither the dexamethasone nor the glycated albumin had a proapoptotic effect (Fig. 8A). Then, we noted that both RAGE (Fig. 8B) and galectin-3 (Fig. 8C) antagonists, FPS-ZM1 and GCS-100, respectively, inhibited the apoptosis of mast cells induced by the combination of dexamethasone and glycated albumin in vitro.

We also evaluated if glycated albumin is able to induce mast cell degranulation in vitro in our system. We showed that neither dexamethasone nor glycated albumin or a combination of dexamethasone plus glycated albumin was able to induce release of β -hex by mast cells in vitro in a condition where a stimulation with DNP-BSA provoked an intense mast cell degranulation (Fig. 8D).

4 | DISCUSSION

In the present study, we investigated the contribution of the AGEs to the GC-induced reduction of mast cell number and reactivity. Aminoguanidine, an AGE inhibitor, restored basal pleural numbers in GC-treated rats and mast cell reactivity in vitro, in a strong correlation with decreased levels of apoptotic mast cells. In contrast, aminoguanidine did not alter the loss of body weight, skeletal muscle atrophy, and immunosuppression induced by GC. GC treatment increased the expression of AGE receptors, RAGE and galectin-3, in mast cells through a mechanism sensitive to aminoguanidine treatment. Moreover, the selective antagonists of RAGE, FPS-ZM1, or galectin-3, GCS-100, blocked the GC-induced mastocytopenia apparent in the celomic cavity and mesenteric tissue, and the glycated albumin-induced mast cell apoptosis in vitro, in cells pretreated with GC, without modifying other GC-induced systemic changes, such as body weight loss and blood lymphopenia. These findings suggest that GC therapy can up-regulate RAGE and galectin-3 on mast cells, which, following exposure to AGEs undergoes apoptosis, leading to tissue and celomic mast cell depletion.

Mast cells of diabetic rats are hyporesponsiveness to immunological and nonimmunological stimuli,^{8,18} closely associated with a high rate of apoptosis through a mechanism correlated with hypercorticism.^{19,20} In addition, high level of apoptosis observed

in the mast cells of diabetic rats was associated with increased AGE formation.¹⁹ Thus, we hypothesized that the GC-induced mastocytopenia and suppression of mast cell activation are mediated by the AGEs/RAGEs signaling axis. In the present study, the AGE formation inhibitor, aminoguanidine, abolished both the dexamethasone- and prednisolone-induced reduction in the pleural mast cell population. In addition, aminoguanidine restored responsiveness to either allergen or compound 48/80 challenge of purified mast cells from both dexamethasone- and prednisolone-treated rats. These data indicate that aminoguanidine blocks the effect of structurally distinct GCs on mast cells, consistent with previous studies demonstrating that the inhibition of AGE formation also restored mast cell numbers and reactivity in diabetic rats, in which a sustained increase in the circulating levels of corticosterone was observed.¹⁹ However, AGEs lead to activation and degranulation of mast cells in vitro directly,²⁷ an opposite effect to what we propose in this work. In order to elucidate this question, we incubated a cell line of mast cells with dexamethasone and then stimulated these cells with glycated albumin in vitro. We noted that neither glycated albumin nor a combination of dexamethasone plus glycated albumin was able to induce mast cell activation in vitro. A possible explanation for these data is that not all AGEs are able to activate mast cells in vitro directly, such as maltosyl-BSA and lactosyl-BSA.²⁷

In addition to the inhibition of the AGEs formation, aminoguanidine exhibits a high spectrum of activity, including the suppression of inducible NOS.²⁸ Treatment with aminoguanidine at doses that inhibit inducible NOS, but not AGE formation,²⁹ did not alter the reduction in pleural mast cell numbers in dexamethasone-treated rats. Similar results were obtained using the classical nonselective NOS inhibitor L-NAME, reinforcing the idea that the restoration of mast cell numbers in GC-treated rats induced by aminoguanidine is independent of its inhibitory effect upon NOS.

