CD25+CD4+ regulatory T cells generated by exposure to a model protein antigen prevent allograft rejection: antigen-specific re-activation in vivo is critical for bystander regulation

Running title: Bystander regulation can prevent allograft rejection

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M.K. is a Wellcome Trust Research Fellow. K.J.W. holds a Royal Society Wolfson Research Merit Award. This work was funded by The Wellcome Trust and The Roche Organ Transplantation Research Foundation.

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Total text word count: 4293
Abstract word count: 194
Scientific heading: Transplantation

The approach described in this manuscript is subject to patent protection and the intellectual property rights are being commercialised by the University of Oxford.
Abstract

The importance of CD25⁺CD4⁺ regulatory T cells (Treg) in the control of immune responses is established, but their antigen specificity in vivo remains unclear. Understanding Treg specificity requirements will be important if their potential is to be developed for immunotherapy. Pre-treatment of recipient mice with donor alloantigen plus anti-CD4 antibody generates CD25⁺CD4⁺ Treg with the capacity to prevent skin allograft rejection in adoptive transfer recipients. Here we demonstrate that although this regulation can be antigen-specific, re-activation with the original tolerising alloantigen allows the Treg to suppress rejection of third party allografts. Aware of the limitations of alloantigen pre-treatment, we asked whether graft-protective Treg could be generated against unrelated, non-graft antigens. We demonstrate that bystander regulation also extends to CD25⁺CD4⁺ Treg generated in vivo by exposure to nominal antigens under anti-CD4 antibody cover. Providing these Treg are re-exposed to the tolerising antigens before adoptive transfer, they prevent the rejection of fully allogeneic skin grafts. That this might form the basis of a clinically relevant tolerance induction strategy is demonstrated by the fact that when combined with sub-therapeutic anti-CD8 antibody, Treg generated in response to non-graft antigens facilitate the acceptance of cardiac allografts in primary recipients.
**Introduction**

The existence of lymphocytes with suppressive capacity was first described over thirty years ago, but in recent years there has been renewed interest in the identification and characterisation of regulatory T cells (Treg) that can regulate immune responses particularly with a view to harnessing their potential for immunotherapy. Several surface markers have been identified that can be used to enrich regulatory cells, one of which is CD25, the α subunit of the IL-2 receptor. CD25^+CD4^+ Treg with the capacity to regulate responses in vitro have been identified in both mice and humans. CD25^+CD4^+ Treg can suppress the proliferation and / or effector activity of both CD4^+ and CD8^+ cells, can prevent the development of autoimmune disease, and have been shown to play a role in tumor immunity and transplantation tolerance.

We have previously shown that CBA mice pre-treated with donor-specific transfusion (DST) under cover of either depleting or non-depleting anti-CD4 antibody accept fully allogeneic cardiac grafts indefinitely, and as demonstrated in several other transplant models, they contain CD25^+CD4^+ Treg with the capacity to suppress allograft rejection. However, we have recently shown that without further treatment, the non-depleting anti-CD4/DST protocol generates CD25^+CD4^+ Treg from CD25 negative precursors prior to transplant that are able to prevent the rejection of donor-type skin allografts in a sensitive adoptive transfer model. Significantly, equivalent numbers of CD25^+CD4^+ cells from naïve mice or from mice pre-treated with only either the anti-CD4 antibody or DST components of the combined therapy are unable to regulate under these conditions, demonstrating that these Treg arise as a consequence of the full pre-treatment protocol. In common with naturally-occurring CD25^+CD4^+ Treg, regulation by these alloantigen-induced cells in vivo is dependent on IL-10 and CTLA-4.

*In vitro* studies have demonstrated that, although Treg require activation via their T cell receptors to regulate, once activated they can inhibit responses in an antigen non-specific or ‘bystander’ manner. In this study we have attempted to exploit this bystander effect in allotransplantation and have developed a strategy in which Treg are driven and re-activated by soluble protein antigens completely unrelated to the eventual allograft. CD25^+CD4^+ T cells generated in this manner have the ability to prevent allograft rejection, suggesting that such an approach might form the basis of protocols with clinical potential.
Materials and methods

Mice
CBA.Ca (CBA, H2\(^{k}\)), C57BL/10 (B10, H2\(^{b}\)), BALB/c (BALB, H2\(^{d}\)), and CBA-recombination-activating gene 1 knockout (CBA-Rag\(^{-/-}\), H2\(^{k}\), kindly provided by Dr. D. Kioussis, Division of Molecular Immunology, National Institute for Medical Research, Mill Hill, London, U.K.) mice were obtained from and housed in the Biomedical Services Unit, John Radcliffe Hospital (Oxford, U.K.). Sex-matched mice between 6 and 12 weeks of age at the time of first experimental procedure were used in all experiments.

