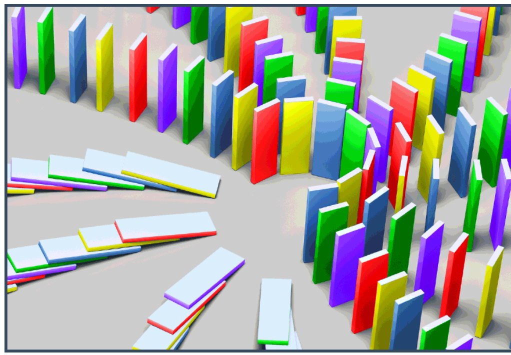
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DNA Fusion Vaccines Induce Targeted Epitope-Specific CTLs against Minor Histocompatibility Antigens from a Normal or Tolerized Repertoire[1]

Jason Rice,[2]* Sarah Buchan,* Hamlata Dewchand,† Elizabeth Simpson,† and Freda K. Stevenson*

We have designed DNA fusion vaccines able to induce high levels of epitope-specific CD8$^+$ T cells, using linked CD4$^+$ T cell help. Such vaccines can activate effective immunity against tumor Ags. To model performance against minor histocompatibility (H) Ags important in allogeneic hemopoietic stem cell transplantation, responses against the H2D^b-restricted Uty and Smcy male HY epitopes have been investigated. Vaccination of females induced high levels of tetramer-specific, IFN-γ-producing CD8$^+$ T cells against each epitope. Vaccines incorporating a single epitope primed effector CTL able to kill male splenocytes in vitro and in vivo, and HY^{Db}*Uty*-specific vaccination accelerated rejection of syngeneic male skin grafts. Priming against either epitope established long-term memory, expandable by injection of male cells. Expanded CD8$^+$ T cells remained specific for the priming HY epitope, with responses to the second suppressed. To investigate vaccine performance in a tolerized repertoire, male mice were vaccinated with the fusion constructs. Strikingly, this also generated epitope-specific IFN-γ-producing CD8$^+$ T cells with cytotoxic function. However, numbers and avidity were lower than in vaccinated females, and vaccinated males failed to reject CFSE-labeled male splenocytes in vivo. Nevertheless, these findings indicate that DNA fusion vaccines can mobilize CD8$^+$ T cells against endogenous minor H Ags, even from a profoundly tolerized repertoire. In the transplantation setting, vaccination of donors could prime and expand specific T cells for in vivo transfer. For patients, vaccination could activate a potentially less tolerized repertoire against similar Ags that may be overexpressed by tumor cells, for focused immune attack. ***The Journal of Immunology,*** **2004, 173: 4492–4499.**

Transplantation of HLA-matched allogeneic hemopoietic stem cells to leukemic patients provides significant clinical benefit (1). Donor stem cells repopulate the immune system of the patient following damage by intensive antileukemic chemotherapy, and the transferred T cells can eliminate residual tumor cells, known as the graft-vs-leukemia (GvL)[3] effect (2). In the situation in which donor and recipient are HLA matched, the target Ags are predominantly minor histocompatibility (H) Ags, consisting of peptide epitopes derived from polymorphic proteins (3). The problem is that the desired GvL effect, mediated by donor T cells, can be marred by an accompanying graft-vs-host (GvH) response due to parallel attack on recipient minor H Ags expressed by other host cells. Donor lymphocytes are often infused to treat leukemic relapse donor lymphocyte infusion (DLI) after the initial hemopoietic stem cell transplant (4) has established chimerism, and in this setting GvH can be diminished. Although polyclonal DLI treatment can succeed, the strategy of transferring selected minor H-specific T cells would be more attractive. For leukemia, the aim would be to focus immune attack on minor H Ags expressed preferentially on cells of hemopoietic origin.

One strategy has been to generate selected allogeneic minor H-specific CD8$^+$ T cells for transfer to the recipient (5). This approach is based on the successful use of EBV-specific CTL for adoptive therapy of EBV-related B cell malignancies (6), in which priming in vivo has already occurred. However, it is technically demanding and expensive, and priming a naive repertoire in vitro is very difficult. An alternative approach, transferring T cells genetically engineered to express specific TCR genes, has been proposed (7), but this remains a challenging procedure.

A simpler approach would be to vaccinate the donor and transfer immune cells either during the transplant or in the setting of DLI. Vaccination would increase the precursor frequency of the effector cells, facilitating their selection using tetramers and the removal of other T cells capable of developing undesirable GvH activity. Transfer of T cells selected from mice primed with an immunodominant minor H peptide can result in GvL without accompanying GvH (8). In this setting, use of CD8$^+$ T cells specific for the immunodominant minor H Ag avoided the problem of epitope spreading to include other Ags presented at an inflammatory site (9). Selective activity of minor H-specific CD8$^+$ T cells against tumor cells may be due to the higher level of epitope expression in comparison with normal cells, or a greater sensitivity to lysis.

We have developed powerful DNA fusion vaccines capable of inducing high levels of epitope-specific CD8$^+$ T cells. Using CD8$^+$ T cell epitopes of the HY male minor H Ag, we show in this study that effector cells generated against a single epitope are capable of in vivo cytotoxicity against male cells and of causing accelerated rejection of syngeneic male skin grafts. Immunity is long-lived and focused on the epitope used to prime. The strategy

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[3] Abbreviations used in this paper: GvL, graft-vs-leukemia; DLI, donor lymphocyte infusion; GvH, graft-vs-host; H, histocompatibility.

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of providing high levels of linked T cell help against a separate foreign Ag (tetanus toxin) also allows induction and maintenance of minor H-specific $CD8^+$ T cells in a setting of profound tolerance. Vaccination of transplant donors and/or patients against selected minor H epitopes may now be feasible.

Materials and Methods

Cell lines

Abelson leukemia virus-transformed cloned male and female B cell lines, derived from $(H2^k \times H2^b)F_1$ mice, have been described previously (10). EL4 is a chemically induced T cell lymphoma derived from C57BL/6N mice. Cells were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated FCS (Invitrogen Life Technologies, Paisley, U.K.), 1 mM sodium pyruvate, 2 mM L-glutamine, nonessential amino acids (1% of 100× stock), 25 mM HEPES buffer, and 50 μM 2-ME (complete medium). The EL4-Uty transfectants were previously described (11).

Peptides and tetramers

The immunodominant MHC class I ($H\text{-}2D^b$)-restricted HY peptide, WMHHNMDLI, derived from the *Uty* gene (HY^{Db}*Uty*), and the subdominant $H\text{-}2D^b$-restricted peptide, KCSRNRQYL, derived from the *Smcy* gene (HY^{Db}*Smcy*), have been described previously (11, 12). Peptides were synthesized commercially and supplied at >95% purity (Peptide Protein Research, Southampton, U.K.). Peptide stocks (1 mM) were made up in PBS, filter sterilized, and stored at −20°C. MHC class I tetramers were produced using a modification of the method of Altman et al. (13), as described previously (14).

