

Conversion of Th2 memory cells into Foxp3⁺ regulatory T cells suppressing Th2-mediated allergic asthma

Byung-Seok Kim^a, Il-Kyu Kim^a, Young-Jun Park^a, Yun-Sun Kim^a, Yeon-Jeong Kim^a, Woo-Sung Chang^a, Yoon-Sook Lee^a, Mi-Na Kweon^b, Yeonseok Chung^c, and Chang-Yuil Kang^{a,d,1}

^aLaboratory of Immunology, Institute of Pharmaceutical Sciences, College of Pharmacy, Seoul National University, Seoul 151-742, Korea; ^bMucosal Immunology Section, International Vaccine Institute, Seoul 151-919, Korea; ^cDepartment of Immunology, M. D. Anderson Cancer Center, Houston, TX 77030; and ^dDepartment of Molecular Medicine and Biopharmaceutical Science, Graduate School of Convergence Science and Technology and College of Pharmacy, Seoul National University, Seoul 151-742, Korea

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Genetic and epigenetic programming of T helper (Th) cell subsets during their polarization from naive Th cells establishes long-lived memory Th cells that stably maintain their lineage signatures. However, whether memory Th cells can be redifferentiated into another Th lineage is unclear. In this study, we show that Ag-specific memory Th cells were redifferentiated into Foxp3⁺ T cells by TGF- β when stimulated in the presence of *all-trans* retinoic acid and rapamycin. The “converted” Foxp3⁺ T cells that were derived from Th2 memory cells down-regulated GATA-3 and IRF4 and produced little IL-4, IL-5, and IL-13. Instead, the converted Foxp3⁺ T cells suppressed the proliferation and cytokine production of Th2 memory cells. More importantly, the converted Foxp3⁺ T cells efficiently accumulated in the airways and significantly suppressed Th2 memory cell-mediated airway hyperreactivity, eosinophilia, and allergen-specific IgE production. Our findings reveal the plasticity of Th2 memory cells and provide a strategy for adoptive immunotherapy for the treatment of allergic diseases.

all-trans retinoic acid | immune tolerance | immunotherapy | plasticity | rapamycin

Upon antigen exposure, naive T helper (Th) cells are activated and then undergo several divisions to differentiate into specialized effector T cells, including Th1, Th2, and Th17, each of which has its own characteristic cytokine expression pattern (1). Each Th subset maintains its cytokine expression and represses opposing Th cytokine expression by coordinating the regulation of master transcription factors. Additionally, chromatin remodeling and epigenetic modifications, such as DNA methylation and histone acetylation, of specific cytokine gene loci support the stability of Th cell fates. These epigenetic modifications are inherited by daughter cells as the cell divides, and the modifications also persist in memory cells, thus accounting for the rigid nature of Th lineages (2, 3).

Recently, however, the classical notion that Th cell lineage commitment is irreversible has been challenged (4). For example, it has been reported that Foxp3⁺ regulatory T cells (Tregs) can be converted into Th17 cells under Th17-polarizing conditions, and Th17 cells become IFN- γ -producing cells in the presence of IL-12 (5–7). In addition, Th1 and Th2 memory cells have been reported to be interconvertible under conditions that promote the opposite Th lineage (8). However, whether Th1 and Th2 memory cells can be redifferentiated into Foxp3⁺ Tregs has not been explored.

The clinical potency of Treg-based adoptive immunotherapy has been proven in several disease settings, including transplantation and the treatment of autoimmune and allergic diseases (9–11). With a few successful applications of Tregs in animal studies, at least two phase I clinical trials using Tregs are now being conducted in hematopoietic stem cell transplantation and type 1 diabetes (12). Until now, one of the major obstacles to the clinical application of Treg therapy has been the scarcity of Tregs in human peripheral blood (13). To overcome this problem, TGF- β -mediated induction of Tregs from abundant naive precursors has been used (14). In ad-

dition, *all-trans* retinoic acid (ATRA) has been reported to enhance TGF- β -mediated Foxp3 induction in naive Th cells either by a direct effect on naive Th cells or by an indirect effect on cytokine production of memory Th cells (15–17). Furthermore, rapamycin, a mammalian target of rapamycin (mTOR) inhibitor, can enrich Foxp3⁺ Tregs through Foxp3-mediated induction of Pim 2 (18). However, whether the TGF- β -mediated Foxp3 induction in effector/memory Th cells can be enhanced by ATRA and rapamycin remains to be elucidated.

Here, we show that TGF- β -mediated Foxp3 expression in Ag-specific Th2 memory cells can be enhanced by ATRA and can be further enhanced by rapamycin. The resultant Th2 memory-derived Foxp3⁺ cells can efficiently modulate Th2 memory-induced airway hyperreactivity (AHR) and eosinophilia. These results demonstrate that, with the proper manipulation, Ag-specific Th2 memory cells can be induced to express Foxp3, providing guidance for developing an effective and safe Treg-based immunotherapy.

Results

Ag-Specific Th2 Memory Cells Do Not Express Foxp3 in Response to TGF- β Plus *All-Trans* Retinoic Acid. In naive Th cells, TGF- β induces Foxp3 expression, which is further synergized with ATRA (14, 15, 17). To determine whether Ag-specific Th2 memory cells can also express Foxp3 in response to these factors, we generated ovalbumin (OVA)-specific Foxp3⁺ Th2 memory cells by using naive OVA-specific CD4⁺ Foxp3⁺ T cells isolated from DO11.10-RAG2^{−/−} mice (19). Purified naive Th cells were stimulated with irradiated splenocytes plus the OVA_{323–339} peptides under Th2-polarizing conditions for 3 days. These activated T cells were adoptively transferred into syngeneic BALB/c mice to induce them to become memory T cells (20). Three to 6 weeks later, Th2-polarized donor T cells harbored the phenotype of memory cells (FSC^{low}, CD44^{hi}, CD25⁺, CD127^{low}, CD62L^{hi/low}) and were Foxp3⁺, whereas naive Th cells were FSC^{low}, CD44^{low}, CD25[−], CD127[−], CD62L^{hi}, and Foxp3[−] (Fig. S1A). Functionally, the former promptly produced IL-4 but not IFN- γ upon restimulation with their cognate peptide *ex vivo* (Fig. S1B). Taken together, these results demonstrate that, in the context of both phenotype and function, Th2-polarized donor T cells in the recipient established genuine Ag-specific Th2 memory cells.

When we purified the Th2 memory cells and stimulated them with spleen dendritic cells (DCs) and OVA_{323–339} peptides in the

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¹To whom correspondence should be addressed. E-mail: cykang@snu.ac.kr.

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presence of TGF- β and/or ATRA, little Foxp3 expression was observed (Fig. 1A). We also obtained OVA-specific Th1 memory cells by similar methods (Fig. S1C and D) and found a similar inefficient Foxp3 expression in response to TGF- β and ATRA (Fig. S1E). Therefore, Ag-specific Th2 memory cells as well as Th1 memory cells are resistant to Foxp3 induction in response to TGF- β and ATRA.

Rapamycin and ATRA Synergistically Increase TGF- β -Mediated Foxp3 Induction in Ag-Specific Th2 Memory Cells in the Presence of Effector Cytokine Neutralization. Th1 and Th2 effector cytokines have been shown to inhibit Treg differentiation as well as Th17 differentiation (1, 21, 22). In addition, mTOR signaling attenuates TGF- β -mediated Foxp3-inducing signals (23–25). Therefore, we hypothesized that the resistance of Ag-specific memory Th cells against TGF- β -mediated Foxp3 induction could be due to inefficient TGF- β signaling mediated by effector cytokines and/or the activated mTOR pathway. To test this hypothesis, we added neutralizing anti-IL-4 and anti-IFN- γ Abs to the culture of Th2 memory cells. Notably, a considerable population of Foxp3⁺ cells was induced by TGF- β , anti-IL-4, and anti-IFN- γ , and the Foxp3⁺ cells were significantly increased by ATRA (Fig. 1A). Interestingly, further addition of mTOR inhibitor rapamycin greatly enhanced TGF- β -mediated Foxp3 induction in Th2 memory cells, in which over 60% were Foxp3⁺ (Fig. 1A). Similarly, Foxp3⁺ cells were substantially induced by treating Th1 memory cells with a combination of TGF- β , anti-IFN- γ Ab, ATRA, and rapamycin (Fig. S1E). We observed a similar enhancement of Foxp3 expression by ATRA and rapamycin in an antigen presenting cell (APC)-free system with little effect on cell viability, demonstrating the T-cell intrinsic effects of these reagents (Fig. 1B and C and Fig. S2).

