A Novel Mechanism of Action for Anti-Thymocyte Globulin: Induction of CD4⁺CD25⁺Foxp3⁺ Regulatory T Cells

Marta Lopez, Michael R. Clarkson, Monica Albin, Mohamed H. Sayegh, and Nader Najafian

Transplantation Research Center, Brigham and Women’s Hospital, and Children’s Hospital Boston, Harvard Medical School, Boston, Massachusetts

T cell–depleting agents are being tested as part of clinical tolerance strategies in humans with autoimmunity and transplantation. The immunosuppressive activity of anti-thymocyte globulin (ATG) has been thought to result primarily from depletion of peripheral lymphocytes. Herein is reported for the first time that ATG but not anti-CD52 mAb (alemtuzumab) or the IL-2R antagonists causes rapid and sustained expansion of CD4⁺CD25⁺ T cells when cultured with human peripheral blood lymphocytes. These cells display enhanced expression of the regulatory markers glucocorticoid-induced TNF receptor, cytotoxic T lymphocyte–associated antigen-4 (CTLA-4), and forkhead box P3 and efficiently suppress a direct alloimmune response of the original responder lymphocytes. It is interesting that the cells do not suppress memory responses to the recall antigen mumps. Ex vivo expansion of regulatory T cells is due mainly to conversion of CD4⁺CD25⁻ into CD4⁺CD25⁺ T cells and to a lesser degree to proliferation of natural CD4⁺CD25⁺ T cells. The induction of regulatory T cells depends on production of Th2 cytokines in the generating cultures. These novel data suggest that ATG not only may promote expansion/generation of regulatory T cells but also may be useful in future ex vivo expansion of these cells for cellular therapy in autoimmunity and clinical transplantation.


Polyclonal anti-thymocyte globulin (ATG) is the purified IgG fraction of sera from rabbits, horses, or, more rarely, goats that are immunized with human thymocytes or T cell lines. ATG is used for treatment of various clinical conditions, including prevention or rescue treatment of acute rejection in organ transplantation (1), conditioning for hematopoietic stem cell transplantation, treatment of severe aplastic anemia, various autoimmune diseases, and more recently graft-versus-host disease (2). The immunosuppressive activity of ATG has been thought to result primarily from the depletion of peripheral lymphocytes from the circulating pool through complement-dependent lysis or activation-associated apoptosis (1,3–5). Other potential mechanisms of action include modulation of surface adhesion molecules or chemokine receptor expression (6).

Regulatory T cells (Treg) are specialized T cell subsets that play important roles in maintaining immune homeostasis (7,8). Treg are characterized by the expression of the IL-2 receptor α-chain (CD25) and the transcription factor forkhead box P3 (Foxp3) (9). There exists emerging evidence in both rodents and humans to support important immunoregulatory functions of CD4⁺CD25⁺ Treg in maintaining both self-tolerance and tolerance toward autoantigens (8) and alloantigens (10). Treg also may play a role in preventing human renal autoimmune diseases such as Goodpasture’s disease (11). Our group previously demonstrated that active regulation of the alloimmune responses by Treg may function to maintain hyporesponsiveness to alloantigens in renal transplant patients (12,13). In preclinical animal models, ex vivo expanded Treg protect mice from lethal graft-versus-host disease (14). In fact, a clinical trial was proposed recently to use ex vivo expanded Treg at the time of hematopoietic stem cell transplantation (15,16). Therefore, understanding the conditions that are required for the generation and propagation of Treg would allow development of novel therapeutic strategies for inducing immunologic tolerance in various immune-mediated diseases.

Here we report the novel finding that ATG-mediated immunosuppression is delivered in part via immunologically specific actions involving the generation of Treg, particularly CD4⁺CD25⁢⁰⁺Foxp3⁺ cells. This is due mainly to ATG’s unique ability to convert the CD4⁺CD25⁻ T cells into CD4⁺CD25⁺ T cells. More important, in a mixed lymphocyte reaction, the regulatory function is restricted to autologous responder cells from which Treg originally were generated and does not affect the memory response to recall antigens. Our findings have relevant clinical implications for designing new immunomodulatory protocols for immune-mediated diseases.

