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Minor Antigen Vaccine-Sensitized DLI: In Vitro Responses Do Not Predict In Vivo Effects

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Background. We reported on a pilot study of minor histocompatibility antigen vaccination using constructs expressing male-specific gene disparities of selected mouse CDNA on Y and sex determining region Y in the canine model. We performed reduced-intensity hematopoietic cell transplantation with female donors and male recipients, producing stable mixed donor-recipient hematopoietic chimeras. We then performed a vaccine series in three female transplant donors followed by donor lymphocyte infusion (DLI) into their respective mixed chimeras. One mixed chimera experienced a significant shift in the percentage of donor chimerism, but no response occurred in the other 2 recipients. We then hypothesized that inadequate donor sensitization was responsible for these results. **Methods.** To test this hypothesis, we added 4 monthly booster vaccinations to 2 of the original hematopoietic cell transplantation donors, including the donor that drove the partial response, followed by a second DLI. **Results.** Strong T cell responses were shown by ELISpot and confirmed by intracellular cytokine staining in both donors. A second DLI resulted in a further increase in donor chimerism in the same mixed chimera that experienced the previous increase, but no change in donor chimerism was again seen in the other recipient. Evaluation of RNA expression of the target antigens demonstrated that conversion occurred in the recipient that expressed both selected mouse CDNA on Y and sex determining region Y. **Conclusions.** T cell responses against Y chromosome-encoded disparities were not necessarily sufficient to drive in vivo female antimale responses. Other factors including the presence of specific haplotypes or the heterogeneous expression of the target antigen may affect T cell responses against minor histocompatibility antigens. These results warrant future vaccine studies in a larger transplant cohort using epigenetic modulation of the recipient to promote target gene expression.

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In the major histocompatibility complex (MHC)-matched hematopoietic cell transplantation (HCT) setting, minor histocompatibility antigens (miHAs) are implicated in curative graft-versus-tumor (GVT) responses for patients with hematologic malignancies, as well as the morbidity of graft rejection and graft-versus-host disease (GVHD). Minor histocompatibility antigens are MHC class I- and class II-presented endogenous peptides derived from nonsynonymous disparities within coding regions between the donor and recipient. These include unique Y chromosome disparities (H-Y) in female into male HCT. Genetic disparities that give rise to miHAs including H-Y are only antigenic when presented in the context of specific MHC molecules, a requirement termed HLA-restricted and dog leukocyte antigen (DLA)-restricted, in humans and dogs, respectively. Tissue-selective expression of miHAs suggests that it may be possible to augment and separate GVT responses from GVHD using a miHA vaccine.[1] Although some miHAs are known in humans, formidable obstacles of efficacy, safety, and feasibility currently prevent the translation of our knowledge of miHAs into an established immunotherapy.[2]

We seek to establish a recombinant miHA vaccine in the canine model of allogeneic HCT to provide a large outbred

animal model capable of addressing the challenges faced in implementing a miHA vaccine in human allogeneic HCT. With minimum-intensity conditioning, DLA-identical marrow infusion, and a short course of postgrafting immunosuppression, the canine model produces stable mixed donor-recipient hematopoietic chimeras.[3] This mixed chimerism is a state of tolerance between donor and recipient cells and is not affected by "unsensitized" donor lymphocyte infusions (DLIs).[4,5] However, if the donor is first sensitized to miHAs via recipient-derived skin implants, organ transplantation, or injections of allogeneic peripheral blood mononuclear cells (PBMCs), then a sensitized DLI breaks tolerance resulting in full donor chimerism that is often accompanied by GVHD.[4-7] Thus, stable mixed chimerism provides a reproducible in vivo model to test donor T cell sensitization against recipient miHAs. A graphic reproduction of published results on chimerism analyses after unsensitized and miHA-sensitized DLI into DLA-identical mixed chimeras is provided in Figure 1 as a reference to interpret the results of this pilot study.[4]

A major challenge facing the development of a recombinant miHA vaccine in the canine model is the lack of characterized miHAs. T cell cloning reagents used to characterize miHAs in humans are not yet available in the canine model. Instead, we postulated that making a vaccine encoding large sections of Y chromosome gene disparities may overcome the lack of peptide-level characterization of miHAs in the canine model and allow us to further develop this model through the use of female transplant donors and male transplant recipients.

At the time of vaccine development, the canine genome had only 3 Y chromosome gene sequences available including ubiquitously transcribed tetratricopeptide repeat containing, Y-linked (UTY), selected mouse CDNA on Y (SMCY), and sex determining region Y (SRY). Attempts were made to clone the most disparate sections with respect to their X homologues of the large genes UTY and SMCY, as well as the entire small *SRY* gene. After 2 cloning attempts, 3 domains of canine SMCY encoding approximately 60% of the total disparities with the X-homologue SMCX were cloned, as well as the entire *SRY* gene. These clones were shuttled into DNA expression plasmids and replication-deficient human adenovirus type 5 (rAd5) vectors. Three female HCT donors then received 2 doses of the expression plasmids delivered by particle-mediated epidermal delivery (PMED) 4 weeks apart, and then 4 weeks later received an intramuscular boost of rAd5. Four weeks after the rAd5 boost injection, a DLI into their respective male mixed hematopoietic chimeric recipients resulted in a significant increase in donor chimerism in 1 of 3 hosts, representing the first functional miHA response to a recombinant miHA vaccine in a large animal model.[8]

In our initial vaccine series, we observed relatively weak antigen-specific T cell responses at the time of the first DLI. We hypothesized that "inadequate donor sensitization" may explain why the observed increase in donor chimerism did not reach full donor chimerism and explain the lack of change in donor chimerism in the other 2 recipients. Because 2 of the original donor-recipient transplant pairs were still available, including the pair whose recipient demonstrated an increase in donor chimerism after the first DLI, we were able to address this hypothesis with 4 additional PMED boost injections in the 2 female donors followed by a second DLI into their respective male mixed hematopoietic chimeric recipients.

