Chapter 2

Generating Functional Antigen-specific T Cells in Defined Genetic Background

The work presented here is taken from:
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1. Introduction

T cells provide protection against infections and tumors by recognizing peptides presented on major histocompatibility molecules using a highly specific antigen receptor, the T cell receptor (Fremont et al., 1996; Garcia et al., 1999; Hedrick et al., 1984; Yanagi et al., 1984). The genetic template for this receptor is created during T cell development in the thymus by the V(D)J DNA rearrangement process, which imparts a unique antigen specificity upon each TCR (Fugmann et al., 2000; Hesslein and Schatz, 2001; Schatz et al., 1989). The TCR plays an essential role in T cell function, development, and survival. Genetic lesions that interfere with the generation of antigen receptors, block T cell development and result in immunodeficiencies (Mombaerts et al., 1992; Shinkai et al., 1992; Villa et al., 2001). By defining the specificity of pathogenic T cells, TCRs are also centrally involved in the initiation of autoimmunity and allergies (Ji et al., 1999; Kreuwel and Sherman, 2001).

Because TCRs play such a critical role in T cell development and function, much has been learned about the life history of T cells, and the immune responses that they elicit, by studying the composition and function of these antigen receptors. The ability to generate mice that express a specific TCR on most T cells by transgenesis has enabled the study of T cell development and function *in vivo* (Berg et al., 1989; Bluthmann et al., 1988; Kisielow et al., 1988; Ohashi et al., 1991; Pircher et al., 1989; Schonrich et al., 1991; Sha et al., 1988; Teh et al., 1988). It has also made possible the creation of antigen-specific animal models of many important immunological diseases (Goverman et al.,

1993; Katz et al., 1993; Ohashi et al., 1991). The first successful strategy to generate TCR transgenic mice involved pro-nuclear injection of large fragments of genomic DNA encoding the rearranged and chains of the TCR (Bluthmann et al., 1988). In these mice, the expression of the TCR was driven by the homologous promoter and enhancer elements. Subsequent studies have demonstrated that it is also possible to generate TCR transgenic mice expressing constructs that use heterologous promoters. These can either be T cell-specific, such as the CD2 promoter (Mamalaki et al., 1993), or non-specific, such as the MHC class I promoter, which is expressed in all nucleated cells (Pircher et al., 1989). In TCR transgenic mice that use promoters whose activity is not restricted to T cells, TCRα and TCRβ chains are found in other cells types. However, these proteins are only displayed on the plasma membrane in T cells because these are the only cells that express CD3, a protein complex required for surface expression of TCRs (Oettgen et al., 1986).

Recently, a number of groups have shown that functional expression of a TCR can also be obtained in mature T cells by using retroviral vectors to carry DNA into these cells (Clay et al., 1999; Cooper et al., 2000; Fujio et al., 2000; Kessels et al., 2001; Stanislawski et al., 2001). I n these studies, TCRα and TCRβ genes were introduced and stably expressed in T cells that had been activated with a mitogen. Using this approach, T cells derived from non-specific, heterogeneous populations were converted into antigen-specific T cells capable of responding to protein antigens and tumors (Clay et al., 1999; Cooper et al., 2000; Fujio et al., 2000; Kessels et al., 2001; Stanislawski et al., 2001). These

results suggest that transgenic expression of TCRs might be used, not only to study T cell function, but also as a therapeutic strategy to generate antigen-specific T cells capable of targeting tumor antigens and infectious agents.

While retrovirus-mediated expression of TCR genes shows promise as a strategy to produce antigen-specific T cells, the published approaches have certain limitations. The T cells that are engineered to express TCRs are activated mature cells that already express an endogenous TCR of unknown specificity. Their effector function may be restricted by the conditions under which they are activated *in vitro*, and it is unclear how long they persist *in vivo* (Jamieson and Ahmed, 1989; Opferman et al., 1999). To attempt to overcome these problems, we have tested whether it is possible to generate naïve antigen-specific T cells by expressing TCRα and TCRβ genes in hematopoietic precursor cells derived from RAG1-deficient mice using retroviruses and then injecting these cells into irradiated mice to reconstitute their T cell population. We report here that this approach has been successful and that T cells produced in this manner respond normally to antigen challenge both *in vitro* and *in vivo*. Furthermore, we demonstrate that this strategy can be used to generate T cells with different functional properties by expressing TCRα and TCRβ genes in hematopoietic cells from wild type and IL-2 knockout mice. Our results demonstrate that retrovirus-mediated expression of TCR genes in hematopoietic precursor cells provides an efficient and rapid new approach to generate antigen specific T cells that can be used for basic research and might be valuable in the clinic.

2. Materials and Methods

Mice

C57BL/6 mice were purchased from Charles River, RAG1 and IL-2 knockout mice from Jackson Laboratories. IL-2/RAG1 double knockout mice were generated by breeding IL-2 knockout mice with RAG1 mice. All mice were housed in the Caltech animal facility according to Institute regulations.

MIG-TCR Retroviruses Construction

The generation of the MIG retroviral expression vector has been described before (Van Parijs et al., 1999b). OTII TCRα cDNA and OTII TCRβ cDNA (a generous gift from Drs Francis Carbone and William Heath, Melbourne, Australia) were cloned separately into the unique EcoRI site of MIG. Retroviruses were generated by transfecting HEK 293.T cells with standard calcium phosphate precipitation technique (Pear et al., 1993). Viruses were harvested 48 and 72 hr after transfection.

Generation of the THZ Hybridoma Cell Line and Infection with Retroviruses

Activated mouse CD4+ T cells were fused with the BWZ cell line, which contains a reporter gene (lacZ) that is expressed under the control of the nuclear factor of activated T cells (NFAT) elements of the human interleukin-2 promoter (Sanderson and Shastri, 1994), to generate T-cell hybridomas by standard methodology. The hybridomas were selected and cloned by limiting dilution. One specific clone was lack of TCR expression, while still maintaining CD3 and CD4

expression. This clone was sorted by flow cytometry three times to stabilize the TCR-$CD3^+CD4^+$ phenotype. The resulting T hybridoma line, which we named THZ, can be used to test surface expression and specificity of TCRs. The ability of these TCRs to respond to antigen stimulation can be analyzed by a lacZ assay.

