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Effects of cyclosporin A and a rapamycin derivative (SAR943) on chronic allergic inflammation in sensitized rats

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SUMMARY

Immunomodulators such as cyclosporin A (CsA) and SAR943 (32-deoxorapamycin) inhibit single allergen-induced allergic inflammation such as eosinophilic and lymphocytic infiltration and mRNA expression for interleukin (IL)-4 and IL-5. We examined the effects of CsA and SAR943, administered orally, on asthmatic responses in a rat model of chronic allergic inflammation. Sensitized Brown-Norway (BN) rats were exposed to ovalbumin (OVA) aerosol every third day on six occasions. CsA (5 mg/kg/day), SAR943 (2·5 mg/kg/day) or vehicle (Neoral™) was administered orally, once a day, from days 10 to 21 (a total of 12 doses). We measured eosinophilic and T-cell inflammation in the airways, proliferation of airway cells by incorporation of bromodeoxyuridine (BrdU) and bronchial responsiveness to acetylcholine. CsA had no effects, while SAR943 inhibited airway smooth muscle (ASM, $P < 0{\cdot}05$) and epithelial cell ($P < 0{\cdot}01$) BrdU incorporation, and the number of $CD4^+$ T cells ($P < 0{\cdot}05$), without effects on BHR. ASM thickness was not significantly increased following chronic allergen exposure. Therefore, CsA and SAR943 have no effect on chronic eosinophilic inflammation, while SAR943, but not CsA, had a small effect on the proliferation of ASM and epithelium.

INTRODUCTION

Asthma is a chronic inflammatory airway disease characterized by spontaneous airflow limitation and non-specific bronchial hyperresponsiveness (BHR). Changes to the structure of the airways, in particular alterations to the airway smooth muscle (ASM), may contribute to the irreversible airflow obstruction that is often observed in asthma.[1] Increased numbers of T cells expressing T-helper (Th) type 2 cytokines, such as interleukin (IL)-4 and IL-5, have been identified in the airways of asthmatics.[2,3] Th2 cells may orchestrate asthmatic inflammation by increasing specific immunoglobulin E (IgE) levels through an action of IL-4 on B cells and by increasing the terminal differentiation and activation of eosinophils through the release of IL-5.[4,5] Th2 cells may modulate the development of chronic structural changes in the airways. For example, over-expression of Th2 cytokines (such as IL-13) in the airways of mice leads to features of airway wall remodelling, including ASM hyperplasia,[6,7] and depletion of activated T cells and the elimination of T-cell-specific cytokine production affects the development of pathological changes in airway structure and function.[8]

T-cell immunosuppressants such as cyclosporin A (CsA) have a limited role in the treatment of asthma. CsA has been shown to improve lung function and decrease oral corticosteroid requirements in chronic severe asthmatics and to inhibit the late-phase response following allergen challenge in mild atopic asthmatics.[9–11] At high doses, CsA inhibits BHR and airway wall remodelling after chronic antigen challenge in sensitized cats.[12] CsA may act by suppressing calcineurin, which is important in the signal-transduction pathways necessary for the expression of many cytokines, including IL-2, IL-3, IL-4, IL-5 and granulocyte–macrophage colony-stimulating factor (GM-CSF), and thereby inhibit T-cell activation and proliferation.[13] Rapamycin, another T-cell immunomodulator, acts at a late stage in T-cell activation, and inhibits the proliferation of T cells, whilst CsA is insensitive at this stage of

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Abbreviations: ASM, airway smooth muscle; BHR, bronchial hyperresponsiveness; BrdU, bromodeoxyuridine; CsA, cyclosporin A; OVA, ovalbumin; GM-CSF, granulocyte–macrophage colony-stimulating factor; IgE, immunoglobulin E; IL, interleukin; Th, T helper.

