

Suppression of airway eosinophilia by killed *Mycobacterium vaccae*-induced allergen-specific regulatory T-cells

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Allergic asthma is a chronic inflammatory disease and despite the introduction of potent and effective drugs, the prevalence has increased substantially over the past few decades¹. The explanation that has attracted the most attention is the 'hygiene hypothesis', which suggests that the increase in allergic diseases is caused by a cleaner environment and fewer childhood infections²⁻⁴. Indeed, certain mycobacterial strains can cause a shift from T-helper cell 2 (Th2) to Th1 immune responses, which may subsequently prevent the development of allergy in mice⁵⁻⁷. Although the reconstitution of the balance between Th1 and Th2 is an attractive theory, it is unlikely to explain the whole story, as autoimmune diseases characterized by Th1 responses can also benefit from treatment with mycobacteria and their prevalence has also increased in parallel to allergies⁸. Here we show that treatment of mice with SRP299, a killed *Mycobacterium vaccae*-suspension, gives rise to allergen-specific CD4⁺CD45RB^{Lo} regulatory T cells, which confer protection against airway inflammation. This specific inhibition was mediated through interleukin-10 (IL-10) and transforming growth factor- β (TGF- β), as antibodies against IL-10 and TGF- β completely reversed the inhibitory effect of CD4⁺CD45RB^{Lo} T cells. Thus, regulatory T cells generated by mycobacteria treatment may have an essential role in restoring the balance of the immune system to prevent and treat allergic diseases.

We analyzed the effect of SRP299 treatment on the ovalbumin (OVA)-induced eosinophilic airway inflammation using two different immunization protocols. A single OVA aerosol challenge resulted in a significant increase in the number of eosinophils, neutrophils, lymphocytes and macrophages in bronchoalveolar lavage (BAL) fluid from both the short-term (21 d, two immunizations) as well as the long-term (63 d, four immunizations) protocols (Fig. 1a). Subcutaneous (s.c.) injection of 0.1 mg SRP299 (optimal dose; ref. 9 and data not shown) three weeks before the first OVA immunization significantly reduced the airway eosinophilia using both immunization protocols (Fig. 1a). The inhibitory effect of SRP299 treatment on the allergic inflammation was not restricted to eosinophils but also affected other inflammatory cell types (Fig. 1a).

Cultures of OVA-stimulated splenocytes from saline-treated and OVA-immunized mice (short-term protocol) contained substantial amounts of interleukin-4 (IL-4), IL-5 and IL-10 (Fig. 1b),

whereas the antigen stimulation failed to trigger significant production of interferon- γ (IFN- γ), IL-2 and IL-12. Cultures of splenocytes from immunized and SRP299-treated mice contained significantly higher levels of IL-5 and IL-10, whereas the production of IL-4 was not altered. No significant differences were found in IFN- γ , IL-2 and IL-12 levels, irrespective of treatment, suggesting that the mechanisms by which SRP299 induces the suppression of the allergic inflammatory response is not due to a diversion from allergen-specific T-helper cells (Th2) to Th1 cells.

The increased production of IL-5 and IL-10 in SRP299-treated mice without corresponding increase in Th1-associated cytokines may indicate that SRP299 induces the generation of IL-10-producing regulatory cells that might specifically block the allergic response over a long period of time¹⁰⁻¹². We therefore investigated whether splenocytes from SRP299-treated mice could inhibit airway eosinophilia in immunized recipient mice. Intravenous transfer of 2×10^6 splenocytes from OVA-primed SRP299-treated mice to OVA-immunized recipient mice, 24 h before the antigen challenge, significantly reduced OVA-induced airway eosinophilia (Fig. 2a). A less pronounced inhibitory effect was observed in recipient mice injected with splenocytes from SRP299-treated and PBS-challenged mice. By contrast, the transfer of splenocytes from saline-treated and PBS- or OVA-primed as well as from naive mice into OVA-immunized mice failed to inhibit the antigen-induced airway eosinophilia.

