Chapter 4

Construction Of The Two Arms Of The Anti-tumor T Cell Immunity

1. Introduction

The adaptive immune system of the vertebrates does a magnificent job to protect an animal from most pathogens efficiently and specifically. One of the greatest medical interventions in human history is the development of preventive vaccines aiming to enhance adaptive immune response. For over two centuries, this immunotherapy approach has claimed victories in preventing many infectious diseases that used to plague humankind, resulting in worldwide eradication of diseases such as smallpox. Inspired by these successes, enormous interests and efforts have been devoted to search for methods to arm the immune system against cancer. However, there has been only limited success, mostly due to the fact that cancer, arising from the organisms' own tissues, usually has developed strategies to escape normal immune responses. During last decade, knowledge of the molecular mechanisms underlying the tumor-host interactions has grown rapidly, providing opportunities to manipulate host immunity and target tumor cells for destruction.

One of the crucial progresses has been the identification of tumor antigens capable of stimulating T cell responses (Boon et al., 1994; Boon et al., 1997; Gilboa, 1999; Robbins and Kawakami, 1996; Rosenberg, 1999). In numerous pre-clinical animal models, cytotoxic T cells (CTLs, or CD8 T cells) have been shown to be the major effector cells that mediate tumor rejection. This has been supported by the many adoptive transfer studies in which CTL cell lines and CTL clones specific for tumor antigens, when activated *in vitro*, can mediate antitumor immunity when transferred into tumor-bearing hosts (Riddell and Greenberg,

1995). Recent reports also indicate that peptide immunization resulting in productive antitumor immunity is restricted by MHC class I (Feltkamp et al., 1993; Mandelboim et al., 1995; Mayordomo et al., 1995; Noguchi et al., 1995). Furthermore, depletion of CD8 T cells via antibody or genetic knockout greatly impairs the antitumor immunity induced by most cancer vaccines (Dranoff et al., 1993; Fearon et al., 1990; Golumbek et al., 1991; Lin et al., 1996). The role of CTLs in anti-tumor immunity is manifested by the fact that CTLs perform tumor killing upon direct recognition of tumor antigen peptides presented by tumor's MHC class I molecules. Since last decade, comprehensive progress has been achieved in identifying human tumor antigens recognized by CTLs (Renkvist et al., 2001).

Recently, the other arm of T cell immunity, CD4 helper T cells, has attracted more and more attention. Accumulating evidence shows that CD4 T cells, which are known to play a central role in organizing virtually all antigen specific response, also hold a critical position in orchestrating multiple effector functions in anti-tumor immunity, including activation of CTL, macrophage, and eosinophil and B cells (Pardoll and Topalian, 1998). Much work has focused on the ability of CD4 T cells to help in priming CTLs during anti-tumor immunity, suggesting that there are two mechanisms which deliver help: one, through providing cytokines (such as IL-2); the other, through "cross-priming" using APCs as the bridge, which is mediated by CD40L/CD40 interactions (Bennett et al., 1998; Huang et al., 1994; Ridge et al., 1998; Schoenberger et al., 1998). However, recent studies of cell-based vaccine models suggests that CD4 T cells

may help other effector pathways, including the Th1 effector pathway, which activates macrophages to produce reactive oxygen intermediates, and the Th2 effector pathway, which activates eosinophils to release their granule contents (Hung et al., 1998). Furthermore, a member of the cancer-testis antigens, NY-ESO-1, has been shown to be recognized by CD8 and CD4 T cells as well as antibodies (Jager et al., 1999), suggesting that CD4 T cells play a role in helping B cells to make anti-tumor antibodies. Similarly, the key role of CD4 T cells in productive anti-tumor immunity has been demonstrated by the abrogation of anti-tumor immunity in CD4 knockout mice and mice depleted of CD4 T cells (Pardoll and Topalian, 1998). CD4 T cells recognize tumor antigen peptide presented by MHC class II. Compared to their counterparts recognized by CTLs, fewer tumor antigens recognized by CD4 T cells have been identified (Renkvist et al., 2001). But the list is actively expanding.

Despite of the clear evidence about the existence of tumor antigens and spontaneous or immunization-induced anti-tumor T cell responses, most human tumors remain elusive targets for anti-tumor T cell immunity (Marincola et al., 2003). Mechanisms that cause this immunological failure include: inadequate numbers of anti-tumor T cell precursors, low avidity of their TCRs upon binding to tumor antigens, insufficient costimulation provided by the host, and the tolerogenic attenuation of the T cell immunity by the tumor cells (Chambers et al., 2001; Marincola et al., 2003; Matzinger, 2002; Ochsenbein, 2002; Smyth et al., 2001). Therefore, the major goal of immunotherapy is to maintain in the host a large population of high-avidity anti-tumor T cells that can resist tolerization

(Dudley and Rosenberg, 2003). The existing methods for cancer T cell immunotherapy fall into two major categories: passive immunotherapy and active immunotherapy. In passive immunotherapy, *in vitro* expanded highly tumor reactive T cells are transferred into the host, directly providing effector T cells to target tumor cells (Dudley and Rosenberg, 2003). In active immunotherapy, the host's endogenous T cell repertoire is activated by vaccination to generate anti-tumor effectors for tumor destruction (Berzofsky et al., 2001). We approach the goal from another angle and develop a new method entitled "instructive immunotherapy". In this method, the anti-tumor specificity of T cells are encoded into TCR cDNAs and introduced into hematopoietic stem cells (HSCs) via retrovirus. The virus-transduced HSCs are then transferred into the host. Under the instruction of the TCR transgenes, these HSCs efficiently give rise to T cells with the desired anti-tumor specificity *in vivo*. Taking advantage of the HSC characteristics of longevity and self-renewal, this method can provide the host with a lifelong supply of highly tumor specific T cells in large quantity. In particular, both anti-tumor CD8 CTLs and CD4 helper T cells can be generated in one host using this method, providing the opportunity to achieve the maximal benefits through the collaboration of both arms of the anti-tumor T cell immunity. In real clinic applications, the boundary of the different therapy methods may not be very distinct. Achieving the best therapy effect often requires combination of multiple methods. In regard to this fact, the opportunity of combining our method with the method of cancer vaccination was also explored.

2. Materials and Methods

Mice

C57BL/6J(B6) female mice were purchased from Charles River Breeding Laboratories, and RAG1 deficient female mice in the B6 background were purchased from The Jackson Laboratory. OT2 TCR transgenic mice in B6 background were also purchased from The Jackson Laboratory and then bred into RAG1 deficient background to generate OT2/RAG1 TCR transgenic mice. All mice were housed in the California Institute of Technology animal facility in accordance with institute regulations.

MOT1 and MOT2 Retrovirus

The MOT1 and MOT2 construct were generated from the MIG retrovirus (Van Parijs et al., 1999) by replacing GFP with the OT1 or OT2 TCR beta chain cDNA and inserting the OT1 or OT2 TCR α chain cDNA in the upstream of the IRES (OT1 and OT2 TCR cDNAs are kind gifts of Dr. Carbone and Dr. Heath, Australia). Retroviruses were made in HEK293.T cells using a standard protocol (Pear et al., 1993). Viruses were harvested 36-48 hours after transfection.

Peptides

$OVA_{257-264}$ SIINFEKL peptide (designated as OVAp1) recognized by OT1 TCR, and $OVA_{323-339}$ peptide (designated as OVAp2) recognized by OT2 TCR were all synthesized at Caltech Biopolymer Synthesis Center.

Primary T Cell Infection and Stimulation

Spleen cells were harvested from B6 female mice of 6-8 weeks and activated *in vitro* with 0.5μg/ml anti-CD3 and 0.5μg/ml anti-CD28 Abs (both from Pharmingen). On day 2 of culture, cells were spin-infected with MOT1 or MOT2 retroviruses in the presence of 10μg/ml polybrene for 90min at 2,500rpm at 30ºC. On day 3, cells were collected. Some aliquots of the collected cells were used to assay for the expression of OT1 or OT2 TCRs by flow cytometry. The rest were rested overnight with 10ng/ml rmIL-2 (Biosource International, Camarillo, CA). The next day, the rested cells were tested for their responsiveness to antigen stimulation. Cells infected with MOT1 retroviruses were stimulated with OVAp1 at 0-1μg/ml in the presence of APCs (spleen cells of B6 female mice). The cells infected with MOT2 retroviruses were stimulated with OVAp2 at 0-10μg/ml in the presence of APCs (spleen cells of B6 female mice). On day 3 of stimulation, cell culture supernatants were collected and analyzed for IFN-γ production using ELISA as described before (Yang et al., 2002).

Hematopoietic Stem Cells (HSCs) Isolation, Infection and Transfer

B6 female mice or RAG1$^{-/-}$ female mice were treated with 250μg per gram of body weight of 5-fluorouracil (Sigma). Five days later, bone marrow (BM) cells enriched with HSCs were harvested and cultured for 4 days in RPMI containing 10% FBS with 20ng/ml rmIL-3, 50ng/ml rmIL-6 and 50ng/ml rmSCF (all from Biosource International, Camarillo, CA). On day 2 and 3, the cells were spin infected with MOT1 or MOT2 retroviruses supplemented with 8μg/ml polybrene

for 90min at 2,500rpm, 30ºC. On day 4 of culture, BM cells were collected and transferred by tail vein injection into B6 female hosts or RAG1$^{-/-}$ female hosts that had received 1200 rads or 360 rads whole-body radiation, respectively. Each host received 2-3x10^6 infected BM cells. BM recipient mice were maintained on the mixed antibiotic sulfmethoxazole and trimethoprim oral suspension (Hi-Tech Pharmacal, Amityville, NY) in a sterile environment for 6-8 weeks until analysis or usage for further experiments.

In vitro *T cell Stimulation and Functional Assays*

Spleen cells from BM recipient mice were harvested and cultured at 2x10^5 cells/well in T cell culture medium containing OVAp1 at 0-1μg/ml or OVAp2 at 0-10ug/ml. Three days later, culture supernatants were collected and assayed for IL-2 or IFN-γ production by ELISA, and proliferation was assessed by [^{3}H]-thymidine incorporation as described before (Yang et al., 2002).

Antibodies and FACS Analysis

Fluorochrome-conjugated antibodies specific for mouse CD4, CD8, CD25, CD69, CD62L, CD44, TCRVα2, TCRVβ5.1,5.2 were purchased from BD Pharmingen (San Diego, CA). Surface staining was performed by blocking with anti-CD16/CD32 (mouse Fc receptor, BD Pharmingen, San Diego, CA) followed by staining with fluorochrome-conjugated antibodies. Intracellular staining of TCR was preformed using the Cytofix/Cytoperm^{TM} Kit from BD Pharmingen (San Diego, CA). A FACScan flow cytometer was used for detailed analysis.

T Cell Memory Study

Spleen and lymph node cells from BM recipient mice (B6/MOT1 or B6/MOT2) were harvested and stimulated with 0.1μg/ml OVAp1 or 1μg/ml OVAp2 for 3 days *in vitro*, respectively. The cells were then collected and transferred into RAG1$^{-/-}$ hosts by tail vein injection. Each host received 20-30x10^{6} cells (>10% were activated OT1 or OT2 T cells). Sixteen weeks later, spleen cells were harvested from the hosts and analyzed for the presence of long-lived OT1 or OT2 T cells. Memory phenotype of the OT1 or OT2 T cells was studied by FACS. Memory function was studied by antigen dosage response, antigen time-course response and cytokine proliferation response of the OT1 or OT2 T cells. For antigen dosage response, cells were stimulated with 0-1μg/ml OVAp1 or 0-10μg/ml OVAp2 for 3 days, and the culture supernatants were collected and assayed for IL-2, IL-4 or IFN-γ production by ELISA; proliferation was assessed by [^{3}H]-thymidine incorporation as described before (Yang et al., 2002). For antigen time-course response, cells were stimulated with 0.1μg/ml OVAp1 or 1μg/ml OVAp2, and the culture supernatants were collected and assayed for IL-2, IL-4 or IFN-γ production by ELISA on day 1.5, day 2.5 and day 3.5. In cytokine proliferation response, cells were cultured with 10ng/ml rmIL-2, or 10ng/ml IL-4 or 10ng/ml rmIL-15 (all from BioSource International, Camarillo, CA) for 4 days in the absence of antigen, and proliferation was assessed by [^{3}H]-thymidine incorporation.

Tumor Challenge of Mice

The tumor cell lines EL.4 (C57BL/6, H-2^b, thymoma) and E.G7 (EL.4 cells transfected with the chicken OVA cDNA) (Moore et al., 1988) were used for tumor challenge of mice. 5-10x10^6 EL.4 or E.G7 cells were injected subcutaneously into the left flank of the mice. Tumor size was measured every other day using fine calipers (Manostat Corporation, Switzerland), and is shown as the product of the two largest perpendicular diameters a x b (mm^2). Mice were euthanized when the tumors reached 400mm^2.

