Published in final edited form as:

Transplantation. 2010 August 15; 90(3): 260–269.

A NOVEL CLINICALLY RELEVANT APPROACH TO TIP THE BALANCE TOWARDS REGULATION IN STRINGENT TRANSPLANT MODEL

Francesca D'Addio^{1,2}, Xueli Yuan¹, Antje Habicht^{1,3}, John Williams⁴, Melanie Ruzek⁴, John Iacomini¹, Laurence A. Turka⁵, Mohamed H. Sayegh¹, Nader Najafian¹, and M. Javeed Ansari^{1,6}

¹Transplantation Research Center, Renal Division, Brigham and Women's Hospital and Children's Hospital Boston, Harvard Medical School, Boston, MA

²Nephrology Dialysis and Transplantation Unit, University of Bologna, Italy

³Medical School of Hanover, Department of Nephrology, Hanover, Germany

⁴Genzyme Corporation, Cambridge, MA

⁵University of Pennsylvania, Division of Renal, Electrolyte & Hypertension, Philadelphia, PA

⁶Divisions of Nephrology and Organ Transplantation, Northwestern University Feinberg School of Medicine, Chicago, IL

Abstract

Background—Regulatory T cells (T_{reg}) actively regulate alloimmune responses and promote transplantation tolerance. Thymoglobulin[®], a rabbit polyclonal anti-thymocyte globulin (ATG), is a widely used induction therapy in clinical organ transplantation that depletes peripheral T cells. However, resistance to tolerance induction is seen with certain T cell depleting strategies and is attributed to alterations in the balance of naïve, memory and T_{regs}. The exact mechanism of action of ATG and its effects on the homeostasis and balance between T_{regs} and T-effector-memory cells (T_{em}) are unknown.

Methods—A novel antibody reagent, rabbit polyclonal anti-murine thymocyte globulin (mATG), generated by the same process utilized to manufacture Thymoglobulin[®], was used alone or in

Address correspondence to: Dr. M. Javeed Ansari, M.D., Assistant Professor of Medicine, Divisions of Nephrology and Organ Transplantation, Northwestern University Feinberg School of Medicine, 300 East Superior Street, Tarry Building, Suite 11-723, Chicago, IL 60611, jansari@northwestern.edu, Office Tel: 312-503-2677, Office Fax: 312-503-3366.

Author contributions:

D'Addio: participated in the performance of research, data analysis and writing of the paper.

Yuan and Habicht: participated in the performance of research.

Williams, Ruzek and Iacomini: contributed new reagents and participated in research design.

Turka and Najafian: participated in research design.

Sayegh: participated in research design and writing of the paper.

Ansari: participated in research design, data analysis and writing of the paper.

STATEMENT OF COMPETING FINANCIAL INTERESTS

XY, MJA were recipients of research grants from Genzyme Corporation. MHS is a consultant for Genzyme. MR and JW are employees of Genzyme Corporation.

combination with CTLA4Ig and/or sirolimus (SRL) in a stringent fully MHC-mismatched murine skin allograft model to study graft survival and mechanisms involved.

Results—mATG depletes T cells but preferentially spares CD25⁺ natural T_{regs} which limit skewing of T cell repertoire toward T_{em} phenotype among the recovering T cells. T cell depletion with mATG combined with CTLA4Ig and SRL synergize to prolong graft survival by tipping the T_{reg}/T_{em} balance further in favor of T_{regs} by preserving T_{regs}, facilitating generation of new T_{regs} by a conversion mechanism and limiting T_{em} expansion in response to alloantigen and homeostatic proliferation.

Conclusions—Simultaneous T-cell depletion with ATG and costimulatory blockade, combined with SRL synergize to promote regulation and prolong allograft survival in a stringent transplant model. These results provide the rationale for translating such novel combination therapy to promote regulation in primate and human organ transplantation.

INTRODUCTION

Allograft tolerance may be viewed as a balance between alloreactivity and regulatory mechanisms, where different subsets of T cells can interact to create the appropriate environment for long-term graft acceptance (1). T_{regs}, a T cell subset involved in modulating the immune response (2), have been demonstrated to play a critical role in preventing autoimmune diseases (3), regulating immune response during infectious diseases (4), maintaining feto-maternal tolerance (5), and also preventing allograft rejection in transplantation (6). Consequently, there is great interest in harnessing the power of T_{regs} to therapeutic advantage, and indeed ex-vivo expanded natural CD4⁺CD25⁺ T_{reg} therapy has been used to prevent experimental acute allograft rejection (7). On the other hand T-effector cells are thought to play a major part in mediating acute and chronic allograft rejection (8). It has been proposed that tolerance may be viewed not as an “all or none” phenomenon but rather as a balance between these different subsets of T cells, which can interact to create the appropriate environment for long-term graft acceptance under a variety of immunosuppressive treatments (9).

Various preparations of anti-thymocyte globulin (ATG), a polyclonal T cell depleting agent, have been used clinically since the early 80's as induction therapy and for treatment of acute rejection in solid organ transplantation (10, 11). ATG consists of purified IgG fraction of sera from rabbits or horses. Genzyme's Thymoglobulin[®], produced by immunizing rabbits with fresh human thymocytes, is probably the most widely used and studied polyclonal ATG preparation. Both experimental and clinical studies of Thymoglobulin[®] report profound T cell depletion through complement-dependent lysis or activation associated apoptosis, which is believed to be the key mechanism of its action by reducing the alloreactive T cell repertoire and frequency (11–15). Other potential mechanisms of action include modulation of surface adhesion receptor molecules or chemokine receptor expression (16). However the exact mechanisms of its action are still unknown and an ever-expanding array of target antigens is being identified in ATG preparations (17). We and others have reported that besides profound peripheral T cell depletion, Thymoglobulin[®] modulates the immune response through its effects on the function of many other immune cell types, including, B cells, monocytes/macrophages, dendritic cells (DC) and T_{regs} (17–19). Further, anti-murine