To generate THZ cells that express the OTII TCR, these cells were spin-infected with both MIG/OTII α and MIG/OTII β retroviruses in the presence of 10μg/ml polybrene for 1hr 30mins at 2,500rpm at 30˚C. The efficiency of infection was determined by assaying GFP fluorescence using flow cytometry. The response of infected cells expressing the OTII TCR to residues 323-339 of chicken ovalbumin (OVAp) was assayed by stimulating cells with OVAp presented by B6 spleen cells overnight. The next day, antigen responses were measured by bulk lacZ assay (see below).

Bulk LacZ Assay

Individual cultures of THZ cells cultured in round-bottom 96-well plates were washed once with 100μl PBS, lysed and exposed to the colorogenic β-galactosidase substrate, chlorophenol red β-galactoside (Calbiochem, La Jolla, CA) in 100μl Z buffer (100mM 2-mercaptoethanol, 9mM $MgCl_2$, 0.125% NP-40 in PBS) and incubated at 37˚C overnight. The development of the colored lacZ product was assayed using a plate reader with a 570 nm filter, and a 630nm filter for reference.

Primary CD4+ T Cell Infection and Stimulation

To obtain CD4+ T cells, splenocytes were harvested from 6-8 wks C57BL/6 mice. CD8+ T cells were depleted using Dynalbeads Mouse CD8 (Lyt2) (Dynal ASA, Oslo, Norway) according to the manufacturers instructions. $2x10^6$ $CD4^+$ T cells and spleen APCs were then cultured in 1 ml in a 24-well plate in the presence of 0.5μg/ml anti-CD3 (Pharmingen, San Diego, CA) for 3 days. On day 1 and day 2 of activation, the cells were spin-infected with both MIG/OTII α and MIG/OTII β retroviruses in the presence of 5μg/ml polybrene for 1hr 30mins at 2,500rpm at 30˚C. After each spin infection, the retroviral supernatant was replaced. On day 3, the cells were collected and some were used to assay the expression of OTII TCR on the surface of infected $CD4^+$ T cells by FACS. The rest were cultured overnight with 40U/ml IL-2 (Biosource, Camarillo, CA) at $1x10^6$ cells per ml. The next day, the rested cells were tested for their responsiveness to OVAp by culturing them at $2x10^5$ cells/well in a flat-bottom 96-well plate with $2x10^5$ cells/well of irradiated B6 spleen cells as APC in T cell culture medium containing OVAp at 0, 1, 10μg/ml. Three days later, ^{3}H-thymidine was added to the wells at a final concentration of 0.01mCi/ml followed by overnight culture. The proliferation of the cells was analyzed with a Wallac ^{3}H counter.

Bone Marrow (BM) Stem Cell Isolation, Infection and Transfer

RAG1 knockout mice, in a wild type or IL-2 knockout background, were treated with 250μg/gram body weight of 5-flurouracil (Sigma) dissolved in PBS. Bone marrow cells were harvested 5 days later from the tibia and femur and

cultured for 5 days at $2x10^6$ cells/ml with 20ng/ml rmIL-3, 50ng/ml rmIL-6, and 50ng/ml rmSCF (all from Biosource, Camarillo, CA) in DMEM containing 10% FCS. After 48-72 hr, the BM cells were spin-infected with a mixture of MIG/OTII α and MIG/OTII β retroviruses and 8μg/ml polybrene, for 1hr 30mins at 2,500rpm at 30˚C. After spin infections, the retroviral supernatant was replaced. Recipient $RAG1^{-/-}$ mice of desired genetic background received a total of 480 rads whole body radiation and then received $1\text{-}2x10^6$ infected bone marrow cells by tail vein injection. Bone marrow recipient mice were maintained on the mixed antibiotic TMS (Sulfamethoxazole and Trimethoprim oral suspension; Hi-Tech Pharmacal Co., Amityville, NY) in a sterile environment for 6 to 11 weeks until analysis.

Immunization of Bone Marrow Recipient Mice

Ten weeks after irradiation and receiving bone marrow, mice were immunized by intra-peritoneal injection of 200μg OVAp in 200μl PBS, and then left for 6 days until analysis.

In Vitro T Cell Stimulation and Proliferation Assays

Spleen cells from bone marrow recipient mice were harvested and cultured at $2x10^5$ cells/well in flat-bottom 96-well plate with $2x10^5$ cells/well B6 spleen cells as APC in T cell culture medium containing OVAp at 0, 0.01, 0.1, 1, 10μg/ml. Three days later, culture supernatants were collected and used for IL-2 and interferon (IFN)-γ ELISAs. Proliferation was assessed by adding ^3H-

thymidine to the wells at a final concentration of 0.01mCi/ml followed by overnight culture. Data was collected with a Wallac ^{3}H counter.

IL-2 and IFN-γ ELISA

96-well ELISA plates were coated with 50µl/well of 1µg/ml anti-mIL-2 or anti-mIFN-γ antibody (Pharmingen, San Diego, CA) in carbonate buffer (mix 325ml 0.1M sodium bicarbonate and 50ml 0.1M sodium carbonate, PH to 9.4) for 2hrs at 37ºC. The plates were then washed twice with PBS, blocked by adding 100µl/well of dilution buffer (BBS/2%BSA/0.002% sodium azide), incubated for 30min at 37ºC. After being washed 4 times with PBS, sample supernatants were added to the plates at a final volume of 50µl/well, incubated for 3hrs at 37ºC. The plates were then washed 4 times followed by adding 50µl/well of 1µg/ml of the detecting biotinylated antibody (Pharmingen, San Diego, CA) in dilution buffer and incubated for 45min at RT. The plates were then washed 6 times with PBS, 50µl/well of the Avidin-Alkaline Phosphotase (Pharmingen, San Diego, CA) was added at a dilution of 1:400 in the dilution buffer, and the plates was incubated for 30min at RT. Finally the plates were washed 6 times with PBS. Developing solution Sigma 104 Phosphatase Substrate (Sigma, ST. Louis, MO) was made up at 1mg/ml in DEA buffer right before use and then added at 50µl/well. Data was collected with a plate reader at 405nm.