Dendritic Cell Generation, Antigen Pulsing and Mouse Immunization

Dendritic cells (DC) were generated from bone marrow cultures as described by Lutz MB et al. (Lutz et al., 1999), with some minor modifications. Briefly, bone marrow cells were harvested from B6 female mice (6-8 weeks old) and cultured in 10cm diameter petri dishes at 2x10^6 cells/dish in 10ml R10 medium (RPMI-1640 supplemented with 100U/ml Penicillin, 100µg/ml Streptomycine, 2mM L-glutamin, 50µM 2-mercaptoethanol and 10% FBS) containing 1:30 J558L culture supernatant. J558L is a cell line transfected with the murine GM-CSF gene (Zal et al., 1994) and its culture supernatant used at the source of GM-CSF. On day 3, another 10ml R10 medium containing 1:30 J558L culture supernatant was added into each dish. On day 6 and day 8, half of the culture supernatant was collected and centrifuged, and the cell pellet was resuspended in 10ml fresh R10 medium containing 1:30 J558L culture supernatant and added back into the original culture dishes. On day 9, non-

adherent cells were collected and plated into new 10cm diameter petri dishes at 4-6x10^6 cells/dish in 10ml R10 medium containing 1:60 J558L culture supernatant. LPS (Sigma, 1μg/ml) was added to mature DCs. On day 10, non-adherent cells (usually >80% are mature DCs) were collected and washed once with IMDM/50mM 2-mercaptoethanol and resuspended in 0.8ml of the same medium containing 100μg OVAp1 (or 100μg OVAp1+100μg OVAp2). The cells were then incubated at 37ºC for 3 hours with gentle shaking every 30min. Three hours later, the OVAp1 or OVAp1+2 loaded DCs were washed twice with PBS and used to immunize mice by tail vein injection. Each mouse received about 0.5x10^6 OVAp loaded DCs.

3. Results

Tumor Model

We chose to test the feasibility and potential of this method for cancer immunotherapy in the E.G7 mouse tumor model. E.G7 is a B6 origin mouse thymoma cell line, which was generated by engineering the parental cell line EL.4 to express chicken OVA gene (Moore et al., 1988). Previous studies showed that the $OVA_{257-264}$ peptide (SIINFEKEL), the epitope recognized by CD8 TCR OT1 in the context of MHC class I molecule H-$2K^b$ (Hogquist et al., 1994), is displayed on the E.G7 surface at a density (about 100 H-2Kb/OVAp per cell) similar to tumor antigens on authentic tumor cells (Rotzschke et al., 1991). Therefore, the E.G7 tumor cell-OT1 T cell system has been widely used to study CTLs mediated anti-tumor immune responses (Helmich and Dutton, 2001;

Shrikant et al., 1999). Furthermore, the natural processed epitope $OVA_{323-339}$ peptide presented by MHC class II molecule I-A^b is recognized by CD4 TCR OT2 (Barnden et al., 1998), providing an opportunity to study the anti-tumor CD4 T cell immune response.

Retrovirus Mediates Functional Expression of CD8 and CD4 T cell Receptors (TCRs)

We chose the well characterized CD8 TCR OT1 and CD4 TCR OT2 for our study. Both TCRs were cloned from B6 mice (Barnden et al., 1998; Kelly et al., 1993). OT1 recognizes chicken $OVAp_{257-264}$ (denoted as OVAp1) in the context of the MHC I molecule H-$2K^b$ (Hogquist et al., 1994). OT2 recognizes chicken $OVAp_{323-339}$ (denoted as OVAp2) in the context of the MHCII molecule I-A^b (Barnden et al., 1998). We inserted the OT1 or OT2 TCR α and β chain cDNAs into retroviral vector mouse stem cell virus (MSCV) under the control of viral LTR promoter to generate MOT1 or MOT2 constructs (Figure 4-1A). To achieve the co-expression of the TCR α and β chains, we linked the two cDNAs with IRES (internal ribosome entry site). When MOT1 retroviruses were used to infect *in vitro* activated mouse peripheral T cells, we observed 41% of the cells expressing OT1 TCRs (Figure 4-1B, left). When stimulated with OVAp1, these infected T cells were able to respond as measured by IFN-γ production (Figure 4-1C, left). Similarly, when MOT2 retroviruses were used to infect *in vitro* activated mouse peripheral T cells, 52% of the cells were detected to express OT2 TCRs (Figure 4-1B, right). These infected cells were able to respond to OVAp2

stimulation to produce IFN-γ (Figure 4-1C, right). Our results show that the retroviral constructs MOT1 and MOT2 can efficiently mediate functional expression of the OT1 CD8 and OT2 CD4 TCRs.

Generation of Monospecific CD8 or CD4 T cells by Retrovirus-Mediated Expression of CD8 or CD4 TCR in RAG1$^{-/-}$ HSCs

Previously we showed that we can generate antigen-specific CD4 T cells *in vivo* via retrovirus-mediated expression of TCR cDNAs in RAG1$^{-/-}$ HSCs (Yang et al., 2002). We then tested whether the MOT1 and MOT2 constructs could be used to generate OT1 CD8 and OT2 CD4 T cells. RAG1$^{-/-}$ mice were treated with 5-FU to enrich the HSCs in bone marrow (BM). Five days later, BM cells were harvested and cultured *in vitro* when the cells were infected with MOT1 or MOT2 retroviruses. The transduced HSCs were then collected and transferred separately into irradiated RAG1$^{-/-}$ recipient mice and the resulting mice were designated as RAG1/MOT1 and RAG1/MOT2, respectively. The recipients were allowed to reconstitute their immune system for at least 6 weeks.

Seven weeks after adoptive transfer, the RAG1/MOT1 and RAG1/MOT2 recipients were analyzed. We found about 5% and 7% of cells in BM expressing the OT1 or OT2 transgenic TCRs, respectively (Figure 4-2A). Analysis showed the presence of long-term HSCs, as identified by surface markers c-Kit and Scal-1 (data not shown). Study of the recipients 6 months after adoptive transfer showed the persistence of the transduced HSCs (data not shown). The observation is further confirmed by the persistence of these cells upon the

secondary transfer (data not shown). Taken together, the results suggest that through our method, TCR genes can be transferred into HSCs that maintain the stem cell features of longevity and self-renewal, providing the recipients a life-long source of genetic modified hematopoietic cell progenitors.

We then analyzed the thymii of RAG1/MOT1 and RAG1/MOT2 mice. The majority of the thymocytes expressed the OT1 or OT2 TCR transgenes (64% in RAG1/MOT1 and 84% in RAG1/MOT2) as shown in Figure 4-2B (upper), indicating they developed from the virally transduced HSCs. Further study of the distribution of T cell development markers CD4 and CD8 on thymocytes of the RAG1/MOT1 mice shows a typical pattern for CD8 T cell development. Due to the lack of endogenous TCR rearrangement, T cells cannot be developed in $RAG1^{-/-}$ mice. In thymus, the natural $RAG1^{-/-}$ thymocytes stay at the double negative (DN) stage (Figure 4-2B, lower left). We found that in RAG1/MOT1 mice, T cell development was rescued and the $RAG1^{-/-}$ thymocytes advanced to double positive (DP) stage, followed by CD8 single positive (SP) stage (Figure 4-2B, lower middle). Similarly, thymocytes of RAG1/MOT2 mice showed a rescued CD4 T cell development (Figure 4-2B, lower right).

We then analyzed the peripheral lymph organs (spleen and lymph notes) for the presence of mature T cells. In RAG1/MOT1 mice, we found CD8 T cells to be uniformly expressing OT1 TCRs, and no CD4 T cells were detected (Figure 4-2C, upper). In contrast, we found CD4 T cells to be uniformly expressing OT2 TCR, and no CD8 T cells were detected in RAG1/MOT2 mice (Figure 4-2C, lower).

Our results suggest that by retrovirus-mediated expression of pre-rearranged TCR cDNAs in RAG1$^{-/-}$ HSCs, T cell development can be efficiently rescued. Moreover, the determination of CD4 or CD8 T cell fate is strictly controlled by the nature of the transgenic TCR genes.

Comparison of the Transgenic TCR Expression and T cell Development in Mice Receiving Retrovirus-Transduced RAG1$^{-/-}$ HSCs with Those in the Conventional TCR Transgenic Mice

Traditionally, generation of antigen specific T cells *in vivo* is achieved by making TCR transgenic animals via co-injecting constructs expressing either TCR α chain gene or β chain gene into the fertilized egg (Vonboehmer, 1990). Two approaches have been developed for this purpose. The more straightforward one makes use of heterologous regulatory elements to drive the expression of cDNA genes encoding the separate α and β chains of the TCR (Mamalaki et al., 1993; Pircher et al., 1989; Turner et al., 1996). The other is usually more complex because the natural TCR promoter and enhancer elements are used as part of a genomic TCR transgene expression construct (Berg et al., 1989; Kisielow et al., 1988; Sha et al., 1988). Although the second approach is usually preferred, as it is assumed to more closely mimic the normal developmental expression of the TCR genes in unmanipulated animals (Kouskoff et al., 1995), success has been made using both approaches. The OT2 TCR transgenic mouse has been made using a combination of the two approaches: the cDNA encoding the OT2 α chain was inserted into the pES4 transgene

expression construct that contains the H-2K^b promoter, the IgH chain enhancer and the polyadenylation signal sequence of the human β-globin gene; meanwhile, the OT2 β chain gene was inserted into a genomic-based construct (Barnden et al., 1998).

Our method of retrovirally mediated HSC transfer to generate antigen specific T cells uses minimal genetic materials: cDNAs encoding OT2 TCR α and β chains linked by an IRES sequence are inserted into a retroviral vector under the control of viral LTR promoter. Compared to the large constructs used for making TCR transgenic mice, our approach has the advantage of being easy to handle. More importantly, this method holds therapeutic potential. To evaluate the efficacy of our method, we performed detailed comparison of OT2 TCR expression and T cell development between our RAG1/MOT2 recipient mice and the OT2/RAG1 Tg mice (the conventional OT2 TCR transgenic mice bred into RAG1$^{-/-}$ background, designated as OT2/RAG1 Tg). RAG1 genetic deficiency dose not support endogenous TCR rearrangement and T cell development, providing us a clean background for the intended study.

At first, we examined the OT2 TCR expression in BM, from which the T cell progenitors are derived (Kondo et al., 2003). Since TCRs cannot display on the cell surface without associating with the CD3 proteins, which are only expressed in committed T lineage cells (Oettgen et al., 1986), we used intracellular staining to analyze TCR expression. We found in OT2/RAG1 Tg mice, a large portion (~32%) of the BM cells expressed OT2 α chain (Figure 4-3A, middle). No β chain expression was detected (Figure 4-3A, middle). This

observation is consistent with how the transgenes were constructed: OT2 α chain is under control of H-2K^b promoter and IgH enhancer and β chain is under control of natural TCR promoter and enhancer (Barnden et al., 1998). In the RAG1/MOT2 mice, we found 7% of the BM cells expressing OT2 genes and the α chains always co-localized with the β chains (Figure 4-3A, right), indicating the MOT2 retroviruses effectively mediated co-expressing of OT2 α and β cDNAs in hematopoietic cells.

We then analyzed the thymus. In RAG1$^{-/-}$ control mice, thymocytes stopped at the DN stage due to the failure of rearrangement of endogenous TCRs (Figure 4-3B, upper left). In OT2/RAG1 Tg mice, the development is rescued and the thymocytes show a typical CD4 T cell development pattern (Figure 4-3B, middle left). Similarly, thymocytes in RAG1/MOT2 mice are rescued and can develop CD4 SP T cells (Figure 4-3B, lower left). We followed the OT2 TCR expression in OT2/RAG1 Tg mice through the developmental stages (DN, DP and CD4 SP) and found that the α chain was expressed constantly high through all the three stages; while the β chain expression started from DN stage at a low level, up-regulated slightly in DP stage and reached a high level in CD4 SP stage (Figure 4-3B, middle right). Interestingly, in RAG1/MOT2 mice, both OT2 α and β chain expressions closely resembled the pattern of β chain expression in OT2/RAG1 Tg mice, which is under control of the natural TCR promoter and enhancer (Figure 4-3B, lower right). It is unlikely that viral LTR promoter was regulated in the same way as natural TCR promoters and enhancers along the T cell development (Vonboehmer, 1990). A possible

explanation is that at each developmental stage, the thymocytes expressing the right levels of OT2 TCRs were selected and allowed to advance into the next stage; the others failed and were deleted. When we used MOT2 retroviruses to infect HSCs, we generated a large number of HSCs that expressed OT2 TCR genes at a broad range, due to the differences of viral copy numbers and integration sites each HSC received. In fact, we did observe a heterogeneous TCR expression in BM cells (Figure 4-3A, right) and DN thymocytes of RAG1/MOT2 mice (Figure 4-3B, right). Subsequently, thymocytes expressing a slightly higher level of TCRs were allowed to enter the DP stage. A final selection of thymocytes expressing an even higher level of TCRs led to the generation of CD4 SP T cells (Figure 4-3B, lower right). We observed that the CD4 SP thymocytes accounted for about 2% of the total thymocytes in RAG1/MOT1 mice, much less than the 42% in OT2/RAG1 Tg mice (Figure 4-3B, left), further supporting the “selection for fitness” hypothesis.

At last we analyzed the presence of mature T cells in peripheral lymph organs. As expected, we found only monospecific OT2 CD4 T cells and no CD8 T cells in both OT2/RAG1 Tg mice and RAG1/MOT2 mice (Figure 4-3C). Compared with the OT2 T cells in OT2/RAG1 Tg mice, the OT2 T cells in RAG1/MOT2 mice expressed TCRs in a broader range and with a lower average level, both at the level of protein expression as measured by intracellular staining, (Figure 4-3C, left) and the surface display as measured by surface staining (Figure 4-3C, right). It has been reported previously that lower expression of TCR could impair the ability of T cells to respond to antigens (Homer et al., 1993).

