



Increased immunosuppressive function of CD4⁺CD25⁺Foxp3⁺GITR⁺ T regulatory cells from NFATc2^(-/-) mice controls allergen-induced experimental asthma

Roman Karwot^{a,1}, Caroline Übel^{b,1}, Tobias Bopp^c, Edgar Schmitt^c, Susetta Finotto^{b,*}

^a Laboratory of Cellular and Molecular Immunology of the Lung, Institute of Molecular Medicine, University of Mainz, 55131 Mainz, Germany

^b Laboratory of Cellular and Molecular Lung Immunology of the Lung, Institute of Molecular Pneumology, University of Erlangen-Nürnberg, 91054 Erlangen, Germany

^c Institute of Immunology, University of Mainz, 55131 Mainz, Germany

ARTICLE INFO

Article history:

Received 30 November 2011

Received in revised form

21 December 2011

Accepted 1 January 2012

Keywords:

CD4⁺CD25⁺GITR⁺ T cells
CD4⁺CD25⁺Foxp3⁺ regulatory T cells
NFATc2
pSTAT-5

ABSTRACT

The expansion of effector T cells is tightly controlled by transcription factors like nuclear factor of activated T cells (NFAT) family members that mediate early intracellular responses to T cell receptor-mediated signals. In this study we show that, after allergen challenge, NFATc2^(-/-) mice had augmented number of functionally intact CD4⁺CD25⁺GITR⁺ T regulatory (T regs) cells in the lung. Anti-GITR antibody treatment inhibited T regulatory cell function and enhanced the number of activated lung CD4⁺ T cells associated with increased IL-2 and pSTAT-5 in the airways of NFATc2^(-/-) mice in experimental allergic asthma. This agonistic treatment led to increased inflammation in the lung of NFATc2^(-/-) treated mice. These data indicate that NFATc2^(-/-) mice have increased number of CD4⁺CD25⁺Foxp3⁺ T regulatory cells with induced immunosuppressive function that control allergen-induced experimental asthma.

© 2012 Elsevier GmbH. All rights reserved.

Introduction

The development of allergic immune responses in asthma is mediated by different cell types, including CD4⁺ T helper type 2 cells (Th2), which produce the cytokines IL-4, IL-5, and IL-13 (Neurath et al. 2002; Robinson et al. 1992), and CD4⁺ T helper 17 (Th17) cells which produce the cytokines IL-17A, IL-17F, IL-21 and IL-22 (Nurieva et al. 2007; Song et al. 2008). By contrast, CD4⁺CD25⁺Foxp3⁺ regulatory T cells (T reg) which play a pivotal role in autoimmune diseases have been thought to be decreased or functionally defective in allergic asthma.

The family of nuclear factor of activated T cells (NFAT) of transcription factors is critical in regulating early gene transcription in response to T cell receptor-mediated signals in lymphocytes (Macian 2005; Muller and Rao 2010; Serfling et al. 2007). Mice with a targeted disruption of NFATc2 show increased Th2 cytokines

and maintain the expression of IL-4 transcripts longer than wild-type mice (Bopp et al. 2005; Teixeira et al. 2005). In the present study we have focused on the dissection of NFATc2 function after allergen challenge independent from other family members in a murine model of asthma. We describe herein, that NFATc2^(-/-) mice develop CD4⁺CD25⁺GITR⁺ T regulatory cells in the spleen as well as in the lung with enhanced immunosuppressive function as compared to those obtained from the wild type littermates after allergen challenge in murine asthma models.

Materials and methods

Mice

NFATc2^(-/-) and wild type mice on a Balb/c genetic background were kindly provided to us by Laurie H. Glimcher (Harvard University, Boston, MA). Mice were maintained under specific pathogen free conditions. They entered the experimental protocol at the age of 6–8 weeks.

Allergen sensitization and challenge

According to previously published protocols female Balb/c or NFATc2^(-/-) received i.p. injections of 100 µg OVA/alum on days 0 and 7 and were challenged (50 mg OVA/ml PBS in aerosol solution)

Abbreviations: AHR, airway hyperresponsiveness; BAL, bronchoalveolar lavage; BALF, BAL fluid; MCh, methacholine; N, normal (concentration of ionizable groups); qPCR, quantitative real-time polymerase chain reaction; Ri, airway resistance; *t* test, Student's *t* test.

* Corresponding author at: Laboratory of Cellular and Molecular Lung Immunology, Department of Molecular Pneumology, Building 2, Room 1.025, Hartmannstrasse 14, 91054 Erlangen, Germany. Tel.: +49 09131 8535883; fax: +49 09131 8535977.

E-mail address: susetta.finotto@uk-erlangen.de (S. Finotto).

¹ These two authors share first authorship.

three times on days 14, 15 and 16 (Doganci et al. 2005; Finotto et al. 2002).

Anti-GITR agonistic antibodies treatment, assessment of AHR and BALF recovery

Two-hundred micrograms of agonistic anti-GITR (DTA1) or IgG control antibodies were injected intraperitoneally on days 8 and 11 in the above described model of murine allergic asthma. To assess AHR, we used a whole body plethysmograph as previously described (Doganci et al. 2008; Karwot et al. 2008). Following lung plethysmography, bronchoalveolar lavage (BAL) was performed as previously described (Karwot et al. 2008; Maxeiner et al. 2007).

Protein extraction and Western blot analysis

Tissue proteins were extracted and protein concentration was determined as previously described (Doganci et al. 2005). Western blots were performed with 50 µg of whole lung proteins and antibodies were used as previously indicated. Anti-T-bet monoclonal antibodies were kindly provided by L.H. Glimcher (Harvard Medical School, Boston, MA). Anti-pSTAT-5 was detected by using a cell signaling antibody (Tyr694).

ELISA

Mice IL-2, IL-4, IL-5, IL-6, IL-10 and IFN-γ were detected using a specific sandwich ELISA (OptEIA™, BD PharMingen, Heidelberg, Germany). Murine IL-17A was detected using an ELISA kit (R&D Systems). TGF-β1 analysis was performed by using purified rat anti-mouse, human, pig TGF-β1 as capture antibody (BD PharMingen, Heidelberg, Germany) and biotinylated rat anti-mouse, human, pig TGF-β1 (BD PharMingen) polyclonal antibody. Recombinant TGF-β1 was purchased from R&D Systems. To activate latent TGF-β1, samples were pre-treated with 10 µl of 1 N HCl for 30 min at 37 °C. Samples were then neutralized by adding 10 µl of 1 N NaOH.

