

Elevated receptor for activated C kinase 1 expression is involved in intracellular Ca²⁺ influx and potentially associated with compromised regulatory T cell function in patients with asthma

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Running head: Elevated RACK1 involvement in Ca^{2+} influx in asthma T_{regs}

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Summary

Background Regulatory T cells (T_{regs}) are activated during anergy in response to T cell receptor (TCR) activation and functional immune suppression. Anergy of pediatric T_{regs} is partially dependent on intracellular calcium mobility; following TCR activation, T_{regs} do not exhibit increased intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_{\text{i}}$).

Objective We determined whether $[\text{Ca}^{2+}]_{\text{i}}$ in adult T_{regs} defined their anergy, if intracellular Ca^{2+} movement was linked to regulatory functions, whether $[\text{Ca}^{2+}]_{\text{i}}$ was indicative of asthma pathology, and the potential molecular mechanism responsible for Ca^{2+} movement in T_{regs} .

Methods T_{regs} were purified by the magnetic bead method, and their regulatory functions were assessed by monitoring carboxyfluorescein succinimidyl ester-labeled responder T cell proliferation. The Ca^{2+} response of Fura-2-labeled cells was measured using a video image analysis system. To analyze the functions of T_{regs} at the molecular level, we generated Jurkat Tet-On® clones with doxycycline (Dox)-induced forkhead box P3 (FOXP3) protein

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expression.

Results CD4⁺CD25⁺CD127^{-/low} T_{regs} from participants without asthma did not elicit Ca²⁺ influx in response to TCR activation, exhibited little proliferation, and suppressed proliferation of CD4⁺CD25⁻ T cells. In contrast, under similar conditions, T_{regs} from patients with asthma exhibited increased [Ca²⁺]_i and robust proliferation with partial loss of regulatory functions. FOXP3 protein levels in Tet-On® clones were high after both 2- and 5-day Dox treatment; however, 5-day cells were comparable to T_{regs} from patients with asthma, whereas 2-day cells were similar to T_{regs} from participants without asthma. Increasing [Ca²⁺]_i induced a high level of receptor for activated C kinase 1 (RACK1) expression in 5-day cells.

Conclusions and Clinical Relevance We confirmed that T_{regs} in patients with asthma are functionally impaired and that the abnormal regulatory functions of these cells can be analyzed by [Ca²⁺]_i following TCR engagement. Furthermore, the impaired functioning of T_{regs} evident in patients with asthma may be due to a high level of RACK1.

Introduction

CD4⁺CD25⁺ regulatory T cells (T_{regs}) regulate airway inflammation in asthma caused by the development of T helper cells and eosinophils [1-5]. Two types of T_{regs} participate in the regulation of allergic inflammation—thymus-derived T_{regs} (tT_{regs}) and peripherally derived T_{regs} (pT_{regs})—which function by secreting suppressor cytokines, such as interleukin (IL)-10 and transforming growth factor-β [6–9]. Ling *et al.* demonstrated that the regulatory activity of CD4⁺CD25⁺ T cells was impaired in symptomatic patients with atopic asthma or hay fever [10]. Several studies have investigated the number and/or functions of T_{regs} in patients with asthma [11-14]. Hartl *et al.* indicated that the number of CD4⁺CD25^{high} T cells and *forkhead box P3 (FOXP3)* mRNA expression levels were decreased in the bronchoalveolar lavage (BAL) of children with asthma without inhaled corticosteroid (ICS) treatment [15].

Furthermore, after 4 weeks of ICS treatment, both the number of CD4⁺CD25^{high} T cells and *FOXP3* mRNA expression levels increased. Conversely, other reports have shown that the number of T_{regs} and FOXP3 protein levels did not change in BAL of patients with asthma compared with healthy subjects [16,17]. Because the number of T_{regs} is associated with the degree of airway inflammation and because FOXP3 expression can be induced by corticosteroids, these studies suggested that the increase in CD4⁺FOXP3⁺ T cells was probably the result of these effects combined. These studies also suggested that most T_{regs} in BAL are pT_{regs}. Regarding peripheral blood mononuclear cells (PBMCs), which are less sensitive than BAL, the frequency of FOXP3⁺ cells among CD4⁺CD25^{bright} T cells in adult, steroid-treated patients with atopic asthma was higher than that in steroid-naïve patients with asthma [18]. *FOXP3* mRNA expression in PBMCs increased by specific allergen provocation. These data suggest that both types of T_{regs} function in the regulation of local inflammation and that the impaired function of T_{regs} causes chronic inflammation in allergy.

Ca²⁺ signaling plays a key role in the functioning of lymphocytes, including the regulation of their proliferation [19] and the gene expression of several cytokines, including IL-2 and chemokine (C-X-C motif) ligand 2 [20,21]. The Ca²⁺ release-activated Ca²⁺ (CRAC) channel, which consists of the pore-forming subunit (ORAI1) and the endoplasmic reticulum (ER) Ca²⁺ sensor stromal interaction molecule 1 (STIM1), is a highly Ca²⁺-selective store-operated channel [22,23]. The CRAC channel plays an important role in store-operated Ca²⁺ entry (SOCE) in T cells [24]. In addition, dysfunction of the Ca²⁺/calcineurin pathway following activation of nuclear factor of activated T cells results in Ca²⁺-dependent anergy [25]. Dysfunction of 70-kD ζ-associated protein, phospholipase C-γ, mitogen-activated protein kinase, or the Ca²⁺/calcineurin pathway results in reduced IL-2 transcription following reduced proliferation [26-28]. A decline in intracellular Ca²⁺ concentration ([Ca²⁺]_i) causes low IL-2 production and low expression of T cell activation

markers, such as CD25 and CD69, in response to T cell receptor (TCR) activation [29]. Recently, receptor for activated C kinase 1 (RACK1), a scaffold protein, was reported to act as a modulator of Ca^{2+} release from ER through binding to the inositol 1,4,5-triphosphate receptor (IP_3R) [30]; therefore, RACK1 is another key molecule for SOCE.

In a murine model study, reduced $[\text{Ca}^{2+}]_i$ was observed in anergic $\text{CD4}^+\text{CD25}^+$ T_{regs} following TCR activation [31]. Ca^{2+} unresponsiveness in $\text{CD4}^+\text{CD25}^+$ T_{regs} seems to be due to the long-lasting anergic state of these cells. In the anergic state, these cells also dissociate from their regulatory abilities at the molecular level [32]. We recently demonstrated that T_{regs} from pediatric patients with asthma exhibit aberrant $[\text{Ca}^{2+}]_i$ in response to TCR activation [33]. However, it is unclear whether this affects their suppressive ability. In this study, we focused on Ca^{2+} response to evaluate T_{reg} function in adult patients with asthma and directly compare T_{reg} function and functionality. We observed that TCR activation resulted in increased $[\text{Ca}^{2+}]_i$ in T_{regs} from adult patients with asthma compared with participants without asthma, and that this altered Ca^{2+} response reflected the abnormal proliferation of cells and compromised regulatory ability.

Materials and Methods

Subjects

Between November 2002 and October 2004, we recruited 50 patients with asthma from the Pulmonary Internal Outpatient Department at Showa University Fujigaoka Hospital, Tokyo, Japan and 23 volunteers without asthma as controls from Showa University through advertisement. Their characteristics are presented in Table 1. All volunteers answered a standard questionnaire regarding their allergy symptoms. Their atopic status was determined on the basis of serum total IgE levels, serum-specific IgE, and a history of allergic symptoms. The proportions of atopy among participants with and without asthma were 78% and 22%,

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respectively. Atopy in participants without asthma included hay fever (out of season) and, in 5 patients, isolated positive serum specific IgE for aeroallergens (mites, cat and dog dander, grass pollen, and *Aspergillus*) without allergic symptoms. Asthma was defined in accordance with the criteria established by the 1998 Japanese guidelines [34] and the Global Initiative for Asthma as revised in 2002 [35]. The severity of asthma was determined by evaluating daily and nocturnal asthma symptoms, peak expiratory flow, and forced expiratory volume in 1 s. During the study, all patients with asthma were stable and did not show any symptoms of asthma. β -agonists were not used 6 months before or after the study. The symptoms of 45 patients with less severe asthma (below Step 3) were controlled with inhaled corticosteroids; however, 3 of the 5 patients with Step 4 asthma used both inhaled and oral corticosteroids. Ethical approval for this study was granted by the Showa University Fujigaoka Hospital and the Pharmaceutical Ethics Committee of Showa University (2002022) and performed according to the Declaration of Helsinki. Written informed consent was obtained from all participants prior to study initiation.

