DNA Fusion Vaccines Induce Epitope-Specific Cytotoxic CD8+ T Cells against Human Leukemia-Associated Minor Histocompatibility Antigens

Jason Rice,1 Stuart Dunn,1 Karen Piper,2 Sarah L. Buchan,1 Paul A. Moss,2 and Freda K. Stevenson1

1Molecular Immunology Group, Southampton University Hospitals Trust, Southampton, Hampshire, United Kingdom and 2Cancer Research UK Institute for Cancer Studies, The University of Birmingham, Edgbaston, Birmingham, United Kingdom

Abstract

The graft-versus-leukemia effect of allogeneic stem-cell transplantation is believed to be mediated by T-cell recognition of minor histocompatibility antigens on recipient cells. For minor histocompatibility antigens HA-1 and HA-2, normal cell expression is restricted to hemopoietic cells, and boosting the immune response to these antigens may potentiate graft-versus-leukemia effect without accompanying graft-versus-host disease. To increase efficacy, expansion of HA-1- or HA-2-specific CTL before transplantation is desirable. However, primary HA-1- or HA-2-specific CTL expanded in vitro are often of low avidity. An alternative approach is to prime specific CTL responses in vivo by vaccination. Clearly, donor vaccination must be safe and specific. We have developed DNA fusion vaccines able to induce high levels of epitope-specific CTL using linked CD4+ T-cell help. The vaccines incorporate a domain of tetanus toxin (DOM) fused to a sequence encoding a candidate MHC class I binding peptide. This design generates an antitumor CD8+ T-cell response

Introduction

The effectiveness of donor lymphocyte infusion in suppressing hematologic malignancies in patients relapsing after allogeneic stem-cell transplantation has sparked intense immunological and clinical interest. It seems that CTL are the main mediators and target epitopes arise from polymorphic differences between donor and recipient (1, 2). These are termed minor histocompatibility (H) antigens, and several candidate molecules have been described (1). The tissue expression of minor H antigens is variable and those with clinical relevance are expressed exclusively on hematopoietic tissue (3, 4), consequently restricting donor CTL attack to graft-versus-leukemia activity while avoiding graft-versus-host disease (5, 6).

Two important hematopoietic-restricted minor H antigens are HA-1 and HA-2 (7, 8). HLA-A*0201-restricted CTL against immunodominant peptides within these antigens can be induced spontaneously by transplantation. HA-1- or HA-2-specific CTL are able to lyse a variety of tumor target cells in vitro, and expansion of HA-1- and HA-2-specific CD8+ T cells following donor lymphocyte infusion was found to correlate with disease remission in the absence of graft-versus-host disease (9). In addition, HA-1- and HA-2-specific CTL do not induce tissue damage in a skin explant model, in contrast to CTL specific for peptides derived from ubiquitously expressed H-Y proteins (10). These findings have encouraged strategies to generate HA-1- or HA-2-specific CTL in vitro before adoptive T-cell therapy (11). However, in addition to the technical difficulties inherent in ex vivo priming, T cells generated in this way often show low functional avidity (12).

An alternative approach is to generate a high-avidity minor H antigen–specific immune response in vivo through the use of vaccination. Clearly, priming the donor in vivo could generate specific T-cell responses for direct transfer or for expansion in vitro, but no vaccine is currently available.

Gene-based vaccines provide an effective strategy for inducing prophylactic and therapeutic immunity in preclinical models of infectious disease and cancer. Relatively simple to manipulate and manufacture compared with cellular or viral vaccines, DNA vaccination ensures that the immune response will become focused on vaccine-encoded antigens and, unlike protein or peptide vaccines, it does not require codelivery with an adjuvant to confer immunogenicity (13). Importantly, DNA vaccines seem to be safe and they have already entered pilot clinical trials (14, 15). Our group has developed DNA fusion vaccines which encode two functional elements: one to stimulate high levels of CD4+ T-cell help using a sequence (fragment C) from tetanus toxin, and the other consisting of the tumor antigen sequence (16, 17). By using a fused molecule that is not tumor derived (fragment C), it is possible to engage a large repertoire of Th cells that will not have been reduced or tolerized by the presence of tumor, a strategy that has proved to be critically important for tumor vaccines (17, 18). Initially, we are testing idiotypic antigens, encoded by the immunoglobulin variable region genes, fused to the fragment C sequence in patients with lymphoma (14); the vaccines seem to be very safe even following repeated vaccination in patients already immune to tetanus toxin (14). In addition, allogeneic transplant...
donors are being vaccinated against idiotypic determinants before stem-cell donation to recipient patients with myeloma (14). It should be emphasized that although fragment C contains a number of CD4+ T-cell epitopes, these are all specific for tetanus toxin and there is no increase in nonspecific CD4+ T-cell reactivity.

