Isolation of TCR genes with tumor-killing activity from tumor-infiltrating and circulating lymphocytes in a tumor rejection cynomolgus macaque model

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To develop effective adoptive cell transfer therapy using T cell receptor (TCR)-engineered T cells, it is critical to isolate tumor-reactive TCRs that have potent anti-tumor activity. In humans, tumor-infiltrating lymphocytes (TILs) have been reported to contain CD8+PD-1+ T cells that express tumor-reactive TCRs. Characterization of tumor reactivity of TILs from non-human primate tumors could improve anti-tumor activity of TCR-engineered T cells in preclinical research. In this study, we sought to isolate TCR genes from CD8+PD-1+ T cells among TILs in a cynomolgus macaque model of tumor transplantation in which the tumors were infiltrated with CD8+ T cells and were eventually rejected. We analyzed the repertoire of TCRα and β pairs obtained from single CD8+PD-1+ T cells in TILs and circulating lymphocytes and identified multiple TCR pairs with high frequency, suggesting that T cells expressing these recurrent TCRs were clonally expanded in response to tumor cells. We further showed that the recurrent TCRs exhibited cytotoxic activity to tumor cells _in vitro_ and potent anti-tumor activity in mice transplanted with tumor cells. These results imply that this tumor transplantation macaque model recapitulates key features of human TILs and can serve as a platform toward preclinical studies of non-human primate tumor models.

INTRODUCTION

Immune checkpoint blockade therapies have shown effectiveness in treating human cancers with high levels of somatic mutations.[1,2] In addition, adoptive transfer of tumor-specific lymphocytes or T cell receptor (TCR)-engineered T cells has mediated effective anti-tumor responses in several types of cancer.[3,4] To develop effective adoptive cell transfer therapy using TCR-engineered T cells, it is critical to isolate tumor-reactive TCRs that have potent anti-tumor activity. Several studies have shown that neoantigens derived from somatic mutations could activate and enrich tumor-reactive T cells, which could mediate objective clinical responses.[5–7] In humans, tumor-infiltrating lymphocytes (TILs) have been reported to contain CD8+ T cells (cytotoxic T

lymphocytes, CTLs) that are reactive with mutated tumor-associated antigens.[8–11] Indeed, TILs isolated from patients can be expanded _ex vivo_ and used for adoptive T cell therapies, which could mediate objective clinical responses.[6,12–14] In particular, PD-1 and/or CD137 (4-1BB)-positive T cells in human TILs have shown to express tumor-reactive TCRs against shared tumor antigens and neoantigens.[15–17] However, it has been shown that PD-1-positive T cells in TILs are exhausted and functionally impaired.[15] Furthermore, some patients do not respond to the adoptive transfer therapy of mutation-reactive TILs, probably because the adoptively transferred T cells are highly differentiated with little proliferative potential.[18] To overcome this problem, it is plausible that TCR genes could be isolated from tumor-specific TILs and be introduced into CTLs in peripheral blood lymphocytes (PBLs) without impaired function to generate tumor-reactive T cells that exhibit potent anti-tumor activity.

The development of these immune-based cancer therapies requires an animal model with an immune system similar to that of humans. Mouse tumor models have provided important insights into anti-tumor immune responses; however, substantial differences between the mouse and human immune systems hamper the clinical translation of the results obtained in mouse tumor models.[19] In addition, off-target toxicity with tumor-reactive TCRs cannot be predicted by using mouse models. Thus, tumor models utilizing non-human primates, which are phylogenetically closer to humans than any other laboratory animals, are essential for the development and optimization of novel immunotherapies.[20] Nonetheless, non-human primate tumor models have not been developed so far, since it is very difficult to transform primate cells into cancer cells. In fact, the mechanisms underlying cellular transformation are
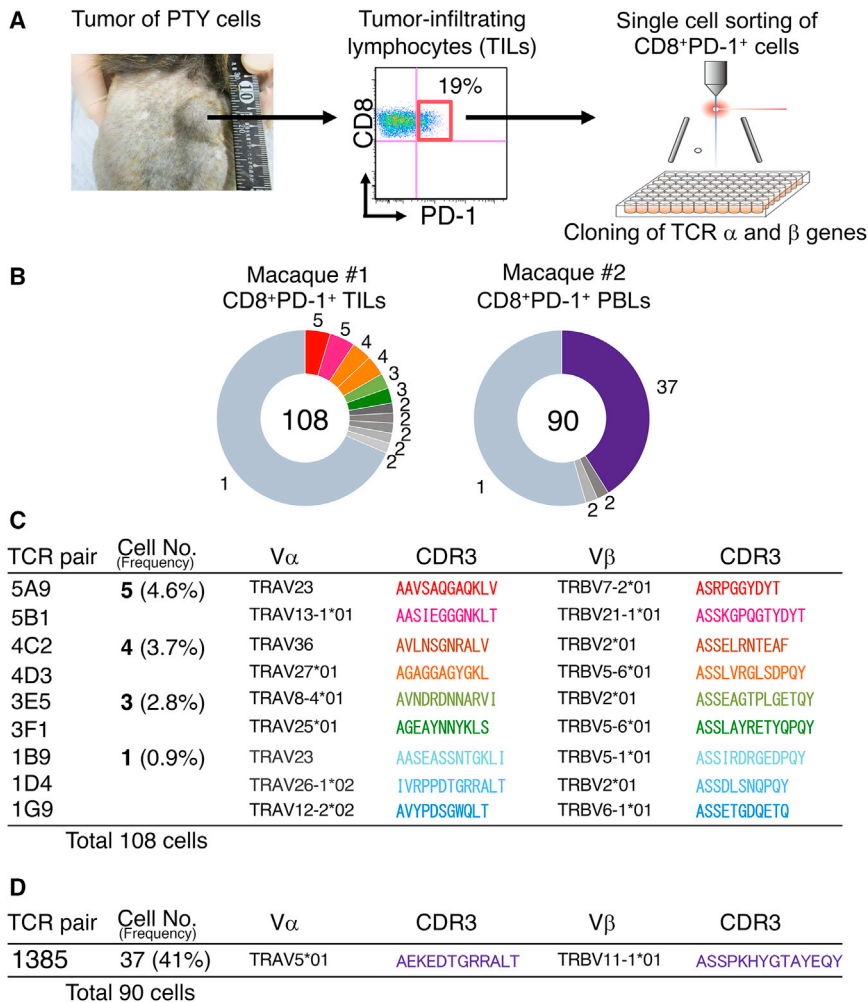
Figure 1. Isolation of TCR genes and TCR repertoire analysis of CD8+PD-1+ TILs and PBLs in tumor-transplanted cynomolgus monkey

(A) Schematic representation for isolation of TCR genes from single CD8+PD-1+ TILs at day 14 after PTY cell transplantation. (B) TCR repertoire of CD8+PD-1+ TILs (Macaque #1) and PBLs (Macaque #2). The numbers of T cells expressing the same TCR clonotype are indicated around the pie charts. (C) V genes and CDR3 sequences of TCRα and TCRβ pairs isolated from CD8+PD-1+ TILs (Macaque #1). (D) V genes and CDR3 sequences of TCRα and TCRβ pairs isolated from CD8+PD-1+ PB T cells of the macaque that had been taken repeated PTY cell injection (Macaque #2).

In this study, we sought to examine whether TILs and PBLs in this tumor rejection model of cynomolgus macaque may have tumor-killing activity by isolating TCR genes from CD8+PD-1+ T cells in TILs and PBLs and investigating their cytotoxic activity against PTY cells. Multiple TCRs with high frequency showed cytotoxic activity to PTY cells *in vitro* and also exhibited potent anti-tumor activity *in vivo*, implying that this tumor transplantation macaque model recapitulates key features of human TILs and can serve as a platform toward preclinical studies of non-human primate tumor models.

RESULTS

Isolation of TCR genes and TCR repertoire analysis of CD8+PD-1+ TILs and PBLs in tumor-transplanted cynomolgus monkeys

We previously showed that CD8+ TILs in tumors transplanted in cynomolgus monkeys expressed IFNγ and PD-1 along with cytotoxic molecules such as perforin and granzyme B, raising the possibility that CD8+ TILs might be involved in tumor rejection of this tumor transplantation macaque model,[27] although it is not known that CD8+PD-1+ T cells are reactive to tumor cells in non-human primates. To investigate this possibility, we examined whether CD8+ TILs have tumor-killing activity by isolating TCR genes from CD8+ TILs and analyzing their cytotoxic activity against PTY cells. To this end, we injected PTY cells into four separate regions of the back of an MHC-matched heterozygous macaque (Macaque #1) and prepared TILs from the tumors resected at day 14 before they were rejected. To isolate TCRα and β gene pairs from single cells, we performed single-cell sorting of CD8+PD-1+ T cells in TILs, since PD-1-positive T cells in human TILs have been shown to express tumor-reactive TCRs (Figure 1A).[15,16] Then, we succeeded to amplify TCRα and β gene pairs from 108 single CD8+PD-1+ cells among 184 cells analyzed (58.7%) (Figure 1B). Next, we determined the nucleotide sequences of 108 TCRα/β pairs and analyzed their TCR repertoire (Figures 1B and 1C). All the amino acid sequences of complementarity-determining region (CDR) 3 of

different between rodents and primates.[21] For example, in mice, inhibition of one tumor suppressor gene such as p53 and activation of one oncogene such as Kras are sufficient to develop tumors in many cancer models.[22] In contrast, forced expression of at least four cancer-inducing genes is required to convert normal human cells into tumorigenic ones.[21,23–25] Thus, we previously established tumor cell lines from induced pluripotent stem cells (iPSCs) of a cynomolgus macaque carrying a homozygous major histocompatibility complex (MHC) haplotype by overexpressing six oncogenic genes.[26] One tumor cell line, termed PTY cells, exhibited a phenotype of embryonal carcinoma and formed tumors when injected into immunodeficient NOG mice. However, tumors were immunologically rejected within 4–5 weeks when transplanted in MHC-matched heterozygous monkeys. The involvement of humoral immunity as a mechanism of tumor rejection has been shown previously, but we further characterized TILs in this model and found that CD8+ TILs expressed high levels of IFNγ and PD-1 along with cytotoxic molecules such as perforin and granzyme B, implying that these CD8+ TILs might be involved in tumor rejection of this tumor transplantation model.[27]