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activation.[14] Rapamycin also has direct effects in inhibiting the proliferation of other cell types, such as epidermal cells and keratinocytes,[15,16] and therefore such immunomodulators may also act directly on structural cells. Because rapamycin exhibits adverse physicochemical properties, its formulation and administration in an appropriate therapeutic form have been difficult. SAR943 (32-deoxorapamycin), a novel rapamycin derivative with immunosuppressive properties, has greater chemical stability in galenical formulation.[17] Fujitani & Trifilieff have reported on the anti-inflammatory properties of SAR943 in a murine model of single allergen challenge;[18] they also demonstrated that SAR943 inhibited ASM cell proliferation *in vitro*.[18] These results contrast with the lack of effect of SAR943 on the allergic inflammation and BHR induced by single allergen exposure in sensitized rats.[19] We have previously investigated the differential effects of CsA and SAR943[17] on chronic allergen-induced airway asthmatic and proliferative responses in the Brown-Norway (BN) rat model of chronic inflammation. We examined the hypothesis that inhibition of T-cell activation and proliferation may result in a reduction of structural cell proliferation and remodelling. In the present study we focused on the effects of these T-cell immunomodulators on ASM and epithelial cell DNA synthesis, ASM thickening and chronic eosinophilic inflammation following repeated allergen exposure in BN rats actively sensitized to ovalbumin (OVA).

MATERIALS AND METHODS

Sensitization and challenges

Pathogen-free, male BN rats weighing 220–250 g (Harlan, Bicester, UK) were sensitized on days 1, 2 and 3 using intraperitoneal (i.p.) injections of 1 mg/kg OVA prepared in 1 ml of 0·9% sterile saline containing 100 mg $Al(OH)_3$ as adjuvant. On days 6, 9, 12, 15, 18 and 21 after the start of sensitization, animals were exposed to either saline or 1% OVA aerosol for 20 min.

Study design

Four groups were studied:

(1) Sensitized, vehicle-treated and repeatedly exposed to saline aerosol (saline group; $n = 8$). The rats of this group received vehicle (Neoral®; Novartis, Horsham, UK), 1 ml orally, once a day, from day 10 to day 21 of the procedure (a total of 12 doses).
(2) Sensitized, vehicle-treated and repeatedly exposed to OVA (OVA group; $n = 8$). The procedures were the same as described above for the saline group, except that the aerosol was 1% OVA.
(3) Sensitized, CsA treated and OVA exposed (CsA group; $n = 8$). The procedures were the same as described above for the saline group. Rats received 5 mg/kg CsA once a day, from days 10 to 21, administered by gavage 2 hr prior to antigen exposure on days 12, 15, 18 and 21 (60 mg/kg in total).
(4) Sensitized, SAR943 treated and OVA exposed (SAR group; $n = 8$): The procedures were the same as described above for the saline group. Rats received 2·5 mg/kg SAR943 once a day, from days 10 to 21, administered by gavage 2 hr prior to antigen exposure on days 12, 15, 18 and 21 (30 mg/kg in total).

All rats were studied 18–24 hr after exposure to either 1% OVA or 0·9% NaCl aerosol.

Bromodeoxyuridine dosing

5-Bromo-2′-deoxyuridine (BrdU; Sigma Chemicals, Poole, UK) was dissolved in dimethylsulphoxide (DMSO) and diluted with sterile water, giving a final concentration of DMSO of $<7\%$. Rats were injected i.p., with 50 mg/kg BrdU in 1 ml of solution, immediately following the allergen challenges on days 12, 15, 18 and 21, and received a second dose 8 hr later (a total of eight injections).

Measurement of bronchial responsiveness to acetylcholine

Bronchial responsiveness was measured 18–24 hr after the final allergen challenge, as previously described.[20] Briefly, rats were anaesthetized, a tracheostomy was performed and lung resistance measured by the method of von Neergard & Wirz[21] using an in-house developed software program (LabVIEW 2; National Instruments, Austin, TX). Increasing half-$\log_{10}$ concentrations of acetylcholine were administered by inhalation for 45 breaths and the lung resistance was measured. The concentration of acetylcholine required to increase baseline resistance by 200% (PC_{200}) was determined by linear interpolation of log concentration–lung resistance curves.

Tissue collection

Rats were killed using an i.p. overdose of sodium pentobarbitone (500 mg/kg). The lungs were rapidly removed and insufflated with OCT Tissue Tek™ mounting medium (Raymond A. Lamb, London, UK) diluted 1 : 1 with phosphate-buffered saline (PBS). Regions of the left and right lung lobes were mounted on cork blocks with the main bronchi uppermost, snap-frozen in melting isopentane and stored at -25°.