To optimize induction of CD8+ CTL, we have minimized the fragment C sequence to a single domain (DOM) and fused it to a target epitope able to bind to MHC class I (19). For mouse tumor antigens, these vectors induce high levels of tumor-specific CTL supported by CD4+ T cells activated from the large anti–fragment C repertoire (18, 20). Using CD8+ T-cell epitopes of the murine H-Y male minor histocompatibility antigen, we have shown that this strategy can mobilize CD8+ T cells against endogenous minor H antigens, even from a profoundly tolerized male repertoire (20). To test this design for HLA-A*0201-restricted human HA-1- and HA-2-derived peptides, we have generated DNA constructs in which the HA-1 or HA-2 sequences were fused downstream of the minimized fragment C domain and have vaccinated mice transgenic for HLA-A*0201. This primed HLA-A*0201-restricted, minor H antigen–specific CTL that were able to kill human target cells with endogenous expression of HA-1 in a CD8-independent manner. The HA-2 peptide is processed and presented by mouse cells and is therefore potentially tolerizing. However, significant levels of CTL were induced against HA-2, which were also capable of specific killing of human target cells. These findings show that our DNA fusion vaccine design is able to induce functional and effective CTL against clinically significant minor H antigens.

Materials and Methods

Cell lines. Human T2 cells (HLA-A2, TAP−) were maintained in RPMI plus 10% FCS (complete medium; ref. 20). Human EBV-transformed lymphoblastoid B-cell lines were generated from HLA-A*0201-positive individuals with known HA-1 or HA-2 genotype and maintained in complete medium.

Peptides and tetramers. The HLA-A*0201-restricted peptides VLHDDLLEA and YIGEVLVSV from HA-1 and HA-2, respectively, and the fragment C–derived Th peptide p30 (FNNFTVSFWLRVPKVSASHLE) were synthesized commercially and supplied at >95% purity (PPRL, Southampton, United Kingdom; refs. 7, 8). Peptide stocks (1 mmol/L) were made up in PBS, filter sterilized, and stored at −80°C.

Construction of DNA vaccines. The DNA construct p.DOM, encoding the first domain (DOM) of fragment C from tetanus toxin, has been constructed by PCR from HA-1 or HA-2 to the COOH terminus of p.DOM. Each vaccine includes a leader sequence at the NH2 terminus. Vaccine sequences were assembled and inserted into the commercial vector pcDNA3 using HindIII and NotI restriction sites.

β2-microglobulin is covalently linked to the NH2 terminus of chimeric HLA-A2 α1 and α2 domains fused with the murine H-2Dβ c3 domain (21). These mice lack serologically detectable cell-surface expression of mouse endogenous H-2Kβ class I molecules due to targeted disruption of the H-2Dβ and mouse β2-microglobulin genes.

DNA vaccination protocol. HLA-A*0201-transgenic HHD mice were vaccinated at 6 to 10 weeks of age with a total of 50 μg DNA in saline, injected into two sites in the quadriceps muscles on day 0 (22). When indicated, animals were anesthetized and given booster injections together with electroproportion at day 28, as previously described (23). Animal experimentation was conducted within local Ethical Committee and UK Coordinating Committee for Cancer Research (London, United Kingdom) guidelines under Home Office license.

