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RESEARCH ARTICLE

The development and functions of CD4$^+$ T cells expressing a transgenic TCR specific for an MHC-I-restricted tumor antigenic epitope

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It has been reported that the ratio of CD4$^+$ to CD8$^+$ T cells has no bias in a few class I major histocompatibility complex (MHC-I)-restricted T-cell receptor (TCR)-transgenic mice specific for alloantigens or autoantigens, in which most CD4$^+$ T cells express an MHC-I-restricted TCR. In this study, we further showed that more than 50% of CD4$^+$ T cells in MHC-I-restricted P1A tumor antigen-specific TCR (P1ATCR)-transgenic mice could specifically bind to MHC-I/P1A peptide complex. P1A peptide could stimulate the transgenic CD4$^+$ T cells to proliferate and secrete both type 1 helper T cell and type 2 helper T cell cytokines. The activated CD4$^+$ T cells also showed cytotoxicity against P1A-expressing tumor cells. The analysis of TCR α-chains showed that these CD4$^+$ T cells were selected by co-expressing endogenous TCRs. Our results show that CD4$^+$ T cells from P1ATCR transgenic mice co-expressed an MHC-I-restricted transgenic TCR and another rearranged endogenous TCRs, both of which were functional.
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INTRODUCTION

Due to the process of allelic exclusion, the development of T cells is skewed to the CD8$^+$ lineage in most class I major histocompatibility complex (MHC-I)-restricted T-cell receptor (TCR)-transgenic mouse lines, leading to the accumulation of single-positive CD8$^+$ lymphocytes in the thymus and periphery.[1–4] However, exceptions have been reported in MHC-I-restricted alloantigen-[5] and autoantigen-specific[6,7] TCR-transgenic mice, in which there was no differentiation bias for CD8$^+$ T cells. In these transgenic mice, most of the CD4$^+$ T cells expressed an MHC-I-restricted transgenic TCR, which could be detected by specific anti-TCR antibodies, and an additional rearranged endogenous MHC-II-restricted TCR through which the CD4$^+$ T cells could be positively selected to maintain a normal ratio of CD4$^+$ to CD8$^+$ T cells. Moreover, it was found that the CD4$^+$ T cells could be activated through the transgenic TCR or the endogenous TCR by antigen-expressing cells in an MHC-I-restricted alloantigen-specific TCR-transgenic mouse.[5]

CD8 is critical for T-cell recognition of the MHC-I/peptide complex. By binding to MHC-I molecules on antigen-presenting cells, CD8 can strengthen MHC-I-restricted TCR signaling.[8,9] Although it was discovered by antibody staining in several MHC-I-restricted TCR-transgenic mice that the CD4$^+$ T cells expressed MHC-I-restricted transgenic TCRs,[5,6,7] so far, there has not been direct proof to determine whether the MHC-I-restricted transgenic TCRs on the CD4$^+$ T cells bind to the MHC-I/peptide complex.

P1A was the first unmutated tumor antigen identified in mice, which is expressed at high levels in mouse tumor cell lines (such as mastocytoma P815 and plasmacytoma J558) and in immunologically privileged tissues (placenta and testis) and at a low but significant level in normal tissues including hematopoietic tissues.[10–12] An H-2L^d-restricted dominant epitope was identified as P1A$_{35–43}$ nonapeptide (LPYLGWLVF).[13] Although P1A is expressed at low levels in normal tissues, including lymphoid tissues, the P1A-specific TCR (Vα8.3/Vβ1)-transgenic CD8$^+$ T cells develop normally in P1A tumor antigen-specific TCR (P1ATCR)-transgenic mice and remain highly responsive to the P1A antigen.[12] Most of CD4$^+$ T cells also express the MHC-I-restricted transgenic TCR, but the L^d/P1A tetramer could only stain the transgenic CD8$^+$ T cells, not CD4$^+$ T cells.[7] In our study, more than a half of the CD4$^+$ T cells from P1ATCR-transgenic DBA/2 mice could be stained by an L^d/P1A dimer when the amount of dimer for staining was increased to three- to fourfold of the usual dose. Moreover, the P1A peptide could stimulate the CD4$^+$ T cells to show both helper and cytotoxic functions.

MATERIALS AND METHODS

Mice and cell lines

Transgenic mice expressing a TCR specific for the H-2L^d-restricted P1A tumor antigen peptide (P1A$_{35–43}$: LPYLGWLVF) in the BALB/c background have been described previously[12] and backcrossed with

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the DBA/2 background for more than 10 generations. Heterozygous male transgenic mice were crossed with female DBA/2 mice (Vitalriver Experimental Animal Company, Beijing, China) for breeding. The offspring were genotyped by flow cytometry analysis of the expression of the transgenic TCR Vα8.3 chain on $CD8^+$ T cells in peripheral blood mononuclear cells. All of the mice were maintained under specific pathogen-free conditions in the animal facility at the Institute of Biophysics, Chinese Academy of Sciences. All studies involving animals were approved by the Institutional Laboratory Animal Care and Use Committee.

The P815 mastocytoma cell line and the CT26 colon tumor cell line were maintained in complete medium of RPMI 1640 (Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (Thermo Fisher, Beijing, China), 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 2 mM *L*-glutamine, 1 mM sodium pyruvate, 100 U/ml penicillin G and 100 μg/ml streptomycin sulfate.

