

Kinetics of Regulatory T Cells in the Ovalbumin Asthma Model in the Rat

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Key Words

Asthma model • Rat • Regulatory T cells • Kinetics

Abstract

Background: The kinetics of regulatory T cells (T_{Reg}) in allergic diseases such as asthma are only partly known. **Methods:** The asthma model in the Fischer rat with ovalbumin (OVA) sensitization and aerosol challenge was used. The relative and absolute numbers of leukocytes, lymphocytes and T_{Reg} subsets were determined by flow cytometry in the lung interstitium and draining bronchial lymph nodes at different time points after two challenges, and lung function was tested in parallel. **Results:** The progressive number of challenges resulted in increased relative and absolute numbers of lymphocytes and in particular of T_{Reg} . The T_{Reg} number was augmented with each aerosol challenge and was already significantly increased 6 h after the second challenge. The relative (%) and absolute numbers of CD4+ and CD8+ T_{Reg} and dendritic cells showed different kinetics after two challenges. The leukocyte numbers in the lung did not correlate with lung function. **Conclusion:** T_{Reg} increased surprisingly early after challenge in the lung tissue. Relative and absolute numbers of leukocyte subsets should always be calculated. The kinetics of different leukocyte subsets can only be determined when several time points are studied.

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Introduction

Immune reactions have to be tightly regulated to keep a balance between adequate protective immune reactions of short duration, continuous reactions and autoimmune phenomena [1–3]. In healthy people, an adequate immune reaction to allergens depends on sufficient numbers of regulatory T cells (T_{Reg}). If T_{Reg} production is not sufficient, allergic inflammation may evolve [for review see ref. 1–3]. These regulatory interactions in secondary lymphoid organs have attracted enormous interest in recent years. Subsets, in particular CD4+ lymphocytes with regulatory functions, and defining markers for T_{Reg} subsets are still an expanding field of research interest. Furthermore, interactions of the innate and adaptive immune system have been characterized in allergic airway inflammation [4], and the effects of Toll-like receptors in the expansion of T_{Reg} [5] as well as of T-lymphocyte-independent pathways [6] have been described. Recently, the elimination of T_{Reg} was shown to be essential to vaccinate mice effectively [7]. Recently, the phenotype of CD4+ T_{Reg} and their subsets have been reviewed with respect to tolerance to autoantigens and foreign antigens [8], as well as specific functions of allergic lung diseases, e.g. the resolution of airway hyperreactivity [9–11], their effect on the subset composition and dendritic cell function [12], and tolerance phenomena [13].

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Foxp3 has been characterized as an important marker for T_{Reg} in mice but also in humans [14]. However, the kinetics of T_{Reg} during an allergic reaction and potential differences between organ compartments are largely unknown but of major relevance to understand their pathophysiology and potential therapeutic interventions. In a very recent viewpoint article on T_{Reg} , this aspect of the time points and dynamics of T_{Reg} in allergic reactions was not mentioned [15]. Recently, Hartl et al. [16] documented a quantitative and functional impairment in pulmonary T_{Reg} (CD4⁺CD25^{hi}) in children with asthma, compared these cells in the blood with those in the bronchoalveolar fluid and found a decrease in T_{Reg} .

Ovalbumin (OVA) has extensively been used as an antigen in mice (intraperitoneal sensitization followed by a different number of aerogen OVA challenges) [17]. In recent years, the rat has been used as an OVA asthma model as the rat has a number of advantages over the mouse, e.g. the presence of bronchial blood supply similar to that in humans, which is absent in the mouse [for review see ref. 18, 19]. We have previously established the OVA model in Fischer rats to define the effects of CD26 deficiency [20, 21], but also documented the positive or negative effects of early postnatal stimulation or deprivation on asthma in adult life [22]. Phenotypically, T_{Reg} cells have been characterized in the rat [23]. The kinetics of T_{Reg} subsets after the intratracheal injection of the TLR-2 agonist, the lipopeptide MALP-2, have been characterized in rats, and a kinetic pattern has been documented, which was different for the various compartments of the lung [24]. Lymphocytes and other immune cells are found in specific subset compositions in the different compartments of the lung [25], which was also documented recently for resolution of airway inflammation with long-term aerosol challenge [11]. Lung function is a critical parameter in asthma models and, therefore, body plethysmography was carried out in parallel.

The importance of regional lymph nodes in mucosal tolerance and in protective immune reactions initiated at the mucosal surface has recently been summarized [26] and documented in allergic airway diseases in short-term as well as long-term aerosol challenge [11]. Thus, the lung and draining mediastinal lymph nodes were included in this study.

The aims of this study were to define whether (1) T_{Reg} change in number and subset composition with time after allergen challenge in the lung, (2) repetitive intratracheal allergen challenges influence the T_{Reg} pattern and (3) the draining bronchial lymph nodes are involved in the T_{Reg} responses.

Material and Methods

Animals

Female Fischer rats, 18–20 weeks old (supplied by Charles River, Sulzfeld, Germany), were kept under specific pathogen-free conditions at constant room temperature and a 12-hour dark-light cycle. The animals had free access to pelleted food and water. The experiments had been approved by the local authorities.

Procedure

The rats (mean weight 213 ± 4 g) were sensitized with 500 μ g OVA (grade V; Sigma, St. Louis, Mo., USA) and 20 mg of aluminum hydroxide in 500 μ l saline by a first subcutaneous injection in the right hind leg and a second challenge for sensitization in the left hind leg after 7 days. Simultaneously, 5×10^9 heat-inactivated *Bordetella pertussis* bacteria in 1 ml 0.9 NaCl i.p. were given as an adjuvant. The OVA challenge was performed using a head-out bodyplethysmograph [27]. The protocol is outlined in figure 1a.