Notably, addition of ATRA or rapamycin 3 days after restimulation failed to increase the Foxp3⁺ population (Fig. S3A–C). Therefore, both signals are required during the early phase of T cell receptor (TCR) restimulation to induce optimal expression of Foxp3 in Th2 memory cells.

Foxp3⁺ T Cells Converted from Th2 Memory Cells Do Not Express GATA-3 and Th2 Cytokines. We next compared gene expression profiles among naive Th cells, Th2 memory cells, and Th2 memory-derived Tregs (we will refer to the Th2 memory cells

restimulated in the presence of TGF- β , anti-IL-4, anti-IFN- γ , ATRA, and rapamycin as Th2 memory-derived Tregs because we discovered that they are regulatory T cells, as described below). Purified Th2 memory cells expressed high levels of Th2 signature genes, including *Gata3*, *Irf4*, *Il4*, *Il5*, *Il13*, and *Ccr4*, at the transcript level. In contrast, Th2 memory-derived Tregs profoundly down-regulated all these Th2 signature genes except *Ccr4* with high expression of Foxp3 (Fig. S4).

To confirm these findings at the protein level, we costained the cells with Foxp3 and GATA-3 or IL-4 or IL-13. Consistent with a previous report (26), TGF- β down-regulated GATA-3 expression in Th2 memory cells. Interestingly, TGF- β plus anti-IL-4 almost completely abrogated GATA-3 expression in Th2 memory cells (Fig. S5). Similarly, TGF- β plus anti-IFN- γ almost completely suppressed T-bet expression in Th1 memory cells (Fig. S1E). In addition, TGF- β and anti-IL-4 significantly decreased the proportion of either IL-4- or IL-13-producing cells within the Th2 memory cell population (Fig. S5). As a result, Th2 memory-derived Tregs contained few GATA-3-expressing and IL-4- or IL-13-producing cells (Fig. 2A and B). This observation was further confirmed by measuring levels of IL-4, IL-5, and IL-13 from the T-cell culture supernatant after restimulation (Fig. 2C).

Recent studies have identified IL-9-producing cells whose polarization is mediated by TGF- β and IL-4 signals (27, 28). Interestingly, a considerable proportion of Th2 memory cells produced IL-9 when restimulated in the presence of TGF- β . In contrast, Th2 memory-derived Tregs produced little IL-9, probably due to the complete blockade of the IL-4 signal (Fig. 2D).

We then determined whether Th2 memory-derived Tregs bear Th2-type integrin and homing receptors. As expected, naive Th cells expressed the lymph node-homing receptor CCR7 but not the lung-homing receptor CCR4 (Fig. S6A). Conversely, Th2 memory-derived Tregs down-regulated CCR7 and highly expressed CCR4 (Fig. S6A). Also, Th2 memory-derived Tregs highly expressed CD103 (α E), which may contribute to their retention and/or accumulation in the lung tissue (Fig. S6A and B) (29). These observations led us to examine the lung-homing capacity of the Th2 memory-derived Tregs. Two days after transfer into congenic mice, both Th2 memory-derived Tregs and naive Th cells were detectable in the spleen. In contrast, only Th2 memory-derived Tregs were detected in the lung, which correlated with

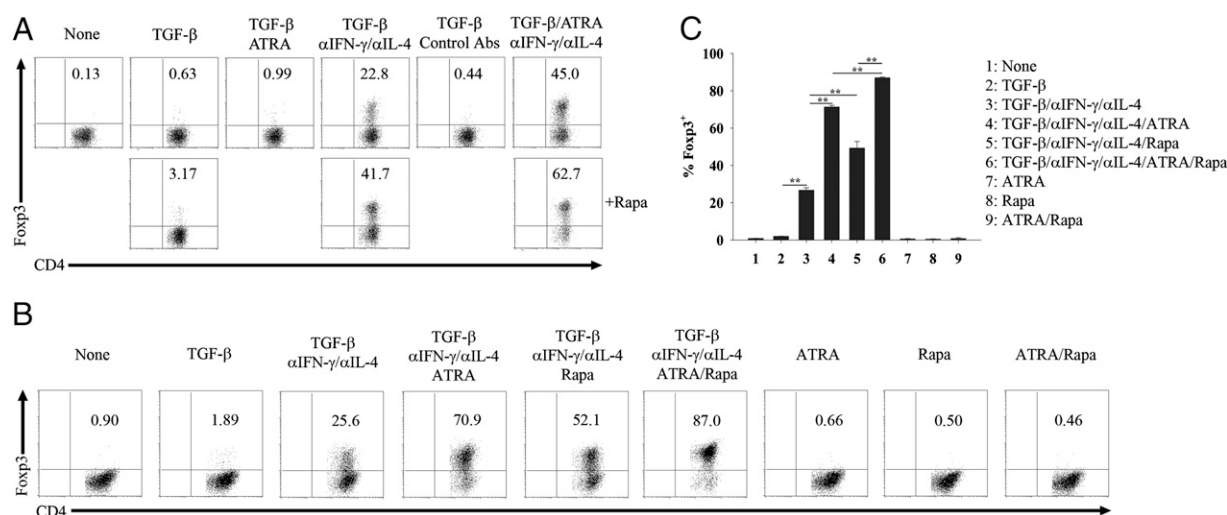


Fig. 1. TGF- β mediates the induction of Foxp3 in Ag-specific Th2 memory cells with sequential addition of ATRA and rapamycin in the presence of IL-4 and IFN- γ neutralization. (A) Purified OVA-specific Th2 memory cells were stimulated with spleen DCs and OVA_{323–339} peptides under indicated conditions. TGF- β (20 ng/mL), ATRA (100 nM), and rapamycin (Rapa; 20 nM) were added at the indicated concentrations. At day 4 of culture, Foxp3 expression levels in gated CD4⁺KJ1-26⁺ cells were analyzed by flow cytometry. (B) Purified OVA-specific Th2 memory cells were stimulated with plate-bound anti-CD3 (2 μ g/mL) and soluble anti-CD28 (1 μ g/mL) Abs under the indicated conditions. The final dose of TGF- β was 5 ng/mL. (C) The mean $\pm$ SEM percentage of Foxp3⁺ cells in each group in B are plotted (** P < 0.005). Data are representative of at least three independent experiments.

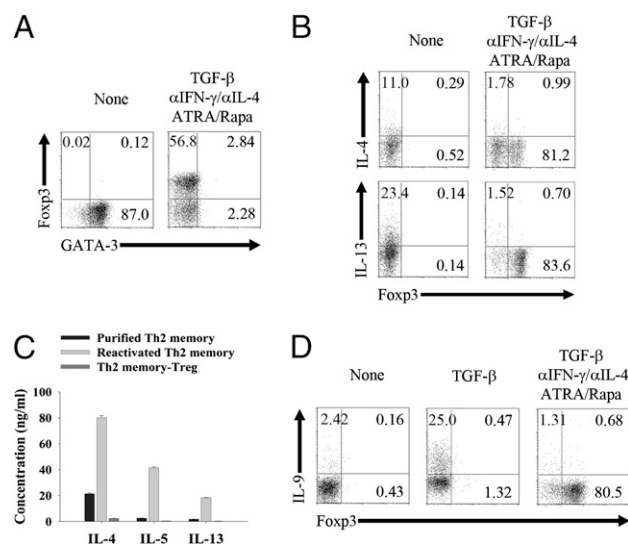


Fig. 2. Ag-specific Th2 memory-derived Foxp3⁺ cells lose their Th2-type effector function. (A) Purified OVA-specific Th2 memory cells were stimulated with spleen DCs and OVA_{323–339} peptides under the indicated conditions for 4 days, and the correlation of Foxp3 and GATA-3 expression on gated CD4⁺KJ1-26⁺ cells was analyzed by flow cytometry. (B) OVA-specific Th2 memory cells were cultured as indicated in the presence of anti-CD3 and anti-CD28 Abs for 5 days. The resultant cells were stained for Foxp3 and either IL-4 or IL-13 after restimulation. (C) Purified OVA-specific Th2 memory, reactivated Th2 memory (“None” in B), and Th2 memory-derived Tregs (TGF- β / α IL-4/ α IFN- γ /ATRA/Rapa in B) were restimulated for anti-CD3 Ab (2 μ g/mL) for 24 h. Culture supernatants from triplicate samples were analyzed for cytokine production by ELISA. (D) OVA-specific Th2 memory cells were cultured as indicated in the presence of anti-CD3 and anti-CD28 Abs for 5 days. The resultant cells were stained for Foxp3 and IL-9 after restimulation. Data are representative of at least three independent experiments.

their high expression of CCR4; however, the proportion of Foxp3-expressing cells within this lung Treg population was lower than that of the spleen population (Fig. S6C). Notably, the Foxp3⁺ proportion of Th2 memory-derived Tregs in the lung was significantly increased after intranasal (i.n.) administration of their cognate antigen (Fig. S6D). Therefore, the Th2 memory-derived Tregs maintained the lung-homing capacity of Th2 memory cells even with diminished Th2 gene profiles.