We next sought to identify the cell population responsible for the inhibition of the allergic response. We purified T cells from the spleen of OVA-primed SRP299-treated mice and tested their regulatory activity in OVA-immunized recipient mice. Only treatment of mice with 2×10^6 purified T cells significantly reduced the antigen-induced airway eosinophilia (Fig. 2a). Furthermore, only CD4⁺ and not CD8⁺ T cells were able to do so. The regulatory activity of the CD4⁺ T cells from SRP299-treated mice is highly potent as cell numbers as low as 0.25×10^6 significantly suppressed allergen-induced eosinophilic inflammation (Fig. 2b). The BAL fluid of mice immunized and challenged following the transfer of purified CD4⁺ T cells from SRP299-treated mice contained significantly increased levels of IL-10, whereas no significant changes in IL-4 or transforming growth factor- β (TGF- β) were observed (Fig. 2c). In addition, transfer of purified CD4⁺ splenocytes from OVA-primed SRP299-treated mice to OVA-immunized and challenged recipient mice significantly re-

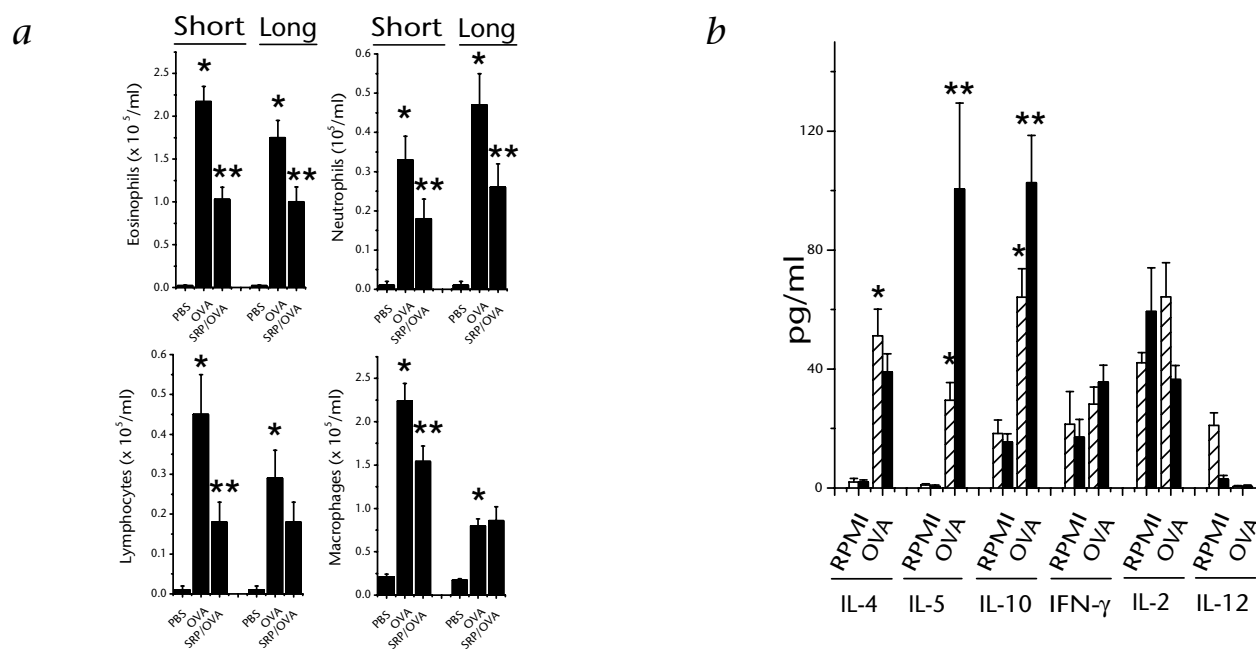


Fig. 1 Effect of SRP299 treatment on the OVA-induced eosinophilic inflammation and cytokine production by splenocytes. **a**, Effect of SRP299 on OVA-induced changes in the cell numbers in mice immunized using a short-term (21 d, two immunizations) and long-term (63 d, four immunizations) protocols. OVA-immunized mice were treated with saline and challenged by aerosol with either PBS (PBS) or OVA (OVA) or treated s.c. with SRP299 21 d before the beginning of immunization and challenged with OVA (SRP/OVA). 48 h after the aerosol PBS or OVA challenge, BAL fluid was collected and cell numbers determined. Data are means $\pm$ s.e.m.; $n = 7$ –10 per group. *, $P < 0.05$; **, $P < 0.05$ versus saline-treated and PBS- or OVA-challenged mice, respectively.

