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A Coreceptor-Independent Transgenic Human TCR Mediates Anti-Tumor and Anti-Self Immunity in Mice


Recent advancements in T cell immunotherapy suggest that T cells engineered with high-affinity TCR can offer better tumor regression. However, whether a high-affinity TCR alone is sufficient to control tumor growth, or the T cell subset bearing the TCR is also important remains unclear. Using the human tyrosinase epitope-reactive, CD8-independent, high-affinity TCR isolated from MHC class I-restricted CD4+ T cells obtained from tumor-infiltrating lymphocytes (TIL) of a metastatic melanoma patient, we developed a novel TCR transgenic mouse with a C57BL/6 background. This HLA-A2–restricted TCR was positively selected on both CD4+ and CD8+ single-positive cells. However, when the TCR transgenic mouse was developed with a HLA-A2 background, the transgenic TCR was primarily expressed by CD3+CD4+CD8− double-negative T cells. TIL 1383I TCR transgenic CD4+, CD8+, and CD4+CD8− T cells were functional and retained the ability to control tumor growth without the need for vaccination or cytokine support in vivo. Furthermore, the HLA-A2*human tyrosinase TCR double-transgenic mice developed spontaneous hair depigmentation and had visual defects that progressed with age. Our data show that the expression of the high-affinity TIL 1383I TCR alone in CD3+ T cells is sufficient to control the growth of murine and human melanoma, and the presence or absence of CD4 and CD8 coreceptors had little effect on its functional capacity.

A adoptive transfer of tumor-infiltrating lymphocytes (TIL) is a promising approach for providing patients with antitumor immunity (1). Alternatively, the use of recombinant viral vectors encoding TCR genes to engineer normal PBL-derived human T cells to provide patients with a source of autologous tumor-reactive T cells is another promising approach that was pioneered by our group (2). We also described the first use of TCR gene-modified T cells in cancer patients (3). This trial, and subsequent trials, suggests that the clinical use of TCR gene-modified T cells for patient treatment is feasible and generally safe (4). However, the therapeutic efficacy of TCR gene-modified T cells has not approached the success observed with TIL (5). Therefore, additional improvements are being actively explored.

Much of what we know about the behavior of T cells in vivo has come from clinical trials and adoptive T cell transfer studies in tumor-bearing mice. In mouse models, the primary source of tumor-reactive T cells has come from TCR transgenic mice that target nonself (6) or mouse self-Ags (7, 8) expressed by tumor cells. A recent study has generated transgenic mice with the entire human TCR-αβ gene loci, whose T cells express a diverse TCR repertoire similar to humans (9). In the current study, we describe a novel TCR transgenic mouse (designated h3T for human TIL 1383I tyrosinase TCR) developed using the HLA-A2–restricted, tyrosinase368–376-reactive human TIL 1383I TCR that is planned to treat patients with advanced melanoma by utilizing a TCR transduction approach. Because TIL 1383I TCR was isolated from a MHC class I-restricted CD4+ T cell, the development of transgenic mice bearing this receptor is also important in understanding T cell selection (10). In C57BL/6 mice, we observed that this HLA-A2–restricted TCR was functionally expressed on both CD4+ and CD8+ T cells. When crossed to HLA-A2 transgenic mice, the resulting h3T-A2 mice had their peripheral lymphoid compartments dominated by a novel CD3+CD4+CD8− T cell effector population expressing the TIL 1383I TCR, and the mice developed spontaneous vitiligo. Our data presented in this study show that h3T and h3TA2 mice are resistant to challenge with HLA-A2* B16 melanoma cells and that their splenocytes mediated rejection of established tumors in vivo irrespective of the presence of the CD4 and CD8 coreceptors. Because the CD4 and CD8 coreceptors are thought to play an important role in stabilizing the peptide–MHC complex and activation of T cells, the transgenic TCR bearing CD4+, CD8+, and CD4−CD8− T cells obtained from h3T and h3TA2 mouse models provided us

A

Abbreviations used in this article: CTX, cyclophosphamide; DN, double-negative; DP, double-positive; ERG, electroretinogram; RPE, retinal pigment epithelium; SF, single-positive; TIL, tumor-infiltrating lymphocytes; TRP-1, tyrosinase-related protein 1.

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a unique opportunity to compare the role of coreceptors in anti-tumor T cell responses.

**Materials and Methods**

**Generation of h3T TCR transgenic mice**

Transgenic mice bearing TIL 1383T TCR reactive to human tyrosinase epitope were developed on a C57BL/6 background. Genomic DNA was isolated from TIL 1383T, a HLA-A2+ human tyrosinase-specific CD4+ T cell line. Fragments containing the genomic V-J and V-D-J regions of the TCR α- and β-chains were cloned, sequenced, and subcloned into the TCR cassette vectors described earlier (11) and co-injected into fertilized C57BL/6 embryos yielding transgenic founder lines using the transgenic core facility at the Medical University of South Carolina. Animals were maintained in pathogen-free facilities and under the approved procedures of the Institutional Animal Care and Use Committee.

**Mice and tumor cells**

HLA-A2+ human melanoma 624-MEL and its HLA-A2–variant 624-28-MEL were established earlier at Surgery Branch, National Cancer Institute (12). T2 cells are transporter associated protein deficient, and their empty surface HLA-A2 molecules were used for direct presentation of epitopes to the Ag-reactive CTL. B16 (H2b) is a tyrosinase-positive murine melanoma. HLA-A2–murine B16 melanoma cells (referred as B16-A2 in text) were obtained from R. Kiessling (Karolinska Institute, Stockholm, Sweden). C57BL/6, HLA-A2, Rag-1−/−, SCID/beige mice were obtained from The Jackson Laboratory.

**Culture medium and reagents**

Human tyrosinase peptide (YMDGTMSQV), murine tyrosinase (YMDGTMSQV), melanoma-associated Ag recognized by T cell (MART-1), and influenza matrix protein (MP) were used as positive control. Human tyrosinase368–376 peptide (GILGVPVFTL) were purchased from Genzyme (Cambridge, MA). Culture medium was IMDM (Life Technologies BRL, Grand Island, NY) supplemented with 10% FBS (Gemini Bioproducts, Calabasas, CA). Fluorochrome-conjugated Abs for CD3 (clone 145-2C11), CD4 (clone GK1.5), CD8 (53-6.7), CD25 (clone 3C7), CD69 (H.12F3), CD44 (clone IM7), CD62L (MEL-14), and CD107a (clone ID4B) were obtained from BioLegend (San Diego, CA). Human Vβ12 Ab (clone S511) was purchased from Thermo Scientific (Rockford, IL). HLA-A2 (clone BB7.2) was obtained from BD Biosciences (San Jose, CA). MHC class I tetramers for human tyrosinase-specific, and human gp100209–217 were purchased from Beckman Coulter (Fullerton, CA).