Materials and Methods

Cells and Antibodies

This study was performed with the approval of the Institutional Review Board for human investigation at the Brigham and Women’s...
Hospital. Blood from healthy volunteers were obtained in heparinized tubes, and peripheral blood lymphocytes (PBL) were isolated by standard Ficolldensity-gradient centrifugation. Two types of ATG were used: A rabbit polyclonal serum raised against human thymocytes (Thymoglobulin; Genzyme, Cambridge MA) or a rabbit polyclonal serum raised against the lymphoblastic Jurkat T cell line (Fresenius, Bad Homburg, Germany). Purified rabbit polyclonal IgG was used as control. To compare the effects of ATG with agents that block the IL-2 receptor α-chain (IL-2Rα) on the surface of activated T lymphocytes, we used either Basiliximab (Simulect, Novartis, NJ), a chimeric (murine/human) mAb (IgG1k), or Daclizumab (Zenapax, Roche, NJ), a humanized IgG1 mAb. In addition, we used alemtuzumab (Campath1-H, human) mAb (IgG1k), or Daclizumab (Zenapax, Roche, NJ), a humanized DNA-derived humanized mAb that is directed against the cell surface glycoprotein CD52, for comparison studies with ATG.

**Generation of Treg**

PBL (1 × 10⁶ per ml) from 10 healthy donors were incubated with ATG (Treg) or rabbit IgG (TControl) in RPMI 1640 medium (Cambrex Bioscience, Walkersville, MD) supplemented with 10% heat-inactivated human serum at 37°C with a PCO₂ 5% for various durations (6 to 96 h). All above agents were used in vitro at concentrations that ranged from 1 to 100 µg/ml.

These cultures are called “generating cultures.” For assessment of the role of cytokines in the expansion of Treg, anti-IL-4, anti-IL-13, and anti-IL-10 mAb each at a concentration of 10 µg/ml were added separately to the generating cultures (17). All of these antibodies were purchased from BD Bioscience (San Jose, CA).

**In Vitro Suppression Assay**

To test the suppression function of cells that were generated under conditions explained (Treg), we set up a mixed lymphocyte reaction (MLR) assay: 1 × 10⁵ cells that were obtained from the generating cultures above were co-cultured (1:1 ratio) with fresh responder (autologous or third-party PBL) and irradiated stimulator cells in a 96-well plate (96-well Cell Culture Cluster, round-bottom culture plate; COSTAR, New York, NY) for 120 h. The cultures were labeled with ³H-thymidine during the last 8 h of culture (Amersham Pharmacia Biotech, Piscataway, NJ). Cells then were harvested, and radioactivity uptake was measured by a scintillation counting machine. In a similar manner, we also tested whether Treg could suppress recall responses to mumps antigens.

**Flow Cytometry**

Cells that were harvested from generating cultures (Treg or TControl) were analyzed using flow cytometric analysis. A total of 2 × 10⁵ cells per sample were stained with anti-human CD4-allophycocyanin (APC), CD25-phycocerythrin (PE), and glucocorticoid-induced TNF receptor (GTRR)-FITC, CD8-APC (BD Bioscience; eBioscience, San Diego, CA). For the intracellular cytokytic T lymphocyte-associated antigen-4 (CTLA-4) staining, cells were permeabilized with Perm Buffer (BD Bioscience) for 20 min at 4°C and labeled with CTLA-4 for 30 min at 4°C. For flow cytometric analysis of Foxp3, 1 × 10⁵ cells first were stained with anti-human CD4-APC and CD25-PE. After washing, cells were resuspended in 1 ml of cold Fix/Perm Buffer (eBioscience) and incubated at 4°C overnight in the dark. After washing twice with 2 ml of permeabilization buffer, cells were blocked with 2% normal rat serum for 15 min. Anti-human Foxp3-FITC (PCH101; eBioscience) then was added, and cells were incubated at 4°C for another 30 min in the dark. Finally, cells were washed with 2 ml of permeabilization buffer and analyzed on a FACSCalibur flow cytometer using CellQuest software (Becton Dickinson, San Jose, CA). For evaluation of the induction of apoptosis/death of CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells, PBL that were incubated with ATG or rabbit IgG were stained with mAb against CD4, CD25, annexin-V, and 7-amino-actinomycin D as per the manufacturer’s instructions (BD Bioscience).

**ELISPOT Assay**

To evaluate the frequency of cytokine-producing cells in the generating culture, we made use of the ELISPOT assay as described previously (12). The resulting spots were counted on a computer-assisted ELISASpot Image Analyzer (Cellular Technology Limited, Cleveland, OH). Cells were tested in triplicate wells. The frequencies then were expressed as the number of spots per million PBL.

**Luminex Assay**

Supernatants from generating cultures (serum-free medium) were tested for the presence of TGF-β by Luminex 100 system (Luminex Corp., Austin, TX). Beadlyte Human Multi-Cytokine Beadmaster Kit and Beadlyte Human TGF-β1/β2 Detection system (Upstate, Charlotteville, VA) were used as per protocol provided by the manufacturer. Plates were analyzed using Multiplex Data Analysis software version 1 (Luminex, Austin, TX).