b, Effect of SRP299 on IL-2, IL-4, IL-5, IL-10, IL-12 and IFN- γ release in response to *in vitro* antigen-mediated activation of splenocytes from OVA-immunized mice. Mice were treated s.c. 21 d before the OVA immunization either with saline (▨) or with SRP299 (■) and killed on day 21 of immunization. Splenocytes were incubated with RPMI alone or were stimulated with OVA and the cytokine levels in the cell-free supernatants were determined by ELISA. Data are means $\pm$ s.e.m. of 5–7 mice per group. **, $P < 0.05$; *, $P < 0.05$ versus saline-treated and RPMI- or OVA-stimulated cells, respectively.

duced subsequent allergen induced bronchial hyperresponsiveness (BHR) to methacholine (Fig. 2d). These data clearly demonstrate that SRP299-induced regulatory T cells not only control the extent of the allergen-induced eosinophilic inflammation, but also affect more pathophysiological readouts such as BHR.

In an attempt to verify the specificity of the CD4⁺ regulatory T cells, we treated cockroach extract antigen (CEA)-immunized mice with CD4⁺ T cells generated in mice treated with SRP299 and immunized and challenged with OVA or CEA. Pretreatment with CD4⁺ T cells from SRP299-treated and CEA-immunized, but not from OVA-immunized mice inhibited the CEA-induced eosinophilic inflammation in CEA-immunized recipient mice (Fig. 2e), demonstrating an allergen-specific effector function of these regulatory T cells. This conclusion is further supported by the observation that the transfer of CD4⁺ T cells from SRP299-treated and OVA-immunized mice into CEA-immunized mice significantly inhibited the allergen-induced eosinophilic inflammation when challenged with both CEA and OVA.

Several studies have demonstrated that the regulatory T cells are predominantly found within the CD4⁺CD45RB^{lo}CD25⁺ subpopulation¹³. The flow-cytometric analysis of the distribution of CD45RB and CD25 expressing CD4⁺ T cells obtained from spleens revealed no significant differences between SRP299-treated or saline-treated, OVA-immunized mice (data not shown). However, the transfer of FACS-sorted CD4⁺ T cells expressing low levels of CD45RB (CD45RB^{lo}) from SRP299-treated, OVA-immunized mice into recipient OVA-immunized mice before challenge significantly reduced the allergen-induced eosinophilic inflammation (Fig. 3a). In contrast, the transfer of

high-level CD45RB-expressing CD4⁺ T cells (CD45RB^{hi}) had no suppressive effect, nor did injection of either CD45RB populations from saline-treated mice.

A characteristic feature of regulatory T cells is their ability to inhibit the proliferation of other T cells *in vitro*^{14,15}. Fig. 3b shows that CD4⁺CD45RB^{lo} T cells from SRP299-treated and OVA-immunized mice added to cultures of CD4⁺ T cells from untreated OVA-immunized mice significantly inhibited the antigen-induced proliferative response *in vitro*. The suppression of the proliferative response of OVA-specific T cells induced by CD4⁺CD45RB^{lo} T cells from SRP299-treated mice was completely reversed by adding neutralizing antibodies against IL-10 and TGF- β .

In an attempt to quantify the number of SRP299-induced regulatory T cells, CD4⁺ T cells from both SRP299- or saline-treated OVA-immunized mice were stimulated *in vitro* with the allergen and the number of IL-10 producing cells were analyzed within the different CD45RB populations using flow cytometry. Fig. 3c shows a representative dot plot of membrane CD45RB and intracellular IL-10 double-stained CD4⁺ T cells, demonstrating that the stimulation of these cells with OVA induces the production of IL-10 in both, CD45RB^{lo} and CD45RB^{hi} cells. The quantitative analysis revealed a significantly increased number of IL-10-producing CD4⁺ cells in cultures from SRP299-treated compared with saline-treated mice (data not shown), which was mainly due to an increase in IL-10-producing CD45RB^{lo} cells (Fig. 3d). Qualitatively similar changes were found measuring the IL-10 levels in supernatants of these cultures (Fig. 3e).