Isolation and functional studies with spleen CD4⁺CD25⁺GITR⁺ T regulatory cells

The functionality and the isolation of CD4⁺CD25⁺GITR⁺ T regulatory cells as well as CD4⁺ target T cells were executed as previously described (Bopp et al. 2005).

FACS analysis and proliferation assay

Antibodies used for FACS analysis were as follows: anti-mouse CD69 (H1.2F3; BD Pharmingen, Heidelberg, Germany), anti-mouse CD44 (Pgp-1, Ly-24, ebioscience, Frankfurt, Germany), anti-mouse CD4 (L3T4, BD Pharmingen, Heidelberg, Germany), anti-mouse CD25 (PC61.5 ebioscience, Frankfurt, Germany), anti-mouse anti-GITR (DTA-1, ebioscience, Frankfurt, Germany), anti-mouse Foxp3 (FJK-16s, Miltenyi Biotec, Bergisch-Gladbach, Germany). The proliferation assay for lung CD4⁺ T cells (CFSE) was performed as previously described (Karwot et al. 2008). The proliferation and suppression assay for the spleen CD4⁺ and T regulatory cells was performed with a thymidine incorporation assay as previously described (Bopp et al. 2005).

Isolation of RNA from CD4⁺ T cells and real-time analysis of Foxp3 mRNA

Sorted and cultured CD4⁺ lung T cells were immediately frozen after culture until RNA was isolated following the manufacturer's instructions and as previously described (RNeasy Micro Kit; QIA-GEN or Tri Reagent, Sigma) (Doganci et al. 2005).

PCR was performed with IQ SYBR Green Supermix (Bio-Rad Laboratories) as previously described (10). Results were normalized using internal control HGPRT. To eliminate amplifications from contaminating genomic DNA, the following primers were designed to span an intron/exon boundary and thus to anneal specifically to cDNA:

Foxp3 forward primer 5'-CTTATCCGATGGCCATCCTGGAAG-3',
Reverse primer 5'-TTCCAGGTGGCGGGTGGTTTCTG-3';
HGPRT forward primer 5'-GTTGGATACAGGCCAGACTTTGTTG-3',
Reverse primer 5'-GAGGGTAGGCTGGCCTATAGGCT-3'.

Statistical analysis

Differences were evaluated for significance ($p < 0.05$) by the Student's two-tailed *t* test for independent events (Excel, PC). Data are given as mean values ± SEM. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Results

Increased regulatory cytokines and CD4⁺CD25⁺Foxp3⁺GITR⁺ lung T cell numbers in NFATc2^(-/-) mice

Recent reports have suggested that CD4⁺CD25⁺Foxp3⁺ regulatory T cells can suppress airway inflammation and AHR in experimental asthma. We previously demonstrated that NFATc2 deficient mice have increased AHR as compared to wild-type littermates in the absence of allergen challenge (Karwot et al. 2008). However, after allergen sensitization and challenge these mice did not show differences in AHR as compared to the wild type littermates indicating to us the presence of an inhibitory cell component induced after allergen challenge. We thus analyzed the regulatory function of T cells in the presence and absence of NFATc2 in a murine model of asthma. In the absence of co-stimulatory anti-CD28 antibodies, lung CD4⁺ T cells lacking NFATc2 released increased amounts of the immune-suppressor cytokine IL-10 (Fig. 1A). Co-stimulation with anti-CD28 antibodies attenuated the differences in IL-10 in the supernatants of lung CD4⁺ T cells of NFATc2^(-/-) mice as compared to those of the wild type littermates. By contrast, TGF-β1, a suppressor cytokine known to induce regulatory T cells, was found elevated with and without anti-CD28 antibodies stimulation in response to allergen challenge (Fig. 1A). This observation suggested the presence of an increased level of T regulatory cytokines in the supernatants of lung CD4⁺ T cells isolated from NFATc2^(-/-) mice.

Since peripheral CD4⁺CD25⁺ T regulatory cells express the forkhead family transcription factor Foxp3, which is up-regulated by TGF-β1 (Fontenot et al. 2003; Ichiyama et al. 2008), we investigated Foxp3 expression in naïve lung CD4⁺CD25⁺ T cells in wild-type and NFATc2^(-/-) mice. FACS analysis revealed a significantly increased number of lung CD4⁺CD25⁺Foxp3⁺ T cells in the lung of NFATc2 deficient mice. These cells also expressed more glucocorticoid induced tumor necrosis factor receptor (GITR) as compared to those isolated from wild-type littermates (Fig. 1B, left panel). Furthermore, total lung CD4⁺ T cells isolated from NFATc2 deficient mice expressed increased Foxp3 mRNA levels both spontaneously (PBS/PBS) and after allergen sensitization (OVA/PBS) or OVA sensitization and challenge (OVA/OVA) as compared to those isolated from wild-type littermates in studies using quantitative RT-PCR (Fig. 1B, right panel).

Taken together, these data suggest up-regulation of T regulatory cells in the lungs of NFATc2^(-/-) mice that inhibits AHR.