MATERIALS AND METHODS

Experimental Animals, DLA Typing, DLA-Identical HCT, and Chimerism Analysis

The donor-recipient HCT pairs in this study were H353-H519 and H592-H597. These dogs were mini-mongrel cross breeds that were raised at the Fred Hutchinson Cancer Research Center, Seattle, WA, and housed in kennels certified by the American Association for Accreditation of Laboratory Animal Care. All study designs were approved by Institutional Animal Care and Use Committee. The DLA typing method was previously described[9-11] and reported.[8] The DLA typing for H353-H519 included DRB1 9/22 and DLA-88 03801/50101, whereas typing for H592-H597 was DRB1 9/15 and DLA-88 01101/01201. Minimal-intensity DLA-identical HCT was previously described.[3] Chimerism analyses were previously described.[12]

Vaccine Preparation, Administration, and DLI

The amino acid sequence of the 4 expression plasmids was previously reported.[8] The 4 plasmids were mixed at equal concentrations, and 2 μg of plasmid DNA was coated onto 0.5 mg of 1 micron gold particles per cartridge, as described.[13] The dogs underwent general anesthesia. Eight cartridges were then delivered to each side of the abdomen lateral to the mammary gland using the PMED device, Powderject XR1, set between 400 to 500 psi, as previously reported.[8] The protocol for DLI was previously described.[14,15] Cell counts with differentials were obtained using an ADVIA 2120i (Siemens, Deerfield, IL). T cell counts were determined using canine-specific antibody for CD3 as described.[4]
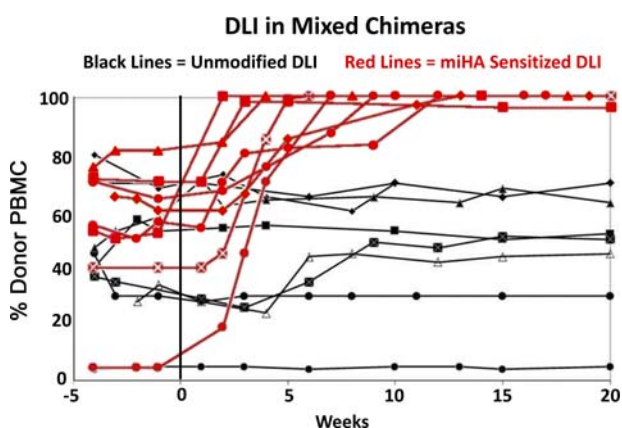
FIGURE 1. Reproduction of published results following unsensitized DLI and miHA-sensitized DLI into eight stable mixed chimeric recipients.[4] The chimerism results were shown as percent donor PBMC on the y axis, with weeks after the DLI shown on the x axis. Eight recipients were infused with unsensitized donor lymphocytes and followed by chimerism analysis (black lines). Eight donors were then sensitized to a miHA via 4 weekly skin grafts from their respective recipients. Eight recipients, 6 of whom had first received an unsensitized DLI, then received a miHA-sensitized DLI 1 week after their respective donor's last skin graft, followed by chimerism analysis (red lines).

Interferon-γ ELISpot, Interferon-γ Intracellular Cytokine Staining, and Peptide Nomenclature

Enzyme-linked immunospot (ELISpots) and intracellular cytokine staining (ICS) were performed as described.[8] The same amount of dimethyl sulfoxide (DMSO) used to dissolve the peptide or peptides was used as a negative control. Phytohemagglutinin at a final concentration of 25 μg/mL was used as a positive control for ELISpots, and phorbol myristate acetate at 100 ng/mL and ionomycin at 1 μg/mL was used as a positive control for ICS. Positive ELISpot responses were defined as greater than 50 spot-forming cells per 10^6 PBMCs on average, with nonoverlapping standard deviations with the DMSO-negative control. Positive ICS responses were based on the percentage of CD3+ CD4+ or CD3+ CD8+ T cells expressing interferon-γ that was determined by placing a gate with an adequate proximity to the DMSO-negative control and maintaining that gate for all samples tested. A positive response was defined as a 4-fold greater response than the DMSO-negative control as long as the DMSO-negative control remained below 0.05%, and the response was greater than or equal to 0.1% interferon-γ–expressing CD3+ CD4+ or CD3+ CD8+ T cells.[16]

The 168 overlapping 15-mer peptide sequences covering the coding sequence of SMCY domain 1 (D1), SMCY domain 2 (D2), SMCY domain 3 (D3), and SRY expression plasmids used in these studies were previously provided.[8] Six overlapping peptides covered SMCY D1 peptide (P) 1 to 6, 40 peptides covered SMCY D2 P1-40, 69 peptides covered SMCY D3 P1-69, and 53 peptides covered SRY P1-53. For the pooled peptide ELISpots and ICS, the 168 peptides were separated into 26 pools that allowed for a 13 × 13 peptide pool matrix as shown in Figures 3A and B.