In a second group of rats (mean weight 160 ± 2 g), the time kinetics of the effects of the aerosol provocation were measured 6, 12, 18 and 24 h after the second challenge (fig. 1b). Control rats were not sensitized but exposed to the allergen on days 13 and 14 and sacrificed 12 h later. After a phase of 4 min of adjustment to the situation, the animals each inhaled 2 ml of a 5% OVA solution for 8 min with a continuous air flow of 200 ml/min. The mean expiratory flow 50 is defined as the time when 50% of the expiratory tidal volume is reached. This is a parameter for bronchoconstriction [27]. The mean of the first 4 min was taken as 100%, so that the relative differences over the following 8 min could be calculated.

After the last provocation, the animals were sacrificed by exsanguination under deep anesthesia. The draining bronchial lymph nodes were excised and used for preparing a cell suspension [24].

The lung tissue from a non-lavaged part of the lung was teased and prepared as described [28]. The cytopspins of cell suspensions were differentiated using the Quick-Diff technique, and at least 400 cells per slide were counted. Routine paraffin histology of the lung was performed, and sections were stained with hematoxylin and eosin.

Flow Cytometry

The antibodies used are shown in table 1. Flow cytometry was performed with a FACSCanto flow cytometer (Becton Dickinson, Heidelberg, Germany) as described previously [28]. The mononuclear cells which were MHC class II⁺ and CD103⁺ were classified as dendritic cells. Foxp3 staining could only be done after incubating the cells with a permeabilizing reagent [24]. A representative FACS pattern is shown in figure 2. All data on T_{Reg} subsets and dendritic cells were from the same animals.

Statistics

For flow-cytometric evaluation, FACSDIVA software (Becton Dickinson) was used. Means $\pm$ SE were calculated, and the t test and the Mann-Whitney U test were applied accordingly to test the significance of the data. $p < 0.05$ was regarded as statistically significant difference.

Fig. 1. Outline of the two protocols. Subsequent to two sensibilizations 7 days apart (group A), one to four challenges with OVA starting on day (d) 13 were always followed by the evaluation (+) 1 day after the last challenge. In group B, the lung was examined (+) 6, 12, 18 or 24 h after the second challenge. Control rats were not sensitized but also exposed to OVA and sacrificed 12 h later.

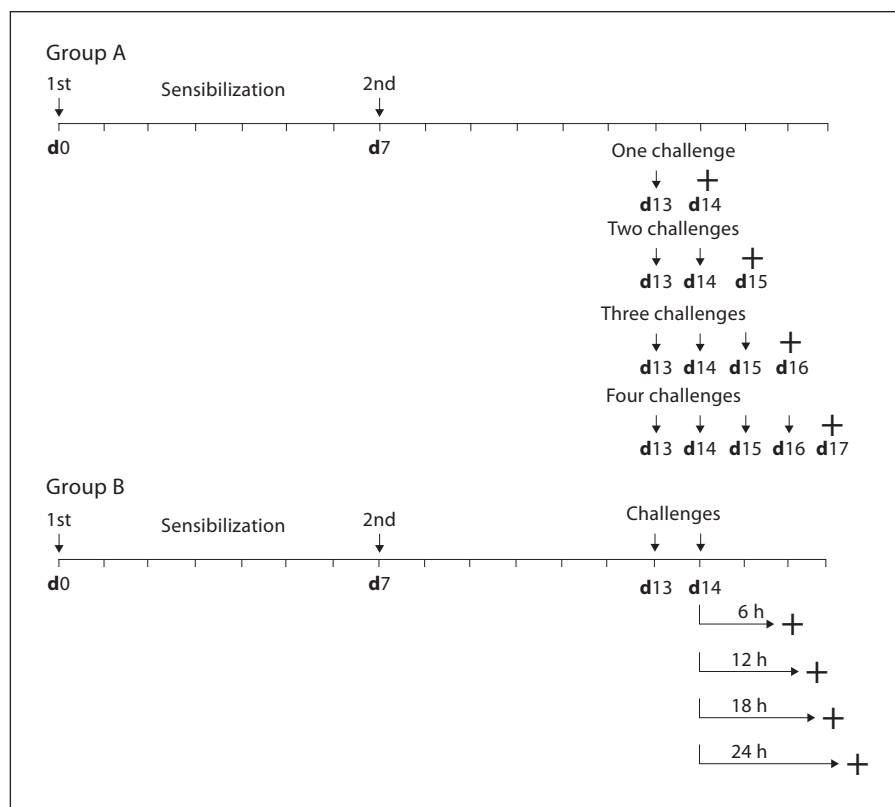


Table 1. Antibodies used

Specificity	Clone	Isotype	Conjugate	Dilution	Supplier
CD4	W3/25	IgG1	biotinylated FITC	1:100 1:1000	Serotec ¹
CD103 (dendritic cell-specific integrin)	Ox 62	IgG1	FITC	1:10	Serotec
CD25	Ox 39	IgG1	biotinylated PE	1:50 1:50	Serotec
MHC class II	Ox 6	IgG1	biotinylated	1:50	Serotec
B cells	Ox 12	IgG1	PE	1:50	Serotec
T lymphocytes (α/β)	R73	IgG1	PE FITC	1:20 1:500	Serotec
Streptavidin-PerCp-Cy5.5				1:500	BD Biosciences ²
Foxp3	150D	IgG1	APC	1:10	BioLegend ³

¹ Kidlington, UK; ² Pharmingen, Heidelberg, Germany; ³ San Diego, Calif., USA.