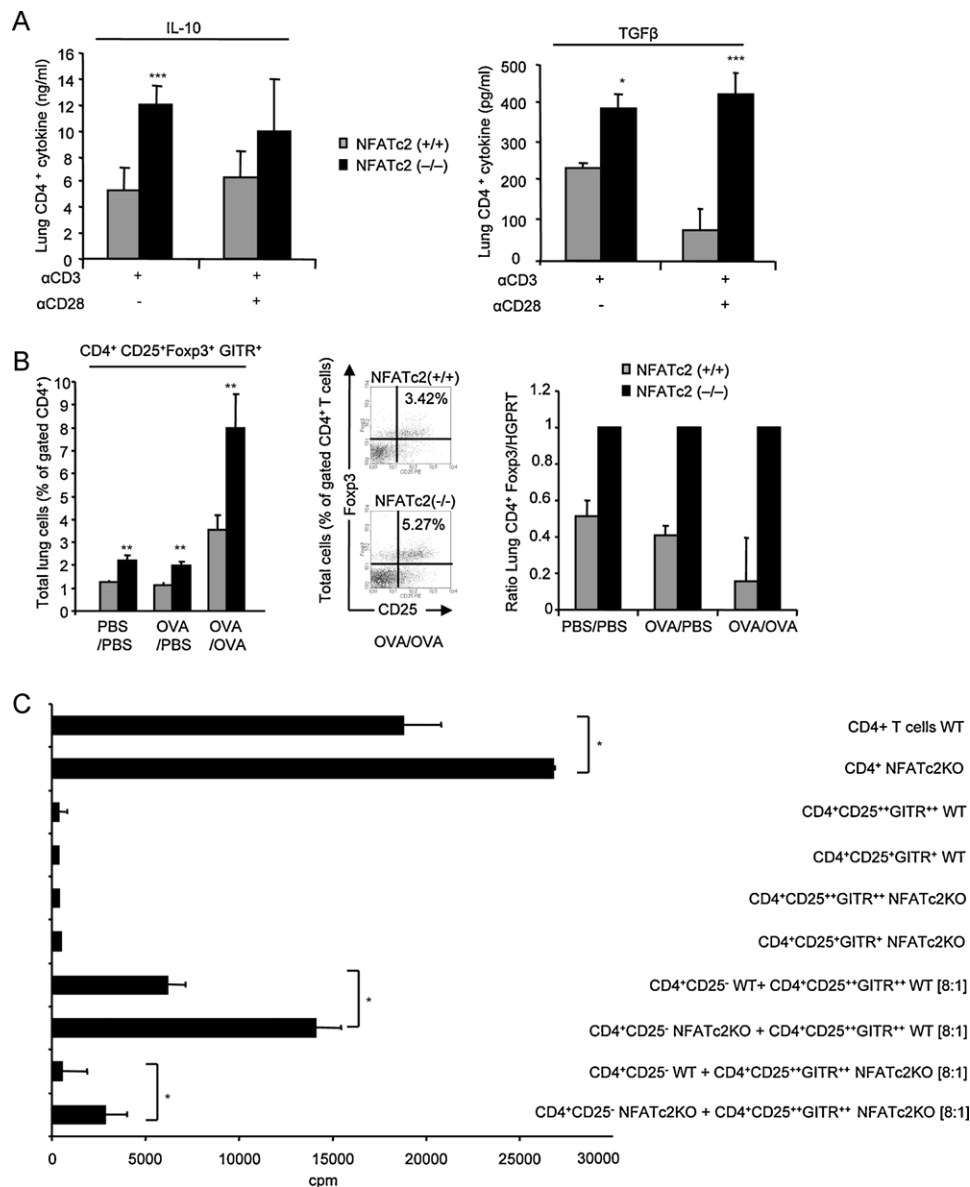


Fig. 1. Increased IL-10/TGF-β1 producing lung CD4⁺ T cells and lung CD4⁺CD25⁺Foxp3⁺GITR⁺ T cells in NFATc2^{-/-} mice after allergen sensitization and challenge. (A) CD4⁺ T cells isolated from the lungs NFATc2^{-/-} mice secreted increased amounts of IL-10 (left panel) and TGF-β1 (right panel) as measured by ELISA ($p=0.0098$ and $p=0.026$ for IL-10 and $p=0.047$ and $p=0.00023$ for TGF-β1 as compared to wild-type mice after anti-CD3/CD28 antibodies challenge, respectively). (B, Left panel) NFATc2^{-/-} mice had significant more lung CD4⁺CD25⁺Foxp3⁺GITR⁺ T cells than wild-type mice as assessed by FACS analysis ($n=4$; $p=0.04$, CD4⁺CD25⁺, $p=0.02$ CD4⁺CD25⁺Foxp3⁺, $p=0.01$ CD4⁺CD25⁺GITR⁺Foxp3⁺). (Right panel) Foxp3 expression in lung CD4⁺ T cells from NFATc2^{-/-} and wild type littermates was analyzed after RNA extraction followed by qPCR. QPCR for Foxp3 in CD4⁺ T cells is shown and reported as ratio between Foxp3 and HGPRT for wild-type and NFATc2^{-/-} CD4⁺ T cells. Lack of NFATc2 led to significant up-regulation of Foxp3 as compared to wild-type littermates. Three experiments were performed in duplicate. (C) Isolation and functional characterization of CD4⁺ CD25⁺GITR⁺ T cells in wild type and NFATc2^{-/-} mice. Upper part. Proliferation of spleen CD4⁺ T cells from wild type and NFATc2^{-/-} mice. Wild-type and NFATc2^{-/-} CD25⁺ T cells were purified using magnetic cell sorting and stained for GITR-FITC and CD25-PE for FACS analysis and after that proliferation was analyzed. (Lower part) Wild-type or NFATc2^{-/-} CD4⁺CD25⁺GITR⁺ T cells were FACS-sorted and then incubated with CD4⁺CD25⁻ T cells from wild-type and NFATc2^{-/-} mice, NFATc2^{-/-} and wild-type CD4⁺CD25⁺GITR⁺ T regulatory cells show comparable suppressive activity on the proliferation of wild-type and NFATc2^{-/-} CD4⁺CD25⁻ T cells. No suppressive effect was seen when CD4⁺CD25⁺GITR⁺ were used instead. Shown are data from one representative experiment of two performed in triplicate using CD4⁺CD25⁺GITR⁺ T cells.