To address this concern, we tested the antigen responsiveness of these OT2 T cells *in vivo* by immunizing the animals with OVAp2 peptide antigen; OT2/RAG1 Tg mice were included as a control. As shown in Figure 4-3C, compared to the OT2 T cells in unchallenged mice, the OT2 T cells in immunized RAG1/MOT2 mice expressed TCRs above a certain level (judged by the intensity of intracellular TCR staining, Figure 4-3C), indicating that these cells responded to antigen stimulation and were preferentially expanded. On the contrary, no such change was observed in immunized OT2/RAG1 Tg mice (Figure 4-3C). The results support the notion that there is a quantitative signal threshold for T cell responsiveness, as reflected in Figure 4-3C by the TCR expression level. In RAG1/MOT2 mice, the unresponsive cells expressing TCRs below the threshold accounted for less than 10% of the total OT2 T cells generated in RAG1/MOT2 mice (Figure 4-3C). For the OT2 T cells above that threshold, the variation on TCR expression level seems not to affect the ability of the T cells to respond, and no obvious expansion advantage was observed for OT2 T cells expressing higher level of TCRs (Figure 4-3C). This observation is also confirmed by the *in vitro* T cell stimulation (data not shown). In spite of the differences, comparison of the naïve OT2 T cells generation and the overall T cell expansion in response to antigen stimulation *in vivo* showed that the efficacy of this method in generating antigen specific T cells and the antigen-induced T cell response is comparable to that of the conventional TCR transgenic technique (Figure 4-3D).

In summary, during T cell development, TCR expression pattern mediated by retroviral LTR promoter resembles closely with that controlled by natural TCR

promoter and enhancer in conventional transgenic mice. T cell development is normal in mice receiving retrovirus-transduced HSCs, and this method is very efficient in generating functional antigen-specific T cells, with an efficacy comparable with the conventional TCR transgenic method.

Generation of Antigen-Specific CD8 or CD4 T Cells by Retrovirus-Mediated Expression of CD8 or CD4 TCR in Wild-type HSCs

Our success in generating antigen-specific CD8 cytotoxic and CD4 helper T cells by retroviral transduction of $RAG1^{-/-}$ HSCs is encouraging, and prompted us to test whether this method also works with wild-type HSCs, which is a critical step leading to test the instructive immunotherapy concept. Using the similar approach, we treated wild-type B6 mice with 5-FU to enrich the HSCs, and harvested BM cells 5 days later. The cells were then infected with either MOT1 or MOT2 retroviruses and transferred into irradiated B6 recipient mice (designated as B6/MOT1 or B6/MOT2). The recipients were allowed to reconstitute their immune system for at least 6 weeks. This approach is illustrated in Figure 4-4A.

Eight weeks after adoptive transfer, we analyzed the B6/MOT1 and B6/MOT2 mice. Similarly as in the RAG1/MOT1 and RAG1/MOT2 mice, we observed the presence of BM cells expressing the transgenic OT1 or OT2 TCRs in B6/MOT1 (5.5%, Figure 4-4B, middle) or B6/MOT2 (3%, Figure 4-4B, right) mice. Analysis exhibited that among these cells were long-term HSCs via stem cell markers c-Kit and Scal-1 staining (data not shown). Study of the recipients 8

months later after adoptive transfer showed the persistence of the transduced HSCs (data not shown). Furthermore, these transduced cells were observed to persist through the secondary transfer (data not shown). These results are consistent with the previous study using RAG1$^{-/-}$ HSCs (Figure 4-2), suggesting that in using our method, TCR genes can be stably transferred into wild-type HSCs without affecting the stem cell features of longevity and self-renewal, and thus providing the recipients a lifelong source of genetic modified hematopoietic cell progenitors.

Our next step was to examine the thymus. We found that 5% or 3% of the thymocytes in B6/MOT1 mice or B6/MOT2 mice, respectively, expressed the transgenic OT1 or OT2 TCRs, indicating that these cells were derived from the transduced HSCs (Figure 4-4C, upper). Study of the surface expression pattern of the developmental markers CD4 and CD8 showed that CD8 SP compartment in B6/MOT1 mice (4.3% compared to 2.0% in B6 control mice) and CD4 SP compartment in B6/MOT2 mice (8.3% compared to 4.5% in B6 control mice) were selectively enriched (Figure 4-4C, lower), suggesting that expression of OT1 or OT2 TCR transgenes in thymocytes could direct them to the appropriate T cell fate.

Finally, we analyzed the periphery. We found that in the spleen, about 25% of the CD8 T cells in B6/MOT1 mice were OT1 T cells, and no CD4 T cells apparently expressed the OT1 TCRs (Figure 4-4D, upper). On the other hand, in the spleen of B6/MOT2 mice, about 8% of the CD4 T cells were OT2 T cells; no CD8 T cells apparently expressed OT2 TCRs (Figure 4-4D). Our results suggest

that our method of retrovirus-mediated transfer of TCR cDNAs into wild-type HSCs is highly efficient in generating the T cells with the desired characteristic (CD4 vs. CD8 T cells) and specificity.

Characterization of the CD8 and CD4 T Cells Generated by Viral Transduction of Wild-type HSCs

Next, we tested whether the CD8 and CD4 T cells generated by viral transduction of wild-type HSCs are functional. At first we tested the OT1 CD8 T cells generated by MOT1-mediated bone marrow transfer. Spleen cells were harvested from B6/MOT1 mice and stimulated with OVAp1 in the culture. Before stimulation, about 25% of the CD8 T cells were OT1 cells that showed naïve CD8 T cell phenotype of $CD25^{-}CD69^{-}CD62L^{high}CD44^{low}$ as measured by surface staining (Figure 5-5A, upper). After stimulation with OVAp1 for 3 days, OT1 T cells expanded to 80% of the total CD8 T cells in the culture (Figure 4-5A, middle). Study of the surface activation markers showed that these OT1 T cells expressed a typical effector CD8 T cell phenotype: CD25highCD69high-CD62LlowCD44high (Figure 4-5A, middle). When compared with OT1 T cells harvested from the conventional TCR transgenic mice (designated as OT1(Tg)), the OT1 T cells generated by retroviral mediated BM transfer (designated as OT1(BMT)) showed comparable proliferation (Figure 4-5B, left) and IFN-γ production (Figure 4-5B, middle) in response to antigenic stimulation.

A unique feature of the adaptive immune system is the ability to generate long-term memory after the initial antigen encounter, thus providing more efficient

protection for the next infection (Sprent and Surh, 2002). This feature is of special interest to us because of its importance for potent tumor immunotherapy. To this end, we tested whether the OT1(BMT) T cells can generate memory. Effector OT1 T cells were collected from culture after stimulation with OVAp1 for 3 days and adoptively transferred into RAG1 recipients. Sixteen weeks later, the recipients were analyzed for the presence of long-lived memory OT1 T cells. As shown in Figure 4-5A (lower), we found about 6% of the recovered CD8 T cells were OT1 T cells. Surface staining of the activation markers showed that these OT1 T cells expressed the featured memory T cell phenotype: $CD25^{-}CD69^{-}CD62L^{high}CD44^{high}$ (Figure 4-5A, lower right). Furthermore, when stimulated with OVAp1 in the culture, these OT1 T cells showed a stronger and faster response as measured by IFN-γ production, compared with the response of the naïve OT1 T cells (Figure 4-5C, left and middle). And when stimulated with cytokines IL-2 and IL-15, these OT1 T cells responded with extensive proliferation, while the naïve OT1 T cells did not (Figure 4-5C, right).

Secondly, we tested the function of the OT2 T cells generated by MOT2 mediated BM transfer (designated as OT2(BMT)). Similarly, spleen cells were harvested from B6/MOT2 mice and stimulated with OVAp2 in the culture. Before stimulation, we detected about 8% of the spleen CD4 T cells expression OT2 TCRs (Figure 4-5D, upper left). Surface staining showed that these OT2 CD4 T cells were of the naïve T cell phenotype: $CD25^{-}CD69^{-}CD62L^{high}CD44^{low}$ (Figure 4-5D, right). After stimulation with OVAp2 for 3 days, these OT2 T cells expanded to 17% of the total CD4 T cells in culture, and expressed the typical

effector CD4 T cell phenotype: $CD25^{high}CD69^{high}CD62L^{low}CD44^{high}$ (Figure 4-5D, middle). When compared with OT2 T cells harvested from the conventional OT2 TCR transgenic mice (designated as OT2(Tg)), these OT2(BMT) cells showed comparable proliferation and IL-2 production (Figure 4-5E, left and middle). Again we tested the ability of these OT2 T cells to generate long-term memory. Effector OT2 CD4 T cells were collected from culture after stimulation with OVAp2 for 3 days and adoptively transferred into RAG1 recipients. Fourteen weeks later, the recipients were analyzed. As shown in Figure 4-5D (lower left), we detected the presence of long-lived OT2 T cells (~4% of the total CD4 T cells). Study of these OT2 T cells showed that they displayed the memory phenotype of $CD25^{-}CD69^{-}CD62L^{high}CD44^{high}$ (Figure 4-5D, lower right). Further analysis showed that compared with naïve OT2 T cells, these OT2 T cells responded to antigen stimulation stronger (Figure 4-5F, upper) and faster (Figure 4-5F, lower), as measured by IL-2, IL-4 and IFN-γ production. Moreover, these OT2 T cells proliferated intensively when stimulated with cytokines IL2, IL4 and IL-15, while the naïve OT2 T cells did not (Figure 4-5E, right).

Taken together, these results reveal that the OT1 CD8 T cells and the OT2 CD4 T cells generated using retrovirus transduction of B6 HSCs are fully normal and functional in all the aspects we tested. In particular, these T cells can generate long-term memory, making our method markedly attractive for immunoherapy.

Imparting into Mouse T Cell Repertoire Both Anti-tumor CD8 Cytotoxic and CD4 Helper T Cell Specificities

Our method of using a population of HSCs as the targets for gene transfer offers the opportunity to divide them into sub-pools and deliver different genes into each one. In the case of imparting anti-tumor specificities to the mouse T cell repertoire, we envisioned that by dividing the HSCs into two sub-pools and transducing one with MOT1 and the other with MOT2 retroviruses, we should be able to generate both OT1 CD8 and OT2 CD4 T cells *in vivo*. The idea was tested as follows: B6 mice were treated with 5-FU to enrich the HSCs. Five days later, BM cells were harvested and divided into two populations. One population of the cells was infected with MOT1 retroviruses and the other with MOT2 retroviruses. The transduced HSCs were then pooled together and transferred into irradiated B6 recipient mice (designated as B6/MOT1+MOT2). The recipients were allowed to reconstitute their immune system for 6 weeks and then were analyzed for the presence of OT1 and OT2 T cells. As expected, we found OT1 CD8 T cells and OT2 CD4 T cells generated in the recipient mice, accounting for about 10% of the peripheral CD8 and 6% of the peripheral CD4 T cells, respectively (Figure 4-6). Further analysis showed that they exhibited completely normal and functional characteristics of T cells (data not shown). Therefore, our method can be used to efficiently impart to the T cell repertoire both anti-tumor CD8 cytotoxic and CD4 helper T cell specificities. A further extension of the method is to impart to the T cell repertoire CD8 and CD4 specificities that recognize multiple epitopes of the tumor antigens, thus providing a new

opportunity to overcome the tendency of tumors towards “epitope escape” (Khong and Restifo, 2002).

Test the Concept of Tumor Immunotherapy in E.G7 Mouse Tumor Model: Suppression of syngenic tumor growth by imparting anti-tumor specificities to mouse T cell repertoire

The role of CD8 cytotoxic T cells (CTLs) in anti-tumor immune response has been very well recognized (Riddell and Greenberg, 1995). The accumulating evidence indicates that CD4 helper T cells are an equally critical component of the anti-tumor immune response (Pardoll and Topalian, 1998). It is now generally believed that optimal tumor immunotherapy requires the activation of both arms of anti-tumor T cell immunity (Pardoll and Topalian, 1998). Our success in generating antigen specific CD8 and CD4 T cells *in vivo* by retroviral transduction of HSCs provides new opportunity for such purpose. Therefore, we tested this concept in the E.G7 mouse tumor model. To evaluate the anti-tumor function of each arm of the T cell immunity and the combination of both, we performed experiments using mice imparted with anti-tumor CD8 specificity (B6 mice receiving B6 HSCs transduced with MOT1, designated as B6/MOT1), or anti-tumor CD4 specificity (B6 mice receiving B6 HSCs transduced with MOT2, designated as B6/MOT2) or both (B6 mice receiving both B6 HSCs transduced with MOT1 and HSCs transduced with MOT2, designated as B6/MOT1+MOT2).

At first we tested the suppression of syngenic tumor growth using the protocol as shown in Figure 4-7A. B6 mice receiving B6 HSCs transduced with

MOT1, MOT2 or the mixture of both were allowed to reconstitute the immune system for 8-10 weeks. E.G7 or the control tumor cells EL.4 were then injected subcutaneously (each mice received $5x10^6$ E.G7 or EL.4 tumor cells). To evaluate the effects of immunization, 4 days after the tumor injection, 8 groups of mice out of 16 were immunized with one dose of dendritic cells (DCs) loaded with OVAp1 to boost anti-tumor CD8 response. Tumor growth was monitored daily, and mice were euthanized when tumors reached the size of $400mm^2$. Four mice were used in each group and the experiments were performed three times.