A

B
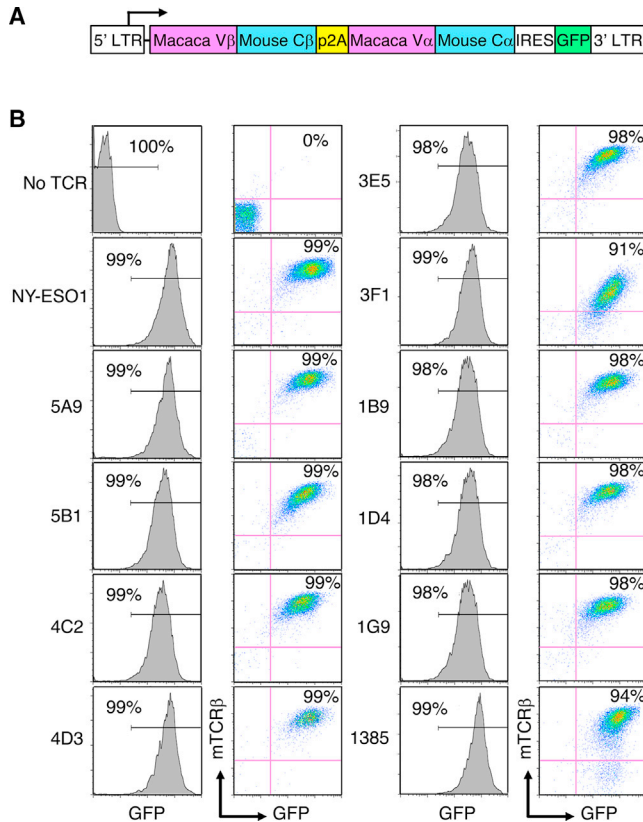
Figure 2. Expression of the TCRs isolated from macaque CD8+PD-1+ T cells on CTLs regenerated from human WT1-T-iPSCs

(A) Schematic of the retroviral vector for exogenous expression of TCR genes. (B) Expression of the exogenous TCRs on regenerated CTLs was examined by the mTCRβ antibody together with GFP.

the TCRα and β chains that were isolated from CD8+PD-1+ TILs are shown in Table S1. We identified two TCR pairs, namely 5A9 and 5B1, both of which were found in five cells among 108 cells (4.6%), two TCR pairs 4C2 and 4D3 found in four cells (3.7%), two TCR pairs 3E5 and 3F1 found in three cells (2.8%), five TCR pairs found in two cells (1.9%), and the rest of the TCR pairs including 1B9, 1D4, and 1G9 were observed only in one cell among 108 cells (0.9%), indicating that CD8+PD-1+ T cells in TILs contain T cells expressing the TCRs with relatively high frequencies, which might be clonally expanded in the tumor.

We also previously reported that repeated PTY cell injections into an MHC-matched heterozygous cynomolgus macaque induced rapid tumor rejection without tumor growth, suggesting that tumor-specific lymphocytes had been expanded and were rapidly recruited from blood to the tumor injection site.[27] Thus, we also sought to isolate TCR pairs from CD8+PD-1+ T cells in PBLs of the MHC-matched heterozygous macaque that had taken repeated PTY cell injections (Macaque #2). We successfully amplified 90 TCR pairs from 184 cells analyzed (48.9%) and identified one highly frequent TCR pair, named 1385, from 37 cells among 90 cells (41.1%), suggesting that T cells ex-

pressing this 1385 TCR clonotype might be clonally expanded by repeated PTY cell injections (Figures 1B and 1D).

Expression of the TCRs isolated from macaque CD8+PD-1+ T cells on CTLs regenerated from human WT1-T-iPSCs

Next, we tested whether the TCR pairs with relatively high frequencies are reactive to PTY cells by expressing these TCRs into CTLs, because these frequently identified TCR pairs might be derived from T cells that were clonally expanded by responding to PTY cells in the tumor. For this experiment, CTLs derived from PBLs of cynomolgus macaques would be an ideal material. However, it is difficult to obtain a great enough number of CTLs from PBLs of cynomolgus macaques due to the inability to activate T cells properly in our experimental setting. We thus took advantage of utilizing iPSCs established from human WT1-antigen-specific T cells (T-iPSCs), since CTLs can be efficiently regenerated from WT1-T-iPSCs and regenerated CTLs can be easily expanded more than 10,000-fold by repeated TCR stimulation while retaining potent cytotoxic activity.[28–30]

To express macaque TCRs in regenerated CTLs, we constructed retroviral vectors, in which TCRβ and TCRα genes connected with the self-cleaving p2A peptide were introduced along with GFP (Figure 2A). To improve the expression of introduced TCRs, constant regions were replaced by murine counterparts modified with interchain disulfide bond as previously described.[31] We transduced regenerated CTLs with the resultant retroviruses and examined the expression of introduced TCRs using the antibody against mouse TCRβ constant region (mTCRβ). The introduced TCRs were efficiently expressed together with GFP in more than 90% of the transduced regenerated CTLs (Figure 2B). We also transduced regenerated CTLs with the human TCR that recognize the cancer testis antigen NY-ESO-1 to use as a negative control of the killing analysis described below.

The TCR pairs with relatively high frequencies have the ability to attack PTY tumor cells in vitro

We next examined whether regenerated CTLs transduced with TCRs show the killing activity against PTY tumor cells. To this end, we produced PTY cells expressing luciferase (PTY-Luc) and then co-cultured PTY-Luc cells with regenerated CTLs transduced with TCRs for 20 h, and the cytotoxic activity was assessed by measuring the viability of the PTY-Luc cells using the luciferase assay as previously described.[32,33] Apparent cytotoxicity against PTY-Luc cells was observed for regenerated CTLs expressing the first and second ranked TCR pairs, 5A9, 5B1, 4C2, and 4D3, found in 5 or 4 cells among 108 cells as well as the 1385 TCR pair that was identified from PBLs of the macaque repeatedly injected with PTY cells (Figure 3A) in a manner dependent on the concentration of effector regenerated CTLs relative to target PTY-Luc cells (Figure 3B). In contrast, the TCR pairs found in 3 or 1 cells and NY-ESO-1-TCR were mostly non-cytotoxic, except that only one TCR pair 1D4 was cytotoxic.

Next, we examined the expression of degranulation marker CD107a on TCR-transduced regenerated CTLs upon co-culture with PTY-Luc
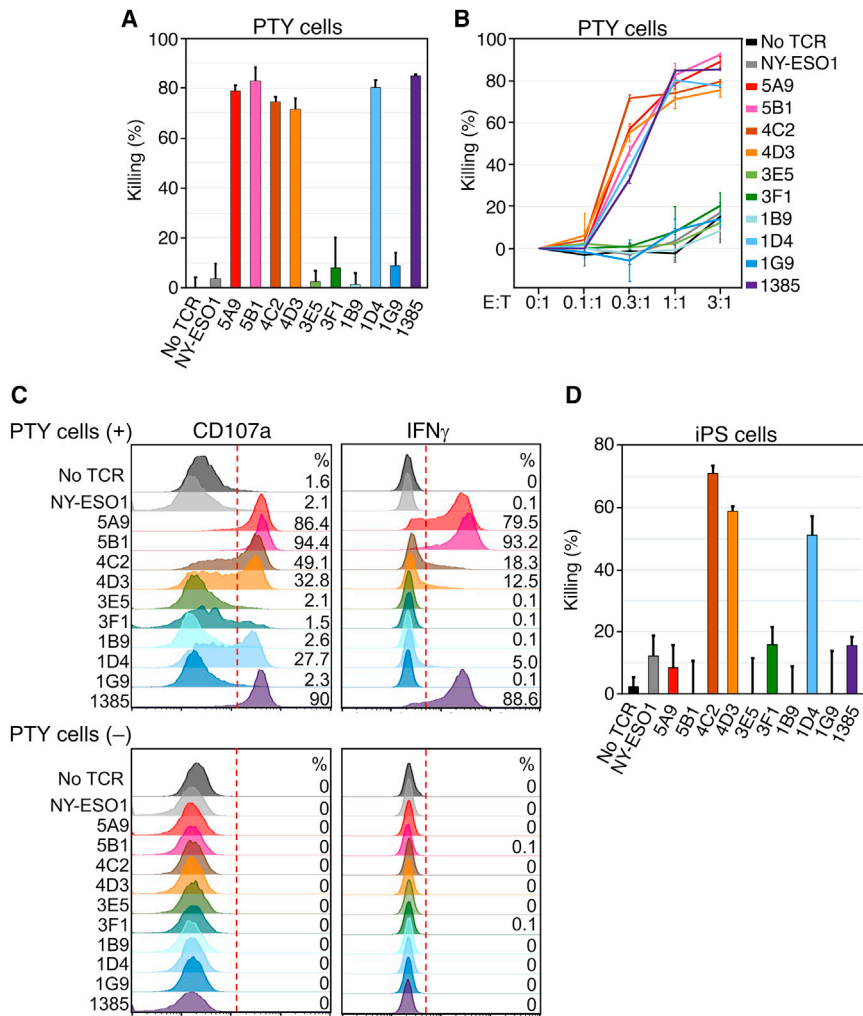
Figure 3. Functional analysis of TCR-transduced regenerated CTLs

