

## Anti-asthmatic potential of a D-galactose-binding lectin from *Synadenium carinatum* latex

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**Extracts from the plant *Synadenium carinatum* latex are widely and indiscriminately used in popular medicine to treat a great number of inflammatory disorders and although the mechanisms underlying these effects remain undefined, the lectin isolated from *S. carinatum* latex (ScLL) is thought to be in part responsible for these anti-inflammatory effects. In order to elucidate possible immunoregulatory activities of ScLL, we investigated the effects of ScLL administration in models of acute and chronic inflammation. Oral administration of ScLL significantly inhibited neutrophil and eosinophil extravasation in models of acute and chronic inflammation and reduced eosinophil and mononuclear blood counts during chronic inflammation. ScLL administration reduced IL(interleukin)-4 and IL-5 levels but increased interferon- $\gamma$  and IL-10 in an asthma inflammatory model, which suggested that it might induce a TH2 to TH1 shift in the adaptive immune response. ScLL also inhibited I $\kappa$ B $\alpha$  degradation, a negative regulator of proinflammatory NF- $\kappa$ B. Taken together, these results provide the first description of a single factor isolated from *S. carinatum* latex extract with immunoregulatory functions and suggest that ScLL may be useful in the treatment of allergic inflammatory disorders.**

**Key words:** anti-inflammatory/asthma/immune response/lectin/*Synadenium carinatum*

### Introduction

Following antigen stimulation, adaptive immune responses differentiate into TH1 or TH2 responses, depending on the

nature of the antigen and the inflammatory milieu (Zhu et al. 2006). The differentiation of naïve CD4+ T cells into TH1 CD4+ or TH2 CD4+ T cells ultimately dictates whether the adaptive immune response will be primarily cytotoxic CD8+ T cell based or whether humoral immunity will predominate. TH2 CD4+ T cells, capable of producing the cytokines IL(interleukin)-4, IL-5, and IL-13, activate humoral- and eosinophil-mediated immunity against extracellular pathogens. However, inappropriate TH2 responses result in allergic and eosinophilic diseases (Zhu et al. 2006), such as asthma (Anderson and Coyle 1994; Hamelmann et al. 1999; Wills-Karp 1999; Zhang et al. 1999; Robinson 2000). The asthma-associated TH2 immune response often results in eosinophilia, which contributes to airway hyper-responsiveness, mucus hypersecretion by goblet cells, and elevated serum immunoglobulin (Ig) E levels (Neurath et al. 2002; Elias 2004).

In conjunction with TH2 adaptive immune cytokines, nuclear factor (NF)- $\kappa$ B also exhibits key regulatory functions in the pathophysiology of asthma (Barnes and Karin 1997). NF- $\kappa$ B activation follows stimulation by a wide range of effectors, including respiratory tract viral infections, tumor necrosis factor (TNF)- $\alpha$ , and IL-1 $\beta$  and results in the increased expression of adhesion molecules, cytokines, and chemokines (Hart et al. 1998; Chung and Barnes 1999; Donovan et al. 1999; Poynter et al. 2004).

Glucocorticoids currently provide the most effective anti-inflammatory therapeutics in the treatment of asthma. Glucocorticoid administration inhibits NF- $\kappa$ B activation (Scheinman et al. 1995) and suppresses the deleterious sequelae associated with many chronic inflammatory diseases such as rheumatoid arthritis, inflammatory bowel disease, autoimmune diseases, and asthma (Eum et al. 1996; Frieri 1999; Belvisi and Hele 2003). In particular, the glucocorticoid dexamethasone attenuates leukocyte migration into the sites of inflammation through the inhibition of proinflammatory cytokine and chemokine synthesis and through the reduction in leukocyte responsiveness (Van der Velden 1998). Although the anti-inflammatory effects of glucocorticoids provide an attractive rationale for their administration in the treatment of inflammatory diseases, the side effects of long-term glucocorticoid treatment reduce their clinical utility (Saklatvala 2002). As a result, the identification of alternative anti-inflammatory therapeutics may enhance treatment strategies of these diseases.

Plant extracts provide a potential source through which novel anti-inflammatory compounds may be identified (Verpoorte 1999; Bielory et al. 2004; Boldi 2004; Clardy and Walsh 2004; Koehn and Carter 2005). Natural compounds that may potentially modulate eosinophil recruitment in acute and chronic inflammation models have been previously

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investigated in our laboratory (Rogerio et al. 2003, 2006). Recently, Souza et al. (2005) isolated and characterized a novel D-galactose (D-gal)-binding lectin, named ScLL (*Synadenium carinatum* latex lectin), extracted from the latex of the *S. carinatum* (Euphorbiaceae). This plant is common in ornamental gardens in Brazil, and an aqueous preparation from its latex has been widely and indiscriminately used in popular medicine to treat a great number of inflammatory disorders, including allergic diseases, without any scientific evidence that could underlie its putative action in the pathophysiology of inflammation.

Plant lectins exhibit diverse expression within the Plantae kingdom and can be found in seeds, leaves, bark, bulbs, rhizomes, roots cotyledon, and tubers, depending on the plant species (Cavada et al. 1998; Wititsuwannakul et al. 1998; Konozy et al. 2002; Oliveira et al. 2002; Yagi et al. 2002; Konozy et al. 2003). Seeds, especially of leguminous species, often contain significant levels of lectin, although lectins isolated from other plant tissues, such as the latex, including ScLL, have been reported. ScLL exists as an oligomer, with subunits of 28 and 30 kDa molecular weight (Souza et al. 2005). ScLL exhibits affinity for  $\beta$ -galactoside-containing glycan ligands as measured by the inhibitory capacity of a library of glycans on ScLL-induced agglutination of red blood cells (Souza et al. 2005).

