Supplemental information, Hardy et al.

Recombinant follistatin

Follistatin 288 (FS288) was produced using the follistatin expressing plasmid (pSV2HF288), a gift from Professor Shunichi Shimasaki (University of California, San Diego, USA). The FS288 gene was amplified from pSV2HF288 by PCR and sub-cloned into pAPEX3P vector. The pAPEX3P-FS288 plasmid was transfected into 293EBNA cells, and puromycin-resistant cells expanded to form the stable 293EBNA FS cell line. FS288 was purified from conditioned media of cultured 293EBNA FS cells by successive rounds of chromatography through heparin-Sepharose affinity (5 ml Hi-Trap Heparin column, GE Healthcare Bio-Sciences), size exclusion (Superdex 200 prep grade, Hi-load 16/60) and RP-HPLC (Reversed Phase, OD-300, Aquapore ODS, C-18, 7 um, 300 A, 10 cm, 2.1 mm i.d. Brownlee Cartridge Column; PerkinElmer) columns.

Follistatin luciferase bioassay

To assess the ability of recombinant follistatin to inhibit activin activity, HEK293T cells, plated on poly-lysine-coated 24-well plates at a density of 150,000 cells per well, were co-transfected with 50 ng A3-luc (a Smad2-responsive luciferase reporter), 25 ng FAST-2 (a transcriptional co-activator), and 25 ng β-galactosidase (to normalise for transfection efficiency). Transfections were performed under optimized conditions using lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA). 16 h post-transfection, cells were treated with activin-A and increasing doses of recombinant human FS288 (rhFS288) for ~16 h. Cells were harvested in solubilization buffer (1% Triton X-100, 25 mM glycylglycine (pH 7.8), 15 mM MgSO4, 4 mM EGTA, and 1 mM dithiothreitol), and luciferase reporter activity was measured (Victor2 Multilabel Counter; Perkin Elmer, Waltham, MA) and normalized relative to β-galactosidase activity.

Bronchoalveolar lavage, differential counts, and tissue sampling

Methods were as described previously [1]. Blood was collected from the inferior vena cava, and serum separated by centrifugation. Bronchoalveolar lavage (BAL) was performed

with 0.4 ml 1% FCS in PBS followed by three lavages of 0.3 ml. Viable lung-draining lymph node (LN) cells and BAL leukocytes were counted in a hemocytometer. For differential cell counts, BAL cytospots were stained with Giemsa (Merck, Kilsyth, Victoria, Australia), and 200 cells were identified by morphologic criteria. Lungs were formalin fixed prior to paraffin embedding.

Lung tissue digests

Tissue digestion was performed as described [2] with modifications. The right ventricle was perfused with 5 ml Ca^{2+}/Mg^{2+}-free HBSS (Invitrogen #14175095) with 0.01 M EDTA, pH 7.2. Lung and draining LN were chopped with a tissue chopper (Mickle Laboratory Engineering Co. Ltd, Gomshall, Surrey, UK). Tissue fragments were digested in collagenase type III (1 mg/ml; Worthington, Lakewood, NJ, USA) and DNase type I (0.025 mg/ml; Roche Diagnostics, Sydney NSW #1284932) in a volume of 7 ml at 25°C by manual pipetting for 20 minutes. The reaction was stopped by adding one 10^{th} volume of 0.01 M EDTA and mixing for 5 minutes. The cell suspension was filtered through a 70 μm cell strainer (BD Falcon) and underlaid with 1 ml 0.01 M EDTA in FCS prior to centrifugation (350 *g*, 4°C). The cell pellet was resuspended in red cell lysis solution for 3–5 min (#R7757, Sigma-Aldrich, St. Louis, MO, USA), diluted to 10 ml in RPMI and 10% FCS, and underlaid with 1 ml 0.01 M EDTA in FCS prior to centrifugation (350 *g*, 4°C). Cells were resuspended in staining buffer [3% FCS, 3% pooled normal mouse serum, 5 mM EDTA (pH 7.2) and 0.1% Na-Azide in Ca^{2+}/Mg^{2+}-free HBSS], and viable cells counted in a haemocytometer.