Cells

PBMCs were separated from ethylenediaminetetraacetic acid (EDTA)-treated venous blood samples by density gradient centrifugation using Lymphoprep™ (Axis-Shield PoC AS, Oslo, Norway). Whole blood was diluted with an equal volume of phosphate-buffered saline (PBS), and 9 mL of the diluted blood was carefully layered with 4.5 mL Lymphoprep™ in a 15-mL centrifuge tube. The tube was centrifuged at 800 $\times g$ for 20 min at room temperature. CD4⁺CD25⁺ T cells, CD4⁺CD25⁻ T cells (responder cells), and CD2⁻ cells (feeder cells) were isolated from PBMCs using a MidiMACS™ Separation Unit (MACS; Miltenyi Biotec, Bergisch Gladbach, Germany). CD4⁺ T cells were purified from PBMCs (1×10^7) using CD4⁺ T Cell Isolation Kit II (Miltenyi Biotec) according to the manufacturer's instructions.

After collecting the flow-through from a negative selection of CD4⁺ T cells, 1×10^7 cells were incubated for 15 min at 4°C with a 2-fold dilution of anti-CD25 microbeads (the modified point) according to the manufacturer's instructions (Miltenyi Biotec). After magnetic selection, CD4⁺CD25⁺ and CD4⁺CD25⁻ T cell fractions were used as T_{regs} and responder cells, respectively. CD2⁻ cells were isolated from PBMCs (1×10^7) with anti-CD2 microbeads (Miltenyi Biotec). The purity of cell populations was measured by flow cytometry (EPICS Elite; Beckman Coulter, Inc., Miami, FL, USA).

Isolation of CD4⁺CD25⁺CD127^{-low} T cells

CD4⁺ T cells (1×10^6) were incubated for 10 min at 4°C with biotin-labeled anti-CD127 antibody (eBioscience, Inc., San Diego, CA, USA) and washed 1 time in MACS buffer. The cells were then incubated for 15 min at 4°C with Streptavidin microbeads and washed 1 time. CD4⁺CD127^{-low} T cells were obtained using a MACS separation unit. The CD4⁺CD127^{-low} T cells were positively selected using anti-CD25 microbeads. Purity of FOXP3⁺ cells among CD4⁺CD25⁺CD127^{-low} T cells was measured by flow cytometry.

Real-time PCR quantification of mRNA expression

Total RNA was obtained from CD4⁺CD25⁺, CD4⁺CD25⁺CD127^{-low}, and CD4⁺CD25⁻ T cells using RNAiso Plus reagent (Takara Bio Inc., Otsu, Japan). Total RNA (0.5 µg) was reverse transcribed to cDNA using the ReverTra Ace-α® (Toyobo Co., Ltd., Osaka, Japan) protocol with random primers. Real-time PCR was performed using a StepOne™ Real-Time PCR System (Life Technologies Co., Carlsbad, CA, USA) using the SYBR® Green (Molecular Probes Inc., Eugene, OR, USA) or TaqMan® (Applied Biosystems, Inc., Foster City, CA, USA) method. Analyses were performed using specific primers for *FOXP3* (NM_014009), *STIM1* (NM_003156), *ORAI1* (NM_032790), *RACK1* (NM_006098), and

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β -actin (NM_001101). SYBR® Green real-time PCR was performed for *FOXP3* and β -actin according to methods described previously [28]. *STIM1* (Hs00963373_m1), *ORAI1* (Hs00385627_m1), *RACK1* (Hs00272002_m1), and β -actin (Hs99999903_m1) were quantified using specific TaqMan® probe primer sets (Life Technologies Co.), and TaqMan® real-time PCR was performed according to the manufacturer's instructions (Life Technologies Co.). Individual data were normalized to β -actin expression.

Flow cytometry

Freshly isolated PBMCs were washed 2 times in PBS containing 5% fetal bovine serum (FBS), 2 mM EDTA, and 0.1% sodium azide [fluorescence-activated cell sorting (FACS) buffer] and were blocked with human IgG (Sigma-Aldrich Corporation, St. Louis, MO, USA) for 15 min at 4°C. After Fc blocking, the cells were incubated in 20 μ L FOXP3 staining solution [PBS, 0.5% bovine serum albumin (BSA), 2 mM EDTA, and 0.5% Tween 20] containing anti-FOXP3 (259D) monoclonal antibodies (mAbs). Intracellular FOXP3 staining was performed as described previously [29], and the surface antigens CD4, CD25, and CD127 were stained with two or three appropriately diluted PC5-, PC7-, or PE-labeled mAbs against human CD4 (13B8.2), CD25 (M-A251), and CD127. Unlabeled anti-FOXP3 (259D) and biotin-conjugated anti-CD127 (eBioRD5) mAbs were used as primary antibodies, and the cells were labeled with Alexa Fluor® 488- or PC5-streptavidin-labeled secondary antibodies, respectively. The cells were incubated with labeled and unlabeled primary antibodies for 15 min at 4°C and then washed 3 times with FACS buffer. For fluorochrome-nonconjugated primary antibodies, the cells were incubated with fluorochrome-conjugated secondary antibodies for 15 min at 4°C and then washed 2 times with FACS buffer. PC5-CD4, PC7-CD4, and PE-CD25 antibodies were obtained from Beckman Coulter, Inc., and biotin anti-CD127 specific mAb (eBioRD5) was obtained from eBioscience, Inc. The samples,

suspended in 350 μ L FOXP3 staining solution, were subjected to flow cytometry (EPICS XL; Beckman Coulter, Inc.) and analyzed using FlowJo software (TreeStar, Inc., Ashland, OR, USA). During flow cytometric analysis, the CD25⁺ population was gated for high level expression, thereby increasing the density of T_{regs}.

Measurement of changes in $[Ca^{2+}]_i$

Changes in $[Ca^{2+}]_i$ were measured as described previously [33,36]. Coverslips were coated with Cell-Tak™ (BD Biosciences, San Jose, CA, USA) to loosely fix the cells according to the manufacturer's instructions. The calcium indicator Fura-2 AM (5 μ M) was added to the cells, and the cells were incubated for 40 min while they adhered to glass coverslips (3 $\times$ 10 mm, thickness 0.12–0.17 mm; Matsunami Glass Ind., Ltd., Osaka, Japan). Freshly isolated cells and FOXP3 stable clones were incubated in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 5% AB Serum (Dainippon Sumitomo Pharma Co., Ltd., Osaka, Japan) and 10% FBS, respectively. The cells on the coverslips were washed and perfused with 0.1% BSA and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-buffered saline, which contained 107 mM NaCl, 6 mM KCl, 1.2 mM MgSO₄, 2 mM CaCl₂, 1.2 mM KH₂PO₄, 11.5 mM glucose, and 20 mM HEPES adjusted to pH 7.4 with NaOH. The coverslips were then placed in a perfusion chamber mounted on a microscope stage. Fluorescence images of the cells were recorded and analyzed using a video image analysis system (Meta Fulora; Nippon Ropper, Tokyo, Japan). Fura-2 fluorescence at an emission wavelength of 510 nm (bandwidth, 50 nm) was observed at room temperature by exciting Fura-2 at 340 nm (bandwidth, 10 nm) and 380 nm (bandwidth, 13 nm). Fluorescence (340/380) ratio images in individual cells were obtained on a pixel-by-pixel basis. TCR was activated by adding 5 μ g/mL α -CD3 (UCHT-1; BD Biosciences) and 5 μ g/mL of secondary antibody contained in perfusion solution. Cytosolic Ca²⁺ concentration was measured by the

two-wavelength excitation method (340/380). The delta ratio was recorded as the 340/380 ratio of peak minus average basement. Data were presented as means $\pm$ standard deviation (SD).

