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Ex Vivo Generation of Human Alloantigen-Specific Regulatory T Cells from CD4^{pos}CD25^{high} T Cells for Immunotherapy

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Introduction

Regulatory T cells (Treg) play a critical role in various immunological processes. These cells dampen immune responses, which is important in maintenance of (self-) tolerance and homeostasis. Immunotherapy based on Treg (either in vivo facilitation of Treg or infusion of ex vivo isolated / manipulated Treg) is therefore a potential treatment for many immune disorders. Such an approach has been proven successful in animal models of stem cell transplantation [1–7], solid organ transplantation [8,9], auto-immunity [10–14] and even in infertility [15]. Treg therapy in human patients will require high cell numbers, phenotype, function and antigen-specificity before and after expansion. Our hypothesis, we alternated these two stimulation methods in previous findings, we hypothesized that it would be beneficial to combine polyclonal stimulation, to boost expansion, with allogeneic stimulator cells and T cell growth factors a very high degree of alloantigen-specificity was reached in magnetic bead isolated human CD4^{pos}CD25^{high} Treg. Efficient increases in cell numbers were obtained. Primary allogeneic stimulation appeared a prerequisite in the generation of alloantigen-specific Treg, while secondary allogeneic or polyclonal stimulation with anti-CD3 plus anti-CD28 monoclonal antibodies enriched alloantigen-specificity and cell yield to a similar extent.

Conclusions/Significance: The ex vivo expansion protocol that we describe will very likely increase the success of clinical Treg-based immunotherapy, and will help to induce tolerance to selected antigens, while minimizing general immune suppression. This approach is of particular interest for recipients of HLA mismatched transplants.

Abstract

Background: Regulatory T cell (Treg) based immunotherapy is a potential treatment for several immune disorders. By now, this approach proved successful in preclinical animal transplantation and auto-immunity models. In these models the success of Treg based immunotherapy crucially depends on the antigen-specificity of the infused Treg population. For the human setting, information is lacking on how to generate Treg with direct antigen-specificity ex vivo to be used for immunotherapy.

Methodology/Principal Findings: Here, we demonstrate that in as little as two stimulation cycles with HLA mismatched allogeneic stimulator cells and T cell growth factors a very high degree of alloantigen-specificity was reached in magnetic bead isolated human CD4^{pos}CD25^{high} Treg. Efficient increases in cell numbers were obtained. Primary allogeneic stimulation appeared a prerequisite in the generation of alloantigen-specific Treg, while secondary allogeneic or polyclonal stimulation with anti-CD3 plus anti-CD28 monoclonal antibodies enriched alloantigen-specificity and cell yield to a similar extent.

Conclusions/Significance: The ex vivo expansion protocol that we describe will very likely increase the success of clinical Treg-based immunotherapy, and will help to induce tolerance to selected antigens, while minimizing general immune suppression. This approach is of particular interest for recipients of HLA mismatched transplants.


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Competing Interests: The authors have declared that no competing interests exist.

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Results

Optimal conditions for primary and secondary expansion cycles

With the objective to obtain the highest numbers of functionally active Treg with optimal direct alloantigen-specificity, we devised four expansion strategies, employing polyclonal and alloantigen-specific stimulation in two subsequent cycles of Treg expansion (Figure 1). The cells that were obtained with these expansion strategies were compared in terms of absolute cell numbers, phenotype, suppressive capacity, antigen-specificity and anergy. Prior to analysis of the four selected Treg expansion strategies, individual expansion cycles were optimized, with regard to strength and mode of stimulation.

Cell sorting based on multiple surface markers such as CD25, CD127, CD62L and CD27 yields a high purity of FoxP3<sup>+</sup> Treg. However, these tools are not available for clinical grade purposes. Therefore, we specifically chose to use magnetic bead isolation for purification of Treg, as this most closely fits with the currently available GMP isolation tools. Cells isolated by this procedure expressed CD25 and FoxP3, but not CD127 (Figure 2A), thus displaying a typical Treg phenotype [23-26]. In contrast, CD4<sup>+</sup>CD25<sup>-</sup> conventional T cells (Tconv) did not express FoxP3 or CD25, and were positive for CD127. The majority of the isolated Treg as well as Tconv expressed the differentiation marker CD27, not its ligand CD70, and were positive for CD62L.