Foxp3⁺ T Cells Converted from Th2 Memory Cells Are Regulatory T Cells. To characterize the Th2 memory-derived Foxp3⁺ cells in the context of regulatory T cells, we next analyzed the surface expression of Treg-specific markers, including CD25, CD39, FR4, GITR, PD-1, and ICOS, even though most of these molecules are expressed on activated T cells as well. Compared with reactivated Th2 memory cells, Th2 memory-derived Foxp3⁺ cells expressed slightly reduced levels of CD25, CD39, GITR, PD-1, and ICOS. Interestingly, however, Th2 memory-derived Foxp3⁺ cells highly expressed FR4, a recently discovered Treg-specific surface marker (30), whereas reactivated Th2 memory cells did not (Fig. 3A). Therefore, the Foxp3⁺ T cells derived from Th2 memory cells are phenotypically regulatory T cells.

Next, we analyzed the ability of Th2 memory-derived Tregs to suppress the activity of Th2 memory responder cells. As shown in Fig. 3B, no more than 20% of Th2 memory-derived Tregs divided more than three times, whereas over 90% of Th2 memory cells did. Similarly, Th2 memory-derived Tregs produced little IL-4, IL-5, and IL-13, whereas Th2 memory cells produced a significant amount of these cytokines (Fig. 3C). In addition, Th2 memory-derived Tregs actively suppressed proliferation and cytokine production of Th2 memory cells in a Treg-to-T effector ratio-dependent manner (Fig. 3B and C). Also, the suppressive potency of

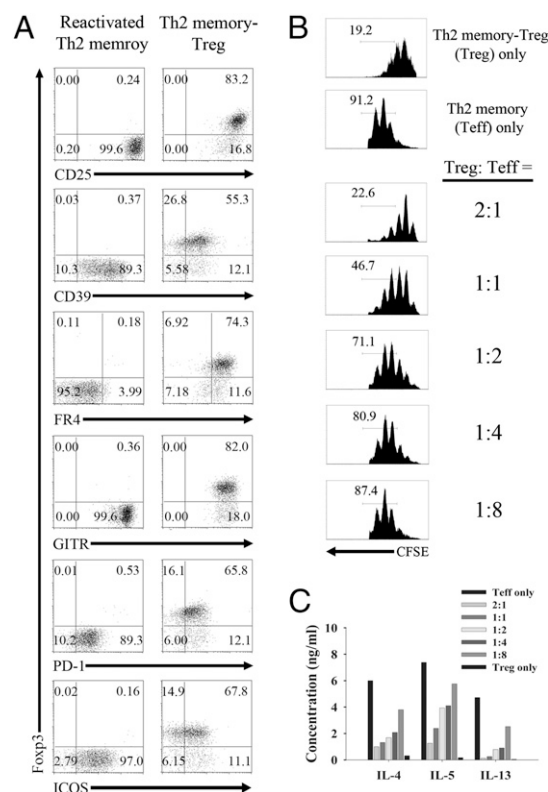


Fig. 3. Ag-specific Th2 memory-derived Foxp3⁺ cells are genuine regulatory T cells. (A) Reactivated Th2 memory cells or Th2 memory-derived Tregs were analyzed by flow cytometry for surface expression of CD25, CD39, FR4, GITR, PD-1, and ICOS, together with Foxp3. (B and C) OVA-specific Th2 memory-derived Treg-mediated suppression of proliferation (B) and cytokine production (C) of OVA-specific Th2 memory cells (T effectors; Teff) were examined as described in SI Materials and Methods. Numbers inside the histograms indicate the percentage of cells dividing more than three times in each group. Data are representative of at least three independent experiments.

Th2 memory-derived Tregs (80% Foxp3⁺) was two times higher than naive-induced Tregs (90% Foxp3⁺) (Fig. S7). Moreover, Th2 memory-derived Tregs stably expressed Foxp3 and produced few Th2 cytokines after restimulation under Th2-polarizing conditions (Fig. S8A). It is noteworthy that half of the Foxp3⁺ cells reexpressed GATA-3 under these conditions. More importantly, they also retained suppressive activity against Th2 memory cells, although their potency was slightly reduced (Fig. S8B and C). Therefore, Th2 memory-derived Foxp3⁺ cells are bona fide regulatory T cells with potent and stable suppressive activity.

Ag-Specific Th2 Memory-Derived Regulatory T Cells Ameliorate Th2 Memory-Mediated Airway Hyperreactivity and Eosinophilic Inflammation. Finally, we determined whether the Ag-specific Th2 memory-derived Tregs could suppress Th2 memory-mediated inflammation in vivo. To this end, we adoptively transferred Th2 memory-derived Tregs into Th2 memory-bearing recipients 1 day before i.n. administration of allergen. When we analyzed the mice after airway allergen challenge, Th2 memory-derived Tregs significantly suppressed Th2 memory-mediated AHR and eosinophilia in bronchoalveolar lavage fluid (BALF) (Fig. 4A and B). The level of OVA-specific IgE in sera was also greatly decreased by Th2 memory-derived Tregs (Fig. 4C).

As depicted in Fig. 4D, IL-4-, IL-5-, and IL-13-producing OVA-specific CD4⁺ T cells in the draining mediastinal lymph node (MdLN) were significantly decreased by Th2 memory-derived Tregs. More importantly, more than 70% of the transferred

