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An Antibody Combination That Targets Activated T Cells Extends Graft Survival in Sensitized Recipients

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Memory T cells are the very essence of adaptive immunity with their rapid and efficient response to antigen rechallenge and long-term persistence. However, it is becoming increasingly evident that when primed with self or transplanted tissue, these cells play a key role in causing and perpetuating tissue damage. Furthermore, current treatments, which efficiently control the naive response, have limited effects on primed T cells. We have used a treatment based on a combination of antibodies specific for molecules expressed by activated T lymphocytes to selectively remove these cells. This approach, which we termed multi-hit therapy, leads to cumulative binding of antibodies to the target T cells and a striking prolongation of skin graft survival in presensitized recipients in a stringent skin transplant model. The findings are consistent with the depletion of graft-specific CD4+ and CD8+ T cells, although other modes of action, such as T-cell regulation and altered migration could play a role. In conclusion, our therapeutic strategy controls primed T cells which are a major driving force in the pathology of many autoimmune diseases and in transplant rejection.

Key words: Antibody therapy, sensitized recipients, T-cell memory, transplantation immunology

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Introduction

In the clinical transplantation setting the T-cell response to allogeneic tissue comprises both naïve and primed (memory) T cells. The latter are generated in response to previous exposure to alloantigen (transplants, blood transfusions, pregnancies) or to microbial antigens but which cross-react and respond to the transplanted tissue (1–4).

It is thought that 40–50% of peripheral blood T cells in adult humans are of memory phenotype (5). This is in contrast to most experimental transplant models which use recipients that are maintained in artificially clean environments and thus have a largely naïve immune repertoire. Recently, it has been demonstrated in rodent models that viral or bacterial infection before transplantation results in an increase in primed/memory T cells, heterologous immunity and resistance to treatments that can efficiently induce tolerance to specific allografts in naive animals (6,7).

As primed T cells have a lower activation threshold and an altered dependence on costimulatory signals compared with naive T cells, this would be predicted to contribute to their resistance to treatments based on costimulation blockade (8). Indeed, whereas treatment based on CD154-specific antibody alone (targeting the CD154-CD40 pathway) or in combination with CTLA-4-Ig (blocking the CD28-CD80/86 pathway) has proved highly effective in a number of transplant models, including kidney grafts in nonhuman primates (9), the primed immune response is highly resistant to such therapy (1,8,10).

Furthermore, in the majority of autoimmune disease models treatments are generally more effective if applied early during an autoantigen challenge rather than at a later stage when the disease has fully developed and is increasingly refractory to therapy (11). Thus, primed T cells play an important role in both transplant rejection and autoimmune diseases and their selective depletion would have considerable beneficial effects (12).

Materials and Methods

Multi-hit therapy

Antibody reagents were injected intraperitoneally (i.p.) on the day of challenge (grafting) (Day 0) and 4 days later. They consisted of antimouse CD154-specific antibody MR1 (250 μg/dose, ATCC), CD70-specific antibody FR70 (13) (500 μg/dose) and/or CD8-specific antibody YTS169.4.2.1 (5 μg/dose, ECACC). Rapamycin was prepared in 0.5% CMC/PBS and administered i.p. at the times and dose stated.

Tetramers

HY-specific CD8+ T cells were identified using PE-conjugated H-2D^b tetramer containing the immunodominant HY peptide WMHHNMDLI (Beckman Coulter).

Tetramer staining peripheral blood

Peripheral blood (100–150 μl) was collected into 1.5 mL Eppendorf tubes containing 200 μl blood buffer (0.01 M EDTA, 500 U sodium heparin in sterile

PBS). Red blood cells were lysed by addition of 1 ml lysis buffer (0.84% NH_4Cl in dH_2O) followed by incubation at RT for 10 min. Samples were centrifuged at 3000 rpm for 4 min, the supernatant aspirated and cell pellet resuspended in 200 μl of RPMI/10%FCS. The cells were then aliquoted into 96-V-well plates, centrifuged for 3 min at 1800 rpm and resuspended in 20 μl/well RPMI/10%FCS. Tetramer was added to the wells as required and left at 37°C for 15 min. Antibody reagents were then added to the appropriate wells and the plate incubated on ice for 20 min. Cells were spun down, supernatant discarded before washing once with PBS/2%FCS, and once with PBS alone. Final resuspension was in PBS for immediate analysis by flow cytometry (BD FacScan and Cell Quest software).

Tetramer staining splenocytes

Mouse splenocytes were enriched for T cells by depletion of IgG+ cells using sheep antimouse IgG Dynabeads (Dynal) for 20 min at RT with rotation. Contaminating IgG+ cells bound to the Dynabeads were removed using a magnet. The remaining T-cell-enriched preparation was used for tetramer staining (10^6 cells/well). Cells were preblocked in PBS/2%FCS containing rat antimouse CD16/CD32 antibody (0.5 μg/10^6 cells, BD) for 10 min at RT. Tetramer (3 μl/well) was then added directly and incubated for 10 min at 37°C. Antibody reagents were then added as required and incubation continued for a further 10 min at 37°C. Cells were then spun down and washed once with PBS/2%FCS, and once with PBS alone before final suspension in PBS and immediate flow cytometric analysis.

Phenotyping splenocytes

Antibody reagents from BD Biosciences (Oxford, UK) were CD8-FITC, CD8-CyChrome, CD4-FITC, CD69-PE and CD154-PE. Reagents from Serotec Ltd, (Oxford, UK) were CD154-FITC, CD70-FITC and CD44-FITC. Cells were stained in PBS/2%FCS. The required combination of antibodies (1 μg/10^6 cells) was added and cells incubated for 20 min on ice. Cells were then washed twice with PBS/2%FCS and resuspended in fixing solution before flow cytometric analysis as described in figure legends.

Detection of donor-specific (alloreactive) antibodies

Sera from skin transplant recipients (C57BL/6) were analysed for the presence of donor (BALB/c)-specific, alloreactive IgG and IgM antibodies. BALB/c splenocytes (donor cells) were preblocked by incubation with PBS/2%BSA/5% normal goat serum containing rat antimouse CD16/CD32–Fc block (0.5 μg/10^6 cells) for 20 min on ice. Hamster antimouse CD3-PE (BD); 1 μg/10^6 cells) was then added directly to the cells to enable subsequent gating on CD3+ T cells. After further incubation for 20 min on ice cells were washed twice using PBS/2%BSA. Recipient's serum or control (naive or no serum) was then added (5 μl/well, final dilution 1:10) to appropriate wells and incubated as before. Cells were washed twice (PBS/2%BSA) before the addition of goat antimouse IgM- or IgG-FITC (Sigma, UK, diluted 1:150 and 1:200, respectively) for 20 min on ice. Cells were washed again twice in PBS/2%BSA, resuspended in 1% paraformaldehyde and analysed using a FACScan flow cytometer and CellQuest software.