Results

Repetitive OVA Challenge

After the first OVA challenge, the body weight of the rats dropped significantly by about 13% and remained at

this level after the following three challenges by inhalation of OVA (data not shown). During and within the first hour after the provocation, the rats showed a lower respiratory frequency and enforced inspiration.

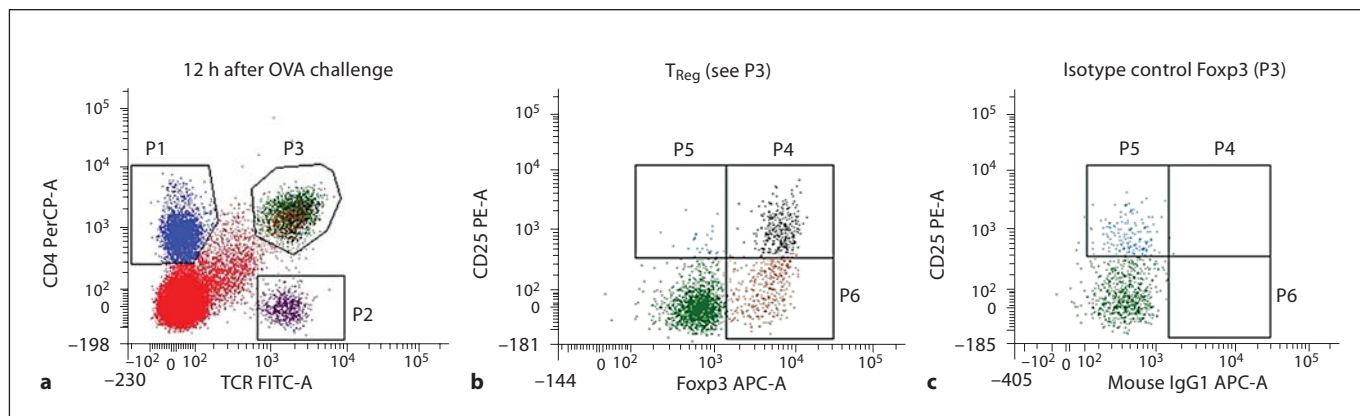


Fig. 2. Representative FACS pattern documenting mononuclear cells 12 h after OVA challenge. **a** P1 = CD4+ monocytes; P2 = CD8+ T cells; P3 = double-positive TCR+CD4+ T cells. **b** TCR+CD4+CD25Foxp3+ cells are in rectangle P4 (these are the

cells shown in P3). Gate P5 documents TCR+CD4+CD25+ T_{Reg} and gate P6 TCR+CD4+Foxp3+ cells. **c** Control staining isotype for Foxp3 documenting the lack of labeled cells in P4 and P6.

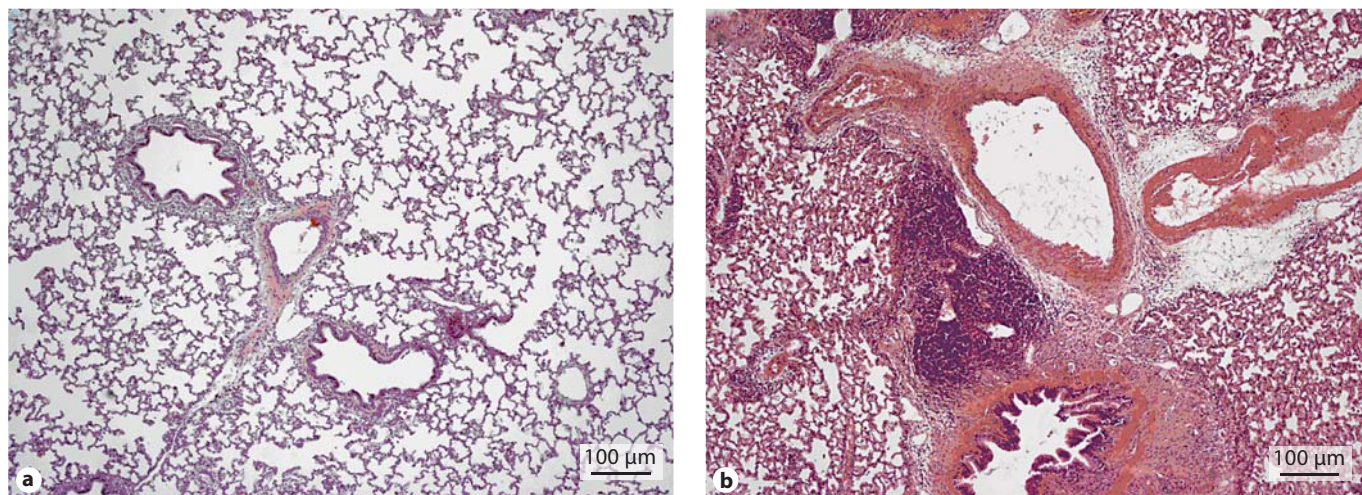


Fig. 3. Histology of a control lung (**a**) and after four challenges (**b**). HE.

Lung Tissue

After repetitive OVA challenge, a massive periarterial and less peribronchial leukocyte accumulation was obvious (fig. 3). In the cell suspensions of the lungs, the total number of leukocytes did not reach significant difference, while the CD4 T-cell number showed a steady increase (fig. 4a). The T_{Reg} subsets CD4+CD25+ and CD4+CD25+Foxp3+ had a comparable kinetic pattern with a continuous increase in absolute numbers with increasing numbers of challenges (fig. 4b). The relative (%) and absolute numbers of the CD4+CD25+Foxp3+ T_{Reg}

after four OVA challenges are documented in figure 5. After the fourth challenge, the absolute T_{Reg} number was seven times higher.