thymocyte globulin (mATG) has also been shown to bind to DC and deplete CD8⁺DC in the NOD mouse (20). However, resistance to tolerance induction is seen with certain T cell depleting strategies and is attributed to alterations in the balance of naive, memory and regulatory T cells (21). Our group has previously demonstrated that Thymoglobulin[®] can expand T_{regs} ex vivo, inviting a fresh look at the tolerogenic potential of this T cell depletion strategy (19). Similarly, SRL, another widely used immunosuppressive agent in clinical transplantation, is thought to expand naturally occurring T_{regs} (22, 23). Further, strategies to block T cell costimulatory pathways prolong allograft survival in rodents and primates through expansion of CD4⁺CD25⁺ regulatory T cells in certain situations, and are currently undergoing clinical development in kidney transplantation (24–28) providing the rationale to combine ATG with SRL and CTLA4Ig to promote regulation and long-term allograft survival. We therefore tested the effect of combined mATG, SRL and B7 T cell costimulatory blockade *in vivo* in a fully allogeneic mouse skin allograft model. Our experiments confirm the previous report that mATG preserves natural T_{regs} (29). Further, we report for the first time that these preserved CD25⁺ natural T_{regs} limit skewing of T cell repertoire toward T-effector-memory cells (T_{em}) phenotype among the recovering T cells. Furthermore, simultaneous T-cell depletion combined with SRL and costimulatory blockade synergize to prolong allograft survival in a stringent transplant model by further limiting the alloreactive T_{em} cell pool and that this effect is dependent on CD25⁺ T_{regs}. These data emphasize the advantage of a novel strategy combining mATG with T cell costimulatory blockade to preserve T_{regs} and limit homeostatic proliferation of effector and memory T cells and provide the rationale to translate such a strategy to promote regulation in human organ transplantation.

METHODS

Mice

C57BL/6 (B6, H-2b), BALB/c (H-2d), B6.PL-*Thy1^a*/CyJ (Thy1.1 B6) and B6.C.H-2 bm12 (bm12) mice were purchased from Jackson Laboratory. ABM-Foxp3GFP^{rep} mice were generated by inter-breeding ABM and Foxp3GFP^{rep} mice, both on the B6 background and bearing the Thy 1.2 allele. All mice were housed in accordance with institutional guidelines.

Generation and characterization of mATG

mATG was generated in a manner analogous to the commercial ATG product (Thymoglobulin[®]) by immunizing rabbits with a mixture of thymocytes from 8 different strains of mice (C57BL/6, BALB/c, DBA/2, 129, C3H, SJL, Swiss Webster, ICR) as described previously (30). Thymocyte suspensions were prepared from thymi extracted from the various donor mice and at least 25 New Zealand rabbits were immunized twice, 2 weeks apart, and terminally bled 2 weeks following the second immunization (Millbrook Immunoserv). Total rabbit IgG from the resulting serum was pooled and purified with a process analogous to Thymoglobulin[®]. Control rabbit IgG was similarly purified from whole normal rabbit serum. mATG contains cytotoxic antibodies directed against antigens expressed on murine T and non-T lymphocytes as well as a wide variety of antigens as described previously (13, 18, 31, 32).

Treatment Protocols

mATG or control IgG was administered at a dose of 0.5 mg on day 0 and day 4 after transplantation as induction therapy; CTLA4Ig (Abatacept) is a human IgG1 fusion protein obtained from Bristol-Myers Squibb and was administered at a dose of 0.5 mg on day 0 and 0.25 mg on days 2, 4, 6, 8 and 10. Sirolimus was a generous gift of Wyeth Pharmaceuticals and administered at a dose of 0.3 mg/kg/day on days 0, 2, 4, 6, 8, and 10. CD25⁺ T cell depletion was achieved by treating mice pre-operatively with 0.5 mg of anti-CD25 mAb (PC61) on days 6 and 1 before transplantation. All treatments were administered by intraperitoneal injection.

Skin Transplantation

Full-thickness trunk skin grafts (1 cm²) harvested from BALB/c donors were transplanted onto the flank of B6 recipient mice, sutured with 6.0 silk, and secured with dry gauze and a bandage for 7 days. Skin graft survival was monitored daily thereafter, and rejection was defined as complete graft necrosis. For mechanistic studies bm12 skin was harvested and transplanted onto the flank of Thy 1.1 B6 recipients mice with the same technique.

Flow Cytometry Analysis

Recipient lymphocytes were isolated from the spleens and draining lymph nodes 7 days after transplantation. Cells were stained with anti-CD3 FITC, anti-CD19 PE, anti-CD8 APC and anti-CD4 PerCP to assess the depletion effect of mATG; T_{em} cells were determined by staining with anti-CD4 PerCP, anti-CD8 FITC, anti-CD62L APC, and anti-CD44 PE (all from BD Pharmingen) and analyzed for cells bearing the CD44^{hi}CD62L^{lo} phenotype. Cells were also stained with NK1.1 PE, anti-CD11c APC and anti-CD80 PE, anti-CD11b FITC and anti-F4/80 PE to identify NK cells, dendritic cell and macrophages respectively. T_{regs} were detected by staining with anti-CD4 FITC, anti-CD25 PE and intracellular staining with Foxp3 APC (eBioscience) as per manufacturer's instructions. Flow cytometry was performed using a FACSCaliburTM flow cytometry system and analyzed using CellQuestTM software (BD Biosciences). Results are representative of at least three independent experiments.

ELISPOT assay

Immunospot plates (Cellular Technology, Cleveland) were coated with anti-mouse IFN- γ or IL-4. Recipient splenocytes plus irradiated (30 Gy) allogeneic splenocytes (0.5×10^6 each) were cultured for 24 h (IFN- γ) or 48 h (IL-4), incubated with biotinylated anti-mouse IFN- γ or IL-4 and streptavidin horseradish peroxidase (DAKO), and developed with 3-amino-9-ethylcarbazole (Sigma-Aldrich). The resulting spots were counted on a computer-assisted immunospot image analyzer (T Spot Image Analyzer, Cellular Technology). mAbs were from Pharmingen.

