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Brief communication

Deletion of naïve T cells recognizing the minor histocompatibility antigen HY with toxin-coupled peptide-MHC class I tetramers inhibits cognate CTL responses and alters immunodominance

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ABSTRACT

Alloreactive T-cell responses directed against minor histocompatibility (H) antigens, which arise from diverse genetic disparities between donor and recipient outside the MHC, are an important cause of rejection of MHC-matched grafts. Because clinically significant responses appear to be directed at only a few antigens, the selective deletion of naïve T cells recognizing donor-specific, immunodominant minor H antigens in recipients before transplantation may be a useful tolerogenic strategy. We have previously demonstrated that peptide-MHC class I tetramers coupled to a toxin can efficiently eliminate specific TCR-transgenic T cells in vivo. Here, using the minor histocompatibility antigen HY as a model, we investigated whether toxic tetramers could inhibit the subsequent priming of the two H2-D^b-restricted, immunodominant T-cell responses by deleting precursor CTL. Immunization of female mice with male bone marrow elicited robust CTL activity against the Uty and Smcy epitopes, with Uty constituting the major response. As hypothesized, toxic tetramer administration prior to immunization increased survival of cognate peptide-pulsed cells in an in vivo CTL assay, and reduced the frequency of corresponding T cells. However, tetramer-mediated decreases in either T-cell population magnified CTL responses against the non-targeted epitope, suggesting that D^b-Uty^+ and D^b-$Smcy^+$ T cells compete for a limited common resource during priming. Toxic tetramers conceivably could be used in combination to dissect manipulate $CD8^+$ T-cell immunodominance hierarchies, and to prevent the induction of donor-specific, minor H antigen CTL responses in allotransplantation.

1. Introduction

Host immune responses to donor antigens constitute one of the major mechanisms underlying chronic rejection of transplanted organs [1]. Matching MHC antigens reduces anti-donor T-cell responses and improves long-term survival of allografts, such as kidneys [2], but does not provide complete tolerance nor obviate the need for lifelong immunosuppressive therapy [3,4], which in itself can contribute to transplant dysfunction and demise [5]. Diverse non-MHC polymorphisms across the donor genome give rise to minor histocompatibility (H) antigens, which, excepting those encoded by mitochondrial DNA, are presented by classical class I and II molecules to alloreactive T cells [6]. While the frequency of T cells recognizing these epitopes is only a small fraction of those that react to donor MHC molecules, most minor H antigen disparities in outbred populations cannot readily be circumvented by matching, and consequently, these donor-reactive T-cell responses can be clinically significant causes of rejection [7].

A variety of immunomodulatory agents, often in combination, are chronically administered to graft recipients to suppress alloreactive T-cell responses, including anti-metabolites (e.g., mycophenolate), and inhibitors of the calcineurin and mammalian target of rapamycin (mTOR) pathways. While effective, it has also become clear that, during the initial induction of transplantation tolerance, deletion of anti-donor T cells is optimally needed to reduce the number of alloreactive effectors to levels that can be controlled by pharmacologic maintenance therapy and peripheral physiologic regulatory mechanisms [8,9]. Accordingly, antibodies against T-cell surface markers have been used as depleting agents for bulk T cells, specific subsets, or those of particular activation

Abbreviations: APC, allophycocyanin; CFSE, carboxyfluorescein diacetate succinimidyl ester; IP, intraperitoneally; IV, intravenously; H, histocompatibility; LCMV, lymphocytic choriomeningitis virus; MFI, mean fluorescence intensity; PBSE, Pacific Blue succinimidyl ester; PBL, peripheral blood lymphocyte; PE, phycoerythrin; SAP, saporin; *Yeti*, YFP-enhanced transcript for IFN-γ; YFP, yellow fluorescent protein.

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status, in both clinical patients (antithymocyte globulin; anti-CD2 and -CD52 mAbs) and experimental models (anti-TCRαβ, -CD3ε, -CD4, -CD8α, -CD25, -CD28, -CD45, -CD154 and -CD223 mAbs) [10]. However, wholesale elimination of polyclonal T cells can result in the loss of Tregs, compromising transplantation tolerance, as well as the deletion of protective T cell responses, increasing the risk of opportunistic infections. Ideally, to induce graft tolerance, only donor-specific T cells would be deleted. At first glance, minor H antigen differences would appear too numerous and diverse to permit such an approach, but fortunately, these antigens are limited by immunodominance mechanisms [6], and hence, are rational targets for intervention. The great majority of minor H antigens in humans [7] and mice [3] are MHC class I-restricted, and their cognate $CD8^+$ T cells can be visualized with fluorescently labeled peptide-MHC (pMHC) class I tetramers [11,12]. Logically, the next step is to determine whether such tetramers can be employed to mediate antigen-specific depletion of these alloreactive T cells. We and others have previously demonstrated that class I tetramers can be used to selectively deliver a lethal hit to $CD8^+$ T cells [13–15]. In two models, injection of "toxic tetramers"(tetramers that were coupled to the ribosome-inactivating phytotoxin, saporin [SAP]) eliminated >75% of adoptively transferred, TCR-transgenic $CD8^+$ T-cell targets, and by removing pathogenic T cells in this same manner, the progression of spontaneous type 1 diabetes mellitus in nonobese diabetic mice could be significantly delayed [13,16].

In this study, we evaluated the capability of toxic tetramers to selectively delete murine alloreactive T cells that recognize minor H antigen, HY [17]. In addition to serving as a useful model, HY is also the most clinically important minor H antigen in solid organ transplantation, associated with the decreased survival of kidney, liver, heart and bone marrow grafts [18–21]. Administration of SAP-conjugated tetramers specific for the two immunodominant epitopes, Uty and Smcy, significantly decreased CTL responses elicited by subsequent immunization. Interestingly, targeting either T-cell specificity had the unintended effect of amplifying CTL responses against the other epitope, suggesting that toxic tetramers could serve as a unique tool to facilitate the discovery of additional subdominant minor H antigen epitopes, an important goal in transplantation tolerance studies [3]. Further, the ability to eliminate specific alloreactive precursors prior to exposure to donor-origin tissue illustrates a new and potentially useful therapeutic strategy for the induction of CTL tolerance to minor H antigens.

