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

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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.

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.

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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; QIAGEN 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′-CTTATCCGATGGGCCATCCTGGAAG-3′,
Reverse primer 5′-TTCCAGGTGGCGGGGTGGTTTCTG-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.