Suppression Assay

Spleens and lymph nodes were harvested from B6 recipients of BALB/c skin 7 days post-transplantation, pooled single-cell leukocyte suspensions were prepared. CD4⁺ T cells were purified by magnetic bead negative selection (Miltenyi) with >90% purity. Then, CD25⁺ T

cells were isolated from the enriched CD4⁺ according to the manufacturers protocol (Miltenyi). The purity was consistently >95% for CD4⁺CD25⁻ and CD4⁺CD25⁺ T cell preparations. Isolated 0.4×10⁶ CD4⁺CD25⁻ T cells (T_{eff}) were incubated with 0.4×10⁶ irradiated (30 Gy) BALB/c allogeneic splenocytes (stimulators) in the IFN-γ ELISPOT assay cultures as described above. Decreasing numbers of sorted CD4⁺CD25⁺ T cells (T_{regs}) starting at 0.4×10⁶ from mATG or Control Ig treated recipients were added to the cultures in 1:1, 1:1/4, 1:1/16 T_{eff}/T_{reg} ratios. After 24 hours, frequency of IFN-γ producing cells was determined by counting spots. The percentage inhibition of IFN-γ production was calculated by applying the following formula:

$$[(T_{\text{eff}} \text{ IFN-}\gamma \text{ spots}) - (T_{\text{eff}} + T_{\text{reg}} \text{ IFN-}\gamma \text{ spots})] / (T_{\text{eff}} \text{ IFN-}\gamma \text{ spots}) \times 100.$$

Adoptive transfer of Thy1.2⁺Foxp3GFP^{rep} TCR-tg T cells

Adoptive transfer of Thy1.2⁺Foxp3GFP^{rep} TCR-tg T cells was performed as previously described in the ABM TCR-tg system (33). Briefly, spleens and lymph nodes were harvested from ABM-Foxp3GFP^{rep} mice and pooled single-cell leukocyte suspensions were prepared. CD4⁺ T cells were purified by magnetic bead positive selection (Miltenyi) with > 90 % purity. ABM-Foxp3GFP^{rep} CD4⁺ T cells express CD90.2 (Thy 1.2⁺) on their surface. More than 90 % of CD4⁺ T cells expressed the tg TCR, and approximately 2% of CD4⁺ T cells were GFP⁺. CD4⁺ isolated cells were then flow-sorted for GFP^{+/−} cells with a > 98% purity. 0.3 × 10⁶ Thy1.2⁺Foxp3GFP positive or negative cells were then injected intraperitoneally into Thy 1.1 B6 mice one day before skin transplantation (day −1). Recipients were sacrificed on day 7 following transplantation and draining axillary lymph nodes and spleens were collected, single-cell leukocyte suspensions were prepared and Thy1.2⁺Foxp3GFP^{+/−} TCR-tg T cells were identified by flow cytometry.

Immunohistochemistry staining

Skin graft samples were obtained at each time point in each treatment group, fixed in formalin 10% and stained with H&E for evaluation of cellular infiltration.

Statistics

Graft survival was expressed graphically by the Kaplan-Meier method, and statistical differences in survival between groups were assessed by the log-rank test. Student *t* test was used for comparison of means. *P*<0.05 was considered statistically significant.

RESULTS

mATG prolongs allograft survival by depleting naïve T cells and preserving natural T_{regs}

We first examined the depletion effect of mATG in naïve mice. As expected, mATG caused profound (> 85%) depletion of CD3⁺ (0.2×10⁶ vs. 2.8×10⁶; *p* < 0.00002), CD4⁺ (0.15×10⁶ vs. 1.8×10⁶; *p* < 0.00001) and CD8⁺ (0.05×10⁶ vs. 0.88×10⁶; *p* < 0.00002) cells in the lymph nodes compared to control immunoglobulin (Ig) treated mice. Similar changes were seen in the spleen [CD3⁺ (1.8×10⁶ vs. 19.8×10⁶; *p* < 0.00001), CD4⁺ (1.3×10⁶ vs. 12.9×10⁶; *p* < 0.00002) and CD8⁺ (0.5×10⁶ vs. 6.2×10⁶; *p* < 0.000003)]. There was no decrease in the number of CD19⁺ cells with mATG in the LN (4.2×10⁶ vs. 3.5×10⁶; *p* = NS) or spleen

(29×10^6 vs. 34×10^6 ; $p = \text{NS}$). Next, we examined the kinetics of depletion and recovery of lymphocyte populations following mATG in the full MHC-mismatched skin transplant recipients treated with or without mATG induction therapy. We noted profound ($> 80\%$) and persistent depletion of $\text{CD}3^+$, $\text{CD}4^+$ and $\text{CD}8^+$ T cells in the graft draining lymph nodes particularly at day 7, but also at 14 and 28 days after transplantation compared to control Ig treated allograft recipients (Figure 1A). Similar changes were seen in spleen (Figure 1A). Despite the profound depletion of T cells, we noted relative preservation of number of $\text{Foxp}3^+$ natural T_{regs} in the lymph nodes (LN) of naïve mice at day 7 post-mATG induction therapy (0.17×10^6 vs. 0.14×10^6 ; $p = \text{NS}$) and throughout the time-course in mATG treated skin allograft recipients compared to control Ig treated recipients, resulting in significantly higher percentage of T_{regs} at days 7 and 14 post-transplantation; and in due course declining to levels seen in control recipients by day 28 post-transplantation (Figure 1B). Similar changes were seen in spleen (Figure 1B). Next, we assessed the function of these preserved T_{regs} *in vitro* and noted that T_{regs} from mATG treated mice suppress alloantigen-specific $\text{IFN-}\gamma$ production more efficiently compared to T_{regs} from Control Ig treated mice at each of the $\text{T}_{\text{eff}}/\text{T}_{\text{reg}}$ ratios tested (Figure 1C). This preservation of functional T_{regs} with mATG however, led to only modest prolongation of the stringent full MHC-mismatched skin allograft survival in recipients treated with mATG induction therapy compared to Control Ig treated recipients [median graft survival time (MST) = 15.5 days vs. 9 days respectively; $p < 0.0004$] (Figure 1D).

mATG synergizes with CTLA4Ig and SRL to prolong allograft survival

In an attempt to overcome the rejection of skin allograft despite profound T cell depletion with mATG induction therapy, we initially combined CTLA4Ig with mATG and noted significant prolongation of full MHC-mismatched skin allograft survival in recipients treated with mATG induction therapy and short course CTLA4Ig compared to recipients treated with mATG induction therapy alone (MST 36 days vs. 15.5 days respectively; $p < 0.003$) (Figure 2A). Next, we examined the effect of adding short-course sirolimus (SRL) to this regimen. Interestingly, there was significant prolongation of graft survival in recipients treated with combined mATG induction, short-course CTLA4Ig and SRL therapy compared to mATG induction and short-course CTLA4Ig (MST 58 vs. 36 days respectively; $p < 0.001$) or short-course CTLA4Ig and SRL therapy without mATG induction (MST 58 vs. 17 days respectively; $p < 0.0007$) (Figure 2A). We then examined the allospecific cytokine production, by splenocytes from full MHC-mismatched skin allograft recipients, 7 days after transplantation. The inhibition of graft rejection with mATG induction therapy alone or in combination with short-course CTLA4Ig, SRL, or CTLA4Ig + SRL was associated with almost complete inhibition of $\text{IFN-}\gamma$ and significant inhibition of IL-4 by splenocytes from these recipients (Figure 2B). Histological examination showed well-preserved grafts with no evidence of rejection in grafts harvested from recipients treated with combination therapy compared to control Ig or mATG induction therapy alone (Figure 2C).

