



Full length article

Regulation of allergic airway inflammation by adoptive transfer of CD4⁺ T cells preferentially producing IL-10

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ABSTRACT

Anti-inflammatory pharmacotherapy for asthma has mainly depended on the inhalation of glucocorticoids, which non-specifically suppress immune responses. If the anti-inflammatory cytokine interleukin (IL)-10 can be induced by a specific antigen, asthmatic airway inflammation could be suppressed when individuals are exposed to the antigen. The purpose of this study was to develop cellular immunotherapeutics for atopic diseases using IL-10-producing CD4⁺ T cells. Spleen cells isolated from ovalbumin (OVA)-sensitized mice were cultured with the antigen, OVA and growth factors, IL-21, IL-27 and TGF- β for 7 days. After the 7-day culture, the CD4⁺ T cells were purified using a murine CD4 magnetic beads system. When the induced CD4⁺ T cells were stimulated by OVA in the presence of antigen-presenting cells, IL-10 was preferentially produced in vitro. When CD4⁺ T cells were adoptively transferred to OVA-sensitized mice followed by intratracheal OVA challenges, IL-10 was preferentially produced in the serum and bronchoalveolar lavage fluid in vivo. IL-10 production coincided with the inhibition of eosinophilic airway inflammation and epithelial mucus plugging. Most of the IL-10-producing CD4⁺ T cells were negative for Foxp3 and GATA-3, transcription factors of naturally occurring regulatory T cells and Th2 cells, respectively, but double positive for LAG-3 and CD49b, surface markers of inducible regulatory T cells, Tr1 cells. Collectively, most of the induced IL-10-producing CD4⁺ T cells could be Tr1 cells, which respond to the antigen to produce IL-10, and effectively suppressed allergic airway inflammation. The induced Tr1 cells may be useful for antigen-specific cellular immunotherapy for atopic diseases.

1. Introduction

Asthma is characterized by chronic airway inflammation (Djukanovic et al., 1990). CD4⁺ T cells and their products, such as interleukin (IL)-4, IL-5, and IL-13, play central roles in orchestrating inflammation, especially through infiltration by eosinophils into the airway (Robinson, 2010). Anti-inflammatory pharmacotherapy for asthma has mainly depended on the inhalation of glucocorticoids. However, non-specific immunosuppressive effects have become a problem in treatment with glucocorticoids. If anti-inflammatory molecules, such as IL-10 (Sabat et al., 2010), can be induced by a specific antigen, asthmatic airway inflammation could be suppressed when individuals are exposed to the antigen.

We have demonstrated that multiple intratracheal administrations

of an antigen, ovalbumin (OVA), induced IL-10-producing CD4⁺ T cells in vivo in a murine model of asthma (Nabe et al., 2012). The induced IL-10-producing CD4⁺ T cells were able to be activated by the specific antigen in vivo and in vitro (Nabe et al., 2012). Thus, we supposed that the endogenously induced IL-10-producing CD4⁺ T cells may be an induced subtype of regulatory T (Treg) cells although the cells did not express Foxp3 (Nabe et al., 2012), which is a transcription factor of naturally occurring Treg (nTreg) cells (Hori et al., 2003). Additionally, we demonstrated that in vivo neutralization of IL-10 function by an anti-IL-10 receptor antibody augmented allergic airway inflammation, indicating that IL-10 functioned as a negative regulator in asthma pathogenesis (Nabe et al., 2012). Thus, IL-10-producing CD4⁺ T cells differentiated in vitro could be utilized as a biotherapeutic agent for antigen-specific cellular immunotherapy.

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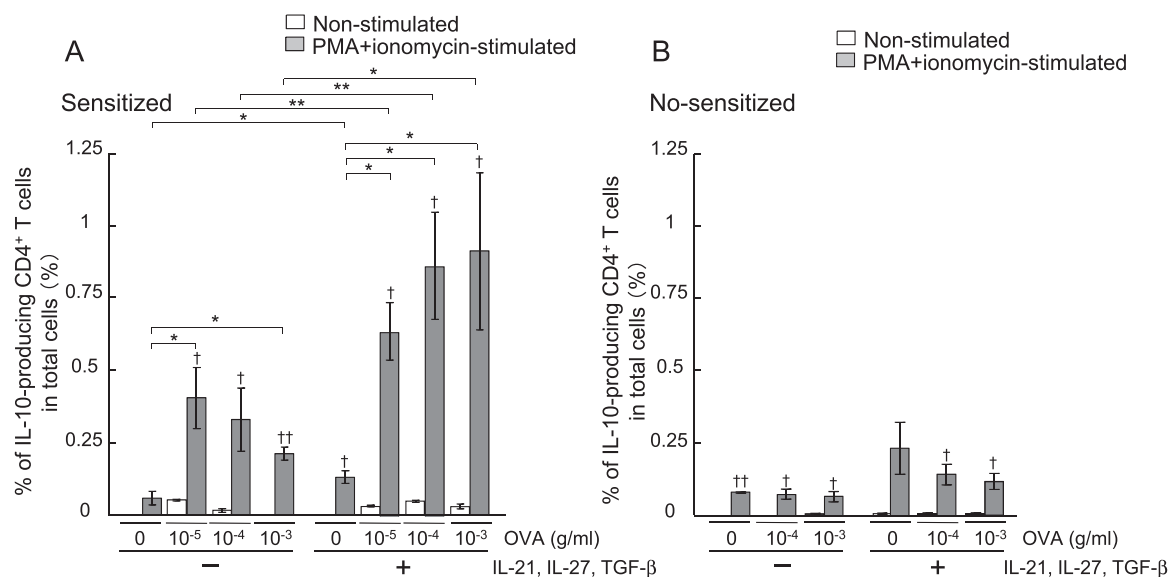


Fig. 1. Antigen-induced proliferation of IL-10-producing CD4⁺ T cells from splenocytes isolated from sensitized and non-sensitized mice. (A and B) Splenocytes isolated from sensitized (A) or non-sensitized (B) mice were incubated with OVA in the presence or absence of IL-21, IL-27, and TGF- β for 7 days, followed by stimulation with PMA + ionomycin for 6 h. Each column represents the mean $\pm$ S.E.M. of 3 experiments. † and ††: $P < 0.05$ and 0.01 vs each value without stimulation. * and **: $P < 0.05$ and 0.01 .

Table 1

Changes of the proportion of the IL-10-producing CD4⁺ T cells in spleen cells or CD4⁺ T cells after 7-day culture or before culture.

	Number of cells in a flask ($\times 10^7$ cells/flask)	% of IL-10 ⁺ CD4 ⁺ T cells	
		In spleen cells	In CD4 ⁺ T cells
Before culture	9.0	0.3 $\pm$ 0.01	1.4 $\pm$ 0.13
After 7-day culture	5.9 $\pm$ 0.4 ^c	0.9 $\pm$ 0.3 ^a	10.0 $\pm$ 2.2 ^b

Splenocytes isolated from sensitized mice were incubated with OVA in the presence of IL-21, IL-27, and TGF- β for 7 days, followed by stimulation with PMA + ionomycin for 6 h. The cell culture was started at a cell concentration of 9.0×10^7 cells/flask. Each value represents the mean $\pm$ S.E.M. of 3–4 experiments. a, b and c: $P < 0.05$, 0.01 and 0.001 vs each value before culture.

In this study, we experimentally evaluated a conventional protocol for antigen (OVA)-induced proliferation of IL-10-producing CD4⁺ T cells, which are responsive to OVA and can be utilized for antigen-specific immunotherapy. It was assessed whether the induced CD4⁺ T cells preferentially produced IL-10 in response to the specific antigen in vitro and in vivo, and whether adoptive transfer of the cells to OVA-sensitized mice attenuates allergic airway inflammation in vivo. Moreover, phenotypes of the in vitro induced IL-10-producing CD4⁺ T cells were characterized immunologically.

