

***Mycobacterium vaccae* induces a population of pulmonary CD11c⁺ cells with regulatory potential in allergic mice**

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The hygiene hypothesis proposes that common, harmless microorganisms, present throughout our evolutionary history, have helped to develop immunoregulatory mechanisms that prevent inappropriate immune responses by the host. Using a mouse model of allergic pulmonary inflammation, we report that treatment with an ubiquitous saprophytic mycobacterium, *Mycobacterium vaccae*, significantly reduces allergic inflammation by decreasing type 2 responses such as eosinophilia and IL-4 expression. Rather than observing an increase in type-1 cytokine expression, we found elevated production of IL-10 in the lungs suggesting a role for regulatory T cells. Since induction of these cells may be dependent on APC, we investigated the effects of *M. vaccae* treatment on pulmonary CD11c⁺ cells. Increased levels of IL-10, TGF- β and IFN- α mRNA were detected in CD11c⁺ cells from *M. vaccae*-treated allergic mice. We propose that *M. vaccae*-induced CD11c⁺ cells have a potential regulatory role at the site of inflammation through their secretion of immunomodulatory cytokines.

Key words: *M. vaccae* / CD11c⁺ / Regulatory T cells / Pulmonary inflammation / Hygiene hypothesis

Received	14/10/03
Revised	9/12/03
Accepted	8/1/04

1 Introduction

Allergic asthma has been defined as a disease of immunodysregulation, where the pathology is a direct consequence of excessive type-2 immune responses to allergens in the lungs. Disease is associated with increased secretion of type-2 cytokines such as IL-4, IL-5 and IL-13, which among other things, promote enhanced IgE levels, induce pulmonary eosinophilic inflammation and increase mucus production in the airways [1]. The recent dramatic rise in the prevalence of allergies in developed countries was at first blamed on an imbalance of the immune system which, lacking exposure to type-1 inducing infections and hence a Th1 cytokine environment, was prone to develop extensive type-2 responses to harmless allergens [2]. Upon closer inspection, however, it has become apparent that diseases associated with excessive type-1 immune responses, such as type-

1 diabetes, multiple sclerosis and inflammatory bowel disease, have been rising simultaneously [2, 3]. Immunopathologies characterized by inappropriate Th1 or Th2 responses to self-antigens, gut content or common allergens, may be caused by a lack of regulatory immune mechanisms such as defective regulatory T cells (Tregs) in the host [4]. Indeed, severe deficiency in Tregs, as seen in patients suffering from a genetic disorder characterized by mutations in the transcription factor Foxp3, which is required for Tregs development, results in a fatal autoimmune and inflammatory syndrome in humans [5, 6]. Similar results have been observed in the Foxp3-mutant scurfy mouse model, which suffers from a rapidly lethal autoimmune syndrome [7–9].

Several theories have been put forward to explain the increased prevalence of asthma and allergies in developed countries. The most widely accepted is Strachan's hygiene hypothesis [10], which in its most current form proposes that the increase in asthma observed in developed countries is due to a decrease in exposure to once common harmless organisms [11–13]. For example, saprophytic mycobacteria, once ubiquitously found in mud and water, have been present throughout our evolutionary history, and have played a critical role in shaping our

[DOI 10.1002/eji.200324659]

Abbreviations: Tregs: Regulatory T cells **BAL:** Bronchoalveolar lavage

immune system. In particular, they may instruct the immune system on how to appropriately deal with avirulent microbes. In contrast to virulent pathogens, which require a prompt immune response, exposure to harmless microorganisms may trigger regulatory mechanisms that prevent a potentially harmful immune response from being mounted to these “Old Friends” [13, 14]. Once in place, these mechanisms may also be used by the host to prevent inappropriate responses to harmless antigens. In effect, these “Old Friends” act as adjuvants for induction of regulatory responses. For example, recent studies have shown that childhood exposure to harmless organisms such as routinely occur on farms, protects against the development of severe symptoms of allergic pulmonary inflammation [12, 15].

Triggering mechanisms such as the induction of Tregs through exposure to these “Old Friends” may be exploited therapeutically to prevent the development of imbalanced immune responses to common allergens or self-antigens. In experimental models of pulmonary inflammation, a saprophytic mycobacterium, *Mycobacterium vaccae*, has shown particular therapeutic potential. Treatment with *M. vaccae* significantly reduces murine allergic pulmonary inflammation by inducing CD4⁺ CD45RB^{low} Tregs that secrete IL-10 and TGF- β [16, 17]. These cells are allergen-specific, and upon passive transfer can protect recipient allergic mice from airway inflammation by significantly reducing eosinophilia in the lungs [17]. In clinical studies, treatment with *M. vaccae* has shown encouraging results in human patients suffering from atopic asthma and atopic dermatitis [18, 19].