Beneficial effect of mATG alone or in combination with CTLA4Ig and/or SRL on allograft survival is dependent on $\text{CD}25^+$ T cells

To determine if prolonged allograft survival with mATG alone or in combination with CTLA4Ig and/or SRL is dependent on T_{regs} , we administered anti- $\text{CD}25$ mAb to the

recipients prior to full MHC-mismatched skin transplantation. CD25⁺ T cell depletion prior to transplantation led to abrogation of the beneficial effect of mATG induction therapy alone or in combination with CTLA4Ig and/or SRL on allograft survival (Figure 3A), indicating that T_{regs} are required for the graft prolonging effect of mATG. Next, to investigate whether T_{reg} generation by the thymus or peripheral conversion of naïve T cells to T_{regs} and/or expansion of preexisting T_{regs} in the periphery is responsible for the beneficial effects of mATG, we performed thymectomy in the recipients 3 weeks prior to transplantation. Interestingly, thymectomy prior to transplantation did not affect the graft prolonging effect of mATG induction therapy whilst CD25⁺ T cell depletion in thymectomized recipients did abrogate the graft prolonging effect of mATG (Figure 3B), indicating that thymic export of T_{regs} is not required, whilst the presence of peripheral T_{regs} (expansion of preexisting T_{regs} and/or peripheral conversion of naïve T cells to T_{regs}) is required for the beneficial effect of mATG on allograft survival.

CTLA4Ig and Sirolimus inhibit effector-memory differentiation and promote regulation when combined with mATG

To further understand the mechanisms of graft prolongation with mATG-based combination therapy, we utilized the TCR-transgenic-reporter mouse model. 0.3×10^6 CD4⁺Thy1.2⁺ABMFoxp3GFP⁺ or CD4⁺Thy1.2⁺ABMFoxp3GFP⁻ cells were injected in Thy1.1⁺C57BL/6 mice with or without bm12 skin transplantation. In these mice we first studied the effect of mATG induction therapy on antigen-specific T_{reg} (Thy1.2⁺Foxp3GFP⁺) population by analyzing the adoptively transferred CD4⁺Thy1.2⁺ABMFoxp3GFP⁺ cells. In keeping with our previous *in vitro* observations with Thymoglobulin (19), in the case of CD4⁺Thy1.2⁺ABMFoxp3GFP⁺ cell-adoptive transfer, we found mATG induction therapy preserved Thy1.2⁺Foxp3GFP⁺ cells and promoted their expansion *in vivo* compared to control Ig treatment (0.4×10^6 vs. 0.04×10^6 respectively; $p < 0.03$) (Figure 4A). Further, in the case of CD4⁺Thy1.2⁺ABMFoxp3GFP⁻ cell-adoptive transfer, mATG significantly promoted conversion of Thy1.2⁺Foxp3GFP⁻ cells to Thy1.2⁺Foxp3GFP⁺ cells compared to control Ig treatment (0.53×10^6 vs. 0.26×10^6 respectively; $p < 0.04$) (Figure 4B). Despite the preferential preservation of Thy1.2⁺Foxp3GFP⁺ cells and conversion of Thy1.2⁺Foxp3GFP⁻ cells to Thy1.2⁺Foxp3GFP⁺ cells with mATG, the effect of mATG induction therapy on skin allograft survival was modest (Figure 1D). We reasoned that rejection might be occurring due to an increase in the T_{em} (Thy1.2⁺Foxp3GFP⁻CD44^{hi}CD62L^{lo}) populations in response to the alloantigen and/or homeostatic proliferation in the aftermath of T cell depletion with mATG induction therapy. Interestingly, combining mATG induction therapy with short-course CTLA4Ig and SRL either individually or together affords significant synergy in promoting allograft survival (Figure 2A). To understand the mechanisms of this synergy, we next studied the effect of mATG induction therapy in combination with CTLA4Ig and SRL individually or together on antigen specific T_{reg} and T_{em} populations and evaluated the T_{reg}/T_{em} ratio in various treatment groups 7 days after bm12 skin transplantation utilizing the ABM-Foxp3GFP^{rep} model. Interestingly we found that combining CTLA4Ig or SRL with mATG induction therapy led to not only an increase in Thy1.2⁺Foxp3GFP⁺ cells by preservation and conversion but also a significant inhibition of generation of Thy1.2⁺Foxp3GFP⁻ cells with the T_{em} phenotype resulting in a favorable T_{reg}/T_{em} ratio (Figure 4C).

mATG preserves T_{regs} which limit homeostatic proliferation of memory cells following T cell depletion and the prolonged allograft survival is dependent on favorable $T_{\text{reg}}/T_{\text{em}}$ balance

We investigated whether the T_{regs} spared by mATG limit homeostatic proliferation of T_{em} cells and affect graft survival, by pre-depleting $CD25^{+}$ cells and then transplanting B6 mice with full MHC-mismatched BALB/c skin grafts or not and treated with mATG induction therapy alone or in combination with CTLA4Ig+SRL. We found that T cells that emerge in the aftermath of depletion with mATG are more skewed towards a T_{em} ($CD44^{\text{hi}}CD62L^{\text{lo}}$) phenotype in both naïve and transplanted mice treated with mATG or mATG+CTLA4Ig+SRL when $CD25^{+}$ cells are pre-depleted compared to controls (with no $CD25^{+}$ cell depletion) (Table 1). Consequently, $T_{\text{reg}}/T_{\text{em}}$ balance in naïve and transplanted mice is switched in favor of T_{em} cells with $CD25$ pre-depletion and results in accelerated allograft rejection, most dramatically in the mATG+CTLA4Ig+SRL group (Table 1).

