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ATG BASED COMBINATION THERAPY – A NOVEL CLINICALY RELEVANT APPROACH TO PROMOTE REGULATION AND INDUCE LONG-TERM ALLOGRAFT SURVIVAL

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ABSTRACT

Regulatory T cells (T_{reg}) actively regulate alloimmune responses and promote transplantation tolerance. Polyclonal anti-thymocyte globulin (ATG), a widely used induction therapy in clinical organ transplantation, 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 regulatory T cells. Here we report a novel reagent, murine ATG (mATG), depletes T cells but preferentially spares $CD25^+$ natural T_{regs} which limit skewing of T cell repertoire toward T-effector-memory (T_{em}) phenotype among the recovering T cells. T-cell depletion with mATG combined with CTLA4Ig and Sirolimus 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. These results provide the rationale for translating such novel combination therapies to promote tolerance in primate and human organ transplantation.

INTRODUCTION

Allograft tolerance may be viewed as a balance between pro-inflammatory and anti-inflammatory forces, where different subsets of T cells can interact to create the appropriate environment for long-term graft acceptance. Regulatory T cells (T_{regs}), a T cell subset involved in modulating the immune response (32), have been demonstrated to play a critical role in preventing autoimmune diseases (30), regulating immune response during infectious diseases (29), maintaining feto-meternal tolerance (1), and also preventing allograft rejection in transplantation (15). Consequently, there is great interest in harnessing the power of Tregs to therapeutic advantage and indeed ex-vivo expanded natural $CD4^+CD25^+$ T_{reg} therapy has been used to prevent experimental acute allograft rejection (38). On the other hand T effector cells are thought to play a major part in mediating acute and chronic allograft rejection (4). 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 a long-term graft acceptance under a variety immunosuppressive treatment (28).

ATG, a polyclonal T cell depleting agent, has been used clinically since the early 80’s as induction therapy and for treatment of acute rejection in solid organ transplantation (2, 33). It consists of purified IgG fraction of sera from rabbits or horses. Rabbit ATG, 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 ATG 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 (13, 21, 2, 20, 16). Other potential mechanisms of action include modulation of surface adhesion receptor molecules or chemokine receptor expression (7). However the exact mechanisms of its action are still unknown and an ever-expanding array of target antigens is being identified in ATG preparations (24). Others and we have reported that besides profound peripheral T cell depletion, ATG 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 regulatory T cells (T_{regs}) (27, 19, 24). 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 Treg cells (26). Our group has previously demonstrated that low dose ATG can expand T_{regs} ex vivo, inviting a fresh look at the tolerogenic potential of this T cell depletion strategy (19). Similarly, sirolimus (SRL), another widely used immunosuppressive agent in clinical transplantation, is thought to expand naturally occurring T_{regs}. Further, strategies to block T cell costimulatory pathway 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 (8, 9, 34, 35, 17) 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 ATG, SRL and costimulatory blockade in vivo in a fully allogeneic mouse skin allograft model. This is the first report to establish that mATG preserves $CD25^+$ natural Tregs which limit skewing of

T cell repertoire toward Teff/mem phenotype among the recovering T cells and that simultaneous T-cell depletion combined with SRL and costimulatory blockade synergize to prolong allograft survival by limiting the effector-memory alloreactive T-cell pool and that this effect is dependent on $CD25^+$ Tregs in a clinically relevant stringent transplant model. These data emphasize the advantage of a novel strategy combining mATG with T cell costimulatory blockade to preserve Tregs and limit homeostatic proliferation of effector and memory T cells and provide the rationale to translate such a strategy to promote tolerance in human organ transplantation.

RESULTS

Generation and characterization of mATG

Murine ATG was generated 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). Thymocyte suspensions were prepared from extracted thymuses and New Zealand white rabbits were immunized twice, 2 weeks apart and terminally bled 2 weeks following second immunization (Millbrook Immunoserv, Amherst, MA). Total rabbit IgG from the resulting serum was purified with a process analogous to that used for Thymoglobulin. Control rabbit IgG was similarly purified from whole normal rabbit serum (Sigma-Aldrich, St Louis, MO).