It remains to be determined whether *M. vaccae* influences the development of other cells with regulatory functions. For instance, induction of Tregs is likely to be dependent on APC triggered by certain external stimuli. In this study we investigated whether *M. vaccae* acts on pulmonary APC, such as CD11c⁺ cells. Interest in these cells was raised by recent reports suggesting that pulmonary dendritic cells (DC) from mice exposed to respiratory antigen produced IL-10. In the presence of this cytokine, DC stimulate the development of CD4⁺ Tregs which also produce IL-10, mediate tolerance and prevent the development of airway hyperreactivity and asthma [20, 21]. We report that treatment with *M. vaccae* significantly reduces pulmonary inflammation in the lungs of allergic mice. This beneficial effect is not due to increased type-1 cytokine expression. Rather, it correlates with the development of CD11c⁺ cells which produce high levels of immunoregulatory cytokines such as IL-10, TGF- β and IFN- α . These results show that *M. vaccae* plays a critical role in CD11c⁺ APC induction, which may lead to the previously reported allergen-specific Treg-mediated protection. This is the first study to show

that exposure to a non-pathogenic organism such as *M. vaccae* can induce CD11c⁺ APC with regulatory potential.

2 Results

2.1 *M. vaccae* treatment inhibits pulmonary cellular inflammation in allergic mice

Following sensitization with ovalbumin and alum, intratracheal challenge with ovalbumin induced a dramatic influx of cells in the lungs as measured by the number of cells recovered in the bronchoalveolar lavage (BAL) fluid. This cellular infiltrate was caused by the allergen challenge, as allergic mice challenged with saline showed no symptoms of pulmonary inflammation. Allergen-challenged allergic mice treated with *M. vaccae* showed a significant reduction in the total cellular infiltrate compared to mice treated with saline (Fig. 1A).

Allergic pulmonary inflammation was associated with a large influx of eosinophils, neutrophils, lymphocytes and monocytes/macrophages. We found that mice treated with *M. vaccae* presented a significant reduction in the number of eosinophils recovered in the BAL fluid. The decreased eosinophilia did not result in neutrophilia or in an increase of monocytes or macrophages at the site of inflammation (Fig. 1B). These results suggested that pretreatment of allergic mice with *M. vaccae* significantly reduced the severity of the cellular inflammation following allergen challenge.

2.2 *M. vaccae* treatment reverses the localized Th2 response associated with allergy without switching to a Th1 response

Allergic inflammation is characterized by an excessive type-2 response. We measured total IgE levels in allergic mice and found that even though levels were significantly increased compared to negative control mice, there were no significant differences between treatment groups (763 $\pm$ 3 ng/ml in saline-treated compared to 726 $\pm$ 1 ng/ml in *M. vaccae*-treated mice, data representative of two separate experiments). Hence, we decided to investigate the immune response at the site of inflammation as this is most relevant for disease pathology. Ovalbumin challenge in allergic mice induced a significant increase in IL-4 mRNA levels in whole lung tissue, compared to allergic mice challenged with saline (Fig. 2A). To determine whether *M. vaccae* influences the cytokine milieu developed in allergic mice we measured mRNA levels of type-1 and type-2 cytokines. Treatment with *M. vaccae* significantly lowered the amounts of IL-4

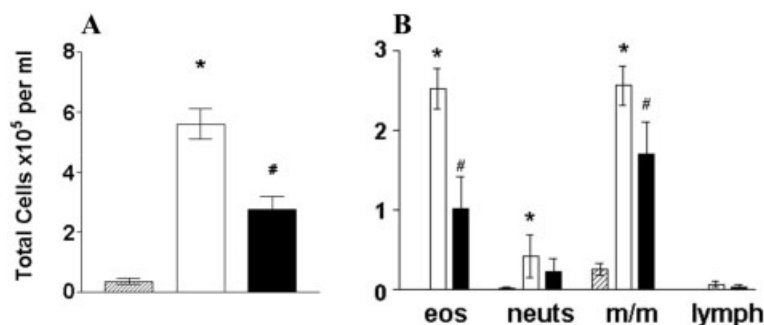


Fig. 1. Treatment with *M. vaccae* reduces both the total number of cells recovered in the BAL fluid (A) and the influx of eosinophils, neutrophils, and monocytes/macrophages in the BAL fluid (B) of allergic mice challenged with ovalbumin. Animals were treated with *M. vaccae* (black bar) or saline (white bar). Allergic mice were challenged with ovalbumin to induce pulmonary inflammation. As negative control, saline treated allergic mice were challenged with saline (shaded bar). Data are expressed as mean $\pm$ SEM of 4–8 mice per group. * $p < 0.01$ compared with negative control. # $p < 0.01$ compared with saline-treated, ovalbumin-challenged mice. These results are representative of at least three separate experiments.

message in lung tissue, compared to treatment with saline (Fig. 2A). As the decrease in IL-4 may be due to an increased type-1 environment, IFN- γ and IL-12 mRNA