DISCUSSION

mATG, like the other preparations of anti-thymocyte globulins, depletes T cells effectively both in naïve and transplanted animals (Figure 1A). In keeping with our previous studies with Thymoglobulin (19), T_{regs} were preferentially spared from depletion with mATG (Figure 1B). Minamimura et al. reported that naïve $CD25^{+}$ T cells are resistant to depletion by anti-lymphocyte serum and this is thought to be due to their higher expression of anti-apoptotic gene $Bcl-X_L$. Interestingly, on further analysis, $Bcl-X_L$ protein expression by flow cytometry appeared to be highest in $Foxp3^{+}$ cells regardless of IL-2R α -chain ($CD25$) expression (34), explaining the relative sparing of $Foxp3^{+}$ cells from the depletion effect of mATG in our studies (Figure 1B).

The fate of the alloimmune response is dependent on the balance between alloreactive T_{em} and T_{regs} *in vivo*. mATG prolonged allograft survival by profound T cell depletion and preferential sparing of T_{regs} (Figure 1B and 1C) and this effect was independent of recent thymic immigrants but dependent on expansion of preexisting T_{regs} and/or peripheral conversion of naïve T cells to T_{regs} (Figure 3B). Of note, the prolongation of graft survival with mATG treatment was modest. This is of particular clinical relevance since homeostatic proliferation is thought to be a barrier to transplantation tolerance induction in both reconstituted *scid* mice and WT mice treated with T cell-depleting antibodies (35). Further, in an appropriate environment, $Foxp3^{+}$ T_{regs} can differentiate into effector cells (36–38). Recently it was demonstrated that $Foxp3^{+}$ cells consists of a heterogeneous population and $Foxp3^{+}CD25^{-}$ cells retain developmental plasticity and preferentially proliferate into cells with effector phenotype in a lymphopenic or inflammatory environment but maintain some regulatory function (39). On the other hand, it has been noted that naïve T cells readily express $Foxp3$ in response to alloantigen (40). In keeping with this we noted significant conversion of adoptively transferred T_{regs} into T_{em} cells and vice versa (Figure 4A and 4B) in the context of transplantation and treatment with Control Ig or mATG. However, our data suggests that mATG, unlike Control Ig and other T cell depleting therapies (21), preserves T_{regs} and limits conversion to/expansion of T_{em} cells (Figure 1B and 4). Further, these preserved T_{regs} themselves undergo homeostatic proliferation as suggested by an elegant

study that showed CD4⁺CD25⁺ T_{reg} cells are also capable of undergoing homeostatic proliferation in a lymphopenic environment and acquire memory-like phenotype (CD44^{high}CD45RB^{low} expression) with loss of CD25 and CD62L expression but exhibit substantially augmented suppressive function (41). Furthermore, T_{regs} are thought to play a major role in regulating lymphopenia-induced T cell proliferation and acquisition of T_{em} phenotype (42). Moreover, *ex-vivo* expanded antigen-specific natural T_{regs} synergize with host T cell depletion and promote long-term allograft acceptance (43). In keeping with these, our data suggests that mATG preserves CD25⁺ natural T_{regs} which limit skewing of T cell repertoire toward T_{em} phenotype among the recovering T cells by inhibiting both lymphopenia-induced and alloantigen-induced proliferation of T cells (Table 1).

B7/CD28 blockade with CTLA4Ig, on the other hand, has also been demonstrated to be effective in controlling the homeostasis of T_{em} cells by reducing expansion of central memory cells and migration of activated memory CD4 cells to the periphery (44). Further, costimulation requirement of alloreactive T cells is critically dependent on their precursor frequency, where recipients with low CD4⁺ and CD8⁺ donor-reactive T cell frequencies exhibited long-term skin graft survival upon CD28/CD154 blockade, whereas simultaneously raising the frequency of CD4⁺ T cells to 0.5% and CD8⁺ T cells to 5% precipitated graft rejection despite CD28/CD154 blockade (45). These, taken together with our data (Figure 2A and 4), indicate that T cell depletion with mATG may limit the precursor frequency of alloreactive T cells and render them more susceptible to costimulation blockade. Further, adding SRL to costimulation (B7-CD28 and CD40-CD40L pathways) blockade prolonged skin and heart graft survival in murine models by two main mechanisms: inhibition of proliferation of alloreactive T cells and induction of apoptosis of dividing alloreactive T cells (46). A more recent study in human renal transplant recipients demonstrated that combined treatment with ATG, SRL and mycophenolate mofetil was associated with a progressive increase in T_{reg} population both in the periphery and in the graft infiltrating cells with concomitant decrease of donor responsiveness *in vitro* (47). Moreover, both CTLA4Ig and SRL inhibit lymphopenia-induced and alloantigen-induced proliferation of T cells (48, 49). Consistent with these data, we observed significant synergy on combining mATG with CTLA4Ig and SRL individually or together (Figure 2A and 4). The beneficial effects of this combination therapy were partly dependent on CD25⁺ T_{regs} indicating that T_{regs} are required for the graft prolonging effect of combination therapy involving mATG (Figure 3A). These data emphasize the advantages of this novel strategy of combining mATG with T cell costimulatory blockade and SRL to preserve T_{regs} and limit homeostatic proliferation of effector and memory T cells to promote regulation in human organ transplantation.

In summary, our data demonstrate that the combination of mATG with costimulation blockade and sirolimus represents an effective strategy in preventing allograft rejection and promoting graft acceptance in a full MHC-mismatched stringent skin transplant model. The effectiveness of this combination strategy depends not only on the depletion effects of mATG but also on generating a favourable T_{reg}/T_{em} balance. These data will serve as the basis of further studies in non-human primates to develop a clinically applicable protocol to promote regulation in transplantation.

Acknowledgments

Funding sources:

This work was supported by grants from the NIH:

RO1AI-51559, R01AI-37691 and PO1-AI41521 (MHS).

FD was supported by American Society of Transplantation (AST) Basic Science Fellowship Grant Award

XY was supported in part by American Society of Transplantation (AST) Basic Science Faculty Development Grant Award.

MJA is supported in part by the AST-Wyeth Basic Science Faculty Development Grant Award and NIH Grant K08 AI080836-01.