Reagents and monoclonal antibodies
The following monoclonal antibodies (mAb) were used for cell purification, flow cytometry, and in vivo administration. The hybridoma TIB120 (anti-major histocompatibility complex (MHC) class II) was obtained from American Type Culture Collection, Manassas, Virginia; YTS169 (anti-CD8) and YTS177.9 (anti-CD4)\(^{30}\) were kindly provided by Professor H. Waldmann (Sir William Dunn School of Pathology, Oxford, U.K.). RM4-5 (anti-CD4)-cytochrome, 16A (anti-CD45RB)-phycoerythrin, 7D4 (anti-CD25)-biotin, and streptavidin-phycoerythrin were purchased from Pharmingen (San Diego, California).

Human gamma globulin (HGG) was purchased from Sigma-Aldrich (St. Louis, Missouri) and was heat aggregated at 63 °C for 25 minutes and then incubated overnight on ice prior to use.

In vivo pre-treatment protocol
Adult CBA mice received 200 \(\mu\)g of the anti-CD4 mAb YTS177 intravenously on days -28 and -27. On day -27 they also received 250 \(\mu\)l of allogeneic (B10 or BALB) blood or 500 \(\mu\)g of HGG intravenously. In some experiments a further dose of allogeneic blood or HGG was administered on day -1. On day 0, either spleens were harvested for isolation of CD25\(^{+}\)CD4\(^{+}\) cells, or 200 \(\mu\)g of the anti-CD8 mAb YTS169 was administered intravenously and a B10 cardiac allograft performed.

Cell purification
CD45RB\(^{hi}\)CD4\(^{+}\) T cells were isolated from lymph nodes and spleens of naive CBA mice, and CD25\(^{+}\)CD4\(^{+}\) T cells were obtained from spleens of animals pre-treated with
YTS177 and allogeneic blood or HGG. Populations were purified by negative selection using magnetic beads followed by FACS sorting as previously described. On re-analysis, all populations were > 95% pure.

Cell adoptive transfer and skin transplantation
CBA-Rag^-/- mice were reconstituted intravenously with 10^5 CD45RB^{high}CD4^+ cells with or without 2×10^5 CD25^-CD4^+ cells. The following day full thickness B10 or BALB tail skin allografts were transplanted onto graft beds prepared on the flanks of the reconstituted mice. Allografts were monitored and graft survival between groups was compared using the log rank test using software developed and kindly provided by Dr. S. Cobbold, Sir William Dunn School of Pathology, Oxford, U.K.

Cardiac transplantation
Heterotopic cardiac allografts were performed as previously described. Graft function was followed by abdominal palpation and confirmed at post-operative day 100 by laparotomy. Allograft survival between groups was confirmed using the log rank test.

Serum anti-HGG antibody ELISA
Plate-bound HGG was used to capture serum anti-HGG antibody, which was then quantified using horseradish peroxidase-conjugated rabbit anti-mouse IgG and IgM (Jackson ImmunoResearch Laboratories, West Grove, Pennsylvania) followed by ABTS (2,2’-azino-bis(2-ethyl-benzthiazoline-6-sulfonic) acid). Absorbance at 405 nm was read and results are presented as the mean of duplicate wells ± SD.
Results

_Alloantigen-induced CD25^+CD4^+ cells regulate the rejection of donor-specific skin grafts_