ELISPOT. To assess priming of minor H antigen–specific CD8+ T cells, vaccinated HHD transgenic mice were sacrificed on day 15 or 16. To monitor the potential to boost existing minor H antigen–specific CD8+ T-cell responses, HHD mice were vaccinated at day 0 with DNA alone, then boosted at day 28 with DNA plus electroporation, as previously described (23); splenocytes were harvested on day 36 to monitor CD8+ T-cell responses. Vaccine-specific IFNγ secretion by splenocytes from individual mice was assessed ex vivo (BD ELISPOT Set, BD PharrMingen, San Diego, CA) as previously described (20). Splenocytes were incubated with the HLA-A*0201-restricted HA-1 or HA-2 peptides for 24 hours to assess CD8+ T-cell responses; the p30 peptide (derived from the fragment C fusion domain) was used to assess CD4+ T-cell responses. Triplicate samples were tested with a range of HA-1 or HA-2 peptide concentrations; control samples were incubated without peptide. Peptide-specific ELISPOT responses of >50 spot-forming cells per 106 splenocytes and more than twice baseline values observed in the absence of peptide were considered positive.

Generation and assay of HA-1- and HA-2-specific CD8+ T-cell cultures. To assess priming for CTI responses, vaccinated mice were sacrificed at day 16 and spleens were removed. Single-cell suspensions were made from individual spleens in complete medium. Splenocytes were washed, counted, and resuspended at 3 × 106 cells/mL; 15 mL were added to upright 25-cm² flasks together with recombinant human interleukin-2 (IL-2; 20 IU/mL; Perkin-Elmer, Foster City, CA) and peptide (1 μmol/L). Following 6 days of stimulation in vitro, cytolytic activity of the T-cell cultures was assessed by standard 5-hour 3H-release assay as previously described (20). Target cells (T2) were chromium 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% NP40 – release by targets alone] × 100%.

To expand CTL lines for testing against a panel of human target cells, splenocytes from groups of two vaccinated mice were pooled, resuspended at 3 × 106 cells/mL, and initially stimulated for 1 week with free peptide (1 μmol/L) and recombinant human IL-2 (20 IU/mL). Subsequently, T cells (2 × 105–5 × 106 per well) were restimulated every 7 days with irradiated

Figure 1. Schematic diagram indicating DNA fusion vaccine design. The control vaccine p.DOM contains sequence encoding the NH2-terminal domain (DOM) of fragment C, including the p30 CD4+ Th epitope, p.DOM-HA-1 and p.DOM-HA-2 include DNA sequence encoding human HLA-A*0201-restricted CTL epitopes from HA-1 and HA-2, respectively, linked to the COOH terminus of DOM. Each vaccine includes a leader sequence at the NH2 terminus. Vaccine sequences were assembled and inserted into the commercial vector pcDNA3 using HindIII and NotI restriction sites.
(2,500 rad), peptide-pulsed female splenocytes (5 × 10^6 per well) and recombinant human IL-2 (20 IU/mL) in 24-well tissue culture plates. After a total of 3 weeks expansion in vitro, cytotoxicity was assessed by ^51^Cr-release assay; target cells included T2 cells pulsed with HA-1 or HA-2 peptide, as well as HLA-A*0201+ human EBV-transformed lymphoblastoid B cells from individuals with known HA-1 or HA-2 genotype.

For blocking experiments, mice were primed with DNA at day 0 and boosted with DNA plus electroporation at day 28. Splenocyte cultures from individual mice were established at day 36 and stimulated with free peptide, then with irradiated peptide-pulsed female splenocytes 7 days later, as described above. After 2 weeks in vitro, cytotoxicity was assessed using peptide-pulsed T2 cells as targets; following labeling with peptide and ^51^Cr, target cells were incubated with mouse anti-HLA-A2 antibody (BB7.2, Serotec, Oxford, United Kingdom) or isotype control at 20 μg/mL (30 minutes, 37°C) before plating out with T cells. The antibodies were included in assay wells at 10 μg/mL.