**Cytokine release assay**

Cytokine release by effector cells was measured by culturizing 1 × 10^6 to 1 × 10^7 effector cells in a 1:1 ratio with melanoma tumor cells or peptide-pulsed T2 cells, as described previously (13). After 16–24 h, culture supernatants were harvested and cytokine concentrations were measured by sandwich ELISA per the manufacturer’s protocol (R&D Systems) using a spectrophotometer (BioTek, Winoski, VT).

**Flow cytometry**

Staining for cell surface markers was performed by incubation of Abs at a 1:100 dilution in FACS buffer (2% FCS, PBS) for 30 min at 4°C. Samples were acquired on a FACScan or FACSCalibur (BD Biosciences) and analyzed with FlowJo software (Tree Star). FACSaria cell sorter (BD Biosciences) was used to obtain >99% Vβ12+CD4+ or Vβ12+CD8+ T cells from h3T mice splenocytes and Vβ12+CD4+CD8+ T cells from h3T-A2 mouse splenocytes.

**Adoptive transfer and tumor treatment**

HLA-A2 mice were s.c. injected with 2.5 × 10^5 B16-A2 cells, and palpable tumors were treated with cyclophosphamide (4 mg/mouse) 1 d after adoptively transferring 1.0 × 10^6 Vβ12+ freshly isolated splenocytes from h3T or h3T-A2 mice. Tumors were measured with calipers twice per week. For lung metastases experiments, HLA-A2 mice i.v. injected with 2.5 × 10^5 B16-A2 melanoma cells were administered cyclophosphamide (4 mg/mouse) 4 d after tumor inoculation. One day later, mice were left untreated or i.v. adoptive transfer of freshly isolated 1.0 × 10^6 Vβ12+ fresh splenocytes from h3T or h3T-A2 mice. Animals were sacrificed 3 wk after adoptive transfer, and tumor foci were counted. To test the ability of h3T- and h3T-A2-derived T cells in treating human tumors, SCID/Beige mice s.c. injected with 5 × 10^6 624 MEL cells and palpable tumors were treated with i.v. adoptive transfer of 1 × 10^7 freshly sorted h3T CD4+Vβ12+, h3T CD8+Vβ12+, or h3T-A2 CD4+CD8+ Vβ12+ T cells. Tumors were measured with calipers twice per week. All experiments were performed at least twice.

**In situ RT-PCR**

Human Vβ12 TCR mRNA expression was examined in formalin-fixed, paraffin-embedded eye sections from HLA-A2- and h3T-A2 mice using the in situ RT-PCR methods, as previously described (14). After removing the anterior segment of eye and the lens, eyes were further fixed in 4% paraformaldehyde for another 2 h. Eyes were transferred to a 30% sucrose solution for 12–14 h and cut into 10-μm-thick cryosections. Furthermore, the tissue was dewaxed in xylene, gradually rehydrated with alcohol, and washed in PBS before proteinase K treatment for 27 min at room temperature. Proteinase K treatment was inactivated by incubating slides at 105°C for 3 min. This was then followed by DNaase treatment at 37°C overnight. The in situ RT-PCR procedure was then performed using the forward primer (5′-ATGCGTAACACCCAGAGCAGAGGCA-3′) and reverse primer (5′-TATACGATGCTGGAGGAGGGA-3′). The iScript one-step RT-PCR kit (Bio-Rad, Hercules, CA) was used to form the cDNA copy of the template and to amplify. After amplification, slides were washed in 2× SSC buffer for a total of three times, and then amplicons were detected by in situ hybridization methods, as previously described (14). Hybridizations were performed by using a green fluorescent probe (5′-ACATGTGCGCTGCGTCTGATTGTTTAC-3′), specific for human Vβ12 chain. Hybridizations were performed using EKONO buffer (G-Biosciences, Maryland Heights, MO) at 95°C for 5 min, then 42°C overnight. RT-PCR and hybridization reactions were performed in a MJR-Bio-Rad Twin Tower, PCT-200 theromocler (Waltham, MA). The cycling reactions started with 30 min at 50°C, then 95°C for 5 min (cDNA step), followed by 42 cycles of cDNA amplification at 95°C denaturing, 60°C annealing, and 72°C extension. Subsequently, images were acquired on Olympus BX61 motorized microscope equipped with Olympus DP72 digital camera and CellSens digital imaging software.

**Histology**

The 8-μm cryosections were cut from Tissue-Tek OCT compound (Sakura Finetek USA, Torrance, CA) embedded tissue of pigmented, nontransgenic control mice, and depigmented, h3T-A2 double-transgenic mice, fixed in cold acetone. After blocking with a specific Ab binding using Superblock and biotin block (ScyTek, Logan, UT), sections were stained with mouse mAb T99 to tyrosinase-related protein 1 (TRP-1; Covance, Princeton, NJ) and biotinylated Armenian hamster mAb to CD3e (BD Pharmingen, San Jose, CA), followed by a combination of alkaline phosphatase-labeled secondary goat antiserum to mouse IgG2a (Southern Biotechnology Associates, Birmingham, AL) and HRP-labeled streptavidin (Southern Biotechnology Associates). Immunoenzymatic staining was completed using Fast blue BB as a substrate for alkaline phosphatase (Sigma-Aldrich, St. Louis, MO), followed by detection of peroxidase using aminoethylcarbazole (Sigma-Aldrich) with hydrogen peroxide in sodium acetate buffer, essentially as previously described (15). Following the similar procedure as described above, immunohistochemical staining of mouse eyes, the sections were performed using CD3e biotinylated (145-2C11; BD Pharmingen), CD25 biotinylated (PC61; BioLegend), Trp-1 (Ta9; Covance), CD68 (FA-11; Abcam), and Vβ12-FITC (S511; Thermo Fisher Scientific), and detected using secondary Abs streptavidin HRP (Southern Biotechnology Associates), anti-mouse IgG2a HRP (Southern Biotechnology Associates), anti-rat HRP (Southern Biotechnology Associates), and anti-FITC HRP (MyBioSource), respectively. Slides were developed in 3-amino-9-ethylcarbazole (A5754; Sigma-Aldrich) substrate solution to detect Ags and counterstained with hematoxylin for nuclear localization.