2. Materials and methods

2.1. Mice

C57BL/6J (B6) mice (Thy1.1 and Thy1.2) were purchased from The Jackson Laboratory (Bar Harbor, ME). Yellow fluorescent protein (YFP)-enhanced transcript for IFN-γ (*Yeti*) [22] and TCR-transgenic B6.D2TgN(*Tcr-Lcmv*)327Sdz/Fre (P14) [23] and B6 TgN(*Tcr-HY*) [24] mice were bred in-house. All mice were housed in an Association for Assessment and Accreditation of Laboratory Animal Care-accredited, specific pathogen-free facility. The mice were typically used at 6–8 weeks of age in experiments that were approved by the Institutional Animal Care and Use Committee of the University of North Carolina–Chapel Hill and adhered to published principles of laboratory animal care.

2.2. Cell preparation

Using aseptic technique, cell preparations of bone marrow flushed from tibias and femurs, or spleens disaggregated between glass slides, were depleted of erythrocytes with ammonium chloride lysis buffer (0.15 M NH_4Cl_4, 1 mM $KHCO_3$, 0.1 mM Na_2EDTA), passed through a 40 μm nylon cell strainer (BD Biosciences, Franklin Lakes, NJ, USA), and resuspended in PBS containing 0.5% FBS. In some experiments, splenocytes were depleted of $CD8\alpha^+$ T cells or $B220^+$ B cells using the appropriate microbead kit and a QuadroMACS magnetic separator (Miltenyi Biotec, Auburn, CA, USA). To isolate peripheral blood lymphocytes (PBLs), venous blood was collected from the superficial temporal vein with a Goldenrod lancet (MEDIpoint Inc., Mineola, NY, USA), diluted into PBS containing 0.15% EDTA, layered over Histopaque-1083 (Sigma-Aldrich, St. Louis, MO, USA) and centrifuged at 800 ×*g* for 20 min; cells at the interface were collected, washed and resuspended in FACS buffer (2% FBS and 0.1% NaN_3 in PBS) prior to analysis.

2.3. Immunization for eliciting anti-HY T-cell responses

Female mice were administered a single-cell suspension of fresh, syngeneic male cells (bone marrow or splenocytes) in 200 μL PBS intraperitoneally (IP) or intravenously (IV, via the lateral tail vein).

2.4. Peptide-MHC class I tetramer preparation

The H2-D^b-restricted peptides $Smcy_{738–746}$ (KCSRNRQYL; referred to as Smcy), synthesized by GenScript (Piscataway, NJ, USA), and $Uty_{246–254}$ (WMHHNMDLI; referred to as Uty) and the lymphocytic choriomeningitis virus (LCMV) glycoprotein-derived altered peptide ligand $gp33_{33–41}$C9M (KAVYNFATM; referred to as gp33C9M), produced at the UNC-CH Peptide Synthesis Facility, were each dissolved in dimethyl sulfoxide at 10 mg/mL. To generate pMHC class I complexes, peptides were individually incubated in folding buffer (100 mM Tris, pH 8.0; 400 mM L-arginine; 5 mM reduced glutathione; 0.5 mM oxidized glutathione; and protease inhibitors) with H2-D^b heavy chain purified from *Escherichia coli* inclusion bodies, and human beta-2 microglobulin, at 10 °C for 48–72 h. Folded complexes were subsequently concentrated with an Amicon stirred ultrafiltration cell (EMD Millipore, Billerica, MA, USA) and purified by gel filtration chromatography. After biotinylation with the BirA enzyme, pMHC class I tetramers were prepared by the fractional addition (1/4 of the total amount every 10 min) of streptavidin (SA)-SAP (Advanced Targeting Systems, San Diego, CA, USA; 2.5 molecules of SAP per molecule of SA), or phycoerythrin (PE) or allophycocyanin (APC)-conjugated SA (Leinco Technologies, St Louis, MO, USA) at a 5:1 or 6:1 (pMHC: streptavidin) molar ratio, as described [13].

2.5. Peptide-MHC class I tetramer administration

Prior to injection, pMHC class I tetramers were sterilized by passage through a 0.22 μm centrifugal filter unit (Ultrafree-MC; EMD Millipore). Mice received 2 IV injections of unmodified or SAP-conjugated D^b-tetramers (diluted to 200 μL in PBS) via the lateral tail vein.

2.6. In vivo CTL assay

To prepare target cells, syngeneic female B6 splenocytes were incubated for 1 h at 37 °C in R-10 medium (10% FBS, 5×10^{-5} M 2-mercaptoethanol, 2 mM L-glutamine, 100 μg/mL streptomycin and 100 IU/mL penicillin in RPMI 1640) containing 10 μg/mL Uty, Smcy, both, or no peptides. After extensive washing, peptide-pulsed cells were labeled with 30 μM Pacific Blue succinimidyl ester (PBSE) and varying concentrations (none, or 0.05, 0.5 or 2.5 μM) of carboxyfluorescein diacetate succinimidyl ester (CFSE; both dyes from Invitrogen, Carlsbad, CA, USA) for 10 min at 22 °C, and subsequently quenched with FBS. Labeled, pulsed splenocytes were resuspended in PBS (2×10^7 cells/mL), combined in equal ratios, and adoptively transferred IV (200 μL) into HY-sensitized (14 days post-immunization) and naïve female B6 recipients. To assess CTL activity, differential target cell survival in the spleen was measured by flow cytometry 18–24 h after transfer; any immunized mice that had complete recovery of all targets were considered priming failures and

excluded from analysis to prevent overestimating the protective effects of tetramers.