Draining Lymph Nodes

The total number of lymphocytes, CD4 T cells and CD4+ monocytes showed a different pattern from that of the lung tissue. There was an increase after the first and second challenge, followed by a drop after the third challenge and a second elevation after the fourth (fig. 4c). Only the levels after the fourth challenge were signifi-

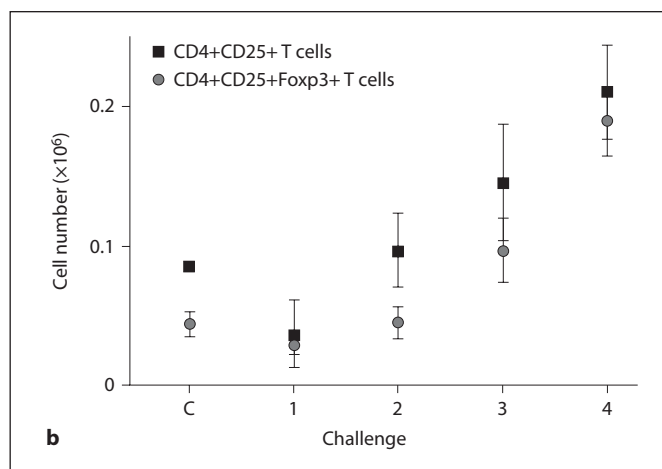
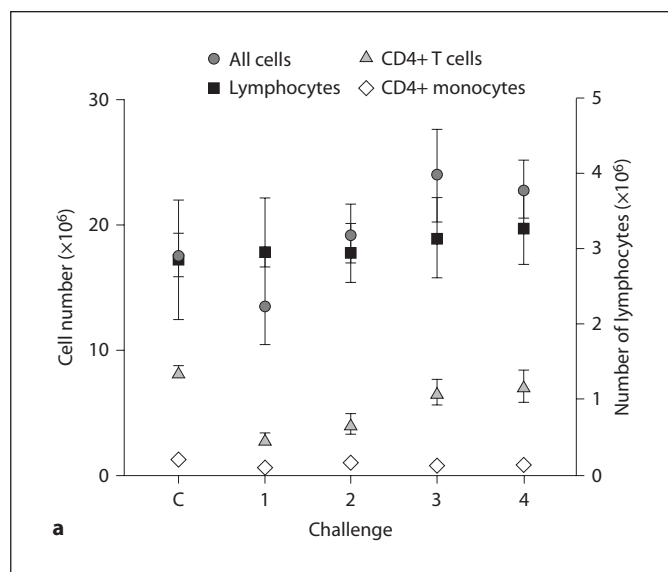
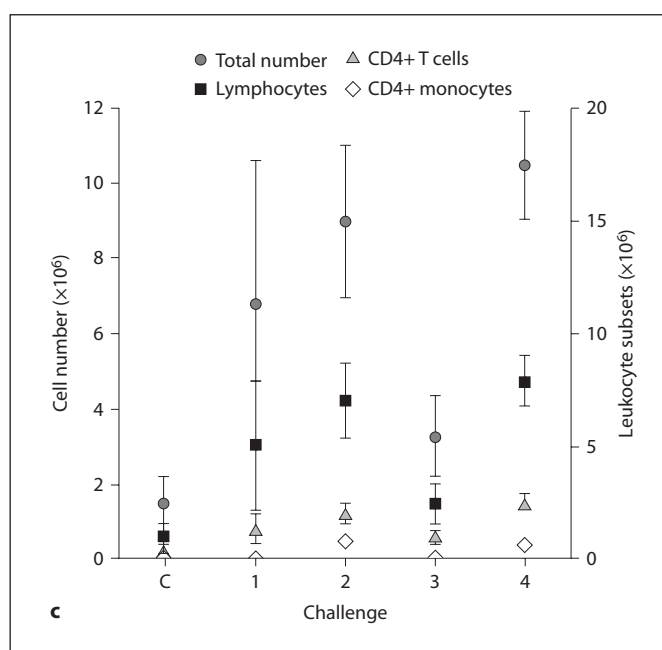


Fig. 4. **a** Total number of leukocytes, lymphocytes and monocytes in the lung tissue after one to four OVA challenges. **b** Number of T_{Reg} (CD4+CD25+ and CD4+CD25+Foxp3+) after one to four challenges in the lung tissue. **c** Total number of leukocytes and their subsets in the draining bronchial lymph nodes after one to four OVA challenges (* $p < 0.05$). Means $\pm$ SE. C = Control.



cantly increased compared with the control. The relative numbers revealed a similar pattern (data not shown). However, the T_{Reg} subsets CD4+CD25+Foxp3+ showed neither a significant difference in relative nor in absolute numbers (data not shown).

Lung Function

The expiratory flow 50 was most dramatically reduced after the first OVA challenge and lasted for the whole time of measurement. The second challenge had the least effect and the third and fourth challenges showed an intermediate pattern (fig. 6).

Time Kinetics after Two OVA Challenges

While the CD4+CD25+ T cells and CD4+Foxp3+ T cells showed no specific pattern from 6 to 24 h after the second challenge, the relative numbers of T_{Reg} (CD4+CD25+Foxp3+) were highest at 12 h (fig. 7a, b). CD8+ T_{Reg} had a similar pattern, including a maximum at 12 h (fig. 7c, d). Dendritic cells revealed a different pattern with significantly less dendritic cells after provocation, followed by a gradual increase to pre-provocation levels in relative and absolute numbers (fig. 7e, f).