**Mouse melanocyte culture**

Strips of HLA-A2 transgenic or C57BL/6 mouse skin adding up to 3 × 2 cm were incubated in a mixture of 30 U/ml DNase I (Roche Diagnostics, Indianapolis, IN), 50 μg/ml thermolysin, 0.1 mg/ml trypsin, and 0.5 mg/ml collagenase IV (all from Sigma-Aldrich) in RPMI 1640 (Mediatech, Manassas, VA), shaking overnight at room temperature. Tissue was passed through a 70 μm cell strainer (BD Biosciences, Bedford, MA), and resulting cells were plated in Ham’s F12 (HyClone Laboratories, Logan, UT) medium with 10% FBS (Mediatech), 0.1% bovine pituitary extract (Invitrogen, Carlsbad, CA), 16 mM PMA (Sigma-Aldrich), 0.1 mM iso- butyl methyl xanthine (Sigma-Aldrich), and 100 IU/ml penicillin/100 μg/ml streptomycin/100 μg/ml amphotericin (Invitrogen). Established cultures were treated with 50 μg/ml G418 sulfate (Invitrogen) for 4 d to
remove cells other than melanocytes that are insensitive to this agent (16). Cells were split 1:4 at confluency and used in experiments.

**Electroretinography**

To quantitate retinal function, we performed electroretinograms (ERG), as previously described (17). Briefly, mice were dark-adapted overnight, and the following day they were anesthetized with ketamine and xylazine. Pupils were dilated with a phenylephrine HCl (2.5%) and tropicamide (1%; Akorn, Buffalo Grove, IL). A needle ground electrode was placed s.c. in the back of the animal, and a reference electrode on the tongue. A stimulus intensity series of ERG was recorded in response to single-flash intensities using 40-dB attenuation (low-intensity flash) through no attenuation (high-intensity flash). The single-flash responses were an average of three flashes, with an interstimulus interval of 2 min to ensure that ERG amplitudes at a given intensity were identical between the first and the last hash. Each single-flash ERG response was measured using a contact lens containing a gold-rod electrode held in place by a drop of methylcellulose. ERG were recorded using UTAS-2000 system (LKC Technologies, Gaithersburg, MD). Corneal electrical responses to a single, 10 μs white-light flash were delivered by a Ganzfeld stimulator.

**Results**

**Development and characterization of h3T mice**

The TIL 1383I TCR has high affinity for HLA-A2/tyrosinase368–376 complexes, and T cells transduced to express this TCR could make potent antitumor effectors for treating melanoma patients. However, what remains unclear is how this unique MHC class I-restricted TCR developed and how effective T cells expressing the TIL 1383I TCR would be in treating patients with advanced melanoma.

To address these and other questions, we generated a TCR transgenic mouse encoding the TIL 1383I TCR and named it h3T (short for hTTT or human TIL 1383I TCR). The h3T mouse was developed with the C57BL/6 background, so the T cells would be syngeneic with the mouse B16 melanoma, and to take advantage of the numerous transgenic and knockout strains available with the H-2b haplotype. As shown in Fig. 1A, the human Vb12 transgene was detected on double-positive (DP) thymocytes in transgenic (h3T), but not in nontransgenic (C57BL/6) littermates, indicating the human TCR transgenes could be expressed on mouse T cells. Analysis of the thymocyte subsets revealed that, compared with C57BL/6 littermates, h3T mice have an increased frequency of CD4+CD8+ double-negative (DN) (23.3 versus 2.4%) thymocytes, a decreased frequency of CD4+CD8+ DP (69.8 versus 87.3%) and CD4−CD8− single-negative (SN) (4.2 versus 8.0%) thymocytes, and a similar frequency of CD4+CD8− SP (2.7 versus 2.4%) thymocytes (Fig. 1B). These results suggested that the expression of the TIL 1383I TCR affected T cell development in the thymus.

Given the altered thymocyte distribution in the h3T mice, we examined human Vb12 expression on their mature T cells. Despite lacking HLA-A2 expression, Vb12+CD8+ and Vb12+CD4+ T cells were present in the spleens of h3T mice (Fig. 1C). These mouse CD4 and CD8 SP T cells expressed the properly assembled human TIL 1383I TCR, as indicated by staining with HLA-A2/tyrosinase368–376 tetramers, but not control HLA-A2/gp10025–33 tetramers (Fig. 1D). Therefore, the TIL 1383I TCR was expressed on the surface of mature T cells in h3T mice even in the absence of HLA-A2 expression in their thymus.

To determine whether h3T T cells were Ag reactive, unfraccionated splenocytes from h3T and C57BL/6 littermates were cocultured overnight with a panel of stimulators, and the amount of IFN-γ released was measured using ELISA. T2 cells loaded with the mouse or human tyrosinase peptide stimulated h3T splenocytes to secrete IFN-γ (Fig. 1E). More importantly, h3T splenocytes recognized HLA-A2+ B16-A2 (mouse) and 624 MEL (human) cells, but not HLA-A2− B16 and 624-28 MEL cells. In addition to IFN-γ, h3T T cells were polyfunctional and secreted cytokines IL-2, TNF-α, and GM-CSF upon Ag stimulation (Supplemental Fig. 1A). Importantly, the cytokine secretion observed was from h3T-derived naive T cells that had not undergone any prior activation with cytokines or TCR engagement as required in the other widely used transgenic mouse model Pmel that expresses a mouse MHC class I-restricted TCR specific for gp10025–33 (7). Of particular importance is the fact that these fresh naive splenic T cells recognized naturally processed Ag on the surface of tumor cells. Also, despite developing in the thymus of H-2b mice, they do not recognize B16 melanoma cells (Fig. 1E) or other H-2b tumor cell lines (data not shown). These results indicate that h3T mice possess multifunctional transgenic T cells with the HLA-A2–restricted tyrosinase reactivity of the parent TIL 1383I T cells.

The TIL 1383I TCR is CD8 independent, meaning that both CD4+ and CD8+ T cells transduced to express the TIL 1383I TCR can recognize HLA-A2+ melanoma cells. Therefore, it was important to determine whether both the CD4+ and CD8+ T cell subsets from h3T mice were functional. Spleens from h3T mice were FACS sorted to purify the individual Vb12+CD8+ and Vb12+CD4+ T cell populations for use in functional assays. When cocultured with mouse or human tyrosinase peptide-loaded T2 cells or 624-MEL tumor cells, the purified CD4+ T cells principally secreted IL-2 and the CD8+ T cells principally secreted IFN-γ (Fig. 1F). And finally, despite being able to recognize processed Ag presented on tumor cells, h3T T cells are similar to the parent TIL 1383I T cells, and TIL 1383I TCR-transduced CD4+ and CD8+ T cells having relatively low functional avidity by requiring T2 cells to be loaded with ≥10 nM peptide to stimulate IFN-γ or IL-2 secretion (Supplemental Fig. 1B). Therefore, despite lacking HLA-A2 expression, h3T mice contain multifunctional CD4+ and CD8+ T cells that have properties similar to TIL 1383I TCR-transduced CD4+ and CD8+ human T cells.

