

Chapter 6

IL-2 Regulates CD4 T Cell Memory Generation

1. Introduction

The immune system has many remarkable characteristics but none is more remarkable than its ability to set aside memory cells that maintain a library of all previous immunologic exposures. This library ensures that when an animal encounters a specific pathogen for a second or greater time, the system will respond rapidly and robustly.

The generation and long-term maintenance of memory T-cells has been a subject of intense study and debate. Currently there are two general memory generation models in the literature (Farber, 1998): 1) memory T-cells are derived from effector T-cells that have regressed to a quiescent stage through an unknown means (“effector” model); 2) memory T-cells are developed by a pathway distinguishable from the effector pathway and thus naïve cells need not pass through an intermediate effector stage (“no effector” model). These two models have been extensively evaluated for T-cell memory development. Although there is evidence that effector differentiation is not a prerequisite for CD8 T-cell memory generation (Manjunath et al., 2001), much recent data indicate that generation of CD4 and CD8 effector T-cells is necessary for production of effective and long-term T-cell memory (Kaech and Ahmed, 2001; Oehen and Brduscha-Riem, 1998; Oehen and Brduscha-Riem, 1999; Opferman et al., 1999; van Stipdonk et al., 2001; Yang and Baltimore, 2003).

An implication of the “effector” model is that memory generation must be intrinsically linked to the three phases of the immune response: initiation, clonal expansion, and contraction (Schluns and Lefrancois, 2003). The initiation phase

of the T-cell response begins with recognition via the cell surface T-cell receptor (TCR) of a specific antigen presented by a major histocompatibility complex (MHC) protein on the surface of an antigen-presenting cell (APC). This in turn triggers the clonal expansion phase of the response. Subsequently, apoptosis occurs in most of activated effector T-cells, resulting in a subpopulation of antigen-specific T-cells persisting in the host as memory T-cells. It should be noted that the kinetics and efficiency of CD4 and CD8 T-cells in response to antigen stimulation are different (Gett et al., 2003; Schluns and Lefrancois, 2003; Seder and Ahmed, 2003). For naïve CD8 T-cells, a brief encounter with an APC is sufficient to launch extensive proliferation and homogenous differentiation. However, naïve CD4 T-cells appear to require a longer antigen exposure and respond in a more heterogeneous fashion, complicating the study of CD4 T-cell memory generation.

It has been proposed that CD4 T-cells follow a progressive differentiation model (Gett et al., 2003; Iezzi et al., 1999; Langenkamp et al., 2002; Reinhardt et al., 2001; Sallusto et al., 1999). This model suggests that “signal strength”, which consists of affinity and concentration of antigen, duration of interaction, and availability of co-stimulation, regulates the progressive differentiation and determines CD4 T-cell fate (Gett et al., 2003). Although “T-cell fitness”, the ability of stimulated T-cells to persist in the host upon the clearance of antigen, has been introduced to characterize the “signal strength”, it remains unknown whether there is a molecular signature of this “signal strength”. More precisely,

can we identify a particular signaling pathway through which “signal strength” acts?

It is known that cytokines can affect T-cell proliferation and survival at many stages of the immune response (Janeway et al., 2001). Thus, it is conceivable that cytokines are also key molecular mediators in the regulation of generation and maintenance of T-cell memory. For CD8 T-cells, it has become clear that the members of the common cytokine receptor γ chain (γ c) family, including interleukin 2 (IL-2), IL-7 and IL-15, contribute significantly to memory T-cell production and differentiation (Schluns and Lefrancois, 2003). It remains controversial whether cytokines are required for generation and maintenance of CD4 memory T-cells (Homann et al., 2001; Hurez et al., 2003; Lantz et al., 2000; Saparov et al., 1999; Schluns and Lefrancois, 2003). No cytokine-driven signaling pathway has been shown to mediate memory cell production and survival for either CD4 or CD8 T-cells. And it is unclear whether there is a specific signaling pathway to regulate T-cell memory.