To determine the antigen specificity of CD25^+CD4^+ cells from pre-treated mice, we isolated CD25^+CD4^+ cells from CBA (H2^k) mice pre-treated with the anti-CD4 antibody YTS177 and blood from B10 (H2^b) mice, and transferred them into syngeneic immunodeficient CBA-Rag^-/- recipients together with CD45RB^high^CD4^+ cells from naïve CBA donors as an effector population. One day later these recipients were transplanted with B10 skin grafts (Figure 1). As we have previously shown, 23 animals reconstituted with effector cells alone acutely rejected B10 skin allografts, but the co-transfer of CD25^+CD4^+ cells isolated from mice pre-treated with YTS177 and donor-specific B10 blood prevented this rejection. In contrast, CD25^+CD4^+ cells isolated from mice pre-treated with YTS177 and third party BALB (H2^d) blood had no such protective effect, with all B10 skin allografts being rejected acutely. We postulated that in this model regulation is either donor-specific, or that pre-treatment with BALB blood in combination with anti-CD4 therapy is incapable of generating Treg. In order to distinguish between these two possibilities, we harvested CD25^+CD4^+ cells from mice pre-treated with YTS177 and BALB blood and examined their ability to regulate the rejection of donor-specific BALB skin allografts mediated by CD45RB^high^CD4^+ effector cells in CBA-Rag^-/- recipients. As with the B10 model, rejection of these BALB skin grafts was prevented completely with all grafts surviving for >100 days (Figure 1) and showing abundant hair growth and normal histology (not shown). The survival of these BALB skin grafts is significant in the context of this model for two reasons. Firstly, it shows that the _in vivo_ generation of Treg with the capacity to control rejection is not limited to a single donor-recipient strain combination, thereby ruling out a simple defect in generation as a trivial explanation for the inability of CD25^+CD4^+ cells from mice pre-treated with YTS177 and BALB blood to protect B10 (H2^b^) skin grafts (Figure 1). Secondly, the data suggest that regulation is alloantigen-specific, and it is the specificity of regulation that forms the basis of the remainder of this study.

_When specifically re-challenged in vivo, alloantigen-induced CD25^+CD4^+ cells can regulate the rejection of third party skin allografts_

The generation of Treg using protocols that depend on pre-treatment with donor-specific alloantigen will likely attract only limited clinical interest because of the
difficulty of donor-specific pre-treatment in the majority of transplant recipients. However, work from other laboratories has shown that activation of CD25+CD4+ Treg by their cognate peptide-MHC ligand can enable them to suppress responses against other antigens in vitro. In at least one of these examples it was possible to rule out T cell cross-reactivity as the mechanism, leaving bystander regulation as the most likely explanation. We therefore asked whether it might be possible to exploit deliberately this bystander mechanism in transplantation. Since the data in Figure 1 had shown that it was possible to generate CD25+CD4+ Treg in response to BALB alloantigen challenge under anti-CD4 antibody cover, we asked whether re-exposure to a second BALB alloantigen challenge would then allow these CD25+CD4+ cells to regulate the rejection of third party B10 skin allografts. CBA mice were pre-treated with BALB blood under the cover of YTS177 and one day before purifying CD25+CD4+ cells for adoptive transfer these cell donors were given a second BALB blood transfusion with the objective of re-activating BALB-reactive CD25+CD4+ Treg generated by the pre-treatment protocol (Figure 2A). Day -1 was chosen for the time of alloantigen re-challenge because in an anti-CD4/DST + DST re-activation protocol in primary heart graft recipients, re-activation of regulatory cells at day -1 was highly effective in protecting the graft from transplant-associated vasculopathy. CBA-Rag-/- mice were reconstituted with these CD25+CD4+ T cells together with CD4+CD45RBhigh cells as an effector population and transplanted one day later with B10 (H2b) skin grafts. In distinct contrast to the result described above where CD25+CD4+ cells from mice pre-treated with YTS177 and BALB blood without re-activation were unable to regulate the rejection of B10 skin grafts (Figure 1), administration of a second BALB blood transfusion to the pre-treated mice the day before adoptive transfer allowed the transferred CD25+CD4+ T cells to prevent completely the rejection of the third party B10 skin allografts, with all grafts surviving for >100 days (Figure 2B). These skin grafts had good hair growth (Figure 2C) and normal histology (Figure 2D) and were essentially indistinguishable from those B10 skin grafts protected by donor-specific Treg (Figure 1C and D), demonstrating the efficiency of this antigen non-specific regulation.

**CD25+CD4+ T cells tolerised to unrelated soluble protein antigens in vivo can prevent the rejection of fully allogeneic skin grafts**

