Aurintricarboxylic acid promotes the conversion of naive CD4$^{+}$CD25$^{-}$ T cells into Foxp3-expressing regulatory T cells

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Received 02 November 2010, accepted 16 June 2011

Abstract

Naive peripheral CD4$^{+}$CD25$^{-}$ T cells can be converted into Foxp3-expressing regulatory T cells under appropriate stimulation conditions. Considering that continuous exposure to antigens is one of the prerequisites for the differentiation and maintenance of Treg cells, we investigated whether preventing activation-induced cell death while providing continuous TCR stimulation could promote the expression of Foxp3 in murine naive CD4$^{+}$ T cells. Among the several anti-apoptotic agents tested, aurintricarboxylic acid (ATA) was found to induce the in vitro conversion of naive CD4$^{+}$ T cells into Foxp3$^{+}$ Treg cells with suppressive activity. Neutralizing studies with an antibody against transforming growth factor (TGF)-β revealed that ATA requires the presence of TGF-β to induce Foxp3 expression in naive CD4$^{+}$CD25$^{-}$ T cells. Although ATA itself did not activate the Smad signaling pathway, it down-regulated the extracellular signal-regulated kinase and mammalian target of rapamycin signaling cascade in activated T cells. Lastly, combined exposure to ATA and TGF-β had a synergistic effect on the rate of induction and maintenance of Foxp3 expression. These results indicate that ATA could be exploited to efficiently prepare inducible regulatory T cells in vitro and may aid in more precisely identifying the specific signaling pathways that drive Foxp3 expression in T cells.

Keywords: apoptosis, mTOR, inducible regulatory T cells, TGF-β

Introduction

Regulatory T lymphocytes (Tregs) play a crucial role in immune homeostasis and the prevention of autoimmune diseases (1). Their activity is also associated with diverse physiological and pathological conditions, such as maternal accommodation of the fetus during pregnancy, immune evasion of cancer, chronic microbial infection and allergy (2). Knowledge of Treg differentiation processes and an ability to manipulate functional Tregs is thus important for designing preventive and therapeutic strategies against various diseases.

There are two major classes of Tregs in the body: naturally occurring Tregs (nTregs) and adaptive/inducible Tregs (iTregs) (3). Although CD25 expression on the cell surface membrane was initially used as a marker for Tregs, nuclear expression of the transcription factor Foxp3 is regarded as a more precise Treg identifier (4–7). Studies of the rare human autoimmune disorder IPEX (immune-dysregulation polyendocrinopathy enteropathy X-linked) and scurfy mice (which exhibit fatal autoimmune lesions) led to the discovery that Foxp3 is required for the development and regulatory function of Tregs (8, 9). Since the identification of Foxp3 as a lineage-specifying factor for Tregs, numerous studies have sought to determine the developmental process leading to Foxp3-expressing Tregs. A small fraction of developing single CD4$^{+}$ T lymphocytes acquires Foxp3 expression in the thymic environment (supplemented with IL-2) upon engagement of both TCR with self-peptide/MHC complexes (signal 1) and CD28 with B7 molecules (signal 2) on thymic antigen-presenting cells (10, 11). Although most Foxp3$^{+}$ Tregs found in peripheral lymphoid tissues are thought to
come from these thymically derived nTregs (12–14), it has been shown that Tregs can also be generated from naive T lymphocytes in peripheral lymphoid tissues in vivo by manipulations, such as continuous stimulation with suboptimal levels of an agonistic peptide, mucosal delivery of antigen or delivery of antigen cross-linked to DEC-205 (15–17). In addition, naive CD4+CD25− T cells can be converted to Foxp3+ iTregs, if they are activated in vitro by TCR stimulation in the presence of transforming growth factor (TGF)-β (18). Recently, additional molecules with the capacity to induce Foxp3 expression in naive CD4+ T cells have been identified (19–24), whereas other laboratories have provided insights into the molecular basis of Foxp3 gene expression in T cells. These latter studies have shown that recruitment of NFAT, cRel, Stat5, Runx-1 and CREB to the promoter and regulatory elements of the Foxp3 gene are required for Foxp3 expression (25, 26). Epigenetic modifications at the regulatory elements of the Foxp3 locus, such as CpG demethylation, are also important for efficient expression of Foxp3 (27). However, a full picture of the molecular mechanisms involved in regulating Foxp3 expression requires not only the identification of additional regulatory elements and transcription factors but also the identification of signaling pathways that lead to transcriptional complex formation.