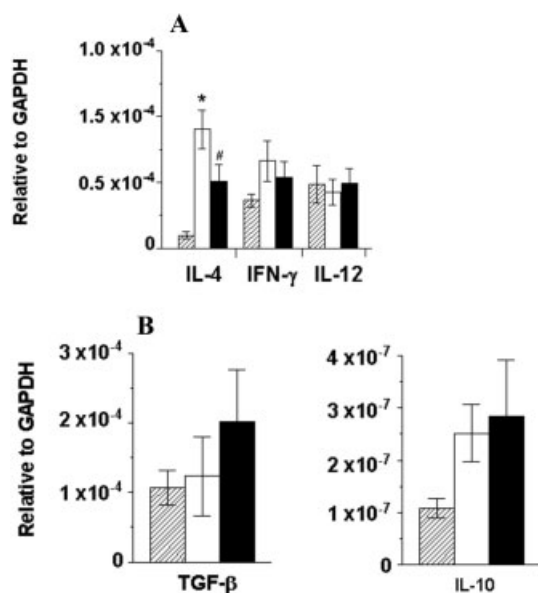


Fig. 2. Treatment with *M. vaccae* reduces IL-4, but not IFN- γ and IL-12 message (A) and does not alter expression levels of IL-10 and TGF- β message (B) in whole lung tissue of allergic mice. Animals were treated with *M. vaccae* (black bar) or saline (white bar). Mice were challenged with ovalbumin to induce pulmonary inflammation. As negative control, saline treated allergic mice were challenged with saline (shaded bar). Data are expressed as mean $\pm$ SEM of 4–8 mice per group. * $p < 0.01$ compared with negative controls. # $p < 0.05$ compared with saline-treated, ovalbumin-challenged mice. These results are representative of at least three separate experiments.

levels were measured in whole lung tissue. We found no significant increase in the message level for either IFN- γ or IL-12 regardless of treatment (Fig. 2A). Similar results were obtained for IL-12 and IFN- γ message in cells recovered in the BAL fluid (data not shown). These findings suggested that the less predominant type-2 response at the site of inflammation in mice treated with *M. vaccae* was not caused by a switch to a type-1 cytokine environment.

2.3 *M. vaccae* treatment leads to an increase in IL-10 secretion into the BAL

To determine whether the decrease in IL-4 mRNA in the lungs correlated with an increase in immunoregulatory cytokines, we measured IL-10 and TGF- β mRNA levels in whole lungs. We found that both cytokines were expressed to similar levels and detected no significant differences between saline and *M. vaccae*-treated animals (Fig. 2B). We measured protein levels of these two cytokines in the BAL fluid. Whereas we found no evidence of any changes in TGF- β protein levels regardless of treatment (1095 $\pm$ 70 pg/ml in saline-treated compared to 1330 $\pm$ 225 pg/ml in *M. vaccae*-treated mice, data representative of two separate experiments) we found that *M. vaccae* treatment did significantly increase IL-10 protein levels in the BAL fluid of allergic mice (Fig. 3).

2.4 *M. vaccae* treatment induces the development of a population of CD11c⁺ cells, characterized by increased expression of immunoregulatory cytokines at the site of inflammation

Due to the large and varied cellular populations found in whole lungs, data on cytokines expression may be difficult to interpret. Thus we focused on CD11c⁺ cells,

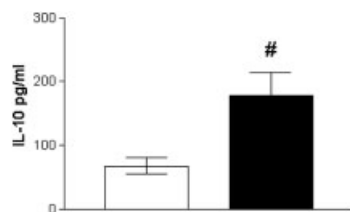


Fig. 3. Treatment with *M. vaccae* significantly alters IL-10 protein levels in the BAL fluid of allergic mice. IL-10 protein levels are increased in the BAL fluid of *M. vaccae* treated animals (black bar) compared to those in the BAL fluid of saline treated mice (white bar). Data are expressed as mean $\pm$ SEM of 4–8 mice per group. [#] $p < 0.05$ compared with saline-treated, ovalbumin-challenged mice. These results are representative of at least three separate experiments.

which are known to produce IL-10 and are reported to play a critical role in the induction of Tregs [20, 21]. To determine whether treatment with *M. vaccae* effects the cytokine expression of CD11c⁺ cells, we isolated CD11c⁺ cells from mice treated with either saline or *M. vaccae* and compared their cytokine expression. In addition to IL-10 and TGF- β , we determined IFN- α expression as this cytokine is characteristic of DC with regulatory functions [22, 23]. We found that *M. vaccae* treatment significantly increased the expression of not only IL-10 and TGF- β but also of IFN- α mRNA in CD11c⁺ cells (Fig. 4). To confirm a specific effect of *M. vaccae* on immunoregulatory cytokines, we measured IL-12 expression in the

CD11c⁺ population. No significant difference was reported in IL-12 levels, regardless of treatment (Fig. 4).

3 Discussion

The effects of treatment with *M. vaccae* in an animal model of allergic pulmonary inflammation were evaluated. In accordance with previous studies, we report that this treatment dramatically ameliorates classic symptoms of allergic inflammation by reducing total cellular infiltration and eosinophilia in the lungs of allergic mice. The beneficial effects of *M. vaccae* are not dependent on an increase in type-1 cytokines, rather they correlate with production of IL-10. In addition, we describe a population of pulmonary CD11c⁺ cells that express high levels of the immunoregulatory cytokines IL-10, TGF- β and IFN- α following treatment. This is the first report to show that a preparation of heat-killed mycobacteria induces the development of a specific population of APC considered to have regulatory functions.