2. Materials and methods

2.1. Sensitization and challenge of mice

For the harvest of splenocytes, 6-week-old BALB/c mice (Japan SLC, Hamamatsu, Japan) were sensitized by i.p. injections of OVA, (Grade V; Sigma Chemical Co., St. Louis, MO, USA) + Al(OH)₃ at 50 μ g OVA adsorbed to 2 mg Al(OH)₃/animal on days 0, 14, and 28 (Nabe et al., 2005, 2011a, 2011b, 2012). As a negative control, non-sensitized mice were used (WAKO, Osaka, Japan).

For the preparation of antigen-presenting cells in the lungs, the i.p. sensitized mice were intratracheally challenged with 2% OVA (25 μ l/animal) once a day on days 35, 36, and 37 during inhaled anesthesia with isoflurane.

For CD4⁺ T cell adoptive transfer recipients, the i.p. sensitization was conducted on day 0, followed by multiple intratracheal challenges

Table 2

Effects of adoptive transfer of CD4⁺ T cells from donor sensitized mice on the production of OVA-specific IgE antibody in the sera of sensitized, challenged recipient mice.

Group	Concentration of OVA-specific IgE (μ g/ml)
Sensitized, non-challenged	1.4 $\pm$ 0.5
Sensitized, challenged	
+ PBS	1.8 $\pm$ 1.0
+ Freshly isolated splenic CD4 ⁺ T cells	4.8 $\pm$ 2.5
+ 7-day cultured splenic CD4 ⁺ T cells	0.8 $\pm$ 0.5

CD4⁺ T cells were adoptively transferred before the 1st challenge, followed by 4 OVA challenges. Serum was harvested at 4 h after the 4th challenge. Each value represents the mean $\pm$ S.E.M. of 3–8 animals.

with 2% OVA on days 7, 8, 9, and 12.

These studies were approved by both Experimental Animal Research Committees of Setsunan University and Kyoto Pharmaceutical University.

2.2. Culture of spleen cells

On the 35th day after the 1st sensitization, the spleen was isolated, and then disrupted on a cell strainer in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 50 μ M 2-mercaptoethanol, 100 μ M MEM non-essential amino acid, 100 U/ml penicillin, 100 μ g/ml streptomycin, and 10% FBS. Collected cells were treated with ACK lysis buffer, and suspended in medium at 3×10^6 cells/ml, followed by incubation in 5% CO₂ at 37 °C for 7 days in the presence or absence of OVA or a mixture of cytokines, mouse IL-21 and IL-27 (both 2.5×10^{-9} g/ml, R & D Systems, Minneapolis, MN, USA), and TGF- β (3×10^{-9} g/ml, Santa Cruz Biotechnology, Santa Cruz, CA, USA). These three cytokines have been reported to help with the proliferation of IL-10-producing CD4⁺ T cells (Awasthi et al., 2007; Stumhofer et al., 2007; Pot et al., 2009).

In order to evaluate ability of the 7-day cultured cells in IL-10 production, the cells (10^6 cells/ml) were incubated with a mixture of PMA (10^{-8} g/ml) and ionomycin calcium salt (10^{-6} g/ml) (Sigma-Aldrich, St. Louis, MO, USA) or PBS at 37 °C for 6 h in the presence of a protein transport inhibitor, monensin sodium salt (2 μ M, Calbiochem, San Diego,

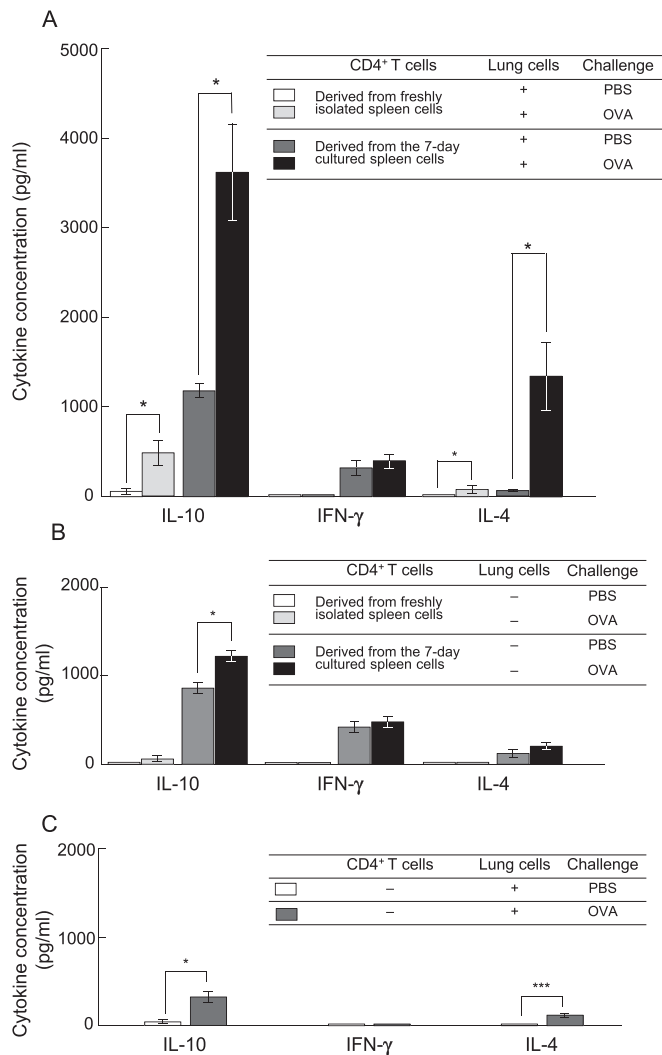


Fig. 2. In vitro antigen-induced production of IL-10, IFN- γ , and IL-4 from CD4⁺ T cells of sensitized mice in the presence (A) or absence (B) of lung cells, and (C) that from lung cells in the absence of CD4⁺ T cells. Splenocytes isolated from sensitized mice were incubated with OVA in the presence of IL-21, IL-27, and TGF- β for 7 days, followed by purification of CD4⁺ T cells using Dynabeads mouse CD4. As control cells, CD4⁺ T cells were purified from freshly isolated splenic cells from sensitized mice. Lung cells were obtained from the lung of sensitized and challenged mice. Each column represents the mean $\pm$ S.E.M. of 3 experiments. * and ***: $P < 0.05$ and 0.001 .

CA, USA). As a negative control, the spleens of non-sensitized mice were isolated. The freshly isolated spleen cells were stimulated with PMA + ionomycin.

IL-10-producing cells were analyzed by a flow cytometer: After incubation with anti-Fc γ receptor II/III (2.4G2), the cells were incubated with FITC anti-CD4 (H129.19) (BD Biosciences, San Diego, CA, USA) or PerCP anti-CD4 (GK1.5) (BioLegend, San Diego, CA, USA), and eFluor660 anti-CD3e (17A2) (eBioscience, San Diego, CA, USA) for 20 min. After washing, the cells were incubated with BD fixation/permeabilization solution (BD Biosciences) for 12–18 h, and then, washed with BD Perm/WashTM buffer (BD Biosciences), followed by incubation with FITC or PE anti-IL-10 (JES5-16E3) for 40 min. Each sample was analyzed using FACSCalibur and Cell Quest Pro version 6.0, or FACSARIATM Fusion and FACSDivaTM version 8.0.1 (BD Biosciences).

2.3. Purification of CD4⁺ cells from 7-day cultured spleen cells

CD4⁺ cells in the 7-day cultured spleen cells or freshly isolated spleen cells were purified using Dynabeads mouse CD4 and

DETAHaBEAD (Invitrogen, Carlsbad, CA, USA) in accordance with the protocol provided by the manufacturer. The ratio of CD4⁺ T cells in the collected CD4⁺ cells was $84 \pm 3\%$ ($n = 16$).