Immunosuppressive function of spleen CD4⁺ CD25⁺ Foxp3⁺ GITR⁺ T regulatory cells in the presence or absence of NFATc2

We then examined the immunosuppressive function of CD4⁺CD25⁺GITR⁺ spleen cells. To this aim, we isolated CD4⁺CD25⁺GITR⁺ and CD4⁺CD25⁻ T cells directly from the spleen of wild-type and NFATc2^{-/-} mice by FACS-sorting. Although not a specific marker for CD4⁺CD25⁺ Foxp3⁺ T reg cells, GITR is preferentially expressed by T reg cells (McHugh et al. 2002; Shimizu et al. 2002). Spleen CD4⁺ T cells were co-cultured for 4 days at different ratios with CD4⁺CD25⁺GITR⁺ or CD4⁺CD25⁺GITR⁺ cells. These

co-cultures were then labelled with [³H]-thymidine overnight and the proliferation was analyzed. As shown in Fig. 1C, NFATc2^{-/-} CD4⁺ T cells proliferated more rapidly than those isolated from wild-type littermates and T reg cells from both strains did not proliferate at all. A statistically significant inhibition of both proliferation of wild-type and NFATc2^{-/-} target CD4⁺ T cells was seen after co-culture with wild-type CD4⁺CD25⁺GITR⁺ cells at the physiological ratio of 8:1. At the same ratio, CD4⁺CD25⁺GITR⁺ T cells (Fig. 1C) from the spleen of NFATc2^{-/-} mice inhibited the proliferation of wild-type and NFATc2^{-/-} CD4⁺CD25⁻ target T cells even more effectively than T regulatory cells isolated from the

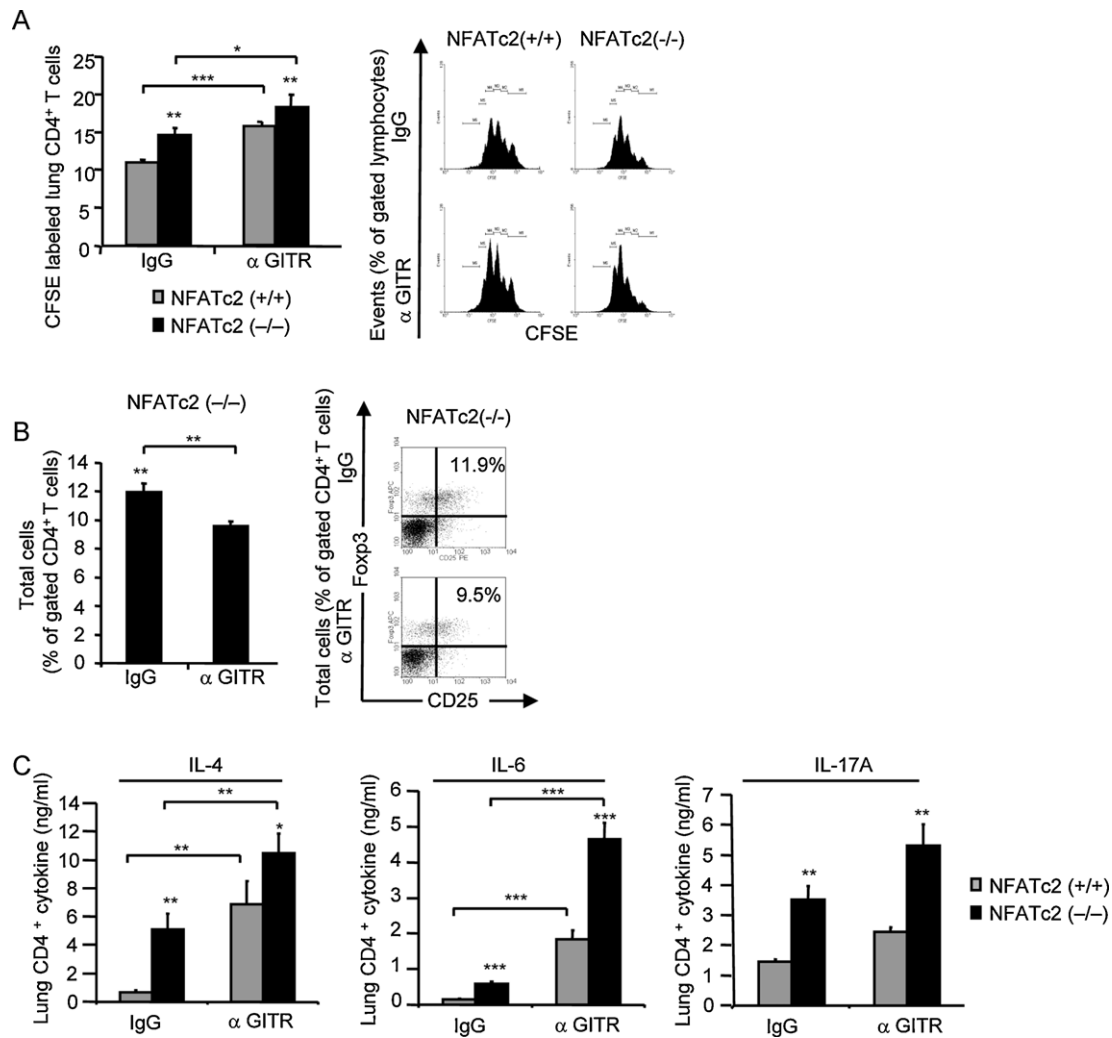


Fig. 2. Anti-GITR antibody treatment decreases CD4⁺CD25⁺Foxp3⁺ T cells and induced CD4⁺ T effector cell proliferation in the airways of NFATc2^{-/-} mice after allergen challenge. (A) Increased proliferation of CD4⁺ T cells isolated from the lungs of NFATc2^{-/-} mice before and after anti-GITR antibody treatment as shown after FACS analysis ($n=4$ per group; $p=0.0019$; $p=0.0063$). This experiment was performed twice. (B) Decreased numbers of CD4⁺CD25⁺Foxp3⁺ T regulatory cells in the lungs of NFATc2^{-/-} mice after anti-GITR antibody treatment ($n=6$; $p=0.028$). (C) Increased IL-4 ($n=4$, $p=0.0042$; $p=0.033$), IL-6 ($n=4$, $p=0.00044$; $p=0.001$) and IL-17 ($n=4$; $p=0.011$ $p=0.0014$) levels in the supernatant of CD4⁺ T cells isolated from lungs of NFATc2^{-/-} mice after anti-GITR antibody treatment and after overnight anti-CD3/CD28 antibody incubation.

wild type mice. The results indicated that NFATc2^{-/-} mice develop functionally intact immunosuppressive CD4⁺CD25⁺Foxp3⁺ T regulatory cells in the spleen. These results suggested that allergen challenge in the absence of NFATc2 leads to increased T regulatory cells which inhibit activation of CD4⁺ T effector function (Diehn et al. 2002; Maloy and Powrie 2001).