M. vaccae improves the pulmonary inflammation induced by allergen challenge in allergic mice. The decrease in type-2 responses exemplified by the reduced pulmonary eosinophilia, is not associated with an increase in type-1 responses in the lungs. We report no changes in the expression levels of IL-12 and IFN- γ mRNA regardless of treatment. This confirms previous work by Zuany-Amorim and colleagues who showed in a

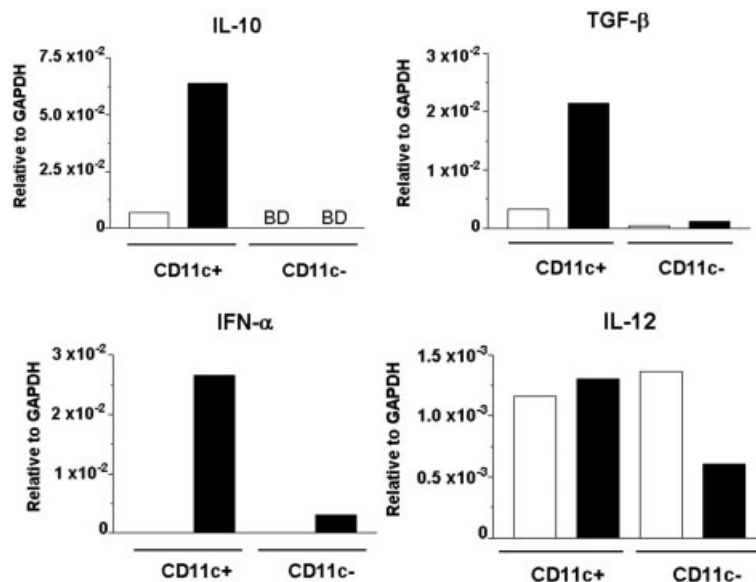


Fig. 4. Treatment with *M. vaccae* increases expression levels of IL-10, TGF- β and IFN- α but not IL-12 mRNA from CD11c⁺ cells separated from lung tissue of allergic mice. Animals were treated with *M. vaccae* (black bar) or saline (white bar). Mice were challenged with ovalbumin to induce pulmonary inflammation. Lungs were pooled and CD11c⁺ cells separated by positive selection. RT-PCR analysis was done in duplicate and data expressed relative to the housekeeping gene GAPDH. BD, below detection limit. These results are representative of at least three separate experiments.

similar model that the beneficial effects of *M. vaccae* are independent of IFN- γ [16]. Hence, rather than affecting the Th1/Th2 balance, *M. vaccae* regulates the allergic host immune responses through the induction of Tregs [17]. Indeed, passive transfer of *M. vaccae*-induced CD4⁺ CD45RB^{low} cells prevents development of allergic responses in mice [17]. We were not surprised by the failure of *M. vaccae* to reduce total IgE levels in allergic mice soon after treatment. Other groups have made similar observations [16, 24]. The lack of effect on IgE may be explained by a recent study showing the prolonged survival of memory plasma cells in specific areas of the bone marrow and spleen [25]. Hence, changes in IgE levels may only be observed during longer follow up periods.

Our data support previous reports showing that *M. vaccae* treatment is associated with the preferential production of immunoregulatory cytokines such as IL-10, TGF- β and, as reported here, IFN- α [17, 24]. Despite an increase in IL-10 protein levels in the BAL fluid of *M. vaccae*-treated mice, no significant changes in IL-10 and TGF- β mRNA were detected in whole lung of allergic mice. This inconsistency may be due to the more varied and heterogeneous cell types found in lung tissues compared to BAL fluid. Hence, the similar mRNA levels detected in whole tissue may mask significant differences in specific cell populations. It is vital, therefore, to determine the effects of *M. vaccae* on defined cell populations at the site of inflammation. Since induction of Tregs might depend upon specific APC, we focused our attention on pulmonary CD11c⁺ cells. Previous studies have shown that following intra-nasal exposure to antigen, these cells express high levels of IL-10 mRNA. The presence of IL-10 influences T cell development as antigen-specific CD4⁺ T cells cultured *in vitro* in the presence of CD11c⁺ cells acquire a Treg phenotype. Further work has also showed that adoptive transfer of IL-10-producing CD11c⁺ cells induces unresponsiveness in recipient mice. Thus, there is convincing evidence to suggest that these cells play a critical role in the regulation of the immune responses in the lungs [21, 26].