Results from one representative experiment were shown in Figure 4-7B. In B6 control mice that were not imparted with anti-tumor specificities, E.G7 grew up at the similar rate as EL.4 tumor cells, resulting in visible solid tumors in one week. And they reached the size of $400mm^2$ in about 3 weeks. In sharp contrast, E.G7 tumor growth was greatly suppressed in B6 mice imparted with anti-tumor CD8 T cell specificity (B6/MOT1 mice). In half of the B6/MOT1 mice, total tumor suppression was observed for as long as the experiment ran (up to 200 days). For the other half of the mice, the tumor growth was suppressed for about 18 days but then finally progressed. We analyzed the OT1 T cells harvested from these tumor-bearing mice and found that these cells could not respond when stimulated with antigen *in vitro* (data not shown), apparently having been attenuated by tumor tolerance mechanisms. We speculated that booster immunization to active OT1 T cells could help. As expected, with a single dose of immunization with DC loaded with OVAp1, complete tumor suppression was observed for all the mice without recurrence for as long as the experiment ran

(up to 200 days). On the contrary, EL.4 tumor grew up in B6/MOT1 mice at the same rate as in B6 control mice, regardless of immunization or not, indicating that the suppression of E.G7 tumor growth is tumor-antigen specific and is mediated by the anti-tumor OT1 T cells.

Interestingly, we also observed significant tumor suppression in mice imparted with anti-tumor CD4 specificity (B6/MOT2 mice). As shown in Figure 4-7B, complete tumor suppression was observed in one out of four of the animals. In the other 3 mice, tumor growth was suppressed for 10-20 days and then progressed. Study of the OT2 T cells recovered from the tumor-bearing mice also showed that they could not respond to antigen stimulation *in vitro* (data not shown), suggesting they had been subjected to the similar tumor tolerance mechanisms. We have tested the E.G7 tumor cells previously and found that they were MHC class II negative, and OT2 T cells could not recognize and respond to them *in vitro* (data not shown). Therefore, the tumor suppression we observed in B6/MOT2 mice could not be mediated by the direct recognition of the E.G7 tumor cells by the OT2 T cells. This phenomenon of CD4 T cell mediated suppression of MHC class II negative tumors has also been reported in several other cases, such as the FBL-3 murine leukemia tumor model (Pardoll and Topalian, 1998). The working model proposed is that tumor antigens released at the tumor sites are ingested, processed and presented by macrophages. The tumor specific CD4 T cells recognize the tumor antigens, get activated and prime multiple arms of the anti-tumor immunity, including CTL activation, macrophage activation and eosinophil activation (Pardoll and Topalian, 1998). In the

B6/MOT2 mice, it seems that the anti-tumor CTL activity plays an important role. When we immunized these mice with one dose of DC pulsed with OVAp1 (the epitope recognized by CD8 T cells) to activate the anti-tumor CTL response, we observed total suppression of tumor growth in half of the B6/MOT2 mice. In the other half of the mice, tumor grew up to a nearly detective size and regressed soon. In all the mice, no tumor recurrence was observed as long as the experiment went on (up to 200 days). EL.4 tumor grew up in B6/MOT2 mice at the same rate as in the B6 control mice, with or without immunization, suggesting that the tumor suppression observed in these mice is tumor antigen specific and mediated by the imparted OT2 CD4 T cell anti-tumor specificity.

When we analyzed the mice imparted with both anti-tumor CD8 and CD4 T cell specificities (B6/MOT1+MOT2), we observed a combinatory effect. Complete tumor suppression was observed in half of the animals. For the other half, tumor growth was suppressed for about 18 days and then progressed, but with a growing rate slower than that observed in B6/MOT1 mice and B6/MOT2 mice. It took longer for these mice to reach the tumor size of 400mm^2 (about 50 days after tumor challenge) than the tumor-bearing mice in the groups of B6/MOT1 and B6/MOT2 (about 36-38 days after tumor challenge) (data not shown). Furthermore, obvious lesions were observed on most of the tumors, suggesting the presence of active anti-tumor immunity (data not shown). Nevertheless, the final progress of the tumors in half of the animals indicates the existence of tumor tolerance, which is confirmed by the much reduced response to antigen stimulation *in vitro* of the OT1 and OT2 T cells recovered from these

mice (data not shown). However, our results suggest that imparting to the mouse T cell repertoire both anti-tumor CD8 and CD4 T cell specificities did have an advantage over imparting only one of the two arms. As expected, immunization of the B6/MOT1+MOT2 mice with one dose of DCs loaded with OVAp1 completely suppressed the E.G7 tumor growth. For the control, the observation of EL.4 tumor growing up in B6/MOT1+MOT2 mice at the same rate as in B6 control mice, regardless of immunization or not, suggests that the E.G7 tumor suppression is tumor-specific and mediated by the anti-tumor OT1 CD8 and OT2 CD4 T cell specificities imparted into the B6/MOT1+MOT2 mice.

Testing the Concept of Tumor Immunotherapy in E.G7 Mouse Tumor Model: Eradication of the established solid tumor by reversal of the functional tumor tolerance via construction of the two arms of anti-tumor T cell immunity

We have showed in Figure 4-7B that imparting to the mouse T cell repertoire either CD8 cytotoxic or CD4 helper T cell specificity efficiently suppressed syngenic tumor growth, and imparting both achieved even better suppression. This encouraged us to test further the possibility to eradicate established solid tumors by constructing both arms of the anti-tumor T cell immunity. Accumulating evidence showed that the presence of CD4 T cells' help would be critical for maximizing the anti-tumor CTL response and generating the optimal immunotherapy (Pardoll and Topalian, 1998). The help from anti-tumor CD4 T cells could be direct help by providing CTLs cytokines like IL-2; or indirect

help by enhancing APC functions by CD40L/CD40 interaction (Pardoll and Topalian, 1998).

The protocol is shown in Figure 4-8A. The experiment included B6 control mice, B6 mice imparted with CTL anti-tumor specificity (B6/MOT1) and B6 mice imparted with both CD8 CTL and CD4 helper T cell anti-tumor specificities (B6/MOT1+MOT2). Mice that received BM transfer were allowed to reconstitute the immune system for 6-10 weeks. The mice were then challenged with E.G7 tumor cells subcutaneously (each mice received $10x10^6$ E.G7 tumor cells). Mice in which tumors grew up were immunized with one dose of DCs loaded with both OVAp1 (epitope recognized by OT1 TCR) and OVAp2 (epitope recognized by OT2 TCR), at the time when tumors reached the size of $30mm^2$. Tumor growth was monitored daily, and mice were euthanized when tumors reached the size of $400mm^2$. Four mice were used in each group and the experiments were performed three times.

Results from one representative experiment are shown in Figure 4-8B. In B6 control mice, E.G7 tumor grew up in 3 days and reached the size of about $30mm^2$ at day 5, when the mice were immunized with one dose of DCs loaded with OVAp1 and OVAp2. The tumor growth was not affected and continued to progress, reaching the size of $400mm^2$ in 20-24 days (Figure 4-8B, left). In B6/MOT1 and B6/MOT1+MOT2 mice, as we observed before (Figure 4-7B), complete tumor suppression was observed in half of the mice, for as long as the experiment ran (up to 150 days). For the other half of the mice, tumor were suppressed for 14-18 days and then progressed (Figure 4-8B, middle and right),

probably due to the tumor tolerance as we observed in Figure 4-7B. On day 18 when tumors were of the size about 30mm^2, each tumor bearing mice was immunized with one dose of DCs loaded with OVAp1 and OVAp2. In B6/MOT1 mice bearing tumors, the tumor was suppressed and remained below the size of 50mm^2 until day 30, but then grew up and reached the size of 400mm^2 in about 50 days (Figure 4-8B, middle). In sharp contrast, for B6/MOT1+MOT2 mice bearing tumor, the tumors shrank after the immunization and then totally disappeared on day 32 (Figure 4-8B, right). These mice kept virtually tumor-free for a long time, with no tumor recurrence observed in majority of them for as long as the experiment ran (up to 150 days). In few cases, tumor recurrence was observed after 90 days (data not shown). We later found that multiple immunizations could prevent tumor recurrence (data not shown).

Our results showed that by imparting to the mouse T cell repertoire both arms of the anti-tumor T cell immunity, combining with immunization to active both arms, established solid vascularized tumors could be efficiently eradicated. In particular, this method could break the existing tumor tolerance, providing new direction for tumor immunotherapy.

4. Discussion

Successful cancer immunotherapy requires the maintenance of a large population of highly tumor specific T cells in the host (Dudley and Rosenberg, 2003). It has been very well defined that the specificity of a T cell is solely determined by the TCRs it expresses. The genetic materials that encode the

TCR are generated through TCR gene rearrangement during T cell development. Therefore, a desired T cell specificity can be endowed by introducing the cognate pre-rearranged TCR genes. Traditionally, this is achieved by pronuclear injection of linear DNA fragments encoding pre-rearranged TCRα and TCRβ genes under the control of homologous or heterologous promoters (Vonboehmer, 1990). Using this method, majority of the T cells in the transgenic animal express the transgenic TCRs. But this method obviously has no therapeutic value.

Recently, several groups reported the functional expression of transgenic TCRs in mature peripheral T cells mediated by retroviral infection (Clay et al., 1999; Cooper et al., 2000; Kessels et al., 2001; Setoguchi et al., 2000; Stanislawski et al., 2001). Using this approach, a heterogenous population of T cells are converted into antigen-specific T cells that can respond to protein antigens and tumors (Clay et al., 1999; Cooper et al., 2000; Kessels et al., 2001; Setoguchi et al., 2000; Stanislawski et al., 2001). Although this approach shows promise for therapy application, the published study shows it has certain limitations. The T cells that have been engineered to express the transgenic TCRs are pre-activated mature T cells that already carry the endogenous TCR genes. As a result, the engineered T cells display at least two specificities. One is of an unknown specificity encoded by the endogenous TCR genes, the other is of the desired specificity encoded by the transgenic TCR genes. The number of specificities could be more due to the possible combination of the endogenous TCR α or β chains with the transgenic TCR β or α chains. Furthermore, the condition under which these T cells are activated *in vitro* also affects their

behavior. All of these factors can greatly limit the effector function of the engineered T cells, and it is unclear how long they can persist *in vivo* (Jamieson and Ahmed, 1989; Opferman et al., 1999).

Our method of “Instructive Immunotherapy” provides new opportunity for T cell immunotherapy. We report the success of imparting anti-tumor specificity into mouse T cell repertoire by retrovirus mediated transfer of TCR cDNAs into HSCs. Using this method, a large population of naïve T cells with the desired specificity can be generated. These antigen specific T cells persist *in vivo* in consistently large numbers, which can account for 25% of the total peripheral CD8 T cells when a model CD8 TCR was introduced, and 8% of the total peripheral CD4 T cells when a model CD4 TCR was introduced. In this method, TCR transgenes are introduced into HSCs and involved in driving the T cell development. As a result, the T cells generated carry a single specificity- the desired anti-tumor specificity. Our results show that these T cells are fully normal and functional in all the aspects we tested, enabling them to conduct the full anti-tumor effector functions. In particular, these cells can generate and maintain long-term memory, making our method especially attractive for immunotherapy.

Choosing HSCs as the target cells also brings several unique advantages to our method. HSC is one of the most accessible adult tissues for gene transfer; HSCs are the ultimate and potent progenitors to extensively proliferate and generate millions of mature T cells in regulated numbers every day from a small number of starting cells; moreover, the ability of HSCs to self-renew allows the transplantation of a small number of HSCs to ensure a lifetime supply of the

antigen-specific T cells (Kondo et al., 2003). Furthermore, the ability to fraction HSCs into subgroups, and transduce each subgroup with different genes, allows for the imparting the T cell repertoire with the specificities of both anti-tumor CD8 cytotoxic and CD4 helper T cells. This provides the opportunity to achieve the maximal therapeutic benefits by the collaboration of both arms of the anti-tumor T cell immunity. In the future, this method can be easily extended to imparting the T cell repertoire with multiple anti-tumor specificities against different epitopes of tumor antigens, thus countering the tendency of tumors towards “epitope escape” by mutating tumor antigens (Khong and Restifo, 2002). Another direction is to construct the B cell immunity by introducing into HSCs the pre-rearranged immunoglobulin genes. Ultimately, the method can be used to construct a complete adaptive immunity, humoral (B cells) and cellular (T cells), with the desired specificity/specificities. We are actively exploring this potential now.

Therefore, by combining gene therapy, hematopoietic stem cell therapy and immunotherapy, our method holds potential for treating cancer. This method can be further adapted to treat chronic infectious diseases, such as cytomegalovirus (CMV) and human immunodeficiency virus (HIV) (Riddell and Greenberg, 2000).