Construction of DNA vaccines

DNA vaccine design is indicated in Fig. 1. Construction of a DNA vaccine containing the gene encoding the first domain (DOM, $TT_{865-1120}$) of FrC (p.DOM) from tetanus toxin, with a leader sequence derived from the V_H of the IgM of the BCL_1 tumor, has been described (15). Two additional DNA vaccines were then constructed using the p.DOM sequence as template: 1) DNA encoding the first domain of FrC, with sequence encoding the HY^{Db}*Uty* CTL motif fused to the C terminus (p.DOM-Uty/D^b); 2) DNA encoding the first domain of FrC with sequence encoding the HY^{Db}*Smcy* CTL motif fused to the C terminus (p.DOM-Smcy/D^b).

p.DOM-Uty/D^b was constructed by PCR amplification of the first domain of FrC, encoded within p.DOM, using the forward primer 5′-TTTTAAGCTTGCCGCCACCATGGGTTGGAGC-3′ and the reverse primer 5′-TTTGCGGCCGCTTAAATTAGATCCATATTATGGTGCATCCAGTTACCCCAGAAGTCACGCAG-3′, which fuses HY^{Db}*Uty*-encoding sequence to the 3′ terminus of DOM. The resulting PCR fragment was gel purified, digested, and cloned into the expression vector pcDNA3 (Invitrogen Life Technologies) using *Hin*dIII and *Not*I restriction sites. p.DOM-Smcy/D^b was constructed in a similar manner, but using the reverse primer 5′-TTTGCGGCCGCTTATAAGTACTGTCGATTCCTTGAGCACTTGTTACCCCAGAAGTCACGCAGGAAGGT-3′, which fuses HY^{Db}*Smcy*-encoding sequence to the 3′ terminus of DOM.

All constructs encode the BCL_1 leader sequence at the N terminus. Vaccine integrity was confirmed by DNA sequencing. Expression and product size were checked in vitro using the TNT T7 coupled reticulocyte lysate system (Promega, Madison, WI).

Mice and DNA vaccination protocol

C57BL/6KaLwRijHsd mice (B6), bred in Southampton from stocks originally obtained from Harlan Netherland (Horst, The Netherlands), were used as responders for all of the experiments, except those involving skin grafting, which used C57BL/6J (B6/J) from Harlan U.K. (Oxford, U.K.). They were vaccinated at 6–10 wk of age with a total of 50 μg of DNA in normal saline, injected into two sites in the quadriceps muscles on the days indicated. Animal welfare and experimentation were conducted in accordance with local Ethical Committee and United Kingdom Coordinating Committee for Cancer Research (London, U.K.) guidelines, under Home Office license.

Generation and assay of HY-specific cytotoxic $CD8^+$ T cells

To assess priming for CTL responses in vaccinated mice, they were sacrificed at day 14 and spleens were removed. Single cell suspensions were made from individual spleens in complete medium. Splenocytes were washed, counted, and resuspended at 3×10^6 cells/ml: 15 ml was added to upright 25-cm^2 flasks together with human rIL-2 (20 IU/ml; PerkinElmer, Foster City, CA) and peptide (10 nM for female splenocytes; 100 nM for male splenocytes). Following 6 days of stimulation in vitro, cytolytic activity of the T cell cultures was assessed by standard 5-h ^{51}Cr release assay, as previously described (15, 16). Target cells, including EL4 cells or Abelson leukemia virus-transformed male and female B cell lines derived from $(H2^k \times H2^b)F_1$ mice, were ^{51}Cr labeled during incubation with or without peptide, as indicated. Specific lysis was calculated by the standard formula ((release by CTL − release by targets alone)/(release by 4% Nonidet P-40 − release by targets alone) × 100%). To maintain CTL lines, splenocytes from vaccinated mice were initially stimulated for 1 wk with free peptide (10 nM) and rIL-2. Subsequently, T cells ($2–5 \times 10^5$/well) were restimulated every 7 days with irradiated (2500 rad), peptide-pulsed female splenocytes (5×10^6/well) and rIL-2 (20 IU/ml) in 24-well tissue culture plates.

Tetramer labeling

Following vaccination, peripheral blood samples (100–200 μl) were collected from individual mice at the times indicated; RBC was removed by hemolysis (Puregene RBC lysis solution; Gentra Systems, Minneapolis, MN); and the remaining cells were washed twice with PBS. Aliquots of cells were labeled in 50 μl of PBS with 0.5–1 μl of either HY^{Db}*Uty*-tetramer-PE or HY^{Db}*Smcy*-tetramer-PE for 10 min at room temperature, then with allophycocyanin-labeled anti-CD8a (Ly-2, 53-6.7) and, where appropriate, FITC-labeled anti-H2-A/E (2G9), for 15 min at 4°C. Cells were then washed twice with PBS, fixed with 1% formaldehyde/PBS for 10 min at 4°C, washed, and analyzed immediately by FACSCalibur, using CellQuest software (BD Biosciences, San Jose, CA). Analyses were performed on lymphocyte populations with MHC class II-positive cells gated out, unless mice had received CFSE-labeled cells, in which case the FITC anti-MHC II Ab was omitted from the labeling step. All Abs were purchased from BD Pharmingen (San Diego, CA).

Skin grafting

Female mice were vaccinated on days 0 and 21. On day 28, they were grafted with syngeneic male skin, according to the method of Billingham (17). After removal of the plaster casts 1 wk later, grafts were observed every 2–3 days and were scored as rejected when <10% viable tissue was present.

In vivo cytotoxic assay

Male and female B6 spleen cells (2×10^7/ml in PBS) were incubated with 5 μM or 0.5 μM CFSE (Molecular Probes, Eugene, OR), respectively, at room temperature for 8 min in the dark. FCS (final concentration 20%) was added to quench the labeling reaction. After washing, the cells were mixed and resuspended in PBS, and 2×10^7 cells in 0.1 ml were injected i.v. to each recipient. Peripheral blood was collected from individual mice at serial time points. After lysis of RBC and blockade of FcR, PBL were stained with anti-CD8a-allophycocyanin and either HY^{Db}*Uty*-tetramer-PE or HY^{Db}*Smcy*-tetramer-PE and analyzed for CFSE expression and tetramer binding by FACS.