Local anti-GITR antibody treatment decreased CD4⁺CD25⁺Foxp3⁺ T cells and induced T-helper type 2 and T-helper 17 effector cells in the airways of NFATc2^{-/-} mice after allergen challenge

Patients with mutations in the transcription factor Foxp3 (IPEX) develop deficiencies in CD4⁺CD25⁺ T reg cells and present severe eczema, elevated IgE, eosinophilia and food allergy (Maloy and Powrie 2001). For a better understanding of the function of the T regulatory cells in the lung, OVA/OVA treated mice were injected intraperitoneally with 200 μg of agonistic anti-GITR antibody which is known to suppress T regulatory cells and thus favour T cell effector function (McHugh et al. 2002; Shimizu et al. 2002). Although GITR is expressed at low levels on resting T, B cells and macrophages, it is expressed at high levels on regulatory T cells

(Shimizu et al. 2002). Anti-GITR antibody treatment was also shown to enhance the expression of the key Th1 (T-bet), Th2 (GATA3) and Th17 (RORγt) transcription factors *in vitro* and *in vivo* (Fujiwara et al. 2007). Anti-GITR antibodies treated mice showed an increased proliferation of lung CD4⁺ T cells in comparison to the IgG treated group (Fig. 2A), suggesting that the number of T reg cells was down-regulated. Consistently, the number of CD4⁺CD25⁺Foxp3⁺ T cells was significantly suppressed by agonistic anti-GITR antibodies treatment in NFATc2^{-/-} mice (Fig. 2B). In addition, we found that anti-GITR treatment significantly up-regulated the Th2 signature cytokine IL-4 (Fig. 2C, left panel). Moreover, we found increased levels of IL-6, a cytokine amongst others produced by Th17 cells, both in wild-type and NFATc2^{-/-} mice (Fig. 2C, middle panel). Although IL-17A was increased in the supernatants of lung CD4⁺ T cells isolated from NFATc2^{-/-} mice, no significant increase was found in wild-type and NFATc2^{-/-} groups after anti-GITR treatment (Fig. 2C, right panel). In summary, we found that anti-GITR treated mice produce a higher amount of cytokine released by T helper effector Th2 and Th17 cells such as IL-4, IL-6 and IL-17A compared to the IgG treated NFATc2^{-/-} and anti-GITR treated wild type mice.

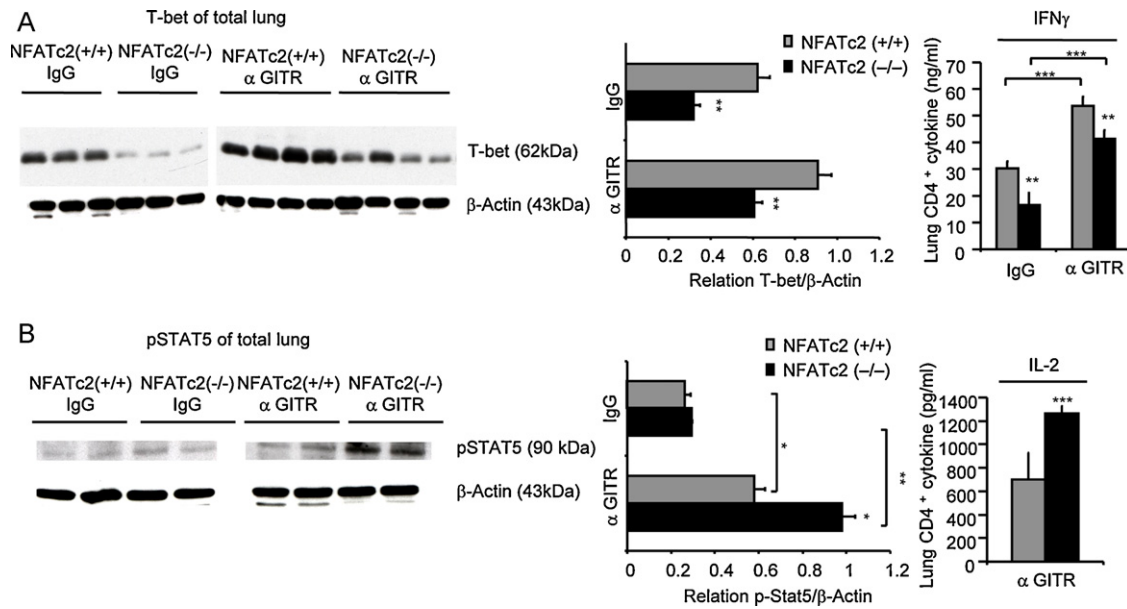


Fig. 3. Anti-GITR antibody treatment increased T-bet and IL-2-pSTAT-5 dependent effector CD4⁺ T cells in the airways of NFATc2^(-/-) mice after allergen challenge. (A) T-bet, the main transcription factor of IFN- γ , and IFN- γ were found decreased in the lungs of NFATc2^(-/-) mice and were significantly up-regulated after anti-GITR antibody treatment. (B) Increased p-STAT5 in the lungs of NFATc2^(-/-) mice as compared to wild-type littermates in a murine model of asthma after antigen sensitization and challenge after agonistic anti-GITR antibodies treatment ($n = 4-6$) $p = 0.00859$ and $p = 0.01725$ for wild type and NFATc2^(-/-) before versus after anti-GITR treatment, respectively; $p = 0.0033$ when anti-GITR antibody treatment in the NFATc2^(-/-) mice was compared to the same treatment in the wild type littermates. IL-2 was also up-regulated in the supernatants of lung CD4⁺ T cells isolated from NFATc2^(-/-) mice treated with anti-GITR antibodies and as compared to wild-type littermates that receive the same treatment ($p = 0.00097$; $n = 6$). The experiments were performed three times. A representative experiment is shown.