**Carboxy-Fluorescein Diacetates Succinimidyl Ester Staining**

To test whether the expansion of Treg by ATG is due to proliferation of preexisting naturally occurring CD4⁺CD25⁺ T cells, we incubated PBL with carboxy-fluorescein diacetates succinimidyl ester (CFSE) in the form of 5 mM stock solution in DMSO at a final concentration of 1 µM for 6 min at room temperature. CFSE-labeled cells were cultured in vitro with phytohemagglutinin (positive control), ATG, and rabbit IgG for 72 h at 37°C. Cells then were stained with anti-human CD4-APC, CD8-PE, and CD25-PE. 7-Amino-actinomycin D was used to exclude death cells.

**Conversion of CD4⁺CD25⁻ to CD4⁺CD25⁺ T Cells**

For testing whether Treg that are generated by ATG are induced from CD4⁺CD25⁻ T cells, PBL were depleted of CD25 by magnetic cell sorting using MACS columns and MACS separators (Miltenyi Biotec, Auburn, CA). CD25-depleted CD4⁺ T cells were then incubated with ATG or rabbit IgG for 24 h. The cells then were harvested and stained for CD25 and regulatory markers. Similarly, their suppressor activity was assessed as described previously.

**Statistical Analyses**

The t test was used for comparison of means between experimental groups examined by flow cytometry and ELISpot assay. Differences were considered to be significant at P < 0.05.

**Results**

**ATG Expands CD4⁺CD25⁺Foxp3⁺ T Cells Ex Vivo**

PBL that were derived from healthy volunteers were incubated with Thymoglobulin or rabbit IgG (10 µg/ml) for various time periods (0, 6, 18, 24, 48, 72, and 96 h). Flow cytometric analysis of harvested cells demonstrated a significant upregulation of CD25 expression that began at 18 h and was maintained beyond 96 h, with peak expression of CD25 at 24 h (percentage of CD4⁺CD25⁺ T cells gated on all viable lymphocytes 20.5 ± 7.8 versus 4.5 ± 1.6; P = 0.002; n = 7; Figures 1A and 2A).

To evaluate the dose-response of ATG and expansion of
CD4+CD25+ T cells, we incubated PBL with ATG or rabbit IgG at various concentrations (1, 5, 10, 50, and 100 μg/ml) for 24 h (percentage of CD4+CD25+ T cells gated on all viable lymphocytes: 1 μg/ml 6.3 ± 0.5, 5 μg/ml 12 ± 4.9, 10 μg/ml 20 ± 6.5, 50 μg/ml 21.7 ± 5, and 100 μg/ml 21 ± 6.7; percentage of CD4+CD25+ T cells with various concentrations of rabbit Ig is between 3 and 5). We found a dose-dependent increase in percentage of CD4+CD25+ T cells using between 1 and 10 g/ml 6.3 ± 0.5, 5 g/ml 12 ± 4.9, 10 g/ml 20 ± 6.5, 50 g/ml 21.7 ± 5, and 100 μg/ml 21 ± 6.7; percentage of CD4+CD25+ T cells with various concentrations of rabbit Ig is between 3 and 5). We found a dose-dependent increase in percentage of CD4+CD25+ T cells using between 1 and 10