The freshly isolated Treg were hyporesponsive upon restimulation (Figure 2B) and able to suppress proliferation of autologous CD4<sup>+</sup>CD25<sup>-</sup> T cells upon stimulation with alloantigen (Figure 2C, D and E), >50% suppression was seen with Tresp:Treg ratios of 4:1 or lower (Figure 2C). Tconv were not anergic, nor suppressive.

For alloantigen-driven expansion, the optimal strength of alloantigen stimulation was determined by titrating irradiated HLA mismatched allogeneic PBMC into Treg cultures, in the presence of exogenous IL-2 and IL-15. In a primary MLR, proliferation was maximal with stimulator:responder ratios of 4:1 (Figure 3A). Secondary stimulation with alloantigen showed similar results (Figure 3B). The data shown in Figure 3B were obtained with Treg primed with alloantigen, but similar results were observed when polyclonally primed Treg were used (data not shown). Consequently, for expansion experiments, a stimulator:responder ratio of 4:1 was used. Interestingly, a higher number of HLA-DRB1 mismatches between stimulator and responder resulted in higher expansion rates (P<0.05) (Figure 3C).

For polyclonal stimulation, CD3 and CD28 triggering, and we compared anti-CD3+ anti-CD28 mAb coated microbeads with platebound anti-CD3+ soluble anti-CD28 mAb stimulation. For primary expansion of Treg, higher proliferation rates were achieved with beads as compared to platebound anti-CD3+ soluble anti-CD28 mAb (Figure 4A). Cell yield after expansion of Treg with either stimulation mode, using optimal dosage, was higher for anti-CD3+ anti-CD28 bead stimulation as compared to platebound anti-CD3+ soluble anti-CD28 stimulation (Figure 4B). To explain these differences, we assessed cell division and survival in Treg cultures for both stimuli (Figure 4C). Upon stimulation with anti-CD3+ anti-CD28 mAb coated beads, the majority of cells were triggered to proliferate as determined by CFSE dilution. Expression of 7AAD, a marker for late apoptotic cells, was low. Bcl-2, an anti-apoptotic protein, was expressed by 70-85% of the cells. Further gating revealed that the dividing cell population specifically expressed Bcl-2. Upon stimulation with platebound anti-CD3+ soluble anti-CD28 mAb, and independent of the concentration used, a large portion of cells was not triggered to proliferate. Again, 7AAD staining was low. The percentage of cells that expressed Bcl-2 was lower as compared to cells stimulated with anti-CD3+ anti-CD28 beads, this correlated with the percentage of dividing cells. The results indicate that the lower cell yield after stimulation of Treg with platebound anti-CD3+ soluble anti-CD28 mAb is merely caused by less efficient triggering of Treg proliferation, and is not the result of enhanced cell death.

These experiments were also conducted for secondary polyclonal stimulation. In this situation, platebound anti-CD3+ soluble anti-CD28 stimulation was superior as a secondary stimulus as compared to anti-CD3+ anti-CD28 microbead stimulation (Figure 5A). Expansion cultures stimulated with platebound anti-CD3+ soluble anti-CD28 yielded more Treg as compared to cultures stimulated with anti-CD3+ anti-CD28 beads (Figure 5B). Attempting to explain these findings, we again assessed cell division and survival in Treg cultures using both stimuli (Figure 5C). In contrast to the results after primary stimulation, both polyclonal stimuli now induced massive proliferation as assessed by CFSE dilution. Also, both 7AAD staining and Bcl-2 expression were comparable between Treg cultures stimulated with either anti-CD3+ anti-CD28 beads or platebound anti-CD3+ soluble anti-CD28 mAb. The data shown in Figure 5 were obtained with Treg primed with anti-CD3+ anti-CD28 beads, similar results were observed when alloantigen primed Treg were used (data not shown). Overall, these data prompted us to use

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Figure 1. Schematic overview of expansion strategies. Treg were expanded in two cycles, in which alloantigen and polyclonal stimulation was alternated, resulting in four distinct strategies: two subsequent cycles with alloantigen stimulation; primary cycle with alloantigen stimulus and secondary cycle with polyclonal stimulus; primary cycle with polyclonal stimulus and secondary cycle with alloantigen stimulus; and two subsequent cycles with polyclonal stimulation.