Reverse Transcription-Polymerase Chain Reaction

RNA was isolated from frozen PBMCs using the RNeasy Plus kit; to maximize genomic DNA elimination, the samples went through the gDNA eliminator with on-column DNase digestion (Qiagen, Venlo, Netherlands). First-strand cDNA synthesis was performed with random hexamers using SuperScript III First-Strand Synthesis System (Thermo Fisher,
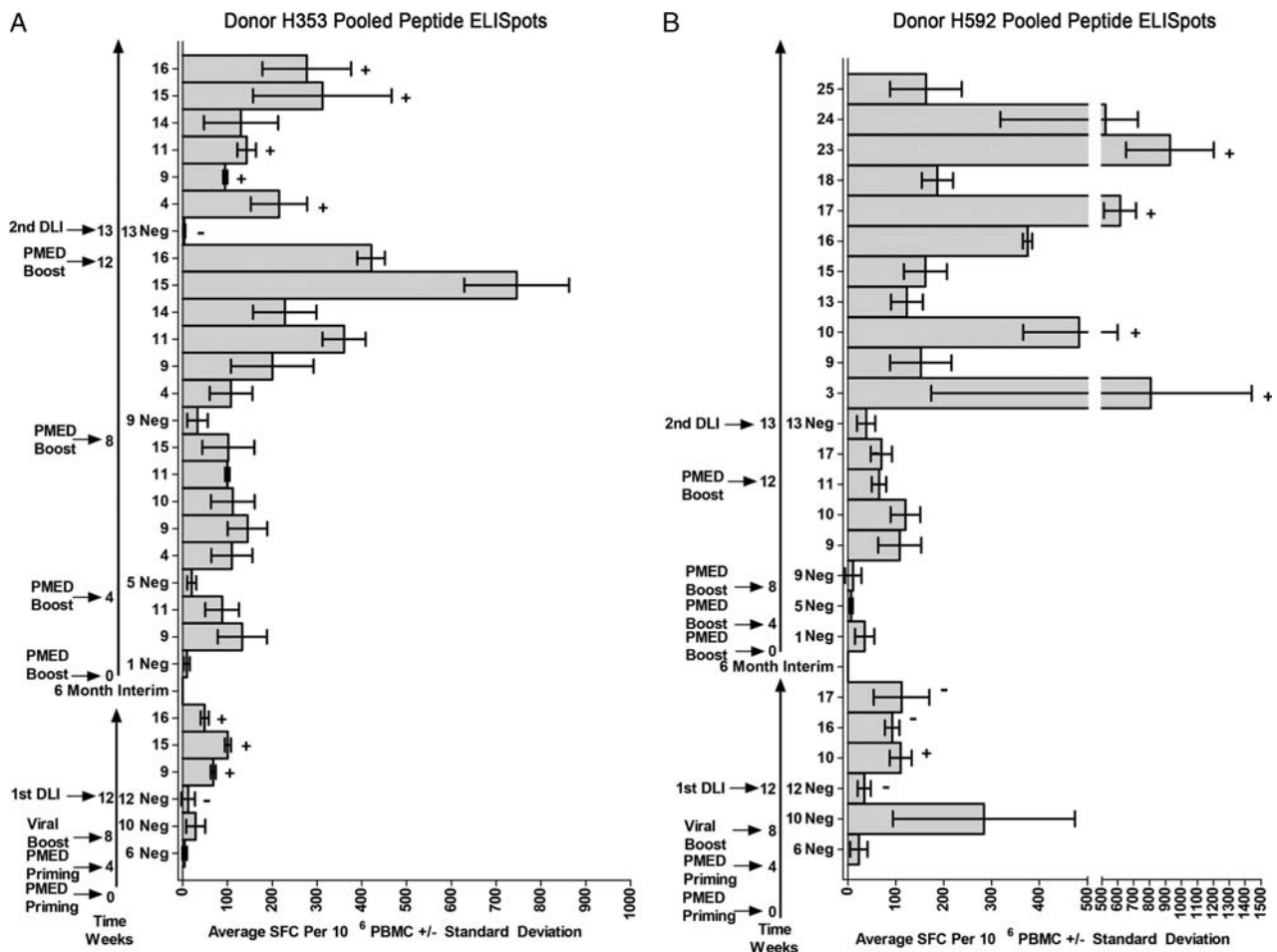
FIGURE 2. Summary of the pooled ELISpot results in the two donors. The entire treatment schema is shown vertically for donors H353 (A) and H592 (B). The 2 long, vertical arrows plot out time in weeks; the numbers to the left indicate the timing of the various rounds of sensitization and subsequent DLIs; the numbers to the right refer to the time at which the ELISpots were performed. The first long vertical arrow represents the first vaccine regimen and first DLI from the previous study, provided as a reference. The second long vertical arrow, following the six-month interim, depicts the four PMED boost sensitizations and the second DLI. The numbers to the right of the 2 long vertical arrows refer to the time at which the ELISpots were performed and were placed next to the DMSO negative (Neg) control for the ELISpot performed at that time point. Only positive pool responses were shown after the DMSO negative control. Specific pooled peptide responses underwent ICS confirmation. A plus sign (+) indicates a positive ICS response and a minus sign (–) indicates a negative ICS response shown in Table 1 and Figure 4.
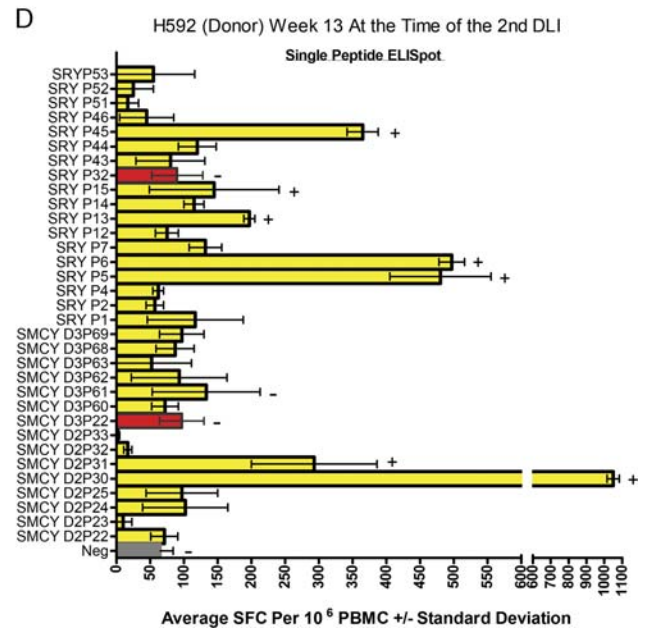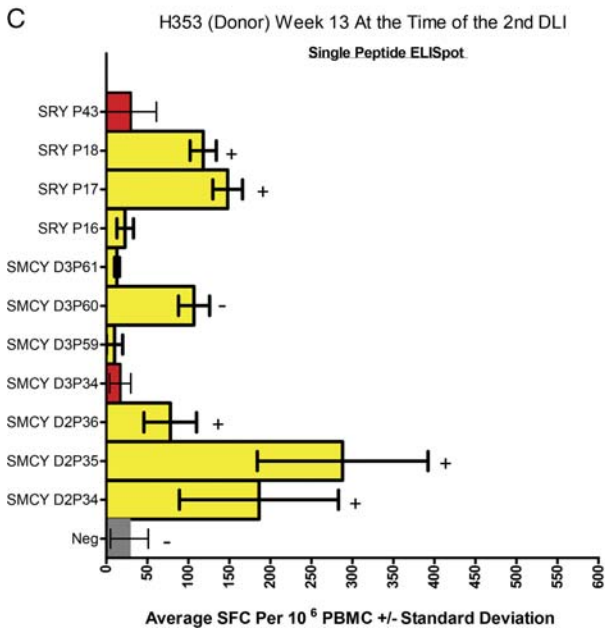
A

Pooled ELISpot Results at the time of the 2nd DLI for H353

Results	Pools	1 (20+/-13)	2 (20+/-20)	3 (15+/-10)	4 (215+/-63)	5 (42+/-33)	6 (15+/-10)	7 (7+/-3)	8 (32+/-12)	9 (95+/-5)	10 (22+/-12)	11 (143+/-21)	12 (47+/-23)	13 (7+/-3)
130+/-83	14	D1 P1	D2 P8	D2 P21	D2 P34	D3 P7	D3 P20	D3 P33	D3 P46	D3 P59	SRY P3	SRY P16	SRY P29	SRY P42
312+/-155	15	D1 P2	D2 P9	D2 P22	D2 P35	D3 P8	D3 P21	D3 P34	D3 P47	D3 P60	SRY P4	SRY P17	SRY P30	SRY P43
277+/-99	16	D1 P3	D2 P10	D2 P23	D2 P36	D3 P9	D3 P22	D3 P35	D3 P48	D3 P61	SRY P5	SRY P18	SRY P31	SRY P44
22+/-16	17	D1 P4	D2 P11	D2 P24	D2 P37	D3 P10	D3 P23	D3 P36	D3 P49	D3 P62	SRY P6	SRY P19	SRY P32	SRY P45
18+/-6	18	D1 P5	D2 P12	D2 P25	D2 P38	D3 P11	D3 P24	D3 P37	D3 P50	D3 P63	SRY P7	SRY P20	SRY P33	SRY P46
10+/-5	19	D1 P6	D2 P13	D2 P26	D2 P39	D3 P12	D3 P25	D3 P38	D3 P51	D3 P64	SRY P8	SRY P21	SRY P34	SRY P47
13+/-10	20	D2 P1	D2 P14	D2 P27	D2 P40	D3 P13	D3 P26	D3 P39	D3 P52	D3 P65	SRY P9	SRY P22	SRY P35	SRY P48
10+/-5	21	D2 P2	D2 P15	D2 P28	D3 P1	D3 P14	D3 P27	D3 P40	D3 P53	D3 P66	SRY P10	SRY P23	SRY P36	SRY P49
7+/-3	22	D2 P3	D2 P16	D2 P29	D3 P2	D3 P15	D3 P28	D3 P41	D3 P54	D3 P67	SRY P11	SRY P24	SRY P37	SRY P50
10+/-8	23	D2 P4	D2 P17	D2 P30	D3 P3	D3 P16	D3 P29	D3 P42	D3 P55	D3 P68	SRY P12	SRY P25	SRY P38	SRY P51
10+/-5	24	D2 P5	D2 P18	D2 P31	D3 P4	D3 P17	D3 P30	D3 P43	D3 P56	D3 P69	SRY P13	SRY P26	SRY P39	SRY P52
17+/-20	25	D2 P6	D2 P19	D2 P32	D3 P5	D3 P18	D3 P31	D3 P44	D3 P57	SRY P1	SRY P14	SRY P27	SRY P40	SRY P53
12+/-13	26	D2 P7	D2 P20	D2 P33	D3 P6	D3 P19	D3 P32	D3 P45	D3 P58	SRY P2	SRY P15	SRY P28	SRY P41	
3+/-3	Negative													
2728+/-207	PHA													