Skin transplantation across a minor histocompatibility (HY) barrier

Full-thickness tail skin segments (~1 cm^2) from syngeneic male C57BL/6 donors were transplanted on to the left flank of female recipients. Recipients were either naïve or presensitized by prior immunization (i.p.) with 10^7 syngeneic male splenocytes (i.p.) day-14. Grafts were held in place by three sutures and bandaged for 7 days. Rejection was defined as the complete loss of viable epidermal graft tissue. Antibody therapy was administered (i.p.) on the day of transplantation (Day 0) and 4 days later. Rapamycin was administered (i.p., 60 μg/dose naïve or 200 μg/dose presensitized recipients) for three consecutive days (Day 0–2).

Skin transplantation across a fully mismatched allogeneic MHC barrier

C57BL/6 mice were immunized (i.p.) with 10^7 BALB/c splenocytes. Fourteen days later, full-thickness BALB/c tail skin segments (~1 cm^2) were transplanted on to the left lateral flanks of C57BL/6 recipient mice, as previously described. Antibody therapy was administered (i.p.) on the day of transplantation (Day 0) and Day 2, 4, 7 and 10 posttransplantation. Rapamycin was administered i.p., 60 μg/dose for 14 consecutive days (Day 0–13).

IL-2 specific ELISPOT assay

All reagents were provided by R&D Systems (Abingdon, UK) and assays were carried out according to manufacturers instructions. In brief MultiScreen-IP 96-well plates (Millipore UK Ltd) were coated (100 μl/well) with antimouse IL-2 capture antibody (1:60 in PBS) overnight at 4°C. Plates were then preblocked by addition (100 μl/well) of PBS/1%BSA for 2 h at RT and washed extensively with sterile PBS. HY-specific recall responses by CD4+ T cells were determined by culture of recipient splenocytes (2×10^5/well) in the presence/absence of HY Ab peptide (10 μM, a kind gift from D. Scott, Imperial College, London, UK) for 24 h at 37°C. Alloreactive recall responses were determined by co-culture of recipient splenocytes (2×10^5/well) in the presence of irradiated donor (BALB/c), third party (CBA/J) or syngeneic (C57BL/6) stimulator cells (1×10^5) for 24 h at 37°C. Cells were then lysed by brief washing with dH_2O and plates washed ×6 with PBS/0.1% Tween-20 (Sigma). Biotinylated antimouse IL-2 detection antibody was then added (100 μl/well 1:60 in PBS/1%BSA) overnight at 4°C. After washing as previously described, streptavidin alkaline phosphatase (100 μl/well 1:60 in PBS/1%BSA) was added for 1 h at RT. Plates were washed with PBS/0.1% Tween-20 and once with dH_2O before addition of BCIP/NBT substrate (Sigma). The reaction was stopped after 30 min in the dark by washing with dH_2O. Plates were air dried before IL-2 spots were enumerated using an automated counter.

Statistical analysis

Survival data were analysed using the Kaplan–Meier method with the log-rank test used to verify the significance of the difference in survival between groups. For all other data statistical analysis in each independent experiment was performed with an unpaired Student's *t*-test. A value of $p < 0.05$ was considered statistically significant.

Results

Presensitization dramatically alters the HY response which becomes refractory to treatment

In this study we have used the well-characterized minor histocompatibility HY model in which female C57BL/6 mice respond to syngeneic male tissue (14). In a naive (primary) system, we found that CD154-specific antibody treatment alone (250 μg on days 0 and 4) results in indefinite acceptance of male HY skin by female recipients (>200 days in five out of six grafts, Figure 1A). Furthermore, CD154 antibody treatment prevented the expansion of HY-tetramer-specific CD8+ T cells in this primary (1°) response (Figure 1B). In contrast these T cells were detected from 10 days post-HY transplant and persisted for >75 days in untreated female recipients (15).

Priming female recipients to HY before rechallenge, i.e. a secondary (2°) immune response, resulted in a dramatic acceleration of male skin graft rejection (Median Survival