Flow cytometry analysis

Anti-CD3, anti-CD8, anti-CD4, anti-Vα2 (eBioscience, San Diego, CA, USA), anti-TCR Vα8.3, anti-Vα8 (BD Pharmingen, San Diego, CA, USA) and anti-Vα11.1/11.2 (BioLegend, San Diego, CA, USA) monoclonal antibodies were used for flow cytometry analysis. H-$2L^d$ Dimer XI was purchased from BD Biosciences (San Diego, CA, USA), and $P1A_{35–43}$ peptide (LPYLGWLVF) and an L^d-restricted control peptide HBV $ENV_{28–39}$ (IPQSLDSWWTSL) were synthesized by GL Biochem Ltd (Shanghai, China). The peptides were incorporated into the dimer according to the manufacturer's instructions. For the blockade of dimer staining, L^d/P1A dimer was incubated with anti-H-$2L^d$ antibody (28-14-8 clone; BD Pharmingen) for 20 min before being added to cells.

Reverse transcription polymerase chain reaction (RT-PCR)

$CD4^+$ T cells in the lymph nodes of P1ATCR-transgenic mice and non-transgenic littermates were stained with the L^d/P1A dimer. The dimer-positive and dimer-negative $CD4^+$ T cells were sorted by making two gates far away from each other to exclude contamination. Total RNA was extracted with TRI reagent (Applied Biosystems, Foster City, CA, USA) and reverse transcribed into cDNA with reverse transcriptase and an oligo-dT primer (Transgene, Beijing, China). To quantitate the cDNA, threefold serial dilutions of the cDNA reactions were subjected to PCR using primers specific for the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase for 35 cycles of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min on a Peltier Thermal Cycler (MJ Research, Waltham, MA, USA). Similar amounts of cDNA were then subjected to PCR using a conserved Cα- or Cβ-specific primer paired with a Vα family-specific primer or Vβ1-specific primer for 35 cycles of 94 °C for 1 min, 55 °C for 1min and 72 °C for 1 min. The primers were chosen according to a previous report.[14] PCR reaction products were electrophoresed on 1.2% agarose gels.

***In vitro* T-cell activation and cytotoxicity assays**

Pooled lymph node and spleen cells were depleted of $CD8^+$ or $CD4^+$ cells using magnetic beads according to the manufacturer's instructions (Miltenyi Biotec, Bergisch Gladbach, Germany) and then labeled with 5 μM carboxyfluorescein succinimidyl ester (CFSE) and stimulated with $P1A_{35–43}$ peptide at different concentrations for 1–3 days. Cytokines in the culture supernatant were detected using the Cytometric Bead Array Mouse Th1/Th2 Cytokine Kit or Inflammation Kit (BD Biosciences) according to the manufacturer's instructions. Two days after stimulation, the stimulated cells were treated with Brefeldin A for 5 h for interferon (IFN)-γ intracellular staining according to the manufacturer's instructions (eBioscience).

The $CD4^+$- or $CD8^+$-depleted spleen and lymph node cells were activated with $P1A_{35–43}$ peptide-pulsed isolated dendritic cells for 3 days and purified by Ficoll. P815 ($P1A^+$ and H-$2L^{d+}$) tumor cells stained with a 5 μM CFSE solution were used as specific target cells, and CT26 ($P1A^-$ and H-$2L^{d+}$) tumor cells stained with a 0.5 μM CFSE solution ($CFSE^{low}$) were used as nonspecific target cells. A mixture of P815 and CT26 tumor cells at a 1 : 1 ratio was incubated with activated $CD8^+$ or $CD4^+$ T cells at different E/T ratios in a round-bottom 96-well plate for 16 h. The cells were harvested and stained with 7-aminoactinomycin D (7-ADD). The CFSE profiles were analyzed using the 7-AAD^-CFSE^+ gate. Cytotoxicity was determined by the following formula: 1-Ratio $(P815/CT26)_{+effector}$/Ratio $(P815/CT26)_{-effector}$. The results are representative of three independent experiments.

Alloresponse

Lymph node cells from P1ATCR-transgenic and non-transgenic DBA/2 mice were labeled with 5 μM CFSE and cocultured with irradiated $CD11c^+$ cells isolated from the splenocytes of C57BL/6 mice by magnetic-activated cell sorting at different ratios for 5 days. The proliferation of $CD4^+$ T cells as monitored by CFSE dilution.

RESULTS

$CD4^+$ T cells expressing an MHC-I-restricted transgenic T-cell receptor (TgTCR) bind to the MHC-I/peptide complex

It has been reported that the $CD4^+$ T cells from P1ATCR-transgenic mice cannot be stained by the L^d/P1A tetramer, even most of these cells express the TgTCR.[5] In our study, we found that approximately 90% of $CD8^+$ T cells were L^d/P1A dimer-positive, while a few $CD4^+$ T cells were weakly stained by the L^d/P1A dimer. However, approximately 60% of $CD4^+$ T cells from P1ATCR-transgenic DBA/2 mice could be strongly stained by the L^d/P1A dimer when the amount of dimer was increased by two- to threefold. The mean fluorescence intensity value of dimer staining increased as the $CD4^+$ T cells were stained with more dimer, but the frequency of positive cells did not increase any further (Figure 1a). The binding of the L^d/P1A dimer to the transgenic $CD4^+$ T cells was specific because the binding could be completely blocked by an anti-H-$2L^d$ monoclonal antibody. In addition, the L^d/P1A dimer could not bind to $CD4^+$ T cells in non-transgenic littermates (Figure 1b).