2.4. Analyses of antigen-induced cytokine production from the purified CD4⁺ T cells in vitro

For the preparation of antigen-presenting cells, lung tissues isolated 3 days after the 3rd challenge on day 40 were digested by incubation with collagenase at 37 °C for 1 h. The single cell suspension (2.6×10^6 cells/0.65 ml) suspended in RPMI1640 medium containing 10% FBS, 100 U/ml penicillin, and 100 μ g/ml streptomycin was combined with the purified CD4⁺ T cells (2.6×10^6 cells/0.65 ml), then the co-cultured cells were incubated with OVA (10^{-3} g/ml) or PBS at 37 °C in 5% CO₂ for 18 h. After the incubation, cell culture supernatant was collected for cytokine measurement.

2.5. Adoptive transfer of the purified CD4⁺ T cells, and effects on antigen-induced airway inflammation in vivo

Purified CD4⁺ T cells (3×10^7 cells/animal) were injected intravenously into sensitized mice 6 days after i.p. sensitization with OVA + Al(OH)₃. The recipient mice were challenged intratracheally with OVA on days 7, 8, 9, and 12. At 4 h after the fourth challenge, the recipient mouse was anesthetized by i.p. injection of pentobarbital and xylazine. Peripheral blood was drawn from the abdominal aorta, and bronchoalveolar lavage fluid (BALF) was obtained from the right lung lobes as reported for the measurement of cytokines in the serum and BALF (Nabe et al., 2005, 2011a, 2011b, 2012). Total and differential leukocyte counts in the BALF were determined after treatment with ACK lysis buffer. The left lobe of lung was isolated for histological studies.

2.6. Histological studies

The left lobe of the lung was fixed with 10% buffered formalin. Tissues were embedded in paraffin, and 4- μ m sections were stained with periodic acid–Schiff (PAS) and Masson trichrome for light microscopic observation. Quantitative analyses of histological changes were conducted as reported previously (Nabe et al., 2011b). Briefly, the degree of epithelial thickening was quantified as follows: the bronchus, the diameter of which was approximately 200–400 μ m, was photographed, and then the epithelial area (μ m²) and the length of the basement membrane (μ m) were measured using Adobe Photoshop CS2 (version 9.0; Adobe Systems, San Jose, CA, USA) and Image J (version 1.49; NIH, Bethesda, MD, USA). The degree of epithelial thickening was expressed by dividing the epithelial area by the length of the basement membrane. Mucus accumulation in the epithelial layer was also quantified by dividing the PAS⁺ area in the epithelium (μ m²) by the length of the basement membrane (μ m). Subepithelial fibrosis was scored using a 4-point graded scale using Masson trichrome-stained sections; 0, no blue fibrotic area existed underneath the basement membrane; 1, 2, and 3 blue fibrotic area existed underneath the basement membrane by approximately 30, 60 μ m, and more than 60 μ m, respectively.

2.7. Assays of levels of cytokines in the cell culture supernatant, serum and BALF

IL-10 was measured using an ELISA kit (R & D Systems). IL-4, IL-5, IL-13, IL-17 and IFN- γ were measured using Ready Set Go! ELISA kits (eBioscience).

2.8. Measurement of OVA-specific IgE in the serum

Amounts of OVA-specific IgE were measured by ELISA using anti-IgE (clone R35-72, BD Biosciences) as a capture antibody, horseradish

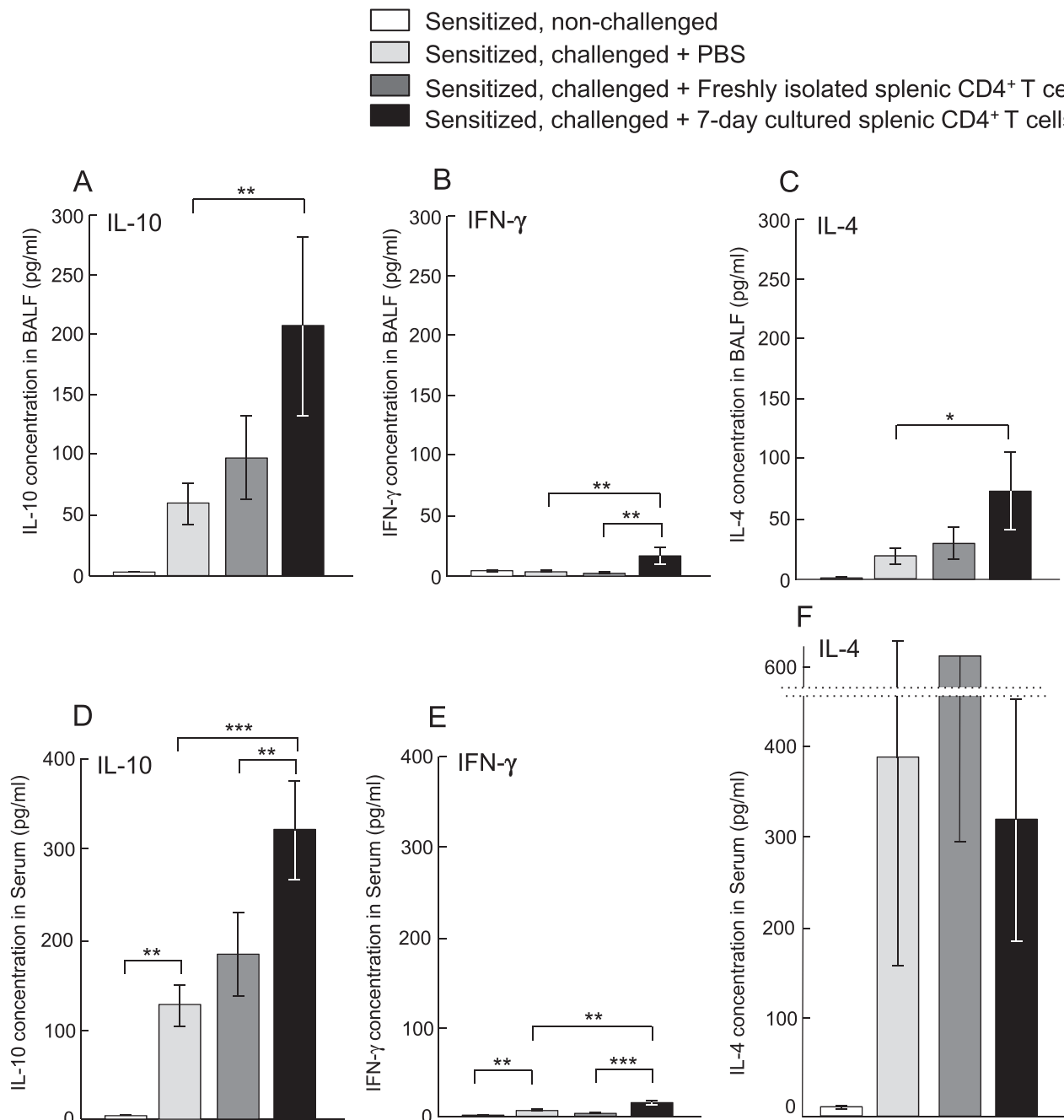


Fig. 3. Effects of adoptive transfer of CD4⁺ T cells from donor sensitized mice on antigen-induced production of IL-10, IFN-γ, and IL-4 in BALF (A, B and C) and serum (D, E and F) in recipient sensitized mice. Splenocytes isolated from sensitized mice were incubated with OVA in the presence of IL-21, IL-27, and TGF-β for 7 days, followed by purification of CD4⁺ T cells using Dynabeads mouse CD4. As control cells, CD4⁺ T cells were purified from freshly isolated splenic cells from sensitized mice. Those CD4⁺ T cells were adoptively transferred before the 1st challenge, followed by 4 OVA challenges. BALF was obtained at 4 h after the 4th challenge. Each column represents the mean ± S.E.M. of 4–9 experiments. *, ** and ***: P < 0.05, 0.01 and 0.001.

peroxidase (HRP)-labeled OVA (Abcam, Cambridge, UK) as a detection reagent, and OVA-specific IgE (clone E-C1, Chondrex, Redmond, WA, USA) as a standard. Color development was performed by incubation with tetramethylbenzidine substrate solution (eBioscience). After addition of H₃PO₄ solution, OD at 450 nm was measured.