BrdU and α-smooth muscle actin immunohistochemistry

Detailed methods have previously been described.[22] Briefly, for the detection of cells undergoing DNA synthesis, cryostat sections were incubated with a primary anti-BrdU monoclonal antibody (mAb) solution (clone BU-1; Amersham International, Bucks., UK). After labelling with a biotinylated rat-adsorbed antiserum to mouse immunoglobulin G (IgG) (Vector Laboratories, Peterborough, UK), BrdU-positive cells were visualized using 3,3-diaminobenzidinetetrachloride solution (Sigma) with glucose oxidase-nickel enhancement to give a black end-product.[23] For the visualization of α-smooth muscle actin, cryostat sections were incubated with a primary anti α-smooth-muscle actin mAb (clone 1A4; Sigma). After labelling with a biotinylated rat-adsorbed antiserum to mouse IgG (Vector Laboratories) the α-smooth-muscle actin staining was visualized using the alkaline phosphatase/anti-alkaline phosphatase method. Nuclei that were not immunoreactive for BrdU were counterstained by application of the fluorescent DNA ligand, 4,6-diamidino-2-phenylindole hydrochloride (DAPI) (Sigma), and mounted under glass coverslips.

Eosinophil major basic protein (MBP) and T-cell immunohistochemistry

Detailed methods have previously been described.[20,22] Briefly, for the detection of eosinophils, we used an IgG1 mAb against human MBP, clone BMK-13 (Monosan, Uden, the Netherlands). The cryostat sections were incubated with BMK-13. After labelling with a biotinylated horse anti-mouse secondary mAb, positively stained cells were visualized using the alkaline phosphatase/anti-alkaline phosphatase method. For staining $CD2^+$, $CD4^+$ or $CD8^+$ T lymphocytes in tissue sections, the sections were incubated with mouse anti-rat CD2, CD4 or CD8 mAb (pan T-cell markers; PharMingen, Cambridge Bioscience, Cambridge, UK). After labelling with a biotinylated goat anti-mouse secondary mAb, positively stained cells were visualized using the alkaline phosphatase/anti-alkaline phosphatase method. All sections were counterstained with Harris Hematoxylin (BDH, Dorset, UK) and mounted in Glycergel (DAKO, Ely, UK). Cellular influx around the five largest airways in each lung section was assessed as the number of positively stained cells in the bronchial submucosa and expressed per mm of basement membrane. MBP-positive cells were enumerated in the lung parenchyma.

Quantification of DNA synthesis and ASM area

Quantification of images was performed as previously described.[20,22] Briefly, DNA synthesis in ASM cells was measured as the number of BrdU-immunoreactive nuclei divided by the total number of nuclei (BrdU + DAPI nuclei) within the α-smooth muscle actin-defined immunoreactive area. Epithelial cell DNA synthesis was measured as the number of BrdU-positive cells per unit length of the epithelium defined by a basement membrane mask. ASM thickness was measured as the total α-smooth muscle actin-immunoreactive area around each airway, expressed per unit length of basement membrane. The five largest airways from a single lung section were used to calculate the DNA synthesis and ASM thickness indices from each treatment group.[22]

Analysis of data

Mean indices were statistically analysed after logarithmic transformation by one-way analysis of variance, followed by *t*-tests with Bonferroni correction, which were used to evaluate significant differences between groups. Values are expressed as means (95% confidence intervals), with *P*-values of $<0{\cdot}05$ considered significant.

RESULTS

Bronchial responsiveness to acetylcholine

Neither CsA nor SAR943 altered body weight and there were no significant differences in the baseline lung resistance (R_L) values following saline challenge in the five experimental groups (data not shown). There was a significant increase in bronchial responsiveness of the sensitized, repeated allergen-exposed and vehicle-treated rats ($-\log PC_{200}$ (M): 2.60; 2.26–2.93. Mean; 95% confidence intervals) compared with sensitized, repeated saline-exposed and vehicle-treated rats ($-\log PC_{200}$ (M): 1.85; 1.55–2.15; $P < 0.001$). Neither CsA ($-\log PC_{200}$ (M): 2.59; 2.43–2.74) nor SAR943 ($-\log PC_{200}$ (M): 2.48; 2.19–2.76) altered BHR (Fig. 1).