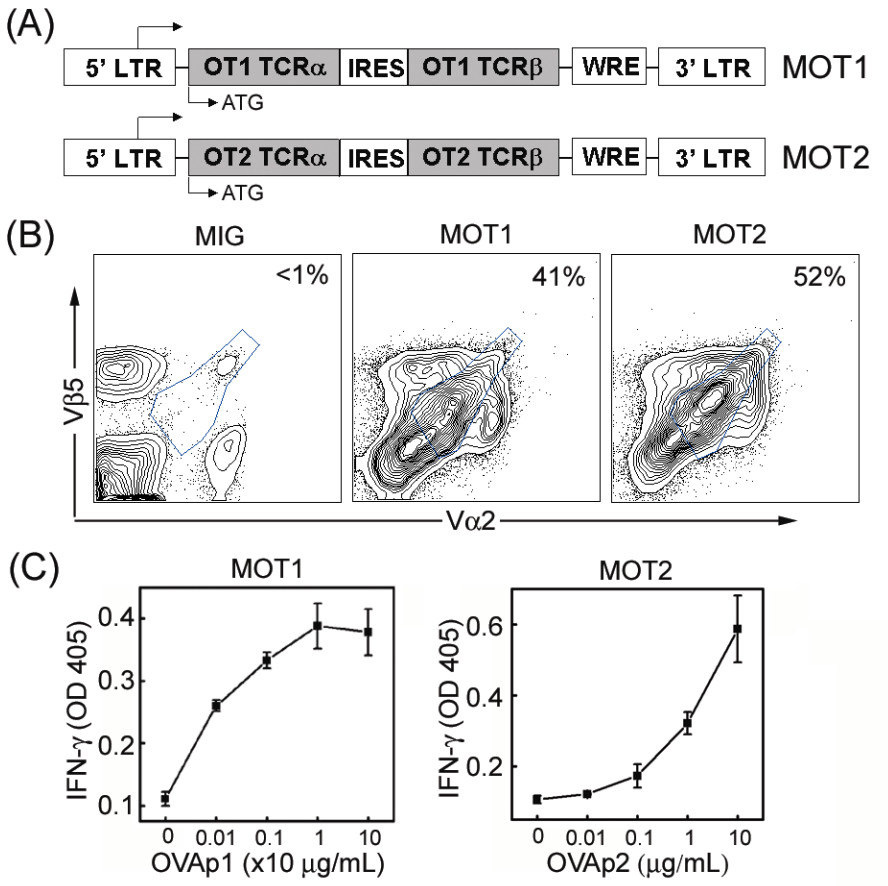
(A)
5' LTR
OT1 TCRα
IRES
OT1 TCRβ
WRE
3' LTR
MOT1
ATG
5' LTR
OT2 TCRα
IRES
OT2 TCRβ
WRE
3' LTR
MOT2
ATG
(B)
MIG
MOT1
MOT2
<1%
41%
52%
Vβ5
Vα2
(C)
MOT1
MOT2
IFN-γ (OD 405)
0.4
0.3
0.2
0.1
0 0.01 0.1 1 10
OVAp1 (x10 μg/mL)
IFN-γ (OD 405)
0.6
0.4
0.2
0 0.01 0.1 1 10
OVAp2 (μg/mL)

Figure 4-1

Figure 4-1: Retrovirus mediated functional expression of OT1 CD8 and OT2 CD4 T cell receptors (TCRs). (A) Schematic representation of the MOT1 and MOT2 retroviruses. MOT1: MSCV derived retrovirus expressing OT1 TCR cDNAs; MOT2: MSCV derived retroviruses expressing OT2 TCR cDNAs; MSCV: murine stem cell virus; LTR: long terminal repeat; IRES: internal ribosomal entry site; WRE: woodchuck responsive element. (B) Retrovirus mediated expression of OT1 and OT2 TCRs on mature mouse T cells. Spleen cells were collected from B6 female mice and stimulated with anti-CD3 +anti-CD28 antibodies (Abs) in the culture then infected with MOT1 or MOT2 retroviruses or the control retroviruses MIG. Surface expression of OT1 and OT2 TCRs was analyzed by staining with anti-mouse TCR Vα2 and Vβ5.1, 5.2 (denoted as Vβ5) Abs. Vα2 is the Vα element used by both OT1 and OT2 TCR α chains; Vβ5 is the Vβ element used by both OT1 and OT2 TCR β chains. MIG: MSCV-IRES-GFP. (C) Functional analysis of OT1 or OT2 TCRs expressed in mature mouse T cells via retroviral transduction. Antigen responses of mature mouse T cells transduced with MOT1 or MOT2 retroviruses. Mature mouse T cells transduced with MOT1 or MOT2 retroviruses were stimulated with OVAp1 or OVAp2. Antigen responses were measured by IFN-γ production.

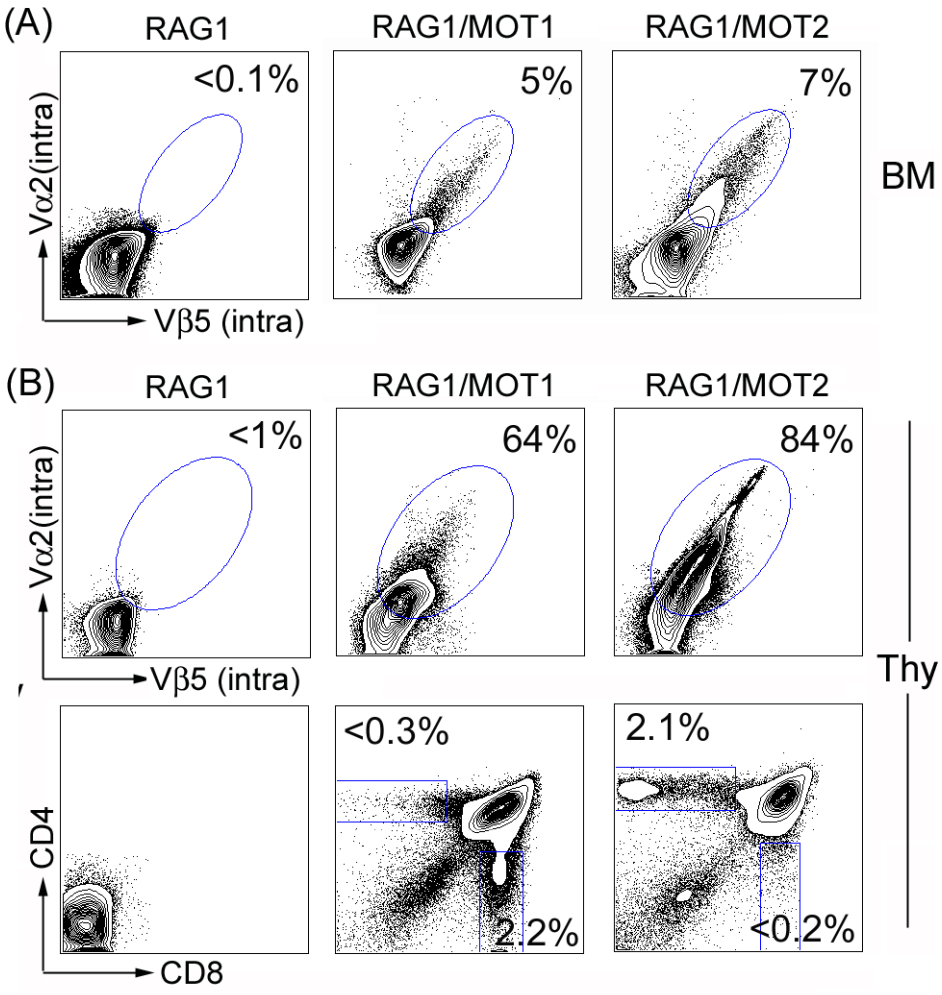

Figure 4-2, A and B

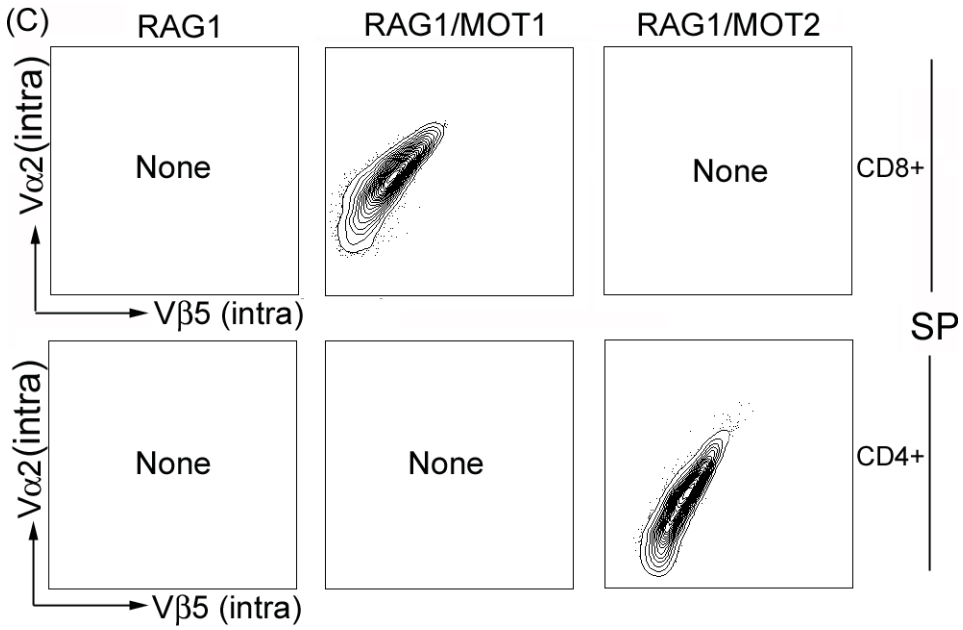
(C)
RAG1
RAG1/MOT1
RAG1/MOT2
Vα2(intra)
None
None
CD8+
Vβ5 (intra)
SP
Vα2(intra)
None
None
CD4+
Vβ5 (intra)

Figure 4-2, C

Figure 4-2: Generation of monospecific CD8 or CD4 T cells by retrovirus-mediated expression of CD8 or CD4 TCR cDNAs in $RAG1^{-/-}$ hematopoietic stem cells (HSCs). $RAG1^{-/-}$ HSCs were transduced with MOT1 or MOT2 retroviruses and then transfered into irradiated RAG1 recipient mice (denoted as RAG1/MOT1 or RAG1/MOT2 mice). The recipient mice were allowed to reconstitute their immune systems for 7 weeks before analysis. Age-matched $RAG1^{-/-}$ mice were included as the negative control. (A) Expression of OT1 or OT2 TCRs in the bone marrow (BM) of RAG1/MOT1 or RAG1/MOT2 mice. Seven weeks after HSC transfer, BM cells were harvested from RAG1/MOT1, RAG1/MOT2 mice and the control RAG1 mice, and analyzed for the OT1 or OT2 TCR expression by intracellular staining using anti-mouse TCR Vα2 and Vβ5 Abs. (B) Development of OT1 CD8 or OT2 CD4 T cells in the thymi of RAG1/MOT1 or RAG1/MOT2 mice. Thymocytes were harvested and analyzed for the expression of OT1 or OT2 TCRs via intracellular staining (upper). T cell development was accessed by the distribution of the CD4 and CD8 development markers on thymocytes (lower). (C) Detection of monospecific OT1 CD8 or OT2 CD4 T cells in the periphery of RAG1/MOT1 or RAG1/MOT2 mice. Spleen cells were harvested and stained with anti-CD8 (upper) or anti-CD4 (lower) Abs to detect the presence of CD4 or CD8 T cells. OT1 or OT2 TCR expression was analyzed by intracellular staining with anti-mouse TCR Vα2 and Vβ5 Abs.