Regulatory activity analysis

Ki-67, a nuclear cell proliferation-associated antigen expressed during all active stages of the cell cycle, was used as a proliferation marker in this study. CD4⁺CD25⁺ T cells and CD4⁺CD25⁻ responder T cells (2×10^4 cells/well in 96-well flat-bottomed plate) were incubated with 30 Gy-irradiated CD2⁻ cells (2×10^5 cells/well) in 200 μ L RPMI 1640 medium supplemented with 5% AB serum for 4 days at 37°C with 5% CO₂. To analyze regulatory functions, CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells were mixed at a 1:1 ratio and incubated in the same well with CD2⁻ cells and 2.5 μ g/mL soluble anti-CD3 (Hit3a) and soluble anti-CD28 as a weak stimulus for polyclonal activation. For a strong stimulus, 5 μ g/mL plate-bound anti-CD3 (UCHT-1) and 2.5 μ g/mL soluble anti-CD28 were used. After culture, the cells were stained with 4 μ L PC5-CD4 mAb (a surface antigen) washed with FACS buffer, fixed and permeated in Cytofix/Cytoperm solution (20 min, 4°C), washed 2 times in Perm/Wash solution, and incubated with 4 μ L fluorescein isothiocyanate-Ki-67 mAb (30 min, 4°C). Stained cells were suspended in 200 μ L FACS buffer and analyzed using a Coulter EPICS Elite System (Beckman Coulter, Inc.).

The baseline proliferation of CD4⁺CD25⁺ T cells was set at a mean value (24.3 ± 3.34) for 5 patients with nonatopic asthma. The percentage of suppression was calculated using the following formula:

$$\% \text{ suppression} = 1 - \left[\frac{\% \text{ of Ki-67-positive cells (cocultured)} - 24.3}{[\% \text{ of Ki-67-positive cells (CD25}^+ + \text{CD25}^- \text{ T cells)]/2} - 24.3} \right]$$

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Autologous responder cells were labeled with the fluorescent dye carboxyfluorescein succinimidyl ester (CFSE; 2.5 μ M; Life Technologies, Co.) and cultured with CD4⁺CD25⁻ T cells or CD4⁺CD25⁺CD127^{-/low} T cells (T_{regs}) at a 2:1 ratio in the presence of α -CD3 (UCHT-1) and α -CD28. After 7–10 days, the amount of CFSE dye dilution was analyzed. Data were represented as the percentage of undivided cells.

Western blotting

FOXP3, ORAI1, STIM1, RACK1, and β -actin proteins were detected by western blotting with specific antibodies for each—anti-FOXP3 (259D) mouse mAb [37], anti-ORAI1 XP rabbit mAb (#3280), anti-STIM1 rabbit antibody (#4916), anti-RACK1 rabbit antibody (#4716; Cell Signaling Technology, Danvers, MA, USA), and anti- β -actin mouse antibody (ab6276; Abcam Plc., Cambridge, UK), respectively—using an Immobilon™ Western Chemiluminescent System (EMD Millipore Corporation, Billerica, MA, USA). The cells were lysed in 100 μ L lysis buffer (Roche Complete, 4 μ L; Roche PhosSTOP, 10 μ L; 1 M Tris-Cl pH 7.4, 2 μ L; 5 M NaCl, 3 μ L; 100 mM PMSF, 0.5 μ L; 10% Triton X-100, 10 μ L; ultrapure water, 70.5 μ L) and sonicated with Vibra Cell™ (Sonics & Materials, Inc., Newtown, CT, USA). After centrifugation at 15,000 rpm for 10 min, the lysates (30 μ g total protein loaded in each lane) were subjected to 10% SDS-PAGE using a miniPAGE Chamber AE6500, and electrotransferred on to a nitrocellulose membrane for 70 min at 20 V. The amount of each protein was measured by densitometric analysis using a GS-800 Densitometer (Bio-Rad Laboratories Inc., Hercules, CA, USA).

Small interfering RNAs

RACK1 small interfering RNAs (siRNAs; s20340, s20341) and negative control #1 siRNAs were purchased from Life Technologies Co. Cells were used for experiments after 48

h of siRNA transfection (final concentration 200 nM) using the Neon® Transfection System (Life Technologies Co.). During the transfection period, antibiotics were completely extracted from RPMI 1640 culture medium supplemented with 10% Tet-On®-approved FBS to enable siRNA transfection. The electroporation settings were as follows: pulse voltage, 1375; pulse width, 10; and pulse no., 3. Precise conditions, such as cell number and reagent volume, were selected according to the manufacturer's instructions.

Establishment of the Tet-On® system for FOXP3 stable clones

The Jurkat Tet-On® cell line was purchased from Takara Bio Inc. We constructed a pTRE-Tight/FLAG-FOXP3 vector to express a FLAG-tagged full-length human *FOXP3* transcript. *FOXP3*-containing vector (3 µg) and a linearized hygromycin resistance gene (600 ng), containing a short dsDNA fragment (Clontech Laboratories, Inc., Mountain View, CA, USA) and thereby providing a selection marker, were electrically transduced into Jurkat Tet-On® cells according to the manufacturer's instructions. The transduced cells were selected using 200 µg/mL hygromycin and were then cloned by the limiting dilution method. We established a Tet-On® FOXP3 hygromycin-resistant stable clone (F2A9) and a hygromycin-resistance only control clone (5G1). The culture medium was RPMI 1640 medium supplemented with 10% Tet-On®-approved FBS (Clontech Laboratories Inc.) and antibiotics.

Statistical analyses

Data were expressed as means ± SD unless otherwise specified. Multiple comparisons were calculated by analysis of variance with Bonferroni/Games-Howell *post-hoc* tests. All statistical tests were performed on a Windows-based computer using SPSS Statistics 21 (IBM Corporation, Somers, NY, USA).

Results

The same proportion of CD4⁺CD25⁺ T cells with different FOXP3 mRNA levels were detected in participants with and without asthma

We measured *FOXP3* transcript expression levels in CD4⁺CD25⁺ T cells and CD4⁺CD25⁻ (purity: 90–95%) T cells using real-time PCR (Fig. 1a). The average *FOXP3*: β -actin ratios in the CD4⁺CD25⁻ T cells of participants with and without asthma were 0.054 ± 0.018 and 0.031 ± 0.013 , respectively, and those in the CD4⁺CD25⁺ T cells of participants with and without asthma were 0.542 ± 0.135 and 0.398 ± 0.148 , respectively. As expected in the purified populations, *FOXP3* mRNA expression in CD4⁺CD25⁺ T cells was significantly higher than that in CD4⁺CD25⁻ T cells ($p < 0.001$). Although *FOXP3* expression in CD4⁺CD25⁺ T cells from patients with asthma was 1.36-fold higher than that in participants without asthma ($p < 0.01$), the proportion of CD45RO⁺ cells (another marker for T_{regs}) among CD4⁺CD25⁺ T cells was approximately 85% in both participants with and without asthma (data not shown). Therefore, no difference was observed in the proportion of CD4⁺CD25⁺CD45RO⁺ T_{regs} between participants with and without asthma. Our flow analysis implied that FOXP3 protein expression in CD4⁺CD25⁺FOXP3⁺ T cells from patients with asthma was similar to that in those from participants without asthma (Supplementary Fig. 1), implying that the translational level of FOXP3 (mean fluorescence intensity [MFI]) in individual cells may be similar between the groups.

Ca²⁺ response in CD4⁺CD25⁺ and CD4⁺CD25⁺CD127^{-low} T cells from participants with and without asthma

Treatment with α -CD3 antibody (UCHT-1), a secondary antibody (Ca²⁺ analysis required crosslinking of α -CD3 with a secondary antibody, because α -CD3 treatment alone did not elicit a sufficiently large Ca²⁺ response), and a TCR stimulator (to distinguish

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CD4⁺CD25⁻ T cells from all participants) resulted in increased oscillations in [Ca²⁺]_i (Fig. 1b, square). In contrast to CD4⁺CD25⁻ T cells, CD4⁺CD25⁺ T cells (proportion of unresponsive cells = 80 ± 11%) from participants without asthma did not exhibit increased [Ca²⁺]_i (Fig. 1b, circle, top left panel; *p* < 0.001 versus the response of CD4⁺CD25⁻ T cells). However, CD4⁺CD25⁺ T cells from patients with asthma showed increased [Ca²⁺]_i following TCR activation (Fig. 1b, bottom left and both panels on the right). Thus, the CD4⁺CD25⁺ T cells of patients with asthma exhibit an abnormal Ca²⁺ response following TCR activation.