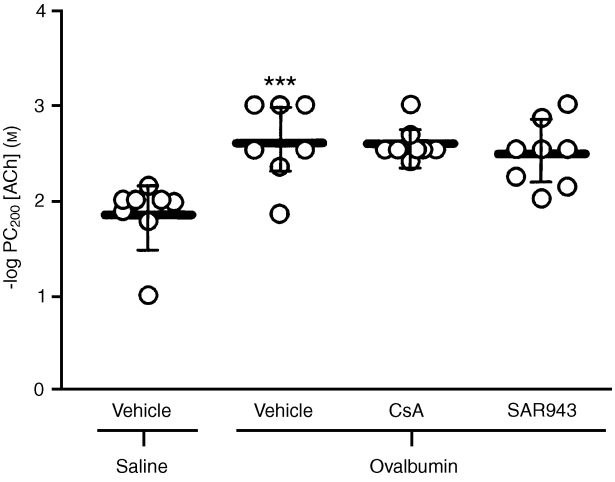

Figure 1. Bronchial responsiveness to acetylcholine in sensitized rats exposed repeatedly to saline or ovalbumin: There was a greater than half-log order increase in the mean -log concentration of acetylcholine required to increase baseline resistance by 200% (PC_{200} value) in rats repeatedly exposed to allergen compared with the saline-exposed group (***$P < 0{\cdot}001$). Neither cyclosporin A (CsA) nor SAR943 attenuated the -log PC_{200} values compared with the vehicle-treated rats. Data are expressed as mean-log PC_{200} values, with bars representing 95% confidence intervals.

ASM and epithelial BrdU indices

There was a significant increase in the ASM cell BrdU index in the sensitized, allergen-exposed and vehicle-treated group (5·24%; 4·08–6·39) compared with the sensitized, saline-exposed and vehicle-treated group (1·77%; 1·18–2·37, $P < 0{\cdot}001$). CsA had no significant effect (3·87%; 2·82–4·92), and SAR943 only a partial effect (2·82%; 1·74–3·90, $P < 0{\cdot}05$; Fig. 2a). Repeated allergen exposure also caused a significant increase in the number of BrdU-positive cells in the epithelium (17·9/mm basement membrane; 14·5–21·2) compared with the sensitized, saline-exposed and vehicle-treated controls (5·1; 4·1–6·2; $P < 0{\cdot}001$). CsA had no effect (18·8, 11·5–26·1), whereas SAR943 inhibited BrdU uptake (10·3; 6·4–14·3, $P < 0{\cdot}01$; Fig. 2b).

ASM thickness

The ASM of the sensitized, allergen-exposed and vehicle-treated group had a greater mean thickness, of 21·6 μm (18·5–24·6), compared with the 18·7 μm (16·4–20·9) of the sensitized, saline-exposed and vehicle-treated rats, but the difference was not significant. Neither CsA or SAR943 had any effect (Fig. 3).

Eosinophil recruitment to the lungs

Repeated exposure to allergen caused a sixfold increase in the number of MBP-positive eosinophils around the airways of