Anti-GITR antibody treatment induced T-helper type 1 effector cells as well as pSTAT-5 in the airways of NFATc2^(-/-) mice after allergen challenge.

T regulatory cells are known to inhibit effector Th1 cells (Fontenot et al. 2003). Consistent with the presence of increased T regulatory cell number in the lungs of NFATc2^(-/-) mice we noticed

a decrease of T-bet in NFATc2^(-/-) mice in a murine model of allergic asthma (Fig. 3A, left panel). In accordance with the above quoted published data, we also found that anti-GITR antibody treatment induced the transcription factor T-bet (Fig. 3A, left and middle panel) in the lungs of treated mice. NFATc2^(-/-) mice were found to express lower levels of T-bet in both IgG and anti-GITR groups.

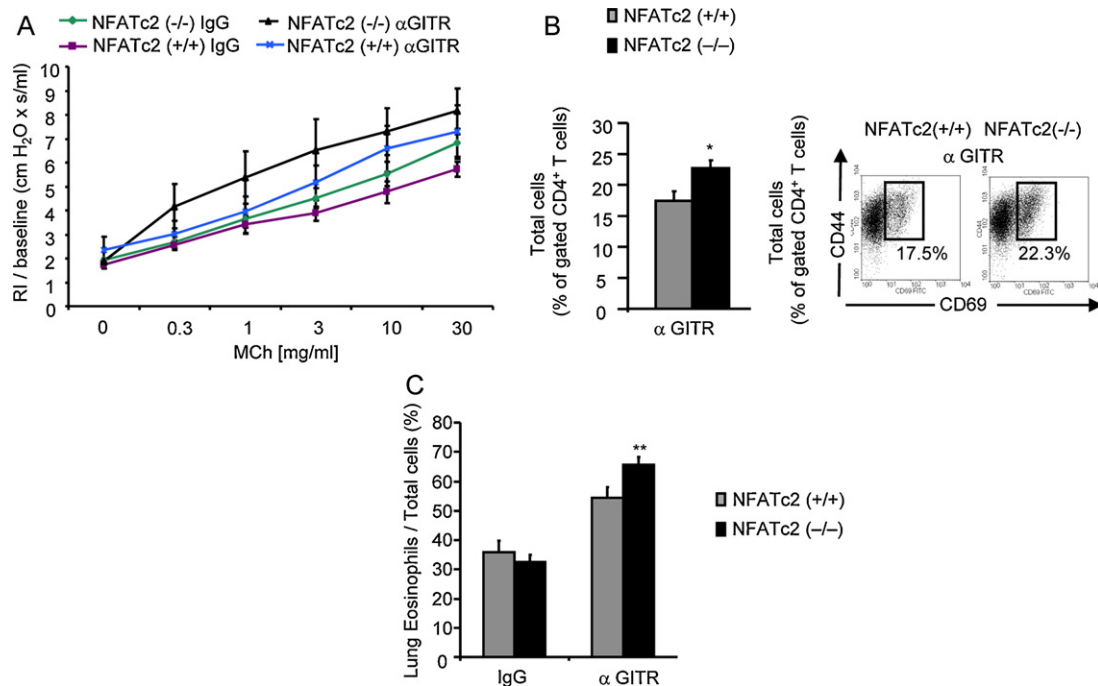


Fig. 4. Local anti-GITR antibody treatment induced T cell activation and eosinophilia in the airways of NFATc2^(-/-) mice. (A) AHR in the lung of wild type and NFATc2^(-/-) mice upon IgG and anti-GITR antibody treatment ($n = 8-9$, $p = 0.022$; $p = 0.043$; $p = 0.032$; $p = 0.016$). (B) Increased activation of lung CD4⁺ T cells after anti-GITR antibody treatment as assessed by quantification of CD4⁺CD44⁺CD69⁺ T cells ($n = 6$; $p = 0.025$). (C) Increased number of eosinophils in the lung of NFATc2^(-/-) mice after anti-GITR antibodies treatment ($n = 4$, $p = 0.0087$). Three experiments were performed and one representative of those is shown.

Consistently, the Th1 signature cytokine IFN- γ was found to be elevated in the supernatants of lung CD4⁺ T cells of NFATc2^(-/-) mice after overnight culture with anti-CD3 and anti-CD28 antibodies (Fig. 3A, right panel). We then looked for a mechanism responsible of the increased effector functions after agonistic anti-GITR antibodies treatment. We reasoned that IL-2, a growth factor known to induce expansion of Th1/Th2/Th17 effector cells, might be up-regulated after agonistic challenge with anti-GITR in NFATc2^(-/-) mice. To investigate this possibility, we looked for phosphorylation of STAT-5, which is located downstream of IL-2 and found that pSTAT-5 was increased in the lungs of NFATc2^(-/-) mice after OVA sensitization and challenge and after additional anti-GITR agonistic antibodies challenge in a murine model of asthma (Fig. 3B) (Fujiwara et al. 2007; Takatori et al. 2005a,b). In confirmation of this, IL-2 was significantly induced in the supernatants of lung CD4⁺ T cells isolated from anti-GITR antibody treated NFATc2^(-/-) mice as compared to those isolated from wild type littermates (Fig. 3B, right panel). Treatment of mice with anti-GITR antibodies enhanced, although not significantly, the AHR in NFATc2^(-/-) mice as well as in wild type littermate controls (Fig. 4A). Interestingly, this treatment was associated with a statistical significant increased number of CD4⁺CD44⁺CD69⁺ activated T cells (Fig. 4B) and eosinophils (Fig. 4C) in the lung of NFATc2^(-/-) mice.