*Figure 1. Anti-thymocyte globulin (ATG) expands CD4+CD25+ Foxp3+ T cells *ex vivo. (A) A representative example of seven experiments demonstrating enrichment of a CD4+CD25+ T cell population at 24 h (peak expression): Cells incubated with Thymoglobulin showed an increase in CD25 expression as compared with cells incubated with rabbit IgG (each at a concentration of 10 μg/ml). Such expanded CD4+CD25+ T cells generated after incubation with Thymoglobulin had significantly enhanced expression of regulatory markers glucocorticoid-induced TNF receptor (GITR; 32 ± 12 versus 6.6 ± 4%; n = 5), intracellular cytotoxic T lymphocyte–associated antigen-4 (CTLA-4) (41.3 ± 19.5 versus 7 ± 1.8%; n = 4), and forhead box P3 (Foxp3; 65.3 ± 21.5 versus 43.8 ± 12.3; n = 5) as compared with control cells (percentage expression of regulatory markers calculated when gating on CD4+CD25+ T cells). (B) A representative example showing a significant increase in the frequency of CD4+CD25+ high population in ATG-treated versus rabbit IgG–treated cells (6.5 ± 2.9 versus 0.7 ± 0.5%; n = 8; P = 0.001). More important, representative examples of two color flow staining (CD25 and each of regulatory markers GITR, CTLA-4, and Foxp3) are shown to compare better the expression of regulatory markers on CD4+CD25+ and CD4+CD25− T cells after incubation with ATG or rabbit IgG. Among all CD4+ T cells (see gate), the percentage of cells that expressed CD25 and GITR (ATG 18.3 ± 6.9, rabbit IgG 0.5 ± 0.4; n = 6; P = 0.001) or CD25 and CTLA-4 (ATG 19.5 ± 6.2, rabbit IgG 1.5 ± 0.4; n = 4; P = 0.008) was markedly enhanced when cells were incubated with ATG versus rabbit IgG. Similarly, gating on all CD4+ T cells, the percentage of cells that expressed CD25 and Foxp3 (%CD4+CD25+Foxp3+ T cells) was significantly increased when cells were treated with ATG versus rabbit IgG (ATG 10.4 ± 2.5 versus rabbit IgG 2.2 ± 0.5; n = 8; P < 0.0001). In contrast, the percentage of CD25+ T cells that expressed GITR (ATG 1.8 ± 1.5%, rabbit IgG 0.3 ± 0.2; n = 6; NS) or CTLA-4 (ATG 5.4 ± 3.7, rabbit IgG 1.3 ± 0.7; n = 4; NS) was minimal. In addition, the percentage of CD4+CD25+ Foxp3+ T cells (gated on CD4+ T cells) was minimal after incubation with ATG versus rabbit IgG (1.1 ± 0.6 versus 0.7 ± 0.4), indicating that ATG did not induce CD4+CD25+Foxp3+ regulatory T cells (Treg).
Figure 2. Expansion of CD4+CD25+ T cells is unique to polyclonal ATG. (A) Peripheral blood lymphocytes (PBL) that were derived from healthy human volunteers were incubated with Thymoglobulin (10 μg/ml) for various time periods (0, 6, 18, 24, 48, 72, and 96 h). Cells then were harvested and underwent flow cytometric analysis. There was significant upregulation of CD25 expression that started at 18 h and was maintained beyond 96 h. (B) T cell depletive agent Campath1-H and anti–IL-2 receptor agents (basiliximab and daclizumab) did not expand CD4+CD25+ T cells in vitro (Figure 2B). Therefore, the expansion observed is unique to polyclonal ATG.

The study of Treg is complicated by a paucity of phenotypic markers to distinguish activated effector T cells from Treg. Expression of molecules such as CTLA-4 and GITR (10) and secretion of regulatory cytokines such as TGF-β (19) and IL-10 (20,21) each have been linked with but not proved to be entirely specific for Treg. Therefore, we first analyzed the surface expression of regulatory markers GITR and CTLA-4 on the expanded CD4+CD25+ T cells (Figure 1A). GITR showed enhanced expression in ATG-induced CD4+CD25+ T cells as compared with CD4+CD25+ T cells that were incubated with rabbit IgG (32 ± 12 versus 6.6 ± 4%; P = 0.005; n = 5). Similar results were observed for intracellular CTLA-4 expression (41.3 ± 19.5 versus 7 ± 1.8%; P = 0.04; n = 4). When gating on all CD4+ T cells, the percentage of cells that expressed both CD25 and GITR (ATG 18.3 ± 6.9, rabbit IgG 0.5 ± 0.4; n = 6; P = 0.001) or CD25 and CTLA-4 (ATG 19.5 ± 6.2, rabbit IgG 1.5 ± 0.4; n = 4; P = 0.008) was markedly enhanced when cells were incubated with ATG versus rabbit IgG (Figure 1B).

In contrast to GITR and CTLA-4, Foxp3, a gene that encodes a forkhead winged helix transcription factor scurfin, is required for Treg development and function (22,23). CD4+CD25+ T cells that were expanded by ATG (at 10 μg/ml) showed higher...
expression of Foxp3 as compared with those that were incubated with rabbit IgG (63.4 ± 12 versus 47 ± 8.3%; P = 0.03; n = 5; Figure 1A). It is interesting that contrary to CD69 expression (see above), the Foxp3 expression declined with increasing dosage of ATG in the generating culture (percentage of Foxp3 expression in CD4⁺CD25⁺ T cells: 50 μg/ml 13 ± 4.2, 100 μg/ml 15.2 ± 0.35). Importantly, gating on all CD4⁺ T cells, the percentage of cells that expressed both CD25 and Foxp3 (percentage of CD4⁺CD25⁺Foxp3⁺ T cells) was significantly increased when cells were treated with ATG versus rabbit IgG (ATG 10.4 ± 2.5 versus rabbit IgG 2.2 ± 0.5; P < 0.0001; n = 8; Figure 1B). In addition, expression of all of the regulatory markers was even markedly more enhanced in the CD4⁺CD25⁺ high population that was expanded after incubation with ATG (GITR 49.4 ± 15.9%, n = 7; CTLA-4 55 ± 24.4%, n = 6; Foxp3 71 ± 14.7%, n = 5).