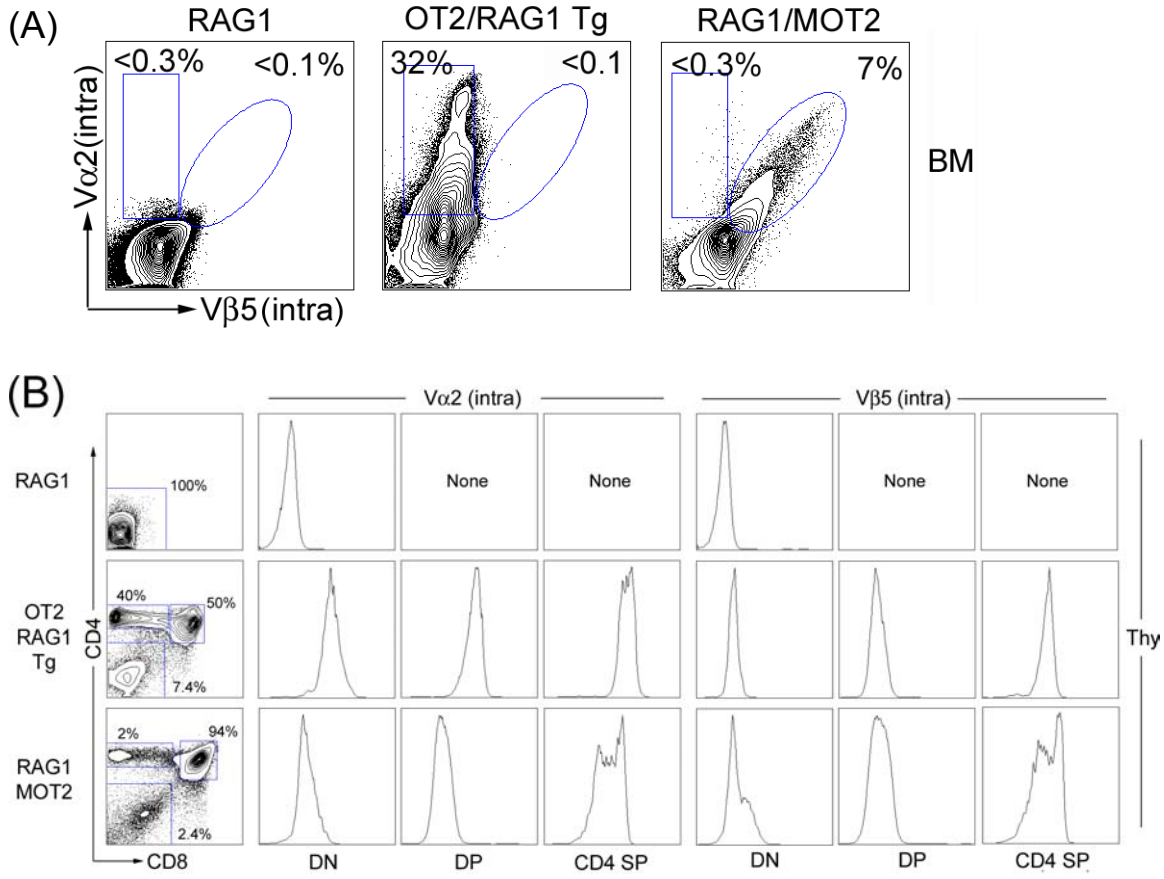

Figure 4-3, A and B

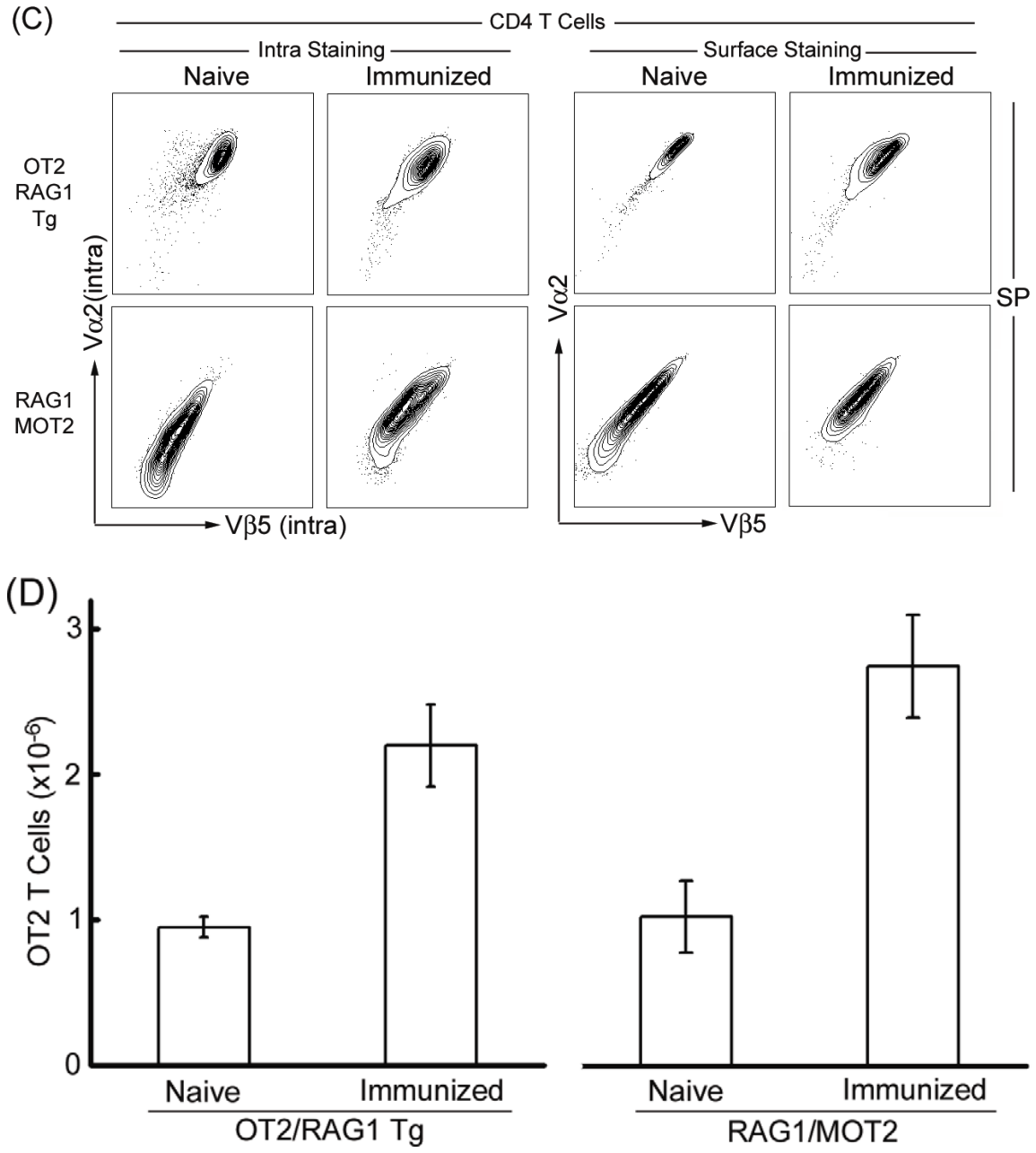

Figure 4-3, C and D

Figure 4-3: Comparison of the transgenic TCR expression and T cell development in mice receiving retrovirus-transduced RAG1$^{-/-}$ HSCs with those in the conventional TCR transgenic mice. RAG1 HSCs were transduced with MOT2 and then transferred into irradiated RAG1 recipient mice (denoted as RAG1/MOT2 mice). The recipient mice were allowed to reconstitute their immune systems for 7 weeks before analysis. OT2/RAG1 Tg mice (conventional OT2 TCR transgenic mice bred into RAG1$^{-/-}$ background) at the same age were used for the study. Age-matched RAG1$^{-/-}$ mice were included as the negative control. (A) Expression of OT2 TCRs in BM of OT2/RAG1 Tg and RAG1/MOT2 mice. BM cells were harvested and analyzed for the expression of OT2 TCR α chain and β chain expression using intracellular staining with anti-mouse TCR Vα2 and Vβ5 Abs. (B) TCR expression and OT2 CD4 T cell development in the thymi of OT2/RAG1 Tg and RAG1/MOT2 mice. Thymocytes were stained with anti-CD4 and anti-CD8 Abs to show the T cell development pattern. OT2 TCR α chain or β chain expression was analyzed using intracellular staining with anti-mouse TCR Vα2 or Vβ5 Ab. The TCR expression in thymocytes at each gated developmental stage (DN, DP, CD4 SP) was shown. DN: double negative; DP: double positive; SP: single positive. (C) Expression of OT2 TCRs in the peripheral OT2 CD4 T cells from OT2/RAG1 Tg and RAG1/MOT2 mice. *In vivo* antigen responsiveness of the OT2 T cells was analyzed by immunizing OT2/RAG1 Tg and RAG1/MOT2 mice with OVAp2 and CFA for 6 days. Spleen cells were harvested from the unchallenged and immunized OT2/RAG1 Tg and RAG1/MOT2 mice. CD4 T cells

were identified by CD4 staining. Protein expression level of OT2 TCR in CD4 T cells was shown by intracellular Vα2 and Vβ5 staining on gated CD4 T cells; surface OT2 TCR expression was shown by surface Vα2 and Vβ5 staining on gated CD4 T cells. (D) Comparable efficacy on OT2 CD4 T cell generation in OT2/RAG1 Tg and RAG1/MOT2 mice. Spleen cells were harvested from unchallenged or immunized (OVAp2+CFA for 6 days) OT2/RAG1 Tg and RAG1/MOT2 mice. OT2 CD4 T cells were identified by co-stain of Vα2 and Vβ5 on gated CD4 T cells.

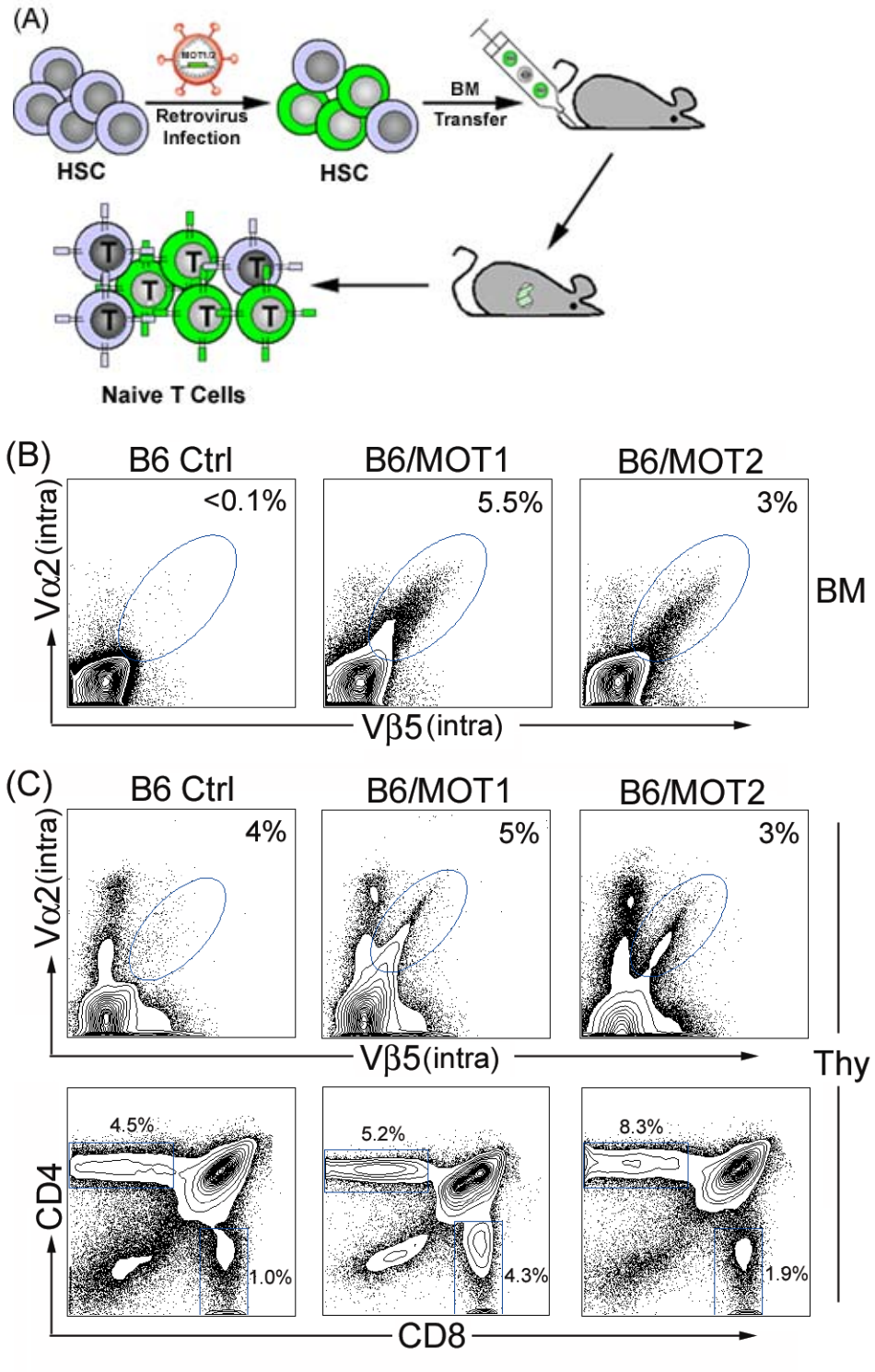

Figure 4-4, A, B and C

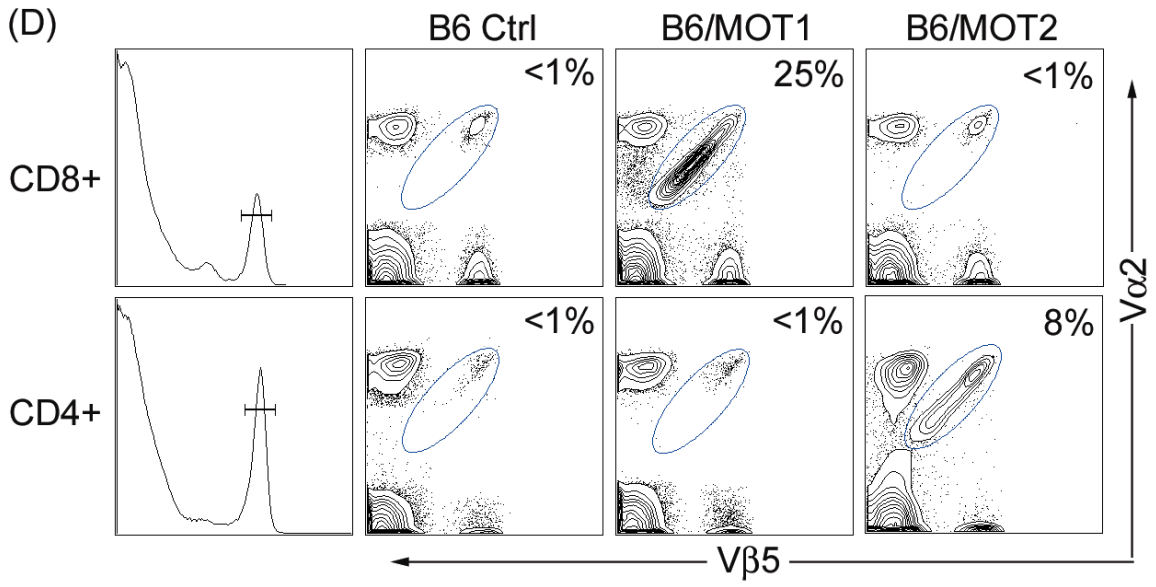

Figure 4-4, D

Figure 4-4: Generation of antigen-specific CD8 or CD4 T cells by retrovirus-mediated expression of CD8 or CD4 TCR cDNAs in wild-type HSCs. (A) Method to generate antigen-specific CD8 or CD4 T cells by retrovirus-mediated expression of CD8 or CD4 TCR cDNAs in wild-type HSCs. Wild-type HSCs are

harvested from 5-FU treated B6 mice and infected with retroviruses expressing TCR cDNAs encoding the desired specificity (e.g. OT1 CD8 TCR, or OT2 CD4 TCR). The transduced HSCs are then transferred into irradiated B6 recipients and allowed to reconstitute the recipients' immune systems for at least 6 weeks. Using this method, the desired specificity can be imparted to the mouse T cell repertoire. (B) Expression of OT1 or OT2 TCRs in the BM of B6/MOT1 or B6/MOT2 mice. Wild-type B6 HSCs were transduced with MOT1 or MOT2 retroviruses and then transferred into irradiated B6 female recipient mice (denoted as B6/MOT1 or B6/MOT2 mice). The recipient mice were allowed to reconstitute their immune systems for 8 weeks before analysis. Age-matched B6 female mice were included as the negative control. Eight weeks after HSC transfer, BM cells were harvested from B6/MOT1, B6/MOT2 mice and the control B6 mice, and analyzed for the OT1 or OT2 TCR expression by intracellular staining of Vα2 and Vβ5. (C) Development of OT1 CD8 or OT2 CD4 T cells in the thymi of B6/MOT1 or B6/MOT2 mice. Thymocytes were harvested and analyzed for the expression of OT1 or OT2 TCRs via intracellular staining (upper). T cell development was accessed by the distribution of CD4 and CD8 development markers on the total thymocytes (lower). (D) Detection of OT1 CD8 or OT2 CD4 T cells in the periphery of B6/MOT1 or B6/MOT2 mice. Spleen cells were harvested and stained with anti-CD8 (upper) or anti-CD4 (lower) Abs to detect CD4 or CD8 T cells. OT1 T cells were identified by co-stain of Vα2 and Vβ5 on gated CD8 T cells. OT2 T cells were identified by co-stain of Vα2 and Vβ5 on gated CD4 T cells.