To extend our knowledge on Tregs, we sought to identify additional conditions that lead to the development of iTregs. Given that most nTregs are continuously stimulated through their self-reactive TCRs without undergoing activation-induced cell death (AICD), we investigated whether continuous stimulation in the presence of anti-apoptotic agents might induce naive T cells to express Foxp3. Interestingly, we found that aurintricarboxylic acid (ATA), which has been reported to be a non-specific enzyme inhibitor (28) and previously reported to be a non-specific enzyme inhibitor (28), is capable of inducing Foxp3 expression in naive CD4+ T lymphocytes. We report here the properties of ATA-induced Foxp3 expression and characterize the signaling pathways that are potentially related to this activity.

Methods

Mice

Adult male C57BL/6 mice were purchased from Japan SLC Inc. OT-II mice were from The Jackson Laboratory. Foxp3.GFP knock-in mice were obtained from Dr Rudensky (Memorial Sloan-Kettering Cancer Center, New York, NY, USA). All mice were housed under specific pathogen-free conditions and underwent the experimental procedure at 6–12 weeks of age in accordance with protocols for animal experimentation approved by the Animal Use and Care Committee of Ulsan University.

Antibodies and reagents

Anti-CD4-FITC (RM-4-5), anti-CD25-PE (PC61), anti-CD62L-PE-Cy5 (MEL-14), anti-Foxp3-allophycocyanin (APC) (FJK-16s), anti-CD3 (145-2C11) and anti-CD28 (37.51) monoclonal antibodies, as well as isotype-matched control antibodies were purchased from BD Pharmingen (San Diego, CA, USA) or eBioscience (San Diego, CA, USA). The neutralizing anti-TGF-β mAb (1D11) was from R&D Systems (Minneapolis, MN, USA). All anti-apoptotic agents (parthenolide, ATA, pifithrin-α and flupirtine maleate) and SB431542 were purchased from Sigma–Aldrich (St. Louis, MO, USA). Recombinant human IL-2 and mouse TGF-β were from Chiron (Amsterdam, The Netherlands) and PeproTech Inc. (Rocky Hill, NJ, USA), respectively.

Isolation of cells

Single-cell suspensions were prepared from the spleen and peripheral lymph nodes of mice. After lysis of red blood cells, CD4+ T cells were purified by negative selection using a magnetic-activated cell sorting (MACS) CD4+ T-cell isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany), according to the manufacturer’s instructions. CD4+ T cells were stained with FITC-anti-CD4, PE-anti-CD25 and PE-Cy5-anti-CD62L mAbs, after which CD4+CD25−CD62L+ T cells or CD4+CD25+CD62L− T cells were isolated using a FACSVantage cell sorter (BD Immunocytometry Systems, San Jose, CA, USA). For Foxp3.GFP knock-in T cells, CD4+CD62L−GFP− T cells were isolated from CD4+ T cells by cell sorting after staining with PE-anti-CD4 and PE-Cy5-anti-CD62L mAbs. In some experiments, CD4+CD25− T cells were prepared by additional staining of spleen/lymph node cells with a biotin-anti-CD25 mAb during MACS CD4+ T-cell isolation. Ovalbumin-presenting cells were prepared from splenocytes of C57BL/6 mice by depletion of T cells using anti-CD90 mAb-conjugated magnetic beads (Miltenyi Biotec). The purity of the isolated cells, as assessed by flow cytometry, was routinely >90% (data not shown).