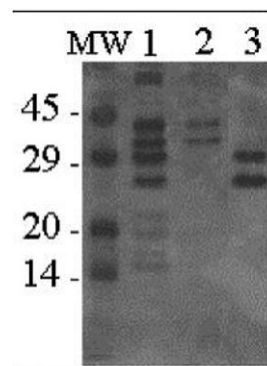
We report here that ScLL exhibits significant immunoregulatory activity. The administration of ScLL inhibited leukocyte trafficking and TH2 cytokine production, and significantly reduced the pathological sequelae associated with the chronic inflammatory disease, asthma, in experimental animal models. These results suggest that ScLL may provide an additional therapeutic possibility in the treatment of inflammatory diseases.

## Results

### Effect of ScLL in acute eosinophilia induced by the F1 fraction

To test the effects of ScLL on models of inflammation, we first purified ScLL from plant extracts as outlined previously (Souza et al. 2005). ScLL existed as two subunits with apparent subunit molecular weights of 28 and 30 kDa, respectively (Figure 1, lane 3). Importantly, ScLL specifically eluted off the column following the addition of the hapten inhibitor galactose (Figure 1), indicating that the purified protein is a lectin, corroborating previous results (Souza et al. 2005).

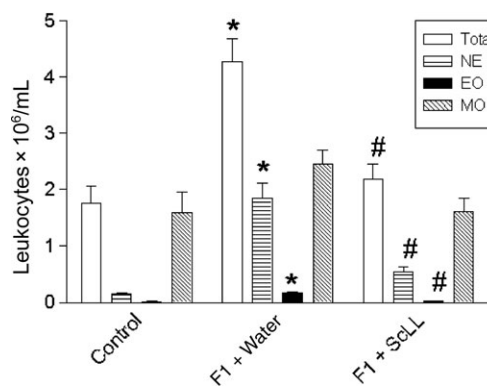
We next sought to determine whether ScLL possessed immunoregulatory activity. To accomplish this, we first sought to examine whether ScLL might modulate leukocyte extravasation in a model of acute peritoneal inflammation. Mice treated with the F1 fraction of *Histoplasma capsulatum*, previously shown to develop acute peritonitis (Medeiros et al. 1999), experienced significant increases in peritoneal leukocyte infiltration. However, the oral administration of ScLL (1 mg/kg) significantly reduced extravasation of leukocytes by nearly 50%. Furthermore, ScLL decreased neutrophil (70%) and eosinophil (86%) cell levels to a greater extent than mononuclear cells, which exhibited no real change following ScLL treatment (Figure 2). These results demonstrate that in this model of acute inflammation, the oral administration of ScLL specifically inhibits neutrophil and eosinophil infiltration (Figure 2).



**Fig. 1.** ScLL purification. Purification of D-gal-binding lectin ScLL from the crude aqueous extract of *S. carinatum* latex. A sample (2 mg) was applied to 3 mL of immobilized D-gal-agarose, previously equilibrated with BBS, and eluted initially with BBS, followed by BBS-D-Gal. Eluted fractions were collected (fraction size: 2 mL; flow rate: 1 mL/min) and monitored spectrophotometrically at 280 nm. Proteins were resolved 15% SDS-PAGE and visualized by silver stain. Lane 1: original crude latex extract; lane 2: flow through; lane 3: ScLL. (MW, molecular weight markers).

### ScLL immunomodulatory effects in murine asthma

Having demonstrated that ScLL exhibits significant inhibition of leukocyte infiltration during an acute inflammatory episode, we next examined immunomodulatory effects of ScLL in a model of chronic inflammation. To accomplish this, mice were immunized with ovalbumin (OVA), followed by subsequent challenges to develop a model of chronic inflammation associated with asthma as outlined previously (Russo et al. 2001). To examine the effects of ScLL on asthma, mice were given ScLL orally at different concentrations (0.25, 0.5, 1.0, 2.0 mg/kg) between the 20th and 22nd day following the first immunization with OVA. The oral administration of ScLL significantly reduced total leukocyte blood counts and mononuclear cell blood counts at all doses tested. Eosinophil numbers also decreased following ScLL administration at concentrations  $>1$  mg/kg. Importantly, ScLL failed to inhibit increases in neutrophil blood counts in this



**Fig. 2.** ScLL administration blocks leukocyte recruitment during acute inflammation. ScLL administration (1 mg/kg) following i.p. inoculation of mice with 100  $\mu$ g of F1 of *H. capsulatum* was accomplished as outlined in the *Materials and methods* section. Cell numbers are expressed as the mean  $\pm$  SEM ( $n = 6$ ). A group inoculated i.p. with PBS was used as control. \* $P < 0.05$  when compared with PBS-treated animals. # $P < 0.05$  when compared with animals treated with F1 + water. NE, neutrophils; EO, eosinophils; MO, mononuclear cells.

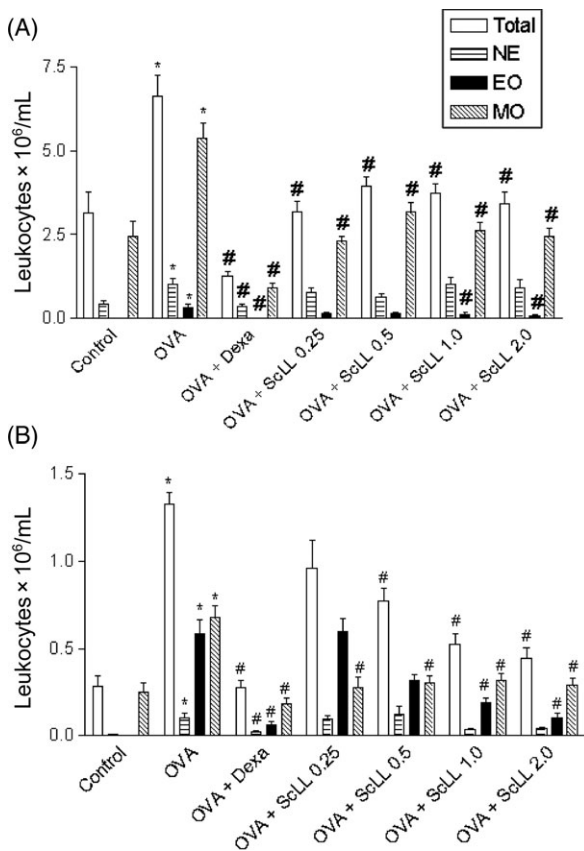
model, although dexamethasone significantly reduced blood counts of all leukocytes, as previously reported (Figure 3A). Consistent with previous results (Russo et al. 2001), the mice immunized and challenged with OVA not only experienced increased leukocyte blood counts, but also exhibited an 11-fold increase in the total number of leukocytes in the bronchoalveolar lavage fluid (BALF) (Figure 3B). Treatment with ScLL (0.5, 1, or 2 mg/kg) also significantly decreased total BALF leukocyte counts, including mononuclear cells (ScLL, all doses tested) and eosinophils (ScLL, 1 and 2 mg/kg) (Figure 3B). Similar results were obtained following dexamethasone treatment (Figure 3B). These results demonstrate that oral administration of ScLL decreases both blood leukocyte levels and leukocyte extravasation in a model of asthmatic inflammation.