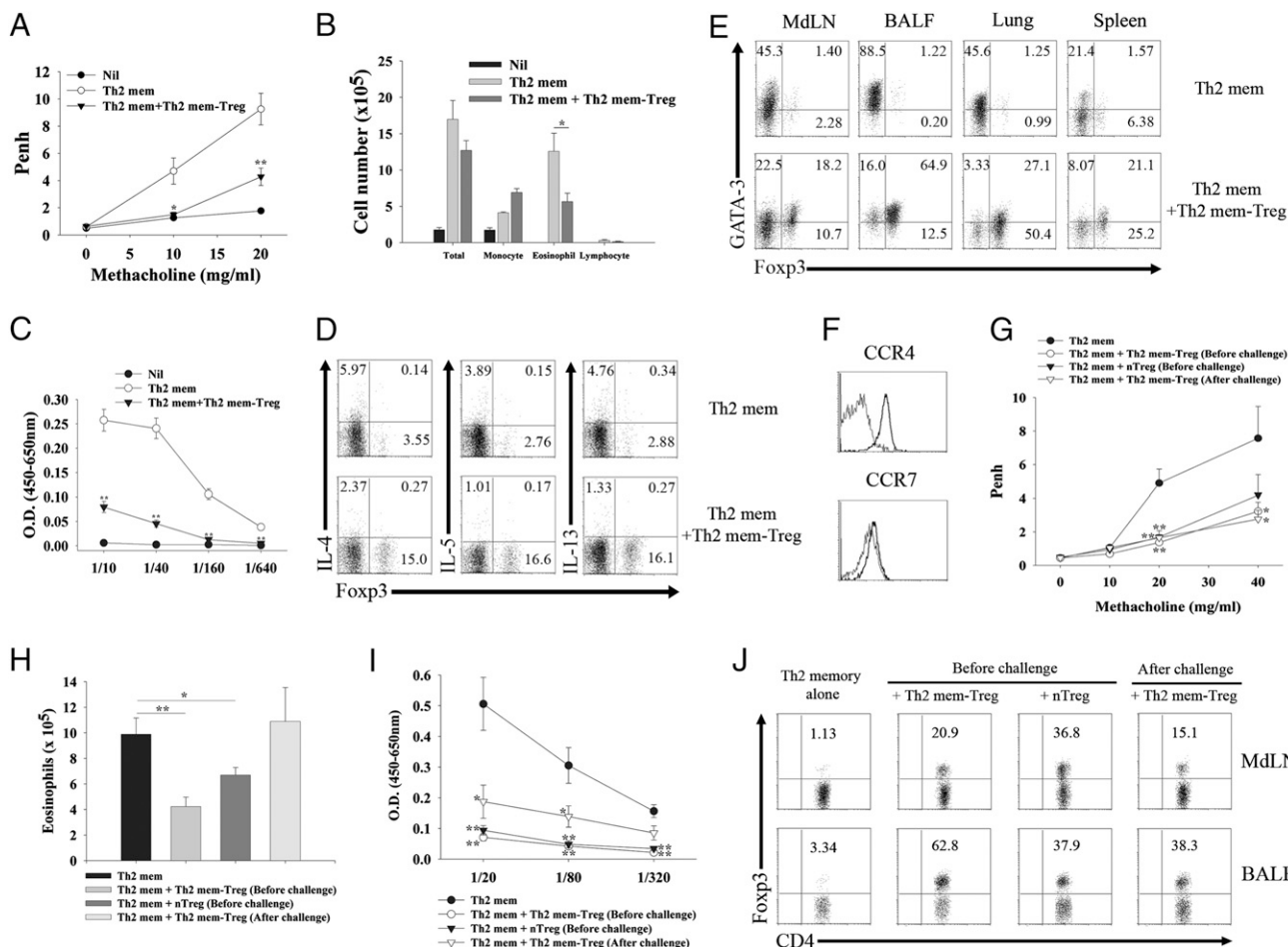


Fig. 4. Th2 memory-derived Tregs can modulate Th2 memory-induced airway hyperreactivity and eosinophilia. (A–E) Th2 memory-mediated airway inflammation was induced as described in Fig. S9A. (A) One day after the last challenge, AHR was analyzed, and the means $\pm$ SEM of the mice in each group are plotted. Values for enhanced pause (Penh) are given (* P < 0.05; ** P < 0.005). (B) Twenty-four hours after AHR analysis, BALF and serum samples were collected. The number of total BALF cells, eosinophils, monocytes, and lymphocytes were analyzed. (C) Serum levels of OVA-specific IgE were detected by ELISA. (D) MdnL cells in each group were restimulated with OVA_{323–339} peptides for 6 h. IL-4, IL-5, or IL-13 production, together with Foxp3 expression on gated CD4⁺KJ1-26⁺ cells, was analyzed by flow cytometry. (E) Foxp3 and GATA-3 expression on gated CD4⁺KJ1-26⁺ cells from MdnL, BALF, lung, and spleen were analyzed by flow cytometry. (F) CCR4 and CCR7 expression on gated CD4⁺KJ1-26⁺Foxp3⁺ cells in BALF from Th2 memory-derived Treg-transferred mice were analyzed by flow cytometry. (G–I) Airway inflammation was induced as described in Fig. S9D. AHR (G), BALF eosinophils (H), and OVA-specific IgE in sera (I) were analyzed. (J) Foxp3 expression of CD4⁺KJ1-26⁺ cells in MdnL and BALF was determined by flow cytometry. These experiments included five to six mice per group. Data are representative of at least two independent experiments.

clonotypic T cells were Foxp3⁺ in BALF and the lung, indicating efficient homing of the Th2 memory-derived Tregs to the site of Th2-mediated inflammation (Fig. 4E). Notably, a majority of the OVA-specific Foxp3⁺ cells coexpressed GATA-3, especially in BALF, with little expression of Th2-type cytokines (Fig. 4D and E), which agrees well with our in vitro observation under Th2-skewing conditions (Fig. S8). Consistent with their efficient homing to the inflamed airway, OVA-specific Foxp3⁺ cells in BALF expressed CCR4 and down-regulated CCR7 (Fig. 4F).

The suppression of Th2 memory cells by Th2 memory-derived Tregs was not due to competition for Ag-bearing APCs, considering that an equal number of naive OVA-specific Th cells did not ameliorate the diseases (Fig. S9A–C). Moreover, this suppression was Ag-specific because the OVA-specific Th2 memory-derived Tregs did not affect the AHR response mediated by the unrelated antigen KLH (Fig. S10A and B).

We then compared the suppressive potency of Th2 memory-derived Tregs and naturally occurring Tregs (nTregs). To this end, we transferred 1.0×10^6 nTregs (>70% OVA-specific, >95% Foxp3⁺) isolated from DO11.10 mice or 7.0×10^5 OVA-specific

Th2 memory-derived Tregs (>99% OVA-specific, >90% Foxp3⁺) into syngeneic mice. When we induced allergic airway inflammation in the recipient mice, we observed that Th2 memory-derived Tregs were at least as potent as nTregs in suppressing Th2 memory-mediated AHR, eosinophilia, and IgE production (Fig. 4G–I). Compared with nTregs, Th2 memory-derived Tregs homed more preferentially to the airway than to the MdnL (Fig. 4J).

We also addressed whether Th2 memory-derived Tregs could suppress a more robust Th2 response in vivo. To this aim, we first intranasally challenged Th2 memory cell-bearing mice to initiate airway inflammation and then transferred Th2 memory-derived Tregs, as depicted in Fig. S9D. Even in this experimental setting, Th2 memory-derived Tregs efficiently suppressed AHR and allergen-specific IgE production (Fig. 4G and I). However, airway eosinophilia was unaffected (Fig. 4H). Again, a significant proportion of OVA-specific Foxp3⁺ T cells were detected in the airway (Fig. 4J).

Collectively, these results demonstrate that Th2 memory-derived Tregs stably express Foxp3 upon adoptive transfer; moreover, they efficiently home to the airway to suppress the ongoing airway inflammation that is mediated by Th2 memory cells.

Discussion

Memory Th cells, especially Th2 memory cells, have been shown to gradually increase in allergic patients with the progression of diseases (31, 32). It has been unclear, however, whether memory Th cells can become Foxp3⁺ cells. Compared with naive Th cells, memory Th cells are known to be resistant to TGF- β -mediated expression of Foxp3 (33). However, after transfer into lymphopenic mice, a preferential conversion of memory Th cells to Tregs compared with that of naive Th cells has been demonstrated (34). Additionally, human Foxp3⁺ Tregs are reported to be derived from memory Th cells, especially in elderly people, and human skin-derived memory Th cells can be converted into Foxp3⁺ Tregs with a suitable manipulation (35, 36).

Our data show that neutralization of IL-4 and IFN- γ is required for TGF- β -mediated Foxp3 induction in Ag-specific memory Th cells. These observations are well supported by several recent studies showing that the TGF- β signal, in the presence of other cytokines, fails to induce Foxp3 expression even in naive Th cells (4, 21, 22). However, blockade of IL-4 and IFN- γ in combination with TGF- β was not sufficient to induce Foxp3 expression in memory Th cells, even with a great reduction of GATA-3 and T-bet, indicating the existence of additional molecular machinery that mediates resistance to TGF- β -mediated Foxp3 expression.

ATRA synergizes with TGF- β to induce the differentiation of Foxp3⁺ cells from naive Th cells by enhancing TGF- β -mediated Smad3 signaling (37), and it inhibits the production of such cytokines as IL-4, IFN- γ , and IL-21 by memory Th cells (16). Our study demonstrates that the addition of ATRA increases Foxp3 expression in memory Th cells in the presence of neutralizing antibodies against IL-4 and IFN- γ . Therefore, in addition to its role in suppressing the production of cytokines, the ATRA signal also makes memory T cells more susceptible to the TGF- β signal.

Rapamycin has been extensively studied in translational research for its capacity to induce tolerance. Tolerance induction by rapamycin is, at least in part, mediated by its role in the selective expansion of the Tregs (38). Also, rapamycin-conditioned DCs can enrich Tregs (39). In addition, the mTOR signaling pathway negatively regulates de novo generation of Tregs (23, 24). Moreover, recent studies have revealed a critical role of mTOR in the generation of memory T cells as well as in the differentiation of effector T cells (25, 40). These observations overall led us to hypothesize that active mTOR signaling in memory Th cells is a critical factor for maintaining the resistance of memory Th cells to Foxp3 induction by TGF- β signaling. Indeed, we observed that addition of rapamycin into the culture greatly increased the proportion of Foxp3⁺ cells in memory Th cells. Kinetic analysis showed that rapamycin acted during the early phase after TCR restimulation, suggesting that it was not the result of selective expansion of induced Foxp3⁺ cells. However, neutralization of IL-4 and IFN- γ was necessary for rapamycin-mediated enhancement of Foxp3 induction in memory Th cells. Therefore, effector cytokines and mTOR work synergistically to inhibit Foxp3 expression in memory Th cells.