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Figure 2. Phenotypical and functional characteristics of freshly MACS-isolated Treg. CD4<sup>pos</sup> T cells were negatively isolated from PBMC and separated into CD25<sup>pos</sup> (Treg) and CD25<sup>neg</sup> (Tconv) T cell fractions by magnetic cell sorting. Data from a typical isolation is shown. (A) Cell surface expression of CD25, CD127, CD27, CD70 and CD62L, and intracellular expression of FoxP3 were analyzed on Treg (black filled histograms) or Tconv (grey line histogram). (B) Proliferative capacity of Treg or Tconv upon stimulation with HLA mismatched gamma irradiated allogeneic PBMC. Treg (black lines) or Tconv (grey lines) were added into these cultures at indicated Tresp:Treg/Tconv ratios. Proliferation was determined by measuring [³H]Thymidine incorporation at day 5. Results are expressed as percentage of [³H]Thymidine incorporation +SD, indexed to [³H]Thymidine incorporation of Tresp and antigen only. (C) Example CFSE of suppression assay as described in Figure 2D. Treg only (grey filled histogram), Tresp+Treg (1:1, black line) and Tresp+Tconv (1:1, grey line) are shown, numbers indicate percentage of proliferating cells, indexed to percentages of proliferating cells in cultures of naive Tresp and antigen only.

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Phenotypic characterization of expanded Treg

A potential risk of Treg expansion is the outgrowth of contaminating cell types such as CD8<sup>pos</sup> T cells or NK-cells. In our experiments, we did not find major contaminations, since typically >90% of the expanded cells were CD4<sup>pos</sup> T cells. The
functional characterization of expanded Treg

Treg retain anergic properties after expansion. One of the hallmarks of Treg is their anergic (hyporesponsive) behavior in vitro. Treg retained their anergic state after expansion, irrespective of the strategy employed, while in contrast Tconv were not anergic. Indeed, Treg did not proliferate upon stimulation in absence of exogenous T cell growth factors, while addition of IL-2 restored the proliferative capacity (Figure 9).

Expanded Treg show enhanced suppressive capacity and can acquire full antigen-specificity within two expansion cycles. The main characteristics of alloantigen-specific Treg should be their strong suppressive capacity when stimulated by target antigen (in transplantation settings this would be donor antigen), in parallel with a lack of suppressive activity in the case of stimulation by other antigens such as fully HLA mismatched third party alloantigen. To assess suppressive capacity, we used both the classic [3H]Thymidine incorporation co-culture suppression assay as well as a CFSE based assay. The latter is used because in some instances it is difficult to draw clear conclusions from the [3H]Thymidine assay when using multiple cell populations.

Figure 10 shows that highly antigen-specific Treg were obtained in two situations. Treg expanded with two cycles of alloantigen stimulation showed very high suppressive activity in target antigen driven MLR (>90% suppression in all ratios tested, Figure 10A) and hardly any suppression of third party antigen driven MLR. The cells obtained after the strategy starting off with a primary allostimulation, followed by secondary polyclonal stimulation, also yielded potent Treg with high antigen-specificity, as indicated by strong suppression of target antigen driven responses (>50% suppression with Tresp:Treg ratios of 16:1 or lower, Figure 10A) and low inhibition of third party HLA mismatched allostimulated MLR. Importantly, these data show that under these circumstances a very high degree of antigen-specificity can already be obtained after two cycles of expansion. This is relevant since we have noticed that multiple expansion cycles, irrespective of the mode of stimulation, can lead to reduced suppressive capacity, cell exhaustion, and subsequent cell death (data not shown). The expansion strategy starting off with a primary polyclonal stimulus followed by a secondary alloantigen stimulus yielded very potent suppressors, showing >90% suppression of target antigen driven MLC at all ratios tested (highest ratio tested 32:1, Figure 10A).

However, this cell population was still capable of suppression in third party alloantigen driven responses, albeit at a much lower efficiency than target antigen driven responses (>50% suppression at Tresp:Treg ratios 2:1 or lower, Figure 10A), indicating a strong enrichment Treg specific for target antigen, but incomplete exclusion of Treg with other specificities. As expected, Treg expanded with two cycles of polyclonal stimulation showed suppression of both target antigen as well as third party alloantigen driven MLR to a similar, moderate degree (>50% suppression at 8:1 and 4:1 or lower, respectively, Figure 10A). Treg displayed similar suppressive activity in CFSE suppression assays, while expanded Tconv were not suppressive (Figure 10B). Note that in the [3H]Thymidine based suppression assays (Figure 10A), the low
counts observed after titration of high numbers of expanded Tconv are not indicative of suppression per se, but rather a result of the very early proliferation of already primed Tconv cells in the co-culture. These cells heavily compete for medium components with the naïve Tresp cells that respond later in time. Tconv present in substantial numbers may indeed lead to distinct culture kinetics with an earlier proliferation peak and a net result of lower numbers at day 5.