Flow cytometry

Non-specific FcR binding was blocked by incubating cells in CD16/CD32 block (BD Biosciences, San Jose, CA, USA). Cells (1×10^6) were surface stained on ice for 20 minutes with the following antibodies: CD3-PE, CD4-V450, CD25-FITC (all from BD Biosciences). Cells were fixed and permeabilized (eBioscience, Inc., San Diego, CA, USA, # 00-5521-00) according to the manufacturer's instructions, prior to intracellular staining with Foxp3-APC (eBioscience). Appropriate isotype control antibodies were used. All dilutions were in

staining buffer (see above). Data was acquired on a LSR II (BD) and analysed on FlowJo (Tree Star, Ashland, OR, USA).

Cytokine ELISPOT

IL-4, IL-5, and IL-13 ELISPOT were performed as described previously [3]. Briefly, ELISPOT plates (Millipore, North Ryde, NSW, Australia) were coated with IL-4, IL-5 (BD Pharmingen, San Jose, CA), or IL-13 antibody (R&D Systems, Minneapolis, MN) and blocked with 10% FCS in RPMI 1640 (Invitrogen, Mt. Waverly, Victoria, Australia). LN cells (0.5×10^6) resuspended in 100 µl RPMI/10% FCS were added to the wells with 10 µl of OVA (final concentration, 25 µg/ml) or RPMI/10% FCS alone; triplicate cultures were used throughout. Plates were incubated for 16 hours at 37°C. Cytokine detection was with biotinylated IL-4, IL-5 (BD Pharmingen), or IL-13 antibodies (R&D Systems) followed by ExtrAvidin–alkaline phosphatase (E-2636; Sigma-Aldrich). Reaction product was developed (170-6432; Bio-Rad, Regents Park, NSW, Australia), and plates were read on an ELISPOT reader (Autoimmun Diagnostika, Strassberg, Germany).

OVA-specific IgE ELISA

OVA-specific IgE was detected as described previously [1]. Briefly, ELISA plates were coated with OVA (10 µg/ml), blocked with 5% skim milk powder/0.05% Tween 20 in PBS for 1.5 hours, and incubated with IgG-depleted serum diluted 1:5 followed by anti-mouse IgE-biotin and streptavidin-peroxidase. Absorbance was read at 490 nm; results are expressed as raw optical density readings minus background (no serum added).

Activin A ELISA

Activin A concentrations were measured using a specific ELISA according to the manufacturer's instructions (Oxford Bio-Innovations, Upper Heyford, Oxfordshire, UK) with modifications as described previously [3-4]. The activin A antibody (E4) was obtained from Professor Nigel Groome (Oxford Brookes University, UK). Note that 1 nM activin A = 25 ng/ml. The limit of activin A detection for serum and BAL fluid samples was 0.01 ng/ml. In some experiments, activin A was measured using a different commercial ELISA kit

according to the manufacturer's instructions (#DY338, R&D Systems). We observe a good correlation between values obtained with these two ELISA systems (data not shown).

Follistatin radioimmunoassay

Follistatin concentrations were measured using a discontinuous radioimmunoassay as described previously [4]. The follistatin rabbit antiserum (#204) used for the immunoassay was developed in house against purified native bovine follistatin. Human recombinant follistatin was used as the standard, and [^{125}I]-rhFS288 was used as the tracer. The assay buffer for the BAL fluid samples was 0.05% BSA/PBS. Note that 1 nM follistatin = 35–45 ng/ml (range due to variable glycosylation and different isoforms). The limit of follistatin detection was 1.04 ng/ml.

TGF-β1 ELISA

TGF-β1 was detected using a TGF-β1 ELISA kit according to the manufacturer's instructions (#DY1679; R&D Systems). Briefly, Costar ELISA plates (Corning Inc., Corning, NY) were coated with TGF-β1 antibody and blocked (5% Tween 20 in PBS with 0.05% NaN_3). Latent TGF-β1 was acid activated with 1 N HCl and neutralized with 1.2 N NaOH/0.5 M HEPES, and samples were adjusted to pH 7.2–7.6. Activated samples and serially diluted TGF-β1 standards were added and incubated for 2 hours. After washing, biotinylated TGF-β1–specific antibody was added for 2 hours. Detection was with streptavidin-horseradish peroxidase, and development was with *o*-phenylenediamine (P5412; Sigma-Aldrich). Absorbance was read at 490 nm. The limit of TGF-β1 detection was 8 pg/ml.