Moreover, TUNEL assay and expression of Bax, a member of Bcl-2 proapoptotic protein, revealed that treatment with aminoguanidine at doses that inhibit AGE formation resulted in a decrease of dexamethasone-induced apoptosis in mast cells. These data are clearly associated with the observed restoration of mast cell numbers and reactivity, and the apoptosis of mast cells contributes to the reduction in population and refractoriness of these cells.⁸ The hypothesis that GC-induced mast cell apoptosis depends on AGEs was also confirmed by the fact that AGEs can induce the apoptosis of mast cells through the activation of their receptors RAGE and galectin-3 in vitro.^{30,31}

Currently, the precise molecular mechanism that distinguishes between the anti-allergic effects and adverse side effects of GCs is not yet known. Thus, we examined whether the blockade of AGE formation could be associated with the adverse effects of GCs, including immunosuppression, altered body weight, and skeletal muscle atrophy.^{9,32} Treatment with aminoguanidine at doses that inhibit AGE formation did not interfere with the loss of body weight, gastrocnemius muscle atrophy, and circulating lymphopenia in dexamethasone-treated rats, suggesting that the GC-induced activation of the AGEs-RAGEs axis is important to the anti-allergic effects of GCs, but not to the generation of their important adverse side effects.

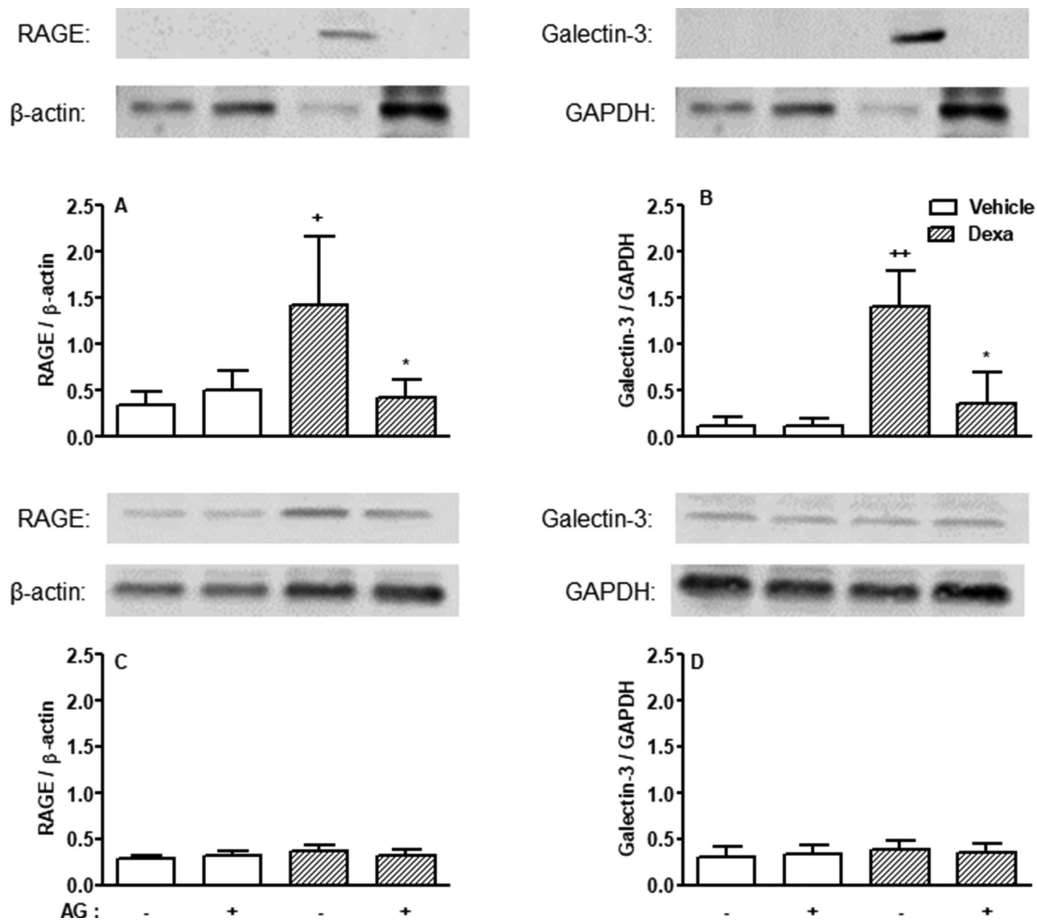


FIGURE 5 Aminoguanidine reduces the expression of RAGE and galectin-3 in mast cells, but not alter their expression in skeletal muscle of dexamethasone-treated Wistar rats. Dexamethasone (0.1 mg/kg) was subcutaneously injected, once a day during 18 consecutive days. Treatment with aminoguanidine (250 mg/kg, oral route) was performed daily for 15 days, starting 3 days after initiating dexamethasone injections. A group of rats was treated daily with aminoguanidine for 15 consecutive days. Untreated rats received an equal amount of vehicle (saline 0.9%). Representative samples of RAGE (A and C) and galectin-3 (B and D) expression in isolated mast cells (A and B) and gastrocnemius muscle (C and D). The expressions of RAGE and galectin-3 were determined by western blot. The data are normalized to β -actin and GAPDH, respectively. The data are expressed as the means $\pm$ SD of 5 rats. + P < 0.05 compared to untreated rats. ++ P < 0.01 compared to untreated rats. * P < 0.05 compared to dexamethasone-treated rats. Dexa, dexamethasone; AG, aminoguanidine