We next sought to determine whether alterations in cytokine levels might accompany ScLL-induced reduction in leukocyte extravasation and blood leukocyte counts. To test this, mice were treated with ScLL, followed by the examination of cytokine levels in BALF and in lung homogenates. ScLL administration significantly decreased IL-4 (ScLL, 2 mg/kg) and IL-5 (all doses tested) levels in BALF (Figure 4A and B). In contrast,

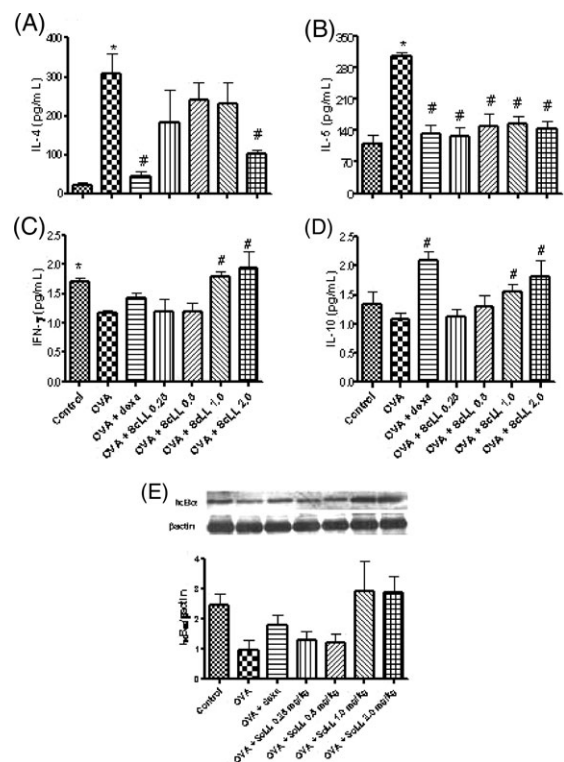
ScLL administration increased interferon (IFN)- $\gamma$  (ScLL at 1 or 2 mg/kg) and IL-10 (ScLL at 1 or 2 mg/kg) levels in the lung homogenates (Figure 4C and D). Significantly, dexamethasone administration reduced levels of IL-4 and IL-5 in BALF and increased the level of IL-10 in lung homogenates in parallel experiments (Figure 4A, B, and D). These results suggest that ScLL may modulate leukocyte trafficking and activity through alterations in cytokine production.

As ScLL administration altered cytokine profiles, we next sought to determine whether ScLL administration might also alter NF- $\kappa$ B regulation, a key factor involved in the orchestration of immune function (Barnes and Karin 1997). As I $\kappa$ B $\alpha$ , a negative regulator of NF- $\kappa$ B, must be degraded prior to NF- $\kappa$ B nuclear translocation (Barnes and Karin 1997), we sought to determine whether ScLL might block I $\kappa$ B $\alpha$  degradation. Indeed, ScLL administration reduced I $\kappa$ B $\alpha$  degradation at doses of 1 and 2 mg/kg (Figure 4E), suggesting that ScLL may in part inhibit inflammation by stabilizing I $\kappa$ B $\alpha$ , thereby reducing NF- $\kappa$ B activity.

As ScLL administration significantly reduced leukocyte extravasation and altered cytokine profiles, we next examined the effect of ScLL administration on the overall pathology of asthma at the histological level. As previously described (Russo et al. 2001), OVA treatment resulted in substantial



**Fig. 3.** ScLL administration alters blood and BALF leukocyte levels. The effect of ScLL treatment on blood (A) and BALF (B) cell numbers of mice immunized and then challenged with OVA was ascertained. The mice were treated 3 times, from the 20th to the 22nd day after the first immunization on day 0 with dexamethasone or increasing doses of ScLL (0.25–2 mg/kg). The samples were collected 24 h after the second OVA challenge. The values are presented as mean  $\pm$  SEM ( $n = 6$  per treatment). \* $P < 0.05$  compared with the control group; # $P < 0.05$  compared with the OVA group. NE, neutrophils; EO, eosinophils; MO, mononuclear cells.

