Immune Rejection in a Humanized Model of Murine Prostate Cancer

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Abstract. *Background/Aim: We attempted to develop a humanized mouse model for prostate cancer to study immune recognition and responses to human prostate-tumor antigens in mice. Materials and Methods: Our study was based on cell lines derived from transgenic adenocarcinoma of the mouse prostate (TRAMP) tumors. TRAMP cells were modified to express a chimeric MHC I molecule comprising the extracellular domains of human HLA-A2.1 with the transmembrane and intracellular domain of K^b. Results: These modified TRAMP cells were immunologically rejected following recognition of human tumor epitopes known to be immunodominant in the context of HLA-A2.1. Immune-compromised SCID-beige mice did not reject these tumors. Conclusion: We conclude that epitopes derived from endogenous murine homologs were being presented by the chimeric MHC class I molecules due to a lack of central tolerance to these epitopes in the mice.*

Transgenic mice that have been engineered to express HLA molecules have been used extensively to model human MHC-restricted anti-tumor immune responses. Particularly popular are transgenic C57BL/6 mice expressing the HLA-A2 allele that is possessed by about 50% of Caucasians. The first such mouse model expressed chimeric HLA-A2.1/K^b that contains the α1 and α2 domains of the HLA-A2.1 molecule, and the α3 domain of K^b (1), being expressed on cells in association with murine beta 2-microglobulin (β2m). An advantage of this model is that while the T-cell receptor interacts with the MHC class I-peptide complex through the α1 and α2 domains of the MHC molecule, murine CD8 co-receptors can interact strongly with a non-polymorphic region within the murine α3 domains (2-4). Immunization of these mice has been shown to induce HLA-A2-restricted $CD8^+$ T-cell responses to relevant human tumor antigenic peptides indicating that the immune system can function to process and present the appropriate HLA-restricted peptides, as can murine tumor cells transduced with the same construct.

Here we sought to extend this humanized murine system to model immune responses to human prostate tumor epitopes of potential clinical relevance. TRAMP C1 and TRAMP C2 mouse prostate cell lines, which were originally derived from the transgenic adenocarcinoma of the mouse prostate (TRAMP) mice that spontaneously develop prostate cancer and closely mimic the development of the human disease (5), were humanized with a lentivirus carrying the chimeric HLA-A2.1/K^b gene (TRAMP C/K^b2.1). Surprisingly, we found that the humanized TRAMP tumor cells were rejected by humanized, immune-competent C57Bl/6-K^b2.1 mice, which had developed immunity directed against known human prostate tumor antigenic epitopes, but they were not rejected by immune-deficient mice.

Materials and Methods

Mice and cell lines. Female or male 6- to 8-week-old C57Bl/6 (K^b), transgenic C57Bl/6-K^b 2.1, or SCID/Beige mice were bred and maintained in a defined-flora, pathogen-free environment in the American Association of Laboratory Animal Care (AALAC)-accredited Animal Facility of the Department of Radiation Oncology, University of California at Los Angeles. Experiments adhered to all local and national animal care guidelines.

TRAMP C1 and TRAMP C2 cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in DMEM (Mediatech, Herndon, VA, USA) with 10% FBS (Omega Scientific, Tarzana, CA, USA), 10,000 IU penicillin, 10,000 μg/ml streptomycin, 25 μg/ml amphotericin (Mediatech), 5 μg/ml insulin and 10^{-8} M dihydrotestosterone (Sigma-Aldrich, St.Louis, MO, USA). Freezing media contained 10% DMSO (Sigma-Aldrich) in FBS. To generate transgenic humanized TRAMP cells, the parental TRAMP C1 or TRAMP C2 were seeded at 100,000 cells/well in a 6-well plate, left to adhere and spin-inoculated twice on two consecutive days with a lentivirus construct carrying the transgene to express the α1 and α2 domains of the human HLA-A2.1

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fused to the murine α3 domain K^b under the control of the CMV promoter. These transgenic TRAMP C1/K^b2.1 and TRAMP C2/K^b2.1 cells were then cultured as above without additional selection pressure. Mice were inoculated *s.c.* on the outer right thigh with single-cell suspensions of cells in PBS from *in vitro* culture or from freshly harvested TRAMP tumor cells. For *in vivo* to *in vivo* injection tumors were harvested from animals and digested with collagenase D (Roche) and DNAse 1 (Type IV; Sigma), and clumps removed by filtration prior to re-injection.