Stimulation cultures of T cells

Purified naive CD4+ T cells were stimulated with plate-bound anti-CD3 (5 μg ml−1) and soluble anti-CD28 (1 μg ml−1) in the presence of 50 U/ml recombinant human IL-2 for 4 days, and different concentrations of anti-apoptotic agents or TGF-β were added to the culture at the beginning of stimulation, unless otherwise indicated. In some stimulation cultures, 20 μg ml−1 of neutralizing anti-TGF-β or isotype control antibodies or different amounts of the Smad signaling inhibitor SB471542 were added to the culture, as indicated in Results. For antigen-specific stimulation, naive OT-II CD4+ T cells were stimulated with ovalbumin-pulsed T-cell-depleted splenocytes from C57BL/6 mice. RPMI1640 medium supplemented with 10% fetal bovine serum (FBS) (Gibco, Invitrogen, Grand Island, NY, USA), 4 mM l-glutamine, 100 U/ml penicillin, 100 μg ml−1 streptomycin, 1 mM sodium pyruvate, 10 μM non-essential amino acids (all from Invitrogen) and 50 μM 2-mercaptoethanol (Sigma–Aldrich) was used in all cultures, except for specifically indicated and signaling studies, in which serum-free medium (AIM-V; Gibco) was used.

Flow cytometry

Single cells were suspended in staining buffer (PBS containing 2% FBS). After incubating with Fc BlockTM Ab (BD Pharmingen), cells were stained for 30 min on ice with an antibody cocktail specific to cell surface molecules. Intracellular Foxp3 was detected with an APC-conjugated anti-mouse Foxp3 staining set (eBioscience), according to the manufacturer’s instructions.
The stained cells were analyzed using a FACSCalibur flow cytometer and CellQuest software (Becton Dickinson).

Real-time PCR

Total RNA was extracted from cells with an RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany), and cDNA was synthesized using a High Capacity RNA-to-cDNA Kit (Applied Biosystems, Foster City, CA, USA). Real-time PCR was performed in triplicate using TaqMan Gene master mix, as described by the manufacturer (Applied Biosystems), and analyzed on an ABIprism 7000 sequence detector system (Applied Biosystems). The levels of Foxp3 mRNA were measured using TaqMan Gene Expression Assays (Mm00475162_m1) and normalized to those of Hprt1 mRNA (Mm00446968_m1).

In vitro suppression assay

CD4+CD25− T cells (5 × 10⁴ cells per well) were stimulated with soluble anti-CD3 (0.25 μg ml⁻¹) and irradiated (25 Gy) syngeneic T-cell-depleted splenocytes (5 × 10⁴ cells per well) in a 96-well round-bottomed plate. Different numbers of FACs-sorted Foxp3.GFP+ T cells induced by TGF-β and/or ATA were added to the above cultures. After a 72-h incubation, which included with the last 8-h pulse with [³H]-thymidine (1 μCi per well; PerkinElmer, Boston, MA, USA), the cells were harvested on filter plates, and [³H]-thymidine incorporation was measured with a TopCount NXT beta counter (PerkinElmer, Downers Grove, IL, USA).

Immunoblotting

For the analysis of Smad2 phosphorylation, CD4+CD25− T cells were first allowed to equilibrate in serum-free medium for 4 h and then were incubated with TGF-β1 (0.5 or 5 ng ml⁻¹) or ATA (70 μM) for 10 min or 2 h at 37°C. For the analysis of p70S6K, p38-MAPK and extracellular signal-regulated kinase (ERK) phosphorylation, CD4+CD25− T cells were stimulated with anti-CD3 (5 μg ml⁻¹) and anti-CD28 (1 μg ml⁻¹) antibodies in the presence of TGF-β1 (2 ng ml⁻¹) or ATA (75 μM) alone or in combination (0.5 or 5 ng ml⁻¹ TGF-β1 + 75 μM ATA). After incubating for the indicated times in serum-free media, cells were lysed by incubating in lysis buffer [20 mM Tris–HCl, 138 mM NaCl, 50% glycerol, 1% Triton X-100, 2 mM EDTA, 1× Protease Inhibitor Cocktail (Sigma), 1 mM Na₃VO₄, 10 mM NaF, 1× Phosphatase Inhibitor Cocktail 1 (Sigma)] and sonicated for 15 s. Protein in cell lysates was quantified using a Bradford Assay (Sigma). Proteins (10 μg per lane) were separated by SDS-PAGE on 8 or 10% polyacrylamide gels and then transferred to polyvinyldene difluoride membranes. After blocking in a 5% non-fat dry milk solution, membranes were probed with the primary antibodies, followed by incubation with HRP-conjugated goat anti-rabbit IgG secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Immunoreactive proteins were detected using an ECL Kit (Amersham, Buckinghamshire, UK).