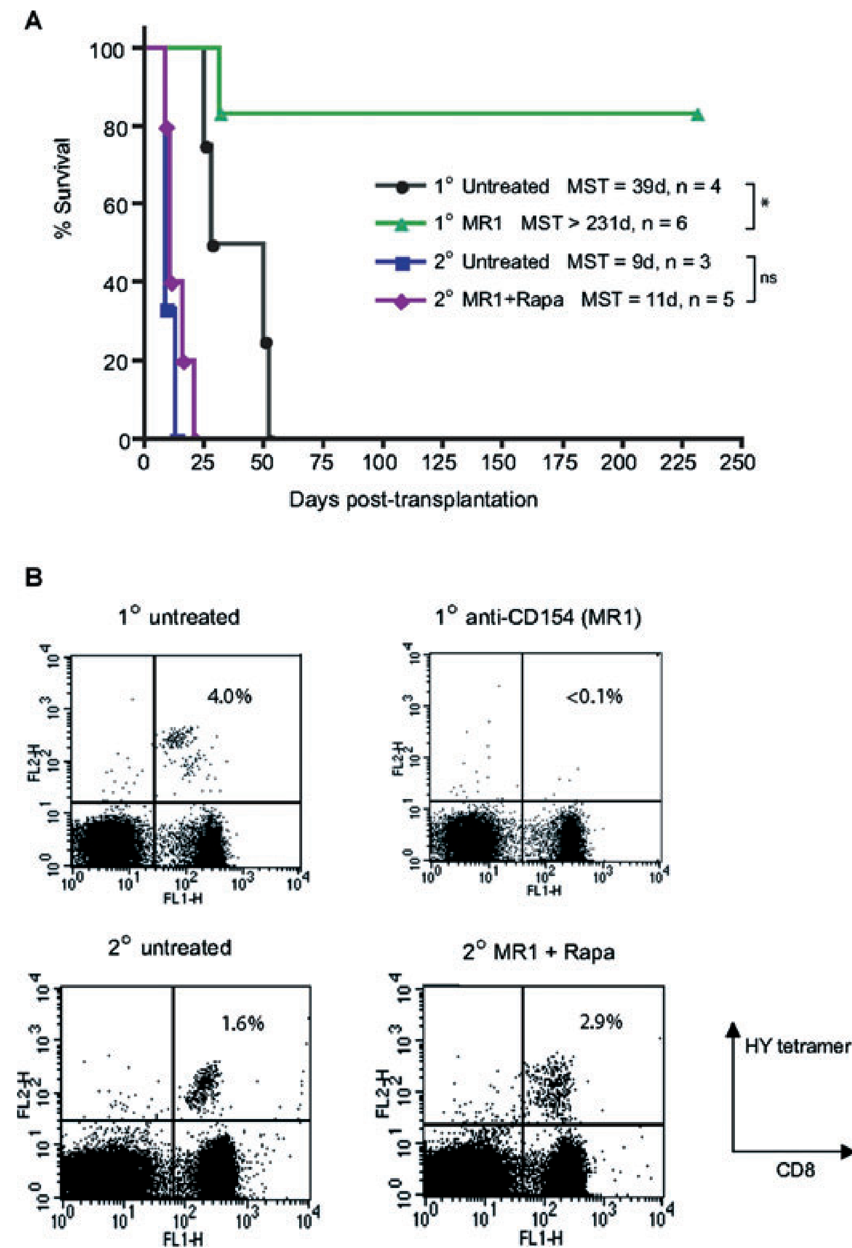

Figure 1: In presensitized female recipients CD154-specific antibody MR1 with or without Rapamycin fails to prevent the rejection of male (HY) skin grafts. (A) Full thickness tail skin grafts from C57BL/6 male donors were transplanted onto syngeneic female mice. Recipients were either naïve (1°) or presensitized (2°) by prior i.p. with 10^7 syngeneic male splenocytes 14 days previously. Antibody therapy (250 μg MR1/dose i.p.) was given on the day of transplantation (Day-0) and 4 days later (Day 4), Rapamycin (60 μg/dose for 1° response or 200 μg/dose for 2° response) was administered (i.p.) for three consecutive days (Days 0–2). MST is shown for untreated and treated groups. Data are representative of three independent experiments. *p = 0.0101; n.s., not significant. P-values are for the experiment shown and are for comparison between the untreated and treated group for a naive and presensitized HY response respectively. (B) Spleens were recovered from recipients on Day-14 following transplantation and splenocytes stained with HY tetramer (H-2D^b/Uty) and CD8-specific antibody. Data shown are for one representative individual from each group, comprising 3–6 mice per group, confirmed in three independent experiments.

Time (MST) = 9 days) compared to a naive (1°) response, MST = 39 days (Figure 1A). In addition, presensitization abolished the ability of CD154-specific antibody with rapamycin to prolong graft survival and prevent expansion of HY-tetramer-specific CD8+ T cells in primed recipients (Figure 1A, B). These findings clearly demonstrate that the presensitized HY model provides a robust and challenging immune response.

Activated CD4+ and CD8+ T cells differentially express CD154

To investigate why presensitization alters CD154-mediated antibody therapy, we analysed the surface phenotype of activated CD4 and CD8 T cells following HY antigen challenge. We consistently found low but detectable CD154 expression is induced in primed CD4+ T cells following challenge by male HY splenocytes (Figure 2A). In contrast, activated CD8+ T cells from primed recipients did not express detectable levels of CD154 upon activation (Figure 2A). Confirmation that cells analysed were activated was provided by the detection of surface CD69 expression (Figure 2A). As the staining in Figure 2A does not discriminate nonspecifically activated T cells, the lack of CD154 expression by HY-specific responders was demonstrated by staining the HY-tetramer-specific CD8+ T-cell population (Figure 2B). The primed/memory phenotype of the HY-tetramer+ T cells was confirmed