Flow cytometry. TRAMP cells were harvested with trypsin and re-suspended in normal goat serum. When indicated, splenocytes were depleted of red blood cells by ACK treatment (Lonza, Walkersville, MD, USA) and re-suspended as a single cell suspension in normal goat serum. A total of 1×10^6 tumor cells or splenocytes were stained for transgene expression with the FITC anti-human HLA-A2 antibody (clone BB7.2, BD Pharmingen) for 30 mins on ice. The parental murine MHC I was detected with PE anti-mouse H2-K^b (clone AF6-88.5, BD Pharmingen). Flow cytometric analysis was performed on 10,000-20,000 events using a FACS Calibur system (BD Biosciences, Mountain View, CA, USA).

IFNγ-ELISPOT. IFNγ-ELISPOT was used to assess the prostate-specific immune response in C57Bl/6-K^b2.1 mice to TRAMP C1/K^b2.1 cells. Male mice were immunized *s.c.* twice, at 5-day intervals, with either 2×10^6 viable or lethally-irradiated (20 Gy) TRAMP C1/K^b2.1 cells. Splenocytes were harvested one week after the second injection and were depleted of red blood cells with ACK treatment (Lonza). 15×10^6/well splenocytes were re-stimulated in 6-well plates at 2.5×10^6/ml in RPMI (Mediatech) with 10% FBS (Omega Scientific), 10,000IU penicillin, 10,000 μg/ml streptomycin, 25 μg/ml amphotericin (Mediatech) in the presence of hIL-2 at 10 U/ml. Re-stimulation was with prostate tumor-specific HLA-A2.1 antigenic peptides, namely six-transmembrane epithelial antigen of the prostate ($STEAP_{86-94}$, FLYTLLREV) and prostate-specific membrane antigen ($PSMA_{27}$, VLAGGFFLL) and survivin (SUR1M2, LMLGEFLKL) dissolved in DMSO with final DMSO concentrations not exceeding 0.1%. Re-stimulated splenocytes were harvested after 48h incubation at 37˚C into X-VIVO 10 medium (Lonza) with the peptides, transferred to MultiScreen-HA plates (Millipore, Bedford, MA, USA) pre-coated with anti-mouse IFNγ antibody (BD Pharmingen) at 1×10^5 cells/well and incubated for an additional 24h at 37˚C. Plates were developed with biotinylated anti-IFNγ antibody (BD Pharmingen), HRP avidin D (Vector Laboratories, Burlingame, CA) and 0.05 M sodium acetate buffer (pH 5.0) containing 0.4mg/ml 3-amino-9-ethyl-carbazole (AEC tablets, Sigma-Aldrich) and 0.012% sodium peroxide (Fisher Scientific, Pittsburgh, PA, USA).

Results

Generation of transgenic TRAMP C/K^b2.1 cells. TRAMP C1 and TRAMP C2 cells were transduced to stably express human HLA-A2.1 using a lentiviral vector without a selection marker since this act as an unwanted immune target *in vivo*. Staining for the human HLA-A2 antigen and FACS analysis however revealed initially only low expression levels with 15-20% of cells being strongly positive (Figure 1A), although expression levels varied over time in culture (Figure 2). Much to our surprise, uncloned transgenic TRAMP C1/K^b2.1 or C2/K^b2.1 cells substantially increased transgene expression once they had been frozen, thawed and re-cultured (Figure 3A). This could be mimicked by exposing cells to DMSO *in vitro* (Figure 3B).