Primary antibodies against the following proteins were used: phospho-Smad2 (Ser465/467), Smad2/3, phospho-p44/42 MAPK (Thr202/Tyr204), p44/42 MAPK, phospho-p38 MAPK (Thr180/Tyr182), p38 MAPK, phospho-p70 S6 kinase (Thr421/Ser424), p70 S6 kinase and β-actin. All primary antibodies were from Cell Signaling Technology (Beverly, MA, USA).

Statistics

Student’s t-test was used to calculate statistical significance for difference in a particular measurement between two groups. A P-value <0.05 was considered statistically significant.

Results

ATA induces Foxp3 expression in CD4+CD25−CD62L+ T cells

In order to determine whether blockade of apoptosis during continuous stimulation could lead to Foxp3 expression in naive CD4+ T cells, we added various anti-apoptotic agents with different mechanisms of action to cultures of CD4+CD25−CD62L+ T cells stimulated with anti-CD3 and anti-CD28 antibodies. An examination of Foxp3 expression after 4 days of culture showed that ATA, alone among the four different anti-apoptotic agents tested, induced Foxp3 expression under these conditions (Fig. 1). The induction of Foxp3 expression by ATA was confirmed at both the mRNA and protein levels (Fig. 2A). Furthermore, we observed that CD4+Foxp3.GFP+ T cells, isolated and purified from Foxp3.GFP knock-in mice, were converted into CD4+Foxp3.GFP+ T cells in the presence of ATA. These results indicate that ATA induces the conversion of Foxp3+ T cells into Foxp3− T cells rather than promoting the preferential expansion of contaminating Foxp3+ T cells originally present in the CD4+CD25−CD62L+ T-cell population (Fig. 2B). Aside from Foxp3 expression, ATA was unable to induce the secretion of IFN-γ, IL-4, IL-17 and IL-10, which are prototypical cytokines of Th1, Th2, Th17 and Tr1 cells, respectively, in naive T cells (Supplementary Figure 1 is available at International Immunology Online).

On further study, we found that ATA induced Foxp3 expression in a dose-dependent manner over the relatively narrow concentration range of 40–80 μM (Fig. 3A) and should be present during the initial period of TCR stimulation for introduction of Foxp3 expression in naive CD4+ T cells. CD4+CD25−CD62L+ T cells from normal B6 mice were stimulated with anti-CD3 and anti-CD28 antibodies in the presence of various anti-apoptotic agents. After 4 days of culture, cells were harvested, counted and stained with an anti-Foxp3 antibody. Outer grey and inner black bars indicate the total number of live cells and Foxp3+ cells, respectively. PA, parthenolide; ATA, aurintricarboxylic acid; PI, pifithrin-α; FM, flupirtine maleate.

![Fig. 1. ATA is capable of inducing Foxp3 expression in naive CD4+ T cells. CD4+CD25−CD62L+ T cells from normal B6 mice were stimulated with anti-CD3 and anti-CD28 antibodies in the presence of various anti-apoptotic agents. After 4 days of culture, cells were harvested, counted and stained with an anti-Foxp3 antibody.](https://academic.oup.com/intimm/article-abstract/23/9/583/710356/fig1)
maximal induction of Foxp3 expression (Fig. 3B). ATA efficiently converted CD62L<sup>+</sup>CD4<sup>+</sup>CD25<sup>−</sup>/C0 T cells to Foxp3<sup>+</sup>Tregs; however, like TGF-β, it was largely ineffective in converting a CD62L<sup>−</sup>/C0 sub-population of CD4<sup>+</sup>CD25<sup>−</sup>/C0 T cells into Foxp3<sup>+</sup>Tregs (Fig. 3C). In addition to polyclonal stimulation by anti-CD3 and anti-CD28 antibodies, antigen-specific stimulation in the presence of ATA induced Foxp3 expression in naive CD4<sup>+</sup>T cells; specifically, OT-II T cells were induced to express Foxp3 upon stimulation with either anti-CD3 and anti-CD28 antibodies or ovalbumin-pulsed T-depleted splenocytes, if supplemented with ATA (Fig. 3D).