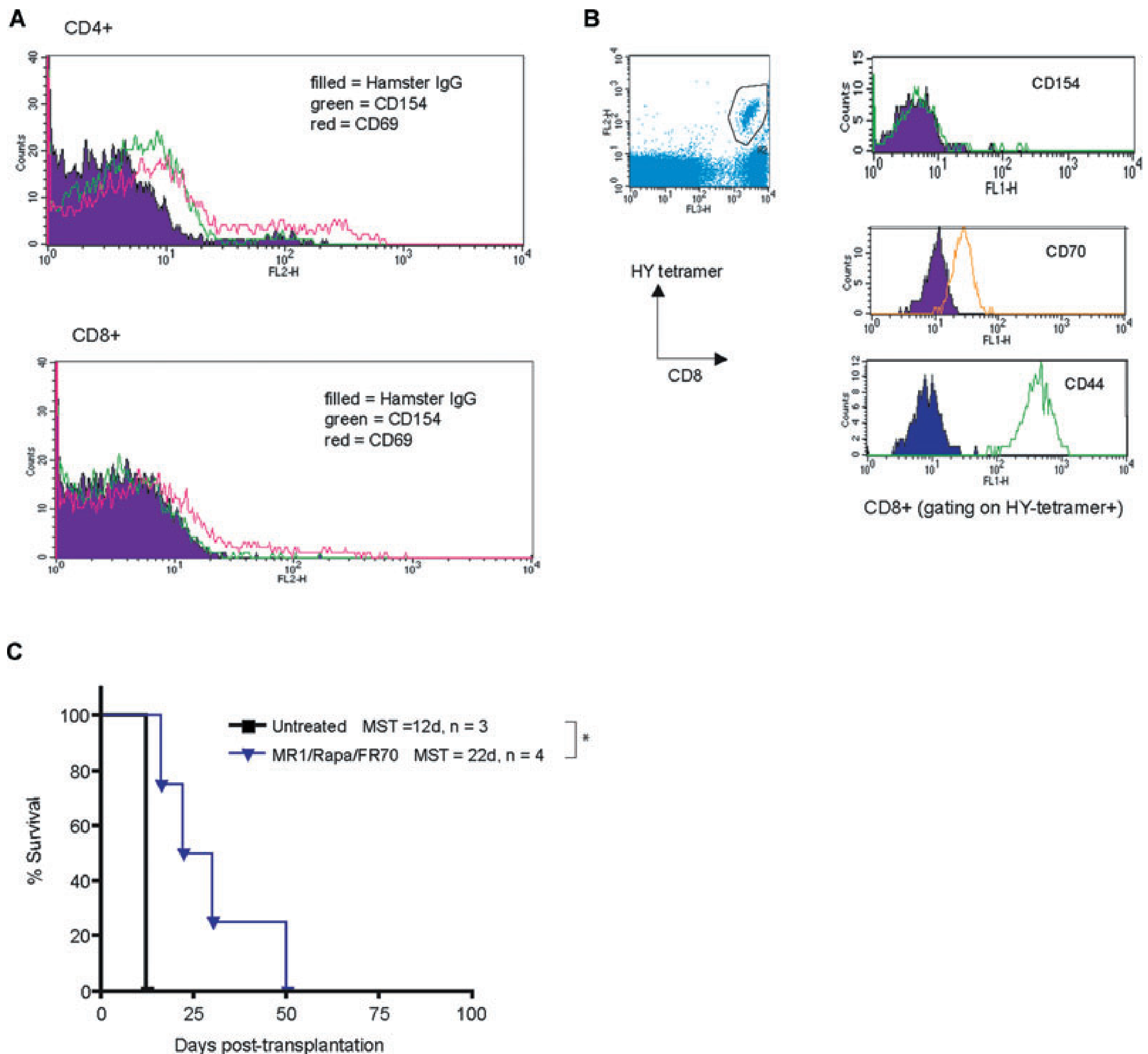

Figure 2: Phenotypic analysis of activated, primed T cells shows CD154 expression by CD4+ but not CD8+ T cells. (A) Spleens were recovered from HY presensitized (10^7 male splenocytes i.p. Day-14) female C57BL/6 mice 24 h following rechallenge in vivo (10^7 male splenocytes i.p.). Staining with CD4- or CD8-specific antibody and antibodies specific for CD154, CD69 or hamster IgG isotype control is shown. (B) Splenocytes from primed and rechallenged (as described in A) female mice were stained with HY tetramer (H-2D^b/Uty), CD8-CyChrome and antibodies specific for CD154, CD70 or CD44. Analysis was carried out gating on HY tetramer+ CD8+ T cells. Histograms in panels A and B show the level of surface expression for each molecule. Data shown are for a representative individual from each group (n = 3) and were confirmed in two independent experiments. (C) Full thickness tail skin grafts from C57BL/6 male donors were transplanted on to syngeneic female mice presensitized by prior i.p. with 10^7 male splenocytes on Day-14. Recipients were untreated or received antibody therapy comprising 250 μg MR1/dose and 250 μg FR70/dose (i.p.) on the day of transplantation (Day 0) and Day 4 and Rapamycin (200 μg/dose i.p.) for three consecutive days (Days 0–2). MST is shown, data are representative of two experiments. *p = 0.0143. The p-value is for the experiment shown and is for comparison between the untreated and treated group.

by the uniformly high CD44 expression by these cells (Figure 2B).

In a primary HY response CD4+ T cell derived help is critical for the development of CD8+ effector T cells, therefore CD154-specific antibody-mediated depletion of CD4+ T-cell responders is sufficient to prevent both CD4+ and CD8+ T-cell responses. However, controlling the cohort of presensitized CD8+ responders clearly requires reagents that specifically target these cells. The surface

phenotyping of the CD8+ HY-tetramer+ T cells showed induced expression of CD70 upon activation (Figure 2B) indicating that CD70 might provide a suitable target molecule.

The potential of CD70-specific antibody (FR70) to control T-cell responses is supported by the observation that as a single reagent in a naive (nonsensitized) HY model, it extended graft survival from MST = 39 days (n = 4) in untreated controls to MST = 77 days (n = 5). However, this was substantially less effective than therapy with CD154-specific antibody alone in a naive HY model (MST > 231 days, n = 6, Figure 1A). In the presensitized HY model, the therapy with CD70-specific antibody alone had no effect on skin graft survival (data not shown).

The triple therapy combination comprising CD154-specific antibody (MR1), FR70 and rapamycin (Rapa) showed modest but significant (p = 0.0143) efficacy in prolonging graft survival in presensitized female mice rechallenged with male skin grafts (MST = 22 days, Figure 2C).

Multihit therapy has striking effects on HY skin transplant survival in presensitized recipients

We hypothesise that activated primed CD8+ T cells may be targeted using multiple antibodies which could include a 'base antibody', which functions to render the target cells sensitive to the effects of a selective antibody such as anti-CD70. A depleting CD8-specific antibody (YTS169.4.2.1) (16) could be used at doses that bind the target cells but do not cause their indiscriminate depletion. Indeed, we found that the use of 5 μg/dose of CD8-specific antibody alone results in variable but incomplete depletion (10–50%) of the CD8+ T-cell population (data not shown).

When used a single reagent in the HY skin transplant model, CD8-specific antibody (5 μg dose on days 0 and 4) prolonged the survival of grafts in the nonsensitized recipients (MST = 94 days, n = 5). However, this anti-CD8 therapy had no effects on graft survival in presensitized recipients (Figure 3A, MST = 13 days).

A combination of CD8- and CD70-specific antibodies could bind to the surface of the same activated, primed CD8+ T cells (multiple hits), thus reaching a threshold for Fc-mediated depletion and overcoming the apparent resistance of these cells to therapeutic control. Similarly, the CD70- and CD154-specific antibodies would be expected to efficiently control the activated CD4+ T-cell responders. The rapamycin added to the antibody combination functions to limit the T-cell responder pool size. Indeed, when used as a single reagent rapamycin (40 μg on days 0–3 postsensitization) prevented the expansion of CD8+ HY-tetramer+ T cells in the naive HY model (3.0–4.5%, n = 3 in the nontreated group vs. 0.2–0.3%, n = 3) in the rapamycin group. Furthermore, in the sensitized HY model rapamycin therapy at higher doses (80–200 μg dose on days 0–3 post-HY antigen rechallenge) resulted in a modest decrease in the expansion of the CD8+ HY-tetramer+ T cells (1.0–14.6%, n = 8 in the untreated group vs. 0.3–5.8%, n = 7 in the rapamycin group). Lower doses of rapamycin (10–60 μg) had no effects on the primed CD8+ HY-tetramer+ T-cell population (data not shown).

Importantly, we found that a combination of four reagents (CD8-, CD154-, CD70-specific antibodies and rapamycin), termed multi-hit therapy, had striking effects on HY skin transplant survival in the presensitized HY model (MST = 84 days, Figure 3A). Extended skin graft survival was associated with the absence of HY-tetramer+ CD8+ T cells in sequential tail bleeds posttransplantation (Figure 3B). These HY-tetramer+ T cells were undetectable in naive recipients (data not shown). Following priming i.p. with HY splenocytes, a population of HY-tetramer+ CD8+ T cells was evident by day 10 and reached a peak 14 days postimmunization (15). Rechallenge with HY antigen resulted in a modest expansion of HY-tetramer+ CD8+ T-cell population from 2.0 to 4.5%, n = 6 to 1.0–14.6%, n = 8.

HY-tetramer+ CD8+ T-cell numbers were low or below the level of detection in tails bleeds from mice with functioning skin grafts, whereas their presence was invariably associated with graft rejection (as illustrated in Figure 3C). The absence of accelerated rejection and the removal of HY-tetramer+ T cells suggest the selective loss of primed T cells since if the responders were merely rendered nonfunctional, their presence would be revealed upon tetramer analysis. Although, it is possible that the treatment has caused the redistribution of the effector T cells into other niches, such as bone marrow and lymph nodes, which were not sampled for HY-tetramer staining.