We found that *M. vaccae*-induced CD11c⁺ cells express high levels of IL-10, TGF- β and IFN- α mRNA. These cytokines play a significant role in the differentiation and effector function of Tregs [22, 27, 28]. In particular, IL-10 and TGF- β not only reciprocally stimulate production of the other but may also modulate each other's functions [29, 30]. It has been hypothesized that in the presence of IL-10 and TGF- β murine T cells become non-responsive and acquire the capacity to suppress T cell responses [27, 31, 32]. Suppression is mediated either by expression of tolerogenic molecules on the T cell surface, for example CTLA-4, or through the production of immuno-

suppressive cytokines such as IL-10 and TGF- β [33–35]. IFN- α has recently been shown to also have immunomodulatory properties. This cytokine upregulates IL-10 production by DC and may act in synergy with this cytokine to promote the generation of T cells with immunosuppressive properties [22, 23, 36]. In addition, it can promote survival of suppressive T cells by preventing deletion of anergic cells [37].

The role of APC in the lungs, and particularly CD11c⁺ cells, is complex. Under certain conditions pulmonary DC induce strongly inflammatory responses to allergen and may be involved in the pathology associated with asthma [38]. In spite of these observations it has become apparent that DC play a largely immunoregulatory role in the lungs. They may directly modulate pulmonary immune responses by production of indoleamine 2,3-dioxygenase (IDO). This enzyme has been shown to suppress T cell responses through tryptophan depletion, which results in the production of toxic catabolites [26, 39]. Moreover, DC residing in mucosal surfaces may preferentially acquire a tolerogenic phenotype and maintain tolerance to allergens by providing a cytokine environment promoting Treg induction [21, 36, 40]. Our data support this second hypothesis by identifying a population of CD11c⁺ cells which express high levels of IL-10, TGF- β and IFN- α following treatment with *M. vaccae*.

In the future we wish to confirm the central role played by CD11c⁺ cells in the induction of Tregs. We are currently investigating whether *M. vaccae*-induced CD11c⁺ cells confer a Treg phenotype to naive CD4⁺ T cells from DO11.10 transgenic mice. Alternatively, CD11c⁺ cells may act as regulatory cells themselves. Thus, by passive transfer into the lungs of recipient allergic mice, we may be able to determine whether these cells directly improve the symptoms of pulmonary inflammation induced by challenge. Further characterization of CD11c⁺ APC role in the immune response is vital. Several distinctive subtypes have already been identified and have shown a variety of functions [38, 41–43]. It is of interest to address further the role of *M. vaccae* in the induction of CD11c⁺ cells.

Heat-inactivated *M. vaccae* has been used in clinical studies of asthma [18] and eczema [19]. Results were encouraging and larger phase II trials are now in progress. It remains to be determined whether the effects in human patients depend on changes in APC phenotype and Treg induction. A therapeutic role for *M. vaccae* is compatible with the current version of the hygiene hypothesis, suggesting that the simultaneous rise in immunoregulatory disorders in the developed countries is attributable to a failure of Treg functions, and not to a Th1/Th2 imbalance.

4 Materials and methods

4.1 Mice

Female 6–8-week-old BALB/c mice were purchased from Harlan (Oxon, GB) and were housed under standard conditions. All experimental protocols complied with the Home Office 1986 Animals Scientific Act.

4.2 Antigens and *M. vaccae* suspension

Ovalbumin was obtained from Sigma (grade V; Dorset, GB). Sterile vials of a heat-killed suspension of *M. vaccae* were provided by SR Pharma (London, GB). The product was manufactured to Good Manufacturing Practice and is currently being tested in phase II clinical trials.

4.3 Murine model of allergic pulmonary inflammation

Mice were treated s.c. on day-21 with either *M. vaccae* (0.1 mg in 100 μ l saline) or with saline alone. All mice were immunized i.p. on days 0 and 12 with 10 μ g ovalbumin in 100 μ l alum (Serva, Heidelberg, Germany). On days 19 and 21, mice were challenged intratracheally (i.t.) with either saline (negative control) or ovalbumin. Where indicated, 3 weeks prior to treatment animals received two further ovalbumin and alum injections 12 days apart. Animals were killed 48 h after the last allergen challenge by i.p. injection of pentobarbitone sodium (240 mg/kg). The trachea was cannulated and the BAL fluid was collected by injecting three times 0.3 ml of RPMI (Invitrogen, Paisley, GB) to which 50 U/ml penicillin and 50 mg/ml streptomycin (Invitrogen) were added. The number of cells recovered in the fluid was determined using a Neubauer chamber.

Cytospins of each BAL sample was performed (Shandon Scientific, Cheshire, GB) and stained with Wright Giemsa Stain (Sigma). A differential count of 200 cells was performed using standard morphological criteria. The remaining BAL fluid was centrifuged and supernatant collected and stored at -20°C for cytokine analysis. Cells were pooled based on treatment and used for PCR analysis.

4.4 Cytokine analysis

Cytokine levels in the BAL fluid were measured using commercially available ELISA Kits (R&D Systems, Oxon, GB). Antibody pairs and protein standards for IL-10 and IL-12 were used according to the manufacturer's instructions.