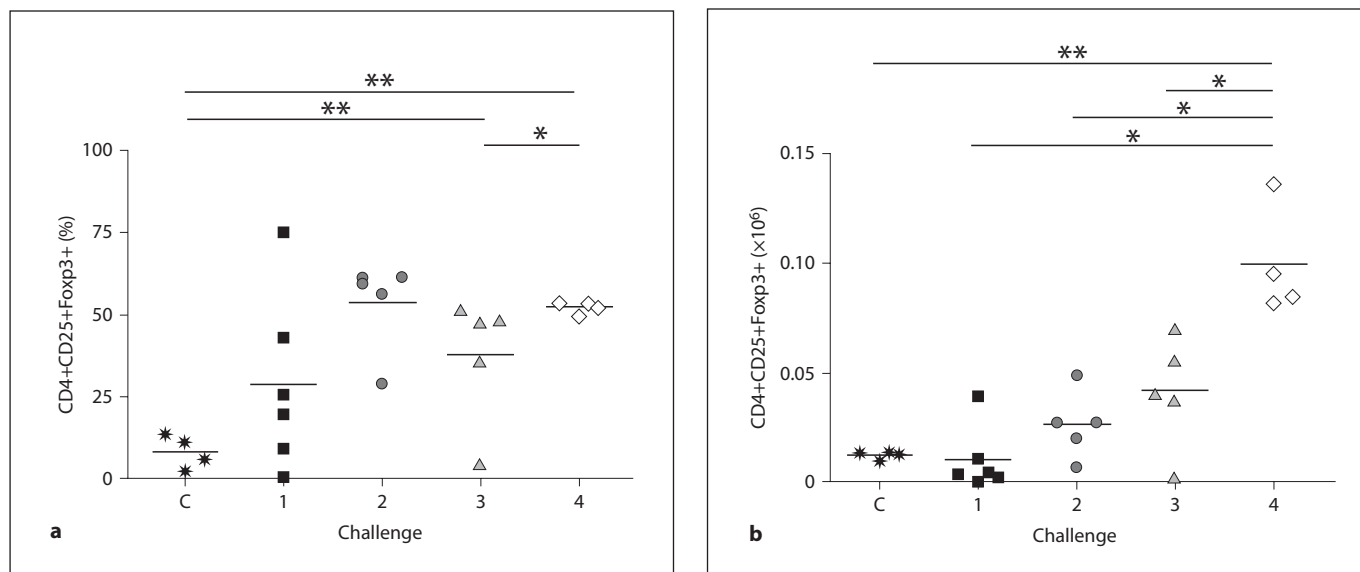


Fig. 5. Relative (%) and absolute numbers of T_{Reg} (CD4+CD25+Foxp3+) in the interstitial tissue of the lung (* $p < 0.05$ vs. numbers of provocation, ** $p < 0.05$ vs. control animals). C = Control.

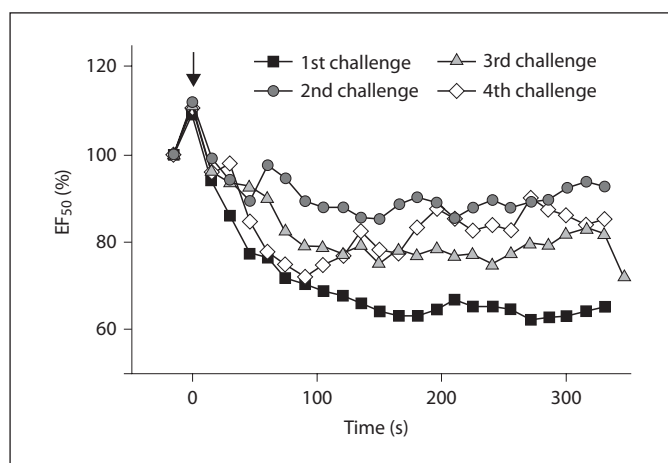


Fig. 6. Lung function test: mean expiratory flow 50 (EF₅₀) after one to four OVA challenges.

Discussion

Despite the continuously increasing description of subsets of T_{Reg} , very little is known about their kinetics. An increase in T_{Reg} numbers during an immune reaction can be caused by an increased influx [29], a local differentiation resulting in the expression of T_{Reg} markers or by local proliferation. In such dynamic processes, the determination of T_{Reg} at a single time point can be mislead-

ing [30]. Furthermore, there are often marked discrepancies between in vitro and in vivo data [30]. In a previous study using deuterium as a label for circulating human T_{Reg} , CD4+CD45RO+CD25^{hi}Foxp3+ cells were highly proliferative with a doubling time of only 8 days in contrast to 199 days for naïve CD4+ cells [31]. The present study shows a dramatic increase in T_{Reg} subsets with the number of challenges in contrast to the number of CD4+ T cells. Recent studies in mice and patients documented a compartmentalization of T_{Reg} subsets in the lamina propria and lymphoid organs in gut inflammation [32]. The different pattern of T_{Reg} in the lung and draining lymph nodes in this study supports such compartmentalization in the respiratory tract.

However, it has been stressed that T_{Reg} in the blood might not be representative of all organs or infected tissues, thus underlining the relevance of studying the tissue of interest and not only the blood [33]. Therefore, we focused on the lung tissue and the draining lymph nodes, which often play a central role in a localized immune reaction [11, 26]. This was also obvious in the present study.

The functional role of T_{Reg} has mainly been discussed in respect to autoimmune and infectious diseases as well as cancer, but much less regarding their role in allergy [34–36]. It is still unclear which T_{Reg} subsets play a role in respiratory diseases and either inhibit eosinophilia or the development of airway hyperreactivity [reviewed in ref.