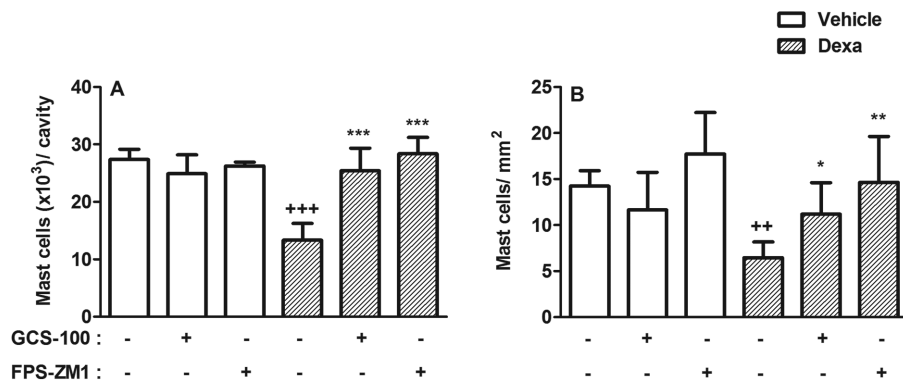


FIGURE 6 Blockade of RAGE or galectin-3 restores the reduction of peritoneal (A) and mesenteric (B) mast cell numbers induced by dexamethasone in Swiss-Webster mice. Dexamethasone (0.1 mg/kg) was subcutaneously injected, once a day during 18 consecutive days. Treatments with galectin-3 antagonist GCS-100 (1 mg/kg, oral route) or RAGE inhibitor FPS-ZM1 (1 mg/kg, i.p.) were performed daily for 15 days, starting 3 days after initiating dexamethasone injections. A group of mice was treated daily with GCS-100 or FPS-ZM1 for 15 consecutive days. Untreated mice received an equal amount of vehicle (DMSO 0.05%). The data are expressed as the means $\pm$ SD of 5 mice. ++ P < 0.01 compared to untreated mice. +++ P < 0.001 compared to untreated mice. * P < 0.05 compared to dexamethasone-treated mice. ** P < 0.01 compared to dexamethasone-treated mice. *** P < 0.001 compared to dexamethasone-treated mice. Dexa, dexamethasone

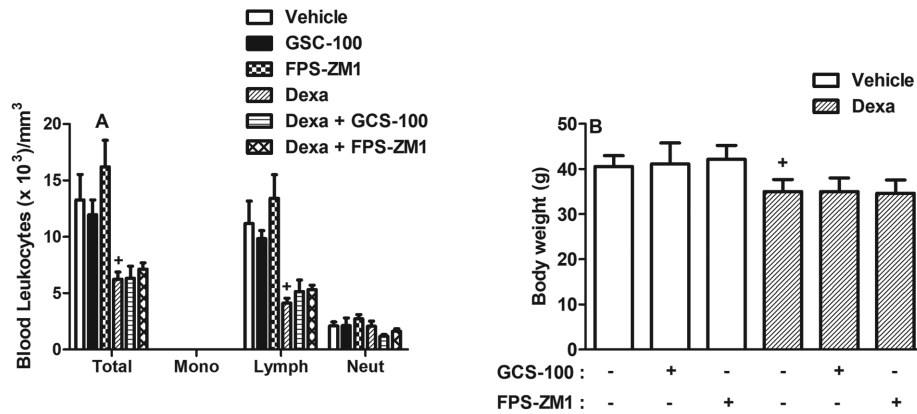


FIGURE 7 Blockade of RAGE or galectin-3 does not alter the reduction of peripheral lymphopenia (A) and body weight (B) induced by dexamethasone in Swiss-Webster mice. Dexamethasone (0.1 mg/kg) was subcutaneously injected, once a day during 18 consecutive days. Treatments with galectin-3 antagonist GCS-100 (1 mg/kg, oral route) or RAGE inhibitor FPS-ZM1 (1 mg/kg, i.p.) was performed daily for 15 days, starting 3 days after initiating dexamethasone injections. A group of mice was treated daily with GCS-100 or FPS-ZM1 for 15 consecutive days. Untreated mice received an equal amount of vehicle (DMSO 0.05%). The data are expressed as the means $\pm$ SD of 5 mice. $+P < 0.05$ compared to untreated mice. Dexa, dexamethasone; Total, total leukocytes; Mono, monocytes; Lymph, lymphocytes; Neut, neutrophils