ELISPOT

ELISPOT analysis was performed using the BD ELISPOT Set (BD Pharmingen), according to the manufacturer's instructions, with slight modification. Briefly, ELISPOT plates were coated overnight with 5 μg/ml anti-mouse IFN-γ mAb, washed, and then blocked with complete medium. Cell suspensions were made in complete medium from spleens of vaccinated mice, and viable cells were selected by density centrifugation. Cells were washed and adjusted to a concentration of 2×10^6/ml in complete medium, then added to the microtiter wells together with either HY^{Db}*Uty* or HY^{Db}*Smcy* peptide to give a final volume of 200 μl containing 2×10^5 cells; control wells received no peptide. Triplicate samples were tested with a range of HY D^b peptide concentrations. After 24-h incubation, cells were lysed with water and washed three times with PBS/0.05% Tween 20, before overnight incubation at 4°C with 1 μg/ml biotinylated anti-mouse IFN-γ mAb in PBS/10% FCS. Plates were washed three times with PBS/0.05% Tween 20, before adding 40 ng of streptavidin-alkaline phosphatase (Mabtech, Nacka, Sweden), in 100 μl of PBS/10% FCS, to each well. Plates were incubated at room temperature for 1 h, and washed (four times) with PBS/0.05% Tween 20 and then with PBS alone (twice) before developing spots using the 5-bromo-4-chloro-3-indolyl phosphate/NBT kit (Zymed Laboratories, San Francisco, CA).

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Results

DNA fusion vaccines induce effector CTL against HY Ags in female mice

The ability of our p.DOM-epitope DNA vaccine to prime CTL responses against HY epitopes was tested by vaccinating female B6 mice with p.DOM-Uty/D^b or p.DOM-Smcy/D^b (Fig. 1). After 14 days, splenocytes from vaccinated mice were stimulated in vitro with relevant peptide for 6 days before assessing lytic activity in a ^{51}Cr release assay. CTL primed with p.DOM-Uty/D^b lysed female target cells pulsed with HY^{Db}*Uty* peptide and killed male target cells expressing endogenous Uty Ag (Fig. 2*a*). Similarly, p.DOM-Smcy/D^b-primed CTL were able to lyse female Abelson-transformed target cells pulsed with HY^{Db}*Smcy* peptide, although these CTL were unable to lyse male Abelson-transformed target cells (Fig. 2*b*), possibly due to poor presentation of the HY^{Db}*Smcy* epitope by these male cells (14). CTL were HY peptide specific, not lysing target cells pulsed with irrelevant peptide (data not shown), and no specific CTL activity was generated by culture of splenocytes in vitro with either HY peptide following vaccination with the control vaccine, p.DOM (data not shown).

To assess the influence of HY DNA vaccines on graft rejection, vaccinated female mice were grafted with syngeneic male skin. Results (Fig. 2*c*) indicate that priming of female recipients with p.DOM-Uty/D^b accelerated male skin graft rejection ($p < 0.002$) compared with mice receiving the control vaccine (p.DOM). However, p.DOM-Smcy/D^b failed to influence rejection times of male skin grafts (data not shown) (see *Discussion*).

Challenge of DNA-primed females with male splenocytes: effect on HY tetramer response

We used tetramers to assess the kinetics of HY-specific $CD8^+$ T cell responses ex vivo in PBL from individual female mice following vaccination with p.DOM-Uty/D^b (Fig. 3*a*) or p.DOM-Smcy/D^b (Fig. 3*b*). At day 10, following DNA vaccination, neither vaccine had induced detectable levels of tetramer-positive cells (~0.1%). However, significant tetramer-positive responses were observed to each epitope at day 17. By day 21, ~1.1% of $CD8^+$ cells stained positive with HY^{Db}*Uty* tetramer in p.DOM-Uty/D^b-vaccinated mice. p.DOM-Smcy/D^b vaccination induced 6.7–8.3% HY^{Db}*Smcy* tetramer-binding $CD8^+$ cells. We then assessed the effect of challenge of DNA-primed female mice by injection of male splenocytes, 21 days after DNA vaccination (Fig. 3, *a* and *b*). Following challenge, HY-specific tetramer-positive $CD8^+$ T cell

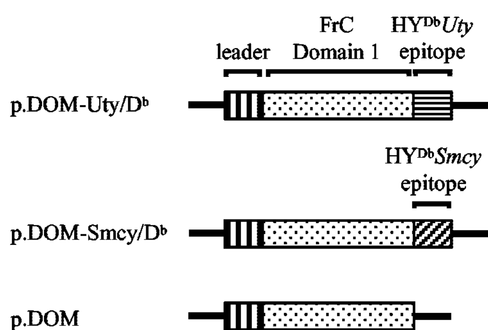

FIGURE 1. Schematic representation indicating DNA vaccine design. Vaccine sequences were assembled and inserted into pcDNA3 vector between *Hin*dIII (5′) and *Not*I (3′) restriction sites. The control vaccine, p.DOM, contains DNA sequence encoding the N-terminal domain (DOM) of fragment C (▦). The vaccines p.DOM-Uty/D^b and p.DOM-Smcy/D^b include DNA encoding the HY^{Db}*Uty* (▤) and HY^{Db}*Smcy* (▨) CTL epitope sequences, respectively, fused to the 3′ terminus of DOM. All constructs encode the BCL_1 leader sequence at the N terminus (▥).

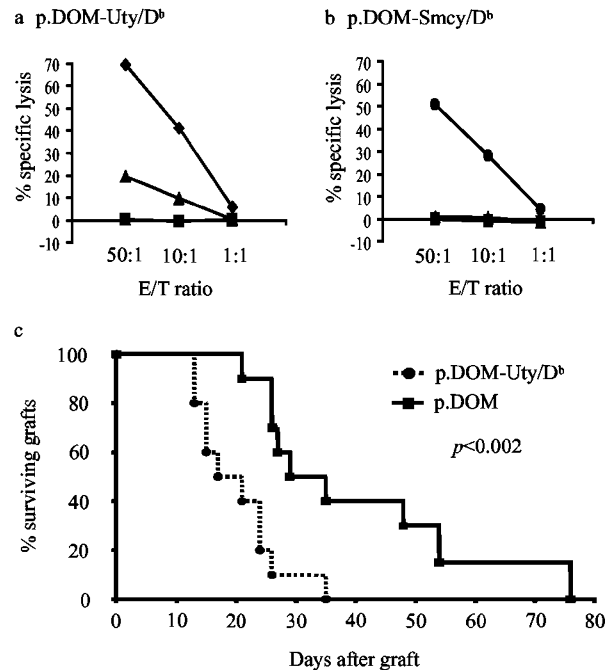

FIGURE 2. DNA fusion vaccines induce effector CTL against HY Ags in female B6 mice and accelerate male skin graft rejection. Female mice were vaccinated with p.DOM-Uty/D^b (*a*) or p.DOM-Smcy/D^b (*b*) DNA vaccines, as indicated. At day 14, splenocytes were cultured with 10 nM HY^{Db}*Uty* (*a*) or HY^{Db}*Smcy* (*b*) peptide for 6 days in vitro, before measuring CTL activity by ^{51}Cr release assay. Targets were Abelson-transformed B cells of male (▲) or female (■) origin, alone, or female cells pulsed with HY^{Db}*Uty* (♦) or HY^{Db}*Smcy* (●) peptide. Representative data from individual mice are shown. Spontaneous ^{51}Cr release from targets was <12%. *c*, Female mice were vaccinated with either p.DOM-Uty/D^b or p.DOM, as indicated, on days 0 and 21, and grafted with syngeneic male skin at day 28. Grafts were scored as having been rejected when <10% viable tissue was visible. Data combined from two experiments are shown.