2.7. Staining of cells and flow cytometric analyses

The following fluorochrome-conjugated mAbs (Ebioscience, San Diego, CA; Invitrogen; or BioLegend, San Diego, CA) were used at pre-determined optimal concentrations (clone names are indicated parenthetically): anti-CD4 (GK1.5), anti-CD8α (53-6.7), anti-CD19 (eBioID3 or 6D5), anti-CD44 (IM7), anti-B220 (RA3-6B2), anti-CD62L (MEL-14), anti-90.1 (Thy1.1;OX-7), anti-90.2 (Thy1.2; 53-2.1) and anti-IFN-γ (XMG1.2). After 10-min incubation with Fc receptor block (anti-mouse CD16/CD32; eBioscience), single-cell suspensions were labeled with fluorochrome-conjugated mAbs or pMHC class I tetramers in FACS buffer in 96-well round-bottom polypropylene plates for 45 min at 4 °C, washed, and fixed in buffered 1% formaldehyde containing FBS. For detection of intracellular IFN-γ, splenocytes were first incubated in R-10 medium containing fluorochrome-conjugated tetramers in 96-well flat-bottom polystyrene plates for 5 h at 37 °C. Brefeldin A (3 μg/mL; eBioscience) was added after the first hour. Cells were permeabilized using a Cytofix/Cytoperm kit (BD Biosciences), performed according to the manufacturer's protocol. List mode data were collected with a FACSCalibur (BD Biosciences) or CyAn ADP cytometer (Beckman Coulter, Brea, CA) and analyzed with Summit software (Dako, Carpinteria, CA). Viable cells were discriminated by forward and side scatter characteristics.

2.8. Statistical analyses

Significant differences in means between groups were calculated using a 2-tailed unpaired *t* test, or 1-way ANOVA with Bonferroni multiple comparisons post-test, using Prism 5.0 (GraphPad Software, San Diego, CA, USA). A *P* value <0.05 was considered significant.

3. Results and discussion

HY is a well-established minor H antigen model system [17,25]. HY antigens are widely expressed proteins encoded by the Y chromosome and consequently, as non-self, are immunogenic in females. Like other H-2^b strains, B6 mice are HY "high responders", and females rapidly and reliably reject syngeneic male tissues, with a typical, accelerated second-set reaction [11]. Since the pioneering work of Billingham and Silvers [26,27], HY incompatibility has provided a frequently used platform for testing strategies to induce tolerance to minor H antigens [28–31], and similarly, was employed in this study to assess the ability of toxic tetramers to inhibit alloreactive CTL responses.

3.1. Kinetics of H2-D^b-restricted, HY-reactive $CD8^+$ T-cell populations elicited by immunization with male bone marrow cells

Both direct and indirect priming are necessary to optimally induce anti-HY CTL responses [11,32]. In early experiments, we injected syngeneic male splenocytes (typically $5–10 \times 10^6$ cells per mouse), but occasionally had female B6 recipients that did not respond (data not shown). To potentially improve immunization efficiency, alternate priming protocols were evaluated. When magnetic separation was used to deplete immunizing splenocytes of either $CD8\alpha^+$ cells, which can act as so-called "veto" cells (donor T cells that delay activation of the host CTL response) [33], or B cells, which have a tolerizing effect on naïve HY-reactive T cells [34], some recipient mice still failed to mount a detectable response (data not shown). Priming with bulk male bone marrow cells has been reported to elicit stronger anti-HY responses than with either splenocytes or dendritic cells, with no differences between IV or IP routes of administration [11]. Similarly, in our hands, IP injection of bone marrow (5×10^6 cells) provided the most robust and consistent anti-HY responses, and this method was used in subsequent experiments.

Anti-HY $CD8^+$ T cells recognize two immunodominant epitopes restricted by H2-D^b, Uty [35] and Smcy [36]; epitopes derived from Uty and Smcy proteins are also HLA class I-restricted HY targets in humans [3]. After priming of B6 female mice, these T-cell populations can be visualized in peripheral blood by pMHC class I tetramer staining (Fig. 1A). It remains unclear which of these two specificities constitutes the major response; D^b-Uty^+CTL are usually considered quantitatively and qualitatively superior