Next, we examined the potential role of IL-10 and TGF- β in the regulation of the *in vivo* inhibitory activity. Pre-treatment of

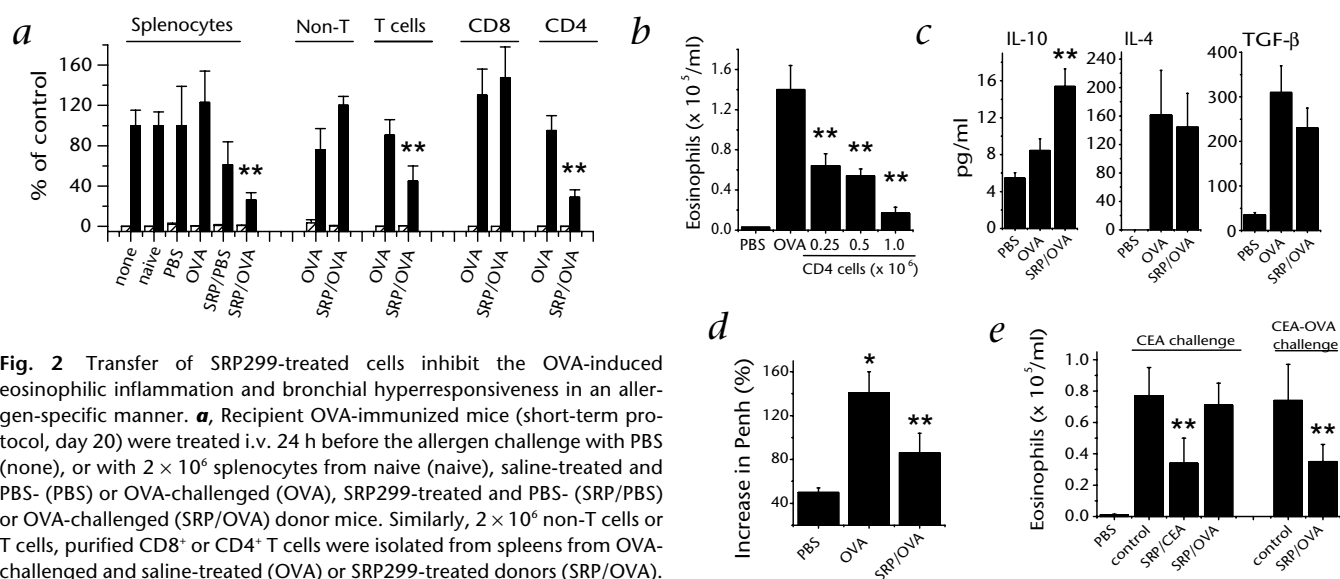


Fig. 2 Transfer of SRP299-treated cells inhibit the OVA-induced eosinophilic inflammation and bronchial hyperresponsiveness in an allergen-specific manner. **a**, Recipient OVA-immunized mice (short-term protocol, day 20) were treated i.v. 24 h before the allergen challenge with PBS (none), or with 2×10^6 splenocytes from naive (naive), saline-treated and PBS- (PBS) or OVA-challenged (OVA), SRP299-treated and PBS- (SRP/PBS) or OVA-challenged (SRP/OVA) donor mice. Similarly, 2×10^6 non-T cells or T cells, purified CD8⁺ or CD4⁺ T cells were isolated from spleens from OVA-challenged and saline-treated (OVA) or SRP299-treated donors (SRP/OVA). Animals were killed after challenge with either PBS (□) or OVA (■). Results are % of OVA-challenged control mice without cell transfer. Data are means $\pm$ s.e.m.; $n = 5-8$ per group. **, $P < 0.05$ versus control OVA-challenged mice (none). **b**, Dose response data of purified CD4⁺ T cells obtained from SRP299-treated and OVA-immunized and challenged mice, adoptively transferred into OVA-immunized recipient mice. Results are number of eosinophils present in BAL fluid obtained 48 h after the challenge with either PBS (PBS) or OVA (all other bars). Data are means $\pm$ s.e.m.; $n = 5-8$ per group. **, $P < 0.05$ versus control OVA-challenged mice (OVA). **c**, BAL fluid IL-10, IL-4 and TGF- β levels in OVA-immunized mice challenged with PBS (PBS) or OVA (OVA) following transfer of no cells or purified CD4⁺ T cells (1×10^6 cells/ml) from SRP299 treated mice as described above (SRP/OVA). Data are means $\pm$ s.e.m.; $n = 5-8$ per group. **, $P < 0.05$ versus control OVA-challenged mice (OVA). **d**, Antigen-induced