In summary, optimal results were obtained with Treg expansion in two subsequent cycles of alloantigen stimulation: efficient increases in cell numbers, yielding highly potent and strictly antigen-specific Treg. The success of Treg expansion with alternated alloantigen and polyclonal stimulation depends on the order of the stimulation cycles. When Treg are expanded by a primary alloantigen stimulus followed by a secondary polyclonal stimulus, cells expand efficiently and show highly potent, strictly antigen-specific suppressive capacity. The reverse strategy with a primary polyclonal stimulus and a secondary alloantigen stimulus, yield high numbers of cells, and enrichment for target antigen-specific Treg. However, Treg specific for other antigens are still present. As expected, two cycles of polyclonal stimulation yielded high numbers of Treg. These cells were suppressive, but no enrichment for alloantigen-specific Treg had taken place.

Discussion

High numbers of Treg will be needed for effective Treg immunotherapy in humans to facilitate tolerance in patients with auto-immunity or after transplantation. Ex vivo Treg expansion can provide the solution to obtain these high numbers. Therapeutic efficiency can be improved by selecting for target-antigen reactive Treg, as indicated by preclinical mouse models designed to study the prevention of autoimmunity [12-14] and graft-versus-host disease [4,6,7]. At the same time, exclusion of Treg with specificities for other antigens lowers the risk for unwanted non-specific immune suppression, thus decreasing the risk on opportunistic infections and tumor growth.

Information on strategies for large scale antigen-specific expansion of human Treg is scant [22], as previous studies on ex vivo Treg expansion have primarily focused on polyclonal stimulation of Treg [16-21]. In the current report, we demonstrate the ex vivo generation of Treg with direct alloantigen-specificity from human naturally occurring CD4posCD25high Treg in as little as two stimulation cycles, requiring primary stimulation with HLA-mismatched allogenic stimulator cells and IL-2 plus IL-15. Experiments were performed with alternating allogenic and polyclonal (anti-CD3 anti-CD28 mAb) stimulation to define the ex vivo conditions resulting in optimal enrichment of alloantigen-specific Treg and high cell yield.

Naturally occurring CD4posCD25high Treg display a polyclonal TCR V-beta pattern [28]. Stimulation of this polyclonal Treg population with allogenic stimulator APC has been shown to specifically activate alloantigen-reactive Treg in mice [1,4,6,7,29,30] and humans [27]. Thus, these studies indicate that it is feasible to generate alloantigen-specific Treg from the naturally occurring CD4posCD25high Treg population. Here, we elaborated on these findings and questioned how to generate high numbers of alloantigen-specific Treg starting with polyclonal CD4posCD25high Treg populations obtained from peripheral blood. We specifically focused on a magnetic bead based method for Treg isolation, so as to fit in with currently available clinical grade isolation tools, with the objective to facilitate easy translation into clinical practice. Clinical grade (Good Manufacturing Practice, GMP) CD4posCD25high Treg isolation by a magnetic bead based method is now feasible using the CliniMACS system [31,32], albeit that Treg purity is suboptimal (50-60%). Our recent data on expansion of CliniMACS isolated Treg confirms that the results reported here can indeed be extrapolated to clinical grade purification (unpublished observations). Clearly, with regard to purity of the starting population magnetic bead isolation is inferior to FACS sorting, but this latter method is not readily available for GMP purposes. We obtained with high purity FACS sorted Treg similar results regarding antigen specificity, but with lower expansion rates and higher overall suppressive capacity (unpublished observations).

To design a successful strategy for obtaining high numbers of alloantigen-specific Treg, we hypothesized that it might be beneficial to combine ex vivo expansion cycles applying polyclonal stimulation, to boost expansion, and cycles applying alloantigen stimulation, to selectively stimulate alloantigen-specific Treg. Our data show that a high degree of alloantigen-specificity could be obtained in as little as two cycles of expansion. To increase alloantigen-specificity even more, it would be an option to repeat alloantigen expansion stimulation for multiple cycles, which in theory would progressively enrich for strictly antigen-specific Treg. However, if Treg populations were expanded for more than two expansion cycles, we observed a loss of suppressive capacity and high cell death (unpublished results).