Although Th2 memory cells produced IL-4 but not IFN- γ at an early time point after restimulation, neutralization of IFN- γ was required for optimal induction of Foxp3⁺ cells in Th2-polarized memory cells. It is likely that some of Th2 memory cells started to produce IFN- γ after 4–5 days of restimulation as recently suggested (41).

Recent studies have elegantly shown that Foxp3⁺ T cells expressing T-bet, IRF4, or STAT3 specifically suppress the Th1, Th2, or Th17 response in vivo, respectively (42–44). The Th lineage-specific inhibition by each Foxp3⁺ Treg subset is, at least in part, mediated by their efficient homing to the site of inflammation. Importantly, most of the transferred Th2 memory-derived Tregs in the present study became Foxp3⁺GATA-3⁺ cells upon the triggering of Th2-type airway inflammation. Moreover, the Th2 memory-derived Foxp3⁺ cells in the inflamed airway maintained CCR4 expression. We propose that Th2 memory-derived Tregs expressing CCR4 ef-

ficiently home to the airway and re-express GATA-3 under the Th2-rich environment, which maintains CCR4 expression and facilitates efficient suppression against pathogenic Th2 cells as IRF4-expressing Tregs (43). Interestingly, the expression level of GATA-3 in Th2 memory-derived Tregs was lower than that in Th2 memory cells, which is similar to the lower expression of T-bet in T-bet⁺ Tregs compared with that in Th1 effector cells (42). These results suggest that low expression of GATA-3 in Th2 memory-derived Tregs was responsible for their efficient homing to the inflamed airway without redifferentiation into Th2 effector cells. The exact mechanism of GATA-3 re-expression in vivo and its function in the suppression of inflammation in our model need to be elucidated in the future.

It has recently been demonstrated that Tregs down-regulate Foxp3 expression under inflammatory conditions (45, 46). Although we showed that Th2 memory-derived Tregs stably maintain Foxp3 expression under a Th2-skewing environment both in vitro and in vivo, it is also important to monitor the stability of Foxp3 expression in these cells in the long term. In addition, we support the notion that Treg-mediated adoptive immunotherapy should be used to maintain remission in inflammatory diseases (13), not only because it is more difficult to inhibit effector function of activated T cells than to inhibit activation of resting T cells, but also because the stability of Foxp3 expression in Tregs could be diminished under strong inflammatory conditions.

Materials and Methods

Mice. Six- to 8-week-old female BALB/c mice were purchased from Charles River Laboratories. DO11.10-RAG2^{-/-} mice were purchased from Taconics. Thy1.1⁺ BALB/c was a kind gift from Charles D. Surh (Scripps Institute). All of the animals were bred and maintained in the animal facility at Seoul National University under specific pathogen-free conditions, and all animal procedures were approved by the Institutional Animal Care and Use Committee at Seoul National University.

Generation and Isolation of Ag-Specific Memory Th Cells. CD4⁺ T cells were purified from pooled spleen and mesenteric lymph nodes of DO11.10-RAG2^{-/-} mice via positive selection with CD4 MACS beads and cultured with OVA_{323–339} peptides (1 μ g/mL) (Peptron) and irradiated (3000 cGy) splenocytes from BALB/c mice. For Th1 polarization, the cells were cultured in the presence of 4 ng/mL IL-12 (eBioscience) and 10 μ g/mL anti-IL-4 Ab (clone HB188, ATCC), and for Th2 polarization, the cells were cultured with 10 ng/mL IL-4 (eBioscience) and 10 μ g/mL anti-IFN- γ Ab (clone HB170, ATCC). Three days later, activated cells were purified by Percoll (GE Healthcare) gradient centrifugation. Purified effector cells were i.v. injected into syngeneic BALB/c hosts (10–20 $\times$ 10⁶ cells/mouse), and persisting Ag-specific Th memory cells were harvested 3–6 weeks posttransfer.

Induction and Analysis of Th2-Mediated Allergic Asthma. OVA-specific Th2-polarized effector cells (5 $\times$ 10⁵ cells for AHR induction, 10–20 $\times$ 10⁶ cells for Th2 memory cell isolation) were adoptively transferred into naive BALB/c mice. Four weeks later, OVA-specific Th2 memory cells were isolated and induced to express Foxp3 by TGF- β , ATRA, anti-IL-4 and anti-IFN- γ Abs, and rapamycin in the presence of anti-CD3 and anti-CD28 Abs. After 5 days of culture, Th2 memory-derived Tregs, containing more than 80% of Foxp3⁺ cells, were isolated and adoptively transferred to Th2 memory-bearing mice (7.0–7.5 $\times$ 10⁵ cells/mouse). OVA-specific CD4⁺CD25⁺ or naive CD4⁺ T cells were injected as controls. In some experiments, Th2 memory-derived Tregs were injected after two i.n. challenges. Twenty-four hours after the Treg transfer, the mice were intranasally challenged with 50 μ g OVA (grade VII, Sigma) four to five times as described in Fig. S9 A and D. AHR, BALF, and IgE levels in sera were assessed as previously described (47). In some experiments, eosinophils in BALF were determined as SSC^{hi}CD3⁻B220⁻Fc ϵ RI⁺CCR3⁺ by flow cytometry. Foxp3 and GATA-3 or CCR4 or CCR7 expression on CD4⁺KJ1-26⁺ cells in BALF, lung, MdLN, and spleen were analyzed by flow cytometry. Detailed descriptions of materials and methods are available in SI Materials and Methods.

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Supporting Information

Kim et al. 10.1073/pnas.0911756107

SI Materials and Methods

Generation and Functional Assay of Tregs. For the induction of Foxp3 in ovalbumin (OVA)-specific memory Th cells, these cells ($3.0\text{--}5.0 \times 10^4$ cells) were cultured with spleen dendritic cells (DCs; 1.0×10^4 cells) in the presence of OVA_{323–339} peptides or with plate-bound anti-CD3 and soluble anti-CD28 Abs. TGF- β , ATRA, anti-IFN- γ /anti-IL-4 Abs, and rapamycin were sequentially added to the culture as indicated in the Fig. 1 A and B.

To assess the suppressive function of induced Tregs, a fixed number of carboxyfluorescein succinimidyl ester (CFSE)-labeled OVA-specific Th2 memory responder cells (5.0×10^4 cells) were cocultured with graded numbers of OVA-specific Tregs induced from Th2 memory cells or naive Th cells for 72 h in the presence of irradiated T cell-depleted splenocytes (2.0×10^5 cells) and the OVA_{323–339} peptides (1 $\mu\text{g/mL}$) in a total volume of 0.2 mL of culture medium using 96-well round-bottom plates. The proliferative response of the responder T cells was measured by CFSE dilution within CD4⁺KJ1-26⁺ cells, and cytokine levels in the culture supernatant were analyzed by ELISA.

Intracellular Cytokine Staining and Flow Cytometry. To analyze the phenotype of transferred OVA-specific memory Th cells, pooled spleen and lymph node cells containing OVA-specific memory Th cells were stained with phycoerythrin (PE)-anti-DO11.10-TCR Ab (KJ1-26; BD Biosciences), APC-anti-mouse CD4 Ab, and FITC-conjugated Abs against CD44, CD127, CD62L, and CD25 or isotype-matched FITC-conjugated control Abs. Expression of each molecule on gated CD4⁺KJ1-26⁺ cells was analyzed. For intracellular cytokine staining, these cells were restimulated with OVA_{323–339} peptides for 6 h. Bredfeldin A (Golgi-Plug; BD Biosciences) was added 4 h before cells were harvested. In parallel, OVA-specific naive CD4⁺ T cells from DO11.10-RAG2^{−/−} mice were adoptively transferred to naive BALB/c mice 24 h before analysis of OVA-specific memory Th cells and then analyzed in the same way. For intracellular cytokine staining of in vitro differentiated cells, cells were restimulated with phorbol 12-myristate 13-acetate (50 ng/mL; Sigma) and ionomycin (500 ng/mL; Sigma) for 4 h with Bredfeldin A. For IL-9 staining, PE-conjugated anti-mouse IL-9 Ab (RM9A4; Biolegend) was used.

Quantitative Real-Time RT-PCR. Total RNA was extracted with the TRIzol reagent (Invitrogen Life Technologies), and cDNA was generated with SuperScript reverse transcriptase and oligo(dT) primers (Invitrogen Life Technologies). The light cyclers optical system (Roche) with SYBR green real-time PCR kit (Takara) was used for analysis of gene expression. Target gene values were calculated relative to GAPDH expression.