**Results**

DNA fusion vaccines induce human minor H antigen-specific T cells detectable *ex vivo*. Fusion gene vaccines were constructed to encode a single domain of fragment C of tetanus toxin (DOM), known to contain the "universal" p30 Th epitope, fused to a sequence encoding either the HA-1 or HA-2 epitope (Fig. 1). HLA-A*0201-transgenic HHD mice were vaccinated with the fusion vaccines (p.DOM-HA-1 or p.DOM-HA-2) or with a control vaccine (p.DOM) without an epitope. T-cell responses in the spleen were measured immediately *ex vivo* by ELISpot assay on day 15 or 16 following a single DNA vaccination (Fig. 2A and B). Vaccination induced HA-1-specific (Fig. 2A) or HA-2-specific (Fig. 2B) CD8+ T-cell responses as measured by the production of IFNγ. In the experiments illustrated, the single injection induced significant responses against the HA-1-specific vaccine in three of five mice (Fig. 2A) whereas one of eight mice responded to the HA-2-specific vaccine (Fig. 2B). The control vaccine (p.DOM) gave no HA-specific responses (Fig. 2A and B; Table 1). A survey of large numbers of mice was then carried out, which gave response frequencies of 11 of 42 (26%) against HA-1, with 6 of 52 (12%) responding to HA-2 (Table 1).

CD4+ T-cell responses against the "promiscuous" MHC class II binding peptide p30, embedded in the fragment C domain, were also detected in a proportion of mice vaccinated with p.DOM-HA-1 and p.DOM-HA-2 or the control vaccine (p.DOM; Fig. 2A and B). This was confirmed in the larger cohort, with 38% of vaccine recipients responding in the p.DOM-HA-1 group and 44% in the p.DOM-HA-2 group. Because the CD4+ T-cell response to p30 is derived from the unmanipulated mouse repertoire, it could be used as an overall indicator of vaccine performance. Of the p30 responders, 69% responded to HA-1 and 26% to HA-2.

Electroporation in a “prime/boost” regimen can improve vaccine-specific T-cell induction. We have previously described a novel homologous prime/boost strategy, combining DNA vaccination and electroporation, to generate superior antitumor immune responses (23). Strikingly, this approach induced robust HA-1-specific CD8+ T-cell responses (measured by IFNγ ELISpot assay) in 100% of mice vaccinated with p.DOM-HA-1 (Fig. 2C; Table 1) and all animals responded to the p30 Th peptide (Table 1). Electroporation also significantly improved the response rate to p.DOM-HA-2, with HA-2-specific CD8+ T cells detected in 42% of mice and p30 responses detected in 100% of mice (Fig. 2D; Table 1). The control vaccine (p.DOM) gave no HA-specific responses (Fig. 2C and D) but four of five mice mounted a response to the p30 Th peptide following electroporation (Table 1). Although the kinetics of CD4+ Th-cell induction following boosting with our vaccines...
have not yet been assessed, the addition of the boost with electroporation improved the overall performance of both vaccines, as well as that of the control vaccine, and also increased the frequency and level of the epitope-specific CD8+ T-cell responses.

**Cytotoxic function of the epitope-specific CTL.** To assess cytolytic activity of HA-1- or HA-2-specific CD8+ T cells, splenocytes from individual vaccinated mice were expanded *in vitro* by stimulation with the relevant human peptide for 6 days before assessing lytic activity in a 51Cr-release assay. CTL from mice primed with p.DOM-HA-1 lysed T2 target cells pulsed with HA-1 peptide to variable extents (Fig. 3A), with lytic activity observed in splenocyte cultures from five of six individual mice tested. Similarly, CTL from HHD mice primed with the p.DOM-HA-2 vaccine were able to lyse T2 cells pulsed with HA-2 peptide (Fig. 3B), with lytic activity observed in splenocyte cultures from three of six individual mice tested. Lysis was completely specific for each HA peptide because, in each case, no lysis of target cells loaded with the reciprocal peptide was observed. No specific CTL activity was generated by culture of splenocytes *in vitro* with either minor H antigen peptide following vaccination with the control vaccine, p.DOM (Fig. 3A and B). The lytic activity of minor H antigen–specific CTL from individual mice primed with p.DOM-HA-1 or p.DOM-HA-2 was significantly blocked by an anti-HLA-A2 monoclonal antibody (Fig. 3C and D), showing the MHC restriction of these T cells. No block in lytic activity was observed using a control antibody (Fig. 3C and D).