populations expanded significantly in vivo in female recipients vaccinated with either p.DOM-Uty/D^b or p.DOM-Smcy/D^b; peak responses from individual mice were observed at day 31 (10 days after challenge) with ~8.2% $CD8^+$ HY^{Db}*Uty* and ~34% $CD8^+$ HY^{Db}*Smcy* tetramer$^+$ cells (Fig. 3, *a* and *b*).

In a second experiment, the p.DOM-epitope DNA fusion vaccines were tested for their ability to induce long-term immunological memory. Data (Fig. 3, *c* and *d*) from individual female mice confirm the kinetics of the primary response to each DNA vaccine, with up to 2.9 and 8.5% of $CD8^+$ T cells staining positive with HY^{Db}*Uty* and HY^{Db}*Smcy* tetramers, respectively, on day 17 (Fig. 3, *c* and *d*). Analysis at additional time points indicated that the proportions of tetramer-positive $CD8^+$ T cells decreased gradually over time in individual mice, with <0.2 and 1.3% cells specific for HY^{Db}*Uty* and HY^{Db}*Smcy*, respectively, remaining at day 49. In this experiment, DNA-primed female mice were then challenged with syngeneic male splenocytes at day 109 after DNA vaccination. The male cells were CFSE labeled and injected with differentially labeled female cells as part of an in vivo cytotoxicity assay. Both DNA vaccines induced long-lasting immunological memory, and challenge at day 109 resulted in significant expansion of HY tetramer-positive $CD8^+$ T cells (Fig. 3, *c* and *d*), with kinetics similar to those observed in DNA-primed female mice challenged with male cells at day 21 (Fig. 3, *a* and *b*).

Challenge of unimmunized female mice with male splenocytes initiates a primary response against HY Ags that are expressed endogenously by male cells (14). This response is also observed in

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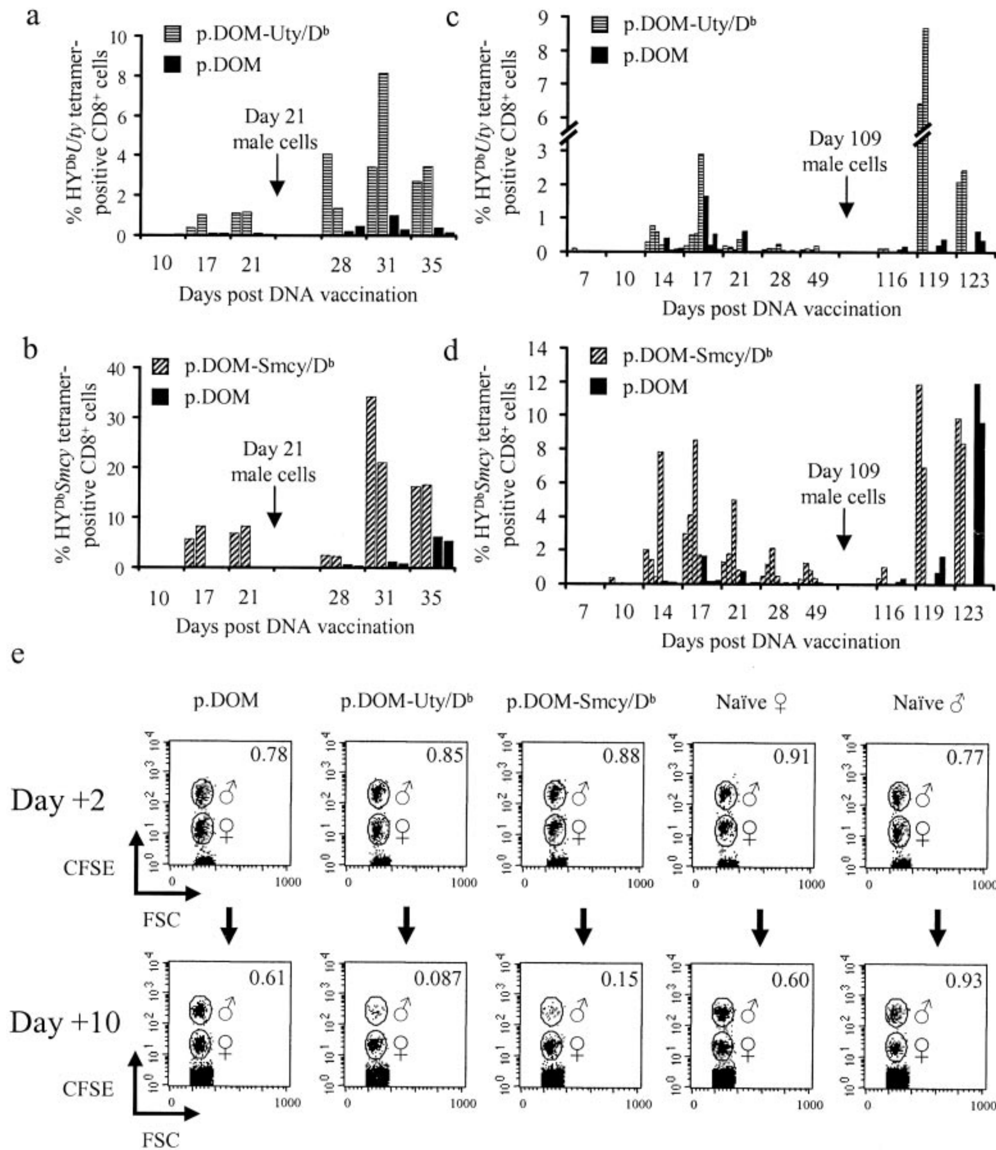