ATA-induced CD4<sup>+</sup>Foxp3<sup>+</sup> T cells acquire immunosuppressive function

To test the suppressive capacity of ATA-induced CD4<sup>+</sup>Foxp3<sup>+</sup> T cells, we stimulated CD4<sup>+</sup>Foxp3.GFP<sup>+</sup> T cells with anti-CD3 and anti-CD28 antibodies in the presence of ATA or TGF-β and sorted Foxp3.GFP<sup>+</sup> T cells after 4 days of culture. Sorted iTregs were then added to stimulation cultures containing responder T cells. ATA-induced iTregs suppressed the proliferation of responder T cells at high Treg:Tresp ratios, albeit less efficiently than nTregs or TGF-β-induced iTregs, as measured by either [<sup>3</sup>H]thymidine incorporation (Fig. 4) or CFSE dilution assay (Supplementary Figure 2 is available at International Immunology Online). These data demonstrate that ATA has the capacity to promote the development of functional Foxp3<sup>+</sup>iTregs.

TGF-β is required for ATA-induced conversion of CD4<sup>+</sup>CD25<sup>−</sup>T cells into Foxp3<sup>+</sup>iTregs

Because TGF-β is well known to induce Foxp3 expression in naive T cells (18), we investigated whether the induction of Foxp3 expression by ATA also requires the involvement of TGF-β. First, we found that addition of a neutralizing anti-TGF-β antibody to stimulation cultures of naive CD4<sup>+</sup>T cells in the presence of ATA completely blocked iTreg conversion (Fig. 5A). Next, to determine whether ATA itself binds to the TGF-β receptor and triggers the TGF-β signaling cascade, we exposed cells to ATA and examined the extent of phosphorylation of Smad2, a downstream target of the TGF-β pathway. In clear contrast to TGF-β, ATA failed to induce the phosphorylation of Smad2 in naive CD4<sup>+</sup>T cells, either after a brief (10 min) or a relative long (2 h) incubation (Fig. 5B), in serum-free media. Although ATA did not directly induce Smad2 phosphorylation, the induction of Foxp3 expression by ATA in serum-containing cultures required the Smad signaling pathway, as evidenced by the fact that small amounts of SB431542, a Smad signaling pathway inhibitor, added to stimulated cultures of naive T cells interfered with ATA-induced generation of Foxp3<sup>+</sup>iTregs (Fig. 5C). Next, we
sought to identify the source of TGF-β involved in the induction of Foxp3 expression by ATA: secreted from T cells or present in the supplemented serum. In serum-free media, ATA, even at high doses, was able to induce neither TGF-β secretion (data not shown) nor Foxp3 expression in naive T cells. However, supplementation with a small amount of TGF-β (0.2 ng/ml) supported a concentration-dependent induction of Foxp3 expression by ATA (Fig. 5D). Collectively, these data support the conclusion that ATA has the capacity to induce Foxp3 expression in naive CD4+ T cells only in the presence of TGF-β.

ATA leads to the early termination of ERK and mammalian target of rapamycin signaling cascade during the induction of iTregs

It has been previously reported that ATA can inhibit a diverse range of intracellular kinases as well as endonucleases (30–34). To gain insight into the molecular mechanisms of Foxp3 induction by ATA, we evaluated the effects of ATA on the MAPK and Akt-mammalian target of rapamycin (mTOR) signaling pathways, which have recently been associated with Foxp3 expression (35–38). CD4+CD25− T cells were stimulated with anti-CD3 and anti-CD28 antibodies in the presence of 70 μM ATA. The percentage of Foxp3+ cells was determined after culturing for 4 days. Data represent five (A), two (B), three (C) and two (D) independent experiments. The Student’s t-test for unpaired data was used to compare values between two groups in C and with ‘None’ in D (**P < 0.05; ***P < 0.005).