Previous work indicated that Foxp3 also can be induced in CD4⁺CD25⁻ T cells and that these cells can function as Treg (24). However, in contrast to CD4⁺CD25⁺ T cells, CD4⁺CD25⁻ T cells that were incubated with ATG or rabbit IgG showed only minimal increase in GITR (5.6 ± 4.4 versus 0) and CTLA-4 (11.5 ± 4.2 versus 0). Similarly, gating on all CD4⁺ T cells, the percentage of CD25⁻ T cells that expressed GITR (ATG 1.8 ± 1.5% rabbit IgG 0.3 ± 0.2; n = 6; NS) or CTLA-4 (ATG 5.4 ± 3.7, rabbit IgG 1.3 ± 0.7; n = 4; NS) was minimal (Figure 1B). In addition, the percentage of CD4⁺CD25⁺Foxp3⁻ T cells (gated on CD4⁺ T cells) was minimal after incubation with ATG versus rabbit IgG (1.1 ± 0.6 versus 0.7 ± 0.4), indicating that after incubation with ATG, CD4⁺CD25⁺ T cells remain Foxp3⁻ (Figure 1B). Although overall there was a slight decrease in the CD8⁺ T cells after incubation with ATG as compared with rabbit IgG (21.96 ± 4.5 versus 25.6 ± 5.1; n = 8; P = 0.02), we found no significant difference in the percentage of CD8⁺CD28⁻ T cells (11.3 ± 5.6 versus 14.9 ± 6; n = 5). In addition, we found no evidence of CD8⁺Foxp3⁺ T cells before or after treatment with ATG or rabbit IgG. Taken together, these data do not indicate expansion of other previously described Treg populations in our model.

Suppressor Function of CD4⁺CD25⁺ T Cells Generated by ATG Is Restricted to Responder Cells

After demonstrating that CD4⁺CD25⁺ T cells that are expanded by ATG can maintain their regulatory phenotype, we next set out to study the actual suppressor function of these cells in vitro. The in vitro suppressive activity of the cells was evaluated by examination of their ability to suppress an MLR to donor alloantigens. We found significant suppression of direct alloimmune responses of the original responders (PBL from which the Treg initially were generated) to stimulator cells by Treg but not by TControl (1:1 ratio, 61.3 ± 7.4 inhibition versus 20.2 ± 18.4%; n = 4; P = 0.01; Figure 3A). It is interesting that the same Treg were unable to suppress an MLR of responder cells that were not autologous to the original responder cells (Figure 3B). We next tested the ability of these Treg to suppress a recall memory response of original responders to mumps antigen. It is interesting that the proliferative response to recall antigen mumps was not inhibited by Treg, indicating that the expanded Treg did not block memory cells to the antigen (mumps alone 4848 ± 969, Treg 5214 ± 900, control 9595 ± 1231; Figure 3C).

Conversion of CD4⁺CD25⁻ into CD4⁺CD25⁺ T Cells Is the Main Mechanism of Expansion of Treg by ATG

The absolute number of CD4⁺CD25⁻ T cells (mean values of cell numbers counted in wells) that were incubated with ATG (before 75,833 ± 31,051, after 639,167 ± 249,448; n = 6; P = 0.002) but not rabbit IgG was dramatically increased (before 90,833 ± 33,229, after 113,333 ± 54,283; NS) after 24 h of incubation. In contrast, there was much more decrease in the number of CD4⁺CD25⁻ T cells that were treated with ATG (before 1878,300 ± 322,020, after 1082,500 ± 301,027; P = 0.005) as compared with rabbit IgG (1991,700 ± 379,548 versus 1861,666 ± 238,362; NS). On the basis of these findings, we hypothesized that the expansion of Treg by ATG may result from one or more mutually nonexclusive and possibly complementary mechanisms: First, ATG may preferentially promote death/apoptosis of CD4⁺CD25⁻ T cells as compared with CD4⁺CD25⁺ T cells, creating a new balance in favor of the latter cells. Second, ATG may promote the proliferation of already existing, naturally occurring CD4⁺CD25⁺ T cells. Third, ATG may be able to convert CD4⁺CD25⁻ T cells into CD4⁺CD25⁺ T cells with regulatory functions (induction of Treg). The first possibility is supported by published data demonstrating that ATG in fact can bind to multiple epitopes on the T cell surface and induce apoptosis in T lymphocytes through Fas-L (CD95L) (3,5). Nevertheless, we found no difference (gating on all lymphocytes without excluding the dead cells) in apoptosis of CD4⁺CD25⁻ T cells and CD4⁺CD25⁺ T cells that were incubated for 24 h with 10 μg/ml ATG (6.7 ± 3.1 versus 5 ± 4.7%) or rabbit IgG (5 ± 3 versus 3.2 ± 2.5%). In fact, the overall viability of the cells was >95% regardless of incubation with ATG or rabbit IgG.