Orange = Single Positive Pool   Yellow = Double Positive Peptides   Red = A Single Positive is Likely Negative

B

Pooled ELISpot Results at the time of the 2nd DLI for H592

Results	Pools	1 (40+/-30)	2 (32+/-26)	3 (808+/-634)	4 (3)	5 (43+/-32)	6 (10+/-5)	7 (88+/-31)	8 (52+/-14)	9 (152+/-64)	10 (483+/-117)	11 (68+/-36)	12 (23+/-28)	13 (123+/-33)
75+/-27	14	D1 P1	D2 P8	D2 P21	D2 P34	D3 P7	D3 P20	D3 P33	D3 P46	D3 P59	SRY P3	SRY P16	SRY P29	SRY P42
162+/-45	15	D1 P2	D2 P9	D2 P22	D2 P35	D3 P8	D3 P21	D3 P34	D3 P47	D3 P60	SRY P4	SRY P17	SRY P30	SRY P43
375+/-10	16	D1 P3	D2 P10	D2 P23	D2 P36	D3 P9	D3 P22	D3 P35	D3 P48	D3 P61	SRY P5	SRY P18	SRY P31	SRY P44
615+/-101	17	D1 P4	D2 P11	D2 P24	D2 P37	D3 P10	D3 P23	D3 P36	D3 P49	D3 P62	SRY P6	SRY P19	SRY P32	SRY P45
187+/-33	18	D1 P5	D2 P12	D2 P25	D2 P38	D3 P11	D3 P24	D3 P37	D3 P50	D3 P63	SRY P7	SRY P20	SRY P33	SRY P46
77+/-51	19	D1 P6	D2 P13	D2 P26	D2 P39	D3 P12	D3 P25	D3 P38	D3 P51	D3 P64	SRY P8	SRY P21	SRY P34	SRY P47
33+/-10	20	D2 P1	D2 P14	D2 P27	D3 P1	D3 P13	D3 P26	D3 P39	D3 P52	D3 P65	SRY P9	SRY P22	SRY P35	SRY P48
40+/-28	21	D2 P2	D2 P15	D2 P28	D3 P1	D3 P14	D3 P27	D3 P40	D3 P53	D3 P66	SRY P10	SRY P23	SRY P36	SRY P49
40+/-13	22	D2 P3	D2 P16	D2 P29	D3 P2	D3 P15	D3 P28	D3 P41	D3 P54	D3 P67	SRY P11	SRY P24	SRY P37	SRY P50
928+/-275	23	D2 P4	D2 P17	D2 P30	D3 P3	D3 P16	D3 P29	D3 P42	D3 P55	D3 P68	SRY P12	SRY P25	SRY P38	SRY P51
523+/-205	24	D2 P5	D2 P18	D2 P31	D3 P4	D3 P17	D3 P30	D3 P43	D3 P56	D3 P69	SRY P13	SRY P26	SRY P39	SRY P52
163+/-75	25	D2 P6	D2 P19	D2 P32	D3 P5	D3 P18	D3 P31	D3 P44	D3 P57	SRY P1	SRY P14	SRY P27	SRY P40	SRY P53
100+/-13	26	D2 P7	D2 P20	D2 P33	D3 P6	D3 P19	D3 P32	D3 P45	D3 P58	SRY P2	SRY P15	SRY P28	SRY P41	
38+/-19	Negative													
1998+/-177	PHA													

Orange = Single Positive Pool   Yellow = Double Positive Peptides   Red = A Single Positive is Likely Negative

C — H353 (Donor) Week 13 At the Time of the 2nd DLI — Single Peptide ELISpot

Peptides (top to bottom): SRY P43, SRY P18 (+), SRY P17 (+), SRY P16, SMCY D3P61, SMCY D3P60 (−), SMCY D3P59, SMCY D3P34, SMCY D2P36 (+), SMCY D2P35 (+), SMCY D2P34 (+), Neg (−)

X axis: Average SFC Per 10⁶ PBMC +/- Standard Deviation (0–600)

D — H592 (Donor) Week 13 At the Time of the 2nd DLI — Single Peptide ELISpot

Peptides (top to bottom): SRYP53, SRY P52, SRY P51, SRY P46, SRY P45 (+), SRY P44, SRY P43, SRY P32 (−), SRY P15 (+), SRY P14, SRY P13 (+), SRY P12, SRY P7, SRY P6 (+), SRY P5 (+), SRY P4, SRY P2, SRY P1, SMCY D3P69, SMCY D3P68, SMCY D3P63, SMCY D3P62, SMCY D3P61 (−), SMCY D3P60, SMCY D3P22 (−), SMCY D2P33, SMCY D2P32, SMCY D2P31 (+), SMCY D2P30 (+), SMCY D2P25, SMCY D2P24, SMCY D2P23, SMCY D2P22, Neg (−)

X axis: Average SFC Per 10⁶ PBMC +/- Standard Deviation (0–1100)

FIGURE 3. Determining single peptide responses from within the pooled ELISpot results in both female donors at the time of the second DLI. Pooled ELISpot results at the time of the second DLI for H353 (A) and H592 (B) were shown in the context of a 13 × 13 overlapping peptide pool matrix with the DMSO-negative and phytohemagglutinin-positive control included as a reference. Each column and each row display the peptides included in a pool. D1, D2, and D3 refer to the 3 domains of SMCY encoded within the vaccine; SMCY was dropped from the spreadsheet for space constraints. The P refers to peptide and the number after the P refers to the overlapping peptide, as discussed in the Materials and Methods section. Results were listed either horizontally or vertically with respect to each pool and expressed as average SFC per 10⁶ PBMCs ± standard deviation. Strongly positive pools were highlighted in orange. Individual peptides found at the intersection of 2 strongly positive pools were highlighted in yellow and underwent single peptide ELISpot confirmation for H353 (C) and H592 (D). Two peptides that overlap a negative and positive pool, thus predicted not to elicit a response, were highlighted in red and included in the single peptide analysis. Specific single peptide responses underwent ICS confirmation. A plus sign (+) indicates a positive ICS response and a minus sign (–) indicates a negative ICS response shown in Table 1 and Figure 4. SFC, spot-forming cell.