To determine whether the dimer-negative $CD4^+$ T cells in P1ATCR-transgenic mice express the P1ATCR transgenes, we used RT-PCR to detect the expression levels of the transgenic TCR Vα8 and Vβ1 chains in dimer-positive and dimer-negative $CD4^+$ T cells. As shown in Figure 1c, the Vβ1 chain was expressed at similar levels in dimer-positive and dimer-negative $CD4^+$ T cells, but dimer-positive $CD4^+$ T cells displayed higher expression of the TCR Vα8 chain. To confirm the difference in the Vα8 chain expression on the surface of the $CD4^+$ T cells, we doubly stained $CD4^+$ T cells from the transgenic mice with the L^d/P1A dimer and an anti-Vα8 antibody. Consistent with the RT-PCR result, the cells in the $CD4^+$ $L^d/P1A^+$ gate had a higher mean fluorescence index for anti-Vα8 staining compared with those cells in $CD4^+$ $L^d/P1A^-$ gate (Figure 1d). These results clearly show that more than a half of the transgenic $CD4^+$ T cells expressed a high level of the Vα8.3–Vβ1 TCR, which could bind to the L^d/P1A complex.

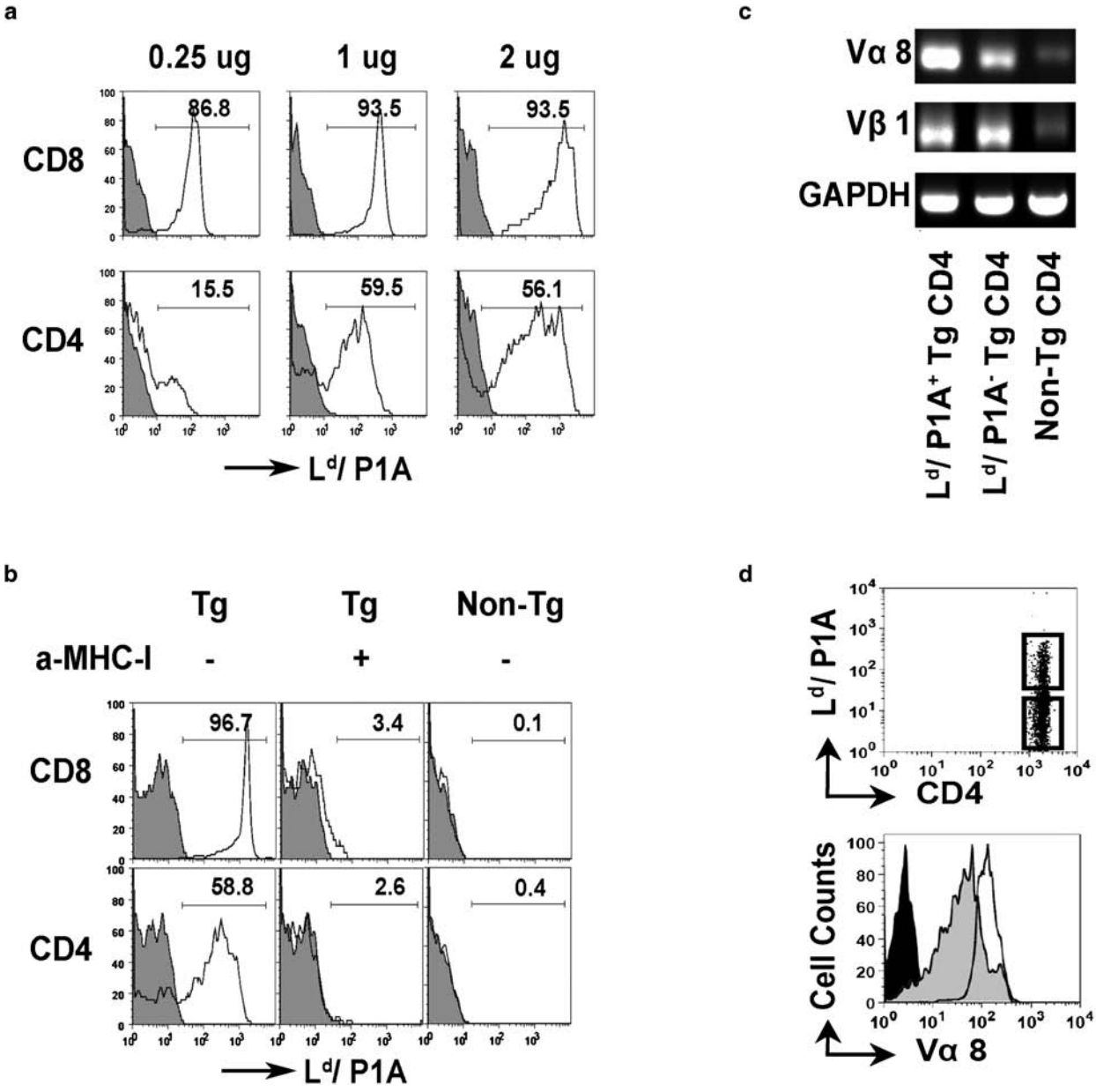

Figure 1 Detection of MHC-I-restricted TgTCR on CD4$^+$ T cells in P1ATCR-transgenic mice. (**a**, **b**) Lymph node cells (1×10^6) from P1ATCR-transgenic or non-transgenic DBA/2 mice were stained with the indicated amounts of L^d/P1A (open) or control L^d/HBV (filled) dimer, anti-CD3, anti-CD4 and anti-CD8 mAb simultaneously. (**a**) Binding of L^d/P1A dimer to CD4$^+$ and CD8$^+$ T cells was analyzed; (**b**) 1 μg of L^d/P1A (open) or control L^d/HBV (filled) dimer was incubated with anti-H-2L^d before staining. The values are the percentages of L^d/P1A dimer-positive cells. (**c**) Expression of TCR Vα8 was detected by RT-PCR in L^d/P1A$^+$, L^d/P1A$^-$ P1ATCR-transgenic CD4$^+$ T cells and non-transgenic CD4$^+$ T cells. GAPDH were used as an internal reference. (**d**) Expression levels of TCR Vα8 transgene on L^d/P1A dimer-positive (open) and dimer-negative T cells (filled, grey) were detected by anti-TCR Vα8 antibody. Rat IgG was used for the isotype control (filled, black). The results are representative of at least three independent experiments. GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IgG, immunoglobulin G; mAb, monoclonal antibody; MHC, major histocompatibility complex; P1ATCR, P1A tumor antigen-specific T-cell receptor; TgTCR, transgenic T-cell receptor; TCR, T-cell receptor.