2.9. Phenotype analyses of the induced IL-10-producing CD4⁺ T cells

After the 7-day culture, cells were incubated with anti-Fcγ receptor II/III (2.4G2). Then, the cells were incubated with FITC anti-CD4 (H129.19) and PE anti-CD25 (3C7) (BD Biosciences), PerCP anti-CD4 (GK1.5),

PerCP/Cy5.5 anti-LAG-3 (C9B7W), AF647 anti-CD49b (HMa2) (BioLegend), and/or eFluor660 anti-CD3e (17A2) for 20 min. After washing, the cells were incubated with BD fixation/permeabilization solution or Fixation/Permeabilization Concentrate (eBioscience) for 12–18 h, and then, washed with BD Perm/Wash™ buffer or Permeabilization Buffer (eBioscience), followed by incubation with FITC or PE anti-IL-10 (JES5-16E3), PE anti-IFN-γ (XMG1.2), PE anti-IL-4 (11B11), PE anti-IL-17A (eBio17B7), AF647 anti-Foxp3 (FJK-16s), eFluor660 anti-T-bet (eBio4B10), and/or eFluor660 anti-GATA-3 (TWAJ) (eBioscience) for 40 min. Each sample was analyzed using FACSCalibur and Cell Quest Pro version 6.0, or FACS Aria™ Fusion and FACSDiva™ version 8.0.1.

Table 3

Effects of adoptive transfer of CD4⁺ T cells from donor sensitized mice on the production of IL-5 and IL-13 in BALF of sensitized, challenged recipient mice.

Group	Concentration of IL-5 (pg/ml)	Concentration of IL-13 (pg/ml)
Sensitized, non-challenged	0.1 ± 0.1	0
Sensitized, challenged		
+ PBS	15.8 ± 4.2 ^b	38.5 ± 9.9 ^b
+ Freshly isolated splenic CD4 ⁺ T cells	23.6 ± 12.9	58.5 ± 28.1
+ 7-day cultured splenic CD4 ⁺ T cells	15.3 ± 3.5	51.6 ± 12.8

CD4⁺ T cells were adoptively transferred before the 1st challenge, followed by 4 OVA challenges. BALF was harvested at 4 h after the 4th challenge. Each value represents the mean ± S.E.M. of 4 or 9 animals.

^b P < 0.01 vs each value sensitized, non-challenged group.

2.10. Statistical analyses

Two groups were compared with the unpaired *t*-test. To compare the four groups, one-way ANOVA was performed, followed by Dunnett's multiple comparison test. A P-value less than 0.05 was considered significant. Statistical analyses were performed with Prism (Version 5.0a; GraphPad, La Jolla, CA, USA) or JMP® Pro (Version 11.2.0, SAS Institute Japan, Tokyo, Japan).

3. Results

3.1. Antigen-induced proliferation of IL-10-producing CD4⁺ T cells

When spleen cells isolated from the OVA-sensitized mice was cultured with OVA for 7 days, the number of IL-10-producing CD4⁺ T cells was increased significantly by incubation with OVA for 7 days,

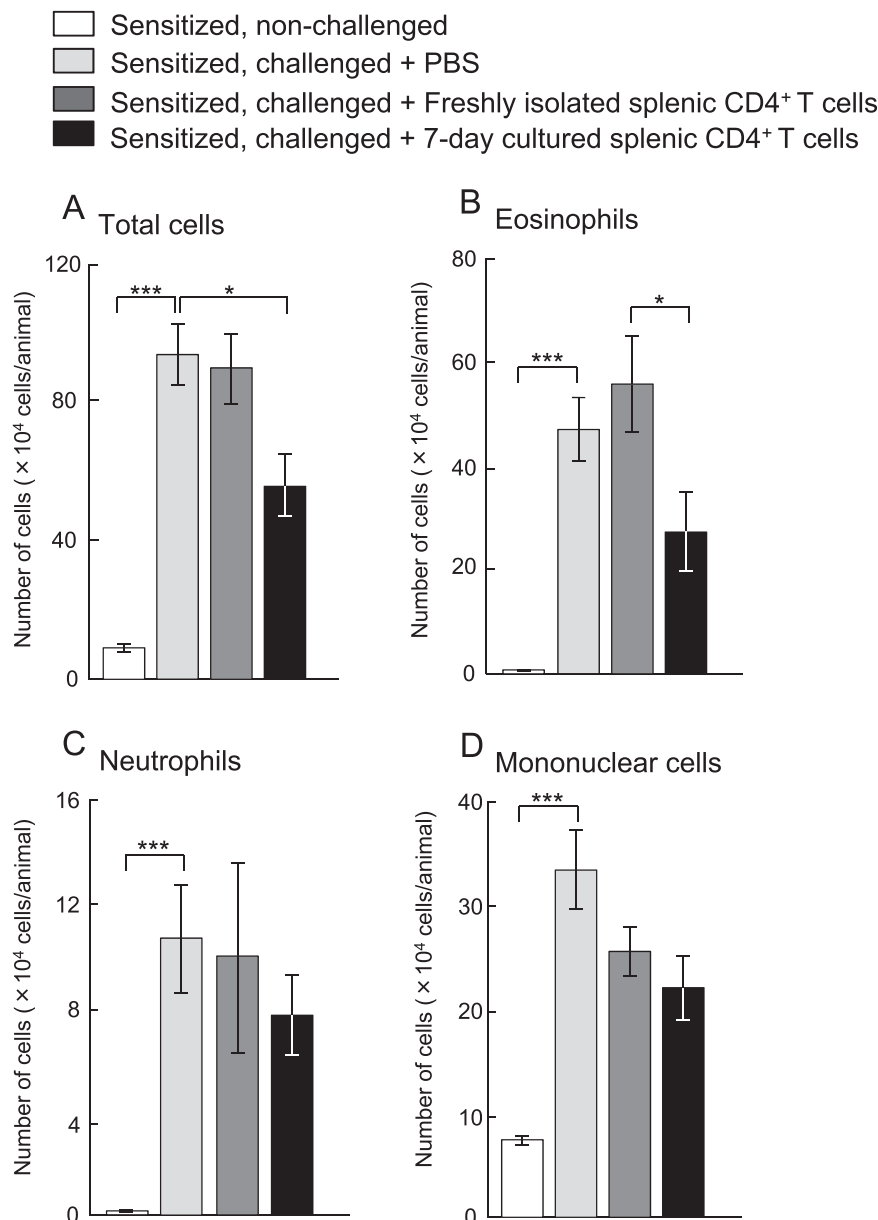


Fig. 4. Effects of adoptive transfer of CD4⁺ T cells from donor sensitized mice on leukocyte infiltration into the BALF in recipient sensitized mice. Splenocytes isolated from sensitized mice were incubated with OVA in the presence of IL-21, IL-27, and TGF-β for 7 days, followed by purification of CD4⁺ T cells using Dynabeads mouse CD4. As control cells, CD4⁺ T cells were purified from freshly isolated splenic cells from sensitized mice. Those CD4⁺ T cells were adoptively transferred before the 1st challenge, followed by 4 OVA challenges. BALF was obtained at 4 h after the 4th challenge. Each column represents the mean ± S.E.M. of 4–9 experiments. * and ***: P < 0.05 and 0.001, respectively.