Mouse HLA-A2-restricted CTL are able to lyse human lymphoblastoid cell lines expressing endogenous antigen. The main question for clinical efficacy was whether or not the CTL would be capable of killing cells expressing the target peptides endogenously. Because the transgenic mice express mouse CD8, which cannot interact with the α3 sequence of the human MHC class I (24), all killing of human cells by the induced CTL has to be CD8 independent. This means that only high-avidity CTL will be able to kill the human cell lines expressing the HA epitopes endogenously. We tested the ability of the minor H antigen–epitope–specific CTL to kill human lymphoblastoid B-cell lines established from HLA-A*0201-expressing patients of defined HA-1 or HA-2 genotype. CTL specificity was verified by lysis of T2 cells loaded with the appropriate peptide (Fig. 4A and B). Importantly, these CTL could kill human lymphoblastoid cell lines endogenously expressing the appropriate minor H antigen without the requirement for peptide loading (Fig. 4A and B). The killing was specific for the appropriate minor H antigen and HA-negative control lymphoblastoid cell lines were lysed when loaded with the appropriate peptide (e.g., 48.3% lysis, at 25:1 effector/target ratio, of HA-1-negative lymphoblastoid cell lines pulsed with HA-1 peptide by HA-1-specific CTL, compared with −1.9% lysis when unpulsed). In this case, therefore, the association of the T-cell receptor with the MHC class I-peptide complex seems to be sufficiently high to overcome the need for the usual strengthening interaction between the MHC class I heavy chain α3 domain and CD8 (25).

**Discussion**

These findings show a vaccine design able to induce functional and effective CTL against clinically significant minor H antigens. They confirm the efficacy of the single epitope design, which focuses the CTL response onto the target peptide (19) and mimics the natural focusing of the immune response against a single, or limited group of, immunodominant determinant(s) (26). It is clear that CTL specific for single epitopes can mediate effective antiviral immunity (27) and they allow strict comparability of the total response among patient groups. For leukemia, the precise immune focusing afforded by the p.DOM-peptide vaccine, coupled with the limited tissue expression of the target minor H antigens (HA-1/HA-2), should restrict CTL attack to graft-versus-leukemia activity while minimizing the opportunity for graft-versus-host disease (5). Although the power of epitope-specific CTL to kill antigen-expressing tumor cells seems to be ensured (18–20), it could be argued that attack on multiple epitopes would be more appealing. However, multiple presentation from a single vaccine gives rise to

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**Table 1. Ex vivo detection of vaccine-specific T lymphocytes in HHD mice**

<table>
<thead>
<tr>
<th>DNA vaccine</th>
<th>Ex vivo peptide stimulation</th>
<th>Vaccine-specific responses</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Priming alone</td>
</tr>
<tr>
<td>p.DOM-HA-1</td>
<td>HA-1</td>
<td>11 of 42 (26%)</td>
</tr>
<tr>
<td></td>
<td>p30</td>
<td>16 of 42 (38%)</td>
</tr>
<tr>
<td>p.DOM-HA-2</td>
<td>HA-2</td>
<td>6 of 52 (12%)</td>
</tr>
<tr>
<td></td>
<td>p30</td>
<td>23 of 52 (44%)</td>
</tr>
<tr>
<td>p.DOM</td>
<td>HA-1 or HA-2</td>
<td>0 of 26 (0%)</td>
</tr>
<tr>
<td></td>
<td>p30</td>
<td>12 of 26 (46%)</td>
</tr>
</tbody>
</table>