Immunohistochemistry

Paraffin sections were dewaxed and rehydrated, and antigens retrieved by immersing slides in 0.01 M citrate buffer (pH 6.0), heating in a 1,000 W microwave oven (high for 2.5 min, low for 5 min), cooling at 4°C for 20 min, and washing in water for 5 min. Endogenous peroxidase was blocked in 3% H_2O_2 for 10 min, and nonspecific binding was blocked for 1 hour (CAS-block [00-8120; Zymed Laboratories, South San Francisco, CA] and 10% normal

rabbit serum). Sections were incubated with mouse monoclonal antibodies specific for the activin βA subunit (E4; $IgG2_b$) (obtained from Professor Nigel Groome) or follistatin (2E6; IgM) at 10 μg/ml overnight at 4℃. After washing, slides were incubated in rabbit anti-mouse IgG_{2b} peroxidase (61-0320; Zymed) or -IgM peroxidase (61-6820; Zymed) diluted 1:500 for 2 hours for activin A and follistatin primary antibodies, respectively. Slides were washed in Tris-buffered saline (TBS), 0.05% Tween-20 (pH 7.5) and then MilliQ H_2O. Reaction product was developed with a 3,3′-diaminobenzidine tetrahydrochloride substrate kit (00-2014; Zymed), and sections were counterstained in Harris' hematoxylin. All wash steps were in TBS/0.05% Tween-20. Antibodies were diluted in 1% BSA/TBS. Nonimmune mouse antibodies of the appropriate immunoglobulin isotype were used for negative controls (02-6800 and 02-6300 for IgM and IgG_{2b}, respectively; Zymed). For TGF-β immunostaining antigens were retrieved in 0.01 M citrate buffer, and endogenous peroxidise blocked (3% H_20_2) prior to staining with TGF-β1−specific antibody (Santa Cruz Biotechnology, Inc. Santa Cruz, CA U.S.A., sc-146), or rabbit IgG isotype control (sc-2027) using the rabbit ABC staining system (sc-2018). For activin receptor staining antigens were retrieved in 50 mM glycine pH 3.5, and endogenous peroxidise blocked (3% H_20_2) prior to staining with antibodies specific for ActRIB/ALK4 (Santa Cruz, N-20, sc-11984), ActRIIA (Santa Cruz, N-17, sc-5667) and ActRIIB (Santa Cruz, N-16, sc-5665) or goat IgG isotype control.

Image analysis

For analysis of PAS, activin A, follistatin and ActRIB staining, images of lung airways (basement membrane circumference 400-700 μm) were captured using a 40 x objective, and the number of positive cells per mm linear basement membrane calculated using appropriately calibrated Fiji Open Source image analysis software (http://fiji.sc). For TGF-β, activin A, ActRIIA and ActRIIB staining integrated pixel density of peroxidase-positive cells was calculated (http://fiji.sc); this type of analysis gave an almost identical pattern of results to that obtained with the frequency analysis. Thickness of the subepithelial smooth muscle in large airways was measured at right angles across the muscle bundle, at 8 equidistant points around the airway (images captured with the 20 x objective: http://fiji.sc).

Figure legends

Figure S1. Schedule of allergen immunisations and follistatin treatments for chronic allergen challenge models. Mice were sensitised i.p. with OVA/alum, while controls received sal/alum. (a) For the chronic time-course experiments mice were challenged 3 times/week with 5, 25 or 100 µg of OVA i.n., and groups of mice killed at the indicated time-points. (b) For the follistatin instillation studies mice were challenged with 25 µg OVA i.n. or OVA mixed with follistatin (0.05, 0.5 or 5 µg) for 5 wk.

Figure S2. High dose follistatin instillation during chronic allergen challenge causes a small decrease in total numbers of allergen-specific Th2 cytokine producing cells in the lung-draining LN. Mice were sensitised as described in Supplementary Figure 1b, and challenged with OVA, or OVA and follistatin; controls received saline instead of OVA or follistatin. (a–c) Absolute numbers of OVA-specific IL-4, IL-5 and IL-13 producing cells in the lung-draining LN. Mean ± sem, n=6–8 mice/group. **, ***; $p < 0.01$, 0.001, respectively.