Stability of ATA-induced Foxp3 expression

We next examined whether Foxp3 could be stably expressed in iTreg cells generated by combined exposure...
Fig. 4. Acquisition of suppressive function by ATA-induced Foxp3^+ T cells. Naive CD4^+CD62L^+Foxp3.GFP^- T cells were stimulated with anti-CD3 and anti-CD28 antibodies in the presence of TGF-β (2 ng ml^{-1})/ATA (70 µM) or TGF-β+ATA. After 4 days of culture, Foxp3.GFP^+ cells were sorted (left panel) and added at different cell ratios to co-cultures of CD4^+CD25^- responder T cells stimulated with anti-CD3 antibodies and T cell-depleted splenocytes. The proliferation of responder T cells was determined after co-culturing for 3 days (right panel). Representative data from three independent experiments are shown. *P < 0.05; **P < 0.005; ***P < 0.0005 compared with Tresp alone.

Fig. 5. ATA-induced Foxp3 expression requires the concomitant presence of TGF-β. (A) CD4^+CD25^-CD62L^+ T cells were stimulated with anti-CD3 and anti-CD28 antibodies in the presence of 60 µM ATA or 2 ng ml^{-1} TGF-β. In some cultures, 20 µg ml^{-1} neutralizing anti-TGF-β or control Ig was added. After 4 days of culture, cells were stained with an anti-Foxp3 antibody. Numbers in the histogram indicate the percentage of Foxp3^+ cells, and the shaded histogram indicates the staining profile with an isotype control antibody. (B) After resting for 4 h in serum-free media, CD4^+CD25^-CD62L^+ T cells were treated with TGF-β (0.5 or 5 ng ml^{-1}) or ATA (70 µM) for 10 min or 2 h at 37°C and then phosphorylated Smad2 was measured by western blotting. (C) CD4^+CD25^-CD62L^+ T cells were stimulated as in (A) and incubated with or without 0.1, 1 or 10 µM SB431542. Cells were stained with an anti-Foxp3 antibody after 4 days of culture. (D) CD4^+CD25^-CD62L^+ T cells were stimulated with anti-CD3 and anti-CD28 antibodies in the presence (closed squares) or absence (open squares) of 0.2 ng ml^{-1} TGF-β. Different amounts of ATA were added to this stimulation cultures, as indicated. After 4 days of culture, cells were stained with an anti-Foxp3 antibody. The cultures were incubated in FBS-containing media (A and C) or serum-free media (B and D). Data A and B are representative of three independent experiments, and Data C and D represent mean ± SEM of three experiments. *P < 0.05; **P < 0.005; ***P < 0.0005 compared with cells in the absence of SB431542 (C) or ATA (D).
to ATA and TGF-β. Naive CD4^+GFP^− T cells from Foxp3.GFP knock-in mice were stimulated with anti-CD3 and anti-CD28 antibodies in the presence of TGF-β alone or TGF-β and ATA. After 4 days of culture, Foxp3.GFP-expressing iTreg cells were sorted and used in secondary stimulation cultures to evaluate the maintenance of Foxp3 expression. When iTreg cells were re-stimulated with anti-CD3 and anti-CD28 antibodies without further addition of TGF-β or ATA, >70% of cells lost their expression of Foxp3, regardless of whether they had initially been generated by incubating with TGF-β alone or TGF-β together with ATA. However, upon re-stimulation with the same supplementation regimen (TGF-β or TGF-β/ATA) as used in the first stimulation cultures, Foxp3 expression was better maintained in iTreg cells originally induced with TGF-β or ATA, >70% of cells lost their expression of Foxp3, regardless of whether they had initially been generated by incubating with TGF-β alone or TGF-β together with ATA. However, upon re-stimulation with the same supplementation regimen (TGF-β or TGF-β/ATA) as used in the first stimulation cultures, Foxp3 expression was better maintained in iTreg cells originally induced with TGF-β or ATA (92.2% Foxp3^+^ cells) than in those induced with TGF-β alone (72.8% Foxp3^+^ cells; Fig. 7). These data indicate that ATA plays a role in both the induction and maintenance of Foxp3 expression in CD4^+^ T cells.