MHC-I-restricted TgTCR signals CD4$^+$ T cells to produce cytotoxic activity in addition to helper functions

Because more than a half of the CD4$^+$ T cells in P1ATCR-transgenic mice bind to L^d/P1A dimer, we wondered whether the P1A peptide could stimulate CD4$^+$ T cell activation. First, we labeled lymph node and spleen cells from P1ATCR-transgenic DBA/2 mice with CFSE and stimulated the cells with different concentrations of the $P1A_{35–43}$ peptide. As shown in Figure 2a, both the CD8$^+$ and CD4$^+$ T cells of P1ATCR-transgenic mice could proliferate under the stimulation of $P1A_{35–43}$ peptide. However, the activation of CD4$^+$ T cells required a higher concentration of the peptide. CD8$^+$ T cells could proliferate when the peptide was at a concentration of 0.01 μg/ml, but the CD4$^+$ T cells did not proliferate until the concentration of the peptide was increased to 0.1 μg/ml. To exclude the possibility that the proliferation of CD4$^+$ T cells was a bystander response resulting from the activation of CD8$^+$ T cells, we depleted CD8$^+$ T cells from the lymph node and spleen cells of P1ATCR-transgenic mice. As shown in Figure 2b, CD4$^+$ T cells could be similarly stimulated to proliferate by $P1A_{35–43}$ peptide

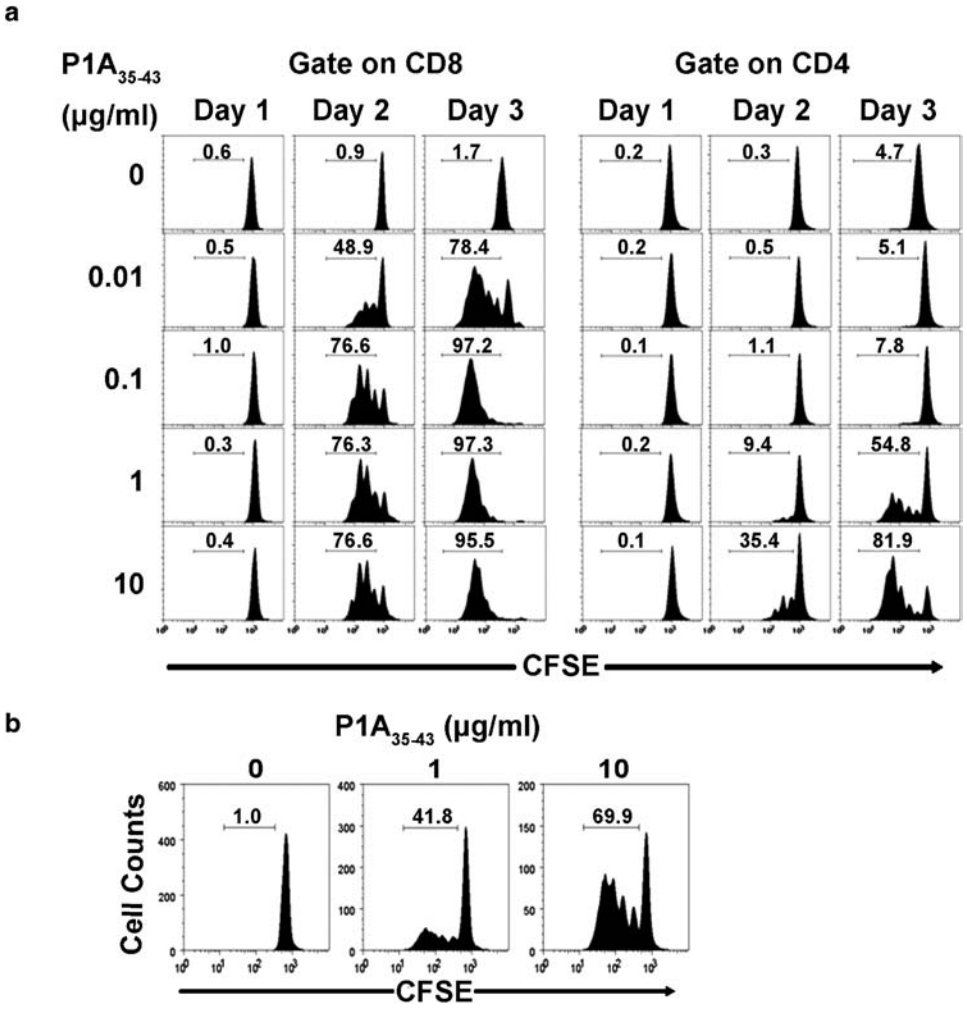

Figure 2 P1A peptide stimulates the proliferation of P1ATCR-transgenic CD4+ T cells. Lymph node and spleen cells from P1ATCR-transgenic DBA/2 mice were labeled with CFSE and stimulated with the indicated concentrations of P1A peptide for 3 days. (**a**) Proliferation, evaluated using CFSE, was analyzed for gated CD8+ (left) and CD4+ (right) T cells. (**b**) CD8-depleted lymph node and spleen cells were stimulated with the indicated concentrations of P1A peptide, and the proliferation of CD4+ T cells was analyzed. The numbers above the gating bars depict the percentages of proliferated cells. The results are representative of at least three independent experiments. CFSE, carboxyfluorescein succinimidyl ester; P1ATCR, P1A tumor antigen-specific T-cell receptor.

in the absence of CD8+ T cells. These results indicate that the MHC-I-restricted TgTCR could deliver signals to CD4+ T cells, although the strength of the CD4+ T cell response was much lower compared to that of CD8+ T cells. The lower response of CD4+ T cells was consistent with the lower avidity of CD4+ T cells for the L^d/P1A dimer compared to that of the CD8+ counterpart.