Recent studies have shown that CD4 T-cells can help generate functional memory CD8 T-cells (Bourgeois et al., 2002; Janssen et al., 2003; Kaech and Ahmed, 2003; Shedlock and Shen, 2003; Sun and Bevan, 2003). In the absence of CD4 cells, although memory CD8 T-cells can be generated, they exhibit poorer secondary responses than memory CD8 T-cells generated with CD4 help. These studies did not examine the detailed molecular mechanisms and signaling pathways involved in the CD4 helping process. Several experiments suggest that CD4 help is achieved by supplying IL-2 to CD8 T-cells (Bourgeois et al.,

2002; Janssen et al., 2003; Wherry et al., 2003). It is unclear how this event is initiated and what other signaling molecules are involved. We and others have shown that generation of effective CD4 T-cell memory is independent of CD8 T-cells (London et al., 2000; London et al., 1999; Yang and Baltimore, 2003). In a physiological setting, a productive immune response always involves both CD4 and CD8 T-cells coordinately developing into effector cells and memory cells. This implies that they may share certain molecular mechanisms for memory generation, which needs to be triggered by CD4 T-cells. Study of CD4 T-cell memory can facilitate the understanding of CD8 T-cell memory development.

Here we show that IL-2 is required for memory generation but not required for CD4 T-cell memory maintenance. IL-2 receptor (IL-2R) functions through two well-established pathways, one leading to the AKT kinase and the other to the STAT5 transcription factor. Using the lentivirus-based genetic tool developed in our laboratory, we are able to show that it is activation of STAT5, not AKT, which is necessary and sufficient for generation of CD4 T-cell memory. Based on the implication of these results, we also propose a “Path to STAT5” memory model.

2. Materials and Methods

Mice

C57BL/6J(B6) female mice were purchased from Charles River Breeding Laboratories, and RAG1 and IL-2 knockout mice and OT2 TCR transgenic mice in the B6 background were purchased from The Jackson Laboratory. IL-2^{-/-}/RAG1^{-/-} mice (denoted as IL-2⁻/RAG⁻), OT2^{Tg}/RAG1^{-/-} mice (denoted as

OT2/RAG⁻), and OT2^{Tg}/RAG1⁻/IL-2⁻ mice (denoted as OT2/RAG⁻/IL-2⁻) were generated by breeding. All mice were housed in the California Institute of Technology animal facility in accordance with institute regulations.

FUGW, FUIGW-AKT* and FUIGW-STAT5* Lentiviruses

FUGW and FUIGW lentiviruses were generated in our laboratory (Lois et al., 2002a). The cDNAs encoding myristoylated *AKT* (denoted as *AKT**, kind gift from Dr. Z. Songyang, Baylor College of Medicine, Houston, TX) (Zhou et al., 1997a) or a constitutively active form of *STAT5* (denoted as *STAT5**) (Onishi et al., 1998a) were cloned into FUIGW to generate FUIGW-*AKT** or FUIGW-*STAT5** lentiviral constructs. Lentiviruses were made in HEK293.T cells by co-transfection of the lentiviral construct (FUGW, FUIGW-*AKT** or FUIGW-*STAT5**), the packaging vectors pRRE and pREV, and the peEco vector encoding the Eco envelope glycoprotein (Takara, Japan) using a standard calcium phosphate precipitation protocol (Pear et al., 1993). Viruses were harvested 36-48 hours after transfection.

Peptides

OVA₃₂₃₋₃₃₉ peptide (denoted as OVAp) recognized by OT2 TCR was synthesized at Caltech Biopolymer Synthesis Center.