Because of this variability in gene expression, we selected for HLA-A2.1-positive cells by cloning. Five out of twenty TRAMP C2/K^b2.1 clones were clearly positive (Figure 1B shows 3 of the 5 positive clones). This situation was not improved by initially selecting positive cells using the MACS magnetic separation system with anti-HLA-A2 antibody (Miltenyi Biotec Inc.). Cloning of enriched TRAMP C1/K^b2.1 cells gave 6 out of 30 clones that expressed high levels of HLA2.1 expression (not shown).

TRAMP C1/K^b2.1 and TRAMP C2/K^b2.1 cells fail to form tumors in C57Bl/6-K^b2.1 mice. Parental TRAMP C1 and TRAMP C2 cells generally become palpable within 4-10 weeks in C57Bl/6 mice. They grow better in male mice, but also form tumors in female mice (Table I). Surprisingly, none of the inocula of cloned or uncloned TRAMP C1/K^b2.1 or C2/K^b2.1 cells injected *s.c.* into male or female humanized C57BL/6-K^b2.1 mice formed tumors even with up to 5×10^6 cells. Attempts to obtain tumor take with cell suspensions injected within Matrigel were similarly fruitless. Overall, none of more than 100 mice that were injected over the course of more than a year grew tumor (Table I). This contrasted with 100% take of the same tumors in SCID/beige mice, indicating that immune rejection was the reason for lack of growth. Even tumors that grew in SCID/beige mice, which were confirmed to still express HLA-A2.1 (not shown), that were harvested and re-injected as a mince into C57Bl/6-K^b2.1 mice failed to grow.

Because TRAMP cells grow well in C57Bl/6-K^b2.1 mice (Table I), we hypothesized that antigens being presented in the context of HLA-A2.1 were responsible for the immune rejection of humanized TRAMP cells by C57Bl/6-K^b2.1 mice. The most obvious candidate antigens were murine prostate antigen homologs, which would imply that central tolerance is not generated to these antigens. To test this hypothesis we identified human prostate antigens for which the HLA-A2.1 immunodominant epitopes are known and that have homologs that are expressed in TRAMP cells that have similar sequences. Of these we chose six-transmembrane epithelial antigen of the prostate (STEAP; 80% homology), prostate-specific membrane antigen (PSMA; 86% homology) and survivin (84% homology) (6-9) as test antigens.

To test the hypothesis, viable or heavily irradiated TRAMP C1/K^b2.1 cells were injected into C57Bl/6-K^b2.1 mice and IFNγ-ELISPOT-responses directed against human immunodominant HLA-A2.1-restricted epitopes on STEAP, PSMA, and survivin (6-9) were measured (Figure 4).