The demonstration that re-activation of Treg prior to adoptive transfer prevented the rejection of third party grafts could have important implications for the design of
tolerance induction strategies in man by allowing more flexibility in the design of pre-treatment strategies. However, concerns over the transmission of blood-borne pathogens may limit the utility of blood or other allogeneic cells as a source of tolerising antigen. Generating Treg specific for a non-cellular protein antigen or antigens could offer an alternative strategy if it could be shown that such cells can regulate responses to alloantigens in vivo. The ability of CD25⁺CD4⁺ T cells to mediate bystander regulation following alloantigen re-exposure (Figure 2) led us to ask whether a similar approach might be successful using simple protein antigens. It has previously been demonstrated that tolerance to soluble antigens such as albumin or human gamma globulin (HGG) can be achieved in mice by intravenous administration under the cover of anti-CD4 antibody. We therefore chose HGG as a candidate for the tolerising antigen for these experiments. In order to determine whether tolerance to HGG could be replicated in our hands we administered HGG under the cover of the anti-CD4 antibody YTS177 based on a previously published protocol and then measured serum anti-HGG antibody concentrations by ELISA. Details of the priming and tolerising protocols are shown in Figure 3A. Positive control mice that received a priming protocol (where HGG was given following but not coincident with YTS177) produced high levels of anti-HGG antibody, whereas mice given HGG under the cover of YTS177 had low antibody titers which were identical to those from naïve mice, indicating tolerance to HGG. Having confirmed that tolerance to HGG could be induced we then asked whether an HGG tolerising plus re-activation protocol based on that using alloantigen shown in Figure 2 (HGG on day -27 under the cover of YTS177 plus HGG re-challenge on day -1) could also induce tolerance to HGG (Figure 3A, bottom panel). Importantly, mice pre-treated according to this protocol also produced background levels of anti-HGG antibody and were therefore judged to be tolerant to HGG (Figure 3B).

Having established that tolerance to HGG can be induced using this protocol, we asked whether CD25⁺CD4⁺ cells generated by pre-treatment with HGG under the cover of YTS177 and specifically re-activated with HGG could regulate the rejection of B10 skin allografts (Figure 4A). Animals reconstituted with CD45RBhighCD4⁺ cells alone all rejected their grafts acutely but in contrast, all mice that received co-transfer of CD25⁺CD4⁺ cells from animals pre-treated with YTS177 and HGG then given a second HGG challenge the day prior to cell isolation (to re-activate the regulatory population) accepted their B10 skin allografts for >100 days (Figure 4B). As determined by both macroscopic (Figure 4C) and histological appearance (Figure 4D), these skin grafts were indistinguishable from those protected by Treg driven by donor-specific
 alloantigen (Figure 1C and D). As important controls, we tested the regulatory capacity of CD25\(^+\)CD4\(^+\) cells from animals pre-treated with YTS177 and HGG but without the second (re-activating) dose of HGG, and from animals pre-treated with HGG in the absence of YTS177 followed by a second dose of HGG prior to cell isolation. In both these groups all B10 skin allografts were rejected acutely.

**Bystander regulation can facilitate the acceptance of primary cardiac allografts**

The data presented in Figure 4 clearly demonstrate that CD25\(^+\)CD4\(^+\) T cells generated and re-activated by exposure to nominal antigens have the capacity to prevent the rejection of fully allogeneic grafts in an adoptive transfer system. However, these data were obtained in immunodeficient recipients where rejection is mediated by the adoptive transfer of limited numbers of CD4\(^+\) effector T cells. In order to determine whether bystander regulation of allograft rejection is effective in a more clinically-applicable model, we asked whether Treg generation and re-activation would allow the survival of fully allogeneic grafts in primary recipients with an intact immune system. Since the anti-CD4/HGG protocol described above was developed from an anti-CD4/donor-specific blood transfusion protocol which allows the long term survival of fully mismatched B10 cardiac allografts without additional therapy\(^{25}\) we used the same heart model to determine the comparative efficacy of the YTS177/HGG + HGG re-activation regimen. CBA mice were pre-treated with HGG under the cover of YTS177 on days -28 and -27, re-exposed to HGG on day -1, and transplanted with B10 hearts on day 0, but disappointingly these hearts were rejected at control rates (median survival time, MST, 8 days, Figure 5). We speculated that, although regulation might be occurring to some extent in these recipients, the precursor frequency of the self-restricted HGG-specific Treg might be too low to have a significant impact on the alloreactive T cell population, expected to be far greater in size.\(^{37}\) We have previously shown that in the absence of anti-CD4 antibody Treg can be also be generated in CBA recipients by successive multiple donor-specific transfusions, whilst a single DST is not sufficient.\(^{38}\) This incremental effect suggested that multiple challenges with HGG combined with the day -1 re-activating dose might increase the efficacy of the basic YTS177/HGG protocol. Therefore, CBA mice were pre-treated with HGG under the cover of YTS177 on days -28 and -27 and re-challenged with HGG on day -1, but also received additional doses of HGG on days -21, -14, and -7. These mice were then transplanted with B10 hearts on day 0. However, these mice also rejected their grafts at control rates (data not shown).
In an attempt to identify adjunctive therapies that might enable Treg generated by the YTS177/HGG protocol to influence the outcome of primary heart transplants we considered three separate strategies. The first was to block CD154 using the anti-CD154 antibody MR1\(^\text{39}\) which as a monotherapy has been shown to be partially effective in preventing cardiac allograft rejection.\(^\text{40}\) The second was to use a short course of sirolimus (rapamycin) on the basis that this immunosuppressive agent, unlike calcineurin inhibitors, appears not to inhibit the function of Treg.\(^\text{41,42}\) The third approach was to target CD8\(^+\) T cells on the grounds that in several experimental models, CD8\(^+\) T cells have been shown capable of mediating allograft rejection independently of CD4\(^+\) help.\(^\text{43-45}\) The additional possibility of using anti-CD4 antibody as adjunctive therapy was rejected on the theoretical basis that this would almost certainly target the CD25\(^+\)CD4\(^+\) Treg population whose regulatory potential we were seeking to augment.