The requirement for 'multiple antibody hits' is supported by the observation that the removal of the CD70-specific antibody substantially decreased the therapeutic effects (Figure 3A). Indeed, use of a lower dose of CD70-specific antibody (250 μg instead of 500 μg) resulted in the significant loss of efficacy (data not shown).

To gain further insight into the mechanisms of graft survival mediated by the multi-hit therapy we retransplanted male skin both to multihit treated recipients with indefinitely surviving grafts (>100 days) and to those that had rejected their grafts after prolonged survival. Despite surgery and in the absence of additional treatment, recipients in the indefinite survival group accepted the second male grafts (>100 days, n = 4), whereas those that had rejected their first graft rapidly rejected their challenge male grafts (MST = 12 days, n = 4). This result was confirmed (n = 4, data not shown) and suggests that both primed T cells and the naive T-cell population have been depleted of HY-specific responders to an extent that the remaining cohort, i.e. new thymic emigrants, is insufficient to reject the second graft. Alternatively, the therapy may have altered the balance between effector T cells and regulatory T cells in favor of the latter and thus insured long-term graft survival.

Multi-hit therapy effects on primed HY-specific CD4+ T cells

To investigate the effects of the multi-hit therapy on primed HY-specific CD4+ T cells we recovered spleen cells from female mice that were HY presensitized, rechallenged and treated with multi-hit therapy as described before. The responder T cells were stimulated *ex vivo* using the HY H2Ab peptide (17), the sole HY epitope presented by H-2A^b MHC class II molecules to CD4+ T cells (17, 18). Stimulation with the HYAb peptide resulted in IL-2 production by T cells obtained from untreated presensitized female mice (Figure 4). In contrast, the multi-hit therapy group showed a complete absence of HYAb peptide-specific T-cell responders (Figure 4).

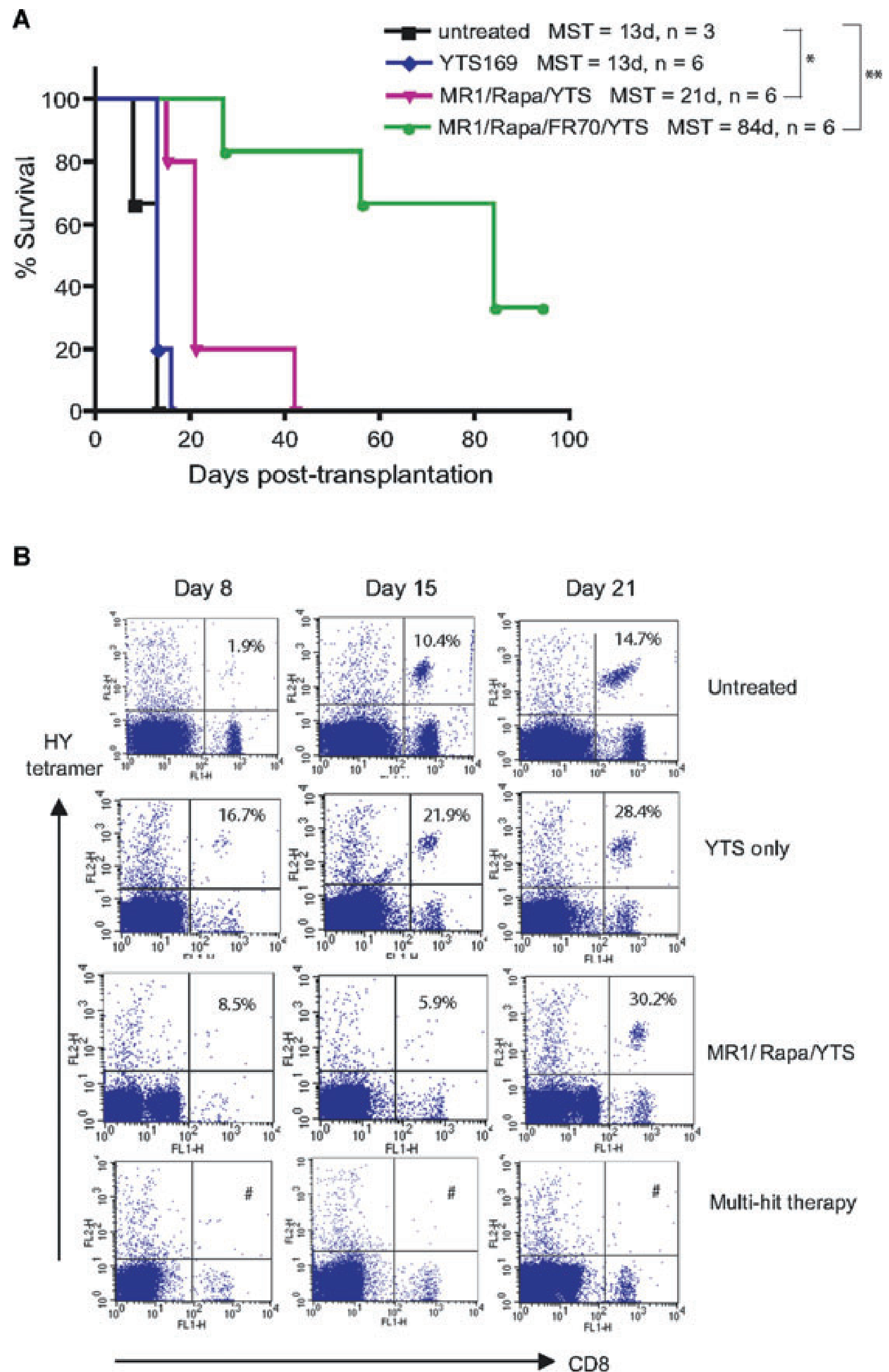

Figure 3: Multi-hit therapy of presensitized recipients results in prolonged skin graft survival and removal of HY-specific T cells. (A) Presensitized female recipients of male skin grafts were untreated or treated with multi-hit therapy comprising antibodies (MR1, YTS169 and FR70 i.p. at 250, 5 and 500 μg/dose, respectively) on Days 0 and 4 and Rapamycin (200 μg/dose) for three consecutive days (Days 0–2). Control groups received YTS169 antibody alone or the MR1/YTS169/Rapamycin combination. The data are representative of three experiments. The p-values are for the experiment shown. *p = 0.0067 untreated versus MR1/Rapa/YTS group; **p = 0.0033 untreated versus MR1/Rapa/FR70/YTS group. (B) Peripheral blood from recipient female mice obtained by sequential tail bleeds was stained for the presence of HY-specific CD8$^+$ T cells using HY tetramers (H-2D^b/Uty). Data shown are for one representative individual from each group (n = 3–6), confirmed in three independent experiments. #Staining was indistinguishable from the background obtained in the absence of tetramers. (C) Peripheral blood samples obtained from presensitized female recipient mice treated with multihit therapy were stained as described in B. Data shown are from representative individual recipients, confirmed in three independent experiments.