NOTE: HHD mice were vaccinated with p.DOM-HA-1, p.DOM-HA-2, or the control vaccine (p.DOM). To monitor immune priming, mice were vaccinated at day 0 only, with splenocytes harvested at days 15 to 17 (20); to monitor the potential to boost existing T-cell responses, mice were vaccinated at day 0 with DNA alone, then boosted at day 28 with DNA plus electroporation (23), with spleens harvested at day 36. T-cell responses were measured directly *ex vivo* by ELISpot assay. Splenocytes were incubated with the HLA-A*0201-restricted HA-1 or HA-2 peptides for 24 hours to assess CD8+ T-cell responses; the p30 peptide (derived from the fragment C fusion domain, DOM) was used to assess vaccine-specific CD4+ T-cell responses. The number of mice demonstrating peptide-specific effector T-lymphocyte priming out of the total mice tested is shown, with the percentage responding in parentheses. Peptide-specific ELISpot responses of >50 spot-forming cells per 10⁶ splenocytes and greater than twice baseline values observed in the absence of peptide were considered positive.
the problem of epitope competition, especially at the point of boosting (28). Thus, it may be more appropriate to administer multiple vaccines, each encoding a single target CTL epitope. For infectious diseases, this may be achieved by peptide vaccination (27). However, this approach is less appealing for cancer therapy due to the poor immunogenicity of many peptides derived from tumor-associated antigens, necessitating their delivery with adjuvant and/or the manipulation of "anchor" residues to improve MHC class I binding or T-cell recognition (29, 30). Vaccination with native tumor-associated antigen–derived peptide has generally yielded disappointing results and, in our experience, does not compare favorably with the p.DOM-peptide DNA vaccine design (data not shown). Gene-based vaccines also permit the simple insertion of DNA encoding "additive" molecules to amplify or direct immune outcome.

The p.DOM-peptide vaccine design is very effective at inducing antigen-specific CD8+ T cells in normal mice, with typically 75% to 100% of animals responding to priming alone (20, 25), and it can even mobilize T cells remaining within a profoundly tolerantized polyclonal repertoire (20). The lower response rates generally observed in vaccinated HHD transgenic mice following priming injections probably reflect the reduced cell-surface expression of transgenic HHD molecules in these animals, in addition to a reduced peripheral CD8+ T-cell count (~5-fold) compared with normal mice (21). However, although defective in certain ways, the HHD mice represent an intermediate step between testing vaccine design and clinical application.

The modest response rate observed against the HLA-A2-restricted HA-2 epitope might also result from expression of a mouse orthologue of HA-2. The protein sequence of mouse myosin IG shows 91% identity to that of human myosin IG. The human HA-2 epitope is completely identical to amino acid residues 47 to 55 of mouse myosin IG (accession no. NP_488534). In fact, human HA-2 specific T cells recognize HLA-A2/Kb-transduced mouse hematopoietic cells, showing that the mouse HA-2 epitope is processed and presented in the context of HLA-A2/Kb.4 In contrast, the human HA-1 epitope V1.HDDLEA differs from the nonamer in the mouse, which has amino acid sequence V1.QDIDDLEA.5 Consequently, human HA-1-specific T cells do not recognize HLA-A2/Kb-transduced mouse hematopoietic cells. The contrasting sequence identity of each minor H antigen epitope between human and mouse and their expression in murine tissues no doubt influence the circulating repertoire of CD8+ T cells reactive against each epitope. This may account for the disparity in efficacy between p.DOM-HA-1 and p.DOM-HA-2 at inducing minor H antigen–specific CD8+ T cells in HLA-A2-transgenic HHD mice.

Although this study establishes that our DNA fusion vaccine strategy can successfully induce HLA-A2.1-restricted CTL against human tumor-specific epitopes, clinical success depends on the translation of observations in transgenic mice to human subjects. However, the efficacy of DNA vaccine performance in humans has been questioned (31, 32). This is partly due to dose and volume of DNA, which are difficult to scale up for human application (14). Physical strategies to overcome these problems, including electroporation or microparticle formulation, show promise and are moving into the clinic (33, 34). Another option is the prime/boost approach wherein priming with DNA is followed by boosting with an anti-HLA-A2 antibody or an isotype control, as indicated. Columns, mean specific lysis pooled from individual mice (n = 3); bars, SE. Groups were compared using the unpaired t test.