References

1. Zheng XX, Sanchez-Fueyo A, Domenig C, Strom TB. The balance of deletion and regulation in allograft tolerance. *Immunol Rev.* 2003; 196:75. [PubMed: 14617199]
2. Shevach EM, DiPaolo RA, Andersson J, Zhao DM, Stephens GL, Thornton AM. The lifestyle of naturally occurring CD4⁺ CD25⁺ Foxp3⁺ regulatory T cells. *Immunol Rev.* 2006; 212:60. [PubMed: 16903906]
3. Sakaguchi S, Ono M, Setoguchi R, et al. Foxp3⁺ CD25⁺ CD4⁺ natural regulatory T cells in dominant self-tolerance and autoimmune disease. *Immunol Rev.* 2006; 212:8. [PubMed: 16903903]
4. Rouse BT, Sarangi PP, Suvas S. Regulatory T cells in virus infections. *Immunol Rev.* 2006; 212:272. [PubMed: 16903920]
5. Aluvihare VR, Kallikourdis M, Betz AG. Regulatory T cells mediate maternal tolerance to the fetus. *Nat Immunol.* 2004; 5 (3):266. [PubMed: 14758358]
6. Joffre O, Santolaria T, Calise D, et al. Prevention of acute and chronic allograft rejection with CD4⁺CD25⁺Foxp3⁺ regulatory T lymphocytes. *Nat Med.* 2008; 14 (1):88. [PubMed: 18066074]
7. Xia G, He J, Zhang Z, Leventhal JR. Targeting acute allograft rejection by immunotherapy with ex vivo-expanded natural CD4⁺ CD25⁺ regulatory T cells. *Transplantation.* 2006; 82 (12):1749. [PubMed: 17198271]
8. Bingaman AW, Farber DL. Memory T cells in transplantation: generation, function, and potential role in rejection. *Am J Transplant.* 2004; 4 (6):846. [PubMed: 15147417]
9. Rothstein DM. Immunosuppression and regulation: cast in new light? *J Am Soc Nephrol.* 2006; 17 (10):2644. [PubMed: 16971654]
10. Starzl TE, Marchioro TL, Hutchinson DE, Porter KA, Cerilli GJ, Brettschneider L. The clinical use of antilymphocyte globulin in renal homotransplantation. *Transplantation.* 1967; 5(4):Suppl:1100.
11. Beiras-Fernandez A, Thein E, Hammer C. Induction of immunosuppression with polyclonal antithymocyte globulins: an overview. *Exp Clin Transplant.* 2003; 1 (2):79. [PubMed: 15859913]
12. Genestier L, Fournel S, Flacher M, Assossou O, Revillard JP, Bonnefoy-Berard N. Induction of Fas (Apo-1, CD95)-mediated apoptosis of activated lymphocytes by polyclonal antithymocyte globulins. *Blood.* 1998; 91 (7):2360. [PubMed: 9516135]
13. Michallet MC, Preville X, Flacher M, Fournel S, Genestier L, Revillard JP. Functional antibodies to leukocyte adhesion molecules in antithymocyte globulins. *Transplantation.* 2003; 75 (5):657. [PubMed: 12640305]
14. Lowsky R, Takahashi T, Liu YP, et al. Protective conditioning for acute graft-versus-host disease. *N Engl J Med.* 2005; 353 (13):1321. [PubMed: 16192477]
15. Kang SM, Tang Q, Bluestone JA. CD4⁺CD25⁺ regulatory T cells in transplantation: progress, challenges and prospects. *Am J Transplant.* 2007; 7 (6):1457. [PubMed: 17511675]
16. Brennan DC. Faith supported by reason: mechanistic support for the use of polyclonal antibodies in transplantation. *Transplantation.* 2003; 75 (5):577. [PubMed: 12664887]

17. Mohty M. Mechanisms of action of antithymocyte globulin: T-cell depletion and beyond. *Leukemia*. 2007; 21 (7):1387. [PubMed: 17410187]
18. Rebellato LM, Gross U, Verbanac KM, Thomas JM. A comprehensive definition of the major antibody specificities in polyclonal rabbit antithymocyte globulin. *Transplantation*. 1994; 57 (5): 685. [PubMed: 7908150]
19. Lopez M, Clarkson MR, Albin M, Sayegh MH, Najafian N. A novel mechanism of action for antithymocyte globulin: induction of CD4+CD25+Foxp3+ regulatory T cells. *J Am Soc Nephrol*. 2006; 17 (10):2844. [PubMed: 16914538]
20. Huang Y, Parker M, Xia C, et al. Rabbit polyclonal mouse antithymocyte globulin administration alters dendritic cell profile and function in NOD mice to suppress diabetogenic responses. *J Immunol*. 2009; 182 (8):4608. [PubMed: 19342635]
21. Neujahr DC, Chen C, Huang X, et al. Accelerated memory cell homeostasis during T cell depletion and approaches to overcome it. *J Immunol*. 2006; 176 (8):4632. [PubMed: 16585554]
22. Battaglia M, Stabilini A, Roncarolo MG. Rapamycin selectively expands CD4+CD25+FoxP3+ regulatory T cells. *Blood*. 2005; 105 (12):4743. [PubMed: 15746082]
23. Basu S, Golovina T, Mikheeva T, June CH, Riley JL. Cutting edge: Foxp3-mediated induction of pim 2 allows human T regulatory cells to preferentially expand in rapamycin. *J Immunol*. 2008; 180 (9):5794. [PubMed: 18424697]
24. Taylor PA, Noelle RJ, Blazar BR. CD4(+)CD25(+) immune regulatory cells are required for induction of tolerance to alloantigen via costimulatory blockade. *J Exp Med*. 2001; 193 (11):1311. [PubMed: 11390438]
25. van Maurik A, Herber M, Wood KJ, Jones ND. Cutting edge: CD4+CD25+ alloantigen-specific immunoregulatory cells that can prevent CD8+ T cell-mediated graft rejection: implications for anti-CD154 immunotherapy. *J Immunol*. 2002; 169 (10):5401. [PubMed: 12421913]
26. Coenen JJ, Koenen HJ, van Rijssen E, Hilbrands LB, Joosten I. Tolerizing effects of co-stimulation blockade rest on functional dominance of CD4+CD25+ regulatory T cells. *Transplantation*. 2005; 79 (2):147. [PubMed: 15665762]
27. Coenen JJ, Koenen HJ, van Rijssen E, Boon L, Joosten I, Hilbrands LB. CTLA-4 engagement and regulatory CD4+CD25+ T cells independently control CD8+-mediated responses under costimulation blockade. *J Immunol*. 2006; 176 (9):5240. [PubMed: 16621989]
28. Larsen CP, Knechtle SJ, Adams A, Pearson T, Kirk AD. A new look at blockade of T-cell costimulation: a therapeutic strategy for long-term maintenance immunosuppression. *Am J Transplant*. 2006; 6 (5 Pt 1):876. [PubMed: 16611323]
29. Simon G, Parker M, Ramiya V, et al. Murine antithymocyte globulin therapy alters disease progression in NOD mice by a time-dependent induction of immunoregulation. *Diabetes*. 2008; 57 (2):405. [PubMed: 18039815]
30. Ruzek MC, Waire JS, Hopkins D, et al. Characterization of in vitro antimurine thymocyte globulin-induced regulatory T cells that inhibit graft-versus-host disease in vivo. *Blood*. 2008; 111 (3):1726. [PubMed: 18025149]
31. Bonnefoy-Berard N, Vincent C, Revillard JP. Antibodies against functional leukocyte surface molecules in polyclonal antilymphocyte and antithymocyte globulins. *Transplantation*. 1991; 51 (3):669. [PubMed: 2006524]
32. Bourdage JS, Hamlin DM. Comparative polyclonal antithymocyte globulin and antilymphocyte/antilymphoblast globulin anti-CD antigen analysis by flow cytometry. *Transplantation*. 1995; 59 (8):1194. [PubMed: 7732566]
33. Sayegh MH, Wu Z, Hancock WW, et al. Allograft rejection in a new allospecific CD4+ TCR transgenic mouse. *Am J Transplant*. 2003; 3 (4):381. [PubMed: 12694059]
34. Minamimura K, Gao W, Maki T. CD4+ regulatory T cells are spared from deletion by antilymphocyte serum, a polyclonal anti-T cell antibody. *J Immunol*. 2006; 176 (7):4125. [PubMed: 16547248]
35. Wu Z, Bensinger SJ, Zhang J, et al. Homeostatic proliferation is a barrier to transplantation tolerance. *Nat Med*. 2004; 10 (1):87. [PubMed: 14647496]