bronchial hyperreactivity to inhaled methacholine measured as increase in Penh from PBS- (PBS) or OVA-challenged mice (OVA) following transfer of none or purified CD4⁺ T cells (1×10^6 cells/ml) from SRP299-treated mice as described above (SRP/OVA). Data are means $\pm$ s.e.m.; $n = 10$ per group. **, $P < 0.05$ versus control OVA-challenged mice. **e**, Mice pre-immunized with CEA were i.v. treated with PBS (control) or with 1×10^6 CD4⁺ T cells from SRP299-treated and OVA-immunized and challenged mice (SRP/OVA) or SRP299-treated and CEA-immunized and challenged mice (SRP/CEA). BAL was performed 48 h after the intranasal challenge with CEA alone (CEA challenge) or with OVA and CEA (CEA-OVA). For the latter, mice received an aerosol challenge of OVA followed by an intranasal challenge with CEA, 6 h later. Data are means $\pm$ s.e.m.; $n = 5-7$ per group. **, $P < 0.05$ versus PBS-treated and CEA-challenged mice.

recipient mice with neutralizing antibodies to either IL-10 or TGF- β significantly reduced the CD4⁺CD45RB^{lo} T-cell-mediated suppressive effect, suggesting that both cytokines are required for the regulation of the eosinophilic inflammation by regulatory T cells (Fig. 3f). Indeed, treatment with antibodies against both IL-10 and TGF- β completely reversed the inhibition due to SRP299-induced OVA-specific CD4⁺CD45RB^{lo} T cells. In contrast, neither the anti-cytokine nor the isotype-matched control antibodies had any effect on the OVA-induced inflammation in control mice. Taken together, these findings demonstrate that SRP299 treatment gives rise to regulatory CD4⁺CD45RB^{lo} T cells, which display a specific protective effect on allergic pulmonary inflammation through IL-10 and TGF- β production.

Down-regulation of allergic responses by *mycobacteria* *in vivo* has been observed in numerous cases. However, the mechanism by which *mycobacteria* inhibit the inflammatory response remains unclear and various mechanisms, such as the induction of Th1 cytokines such as IFN- γ , have been proposed⁵. However, we were unable to demonstrate any significant changes in IFN- γ production following SRP299 treatment. In addition, the inhibitory effect was not confined to eosinophils but also affected all other cell types, suggesting that mechanisms other than simple changes in the balance between Th1 and Th2 must be responsible for the observed effects.

It is now well established that regulatory T cells can inhibit harmful immunopathological responses, but many key aspects, such as their antigen specificities, the cellular and molecular pathways involved in their development and mechanism of ac-

tion remain unresolved. Different regulatory T-cell populations have been described in both the murine and human systems¹³. For example, regulatory T cells induced *in vitro* produce high levels of IL-10 but not IL-4, do not proliferate after stimulation and inhibit antigen-specific proliferation and cytokine production in a cell contact-dependent manner¹⁶. In addition, IL-10-producing regulatory T cells were shown to actively modulate a Th2-type response and immunoglobulin E (IgE) production¹⁷. Moreover, the protective mechanism by which CD4⁺CD45RB^{lo} regulatory T cells inhibit the development of inflammatory bowel disease or prevent graft rejections in adoptive transfer experiments is critically dependent on the production of IL-10 and TGF- β (refs. 18,19). The regulatory T-cell described here is characterized as CD4⁺CD45RB^{lo} T-cell subset producing and/or inducing the production of IL-10 and TGF- β . It is capable of inhibiting antigen-mediated proliferative responses and specifically suppresses allergen-induced eosinophilic inflammation. The phenotype and cytokine profile is therefore in line with the regulatory T cells described elsewhere¹³. Furthermore, treatment with SRP299 gives rise to the development of newly generated, allergen-specific regulatory T cells. This was clearly shown in the experiments where the adoptive transfer of purified CD4⁺ T cells from SRP299-treated, OVA-immunized mice into CEA-immunized and challenged mice had no effect on the eosinophilic inflammation, whereas the double challenge with both CEA and OVA induced a significant inhibition. The IL-10 and TGF- β dependency of the observed inhibition was clearly demonstrated using neutralizing antibodies. In contrast, however, only small

increases in IL-10, but not TGF- β were found in BAL fluid or cell cultures from SRP299-treated mice. It is reasonable to assume that data generated with neutralizing antibodies best reflect the underlying mechanisms as cytokine levels in supernatants and diluted BAL fluid do not account for cell-cell interactions, temporal relationships between production and utilization or responses specific to the target organ.