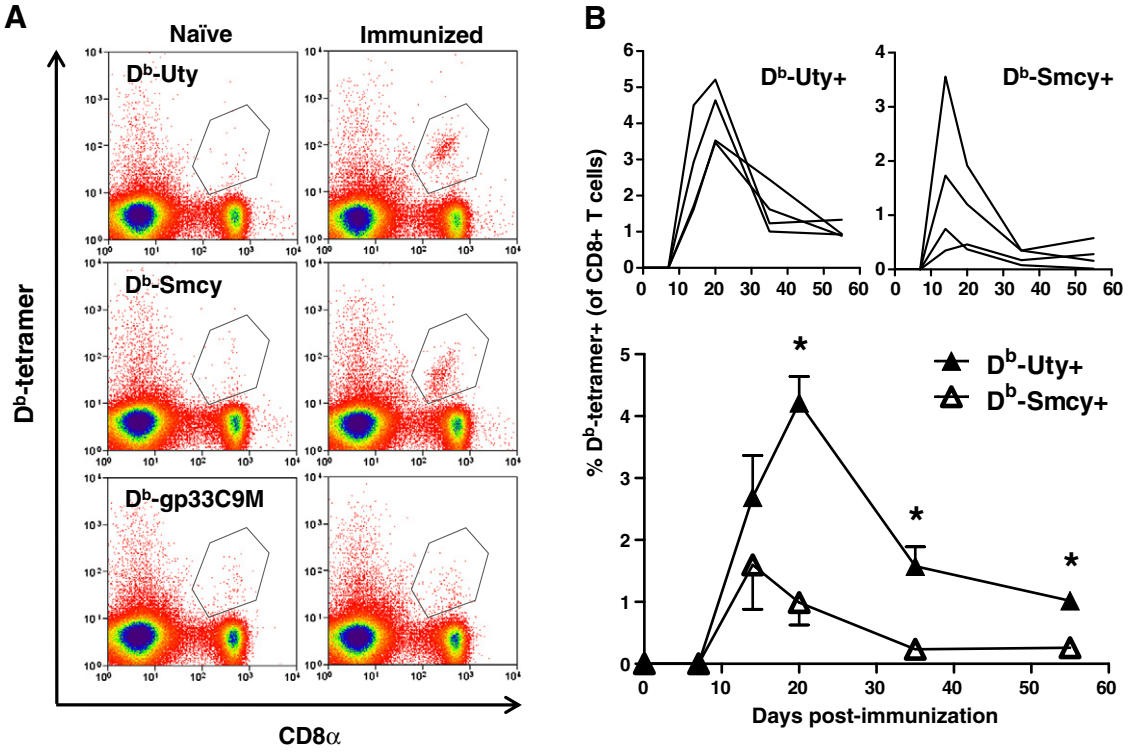

Fig. 1. Upon exposure to male antigen, D^b-Uty^+ $CD8^+$ T cells exhibit a more vigorous, prolonged expansion than do D^b-$Smcy^+$ $CD8^+$ T cells. (A) Flow cytometric dot-plots showing the two immunodominant, HY-specific $CD8^+$ T-cell responses in the peripheral blood of a representative female B6 mouse 14 days after IP administration of syngeneic male bone marrow cells ("immunized"). In all experiments, the D^b-restricted, LMCV altered peptide ligand gp33C9M tetramer, and naïve female mice, which do not have detectable HY-reactive $CD8^+$ T cells in peripheral blood [47], served as negative controls. (B) The kinetics of circulating D^b-Uty^+ and D^b-$Smcy^+$ $CD8^+$ T cells after immunization; T-cell expansion and contraction in individual mice are shown in the top graphs (note different y-axis scales). Mean peripheral anti-HY responses ($n = 4$) over time are shown below. The D^b-Uty^+ $CD8^+$ T-cell percentages were significantly greater at days 20, 35 and 55 ($^*P<0.05$, by two-tailed *t*-test). Error bars indicate SEM. The data represents one of two independent experiments that yielded the same results.

[11,37,38], but other studies have found the converse, with D^b-Smcy$^+$ CTL predominating [33,39]. The graphs at the top of Fig. 1B depict the changes in the frequency of circulating HY-reactive CTL in individual mice after priming, and the bottom graph shows average responses over time. Initially, increases in circulating D^b-Uty$^+$ and D^b-Smcy$^+$ CTL were similar, with no significant difference in frequency (or cell number — not shown) at 14 days post-immunization. However, the Uty-reactive T-cell population continued to expand for a longer period than did the Smcy-reactive population, and as a consequence, D^b-Uty$^+$ CTL ultimately reached a higher peak frequency and remained at significantly greater levels throughout the contraction phase.

3.2. Characterization of H2-D^b-restricted anti-HY CTL responses

We next sought to compare the effector functions of the two HY-reactive specificities. Splenocytes were collected at 14 days post-immunization, when T cells were sufficiently numerous to permit evaluation, and before either population began contracting. In response to TCR ligation by cognate tetramer, approximately equal proportions of T cells produced nearly identical amounts of IFN-γ, as shown by intracellular staining (Supplemental Fig. 1A). Because ex vivo re-stimulation is necessary to induce cytokine production, this assessment could have failed to disclose differences between the two specificities in their IFN-γ responses to the priming inoculum. To circumvent this potential limitation, we also immunized B6-background, IFN-γ reporter (*Yeti*) mice that produce YFP upon transcription of the locus [22,40]. The cumulative fraction of each activated, tetramer$^+$ T-cell population that had been induced to produce IFN-γ by male antigen exposure can now be seen to be much greater than that revealed by intracellular staining; once again, however, the two specificities were not significantly different (Supplemental Fig. 1B). Additional analyses of D^b-Uty$^+$ and D^b-Smcy$^+$ CTL from immunized B6 mice also demonstrated equivalent levels of TNF-α and granzyme B (by intracellular staining; not shown).

Finally, we directly compared cytotoxic activity at 14 days post-immunization. At this time point, in addition to having detectable circulating tetramer$^+$ T cells, female Thy1.2$^+$ mice had also cleared an immunizing inoculum of Thy1.1$^+$ male splenocytes (Supplemental Fig. 2A). To measure CTL responses, an in vivo assay was used [41]. All peptide-pulsed targets, identified by differential dye staining, were equally recovered in naïve female mice; a representative dot-plot is shown in Fig. 2A. Two weeks after a priming injection of bone marrow cells, targets pulsed with Smcy or Uty (or both) peptides were readily eliminated. Responses against Uty were significantly greater in all experiments (Fig. 2B). On average, 33% of Smcy-pulsed targets survived (vs. unpulsed), while <2% Uty-pulsed cells could be recovered. Because the frequencies of D^b-Uty$^+$ and D^b-Smcy$^+$ CTL were not different at this time point (Fig. 1B), this observation suggests that anti-Uty CTL are more efficient in vivo on a per-cell basis.

3.3. Toxin-coupled D^b-Uty and D^b-Smcy tetramers selectively inhibit anti-HY CTL responses in vivo

We then investigated whether HY-reactive CD8$^+$ T cells could be removed in vivo by administration of cognate toxic tetramers. In particular, we wished to delete naïve T cells, as such an approach mimics a possible therapeutic intervention that could be initiated prior to allotransplantation. Moreover, naïve T cells appear to be generally more sensitive than effector cells to the toxic effects of SAP-conjugated tetramers (our unpublished data). Additionally, with this strategy, the number of target T cells is quite small: at a typical CD8$^+$ T-cell precursor frequency of 10^{-5}, only ~200–500 specific T cells would need to be eliminated in an individual mouse [42]. However, this extremely small number also means that deletional effects could not be directly assessed. Rather, as an indirect measure, we sought to determine whether toxic tetramer administration would remove sufficient precursor T cells to substantially reduce (or ideally, abolish) CTL responses elicited by immunization. This outcome should result in increased survival of the corresponding target cell in the in vivo CTL assay. The design of experiments to test this prediction is shown in Fig. 3A. The dose of toxic tetramer (33 pmol) was based on our previous work [13,16], and in vivo preliminary studies with D^b-Uty-SAP (not shown). At this dosage, mice did not exhibit clinical signs of illness. To potentially enhance the efficacy of T-cell deletion, 2 injections of tetramer were given, 5 days apart. While even more closely spaced treatments might appear advantageous, CD8$^+$ T cells can become temporarily refractory to tetramer binding after antigen exposure [43], and consequently, the optimal interdose interval was uncertain. To determine whether this binding resistance effect occurs after tetramer administration, we used D^b-gp33$^+$ CD8$^+$ T cells (from an LCMV TCR-transgenic P14 mouse) as a surrogate target. Two days after the injection of cognate D^b-gp33C9M tetramer, approximately one-third of P14 T cells are unable to bind tetramer in vitro (Supplemental Fig. 2B). A follow-up experiment demonstrated that D^b-gp33C9M tetramer binding rebounded by 5 days post-injection (Supplemental Fig. 2C). It should be noted that fluorophore-labeled tetramers likely overestimate resistance to SAP-conjugated tetramers, as the former are used optimally in the ≥5 nM range, while toxic tetramers are generally effective at subnanomolar doses [13]. Based on these data, toxic tetramer doses were separated by 5 days; this interval also allows the second dose to be administered after any acute adverse hepatic effects of SAP had peaked (at day 2) [13].