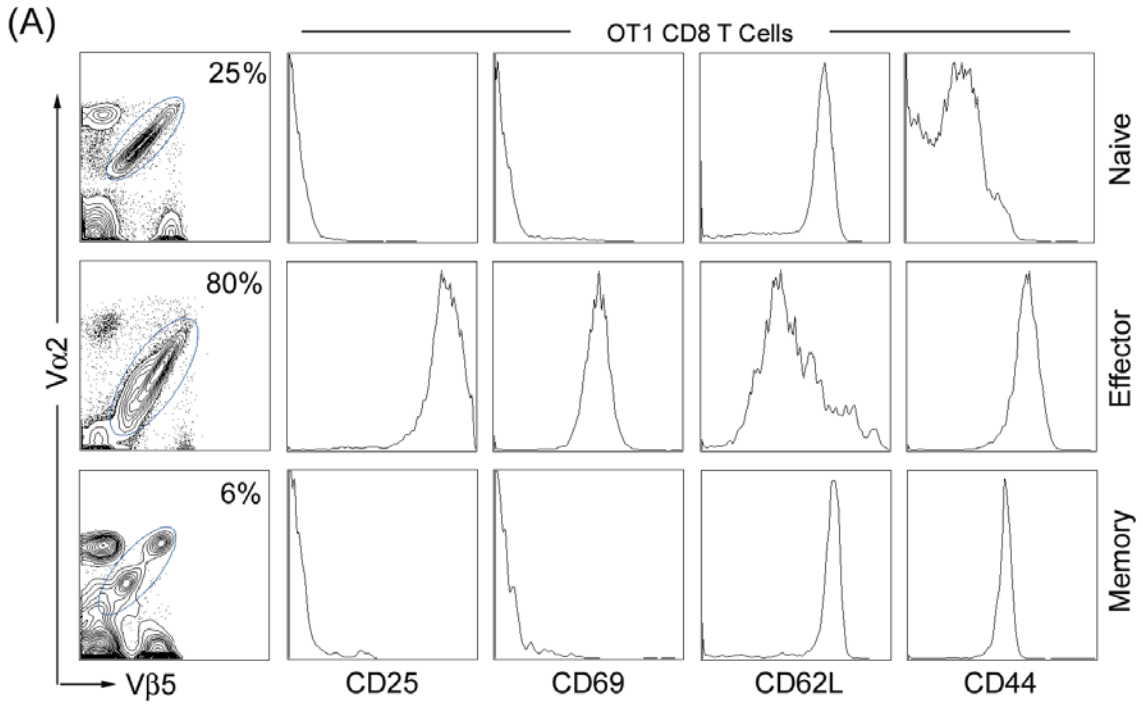
(A)
OT1 CD8 T Cells
25%
80%
6%
Vα2
Vβ5
CD25
CD69
CD62L
CD44
Naive
Effector
Memory

Figure 4-5, A

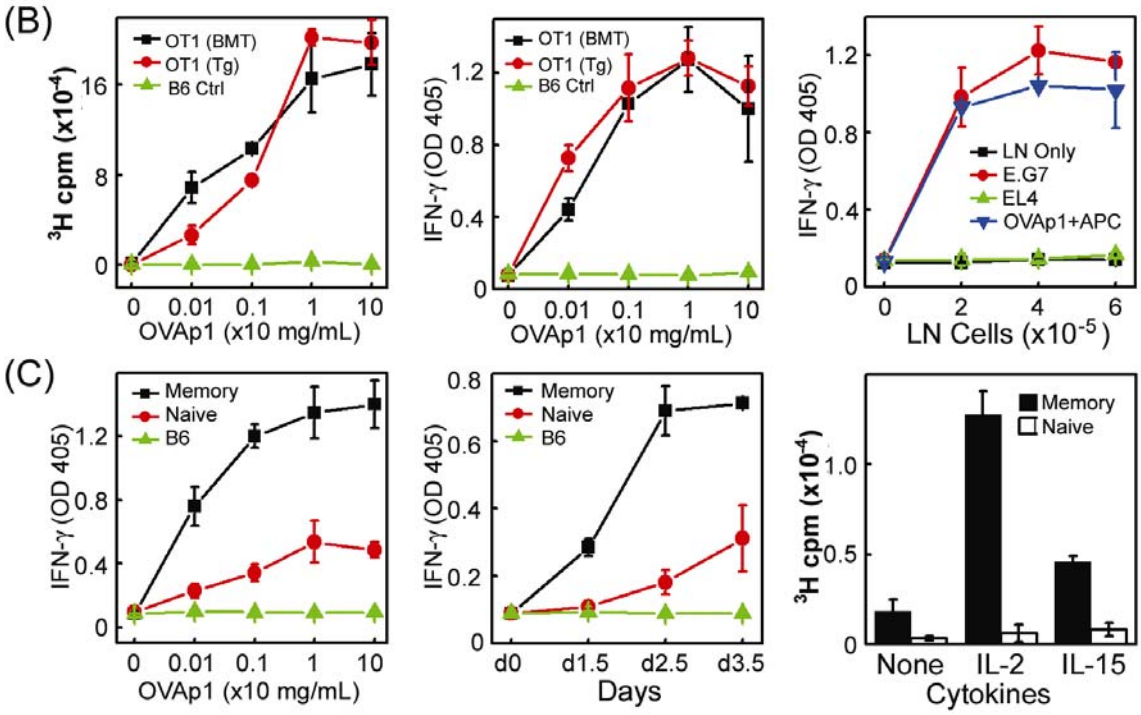

Figure 4-5, B and C

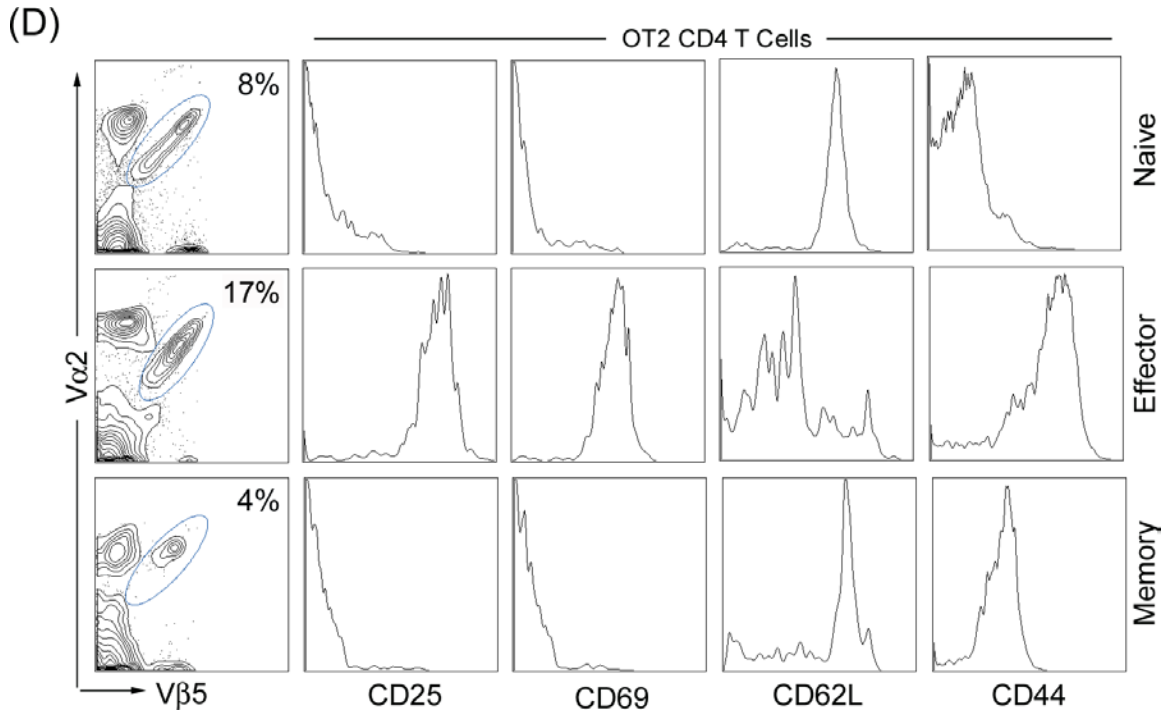
(D)
OT2 CD4 T Cells
8%
17%
4%
Naive
Effector
Memory
Vα2
Vβ5
CD25
CD69
CD62L
CD44

Figure 4-5, D

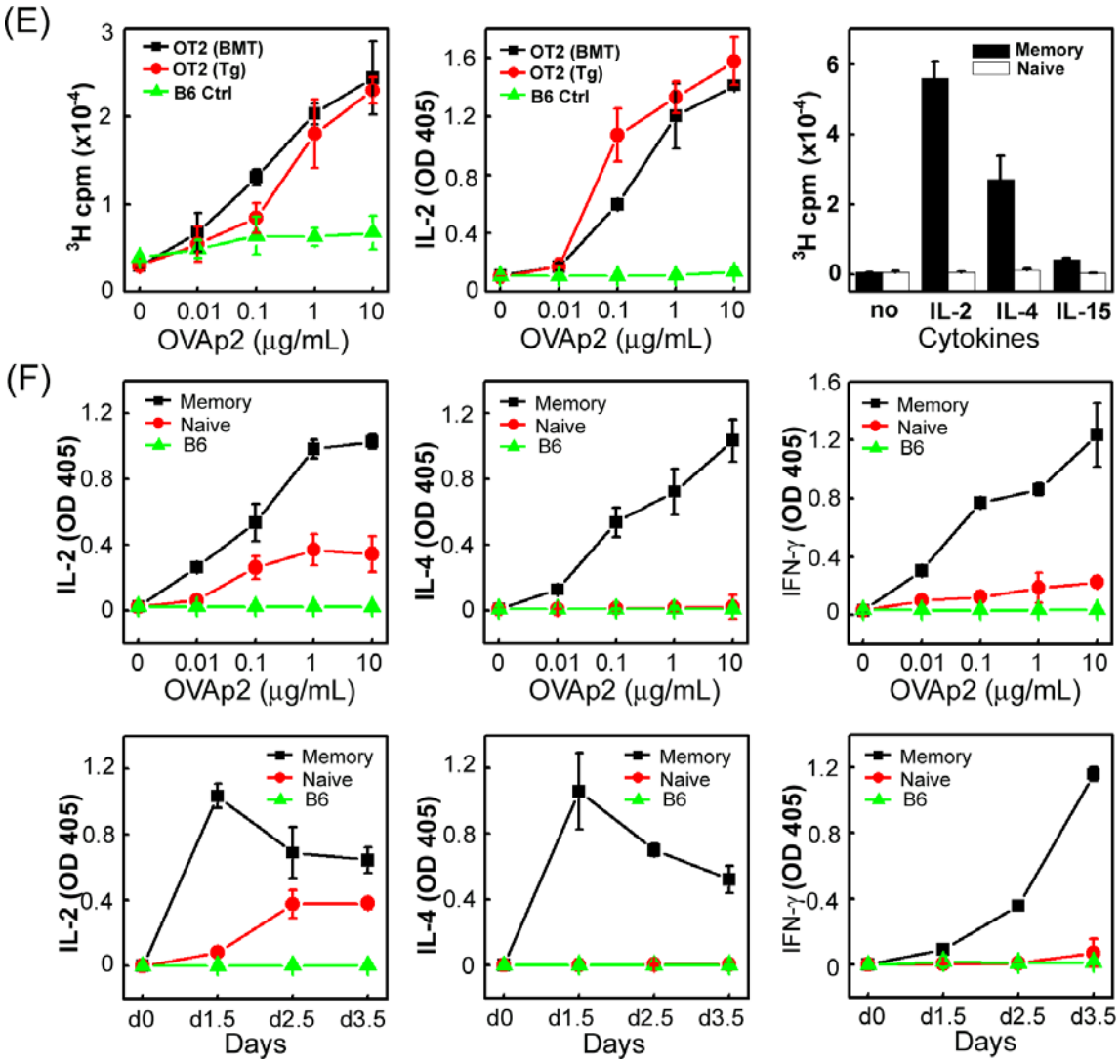

Figure 4-5, E and F

Figure 4-5: Characterization of the CD8 and CD4 T cells generated by retrovirus-mediated expression of CD8 or CD4 TCR cDNAs in wild-type HSCs. Wild-type B6 HSCs were transduced with MOT1 or MOT2 retroviruses and then transferred into irradiated B6 female recipient mice (denoted as B6/MOT1 or B6/MOT2 mice). The recipient mice were allowed to reconstitute their immune systems for 8 weeks before analysis. OT1 CD8 T cells or OT2 CD4 T cells harvested from B6/MOT1 or B6/MOT2 mice 8 weeks after HSC transfer were considered to be naïve. They were stimulated with OVAp1 or OVAp2 *in vitro* for 3 days to generate effector OT1 or OT2 T cells, which were then transferred into $RAG1^{-/-}$ recipient mice. Sixteen or fourteen weeks later, the recipient mice were analyzed for the presence of memory OT1 or OT2 T cells.