Discussion

We previously demonstrated that NFATc2 deficient mice have increased AHR as compared to wild-type littermates in the absence of allergen challenge (Karwot et al. 2008). In the present study we report increased number of CD4⁺CD25⁺Foxp3⁺GITR⁺ regulatory T cells in the lung of NFATc2^(-/-) mice after allergen challenge in a murine model of allergic asthma. However, patients with mutations in the transcription factor Foxp3 (IPEX) develop deficiencies in CD4⁺CD25⁺ T reg cells and present severe eczema, elevated IgE, eosinophilia and food allergy (Maloy and Powrie 2001). The CD4⁺CD25⁺ T regulatory cells in the periphery are known to specifically express the forkhead family transcription factor Foxp3, which is believed to be up-regulated by TGF- β 1 (Fontenot et al. 2003; Ichiyama et al. 2008). The increased IL-10 and TGF- β 1 levels in the supernatants of isolated CD4⁺ T cells from the lungs of NFATc2^(-/-) mice could be a sign of an up-regulation of regulatory T cells. Investigation of the regulatory population by FACS analysis shows an increased number of lung CD4⁺CD25⁺Foxp3⁺GITR⁺ T regulatory cells. To obviate a dysfunction of the T regulatory cells in the NFATc2 deficient mice we examined the immunosuppressive function of CD4⁺CD25⁺GITR⁺ spleen cells. The results indicated that NFATc2^(-/-) mice develop functionally intact immunosuppressive CD4⁺CD25⁺GITR⁺ T regulatory cells in the spleen. These results suggested that allergen challenge in the absence of NFATc2 leads to increased T regulatory cells which inhibit activation of CD4⁺ T effector function (Diehn et al. 2002; Maloy and Powrie 2001). For a better understanding of the function of the T regulatory cells in the lung, OVA/OVA treated mice were injected with agonistic anti-GITR antibody which is known to suppress T regulatory cells and thus favors a T cell effector function (McHugh et al. 2002; Shimizu et al. 2002). Although GITR is expressed at low levels on resting T, B cells and macrophages, it is expressed at high levels on regulatory T cells (Shimizu et al. 2002). Anti-GITR antibody treatment was also recently shown to enhance the expression of the key Th1 (T-bet), Th2 (GATA3) and Th17 (ROR γ t) transcription factors *in vitro* and *in vivo* (Ichiyama et al. 2008; Fujiwara et al. 2007). In this paper we confirmed an up-regulation of T-bet after anti-GITR agonistic treatment. Indeed we found that anti-GITR treated mice produce a higher amount of cytokine released by T helper effector cells such as IL-4, IL-6 and IFN- γ compared to the IgG treated NFATc2^(-/-)

and anti-GITR treated wild type mice. The anti-GITR treatment led also to a significant up-regulation of CD44⁺CD69⁺ activated CD4⁺ T cells and to a significant down-regulation of CD4⁺CD25⁺Foxp3⁺ T regulatory cells in the lung of NFATc2 deficient mice which resulted in induced AHR and airway eosinophilia, demonstrating that CD4⁺CD25⁺Foxp3⁺GITR⁺ T regulatory cells and/or activation of lung CD4⁺GITR⁺ T effector cells control AHR. Moreover, we report here that agonistic challenge of GITR on T regulatory cells in the absence of NFATc2 leads to an increase of IL-17A implying an NFATc2 dependent inhibition of IL-17A by T regulatory cells. We thus looked for a common regulator of T helper effector cells. We previously demonstrated NFATc2 deficiency is accompanied by the down-regulation of IL-2 and IFN- γ production by CD8⁺ T cells which led to the expansion of the long lived memory CD8⁺CD122 (IL-2R β)⁺ IL-7R α ⁺ T cells (Karwot et al. 2008). Furthermore we demonstrated in an experimental tumour model that agonistic anti-GITR treatment in absence of NFATc2 induced CD4⁺CD127⁺ memory T cells and reduced T regulatory cells and restored IL-2 levels in CD8⁺ T cells (Maxeiner et al. 2009). We thus hypothesized that, also in asthma, in the absence of NFATc2, agonistic anti-GITR antibody treatment would reduce regulatory T cells and enhance AHR and airway inflammation through effectors T cells by inducing IL-2 signal transduction. We found that, *in vivo* treatment with anti-GITR antibodies led to up-regulation of IL-2 and pSTAT-5, downstream of IL-2R, in NFATc2 deficient mice as compared to wild type littermates in a murine model of allergic asthma after allergen sensitization and challenge. Moreover, in absence of NFATc2, agonistic GITR challenge enhanced airway eosinophilia significantly resulting in repression of the number of regulatory T cells and inducing memory-effectors function probably via activation of IL-2 and IL-2 signal transduction, namely pSTAT-5 (Maloy and Powrie 2001; Takatori et al. 2005a,b). These results thus show how in the absence of NFATc2 the balance between effector pathological and protective T regulatory cells can be easily shifted to a pathological side indicating that tailored therapy might be considered in allergic asthma caused by the absence of NFATc2.

Acknowledgments

The authors thank Françoise Burri, Joachim Maxeiner, Petra Schuster and Monika Zwicklbauer for their superb technical help in this study.

This study is supported by a SFB grant (TR-52/SFB643Mainz/Erlangen).

References

- Bopp, T., Palmethofer, A., Serfling, E., Heib, V., Schmitt, S., Richter, C., Klein, M., Schild, H., Schmitt, E., Stassen, M., 2005. NFATc2 and NFATc3 transcription factors play a crucial role in suppression of CD4⁺ T lymphocytes by CD4⁺CD25⁺ regulatory T cells. *The Journal of Experimental Medicine* 201, 181.
- Diehn, M., Alizadeh, A.A., Rando, O.J., Liu, C.L., Stankunas, K., Botstein, D., Crabtree, G.R., Brown, P.O., 2002. Genomic expression programs and the integration of the CD28 costimulatory signal in T cell activation. *Proceedings of the National Academy of Sciences of the United States of America* 99, 11796.
- Doganci, A., Eigenbrod, T., Krug, N., De Sanctis, G.T., Hausding, M., Erpenbeck, V.J., Haddad el, B., Lehr, H.A., Schmitt, E., Bopp, T., Kallen, K.J., Herz, U., Schmitt, S., Luft, C., Hecht, O., Hohlfeld, J.M., Ito, H., Nishimoto, N., Yoshizaki, K., Kishimoto, T., Rose-John, S., Renz, H., Neurath, M.F., Galle, P.R., Finotto, S., 2005. The IL-6R alpha chain controls lung CD4⁺CD25⁺ Treg development and function during allergic airway inflammation *in vivo*. *The Journal of Clinical Investigation* 115, 313.
- Doganci, A., Karwot, R., Maxeiner, J.H., Scholtes, P., Schmitt, E., Neurath, M.F., Lehr, H.A., Ho, I.C., Finotto, S., 2008. IL-2 receptor beta-chain signaling controls immunosuppressive CD4⁺ T cells in the draining lymph nodes and lung during allergic airway inflammation *in vivo*. *Journal of Immunology* 181, 1917.
- Finotto, S., Neurath, M.F., Glickman, J.N., Qin, S., Lehr, H.A., Green, F.H., Ackerman, K., Haley, K., Galle, P.R., Szabo, S.J., Drazen, J.M., De Sanctis, G.T., Glimcher, L.H., 2002. Development of spontaneous airway changes consistent with human asthma in mice lacking T-bet. *Science* 295, 336.