To address the possibility of the proliferative effect of ATG on already existing, naturally occurring Treg, we cultured in vitro CFSE-labeled PBL with ATG or rabbit IgG for 72 h (10 μg/ml). We found three to four discrete division cycles in CD4⁺CD25⁻ cells (proliferative cells 16 ± 9%; n = 3; P = 0.01) but only one cycle of division in a CD4⁺CD25⁻ subpopulation that was incubated with ATG (7.4 ± 7.7%) and none at all with rabbit IgG, suggesting a moderate degree of expansion of CD4⁺CD25⁺ T cells. It is possible that some of the proliferating CD4⁺CD25⁺ T cells simply may be activated T cells rather than proliferating naturally occurring Treg. Nevertheless, whereas there is an eight-fold expansion of CD4⁺CD25⁺ T cells at 24 h with ATG, proliferating cells make up only approximately 16% of the CD25⁺ population at 72 h. This indicates that proliferation is unlikely to contribute significantly to the expansion of the cells. In contrast, the CD8⁺ T cells that were incubated with ATG did not show proliferation at all (Figure 4).

To determine whether ATG is capable of converting CD4⁺CD25⁻ cells into CD4⁺CD25⁺ cells with regulatory properties, we first depleted naturally occurring CD25⁺ T cells from PBL ex vivo with magnetic bead separation (before 5.9 ± 2.9 versus after depletion 0.6 ± 0.9%; Figure 5A). Such CD25-
depleted T cells then were incubated for 24 h with ATG or rabbit IgG, after which the induction of CD25+ T cells was evaluated by flow cytometry. We found not only significant upregulation of CD25 expression on CD4+ T cells that were incubated with ATG but not rabbit IgG (18.7 ± 4 versus 3.4 ± 1.8%; n = 5; P = 0.02) but also clear expansion of the CD4+CD25+Foxp3+ T cells (gating on CD4+ T cells: ATG 8.2 ± 0.3 versus rabbit IgG 0.6 ± 1.1%; n = 3; P = 0.003; Figure 5). These newly induced Treg showed high expression of Foxp3 similar to naturally occurring CD4+CD25+ T cells that were expanded by ATG (gating on CD4+CD25+ T cells 52.6 ± 13.8 versus 63.4 ± 12%; n = 3; P = 0.57) and also were equally capable of suppressing the proliferative response in an MLR (44 ± 14 versus 5 ± 7%; n = 3; P = 0.01).

**Th2 Cytokines Play a Critical Role in the Expansion of Treg by ATG**

Next, to evaluate the role of regulatory cytokines, we determined the frequency of cytokine-producing cells (IL-4, IL-5, IL-10, IL-13, and IFN-γ) by incubating PBL that were isolated from healthy volunteers with either ATG or rabbit IgG in ELISPOT plates for 48 h (Figure 6A). Expansion of Treg by ATG was accompanied by significant increase in IL-4 (64.5 ± 34.1 versus 13 ± 9.5; P = 0.01), IL-5– (137 ± 19.7 versus 45.8 ± 46.7; P = 0.004), and IL-10–producing PBL (247.8 ± 65.9 versus 30.2 ± 20.5; n = 3; P = 0.0003). Although the IL-13 production also was higher in the presence of ATG as compared with rabbit IgG, the difference did not reach statistical significance (data not shown). The analysis of TGF-β1 (131.9 ± 39.6 versus 307 ± 112 pg/ml; n = 7; NS) and TGF-β2 (28.6 ± 7.2 versus 38.3 ± 9.5; n = 6; NS) in the culture supernatants of PBL that were incubated with ATG or rabbit IgG did not reveal any significant difference. Even though the frequency of IFN-γ–producing cells overall was low, it still was slightly more in ATG-incubated cells as compared with controls (28.7 ± 20.22 versus 14.6 ± 10.3; n = 4; P = 0.003). To confirm the functional role of Th2 cytokines in expansion of Treg, we added each anti–IL-4, anti–IL-13, and anti–IL-10 antibodies to the generating cultures (each at 10 µg/ml) and analyzed the percentage of CD4+CD25+Foxp3+ T cells (Figure 6B). Neutralization of each of the cytokines resulted in reversal of Treg expansion by ATG (anti–IL-4 4.2 ± 0.4%, anti–IL-13 4.1 ± 1.2%, anti–IL-10: 3.8 ± 0.3%, n = 2, P < 0.01 for all versus ATG alone 10.4 ± 2.5%, rabbit Ig alone 2.2 ± 0.5, P < 0.0001).