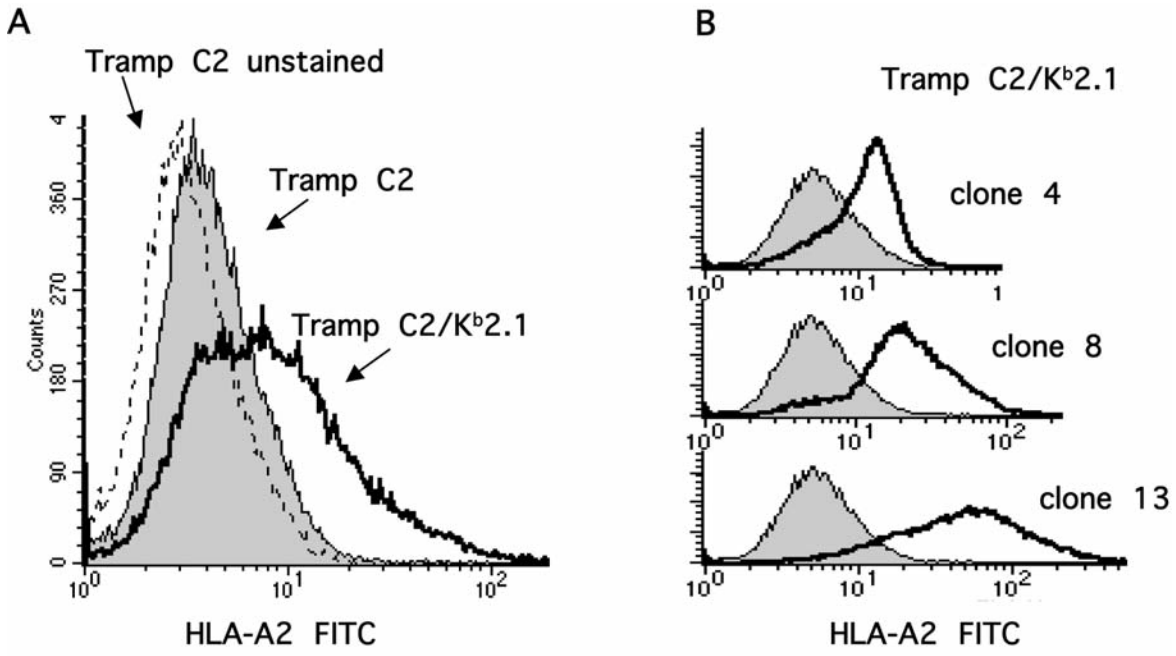

Figure 1. *Viral transformation and clonal selection to establish stable TRAMP C2/K^{b}2.1 cells. TRAMP C2 cells were transformed with a lenti virus carrying the human HLA2.1 gene. A: FACS analysis with the FITC-anti human HLA-A2 antibody (clone BB7.2) indicated that about 20% of treated cells were clearly HLA2 positive, which was further improved by clonal selection (B). Dashed line=unstained control; Gray=parental TRAMP C2; black line=individual TRAMP C2/K^{b}2.1 clones.*

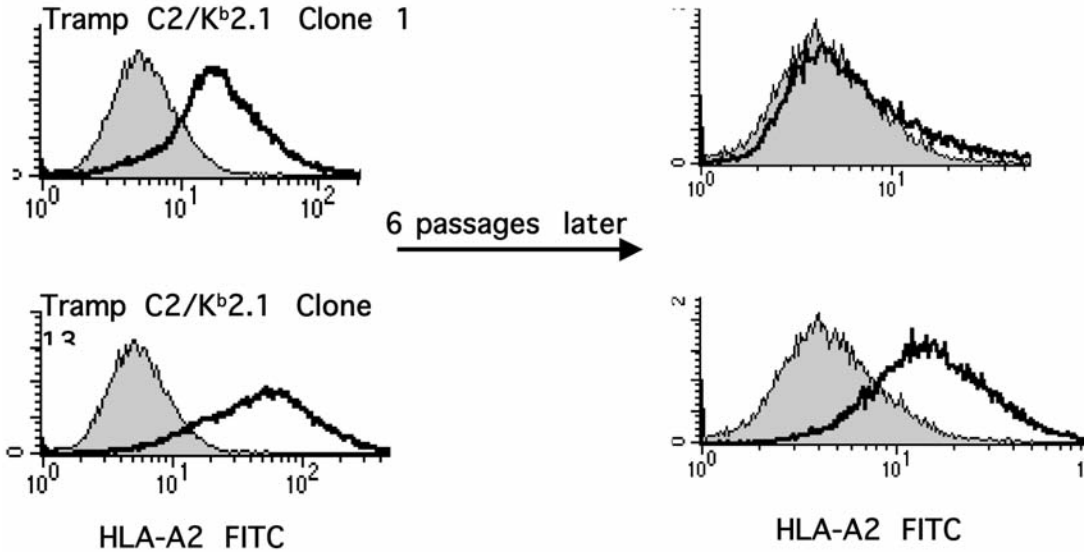

Figure 2. *Not all TRAMP C2/K^{b}2.1 clones maintained long-term HLA2.1 expression. HLA2.1 expression was assessed by FACS analysis with the FITC-anti human HLA-A2 (clone BB7.2). TRAMP C2/K^{b}2.1 clone 1 (top) lost HLA2.1 expression during in vitro culture while clone 13 (bottom) remained positive after 6 passages and even after 18 passages (not shown).*

ELISPOT analysis showed that significant immune responses had been generated against these peptides, especially in the case of survivin, whether viable or heavily irradiated TRAMP C1/K^{b}2.1 cells were injected. While it is not currently possible to say definitively which, if any, of these are rejection antigens, mice were clearly able to respond to these human epitopes and further analysis is warranted to determine this property.