Figure S3. Follistatin instillation during chronic allergen challenge inhibits BAL fluid levels of IL-13. Mice were sensitised as described in Supplementary Figure 1b, and challenged with OVA, or OVA and follistatin; controls received saline instead of OVA or follistatin. BAL fluid concentrations of IL-13. Mean ± sem, n=5–7 mice/group. *, **; $p < 0.05$, 0.01, respectively.

Figure S4. Chronic allergen challenge increases numbers of $CD4^+CD25^+Foxp3^+$ Treg in the lung, and this is not affected by follistatin. Mice were sensitised as described in Supplementary Figure 1b, and challenged with OVA, or OVA and follistatin; controls received saline instead of OVA or follistatin. Lung leukocytes were isolated by collagenase/DNase digestion, and gated on viable $CD3^+$ cells. Data shows proportions (a) and numbers (b) of $CD4^+CD25^+Foxp3^+$ cells. Mean ± sem, n=3–4 replicates/group (pools of 2–3 mice per replicate). ***; $p < 0.001$.

Figure S5. Chronic allergen challenge causes loss of activin A immunoreactivity in airway epithelium. Mice were sensitised as described in Supplementary Figure 1b, and challenged with OVA, or OVA and follistatin; controls received saline instead of OVA or follistatin. Formalin-fixed lung sections were stained immunohistochemically with antibody to activin βA subunit. Representative micrographs, original magnification 400 x. (b) Airway epithelial activin A staining intensity in small-medium airways. Mean ± sem, n=3 mice/group.

Figure S6. Chronic allergen challenge causes loss of ActRIIA and ActRIIB immunoreactivity in airway epithelium. Mice were sensitised as described in Supplementary Figure 1b, and challenged with OVA, or OVA and follistatin; controls received saline instead of OVA or follistatin. Formalin-fixed lung sections were stained immunohistochemically with antibodies to ActRIIA and ActRIIB. Representative micrographs, original magnification 400 x. Airway epithelial ActRIIA (b) and ActRIIB (c) staining intensity in small-medium airways. Mean ± sem, n=3 mice/group.

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2. Vremec D, Zorbas M, Scollay R, et al. The surface phenotype of dendritic cells purified from mouse thymus and spleen: investigation of the CD8 expression by a subpopulation of dendritic cells. *J Exp Med.* 1992;**176**:47-58.