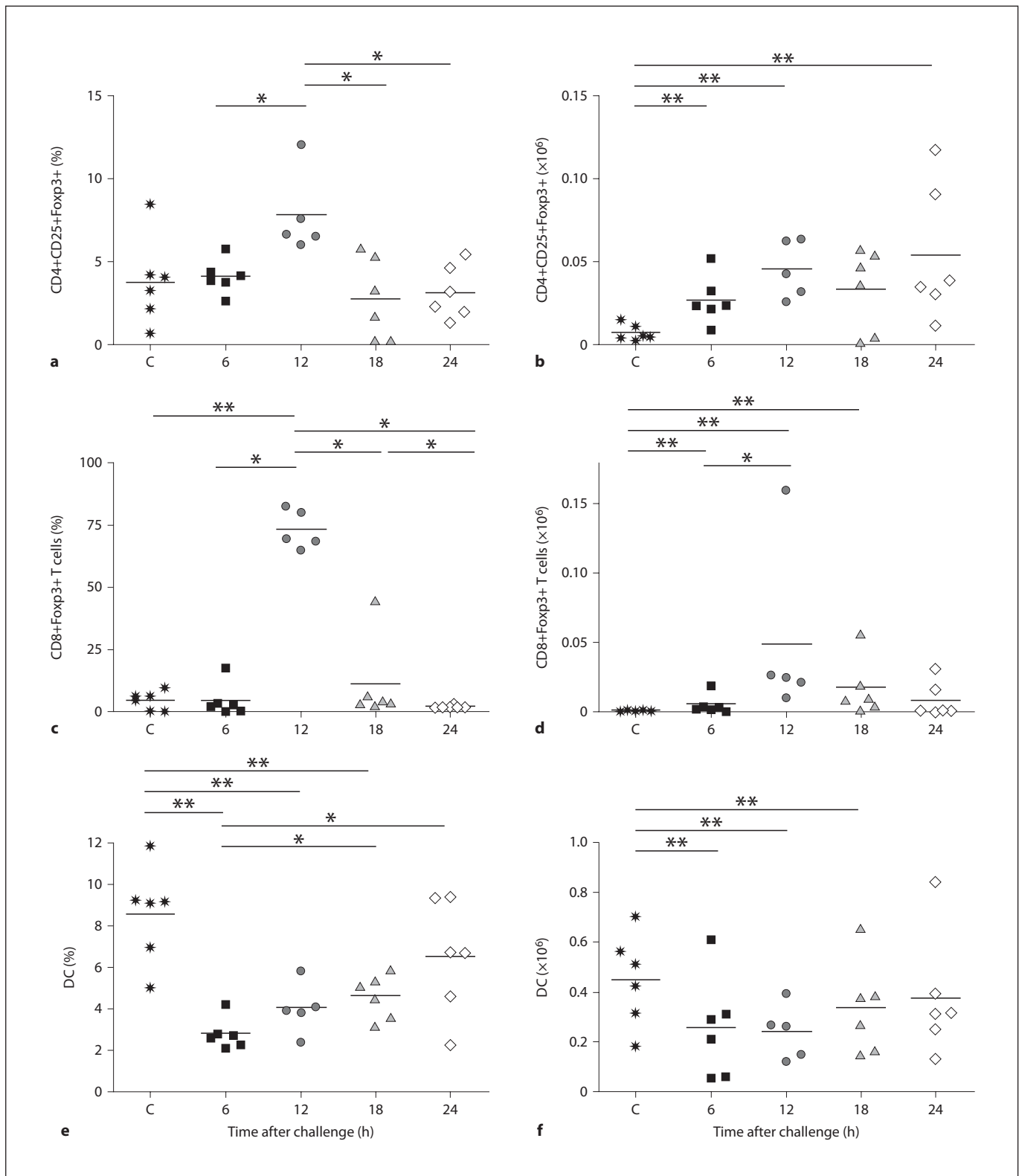


Fig. 7. Comparison of relative (%) numbers (**a**, **c**, **e**) of T_{Reg} subsets and dendritic cells (DC) and absolute numbers (**b**, **d**, **f**) at different time points after two OVA challenges (* $p < 0.05$ vs. the respective time point, ** $p < 0.05$ vs. control animals) in the lung tissue. C = Control.

35]. We focused on the kinetics of dendritic cells after OVA challenge, as T_{Reg} seem to play a major role in the protection against experimentally induced asthma via dendritic cells [12]. There was a different pattern after challenge of Foxp3+ T_{Reg} and dendritic cells (fig. 7). Thus, one should not extrapolate from one cell type to another.

In many asthma models, previously sensitized animals receive only a single challenge, but protective T_{Reg} activity depends on continuing allergen stimulation [37], as is the case in patients who are repeatedly exposed to house dust mites, for example. The data of the present study document the steadily increasing T_{Reg} number with the number of challenges (fig. 4). CD8+ T_{Reg} have been studied, for example, following in vitro stimulation of blood lymphocytes, characterizing the cytokine stimulation [38]. To our knowledge this subset has not been studied before in an allergy model. The increase of this cell type was concurrent with the increase in CD4+ T_{Reg} (fig. 7).

In a very recently published study, mice were exposed to OVA aerosol for 1 h/day for 42 days. This protocol resulted in significantly more T_{Reg} , in a tenfold increase of Foxp3+ T_{Reg} in the bronchoalveolar lavage fluid and significant increases in T_{Reg} in the draining bronchial lymph nodes in contrast to inguinal lymph nodes [11]. This much longer repetitive exposure to OVA might be the reason for these findings in the draining lymph nodes, as in the present study T_{Reg} were not significantly increased in the draining lymph nodes. The only study on T_{Reg} in asthma in humans by Hartl et al. [16] documented the absence of a difference in CD4⁺CD25^{hi} T_{Reg} in the blood and, surprisingly, a reduction in asthmatic children in the bronchoalveolar lavage fluid. However, all data were only given in relative numbers. Furthermore, the chil-

dren were not exposed to the allergen before the study. There was a dramatic effect of inhaled corticoids. Thus, the data obtained in patients are difficult to compare to the defined allergen exposure in the present study.