In the anti-CD154 experiments, CBA mice were pre-treated with the YTS177/HGG + HGG re-challenge protocol, but in addition received a sub-therapeutic course of the anti-CD154 antibody MR1 (2 mg/kg intraperitoneally on days -27, -25, -23, and -21). These animals were then transplanted at day 0 with B10 cardiac allografts. The anti-CD154 antibody was administered in the pre-treatment rather than the peri-transplant period to avoid the possibility of subjecting the Treg population to CD154 blockade. In the rapamycin experiments, CBA mice were pre-treated with the YTS177/HGG + HGG re-challenge protocol, transplanted with B10 hearts at day 0, and then given either of two sub-therapeutic courses of sirolimus (200 μg/kg/dose intraperitoneally on days +1, +3, +5, and +7, or 200 μg/kg/dose intraperitoneally on days -1, 0, +1, +3, +5, +7, +9, +11, and +13). We were disappointed to find, however, that neither the YTS177/HGG + anti-CD154 nor the YTS177/HGG + rapamycin protocols had any effect on graft outcome compared with relevant controls which received the same therapy in the absence of HGG challenge (data not shown).

In the light of the observation that in several transplant models independent targeting of CD8\(^+\) T cells can convert ineffective into effective therapeutic protocols,\(^\text{43-45}\) we asked whether targeting CD8\(^+\) T cells with a sub-optimal dose of anti-CD8 antibody would have a similar beneficial effect in the YTS177/HGG + HGG re-challenge protocol. CBA mice were pre-treated with YTS 177 on days -28 and -27 with or without HGG at day -27 and day -1, transplanted with B10 cardiac allografts on day 0, and given a single dose (8 mg/kg on day 0) of the depleting anti-CD8 antibody YTS169. As shown in Figure 5, whilst addition of this single dose of anti-CD8 antibody to control mice pre-treated with anti-CD4 antibody in the absence of HGG had little
impact on graft outcome (MST 26 days), adding the anti-CD8 antibody to the YTS177/HGG + HGG re-challenge protocol led to indefinite graft survival in 3 out of 4 recipients (MST >100 days, Figure 5B), with these long term surviving hearts showing neither distortion of myocardial architecture (Figure 5C) nor significant intimal proliferation (Figure 5D). Thus, Treg generated against non-graft antigens and then re-activated prior to transplantation can, in the presence of sub-therapeutic anti-CD8 antibody, prevent allograft rejection in primary recipients with an intact immune system, demonstrating that under appropriate conditions it is possible to exploit the potential of bystander regulation in primary allograft recipients.
Discussion

In recent years much progress has been made in defining the phenotypic and functional properties of CD25⁺CD4⁺ Treg. One characteristic that has been demonstrated in vitro by several groups is that these Treg require activation via their T cell receptors in order to exert regulatory activity but that, once activated, they are able to regulate in an antigen non-specific manner.²,⁴,⁶ The data presented here are, to our knowledge, the first to demonstrate that following their generation in response to either alloantigen or nominal antigen given under the cover of anti-CD4 antibody, re-exposure of these CD25⁺CD4⁺ Treg to the original tolerising antigen allows them to regulate allograft rejection in vivo in a manner that is antigen non-specific.