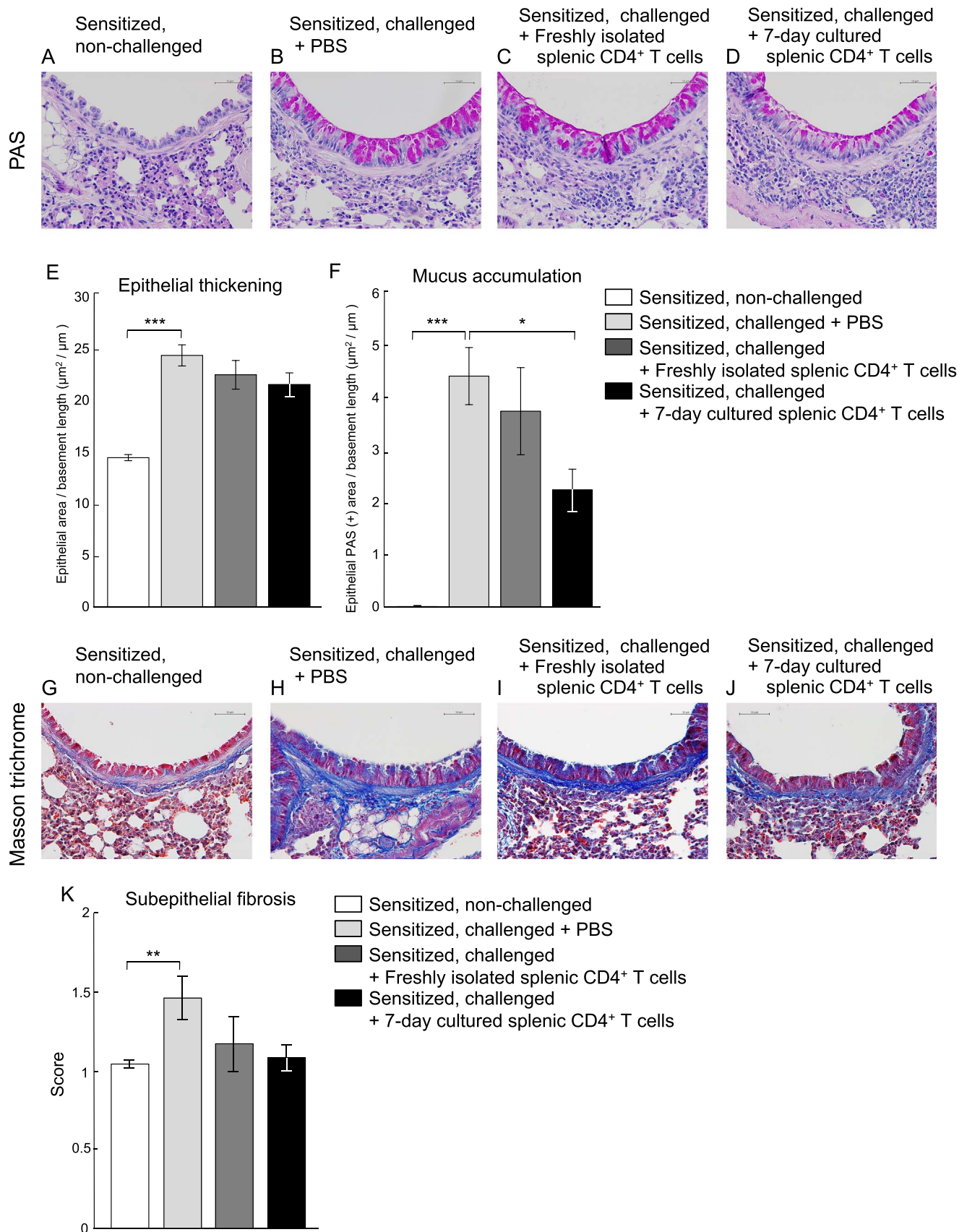


Fig. 5. Effects of adoptive transfer of CD4⁺ T cells from donor sensitized mice on antigen-induced development of airway remodeling in recipient sensitized mice. Splenocytes isolated from sensitized mice were incubated with OVA in the presence of IL-21, IL-27, and TGF- β for 7 days, followed by purification of CD4⁺ T cells using Dynabeads mouse CD4. As control cells, CD4⁺ T cells were purified from freshly isolated splenic cells from sensitized mice. Those CD4⁺ T cells were adoptively transferred before the 1st challenge, followed by 4 OVA challenges. Lung tissue was isolated at 4 h after the 4th challenge, followed by staining with periodic acid-Schiff (PAS, A–D) and Masson trichrome (G–J). The degree of epithelial thickening was quantified using PAS-stained sections by dividing the epithelial area in a bronchus by the length of basement membrane (BM) (E). Degree of mucus deposition was calculated by dividing PAS⁺ area in a bronchus by the length of BM (F). The degree of subepithelial fibrosis was scored using Masson trichrome-stained sections (K). Scale bar represent 50 μm . Each column represents the mean $\pm$ S.E.M. of 4–9 animals. *, ** and ***: $P < 0.05$, 0.01 and 0.001, respectively.

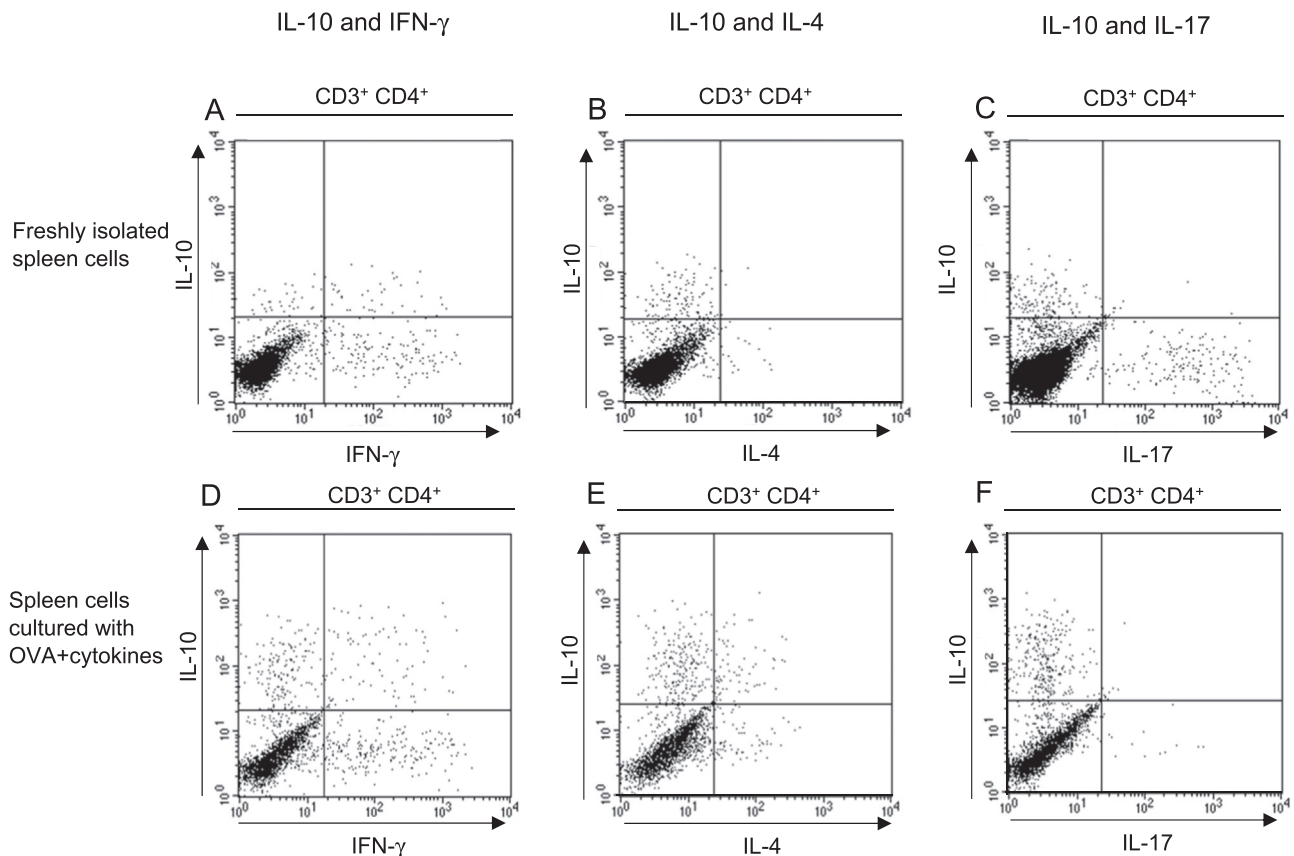


Fig. 6. Flow cytometry analyses of antigen-induced proliferation of IL-10-, IFN- γ -, IL-4-, and IL-17-producing CD4⁺ T cells from splenocytes isolated from sensitized mice. (D, E, and F) Splenocytes isolated from sensitized mice were incubated with OVA in the presence of IL-21, IL-27, and TGF- β for 7 days, followed by stimulation with PMA + ionomycin for 6 h. (A, B and C) Splenocytes isolated from the sensitized mice were not treated with the 7-day culture, but were stimulated with PMA + ionomycin for 6 h.