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 (day 7 after depletion with mATG). As expected, mATG caused profound (> 85%) depletion of $CD3^+$ (193050±62 vs. 2822639±18; $p < 0.00002$), $CD4^+$ (147537±45 vs. 1850720±100; $p < 0.00001$) and $CD8^+$ (53574±16 vs. 879043±58; $p < 0.00002$) cells in the lymph nodes compared to control immunoglobulin (Ig) treated mice. Similar changes were seen in the spleen (data not shown). Interestingly, there was no decrease in the number of $CD19^+$ cells with mATG (4167366±35 vs. 3434978±77; p = 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 $CD3^+$, $CD4^+$ and $CD8^+$ T cells in the graft draining lymph nodes particularly at day 7, but also 14 and 28 days after transplantation compared to control Ig treated allograft recipients (Figure 1A). Similar changes were seen in spleen (data not shown). Again, there was no decrease in the number of $CD19^+$ cells in allograft recipients treated with mATG induction therapy compared to control Ig at each of these time-points (Figure 1A). Despite the profound depletion of T cells, we noted significant preservation of number of $Foxp3^+$ natural T_{regs} in naïve mice at day 7 post-mATG induction therapy (166500±35770 vs 136900±15960, p=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 day 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 (data not shown). This preservation of 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 survival time (MST) = 15.5 days vs. MST = 9 days respectively; p < 00004] (Figure 1C).

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 stringent 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. MST = 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 days vs. MST = 36 days respectively; $p < 0.001$) (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 was associated with almost complete inhibition of IFN-γ and low but detectable production of IL-4 by splenocytes from recipients treated with mATG induction therapy alone or in combination with short-course CTLA4Ig+SRL (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 CD25$^+$ 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-CD25 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 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 a novel TCR-transgenic-reporter mouse model termed ABM-Foxp3GFP^{rep} [with a TCR that utilizes the Vα2.1 and Vβ8.1 chains and specifically recognizes the MHC class II molecule I-A^{bm12} – anti-bm12 (ABM) and expresses Foxp3-green fluorescence protein (GFP)]. Utilizing microbeads system, we first isolated $CD4^+$ cells from Thy1.2$^+$ABM-Fox3GFP^{rep} mice and sorted for GFP^+ and GFP^- populations by flow cytometry with > 98% purity. Then, $0.3x10^6$

$CD4^+Thy1.2^+ABMFoxp3GFP^+$ 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 (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 (391178±92 vs. 42911±19 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 (529341±97 vs. 262120±96 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. 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} ($Thy1.2^+Foxp3GFP^+$) and T_{em}

(Thy1.2^{+}Foxp3GFP^{-}CD44^{hi}CD62L^{lo}) 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 lead 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 (Thy1.2^{+}Foxp3GFP^{-}CD44^{hi}CD62L^{lo}) 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_{reg}/T_{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 with full MHC-mismatched 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^{hi}CD62L^{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_{reg}/T_{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 (19), T_{regs} were preferential spared from depletion with mATG (Figure 1B). Naïve $CD25^+$ T cells are resistant to depletion by ALS 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 expression appeared to be highest in $Foxp3^+$ cells regardless of IL-2R α-chain (CD25) expression (22), 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 particular clinical relevance since homeostatic proliferation is thought to be barrier to transplantation tolerance induction in both reconstituted *scid* mice and WT mice treated with T cell-depleting antibodies (36). However, our data suggests that mATG, unlike other T cell depleting therapies (26), preserves Tregs (Figure 1B and 4). Further, these preserved T_{regs} themselves undergo homoestatic proliferation as suggested by an elegant study which 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 (12). Furthermore, T_{regs} are thought to play a major role in regulating lymphopenia-induced T cell proliferation and acquisition of T_{em} phenotype (10). Moreover, ex-vivo expanded antigen-specific natural Tregs synergize with host T cell depletion and promote long-term allograft acceptance (37). 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 (25). 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 (11). 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 (18). 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* (3). Moreover, both CTLAIg and SRL also inhibit lymphopenia-induced and alloantigen-induced proliferation of T cells (14, 23). 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 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 advantage 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 tolerance 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 tolerance in 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 The Jackson Laboratory. Foxp3GFP-ABM TCR-tg mice were generated by breeding Foxp3GFP reporter mice, that expresses Foxp3-green fluorescence protein (GFP), and ABM (anti-bm12) TCR-tg mice, a TCR-transgenic mouse reactive to I-A^{bm12} (with a TCR that utilizes the Vα2.1 and Vβ8.1 chains and specifically recognizes the MHC class II molecule I-A^{bm12}), both on the C57BL/6 background and bearing the Thy 1.2 allele. Foxp3GFP-ABM mice were maintained as a breeding colony in our animal facility. All mice were used at 6–12 weeks of age and were housed in accordance with institutional and National Institutes of Health guidelines.