Next, we compared the cytokine production of CD4+ T cells with that of CD8+ T cells by depleting CD8+ or CD4+ T cells from the lymph node and spleen cells of P1ATCR-transgenic mice and stimulating the remaining cells with $P1A_{35–43}$ peptide. As shown in Figure 3a, both the non-CD8+ and non-CD4+ populations of the lymph node and spleen cells could be stimulated with P1A peptide to secrete IFN-γ, TNF-α, IL-2 and IL-6. However, the cytokine production of the CD4+ T cells was much lower than that of CD8+ T cells. The production of IFN-γ by CD4+ T cells was confirmed by intracellular staining (Figure 3b). In addition, the activated CD4+ T cells also produced Th2 cytokines including IL-4, IL-5 and IL-10, which could hardly be detected in non-CD4+ cells.

Last, we checked whether this population of activated CD4+ T cells had cytotoxic activity against tumor cells expressing P1A antigen. We performed an *in vitro* killing assay using a mixture of P815 (H-2^d, P1A+ and CFSE^{hi}) and CT26 (H-2^d, P1A− and CFSE^{low}) tumor cells as targets. As shown in Figure 3c, the CD4+ T cells activated with P1A peptide were able to kill P815 tumor cells, although the activity was much lower than that of activated CD8+ T cells. Because P815 tumor cells do not express MHC-II molecules,[15] the killing activity of CD4+ T cells should be MHC-I-mediated. These results show that CD4+ T cells acquired both helper and cytotoxic functions through MHC-I TCR signaling.

Development of the transgenic CD4+ T cells requires rearranged endogenous TCRs, which are functional

The P1ATCR-transgenic mice in a RAG-$1^{-/-}$ B10.D2 background[7] and a RAG-$1^{-/-}$ Balb/c background (data not shown) do not have CD4+ T cells, indicating that the transgenic CD4+ T cells are selected through co-expression of a second rearranged TCR. To further analyze the dual TCRs on the CD4+ T cells in P1ATCR-transgenic DBA/2 mice, we randomly tested the expression of TCR Vα1, 2, 3, 4 and 5 in transgenic CD4+ T cells by RT-PCR. As shown in Figure 4a, both L^d/P1A dimer-positive and dimer-negative CD4+ T cells also