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Targeting of donor/patients with a minor H antigen mismatch would be optimal for immune induction, avoiding the issue of tolerance in the donor. The number of donor/recipient pairs differing appropriately at either of these polymorphisms is small but ~10% to 15% of transplant pairs would be eligible for vaccination against HA-1, with perhaps 5% of pairs being suitable for HA-2 immunization. However, these numbers would certainly be feasible for a small clinical trial to show proof of principle. The safety of DNA vaccination per se seems to be ensured; indeed, there should be no risk to the donor and the only side effect of our approach would be to enhance preexisting immunity against *Clostridia tetani*, as is obtained by a boost of the conventional tetanus toxoid vaccine. These peptide-specific vaccines will induce a focused CTL response in donors, but because multiparous women remain healthy in spite of apparent priming against minor H antigens from the fetus (38, 39), immune side effects again are unlikely (40). An alternative or additional approach would be to vaccinate patients either before or following donor lymphocyte infusion. As graft-versus-host disease is mediated by an allogeneic immune response against minor histocompatibility antigens, there is no theoretical risk of increased graft-versus-host disease after T-cell infusion because transfer would take place in the context of donor lymphocyte infusion when the recipient antigen-presenting cells have been largely replaced by donor antigen-presenting cells (41–43).

In summary, DNA fusion vaccines can induce effective HLA-A*0201-restricted CTL against human minor H antigens in HHD transgenic mice. To reproduce this in transplant donors is the next goal, and combination with electroporation or microparticle formulation should be safe and testable. Preexisting immunity against tetanus toxoid has no effect on induction of CTL by this strategy (18). In addition, once primed via harnessing of CD4+ T cells from the large antimicrobial repertoire, CTL seem not to require further CD4+ stimulation via fragment C, and expansion with peptide *in vitro*, if required, is straightforward (18, 20). Alternatively, expansion *in vivo* in patients is likely on transfer and can be boosted by vaccination on a continuing basis (44). Patients with other HA-1-expressing tumors, including myeloma (45, 46) and some epithelial cancers (47), could also be vaccinated. Thus, DNA fusion vaccines offer a novel approach to induce alloreactive CD8+ T-cell responses which are capable of mediating graft-versus-leukemia responses in the stem-cell transplant setting.

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Figure 4. DNA fusion vaccines induce high-affinity HLA-A*0201-restricted CTL able to lyse human lymphoblastoid cell lines expressing endogenous antigen. HHD mice were vaccinated with either p.DOM-HA-1 (A) or p.DOM-HA-2 (B). At day 14, splenocytes were harvested and used to establish HA-1-specific (A) or HA-2-specific (B) CTL cell lines *in vitro*; cells were stimulated with 1 μmol/L of relevant peptide for 7 days, then weekly with irradiated, peptide-pulsed female splenocytes for 2 weeks, before measuring CTL activity by 51Cr-release assay. Targets included T2 cells pulsed with HA-1 or HA-2 peptides or human HLA-A*0201* EBV-transformed lymphoblastoid cell lines (EBV-LCL) that were either positive or negative for endogenous expression of HA-1 or HA-2, as indicated. Points, mean of each sample in triplicate; bars, SE. Representative data from one of two CTL lines, each giving similar results.

One solution may be our recent demonstration that the priming qualities of DNA fusion vaccines, integrated with the improved antigen expression offered by electroporation, can be combined in a novel homologous prime/boost approach to generate superior antitumor immune responses, including CTL induction (23). Therefore, boosting may not require viral vectors but simply a physical change in vaccine delivery, facilitating application to the cancer clinic. Using this approach, we have shown that the frequency of HHD mice responding to our DNA vaccines can be significantly improved, even in the setting of immunologic tolerance, with HA-2-specific CD8+ T cells detected *ex vivo* in 42% of HHD mice vaccinated with p.DOM-HA-2. Electroporation devices are now acceptable for either positive or negative for endogenous expression of HA-1 or HA-2, as indicated. Points, mean of each sample in triplicate; bars, SE. Representative data from one of two CTL lines, each giving similar results.

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