In vitro OT2 T cell Stimulation and Functional Assays

Spleen cells from OT2/RAG⁻ or OT2/RAG⁻/IL-2⁻ mice were harvested and cultured at 2x10⁵ cells/well in T cell culture medium containing 0.5µg/ml anti-CD3, or 0.5µg/ml anti-CD3 +0.5µg/ml anti-CD28 (both from BD Pharmingen, San Diego, CA), or 1µg/ml OVAp (with 2x10⁵ cells/well B6 spleen cells as APCs), with or without 10ng/ml rIL-2 (BioSource International, Camarillo, CA). Three days later, culture supernatants were collected and assayed for IL-2 or IFN-γ production by ELISA, and proliferation was assessed by [³H]thymidine incorporation as described before (Yang et al., 2002a). The surface phenotype of the activated OT2 CD4 T cells was analyzed by flow cytometry.

Naïve OT2 CD4 T Cell Infection and Stimulation

Naïve OT2 CD4 T cells were harvested from the spleens of OT2/RAG⁻ or OT2/RAG⁻/IL-2⁻ mice and spin-infected with the lentiviruses in the presence of 10µg/ml polybrene for 90min at 2,500rpm, 30°C. After infection, the lentiviral supernatant was removed and replaced with fresh medium containing APCs (B6 spleen cells) and 1µg/ml OVAp. The cells were stimulated for 3 days and then used for analysis and further adoptive transfer.

Antibodies and FACS Analysis

Fluorochrome-conjugated antibodies specific for mouse CD4, CD25, CD69, CD62L, CD44, TCRVα2, TCRVβ5.1,5.2, IL-7Rα 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. Anti-phospho-AKT and anti-phospho-STAT5 antibodies were purchased from Cell Signaling Technology and the intracellular staining was done following the protocol provided by the manufacture. Analyses were performed on a FACScan flow cytometer.

T Cell Memory Study

Spleen cells were harvested from OT2/RAG⁻ or OT2/RAG⁻/IL-2⁻ mice. If indicated, the naïve OT2 CD4 T cells were spin-infected with FUGW, FUIGW-AKT* or FUIGW-STAT5* lentiviruses in the presence of 10µg/ml polybrene for 90min at 2,500rpm, 30°C. The cells were then stimulated with 1µg/ml OVAp (using B6 spleen cells as APCs), with or without adding 10ng/ml rmlL-2 (BioSource International, Camarillo, CA), for 3 days *in vitro*. On day 3, the cells were collected. Some were used to assay the surface phenotype, and if applied, the lentiviral infection efficiency (indicated by GFP expression), of the OT2 CD4 effector cells by flow cytometry. The rest were transferred into IL-2⁻/RAG⁻ hosts by tail vein injection. Each host received 30-40x10⁶ cells (>10% were OT2 CD4 effector T cells). Five mice were used for each experimental group. Four to sixteen weeks later, spleen cells were harvested from the hosts and analyzed for the presence of long-lived OT2 T cells. Memory phenotype of the OT2 T cells was studied by flow cytometry. Memory function was studied by antigen dosage response, antigen time-course response and cytokine proliferation response of the recovered OT2 T cells. For antigen dosage response, cells were stimulated with 0-10µg/ml OVAp 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., 2002a). For antigen time-course response, cells were stimulated with 1 μ g/ml OVAp, 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 (or day 2, day 3 and day 4). For assay on cytokine proliferation response, cells were cultured with 10ng/ml rIL-2, or 10ng/ml IL-4 or 10ng/ml rIL-15 (all from BioSource International, Camarillo, CA) for 4 days in the absence of antigen, and proliferation was assessed by [3 H]thymidine incorporation.

3. Results

Generation and Characterization of Naïve IL-2 Deficient CD4 T-cells

A classical approach to studying the contribution of IL-2 to memory generation or persistence in CD4 T-cells would be to knock out the IL-2 gene in mice and study the CD4 T-cells. Unfortunately, IL-2 KO mice develop autoimmune diseases right after birth. CD4 T-cells harvested from these mice are predominantly pre-activated autoreactive CD4 T-cells, which are not appropriate for memory study. A homogenous population of naïve IL-2 deficient CD4 T-cells with known antigen specificity would provide the best material for such a study. To achieve this, we decided to cross IL-2 $^-$ mice with RAG knockout mice. We use a “-“ to represent homozygous knockout and a “+” to represent transgenic. Autoimmune disease was completely eliminated in the resulting IL-2 $^-$ /RAG $^-$ mice. We also crossed the OT2 T-cell receptor (TCR) transgenic mice