**Discussion**

Our data demonstrate for the first time that ATA promotes the development and maintenance of iTreg cells. We found that ATA, if present at an early stage of T-cell stimulation and supplemented with a minimal amount of TGF-β, efficiently induced the expression of Foxp3 in naive CD4^+^ cells but not in effector/memory T cells. ATA-induced Foxp3^+^ T cells acquired immunosuppressive activity, and their Foxp3 expression was well maintained upon repeated stimulation in the presence of ATA and TGF-β. Furthermore, we showed that ATA induced early termination of the Akt-mTOR signaling cascade following activation by TCR engagement in naive CD4^+^ T cells. This is notable because there is strong evidence that disruption of Akt-mTOR signaling is critical for the conversion of these cells into Foxp3^+^ iTreg cells (37, 38). Thus, our study provides an additional tool for generating iTreg cells and might further our understanding of the molecular mechanisms of iTreg cell development.

Our initial hypothesis was that Foxp3^+^ iTreg cells could be generated from naive CD4^+^ T cells if the latter cells received continuous stimulation but escaped AICD. Although Fas-mediated cell death has been shown to be a major mechanism of AICD in T cells (39), inhibition of this pathway by parthenolide failed to induce Foxp3 expression in activated T cells. In addition, blocking other potential mechanisms of AICD, such as p53- or reactive oxygen species-mediated apoptosis, was also ineffective. In contrast, ATA, which is known as an anti-apoptotic agent owing to its ability to inhibit DNA fragmentation (34), was revealed in our study to induce Foxp3 expression in activated T cells. In addition, blocking other potential mechanisms of AICD, such as p53- or reactive oxygen species-mediated apoptosis, was also ineffective. In contrast, ATA, which is known as an anti-apoptotic agent owing to its ability to inhibit DNA fragmentation (34), was revealed in our study to induce Foxp3 expression in activated T cells. Although it has been shown that ATA inhibits activation-induced apoptosis in immature thymocytes and some T-cell hybridomas (29), our results indicate that, at the concentrations that induce Foxp3 expression, ATA had a stronger effect on the activation of naive T cells in response to TCR engagement than it did on the apoptotic death of pre-activated T cells. A higher proportion of cells remained as small resting cells or became CD25-negative.
weakly positive cells after activation in the presence of ATA, whereas the proportion of dead cells was not greatly diminished in fully activated blast-like cells for 4–5 days after activation (data not shown). Nonetheless, T-cell apoptosis induced by serum deprivation was efficiently prevented by ATA (data not shown), as previously observed in other cell types (40–42). Therefore, it might be inappropriate at the moment to conclude that the anti-apoptotic activity of ATA resulting from its prevention of DNA fragmentation is the mechanism by which ATA induces Foxp3 expression in activated T cells. In addition to its ability to inhibit DNA fragmentation, ATA has been reported to inhibit other cellular activities, including enzymatic activities of a range of intracellular kinases and nucleases (30–33), protein synthesis initiation (43) and protein–nucleic acid interactions (44). One of these additional activities, especially anti-kinase activity, might prove to be more closely related to the ability of ATA to induce Foxp3 expression, as discussed below.

In the initial stage of our study, we thought that ATA induced Foxp3 expression in T cells independent of TGF-$\beta$ activity because this phenomenon was observed in stimulation cultures in the absence of exogenously added TGF-$\beta$. However, the observation that ATA-induced Foxp3 expression was completely inhibited by a neutralizing antibody to TGF-$\beta$ or a Smad signaling inhibitor demonstrated that TGF-$\beta$ was involved in the induction of Foxp3 expression by ATA. Since ATA failed to induce not only TGF-$\beta$ secretion but also Foxp3 expression in T cells cultured in serum-free media, it is highly likely that even the miniscule amount of TGF-$\beta$ present in serum is sufficient to synergize with ATA to induce Foxp3 expression. This possibility is supported by our observation that T cells express Foxp3 in response to ATA when cultured in serum-free media supplemented with a small amount of exogenous TGF-$\beta$ (Fig. 5D). Because ATA itself does not induce Smad2 phosphorylation, we could exclude the Smad signaling pathway.pA target of ATA.