**Influence of HLA-A2 expression on transgenic T cells in h3T mice**

Given that h3T T cells were positively selected in the absence of HLA-A2 expression in C57BL/6 mice, the next question was to assess the impact of HLA-A2 on T cell development and function. We crossed h3T mice to HLA-A2 transgenic mice (expressing the complete HLA-A2 molecule) and analyzed the resulting progeny (h3T-A2). In contrast to h3T mice, the human Vb12 transgene was expressed on the DN thymocytes in h3T-A2 mice, but not on DP thymocytes (Fig. 2A). These DN cells represented a higher proportion of the h3T-A2 thymus compared with their HLA-A2 nontransgenic littermates (~60 versus 6%; Fig. 2B). The increased frequency of DN cells in the thymus of h3T-A2 mice appeared to be at the expense of the DP cells (~26 versus 68%) with only a slight reduction in the CD4 SP (∼7 versus 18%) and CD8 SP (∼3 versus 6%) cells. These results indicate that HLA-A2 expression had a profound effect on T cell development in the thymus of h3T-A2 mice.

Because of the altered thymocyte distribution in h3T-A2 mice, we examined the distribution of Vb12+ T cells in their spleens and lymph nodes. The vast majority of the T cells (~80%) in the spleen of h3T-A2 mice expressed the human Vb12 transgene (Fig. 2C). However, only 8–10% of the splenic T cells were CD8+ SP T cells, with very few (generally <1%) being CD4+ SP T cells. The remaining Vb12+ T cells in the spleen of h3T-A2 mice (generally >90%) expressed neither the CD4 nor CD8 coreceptor. Four-color staining with anti-Vb12, anti-CD3, anti-CD4, and anti-CD8 determined the majority of the Vb12+ T cells were indeed...
CD4/CD8 DN T cells with a minor population of CD8+ SP T cells, whereas the Vβ12+ T cells were mostly SP T cells with the majority being CD4+ T cells (Supplemental Fig. 2A). As seen with the h3T mice, expression of the Vβ12 transgene represented properly assembled TIL 1383I TCRs because the same proportion of Vβ12+ T cells (∼80%) stained with HLA-A2/tyrosinase368–376 epitope-reactive cognate tetramer (upper panel) or human gp100209–217 epitope-reactive control tetramer reagent (lower panel). (E) IFN-γ was measured by ELISA using supernatant obtained after overnight stimulation of h3T splenocytes as effectors and human or mouse tyrosinase peptide-pulsed T2 cells, HLA-A2-transduced mouse melanoma B16 used untreated or after treatment with IFN-γ overnight, and HLA-A2+ human melanoma 624 MEL as cognate stimulators. MART-127–35 peptide-pulsed T2 cells, mouse melanoma B16 untreated or after treatment with IFN-γ overnight, and HLA-A2+ human melanoma 624-28 MEL served as controls. (F) Supernatants obtained after overnight stimulation of sorted Vβ12+CD4+ and Vβ12+CD8+ effector T cells from h3T mouse were cocultured with human or mouse tyrosinase peptide-pulsed T2 cells and HLA-A2+ human melanoma 624 MEL as stimulators. Flu-MP38–36 peptide-pulsed T2 cells and HLA-A2+ human melanoma 624-28 MEL served as controls. Data shown in (A)–(E) are from one representative of five experiments performed, and in (F) from one of two experiments.

Functionally, h3T-A2 T cells are similar to h3T T cells in that fresh splenocytes secrete IFN-γ (Fig. 2E) and other cytokines (Supplemental Fig. 2B) when stimulated with tyrosinase peptide-loaded T2 cells or HLA-A2+ tyrosinase+ tumor cells. Despite the high-affinity TIL 1383I TCR and the expression of HLA-A2 during T cell development, h3T-A2 mice possess multifunctional tumor-reactive T cells in their periphery. Given the altered T cell subset distribution in h3T-A2 mice, we wanted to determine whether the Vβ12+CD8+ T cells and in particular the Vβ12+ DN T cells were functional. The h3T-A2 splenocytes were FACS sorted to purify the Vβ12+CD8+ T cells and in particular the Vβ12+ DN T cells for use in functional assays. The Vβ12+ CD8+ T cells secreted IFN-γ, IL-2, TNF-α, and GM-CSF when stimulated with tyrosinase368–376 peptide-loaded T2 cells and HLA-A2+ human melanoma cells (Fig. 2F). The Vβ12+CD8+ T cells secreted low levels of IFN-γ and TNF-α and no IL-2 when stimulated with peptide-pulsed targets and melanoma cells. These results indicate that despite
the expression of a CD8-independent TCR with high affinity for Ag and the HLA-A2 restriction element, h3T-A2 mice have functional transgenic CD8+ T cells. Also, these mice have a unique population of DN T cells that are HLA-A2 restricted and tyrosinase reactive.

**Phenotypic characterization of h3T and h3T-A2 splenocytes**

To investigate any phenotypic differences between the transgenic TCR-bearing splenic T cells from both h3T and h3T-A2 mice, we evaluated baseline levels of the activation/effector markers CD25, CD44, and CD69. Transgenic h3T mouse-derived Vβ12+CD4+ T cells showed a slightly higher level of CD25 expression and a reduced CD62L expression as compared with h3T Vβ12+CD8+ and h3T-A2 Vβ12+CD4+ CD8+ (Fig. 3A). Thus, h3T Vβ12+CD8+ and h3T-A2 Vβ12+CD4+ CD8+ T cells exhibited a naive phenotype, whereas h3T Vβ12+CD4+ T cells showed an activated phenotype. Furthermore, the expression level of human Vβ12 on various subsets of T cells from h3T and h3T-A2 mice was found to be almost similar (Fig. 3B). An evaluation of the cytolytic ability as measured by upregulation of CD107a expression upon Ag stimulation revealed that TIL 1383I TCR-bearing Vβ12+CD4+ T cells, h3T Vβ12+CD8+ T cells, and h3T-A2 Vβ12+CD4+ CD8+ are cytolytic, although not to the same extent (Fig. 3C). However, despite having large numbers of functional cytolytic tyrosinase-reactive CD4+ and CD8+ T cells in their periphery, h3T mice remain pigmented throughout their life. In contrast, h3T-A2 mice are born pigmented, and, starting about the time of weaning (3–4 wk of age), they begin to exhibit vitiligo that progresses with age, leading to complete depigmentation (Fig. 3D).