To determine the mechanism underlying increased [Ca²⁺]_i in the CD4⁺CD25⁺ T cells of patients with asthma, the cells were stimulated with α-CD3 in the absence or presence of extracellular Ca²⁺. As shown in Figure 1c, although TCR stimulation without extracellular Ca²⁺ (0Ca) did not result in increased [Ca²⁺]_i, the presence of 2 mM Ca²⁺ (2Ca) extracellularly increased [Ca²⁺]_i in both the CD25⁻ and CD25⁺ T cells of patients with asthma. These data suggest that increase in [Ca²⁺]_i following TCR activation is attributable to Ca²⁺ influx primarily from the extracellular space. Increased [Ca²⁺]_i in CD4⁺CD25⁻ T cells following TCR activation was also induced by Ca²⁺ influx from the extracellular space. These data were similar to that obtained using the CD4⁺CD25⁻ T cells of participants without asthma (data not shown). Therefore, SOCE activity in CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells in patients with asthma was similar and did not reflect the differences observed between these populations.

Our isolated CD4⁺CD25⁺ T cell populations were of insufficient purity (81–95%) to functionally characterize FOXP3⁺ T_{regs}; therefore, we used CD4⁺CD25⁺CD127^{-/low} T cells (purity: 90–97.8%) to obtain a sufficiently pure population of FOXP3⁺ T_{regs} for our studies (Supplementary Fig. 2). Through intracellular Ca²⁺ analysis, the unresponsive state of CD4⁺CD25⁺CD127^{-/low} T cells was confirmed to be similar to that of CD4⁺CD25⁺ T cells (Fig. 3a, b). These data indicate that the frequency of unresponsive cells is dependent on the

purity of T_{regs} in CD4⁺CD25⁺CD127^{-/low} and CD4⁺CD25⁺CD45RO⁺ T cell populations.

Characteristics of study participants

All patients with asthma exhibited increasing [Ca²⁺]_i in T_{regs} in response to TCR stimulation, in contrast to participants without asthma, who did not. Of 20 patients with asthma, 19 were atopic. The Ca²⁺ response of T_{regs} was divided into poor (<0.1) and good (>0.1) based on the delta ratio of the plateau phase. Age had no effect on the Ca²⁺ response of T_{regs} (Supplementary Table 1). The number of patients with asthma exhibiting both responses was the same. Slightly more subjects exhibited a poor response with regard to severity and, among patients with Step 2 asthma, 60% exhibited a poor response and 40% a good response. T_{regs} from 3 patients using high doses (1,600 µg) of ICS exhibited a poor response; thus, inhaled steroids likely inhibit airway inflammation and help recover T_{reg} functions. However, T_{regs} from 1 patient with Step 4 asthma showed a good response, which may reflect uncontrollable airway inflammation in this patient, despite inhaled and oral steroid use.

Proliferation and reduced suppressive activity of CD4⁺CD25⁺ and CD4⁺CD25⁺CD127^{-/low} T cells from patients with asthma

T cell proliferation was assessed using the cell cycle marker Ki-67 (Fig. 2) or CFSE (Fig. 3c). Using soluble α-CD3 (Hit3a) and α-CD28 stimulation, the proportions of Ki-67-positive cells in cultures of CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells in participants without asthma were 26.2% ± 0.212% and 61% ± 1.84%, respectively (Fig. 2b). In cocultures of CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells, which were analyzed for regulatory activity affecting CD4⁺CD25⁻ T cell proliferation, the number of Ki-67-positive cells was reduced to the basal level of CD4⁺CD25⁺ T cells (85.5% inhibition; *p* < 0.05). Using plate bound α-CD3 (UCHT-1) and α-CD28 stimulation, the proportions of Ki-67-positive cells among

CD4⁺CD25⁺ T cells, CD4⁺CD25⁻ T cells, and cocultures were similar to those obtained using soluble α -CD3 (Hit3a) and α -CD28 stimulation (80.3% inhibition; $p < 0.05$). These data indicate that CD4⁺CD25⁺ T cells from participants without asthma proliferate less and exhibit suppressive activity. However, CD4⁺CD25⁺ T cells from Patient No. 1 with asthma (**representative of 4 patients**) showed similar levels of proliferation ($61.7\% \pm 4.67\%$) to CD4⁺CD25⁻ T cells, whereas the percentage inhibition of stimulation using both anti-CD3 antibodies (Hit3a and UCHT-1) in co-cultures declined to 35.8% ($p < 0.05$) and 14.6% (no significant difference), respectively. Thus, stimulation of CD4⁺CD25⁺ T cells from Patient No. 1 with asthma using α -CD3 or α -CD28 without additional IL-2 resulted in cell expansion and reduced regulatory functions. In Patient No. 2 with asthma (**representative of 5 patients**), stimulation of CD4⁺CD25⁺ T cells with soluble α -CD3 resulted in reduced proliferation, similar to that observed in the cells of participants without asthma, and an inhibition rate of approximately 100% ($p < 0.05$). Plate-bound α -CD3 stimulus resulted in less proliferation but no suppressive activity (-5.25% ; no significant difference). Thus, we observed two types of suppressive activity patterns in T_{regs} of patients with asthma. Moreover, the type of regulatory activity was compatible with the type of Ca²⁺ response; i.e., regulatory function of cells from Patient No.1 showed a good response and cells from Patient No.2 exhibited a poor response.

Coculture of CD4⁺CD25⁺CD127^{-low} T cells and responder cells showed that the percentage of undivided cells in patients with asthma (18 ± 2.24) was less than that in participants without asthma (49.05 ± 0.69 ; Fig. 3c). We concluded that T_{regs} from a proportion of patients with asthma exhibited reduced regulatory functions, which coincided with increasing [Ca²⁺]_i in CD4⁺CD25⁺ and CD4⁺CD25⁺CD127^{-low} T cells.

Characteristics of FOXP3⁺ Jurkat Tet-On® cells and analysis of FOXP3 repressor functions in these cells

The percentage of FOXP3⁺ cells increased among F2A9 clones but not in the controls (5G1) in a dose-dependent manner following doxycycline (Dox) treatment (data not shown), and the maximum rate was achieved at 1 µg/mL Dox. Expression of the CD25 antigen, transcription of which is positively regulated by FOXP3, increased (Geom.MFI: whole cells, 0.23; FOXP3^{low}, 0.37; FOXP3^{high}, 1.01) in F2A9 cells but not in control 5G1 cells (Fig. 4a). FOXP3 protein levels in F2A9 cells increased in a time-dependent manner and remained at high levels for 5 days (Fig. 4b). The CD25⁺ population among the F2A9 clones contained a higher frequency of FOXP3⁺ cells than unpurified clones (Geom.MFI: F2A9 clone, 0.30; CD25⁺ F2A9 clone, 1.8; data not shown). The Ca²⁺ response of FOXP3⁺ cells differed between Dox-treated 2-day (Dox 2-day) and 5-day (Dox 5-day) cells. Control cells responded positively to TCR stimulation, but Dox 2-day cells did not (Fig. 5a and b). Despite similar FOXP3 expression levels at 8 and 24 h in Dox 2-day cells (reflecting a similar Ca²⁺ response), Dox 5-day cells (c) responded in a manner reminiscent of the control cells.

Because the Ca²⁺ response to TCR stimulation of T cells is determined by CRAC channels, we measured both *ORAI1* and *STIM1* expression in these two cell types. *STIM1* mRNA expression in Dox 2-day cells tended to be lower than that of control cells (Fig. 6a, c). In Dox 2- and 5-day cells, *ORAI1* tended to exhibit reduced transcription and protein levels (Fig. 6b, c). FOXP3⁺ T_{regs} consistently tended to express low levels of *ORAI1*, with no difference between Dox 2- and 5-day cells; therefore, we could not explain the difference between these cells with regard to Ca²⁺ signaling.

IL-2 mRNA expression in Dox 2-day cells was repressed compared with control cells, but in Dox 5-day cells, there was no significant difference in *IL-2* mRNA expression compared with control cells (Fig. 5e). Because RACK1 functionally regulates SOCE, we also

analyzed *RACK1* mRNA and protein expression. Dox 5-day cells expressed 2.41- and 4.51-fold higher *RACK1* transcripts than control 5-day and Dox 2-day cells, respectively (Fig. 7a). Dox 5-day cells expressed noticeably higher levels of RACK1 protein than other samples (Fig. 7c). We investigated whether nonfunctional T_{regs} in patients with asthma also exhibited increased *RACK1* transcripts. CD4⁺CD25⁺CD127^{-low} T cells from patients with asthma expressed 1.52-fold higher *RACK1* levels on average than those from participants without asthma, and these levels were 1.97-fold higher than those expressed by the whole CD4⁺ population from patients with asthma (Fig. 7b).