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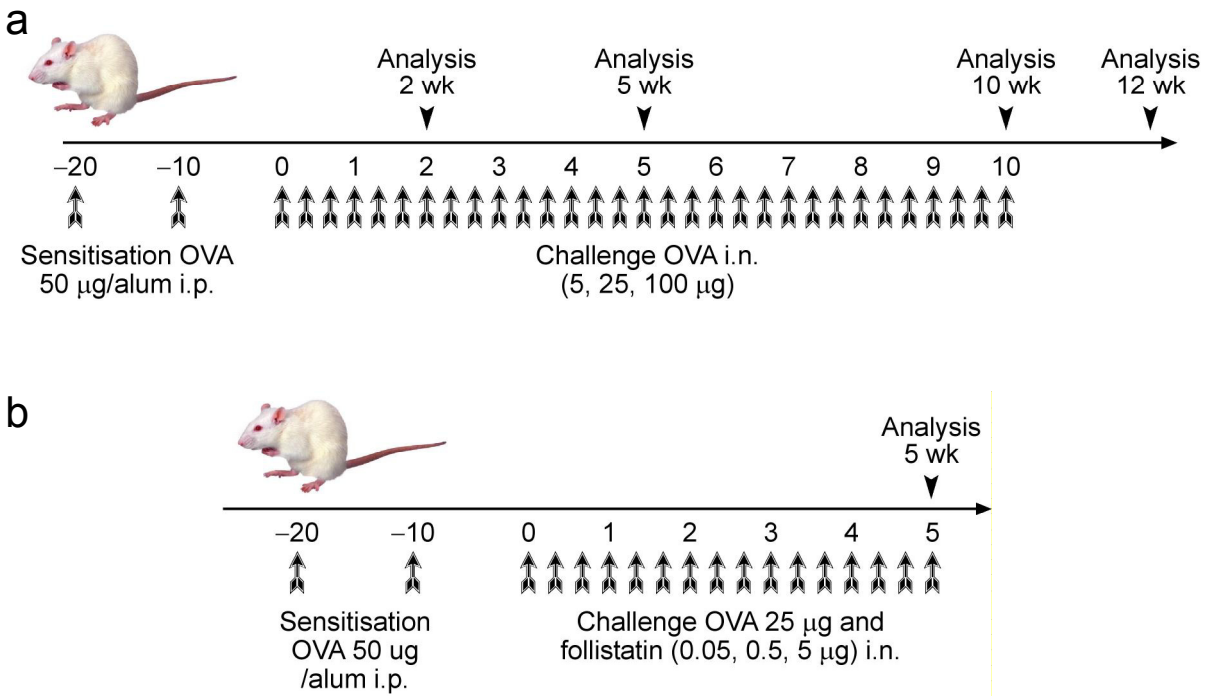
Fig. S1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2 wk
Analysis
5 wk
Analysis
10 wk
Analysis
12 wk
–20
–10
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1
2
3
4
5
6
7
8
9
10
Sensitisation OVA
50 μg/alum i.p.
Challenge OVA i.n.
(5, 25, 100 μg)
b
Analysis
5 wk
–20
–10
0
1
2
3
4
5
Sensitisation
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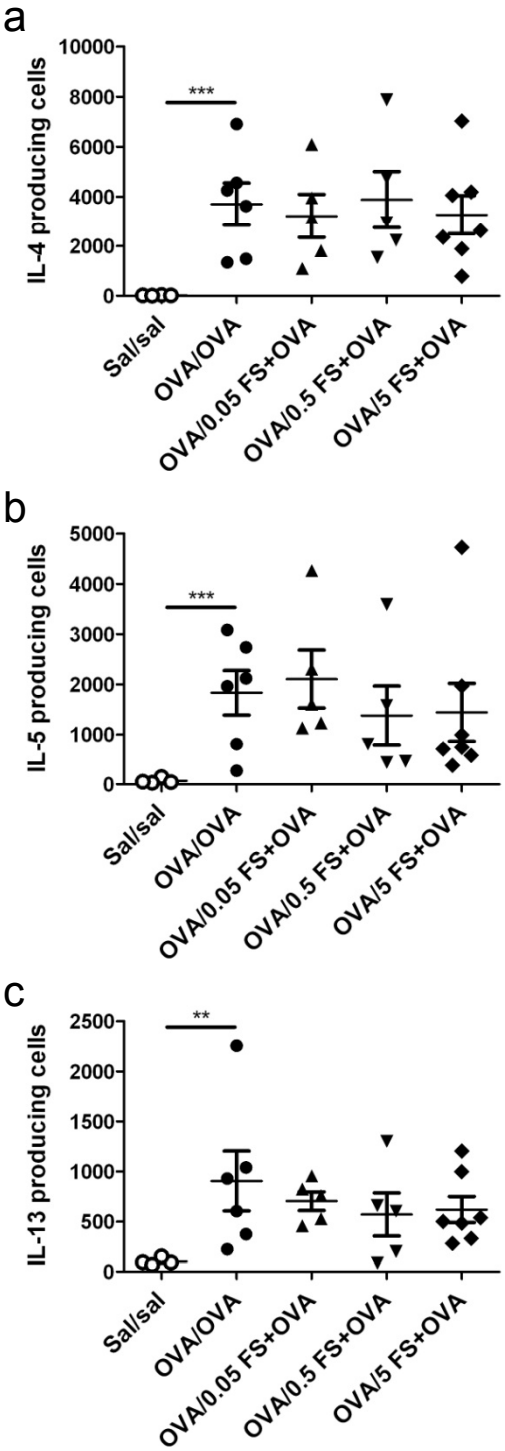
/alum i.p.
Challenge OVA 25 μg and
follistatin (0.05, 0.5, 5 μg) i.n.