(A) Cytotoxicity of TCR-transduced regenerated CTLs against PTY cells upon co-culture with PTY-Luc cells for 20h at E:T ratio of 1:1. The human TCR that recognizes the NY-ESO-1 antigen was used as a negative control. (B) Cytotoxicity of TCR-transduced regenerated CTLs against PTY cells at different E:T ratios. Representative data (mean ± SD) from two independent experiments are shown in (A) and (B). (C) Expression of CD107a and IFNγ of TCR-transduced regenerated CTLs upon co-culture with PTY-Luc cells (+) or without PTY-Luc cells (−) for 6 h at E:T ratio of 1:1. (D) Cytotoxicity of TCR-transduced regenerated CTLs against iPSCs upon co-culture with iPSCs-Luc cells for 20 h at E:T ratio of 3:1.

cells for 6 h, since CD107a expression on CTLs is associated with the perforin-granzyme-mediated activation of apoptosis of target cells.[34] Regenerated CTLs expressing the TCRs with killing activity such as 5A9, 5B1, 4C2, 4D3, 1D4, and 1385, but not those expressing the TCRs without killing activity and NY-ESO1-TCR, expressed CD107a when co-cultured with PTY-Luc cells, but did not express CD107a when cultured alone (Figure 3C). We also examined IFNγ expression by intracellular staining and found that regenerated CTLs expressing 5A9, 5B1, and 1385 TCRs markedly expressed IFNγ when co-cultured with PTY-Luc cells, but did not express IFNγ when cultured alone (Figure 3C). In addition, regenerated CTLs expressing 4C2, 4D3, and 1D4 TCRs marginally expressed IFNγ, whereas regenerated CTLs expressing other TCRs without cytotoxic activity did not. It might be plausible that IFNγ expression was less efficiently detected than CD107a expression because higher TCR affinity is required to express IFNγ compared to CD107a expression.[35] These results indicate that the TCRs with cytotoxic activity have the ability to mediate the expression of CD107a and IFNγ in response to PTY cells.

Multiple TCR pairs with cytotoxic activity specifically react with PTY cells, but not with the parental iPSCs
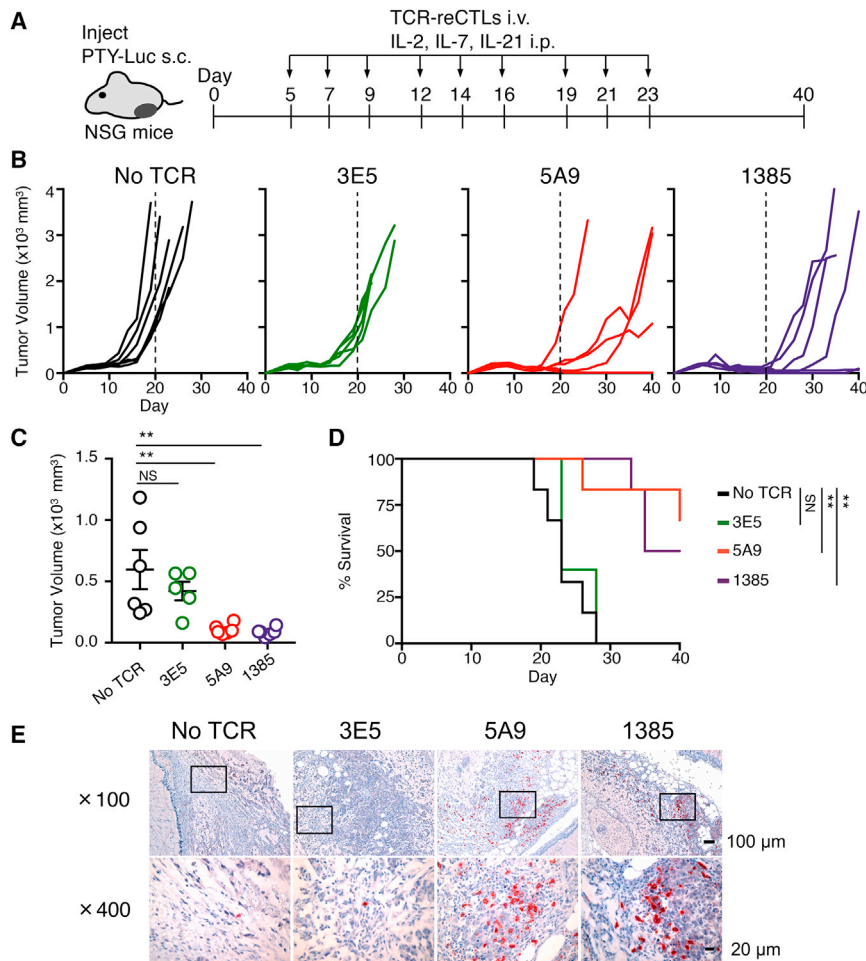
Even though transplantation was carried out between MHC-matched macaques, macaques are out bred, so many gene loci other than the MHC locus, which include minor histocompatibility antigen loci, are polymorphic. Thus, one might argue that the TCRs with cytotoxic activity may recognize minor histocompatibility antigens, rather than tumor-specific antigens or neoantigens. To address this issue, we examined the reactivity of the TCRs with cytotoxic activity to the parental iPSCs, which we used to establish PTY cells by overexpressing six oncogenic genes. Among TCRs that are capable of killing PTY cells, 5A9, 5B1, and 1385 TCRs do not react with the parental iPSCs, but specifically react with PTY cells (Figures 3A and 3D), indicating that these TCRs do not react with minor histocompatibility antigens, but are likely to react with tumor-specific antigens. However, the remaining TCRs that have killing ability, 4C2, 4D3, and 1D4, react with both PTY and iPSCs (Figures 3A and 3D), so we cannot completely exclude the possibility that these TCRs react with minor histocompatibility antigens. We have also examined whether the PTY-specific TCRs, 5A9, 5B1, and 1385, react to the products of six oncogenic genes that we introduced into the parental iPSCs. We have confirmed that T cells expressing these TCRs do not kill iPSCs that overexpress the respective oncogenic genes one by one (data not shown), suggesting that these TCRs are likely to react with tumor-specific antigens other than the six oncogenic gene products. However, we cannot strictly conclude that these TCRs are tumor-specific unless we identify the peptides that these TCRs recognize and confirm that the peptides are tumor-specific antigens.

TCRs able to kill PTY cells *in vitro* show potent anti-tumor activity *in vivo*

We further assessed whether regenerated CTLs expressing the TCRs with cytotoxic activity *in vitro* show anti-tumor activity *in vivo*. To

mors when regenerated CTLs expressing the 5A9 and 1385 TCR were transferred, but not when regenerated CTLs expressing non-exogenous TCR or the 3E5 TCR were transferred (Figure 4E). These results indicate that upon injection into tumor-bearing mice, regenerated CTLs expressing the TCRs with cytotoxic activity can infiltrate the tumor and show anti-tumor activity by responding to PTY cells *in vivo*.

DISCUSSION

In this study, we first isolated TCR genes from CD8^{+}PD-1^{+} T cells infiltrating the tumor that is eventually rejected in MHC-matched macaques because PD-1-positive T cells in human TILs have been shown to express tumor-reactive TCRs. We succeeded to obtain multiple tumor-reactive TCRs from the PD-1-positive population of CD8^{+} TILs and PBLs in this tumor transplantation macaque model. This finding indicates that PD-1 is useful as a marker for tumor-reactive T cells even in non-human primates. However, several studies have reported that tumor-reactive TCRs can also be successfully isolated from CD137-positive human and mouse TILs.[17,36,37] In line with this notion, it has been reported that clonally expanded T cells expressing the same clonotypic TCR are present in PD-1^{+}CD137^{+}CD8^{+} TILs in patients of multiple cancer types.[36] Because PD-1 and CD137 are expressed on activated mouse and human T cells,[38,39] their finding implies that those clonally expanded populations responded to certain tumor components such as tumor antigens.[36] Although we have not utilized CD137 as a prospective identification marker, it would be feasible to use CD137 instead of PD-1 or in combination with PD-1 for isolation of tumor-reactive TCRs in our macaque model.

this end, we transplanted immunocompromised NSG mice with PTY-Luc cells subcutaneously, and after five days of transplantation, regenerated CTLs that were not transduced (No TCR) and regenerated CTLs expressing the 3E5 TCR that did not show killing activity, the 5A9 and 1385 TCR that showed killing activity *in vitro* were transferred intravenously, and IL-2, IL-7, and IL-21 were administered intraperitoneally as shown in Figure 4A. When regenerated CTLs expressing the 3E5 TCR were transferred, tumor growth was not inhibited (Figures 4B and 4C), and the survival of mice was not prolonged when compared with regenerated CTLs that did not express exogenous TCR (Figure 4D). In contrast, when regenerated CTLs expressing the 5A9 and 1385 TCR were transferred, tumor growth was significantly inhibited (Figures 4B and 4C), and the survival of mice was also significantly prolonged (Figure 4D), indicating that regenerated CTLs expressing the TCRs able to kill PTY cells *in vitro* also show potent anti-tumor activity *in vivo*.