4.5 CD11c cells separation

Lungs were pooled in groups based on treatment and digested with collagenase D (75 U/ml, Boehringer, Brack-

nall, GB) for 1 h at 37°C . Tissue was forced through a $70\text{-}\mu\text{m}$ sieve and red blood cells removed by hypotonic lysis. Cells were separated with CD11c microbeads by positive selection, according to the manufacturer's instructions (Miltenyi Biotec, Bergisch-Gladbach, Germany). Briefly, cells were incubated in cold buffer (0.5% BSA in PBS) with CD11c microbeads for 15 min at 4°C . Cells were applied onto a MS+ separation column. Both the positive fraction enriched for CD11c⁺ cell and the negative fraction were collected. Cells were stored at -80°C until further analysis.

4.6 PCR analysis

RNA was extracted using a commercially available kit RNeasy, (Qiagen, Crawley, GB) according to manufacturer's instructions. Briefly, whole tissue was disrupted using a homogenizer and placed through an RNA-binding column. CD11c-negative and -positive fractions were passed through a shredder column. cDNA was obtained from eluted RNA with the Omniscript kit (Qiagen, GB), using Oligo-dT and Rnasin (Promega, Southampton, GB). Samples were stored at -20°C .

4.7 Quantitative real-time PCR

cDNA samples were analyzed using the ABI7000 real-time PCR machine (Applied Biosystems, Warrington, GB) and Quantitect Sybr Green Master Mix (Qiagen). Primers for GAPDH (forward: CATTGTGGAAGGGCTCATGA, reverse: GGAAGGCCATGCCAGTGAGC), IL-10 (forward: TTTGAAT-TCCCTGGGTGAGAAG, reverse: ACAGGGGAGAAATCGAT-GACA), TGF- β (forward: ACAGGGCTTTTCGATTCAGCGC, reverse: CCCTTGGGCTCGTGGGGTCTCT), IL-4 (forward: CGTCCTCACAGCAACGGAGA, reverse: GCAGCTTATC-GATGAATCCAGG), IL-12 (forward: GACATCATCAAACCA-GACCCG, reverse: TTACACCCCTCCTCTGTCTCCTT), IFN- γ (forward: GGTGACATGAAATCCTGCAGA, reverse: CCTCAAACCTGGCAATACTCATGA), and IFN- α (forward: CTCTGTGCTTTCCTGATGGT, reverse: AGAGAGGGAGT-CTCCTCATT) were designed using Primer Express software (Applied Biosystems). Primers were obtained from Eurogentec (Romsey, GB). Standards for each gene were set up using a PCR amplicon, and were serially tenfold diluted. The standard curve generated was used to extrapolate the copy number obtained from each sample. All genes were normalized to GAPDH.

4.8 Statistical analysis

Results are expressed as the mean $\pm$ SEM. Data were subjected to a normality test to ensure a normal distribution. One-way ANOVA or Student's *t*-test were used to determine statistically significant differences between the groups. A *p* value of less than 0.05 was considered significant.

Acknowledgements: We would like to thank Steve Harrison and David Farrer for critically reading the manuscript. This work was supported by SR Pharma.