**Discussion**

We and others previously reported that active regulation of the alloimmune responses by Treg may function to maintain hyporesponsiveness to alloantigens in stable renal transplant patients (12,13). A recent study of renal transplant patients who were experiencing acute rejection demonstrated increased levels not only of mRNA of CD25 and perforin (markers of activated and cytotoxic T cells) but also of Foxp3 (functional marker of Treg) in samples of urinary lymphocytes (25). More interesting, higher levels of Foxp3 mRNA were associated with improved probability of reversibility of acute rejection and lower risk for graft failure 6 mo after rejection episode. These
findings are consistent with the hypothesis that Treg also can serve to limit anti-graft immunity during acute rejection. Taken together, the above studies suggest that drugs that enhance the generation of Treg or administration of Treg themselves may improve the outcome of both acute rejection episodes and the long-term survival of allografts overall. In this article, we report the novel finding that ATG can lead to expansion of CD4\(^+\)CD25\(^+\) Foxp3\(^+\) Treg from naïve CD4\(^+\)CD25\(^-\) T cells. Our data are in agreement with several other studies showing generation of Foxp3-expressing CD4\(^+\)CD25\(^+\) T cells from CD4\(^+\)CD25\(^-\) precursors in the periphery (24,26). In contrast to mouse, in which Foxp3 could be induced on Foxp3\(^-\) cells under specialized conditions (e.g., activation plus TGF-β) (24), human CD25\(^-\) T cells seemed to upregulate both CD25 and Foxp3 (26). Therefore, activation of human CD4\(^+\)CD25\(^-\) T cells led to generation of regulatory CD4\(^+\)CD25\(^+\)Foxp3\(^+\) T cells (26), raising the possibility that ATG in a similar manner may expand the Treg by initially activating the CD4\(^+\)CD25\(^-\) T cells. Although deletion of T cells by apoptosis has been demonstrated to promote immunoregulation (27), we did not observe significant apoptosis after 24 h of incubation of PBL with ATG at the concentration of 10 μg/ml, suggesting that apoptosis is unlikely to contribute. Furthermore, whether the original CD4\(^+\)CD25\(^-\) T cells are truly naïve T cells or regulatory cells that were generated initially in thymus and later lost the CD25 expression and then recovered their suppressive activity again upon activation remains unclear (9). It is interesting not only that this process is accompanied by an increase in the Th2 cytokines in the generating culture but also that in fact neutralization of each of the Th2 cytokines can significantly hamper the Treg-expanding effects of ATG. While Th2 cytokines have the capacity to promote Treg by various mechanisms such as altering T cell activation, affecting apoptosis or cell proliferation, it is likely that they promote the conversion of CD4\(^+\)CD25\(^-\) T cells into Treg, as conversion is established clearly as the main contributor to Treg expansion. These findings are very intriguing and supported by recent published data demonstrating that IL-4R α-chain–binding cytokines, such