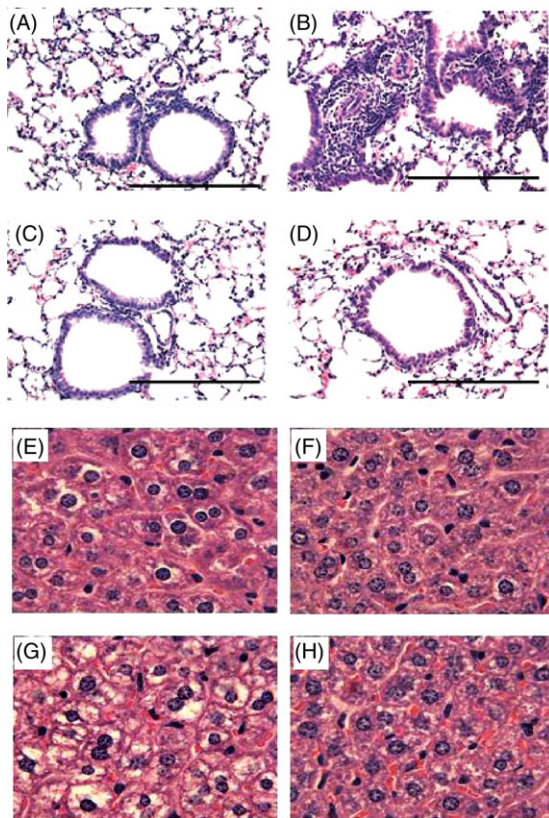


**Fig. 4.** ScLL administration modulates cytokine production. ScLL-induced alterations of IL-4 (A) and IL-5 (B) in BALF and IFN- $\gamma$  (C) and IL-10 (D) in the lung were measured as outlined. (E) I $\kappa$ B $\alpha$  western blot analysis in the lung homogenates. In each panel, the mice were treated 3 times, from the 20th to the 22nd day following the first immunization on day 0 with dexamethasone or increasing doses of ScLL (0.25–2 mg/kg) as indicated and the samples were collected 24 h after the second OVA challenge. The values are mean  $\pm$  SEM ( $n = 6$  per group for cytokine analysis and  $n = 3$  per group for I $\kappa$ B $\alpha$  western blot analysis).  $\beta$ -Actin was used as a loading control during the western blot analysis. \* $P < 0.05$  compared with the control group; # $P < 0.05$  compared with the OVA group.



peribronchovascular inflammation (Figure 5B). In contrast, the administration of either dexamethasone (Figure 5C) or ScLL (1 mg/kg, Figure 5D) markedly reduced leukocyte infiltration. Similar inflammatory reduction followed the administration of ScLL at 2 mg/kg (data not shown). Taken together, these results demonstrate that ScLL administration not only reduced leukocyte infiltration and altered cytokine levels, but also reduced the overall pathology associated with asthma in this model.

As the pathological side effects prevent glucocorticoid treatment from delivering optimal therapeutic benefit to patients with asthma, we next examined whether ScLL administration occurred in the presence or absence of liver injury, a common pathological side effect of glucocorticoids. Glucocorticoid-treated mice exhibited pathological alterations in the hepatic lobule, such as sinusoidal distortion (Figure 5G). Furthermore, hepatocytes displayed nuclear shrinkage and plasmatic vacuolization typical of hydropic degeneration (Figure 5G). However, no such alterations were observed following ScLL administration (Figure 5H), which suggested that ScLL administration reduced the pathology associated with asthma, although it failed to induce the same hepatocellular pathology that accompanied glucocorticoid treatment.



**Fig. 5.** Leukocyte infiltration is reduced in the lung parenchyma of mice treated with ScLL, which failed to induce liver tissue pathology. BALB/c mice were immunized, challenged with OVA, and treated as outlined. Twenty-four hours after the last OVA challenge, the lungs and livers were removed, fixed, and paraffin-embedded, followed by staining with HE. Representative lung and liver sections from controls [(A), (E)], OVA-immunized and challenged mice with no treatment [(B), (F)], and the mice treated with dexamethasone [(C), (G)] or ScLL at 1 mg/kg (D) or 2 mg/kg (H) are depicted in the figure. Scale bar 100  $\mu$ m.

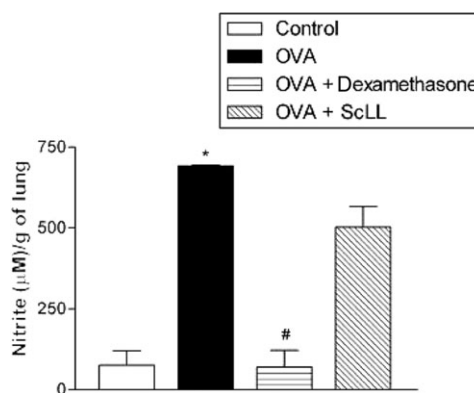
As ScLL administration reduced the pathology associated with asthma, we next examined whether ScLL might also modulate nitric oxide (NO) production, an important inflammatory mediator of asthmatic inflammation (Saleh et al. 1998; Ricciardolo 2003). Interestingly, ScLL (1 mg/kg) administration failed to affect NO production (Figure 6), although dexamethasone decreased NO levels as reported previously (Figure 6). These results demonstrate that although ScLL reduces pathology associated with asthma, this reduction probably occurs through NO-independent mechanisms.

#### *Resistance of ScLL to pepsin digestion and acidic denaturation*

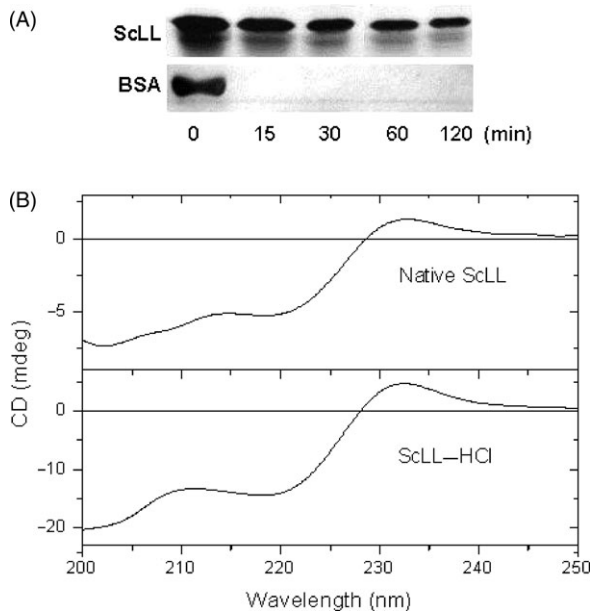
As the anti-inflammatory effects of ScLL occurred following oral administration, we next examined whether ScLL may be resistant to gastric digestion. To accomplish this, we determined the sensitivity of ScLL to *in vitro* treatment with pepsin under acidic conditions. ScLL exhibited significant resistance toward pepsin, even after 2 h of incubation, whereas bovine serum albumin (BSA) experienced complete degradation within 15 min in parallel assays (Figure 7A). ScLL also displayed very little change in tertiary structure following acidification, as indicated by circular dichroism (CD) (Figure 7B), demonstrating resistance to acid-induced denaturation. Finally, ScLL treated with acid and pepsin retained hemagglutinating activity at high concentrations (24–48  $\mu$ g/mL), but not at lower doses (3–12  $\mu$ g/mL) (data not shown), suggesting that the higher doses required in these studies may reflect doses needed to deliver a minimal concentration of ScLL successfully to the intestine following passage through the gastric milieu.