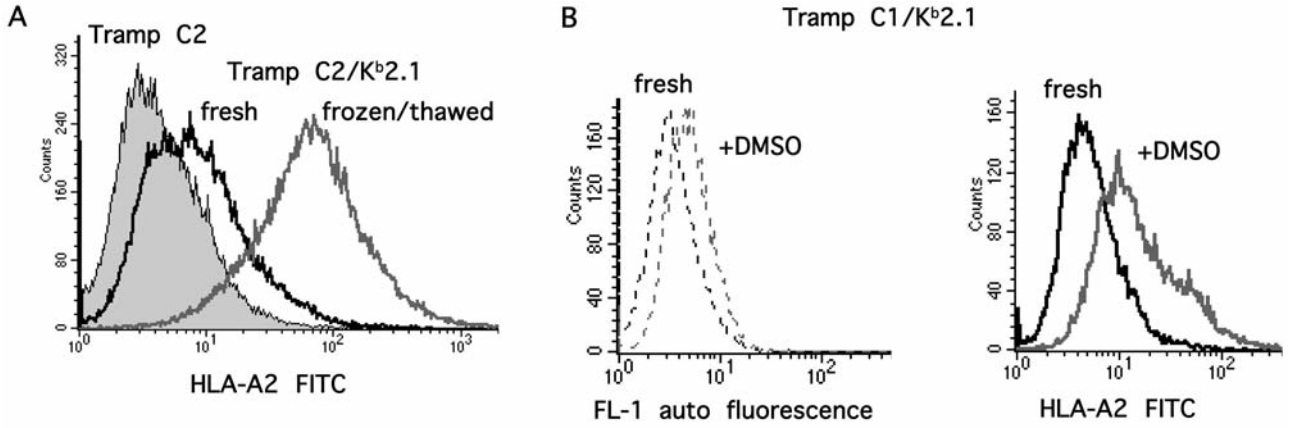

Figure 3. *Surface levels of HLA-2.1 expression increase in TRAMP C/K^{b}2.1 cells following freezing/thawing and/or exposure to DMSO. A: TRAMP C2/K^{b}2.1 cells were stored in 10%DMSO/FCS at –80°C for several weeks and subsequently cultured under standard in vitro conditions for several days and then stained as above. B: TRAMP C1/K^{b}2.1 were cultured in the presence of 3% DMSO for 48h. Cells were stained with the FITC-anti human HLA-A2 antibody (clone BB7.2) (Right panel). DMSO also increases autofluorescence as seen in the unstained samples (left panel). Dashed line=unstained control; gray=parental TRAMP C2; black line=TRAMP C2/K^{b}2.1; gray line=TRAMP C2/K^{b}2.1 frozen/thawed or exposed to DMSO.*

Table I. *Summary of tumor growth or the absence thereof in mice injected with TRAMP tumor cells. Transgenic mice were injected s.c. with indicated number of clonally selected or uncloned TRAMP C1 or C2 cells expressing the chimeric K^{b}2.1. Tumor take of parental TRAMP C1 and TRAMP C2 in C57Bl/6 served as control.*

TRAMP cells	Quantity injected ×10^6	Mouse strain	Mouse gender	Tumor take
C2/K^{b}2.1 cloned	1	C57Bl/6-K^{b}2.1	Female	0/15
C2/K^{b}2.1 cloned	3	C57Bl/6-K^{b}2.1	Female	0/10
C2/K^{b}2.1 cloned	5	C57Bl/6-K^{b}2.1	Female	0/6
C2/K^{b}2.1 cloned	5 in matrigel	C57Bl/6-K^{b}2.1	Female	0/4
C2/K^{b}2.1 cloned	5	C57Bl/6-K^{b}2.1	Male	0/4
C2/K^{b}2.1 cloned	5	SCID beige	Male	4/4
C2/K^{b}2.1 *in vivo*	tumor bids from SCID	C57Bl/6-K^{b}2.1	Female	0/12
C2/K^{b}2.1 uncloned	5	C57Bl/6-K^{b}2.1	Female	0/4
C1/K^{b}2.1 cloned	3-5	C57Bl/6-K^{b}2.1	Female	0/24
C1/K^{b}2.1 uncloned	4	C57Bl/6-K^{b}2.1	Female	0/4
C1/K^{b}2.1 uncloned	3-4	C57Bl/6-K^{b}2.1	Male	0/17
Parental C2	1	C57Bl/6	Female	0/3
Parental C2	3	C57Bl/6	Female	3/3
Parental C2	tumor bids from Bl6	C57Bl/6-K^{b}2.1	Female	4/4
Parental C1	3	C57Bl/6	Male	3/3
Parental C1	3	C57Bl/6	Female	2/8