36. Kaspirowicz DJ, Droin N, Soper DM, Ramsdell F, Green DR, Ziegler SF. Dynamic regulation of FoxP3 expression controls the balance between CD4⁺ T cell activation and cell death. *Eur J Immunol.* 2005; 35 (12):3424. [PubMed: 16285010]
37. Xu L, Kitani A, Fuss I, Strober W. Cutting edge: regulatory T cells induce CD4⁺CD25⁺Foxp3⁺ T cells or are self-induced to become Th17 cells in the absence of exogenous TGF- β . *J Immunol.* 2007; 178 (11):6725. [PubMed: 17513718]
38. Yang XO, Nurieva R, Martinez GJ, et al. Molecular antagonism and plasticity of regulatory and inflammatory T cell programs. *Immunity.* 2008; 29 (1):44. [PubMed: 18585065]
39. Komatsu N, Mariotti-Ferrandiz ME, Wang Y, Malissen B, Waldmann H, Hori S. Heterogeneity of natural Foxp3⁺ T cells: a committed regulatory T-cell lineage and an uncommitted minor population retaining plasticity. *Proc Natl Acad Sci U S A.* 2009; 106 (6):1903. [PubMed: 19174509]
40. Gao W, Lu Y, El Essawy B, Oukka M, Kuchroo VK, Strom TB. Contrasting effects of cyclosporine and rapamycin in de novo generation of alloantigen-specific regulatory T cells. *Am J Transplant.* 2007; 7 (7):1722. [PubMed: 17511761]
41. Gavin MA, Clarke SR, Negrou E, Gallegos A, Rudensky A. Homeostasis and anergy of CD4⁺CD25⁺ suppressor T cells in vivo. *Nat Immunol.* 2002; 3 (1):33. [PubMed: 11740498]
42. Curotto de Lafaille MA, Shen S, Olivares-Villagomez D, Camps-Ramirez M, Lafaille JJ. Do regulatory T cells play a role in the control of homeostatic proliferation? *Int Rev Immunol.* 2005; 24 (3-4):269. [PubMed: 16036378]
43. Xia G, He J, Leventhal JR. Ex vivo-expanded natural CD4⁺CD25⁺ regulatory T cells synergize with host T-cell depletion to promote long-term survival of allografts. *Am J Transplant.* 2008; 8 (2):298. [PubMed: 18190656]
44. Ndejmbi MP, Teijaro JR, Patke DS, et al. Control of memory CD4 T cell recall by the CD28/B7 costimulatory pathway. *J Immunol.* 2006; 177 (11):7698. [PubMed: 17114440]
45. Ford ML, Koehn BH, Wagener ME, et al. Antigen-specific precursor frequency impacts T cell proliferation, differentiation, and requirement for costimulation. *J Exp Med.* 2007; 204 (2):299. [PubMed: 17261633]
46. Li Y, Li XC, Zheng XX, Wells AD, Turka LA, Strom TB. Blocking both signal 1 and signal 2 of T-cell activation prevents apoptosis of alloreactive T cells and induction of peripheral allograft tolerance. *Nat Med.* 1999; 5 (11):1298. [PubMed: 10545997]
47. Bestard O, Cruzado JM, Mestre M, et al. Achieving donor-specific hyporesponsiveness is associated with FOXP3⁺ regulatory T cell recruitment in human renal allograft infiltrates. *J Immunol.* 2007; 179 (7):4901. [PubMed: 17878390]
48. Hagen KA, Moses CT, Drasler EF, Podetz-Pedersen KM, Jameson SC, Khoruts A. A role for CD28 in lymphopenia-induced proliferation of CD4 T cells. *J Immunol.* 2004; 173 (6):3909. [PubMed: 15356139]
49. Minamimura K, Sato K, Yagita H, Tanaka T, Arai S, Maki T. Strategies to induce marked prolongation of secondary skin allograft survival in alloantigen-primed mice. *Am J Transplant.* 2008; 8 (4):761. [PubMed: 18261171]

Fig. 1B

■ Control Ig
▲ mATG

Fig. 1C

Figure 1. mATG depletes naïve T cells, preserves functional natural T_{regs} and prolongs allograft survival

A. Line graphs depicting absolute numbers of CD3⁺, CD4⁺, CD8⁺ and CD19⁺ cells in LN (left column) and spleen (right column) on days -1, 7, 14 and 28 in relation to transplantation with mATG induction or control Ig treatment. **B.** Line graphs depicting absolute numbers and percentage of T_{regs} in LN (left column) and spleen (right column) on days -1, 7, 14 and 28 in relation to transplantation with mATG induction or control Ig treatment. **C.** Bar graph depicting suppression of IFN- γ production, from a maximum of 140 IFN- γ spots by T_{eff} from transplanted untreated recipients, by T_{regs} from mATG or Control Ig treated recipients. **D.** Kaplan-Meier graphs depicting full MHC-mismatched skin allograft survival with mATG induction or control Ig treatment.