The following primer pairs were used: *Gata3* sense (AGC-ACAGAAGGCAGGGAGTG), *Gata3* antisense (GGTCCCC-ATTAGCGTTCCTC), *Irf4* sense (CAGGACTACAATCG-TGAGGA), *Irf4* antisense (AAGTAGGAGGATCTGGCTTG), *Foxp3* sense (CAGCTGCCTACAGTGCCCTAG), *Foxp3* antisense (CATTTGCCAGCAGTGGGTAG), *Il4* sense (TCG-GCATTGTTGAACGAGGTC), *Il4* antisense (CAGTGATGTG-GACTTGGACTCA), *Il5* sense (CACCAGCTATGCATTGGA-GAAATC), *Il5* antisense (TCTGTACTCATCACCAAGG-AAC), *Il13* sense (AGCATGGTATGGAGTGTGGACCTG), *Il13* antisense (CAGTTGCTTTGTGTAGCTGAGCAG), *Ccr4* sense (AACAGAGCAGTGCGCATGAT), *Ccr4* antisense (CG-TTGACGGCGTCCAGAA), *Ccr7* sense (AAAGCACAGCC-TTCCTGTGT), *Ccr7* antisense (AGTCCACCGTGGTATTCT-CG), *Cd103* sense (GCGGTTGTCAGAGCTGCCTC), *Cd103* antisense (AGTGGTCCAGGGTGGGGTTG), *GAPDH* sense (AACGACCCCTTCATTGAC), and *GAPDH* antisense (TCCAC-GACATACTCAGCAC).

OVA and KLH Sensitization and Challenge. BALB/c mice were sensitized by i.p. injection of 50 μg of OVA or KLH adsorbed to 1 mg of alum (Pierce) on days 0 and 14. On day 28, 5.0×10^5 OVA-specific Th2 memory-derived Tregs were transferred i.v. into mice. All of the mice were i.n. challenged with 50 μg of OVA or KLH on days 29, 30, 31, 34, and 35. Airway hyperreactivity (AHR) was assessed 24 h after the last challenge.

Statistical Analysis. Results are expressed as the mean $\pm$ SEM. Statistical analyses were performed using Student's *t* test. Results with values of $P < 0.05$ were considered to be statistically significant.

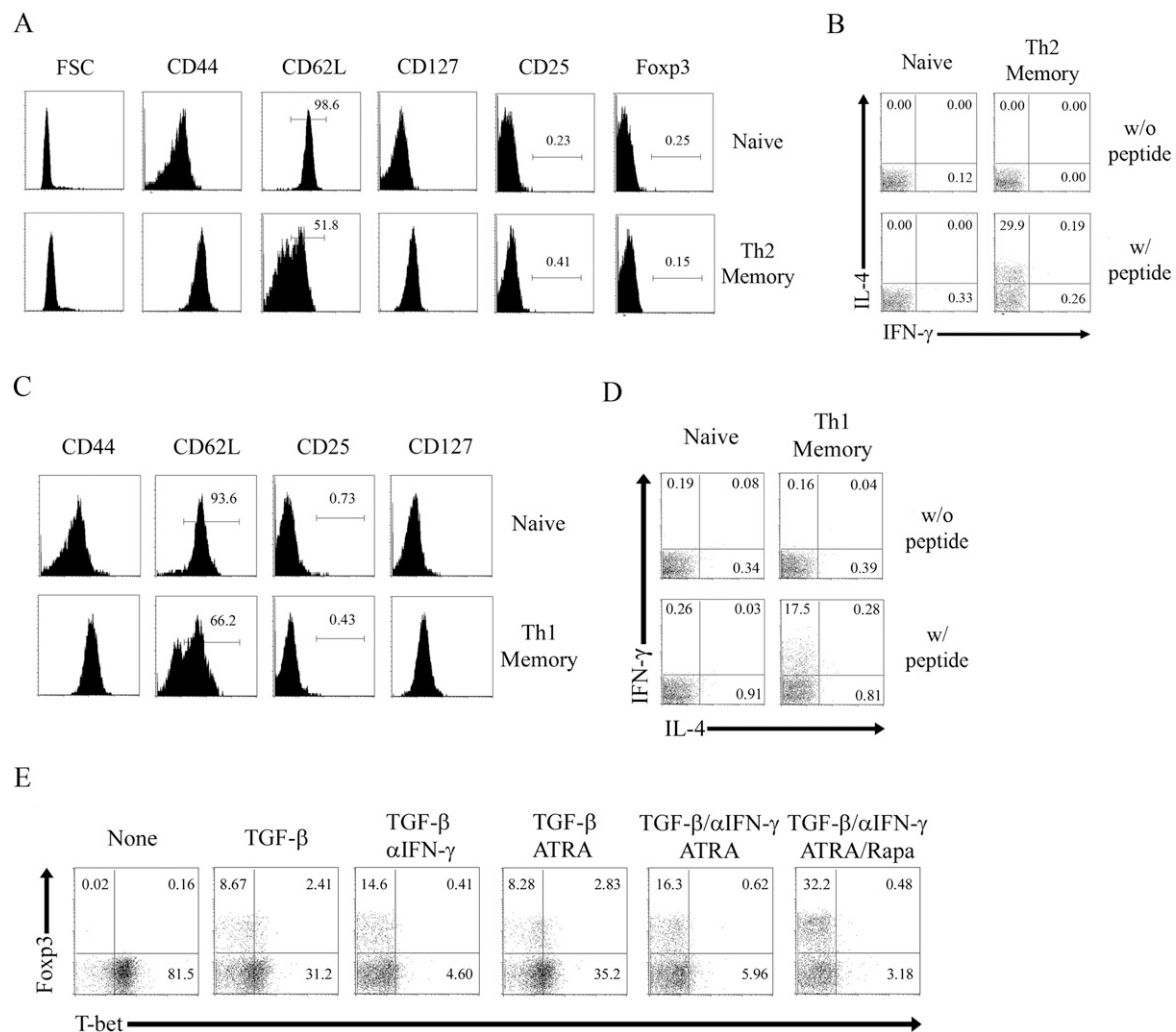


Fig. S1. Phenotypic and functional analysis of Ag-specific Th2 and Th1 memory cells. (A and B) OVA-specific Th2 effector cells were adoptively transferred into syngeneic BALB/c mice. Six weeks posttransfer, cell surface phenotype and cytokine-producing capacity of OVA-specific Th2 memory cells were analyzed. As a control, OVA-specific naive CD4⁺Foxp3[−] cells from DO11.10-RAG2^{−/−} mice were transferred into another BALB/c host 24 h before analysis of Th2 memory cells. (A) Cell surface expression of CD44, CD62L, CD127, CD25, and Foxp3 on gated CD4⁺KJ1-26⁺ naive or Th2 memory cells from pooled spleen and lymph node lymphocytes. The numbers inside the histograms indicate the percentage of gated cells among CD4⁺KJ1-26⁺ cells. (B) Pooled spleen and lymph node cells containing OVA-specific naive or Th2 memory cells were either stimulated or not stimulated with the OVA_{323–339} peptides for 6 h. IL-4 and IFN-γ production from each cell population was assessed by intracellular cytokine staining. The plots shown are gated on CD4⁺KJ1-26⁺ cells with quadrants drawn on the basis of isotype controls. (C) Three weeks after transfer of Th1 effectors, cell surface expression of CD44, CD62L, CD25, and CD127 on gated CD4⁺KJ1-26⁺ cells was analyzed by flow cytometry. As a control, OVA-specific naive CD4⁺Foxp3[−] cells were analyzed. (D) Cytokine production of OVA-specific naive Th or Th1 memory cell-bearing mice was analyzed as in B. (E) Purified OVA-specific Th1 memory cells were stimulated with spleen DCs and OVA_{323–339} peptides under the indicated conditions. T-bet and Foxp3 expression on gated CD4⁺KJ1-26⁺ cells are shown. Data are representative of at least two independent experiments.