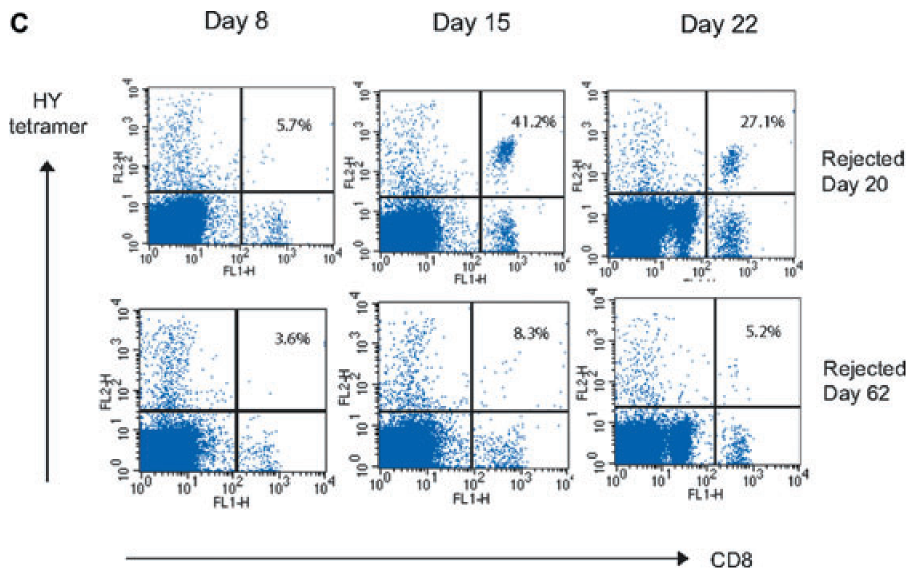

Figure 3: Continued.

Multi-hit therapy prolongs skin graft survival in presensitized MHC mismatched recipients

By adding a presensitization step to the highly stringent, fully MHC mismatched BALB/c to C57BL/6 skin transplant model (19) we have generated a setting where, to our knowledge, none of the existing treatments used to prolong graft survival have shown any beneficial effects. However, the multi-hit combination therapy resulted in a significant prolongation ($p < 0.0001$) of skin graft survival in presensitized recipients (Figure 5A) in this allogeneic strain combination. Notably, in 4 out of 11 mice from the multi-hit treatment group skin grafts were present for >50 days, a striking result in such a challenging setting.

To investigate the effects of multi-hit therapy on the alloresponse, treated mice which retained their allografts on day 56 posttransplant were sacrificed and their T-cell responses analysed *ex vivo* using an IL-2-specific ELISPOT assay. Responder splenocytes from mice of the multi-hit therapy group showed a significant decrease in the number of donor-specific IL-2 producing T cells in contrast to those from untreated controls (Figure 5B). Importantly, the

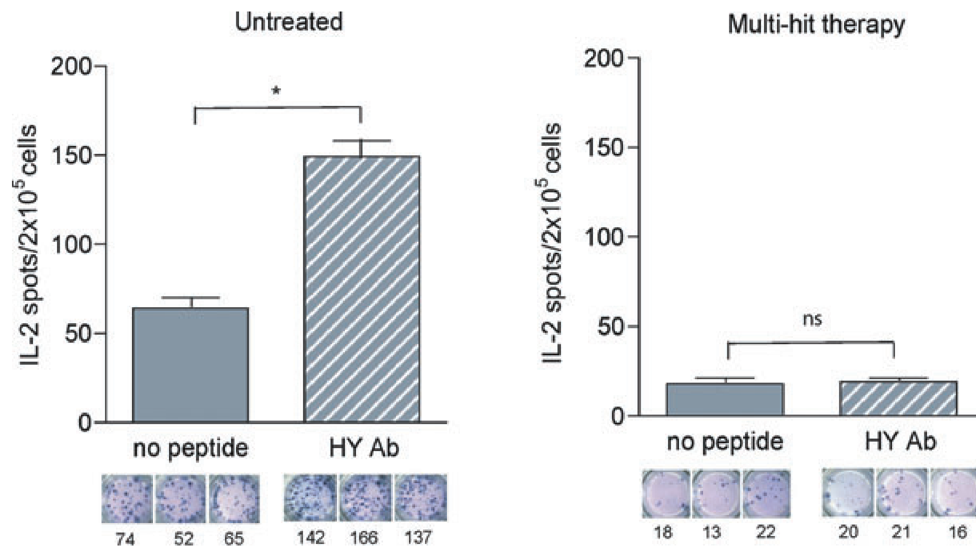

Figure 4: The selective removal of primed CD4+ T cells. Presensitized female mice were re-challenged with HY antigen and treated with multi-hit therapy (n = 4) as described in Figure 3A, or left untreated (n = 3). Splenocytes were recovered 3 weeks later and cultured *ex vivo* in the presence/absence of HY Ab peptide (10 μM) for 24 h. IL-2 production was detected by standard ELISPOT assay. Images of triplicate ELISPOT wells and the number of spots per 10^5 responders are shown. Results are for one representative individual from each group (n = 3–4), confirmed in two independent experiments. *P = 0.0015; n.s., not significant. P-values describe the experiment shown.