Waltham, MA). Polymerase chain reaction (PCR) was performed using heat-activated immomix (Bioline, London, UK). To prevent carry over DNA contamination, 2'-deoxyuridine 5'triphosphate was spiked into each PCR reaction, and all subsequent PCR reactions underwent an initial uracil-DNA glycosylase incubation step (Amresco, Solon, OH). Gene-specific primers were created using sequences from Canis Familiaris GenBank reference sequence DQ156494.1 for SMCY and sequence AF107021.1 for SRY. All the primer sets spanned introns with the exception of the *SRY* gene, which has no introns.

RESULTS

Extra Rounds of Sensitization Produce Strong Pooled Peptide Responses in Both Donors

The entire vaccine schema for the 2 female donors H353 and H592 is shown vertically in Figures 2A and B. The initial vaccine series consisted of priming these donors with 2 monthly rounds of the expression plasmids delivered by PMED, which was followed by a boost from an injection of rAd5 viral vectors encoding the same disparities. Overlapping peptide pools that covered the entire coding sequence of the expression plasmids were used to evaluate responses by interferon-γ ELISpot and ICS.[13] At the end of the initial vaccine series and at the time of the first DLI, only a few peptide pools were weakly positive by ELISpot and able to be confirmed by ICS. No positive responses were seen for individual peptides at the time of the first DLI for either donor (data not shown).

Based on the review of a vaccine series used to validate the expression plasmids by PMED in 2 test females,[8] it was thought that strong ELISpot responses would be detected with 2 extra PMED boosts because these donors were already previously sensitized. Two additional PMED boosts for a total of four injections would then allow an assessment of the number of rounds of sensitizations necessary to reach maximal in vitro responses. Additionally, based on preliminary validation studies, the maximum ELISpot response after PMED boosting was observed 1 week after the injection, and began to decrease after that time (unpublished results). Therefore, ELISpot responses were assessed 1 week after each PMED boost.

At the time of the second DLI into their respective mixed chimeric recipients, multiple strong pooled peptide responses were observed for both female donors. For H353, the in vitro responses peaked after the third PMED boost, but were still increasing after the fourth PMED boost for H592. Sufficient samples were available to confirm a variety of CD4+ and CD8+ T cell responses to many of these pooled peptides in both female donors (Table 1 and Figure 4).

Antigen-Specific Responses to Individual Peptides in Both Donors

The strong pooled peptide results suggested that it would be possible to detect in vitro responses to individual peptides. From the onset of these studies, the 26 peptide pools were arranged into a 13 × 13 matrix, shown in a spreadsheet format for the pooled peptide ELISpot performed at the time of the second DLI for both female donors in Figures 3A and B. The matrix allows for a quick determination of candidate peptides that were contributing to the responses seen in the

pooled ELISpot and ICS assays. Strongly positive pools were highlighted in orange. Individual peptides at the intersection of 2 positive pools were highlighted in yellow and underwent a single peptide ELISpot confirmation (Figures 3C and D). As further validation of the matrix, 2 peptides at the intersection of a positive and negative pool, thus predicted to produce a negative response, were highlighted in red and used as negative controls in the single peptide ELISpot. Many of these ELISpot responses were confirmed by ICS (Table 1 and Figure 4).

A relatively few peptides were responsible for the majority of the peptide pool responses in both donors at the time of the second DLI, and demonstrated that a detectable immune response was generated to a relatively small amount of the encoded disparities. For example, 6 peptides accounted for the majority of the pooled peptide responses for H353. These 6 peptides implicated three small sections within 3 of the 4 expression plasmids used in the vaccine including SMCY domain 2 overlapping peptides 34 to 36, SMCY domain 3 peptide 60, and SRY overlapping peptides 17 and 18. The ICS showed a predominantly CD4+ response to SMCY domain 2 overlapping peptides 35 to 36, but a detectable CD8+ T cell response was also shown for SMCY domain 2 peptides 34 and 36. The ICS response for SMCY domain 3 peptide 60 did not reach significance. Finally, SRY peptides 17 and 18 both demonstrated a CD4+ response, whereas a CD8+ response was also shown for SRY peptide 18. In a similar fashion, the majority of peptide pool responses for H592 were attributable to small sections of the encoded disparity. Using the 13 × 13 matrix, 31 yellow highlighted peptides were tested. Single peptide ELISpot implicated SMCY domain 2 overlapping peptides 30 to 31, and several SRY covering peptides. The ICS confirmed that these peptides induced various CD4+ and CD8+ T cell responses. In summary, CD4+ and CD8+ T cell responses were shown against sections of SMCY and SRY at the time of the second DLI for both donors.

Chimerism and Evaluation of GVHD After Vaccine-Sensitized DLI

After the first DLI, H519 experienced an increase in donor chimerism, donor PBMC increased from 50% to 70%, and donor granulocytes increased from 30% to 90% (red lines, Figures 5A and B); no change in the percent donor chimerism occurred in the other 2 recipients H597 and H382. In the second DLI, H519 received 0.4×10^8 CD3+ T cells/kg and H597 received 0.34×10^8 CD3+ T cells/kg. H519 experienced a further increase in donor chimerism with donor PBMC increasing from 70% to 90%, and donor granulocytes increased from 90% to 100% (green lines, Figures 5A and B). No change in donor chimerism was again observed for H597. No clinical or pathologic evidence of GVHD was seen in either H519 or H597 at necropsy.

Assessing the Immune Responses in the 2 Mixed Chimeric Recipients

A major goal of this study was to isolate a T cell response to an individual peptide in a recipient that underwent an increase in donor chimerism after a vaccine-sensitized DLI as evidence of peptide-level characterization of miHA. No positive peptide pool responses were seen in any of the 3

TABLE 1.