(A) Patterns of surface activation markers on OT1 CD8 T cells generated in B6/MOT1 mice at the naïve, effector or memory stages measured by FACS staining. OT1 T cells were identified by co-stain of Vα2 and Vβ5 on gated CD8 T cells. Surface markers studied are indicated below each column of results. (B) Functional analysis of the naïve OT1 CD8 T cells generated in B6/MOT1 mice. Proliferation (left) and IFN-γ production (middle) in response to OVAp1 stimulation, and IFN-γ production in response to E.G7 tumor cells (right) are shown. The responses were compared with those of conventional transgenic OT1 T cells. B6 spleen cells were included as a negative control. OT1 T cells from B6/MOT1 mice were denoted as OT1(BMT) (bone marrow transfer), while the OT1 T cells from conventional OT1 TCR transgenic mice were denoted as OT1(Tg). (C) Functional analysis of memory OT1 T cells derived from B6/MOT1

mice. Dosage response (left) and time-course response (middle) to OVAp1 stimulation, and proliferation response to cytokine stimulation (right) are shown. The responses were compared with those of the naïve OT1 T cells. B6 spleen cells were included as a negative control. (D) Patterns of surface activation markers on OT2 CD4 T cells generated in B6/MOT2 mice at the naïve, effector or memory stages measured by FACS staining. OT2 T cells were identified by co-stain of Vα2 and Vβ5 on gated CD4 T cells. Surface markers studied are indicated below each column of results. (B) Functional analysis of the naïve OT2 CD4 T cells generated in B6/MOT2 mice. Proliferation (left) and IL-2 production (middle) in response to OVAp2 stimulation, and proliferation of the memory OT2 T cells in response to cytokine stimulation (right) are shown. The responses were compared with those of conventional transgenic OT2 T cells. B6 spleen cells were included as a negative control. OT2 T cells from B6/MOT2 mice were denoted as OT2(BMT) (bone marrow transfer), while the OT2 T cells from conventional OT2 TCR transgenic mice were denoted as OT2(Tg). (C) Functional analysis of memory OT2 T cells derived from B6/MOT2 mice. Dosage response (upper) and time-course response (lower) to OVAp2 stimulation as measured by IL-2, IL-4 and IFN-γ production, and proliferation in response to cytokine stimulation (E, right) are shown. The responses were compared with those of the naïve OT2 T cells. B6 spleen cells were included as a negative control.

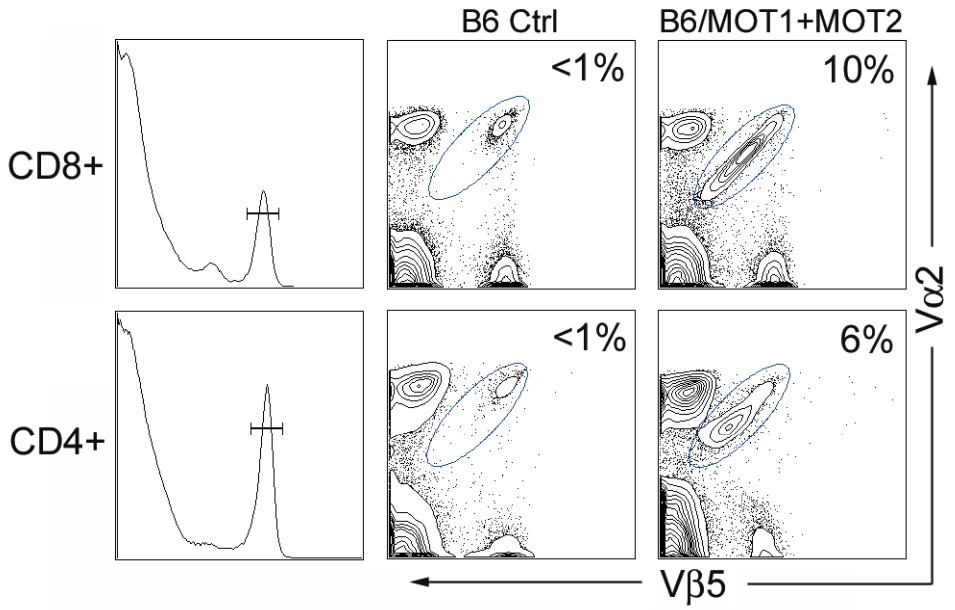

Figure 4-6

Figure 4-6: Imparting to mouse T cell repertoire both anti-tumor CD8 cytotoxic and CD4 helper T cell specificities. BM cells were harvested from 5-FU treated B6 wild-type female mice and were divided into two sub-populations. One population of the cells were transduced with MOT1 retroviruses and the other population of cells were transduced with MOT2 retroviruses. The two populations were then pooled together and transferred into irradiated B6 female recipient mice (denoted as B6/MOT1+MOT2 mice). B6 female mice at the same age were included ad a negative control. The recipients were allowed to reconstitute their immune systems for 6 weeks. At week 6, spleen cells were harvested and the presence of OT1 CD8 and OT2 CD4 T cells were analyzed. OT1 T cells were identified by co-stain of Vα2 and Vβ5 on gated CD8 T cells (upper). While OT2 T cells were identified by co-stain of Vα2 and Vβ5 on gated CD4 T cells (lower).

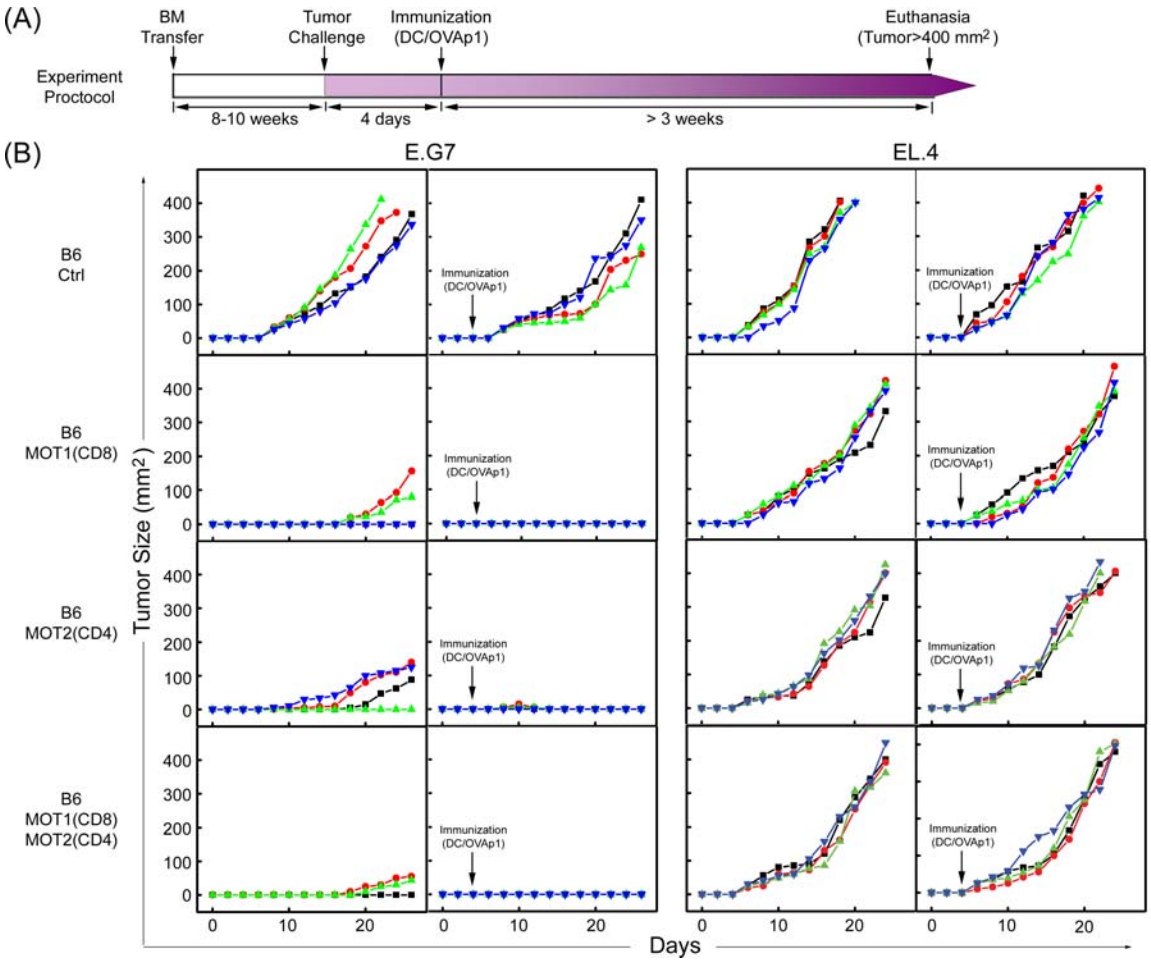
(A)
BM Transfer
Tumor Challenge
Immunization (DC/OVAp1)
Euthanasia (Tumor>400 mm2)
Experiment Proctocol
8-10 weeks
4 days
> 3 weeks
(B)
E.G7
EL.4
B6 Ctrl
B6 MOT1(CD8)
B6 MOT2(CD4)
B6 MOT1(CD8) MOT2(CD4)
Tumor Size (mm2)
Immunization (DC/OVAp1)
Days

Figure 4-7

Figure 4-7: Suppression of syngenic tumor growth by imparting anti-tumor CD8 cytotoxic or/and CD4 helper T cell specificity/specificities to mouse T cell repertoire. B6 recipient mice receiving MOT1 transduced wild-type HSCs (denoted as B6/MOT1), B6 recipient mice receiving MOT2 transduced wild-type HSCs (denoted as B6/MOT2), B6 recipient mice receiving both MOT1 transduced and MOT2 transduced wild-type HSCs (denoted as B6/MOT1+MOT2) were used in the experiments. By the time the experiments started, all recipients were allowed to reconstitute their immune system for 6-8 weeks. Age-matched B6 females were included as a control. (A) Protocol for the suppression of syngenic tumor growth experiment. (B) Solid tumor growth in mice receiving different treatments. Tumor size is shown as the product of the two largest perpendicular diameters a x b (mm^2). Mice were euthanized when the tumors reached 400mm^2.

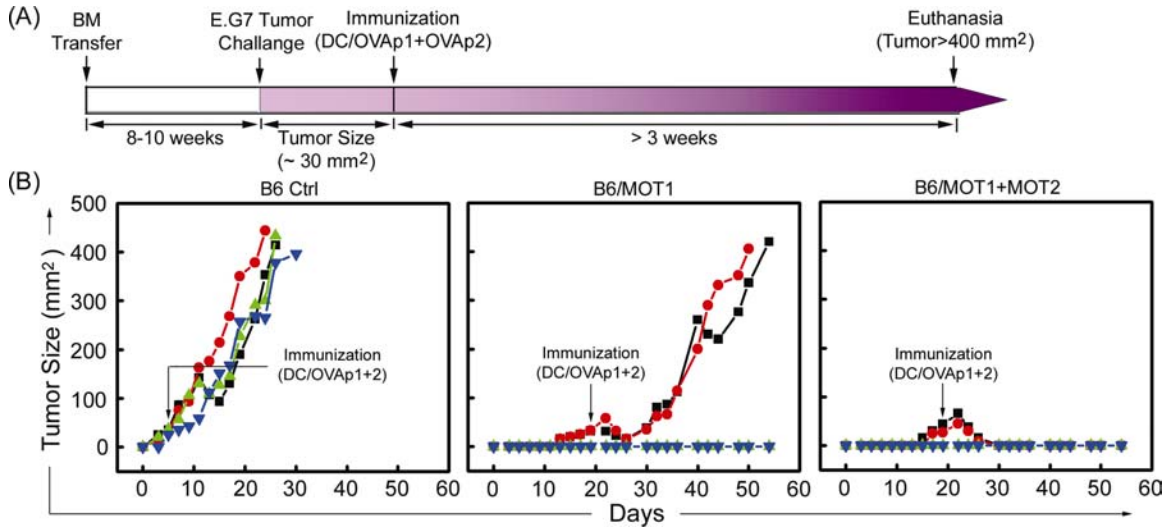
(A)
BM Transfer
E.G7 Tumor Challange
Immunization (DC/OVAp1+OVAp2)
Euthanasia (Tumor>400 mm2)
8-10 weeks
Tumor Size (~ 30 mm2)
> 3 weeks
(B)
B6 Ctrl
B6/MOT1
B6/MOT1+MOT2
Tumor Size (mm2)
Immunization (DC/OVAp1+2)
Days

Figure 4-8

Figure 4-8: Reversal of tumor tolerance and eradication of the established solid tumors by constructing both arms of the anti-tumor T cell immunity. B6 recipient mice receiving MOT1 transduced wild-type HSCs (denoted as B6/MOT1), and B6 recipient mice receiving both MOT1 transduced and MOT2 transduced wild-type HSCs (denoted as B6/MOT1+MOT2) were used in the experiments. By the time the experiments started, all recipients were allowed to reconstitute their immune system for 6-8 weeks. Age-matched B6 females were included as a control. (A) Protocol for the eradication of established solid tumor experiment. (B) Solid tumor growth in mice receiving different treatments. Tumor size is shown as the product of the two largest perpendicular diameters a x b (mm^2). Mice were euthanized when the tumors reached $400mm^2$.

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