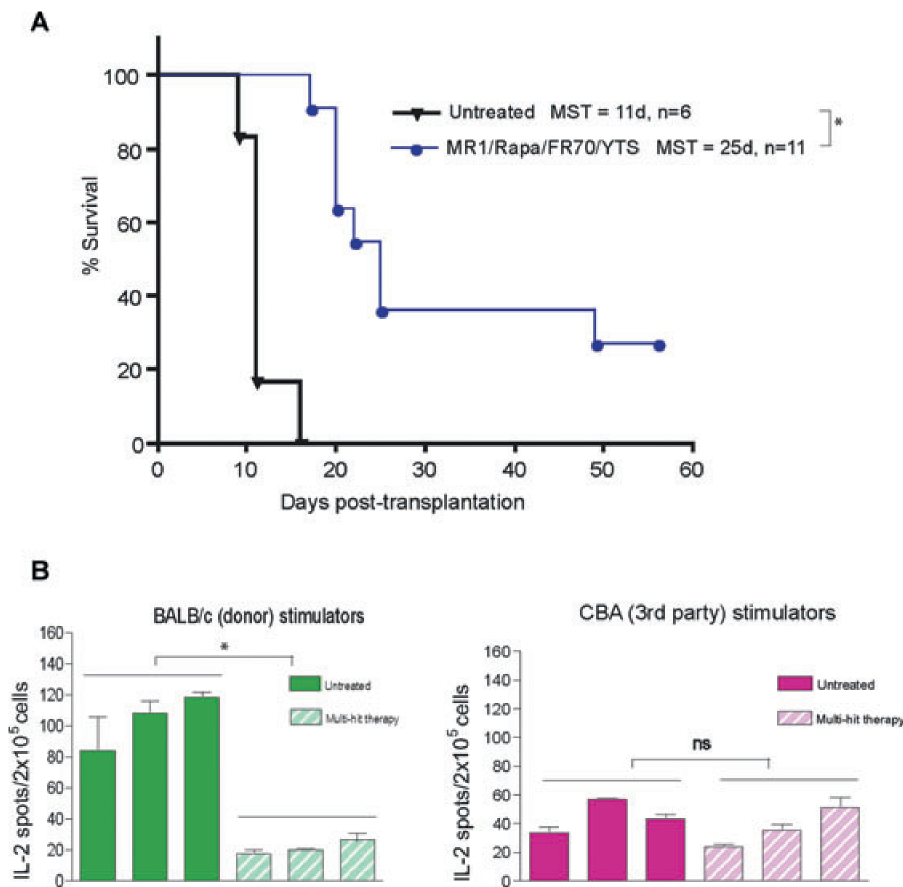

Figure 5: Multi-hit therapy prolongs survival of MHC mismatched allogeneic skin grafts in presensitized recipients. (A) Full thickness tail skin segments from BALB/c donors were transplanted on to C57BL/6 recipient mice presensitized by prior i.p. with 10^7 BALB/c splenocytes on day-14. Following transplantation (day 0) recipients were either untreated or given multi-hit therapy comprising antibodies i.p. (MR1, YTS169 and FR70 at 250, 5 and 500 μg/dose, respectively) on Day 0, 2, 4, 7 and 10 with Rapamycin (60 μg/dose i.p.) for 14 consecutive days (Days 0–13). MST is shown, data are representative of three independent experiments. $^*p < 0.0001$ is for the experiment shown and is a comparison between the untreated and treated group. (B) The spleens were recovered from multi-hit treated recipient mice with allogeneic skin grafts present at day 56 (n = 3) and untreated controls (n = 3). Responder splenocytes (2×10^5/well) were cultured in the presence of (1×10^5) irradiated donor (BALB/c) or third party (CBA/J) stimulator cells for 24 h. IL-2 production was detected by standard ELISPOT assay and data shown are the mean number of spots per triplicate wells ± s.e.m., for three different mice from each treatment group. $^*p < 0.0001$; n.s., not significant. P-value is for the experiment shown and is for comparison of the untreated and multi-hit treated response vs. donor (BALB/c) or third party (CBA) stimulators. Data are representative of three independent experiments.

response to third (3rd) party CBA stimulators was unaffected by therapy (Figure 5B).

Donor-specific antibodies in presensitized recipients of skin allografts

Sera from presensitized skin transplant recipients (C57BL/6), treated with multi-hit therapy (n = 3) and untreated controls (n = 3), were collected pretransplant (Day 0) and in the posttransplant period (Day 10 and Day 20). The presence of donor (BALB/c)-specific, alloreactive antibodies was investigated by flow cytometry gating on CD3+ T cells and using mouse IgM- or IgG-specific antibody. The serum from each individual mouse was tested separately. Presensitization of the recipient (C57BL/6) mice (Day 0) resulted in the production of donor-specific IgM antibodies (geometric mean fluorescence intensity—MFI range = 32–45). The background staining (no serum) ranged MFI = 7–15. In the posttransplant period (Day 20), IgM alloantibodies decreased in both treated (MFI = 13–20) and control (MFI = 11–13) groups (data not shown).

The levels of donor-specific IgG antibodies were relatively high in presensitized mice (Day 0, MFI = 234–457, Figure 6, background MFI = 14–54). In the posttransplant period (Day 20), alloreactive IgG were clearly detectable in the treated mice, although the antibody levels were decreased

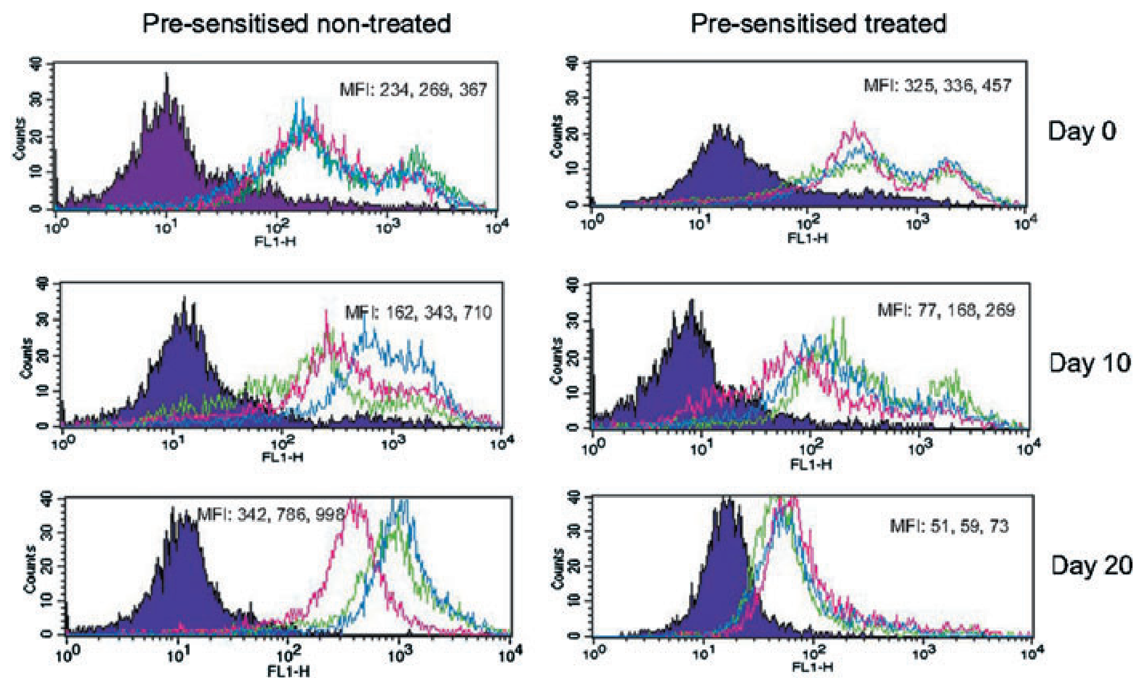

Figure 6: Donor-specific alloantibodies in presensitized skin transplant recipients. The individual sera from six presensitized C57BL/6 recipients of allogeneic (BALB/c) skin were obtained by tail bleeds on Days 0 (pretransplant), Day 10 and Day 20 posttransplant. The presence of donor (BALB/c)-specific IgG was detected, gating on CD3+ cells, following incubation of donor splenocytes with the sera and staining with antimouse IgG-FITC (thin lines). Histograms were generated by flow cytometric analysis and geometric MFI values are shown. Each colored line represents serum from a single mouse from the group and the background staining (no serum) is shown as a solid filled peak. Data are representative of two experiments.