## Discussion

The present study demonstrates that ScLL exhibits significant immunomodulatory activity, providing the first description of a single factor isolated from ScL extracts with anti-inflammatory properties. Furthermore, these results also provide possible mechanisms of ScLL action and implicate



**Fig. 6.** ScLL fails to alter NO production during asthma inflammation. Nitrite production in the lung homogenates of mice immunized and challenged with OVA. The mice were treated 3 times, from the 20th to the 22nd day following the first immunization on day 0 with dexamethasone or ScLL (1.0 mg/kg). The lung was collected 24 h after the second OVA challenge and nitrite was determined by the Griess assay. The values are mean  $\pm$  SEM ( $n = 5$  per group). \* $P < 0.05$  compared with the control group; # $P < 0.05$  compared with the OVA group.



**Fig. 7.** Time course of in vitro ScLL digestion. **(A)** The samples of ScLL and BSA (15  $\mu$ g of dried protein for both) were treated with pepsin at acidic pH for different periods of time (0, 15, 30, 60, and 120 min) to compare the kinetics of protein degradation in stomach conditions. The resulting reaction was examined by 15% SDS-PAGE in reducing conditions. **(B)** Far UV-CD spectra of ScLL in the presence or absence of HCl (pH 2.2). CD spectra of native ScLL or ScLL treated with HCl were recorded from 200 to 250 nm in a 1-mm-path-length cuvette as the average of 16 scans at 25  $^{\circ}$ C.

ScLL as a possible therapeutic in the treatment of chronic inflammatory diseases, such as asthma.

In general, plant materials exhibit potent anti-inflammatory properties, suggesting a possible source of novel therapeutics (Rogerio et al. 2003, 2006; Sa-Nunes et al. 2006). However, many studies examine the therapeutic potential of plant extracts, making it difficult to decipher the actual individual components responsible for these effects. As a result, we examined ScLL, derived from ScL extract, on immune function.

The importance of carbohydrate and carbohydrate-binding protein interaction in various aspects of immune function has been well documented (Barral-Netto et al. 1996; Braun et al. 2001; Lavelle et al. 2002; Dias-Baruffi et al. 2003; Huber et al. 2005; Lavelle 2006; Patchell and Dorscheid 2006; Stowell et al. 2007). For example, leukocyte endothelial adhesion requires carbohydrate interaction, which is a prerequisite for the movement of leukocytes from blood into tissues, a characteristic feature of inflammation (Colditz 1985; Tedder et al. 1995). The regulation of these interactions occurs through many factors, including macrophage-released neutrophil chemotactic factor (MNCF), which itself exhibits lectin properties (Dias-Baruffi et al. 1993).

Besides the documented roles of endogenous mammalian lectins in the regulation of immune function, other plant lectins, in addition to ScLL, exhibit potent immunomodulatory function. For example, the *Vatairea macrocarpa* galactose/*N*-acetylgalactosamine-binding lectin induces a cellular inflammatory response and induces cultured macrophages to release chemotactic mediators in vitro (Alencar et al. 2007).

In contrast, glucose-mannose and *N*-acetylglucosamine-binding plant lectins inhibit neutrophil infiltration in several experimental models of inflammation (Assreuy et al. 1997, 1999; Alencar et al. 1999). The *N*-acetyl-D-glucosamine-specific lectin from *Araucaria angustifolia* seeds, in addition to being antibacterial, also exhibits anti-inflammatory activity (Santi-Gadelha et al. 2006). Therefore, it is believed that exogenous lectins, which present similar characteristics to endogenous lectins, may provide therapeutic potential in the treatment of inflammatory disease.

One common pathological immune response, which requires chronic treatment, is asthma. In general, asthma severity often correlates with the severity of lung eosinophil infiltration (Wardlaw et al. 1995). Eosinophils contain four principal cationic proteins responsible for inducing lung tissue damage (Abu-Ghazaleh et al. 1992). In addition, eosinophils release cytokines such as TNF- $\alpha$ , granulocyte-macrophage colony-stimulating factor, IL-4, IL-13, and IL-5, as well as chemokines, including CXCL8, CCL5, and CCL11 (Braun et al. 1993; Dubucquoi et al. 1994; Levi-Schaffer et al. 1995; Lim et al. 1996; Moller et al. 1996; Nakajima et al. 1998; Woerly et al. 2002), which exhibit significant proinflammatory and immunoregulatory roles. Eosinophils also elaborate cysteinyl-leukotrienes (Weller et al. 1983; Bandeira-Mello and Weller 2003), which induce bronchoconstriction, mucus hypersecretion, and increased vascular permeability in asthma (Drazen 1998; Holgate and Sampson 2000). As a result, factors that target eosinophil function will probably be useful in the treatment of asthma-related inflammatory diseases.