Following the second tetramer injection, CTL precursors were expanded by injection of male bone marrow, and cytotoxic responses compared (Fig. 3B, C). In mice injected with D^b-gp33C9M-SAP, the survival of Uty, Smcy, and Uty/Smcy-pulsed targets was significantly decreased, similarly to the PBS-treated control mice, showing that the administration of non-cognate pMHC molecules or SAP did not exert a non-specific effect on the induction of CTL responses. On the other hand, toxic D^b-Uty and D^b-Smcy tetramers protected their corresponding targets. Within both treatment groups, the recovery of cognate peptide-pulsed cells was not significantly different from that of unpulsed targets, demonstrating a reduction in CTL activity. A repetition of this experiment yielded the same results. When data from the two experiments were analyzed together, significant protective effects were also observed across the treatment groups (Fig. 4A). Administration of D^b-Uty-SAP resulted in ~46% Uty-pulsed target survival (vs. unpulsed), compared to 1–2% in other treatment groups. As noted previously (Fig. 2B),

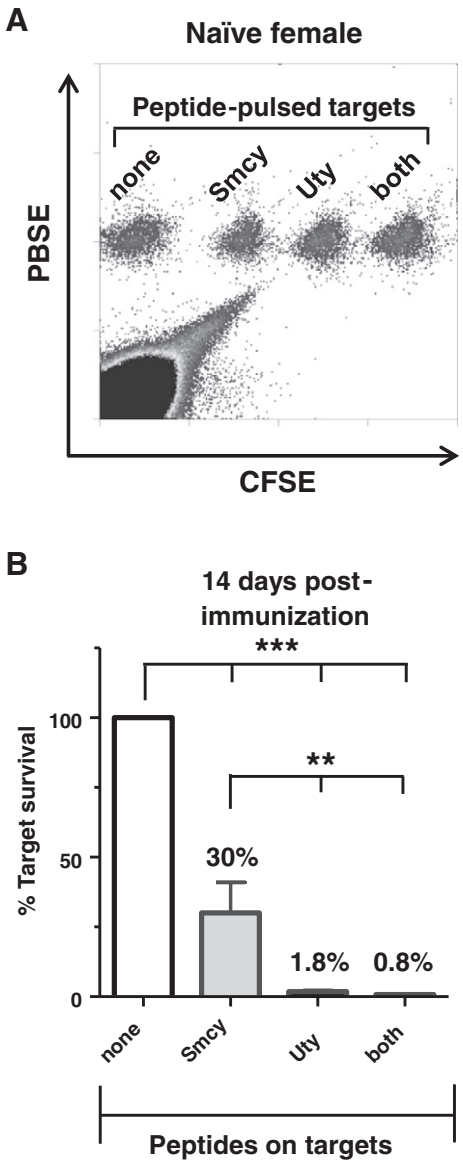

Fig. 2. CTL activity against Uty-bearing targets is more efficient than responses against targets pulsed with Smcy peptide. (A) Representative dot-plot demonstrating the recovery of unpulsed control ("none") and peptide-pulsed target cells, discriminated by labeling with different combinations of PBSE and CFSE dyes, from the spleen of a naïve B6 mouse 18 h after IV adoptive transfer. (B) Anti-Uty CTL responses primed by immunization are significantly more efficient than anti-Smcy CTL at removing cognate target cells. Fourteen days after a single injection of male bone marrow, mice were challenged with a mixture of fluorescently labeled, peptide-pulsed, female-origin splenocytes; 24 h later, spleens were harvested and targets were enumerated by flow cytometry. To permit comparison between animals and across experiments, the survival of each peptide-pulsed group was expressed as a percentage of the unpulsed control cells recovered, and all were significantly decreased (***$P < 0.05$), demonstrating CTL activity against these immunodominant epitopes. However, significantly more Uty-pulsed targets were eliminated than those pulsed with Smcy alone (**$P < 0.05$; both analyses by ANOVA with Bonferroni multiple comparisons post-test). No CTL responses were observed in naïve control mice (not shown). Error bars in graphs indicate SEM. The graph in (B) represents data combined from four separate experiments.