(MFI = 51–73, Figure 6, background MFI = 11–18). In contrast, alloreactive IgG antibodies in the untreated mice were increased (MFI = 342–998, background MFI = 9–12) compared to Day 0 levels (Figure 6).

Discussion

This paper describes the development of a novel multi-hit therapy to target activated T cells. This approach shows striking potential as a treatment for presensitized transplant recipients. Our data suggest that the multi-hit therapy when administered during antigen rechallenge can efficiently control primed T-cell responders. We demonstrate this in two model systems, in one of which the antigen specific epitopes are known, allowing accurate identification and measurement of responder cells. We propose that the same principles may apply to the challenging clinical setting, where multi-hit therapy could have wide implications in controlling primed T cells in both the transplant and autoimmunity settings.

We show that priming dramatically changes the nature of the immune response and the ability of treatments to control it. Whereas the naive immune response to the HY transplantation antigen is susceptible to CD154-specific antibody or rapamycin as single reagents, priming makes this response resistant to both reagents individually and their potent combination. This is surprising in view of the efficacy shown by the CD154-specific antibody and rapamycin combination which is capable of extending graft survival in one of the most stringent naive transplant models, that of fully MHC mismatched BALB/c to C57BL/6 skin allografts (19–21).

Our previously published data demonstrated that CD154-specific antibody works primarily by depleting activated T cells (20), although blocking of the CD40-CD154 interaction may also contribute to its effects (22). More recent studies have confirmed that Fc-mediated T-cell depletion plays a critical role in the effects of CD154-specific antibody, particularly in stringent transplant settings (23). Importantly, however, trials using CD154-specific antibody in humans identified thromboembolic side effects (24). Consequently, therapeutic formulations that do not include CD154-specific antibody are preferable in the clinic. In principle, multi-hit therapy is not dependent on a particular molecular pathway and it should be possible to substitute

anti-CD154 with another antibody specific for the activated T-cell targets without appreciable loss of efficacy. This putative flexibility of the multi-hit approach may have important advantages for this therapeutic strategy and it is currently being investigated.

CD70-specific antibody treatment has recently been shown to diminish the expansion of effector/memory CD8+ T cells in CD28-deficient mice, presumably by blocking the CD27-CD70 costimulatory pathway (25). Our data do not exclude a contribution of antibody-mediated blockade in the efficacy of the therapy. However, we have used a nontransgenic transplant model with a fully functional CD28-CD80/86 pathway where the effects of CD27-CD70 blockade are modest (25) and thus are unlikely to play a major role in the effects of multi-hit therapy. Furthermore, the addition of CD70-specific antibody to the multihit therapy combination was critical in achieving the removal of HY-tetramer+ T cells, which suggests that depletion of primed, activated T cells is a key mechanism of its *in vivo* action. This is supported by a recent report that CD70-specific antibody can deplete *in vivo* tumor targets that express CD70 and that this effect is dependent on a functional Fc region of the antibody (26). However, as we have not excluded the prevention of T-cell expansion or the induction of cell death as alternative mechanisms for the therapeutic effects of anti-CD70, this important question remains to be elucidated.

Multi-hit therapy targets activated T cells and thus, if administered during an acute viral infection, it could remove virus-specific effector and memory T cells. However, viral infections that go unnoticed before the multi-hit therapy is applied are likely to be less fulminate and thus could persist without severe symptoms until therapeutic antibody levels decline in 2–4 weeks time (27). After which, a persistent viral infection is likely to present a stronger immune stimulus than the noninflammed transplant. Consequently, new virus-specific T-cell emigrants can mount an effective defence and generate a new virus-specific memory population.

A number of therapeutic successes in controlling primed alloreactive T cells in transplantation have been reported (28,29), however these have all been obtained in less stringent models such as a cardiac transplantation model (29) or using the adoptive transfer of responder T cells (28). Interestingly, an earlier study by Cobbold et al. (30) prolonged survival of skin transplants by presensitized mice, however the mechanism responsible for the efficacy of this treatment, a combination of depleting, followed by nondepleting CD4- and CD8-specific antibodies, remains unclear (30).

The presensitization with allogeneic splenocytes results in the generation of donor-specific antibodies of both IgG and IgM subtypes. The titer of IgM alloantibodies decreases in the posttransplant period in both treated and untreated skin allograft recipients. However, after transplantation the levels of donor-specific IgG antibodies increases in untreated mice. In contrast, the multi-hit therapy prevents this increase and even reduces the level of IgG alloantibodies (Figure 6). Nevertheless, donor-specific antibodies clearly persist in treated mice.

It is possible that the persistence of alloantibodies in mice treated with multi-hit combination may cause chronic injury and result in late graft loss. The development of therapies that target antibody producing B cells could be used to address this. Furthermore, such therapies may have synergistic effects with treatments aimed at T cells.

Several studies (31–34) have shown that primed/memory T cells have increased resistance to depleting antibodies, although the mechanisms involved are not well understood. Here, we demonstrate that this increased resistance of primed T cells to presumed Fc-mediated depletion can be overcome by targeting these cells with multiple antibody hits resulting in their removal.

Although a potential role for regulatory T cells (Treg) has not been addressed in this study, Treg could contribute to treatment efficacy by controlling putative effector T cells that escape therapy, as well as new thymic emigrants. This is supported by earlier studies which suggested that the individual components used in our multi-hit therapy, such as CD154-specific antibody, may promote Treg induction (35,36).

As the naïve T cell responders appear more sensitive to therapy than primed T cells (1,4,8), it may be presumed that the multi-hit therapy also controls any primary response mounted by activated naïve T-cell responders. This will contribute to the overall efficacy of the therapy.

We believe that this study is the first which describes a therapeutic strategy that controls primed T cells to prolong graft survival in sensitized recipients. This striking effect has been achieved when mice were primed with cognate transplantation antigens and also reproduced in a highly stringent fully MHC mismatched skin transplant model.

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Conflict of Interest Statement. The authors have no financial considerations or conflicts of interest to declare.

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