In this study, ScLL displayed potent anti-inflammatory activity, inhibiting leukocyte accumulation in models of acute and chronic eosinophilia. Tissue eosinophil accumulation during allergic reaction primarily requires the actions of IL-5 (Das et al. 1995; Jonkers and Van der Zee 2005). In addition, this cytokine accentuates terminal differentiation, precursor proliferation, bone marrow release, and activation of mature eosinophils (Sanderson et al. 1985; Clutterbuck and Sanderson 1988; Yamaguchi et al. 1988; Faccioli et al. 1996). Similarly, IL-4, in addition to being a product of TH2 cells, also exhibits autocrine TH2 cell growth and development (Chatila 2004). Importantly, TH2 responses enhance eosinophil immune function. Consistent with this, intense exposure to allergen in the absence of IL-4 results in either failure of TH2 development or poor survival in vivo (Brusselle et al. 1995; Corry et al. 1998). Thus, ScLL may inhibit eosinophil recruitment and activity by reducing IL-4- and IL-5-dependent hypersensitivity responses in chronic airway inflammation. The ability of ScLL to induce increased IFN- $\gamma$  production, a TH1 cytokine that inhibits TH2 immune responses, may also provide some rationale for the decreased pathology observed following administration. In addition, enhancement of the TH1 profile, induced by ScLL administration, may also be useful in the treatment of other diseases, including neoplasia and viral infection, where TH1-driven CD8+ cytotoxic T cell immune responses are desired.

In addition to reducing IL-4 and IL-5 levels and increasing the IFN- $\gamma$  level, ScLL administration also resulted in increased levels of IL-10. Previous studies demonstrate that IL-10 exhibits key regulatory effects on immune activation, including TH2 cell, mast cell (Arock et al. 1996; Royer et al. 2001), and eosinophil activation (Takanaski et al. 1994). IL-10 also

serves as a key effector of regulatory T cell activity (Asseman et al. 1999), suggesting that ScLL may also favorably modulate peripheral tolerance. Interestingly, *Viscum album* lectin also induces IL-10 production in peripheral blood mononuclear cells (Hostanska et al. 1995), suggesting that other plant lectins with therapeutic potential may signal similar responses.

In addition to modulating cytokine production, ScLL administration also altered factors involved in NF- $\kappa$ B activation. NF- $\kappa$ B activation requires phosphorylation and degradation of I $\kappa$ B $\alpha$  (Jobin et al. 1999; Manna et al. 2000). Importantly, ScLL administration inhibited I $\kappa$ B $\alpha$  degradation, suggesting that ScLL reduced NF- $\kappa$ B activation. These results demonstrated that besides the modulation of cytokine production in the TH1/TH2 axis, ScLL also exerts steroid-like effects by inhibiting mechanisms of NF- $\kappa$ B activation.

Accumulating evidence indicates that inflammatory diseases of the respiratory tract are commonly associated with elevated production of NO (Saleh et al. 1998; Ricciardolo 2003). Indeed, in the present work, asthmatic-like animals presented a high production of NO in the lungs and, although acute inhibition of NOS2 activity inhibits asthma-like responses (Landgraf et al. 2005), we did not observe a significant decrease in NO production in the allergic-like mice treated with ScLL. These results suggest that even though ScLL is capable of reducing airway inflammation, this probably does not occur through a reduction in NO in this model. Importantly, although we did not examine other cytokines, including IL-8, leukotrienes, or other chemokines, such as eotaxin, these molecules play an important role in eosinophil recruitment and activation (Lampinen et al. 2004), suggesting that they may also be modulated following ScLL treatment. Future studies will examine the effect of ScLL on these and other factors in order to elucidate further the anti-inflammatory effects of ScLL administration.

ScLL administration by the oral route raised the possibility that ScLL peptides may be responsible for the observed anti-inflammatory effects due to protein degradation during gastric passage. However, ScLL, at the therapeutic concentrations used in this study, displayed resistance to both pepsin degradation and acid-induced denaturation. Previous results demonstrated similar resistance to acid-induced denaturation for plant lectins (Shi et al. 1993). Furthermore, ScLL retained carbohydrate-recognition activity following pepsin treatment and acidification, suggesting that the protein probably enters the proximal intestine functionally intact. The high degree of ScLL glycosylation (Souza et al. 2005) may in part account for its resistance to degradation (Porto et al. 2007). Similar results demonstrated resistance to gastric digestion for other plant proteins, such as the plant proteinase bromelain (Smyth et al. 1961, 1962; Izaka et al. 1972), which also exhibits anti-inflammatory properties (Castell et al. 1997; Hale et al. 2005). However, future studies will be needed to examine whether the carbohydrate-recognition properties of ScLL are indeed responsible for its anti-inflammatory effects.

Current pharmacological therapies of asthma include treatment with antihistamines, glucocorticoids, and  $\beta$ 2-agonists (Barnes 2000; Holgate and Broiede 2003). Of these, glucocorticoids provide the most effective current treatment of asthma, although chronic use results in deleterious side effects (Barnes and Pederson 1993). ScLL may provide an attractive alternative to glucocorticoid administration, as it reduced the

pathology associated with asthma, without inducing side effects, such as pathological changes in the liver observed following glucocorticoid treatment.

In summary, the present results provide the first description of a single factor isolated from ScL extracts that exhibits significant therapeutic potential in the treatment of allergic disease. These results also further highlight plant materials as a resource in the identification of novel therapeutics in the treatment of human disease. Furthermore, these studies reinforce previous results suggesting that plant lectins harbor substantial immunomodulatory potential. Future studies will examine in further detail the effects of ScLL treatment, including possible molecular mechanisms underlying its effects.

## Materials and methods

### Animals

Female BALB/c mice weighing 15–20 g were obtained from the animal facilities of the Faculdade de Ciências Farmacêuticas de Ribeirão Preto, Universidade de São Paulo, Brazil, and were maintained under standard laboratory conditions. All experiments were conducted in accordance with the guidelines of the Animal Care Committee of the university.