- Fontenot, J.D., Gavin, M.A., Rudensky, A.Y., 2003. Foxp3 programs the development and function of CD4⁺ CD25⁺ regulatory T cells. *Nature Immunology* 4, 330.
- Fujiwara, M., Hirose, K., Kagami, S., Takatori, H., Wakashin, H., Tamachi, T., Watanabe, N., Saito, Y., Iwamoto, I., Nakajima, H., 2007. T-bet inhibits both TH2 cell-mediated eosinophil recruitment and TH17 cell-mediated neutrophil recruitment into the airways. *The Journal of Allergy and Clinical Immunology* 119, 662.
- Ichihama, K., Yoshida, H., Wakabayashi, Y., Chinen, T., Saeki, K., Nakaya, M., Takaesu, G., Hori, S., Yoshimura, A., Kobayashi, T., 2008. Foxp3 inhibits RORgammat-mediated IL-17A mRNA transcription through direct interaction with RORgammat. *The Journal of Biological Chemistry* 283, 17003.
- Karwot, R., Maxeiner, J.H., Schmitt, S., Scholtes, P., Hausding, M., Lehr, H.A., Glimcher, L.H., Finotto, S., 2008. Protective role of nuclear factor of activated T cells 2 in CD8⁺ long-lived memory T cells in an allergy model. *The Journal of Allergy and Clinical Immunology* 121, 992.
- Macian, F., 2005. NFAT proteins: key regulators of T-cell development and function. *Nature Reviews. Immunology* 5, 472.
- Maloy, K.J., Powrie, F., 2001. Regulatory T cells in the control of immune pathology. *Nature Immunology* 2, 816.
- Maxeiner, J.H., Karwot, R., Hausding, M., Sauer, K.A., Scholtes, P., Finotto, S., 2007. A method to enable the investigation of murine bronchial immune cells, their cytokines and mediators. *Nature Protocols* 2, 105.
- Maxeiner, J.H., Karwot, R., Sauer, K., Scholtes, P., Boross, I., Koslowski, M., Tureci, O., Wiewrodt, R., Neurath, M.F., Lehr, H.A., Finotto, S., 2009. A key regulatory role of the transcription factor NFATc2 in bronchial adenocarcinoma via CD8⁺ T lymphocytes. *Cancer Research* 69, 3069.
- McHugh, R.S., Whitters, M.J., Piccirillo, C.A., Young, D.A., Shevach, E.M., Collins, M., Byrne, M.C., 2002. CD4⁺CD25⁺ immunoregulatory T cells: gene expression analysis reveals a functional role for the glucocorticoid-induced TNF receptor. *Immunity* 16, 311.
- Muller, M.R., Rao, A., 2010. NFAT, immunity and cancer: a transcription factor comes of age. *Nature reviews. Immunology* 10, 645.
- Neurath, M.F., Finotto, S., Glimcher, L.H., 2002. The role of Th1/Th2 polarization in mucosal immunity. *Nature Medicine* 8, 567.
- Nurieva, R., Yang, X.O., Martinez, G., Zhang, Y., Panopoulos, A.D., Ma, L., Schluns, K., Tian, Q., Watowich, S.S., Jetten, A.M., Dong, C., 2007. Essential autocrine regulation by IL-21 in the generation of inflammatory T cells. *Nature* 448, 480.
- Robinson, D.S., Hamid, Q., Ying, S., Tsicopoulos, A., Barkans, J., Bentley, A.M., Corrigan, C., Durham, S.R., Kay, A.B., 1992. Predominant TH2-like bronchoalveolar T-lymphocyte population in atopic asthma. *The New England Journal of Medicine* 326, 298.
- Serfling, E., Berberich-Siebelt, F., Avots, A., 2007. NFAT in lymphocytes: a factor for all events? *Science's STKE: Signal Transduction Knowledge Environment*, pe42.
- Shimizu, J., Yamazaki, S., Takahashi, T., Ishida, Y., Sakaguchi, S., 2002. Stimulation of CD25⁺CD4⁺ regulatory T cells through GITR breaks immunological self-tolerance. *Nature Immunology* 3, 135.
- Song, C., Luo, L., Lei, Z., Li, B., Liang, Z., Liu, G., Li, D., Zhang, G., Huang, B., Feng, Z.H., 2008. IL-17-producing alveolar macrophages mediate allergic lung inflammation related to asthma. *Journal of Immunology* 181, 6117.
- Takatori, H., Nakajima, H., Hirose, K., Kagami, S., Tamachi, T., Suto, A., Suzuki, K., Saito, Y., Iwamoto, I., 2005a. Indispensable role of Stat5a in Stat6-independent Th2 cell differentiation and allergic airway inflammation. *Journal of Immunology* 174, 3734.
- Takatori, H., Nakajima, H., Kagami, S., Hirose, K., Suto, A., Suzuki, K., Kubo, M., Yoshimura, A., Saito, Y., Iwamoto, I., 2005b. Stat5a inhibits IL-12-induced Th1 cell differentiation through the induction of suppressor of cytokine signaling 3 expression. *Journal of Immunology* 174, 4105.
- Teixeira, L.K., Fonseca, B.P., Vieira-de-Abreu, A., Barboza, B.A., Robbs, B.K., Bozza, P.T., Viola, J.P., 2005. IFN- γ production by CD8⁺ T cells depends on NFAT1 transcription factor and regulates Th differentiation. *Journal of Immunology* 175, 5931.