3. Results

Functional Expression of the OTII TCR in T Cell Lines and Primary CD4$^+$ T Cells Using Retroviruses

We have previously generated a bicistronic retroviral expression vector MSCV IRES GFP (MIG) that allows efficient expression of genes in primary cells of hematopoietic origin (Van Parijs et al., 1999b). This retroviral vector expresses both GFP, to mark infected cells, and a test gene. To examine whether we could use retroviruses to functionally express TCRs in T cell populations, we cloned the OTII T cell receptor (TCR) α or β chain cDNAs into MIG using its unique EcoRI site (Figure 2-1A). The OTII TCR is a well-defined class II MHC-restricted TCR that responds to residues 323-339 of chicken ovalbumin (OVAp) (Van Parijs et al., 1999a). MIG retroviruses expressing the cDNA for the OTII TCR α or β chain were used to co-infect the THZ hybridoma line. This cell line expresses CD3, so it can express TCRs on its surface. It also contains a reporter gene (lacZ) that is expressed under the control of the nuclear factor of activated T cells (NFAT) element of the human interleukin-2 promoter, and can be used to assay TCR signaling (Jamieson and Ahmed, 1989). The infected THZ cells (marked by GFP fluorescence) expressed the β chain of the OTII TCR at the cell surface (Figure 2-1B, left panel), and expressed the lacZ reporter gene when stimulated with OVAp presented by APCs (Figure 2-1C, left panel), indicating that we had achieved functional expression of TCR in this hybridoma line.

We next tested whether we could express the OTII TCR in primary T cells using retroviruses. In these experiments, purified CD4+ T cells from wild type

C57BL/6 mice were activated with an antibody to CD3ε and infected with MIG OTIIα and MIG OTIIβ viruses. The infected T cells (marked by GFP fluorescence) expressed the β chain of the OTII TCR at the cell surface (Figure 2-1B, right panel), and proliferated when cultured with OVAp presented by APCs (Figure 2-1C, right panel), indicating that we had achieved functional expression of TCR in primary $CD4^+$ T cells.

Generation of OTII TCR Transgenic T cells in vivo Following Retrovirus-Based Gene Delivery into Bone Marrow Precursor Cells

We have previously developed a strategy to infect bone marrow precursor cells with the MIG retrovirus (Van Parijs et al., 1999a). To test whether it is possible to generate functional T cells by expressing TCRs in these cells, we harvested bone marrow from wild type and IL-2 knockout RAG1-deficient mice and infected these with retroviruses expressing OTIIα and OTIIβ. These cells were injected into lethally irradiated RAG1-deficient recipient mice and allowed to reconstitute their immune system, thus producing OTII monospecific mice (see below).

To determine whether retrovirus-mediated expression of the OTII TCR could drive T cell development, we harvested and stained thymocytes 11 weeks after the mice received bone marrow cells. The first indication that T cell development had been rescued was that the cellularity of the thymi derived from mice expressing OTIIα and β chains was greatly increased compared to those from control mice that received bone marrow precursor cells infected with the

empty MIG vector (data not shown). As shown in Figure 2-3A, the majority (>80%) of cells in the thymi of mice receiving OTII-expressing cells were GFP positive. These thymocytes showed the expected distribution of CD4 and CD8 markers for a developing class II-restricted T cell. Consistent with the absence of endogenous TCRs, all mature T cells in the thymus were CD4 single positive. The development of OTII T cells was identical in wild type and IL-2-deficient backgrounds, supporting the notion that IL-2 is not required for $CD4^+$ T cell development (Sadlack et al., 1993; Suzuki et al., 1995; Willerford et al., 1995).

We also tested whether there were mature $CD4^+$ T cells in the peripheral lymphoid organs of the OTII monospecific mice. From 30 to 60% of the cells in the lymph nodes and spleens (data not shown) of these mice were GFP positive (Figure 2-3B). More than 80% of these cells were mature $CD4^+$ T cells that expressed the OTII Vβ element, Vβ5. Similar results were observed in both wild type and IL-2-deficient backgrounds. These observations demonstrated that retrovirus-mediated expression of TCR cDNAs in bone marrow precursor cells could drive normal T cell development.

To determine the kinetics with which monoclonal T cells populated recipient mice following bone marrow transfer, we performed a time course experiment. The earliest time point at which we observed mature (CD4 single positive) T cells in the thymus of recipient mice was 6 weeks after reconstitution (data not shown). However, no T cells were detectable in the peripheral lymphoid organs of these mice yet. At 8 weeks we started to observe small numbers of monoclonal CD4 single positive T cells in the lymph nodes and

spleen. These cells were able to respond to OVAp, indicating that they were mature. By 10 to 11 weeks the reconstitution of the T cell compartment was complete, and substantial numbers of functional T cells were detectable in all lymphoid organs analyzed.

Normal in vitro and in vivo Antigen Responses of OTII TCR Transgenic T Cells

To test whether the mature T cells present in OTII monospecific mice were functional, we stimulated cells from lymph nodes and spleens derived from wild type and IL-2 knockout mice with increasing concentrations of OVAp. OTII T cells derived from wild type mice proliferated and secreted IL-2 when stimulated with OVAp (data not shown). IL-2 knockout OTII T cells did not secrete IL-2 or the effector cytokine IFN- upon antigen stimulation, and showed a weak proliferative response to antigen (Figure 2-3C). Addition of exogenous IL-2 rescued proliferation and cytokine production (Figure 2-3C). These results demonstrate that the mature T cells found in OTII monospecific mice are functionally normal, and that T cells with defined genetic lesions exhibit the expected effects of this mutation.