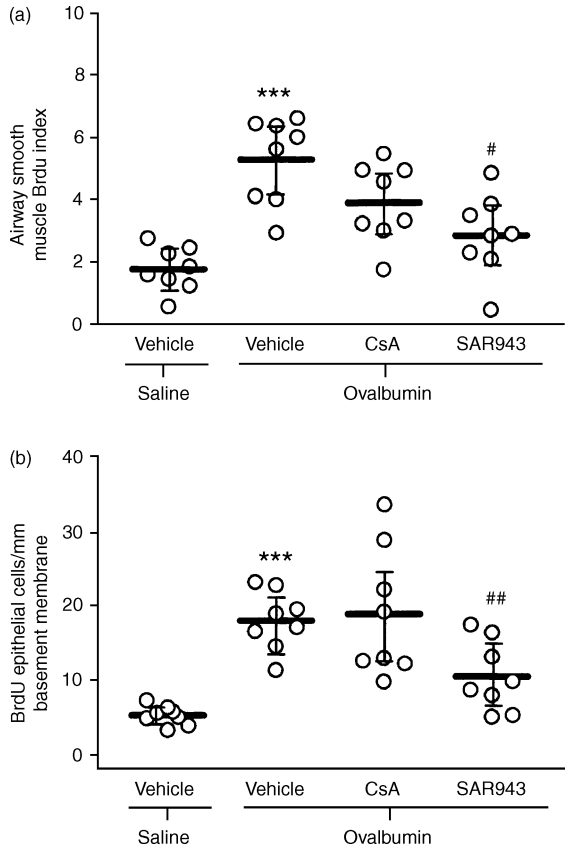

Figure 2. Effects of cyclosporin A (CsA) and SAR943 on airway smooth muscle (ASM) cell (a) and epithelial cell (b) bromodeoxyuridine (BrdU) incorporation following repeated allergen challenge. (a) There was an approximate three-fold increase in the mean BrdU index of the ASM cells of repeated allergen-exposed compared with repeated saline-exposed rats (***$P < 0{\cdot}001$). SAR943, but not CsA, caused significant attenuation of the ASM BrdU index compared with vehicle-treated rats (#$P < 0{\cdot}05$). (b) There was a greater than two-fold increase in epithelial cell BrdU incorporation after repeated allergen challenge (***$P < 0{\cdot}001$ compared with repeated saline-exposed rats). SAR943, but not CsA, caused significant attenuation of epithelial BrdU incorporation compared with vehicle-treated rats (##$P < 0{\cdot}01$). Horizontal bars represent the mean values for each group, with the bars representing the 95% confidence intervals.

sensitized, allergen-exposed and vehicle-treated rats (36·6 cells/mm of basement membrane; 28·9–44·2) compared with the sensitized, saline-exposed and vehicle-treated group (6·0 cells/mm of basement membrane; 3·9–8·2; $P < 0{\cdot}001$). Neither CsA (24·61 cells/mm of basement membrane; 13·43–35·80) nor SAR943 (36·32 cells/mm of basement membrane; 23·09–49·54) attenuated the number of eosinophils (Fig. 4a). Similar results were observed in the parenchyma (Fig. 4b).

T-cell counts

$CD2^+$, $CD4^+$ and $CD8^+$ T-cell counts were increased following chronic allergen exposure. CsA had no significant effect on these cell counts, but SAR943 caused a significant reduction in the number of $CD4^+$ T cells (Fig. 5).

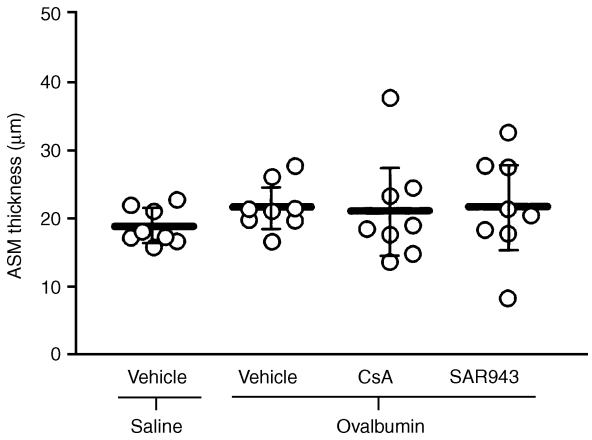

Figure 3. Airway smooth muscle (ASM) thickness after repeated allergen exposure. Following repeated exposure to allergen there was no significant increase in ASM area per unit length of basement membrane compared with rats exposed repeatedly to saline. Both cyclosporin A (CsA) and SAR943 were without significant effect. Data are expressed as mean values, with bars representing 95% confidence intervals.

DISCUSSION

Following repeated allergen exposure of sensitized BN rats, significant increases were detected in both ASM and epithelial-cell DNA synthesis (as measured by BrdU incorporation), as previously reported.[20,22] Treatment of sensitized and repeatedly allergen-exposed rats with a derivative of rapamycin, SAR943, significantly attenuated ASM and epithelial-cell DNA synthesis, which are features of airway remodelling. However, no change in ASM thickness was observed in the present study following repeated exposure to allergen. CsA was without effect on ASM and epithelial-cell DNA synthesis.

Both CsA and SAR943 had no effect on tissue eosinophilic infiltration, while SAR943 reduced the number of $CD4^+$ T cells. Similar results were previously obtained in our laboratory with CsA.[24,25] Because SAR943 concomitantly reduced $CD4^+$ T-cell numbers and decreased ASM proliferation, one may postulate that T cells may be linked to ASM hyperplasia as they can induce ASM cell proliferation.[26]

The dose of CsA employed in our study was effective in inhibiting both alloantibody and cellular immunity responses in various models in the rat.[27–30] Our data are similar to those reported previously regarding the effect of CsA in single-allergen exposure of sensitized BN rats,[24,25] where no inhibition of allergen-induced BHR by CsA was detected, although there was a significant reduction in eosinophils, $CD4^+$ T cells and $CD8^+$ T cells in the airways submucosa.[24] In the present study, CsA caused a trend towards a reduction of eosinophils, T cells and BrdU incorporation into the muscle, but this was not significant. Padrid *et al*. showed a reduction in eosinophilic cellular inflammation and in the thickness of ASM layers in chronic antigen-challenged cats using a higher dose of CsA.[12] It is not clear why there should be a difference in the responsiveness to CsA between these two different chronic models. One possibility is that the differential effects of CsA on cytokine expression may determine the overall response. Therefore, in