Summary of the intracellular cytokine staining results[a]

Dog	Corresponding ELISpot figure	Time point, wk	Condition	CD3 + CD4 + %IFN-γ	CD3 + CD8 + %IFN-γ
H353	2A	12 (First DLI)	DMSO negative	0.03	0.05
			PMA/ionomycin	1.5	2.18
			Pool 9	0.11	0.04
			Pool 15	0.11	0.04
			Pool 16	0	0.37
	2A	13 (Second DLI)	DMSO negative	0.01	0.01
			PMA/ionomycin	21.2	35.1
			Pool 4	0.1	0.12
			Pool 9	0.17	0.02
			Pool 11	0.17	0.04
			Pool 14	0.11	0.13
			Pool 15	0.31	0.51
			Pool 16	0.06	0.14
	3C	13 (Second DLI)	SMCY D2P34	0.04	0.1
			SMCY D2P35	0.13	0.06
			SMCY D2P36	0.21	0.1
			SMCY D3P60	0.01	0.07
			SRY P17	0.17	0.07
			SRY P18	0.14	0.18
H592	2B	12 (First DLI)	DMSO negative	0.02	0
			PMA/ionomycin	1.24	4.62
			Pool 10	0.03	0.13
			Pool 16	0.02	0.02
			Pool 17	0.04	0.05
H592	2B	13 (Second DLI)	DMSO negative	0.02	0.03
			PMA/ionomycin	10.7	27.6
			Pool 3	0.22	0.65
			Pool 10	0.27	0.65
			Pool 17	0.19	0.47
			Pool 23	1.16	3.94
	3D	13 (Second DLI)	SMCY D2P30	0.57	1.50
			SMCY D2P31	0.01	0.46
			SMCY D3P22	0.02	0.04
			SMCY D3P61	0.02	0.05
			SRY P5	0.5	0.53
			SRY P6	0.47	1.00
			SRY P13	0.28	0.4
			SRY P15	0.45	0.82
			SRY P32	0.05	0.04
			SRY P45	0.55	0.46
H519	6	13 (After first DLI)	DMSO negative	0.01	0
			Pool 15	0.19	0.07
			Pool 16	0.05	0.2
H519	6	1 (After second DLI)	DMSO negative	0.01	0.02
			PMA/ionomycin	10.1	18.9
			Pool 9	0.06	0.46
			Pool 11	0.08	0
			Pool 12	0.04	0.09
			Pool 15	0.19	0.06

TABLE 1. (Continued)

| Dog	Corresponding ELISpot figure	Time point, wk	Condition	CD3 + CD4 + %IFN-γ	CD3 + CD8 + %IFN-γ
	No figure	1 (After second DLI)	SMCY D2P35	0.07	0.09
			SMCY D3P60	0.01	0.14
			SRY P17	0	0.65
			SRY P18	0.07	0.55

[a] The actual flow cytometry plots for the underlined percentages are provided in Figure 4.
PMA, phorbol myristate acetate.

recipients 1 week and 5 weeks after the first DLI. However, after H519 experienced an increase in donor chimerism, an ELISpot performed 13 weeks after the DLI was positive for pools 15 and 16 (Figure 6). These responses were confirmed by ICS with pool 15 producing a CD4+ T cell response and pool 16 producing a CD8+ response (Table 1). No positive responses were seen for single peptides from within pools 15 and 16 by ELISpot after 2 attempts (data not shown).

The second vaccine-sensitized DLI provided another opportunity to demonstrate an immune response in a recipient that underwent an increase in donor chimerism. In contrast to the first DLI, responses were evident within 1 week after the second DLI into recipient H519 with positive peptide pools by ELISpot to 9, 11, 12, and 15 (Figure 6). The ICS analysis showed that pool 9 produced a CD8+ response, whereas pool 15 demonstrated a CD4+ response, and pools 11 and 12 were negative (Table 1, Figure 4). Two single peptide ELISpots failed to show a positive response to peptides shown to produce a response in the vaccinated donor H353. An attempt was then made to isolate a response to a single peptide using ICS, because differences exist with respect to response detection between ELISPOT and ICS.[17,18] Assaying these same peptides by ICS showed a CD8+ T cell response for SMCY domain 3 peptide 60, SRY peptide 17, and SRY peptide 18 (Table 1 and Figure 4). Further characterization of the memory or naive T cell subset analysis of these responses awaits the development of that capability in the canine model. By 5 weeks after the second DLI, only pool 11 remained weakly positive by ELISpot, and no positive responses were seen after that time (Figure 6). No ELISpot responses were observed 1 week and 5 weeks after the second DLI for recipient H597 (not shown). In summary, T cells were shown to respond to peptides encoded from within SMCY and SRY in the recipient that experienced an increase in donor chimerism.