In the final set of experiments, we analyzed the response of OTII monospecific T cells to antigen stimulation *in vivo*, by immunizing mice with OVAp. Cells from the lymph nodes and the spleen were harvested 6 days later and counted, stained with antibodies to activation markers, and restimulated *in vitro*. In both the wild type and IL-2-deficient backgrounds, there was a significant

increase in T cell number following immunization (Figure 2-4A). The accumulating cells expressed surface markers typical of activated T cells ($CD62L^{low}$ and $CD44^{high}$) (Figure 2-4A) (Huet et al., 1989; Mobley et al., 1994). Interestingly, we noticed that immunization led to a preferential expansion of GFP^{high} cells (Figure 2-4B). Since the expression of GFP correlates with the expression of TCR in our vector system (Van Parijs et al., 1999b), this indicates that antigen exposure selects for T cells that express higher levels of TCR in the mice. Upon restimulation with OVAp *in vitro,* wild type OTII T cells from immunized mice proliferated vigorously and secreted the effector cytokine, IFN-γ (Figure 2-4C). As expected, immunized IL-2-deficient T cells showed reduced proliferation and IFN-γ production, unless exogenous IL-2 was added to the cultures (Figure 2-4C). These results demonstrated that retrovirus-mediated expression of TCR cDNAs in bone marrow precursor cells could give rise to functionally mature T cells with different genetic backgrounds that respond normally to antigen exposure *in vivo*.

4. Discussion

We report here on a new strategy to generate functional antigen-specific T cells by expressing TCRα and β chain cDNAs in RAG1-deficient hematopoietic precursor cells using a retrovirus-based vector, and then injecting these cells into lethally irradiated host mice. The T cells that develop in these mice respond normally to peptide antigen when stimulated *in vitro*, and can be expanded and activated *in vivo* by immunization. Our approach allows the rapid and efficient

generation of antigen-specific T cells in different genetic backgrounds and provides a new tool to study T cell function. It may be possible to adapt this strategy to generate T cells with prophylactic and therapeutic value.

TCR-Expressing Retroviruses Drive T Cell Development

The ability to impart upon T cells a defined antigen-specificity by transgenic expression of TCR genes has proven extremely valuable as an approach to study the immune system. Traditionally, this has been accomplished by injecting linear DNA fragments that express TCR α and TCR β genes under the control of homologous or heterologous promoters into the nuclei of embryos. Recent studies have shown that it is also possible to functionally express TCRs directly in mature T cells using retroviruses. This finding suggests that TCR transgenesis might be used for therapeutic, as well as scientific, applications.

Our current findings demonstrate that it is possible to obtain functional antigen-specific T cells *in vivo* by retrovirus-mediated expression of TCRα and β cDNAs in hematopoietic precursor cells. We accomplished this using a well-studied retroviral vector, based on the murine stem cell virus (MSCV, (Hawley et al., 1994). The advantage of MSCV is that it maintains long-term and stable expression in hematopoietic precursor cells and their differentiated progeny (Cherry et al., 2000). Importantly, the levels of TCRα and TCRβ cDNAs obtained from this vector in the T cell compartment are sufficiently high to drive T cell development and function. We have detected large numbers of mature antigen-

specific T cells up to 3 months after injection of infected hematopoietic precursor cells. Because these cells are derived from long-lived progenitor cells, it is likely that they will be continually generated and persist for many months, possibly even for the lifetime of the mouse (Van Parijs et al., 1999b). Previous attempts to generate monospecific T cell populations using other oncoretrovirus-based vectors have failed to show detectable or functional expression of these receptors, presumably because the vectors were silenced during hematopoietic development (Huet et al., 1989).

The only significant drawback of the retroviral vectors that we have used in this study is that they can only introduce DNA into cells that are proliferating. Hematopoietic stem cells are usually quiescent. To induce them to proliferate, they have to be harvested from mice that have been treated with the toxic agent, 5-fluorouracil, and then cultured with cytokines *in vitro*. In contrast, lentivirus-based vectors are able to express genes in non-cycling cells (Lois et al., 2001). We have recently developed protocols to express TCR genes in unmanipulated hematopoietic precursor cells using well-defined HIV-derived vectors (data not shown), and are testing whether these cells can be used to generate TCR transgenic T cells.

Use of Retroviruses to Study T Cell Development and Function

Traditional TCR transgenic mice are designed to express a complex genomic fragment of the TCRα and TCRβ locus, not the cDNA for these genes (Kouskoff et al., 1995). In fact, transgenic mice have been created with the

TCRα and TCRβ cDNAs for the OTII TCR that we use here, but these did not show surface expression of this TCR or produce any OTII T cells (Barnden et al., 1998). It is thought that genomic fragments produce better-regulated expression of TCRs, leading to more efficient generation of transgenic T cells. The fact that we have been successful with cDNAs may reflect the circumstance that in each experiment we generate a large number of hematopoietic precursor cells that express TCR genes at different levels. Any individual retrovirus transgenic mouse receives about one million of these cells. It is likely that during thymic development only those progenitor cells that express an appropriate amount of TCR proteins give rise to mature T cells. Supporting the idea that populations of T cells can be selected on the basis of the level of TCR that they express in our monospecific T cell mice, we observed that immunization led to an increase in GFP^{high} (TCR^{high}) cells. It is likely that the limited amount of cognate peptide antigen present in immunized mice led to a preferential expansion of T cells expressing high amounts of the OTII TCR.

The available data suggest that neither transgenic nor retrovirus-mediated expression of TCRs faithfully reproduce the patterns of endogenous TCR expression. How this impacts the development of T cells remains unclear. However, a growing body of anecdotal and direct evidence suggests that the timing and level of TCR expression plays an important role in determining the fate of a T cell (Bruno et al., 1996; Lacorazza et al., 2001; Liu et al., 1996; Vonboehmer, 1990; Vonboehmer et al., 1991). New retroviruses that drive expression of genes under the control of tissue-specific and regulated promoters

should provide a novel and efficient tool to further address this important question in T cell biology (Lois et al., 2001).

An additional advantage of using retroviruses to study T cell development is that they can be used to introduce TCR genes into hematopoietic stem cells derived from mice of different genetic backgrounds or with defined genetic lesions. This has enabled us to generate antigen-specific T cells that are deficient for IL-2, and should allow the rapid and efficient evaluation of genetic effects more generally on the development and function of antigen-specific T cells.