even in the absence of exogenous cytokines, IL-21, IL-27, and TGF- β (Fig. 1A). Culturing with the cytokine mixture in the absence of OVA also significantly increased the number of IL-10-producing CD4⁺ T cells, although the degree was relatively small (Fig. 1A). Interestingly, when cultured with both OVA and the cytokines, OVA-induced proliferation of IL-10-producing CD4⁺ T cells was synergistically augmented (Fig. 1A). After the 7-day culture, the proportion of IL-10-producing cells in the CD4⁺ T cells were significantly increased by approximately 7 times (Table 1).

When spleen cells isolated from non-sensitized mice were incubated with OVA in the presence of IL-21, IL-27, and TGF- β , IL-10-producing CD4⁺ T cells did not proliferate (Fig. 1B).

3.2. Antigen-induced activation of the purified CD4⁺ T cells in vitro

As shown in Fig. 2B, when 7-day cultured CD4⁺ T cells were incubated in the absence of lung cells, a large amount of IL-10 was produced without antigen stimulation, even though only small amounts of IL-10 were detected from CD4⁺ T cells derived from freshly isolated spleen. Upon antigen stimulation, IL-10 production from 7-day cultured CD4⁺ T cells significantly increased, suggesting that the purified CD4⁺ cell fraction may contain antigen-presenting cells (Fig. 2B). IL-4 and IFN- γ were also produced spontaneously from 7-day cultured CD4⁺ T cells, but they were not further increased by the antigen challenge (Fig. 2B). IL-17 levels in the culture supernatant were close to the minimum detectable level for the ELISA (data not shown).

Regarding antigen-induced cytokine production in the presence of lung cells including antigen-presenting cells, the production of IL-10 and IL-4 from both 7-day cultured and freshly isolated splenic CD4⁺ T

cells was significantly enhanced by the antigen challenge (Fig. 2A). As shown in Fig. 2C, when the lung cells were incubated with OVA in the absence of cultured CD4⁺ T cells, significant increases in IL-10 and IL-4 were induced. However, the co-culture of CD4⁺ T cells and lung cells showed synergistic increases in the levels of IL-10 and IL-4. IL-10 production in particular was markedly augmented by the co-culture (Fig. 2A). IFN- γ production was not increased by the antigen challenge in any of the examples studied here (Fig. 2A).

3.3. Effects of adoptive transfer of purified CD4⁺ T cells on antigen-induced cytokine production, airway inflammation, and antigen-specific IgE production in vivo

In comparison with the non-challenged group, IL-10 increased in both BALF and serum after multiple antigen challenges even without cell transfer (Fig. 3A and D). The antigen-induced increase in IL-10 levels was not significantly affected by the transfer of CD4⁺ T cells derived from freshly isolated splenic cells (Fig. 3A and D). As expected, the transfer of CD4⁺ T cells derived from 7-day cultured splenic cells caused significant increases in IL-10 levels in BALF and serum (Fig. 3A and D). IFN- γ levels in BALF and serum also increased with the transfer of 7-day cultured splenic CD4⁺ T cells, whereas the amounts of IFN- γ were considerably lower and close to the minimum ELISA values (Fig. 3B and E). IL-4 levels in BALF also increased in the animals that received 7-day cultured splenic CD4⁺ T cells, but the amounts were relatively small (Fig. 3C). Relatively high amounts of IL-4 were detected in the serum, but cell transfer did not augment the antigen-induced increase in IL-4 (Fig. 3F). Moreover, IL-5 and IL-13 levels in BALF were not significantly increased by the transfer of 7-day cultured splenic CD4⁺ T cells (Table 3). IL-17 was not detected in either BALF or serum (data not shown).

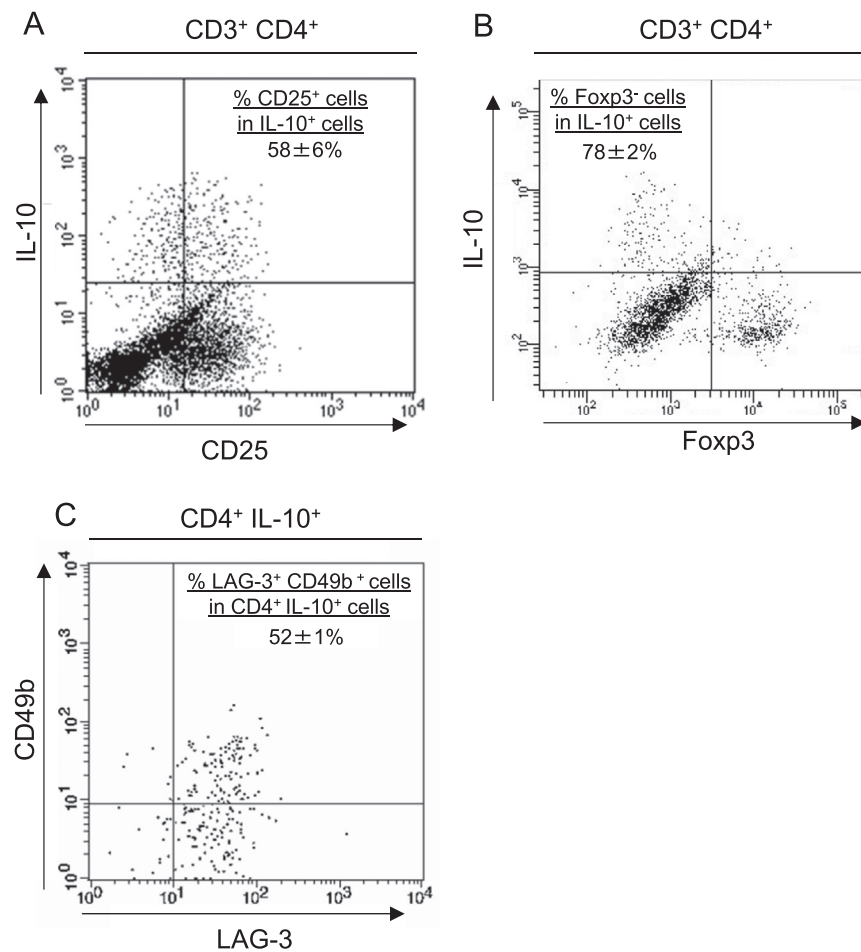


Fig. 7. (A, B and C) Flow cytometry profiles showing expression of cell surface CD25 (A), intracellular Foxp3 (B), and surface LAG-3 and CD49b (C) in IL-10-producing CD4⁺ T cells. Splenocytes isolated from sensitized mice were incubated with 10^{-3} g/ml OVA in the presence of the cytokines for 7 days, followed by stimulation with PMA + ionomycin for 6 h. Numbers in respective panels (A, B and C) are the mean $\pm$ S.E.M. of 3–5 experiments.

As shown in Fig. 4, compared with the non-challenged mice, the number of leukocytes was markedly increased in the BALF of sensitized-challenged mice (Fig. 4A). More than 50% of the infiltrated leukocytes consisted of eosinophils (Fig. 4A and B). The infiltration of eosinophils was significantly suppressed by the transfer of CD4⁺ T cells derived from 7-day cultured splenic cells. Increases in the numbers of neutrophils and mononuclear cells were also slightly reduced, but the effects were not statistically significant (Fig. 4C and D).