The phenomenon of regulation in the specific setting of transplantation is attracting renewed interest because active, self-sustaining regulation of rejection responses may be a route to drug-independent long term graft survival. A major limiting factor in the clinical use of protocols involving pre-treatment of patients with antigen prior to transplantation is that, except for live donor transplantation, neither the timing of the procedure nor the identity of the donor is known in advance. The observation that CD25⁺CD4⁺ cells from mice pre-treated with YTS177 and unrelated blood can prevent the rejection of third-party skin allografts (Figure 2) demonstrates that regulation can be achieved by the administration of antigens that are not necessarily expressed by the graft itself. The fact that regulation was only observed following re-challenge of the CD25⁺CD4⁺ cell donors with the tolerising antigen is entirely consistent with the in vitro observations of others that naturally-occurring CD25⁺CD4⁺ Treg have the capacity for bystander regulation.²,⁴,⁶

The potential for the clinical transmission of infectious agents by administration of human blood or blood products led us to consider alternative antigens for the generation and re-activation of Treg. The fact that Treg generated by administration of a non-cellular antigen such as HGG can prevent the rejection of skin allografts in an adoptive transfer model (Figure 4) and can influence the survival of heart allografts in immunocompetent recipients (Figure 5) appears to support a proof of principle. Clinical protocols could be explored in which patients awaiting transplantation are given a well-defined, quality-controlled non-cellular antigen or antigens combined with transient immunotherapy to generate Treg. These regulatory cells would be maintained by routine antigen re-challenge then re-activated by the same tolerising antigens immediately prior to transplantation. When combined with a suitable adjuvant therapy such a strategy may facilitate graft acceptance and reduce the need for long term maintenance.
immunosuppression. An additional approach being investigated in this laboratory is the generation of CD8+ Treg. The ability of CD8+ T cells to undergo priming to exogenous antigen suggests that, in principle at least, it may be possible to generate CD8+ Treg using an anti-CD8/HGG + HGG re-activation protocol. If, as suggested in Figure 5, targeting the CD8 arm of the response is essential in immunocompetent recipients, then generating Treg of both subsets in a combined antibody protocol may be a way of achieving long term graft survival in primary allograft recipients without additional therapy.

Our observations in this model not only suggest potential strategies for immunotherapy but also shed some light on the mechanisms involved in regulation in vivo. In the situation where an allograft expresses the same alloantigens as those in the tolerising protocol (for example in the anti-CD4/DST protocol without a DST antigen re-challenge, Figure 1), it seems likely that Treg will be capable of responding to alloantigens expressed on the graft itself and / or on resident donor antigen presenting cells encountered in the draining lymph nodes. This alloantigen-specific re-activation could then lead to the attenuation of allo-aggressive responses. However, in the model where Treg driven by alloantigen under anti-CD4 cover and re-activated by re-exposure to the same alloantigens have the ability to regulate the rejection of third-party allografts (Figure 2) both cross-reactivity and bystander regulation seem possible. However, of the two possibilities, cross-reactivity seems the less likely in the day -1 alloantigen re-activation protocol because without antigen re-exposure no regulation occurs. Whilst it is possible to suggest how re-activation could result in bystander regulation (non-specific secretion of IL-10 perhaps) it is more difficult to explain why antigen re-exposure one day before transplant might enhance cross-reactivity. Finally, in the anti-CD4/HGG + HGG re-activation protocol, CD4+ Treg driven by soluble protein antigens will almost certainly have responded to processed peptides presented by self-MHC class II via the indirect pathway. Cross-reactivity between these indirect CD4+ Treg and intact alloantigens expressed by the graft and / or donor antigen presenting cells via the direct pathway seems extremely unlikely. We propose that these re-activated cells regulate by a bystander mechanism mediated possibly by cytokines such as IL-10, secreted in a restricted local micro-environment.

The duration of graft protection offered by these non-graft-specific Treg is likely to be fairly short-lived since these cells are unlikely to remain activated for a significant length of time in the absence of specific antigen stimulation. However, it is probable that an initial period of graft protection mediated by bystander regulation could lead to
operational tolerance in the alloreactive T cell population by a variety of established mechanisms such as infectious tolerance, IL-10 mediated immunosuppression, competition for co-stimulatory ligands, or down-regulation of antigen presenting cell function.\textsuperscript{50-53}

One potential concern over Treg that mediate bystander regulation is the impact that these cells might have on protective immune responses to pathogens. However, in a model in which bystander regulation has been deliberately induced using a protocol analogous to that shown in Figures 2 and 4, we have recently shown that protective responses to influenza virus are undiminished when compared with those in normal un-manipulated mice.\textsuperscript{34} Clearly this is encouraging, but this conclusion has to be verified with other potential pathogens. This is currently under investigation in our laboratory.