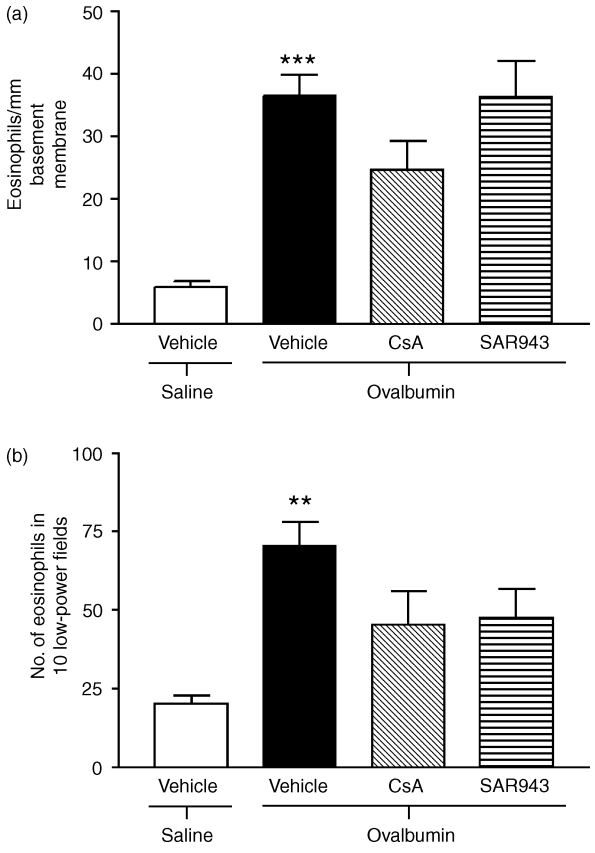

Figure 4. Effects of cyclosporin A (CsA) and SAR943 on eosinophil major basic protein (MBP)-positive eosinophil recruitment to the airways (a) and parenchyma (b) following repeated allergen challenge. (a) There was a significant increase in the number of MBP-positive eosinophils around the airways following repeated allergen exposure of rats compared with repeated saline exposure (***$P < 0{\cdot}001$). Neither CsA nor SAR943 attenuated eosinophil recruitment to the airways as compared with the vehicle-treated group. (b) There was also a significant increase in MBP-positive eosinophil recruitment into the parenchyma of repeatedly allergen-exposed, compared with repeatedly saline-exposed, rats (**$P < 0{\cdot}01$). Neither CsA nor SAR943 altered eosinophil recruitment compared with vehicle-treated rats. Data are expressed as mean values ± standard error of the mean (SEM).