Some mice in another similarly designed experiment were sacrificed on day 8 (after twice CTL injection on day 5 and 7), and tumor lesions were examined by immunohistochemistry. Infiltration of human CD8-positive T cells with blastic appearance was observed in the tu-

Likewise, tumor-reactive TCRs can also be successfully isolated from CD8^{+}PD-1^{+} TILs in human melanoma.[16,40] However, a recent study reported that tumor reactivity of TCRs isolated from TILs in human ovarian and colorectal cancers was low and variable between individuals, and some patients did not show reactivity, even though they

were frequently derived from PD-1-expressing CD8^{+} lymphocytes.[41] Thus, we consider that the possible reasons for the effective tumor reactivity of TCRs in our macaque model are as follows. First, naturally occurring human cancers are formed by overcoming their immune system,[42] whereas in our tumor transplantation macaque model, tumor cells that have not been exposed to the immune system are transplanted into the macaques, in which their immune system can effectively respond to tumor cells, thereby enabling the rejection of tumor. Second, the immune system of macaques might have higher reactivity to tumors than that of humans, based on the observation that spontaneous neoplasms and malignant tumors in cynomolgus monkeys are extremely rare.[43] We further showed that the multiple TCRs with relatively high frequency isolated from macaque TILs exhibited apparent cytotoxicity against PTY cells both *in vitro* and *in vivo*, suggesting that tumor-infiltrating CD8^{+} T cells are likely to contribute to the rejection of tumor in this tumor transplantation macaque model. In addition, we previously reported that humoral immunity, including autoantibodies against a heat shock protein GRP94, plays a role in cancer immune surveillance.[26] Thus, these multiple mechanisms including humoral immunity and T-cell-mediated cytotoxicity might be another reason for the efficient rejection of tumors in this macaque model.

Furthermore, we previously showed that repeated PTY cell injection into the same cynomolgus macaque induced rapid tumor rejection without tumor growth, suggesting that tumor-specific lymphocytes had been expanded and were rapidly recruited from blood to the tumor injection site.[27] Indeed, we could identify the highly frequent TCR pair 1385 from CD8^{+}PD-1^{+} PBLs that showed potent cytotoxic activity, suggesting that T cells expressing the 1385 TCR clonotype were clonally expanded by repeated PTY cell injection and were rapidly recruited from blood to the tumor injection site, thereby enabling the rapid elimination of PTY cells. Although the 1385 TCR was isolated from PD-1^{+} PBLs of an artificial repeated injection macaque model, recent studies reported that neoantigen-reactive TCRs were successfully isolated from PD-1^{+} PBLs of patients with naturally occurring melanoma and gastroesophageal cancer,[40,44] suggesting that PD-1^{+} PBLs would serve as an attractive cell source for isolation of tumor-reactive TCRs. In line with our data, tumor-reactive TCRs were identified based on their frequency in the CD8^{+} TIL compartment of fresh human tumor samples, enabling the isolation of tumor-reactive TCRs without prior knowledge of their antigen specificity.[36,37,45] However, in the case of cancers with low mutational burden, in which tumor-reactive T cells are rare, it would be difficult to identify tumor-reactive TCRs simply based on their frequency. For these types of cancers, it would be necessary to identify TCRs that can respond to target mutated peptides and/or to autologous tumor cells.

Because of the differences in the immune systems of mice and humans, it is necessary to study cancer immunity using non-human primates, which have immune systems more similar to those of humans. Along with the present study, we have shown that the macaque model may be useful for analyzing TILs that generally play major roles in the immunological rejection of tumors and optimizing the efficacy of new immunotherapies.[27] Furthermore, the mechanisms underlying cellular transformation are different between rodents and primates.[21] Whereas in mice, inactivation of one tumor suppressor gene and activation of one oncogene are sufficient to cause cancer, forced expression of at least four cancer-inducing genes is required to convert normal human cells into tumorigenic ones.[21,23–25] Therefore, the generation of monkeys in which the p53 gene is mutated by genome editing has already been reported.[46] However, it is unlikely that p53 deficiency alone will cause the development of tumors. Although non-human primate models capable of developing tumors in the presence of a normal immune system have not yet been reported, we have recently succeeded in generating a model of cynomolgus macaques in which four cancer-inducing genes can be inducibly expressed by doxycycline. If tumors can be developed by overcoming the immune system in this macaque model, it will be very useful for optimizing the efficacy of new immunotherapies and for future human applications.

In this study, we took advantage of utilizing CTLs regenerated from WT1-T-iPSCs since regenerated CTLs can be easily expanded by TCR stimulation while retaining potent cytotoxic activity.[28–30] However, producing regenerated CTLs from WT1-T-iPSCs is a very time-consuming process because WT1-T-iPSCs were first established from WT1 antigen-specific CTLs by reprogramming, and then CTLs were regenerated from WT1-T-iPSCs. Thus, we have developed a rapid method, in which tumor antigen-specific TCR genes were directly introduced into iPSCs to produce TCR-iPSCs, and then CTLs were regenerated from TCR-iPSCs.[28–30] Furthermore, in this study, we were able to show for the first time to our knowledge that it is possible to directly introduce exogenous TCR genes into regenerated CTLs and obtain large numbers of tumor-killing CTLs quite rapidly. It is also feasible that if we use CTLs regenerated from iPSCs homozygous for frequent HLA-haplotypes, those CTLs can be transferred into HLA haplotype-heterozygous recipients with minimal immune reaction. Combined with our single-cell TCR cloning method, these approaches would be very useful, and there is a lot of potential for future applications for human cancer. In summary, this study provides the first evidence that cynomolgus macaque would be an ideal model for cancer immunotherapy in terms of being not only evolutionarily close to humans but also having a functional similarity in T cell response.

MATERIALS AND METHODS
Cell lines
Plat-E[47] PG13[48] and PTY cells[26] were maintained in Dulbecco's MEM medium (DMEM) (FUJIFILM Wako, 044-29765) supplemented with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific) and 1X Penicillin Streptomycin Glutamine (PSG) (Thermo Fisher Scientific, 10378-016). Plat-E was kindly provided from Professor Toshio Kitamura (University of Tokyo) and PG13 was purchased from the American Type Culture Collection. Regenerated CTLs, which were regenerated from #3-3-WT1-T-iPSCs (HLA-A*24:02-restricted), were maintained in αMEM medium (Thermo Fisher Scientific, 11900-073) supplemented with 20% FBS, human Interleukin-7 (hIL-7)

(5 ng/mL; PeproTech, AF-200-07), hIL-21 (10 ng/mL; PeproTech, AF-200-21), and ascorbic acid (100 µM; nacalai tesque, 13571-56).[28,29] An autologous lymphoblastoid cell line (LCL) was established from peripheral blood of a healthy donor from whom #3-3-WT1-T-iPSCs were established as described[29] and maintained in RPMI 1640 medium (FUJIFILM Wako, 189-02025) supplemented with 10% FBS and 1X PSG. A luciferase expressing cell line, PTY-Luc, was established by lentiviral transduction of pHIV-Luc-ZsGreen (Plasmid #39196, Addgene) as described.[28]

Animal care

All protocols for animal experiments were approved by the Shiga University of Medical Science Animal Experiment Committee (Permit numbers: 2019-9-7, 2019-9-7H1, and 2019-9-7H2). The animal experiments were carried out in strict accordance with the Guidelines for the Husbandry and Management of Laboratory Animals of the Research Center for Animal Life Science at Shiga University of Medical Science, the guidelines of an Institutional Animal Care and Use Committee and Standards Relating to the Care and Fundamental Guidelines for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology, Japan. Regular veterinary care and monitoring, balanced nutrition, and environmental enrichment were provided by the Research Center for Animal Life Science at the Shiga University of Medical Science.

Tumor transplantation in cynomolgus macaques

Cynomolgus macaques (*Macaca fascicularis*), both MHC homozygous and heterozygous for a particular set of Mafa haplotype alleles called HT1, were identified in the Filipino macaque population. Establishment of iPSCs from an MHC homozygous cynomolgus macaque and generation of iPSC-derived tumors cells (PTY cells) were performed as described.[26,27] Under ketamine/xylazine anesthesia, 2×10^7 PTY cells were injected into the subcutaneous tissue of four separate regions of the back of an MHC-matched heterozygous macaque (Macaque #1), and TILs were prepared from the tumors resected at day 14 before they were rejected. In one experiment, PTY cells were repeatedly transplanted into another individual macaque (Macaque #2), from which PBLs were prepared.