To test whether RACK1 regulates the activity of CRAC channels in Dox 5-day cells, we silenced its expression using two independent siRNAs. Figure 8 (a, b) shows that knockdown using both siRNAs 1 and 2, which bind to different regions of *RACK1*, reduced *RACK1* mRNA and protein expression but had no effect on STIM1 and ORAI1 protein expression. *RACK1* silencing effectively diminished Ca²⁺ response in Dox 5-day cells following TCR stimulation, which did not occur with a control siRNA (Fig. 8c).

Discussion

In the peripheral blood in humans, selective markers for T_{regs} include FOXP3, intracellular cytotoxic T-lymphocyte antigen 4, and coexpression of surface CD25 and CD45RO antigens [5,38,41]. Although human CD4⁺CD25⁺ T_{regs} represent approximately 5–10% of the CD4⁺ T cells present in the blood, Baecher–Allan *et al.* indicated that the proportion of CD25-expressing cells among CD4⁺ T cells was approximately 1–2%, and that only these cells function as T_{regs} [38]. To analyze the functions of CD4⁺CD25^{high} T_{regs}, it is necessary to purify a large population of cells from a large volume of peripheral blood. However, it was not possible to purify CD4⁺CD25^{high} T cells from the peripheral blood of many patients with asthma because, for ethical reasons, only a small volume of blood could

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be obtained. Therefore, in this study, we employed a modified method, in which we were able to evaluate both the proportion and function of CD4⁺CD25⁺ T_{regs} in populations purified from a small volume of blood using a Ca²⁺ fluorescence imaging technique.

Many *in vitro* and *in vivo* studies have been conducted on anergy, which is characterized by reduced IL-2 production and proliferation following TCR activation. Ca²⁺ signaling occurs prior to IL-2 production and proliferation, and immediately after TCR activation. We focused on the Ca²⁺ response to TCR stimulation to analyze the anergic state of CD4⁺CD25⁺ T_{regs}. Consistent with a murine model study [31], we demonstrated that CD4⁺CD25⁺CD127^{-low} T_{regs} from participants without asthma maintain a Ca²⁺-unresponsive state following TCR stimulation; therefore, human T_{regs} from adults may exhibit Ca²⁺-dependent anergy. However, CD4⁺CD25⁺CD127^{-low} T_{regs} from adult patients with asthma elicit a Ca²⁺ response following TCR activation. Unlike the cells of participants without asthma, TCR stimulation led to the proliferation of responder cells (CD4⁺CD25⁻ T cells), resulting in a decline in anergy and regulatory functions in the T_{regs} of patients with asthma. Ca²⁺ influx in T_{regs} paralleled their proliferation and decreased regulatory functions, indicating that even incompletely purified T_{regs}, such as CD4⁺CD25⁺ T cells, can be assessed for their regulatory functions using Ca²⁺ analysis combined with analysis of the proportion of CD45RO⁺ cells by flow cytometric analysis. Our previous report [33] suggested that the Ca²⁺ response of CD4⁺CD25⁺CD45RO⁺ T cells (the area of CD25⁺ cells was set at a higher level in flow cytometric analysis) correlated with childhood asthma symptoms. This Ca²⁺ response was elicited with anti-CD3ε antibody, which can activate T cells via CD3 complexes, and the Ca²⁺ assay used obtained data from individual cells. We analyzed the anergy status of all T_{regs} using this Ca²⁺ assay, and found that cells exhibiting normal function were included among T_{regs} from patients in the poor response group (data not shown). Allergen-specific immune responses play a critical role in patients with asthma. Allergens exacerbate airway inflammation, and the Ca²⁺

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response may be elicited by specific allergens; however, allergen-specific T_{regs} are able to suppress allergen-activated T effector or dendritic cells [9]. Therefore, elucidating the functions of allergen-specific T_{regs} is crucial in patients with asthma. We broadly determined the general functions of T_{regs} from patients with asthma, but further study is necessary to investigate the functions of allergen-specific T_{regs} .

Impaired Ca^{2+} regulation in $\text{CD4}^+\text{CD25}^+$ T_{regs} is implicated in severe asthma. Because asthma chronicity and the duration of therapeutic interventions differ between adults and children, there are fewer parallels between Ca^{2+} response and symptoms in adults than in children. Miyara *et al.* demonstrated that FOXP3⁺ T cells from healthy adult volunteers could be divided into three classes based on the levels of FOXP3 and CD45RA expression [42]. The FOXP3^{low}CD45RA⁺ population represents an immature phenotype of T_{regs} , whereas the FOXP3^{high}CD45RA⁻ population (nearly equivalent to CD45RO⁺ cells) is a mature phenotype in $\text{CD4}^+\text{CD25}^{\text{high}}$ T cells. In adults, the latter is the predominant phenotype, but the former is predominant in children. Furthermore, we reported that the immature population accounted for more than half of T_{regs} in children and was likely to be Ca^{2+} responsive to TCR stimulation [33]. The FOXP3^{high}CD45RO⁺ population in children may differentiate into the mature population in adults. This does not contradict the observation that ease of Ca^{2+} increase in the cells of children correlates well with asthma severity.

Our data show that mRNA and protein levels of FOXP3 in $\text{CD4}^+\text{CD25}^+$ T cells remained unchanged or increased in patients with asthma, but we could not determine the reason for this difference. One possible explanation for this discrepancy is that differences exist between the populations in these experiments. Furthermore, the relatively high expression of *FOXP3* mRNA in $\text{CD4}^+\text{CD25}^+$ T cells from patients with asthma may be caused by ICS treatment. Increased FOXP3 transcription in CD4^+ T cells and PBMCs was shown to be caused by glucocorticosteroid treatment *in vitro*, and no difference in this effect

was evident between healthy subjects and patients with asthma [18,43]. The mechanism by which steroids regulate FOXP3 transcription remains unclear and controversial.

We developed a model system to analyze the molecular mechanisms of impaired Ca^{2+} regulation in the T_{regs} of patients with asthma using Jurkat Tet-On® FOXP3⁺ cell clones. Dox 2-day cells, unlike Dox 5-day cells, exhibited adequate functions, such as the repression of IL-2 transcription, despite both cell types displaying functional levels of FOXP3 protein. Furthermore, both Dox 2- and 5-day cells exhibited a Ca^{2+} response similar to that of $\text{CD4}^+\text{CD25}^+\text{CD127}^{\text{low}}$ T_{regs} from participants with and without asthma. We speculated that similar mechanisms of impaired Ca^{2+} regulation lead to the loss of regulatory functions in $\text{CD4}^+\text{CD25}^+\text{CD127}^{\text{low}}$ T_{regs} from patients with asthma and Dox 5-day cells, because both cells exhibit reduced IL-2 repression and increased RACK1 expression. Although ORAI1 protein levels were comparable in both Dox 2- and 5-day cells, Ca^{2+} influx in Dox 5-day cells following TCR stimulation was identical to that of control cells. Therefore, ORAI1 levels could not account for the functional difference observed between the two time points. Recently, it was reported that agonist-stimulated Ca^{2+} mobilization in human embryonic kidney-293 and HeLa cells is mediated by a protein complex consisting of IP₃R, RACK1, STIM1, ORAI1, and transient receptor potential cation channel, subfamily C [44]. We hypothesized that, because CRAC channels interact with the RACK1 scaffold protein to regulate CRAC channel assembly, RACK1 may have a role in the regulation of T_{reg} function. Ca^{2+} response in Dox 5-day cells was significantly blocked by RACK1 knockdown using two independent siRNAs. Increased *RACK1* expression in Dox 5-day cells, similar to that observed in patients with asthma, may contribute to their impaired Ca^{2+} regulation and loss of regulatory functions.

RACK1 transcription is positively controlled by a transcription factor, nuclear factor- κB (NF- κB) [45]. Furthermore, tumor necrosis factor- α , the levels of which are increased in

patients with asthma, is a known NF- κ B activator. In contrast to NF- κ B, glucocorticoids used in asthma treatment can decrease RACK1 expression [45]. Although the symptoms of asthma are relieved by ICS treatment, chronic inflammation is sustained in the airways of patients with asthma. Therefore, corticosteroids may not effectively reduce *RACK1* transcription in T_{regs} of patients with asthma. The proliferation of bronchial smooth muscle cells was inhibited by the glucocorticoid receptor and the CCAAT/enhancer binding protein α (C/EBP α) complex in participants without asthma; however, the complex could not function because of the impaired translation of C/EBP α in the bronchial smooth muscle cells of patients with asthma [46]. The molecular mechanisms underlying increased RACK1 expression require further investigation.