**Melanocyte destruction in h3T-A2 mice**

The presence of tyrosinase-reactive T cells in h3T and h3T-A2 mice led us to further investigate their ability to mediate autoimmunity by the destruction of melanocytes in vitro and in vivo. The
expression level on the gated T cells, as indicated on staining with isotype control, and open histogram indicates the cell surface CD8 Ab along with the indicated cell surface Ab. Gray histogram indicates chromo-conjugated TCR-specific human anti-V\_b12 expression level on the gated T cells, as indicated on staining with isotype control, and open histogram indicates the cell surface CD8 Ab along with the indicated cell surface Ab. Gray histogram indicates staining with isotype control, and open histogram indicates the cell surface expression level on the gated T cells, as indicated on left. (B) Splenocytes from h3T and h3T-A2 mice were stained using fluorochrome-conjugated TCR-specific human anti-V\_b12, anti-CD4, and anti-CD8 Ab. Gray histogram indicates staining with isotype control, and open histogram indicates the V\_b12 expression level on the gated T cells, as indicated on top. (C) The h3T and h3T-A2 splenocytes were stimulated overnight with human tyrosinase cognate peptide-pulsed T2 cells. CD107a expression was determined by flow cytometry on V\_b12+CD4\^+, V\_b12+CD8\^+, and V\_b12+CD4\^+CD8\^+ T cells. MART-1\_27-35 peptide-pulsed T2 cells were used as control. Gray histogram indicates staining with isotype control, and open histogram indicates the CD107a expression level on the gated T cells, as indicated on top. (D) Spontaneous depigmentation at 10 wk of age in h3T-A2 mice as compared with age-matched C57BL/6, h3T, and HLA-A2 controls.

**FIGURE 3.** Phenotypic characterization of h3T and h3T-A2 splenocytes. (A) Splenocytes from h3T and h3T-A2 mice were stained using fluorochrome-conjugated TCR-specific human anti-V\_b12, anti-CD4, and anti-CD8 Ab along with the indicated cell surface Ab. Gray histogram indicates staining with isotype control, and open histogram indicates the cell surface expression level on the gated T cells, as indicated on left. (B) Splenocytes from h3T and h3T-A2 mice were stained using fluorochrome-conjugated TCR-specific human anti-V\_b12, anti-CD4, and anti-CD8 Ab. Gray histogram indicates staining with isotype control, and open histogram indicates the V\_b12 expression level on the gated T cells, as indicated on top. (C) The h3T and h3T-A2 splenocytes were stimulated overnight with human tyrosinase cognate peptide-pulsed T2 cells. CD107a expression was determined by flow cytometry on V\_b12+CD4\^+, V\_b12+CD8\^+, and V\_b12+CD4\^+CD8\^+ T cells. MART-1\_27-35 peptide-pulsed T2 cells were used as control. Gray histogram indicates staining with isotype control, and open histogram indicates the CD107a expression level on the gated T cells, as indicated on top. (D) Spontaneous depigmentation at 10 wk of age in h3T-A2 mice as compared with age-matched C57BL/6, h3T, and HLA-A2 controls.

progressive vitiligo observed in h3T-A2 from 2 wk onward is due to melanocyte destruction because there is no evidence of pigmented cells remaining in the skin of h3T-A2 mice after active depigmentation (Fig. 4A). By comparison with non-TCR transgenic mice, h3T-A2 mice show an increase in depigmentation with age (Fig. 4B). Whereas depigmentation was noticeable at the time of weaning, it continually progressed to be ∼60–70% by 30 wk of age in h3T-A2 mice. Splenocytes from h3T-A2 mouse also secreted IFN-γ when stimulated with HLA-A2\^*, but not HLA-A2\^-mouse (Fig. 4C) and human (Fig. 4D) melanocytes in vitro. Cytotoxicity of these tyrosinase-reactive and HLA-A2\^-restricted T cells was also confirmed by the loss of melanocytes from the hair follicles of depigmenting mice, as demonstrated by loss of reactivity with Abs to TRP-1 (Fig. 4E). Furthermore, infiltration of the skin by Ag-specific, V\_b12-expressing T cells was also observed (Fig. 4F, lower panel). These results indicate that the expression of the TIL 1383I TCR on h3T and h3T-A2 T cells can lead to melanocyte destruction resulting in autoimmunity. However, in experiments performed to evaluate whether vitiligo could be transferred by adoptively transferring 1 × 10\(^6\) transgenic T cells in HLA-A2 or Rag-A2 mice, we did not notice any development of vitiligo despite the persistence of cells after 90 d of injection (Supplemental Fig. 2C).

Clinical trials using TCR-transduced T cells have also found that T cells bearing high-affinity TCRs can induce reversible ocular and auditory changes in some patients (18–21). Despite their coat color changes, h3T-A2 mice initially have pigmented eyes, suggesting the melanocytes in the eye may be immune to destruction by h3T-A2 T cells. To determine whether vision changes occur in h3T-A2 mice, a stimulus intensity series of ERG were recorded in 12-mo-old age-matched wild-type and h3T-A2 mice in response to single-flash intensities using 44.3-dB attenuation (low-intensity flash) to no attenuation (high-intensity flash; 0 dB). Although h3T-A2 mice had mild changes in a-wave amplitudes, their b-waves and oscillatory potentials were attenuated by >58% compared with the wild-type mice (Fig. 5A). In h3T-A2 mice, b-wave amplitudes were significantly reduced at −30 dB to 0 dB when compared with wild-type mice (wild type 121 ± 11 versus h3T-A2 80 ± 21, n = 6–12, p = 0.073; −40 dB), (wild type 278 ± 25 versus h3T-A2 141 ± 29, n = 6–15, p = 0.006; −30 dB), (wild type 370 ± 31 versus h3T-A2 129 ± 39, n = 5–15, p = 0.0007; −20 dB), (wild type 526 ± 37 versus h3T-A2 217 ± 44, n = 5–15, p = 0.0003; −10 dB), and (wild type 667 ± 41 versus h3T-A2 278 ± 38, n = 8–13, p = 0.0001; 0 dB). At lower flash intensities, b-wave amplitudes were attenuated more drastically in h3T-A2 mice. Despite the presence of pigment in their eyes, we have seen 34–65% loss in b-wave amplitudes in 1-y-old h3T-A2 mice when compared with the wild-type mice, indicating some visual defects in h3T-A2 mice. No significant differences were noticed in mice younger than 1 y old (data not shown). To further confirm the infiltration of transgenic T cells in the retina of h3T-A2 animals, we performed in situ hybridization using specific probes for human V\_b12 chain. Expression of RNA transcript for V\_b12 was distinctly observed in the retina of h3T-A2 mice as compared with retina of age-matched HLA-A2 nontransgenic mice. Most of the V\_b12 mRNA staining was detected in the inner nuclear layer (Fig. 5B, white arrows), which was migrating to the outer plexiform layer. Additionally, bright field pictures demonstrate that retinal layers were intact in HLA-A2 and greatly disturbed only in the h3T-A2 mice. In addition, images from h3T-A2 mice of a retracting eye showing infiltration by transgenic T cells and by macrophages with interruption of the retinal pigment epithelium (RPE) in comparison with the intact contralateral eye (Fig. 5C) convincingly implicate transgenic T cell infiltration for the pathology of eye.