TIL preparation and single-cell RT-PCR

Leukocytes were isolated from peripheral blood after the lysis of red blood cells with ammonium-chloride-potassium (ACK) buffer. For TIL isolation, resected tumors were minced and then incubated in RPMI 1640 containing 2 mg/mL collagenase D (Roche, 11088858001) and 100 µg/mL DNase I (Roche, 4536282001) at 37°C for 1 h. Single-cell RT-PCR was performed essentially as described.[49] CD8+PD-1+ T cells were single-cell-sorted by FACSAria III (Becton Dickinson) into individual wells of 96-well PCR plates (Bio-bik, 3401-00) that contained 10 µL of Lysis/Binding Buffer (Thermo Fisher Scientific, DB61021) composed of 33 µg of Dynabeads Oligo(dT)$_{25}$ (Thermo Fisher Scientific, DB61021) and 1 pmol of each RTα, RTβ1, and RTβ2 primer. After 5 min incubation,

the Lysis/Binding Buffer was removed using Dynal MPC-9600 (Thermo Fisher Scientific, 120.06D). Ten microliters of the reverse transcription solution containing 100 U SuperScript III (Thermo Fisher Scientific, 18080-051), 5 mM MgCl$_2$, 0.5 mM each dNTP, 10 mM DTT, 20 U RNaseOUT (Thermo Fisher Scientific, 18080-051), 0.2% Triton X-100, and 1X First-Strand Buffer (Thermo Fisher Scientific, 18080-051) were added in each well, and incubated at 50°C for 50 min. The solution was replaced with 10 µL of the G-tailing solution containing 0.8 U terminal deoxynucleotidyl transferase (TdT) (New England Biolabs, M0315), 0.5 mM dGTP, 0.25 mM CoCl$_2$, 5 U Qiagen RNase inhibitor (Qiagen, 129916), 5 U Porcine liver Ribonuclease Inhibitor (Takara, 2311A), 0.2% Triton X-100, and 1X TdT buffer (New England Biolabs, M0315) and incubated at 37°C for 40 min. The solution was replaced with 19 µL of the first PCR solution. The first PCR was performed using KOD-FX polymerase (Toyobo, KFX-101) with F1st, R1stα, R1stβ1, and R1stβ2 primers. The resultant PCR mixtures were diluted 4-fold with water, and 0.5 µL of the diluted PCR mixtures was added to 12 µL of the second PCR solution. The second PCR was performed using KOD-FX polymerase with F2nd primer and a primer specific for the constant region of TCRα (R2ndα), TCRβ1 (R2ndβ1), or TCRβ2 (R2ndβ2). The second PCR products were analyzed with the R2ndα, R2ndβ1, or R2ndβ2 primer by direct sequencing. The TCR repertoire was analyzed with reference to human TCR gene sequence using the IgBLAST (https://www.ncbi.nlm.nih.gov/igblast/). Several clones were selected based on their frequencies and subcloned into the pENTR1A vector (Thermo Fisher Scientific). The resulting vectors, pENTR1A-macaque TCRα and pENTR1A-macaque TCRβ, were used for retroviral vector construction. All the oligonucleotide sequences and PCR protocols are described in Table S2.

Construction of the TCR expression vectors

Variable region genes of the selected macaque TCRα (maVα) and TCRβ (maVβ) pairs were amplified by PCR from pENTR1A-macaque TCRα and pENTR1A-macaque TCRβ, respectively. The codon-optimized mouse TCRα constant region (muCα) and the codon-optimized mouse TCRβ constant-2 region linked to the self-cleaving p2A peptide (muCβ2-p2A) were synthesized (Genscript) and subcloned into the pENTR1A vector (pENTR1A-muCα and pENTR1A-muCβ2-p2A). Both of the mouse-constant regions were modified to contain an additional cysteine residue to increase exogenous TCR pairing and decrease mispairing with the endogenous TCR.[31] The muCβ2-p2A fragment was excised from the pENTR1A-muCβ2-p2A by AflII and EcoRI digestion. The pENTR1A-muCα vector was linearized by HindIII digestion. Then the three fragments of maVβ, muCβ2-p2A, and maVα were assembled into the linearized pENTR1A-muCα by Gibson Assembly Master Mix (New England Biolabs, E2621S). The 1G4 TCR gene specific for the human cancer testis antigen NY-ESO-1[50] was constructed as described above. The TCRβ-p2A-TCRα gene blocks were transferred to pMXs-RfA-IRES-EGFP by Gateway LR reaction (Thermo Fisher Scientific, 11791-020). The resulting vector, pMXs-TCRβ-p2A-TCRα-IRES-EGFP, was used for retrovirus production.

Retrovirus production and retroviral transduction of regenerated CTLs

The constructed plasmid vector, pMXs-TCRβ-p2A-TCRα-IRES-GFP was then transfected into Plat-E cells using polyethylenimine (Polysciences, Inc., 24765-1). The culture supernatants collected 72 h after transfection were used to infect PG13 cells in the presence of polybrene (4 μg/mL; Sigma, H9268). The supernatants of PG13 cells collected 14–21 days after infection were used to transduce CTLs regenerated from human iPSCs. Regenerated CTLs (2×10^5 cells/well in 24-well plates) were co-cultured with 50 Gy irradiated HLA-A*24:02$^+$ LCL cells loaded with 100 nM WT1 synthetic peptide (CYTWNQMNL, Eurofins Genomics). After 3 days of co-culture, regenerated CTLs were collected and resuspended in retroviral supernatants from the infected PG13 cells, seeded at the density of 5×10^4 cells/well in 24-well plates coated with RetroNectin (Takara Bio Inc., T100) and centrifuged at $1,220 \times g$ at 32°C for 2 h, followed by incubation at 37°C in 5% CO_2. The viral supernatant was replaced with αMEM medium 1 h after incubation. Regenerated CTLs were cultured at 37°C in 5% CO_2 and subjected for flow cytometric analysis to evaluate the efficiency of TCR transduction 3 days after infection or for *in vitro* cytotoxicity assay and *in vivo* anti-tumor activity assay 10–14 days after infection.

Flow cytometry

The monoclonal antibodies (mAbs) used for flow cytometric analyses were purchased from BD Biosciences, eBioscience, or BioLegend, and included those to CD3 (SP34-2), CD4 (OKT4), CD8a (SK1 and HIT8a), CD45 (D058-1283), PD-1 (EH12.2H7), CD107a (H4A3), IFNγ (4S.B3), and mTCRβ (H57-579). Seven-aminoactinomycin D (7-AAD) was used to distinguish between live and dead cells. For analysis and single-cell sorting of PBLs, cells were incubated with human Fc receptor blocking reagent (Miltenyi Biotec) for 10 min, followed by staining with mAbs for 60 min on ice and washing. For analysis of regenerated CTLs, cells were incubated with mAbs for 30 min on ice and washed. Data were acquired on a FACSAria III or a FACSCalibur (BD Biosciences) and were analyzed using FlowJo software (Tree Star).

In vitro cytotoxicity assay using the luciferase assay

The cytotoxicity of regenerated CTLs transduced with TCR genes was determined by luciferase assay as described.[28] Briefly, TCR-transduced regenerated CTLs and PTY-Luc cells (1×10^4) were seeded in 96-well plates (Corning, 3917) at the indicated effector to target (E:T) ratio and cultured for 20 h. The luciferase activity was measured using Bright-Glo Luciferase Assay System (Promega, E2610) and expressed as relative light units (RLU). Target cells alone were cultured to measure the maximal luciferase activity (RLUmax). The percentage of lysis was calculated as follows: % lysis = (1 − (RLU)/(RLUmax)) × 100.

Analyses of the expression of CD107a and IFNγ

TCR-transduced regenerated CTLs (1×10^5) and PTY-Luc cells (1×10^5) were seeded in round-bottom 96-well plates (Falcon, 353077) and cultured in the presence of anti-CD107a antibody for CD107a staining or 2 μM monensin for IFNγ intracellular staining, respectively. Six hours later, cells were collected and stained with anti-CD8 antibody to distinguish regenerated CTLs from PTY-Luc cells, and CD107a expression was analyzed by flow cytometry. For intracellular IFNγ staining, cells were stained with anti-CD8 antibody, fixed with 4% paraformaldehyde/PBS for 5 min at room temperature, and permeabilized with the saponin solution containing 0.1% saponin, 0.1% BSA, and 10 mM HEPES (pH 7.5), followed by staining with anti-IFNγ antibody and subsequent flow cytometric analysis.

In vivo anti-tumor activity assay using NSG mice

Female NSG mice were purchased from the Jackson Laboratory and Charles River Japan Inc. All mice were 8 weeks old at the beginning of each experiment. On day 0, 5×10^6 PTY-Luc cells suspended in 100 μL of PBS were mixed with an equal volume of Matrigel (Corning, 356237) and injected subcutaneously into the lateral abdomen of mice. On day 5, tumor engraftment was confirmed by measurement of tumor size, and each mouse was uniformly allocated to the control or TCR group based on the size of tumors. The mice in the control and TCR group were intravenously injected with untransduced regenerated CTLs and TCR-transduced regenerated CTLs, respectively, three times per week for three weeks (5×10^6 cells/injection). Cytokines (hIL-2 [PeproTech, AF-200-02] 40 ng, hIL-7 40 ng, hIL-21 40 ng/body) were injected intraperitoneally just after regenerated CTLs injection. Tumor-bearing mice were sacrificed when the tumor reached 20 mm in size for ethical considerations. Tumor volume was calculated by the formula $ab^2/2$ (a, width; b, length) as described.[26]

Immunohistochemistry of TILs

PTY-Luc cells were injected into NSG mice as described above. Regenerated CTLs and cytokines were injected into NSG mice on day 5 and day 7 after PTY-Luc cell injection. Tumors were removed on day 8 and fixed overnight at 4°C in formalin, followed by paraffin embedding. Immunohistochemistry was performed essentially as described.[26] Briefly, formalin-fixed and paraffin-embedded graft tissues were sectioned, followed by deparaffinization and rehydration. Sections were incubated with anti-CD8 antibody (clone: SP16, abcam 101500) overnight at 4°C, followed by incubation with the secondary antibody conjugated with alkaline phosphatase (NICHIREI Bioscience 424261) for 1 h at room temperature, and the immunoreaction was visualized by Fast Red II (NICHIREI Bioscience 415261). Then, sections were counter-stained with hematoxylin according to a standard protocol.