The molecular mechanisms governing Foxp3 expression in Treg cells are not yet completely understood. Several transcription factors and enhancers are known to be involved in the transcriptional regulation of Foxp3 (26, 45). In addition, epigenetic modifications, such as DNA methylation and
acetylation, also contribute to this regulation (27). The linkages between most of these specific regulatory components and specific upstream signaling pathways have not yet been established, but a role for some signaling pathways in Foxp3 expression has been demonstrated. It was reported that inhibition of ERK induces Foxp3 expression in T cells, possibly by promoting down-regulation of DNA methyltransferase expression (35). Because ATA inhibits ERK activation, especially at later time points, it might induce Foxp3 expression through this mechanism. One possible alternative is that ATA acts through inhibition of the mTOR pathway, as shown in this study, to induce the expression of Foxp3. This possibility is supported by several recent reports demonstrating that inhibition of the PI3K-Akt-mTOR pathway promotes Foxp3 expression, whereas constitutive PI3K-Akt-mTOR activity antagonizes Foxp3 induction (37, 38).

Since the discovery that TGF-β has the capacity to convert naive T cells into Foxp3+ Treg cells (18), several other molecules have been suggested to have the ability to induce Foxp3 expression. Among these are vasoactive intestinal peptide (24), retinoic acid (46-48), PD-L1 (19, 49), rapamycin (23), activin A (22), simvastatin (20) and bone morphogenetic proteins (21). Most of these molecules are known to promote TGF-β activity without possessing the innate capacity to induce Foxp3 expression. Although the molecular mechanisms responsible for the activity of these molecules have not been thoroughly investigated, it is interesting to note that PD-L1-mediated iTreg development is associated with down-regulation of the phosphorylated forms of Akt, mTOR, S6K and ERK2 (19), effects that are also observed in T cells treated with ATA. Another interesting finding is that inhibition of the PI3K-Akt-mTOR pathway is commonly associated with exposure to vasoactive intestinal peptide, PD-L1, rapamycin and ATA (19, 23, 50). Thus, down-regulation of the PI3K/Akt/mTOR pathway seems to be a key signaling requirement for Foxp3 expression. A recent report further supports this possibility, showing that Foxo3a, which is a potential downstream target of both Akt and mTOR pathways, induces Foxp3 expression by directly binding to the Foxp3 promoter (51).

There is considerable interest in the ex vivo generation of Treg cells as a therapy for autoimmune diseases and transplant rejection. Adoptive transfer of TGF-β-induced iTreg cells is effective in the control of allergies (18) and graft-versus-host disease (52). However, several issues must be resolved before this type of cell therapy becomes applicable to the clinic, including ensuring the purity and stability of prepared iTreg cells. Upon stimulation in the presence of TGF-β, naive CD4+ T cells are not all converted into Foxp3+ Treg cells, and even converted cells can lose Foxp3 expression and acquire effector function after re-stimulation (53, 54). Considering that repeated stimulation of naive CD4+ T cells in the presence of the combination of TGF-β and ATA yields a relatively pure population of Foxp3+ T cells (Fig. 7) and these cells appear resistant to acquire effector cytokine-producing ability even after co-culture with responder T cells in the absence of TGF-β and ATA (Supplementary Figure 3 is available at International Immunology Online), ATA could be used for the efficient preparation of iTreg cells.

### Supplementary data

Supplementary Figures are available at *International Immunology* Online.

### Funding

Korea Research Foundation Grant funded by the Korean Government (KRF-2008-313-E00227); grant of the Korea Healthcare Technology R&D Project, Ministry for Health, Welfare & Family Affairs, Republic of Korea (A040004).

### Acknowledgements

We thank Dr Rudensky, Memorial Sloan-Kettering Cancer Institute, for providing Foxp3:GFP knock-in mice and Hye-Jin Chung for her cell-sorting expertise.

### References

Induction of Foxp3 expression by ATA