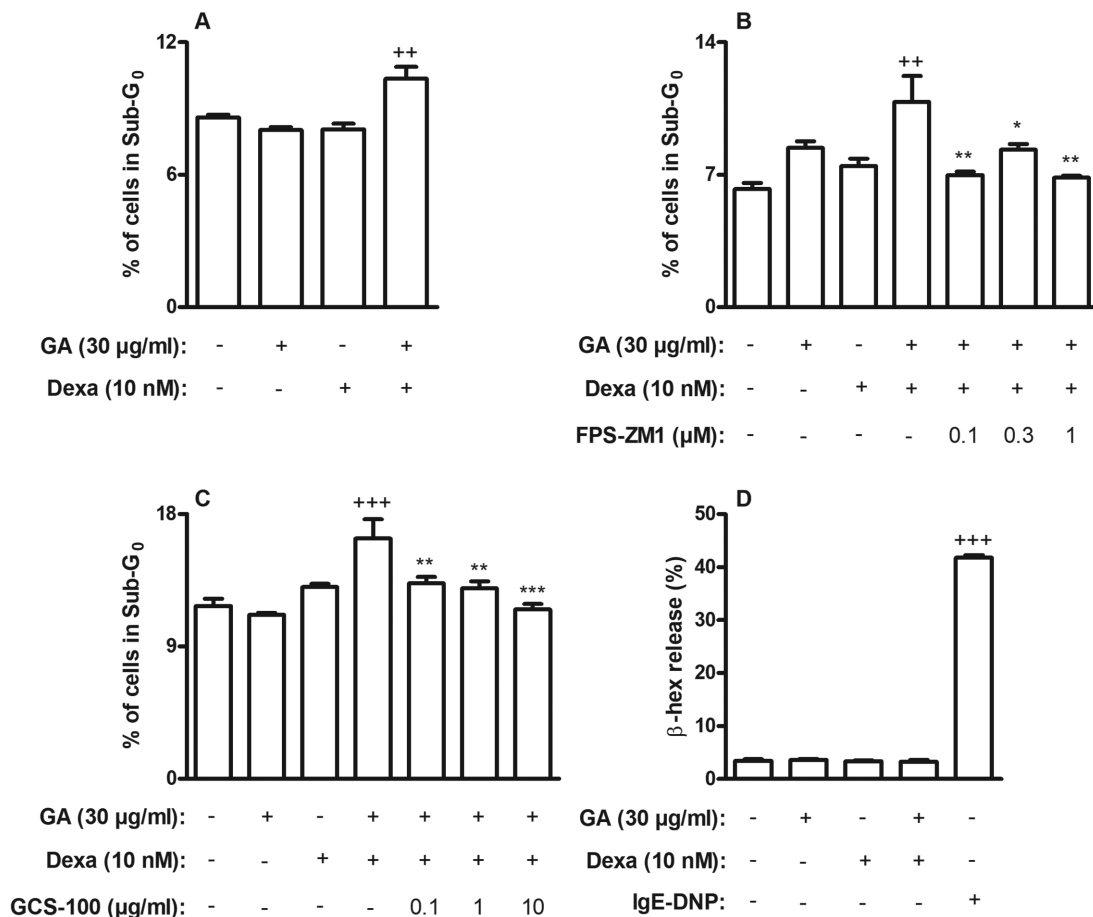


FIGURE 8 Blockade of RAGE or galectin-3 inhibits the glycated albumin-induced apoptosis of mast cells pretreated with GC in vitro. RBL-2H3 cells (1.5×10^5 cells/well) were pretreated with dexamethasone (10 nM) for 24 h and then stimulated with glycated albumin (30 $\mu\text{g/ml}$) for 24 h (A). In some experiments, the cells were treated with RAGE inhibitor FPS-ZM1 (0.1–1 μM ; B) or galectin-3 antagonist GCS-100 (0.1–10 $\mu\text{g/ml}$; C) 30 min before stimulation with glycated albumin. The apoptosis rate was attested by percentage of cells in Sub-G₀ cell cycle. (D) RBL-2H3 cells (1.25×10^5 cells/well) were pretreated with dexamethasone (10 nM) for 24 h and then stimulated with glycated albumin (30 $\mu\text{g/ml}$) for 45 min. In positive control, RBL-2H3 cells were sensitized with IgE anti-DNP (1 $\mu\text{g/ml}$) and stimulated with DNP-BSA (10 ng/ml) 20 h later. The β -hex levels were quantified 45 min after the stimuli using a spectrophotometer at 405 nm. Data points are means $\pm$ SD of triplicate determinations from a single experiment and are representative of 2 experiments. $++P < 0.01$ compared to untreated cells. $+++P < 0.001$ compared to untreated cells. $*P < 0.05$ compared to cells treated with Dexa plus glycated albumin in vitro. $**P < 0.01$ compared to cells treated with Dexa plus glycated albumin in vitro. $***P < 0.001$ compared to cells treated with Dexa plus glycated albumin in vitro. Dexa, dexamethasone; GA, glycated albumin