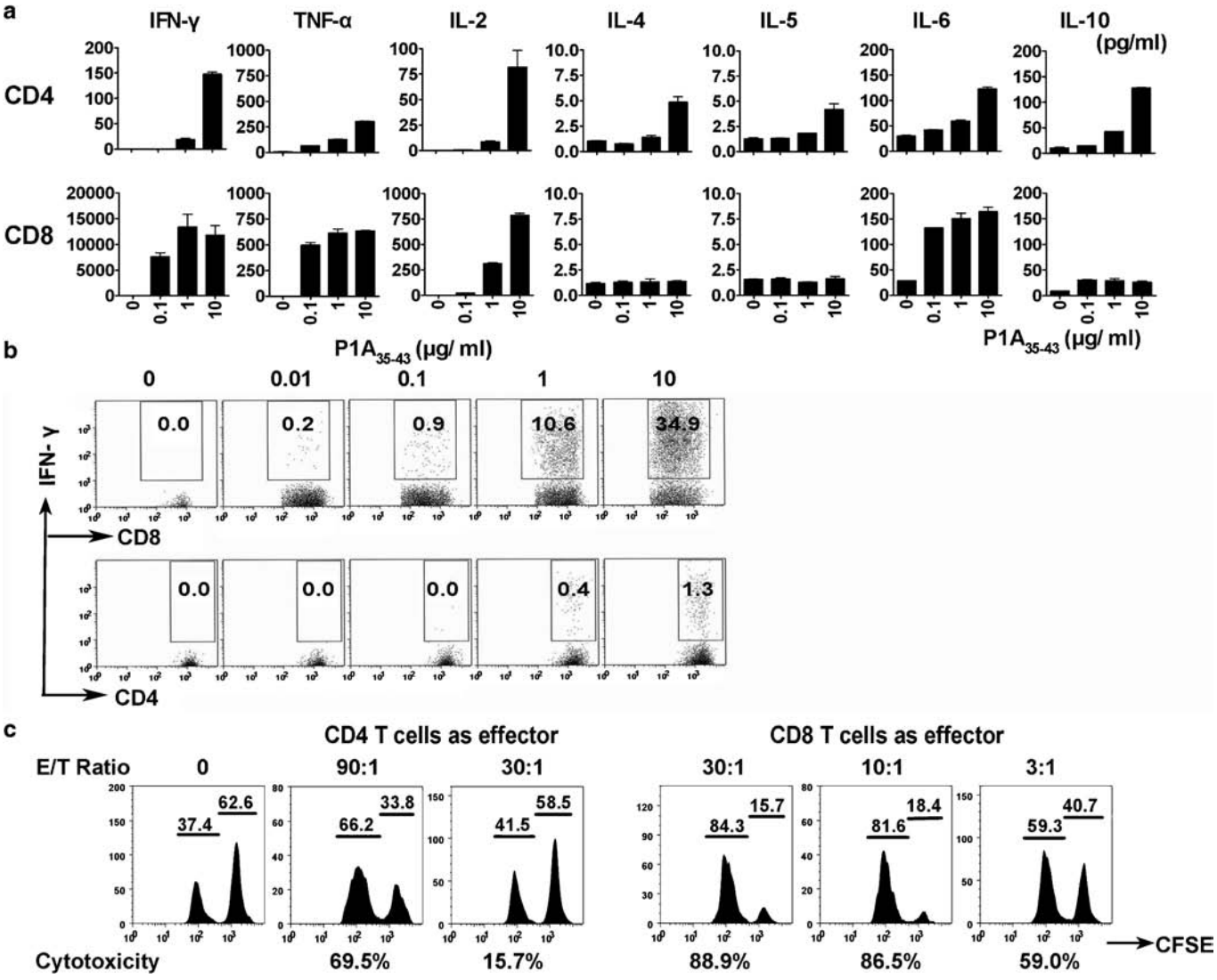

Figure 3 Cytokine production and P1A-specific cytotoxicity of P1ATCR-transgenic CD4$^+$ and CD8$^+$ T cells stimulated with P1A peptide. CD8- or CD4-depleted splenocytes from P1ATCR-transgenic DBA/2 mice were stimulated with the indicated concentrations of P1A peptide for 3 days. (**a**) Cytokines in the supernatants were detected using Cytometric Bead Array Kits. The values in the graphs indicate the mean±s.e.m. values of triplicates. (**b**) The expression of IFN-γ by CD8$^+$ or CD4$^+$ T cells was analyzed by intracellular staining after stimulation with the indicated concentrations of P1A peptide for 2 days. The percentages of IFN-γ$^+$ cells among the gated CD4$^+$ and CD8$^+$ T cells are shown. (**c**) Activated CD8$^+$ or CD4$^+$ splenocytes were incubated at the indicated E/T ratios with CFSE-labeled specific target P815 cells (P1A$^+$ and H-2L^{d+}) and nonspecific target CT26 cells (P1A$^-$ and H-2L^{d+}). After 16 h, the CFSE profiles were analyzed in 7-AAD$^-$CFSE$^+$ cells to determine the level of cytotoxicity. The numbers above the gating bars indicate the percentages of CT26 cells (left) and P815 cells (right) among the total 7-AAD$^-$CFSE$^+$ cells in each panel. The results are representative of at least three independent experiments.

expressed TCR Vα1, 2, 3, 4 and 5 genes in addition to the TCR Vα8 transgene. Although the CD4$^+$ T cells from transgenic mice expressed a much higher level of the TCR Vα8 transgene, the expression pattern of endogenous TCR α-chains at the RNA level was similar to those in normal CD4$^+$ T cells except that L^d/P1A dimer-negative CD4$^+$ T cells expressed a higher level of the Vα1 chain and a lower level of the Vα2 chain. It has been reported that allelic exclusion of the TCR α chain is mainly maintained by a post-translational mechanism,[16,17] so we next detected the surface expression of TCR α-chains on transgenic CD4$^+$ T cells. Because of the limited types of monoclonal antibodies to mouse TCR Vα chains, we could only determine the expression levels of Vα2 and Vα11. As shown in Figure 4b, 17.5% of CD4$^+$ T cells expressed both the Vα2 and Vα8.3 chains, and 94.2% of Vα2$^+$CD4$^+$ T cells co-expressed Vα8.3. Almost all Vα11$^+$CD4$^+$ T cells co-expressed Vα8.3, although these cells were a very small population of the CD4$^+$ T cells (0.7% of CD4$^+$ T cells). Although more than 90% of the CD4$^+$ T cells in the transgenic mice expressed the TCR Vα8.3 chain, they expressed the Vα2 chain in a comparable frequency to that in non-transgenic mice (Figure 4c). Thus, the transgenic CD4$^+$ T cells co-expressed an MHC-I-restricted TgTCR as well as a rearranged endogenous TCR, and the latter might permit positive selection of CD4$^+$ T cells on MHC-II products.

To test the function of the endogenous TCR, we used an allore-sponse assay to compare the polyclonal response of transgenic CD4$^+$ T cells to alloantigen with that of normal CD4$^+$ T cells. Lymph node cells from P1ATCR-transgenic DBA/2 mice and littermates were labeled by CFSE and cocultured with irradiated isolated CD11c$^+$ cells from C57BL/6 mice. As shown in Figure 5, the transgenic CD4$^+$ T cells