The results of the lung function might be due to mast cells as documented for the gut. Rioux and Wallace [39] studied mast cells and their proteases after repeated oral OVA challenge and found a reduction of the number of mast cells after the first exposure. This might also be the case in the lung. It is possible that after the second and the following exposures to OVA, the mast cells have not yet filled up their granules sufficiently.

The present data also support the relevance of giving absolute and relative numbers for T_{Reg} subsets as patterns can be different, leading to a wrong interpretation (fig. 5). Furthermore, the kinetic pattern of T_{Reg} determined at different times after a local challenge is critical for interpretation, although compared with dendritic cells the differences in CD4+ and CD8+ T_{Reg} with time are still difficult to interpret.

In conclusion, not only different subsets of T_{Reg} but also several challenges and points in time after a challenge have to be studied to enable a meaningful interpretation in allergy. This might be a basis for new therapeutic strategies in expanding specific T_{Reg} , as outlined recently [40].

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References

- 1 Mills KH: Regulatory T cells: friend or foe in immunity to infection? *Nat Rev Immunol* 2004;4:841–855.
- 2 Jiang H, Chess L: Regulation of immune responses by T cells. *N Engl J Med* 2006;354:1166–1176.
- 3 Nagler-Anderson C, Bhan AK, Podolsky DK, Terhorst C: Control freaks: immune regulatory cells. *Nat Immunol* 2004;5:119–122.
- 4 Beisswenger C, Kandler K, Hess C, Garn H, Felgentreff K, Wegmann M, Renz H, Vogelmeier C, Bals R: Allergic airway inflammation inhibits pulmonary antibacterial host defense. *J Immunol* 2006;177:1833–1837.
- 5 Suttmüller RP, den Brok MH, Kramer M, Bennink EJ, Toonen LW, Kullberg BJ, Joosten LA, Akira S, Netea MG, Adema GJ: Toll-like receptor 2 controls expansion and function of regulatory T cells. *J Clin Invest* 2006;116:485–494.
- 6 Niu N, Le Goff MK, Li F, Rahman M, Homer RJ, Cohn L: A novel pathway that regulates inflammatory disease in the respiratory tract. *J Immunol* 2007;178:3846–3855.
- 7 Grauer OM, Suttmüller RP, van Maren W, Jacobs JF, Bennink E, Toonen LW, Nierkens S, Adema GJ: Elimination of regulatory T cells is essential for an effective vaccination with tumor lysate-pulsed dendritic cells in a murine glioma model. *Int J Cancer* 2008;122:1794–1802.