Treatment Protocols

Murine Anti-Thymocyte globulin (mATG) is a purified, pasteurized, gamma immune globulin, obtained by immunization of rabbits with mouse thymocytes. It 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 (21, 5, 6, 27). mATG 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 cm^2) 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 (DLN) 7 days after transplantation, and red cells were lysed with ACKLysis buffer (BioWhittaker). Cells were stained with anti-CD3-fluorescein isothiocyanate (FITC), anti-CD19-phycoerytrin (PE), anti-CD8 allophycocyanin (APC) and anti-CD4-Peridinin Chlorophyl Protein (PerCP) to assess the depletion effect of mATG; T effector-memory cells were determined by staining with anti-CD4 PerCP, anti-CD8 FITC, anti-CD62 ligand (CD62L) APC, and anti-CD44 PE (all from BD Pharmingen) and analyzed for cell bearing the $CD44^{hi}CD62L^{low}$ 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. Regulatory T cells (Tregs) were detected by staining with anti-CD4 FITC, anti-CD25 PE and intracellular staining with Foxp3 APC (eBioscience) as per manufacturers instructions. Flow cytometry was performed using a FACSCaliber flow cytometry system (BD Biosciences) and analyzed using CellQuest software (BD Biosciences). Results are representative of three experiments.

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 (31). Briefly, spleens and lymph nodes were harvested from Foxp3GFP-ABM mice and pooled single-cell leukocyte suspensions were prepared. CD4^{+} T cells were purified by magnetic bead positive selection (Miltenyi Biotec, Auburn, CA). Typically, CD4^{+} T cells were isolated to > 90 % purity. An aliquot of cells was stained with anti-CD4, anti-TCR Vα2.1, and anti-TCR Vβ8.1, 8.2 and analyzed by flow cytometry to determine the percentage of ABM TCR-tg CD4^{+} T cells. ABM TCR-tg CD4^{+} T cells express CD90.2 (Thy 1.2+) on their surface. Typically, > 90% of CD4^{+} T cells expressed the tg TCR, and 2% of CD4^{+} T cells was determined as GFP^{+}. CD4^{+} isolated cells were then flow sorted for GFP$^{+/-}$ cells with a 97% of purity. 0.3 x 10^{6} Thy1.2^{+}Foxp3GFP positive or negative cells were then injected intraperitoneally into Thy 1.1 B6 mice one day before skin transplantation (day –1). On day 7 following transplantation draining (axillary, lateral

axillary) lymph nodes and spleens were subsequently collected, single-cell leukocyte suspensions were prepared and Thy1.2^{+}Foxp3GFP$^{+/-}$ TCR-tg T cells were identified by flow cytometry.

ELISPOT assay

The technique for enzyme linked immunospot (ELISPOT) analysis was adapted to measure IFN-γ and IL-4 secreting cells. Immunospot plates (Cellular Technology) were coated with capture antibodies against IFN-γ (R4-6A2) or IL-4 (11B11) in sterile PBS overnight. The plates were then blocked for 1 h with sterile PBS containing 1% BSA-fraction V and washed three times with sterile PBS. Splenocytes (0.5 x 10^6 in 200 µl of HL-1 medium containing 1% L-glutamine) were then placed in each well in the presence of 0.5 x 10^6 irradiated (30 Gy) syngeneic or allogeneic splenocytes and cultured for 24 h (for IFN-γ), or 48 h (IL-4) at 37°C in 5% CO2. After washing with PBS followed by washing with PBS containing 0.05% Tween (PBST), biotinylated rat anti-mouse IFN-γ (XMG1.2) and IL-4 (BVD6-24G2) detection mAb was added overnight. All Abs were purchased from BD Pharmingen. The plates were then washed four times in PBST, followed by 2 h of incubation with HRP-conjugated streptavidin (DAKO) diluted at 1/2000 in PBS/1% BSA. After washing three times with PBST followed by PBS, the plates were developed using 3-amino-9-ethyl-carbazole (Sigma-Aldrich). The resulting spots were counted on a computer-assisted ELISPOT image analyzer (Cellular Technology), and frequencies were expressed as the number of cytokine-producing spots

per 0.5 x 10^6 splenocytes (mean ± SD). Results are representative of three separate experiments.

Immunohistochemistry staining

Skin graft samples were obtained at each time point in each group of treatment; part of the skin graft was fixed in OCT and part in formalyn 10%, they were sectioned and stained with H&E for evaluation of cellular infiltration. The infiltrating cells were stained for CD4, CD8, macrophages (Mac1) and Foxp3 following the standard protocol.

Statistics

Kaplan-Meier survival graphs were constructed and a log rank comparison of the groups was used to calculate *p* values. Student's *t* test was used for comparison of means between experimental groups examined by FACS analysis or ELISPOT assay. Differences were considered to be significant at p values < 0.05.