Fig. 5 shows histological changes in the lung tissue of the respective groups. Compared with the histology of the lung tissue isolated from non-challenged mice (Fig. 5A), epithelial changes in the bronchus such as epithelial thickening and mucus accumulation were evidently induced in the lung tissue of challenged mice (Fig. 5B, E, and F). Neither epithelial thickening nor mucus accumulation was decreased by the transfer of CD4⁺ T cells derived from freshly isolated splenic cells (Fig. 5C, E, and F). The transfer of CD4⁺ T cells derived from 7-day cultured splenic cells significantly reduced mucus accumulation (Fig. 5D and F). Epithelial thickening tended to be decreased by the transfer of 7-day cultured CD4⁺ T cells, although the effect was not statistically significant (Fig. 5D and E). In addition, subepithelial fibrosis was also significantly induced by the antigen challenges (Fig. 5G, H, and K). The subepithelial fibrosis tended to be reduced by the transfer of any CD4⁺ T cells (Fig. 5I, J, and K).

OVA-specific IgE levels in sera were also measured. As shown in Table 2, with transferred freshly isolated splenic CD4⁺ cells, OVA-specific IgE levels of the recipient mice tended to be increased. In contrast, the transfer of 7-day cultured splenic CD4⁺ T cells tended to be decreased. These changes were not statistically significant.

3.4. Phenotype of the induced IL-10-producing CD4⁺ T cells

The cytokine profile of the 7-day cultured cells in response to PMA + ionomycin was evaluated in comparison with that of freshly isolated spleen cells. CD4⁺ T cells in the freshly isolated spleen cells included a proportion of IL-10⁺ and IFN- γ ⁺ cells (Fig. 6A), but only a few IL-4⁺ cells (Fig. 6B), and a relatively large population of IL-17⁺ cells (Fig. 6C). After 7-day culture with OVA, IL-21, IL-27, and TGF- β , the levels of IL-10-producing CD4⁺ T cells increased dramatically (Fig. 6D, E, and F). In addition, a similar level of IFN- γ -secreting cells including double producers of IL-10 and IFN- γ was induced by the 7-day culture (Fig. 6D). As shown in Fig. 6E, IL-4-producing CD4⁺ T cells also increased, but the degree was relatively small (Fig. 6D). In contrast, the number of IL-17-producing CD4⁺ T cells disappeared in the 7-day cultures (Fig. 6F).

Approximately 40% of the IL-10-producing CD4⁺ T cells was negative for CD25, a surface marker of naturally occurring regulatory T (nTreg) cells (Fig. 7A), and approximately 80% of the IL-10-producing CD4⁺ T cells were negative for Foxp3, a transcription factor of nTreg cells (Fig. 7B). Additionally, approximately 50% of the IL-10-producing CD4⁺ T cells were double positive for CD49b and LAG-3, which are surface markers of inducible of regulatory T cells, Tr1 cells (Gagliani et al., 2013) (Fig. 7C).

Most of both IL-10⁺ IFN- γ ⁺ cells and IL-10[−] IFN- γ ⁺ cells were positive for T-bet (Fig. 8A, C, and D), a transcription factor of Th1 cells (Szabo et al., 2000). Of note, more than 80% of IL-10⁺ IFN- γ [−] cells were also T-bet-positive, even though the cells possessed no capability to produce the typical Th1 cytokine IFN- γ (Fig. 8A and B).

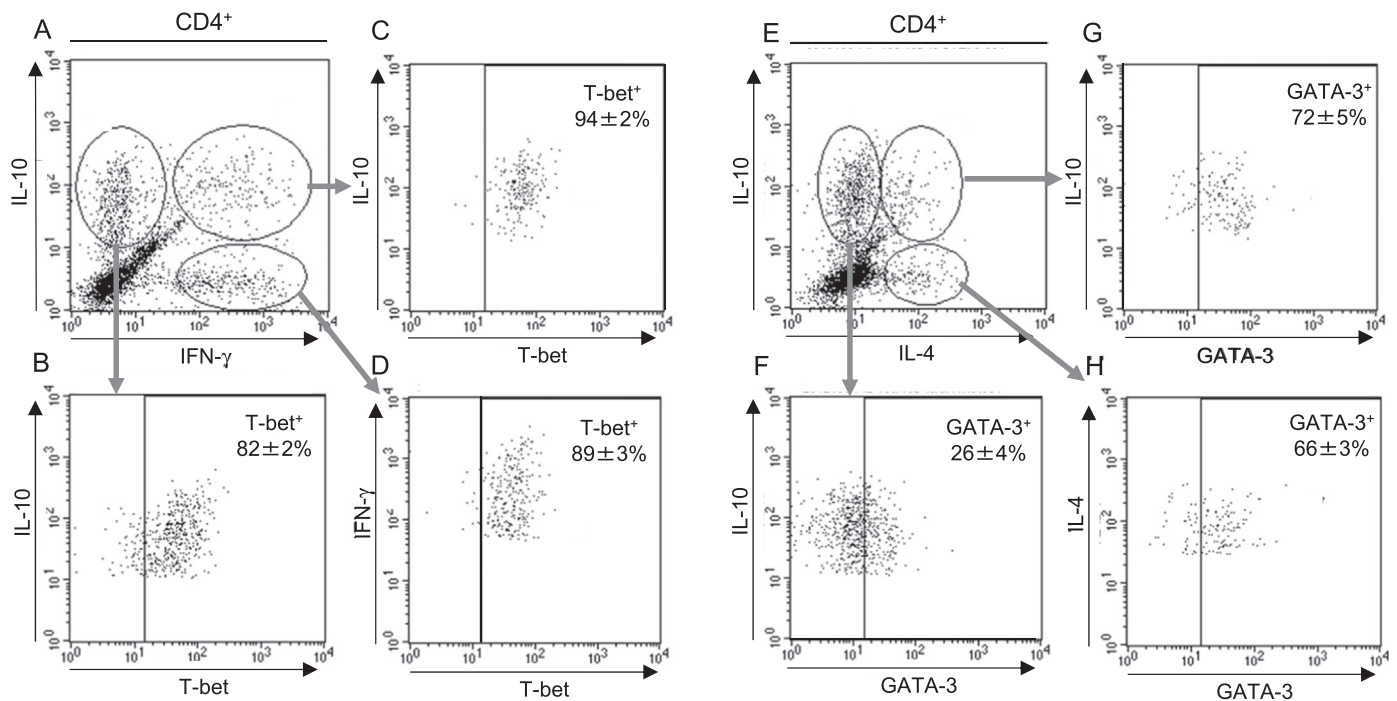


Fig. 8. (A, B, C, and D) Flow cytometry analyses of expression of intracellular T-bet in IL-10⁺ IFN-γ⁻ (B), IL-10⁺ IFN-γ⁺ (C), and IL-10⁻ IFN-γ⁺ (D) CD4⁺ T cells induced from splenocytes of sensitized mice. (E, F, G, and H) Flow cytometry analyses of expression of intracellular GATA-3 in IL-10⁺ IL-4⁻ (F), IL-10⁺ IL-4⁺ (G), and IL-10⁻ IL-4⁺ (H) CD4⁺ T cells induced from splenocytes of sensitized mice. Splenocytes isolated from sensitized mice were incubated with OVA in the presence of IL-21, IL-27, and TGF-β for 7 days, followed by stimulation with PMA + ionomycin for 6 h. Each value in the respective panels is the mean ± S.E.M. of 4 experiments of % T-bet⁺ cells in CD4⁺ T cells producing IL-10 and/or IFN-γ, and % GATA-3⁺ cells in CD4⁺ T cells producing IL-10 and/or IL-4.