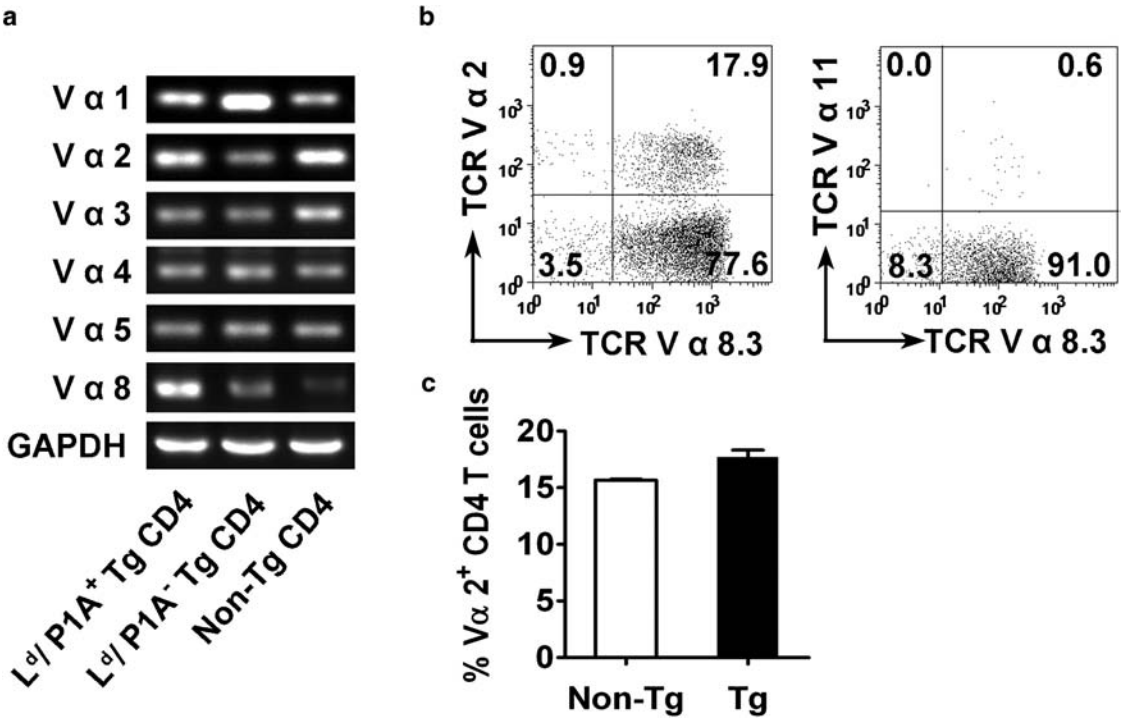

Figure 4 Analysis of endogenous TCRs in P1ATCR-transgenic CD4$^+$ T cells. (**a**) RT-PCR was performed with 1×10^6 sorted L^d/P1A$^+$ and L^d/P1A$^-$CD4$^+$ P1ATCR-transgenic T cells and non-transgenic CD4$^+$ T cells using a conserved Cα-specific primer paired with Vα1-, 2-, 3-, 4-, 5- and 8-specific primers. PCR products were electrophoresed on a 1.2% agarose gel. (**b**) CD4$^+$ T cells were stained with anti-TCR Vα2 and Vα8.3 mAbs (left) or anti-TCR Vα11.1/11.2 and Vα8.3 mAbs (right). (**c**) The frequencies of Vα2$^+$CD4$^+$ T cells among the total CD4$^+$ T cell population of PBMCs from transgenic and non-transgenic DBA/2 mice. The statistical results show the mean±s.e.m. values of five mice for each group. The results are representative of at least three independent experiments. GAPDH, glyceraldehyde 3-phosphate dehydrogenase; mAb, monoclonal antibody; P1ATCR, P1A tumor antigen-specific T-cell receptor; PBMC, peripheral blood mononuclear cell; RT-PCR, reverse transcription polymerase chain reaction; TCR, T-cell receptor.

responded to the H-2^b alloantigen in a manner similar to that of the normal CD4$^+$ T cells, although the alloresponse of L^d/P1A$^+$ CD4$^+$ T cells was slightly weaker than those of L^d/P1A$^-$ transgenic CD4$^+$ T cells and normal CD4$^+$ T cells. These results suggest that endogenous TCRs could also deliver antigen-specific signals to these TCR-transgenic CD4$^+$ T cells. Thus, both the transgenic and the endogenous TCRs on P1ATCR-transgenic CD4$^+$ T cells were functional.

DISCUSSION

Different from most MHC-I-restricted TCR-transgenic mice,[1–4] the P1ATCR-transgenic mice in the DBA/2 background contain a normal percentage of CD4$^+$ T cells in the periphery. Although most of the CD4$^+$ T cells express the MHC-I-restricted transgenic TCR, Shanker *et al.* could not stain the CD4$^+$ T cells with the L^d/P1A tetramer.[7] In our studies, we also found that few CD4$^+$ T cells were weakly positive for L^d/P1A dimer staining using a normal dose at which most CD8$^+$ T cells were strongly positive. However, when a quadruple dose of dimer was used, more than 50% of the CD4$^+$ T cells became L^d/P1A dimer-positive in the periphery of P1ATCR-transgenic DBA/2 mice. All of the transgenic CD4$^+$ T cells expressed the same level of the TCR Vβ1 transgene. Compared to the L^d/P1A dimer-positive CD4$^+$ T cells, the dimer-negative CD4$^+$ T cells expressed a lower level of the TCR Vα8 transgene at both the RNA and surface-protein levels, which might decrease these cells' TCR avidity to the L^d/P1A complex.

Furthermore, the P1A peptide could stimulate the activation of the transgenic CD4$^+$ T cells. Under the stimulation of the P1A peptide, both P1ATCR-transgenic CD8$^+$ and CD4$^+$ T cells could secrete IL-2, IFN-γ, tumor-necrosis factor-α and IL-6, but only the CD4$^+$ T cells produced IL-4, IL-5 and IL-10. Meanwhile, the activated CD4$^+$ T cells also showed specific cytotoxic activity to P815 tumor cells. Compared to those of CD8$^+$ T cells, the CD4$^+$ T cell responses were much lower, which was reflected by the need for a higher concentration of peptide for stimulation. This need for a higher peptide concentration may result from a weaker interaction between the TCR and the MHC-I/peptide complex without CD8$^+$ help or/and a less condensed transgenic TCR expressed by CD4$^+$ T cells than by CD8$^+$ T cells.