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FIGURES AND TABLES

FIGURES

Figure 1: mATG depletes naïve T cells, preserves natural Tregs and prolongs allograft survival

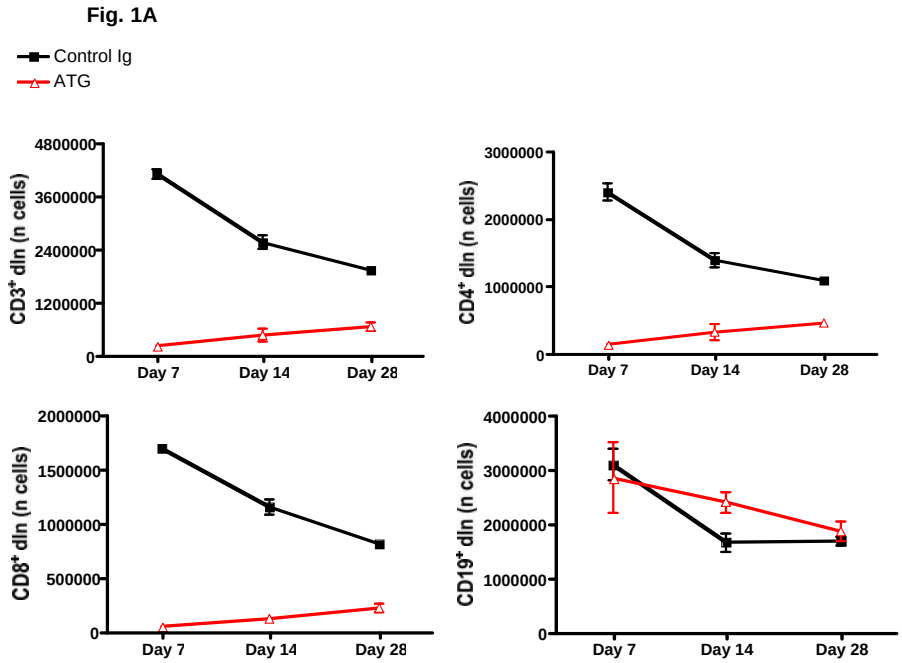

Fig.1A. Line graphs depicting absolute numbers of $CD3^+$ (upper left panel), $CD4^+$ (upper right panel), $CD8^+$ (lower left panel) and $CD19^+$ (lower right panel) cells 7, 14 and 28 days after transplantation with mATG induction or control Ig treatment.

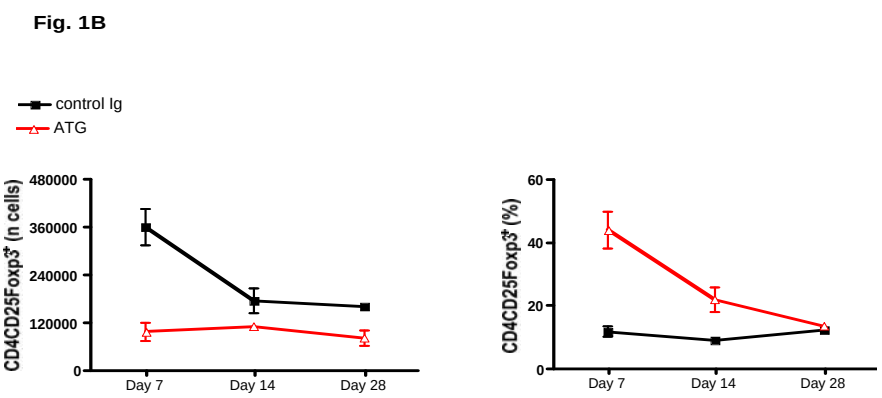

Figure 1B. Line graphs depicting absolute numbers (left panel) and percentage (right panel) of T_{regs} 7, 14 and 28 days after transplantation with mATG induction or control Ig treatment. **C.** Kaplan-Meier graphs depicting full MHC-mismatched skin allograft survival with mATG induction or control Ig treatment.