In conclusion, regulatory CD4⁺ T cells generated by SRP299 treatment may have an essential role in restoring the balance of the immune system to prevent allergic diseases and offer advantages over treatments aimed at inducing a shift from an allergen-specific Th2 to Th1 response. Preliminary clinical studies with *M. vaccae* treatment in adults with asthma and rhinitis or in children with atopic dermatitis demonstrated clinical benefits as measured by reduction in use of rescue medication, severity of disease or inhibition of the allergen-induced late-phase response^{20–22}. In contrast, however, another study using lower doses of *M. vaccae* in asthmatics did not show any beneficial clinical effects²³. All patients in this study were prescribed inhaled glucocorticosteroids and it is possible that the induction and possible effector function of regulatory T cells might be suppressed by this treatment. Moreover, our studies also demonstrated that allergen exposure is absolutely required to generate allergen-specific regulatory T cells. Therefore, one of the critical elements determining success or failure of a clinical study might be the level and duration of natural exposure to allergens during

the treatment period. The new insights into the mechanisms of action of SRP299 treatment as described here should help to design better clinical studies and to understand the dysregulation of the immune response resulting in allergic diseases.

Methods

Animals. Female BALB/c mice (20–25 g, 5–6-wk-old) were obtained from Harlan (Oxon, UK). All experiments complied with the Home Office 1986 Animals Scientific Act and were approved by the NHRC Animal Welfare Committee.

Immunization and airway challenge. Short-term model: mice were immunized intraperitoneally (i.p.) on days 0 and 14 with 10 μ g OVA (Grade V, Sigma) or CEA (Hollister-Stier Laboratories, Spokane, Washington) in 0.2 ml of alum (Serva, Heidelberg, Germany). On day 21, OVA-immunized mice were exposed for 20 minutes to an aerosol of OVA (50 mg/ml) or PBS. CEA-immunized mice were challenged with 10 μ g CEA in 50 μ l PBS or PBS alone administered intranasally. Long-term model: mice were immunized i.p. on days 0, 14 and 42 and s.c. on day 56 with 10 μ g OVA in 0.2 ml of alum and challenged with OVA on day 63 as described above.

Treatment with SRP299. Mice were treated s.c. with 0.1 mg SRP299 (sterile vials containing a suspension of heat-killed *M. vaccae* at 10 mg/ml, SR Pharma) in 200 μ l saline or with saline alone three weeks before OVA immunization.

Bronchoalveolar lavage (BAL) fluid. Animals were i.p. anesthetized with 60 mg/kg pentobarbitone sodium. The trachea was cannulated and BAL was collected by injecting 0.4 ml PBS three times into the lung. Total cell count was determined and differential cell counts were performed on cytospin slides stained with Diff-Quik (Baxter Dade AG, Duding, Switzerland) counting 200 cells. BAL cytokine levels were assessed using ELISA kits (R&D Systems, Oxon, UK). The detection limits were the following: IL-2 (3 pg/ml), IL-4 (2 pg/ml), IL-5 (7 pg/ml), IL-10 (4 pg/ml), IL-12 (4 pg/ml), IFN- γ (2 pg/ml) and TGF- β (7 pg/ml).