Regarding GATA-3, a transcription factor for Th2 cells (Zhang et al., 1997; Zheng and Flavell, 1997), more than 70% of IL-10⁺ IL-4⁻ cells were negative for it (Fig. 8E and F). As expected, most of the IL-10⁺ IL-4⁺ cells and IL-10⁻ IL-4⁺ cells were positive for GATA-3 (Fig. 8E, G, and H).

4. Discussion

In order to develop a cellular immunotherapy for atopic diseases, we experimentally induced IL-10-producing CD4⁺ T cells in vitro by stimulating spleen cells isolated from OVA-sensitized mice with OVA. Stimulation with OVA induced the proliferation of IL-10-producing CD4⁺ T cells, and stimulation with a combination of OVA and the cytokines, IL-21, IL-27, and TGF-β exerted synergistic effects on the proliferation. It has been reported that IL-10-producing CD4⁺ T cell differentiation was promoted by IL-27 (Awasthi et al., 2007; Stumhofer et al., 2007). Moreover, TGF-β amplified the generation of IL-10-secreting T cells by IL-27 (Pot et al., 2009). Additionally, IL-21 acts as an autocrine growth factor for the expansion and/or maintenance of IL-27-induced IL-10-producing cells (Awasthi et al., 2007). Thus, we used these three kinds of cytokines to effectively proliferate IL-10-secreting CD4⁺ T cells.

CD4⁺ T cells derived from 7-day cultured spleen cells were activated by a specific antigen, OVA, in the presence of antigen-presenting cells in vitro to produce large amounts of IL-10. In addition, when CD4⁺ T cells were transferred into asthmatic mice, and the recipient mice were challenged with OVA, IL-10 production was augmented and allergic airway eosinophilia and mucus accumulation in the bronchial epithelium were attenuated in vivo. From these results, the CD4⁺ T cells definitely responded to OVA and produced IL-10, indicating that the regulatory cytokine is generated when exposed to the specific antigen. In preliminary experiments, we found that multiple airway antigen challenges were required to detect significant increases in IL-10 in BALF and serum from the recipient mice. This finding implied that multiple antigen challenges may further proliferate the transferred

CD4⁺ T cells in vivo, and recruit the cells to the local airway sites, including the regional lymph nodes and lungs.

In the present culture conditions, approximately 80% and 40% of the IL-10-producing CD4⁺ T cells were negative for Foxp3 and CD25, respectively. Both molecules are expressed by naturally occurring regulatory T (nTreg) cells (Hori et al., 2003). Thus, the features of the induced IL-10-producing CD4⁺ T cells were considerably different from those of nTreg cells. On the other hand, 50% of the induced IL-10-producing CD4⁺ T cells were double positive for LAG-3 and CD49b, surface markers of type 1 regulatory T (Tr1) cells (Gagliani et al., 2013). Unlike nTreg cells, whose antigen specificity is still unclear, induced IL-10-producing CD4⁺ T cells, such as Tr1 cells, can respond to a specific antigen through TCR activation (Cottrez et al., 2000), and, therefore, may have a therapeutic advantage for immunotherapy over Foxp3⁺ cells, as suggested by Awasthi et al. (Awasthi et al., 2007).

On the other hand, Treg cells have been shown to infiltrate tumors, and are thought to limit anti-tumor immunity by suppressing natural killer CD8⁺ or CD4⁺ effector T cells (Teng et al., 2010; Li et al., 2010; Liu et al., 2016), resulting in the augmentation of tumor growth. Thus, adoptively transferred Treg cells may also suppress anti-tumor immunity if Treg cells are continuously activated in vivo. However, as demonstrated in the present in vivo study, the induced Tr1 cells were activated when recipient mice were exposed to the specific antigen, suggesting that the Tr1-like cells might become a safe tool to suppress antigens specifically induced in allergic reactions, but not anti-tumor immunity. Thus, in vitro induced Tr1-like cells may be utilized for antigen-specific immunotherapy for allergic airway inflammation.

In the current induction method, not only IL-10⁺ but also IFN-γ⁺ cells proliferated preferentially. Even before the 7-day culture, a marked population of IL-10⁻ IFN-γ⁺ cells already existed. After the 7-day culture with OVA in the presence of the cytokines, not only IL-10⁻ IFN-γ⁺ cells, but also IL-10⁺ IFN-γ⁺ cells, both of which were T-bet-positive, emerged prominently. Th1 cells producing both IL-10 and IFN-γ are induced during certain infections, and IL-10 produced from Th1 cells suppressed acquired immunity in mice (Anderson et al.,

2007; Jankovic et al., 2007). The switch to IFN- γ ⁺ IL-10⁺ Th1 cells from IFN- γ ⁺ Th1 cells also occurred during the induction of tolerance to antigens (Cope et al., 2011). These switched Th1 cells maintained T-bet expression (Gabryšová et al., 2009), and were also induced by high doses of antigen exposure in humans (Meiler et al., 2008). In mice, strong TCR ligation conditions, such as the usage of a high antigen concentration as in the present study, could also switch Th1 cells to IL-10-secreting Th1 cells in mice (Saraiva et al., 2009). However, whether IFN- γ ⁺ IL-10⁺ T-bet⁺ cells are Th1 cells is unclear, because Tr1 cells may also express T-bet, although there is a conflicting evidence in this regard (Cope et al., 2011). Moreover, Tr1 cells expressed T cell immunoglobulin and mucin domain-3 (TIM-3), which is a Th1-specific surface protein (White and Wraith, 2016). Taken together, the IL-10-producing CD4⁺ T cells induced in this study may include not only Tr1 cells but also “suppressive Th1 cells”; however, even if the suppressive Th1 cells existed, IFN- γ may not be related to the suppression of allergic airway eosinophilia because IFN- γ was not prominently produced from CD4⁺ T cells by antigen challenge in this study.

IL-4-secreting CD4⁺ T cells were induced relatively weakly by the present culture conditions. In contrast, IL-17-secreting CD4⁺ T cells were decreased in the 7-day culture. In mice, IL-21 and TGF- β function to induce the Th17 lineage, whereas IL-27 inhibits the differentiation of this lineage (Ivanov et al., 2007). In addition, IL-27 has broad inhibitory effects on Th2 and Th17 subsets of T cells (Jankowski et al., 2010). On the other hand, when the 7-day cultured CD4⁺ T cells were stimulated with the antigen in the presence of antigen-presenting cells in vitro, both IL-10 and IL-4 were clearly produced, and the degree of IL-10 production being more marked than that of IL-4 production. This profile of cytokine production was identical to the results obtained using PMA + ionomycin as a stimulant. In contrast, the transferred CD4⁺ cells produced a large amount of IL-4, especially in the serum of recipient mice. Th2 cells, including in the transferred CD4⁺ cells, may limit the regulatory functions of Tr1 cells. Other purification methods such as sorting LAG-3⁺ CD49b⁺ cells, Tim-3⁺ cells or excluding Th2 cells using antibodies against Th2 cell markers may result in further suppression of allergic airway inflammation.

The transfer of 7-day cultured CD4⁺ cells significantly suppressed allergic airway eosinophilia and mucus accumulation in the epithelium. However, serum OVA-specific IgE levels were not significantly reduced by the transfer. Airway eosinophilia and mucus accumulation had never been induced prior to the 1st challenge, when the CD4⁺ cells were injected. In contrast, the amount of OVA-specific IgE was already high at the time before the 1st challenge because the mice had been i.p. sensitized with OVA + Al (OH)₃. If the CD4⁺ cells are injected before the sensitization with OVA + Al (OH)₃, the elevation of OVA-specific IgE may be suppressed.

5. Conclusion

When spleen cells isolated from sensitized mice were cultured with the specific antigen in the presence of IL-21, IL-27, and TGF- β , Tr1 cells and/or suppressive Th1 cells, which produced IL-10, were induced. These suppressive CD4⁺ T cells could be utilized for the development of antigen-specific cellular immunotherapy in experimental studies.

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