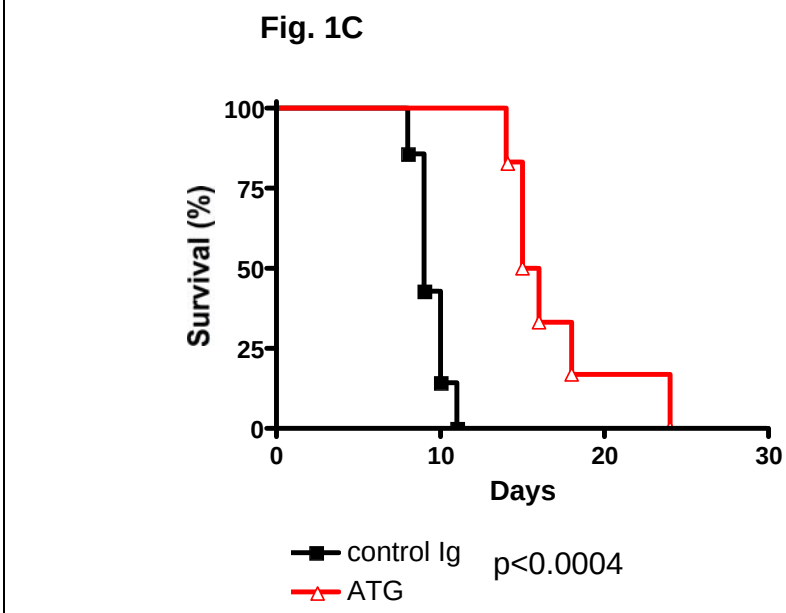

Figure 1C. Kaplan-Meier graphs depicting full MHC-mismatched skin allograft survival with mATG induction or control Ig treatment.

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

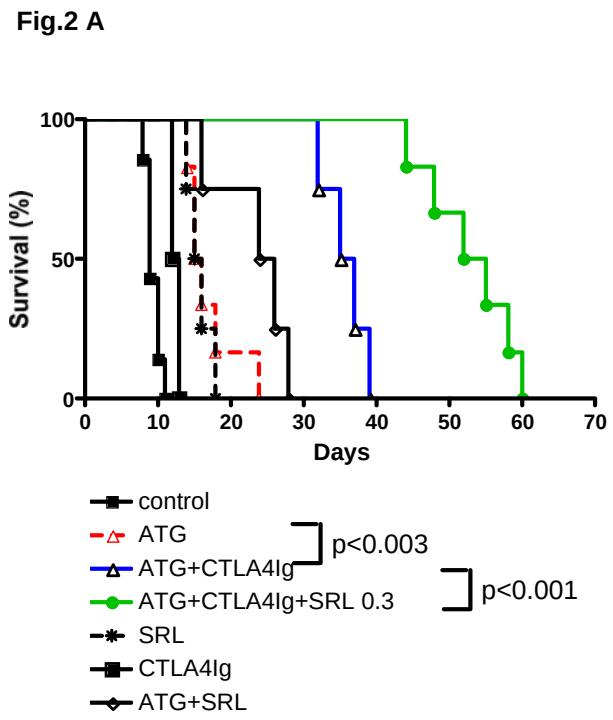

Figure 2A. Kaplan-Meier graphs depicting full MHC-mismatched skin allograft survival with mATG in combination with CTLA4Ig and/or SRL or mATG induction alone.

Fig. 2B

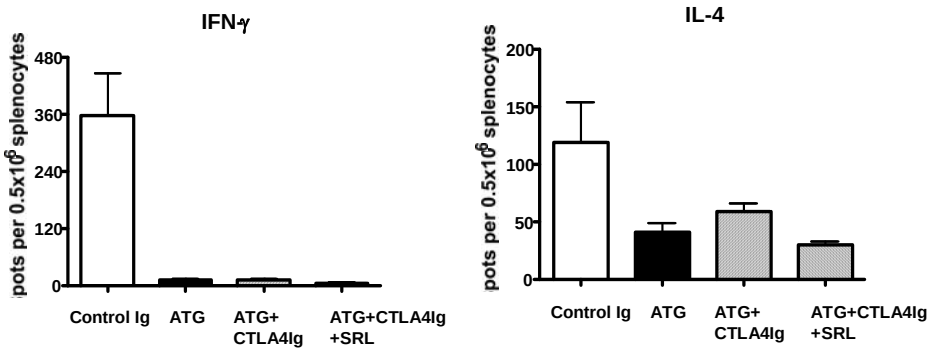

Figure 2B. Bar graphs depicting production of allospecific cytokine IFN-γ (left panel) and IL-4 (right panel), by ELISPOT assay with splenocytes from full MHC-mismatched skin allograft recipients, 7 days after transplantation with various treatments.