References

- Umetsu, D. T., McIntire, J. J., Akbari, O., Macaubas, C. and Dekruyff, R. H., Asthma: an epidemic of dysregulated immunity. *Nat. Immunol.* 2002. **3**: 715–720.
- Bach, J. F., The effect of infections on susceptibility to autoimmune and allergic diseases. *N. Engl. J. Med.* 2002. **347**: 911–920.
- Stene, L. C. and Nafstad, P., Relation between occurrence of type 1 diabetes and asthma. *Lancet* 2001. **357**: 607–608.
- Rook, G. A. and Brunet, L. R., Give us this day our daily germs. *Biologist (London)* 2002. **49**: 144.
- Wildin, R. S., Ramsdell, F., Peake, J., Faravelli, F., Casanova, J. L., Buist, N., Levy-Lahad, E., Mazzella, M., Goulet, O., Perroni, L., Bricarelli, F. D., Byrne, G., McEuen, M., Proll, S., Appleby, M. and Brunkow, M. E., X-linked neonatal diabetes mellitus, enteropathy and endocrinopathy syndrome is the human equivalent of mouse scurfy. *Nat. Genet.* 2001. **27**: 18–20.
- Bennett, C. L., Christie, J., Ramsdell, F., Brunkow, M. E., Ferguson, P. J., Whitesell, L., Kelly, T. E., Saulsbury, F. T., Chance, P. F. and Ochs, H. D., The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. *Nat. Genet.* 2001. **27**: 20–21.
- Hori, S., Nomura, T. and Sakaguchi, S., Control of regulatory T cell development by the transcription factor Foxp3. *Science* 2003. **299**: 1057–1061.
- Fontenot, J. D., Gavin, M. A. and Rudensky, A. Y., Foxp3 programs the development and function of CD4⁺CD25⁺ regulatory T cells. *Nat. Immunol.* 2003. **4**: 330–336.
- Khattari, R., Cox, T., Yasayko, S. A. and Ramsdell, F., An essential role for Scurfin in CD4⁺CD25⁺ T regulatory cells. *Nat. Immunol.* 2003. **4**: 337–342.
- Strachan, D. P., Butland, B. K. and Anderson, H. R., Incidence and prognosis of asthma and wheezing illness from early childhood to age 33 in a national British cohort. *Br. Med. J.* 1996. **312**: 1195–1199.
- Rook, G. A., Clean living increases more than just atopic disease. *Immunol. Today* 2000. **21**: 249–250.
- Braun-Fahrlander, C., Riedler, J., Herz, U., Eder, W., Waser, M., Grize, L., Maisch, S., Carr, D., Gerlach, F., Bufe, A., Lauener, R. P., Schierl, R., Renz, H., Nowak, D. and von Mutius, E., Environmental exposure to endotoxin and its relation to asthma in school-age children. *N. Engl. J. Med.* 2002. **347**: 869–877.
- Rook, G. A., Martinelli, R. and Rosa Brunet, L., Innate immune responses to mycobacteria and the downregulation of atopic responses. *Curr. Opin. Allergy Clin. Immunol.* 2003. **3**: 337–342.
- Rook, G. A. W., Adams, V., Hunt, J., Palmer, R., Martinelli, R. and Rosa Brunet, L., Mycobacteria and other environmental organisms as immunomodulators for immunoregulatory disorders. *Springer Semin. Immunopathol.* 2003, in press.
- Riedler, J., Braun-Fahrlander, C., Eder, W., Schreuer, M., Waser, M., Maisch, S., Carr, D., Schierl, R., Nowak, D. and von Mutius, E., Exposure to farming in early life and development of asthma and allergy: a cross-sectional survey. *Lancet* 2001. **358**: 1129–1133.
- Zuany-Amorim, C., Manlius, C., Trifilieff, A., Brunet, L. R., Rook, G., Bowen, G., Pay, G. and Walker, C., Long-term protective and antigen-specific effect of heat-killed *Mycobacterium vaccae* in a murine model of allergic pulmonary inflammation. *J. Immunol.* 2002. **169**: 1492–1499.
- Zuany-Amorim, C., Sawicka, E., Manlius, C., Le Moine, A., Brunet, L. R., Kemeny, D. M., Bowen, G., Rook, G. and Walker, C., Suppression of airway eosinophilia by killed *Mycobacterium vaccae*-induced allergen-specific regulatory T cells. *Nat. Med.* 2002. **8**: 625–629.
- Camporota, L., Corkhill, A., Long, H., Lordan, J., Stanciu, L., Tuckwell, N., Cross, A., Stanford, J. L., Rook, G. A., Holgate, S. T. and Djukanovic, R., The effects of *Mycobacterium vaccae* on allergen-induced airway responses in atopic asthma. *Eur. Respir. J.* 2003. **21**: 287–293.
- Arkwright, P. D. and David, T. J., Intradermal administration of a killed *Mycobacterium vaccae* suspension (SRL 172) is associated with improvement in atopic dermatitis in children with moderate-to-severe disease. *J. Allergy Clin. Immunol.* 2001. **107**: 531–534.
- Akbari, O., Freeman, G. J., E. H. Meyer, E. H., Greenfield, E. A., Chang, T. T., Sharpe, A. H., Berry, G., DeKruyff, R. H. and Umetsu, D. T., Antigen-specific regulatory T cells develop via the ICOS-ICOS-ligand pathway and inhibit allergen-induced airway hyperreactivity. *Nat. Med.* 2002. **8**: 1024–1032.
- Akbari, O., Dekruyff, R. H. and Umetsu, D. T., Pulmonary dendritic cells producing IL-10 mediate tolerance induced by respiratory exposure to antigen. *Nat. Immunol.* 2001. **2**: 725–731.
- Levings, M. K., Sangregorio, R., Galbiati, F., Squadrone, S., de Waal, R. and Roncarolo, M. G., IFN- α and IL-10 induce the differentiation of human type 1 T regulatory cells. *J. Immunol.* 2001. **166**: 5530–5539.
- Ito, T., Amakawa, R., Inaba, M., Ikehara, S., Inaba, K. and Fukuhara, S., Differential regulation of human blood dendritic cell subsets by IFNs. *J. Immunol.* 2001. **166**: 2961–2969.
- Hopfenspirger, M. T. and Agrawal, D. K., Airway hyperresponsiveness, late allergic response, and eosinophilia are reversed with mycobacterial antigens in ovalbumin-prensensitized mice. *J. Immunol.* 2002. **168**: 2516–2522.
- Cassese, G., Arce, S., Hauser, A. E., Lehnert, K., Moewes, B., Mostarac, M., Muehlinghaus, G., Szyska, M., Radbruch, A., and Manz, R. A., Plasma cell survival is mediated by synergistic effects of cytokines and adhesion-dependent signals. *J. Immunol.* 2003. **171**: 1684–1690.
- Swanson, K. A., Zheng, Y., Heidler, K. M., Mizobuchi, T. and Wilkes, D. S., CD11c⁺ cells modulate pulmonary immune responses by production of Indoleamine 2,3 dioxygenase 1. *Am. J. Respir. Cell Mol. Biol.* 2003, in press.
- Levings, M. K., Bacchetta, R., Schulz, U. and Roncarolo, M. G., The role of IL-10 and TGF- β in the differentiation and effector function of T regulatory cells. *Int. Arch. Allergy Immunol.* 2002. **129**: 263–276.
- Read, S. and Powrie, F., CD4⁺ regulatory T cells. *Curr. Opin. Immunol.* 2001. **13**: 644–649.
- Seder, R. A., Marth, T., Sieve, M. C., Strober, W., Letterio, J. J., Roberts, A. B. and Kelsall, B., Factors involved in the differentiation of TGF- β -producing cells from naive CD4⁺ T cells: IL-4 and IFN- γ have opposing effects, while TGF- β positively regulates its own production. *J. Immunol.* 1998. **160**: 5719–5728.
- Kitani, A., Fuss, I. J., Nakamura, K., Schwartz, O. M., Usui, T. and W. Strober, W., Treatment of experimental (trinitrobenzene sulfonic acid) colitis by intranasal administration of transforming growth factor (TGF)- β 1 plasmid: TGF- β 1-mediated sup-