**The h3T and h3T-A2 T cells are therapeutic in vivo**

The presence of highly Ag-reactive T cells in h3T and h3T-A2 mice capable of mediating vitiligo suggested that these T cells might also have strong antitumor activity in vivo. To this end, we tested the ability of transgenic T cells to protect against tumor challenge.
Both h3T and h3T-A2 mice were resistant to s.c. challenges of $2.5 \times 10^5$ B16-A2 tumor cells, but solid tumors formed and grew progressively when challenged with the same number of B16 tumor cells (Fig. 6A). Similarly, h3T and h3T-A2 mice were resistant to i.v. challenges of $2.5 \times 10^5$ B16-A2 tumor cells, but lung metastases formed when challenged with the same number of B16 tumor cells (Fig. 6B). The HLA-A2 transgenic mice without the TIL 1383I TCR showed progressive growth of B16-A2 tumor cells (Supplemental Fig. 3A). These results indicate that transgenic T cells expressing the TIL 1383I TCR can protect mice against tumor challenge.

Tumor protection is just one measure of antitumor effectiveness in vivo. The real test of an effector T cell is in the therapeutic setting. HLA-A2 transgenic mice bearing established s.c. B16 or B16-A2 tumors were treated with $1 \times 10^5$ h3T or h3T-A2 T cells i.v. after pretreatment with 4 mg/mouse cyclophosphamide (CTX).
The adoptive transfer of h3T or h3T-A2 T cells had no impact on the growth of B16 tumors, but led to rejection or delayed growth of established B16-A2 tumors (Fig. 6C) until 7 wk. When \(1 \times 10^6\) h3T splenocytes were transferred alone in absence of CTX, the tumors grew at the same rate as in untreated control, whereas in the CTX-administered animals h3T transgenic T cells controlled the tumor growth (Supplemental Fig. 3B). Mice challenged i.v. with B16 or B16-A2 tumor cells were also treated with h3T or h3T-A2 T cells to measure the impact on established lung metastases. Splenic T cells from h3T or h3T-A2 transgenic strains effectively treated mice bearing established B16-A2 lung metastases, but were ineffective in mice bearing established B16 lung metastases (Fig. 6D). These results indicate that h3T and h3T-A2 T cells are potent antitumor effectors in vivo.

We have shown that the TIL 1383I TCR can be expressed on mature CD4\(^+\) (h3T), CD8\(^+\) (h3T and h3T-A2), and CD4\(^-\)CD8\(^-\) (h3T-A2) T cells, and each of these populations functions in vitro. We wanted to determine whether each of the subsets had antitumor activity in vivo and the transgenic T cells can control growth of human melanoma. Groups of five SCID/beige mice were challenged s.c. with the HLA-A2\(^+\) human melanoma 624 MEL. Once palpable tumors were established, mice were treated i.v. with FACS-sorted (≥98% pure) subset of TIL 1383I TCR-bearing T cells. As shown in Fig. 6E, mice treated with purified V\(\beta12\)\(^+\)CD4\(^+\), V\(\beta12\)\(^+\)CD8\(^+\), or V\(\beta12\)\(^+\)CD4\(^-\)CD8\(^-\) T cells had a statistically significant reduction in tumor growth compared with untreated mice. In addition, these TIL 1383I TCR-bearing T cell subsets can be traced even 3 mo after adoptive transfer in the
peripheral blood of both immunocompetent HLA-A2 (Supplemental Fig. 4A) and immunodeficient SCID/beige recipient mice (Supplemental Fig. 4B) with CD62L+CD44+ phenotype. These results indicate that each of the mature T cell subsets in h3T mice that express the TIL 1383I TCR has antitumor activity in vivo.

Discussion

The introduction of cloned genes into the germline of mice has proven to be a powerful tool to investigate the role of the respective gene products on the immune system. Specificity of the TCR may control T cell fate by specific receptor–ligand interactions either at early or late stages of T cell development (22). Earlier studies for analyzing the role of TCR were carried out by developing transgenic mice with the TCRs of interest (23). Given the belief that T cells bearing high-affinity TCR would be more effective at targeting self-Ags expressed by tumors, we developed a novel TCR transgenic mouse using HLA-A2–restricted high-affinity TCR reactive to human tyrosinase-derived peptide YMDGTMSQV isolated from class I-restricted CD4+ T cells of TILs of a patient with metastatic melanoma (24). Because the TCR used to develop our TCR transgenic mice was HLA-A2 restricted, TIL 1383I TCR-expressing T cells in our C57BL/6J founder were not expected to be positively selected in the thymus and to be found in the periphery. However, we have found the expression of the transgenic in the CD4+ and CD8+ T cells in peripheral blood and lymphoid organs of C57BL/6 mice. Twenty-five percent of CD4+ and 75% of CD8+ T cells in the h3T mouse spleens. Four weeks after tumor injection, lungs were harvested from mice, and the number of metastatic foci was counted. The experiment was repeated three times with similar results. (E) Human HLA-A2+ (624 MEL) and HLA-A2* (624-28 MEL) human melanomas were established in SCID/beige mice before palpable tumors were treated by adoptively transferring purified populations of 10^5 VÎ²12+CD4+, VÎ²12+CD8+, and VÎ²12+CD4–CD8– h3T T cells. Mice that received PBS were used as controls. Tumor growth was measured using digital calipers every fourth day. Data in figure demonstrate mean tumor size at each time point per group. To compare growth trajectories across groups, random effects linear regression models were fit with log of tumor size as the outcome to adhere to linearity assumptions. Log of tumor size was regressed on time, and statistical significance of differences in slopes (compared with the reference group) was assessed by p values. The p values <0.05 were considered to be statistically significant. Data represent two independent experiments. n = 5 mice/group.
noma cells, or melanocytes, and both murine B16-A2 and human melanoma.