### Latex protein extraction

Proteins were extracted from fresh latex by gentle shaking with deionized water, in a 1:5 ratio, for 48 h at 4 °C. The mixture was centrifuged (3500g, 30 min, 4 °C) and filtered through nitrocellulose membranes (0.45 mm pore size; Merck, Göttingen, Germany) to yield a crude extract. Protein concentration was determined according to Lowry et al. (1951), and the extract was stored at –20 °C until utilized (Souza et al. 2005).

### Affinity chromatography

The galactose-binding lectin was purified using a D-gal-immobilized agarose column (Pierce Chemical Company, Rockford, IL) equilibrated with borate buffer solution (BBS, 0.05 M, pH 7.2). Lectin elution was performed using 0.4 M D-gal in BBS (BBS-D-Gal). The effluent was pooled, concentrated, and dialyzed against tris-buffered saline (TBS). Only one round of chromatography was necessary to obtain the pure lectin, although several rounds were used to obtain sufficient quantities to conduct these studies.

### Polyacrylamide gel electrophoresis

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed using 15% polyacrylamide gels according to the discontinuous Tris–glycine system described by Laemmli (1970). Proteins and molecular markers (Amersham Pharmacia Biotech, UK) were run under denaturing conditions and visualized by silver nitrate staining.

### *H. capsulatum* preparation and fractionation of fungus cell wall

The *H. capsulatum* strain was isolated from a patient at the Hospital das Clínicas, Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo. The live mycelia phase was obtained by culturing fungi at 25 °C on Sabouraud dextrose agar plates (Difco, Detroit, MI). Yeast forms of the



fungus were cultured at 37 °C in BHI-agar medium (Difco Laboratories, Sparks, MD) supplemented with 5% sheep blood for 15 days. The cell wall of the dead yeast was disrupted by ultrasonic vibration at 200 W for 3 min. This process was repeated 6 times and extensive disruption was confirmed by microscopy. The disrupted cell wall was collected and washed 3 times with distilled water by centrifugation at 5000g for 5 min. Lipids were extracted by repeated soaking of the walls in chloroform/methanol (2:1, v/v) at room temperature for 2 h with shaking. The extracts were separated by centrifugation at 5000g for 5 min and the insoluble residue was extracted 3 more times as described earlier. The resulting insoluble cell wall residue was fractionated as previously described (Silva and Fazioli 1985), with methanol, followed by acetone and diethyl ether. After drying, the resulting residue was suspended in 1 N NaOH and gently stirred at room temperature for 1 h. After centrifugation at 5000g for 10 min, the supernatants were collected and the procedure was repeated 4 times. The alkaline-insoluble sediment was washed with water until it reached pH 7.0 and then washed with ethanol, followed by acetone and diethyl ether. After drying, the resulting white powder was designated as F1 from *H. capsulatum*.

#### *ScLL treatment in peritonitis induced by F1*

In order to induce leukocyte recruitment into the peritoneal cavity, 100 µg of F1 was suspended in 1 mL of phosphate-buffered solution (PBS) and injected intraperitoneally (i.p.) into the mice. Control animals received PBS. Animals injected with F1 were treated orally (p.o.) (0.3 mL) daily with water or lectin (1 mg/kg). This dose was chosen on the basis of our experience of treatment of mice with other secondary metabolites of plants. The first treatment was conducted 1 h before i.p. injection and the last, 1 h before death. Twenty-four hours after the injection of F1, the animals were submitted to euthanasia and cells from the peritoneal cavities harvested by injection of 3 mL of PBS containing 0.5% sodium citrate (PBS/SC). The abdomens were gently massaged and a blood-free cell suspension was carefully withdrawn utilizing a syringe. Abdominal washings were placed in plastic tubes and total cell counts were performed immediately in a Neubauer chamber. Differential counting was obtained using Rosenfeld-stained cytospin preparations (Faccioli et al. 1990).

#### *Immunization and induction of allergic airway response*

The mice were immunized on days 0 and 7 by subcutaneous injection of 4 µg of OVA (grade III) and 1.6 mg of aluminum hydroxide in 0.4 mL of saline. Two intranasal OVA challenges (days 14 and 21) were performed with 10 µg of OVA in 50 of µL saline delivered into the nostrils under light anesthesia with the aid of a micropipette. The control group consisted of non-immunized mice that received two intranasal instillations of OVA. All analyses were performed 24 h after the last challenge (Russo et al. 2001).

#### *Lectin and dexamethasone treatment in murine model of asthma*

In order to determine the therapeutic effects of lectin in the murine model of asthma, the animals were treated with differential doses of ScLL or dexamethasone from day 20 to 22 (total of three doses) after the first immunization with OVA (day 0). The following protocol was adopted: the mice

immunized (on days 0 and 7) and challenged (on day 14 and 21) with OVA were randomly divided into six groups (six animals/group) and designated OVA (untreated mice), OVA + dexa (mice receiving subcutaneous injections of 1 mg/kg dexamethasone, 0.25 µL), and OVA + ScLL (mice receiving oral lectin by gavage, in the following doses: 0.25, 0.5, 1, or 2 mg/kg in 0.3 mL).

#### *Evaluation of leukocyte influx into the bronchoalveolar space*

The mice were sacrificed by an overdose of sodium pentobarbitone. Subsequently, a polyethylene cannula was introduced into the trachea and PBS–0.5% SC was instilled in three aliquots (0.3, 0.3, and 0.4 mL) in a total volume of 1 mL. Lavage fluid was recovered and placed on ice. Total cell counts were immediately performed in a Neubauer chamber. Differential counts were obtained by using Rosenfeld-stained cytospin preparations. Following centrifugation (405g), the supernatants of BALF were stored at –70 °C for subsequent cytokine determination.