Overall, the data obtained in this study suggest that the ability to generate Treg populations by controlled exposure to defined antigens may have important implications for organ transplantation, bone marrow transplantation and for autoimmune disease, where attenuation of immune responses remains an important goal.
References


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Figure legends

Figure 1: Alloantigen-induced CD25+CD4+ cells can regulate skin allograft rejection

(A) Pre-treatment and adoptive transfer protocol. CBA mice were pre-treated with 200 μg YTS177 on days -28 and -27 plus 250 μl allogeneic (B10 or BALB) blood on day -27. On day 0, 2×10^5 CD25+CD4+ spleen cells from these donors were adoptively transferred into CBA-Rag^-/- recipients together with 10^5 CD45RB^{high}CD4+ cells from naïve CBA mice. One day later these reconstituted mice were transplanted with B10 or BALB skin grafts. (B) Effect of CD25+CD4+ cells on CD45RB^{high}CD4+ -mediated skin allograft rejection. Reconstitution with: △ CD45RB^{high}CD4+ cells only, B10 graft (group i: MST 20 days, n = 4); CD45RB^{high}CD4+ cells only, BALB graft (group ii: MST 12 days, n = 4); CD45RB^{high}CD4+ cells + CD25+CD4+ cells from YTS177/B10 blood pre-treated mice, B10 graft (group iii: MST > 100 days, n = 4; P < 0.05 versus group i); CD45RB^{high}CD4+ cells + CD25+CD4+ cells from YTS177/BALB blood pre-treated mice, BALB graft (group iv: MST > 100 days, n = 4; P < 0.05 versus group ii); CD45RB^{high}CD4+ cells + CD25+CD4+ cells from YTS177/BALB blood pre-treated mice, B10 graft (group v: MST 25 days, n = 4; P < 0.05 versus groups iii and iv). (C) Representative B10 skin graft 100 days post transplant from group iii. (D) Histology of graft in (C) (formalin-fixed paraffin section stained with H&E).

Figure 2: Activated alloantigen-induced CD25+CD4+ cells can regulate third party skin allograft rejection

(A) Pre-treatment and adoptive transfer protocol. CBA-Rag^-/- mice were reconstituted with 10^5 CD45RB^{high}CD4+ cells with or without 2×10^5 CD25+CD4+ cells from CBA mice pre-treated with 200 μg YTS177 and 250 μl BALB blood followed by a further dose of BALB blood the day prior to cell isolation. The reconstituted mice then received a B10 skin graft the following day. (B) Effect of CD25+CD4+ cells on CD45RB^{high}CD4+ -mediated rejection of B10 skin grafts. CD45RB^{high}CD4+ cells only (MST 17 days, n = 4); CD45RB^{high}CD4+ plus CD25+CD4+ cells (MST > 100 days, n = 4; P < 0.05). (C) Representative skin graft 100 days after transplantation onto mouse reconstituted with both CD45RB^{high}CD4+ and CD25+CD4+ cells. (D) Histology of graft in (C) (H&E).
Figure 3: Tolerance to HGG can be induced by administration under the cover of anti-CD4 therapy

(A) CBA mice were pre-treated as follows and serum harvested at day 0 for ELISA analysis: tolerising protocol – 200 μg YTS177 on days -42, -41, and -40, and 500 μg HGG on days -41, -14, and -7; priming protocol – 200 μg YTS177 on days -42, -41, and -40, and 500 μg HGG on days -14 and -7; YTS177/HGG + HGG re-activation protocol for in vivo adoptive transfer – 200 μg YTS177 on days -28 and -27 and 500 μg HGG on days -27 and -1. (B) Anti-HGG antibody titer at day 0. △ untreated; ⚫ tolerising protocol; ◊ priming protocol; ■ in vivo adoptive transfer protocol. n = 2 in per group, results are presented as mean ± SD.

Figure 4: Activated CD25⁺CD4⁺ cells generated against the unrelated antigen HGG can regulate skin allograft rejection