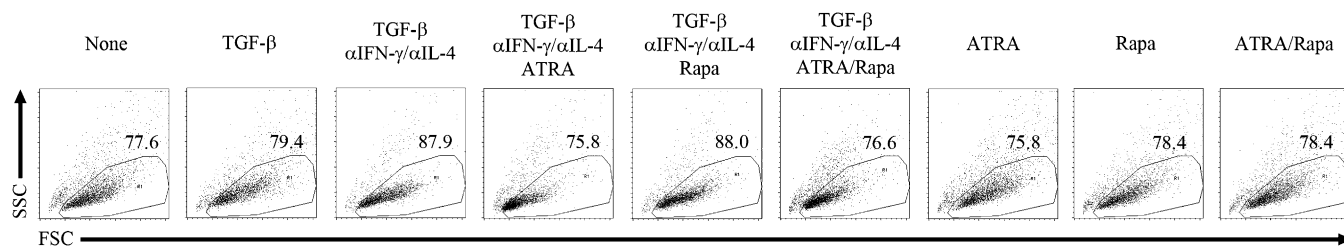


Fig. S2. ATRA and rapamycin do not induce cell death significantly. Purified OVA-specific Th2 memory cells were stimulated with plate-bound anti-CD3 and soluble anti-CD28 Abs under the indicated conditions. TGF-β (5 ng/mL), ATRA (100 nM), and Rapa (20 nM) were used at the indicated doses. Forward scattering (FSC) vs. side scattering (SSC) profile of each group was analyzed by flow cytometry. Data are representative of at least three independent experiments.

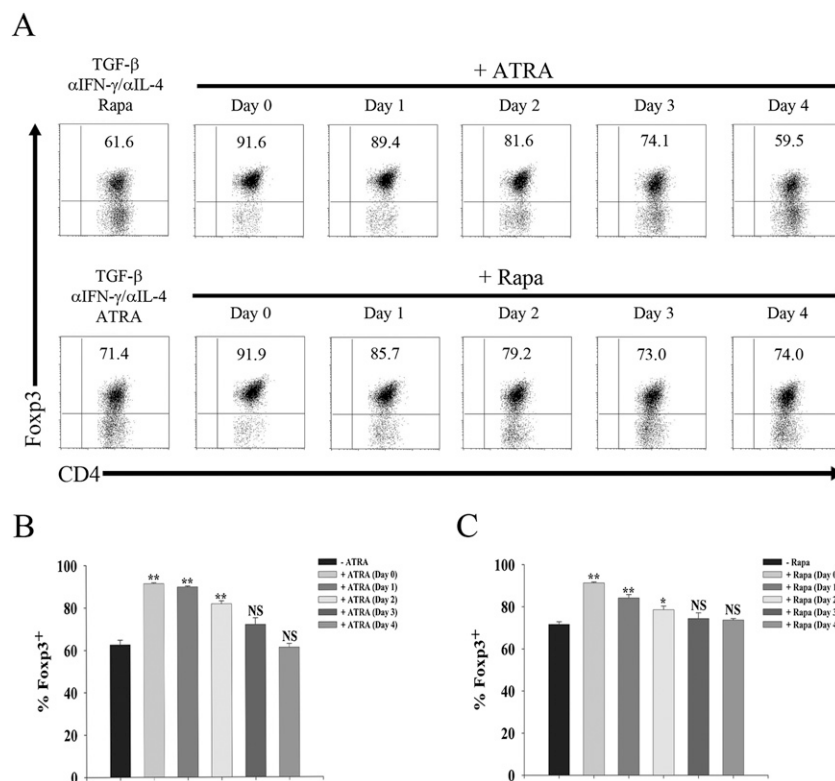


Fig. S3. Kinetic analysis of ATRA and rapamycin addition effects on enhancement of Foxp3 induction in Th2 memory cells. (A) OVA-specific Th2 memory cells were cultured with plate-bound anti-CD3 and soluble anti-CD28 Abs in the presence of TGF- β / α IFN- γ /IL-4/rapamycin or TGF- β / α IFN- γ /IL-4/ATRA. ATRA or rapamycin was added at the indicated time point after T cell receptor stimulation. Foxp3 expression on gated CD4⁺KJ1-26⁺ cells was analyzed at day 6 of culture. (B and C) Mean $\pm$ SEM of the percentage of Foxp3⁺ cells in each group in A are plotted (* P < 0.05, ** P < 0.005; NS, no significant difference). Data are representative of two independent experiments.

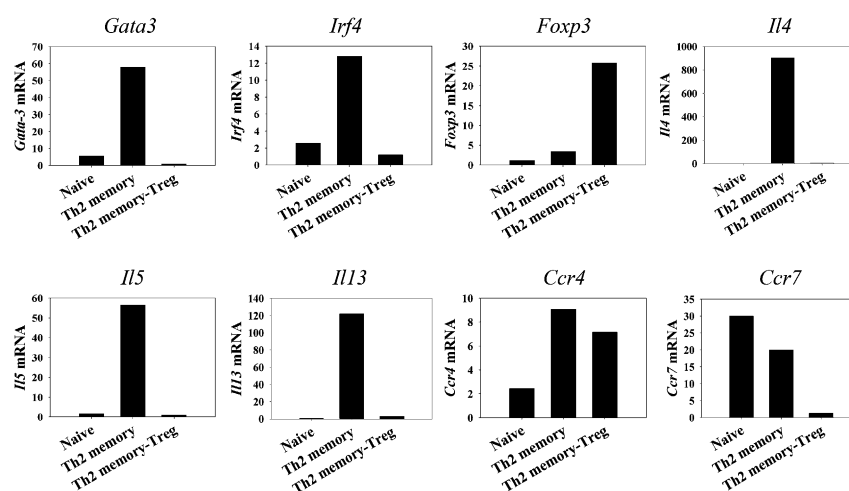


Fig. S4. Gene expression profiles of naive, Th2 memory, and Th2 memory-derived Tregs. Purified OVA-specific naive Th cells, Th2 memory cells, and Th2 memory-derived Tregs (Th2 memory-Treg) were stimulated with plate-bound anti-CD3 Ab (2 μ g/mL) for 4 h, and then total RNA was extracted. Expression of the indicated genes was analyzed by quantitative real-time PCR. Data are representative of at least two independent experiments.

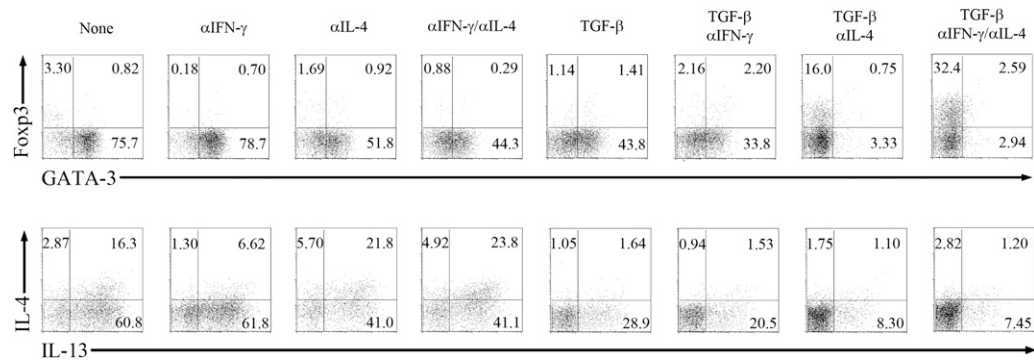


Fig. S5. IL-4 requirement for maintaining Th2 effector function of Th2 memory cells in the presence of TGF- β . OVA-specific Th2 memory cells were cultured as indicated in the presence of plate-bound anti-CD3 and soluble anti-CD28 Abs for 5 days. The resultant cells were stained for Foxp3 and GATA-3 without restimulation (Upper) or IL-4 and IL-13 after restimulation (Lower). Data are representative of two independent experiments.