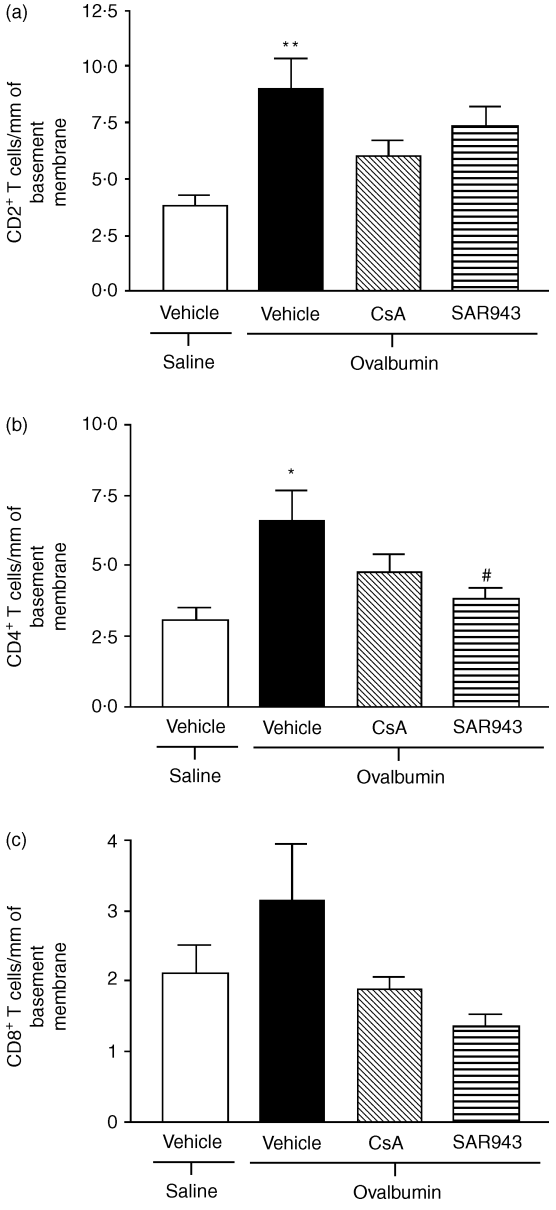

Figure 5. Effects of cyclosporin A (CsA) and SAR943 on $CD2^+$ (a), $CD4^+$ (b) and $CD8^+$ (c) T-cell recruitment to the airways following repeated challenge with allergen. There was a significant increase in the number of (a) $CD2^+$ (**$P < 0{\cdot}01$) T cells and (b) $CD4^+$ (*$P < 0{\cdot}05$) T cells around the airways following repeated allergen exposure of rats compared with repeated saline exposure. Neither CsA nor SAR943 attenuated the recruitment of $CD2^+$ T cells to the airways as compared with the vehicle-treated group (a). SAR943, but not CsA, attenuated the recruitment of $CD4^+$ T cells to the airways, as compared with the vehicle-treated group (#$P < 0{\cdot}05$) (b). Neither CsA nor SAR943 altered $CD8^+$ T-cell recruitment compared with vehicle-treated rats. Data are expressed as mean values ± standard error of the mean (SEM).

our previous study of single allergen exposure, we found that CsA inhibited the expression of Th2 cytokines (such as IL-2, IL-4 and IL-5) in the lung, in addition to the Th1 cytokine, interferon-γ (IFN-γ).[25]

Rapamycin is a macrolide antibiotic and a relatively novel immunosuppressant used in organ transplantation with a 10- to 100-fold stronger potency than CsA in preventing acute rejection of vascularized allograft in animal models.[31,32] In the present study we used a derivative of rapamycin – SAR493 or 32-deoxorapamycin – which has a similar range of action as rapamycin.[17] Rapamycin inhibited growth factor-dependent proliferation of both lymphoid and non-lymphoid cells, including vascular smooth-muscle cells[33] and rat renal mesangial cells, which display functional and morphological features that are similar to those of ASM cells.[34,35] When compared with CsA, rapamycin was much more effective against platelet-derived growth factor-induced vascular smooth-muscle cell proliferation[36] and CsA exerted minimal effects on proliferation of keratinocytes at doses achieved *in vivo* in humans, while rapamycin was effective.[37] Rapamycin has been shown to

inhibit proliferating cell nuclear antigen exposure and blocks the cell cycle in the G1 phase of human keratinocyte stem cells.[16] A direct effect of SAR943 on ASM proliferation has been reported recently.[19] Therefore, in the current study, SAR943 may have had direct inhibitory effects on ASM cell proliferation, but CsA had no effect.

The expression of BHR may be dependent not only on factors affecting muscle phenotype and behaviour, but also on non-muscle components of the allergic response, such as release of mediators that could augment bronchial responsiveness directly without changing muscle phenotype and behaviour. In our model, we have previously shown that both cysteinyl-leukotrienes and endothelin contribute to airway smooth-muscle proliferation, but only cysteinyl-leukotrienes to BHR.[20,38] Fujitani & Trifilieff have recently reported that SAR943, when administered intranasally in mice, inhibited allergen-induced increase in inflammatory cells infiltrating the lungs, epithelial cell proliferation, mucus hypersecretion and associated BHR.[19] It is possible that the lack of greater effect in our model may be related to the more chronic exposure protocol we used or, less likely, to species differences. However, we did find inhibition of ASM proliferation.

In summary, we have shown that the T-cell inhibitor, CsA, and the rapamycin derivative, SAR943, did not inhibit BHR and airway eosinophilic inflammation. However, SAR943 was effective in inhibiting both ASM and epithelial cell DNA incorporation. These studies indicate that rapamycin and its derivatives may have direct antiproliferative effects on structural cells following allergen challenge, irrespective of their T-cell inhibitory effects. The relationship of BHR with ASM is probably very complex.

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