Therapeutic and Prophylactic Uses of Retrovirus-Generated T Cells

As well as providing a new tool to study T cell biology and immune responses, our approach to generate antigen-specific T cells with defined functional properties might be applied in a clinical setting. It has a number of advantages over other published strategies to produce monospecific T cells with retroviruses. Most importantly, our approach allows the production of large numbers of naïve T cells for prolonged periods of time. These cells can be activated into effector T cells both *in vitro* and *in vivo* using a known peptide antigen. By expressing TCRs with the appropriate antigen-specificity, these T cells might provide protection against infectious diseases, such as HIV/AIDS and cancers. Alternatively, they could be used to regulate the development of allergies and autoimmune diseases.

The TCR transgenic T cells generated in this study were derived from hematopoietic precursor cells deficient in the RAG1 gene. These cells were used because they produce lymphoid cells that fail to express endogenous TCR chains. Such endogenous proteins might compete with the retrovirally-expressed TCR for surface expression and reduce the efficiency with which antigen-specific T cells were generated. While our study shows that transgenic T cells can readily be generated from RAG1-deficient progenitor cells, it would be useful, as a model for potential human therapeutic, to be able to generate these cells with wild type cells as well. We have already been able to generate T cells expressing a defined TCRβ chain by retrovirus-mediated gene expression in wild type hematopoietic precursor cells (data not shown), and we are currently testing whether functional antigen-specific T cells can also be obtained from these cells. We are also investigating whether it is possible to generate transgenic T cells by injecting infected progenitor cells into untreated mice, or mice that receive myeloablative treatments that are less severe than whole body irradiation. These treatments are less likely to produce long-lasting damage to the host, and more closely resemble protocols that might be used in humans. Similar approaches have been developed using autologous hematopoietic stem cells to produce long-term microchimerism that permits the transplantation of immunologically incompatible organs (Sykes, 2001).

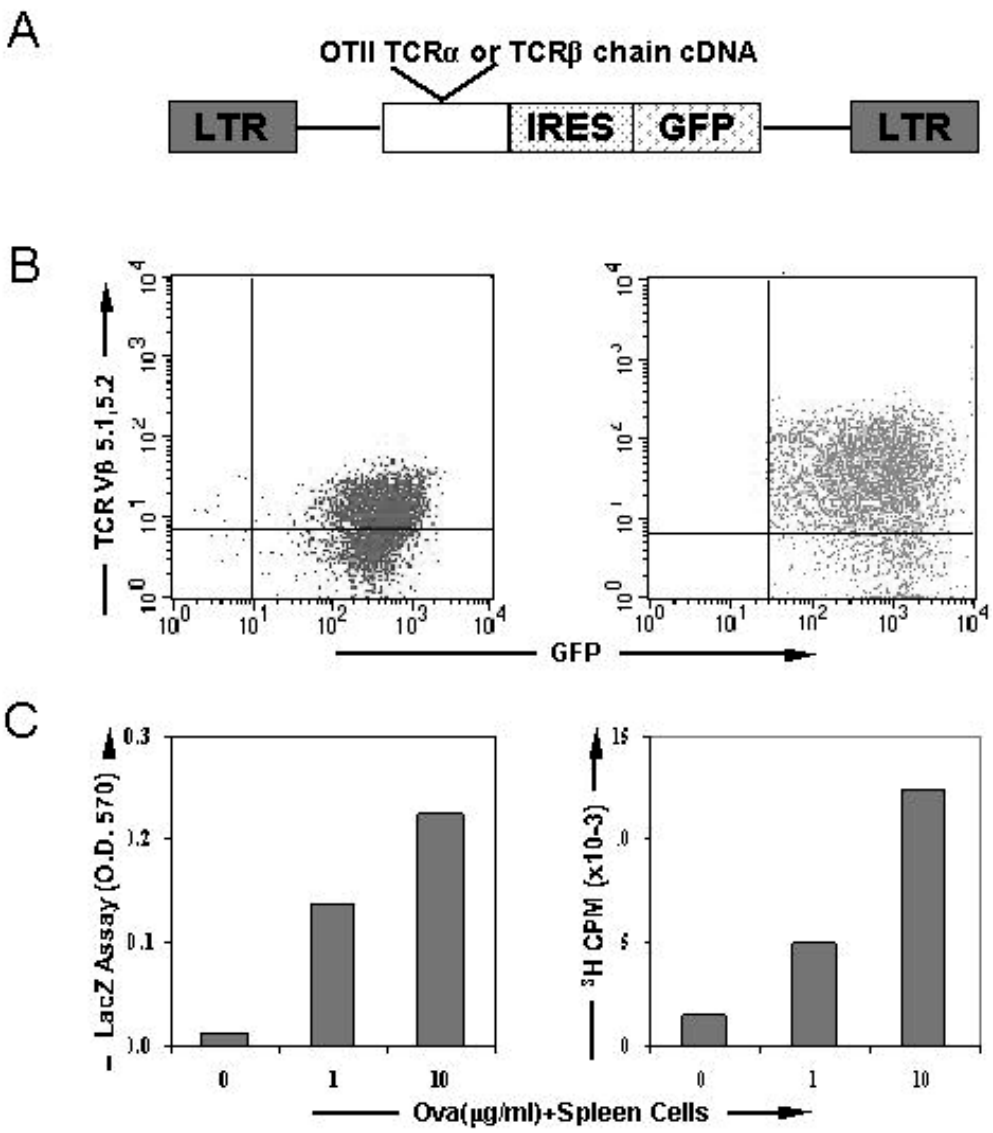
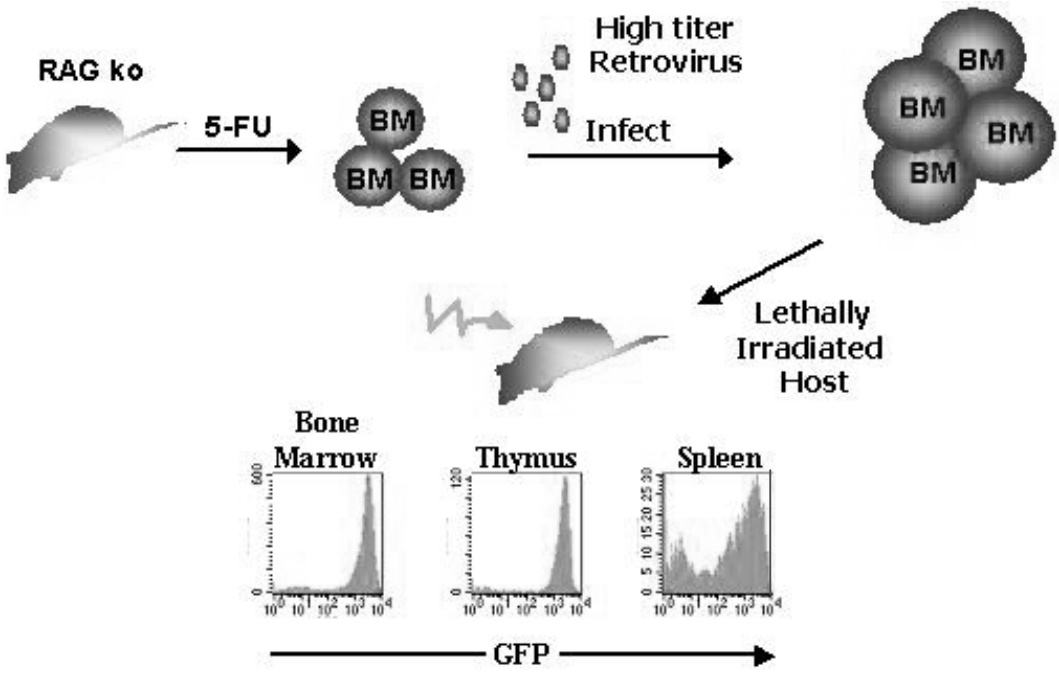