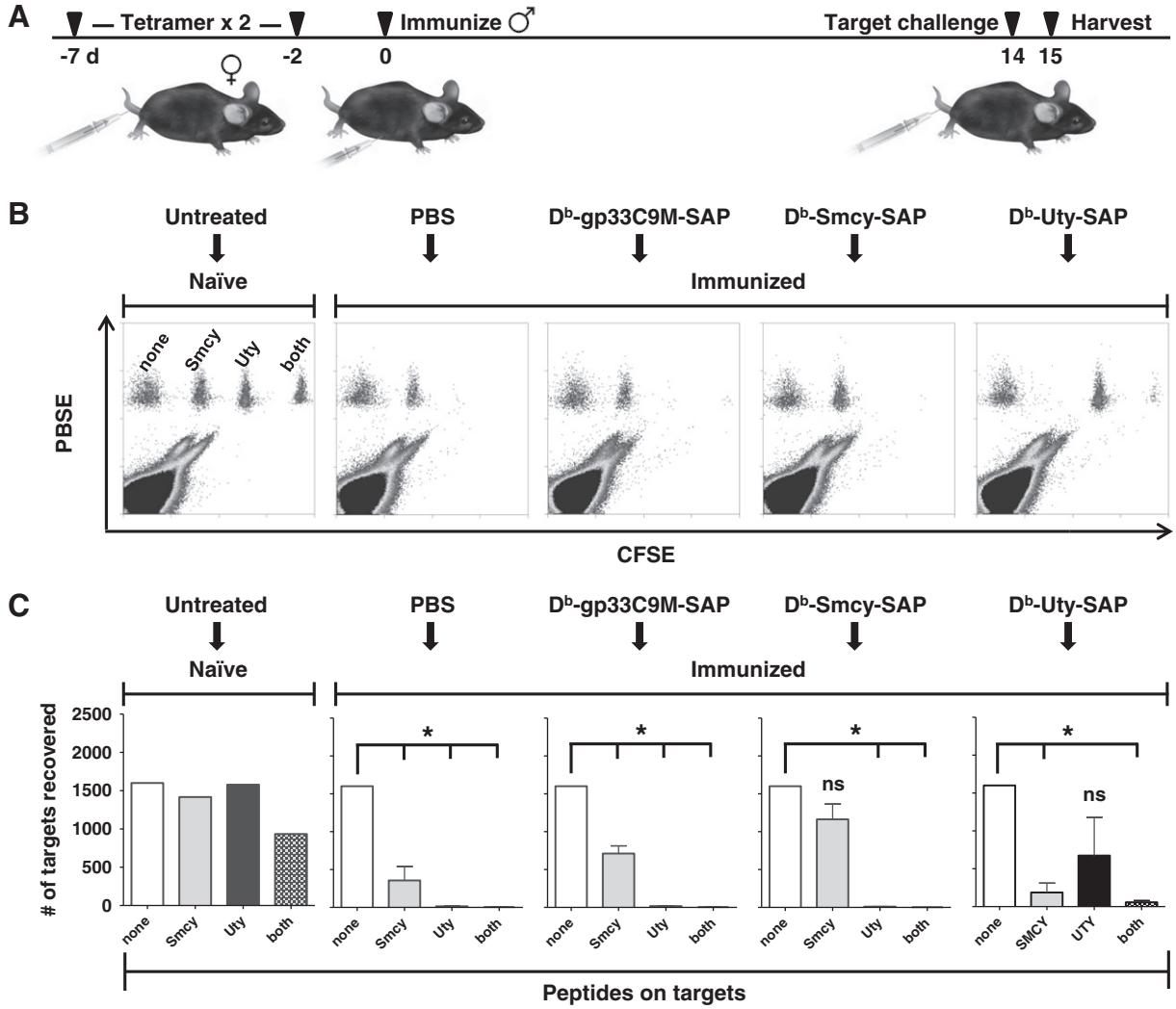

Fig. 3. Administration of SAP-conjugated D^b-Uty and D^b-Smcy tetramers to HY-naïve female mice decreases cognate CTL responses elicited by immunization. (A) Schematic depicting the timeline of experiments. Female B6 mice ($n = 3$ per group) were injected twice, 5 days apart, with cytotoxic tetramers or PBS. Two days after the second treatment, 5×10^6 syngeneic male bone marrow cells were administered IP to prime anti-HY CTL responses, which were subsequently assayed in vivo 14 days later. Representative dot-plots (B) and mean target recovery (C) demonstrate that treatment with D^b-Uty-SAP and D^b-Smcy-SAP tetramers prior to male antigen exposure results in increased survival of transferred target cells bearing the corresponding peptides. The PBS and D^b-gp33C9M-SAP tetramer-treated groups served as negative and antigen-non-specific controls, respectively. To permit comparison between mice, the number of unpulsed control cells recovered from subjects in each treatment group was normalized to the value obtained from untreated, HY-naïve females. All peptide-pulsed targets were significantly reduced, compared to unpulsed control cells, by CTL activity (*$P < 0.05$, by ANOVA with Bonferroni multiple comparisons post-test), except for Smcy-pulsed targets protected by D^b-Smcy-SAP, and Uty-pulsed targets protected by D^b-Uty-SAP (ns, not significant). The data shown is from one of two independent experiments with the same results. Error bars in graphs indicate SEM.

Smcy-pulsed targets are cleared less efficiently by CTL; in mice injected with PBS and D^b-gp33C9M-SAP, the average recovery of these targets ranged from 28 to 39%. With D^b-Smcy-SAP administration, however, survival was increased to 98% of unpulsed control cells.

3.4. Toxic tetramers eliminate cognate T cells

The enhanced recovery of Uty- and Smcy-pulsed targets is presumably the result of toxic tetramer-mediated killing of naïve, HY-reactive T cells. In support of this hypothesis, in a preliminary study, we observed that two injections of unmodified D^b-Uty tetramer, which did not eliminate D^b-Uty$^+$ T cells, also did not protect Uty-pulsed targets (Supplemental Fig. 2D), suggesting that inhibition of priming did not simply result from prior exposure to pMHC alone. Moreover, our previous reports [13,16] and those of others [15] show that SAP-conjugated tetramers selectively bind to, and subsequently kill, cognate T cells. Using an anti-SAP antibody, binding of the D^b-Uty-SAP tetramer to a CD8$^+$ T-cell population in a female mouse sensitized to male antigen can be observed (Supplemental Fig. 2E). Previously, we have demonstrated the killing of Smcy-reactive, TCR-transgenic T cells [24] in vitro with an altered peptide ligand D^b-SmcyC2A-SAP tetramer [13]; dose-dependent killing of these T cells with the native D^b-Smcy-SAP tetramer is shown in Supplemental Fig. 2F. Injected tetramers can gain access to and bind cognate T cells in spleen and lymph node [44], and can selectively eliminate T cells in vivo [13,16]. In the present study, substantially fewer cognate CTL were elicited by immunization in mice that received toxic tetramer injections (Supplemental Fig. 2G), which also supports the notion that CTL precursors are eradicated. In the CTL protection experiments (Fig. 4A), in addition to assessing target survival, we also measured tetramer$^+$ T cells in the spleens of treated mice. As seen in Fig. 4B, in the D^b-Uty-SAP group, D^b-Uty$^+$ CTL were found at only one-third the frequency observed in control PBS-treated mice. With D^b-Smcy-SAP treatment, D^b-Smcy$^+$ CTL were always undetectable (i.e., below the background defined by D^b-gp33C9M tetramer staining). While these findings were consistent across experiments, the reductions in neither HY specificity were statistically significant, likely because of the high variability in tetramer$^+$ T-cell frequencies across mice following immunization, which has been observed by others [11]. It is worthwhile to note that there was no evidence that administration of either SAP-conjugated tetramer primed, rather than deleted, its cognate T-cell pool. The unintended transfer of peptides from tetramers to endogenous MHC class I molecules could lead to this paradoxical adverse effect [45].