To understand how GCs activate the AGEs–RAGEs axis, we quantified the glycemic and plasma levels of glycated albumin. Notably, under the treatment conditions explored here, both dexamethasone and prednisolone did not alter blood glucose and plasmatic fructosamine levels, suggesting that these drugs do not interfere with the circulating levels of glycated albumin. The fact that we do not see changes in the circulating levels of glycated albumin suggests that GCs may induce alterations in the expression of AGE receptors, which could explain, at least in part, why we did not detect modifications in the loss of body weight, gastrocnemius muscle atrophy, and circulating lymphopenia in rats simultaneously treated with GC and aminoguanidine.

In another set of experiments, GC indeed up-regulated the expression of RAGE and galectin-3 on mast cells, consistent with previous findings of the GC-induced elevation of RAGE in human keratinocyte and Kupffer cells, and galectin-3 in macrophages, Clara cells, and Kupffer cells.^{11–14} However, the findings of the present study suggest that this GC effect is likely selective, and does not occur, for example, in gastrocnemius muscle cells. This selectivity might explain why the effect of GCs on mast cells, but not on gastrocnemius muscle, is sensitive to the blockade of the AGEs–RAGEs axis. In addition, the fact that aminoguanidine inhibited the dexamethasone-induced transactivation of both RAGE and galectin-3 in mast cells may explain why aminoguanidine prevents the induction of apoptosis and hyporesponsiveness of mast cell without changing the circulating levels of AGEs. These data are consistent with previous studies showing that aminoguanidine down-regulates the expression of RAGE and galectin-3 under hyperglycemia.^{33,34}

To evaluate whether the effects of GCs on mast cells indeed depend on the up-regulation of RAGE and galectin-3, we treated Swiss-Webster mice with RAGE or galectin-3 antagonist. The results showed that both the RAGE antagonist FPS-ZM1 and galectin-3 inhibitor GCS-100 blocked the dexamethasone-induced reduction in the peritoneal and mesenteric mast cell population. Although our data indicate that GCs induced apoptosis of mast cells through transactivation of AGEs receptors, we cannot assert that it is a direct effect on mast cells once the loss of mast cell numbers following GCs treatment has been reported depending on tissue resident cells, which down-regulate generation and release of stem cell factor,³⁵ or through inhibition of autocrine stimulation of mast cells by IL-4.³⁶ Here, we treated mast cells with dexamethasone for 24 h and then stimulated the cells with glycated albumin in vitro in concentrations at neither dexamethasone nor glycated albumin induced mast cell apoptosis. We showed that the provocation of mast cells previously treated with dexamethasone with glycated albumin induced apoptosis of mast cells in vitro. This increase in mast cell apoptosis rate induced by a combination of GC and glycated albumin in vitro was completely inhibited by treatments with either FPS-ZM1 or GCS-100. These data indicate that the GC-induced mast cell apoptosis depends at least partially on the ability of GCs to induce RAGE/galectin-3 expression and consequently apoptosis of mast cells directly.

In order to understand whether there is a dissociation between the anti-allergic mechanism and the adverse effects of GCs, we analyzed the circulating lymphocyte population and body weight of mice and

observed that both FPS-ZM1 and GCS-100 did not interfere with the loss of body weight and circulating lymphopenia in dexamethasone-treated mice, reinforcing the idea that the GC-induced transactivation of RAGE and galectin-3 is important to the anti-allergic effects but not the adverse side effects of corticosteroid drugs. In summary, these results suggest that the GC-induced hyporeactivity and apoptosis of mast cells depends on the stimulation of RAGE and galectin-3 transactivation. In addition, the stimulation of the AGEs–RAGEs axis is associated with the anti-allergic effects of GCs but apparently has no relationship with the adverse effects of these drugs.

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