A
OTII TCRα or TCRβ chain cDNA
LTR
IRES
GFP
LTR
B
TCR Vβ 5.1,5.2
GFP
C
LacZ Assay (O.D. 570)
3H CPM (x10-3)
0
1
10
Ova(μg/ml)+Spleen Cells

Figure 2-1

Figure 2-1: Functional expression of the OTII TCR in a T cell hybridoma line and primary CD4+ T cells using retroviruses. (A) Schematic representation of the MSCV IRES GFP (MIG) retrovirus construct expressing the cDNA for the OTII TCRα or TCRβ chain. KEY: LTR, long terminal repeat; IRES, internal ribosomal entry site; GFP, green fluorescent protein. (B) Surface expression of the OTII TCRβ chain in infected (GFP+) THZ cells (left panel) and primary CD4+ T cells (right panel). Cells were co-infected with MIG retroviruses expressing the cDNA for the OTII TCRα or β chain, and then stained with a PE-conjugated antibody against TCR Vβ 5.1,5.2, which is the Vβ element used by the OTII TCRβ chain. (C) Functional expression of the OTII TCR in THZ cells (left panel) and primary CD4+ T cells (right panel). Cells were co-infected with MIG retroviruses expressing OTII TCRα chain or OTII TCRβ chain, and restimulated for 48 hours with OVAp in the presence of B6 spleen cells as APCs. Antigen response of THZ cells was assessed by assaying for the induction of β-galactosidase expression and by ^{3}H-thymidine incorporation for primary CD4+ T cells.


RAG ko
5-FU
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BM BM
High titer
Retrovirus
Infect
BM
BM
BM
BM
Lethally
Irradiated
Host
Bone
Marrow
Thymus
Spleen
GFP

Figure 2-2

Figure 2-2: Schematic of our strategy to generate OTII monoclonal T cells *in vivo* using retroviruses. Hematopoietic precursor cells were obtained from wild type and IL-2-deficient RAG knockout mice that had been treated with 5-fluorouracil. These cells were then cultured in the presence of cytokines and co-infected with MIG retroviruses expressing the cDNA for the OTII TCRα or β chain. The infected hematopoietic precursor cells were then transferred into a lethally irradiated host mouse and allowed to reconstitute the immune system. Retrovirally transduced cells were identified by their expression of the green fluorescent protein (GFP).