Discussion

Due to the pivotal role that the MHC plays in the generation of immune responses, transgenic mice carrying HLA alleles have become important models for assessing human tumor epitopes that can be presented in these contexts. C57Bl/6-K^{b}2.1 mice have particular utility because they can be primed to generate HLA-A2.1 restricted cytotoxic T-cells (CTL) that have fine specificity similar to human CTLs supporting the view that xenogeneic MHC can direct determinant selection and dictate the antigen-specific repertoire (1). Similar determinant selection has been shown using HLA gene-transfected tumor cells (10).

Here we report that introducing a chimeric mouse/human HLA-A2.1/K^b gene into murine prostate tumor (TRAMP) cells eliminates their ability to form tumors in immune-competent HLA-A2.1/K^b mice, but not in immune-deficient animals. This finding came somewhat as a surprise since we

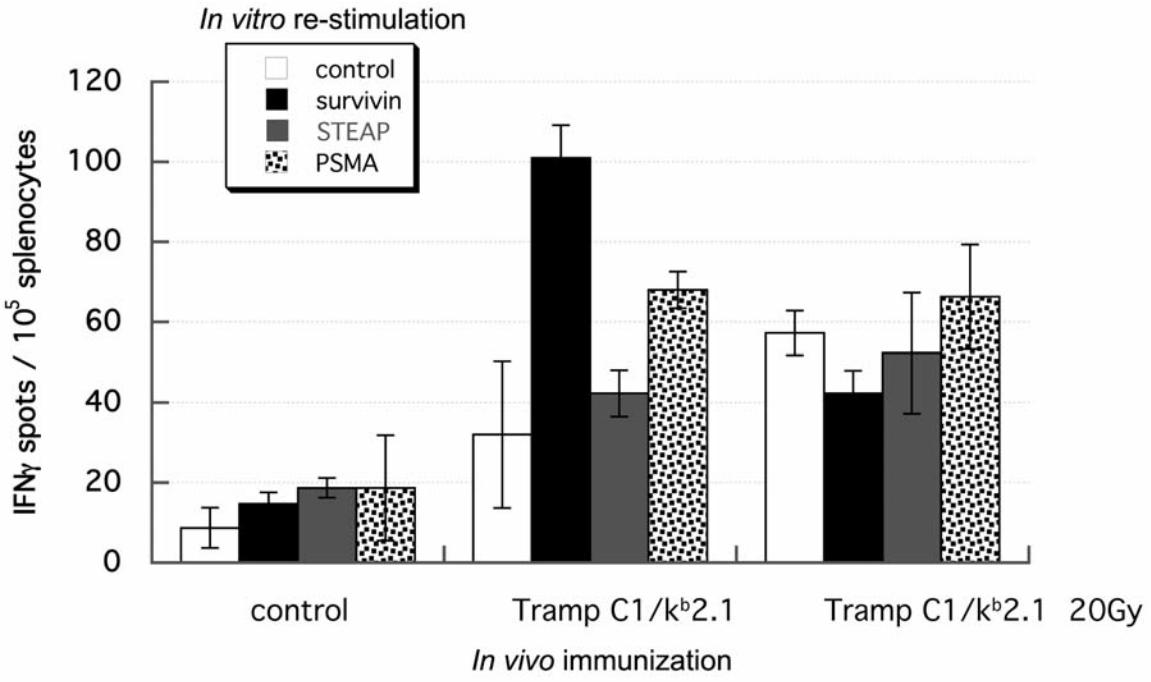

Figure 4. *Splenocyte from male C57Bl/6-K^{b}2.1 mice were tested for IFNγ-production in vitro against a panel of prostate tumor-specific HLA-A2.1 antigenic peptides in an ELISPOT 17 days after two, weekly immunization with viable or lethally-irradiated TRAMP C1/K^{b}2.1 cells.*