Although highest cell yields were expected after expansion with two polyclonal stimulation cycles, our data showed similar cell numbers for all strategies. This result may in part be due to the fact that we opted for a standardized 10 day culture cycle. This choice was made based on the fact that strategies comprising one or more alloantigen stimulation cycles showed optimal expansion at day 10. However, expansion of cells in a second subsequent polyclonal stimulation cycle reached an optimum at day 7, cell death occurred thereafter.

Of note, the strategies described in this report generate Treg with direct alloantigen-specificity. These cells may be especially of benefit for patients receiving an HLA mismatched stem cell graft, where this route of alloantigen-reactivity is important in graft versus host pathology [33]. Although transplantation centers pursue a high degree of HLA-matching to prevent harmful reactions, HLA locus mismatched and haploidentical transplants are increasingly being performed [34]. In these cases current immunosuppressive regimen may benefit from the addition of an antigen-specific component. In solid organ transplantation, next to direct alloantigen recognition, the indirect route of alloantigen presentation clearly contributes to graft rejection [35]. With this in mind, it was recently shown, that it is also feasible to obtain Treg...
with indirect alloantigen-specificity by stimulation with autologous dendritic cells pulsed with allo-HLA-peptides [36].

Recently, the first clinical trials on Treg immunotherapy have been initiated; in these studies, either CliniMACS isolated CD4posCD25high Treg or ex vivo manipulated CD4pos T cell lines containing induced regulatory Tr1 cells are being infused in patients receiving stem cell transplantations [22]. So far, no effects have been reported.
Figure 6. Determination of optimal length of primary and secondary Treg expansion cycles. (A) Optimal length of primary Treg expansion cycles. Treg were stimulated with CFSE-labeled alloantigen (solid line, solid circle) or polyclonal (dotted line, open circle) stimulus in the presence of IL-2 and IL-15. At indicated time points, Treg numbers were counted by FACS (excluding CFSE<sup>+</sup> allogeneic stimulator cells) and related to Treg numbers at initial set up. (B) Optimal length of secondary Treg expansion cycles. Treg were primed with alloantigen (solid lines) or polyclonal (dotted lines) stimulation as indicated in the presence of IL-2 and IL-15 and rested for 2 days prior to restimulation. Cells were restimulated with CFSE-labeled alloantigen (solid circles) or polyclonal stimulus (open circles) in the presence of IL-2 and IL-15. At indicated time points, Treg numbers were counted by FACS (excluding CFSE<sup>+</sup> allogeneic stimulator cells) and related to Treg numbers at initial set up. Data are representative of three independent experiments.
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Figure 7. Treg expansion after primary and secondary cycles with alternated alloantigen or polyclonal stimulation. Treg and Tconv were expanded according the schedule in Figure 1 and rested for two days. Treg numbers were determined and related to Treg numbers at initial set up. Data represent average expansion +SD of seven independent experiments.
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Figure 8. Phenotypical characterization of expanded Treg. Treg and Tconv were expanded according the schedule in Figure 1 and rested for two days. Cell surface expression of CD25, CD127, CD27, CD70 and CD62L, and intracellular expression of FoxP3 was analyzed on Treg (black filled histograms) or Tconv (grey line histogram). Data are representative of four to seven independent experiments.
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In summary, this study has shown that it is feasible to obtain human functional alloantigen-specific Treg in large numbers for immunotherapeutic purposes. This may be a valuable aid in the clinical application of Treg, aiming at clinical tolerance to selected antigens, while minimizing general immune suppression.

Materials and Methods

Treg expansion strategies

To determine the optimal expansion strategy for obtaining high numbers of functionally active human alloantigen-specific Treg from CD4posCD25high Treg, we devised four different strategies, consisting of two subsequent cycles of expansion with alternated polyclonal and alloantigen-specific stimulation (Figure 1).

Cell preparation / isolation

Buffy coats were purchased from Sanquin bloodbank, Nijmegen, The Netherlands. These buffy coats were obtained from healthy human donors upon written informed consent with regard to scientific use.

The current study did not require approval from an ethical committee according to the Dutch Medical Research Involving Human Subjects Act (WMO). PBMC were isolated by density gradient centrifugation (Lymphoprep, Nycomed Pharma, Roskilde, Denmark); CD4pos T cells were negatively selected using mAbs directed against CD8 (RPA-T8), CD14 (M5E2), CD16 (3G8), CD19 (4G7), CD33 (P67.6), CD235a (GA-R2(HIR2) (all from BD Biosciences, SanJose, CA), and CD56 (MOC-1) (Dako, Glostrup, Denmark) combined with sheep-anti-mouse-IgG coated magnetic beads (Dynal Biotech, Oslo, Norway), routinely resulting in a >90% pure CD4pos T cell fraction. CD25high Treg and CD25neg conventional T cells were separated by MACS-sorting, using 10 µl anti-CD25 magnetic microbeads / 10⁶ CD4pos cells (Miltenyi Biotec, Bergisch Gladbach, Germany). HLA typing was performed by serological and DNA based techniques according to international (ASHI/EFI) standards [37].