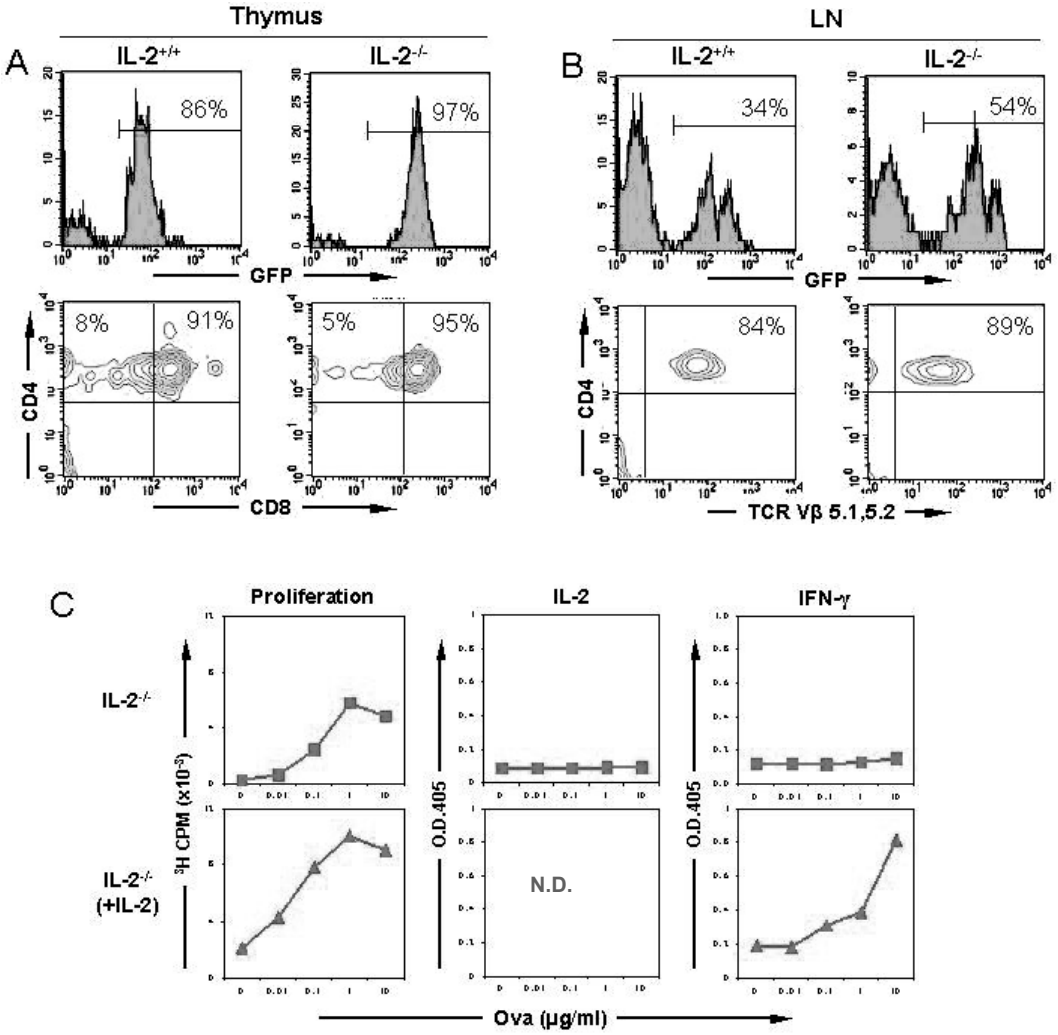
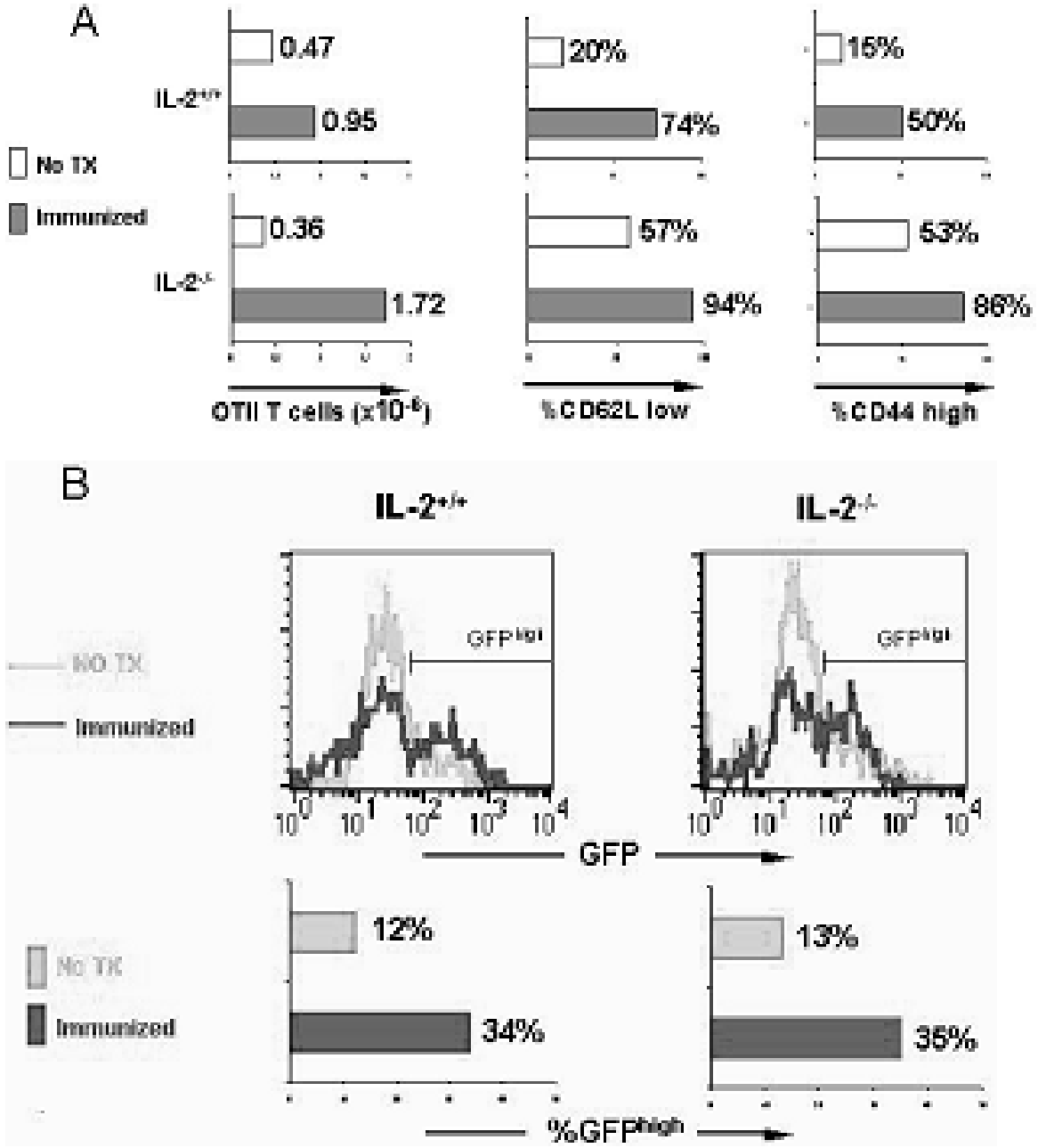


Figure 2-3: Development of functional wild type and IL-2-deficient OTII monoclonal T cells derived from retrovirally-transduced hematopoietic precursor cells. (A) Normal thymic development of OTII monoclonal CD4+ T cells in lethally irradiated mice receiving retrovirally-transduced hematopoietic precursor cells. Thymocytes obtained from lethally-irradiated host mice 11 weeks after