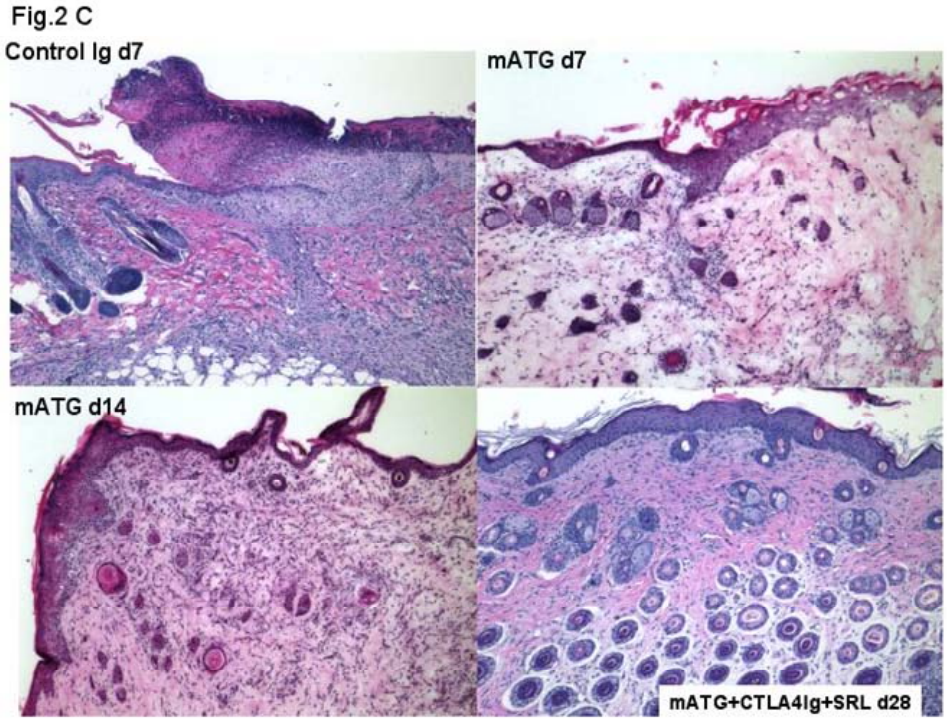

Figure 2C. H and E stained light micrographs depicting features of acute rejection in grafts harvested 7 days after transplantation from recipients treated with control Ig (left upper panel); minimal infiltration of grafts harvested 7 days after transplantation (right upper panel) compared to features of acute rejection in grafts harvested 14 days after transplantation from recipients treated with mATG induction therapy alone; well-preserved graft with no evidence of rejection in grafts harvested from recipients treated with combination therapy (lower right panel).

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

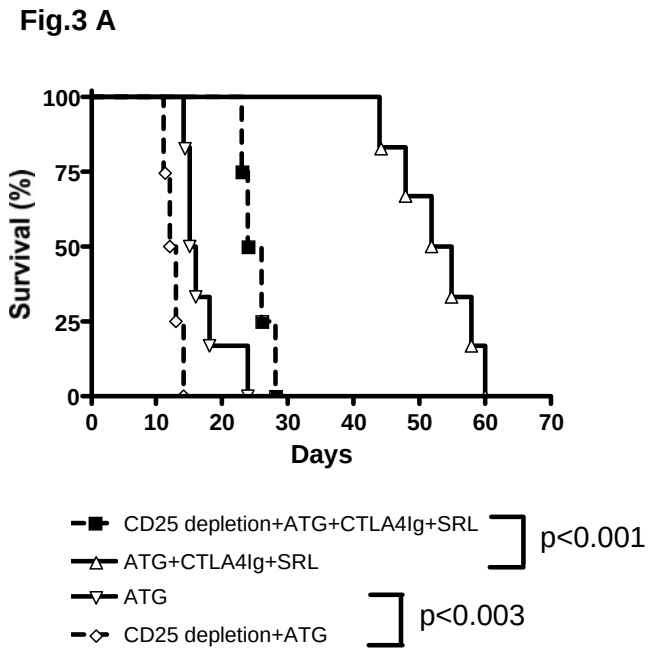

Figure 3A. Kaplan-Meier graphs depicting full MHC-mismatched skin allograft survival with CD25 pre-depletion and mATG in combination with CTLA4Ig and/or SRL or mATG induction alone.

Fig. 3 B

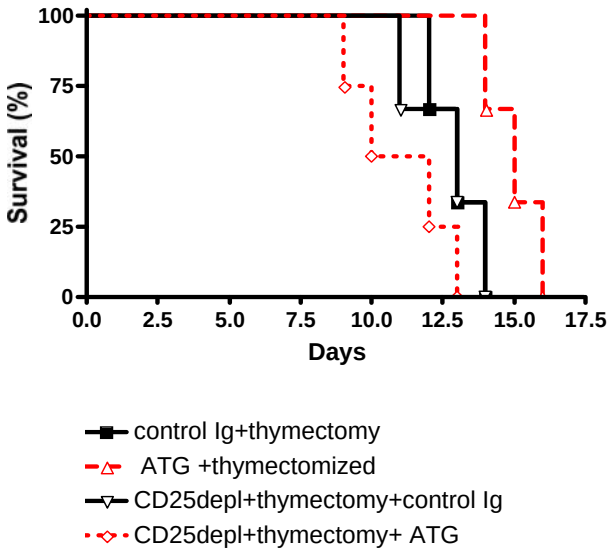

Figure 3B. Kaplan-Meier graphs depicting full MHC-mismatched skin allograft survival in thymectomized or non-thymectomized recipients with or without CD25 pre-depletion and mATG induction therapy.