- 8 Wing K, Suri-Payer E, Rudin A: CD4+CD25+ regulatory T cells from mouse to man. *Scand J Immunol* 2005;62:1–15.
- 9 Stock P, Akbari O, Berry G, Freeman GJ, DeKruyff RH, Umetsu DT: Induction of T helper type 1-like regulatory cells that express Foxp3 and protect against airway hyper-reactivity. *Nat Immunol* 2004;5:1149–1156.
- 10 Kearley J, Barker JE, Robinson DS, Lloyd CM: Resolution of airway inflammation and hyperreactivity after in vivo transfer of CD4+CD25+ regulatory T cells is interleukin 10 dependent. *J Exp Med* 2005;202:1539–1547.
- 11 Carson WF, Guernsey LA, Singh A, Vella AT, Schramm CM, Thrall RS: Accumulation of regulatory T cells in local draining lymph nodes of the lung correlates with spontaneous resolution of chronic asthma in a murine model. *Int Arch Allergy Immunol* 2008;145:231–243.
- 12 Lewkowich IP, Herman NS, Schleifer KW, Dance MP, Chen BL, Dienger KM, Sproles AA, Shah JS, Köhl J, Belkaid Y, Wills-Karp M: CD4+CD25+ T cells protect against experimentally induced asthma and alter pulmonary dendritic cell phenotype and function. *J Exp Med* 2005;202:1549–1561.
- 13 Ostroukhova M, Seguin-Devaux C, Oriss TB, Dixon-McCarthy B, Yang L, Ameredes BT, Corcoran TE, Ray A: Tolerance induced by inhaled antigen involves CD4+ T cells expressing membrane-bound TGF- β and FOXP3. *J Clin Invest* 2004;114:28–38.
- 14 Yagi H, Nomura T, Nakamura K, Yamazaki S, Kitawaki T, Hori S, Maeda M, Onodera M, Uchiyama T, Fujii S, Sakaguchi S: Crucial role of FOXP3 in the development and function of human CD25+CD4+ regulatory T cells. *Int Immunol* 2004;16:1643–1656.
- 15 Sakaguchi S: Regulatory T cells in the past and for the future. *Eur J Immunol* 2008;38:901–903.
- 16 Hartl D, Koller B, Mehlhorn AT, Reinhardt D, Nicolai T, Schendel DJ, Griesse M, Krauss-Etschmann S: Quantitative and functional impairment of pulmonary CD4⁺CD25^{hi} regulatory T cells in pediatric asthma. *J Allergy Clin Immunol* 2007;119:1258–1266.
- 17 Zosky GR, Larcombe AN, White OJ, Burchell JT, Janosi TZ, Hantos Z, Holt PG, Sly PD, Turner DJ: Ovalbumin-sensitized mice are good models for airway hyperresponsiveness but not acute physiological responses to allergen inhalation. *Clin Exp Allergy* 2008;38:829–838.
- 18 Pabst R: Are animal models of asthma useful; in Kay AB, Kaplan A, Bousquet J, Holt P (eds): *Allergy and Allergic Diseases*. Oxford, Blackwell, 2008.
- 19 Tschernig T, Neumann D, Pich A, Dorsch M, Pabst R: Experimental bronchial asthma – the strength of the species rat. *Curr Drug Targets* 2008;9:466–469.
- 20 Kruschinski C, Skripuletz T, Bedoui S, Tschernig T, Pabst R, Nassenstein C, Braun A, von Hörsten S: CD26 (dipeptidyl-peptidase IV)-dependent recruitment of T cells in a rat asthma model. *Clin Exp Immunol* 2005;139:17–24.
- 21 Skripuletz T, Schmiedl A, Schade J, Bedoui S, Glaab T, Pabst R, von Hörsten S, Stephan M: Dose-dependent recruitment of CD25⁺ and CD26⁺ T cells in a novel F344 rat model of asthma. *Am J Physiol Lung Cell Mol Physiol* 2007;292:L1564–L1571.
- 22 Kruschinski C, Skripuletz T, Bedoui S, Raber K, Straub RH, Hoffmann T, Grote K, Jacobs R, Stephan M, Pabst R, von Hörsten S: Post-natal life events affect the severity of asthmatic airway inflammation in the adult rat. *J Immunol* 2008;180:3919–3925.
- 23 Stephens LA, Barclay AN, Mason D: Phenotypic characterization of regulatory CD4+CD25+ T cells in rats. *Int Immunol* 2004;16:365–375.
- 24 Pabst R, Durak D, Roos A, Lührmann, Tschernig T: TLR2/6 stimulation of the rat lung: effects on lymphocyte subsets, natural killer cells and dendritic cells in different parts of the air-conducting compartments and at different ages. *Immunology DOI*: 10.1111/j.1365–2567.2008.02886.x.
- 25 Pabst R: Mucosa-associated lymphoid tissue of the lung: localization, numbers and dynamics of lymphoid cells in the five different compartments; in Busse WW, Holgate ST (eds): *Asthma and Rhinitis*. Oxford, Blackwell, 2000, pp 543–556.
- 26 Kraal G, Samsom JN, Mebius RE: The importance of regional lymph nodes for mucosal tolerance. *Immunol Rev* 2006;213:119–130.
- 27 Glaab T, Taube C, Braun A, Mitzner W: Invasive and noninvasive methods for studying pulmonary function in mice. *Respir Res* 2007;8:63–72.
- 28 Lührmann A, Grote K, Stephan M, Tschernig T, Pabst R: Local pulmonary immune stimulation by the Toll-like receptor 2 and 6 ligand MALP-2 in rats is age dependent. *Immunol Lett* 2007;108:167–173.
- 29 Wei S, Kryczek I, Zou W: Regulatory T-cell compartmentalization and trafficking. *Blood* 2006;108:426–431.
- 30 Walker LSK: CD4+ CD25+ T_{reg}: divide and rule? *Immunology* 2004;111:129–137.
- 31 Vukmanovic-Steijc M, Zhang Y, Cook JE, Fletcher JM, McQuaid A, Masters JE, Rustin MH, Taams LS, Beverley PC, Macallan DC, Akbar AN: Human CD4⁺CD25^{hi}Foxp3⁺ regulatory T cells are derived by rapid turnover of memory populations in vivo. *J Clin Invest* 2006;116:2423–2433.
- 32 Uhlig HH, Coombes J, Mottet C, Izcue A, Thompson C, Fanger A, Tannapfel A, Fontenot JD, Famsdell F, Powrie F: Characterization of Foxp3⁺CD4⁺CD25⁺ and IL-10-secreting CD4⁺CD25⁺ T cells during cure of colitis. *J Immunol* 2006;177:5852–5860.
- 33 Belkaid Y: Regulatory T cells and infection: a dangerous necessity. *Nat Rev Immunol* 2007;7:875–888.
- 34 Taams LS, Palmer DB, Akbar AN, Robinson DS, Brown Z, Hawrylowicz CM: Regulatory T cells in human disease and their potential for therapeutic manipulation. *Immunology* 2006;118:1–9.
- 35 Umetsu DT, Akbari O, DeKruyff RH: Regulatory T cells control the development of allergic disease and asthma. *J Allergy Clin Immunol* 2003;112:480–487.
- 36 Hawrylowicz CM: Regulatory T cells and IL-10 in allergic inflammation. *J Exp Med* 2005;202:1459–1463.
- 37 Strickland DH, Stumbles PA, Zosky GR, Subrata LS, Thomas JA, Turner DJ, Sly PD, Holt PG: Reversal of airway hyperresponsiveness by induction of airway mucosal CD4+CD25+ regulatory T cells. *J Exp Med* 2006;203:2649–2660.
- 38 Jarvis LB, Matyszak MK, Duggleby RC, Goodall JC, Hall FC, Gaston JS: Autoreactive human peripheral blood CD8+ T cells with a regulatory phenotype and function. *Eur J Immunol* 2005;35:2896–2908.
- 39 Rioux KP, Wallace JL: Long-term antigen challenge results in progressively diminished mucosal mast cell degranulation in rats. *Gastroenterology* 1996;111:1516–1523.
- 40 Kang Y, Xu L, Wang B, Chen A, Zheng G: Cutting edge: immunosuppressant as adjuvant for toleragenic vaccination. *J Immunol* 2008;180:5172–5176.