injection of retrovirally-transduced hematopoietic precursor cells were stained with anti-CD4-Cyc and anti-CD8-PE antibodies and analyzed by flow cytometry. The distribution of CD4 and CD8 expression on GFP+ thymocytes is shown. (B) Presence of mature OTII monoclonal CD4+ T cells in the peripheral lymphoid organs of mice receiving retrovirally-transduced hematopoietic precursor cells. Lymph node and spleen (not shown) cells obtained from lethally irradiated host mice 11 weeks after injection of retrovirally-transduced hematopoietic precursor cells were stained with anti-CD4-Cyc and anti-TCR Vβ 5.1,5.2-PE antibodies and analyzed by flow cytometry. The distribution of CD4 and Vβ5.1, 5.2 expression on GFP+ lymph node cells is shown. (C) Normal functional responses of OTII monoclonal CD4+ T cells obtained from the peripheral lymphoid organs of mice receiving retrovirally-transduced hematopoietic precursor cells. Spleen cells obtained from lethally irradiated host mice 11 weeks after injection of retrovirally-transduced hematopoietic precursor cells derived from IL-2-deficient mice were supplemented with B6 spleen cells as APCs and stimulated in vitro with OVAp in the presence or absence of exogenous IL-2. Proliferation was assayed after 72 hours by ^{3}H-thymidine incorporation and cytokine production by ELISA. Data was normalized for the number of GFP+CD4+TCR Vβ 5.1,5.2+ cells present in the starting spleen cell populations. Proliferation and cytokine production was seen with wild type OTII T cells both in the presence and absence of IL-2 (data not shown).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IL-2+/+
IL-2-/-
No TX
Immunized
0.47
0.95
0.36
1.72
OTII T cells (x10-6)
20%
74%
57%
94%
%CD62L low
15%
50%
53%
86%
%CD44 high
B
IL-2+/+
IL-2-/-
No TX
Immunized
GFPhigh
GFP
12%
34%
13%
35%
%GFPhigh

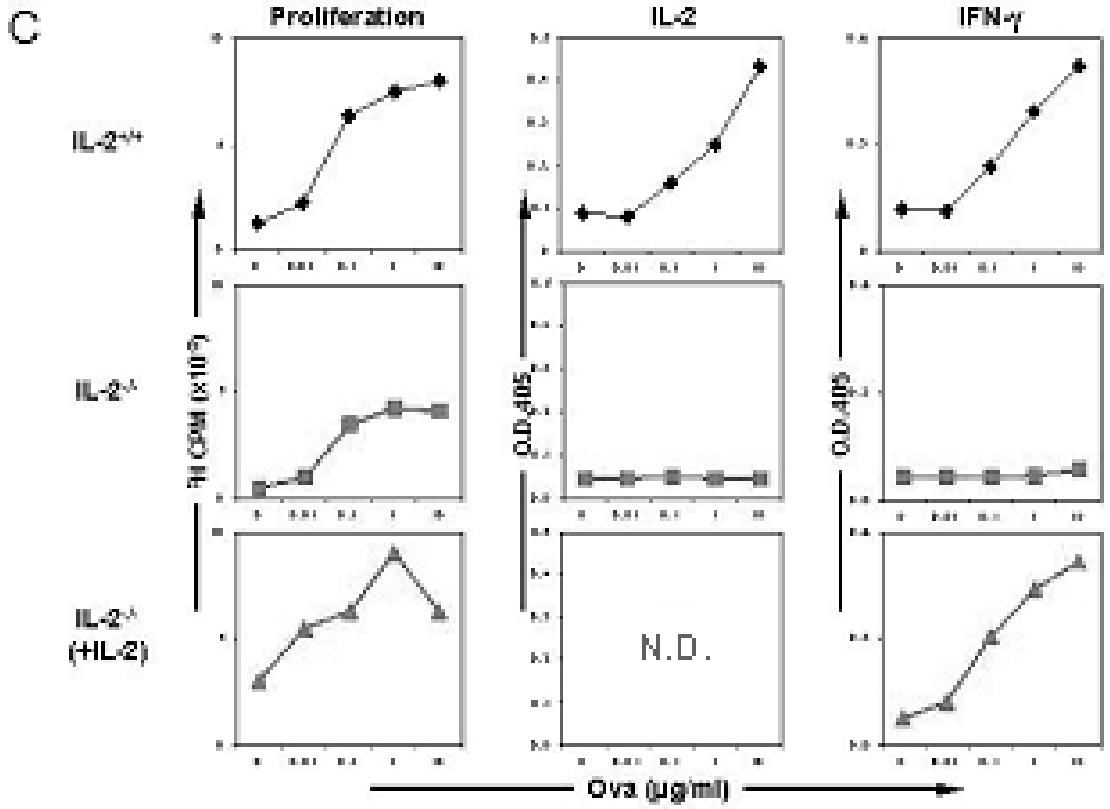

Figure 2-4: *In vivo* responses of wild type and IL-2-deficient OTII monoclonal T cells upon immunization with cognate antigen. (A) T cell expansion and expression of activation markers following immunization. Lethally-irradiated mice host mice were immunized via an intra peritoneal injection of 200μg OVAp (IMM) or left untreated (No TX) 10 weeks after receiving retrovirally-transduced hematopoietic precursor cells. Spleen and lymph node cells were harvested and

counted 6 days later. An aliquot of these cells was stained with anti-CD4-Cyc and anti-TCR Vβ 5.1,5.2-PE, anti-CD62L-PE, or anti-CD44-PE antibodies and analyzed by flow cytometry. The number of OTII monoclonal T cells present in the spleen and lymph nodes of immunized and control mice was determined by multiplying the percentage of GFP+CD4+TCR Vβ 5.1,5.2+ cells by the total number of cells present in these organs. The frequency of activated T cells was determined by gating on GFP+ CD4+TCR Vβ 5.1,5.2+ and $CD62L^{low}$ or $CD44^{high}$ cells. (B) Preferential expansion of GFP^{high} OTII monoclonal CD4+ T cells following immunization. Mice receiving retrovirally-transduced hematopoietic precursor cells were immunized as in (A). Spleen and lymph node cells were collected and stained with anti-CD4-Cyc and anti-TCR Vβ 5.1,5.2-PE antibody and analyzed by flow cytometry. The expression of GFP in Vβ5.1, 5.2+ CD4+ OT.II T cells, and the frequency of GFP^{high} OTII T cells is shown. (C) Effector function of OTII monoclonal CD4+ T cells following immunization. Mice receiving retrovirally-transduced hematopoietic precursor cells were immunized as in (A). Spleen/LN cells were harvested and stimulated *in vitro* with OVAp in the presence of B6 spleen cells as APC. Proliferation was assayed by ^{3}H thymidine incorporation, and cytokine production by ELISA. Data was normalized for the number of GFP+CD4+TCR Vβ 5.1,5.2+ cells present in the starting spleen cell populations.

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