have in-house data that HLA-A2.1-transduced murine lymphoma (Drs Koya and Ribas, unpublished data) and melanoma (11) cells form tumors in the very same transgenic humanized C57Bl/6-K^{b}2.1 mice, albeit growing at a significantly slower rate than parental cells. Our findings were not due to differences in HLA status of the mice or the tumors as they clearly showed expression of both the chimeric mouse/human K^{b}2.1 molecule as well as the murine H-2K^b. The gender of the mice was not a factor. TRAMP cells need very little, if any, androgen *in vitro* and male and female C57Bl/6 mice have comparable levels of androgen (12). In our experience, parental TRAMP C1 and TRAMP C2 take in both male and female standard C57Bl/6 mice although others have noted differences (13), which they attributed to immunological rejection and not hormone-related issues.

The fact that our TRAMP C2/K^{b}2.1 cells were able to form tumors in SCID/beige but not in C57Bl/6-K^{b}2.1 mice shows that they maintained their tumorgenic potential and were being immunologically rejected.

We have already shown that humanized B16/K^{b}2.1 melanoma cells present the human immunodominant MART-127-35 peptide in HLA-A2.1/K^b mice, but still grow, albeit at a slower rate. We therefore hypothesized that HLA-A2.1/K^b mice might generate responses to human prostate antigenic epitopes, implying that central tolerance did not develop to these antigens. Indeed this was the case. We are unable to say at this time that any of these responses were the cause of tumor rejection. Changes in the MHC profile or co-stimulatory molecule expression on TRAMP cells may make the difference between tumor-rejection and tumor take (14, 15), and it may be that the affinity of the specific T-cells that are generated is affected by the type of MHC molecule that is constructed; after all this was one reason given as to why this K^{b}2.1 model was able to generate A2.1-restricted responses (1). It is possible that K^{b}2.1 may not be able to generate self tolerance to tumor-associated antigens in the same way that the parent HLA molecule might, however it seems likely that this is a good model for determining the antigen-related factors that influence the balance between tolerance and tumor immune rejection. It should be noted that a possible trivial explanation for our findings is that during lentivirus treatment of TRAMP cells we re-induced the expression of TAg in these cells. TRAMP was originally derived from SV40 large T antigen (TAg)-driven prostate tumors in transgenic mice. Although the parental TRAMP C1 and C2 cell lines have lost the ability to express TAg (13), naïve Bl6 mice apparently can mount an anti-TAg response that confers tumor protection in TRAMP mice upon adoptive transfer (16). However, there would have to be an unexpectedly major change in Tag expression for this to be the target antigen.

As a side note, we observed that MHC expression following lentiviral insertion varied with time and needs to be carefully followed. Specifically, DMSO or freeze/thawing in the presence of DMSO drastically increases MHC I in our cells.

In fact, this was noted more than 20 years ago with respect to the expression of H-2 antigens on murine lung carcinoma cells making them more immunogenic (17, 18). Enhanced antigen presentation appears to be the result of DMSO chaperoning proteins (19). DMSO -a radical scavenger- is better at forming hydrogen-bonds than water and, by replacing water, can change many cellular structures, including membranes which respond with increased permeability (20), accounting for its ability to induce cell growth and cellular differentiation in embryonic stem cells and in transformed cells (21-23). It was this cellular differentiation that prompted Friend and colleagues (23) to explain their observation that DMSO-treated murine virus-induced leukemic cells are less malignant in mice. In our studies, most of the cells had been in culture without DMSO for some time prior to injection and DMSO-related effects are very unlikely to have played any role in the observed findings.

Acknowledgements

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