While the sparing of Uty- and Smcy-pulsed splenocytes by administration of cognate toxic tetramers prior to immunization serves as proof-of-principle for this approach, the magnitude of this beneficial effect (46% target survival for Uty; 98% for Smcy) would presumably be insufficient to provide durable allograft protection. Ultimately, to be clinically useful, more complete T-cell deletion will be necessary, potentially accomplished by employing a more dose-intense treatment protocol, or a different toxic moiety.

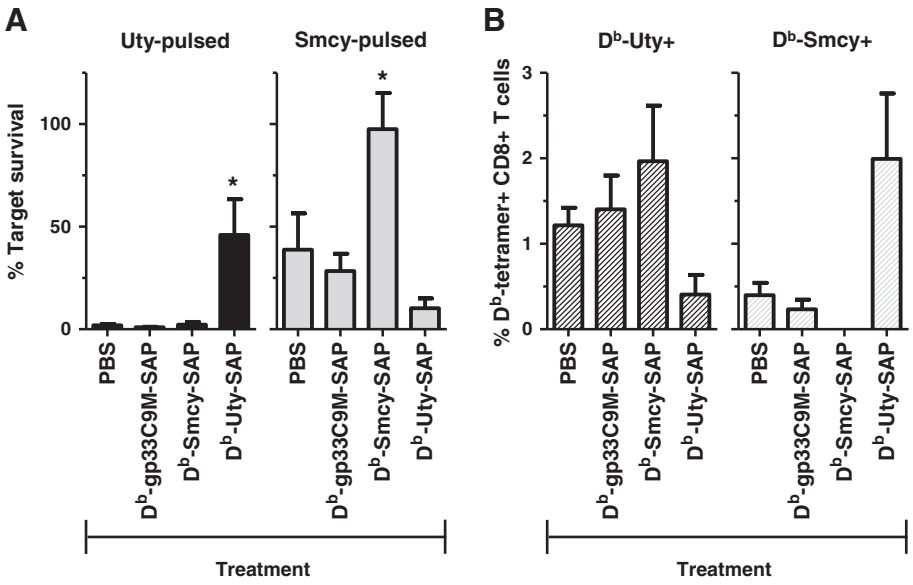

Fig. 4. The protection of Uty- and Smcy-pulsed targets by administration of cytotoxic tetramers is associated with a reduction in cognate CD8$^+$ T cells. (A) Treatment of female mice ($n = 3$ mice per group per experiment) with D^b-Uty-SAP and D^b-Smcy-SAP tetramers before priming significantly decreases specific CTL-mediated elimination of targets (*$P < 0.05$, by ANOVA with Bonferroni multiple comparisons post-test). As described for Fig. 3, data were normalized to cell recovery in HY-naïve mice, and expressed as a percentage to permit comparison between experiments. (B) The expansion of D^b-Uty$^+$ and D^b-Smcy$^+$ CD8$^+$ T cells in the spleens of mice following immunization with male antigen is selectively reduced by prior administration of cytotoxic tetramers. Values in the graph are % tetramer$^+$ T cells, which were determined by subtracting non-specific staining with the PE-labeled D^b-gp33C9M tetramer. In B6 mice, true D^b-gp33$^+$ T cells occur at a frequency of 1 in ~70,000 CD8$^+$ T cells (0.0014%) [42], so essentially all (>99.9%) tetramer staining of this rare population represents false positive (background) signal. Mean differences between the treatment groups were not significant (by ANOVA). The same results were obtained when specific T-cell numbers, rather than % T cells, were compared (not shown). Graphs (A,B) represent combined data from two independent experiments; error bars indicate SEM.

Alternatively, it may be that toxic tetramers in themselves are unable to furnish absolute T-cell tolerance towards minor H antigens, but nonetheless could constitute part of an effective therapeutic regimen towards that end. For example, their pre-transplantation use could reduce CTL precursors sufficiently to make it possible that conventional immunosuppressive therapy could later be tapered or withdrawn without graft harm [46].

In not all pre-transplantation settings will the targeted HY-reactive T-cell population be naïve. Immunodominant CD8$^+$ T cells can be primed by pregnancy with male fetuses in mice (D^b-Uty) and humans (A2-Smcy). These T cells are expanded in number relative to their precursors, and in vitro appear functionally similar to memory T cells elicited by allografting [47]. Whether toxic tetramers directed against HY-reactive CD8$^+$ T cells would be equally effective in reducing CTL responses under these conditions will need to be empirically determined. Further, for some multiparous individuals, deletion of such CD8$^+$ T cells might be superfluous. Multiparity can induce prolonged acceptance of male skin grafts in variable proportions of H-2^b strains of mice, suggesting that T cells primed by this mechanism are sometimes tolerized [47].