Fig. S2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IL-4 producing cells
10000
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2000
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OVA/0.05 FS+OVA
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OVA/5 FS+OVA
b
IL-5 producing cells
5000
4000
3000
2000
1000
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Sal/sal
OVA/OVA
OVA/0.05 FS+OVA
OVA/0.5 FS+OVA
OVA/5 FS+OVA
c
IL-13 producing cells
2500
2000
1500
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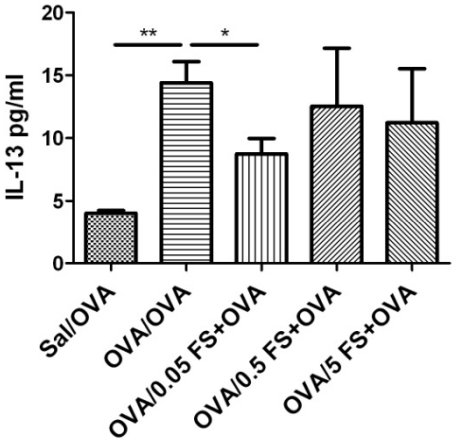
**
Sal/sal
OVA/OVA
OVA/0.05 FS+OVA
OVA/0.5 FS+OVA
OVA/5 FS+OVA

Fig. S3


IL-13 pg/ml
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15
10
5
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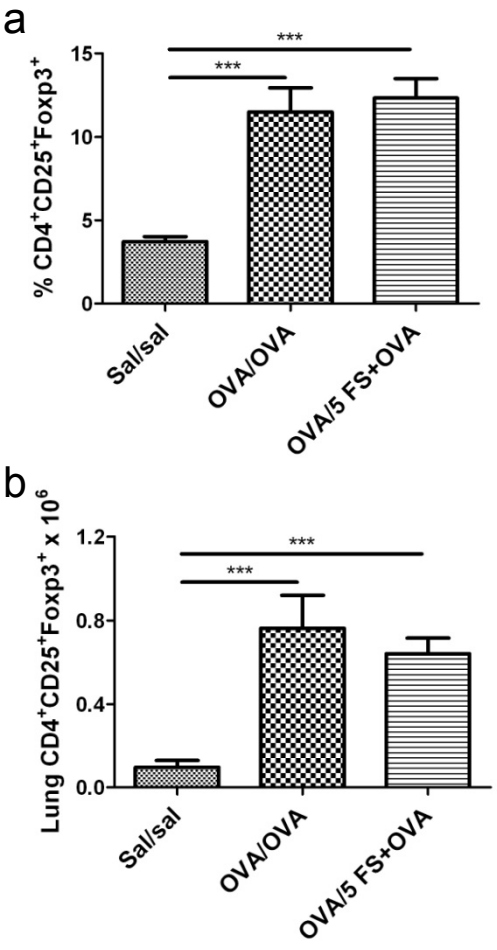
*
Sal/OVA
OVA/OVA
OVA/0.05 FS+OVA
OVA/0.5 FS+OVA
OVA/5 FS+OVA