The development of transgenic mice often results in an unusual phenotype due to forced expression of the TCR αβ-chain (25). In Fas-deficient mice, a significant number of T cells exhibit the DN phenotype (26); however, in the h3T strain the DN phenotype was more prevalent in the double-transgenic h3T-A2 mice that were on correct HLA-matched genetic background. Whether this phenotype is a result of coreceptor downregulation prior to positive selection or that TCR-expressing DN thymocytes bypassed the DP stage and were positively selected needs to be investigated. Notably, another founder animal obtained on C57BL/6 and HLA-A2 background showed the same phenotype of transgenic T cells (data not shown). This consistent set of data confirms that expression of this CD8-independent TCR on both CD4+ and CD8+ T cells and CD3+CD4−CD8− T cells is less likely a random phenomenon related to the forced expression of the human transgene in the mouse genome. Whereas the CD3+CD4−CD8− phenotype may seem to be physiologically less relevant for an effector T cell, peripheral blood mononuclear cells comprise 1–3% of such cells in a normal healthy individual. A recent study has used a novel protocol by which DN T cells can be expanded ex vivo to therapeutic levels in 2 wk from acute myeloid leukemia patients during chemotherapy and induced complete remission. The expanded DN T cells expressed similar or higher levels of IFN-γ, TNF-α, and granzyme B as that seen in bulk activated CD8 T cells from the same patient (27). Another study reported isolation of a human DN T cell clone recognizing a HLA-A2-restricted melanoma-associated antigenic gp100 peptide from the peripheral blood of a melanoma patient (28). Like h3T-A2-derived CD3+CD4−CD8− T cells, this study showed that gp100-specific DN T cell clone was able to confer Ag-specific cytotoxicity against gp100-pulsed target cells as well as HLA-A2+ gp100-expressing melanoma cells. In addition, it has also been shown recently that the CD8 coreceptor enhances susceptibility to TGF-β–mediated immune suppression (29). Thus, in the absence of the coreceptor, the CD4+CD8− T cells from h3T-A2 mice would be less susceptible to immunosuppression and would be able to control tumor growth more efficiently. Together, these data indicate that functionally active Ag-specific DN T cells recognizing MHC class I-restricted tumor-associated Ag contribute to antitumor immunity in vivo.

The immunogenic phenotype of CD4+CD8− T cells is contradictory to other reports that have attributed a regulatory phenotype to this unusual T cell population (30). In contrast, transgenic T cells from h3T-A2 mice with a CD4+CD8− phenotype did not secrete IL-4, IL-5, or IL-10 on Ag stimulation (data not shown). The development of spontaneous depigmentation has been attributed to either self-reactive CD4+ T cells (31) or CD8+ T cells (32). However, the h3T-A2 model carries predominantly αβTCR+CD3+CD4−CD8− T cells. Thus, the mere presence of a high-affinity human tyrosinase-specific functional TCR seems to be sufficient to mediate killing of melanocytes despite differences in anchor residues between human and mouse tyrosinase epitope. An earlier study that established a model using murine tyrosinase-derived homolog (EMDGTMSQV) of human tyrosinase-derived Ag (YMDGTMSQV) in transgenic mice expressing the HLA-A*0201 showed that murine peptide was naturally processed and presented in vivo by the HLA-A*0201 similarly to its human counterpart, and that this presentation leads to incomplete self-tolerance (33). The development of spontaneous depigmentation in our h3T-A2 mice suggests that T cells bearing the receptor reactive with YMDGTMSQV are nontolerant for the EMDGTMSQV, mouse tyrosinase peptide. Whether it is the high-affinity TCR or the loss of coreceptor in the positively selected immunogenic T cells in h3T-A2 mice that is responsible for the nontolerant phenotype is under investigation. It must be noted that despite the positive selection of the tyrosinase TCR in h3T strain, we did not observe any vitiligo in those animals as in case of the h3T-A2 mouse strain. However, these h3T splenocytes recognized HLA-A2+ mouse melanocytes in an in vitro assay. This implies that peripheral T cells recognized the murine tyrosinase Ag when presented in the right context (by HLA-A2+ APCs). Additionally, the loss of melanocytes from the hair follicles of depigmenting h3T-A2 mice was similar to loss of melanocytes from the skin as a hallmark of vitiligo (34). Whereas h3T-A2 mice develop spontaneous vitiligo, adoptive transfer of the splenocytes either in HLA-A2 or Rag-A2 recipient did not result in transfer of vitiligo despite the presence of transgenic T cells even after 90 d. This is in contrast to the FH transgenic mouse model that bears MHC class I-restricted murine tyrosinase TCR or MHC class II-restricted TCR transgenic mouse model in which CD4+ T cells recognize a novel epitope in TRP-1 (8). It is notable that despite the difference in anchor residues between murine and human tyrosinase epitopes, the TIL 1383I transgenic TCR-bearing T cells were able to recognize murine epitope, but vitiligo was still not adoptively transferable. This raises the possibility that other innate mechanisms could also be responsible for vitiligo development in h3T-A2 mice, or, besides adoptively transferring freshly isolated h3T-A2 splenocytes alone, additional stimuli might be required for development of vitiligo (8, 32).

Subsequently, the data obtained for ERG also demonstrate that h3T-A2 mice develop abnormalities in the retina function as compared with age-matched controls. The ERG data suggested that there is a marginal decrease in a-wave amplitudes, whereas major changes were seen in b-wave amplitudes of h3T-A2 mice. These results support the idea that the inner retina of h3T-A2 mice is more severely damaged than photoreceptors in the outer segment of the eye. The causative factors and cellular mechanisms that could be involved in the retina damage in h3T-A2 mice need further investigation. Various studies undertaken to understand the biological role of melanin in the retinal function have used genetically manipulated and spontaneous mutant animal models, for example, oculocutaneous albinism type 1 model that is characterized by congenital hypopigmentation due to a mutation in the tyrosinase gene. These studies in oculocutaneous albinism type 1 (Tryr−2) model have shown that several retinal functional abnormalities are associated with photoreceptor loss. Moreover, adeno-associated virus-mediated retinal gene delivery of the human tyrosinase gene was able to activate melanosome biogenesis and preserve retinal function (35). Tyrosinase-deficient mice lack pigmentation in melanocytes and RPE cells, which ultimately affects the retina function (36). Despite expressing a functional tyrosinase gene in h3T-A2 mice, spontaneous depigmentation due to melanocyte killing can be observed. Our ocular data show 34–65% loss in b-wave amplitudes in h3T-A2 mice when compared with the 1-y-old age-matched wild-type mice. The loss of retina function in h3T-A2 mice could not be only due to hypopigmentation of RPE, because such condition affects mainly outer segment photoreceptor activity (36). In our studies, photoreceptor activity (e.g., a-wave) was marginally affected in h3T-A2 mice, suggesting that RPE pigmentation does not play a key role to maintain retina function in h3T-A2 mice. It is reasonable to speculate that infiltrated T cells could have caused an increased accumulation of cytokines (e.g., TNF-α, IFN-γ, IL-1β) in the eye, which subsequently led to the retinal degeneration. We also observe complete occlusion of one eye in up to 5% of h3T-A2 mice by 14 wk of age. Further studies are required to determine comprehensively the...
mechanisms responsible for retinal degeneration and to address whether the noted defects are specific to tyrosinase TCR transgenic mice.