FIGURE 3. Tetramer response following HY DNA vaccination and challenge with male spleen cells: time course and in vivo cytotoxicity response. Female mice were injected with p.DOM-Uty/D^b, p.DOM-Smcy/D^b, or p.DOM DNA vaccines and challenged 21 (*a* and *b*) or 109 (*c* and *d*) days later by i.v. injection of syngeneic male and female splenocytes differentially labeled with CFSE. *a–d*, Time course of HY-specific CTL responses assessed by staining of serial peripheral blood samples with anti-CD8 and either HY^{Db}*Uty* (*a* and *c*) or HY^{Db}*Smcy* tetramer (*b* and *d*). Each bar indicates the percentage of tetramer$^+$ $CD8^+$ T cells from an individual mouse. Time of CFSE-labeled spleen cell injection is indicated (arrow). *e*, Kinetics of male splenocyte rejection by flow cytometry. Individual mice were bled 2 and 10 days after injection of CFSE-labeled spleen cells (at day 109 following vaccination) to determine the ratios of male to female (♂ or ♀) donor cells surviving in each recipient, as indicated in each panel. Naive male and female mice served as controls; representative data from individual mice are shown.

female mice immunized with the control vaccine (p.DOM), leading to an increase in HY tetramer-positive $CD8^+$ T cells following challenge with male cells (Fig. 3, *c* and *d*). However, priming with the p.DOM-epitope fusion vaccines leads to accelerated HY epitope-specific responses after challenge with male splenocytes (Fig. 3, *c* and *d*).

Challenge of DNA-primed females with male splenocytes: in vivo rejection of male cells

Data (Fig. 3*e*) show that HY DNA-vaccinated females not only displayed vigorous HY-specific tetramer-positive responses, but also rejected CFSE-labeled male splenocytes injected at day 109 in vivo at an accelerated tempo in comparison with females given the control vaccine or naive females. DNA-primed female mice challenged with male splenocytes at day 21 also rejected male splenocytes in a similar manner (data not shown). The partial rejection of male splenocytes 10 days after challenge of naive female recipients, or of females vaccinated with the control vaccine (p.DOM), is consistent with the slower tempo of this primary response to HY Ags on male cells (Fig. 3*e*). It was surprising that females vaccinated with p.DOM-Smcy/D^b could reject male splenocytes, but not skin grafts in an accelerated fashion compared with naive females; as *Smcy* is expressed ubiquitously, this may reflect differences in Ag processing and presentation between the two tissues, or limitations in the effectiveness of T cells with this receptor.

Primed epitope-specific CTL can suppress subsequent primary responses to additional cellular epitopes

The primary response to HY Ags following injection of male cells could be detected 14 days later in PBL from female mice vaccinated with the control DNA vaccine (p.DOM), or in unimmunized

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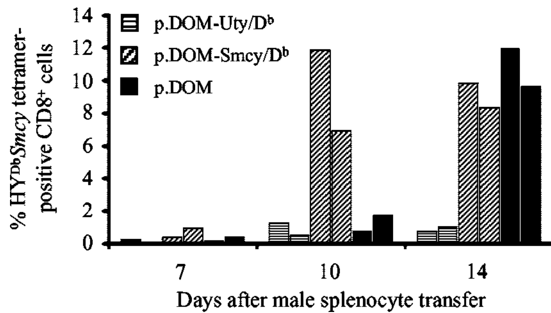

FIGURE 4. On challenge of HY^{Db}Uty-vaccinated females with male splenocytes, the natural CD8$^+$ response against the endogenously expressed Smcy epitope is suppressed. Female mice were vaccinated at day 0 with p.DOM-Uty/D^b, p.DOM-Smcy/D^b, or p.DOM DNA vaccines and challenged 109 days later by i.v. injection containing syngeneic male splenocytes. At the time points indicated, HY^{Db}*Smcy*-specific tetramer responses were assessed in peripheral blood samples. Each bar indicates the percentage of tetramer$^+$ CD8$^+$ T cells from an individual mouse.

females, using HY^{Db}*Smcy*-specific tetramers (Fig. 4, and data not shown). However, priming with p.DOM-Uty/D^b led to suppression of this primary HY^{Db}*Smcy*-specific CD8$^+$ T cell response on challenge with male splenocytes (Fig. 4). This phenomenon was observed in all p.DOM-Uty/D^b-immunized mice, whether challenged with male splenocytes at day 21 or 109 following vaccination. The primary response to HY^{Db}*Uty* 14 days after injection of male splenocytes was too low to permit an evaluation of the influence of a pre-existing HY^{Db}*Smcy* response in female mice (data not shown).

DNA fusion vaccines prime HY-specific CTL in tolerant male mice

Male mice were also assessed for their ability to respond to p.DOM-Uty/D^b and p.DOM-Smcy/D^b DNA vaccines. Surprisingly, both were able to prime HY-specific CTL capable of lysing cognate peptide-pulsed target cells following 6 days of in vitro restimulation with peptide (Fig. 5, *a* and *b*), although the frequencies of male mice responding to vaccination were lower than females (Table I). No HY-specific CTL activity was detected following culture in vitro with either HY peptide after vaccination with the control vaccine (p.DOM; data not shown). The in vitro activity of HY-specific CTL from vaccinated male mice was designated as weak because they lysed peptide-pulsed EL4 target cells, but not EL4 cells transfected to express the Uty epitope endogenously (Fig. 5*c*), whereas comparable HY^{Db}*Uty*-specific CTL of female origin could lyse these target cells (Fig. 5*c*).

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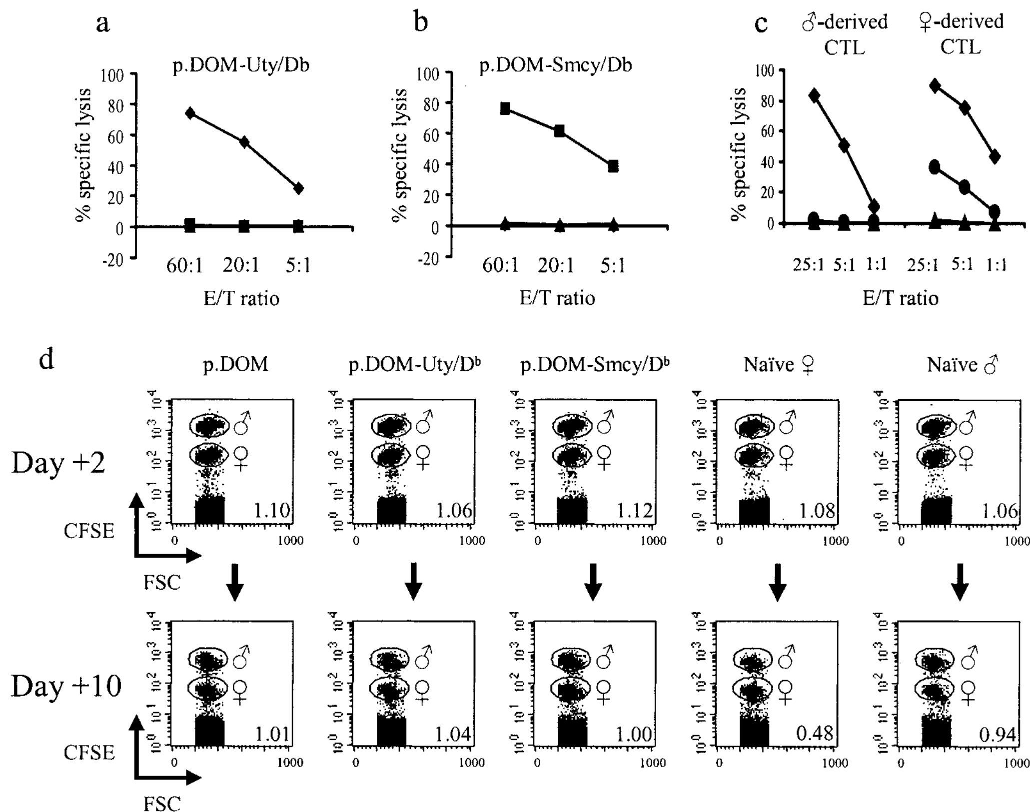