Fig. S4


a
***
***
15
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0
% CD4+CD25+Foxp3+
Sal/sal
OVA/OVA
OVA/5 FS+OVA
b
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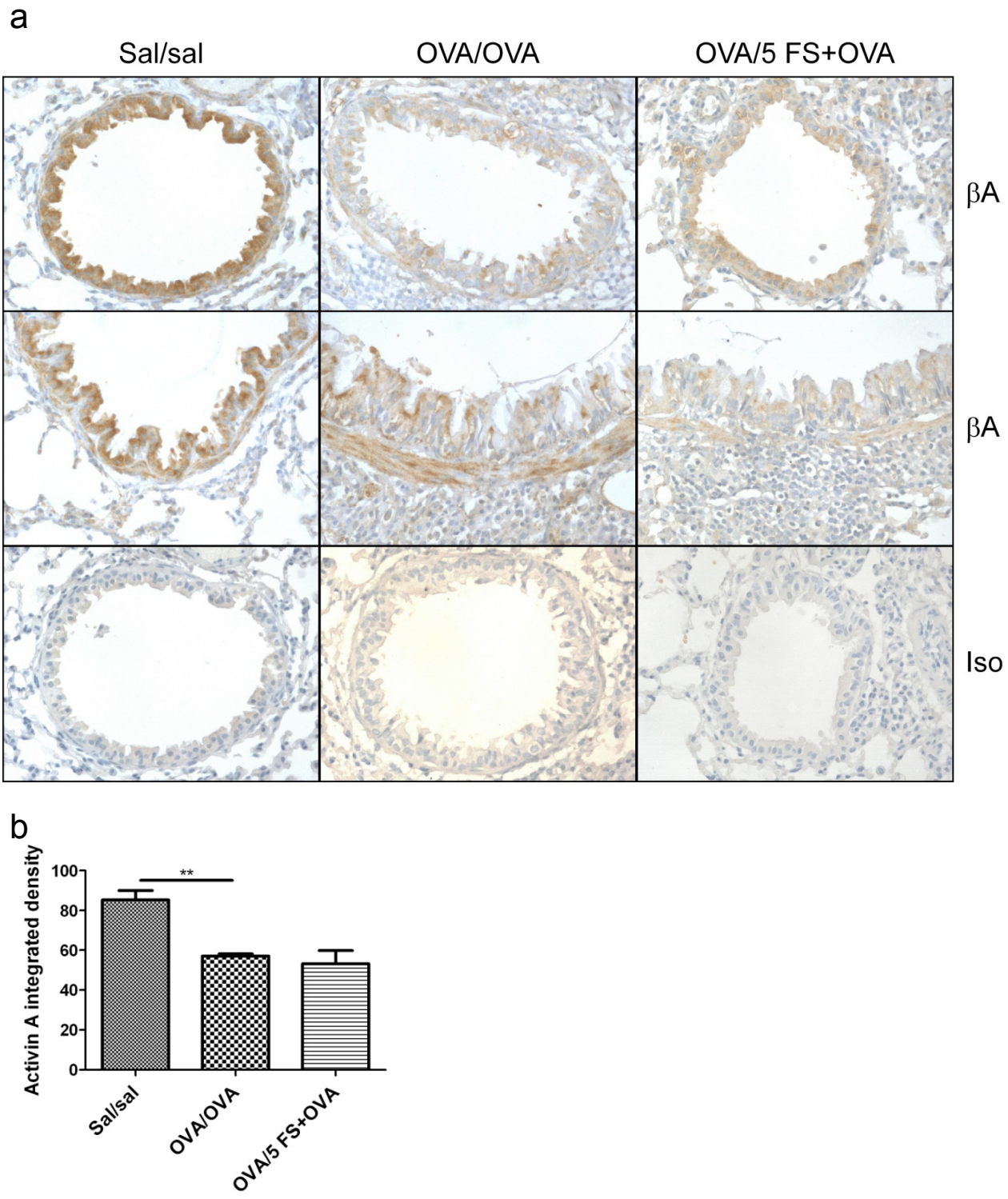
1.2
0.8
0.4
0.0
Lung CD4+CD25+Foxp3+ x 10^6
Sal/sal
OVA/OVA
OVA/5 FS+OVA

Fig. S5


a
Sal/sal
OVA/OVA
OVA/5 FS+OVA
βA
βA
Iso
b
Activin A integrated density
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80
60
40
20
0
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Sal/sal
OVA/OVA
OVA/5 FS+OVA