Because allelic exclusion, particularly for the TCR α-chain, is an incomplete process during thymic selection,[16,17] transgenic TCR-positive CD4$^+$ T cells might be selected through co-expression of a second rearranged endogenous TCR, a hypothesis was supported by the fact that CD4$^+$ T cells could not be detected in P1ATCR-transgenic mice crossed with the Rag1$^{-/-}$ background. To directly analyze the second endogenous TCR on the CD4$^+$ T cells, we detected the expression of TCR Vα2 and TCR Vα11 on the CD4$^+$ T cells by antibody staining and the expression of the Vα1-5 chains by RT-PCR. Although most CD4$^+$ T cells in P1ATCR-transgenic mice highly expressed the TCR Vα8 transgene, they expressed a pattern of TCR Vα1, 2, 3, 4 and 5 genes similar to that in normal mice. Ninety-five percent of Vα2$^+$ and 100% of Vα11$^+$CD4$^+$ T cells simultaneously expressed Vα8.3 transgenes. These results strongly suggest that most CD4$^+$ T cells in P1ATCR-transgenic mice expressed, in addition to the TCR

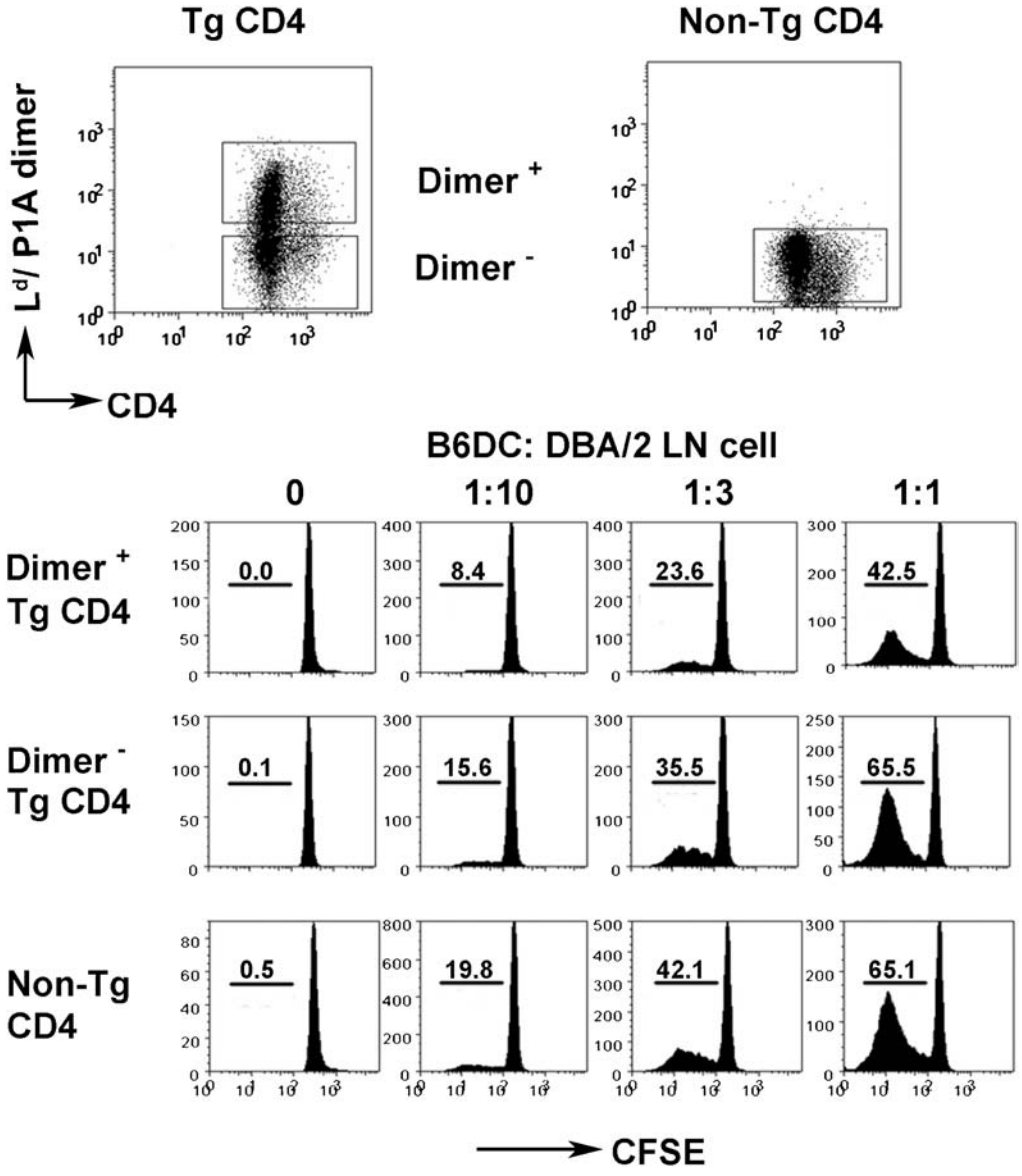

Figure 5 Endogenous TCRs on P1ATCR-transgenic CD4+ T cells are functional. Lymph node cells from P1ATCR-transgenic and non-transgenic DBA/2 mice were labeled with CFSE and cocultured with irradiated CD11c+ splenocytes isolated from C57BL/6 mice at indicated ratios for 5 days. The cells were harvested and stained with anti-CD4 and Ld/P1A dimer. The CFSE profiles were analyzed for gated transgenic Ld/P1A+CD4+ cells, Ld/P1A−CD4+ cells and non-transgenic CD4+ cells. The numbers beside the CFSE histograms indicate the percentages of proliferating cells. The results are representative of at least two independent experiments. CFSE, carboxyfluorescein succinimidyl ester; P1ATCR, P1A tumor antigen-specific T-cell receptor; TCR, T-cell receptor.

Vα8.3 transgene, an endogenous TCR through which they were positively selected.

ACKNOWLEDGEMENTS

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