Figure 4: CTLA4Ig and Sirolimus inhibit effector-memory differentiation and promote favourable $T_{reg}/T_{eff\text{-}mem}$ balance when combined with mATG

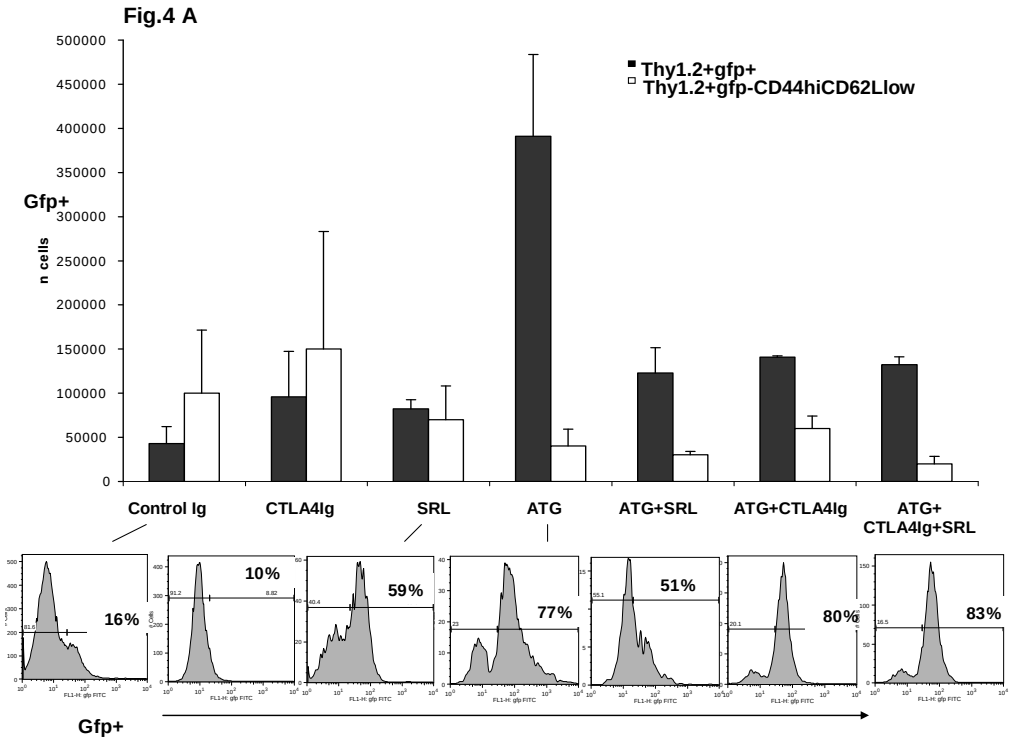

Figure 4A. Bar graphs depicting absolute numbers of Thy1.2^{+}FoxpGFP^{+} or Thy1.2^{+}Foxp3GFP^{-} populations 7 days after transplantation, in both lymph nodes and spleen from recipients adoptively transferred with Thy1.2^{+}Foxp3GFP^{+} cells and treated with mATG induction therapy alone, in combination with CTLA4Ig and/or SRL or control Ig. Representative histograms of Thy1.2^{+}FoxpGFP^{+} cells from recipients in each treatment group are shown below respective bar graphs.