Fig. S6

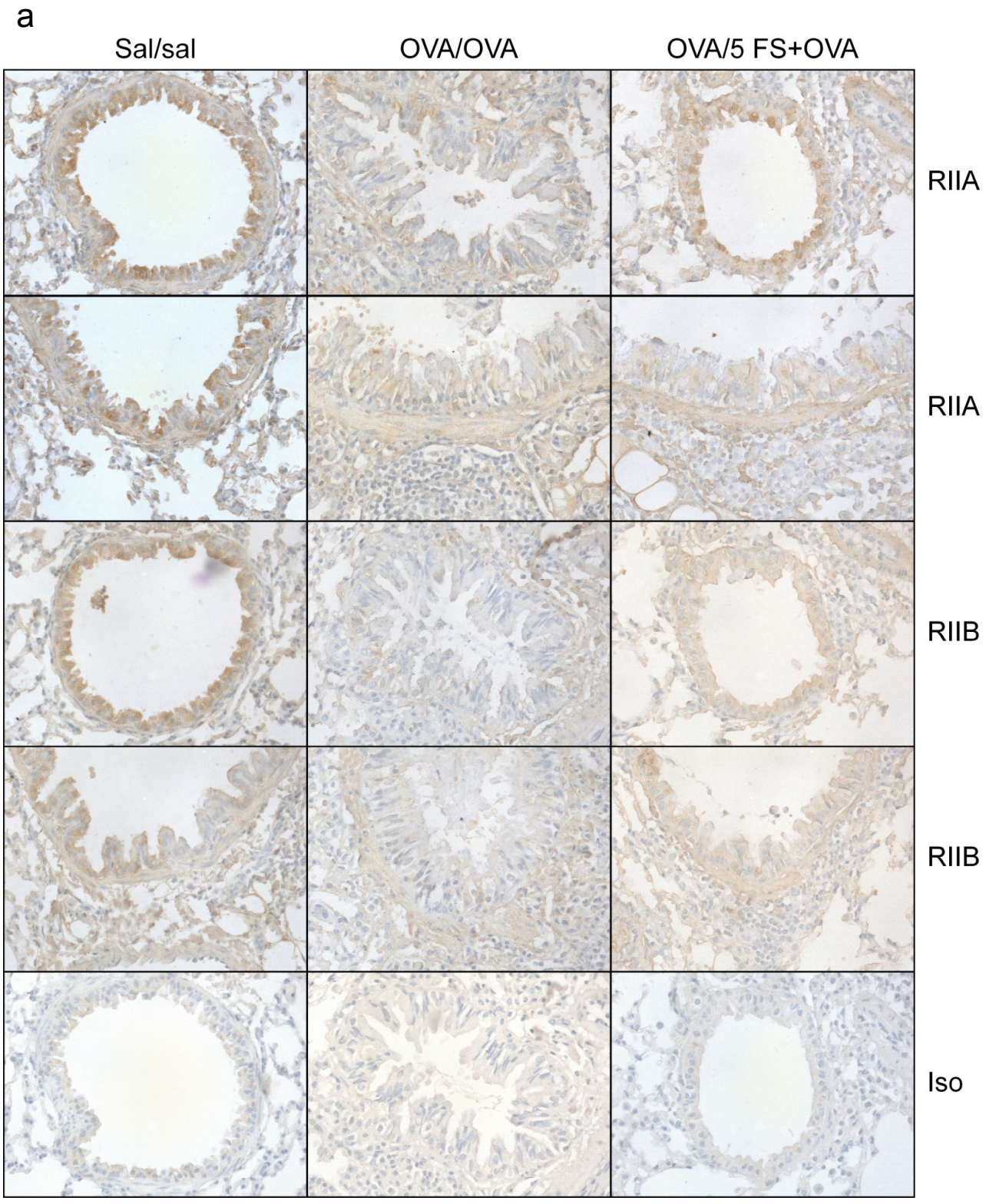
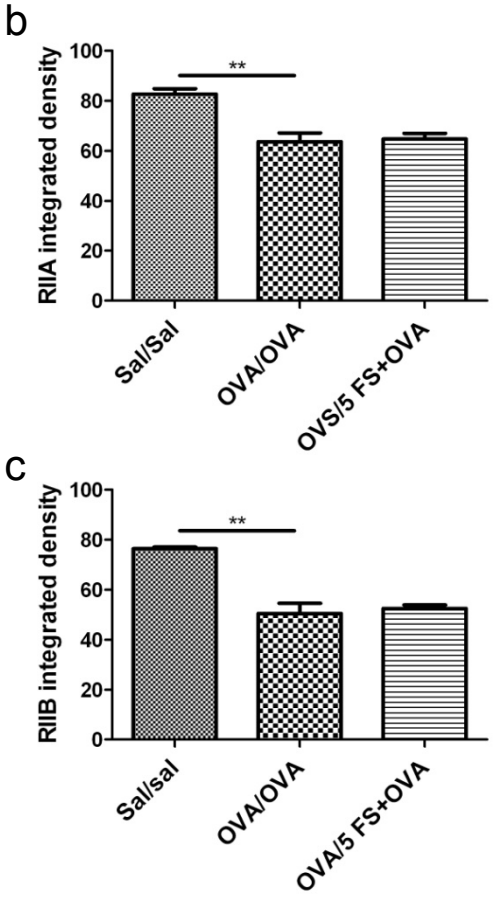


Fig. S6 cont.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RIIA integrated density
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80
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Sal/Sal
OVA/OVA
OVS/5 FS+OVA
c
RIIB integrated density
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Sal/sal
OVA/OVA
OVA/5 FS+OVA