- pression of T helper cell type 1 response occurs by interleukin (IL)-10 induction and IL-12 receptor beta2 chain downregulation. *J. Exp. Med.* 2000. **192**: 41–52.
- 31 Steinbrink, K., Graulich, E., Kubsch, S., Knop, J. and Enk, A. H., CD4⁺ and CD8⁺ anergic T cells induced by interleukin-10-treated human dendritic cells display antigen-specific suppressor activity. *Blood* 2002. **99**: 2468–2476.
 - 32 Ludviksson, B. R., Seegers, D., Resnick, A. S. and Strober, W., The effect of TGF-beta1 on immune responses of naive versus memory CD4⁺ Th1/Th2 T cells. *Eur. J. Immunol.* 2000. **30**: 2101–2111.
 - 33 Maloy, K. J., Salaun, L., Cahill, R., Dougan, G., Saunders, N. J. and Powrie, F., CD4⁺CD25⁺ T(R) cells suppress innate immune pathology through cytokine-dependent mechanisms. *J. Exp. Med.* 2003. **197**: 111–119.
 - 34 Read, S., Malmstrom, V. and Powrie, F., Cytotoxic T lymphocyte-associated antigen 4 plays an essential role in the function of CD25⁺CD4⁺ regulatory cells that control intestinal inflammation. *J. Exp. Med.* 2000. **192**: 295–302.
 - 35 Roncarolo, M. G., Bacchetta, R., Bordignon, C., Narula, S. and Levings, M. K., Type 1 T regulatory cells. *Immunol. Rev.* 2001. **182**: 68–79.
 - 36 Bilsborough, J., George, T. C., Norment, A. and Viney, J. L., Mucosal CD8alpha⁺ DC, with a plasmacytoid phenotype, induce differentiation and support function of T cells with regulatory properties. *Immunology* 2003. **108**: 481–492.
 - 37 Lombardi, G., Dunne, P. J., Scheel-Toellner, D., Sanyal, T., Pilling, D., Taams, L. S., Life, P., Lord, J. M., Salmon, M. and Akbar, A. N., Type 1 IFN maintains the survival of anergic CD4⁺ T cells. *J. Immunol.* 2000. **165**: 3782–3789.
 - 38 Lambrecht, B. N. and Hammad, H., The other cells in asthma: dendritic cell and epithelial cell crosstalk. *Curr. Opin. Pulm. Med.* 2003. **9**: 34–41.
 - 39 Munn, D. H., Sharma, M. D., Lee, J. R., Jhaver, K. G., Johnson, T. S., Keskin, D. B., Marshall, B., Chandler, P., Antonia, S. J., Burgess, R., Slingluff Jr, C. L. and Mellor, A. L., Potential regulatory function of human dendritic cells expressing Indoleamine 2,3-dioxygenase. *Science* 2002. **297**: 1867–1870.
 - 40 Akbari, O., Stock, P., Dekruyff, R. H. and Umetsu, D. T., Mucosal tolerance and immunity: regulating the development of allergic disease and asthma. *Int. Arch. Allergy Immunol.* 2003. **130**: 108–118.
 - 41 Lagranderie, M., Nahori, M. A., Balazuc, A. M., Kiefer-Biasizzo, H., Lapa e Silva, J. R., Milon, G., Marchal, G. and Vargaftig, B. B., Dendritic cells recruited to the lung shortly after intranasal delivery of *Mycobacterium bovis* BCG drive the primary immune response towards a type 1 cytokine production. *Immunology* 2003. **108**: 352–364.
 - 42 Nikolic, T., Dingjan, G. M., Leenen, P. J. and Hendriks, R. W., A subfraction of B220⁺ cells in murine bone marrow and spleen does not belong to the B cell lineage but has dendritic cell characteristics. *Eur. J. Immunol.* 2002. **32**: 686–692.
 - 43 Edwards, A. D., Chaussabel, D., Tomlinson, S., Schulz, O., Sher, A. and Reis e Sousa, C., Relationships among murine CD11c^{high} dendritic cell subsets as revealed by baseline gene expression patterns. *J. Immunol.* 2003. **171**: 47–60.

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