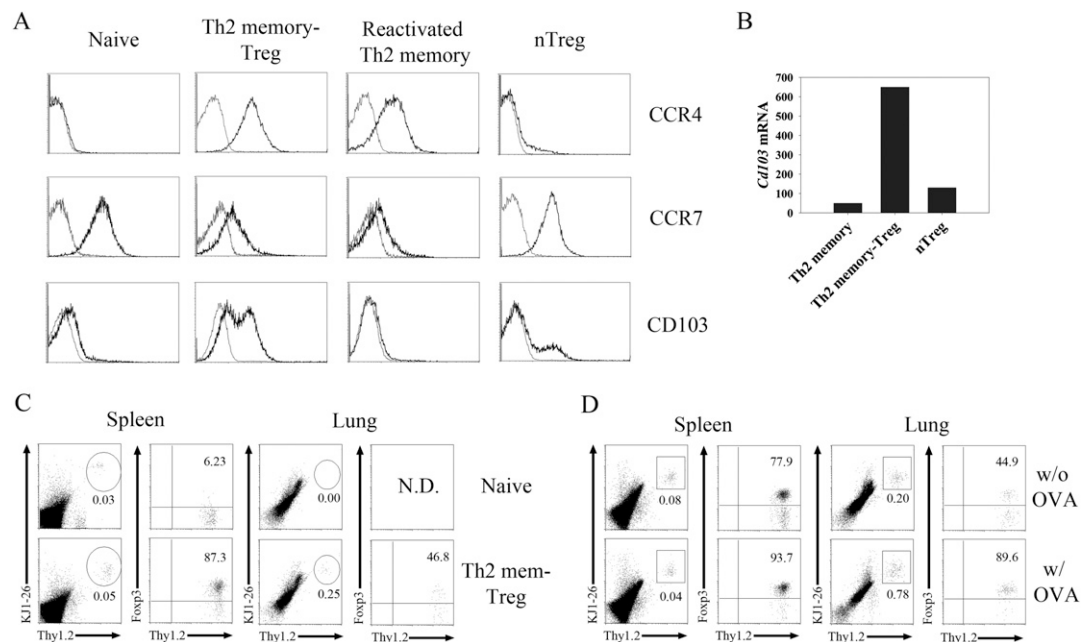
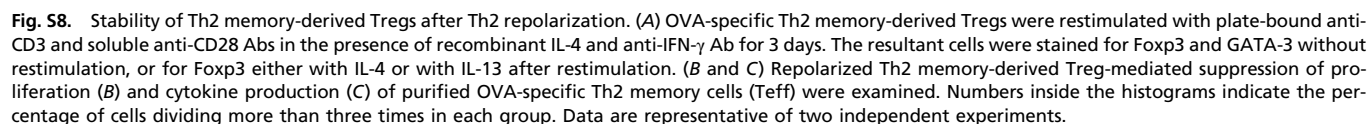


Fig. S6. Chemokine receptor expression and lung-homing capacity of Th2 memory-derived Tregs. (A) Cell surface expression of CCR4, CCR7, and CD103 of OVA-specific naive Th cells, Th2 memory-derived Tregs, reactivated Th2 memory cells, and naturally occurring Tregs (nTregs) were analyzed by flow cytometry (dark, thick line). Isotype controls are expressed as a thin, light line. (B) Purified OVA-specific Th2 memory cells, Th2 memory-derived Tregs, and nTregs were stimulated with plate-bound anti-CD3 Ab (2 μ g/mL) for 4 h, and then total RNA was extracted. Expression of the *Cd103* gene of each cell was analyzed by quantitative real-time PCR. (C) OVA-specific Thy1.2⁺ naive CD4⁺Foxp3⁻ cells and Th2 memory-derived Tregs were adoptively transferred into Thy1.1⁺ BALB/c mice (1.0 $\times$ 10⁶/mouse). The proportion of CD4⁺KJ1-26⁺ cells in spleen and lung cells and their respective Foxp3 expression were analyzed 48 h later. N.D., not detected. (D) OVA-specific Thy1.2⁺ Th2 memory-derived Tregs were adoptively transferred into Thy1.1⁺ BALB/c mice on day 0 (1.0 $\times$ 10⁶/mouse). Half of the mice were intranasally administered with OVA on day 1, 2, 3, 6, and 7. Twenty-four hours after the last i.n. challenge, spleen and lung cells were analyzed as in C. Data are representative of at least two independent experiments.



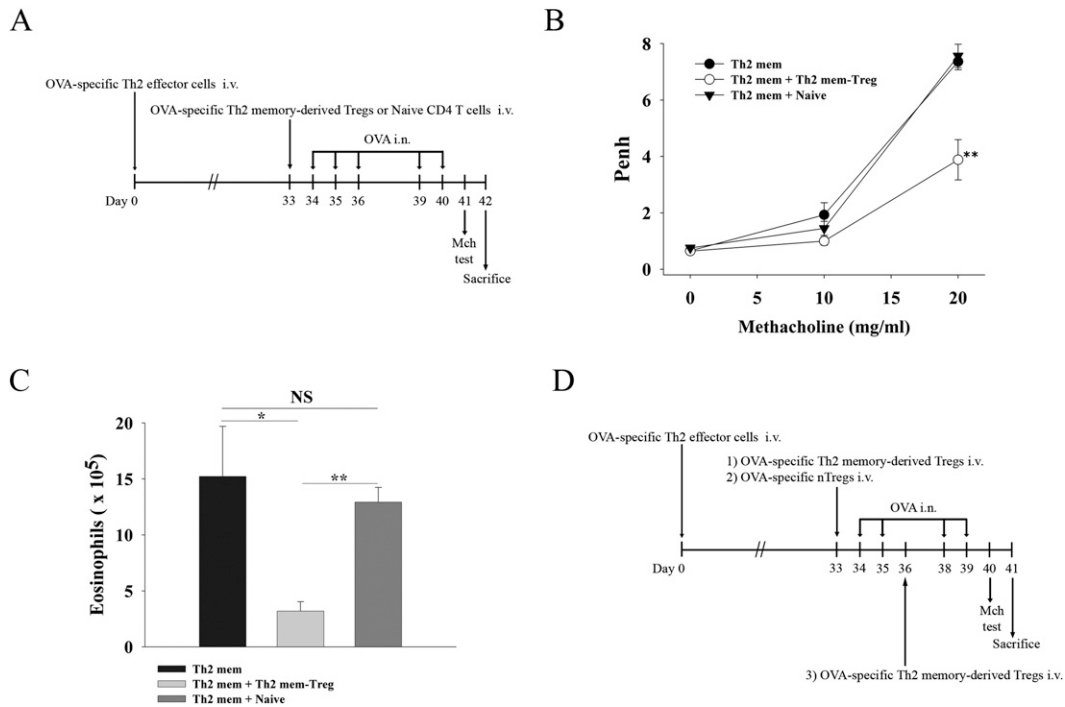


Fig. S9. Experimental scheme for assessment of suppressive activity of Th2 memory-derived Tregs against Th2 memory-induced AHR and eosinophilia. (A) OVA-specific Th2-polarized cells (5.0×10^5) were adoptively transferred into naive BALB/c mice. On day 33, OVA-specific Th2 memory-derived Tregs or OVA-specific naive Th cells (7.5×10^5 each) were transferred into Th2 memory-bearing mice. (B) Twenty-four hours after the transfer, animals were intranasally challenged with OVA five times and analyzed for AHR 24 h after the last challenge. (C) Mice were killed for analysis of bronchoalveolar lavage fluid (BALF) eosinophilia 24 h after AHR analysis ($*P < 0.05$, $**P < 0.005$; NS, no significant difference). (D) Th2 memory-bearing mice were injected with OVA-specific Th2 memory-derived Tregs (7.0×10^5) on day 33 or day 36. OVA-specific nTregs (1.0×10^6) were injected on day 33 as a control. The mice were intranasally challenged with OVA four times on days 34, 35, 38, and 39. The mice were analyzed for AHR 24 h after the last challenge and were killed for analysis of BALF eosinophilia 24 h later. Data are representative of two independent experiments.

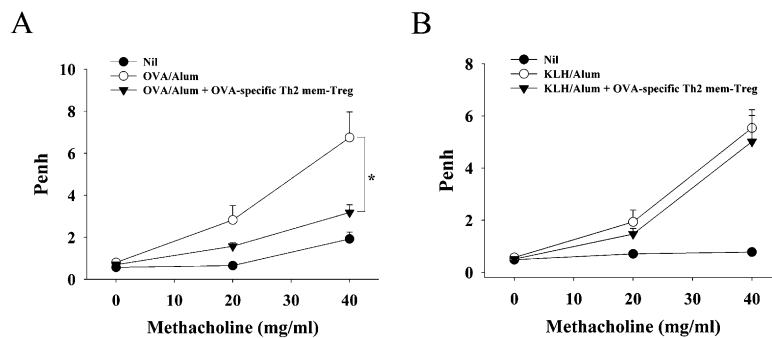


Fig. S10. Th2 memory-derived Treg-mediated suppression is Ag-specific. (A and B) OVA-specific Th2 memory-derived Treg-mediated suppression of AHR in OVA-immunized (A) or KLH-immunized (B) mice was evaluated as described in *SI Materials and Methods*. Values for enhanced pause (Penh) are given ($*P < 0.05$). Data are representative of two independent experiments.