In conclusion, our data show that, following TCR activation, $[\text{Ca}^{2+}]_i$ increased in T_{regs} of patients with asthma, whereas it did not in participants without asthma. This increase in $[\text{Ca}^{2+}]_i$ in T_{regs} was observed in all adult patients with asthma to differing extents. Impaired Ca^{2+} regulation in T_{regs} of patients with asthma mirrored their proliferation and reflected their reduced regulatory functions. The methods described in our study are useful for analyzing the functions of T_{regs} in small volumes of blood. This study also demonstrated that a novel mechanism responsible for increasing RACK1 expression is one of the factors underlying impaired Ca^{2+} regulation in T_{regs} of patients with asthma.

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Figure legends

Fig. 1

Forkhead box P3 mRNA expression and intracellular Ca^{2+} concentration in $\text{CD4}^+\text{CD25}^+$ T cells

$\text{CD4}^+\text{CD25}^+$ and $\text{CD4}^+\text{CD25}^-$ T cells were purified with MACS (see Materials and Methods).

(a) *Forkhead box P3* and β -actin mRNA expression were measured using the SYBR®-green method. (b) Comparison of the Ca^{2+} response in the $\text{CD4}^+\text{CD25}^+$ T cells of participants with and without asthma following T cell receptor stimulation. Changes in intracellular Ca^{2+} concentration (340/380) in individual cells were monitored at 10-s intervals. Results are shown as the mean of 2–3 experiments. A participant without asthma (Nonasthma; upper left), Patient No. 1 with asthma (Asthma Subject No. 1; upper right), Patient No. 2 with asthma (Asthma Subject No. 2; middle-left), and Patient No. 3 with asthma (Asthma Subject No. 3; middle-right) are depicted. Typical patterns of Ca^{2+} response in each group are expressed (nonasthma: 5 participants; asthma: 9 participants). The delta ratio is shown in the bar chart underneath the Ca^{2+} response line charts. Ca^{2+} response of $\text{CD4}^+\text{CD25}^+$ T cells and $\text{CD4}^+\text{CD25}^-$ T cells are shown as circles and squares, respectively. Arrows indicate the start time of α -CD3 and secondary antibody stimulation. (c) Store-operated calcium entry was analyzed in $\text{CD4}^+\text{CD25}^+$ and $\text{CD4}^+\text{CD25}^-$ T cells from patients with asthma. Ca^{2+} release from the endoplasmic reticulum was measured without Ca^{2+} (0Ca: 0 mM Ca^{2+}) following α -CD3 stimulation, and Ca^{2+} influx from the extracellular space was measured with Ca^{2+} (2Ca: 2 mM Ca^{2+}). All results are expressed using 40 randomly selected cells (total: 150–280) with a typical individual pixel pattern. Reproducibility is shown among 3 patients with asthma and 3 without.

Fig. 2

Regulatory activity and proliferative response of CD4⁺CD25⁺ T cells from participants with and without asthma. Cultured cells were collected and stained with fluorescein isothiocyanate-labeled anti-Ki-67 antibody and PC5-labeled anti-CD4 antibody. (a) typical patterns obtained with Ki-67 analysis in the cells of participants without asthma are depicted: CD4⁺CD25⁺ T (left), CD4⁺CD25⁻ T (middle), and mixed culture (right). (b) CD4⁺CD25⁺ T cells, CD4⁺CD25⁻ T cells, and both cells from subjects without asthma, Patient No. 1 with asthma, and Patient No. 2 with asthma were cultured with CD2⁻ cells in the presence or absence of α -CD3 and α -CD28. Typical patterns in each group are shown (nonasthma: 5 participants; asthma: 9 participants). Percentage inhibition was calculated using the formula described in the Methods section.

Fig. 3

Comparison of Ca²⁺ response between CD4⁺CD25⁺CD127^{-/low} T cells from participants with and without asthma following T cell receptor stimulation. (a). Changes in intracellular Ca²⁺ concentration ([Ca²⁺]_i) (340/380) in individual cells were monitored at 10-s intervals. All results are expressed using 40 randomly selected cells (total: 198–241) with a typical individual pixel pattern. Typical patterns in each group are shown (nonasthma: 3 participants; asthma: 3 participants). Arrows indicate the start time of α -CD3 and secondary antibody stimulation. (b) Ca²⁺ responses of CD4⁺CD25⁺CD127^{-/low} T cells from participants with and without asthma are shown as squares and circles, respectively (left). The delta ratio shows increased [Ca²⁺]_i of CD4⁺CD25⁺CD127^{-/low} T cells in patients with asthma (right, $p < 0.001$, versus subjects without asthma). Results shown are the mean of 3–4 experiments. (c) The regulatory function of CD4⁺CD25⁺CD127^{-/low} T cells was analyzed with carboxyfluorescein succinimidyl ester (CFSE)-labeled CD4⁺CD25⁻ T cells as a responder. Typical patterns in

each group are expressed (nonasthma: 3 participants; asthma: 3 participants). Percentage of undivided cells is shown as the frequency of fluorescence intensity. Similar cells were cultured with no stimuli. Thick line: no-stimuli; thin line: stimuli; dashed line: mixed culture of CD4⁺CD25⁺CD127^{-low} T cells and CFSE-CD4⁺CD25⁻ T cells; dotted line: mixed culture of unlabeled CD4⁺CD25⁻ T cells and CFSE-CD4⁺CD25⁻ T cells. The bottom bar chart shows the percentage of undivided cells of the upper flow data as follows: 1: thin line; 2: dashed line; 3: dotted line. These results are from 4 participants without asthma and 5 patients with asthma.

Fig. 4

Features of the established Jurkat Tet-On® clones F2A9 (forkhead box P3⁺) and 5G1 (control). (a) Flow analysis performed in the F2A9 clone stained with Alexa-Fluor® 488–forkhead box P3 (FOXP3) and PE–CD25. Dotted line: whole cells; dashed line: FOXP3^{low} cells; and thin line: FOXP3^{high} cells. (b) FOXP3 protein expression was time dependent with doxycycline treatment of F2A9 cells (upper panel). The amount of protein was measured by densitometric analysis and expressed as a bar chart with the ratio of FOXP3/β-actin (lower panel).

Fig. 5

Comparison of the Ca²⁺ response between 5G1 (control) clone and F2A9 (forkhead box P3⁺) clone following T cell receptor stimulation. The changes in intracellular Ca²⁺ concentration ([Ca²⁺]_i) (340/380) in individual cells were monitored at 10-s intervals. All results are expressed using 40 randomly selected cells (total: 184–214) with a typical individual pixel pattern. Arrows indicated the start time of α-CD3 plus and secondary antibody stimulation. (a) Control Jurkat cells (5G1 clones), (b) forkhead box P3 (FOXP3⁺) 2-day denotes the

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doxycycline (Dox)-treated F2A9 clone after 2 days, and (c) FOXP3⁺ 5-day denotes the Dox-treated F2A9 clones after 5 days. (d) The delta ratio shows increased [Ca²⁺]_i in FOXP3 5-day cells ($p < 0.001$, versus FOXP3 2-day) but not in FOXP3 2-day cells. (e) The repressor function of interleukin-2 transcription was measured under each condition. Control 2- and 5-day (5G1) cells were treated with Dox similarly to control FOXP3⁺ 2- and 5-day cells.

Fig. 6

Expression of Ca²⁺ release-activated Ca²⁺ channel composed molecules, ORAI1 and stromal interaction molecule 1, in control and forkhead box P3⁺ clones. (a) Stromal interaction molecule 1 (*STIM1*) mRNA expression and (b) *ORAI1* mRNA expression were measured by real-time polymerase chain reaction. (c) ORAI1 and STIM1 protein expression were detected by western blotting. The amount of protein was measured by densitometric analysis and expressed as a bar chart with the ratio of ORAI1 or STIM1:β-actin (lower panel). Each experiment was performed 3–7 times.