FIGURE 5. DNA fusion vaccines induce effector CTL against HY Ags in male mice. Male B6 mice were vaccinated with p.DOM-Uty/D^b (*a*) or p.DOM-Smcy/D^b (*b*) DNA vaccines. At day 14, splenocytes were stimulated with 100 nM HY^{Db}*Uty* (*a*) or HY^{Db}*Smcy* (*b*) peptide for 6 days in vitro, before measuring CTL activity by ^{51}Cr release assay. Targets were EL4 cells alone (▲), or EL4 cells pulsed with HY^{Db}*Uty* (♦) or HY^{Db}*Smcy* (■) peptide. Representative data from individual mice are shown. Spontaneous ^{51}Cr release from targets was <11%. *c*, Male (*left panel*) and female (*right panel*) B6 mice were vaccinated with the p.DOM-Uty/D^b DNA vaccine. At day 14, splenocytes were harvested and used to establish HY^{Db}*Uty*-specific T cell lines in vitro; cells were stimulated with 10 nM HY^{Db}*Uty* peptide for 7 days, then weekly with irradiated, HY^{Db}*Uty* peptide-pulsed female splenocytes for 4 wk, before measuring CTL activity by ^{51}Cr release assay. Targets included EL4 cells transfected with the *Uty* gene (EL4-Uty, ●) or mock-transfected EL4 cells either alone (▲) or pulsed with HY^{Db}*Uty* (♦) peptide. Spontaneous release from targets was <10%. *d*, Male mice injected at day 0 with p.DOM-Uty/D^b, p.DOM-Smcy/D^b, or p.DOM DNA vaccines were challenged 14 days later by i.v. injection of syngeneic male and female splenocytes differentially labeled with CFSE. PBL samples were taken from recipients 2 and 10 days later to determine the ratios of male to female (♂ or ♀) donor cells surviving in each recipient, as indicated in each panel. Naive male and female mice served as controls. Representative data from individual mice are shown.

Table I. *Induction of effector T lymphocyte responses in male and female C57BL/6 mice*[a]

Mice	Day 14 In Vitro[b]		Day 14 *ex vivo*[c]		Day 21 *ex vivo*[c]	
	p.DOM-Uty/D^b	p.DOM-Smcy/D^b	p.DOM-Uty/D^b	p.DOM-Smcy/D^b	p.DOM-Uty/D^b	p.DOM-Smcy/D^b
Female	7/8 (87.5%)	8/8 (100%)	6/8 (75%)	6/8 (75%)	2/4 (50%)	4/4 (100%)
Male	10/19 (52.6%)	6/19 (31.6%)	6/8 (75%)	0/8 (0%)	1/4 (25%)	0/4 (0%)

[a] Mice were vaccinated with either p.DOM-Uty/D^b or p.DOM-Smcy/D^b and CD8$^+$ T cell responses were assessed at day 14 or 21, either following a 6-day culture in vitro by ^{51}Cr release assay, [b] or directly ex vivo by ELISPOT assay. [c] The number of mice demonstrating peptide-specific effector T lymphocyte priming of the total mice tested is shown, with the percentage responding in parentheses. Peptide-specific ELISPOT responses greater than twice baseline values observed in the absence of peptide and greater than four times the background levels from mice given the control vaccine (p.DOM) were considered positive; peptide-specific cytolytic responses more than twice background and above 10% specific lysis were also deemed positive.

Although both DNA vaccines could induce HY-specific CTL detectable in vitro, vaccinated male mice were unable to reject CFSE-labeled male splenocytes in vivo (Fig. 5*d*). This was not due to a low frequency of mice responding to the vaccines because they were culled 2 wk after injection of male cells, and HY^{Db}*Uty*-specific and HY^{Db}*Smcy*-specific CTL activity was detected in two of four and four of four of the vaccinated mice, respectively, following culture of splenocytes for 6 days in vitro with the relevant HY peptide (data not shown).