Figure 4. ATG can induce moderate proliferation of preexisting CD4\(^+\)CD25\(^+\) T cells. PBL were labeled with carboxy-fluorescein diacetates succinimidyl ester (CFSE) and cultured in the presence of mitogen phytohemagglutinin, ATG (10 μg/ml) or rabbit IgG (10 μg/ml) for 72 h. The proportion of CFSE cells proliferating were calculated as described in the literature. We found three to four discrete division cycles in CD4\(^+\)CD25\(^+\) cells (proliferative cells 16 ± 9%; n = 3; P = 0.01) but only one cycle of division in a CD4\(^+\)CD25\(^-\) subpopulation that was incubated with ATG (7.4 ± 7.7%) and none at all with rabbit IgG, suggesting a moderate degree of expansion of CD4\(^+\)CD25\(^+\) T cells by ATG. In contrast, the CD8\(^+\) T cells that were incubated with ATG did not show proliferation at all (this is a representative example of three independent experiments).
as IL-4 and IL-13, play a crucial role in generating Foxp3<sup>+</sup>CD25<sup>+</sup> Treg extrathympically (17). In addition, these data point to an intriguing link between the well-established immunoregulatory capacity of Th2 cells and the potent CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells. These results also are important in light of data showing that cyclosporine and tacrolimus, two commonly used immunosuppressive drugs, both inhibit the production of IL-2, an essential growth factor for Treg (28). Conversely, murine T cells that were activated in the presence of rapamycin were highly enriched in Foxp3<sup>+</sup>CD4<sup>+</sup>CD25<sup>+</sup>T<sub>cells</sub> in vitro (29). Nevertheless, our findings here are unique because we clearly demonstrate that ATG can rapidly expand human Treg by promotion of Th2 cytokine production ex vivo.

Although the identification of the exact component of ATG (Genzyme; generated by using thymocytes as the immunogen) that is responsible for the expansion of Treg will require further investigation, our ability to generate Treg using a different ATG (Fresenius; produced with purified and activated CD3<sup>+</sup> lymphoblastic cells as immunogen) suggests a key role for antibodies that are directed against epitopes on T cells. Another unique finding of the study is that ATG-induced Treg significantly suppressed direct alloimmune responses to alloantigens by autologous PBL from which Treg originally were generated. In contrast, nonautologous responders could not be regulated by the Treg. Conversely, the memory response to recall antigen mumps is not affected. This may be due to lack of activation of Treg by mumps antigen or the overall inability of Treg to inhibit the memory responses. Given the great interest in cellular treatment with ex vivo expanded Treg as a means to modulate alloimmune responses without affecting memory responses to infectious agents, these findings are of crucial importance.

Although the in vivo effects of ATG on the induction, expansion, and function of Treg in allograft recipients remain to be characterized fully, recent data demonstrated that CD25<sup>+</sup> T cells are spared from anti-lymphocyte serum–mediated deletion in mice, suggesting the concomitant presence of both T cell depletion and continuous regulatory T cell activity in an in vivo animal model (30). Second, in studying the in vivo effects of ATG in humans, the achieved concentrations of the ATG in blood and lymphoid tissues should be taken carefully into consideration. ATG is used at a dosage of 1 to 1.5 mg/kg per d for 3 to 5 d as induction immunosuppression and also for treatment of steroid-resistant acute rejection, leading to serum levels of the drug between 50 and 100 µg/ml (2,5). This dosing regimen is based on ATG’s efficacy to deplete T cells in the peripheral blood rather than promotion of Treg. There are no human data regarding the achieved concentrations in secondary lymphoid tissues, a potentially important site for induction/expansion of Treg, although the data in primates suggest good penetration into lymph nodes and spleen but minimal penetration into thymus (31). We were able to expand Treg in vitro with a concentration of 10 µg/ml, a dosage that is significantly lower than the levels that currently are achieved in

Figure 5. ATG induces Treg from CD4<sup>+</sup>CD25<sup>−</sup> T cells. PBL were depleted of CD25 using MACS columns. Such CD25-depleted T cells then were incubated for 24 h with ATG or rabbit IgG. Flow cytometric analysis showed an increase in CD25 expression on CD4<sup>+</sup> T cells (representative example of three independent experiments shown). The two-color staining demonstrated also clear expansion of the CD4<sup>+</sup>CD25<sup>−</sup>Foxp3<sup>+</sup> T cells (ATG 8.2 ± 0.3 versus rabbit IgG 0.6 ± 1.1%; n = 3; P = 0.003, gated on all CD4<sup>+</sup> T cells).
blood. In fact, our *in vitro* data may suggest that lower dosages of ATG may lead to expansion of Treg *in vivo*, but clearly further investigation is needed and is currently ongoing.

**Conclusion**

We report a novel mechanism of action for ATG, namely its ability to expand Treg *ex vivo*, mainly by inducing CD4<sup>+</sup>CD25<sup>+</sup> Foxp3<sup>+</sup> T cells. The regulatory function clearly is restricted by the responder cells but does not affect the memory response to recall antigen. These findings are clinically important for two important reasons: First, they may suggest that the therapeutic effect of ATG may be due to both concomitant T cell deletion and continuous regulatory T cell activity; second, they may help us to develop strategies to expand these very rare regulatory cells *ex vivo* for use as cellular treatment in transplantation and autoimmunity.

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