RNA Expression of SMCY and SRY

We then asked the question whether the transplant recipients transcribed the target genes in PBMC at the time of the DLI (Figure 7). RNA was isolated from frozen PBMC samples on all the male mixed chimeric recipients involved in the vaccine studies including H382, H597, and H519. The SMCY gene expression was ubiquitously detected, but SRY was only detected in H519, and this expression remained after the second DLI. We sequenced recipient genomic DNA through the regions corresponding to each primer to eliminate the possibility that an unknown polymorphism inhibited amplification of SRY in some animals. The genomic sequences were perfectly
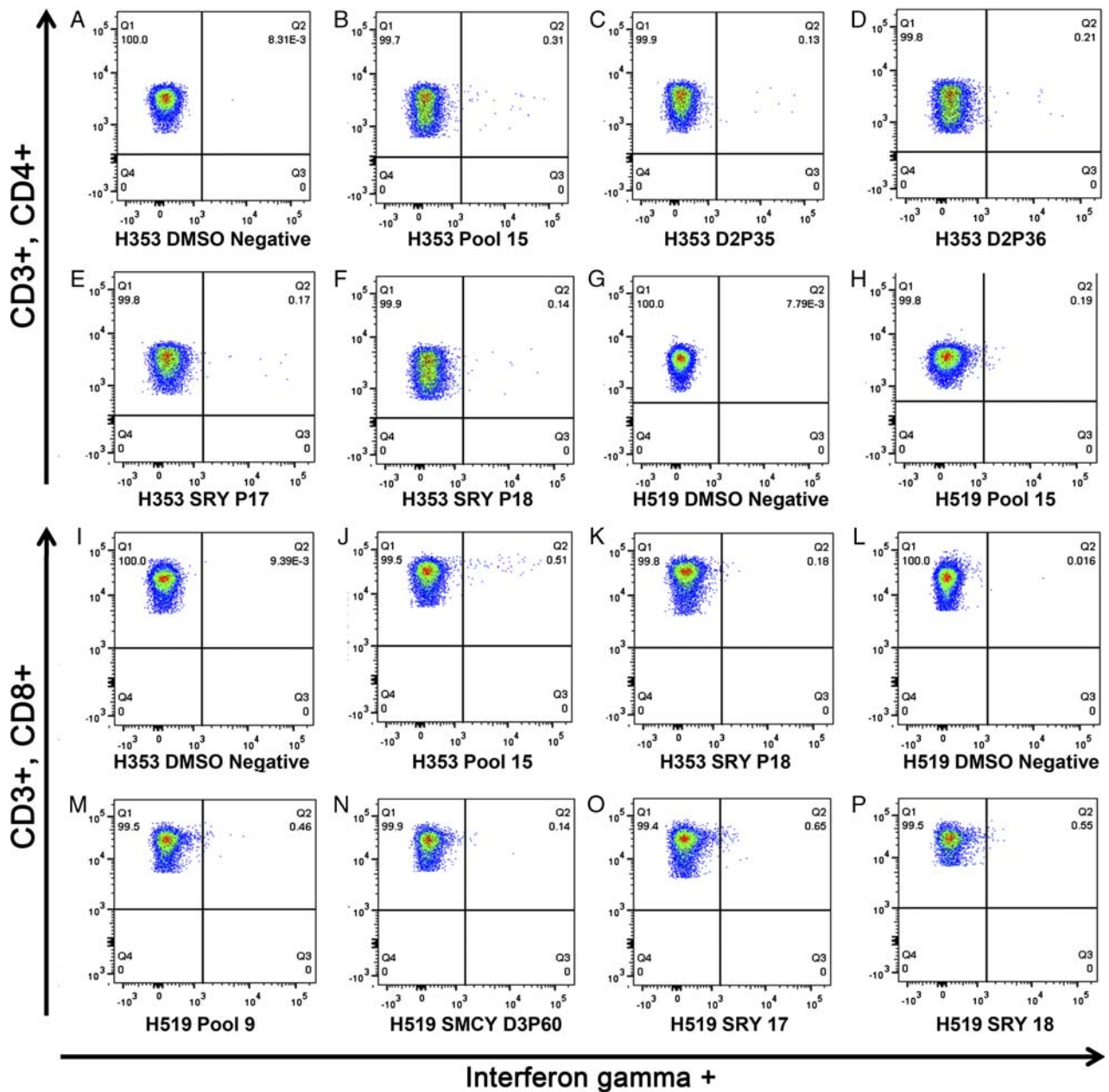
FIGURE 4. Three-color intracellular cytokine staining for interferon-γ. The percentage of interferon-γ CD3 + CD4+ T cells of H353 responding to (A) the DMSO-negative control, (B) pool 15, (C) SMCY D2P35, (D) SMCY D2P36, (E) SRY P17, and (F) SRY P18. The percentage of interferon-γ CD3 + CD4+ T cells of H519 responding to (G) DMSO-negative control, and (H) pool 15. The percentage of interferon-γ CD3 + CD8+ T cells of H353 responding to (I) DMSO-negative control, (J) pool 15, and (K) SRY P18. The percentage of interferon-γ CD3 + CD8+ T cells of H519 responding to (L) DMSO-negative control, (M) pool 9, (N) SMCY D3P60, (O) SRY P17, and (P) SRY P18. The remainder of the data is listed in table format (Table 1).

homologous at all primer positions in all animals, arguing that the observed difference in reverse transcription-PCR between the 2 recipients was due to a difference in expression.

DISCUSSION

This is a second report on a pilot study designed to develop a recombinant miHA vaccine in the canine model of allogeneic HCT. The overall numbers of transplant pairs used in these studies are small, which prevent definitive conclusions. However, important insights into developing immunotherapies targeting genetic disparities can be gleaned

from these studies to address the still unmet clinical goal of boosting GVT responses by targeting specific miHAs.

Our initial vaccine trial demonstrated that adoptive transfer of female donor T cells sensitized with a recombinant vaccine encoding Y chromosome disparities can induce in vivo responses even when the responses are barely detectable by ELISpot and ICS. In this report, we add to our understanding by showing that strong SMCY-specific and SRY-specific CD4+ and CD8+ donor T cell responses did not affect which recipient responded to the vaccine-sensitized DLI. These results are not consistent with an inadequate donor sensitization hypothesis. Instead, future vaccine trials with
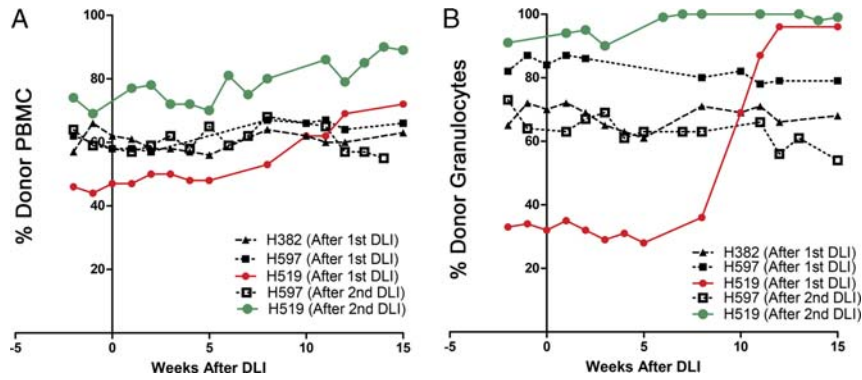
FIGURE 5. The chimerism results were shown as percent donor PBMC (A) and percent donor granulocytes (B) on the *y* axis, with weeks after the DLI shown on the *x* axis. The results for the PMED boosting study were labeled "(After second DLI)", and the chimerism results following the first vaccine study were labeled "(After first DLI)." The chimerism results are shown for recipient H519 after the first DLI (red) and after the second DLI (green).

a larger cohort are necessary to determine whether the observed in vivo response occurred exclusively due to the presence of a specific DLA haplotype or in the setting where recipient cells expressed both SMCY and SRY.

Historical experiments that used skin transplant rejection in congenic mice to map the locus of miHAs provide several insights applicable to the interpretation of our results.[19] First, it is known that not all congenic strains of female mice are able to reject skin grafts from male donors, that is, possess the ability to strongly respond to the genetic disparity encoded within the Y chromosome.[20-23] Instead, the strength, in terms of the rapidity of tissue rejection by H-Y antigens, depends on the presence of specific MHC alleles.[24] Second, tissue rejection or the equivalent in our model of increasing donor chimerism after a vaccine-sensitized DLI likely requires a combination of CD8+ cytotoxic T lymphocytes as well as CD4+ helper T lymphocytes responding to different MHC class I- and class II-restricted epitopes, respectively, in the responding haplotype.[25] Third, it is known that SMCY encodes a miHA in mice and men when presented by specific MHC molecules,[26] hence the justification for cloning sections of SMCY into our vaccine. However, it remains unclear if SMCY encodes a miHA within all of the different MHC haplotypes in outbred populations. Additionally, our vaccine only encodes for 60% of the disparity between SMCX and SMCY; thus a potential miHA encoded within SMCY capable of driving in vivo responses may have been missed. Therefore, it is not surprising that our vaccine-sensitized DLI did not induce an anti-SMCY response capable of inducing an in vivo response in every male recipient, because they all possessed different haplotypes.