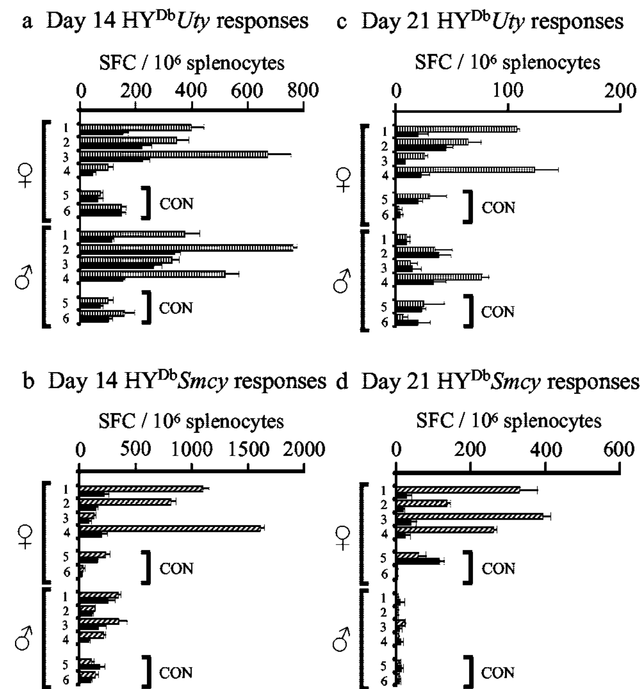

FIGURE 6. Magnitude of anti-HY T cell responses in male and female B6 mice following DNA vaccination. Groups of four (1–4) male and female B6 mice were vaccinated with p.DOM-Uty/D^b (*a* and *c*) or p.DOM-Smcy/D^b (*b* and *d*) DNA vaccines. Mice (5 and 6) given the control vaccine (p.DOM) are indicated (CON). Splenocytes harvested at day 14 (*a* and *b*) or 21 (*c* and *d*) were assessed for IFN-γ secretion by ELISPOT. Peptide-specific responses to p.DOM-Uty/D^b were measured by incubation with HY^{Db}*Uty* peptide (▥); 10 nM for female-derived splenocytes and 100 nM for male-derived splenocytes. Responses to p.DOM-Smcy/D^b were assessed using HY^{Db}*Smcy* peptide (▨); 1000 nM for male-derived splenocytes at day 14, but 100 nM for all other samples. Samples were set up in triplicate. Baseline responses by each mouse (1–6) with no added peptide are indicated (■). Data from individual mice are depicted as mean number of spot-forming cells (SFC) per million splenocytes, and the SEM (error bars).

DNA vaccination activates low avidity HY-specific CTL in male mice

Ex vivo analysis of PBL samples from male mice vaccinated with p.DOM-Uty/D^b or p.DOM-Smcy/D^b failed to detect HY-specific tetramer-positive CD8$^+$ T cells (data not shown). However, T cells secreting IFN-γ in response to HY peptides could be observed using an ELISPOT assay on splenocyte samples from these mice (Table I). Data (Fig. 6) indicate that the number of HY^{Db}*Uty*-specific cells detectable following vaccination with p.DOM-Uty/D^b is similar in males and females at day 14 (Fig. 6*a*), although fewer were observed in males at day 21 (Fig. 6*c*). However, splenocytes from males required exposure to 10-fold higher concentrations of HY^{Db}*Uty* peptide during the ELISPOT assay to detect this level of response. Although HY^{Db}*Smcy*-specific T cells from vaccinated females were detected ex vivo by ELISPOT, none were found in vaccinated male mice (Table I; Fig. 6, *b* and *d*).

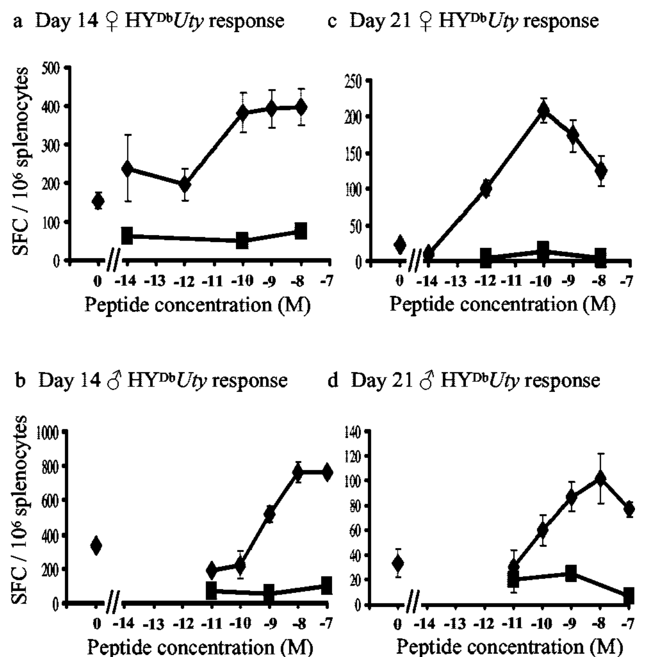

FIGURE 7. DNA vaccination of B6 mice induces lower avidity HY^{Db}*Uty*-specific CTL in males compared with females. Male (*b* and *d*) and female (*a* and *c*) B6 mice were injected with p.DOM-Uty/D^b (◆) or p.DOM (■) DNA vaccines, as indicated. Splenocytes were harvested at day 14 (*a* and *b*) or 21 (*c* and *d*), and the number of HY^{Db}*Uty*-specific spot-forming cells (SFC) secreting IFN-γ was assessed ex vivo by ELISPOT. Representative data from individual mice are shown. Test samples were set up in triplicate for each peptide concentration, and the data indicate mean SFC per million splenocytes, and the SEM (error bars).

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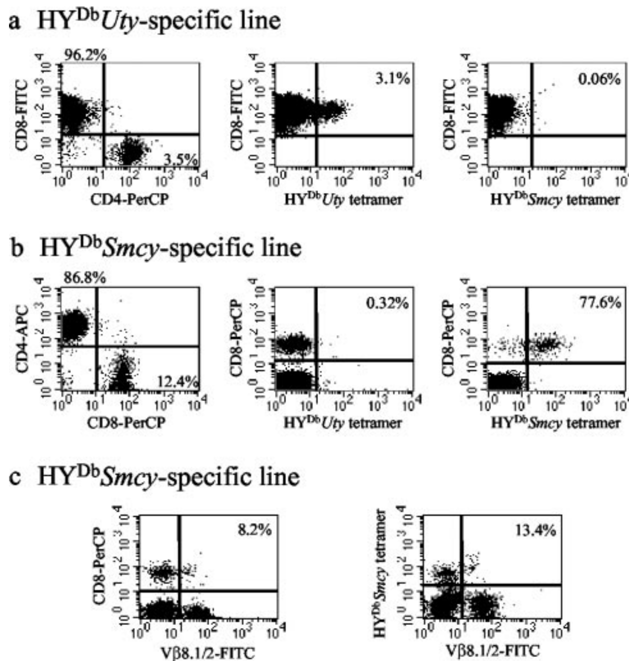

FIGURE 8. HY tetramer-positive cells in spleen cell cultures from DNA-vaccinated males. *a*, An HY^{Db}*Uty* line from a p.DOM-Uty/D^b-vaccinated male B6 mouse following three rounds of restimulation, at 10- to 14-day intervals, with HY^{Db}*Uty* peptide-pulsed female cells in the presence of 20 IU/ml H rIL-2. The *first panel* shows the presence of both CD4$^+$ and CD8$^+$ T cells; the *second* is gated on the CD8$^+$ T cells, showing some of them to be HY^{Db}*Uty* tetramer specific. The *third panel* shows no staining with the HY^{Db}*Smcy* tetramer. *b*, An HY^{Db}*Smcy* line from a p.DOM-Smcy/D^b-vaccinated male treated in vitro as in *a* above, but with HY^{Db}*Smcy* peptide; the CD8$^+$ T cell population is mostly HY^{Db}*Smcy* tetramer positive. *c*, Staining the HY^{Db}*Smcy* tetramer-positive cells in *b* above with Vβ8.1/2 mAb shows a small proportion of them to use this TCR chain.