#### *Lung homogenate*

The lungs of the animals were removed on day 22. Tissue was homogenized (Mixer Homogenizer; Labortechnik, Staufen, Germany) in 1 mL of PBS, centrifuged at 405g for 5 min and the supernatants were stored at –70 °C until assayed (Medeiros et al. 2004).

#### *Histopathological analysis*

After BALF collection, the lungs and livers were removed and immersed in 10% phosphate-buffered formalin, processed, and then embedded in paraffin. The tissues were sliced and 5-µm sections were stained with hematoxylin–eosin (HE) for light microscopy examination.

#### *Measurement of cytokines*

Commercially available enzyme-linked immunosorbent assay antibodies were used to measure IL-4, IL-5, IFN-γ, and IL-10 according to the manufacturer's instructions (BD Pharmingen, San Diego, CA).

#### *Preparation of cytosolic fractions*

The lungs were dissected, snap-frozen in liquid nitrogen, and homogenized in a lysis buffer containing 10 mM HEPES, pH 7.4, 2 mM MgCl<sub>2</sub>, 10 mM KCl, 1 mM phenylmethylsulfonyl fluoride, 1 µg/mL leupeptin, 1 µg/mL pepstatin A, 1 µg/mL leupeptin, 1 µg/mL aprotinin, 1 mM sodium orthovanadate, 10 mM β-glycerophosphate, 50 mM sodium fluoride, and 0.5 mM dithiothreitol. After centrifugation (14 000g for 60 min), the supernatant containing the cytosolic fraction was collected. The protein concentration was determined using the Bio-Rad protein assay kit (Bio-Rad, São Paulo, Brazil).

#### *Western blot analysis*

Equivalent amounts of proteins (50 µg) were mixed in a buffer containing Tris 200 mM, glycerol 10%, SDS 2%, β-mercaptoethanol 2.75 mM, and bromophenol blue 0.04%, and boiled for 5 min. Proteins were resolved in a 10% SDS gel by electrophoresis. After transferring to a polyvinylidene fluoride membrane (GE Healthcare, São Paulo, Brazil), the blots were blocked with 5% fat-free dry milk–TBS buffer

overnight at 4°C and then washed with TBS and 5% Tween-20 (TBST). The membranes were incubated for 2 h at room temperature with 1:1000 dilutions of primary antibodies for  $\beta$ -actin or  $\text{I}\kappa\text{B}\alpha$ , (Santa Cruz Biotechnology, CA). Blots were washed 4 times with TBST for 5 min, followed by incubation with 1:80 000 adjusted peroxidase-coupled biotinylated secondary antibodies (Dako Cytomation, CA) for 1 h. The membranes were washed 4 times with TBST for 5 min and then incubated for 30 min with streptavidin–horseradish peroxidase reagent (Dako Cytomation). The transferred proteins were visualized with an enhanced chemiluminescence detection kit according to the manufacturer's instructions (GE Healthcare).

#### Measurement of nitrite concentration

The concentration of nitrite ( $\text{NO}_2^-$ ) in the lung homogenate was determined by a microplate Griess assay as described previously (Liu et al. 1982), using a standard curve ranging from 1 to 200  $\mu\text{M}$   $\text{NaNO}_2$ . Briefly, 100  $\mu\text{L}$  of the supernatants were incubated with an equal volume of the Griess reagent [1% sulfanilamide (Reagen, Rio de Janeiro, Brazil) in 5% phosphoric acid (MERCK, Darmstadt, German) w/v and 0.1% naphthylethylenediamine dihydrochloride (Sigma Aldrich, Manchester, UK) in water, 1:1, v/v] at room temperature. The  $A_{550}$  was determined with a Titertek Multiskan apparatus (Flow Laboratory, Mclean, VA).

#### Proteolysis and acidic treatment of ScLL

The examination of in vitro digestion of the lectin was accomplished largely as outlined previously (Porto et al. 2007). Briefly, ScLL and BSA (positive control) were individually resuspended (15  $\mu\text{g}$  of dried protein) in 10  $\mu\text{L}$  of 0.1 M sodium acetate, pH 4.0. Pepsin (1 mg/mL; Sigma Chemical Co., St Louis, MO), diluted in 0.1 M acetate buffer, pH 4.0, was then added to the sample, providing a pepsin/substrate ratio of 1:30. The reaction was incubated at 37°C and stopped by raising the pH to 8.6 through the addition of 10  $\mu\text{L}$  of 3 M Tris–HCl. All the samples were kept at the same final volume (20  $\mu\text{L}$ ) and visualized by 15% SDS–PAGE.

#### Far-UV CD

Far-UV CD spectra were recorded at 25°C in a Jasco J715 spectropolarimeter (Jasco Corporation, Japan) at the 220–250 nm range, using 1-mm-path-length cylindrical quartz cells with a 250  $\mu\text{L}$  capacity. The sample protein concentration was 0.6 mg/mL in water (native ScLL) or in 0.1 N HCl at pH 2.2 (ScLL–HCl). The spectra were recorded after accumulation of 16 runs. The CD spectra of buffer only were subtracted to eliminate background effects.

#### Statistical analysis

The results are shown as means  $\pm$  SEM and the statistical differences were analyzed by Mann–Whitney non-parametric test. The results were considered statistically significant when  $P < 0.05$ .

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#### Conflict of interest statement

None declared.

#### Abbreviations

BALF, bronco-alveolar lavage fluid; BBS, borate buffer solution; CD, circular dichroism; D-gal, D-galactose; F1, fraction from the cell wall of a dead yeast form of *Histoplasma capsulatum*; HE, hematoxylin–eosin; IFN, interferon; Ig, immunoglobulin; IL, interleukin; i.p., intraperitoneally; NO, nitric oxide; OVA, ovalbumin; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; p.o., orally; SC, sodium citrate; ScLL, *Synadenium carinatum* latex lectin; SDS, sodium dodecyl sulfate; TBS, tris-buffered saline; TBST, tris-buffered saline and Tween-20; TNF, tumor necrosis factor.

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