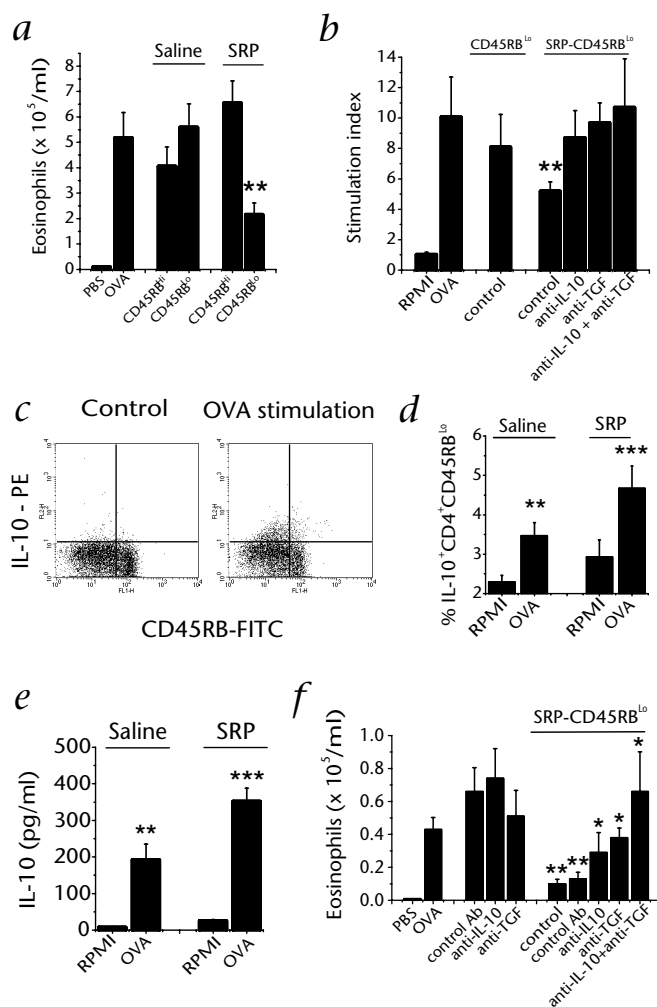


Fig. 3 The inhibitory effect of regulatory T cells resides within the CD4⁺CD45RB^{lo} population and is mediated by IL-10 and TGF- β . **a**, Adoptive transfer of FACS-sorted CD4⁺CD45RB^{hi} or CD45RB^{lo} (0.4×10^6 cells/mouse) T cells from saline- (saline) or SRP299-treated (SRP) and OVA-immunized mice into OVA-immunized recipient mice 24 h before PBS (PBS) or OVA (OVA and all other bars) challenge. Data are means $\pm$ s.e.m. of 9–12 mice per group. **, $P < 0.05$ versus OVA-challenged mice without cell transfer. **b**, *In vitro* proliferative response of OVA-stimulated CD4⁺ T cells from OVA-immunized mice following addition of 1×10^5 purified CD4⁺CD45RB^{lo} T cells from saline-treated (CD45RB^{lo}) or SRP299-treated (SRP-CD45RB^{lo}) and OVA-immunized mice in the absence (control) or presence of neutralizing anti-IL-10 or anti-TGF- β or a combination. Results are SI (^3H -thymidine incorporation induced by OVA/ ^3H -thymidine incorporation in cultures without OVA). Data are means $\pm$ s.e.m. of 3 individual cultures. **, $P < 0.05$ versus OVA-stimulated cultures in the absence of cocultured CD45RB^{lo} cells. **c**, Representative FACS dot plot of double immunofluorescence stained CD4⁺ T cells (FITC-conjugated anti-CD45RB antibodies and intracellular staining with phycoerythrin-conjugated anti-IL-10 antibodies) without (control) or following *in vitro* OVA stimulation. **d**, Quantitative analysis of IL-10 producing, CD4⁺CD45RB^{lo} expressing cells from saline-treated (saline) or SRP299 treated (SRP) and OVA-immunized mice in non-stimulated (RPMI) or OVA (OVA) stimulated cultures. Data are means $\pm$ s.e.m. of cultures from 7–10 mice per group. **, $P < 0.05$; ***, $P < 0.05$ versus RPMI and OVA-stimulated cultures from saline-treated mice, respectively. **e**, IL-10 levels in supernatant from the same cultures as described in **d**. **f**, OVA-immunized mice were treated with PBS or 0.4×10^6 purified CD4⁺CD45RB^{lo} T cells from SRP-treated and OVA-immunized mice (SRP-CD45RB^{lo}). In addition, some of the mice were pretreated with either PBS (control), isotype-matched antibodies (control Ab), anti-IL-10, anti-TGF- β or a combination of anti-IL-10 and anti-TGF- β antibodies. BAL fluid was analyzed 48 h after the PBS (PBS) or OVA challenge. Data are means $\pm$ s.e.m. of 6–8 mice per group. **, $P < 0.05$ versus OVA-challenged mice without cell transfer and antibody treatment; *, $P < 0.05$ versus CD45RB^{lo} transfer control.