Clearly, vaccination of male mice with p.DOM-Uty/D^b leads to priming of a population of HY^{Db}*Uty*-specific CTL capable of secreting IFN-γ and having lytic potential. However, male-derived HY^{Db}*Uty*-specific CD8$^+$ T cells had ~100-fold lower avidity compared with those from vaccinated females, as shown by testing with a range of concentrations of the HY^{Db}*Uty* peptide (Fig. 7). Repeated in vitro stimulation of spleen cell cultures from vaccinated male mice with HY peptide can lead to the expansion of HY-specific CD8$^+$ T cells able to bind HY tetramer: tetramer-positive examples of T cell lines developed after three rounds of in vitro restimulation from individual mice vaccinated with p.DOM-Uty/D^b or p.DOM-Smcy/D^b are shown in Fig. 8, *a* and *b*, respectively. Staining the HY^{Db}*Smcy* tetramer-positive cells for Vβ8.1/2 (Fig. 8*c*) indicates that some, but not all, of them use this TCR chain, which is the one often used by T cells of this specificity by HY-primed females (J. Dyson, personal communication) and the HY-specific TCR transgenic mouse, B2.6.16 (18–20).

Discussion

The male-specific HY Ags are classified as minor H Ags, and represent relevant targets to assess strategies for induction of immunity by DNA vaccines. We have engineered p.DOM-epitope fusion vaccines that have two components. The first is a sequence derived from a domain of a microbial Ag, tetanus toxin, which activates high levels of CD4$^+$ T cell help from the large nontolerized repertoire (21, 22). The linked T cell help then induces and maintains immunity against the fused tumor Ag (21, 23). The second component is designed to induce CD8$^+$ T cell responses against epitopes from intracellular tumor Ags. For this, each vaccine sequence encodes a candidate tumor-derived MHC class I-binding peptide with a fixed C terminus. The fusion protein is directed to the endoplasmic reticulum, where trimming of the N terminus can occur (24). In several models, we have shown that this dual strategy leads to induction of high levels of epitope-specific CTL (15, 16), and others have confirmed the principle (25).

The DNA vaccines expressing each of the two class I-restricted HY peptides were able to induce high levels of epitope-specific CD8$^+$ T cells in females, and the HY^{Db}*Uty*-primed females rejected male skin grafts in an accelerated manner. The failure of HY^{Db}*Smcy*-primed females to do so is consistent with TCR transgenic mice carrying a receptor of this specificity (18, 20) being unable to reject syngeneic male skin grafts (26). This may be due to poor presentation of the HY^{Db}*Smcy* epitope and/or limitations in efficacy of T cells with this receptor. However, their ability to reject male spleen cells is shown in the in vivo cytotoxicity assay (Fig. 3), so clearly, T cell responses directed at a single HY epitope are primed for accelerated rejection, as found for in vivo responses to tumor cells (15, 16). The precision offered by the vaccines allows induction of CTL of selected specificity. Focusing by immunodominance was evident from finding that the immune response following injection of male splenocytes into vaccinated females was modulated to remain specific for the initial inducing peptide, with immunity against the other epitope suppressed. Therefore, if responses against multiple epitopes are desired, injection of separate epitope-specific vaccines might be the best strategy (27, 28).

Another question for fusion vaccines was whether memory CD8$^+$ T cells persisted, and whether epitope-specific CTL could be efficiently expanded on challenge with a source of Ag not expressing the tetanus toxin epitopes used to activate the original Th cells, like male cells. From the results, it appears that memory persists for at least 109 days and that male cells are capable of activating them to become cytolytic effector cells (Fig. 3), making it likely that CD8$^+$ memory T cells primed by vaccination to other minor H Ags or tumor Ags would also respond in this way.

HY genes are expressed in the thymus, and a transgenic model expressing an HY Smcy-specific TCR has been used to assess tolerogenic pressure on a clonal T cell repertoire (18, 20). It is evident that a proportion of cells survives thymic selection and is exported to the periphery, but with reduced affinity due to down-regulation of CD8 (20). More recently, using the same model, anergy has been observed in transgenic T cells following confrontation with Ag in vivo (29). The anergic state was indicated by reduced Ca^{2+} mobilization after TCR engagement, and was reversible on removal of Ag (29). Again in this model, if only the TCRβ was expressed, escape was possible by selection of alternative TCRα genes with less permissive CDR3 loop sequences (30). In each of the cases quoted, tolerance leads to reduced T cell responses and no signs of autoimmunity (31, 32). Mechanisms of active tolerance have been revealed in the setting of transplantation (33). In this study, CD4$^+$ regulatory T cells can control alloreactive CD8$^+$ T cells in vivo by censoring immune effector functions (34). Clearly, multiple mechanisms could be operating to reduce the effectiveness of CD8$^+$ T cells in the presence of Ag, and understanding these has relevance for antitumor immunity.

Vaccination of male mice has to mobilize T cells remaining within a profoundly tolerized polyclonal repertoire. This is a more severe test than most cancers, in which the level of Ag is likely to be low. The fact that the DNA fusion vaccines can activate epitope-specific CD8$^+$ T cells, able not only to produce IFN-γ ex vivo, but to bind tetramer and kill peptide-loaded cells, is encouraging. If in vivo the cells were anergized, this state has apparently been reversed. However, although CD8 expression was normal in

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the HY-specific T cells induced by vaccinating males, TCR affinity, as tested by sensitivity to activation by various concentrations of peptide, was low compared with $CD8^+$ T cells from HY-vaccinated females. This could have contributed to the failure of vaccinated males to reject CFSE-labeled male splenocytes in vivo. The affinity of the antitumor repertoire available in tumor bearers will vary, but tolerance is likely to be less profound. Even low affinity $CD8^+$ T cells, once primed, might be capable of killing tumor cells overexpressing Ag. Also, tumor cell blasts (e.g., leukemic cells) may be more susceptible to lysis by T cell effectors directed at minor H or tumor Ags (8).

For clinical application, an Ag-specific DNA fusion vaccine could be used to induce effector T cells in a hemopoietic stem cell transplant donor, and these selected (e.g., by tetramer) for adoptive transfer. There is clearly scope for using target minor H epitopes preferentially expressed on hemopoietic cells in this clinical setting for treatment of leukemia (5, 35). Alternatively, tumor-bearing patients could be vaccinated to activate the residual T cell repertoire. As the identity of more endogenously derived peptides binding different HLA class I alleles is determined, this approach could be broadened to include individuals of many haplotypes, as well as used to develop strategies for targeting multiple epitopes. Vaccine design is now well advanced, although translation from preclinical to clinical settings requires optimization (22). The next priority is to understand better the immune status of tumor-bearing patients, and how this can be therapeutically modified.

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