Fig. 2A

Fig. 2B

Figure 2. Combination therapy synergizes to prolong graft survival, inhibit allospecific cytokine production and preserves graft histology

A. Kaplan-Meier graphs depicting full MHC-mismatched skin allograft survival with mATG in combination with CTLA4Ig and/or SRL or mATG induction alone. **B.** Bar graphs depicting production of allospecific cytokine IFN- γ (left panel) and IL-4 (right panel), by ELISPOT assay with splenocytes from full MHC-mismatched skin allograft recipients, 7 days after transplantation with various treatments. **C.** H and E stained light micrographs depicting features of acute rejection in grafts harvested 7 days after transplantation from recipients treated with control Ig (left upper panel); minimal infiltration of grafts harvested 7 days after transplantation (right upper panel) compared to features of acute rejection in grafts harvested 14 days after transplantation from recipients treated with mATG induction therapy alone (left lower panel); well-preserved graft with no evidence of rejection in grafts harvested from recipients treated with combination therapy (right lower panel).

Fig. 3A

Figure 3. Prolonged allograft survival with mATG alone or in combination with CTLA4Ig and/or SRL is dependent on CD25⁺ T cells

A. Kaplan-Meier graphs depicting full MHC-mismatched skin allograft survival with CD25 pre-depletion and mATG in combination with CTLA4Ig and/or SRL or mATG induction alone. **B.** Kaplan-Meier graphs depicting full MHC-mismatched skin allograft survival in thymectomized or non-thymectomized recipients with or without CD25 pre-depletion and mATG induction therapy.

Fig.4 A

Fig. 4C

Figure 4. CTLA4Ig and Sirolimus inhibit effector-memory differentiation and promote favourable T_{reg}/T_{em} balance when combined with mATG

A. Bar graphs depicting absolute numbers of Thy1.2⁺Foxp3GFP⁺ or Thy1.2⁺Foxp3GFP⁻ populations 7 days after transplantation, in both lymph nodes and spleen from recipients adoptively transferred with ABM TCR-tg Thy1.2⁺Foxp3GFP⁺ cells and treated with mATG

induction therapy alone, in combination with CTLA4Ig and/or SRL or control Ig. Representative histograms of Thy1.2⁺Foxp3GFP⁺ cells from recipients in each treatment group are shown below respective bar graphs. There is significant preservation of Thy1.2⁺Foxp3GFP⁺ T_{reg} cells and inhibition of Thy1.2⁺Foxp3GFP⁻CD44^{hi}CD62L^{low} T_{em} cells in recipients treated with either mATG induction therapy alone or in combination with SRL, CTLA4Ig or SRL+CTLA4Ig (*p < 0.05 for each group compared with Control Ig treatment). There is no significant difference in either the T_{reg} or T_{em} cells between Control Ig, CTLA4Ig and SRL alone treatment groups (p = NS between each group). **B.** Bar graphs depicting absolute numbers of Thy1.2⁺Foxp3GFP⁺ or Thy1.2⁺Foxp3GFP⁻ populations 7 days after transplantation, in both lymph nodes and spleen from recipients adoptively transferred with ABM TCR-tg Thy1.2⁺Foxp3GFP⁻ cells and treated with mATG induction therapy alone, in combination with CTLA4Ig and/or SRL or control Ig. Representative histograms of Thy1.2⁺Foxp3GFP⁺ cells from recipients in each treatment group are shown below respective bar graphs. There is significant conversion of Thy1.2⁺Foxp3GFP⁻ cells to Thy1.2⁺Foxp3GFP⁺ T_{reg} cells in recipients treated with either mATG induction therapy alone or in combination with SRL or CTLA4Ig (*p < 0.02 for each group compared with Control Ig treatment). There is significant inhibition of Thy1.2⁺Foxp3GFP⁻CD44^{hi}CD62L^{low} T_{em} cells in recipients treated with mATG alone, or in combination with CTLA4Ig or SRL+CTLA4Ig (#p < 0.02 for each group compared with Control Ig treatment). There is no significant difference in either the T_{reg} or T_{em} cells between Control Ig, CTLA4Ig, SRL alone treatment groups (p = NS between each group). **C.** Scatter plots depicting T_{reg}/T_{em} ratios 7 days after transplantation, in both lymph nodes and spleen from recipients adoptively transferred with ABM TCR-tg Thy1.2⁺Foxp3GFP⁺ (left panel) or Thy1.2⁺Foxp3GFP⁻ (right panel) cells and treated with mATG induction therapy alone, in combination with CTLA4Ig and/or SRL or control Ig.

Table 1

mATG preserves T_{regs} which limit homeostatic proliferation of memory cells following T cell depletion

Therapy	Transplant	CD25 pre-depletion	Treg (n)	Teff (n)	Treg/Teff Ratio	MST (days)
mATG	No	No	166500±61	267365±2	0.6	NA
mATG	No	Yes	41585±6 p<0.03	378833±25 p<0.007	0.1; p<0.02	NA
mATG	Yes	No	98214±3	140884±5	0.7	15.5
mATG	Yes	Yes	17377±6 p<0.02	379366±12 p<0.03	0.05; p<0.00001	12.5; p<0.003
mATG+CTLA4Ig+SRL	Yes	No	26441±2	31518±14	0.9	58
mATG+CTLA4Ig+SRL	Yes	Yes	14615±4 p<0.01	200110±60 p<0.009	0.07; p<0.0007	25; p<0.001

All p values are in comparison with no CD25 depletion in the respective group