Data analysis

All the data with error bars are presented as mean ± SE. Difference was assessed using one-way ANOVA or log rank test using Prism software (GraphPad). Values of $p < 0.05$ were considered significant.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.omto.2021.12.003.

AUTHOR CONTRIBUTIONS

K.T., K.K., H.I., A.N., H.S., S.N., K.M., T.K, T.H., K.O., Y.I., H.K., and Y.A. designed the experiments; K.T., K.K., H.I., A.N., H.S., S.N., K.M., T.H., Y.I., H.K., and Y.A. collected, analyzed, and interpreted the data; K.T., K.K., and Y.A. wrote the manuscript, and all authors reviewed the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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Supplemental information

Isolation of TCR genes with tumor-killing activity

from tumor-infiltrating and circulating lymphocytes

in a tumor rejection cynomolgus macaque model

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Supplemental Table S1. V(D)J genes and CDR3 amino acid sequences of the TCR genes isolated from CD8⁺PD-1⁺ T cells in TILs of Macaque

ID	TRBV	TRBD	TRBJ	CDR3 aa seq.	TRAV	TRAJ	CDR3 aa seq.	TCR pair
1.	TRBV7-2*01	TRBD2*01	TRBJ1-2*01	ASRPGGYDYT	TRAV23	TRAJ54*01	AAVSAQGAQKLV	5A9
2.	TRBV7-2*01	TRBD2*01	TRBJ1-2*01	ASRPGGYDYT	TRAV23	TRAJ54*01	AAVSAQGAQKLV	
3.	TRBV7-2*01	TRBD2*01	TRBJ1-2*01	ASRPGGYDYT	TRAV23	TRAJ54*01	AAVSAQGAQKLV	
4.	TRBV7-2*01	TRBD2*01	TRBJ1-2*01	ASRPGGYDYT	TRAV23	TRAJ54*01	AAVSAQGAQKLV	
5.	TRBV7-2*01	TRBD2*01	TRBJ1-2*01	ASRPGGYDYT	TRAV23	TRAJ54*01	AAVSAQGAQKLV	
6.	TRBV21-1*01	TRBD1*01	TRBJ1-2*01	ASSKGPQGTYDYT	TRAV13-1*01	TRAJ10*01	AASIEGGGNKLT	5B1
7.	TRBV21-1*01	TRBD1*01	TRBJ1-2*01	ASSKGPQGTYDYT	TRAV13-1*01	TRAJ10*01	AASIEGGGNKLT	
8.	TRBV21-1*01	TRBD1*01	TRBJ1-2*01	ASSKGPQGTYDYT	TRAV13-1*01	TRAJ10*01	AASIEGGGNKLT	
9.	TRBV21-1*01	TRBD1*01	TRBJ1-2*01	ASSKGPQGTYDYT	TRAV13-1*01	TRAJ10*01	AASIEGGGNKLT	
10.	TRBV21-1*01	TRBD1*01	TRBJ1-2*01	ASSKGPQGTYDYT	TRAV13-1*01	TRAJ10*01	AASIEGGGNKLT	
11.	TRBV2*01	–	TRBJ1-1*01	ASSELRNTEAF	TRAV36	TRAJ29*01	AVLNSGNRALV	4C2
12.	TRBV2*01	–	TRBJ1-1*01	ASSELRNTEAF	TRAV36	TRAJ29*01	AVLNSGNRALV	
13.	TRBV2*01	–	TRBJ1-1*01	ASSELRNTEAF	TRAV36	TRAJ29*01	AVLNSGNRALV	
14.	TRBV2*01	–	TRBJ1-1*01	ASSELRNTEAF	TRAV36	TRAJ29*01	AVLNSGNRALV	
15.	TRBV5-6*01	TRBD2*02	TRBJ2-3*01	ASSLVRGLSDPQY	TRAV27*01	TRAJ52*01	AGAGGAGYGKL	4D3
16.	TRBV5-6*01	TRBD2*02	TRBJ2-3*01	ASSLVRGLSDPQY	TRAV27*01	TRAJ52*01	AGAGGAGYGKL	
17.	TRBV5-6*01	TRBD2*02	TRBJ2-3*01	ASSLVRGLSDPQY	TRAV27*01	TRAJ52*01	AGAGGAGYGKL	
18.	TRBV5-6*01	TRBD2*02	TRBJ2-3*01	ASSLVRGLSDPQY	TRAV27*01	TRAJ52*01	AGAGGAGYGKL	
19.	TRBV2*01	TRBD2*01	TRBJ2-5*01	ASSEAGTPLGETQY	TRAV8-4*01	TRAJ31*01	AVNDRDNNARVI	3E5
20.	TRBV2*01	TRBD2*01	TRBJ2-5*01	ASSEAGTPLGETQY	TRAV8-4*01	TRAJ31*01	AVNDRDNNARVI	
21.	TRBV2*01	TRBD2*01	TRBJ2-5*01	ASSEAGTPLGETQY	TRAV8-4*01	TRAJ31*01	AVNDRDNNARVI	

22.	TRBV5-6*01	TRBD1*01	TRBJ1-5*01	ASSLAYRETYQPQY	TRAV25*01	TRAJ20*01	AGEAYNNYKLS	3F1
23.	TRBV5-6*01	TRBD1*01	TRBJ1-5*01	ASSLAYRETYQPQY	TRAV25*01	TRAJ20*01	AGEAYNNYKLS	
24.	TRBV5-6*01	TRBD1*01	TRBJ1-5*01	ASSLAYRETYQPQY	TRAV25*01	TRAJ20*01	AGEAYNNYKLS	
25.	TRBV5-1*01	TRBD2*01	TRBJ2-1*01	ASSLGWGELNEQF	TRAV8-6*02	TRAJ33*01	AVSGDSNYQLI	
26.	TRBV5-1*01	TRBD2*01	TRBJ2-1*01	ASSLGWGELNEQF	TRAV8-6*02	TRAJ33*01	AVSGDSNYQLI	
27.	TRBV5-5*01	TRBD1*01	TRBJ1-1*01	ASSLGQLNTEAF	TRAV26-1*01	TRAJ43*01	IGRFGNDIR	
28.	TRBV5-5*01	TRBD1*01	TRBJ1-1*01	ASSLGQLNTEAF	TRAV26-1*01	TRAJ43*01	IGRFGNDIR	
29.	TRBV10-1*01	–	TRBJ1-1*01	ASFSSLNTEAF	TRAV25*01	TRAJ22*01	SSIFCDSGWQLT	
30.	TRBV10-1*01	–	TRBJ1-1*01	ASFSSLNTEAF	TRAV25*01	TRAJ22*01	SSIFCDSGWQLT	
31.	TRBV10-1*01	TRBD2*01	TRBJ2-1*01	ASSEDWGGFYGEQF	TRAV38-2	TRAJ53*01	AYRGSSNYKLT	
32.	TRBV10-1*01	TRBD2*01	TRBJ2-1*01	ASSEDWGGFYGEQF	TRAV38-2	TRAJ53*01	AYRGSSNYKLT	
33.	TRBV12-3*01	–	TRBJ2-7*01	ASKKSSYEQY	TRAV6*01	TRAJ34*01	ALHNANKLI	
34.	TRBV12-3*01	–	TRBJ2-7*01	ASKKSSYEQY	TRAV6*01	TRAJ34*01	ALHNANKLI	
35.	TRBV2*01	–	TRBJ2-7*01	ASRLYEQY	TRAV5*01	TRAJ30*01	AENREDKII	
36.	TRBV2*01	TRBD2*01	TRBJ2-4*01	PAVMRRGEAKTLST	TRAV19*01	TRAJ33*01	ALNEAEDSNYQLI	
37.	TRBV2*01	TRBD1*01	TRBJ2-7*01	ATRTDLYEQY	TRAV20*01	TRAJ53*01	AVNSGSSNYKLT	
38.	TRBV2*01	TRBD1*01	TRBJ2-3*01	ASSRTGGMNTDPQY	TRAV22*01	TRAJ32*01	AVLYYGGSGNKLI	
39.	TRBV2*01	–	TRBJ1-5*01	ASSDLSNQPQY	TRAV26-1*01	TRAJ5*01	IVRPPDTGRRALT	1D4
40.	TRBV2*01	–	TRBJ1-1*01	ASSELRNTEAF	TRAV36	TRAJ29*01	CAQFRNRAFV	
41.	TRBV3-1*01	TRBD2*01	TRBJ2-1*01	ASSQDGGADNEQF	TRAV9-1*01	TRAJ28*01	ALNLGYSGAGSYQLT	
42.	TRBV3-1*01	TRBD2*02	TRBJ1-2*01	ASSQGRGNYDYT	TRAV18*01	TRAJ10*01	VLKGASNKLT	
43.	TRBV3-2*01	–	TRBJ2-5*01	ASTSFGDAVETQY	TRAV6*01	TRAJ40*01	ALDASTTGNYKYI	