What about the anti-SRY immune response? Did it play a role in the increase in donor chimerism after DLI? Several lines of evidence suggest a working hypothesis that the increase in donor chimerism after the vaccine-sensitized DLI required SRY expression in the recipient's hematopoietic
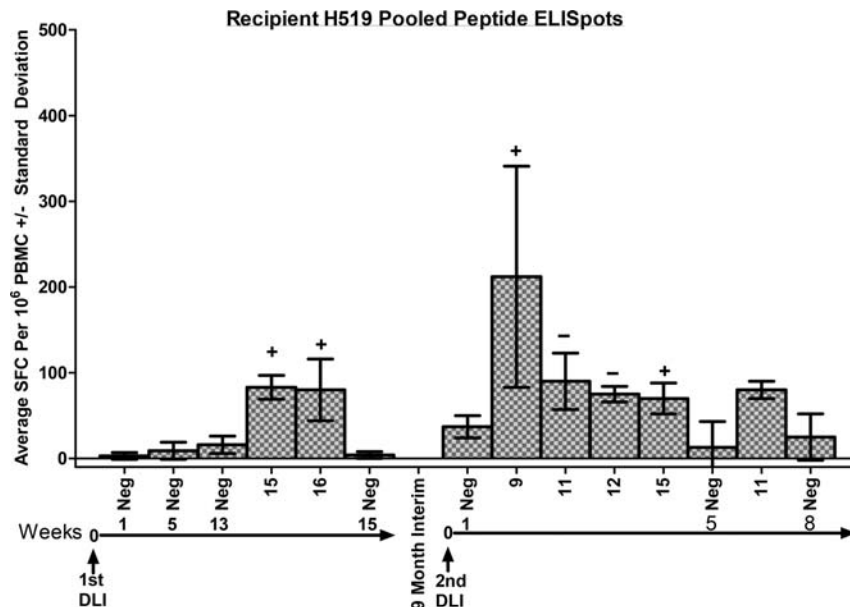
FIGURE 6. Summary of the pooled ELISpot results for recipient H519. The 2 long horizontal arrows represent the time after each DLI, separated by a 9-month interval. The numbers above the arrows refer to the time after the DLI at which the ELISpots were performed and are placed below the DMSO negative (neg) controls for each time point tested. Only positive pools were shown after the DMSO-negative control from that time. Specific pooled peptide responses underwent ICS confirmation. A plus sign (+) indicates a positive ICS response, and a minus sign (–) indicates a negative ICS response shown in Table 1 and Figure 4.
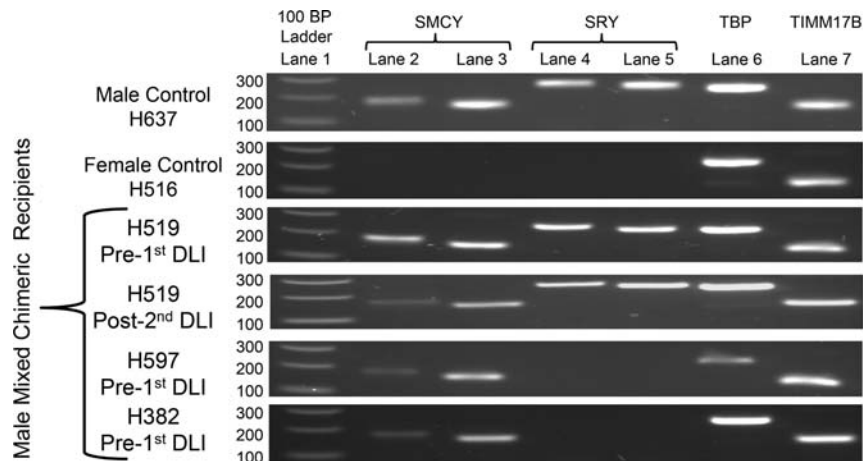
FIGURE 7. Recipient RNA expression of SMCY and SRY by RT-PCR. Lane 1 was loaded with 100 BP DNA ladder. Lanes 2–5 were loaded with reverse transcribed-PCR products from different primer pairs for SMCY and SRY. Lanes 6 and 7 were loaded with RT-PCR products from primer pairs for the housekeeping genes TBP and TIMM17B. Shown were the RT plus (+) samples for a male control, a female control, and the mixed chimeric recipients that underwent DLI. RT minus controls was performed for each sample (not shown). Positive SRY and SMCY bands were verified by sequencing. The following forward and reverse primers were used for SMCY lane 2 (accaactgcaggctgaaacc/agatctgagccctgcacact), SMCY lane 3 (ctgcaggagccctatcattc/agatctgagccctgcacact), SRY lane 4 (agcctcctcctccatgctat/ttagagtaaaaagaagcgtcagcg), SRY lane 5 (ggtaccagtggaaaatgcttacag/cagctgtccgtgtaggtgaa), TBP lane 6 (gaaatgctgaatataatcccaagc/cagctccccaccatgttct), and TIMM17B lane 7 (atcaagggcttccgcaatg/cacagtcgatggtggagaacag).TBP, TATA-box binding protein; TIMM17B, translocase of inner mitochondrial membrane 17 homology B (yeast).

cells. First, both transplant donors showed strong antigen-specific responses to SRY. Second, the transplant recipient that experienced an increase in donor chimerism demonstrated anti-SRY responses and was the only recipient that had SRY expression in their PBMCs. Third, the heterogenous SRY expression in the hematopoietic cells of male dogs provides a plausible explanation for why the responding mixed chimeric recipient did not reach full donor chimerism, that is, a residual population of male cells did not express SRY and were not eliminated. The role that SRY played in these studies cannot be extrapolated from murine studies. The SRY gene does not encode a miHA in mice[27] and, to our knowledge, has not yet been shown to encode a miHA in humans. In mice, SRY expression is tightly regulated by DNA methylation and limited to gonadal tissues with expression that begins to wane by 12.5 days postcoitum, providing a plausible explanation for why SRY does not encode a miHA in mice.[28,29] However, in humans and canines, SRY expression is not limited to the gonads, and the role of SRY beyond testis development has not been determined.[30] These findings require confirmation in a larger cohort and then validation in future vaccine trials by treating the male recipients with a DNA methyltransferase inhibitor, such as 5-azacytidine (Vidaza), to restore SRY expression before vaccine-sensitized DLI.[31]

In conclusion, we developed a recombinant miHA vaccine in the canine model of allogeneic HCT to address the barriers of safety and efficacy currently preventing miHA vaccination in human allogeneic HCT. Future vaccine trials are needed to clarify the role of different DLA alleles and to determine if SRY encodes a miHA in dogs. Once accomplished, this vaccine can then be combined with adjuvants to optimize donor T cell sensitization in the donor, and ultimately devise strategies to directly vaccinate the transplant recipient.

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