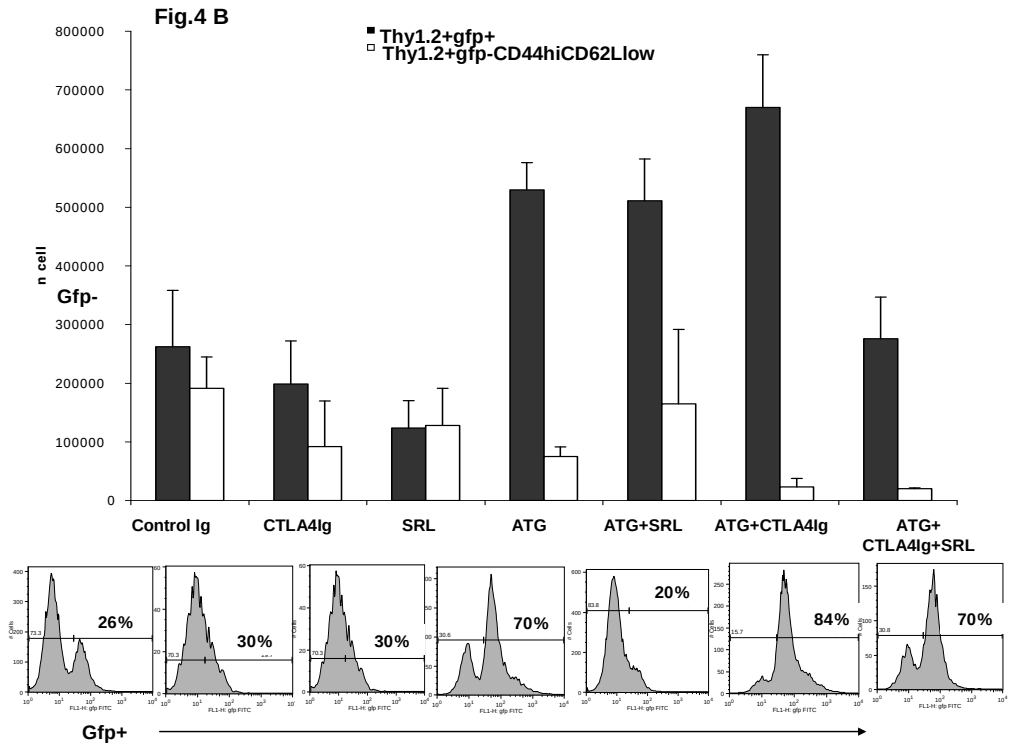

Figure 4B. Bar graphs depicting absolute numbers of Thy1.2^{+}FoxpGFP^{+} or Thy1.2^{+}Foxp3GFP^{-} populations 7 days after transplantation, in both lymph nodes and spleen from recipients adoptively transferred with Thy1.2^{+}Foxp3GFP^{-} cells and treated with mATG induction therapy alone, in combination with CTLA4Ig and/or SRL or control Ig. Representative histograms of Thy1.2^{+}FoxpGFP^{+} cells from recipients in each treatment group are shown below respective bar graphs.

Fig. 4C

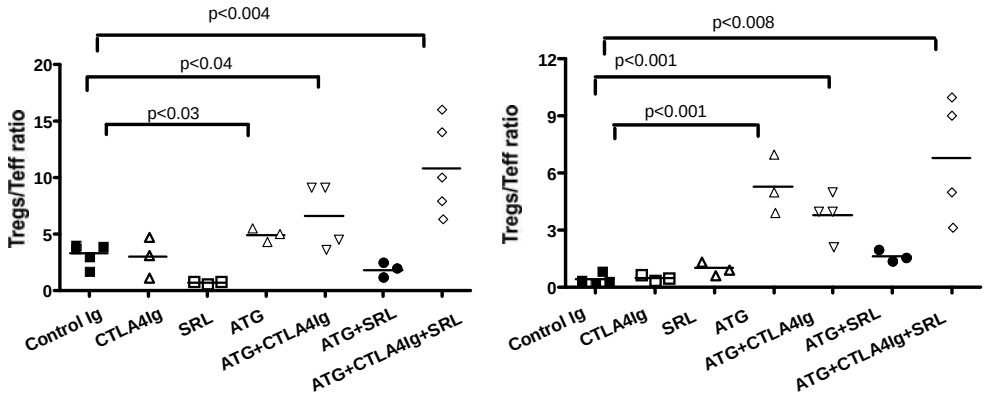

Figure 4C. Scatter plots depicting $T_{reg}/T_{eff\text{-}mem}$ ratios 7 days after transplantation, in both lymph nodes and spleen from recipients adoptively transferred with Thy1.2^{+}Foxp3GFP^{+} (left panel) or Thy1.2^{+}Foxp3GFP^{-} (right panel) cells and treated with mATG induction therapy alone, in combination with CTLA4Ig and/or SRL or control Ig.

TABLES

Table 1: mATG preserves T_{regs} which limit homeostatic proliferation of memory cells following T cell depletion

Therapy	Tx	CD25 pre-depletion	Treg (%)	Teff (%)	Treg/Teff Ratio	MST (days)
mATG	No	No	41±1	41.4±3	1	NA
mATG	No	Yes	18.3±3; p<0.001	55.4±2; p<0.007	0.3; p<0.001	NA
mATG	Yes	No	44±5	31.4±2	1.5	15.5
mATG	Yes	Yes	39±0.5; p=ns	60±5; p<0.02	0.7; p<0.04	12.5; p<0.003
mATG +CTLA4Ig +SRL	Yes	No	33±9	40±10	0.8	58
mATG +CTLA4Ig +SRL	Yes	Yes	5±1; p<0.002	76±1; p<0.001	0.05; p<0.0006	25; p<0.001

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