Traditionally, CD8+ T cytotoxic lymphocytes have been considered necessary for controlling tumor growth. Despite earlier reports that CD4+ T cells alone were capable of protecting experimental animals against solid tumors without providing any CD8+ T cells (37, 38), the role of CD4+ T cells has remained unappreciated. However, recent studies have shown that CD4+ T cells possess an intrinsic ability to orchestrate broad antitumor responses lacking in CD8+ T cells (39). That CD4+ TCR transgenic cells were capable of controlling tumor growth did not raise much discussion due to difference in TCR on CD8+ and CD4+ T cells used in another study (40), and issues about difference in class I and class II epitopes and TCR avidities were raised (41).

Lately, a few studies using class II-restricted gp75/TRP-1 transgenic CD4+ T cells have shown acquisition of cytotoxic activity in vivo and the ability to eradicate large established melanoma in lymphopenic host (8, 42). Our data show that the mere presence of adoptively transferred class I-restricted high-affinity TCR on either CD4+, CD8+, or CD4+ CD8+ −T cells is sufficient to control tumor growth both in immunodeficient SCID/beige recipients that lack T, B, and NK cells and in immunocompetent HLA-A2+ recipients. The persistence of human VB12+ transgenic T cells in mice even months after adoptive transfer is intriguing. Importantly, it should be noted that in our adoptive transfer experiments, TCR transgenic cells from h3T or h3T−A2 mice were neither stimulated ex vivo nor were the tumor-bearing animals given cytokines after T cell transfer for their sustained maintenance. This is in accordance with the study by Hunder et al. (43), who reported successful treatment of a patient with ex vivo generated CD4+ T cell clones recognizing tumor-associated Ag NY-ESO without IL-2 administration. Similarly, a study in mice revealed the capacity of CD4+ T cells to control tumor in the absence of any exogenous cytokine administration (20). Recently, TCR-αβ genes derived after amino acid modifications to increase cell surface expression and reactivity after gene transfer were also used to genetically engineer CD8+ and CD4+ T cells with class I-restricted TCR and had equal tumor regression in a murine melanoma model (44). However, our data suggest that irrespective of the presence or complete absence of CD4 and CD8 coreceptors, the mere presence of a high-affinity TIL 1383 TCR directs its ability to mount an effector immune response. We believe that the novel mouse models reported in this study will not only be useful in designing future tumor immunotherapy protocols, but will also help in understanding the unique thymic selection of the tyrosinase-specific high-affinity TIL 1383 TCR and TCR signaling issues of CD4 and CD8 T cells upon activation with the same Ag.

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Disclosures

The authors have no financial conflicts of interest.


Suppl. Figure 1
Suppl. Figure 2

A

B

C

Suppl. Figure 2
A

Mouse 1

Mouse 2

HLA-A2 with B16

HLA-A2 with B16-A2

h3T-A2 with B16

h3T-A2 with B16-A2

Tumor foci/lung

Weeks after tumor injection

Suppl. Figure 3

B

PBS

h3T

CTX + h3T

Tumor area (mm²)

Weeks after tumor injection
Suppl. Figure 4
Supplementary Figure Legends

**Supplementary Figure S1. Cytokine secretion in h3T mouse T cells.** A) h3T splenocytes were co-cultured overnight with or without human or mouse tyrosinase peptide-pulsed T2 cells. TNF-α and GM-CSF were measured in supernatants by ELISA. B). Vβ12⁺CD4⁺ and Vβ12⁺CD8⁺ T cells were sorted from h3T mouse and co-cultured overnight with T2 cells that were previously pulsed with different concentrations of human tyrosinase peptide. MART-1_{27-35} peptide-pulsed T2 cells were used as control. The concentration of IFN-γ and IL-2 secreted into the culture medium was measured by ELISA.

**Supplementary Figure S2. Co-receptor distribution and cytokine secretion in h3T-A2 mouse T cells.** A). Splenocytes from h3T-A2 mice were stained with fluorochrome conjugated human anti-Vβ12, mouse anti-CD3, anti-CD4 and anti-CD8 antibody. Dot plots on the right panel shows CD4 and CD8 expression on gated CD3⁺Vβ12⁺ and CD3⁺Vβ12⁻ T cell subset. B). h3T-A2 splenocytes were co-cultured overnight with or without human or mouse tyrosinase peptide-pulsed T2 cells. TNF-α and GM-CSF were measured in supernatant by ELISA. (C). Rag-A2 mice received an intravenous adoptive transfer of 1.0 X 10⁶ Vβ12⁺ freshly isolated splenocytes from h3T or h3T-A2 mice. Three months after adoptive T cell transfer, the numbers of Vβ12⁺ cells were determined in peripheral blood of recipient mice.

**Supplementary Figure S3. In vivo anti-tumor efficacy of h3T A2 cells.** (A). Murine melanoma B16 and B16-A2 (2.5 X 10⁵) were intravenously injected into HLA-A2 and h3T-A2 mice. Mice were sacrificed after 21 days and tumor foci were evaluated in the lungs. Left panel shows representative picture of two lungs from each group. Right panel shows the mean number of tumor foci counted per lung (n=5/group). (B). HLA-A2 mice (n = 4/group) were inoculated (sc) with 2.5 x 10⁵ murine B16-A2 melanoma cells and palpable tumors were treated with or without cyclophosphamide (CTX, 4
mg/mouse). Mice that either received CTX or not were adoptively transferred one day later with 1.0 x 10^6 fresh Vβ12+ cells harvested from h3T mice spleens. Tumor sizes were then recorded at the indicated time points. Experiment was repeated twice times with similar results.

**Supplementary Figure S4. Persistence of Vβ12+ T cell subsets.** (A). HLA-A2 mice were subcutaneously injected with 2.5 X 10^5 B16A2 cells and palpable tumors were treated with cyclophosphamide (4 mg/mouse). One day after chemotherapy, mice were left untreated or received an intravenous adoptive transfer of 1.5 X 10^6 Vβ12+ freshly isolated splenocytes from h3T or h3T-A2 mice. The frequency (upper panel) and phenotype (lower panel) of Vβ12+ cells was assessed in spleens harvested seven weeks after adoptive T cell transfer. (B). SCID/beige mice were subcutaneously injected with 5 X 10^6 624 MEL cells and palpable tumors were treated by intravenous adoptive transfer of freshly sorted h3T CD4+Vβ12+, h3T CD8+Vβ12+, or h3T-A2 CD4-CD8-Vβ12+ T cells. Three months after adoptive T cell transfer, the numbers of Vβ12+ cells were determined in spleens of recipient mice.