CD4posCD25high Treg expansion

Cell cultures were performed in 96-well round bottom plates with culture medium consisting of RPMI 1640 supplemented with pyruvate (0.02 mM), penicillin (100 U/ml), streptomycin (100 µg/ml), and 10% human pooled serum (HPS), in a 37°C, 95% humidity, 5% CO₂ incubator.

To optimize Treg expansion conditions, the mode and strength of stimulation, exogenous cytokine concentration, and expansion cycle length were varied in early experiments, as reported in the Results section. For assessment of cell division using CFSE, 1–5x10⁶ cells were labeled with 0.5 µM CFDA-SE (Molecular Probes) prior to stimulation. The final expansion protocols are described below.

For primary and secondary cycle alloantigen-specific expansion, 2.5x10⁴ T cells were cultured with 10⁵ irradiated (30 Gy) HLA mismatched allogeneic PBMC (target alloantigen, ratio alloPBMC:Treg = 4:1).

For first cycle polyclonal expansion, 2.5x10⁴ T cells were cultured with 1.25x10⁵ anti-CD3+ anti-CD28 coated microbeads (Dynal Biotech). For secondary cycle polyclonal expansion, 2.5x10⁴ Treg were cultured with 5 µg/ml platebound anti-CD3 (UCHT1, BD Biosciences, 4 hours in PBS in incubator) plus 1 µg/ml soluble anti-CD28 (CD28.2, BD Biosciences).

Exogenous rhIL-2 (25 U/ml, Chiron, Amsterdam, the Netherlands) and rhIL-15 (10 ng/ml BioSource International, Camarillo, CA) were added to all expansion cultures. Wells were split and fresh medium containing cytokines was added every 3 days. After 10 days, the cells were harvested, washed and rested for 2 days in 5% HPS culture medium with 5 µg/ml rhIL-15 before analysis or further expansion. In all experiments, Tconv were included as control.

Flow cytometry

The phenotype of cells was analyzed by five-color flow cytometry (FC500, Beckman Coulter, Fullerton, CA). For cell surface staining, the following conjugated mAbs were used: CD25(M-A251)-PE, CD70(Ki-24)-PE, CD127(hIL-7R-M21)-AlexaFluor647 (BD Biosciences), CD27(M-T271)-FITC (Dako), CD4-(SFCI12T4D11)-PCy7

![Figure 9. Proliferative capacity of expanded Treg.](image-url)
Figure 10. Suppressive capacity of expanded Treg in [3H]Thymidine incorporation suppression assays. Treg and Tconv were expanded according the schedule in Figure 1 and rested for two days. (A) Suppressive capacity of Treg in target alloantigen and third party alloantigen responses as determined in a MLR co-culture using [3H]Thymidine incorporation. Autologous naive CD4^+CD25^- Tresp cells were stimulated with target alloantigen (same as used for expansion) or third party HLA mismatched gamma irradiated allogeneic PBMC. Expanded Treg (black lines) or Tconv (grey lines) were added into these cultures at indicated Tresp:Treg:Tconv ratios. Proliferation was determined by measuring [3H]Thymidine incorporation at day 5. Results are expressed as percentage of [H]Thymidine incorporation +SD, indexed to [H]Thymidine incorporation of naive Treg and antigen only. Data are representative of seven independent experiments. (B) Suppressive capacity of Treg in target alloantigen and third party alloantigen responses as determined in a MLR co-culture using CFSE dilution. CFSE labeled Autologous naive CD4^+CD25^- Tresp cells were stimulated with PKH26 labeled target alloantigen (same used for expansion) or PKH26 labeled third party HLA mismatched gamma irradiated allogeneic PBMC. Expanded Treg (black lines) or Tconv (grey lines) were added into these cultures at indicated Tresp:Treg:Tconv ratios. Proliferation was determined by measuring CFSE dilution of Tresp at day 5. Results are expressed as percentage of proliferating cells, indexed to percentages of proliferating cells in cultures of naïve Treg and antigen only. Data are representative of three independent experiments.

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Alloantigen-Specific Treg