Cell transfer. 24 h after PBS or OVA challenge (day 22), spleens from saline- or SRP299-treated mice were collected. Single-cell suspensions were prepared by passing cells through a cell strainer. Purified T-lymphocytes, CD4⁺ or CD8⁺ fractions were prepared by magnetic cell sorting (MACS; Miltenyi Biotec, Bergisch Gladbach, Germany). Briefly, splenocytes were incubated with CD90 (Thy1.2), CD4 (L3T4) or CD8a (Ly-2) microbeads, respectively, and desired cells were separated by positive selection¹². The resulting populations were >95% pure as determined by FACS analysis. CD4⁺ T cells were further separated into CD4⁺CD45RB^{lo} and CD4⁺CD45RB^{hi} cells using FITC-conjugated monoclonal antibodies against CD45RB (16A, BD, Oxford, UK) and sorted by flow cytometry (MoFlo, Cytomation). All populations were >99% pure on reanalysis. 24 h before the antigen challenge (day 20), mice were intravenously (i.v.) injected with 50 µl PBS containing the indicated number of the desired cell population.

In vitro cytokine production and proliferation. Splenocytes from SRP299- or saline-treated, OVA-immunized mice (day 20) were cultured at 2×10^5 cells/culture in RPMI supplemented with 10% FBS, 2 mM L-glutamine, 100 µg/ml streptomycin, 100 U/ml penicillin (all from GIBCO, Paisley, UK) at 37 °C, 5% CO₂, in the presence or absence of 100 µg/ml ovalbumin for 72 h. Supernatants were then collected and cytokine levels determined by ELISA.

Purified CD4⁺ T cells (2×10^5 cells) were stimulated by OVA (100 µg/ml) in the presence of T cell-depleted, irradiated (4,000 rad) splenocytes. CD4⁺CD45RB^{lo} T cells from SRP299 or saline-treated mice were added to OVA-primed CD4⁺ T cells in the presence or absence of 60 ng/ml anti-IL-10 (JES052A5, BD) or 10 ng/ml anti-TGF-β (chicken IgY, R&D Systems) antibodies. Proliferation was assessed by [³H]thymidine incorporation (0.5 µCi/well) during the last 16 h of culture and results are expressed as stimulation index (SI, counts of OVA stimulated/counts of non-stimulated cultures).

Intracellular cytokine staining. 2×10^5 splenocytes were cultured with or without OVA for 3 d. For the last 16 h of culture, 3 µM monensin and 5 µg/ml brefeldin A (Sigma) were added. CD4⁺ T cells were then isolated by positive selection using MACS and stained for CD45RB expression with FITC-anti-CD45RB antibodies. Cells were then fixed, permeabilized (Fix&Perm kit, Caltag) and stained for intracellular IL-10 with phycoerythrin-anti-IL-10 antibody (BD).

Determination of BHR. 24 h after challenge, unrestrained conscious mice were placed in a whole-body plethysmographic chamber (BUXCO, EMKA Technologies). After 2 min stabilization, an aerosol of methacholine (Sigma) at 30 mM was delivered for 1 min. The airway resistance was expressed as Penh (enhanced pause)²⁴.

In vivo treatment with antibody. Immunized mice (day 20) were injected i.p. with 0.6 mg of rat IgG1 against mouse IL-10, JES052A5, or with its matched isotype control, LO-DNP-2 (provided by M. Goldman) or with 33 µg of chicken IgY anti-TGF-β or with its matched isotype control or with saline, 2 h before the i.v. injection of 4×10^5 CD4⁺CD45RB^{lo} T cells. BAL was performed 48 h post-challenge.

Statistical analysis. Data were analyzed using microcomputer programs for one-way ANOVA followed by Student's *t*-test for unpaired values. *P* < 0.05 was considered significant. Results are expressed as means ± s.e.m.

Acknowledgments

We thank J. Lee, A. Nicholls and G. Dubois for technical support.

Competing interests statement

The authors declare competing financial interests: see the website (<http://medicine.nature.com>) for details.

RECEIVED 4 FEBRUARY; ACCEPTED 1 APRIL 2002

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