Fig. 7

Ca²⁺ release-activated Ca²⁺ channel regulation molecule and receptor for activated C kinase 1 expression in forkhead box P3⁺ clones and CD4⁺CD25⁺CD127^{-low} T cells. (a) Receptor for activated C kinase 1 (*RACK1*) mRNA expression in control clone and forkhead box P3 (FOXP3⁺) clone and (b) *RACK1* mRNA expression in CD4⁺ T cells and CD4⁺CD25⁺CD127^{-low} T cells derived from participants with and without asthma were measured using real-time polymerase chain reaction. (c) RACK1 protein expression in control and FOXP3⁺ clones detected by western blotting (upper panel). The lower bar chart shows the ratio of RACK1:β-actin proteins. Each experiment was performed 3 times.

Fig. 8

Effect of receptor for activated C kinase 1 knockdown in forkhead box P3 5-day clones. (a) Receptor for activated C kinase 1 (*RACK1*) mRNA expression was measured by real-time polymerase chain reaction and *** indicates $p < 0.001$. (b) RACK1, forkhead box P3 (FOXP3), stromal interaction molecule 1 (STIM1), ORAI1, and β -actin protein expression detected by western blotting (upper panel). The amount of protein was measured by densitometric analysis and expressed as a bar chart with the ratio of each protein/ β -actin (lower panel). ***, $p < 0.001$ (versus none, negative siRNA). (c) Changes in intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) (340/380) in individual cells were monitored at 10-s intervals. All results are expressed using 40 randomly selected cells (total: 131–145) with a typical individual pixel pattern. Arrows indicate the start time of α -CD3 and secondary antibody stimulation. Negative control siRNA-treated (upper), RACK1 siRNA1-treated (middle), and RACK1 siRNA2-treated FOXP3 5-day (lower). The delta ratio shows decreased $[\text{Ca}^{2+}]_i$ of RACK1 siRNA-treated FOXP3 5-day cells ($p < 0.001$ versus negative siRNA). Each experiment was performed 2–4 times.

Table 1**Clinical characteristics of study subjects**

	Asthmatics (n = 50)	Nonasthmatics (n = 23)
Age	54.4 ± 16.7	40.9 ± 14.5
Gender (Male/Female)	27/23	10/13
Type		
Atopic	39	7
Nonatopic	11	16
Severity		
Step 1	4	
Step 2	23	
Step 3	18	
Step 4	5	
Dose of ICS		
CFC-BDP mg/day	712 (0 -1,600)	
Smoking		
Nonsmoker	36	16
Current smoker	14	7