44.	TRBV3-2*01	TRBD1*01	TRBJ1-4*01	ASSQQQATNEKLF	TRAV9-2*01	TRAJ6*01	ALTHPSGGGYVLT	
45.	TRBV5-1*01	–	TRBJ1-4*01	ASSVNEKLF	TRAV12-3*01	TRAJ2*01	AMTLGGTIDKLT	
46.	TRBV5-1*01	TRBD1*01	TRBJ2-3*01	ASSIRDRGEDPQY	TRAV23	TRAJ37*02	AASEASSNTGKLI	1B9
47.	TRBV5-5*01	TRBD1*01	TRBJ1-1*01	ASSLGQLNTEAF	TRAV26-1*01	TRAJ49*01	IVGFGNQL	
48.	TRBV5-6*01	TRBD2*02	TRBJ2-1*01	ASSRLGGAHGEQF	TRAV4*01	TRAJ36*01	LVGDGSWGKQPV	
49.	TRBV5-6*01	TRBD1*01	TRBJ1-5*01	ASSLEQGRNQPQY	TRAV12-3*01	TRAJ24*02	AMRADSWGKLQ	
50.	TRBV5-6*01	TRBD1*01	TRBJ1-5*01	ASRQANREIYQPQY	TRAV25*01	TRAJ20*01	AGEASNNYKLS	
51.	TRBV5-6*01	TRBD1*01	TRBJ2-3*01	ASTLAGRGRSHPQY	TRAV25*01	TRAJ20*01	AGEASNNYKLS	
52.	TRBV5-6*01	TRBD1*01	TRBJ2-7*01	ASSRGRQTYEQY	TRAV38-1*01	TRAJ43*01	AFMIYNNNDIR	
53.	TRBV5-8*01	–	TRBJ2-3*01	ASSLGHPPPQS	TRAV26-1*01	TRAJ43*01	IGRFGNDIR	
54.	TRBV6-1*01	TRBD1*01	TRBJ2-5*01	ASSETGDQETQ	TRAV12-2*02	TRAJ22*01	AVYPDSGWQLT	1G9
55.	TRBV6-1*01	TRBD1*01	TRBJ1-6*02	ASSEYRVSYNSPLH	TRAV14	TRAJ30*01	AMMNRDDKII	
56.	TRBV6-1*01	TRBD1*01	TRBJ2-4*01	ASTPGQTSQNTQY	TRAV20*01	TRAJ9*01	AVQAWTGGFKTV	
57.	TRBV6-1*01	TRBD2*01	TRBJ1-3*01	KWPEVGGFRKHHV	TRAV21*01	TRAJ9*01	AVGYTGGFKTV	
58.	TRBV6-1*01	TRBD1*01	TRBJ2-7*01	ASTKQGPQDEQY	TRAV29	TRAJ52*01	ASCGAGYGKLT	
59.	TRBV6-5*01	TRBD1*01	TRBJ1-1*01	GSSEGVKTEAF	TRAV3*01	TRAJ40*01	AVNEVSGNYKYI	
60.	TRBV6-5*01	TRBD2*01	TRBJ2-5*01	LSPSVTKRPST	TRAV3*01	TRAJ40*01	AVNEVSGNYKYI	
61.	TRBV6-5*01	–	TRBJ2-6*01	GSSVSSGASVLT	TRAV18*01	TRAJ8*01	VVHRLSETR	
62.	TRBV7-2*01	–	TRBJ2-1*01	ASSLLALNEQF	TRAV17*01	TRAJ48*01	VTEGFGNEKLT	
63.	TRBV7-2*01	TRBD2*01	TRBJ1-2*01	ASRPGGYDYT	TRAV19*01	TRAJ52*01	ALNEESWWCWLWKAD	
64.	TRBV7-2*01	–	TRBJ2-1*01	ASSLLALNEQF	TRAV26-2*01	TRAJ48*01	LLGRGVEVRNP	
65.	TRBV7-2*01	–	TRBJ1-2*01	ASRDSMSMDDYT	TRAV29	TRAJ52*01	AARGSGYGKLT	
66.	TRBV7-2*01	TRBD1*01	TRBJ2-5*01	ASSLVGSRVTETQY	TRAV38-1*01	TRAJ42*01	AFMKHEVYGGSQGNLI	
67.	TRBV7-4*01	TRBD1*01	TRBJ2-3*01	ASSGRDRDLADPQY	TRAV18*01	TRAJ4*01	VLPAGGGYDKLI	
68.	TRBV7-6*01	TRBD1*01	TRBJ2-7*01	ASSSLQTDWGDFYEQY	TRAV12-3*01	TRAJ37*02	AMSVTSNTGKLI	
69.	TRBV7-6*01	TRBD1*01	TRBJ2-1*01	ASSPDWGEGSPSEQF	TRAV13-2*01	TRAJ47*02	AEMDYGNKLI	
70.	TRBV7-6*01	TRBD2*01	TRBJ2-4*01	ASSPGPRRGAVNRLY	TRAV16*01	TRAJ54*01	ALSGSLGAQKLV	
71.	TRBV7-9*01	TRBD2*02	TRBJ2-7*01	ASSGSQPSYEQY	TRAV13-1*01	TRAJ33*01	AATPGSNYQLI	

72.	TRBV7-9*01	TRBD1*01	TRBJ2-4*01	ASSLGTPGNTQY	TRAV16*01	TRAJ16*01	ALKSSDGQKLL
73.	TRBV9*01	–	TRBJ2-2*01	PAANFMETPRSCS	TRAV26-1*01	TRAJ22*01	IVRPPSDSGWQLT
74.	TRBV9*01	–	TRBJ2-1*01	ASSEVGYGEQF	TRAV35*01	TRAJ32*01	AGQKGGGGSGNKLI
75.	TRBV9*01	TRBD1*01	TRBJ2-1*01	ATGELGNGDQF	TRAV35*01	TRAJ32*01	AGQKGGGGSGNKLI
76.	TRBV10-1*01	–	TRBJ2-1*01	ASSEINALYGEQF	TRAV8-6*02	TRAJ24*02	AVTTDSWGKLQ
77.	TRBV10-1*01	–	TRBJ1-1*01	ASFSSLNTEAF	TRAV25*01	TRAJ22*01	SSISSDSGWQLT
78.	TRBV10-1*01	–	TRBJ1-1*01	ASFSSLNTEAF	TRAV25*01	TRAJ22*01	STIYCGCGSQLT
79.	TRBV12-3*01	TRBD1*01	TRBJ2-7*01	ASTTPGQVSEQY	TRAV22*01	TRAJ40*01	AVTTGNYKYI
80.	TRBV12-3*01	TRBD1*01	TRBJ2-1*01	ASSLKWGSSYNEQF	TRAV23	TRAJ27*01	AAGDNAGKLT
81.	TRBV13*01	–	TRBJ2-7*01	ASNFVSIYEQY	TRAV2*01	TRAJ27*01	AVLHTNADKLT
82.	TRBV13*01	TRBD1*01	TRBJ1-1*01	TSREGRGLGNEAF	TRAV9-1*01	TRAJ27*01	ALEAGKLT
83.	TRBV14*01	TRBD2*02	TRBJ1-3*01	ASSQAEGTISGNTVY	TRAV2*01	TRAJ11*01	AVEDSGYSTLT
84.	TRBV14*01	TRBD2*02	TRBJ2-3*01	ASSLWEGRDPQY	TRAV2*01	TRAJ23*01	AVEDQDQAGKLI
85.	TRBV14*01	TRBD1*01	TRBJ1-5*01	ASSQGGGSQPQY	TRAV3*01	TRAJ34*01	AVRDPNANKLI
86.	TRBV14*01	TRBD1*01	TRBJ1-2*01	ASTSRDIHDYT	TRAV17*01	TRAJ44*01	ATDSFTGIASKLT
87.	TRBV14*01	TRBD1*01	TRBJ1-5*01	PGGRVAISPS	TRAV19*01	TRAJ32*01	APAGGYGGRGNKLI
88.	TRBV15*01	TRBD1*01	TRBJ2-4*01	ASSKDMGTEGTKTFSS	TRAV26-1*01	TRAJ48*01	IVRARQGGNENLT
89.	TRBV15*02	TRBD1*01	TRBJ1-2*01	ASSKKGQVSHST	TRAV16*01	TRAJ54*01	AQRVITQGAQKLV
90.	TRBV20-1*01	TRBD2*01	TRBJ2-7*01	SAPGLASSYEQY	TRAV9-1*01	TRAJ31*01	ALRQNNNARVI
91.	TRBV20-1*01	TRBD1*01	TRBJ1-2*01	SATLGTEFFRDYT	TRAV12-1*01	TRAJ23*01	AVNMAYNQAGKLI
92.	TRBV20-1*01	TRBD1*01	TRBJ1-3*01	SARMQGTRITVY	TRAV38-2	TRAJ33*01	AYRGNMDSNYQLI
93.	TRBV23-1*01	TRBD1*01	TRBJ2-3*01	ASTGFGDPQY	TRAV9-2*01	TRAJ13*01	ALSSGSYQKVT
94.	TRBV23-1*01	TRBD1*01	TRBJ2-3*01	ASTGFGDPQY	TRAV9-2*01	TRAV9-2*01	ALSSGSTRESS
95.	TRBV23-1*01	TRBD1*01	TRBJ2-3*01	ASKQGWGHRSSV	TRAV18*01	TRAJ33*01	VLRDPMDSNYQLI
96.	TRBV23-1*01	–	TRBJ2-3*01	ASSQGWGTDPQY	TRAV18*01	TRAJ33*01	VLRDPMDSNYQLI
97.	TRBV24-1*01	TRBD1*01	TRBJ1-2*01	ATSDLSITGADYDYT	TRAV12-3*01	TRAJ32*01	ARPGGSGNKLI
98.	TRBV27*01	TRBD2*01	TRBJ2-1*01	ASSGGPGQF	TRAV1-2*01	TRAJ34*01	AVRANKL
99.	TRBV27*01	TRBD1*01	TRBJ1-1*01	ASSKGGGNTEAF	TRAV6*01	TRAJ24*02	APGSDSWGKLQ