(A) CBA mice were pre-treated with the YTS177/HGG + HGG re-activation protocol or with relevant control protocols. At day 0, 2×10⁵ CD25⁺CD4⁺ T cells from these donors were co-transferred with 10⁵ naïve CD45RB⁺⁺CD4⁺ cells into CBA-Rag⁻/⁻ recipients. These reconstituted mice were then transplanted with B10 skin allografts one day later. (B) Skin allograft survival. □ CD45RB⁺⁺CD4⁺ cells only (group i: MST 11.5 days, n = 14); ■ CD45RB⁺⁺CD4⁺ cells + CD25⁺CD4⁺ cells from mice pre-treated with 200 μg YTS177 on days -28 and -27 and with 500 μg HGG on days -27 and -1 (group ii: MST > 100 days, n = 5; P < 0.05 versus group i); ○ CD45RB⁺⁺CD4⁺ cells + CD25⁺CD4⁺ cells from mice pre-treated with 500 μg HGG only on days -27 and -1 (group iii: MST 20 days, n = 5; P = 0.21 versus group i); ◊ CD45RB⁺⁺CD4⁺ cells + CD25⁺CD4⁺ cells from mice pre-treated with 200 μg YTS177 only on days -28 and -27 and 500 μg HGG on day -27 (group iv: MST 21 days, n = 5; P = 0.21 versus group i). (C) Representative skin graft from group ii 100 days post transplant. (D) Histology of graft in (C) (H&E).

Figure 5: Induction of tolerance to HGG followed by HGG re-administration can facilitate the acceptance of primary cardiac allografts

(A) CBA mice were pre-treated with the YTS177/HGG + HGG re-activation protocol or with YTS177 only. These mice were then transplanted with B10 cardiac allografts on day 0 with or without adjunctive anti-CD8 antibody therapy. (B) Cardiac allograft
survival. ○ YTS177/HGG + HGG re-activation protocol (200 µg YTS177 on days -28 and -27 and 500 µg HGG on days -27 and -1; group i: MST 8 days, n = 5); □ 200 µg YTS177 on days -28 and -27 and 200 µg YTS169 on day 0 (group ii: MST 26 days, n = 5; P < 0.05 versus group i); ■ YTS177/HGG + HGG re-activation protocol plus anti-CD8 antibody day 0 (200 µg YTS177 on days -28 and -27, 500 µg HGG on days -27 and -1, and 200 µg YTS169 on day 0; group iii: MST > 100 days, n = 4; P < 0.05 versus groups i and ii). (C) Histology of long term surviving graft from group iii, ■ (H&E). Scale bar = 100 µm. (D) Long term surviving graft from group iii, ■, stained with Elastin van Giessen stain to demonstrate internal elastic lamina (IEL) and outer elastic lamina (OEL). Scale bar = 100 µm.
Figure 1

A

CBA

day -28

YTS177

day -27

YTS177 +

B10 or BALB blood

CBA-Rag

day 0

Splenocytes

CD25^CD4^ fraction

day +1

Naive CD45RB^{high}CD4^ cells

B10 or BALB skin graft

B

% Surviving grafts

Days post transplantation

0 20 40 60 80 100

C

D
Figure 2

A

CBA

YTS177

BALB blood

CD25^+CD4^+ splenocytes

day -28
day -27
day -1
day 0

CBA-Rag

Naïve CD45RB^{high}CD4^+ cells

B10 skin graft

day +1

B

% Surviving grafts

Days post transplantation

0 20 40 60 80 100

C

D
Figure 3

A

**Tolerising protocol**

- Day -42
- Day -41
- Day -40
- Day -14
- Day -7
- Day 0

**CBA**

- YTS177
- YTS177
- YTS177
- HGG
- HGG

**Priming protocol**

- Day -42
- Day -41
- Day -40
- Day -14
- Day -7
- Day 0

**CBA**

- YTS177
- YTS177
- YTS177
- HGG
- HGG

**In vivo adoptive transfer protocol**

- Day -28
- Day -27
- Day -1
- Day 0

**CBA**

- YTS177
- YTS177
- HGG

B

**Anti-HGG antibody concentration**

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Figure 4

A

CBA

day -28
day -27
day -1
day 0

±YTS177
±YTS177
±HGG
±HGG

CD25^+CD4^+ splenocytes

day +1

CBA-Rag

Naïve CD45RB^{hi}CD4^+ cells

B

% Surviving grafts

Days post transplantation

0 20 40 60 80 100

B10 skin graft

C

D
Figure 5

A

CBA

day -28  day -27  day -1  day 0

YTS177  YTS177 ±HGG  ±HGG  B10

cardiac allograft ±YTS169

B

% Surviving grafts

0 20 40 60 80 100

Days post transplantation

C

D

IEL

OEL
CD25+CD4+ regulatory T cells generated by exposure to a model protein antigen prevent allograft rejection: antigen-specific re-activation in vivo is critical for bystander regulation

Mahzuz Karim, Gang Feng, Kathryn J Wood and Andrew R Bushell