Fig.1

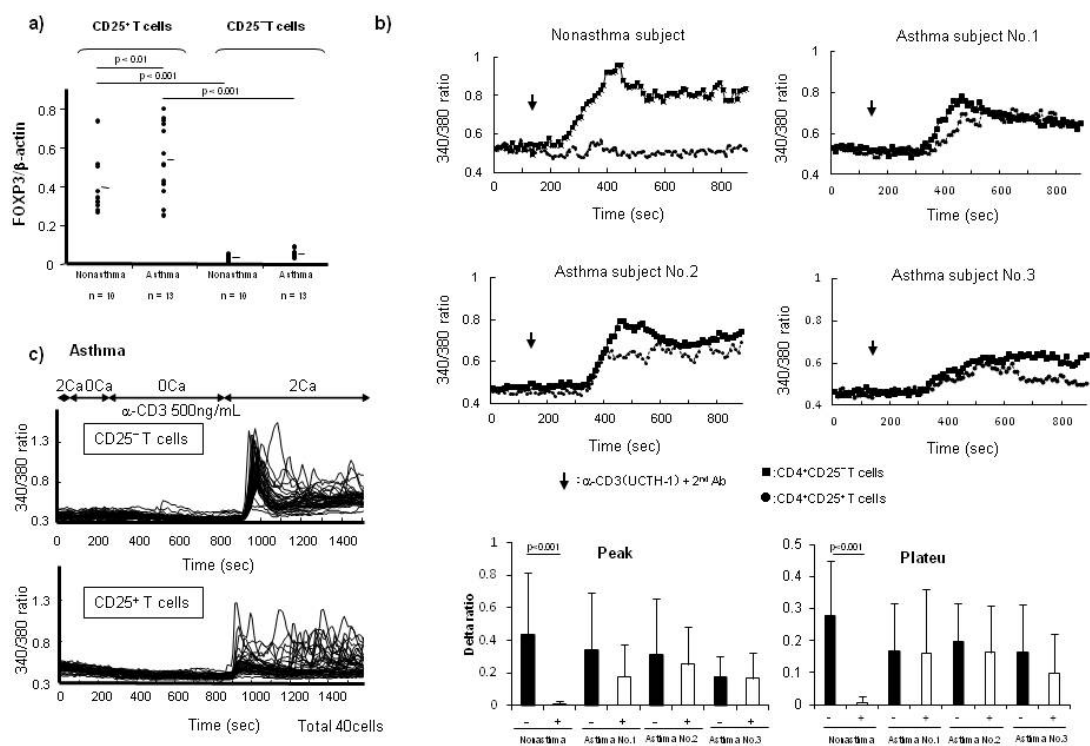


Fig.2

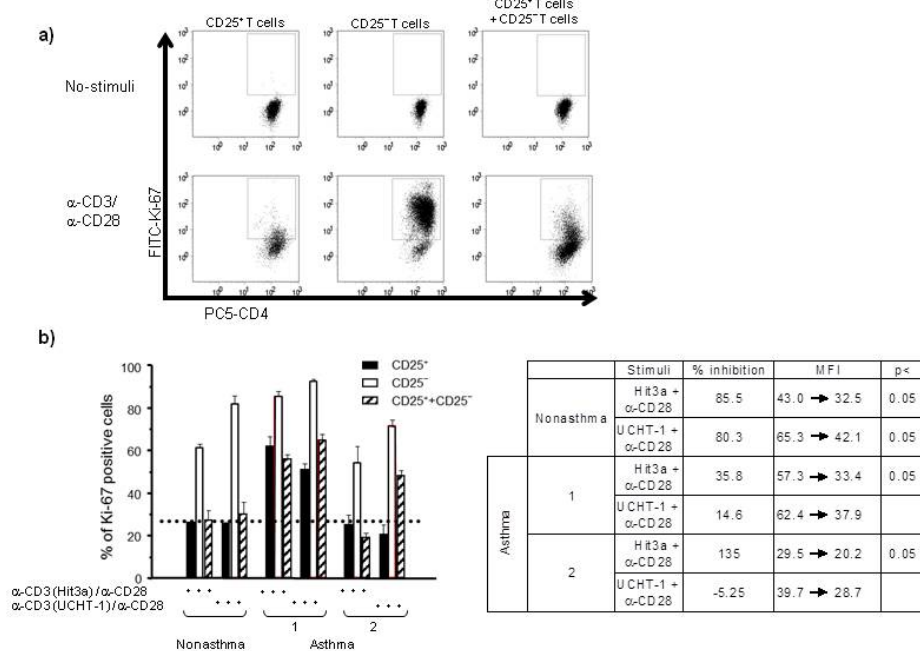


Fig.3

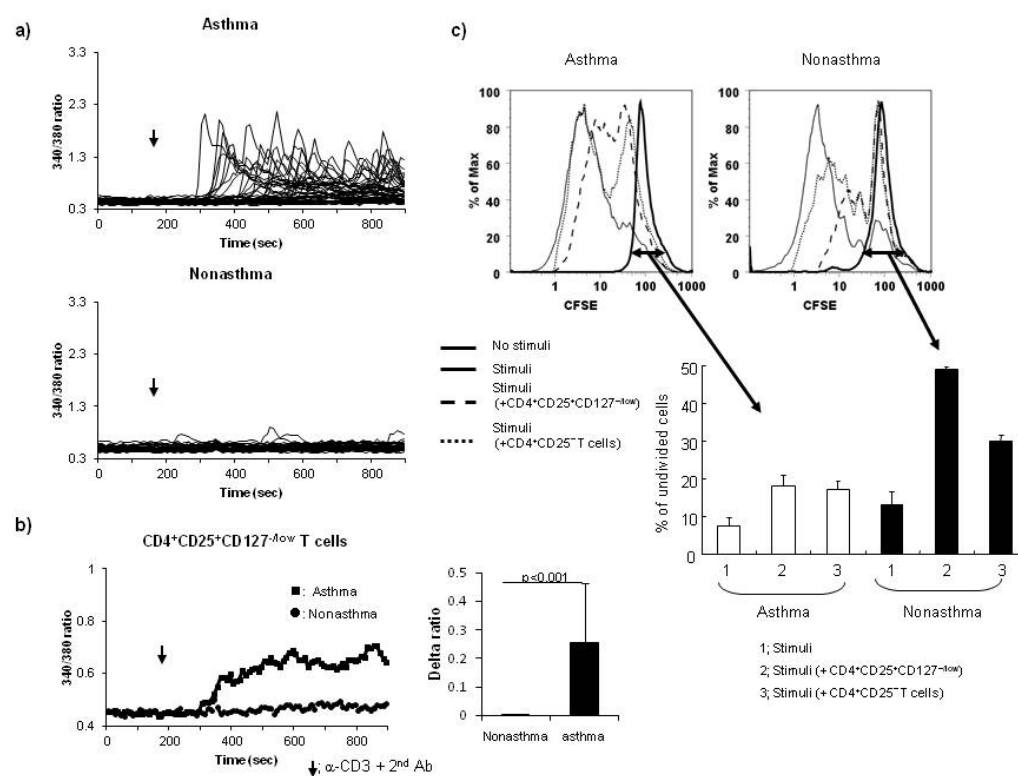


Fig.4

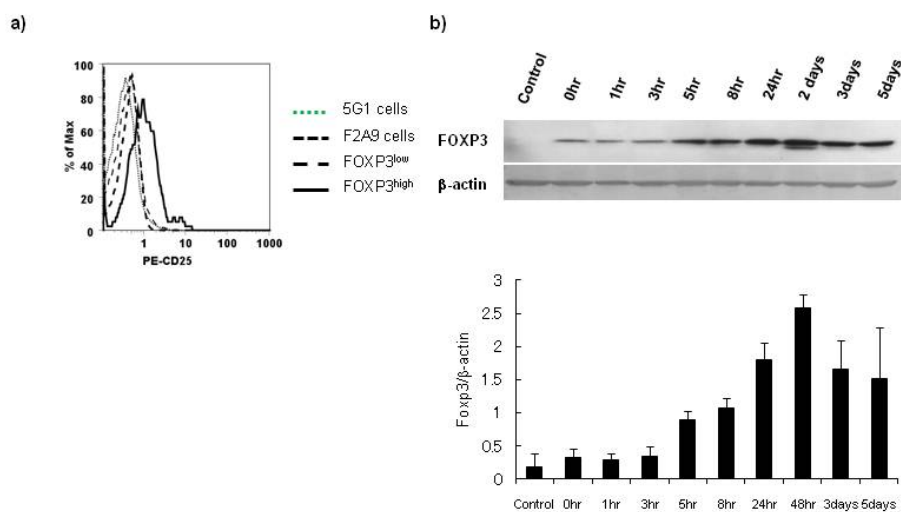


Fig.5

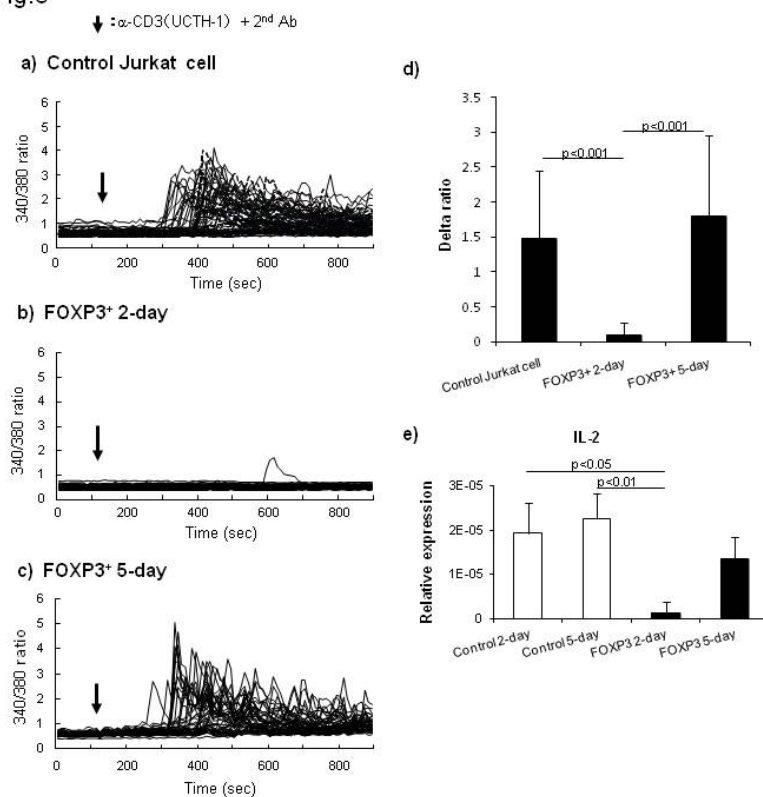


Fig.6

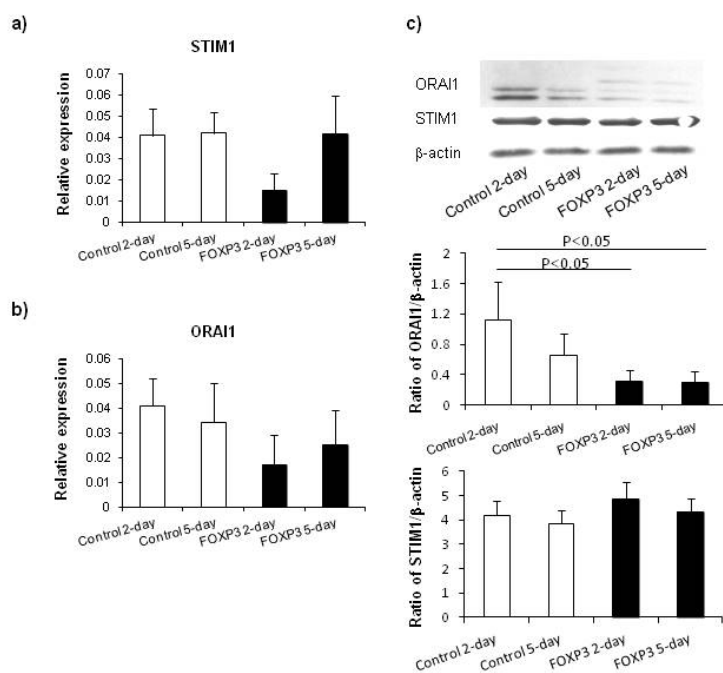


Fig.7

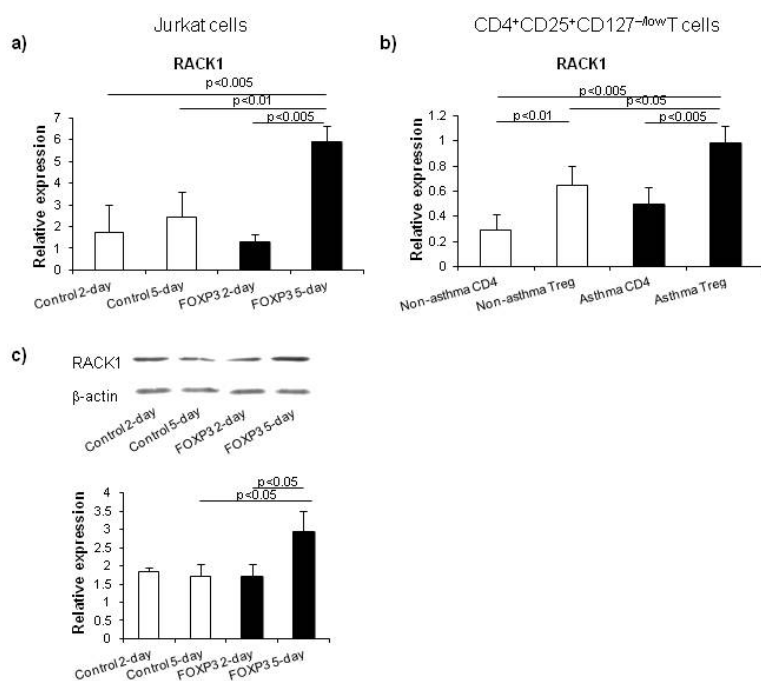


Fig.8