3.5. Toxic tetramers alter the immunodominance hierarchy

As demonstrated previously for other T-cell specificities restricted by H2-D^b [13] or H2-K^d [16], the deletional effect of the toxic tetramers in this study was selective. The D^b-gp33C9M-SAP tetramer had no effect on HY CTL priming. D^b-Uty-SAP did not delete Smcy-reactive T cells, and D^b-Smcy-SAP did not delete Uty-reactive T cells. In fact, the opposite phenomenon was observed: administration of a toxic tetramer of one HY specificity appeared to strengthen the CTL responses of its counterpart. The increase in the D^b-Smcy$^+$ T-cell population with administration of D^b-Uty-SAP is particularly striking. This observation can also be seen in the CTL assay: survival of Smcy-pulsed targets in the D^b-Uty-SAP treatment group was considerably less (~10% vs. unpulsed) than that in mice treated with either PBS or the irrelevant toxic tetramer (Fig. 4A). An additional experiment treating mice with D^b-Uty-SAP (vs. PBS control alone) reveals the same reciprocal increase in Smcy-reactive CTL numbers and activity (Suppl. Fig. 3). This consistent observation implies that the immunodominance hierarchy between the T-cell populations does not depend on absolute precursor frequency, which is thought to be similar [11], but rather, on competition for APC resources between the two species, a well-documented phenomenon [48,49]. It is conceivable, for example, that under normal circumstances, the major CTL response (Uty) efficiently interacts with and deletes [50] or exhausts APCs presenting male epitopes, thereby restraining priming of the minor response (Smcy) [51]. As one might therefore expect, stronger CTL effector function (as we observed for D^b-Uty$^+$ T cells — Fig. 3B), rather than intrinsic proliferative ability, has been correlated with dominant status [52]. Accordingly, in the tetramer-mediated absence of D^b-Uty$^+$ CD8$^+$ T cells, Smcy-reactive T cells have unfettered access to APCs and expand more vigorously. This scenario is consistent with reports that immunodomination of one T cell specificity by another can be overcome by exposure to supraphysiologic numbers of APCs during priming [52–54]. D^b-Smcy$^+$ CD8$^+$ T cells may be more vulnerable than D^b-Uty$^+$ CD8$^+$ T cells to competitive pressures because Smcy binds H2-D^b complexes much less efficiently than does Uty peptide, as shown by RMA-S surface class I stabilization assays (our unpublished data, and [11]). Alternatively, the D^b-Uty$^+$ and D^b-Smcy$^+$ TCRs may bind their cognate pMHC complex with different avidities. Both binding factors have been incriminated in the immunodominance hierarchies of specific CTL [54–57].

For toxic tetramers to become considered as a clinically applicable means of inducing tolerance, the number of T-cell specificities reactive against donor minor H antigens must be limited. Fortunately, this appears to be the case, as there are several mechanisms that greatly restrict the diversity of these alloreactive CTL responses. For some minor H antigens, such as H60, tissue expression is limited to hematopoietic cells. For HY proteins, sequence similarities between peptides derived from some Y chromosome-encoded genes and their X chromosome-encoded paralogs may result in T-cell tolerance [6]. More importantly, for a given antigen, the number of epitopes is severely restricted by immunodominance, which is determined by antigen processing, peptide-MHC binding affinity, TCR availability and other factors. Thus, despite there being 1204 possible nonamer peptides in the Uty protein, the single major focus of CTL responses in B6 mice is the Uty$_{246–254}$ peptide. The end result of these limiting mechanisms can be observed following the immunization of female B6 mice with H2-matched but otherwise disparate male BALB.B spleen cells; >80% of the CTL responses were directed at just four minor H antigens [58]. Finally, it appears unlikely that all immunodominant CTL reactive against donor minor H antigens contribute to graft failure. A recent review of human studies examining minor H antigens in solid organ transplantation patients revealed that, to date, only HY has appeared to play a significant role in rejection [7].

Of course, if the deletion of the major and minor immunodominant HY-reactive CD8$^+$ T cells leads to the expansion of a substantial number of subdominant CTL responses, the need for additional toxic tetramers may become onerous, as the cumulative amount of SAP could be dose-limiting. However, this possibility appears unlikely: Milrain et al. found that, of 54 CTL clones derived from the spleens of male-immunized mice, all recognized either Uty or Smcy [11], so very few other T-cell specificities may emerge. For clinical use, identification of possible subdominant HY-reactive CD8$^+$ T cells in common HLA haplotypes will be essential in assessing feasibility, and conceivably could be accomplished by generating T-cell clones from male-to-female recipients with host-versus-graft or graft-versus-host disease following in vitro, toxic tetramer-mediated elimination of the dominant species. New tetramers resulting from such studies could then be used to investigate the development and impact of these CTL responses in vivo in treated patients.

It is also worthwhile to note that it may not be strictly necessary to delete all HY-reactive CD8$^+$ T cells to achieve tolerance. Repeated injections of the D^b-Smcy tetramer into naïve female B6 recipients prolonged the survival of male skin grafts [30]; this treatment regimen was associated with the generation of antigen-unresponsive, regulatory CD8^{lo} T cells capable of suppressing their naïve cognate peers by TGF-β production [59]. Hence, some specificities may be targeted by nondeletional

means, and therefore, it may be possible to use a mixture of toxic and non-toxic tetramers to induce stable $CD8^+$ T-cell allotolerance. Ultimately, determining the optimal approach for suppressing a complex mixture of minor H antigen-reactive $CD8^+$ T cell responses – whether by providing signal one alone to induce a non-responsive or regulatory phenotype, or by delivering a toxin to simply eliminate the unwanted effector – will need to be made on an empirical, T-cell specificity-by-specificity basis.

4. Conclusions

Our data show that the selective removal of naïve $CD8^+$ T cells by toxic tetramers can reduce T-cell responses in vivo against two immunodominant HY epitopes. This study is the first to formally demonstrate that CTL killing can be modulated by tetramer-mediated delivery of a toxin, and efficacy in an additional antigenic model strengthens the validity of this approach for specific T-cell deletion. The co-administration of SAP-conjugated D^b-Uty and D^b-Smcy tetramers to female recipients could provide effective tolerance of MHC-identical male allografts, although concomitant inactivation or suppression of cytotoxic, HY-reactive $CD4^+$ T-cell responses may also be necessary to protect class II^+ donor cells [33]. In this work, deletion of the T-cell precursor population was associated with reciprocal changes in immunodominance, so combined toxic tetramer treatment may unmask other H2-D^b-restricted subdominant specificities that contribute to anti-HY responses, and subsequently, new tetramers could then be used to eliminate these newly emerged culprits. Ultimately, pre-emptive administration of an optimized panel of toxic (and potentially, non-toxic) tetramers to recipients prior to transplantation could be a useful therapeutic strategy to prevent the induction of CTL responses against multiple minor H antigens that contribute to allograft dysfunction and rejection.

Conflict of interest

The authors declare no financial or commercial conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.trim.2013.10.005.

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