100.	TRBV27*01	TRBD1*01	TRBJ1-5*01	ASTPLGESNQPQY	TRAV8-4*01	TRAJ11*01	AVILNSGYSTLT
101.	TRBV27*01	–	TRBJ2-1*01	ASSLRDFDGEQF	TRAV25*01	TRAJ21*01	AGMDNFNKFY
102.	TRBV27*01	–	TRBJ1-3*01	ASSSWQPSYSGNTVY	TRAV29	TRAJ30*01	GGRDDKII
103.	TRBV28*01	TRBD1*01	TRBJ1-1*01	ASSFWRGVNTEAF	TRAV8-3*02	TRAJ12*01	HNGATGDNKLI
104.	TRBV28*01	TRBD1*01	TRBJ1-1*01	ASSFWRGVNTEAF	TRAV8-3*01	TRAJ50*01	GAGATSYNKLT
105.	TRBV28*01	–	TRBJ2-6*01	ASLDSGASVLT	TRAV12-3*01	TRAJ41*01	AMTHSNSGYALN
106.	TRBV29-1*01	TRBD1*01	TRBJ1-2*01	SVGTGYNYDYT	TRAV4*01	TRAJ30*01	LVGERDDKII
107.	TRBV29-1*01	–	TRBJ2-1*01	SVASLGFSYGEQF	TRAV12-3*01	TRAJ43*01	AMKKDNNDIR
108.	TRBV30*02	TRBD1*01	TRBJ1-1*01	AWKYEGGQTEAF	TRAV36	TRAJ29*01	AYLSGNRALV

Supplemental Table S2. Nucleotide sequence of PCR primers and PCR conditions.

Primer name	TCRβ primer sequence
5A9_b_F	5'-TTCAGTCGACTGGATCCAGCCGCCATGGGCACCAGGCTCCT-3'
5A9_b_R	5'-GGTCACGTTCCTCAGGTCAACAGTTAACTTGGTCCCTG-3'
5B1_b_F	5'-TTCAGTCGACTGGATCCAGCCACCATGTGCCTCAGACTTCT-3'
5B1_b_R	5'-GGTCACGTTCCTCAGGTCCTCTACAACAGTTAACTTG-3'
4C2_b_F	5'-TTCAGTCGACTGGATCCAGCCGCCATGGATACCTGTCTCCT-3'
4C2_b_R	5'-GGTCACGTTCCTCAGGTCCTCTACAACTGTGAGTCTGG-3'
4D3_b_F	5'-TTCAGTCGACTGGATCCAGCCGCCATGGGCCCCGGGCTCCT-3'
4D3_b_R	5'-GGTCACGTTCCTCAGGTCCTCGAGCACTGTCAGCCGGG-3'
3E5_b_F	5'-TTCAGTCGACTGGATCCAGCCGCCATGGATACCTGGCTCCT-3'
3E5_b_R	5'-GGTCACGTTCCTCAGGTCCTCGAGCACCAGGAGCCGCG-3'
3F1_b_F	5'-TTCAGTCGACTGGATCCAGCCGCCATGGGCCCCGGGCTCCT-3'
3F1_b_R	5'-GGTCACGTTCCTCAGGTCCTCTAGGACGGAGAGTCGAG-3'
1B9_b_F	5'-TTCAGTCGACTGGATCCAGCCGCCATGCTGTTTTGCTGGGCA-3'
1B9_b_R	5'-GGTCACGTTCCTCAGGTCCTCGAGCACTGTCAGCCGGG-3'
1D4_b_F	5'-TTCAGTCGACTGGATCCAGCCACCATGGATACCTGGCTCCT-3'
1D4_b_R	5'-GGTCACGTTCCTCAGGTCCTCTAGGACGGAGAGTCGAG-3'
1G9_b_F	5'-TTCAGTCGACTGGATCCAGTTCCCCTTTCATCAATGC-3'
1G9_b_R	5'-GGTCACGTTCCTCAGGTCCTCTAGCACCAGGAGCCGCG-3'
1385_b_F	5'-TTCAGTCGACTGGATCCAGCCGCCATGGGCACCAGGCTCCTCTG-3'
1385_b_R	5'-GGTCACGTTCCTCAGGTCCTCTACGACCGTGAGCCTGG-3'
NYESO1_1G4_b_F	5'-TTCAGTCGACTGGATCCAGCCACCATGTCTATCGGCCTGCTG-3'
NYESO1_1G4_b_R	5'-GGTCACGTTCCTCAGGTCTTCCAGCACGGTCAGTCTGC-3'

	TCRα primer sequence
5A9_a_F	5'-GAGGAGAATCCTGGCCCAATGGACAAGATCTTAGGAGC-3'
5A9_a_R	5'-CGGGCTCTGGGTTCTGGATATTCAGGTTGATGCTCAGCC-3'
5B1_a_F	5'-GAGGAGAATCCTGGCCCAATGACATCCATTCGAGCTGT-3'
5B1_a_R	5'-CGGGCTCTGGGTTCTGGATATTCAGTTCCACTTTTAGCT-3'
4C2_a_F	5'-GAGGAGAATCCTGGCCCAATGATGAAGTGTTCACAGGC-3'
4C2_a_R	5'-CGGGCTCTGGGTTCTGGATATTTGGAATCACAGAAAGTC-3'

4D3_a_F	5'-GAGGAGAATCCTGGCCCAATGGTCCTGAAATTCTCCGT-3'
4D3_a_R	5'-CGGGCTCTGGGTTCTGGATATTTGGATAGACAGTTAAGA-3'
3E5_a_F	5'-GAGGAGAATCCTGGCCCAATGCTCCTGCTGCTCGTCCT-3'
3E5_a_R	5'-CGGGCTCTGGGTTCTGGATATTGGGCTTCACCACCAGCT-3'
3F1_a_F	5'-GAGGAGAATCCTGGCCCAATGCACACATCTGCTTTCCA-3'
3F1_a_R	5'-CGGGCTCTGGGTTCTGGATATTTGCTCTTACAGTTACTG-3'
1B9_a_F	5'-GAGGAGAATCCTGGCCCAATGGACAAGATCTTAGGAGC-3'
1B9_a_R	5'-CGGGCTCTGGGTTCTGGATATCTGGTTGTACTTGTAAAG-3'
1D4_a_F	5'-GAGGAGAATCCTGGCCCAATGAGGCTGGTGGCAAGAGT-3'
1D4_a_R	5'-CGGGCTCTGGGTTCTGGATATTTGGTTGCACTTGGAGTC-3'
1G9_a_F	5'-GAGGAGAATCCTGGCCCAATGATGAAATCCTTGAGAGT-3'
1G9_a_R	5'-CGGGCTCTGGGTTCTGGATATCAGGTACAACGGTCAATT-3'
1385_a_F	5'-GAGGAGAATCCTGGCCCAATGAAGACACTTACTGGATC-3'
1385_a_R	5'-CGGGCTCTGGGTTCTGGATATTTGGTTGCACTTGGAGTC-3'
NYESO1_1G4_a_F	5'-GAGGAGAATCCTGGCCCAATGGAAACCCTGCTGGGCCT-3'
NYESO1_1G4_a_R	5'-CGGGCTCTGGGTTCTGGATGTAGGGGTGCACGATCAGGC-3'

	RT primer sequence
RTα	5'-CAGCGGTGTTTGGCAGCTCTTC-3'
RTβ1	5'-CTGGCAAAAGAAGAATGTAT-3'
RTβ2	5'-ACACAGATTGGGAGCAGGTA-3'

	First PCR primer sequence
F1st	5'-TCAAGCAGTAGCAGCAGTTCGATAAGGATCCCCCCCCCCCCCCDN-3'
R1stα	5'-GAGAGGGAGAGGAGGGGCGAT-3'
R1stβ1	5'-TCACAACGGGTTAGAAGCTC-3'
R1stβ2	5'-GGATGAAGAATGACCCGGGAT-3'

	Second PCR primer sequence
F2nd	5'-AGCAGTAGCAGCAGTTCGATA-3'
R2ndα	5'-AGTAAATAGGCAGACAGAGGT-3'
R2ndβ1	5'-GTGGTCGGGGTAGAAGCCTGT-3'
R2ndβ2	5'-GTGGTCGGGGTAGAAGCCTGT-3'

First PCR condition
1) 94˚C, 2 min
2) 98˚C, 10 sec
3) 68˚C, 30 sec (-2˚C / cycle)
4) 68˚C, 1 min 30 sec
5) Go to 2), Repeat 4 times
6) 98˚C, 10 sec
7) 60˚C, 30 sec
8) 68˚C, 1 min 30 sec
9) Go to 6), Repeat 24 times
10) 68˚C 10 min
11) 12˚C

Second PCR condition
1) 94˚C, 2 min
2) 98˚C, 10 sec
3) 68˚C, 30 sec (-2˚C / cycle)
4) 68˚C, 1 min 30 sec
5) Go to 2), Repeat 4 times
6) 98˚C, 10 sec
7) 60˚C, 30 sec
8) 68˚C, 1 min 30 sec
9) Go to 6), Repeat 29 times
10) 68˚C 10 min
11) 12˚C