with RAG knockout mice to generate OT2/RAG⁻ mice. OT2 is a well-characterized mouse CD4 TCR recognizing chicken OVA_{p323-339} presented by I-A^b (Li et al., 2001). Subsequent interbreeding between IL-2⁻/RAG⁻ mice and OT2/RAG⁻ mice generated OT2/IL-2⁻/RAG⁻ mouse strains. T-cells obtained from OT2/IL-2⁻/RAG⁻ animals were mono-specific and exhibited the same naïve T-cell phenotype as OT2 T-cells from OT2⁺/RAG⁻ mice (Figure 6-1A).

When the naïve CD4 T-cells from animals with normal IL-2 genes were treated with various stimuli, including anti-CD3, anti-CD3 and anti-CD28, or OVA_p and APC, they were activated as shown by proliferation assay, cytokine secretion assay and surface marker staining (Figure 6-1, B and C). However, the cells from IL-2 deficient mice had attenuated T-cell responses, as manifested by proliferation and cytokine production assays after stimulation (Figure 6-1B). Looking at proliferative responses, IL-2 deficient CD4 T-cells could not be stimulated by anti-CD3 antibodies alone or by both anti-CD3 and anti-CD28, implying that even cross-linking T-cell receptor (TCR) and co-stimulation of CD28 cannot activate IL-2 impaired CD4 T-cells (Figure 6-1B). Interestingly, when these cells were co-cultured with antigen-presenting cells (APCs) from wild-type B6 mice loaded with antigen (OVA_p), IL-2⁻ cells could then be activated (Figure 6-1B). Thus the lack of IL-2 signaling to naïve CD4 T-cells can be compensated by interaction with antigen-loaded APCs. For a long time, investigators have been frustrated by the fact that IL-2 KO T-cells could not be effectively activated *in vitro* without adding exogenous IL-2, a result apparently contradictory to the presence of highly activated T-cells in IL-2 KO mice. This is a key reason why the

mechanism of IL-2 deficiency-induced autoimmunity remains an enigma. Initially, we were also concerned whether we were able to find a condition for appropriate activating the naïve IL-2 deficient CD4 T-cells. Our discovery of the activation condition allowed us to continue the study of IL-2 function in CD4 T-cell memory; it could be also useful for those who are interested in the study other IL-2 functions such as self-tolerance.

We also closely examined the patterns of surface activation markers such as CD25, CD69, CD62L and CD44. The naïve OT2 CD4 T-cells from animals in different genetic backgrounds were stimulated with 1 $\mu\text{g}/\text{mL}$ OVA_{p323-339} in the presence of APCs *in vitro*. OT2/IL-2⁻ T-cells proliferated similarly to wild-type OT2. After 3 days, cells were analyzed by flow cytometry. We observed that IL-2 deficiency slightly affected the upregulation of CD25, while the expression of other surface activation markers (CD69, CD62L and CD44) was unaltered (Figure 6-1C, the second row on the left). The expression of CD25 (IL-2 receptor α chain) is upregulated by IL-2 binding to its receptor, which would be lower in the absence of the IL-2. Addition of exogenous recombinant mouse IL-2 (rmIL2, 10 ng/mL) into the culture rescued upregulation (Figure 6-1C, the third row on the left). These results demonstrate that IL-2 deficient CD4 T-cells derived from unchallenged OT2/IL-2⁻/RAG⁻ mice are naïve, and that antigen-loaded APCs can elicit from them an appropriate antigen-specific response. It would be interesting to see whether all aspects of IL-2⁻ CD4 T-cell functions, such as memory generation and persistence, can be achieved as well in absence of IL-2.

An IL-2 Signal Supports CD4 T-cell Memory Generation But Is Not Required for Memory Maintenance

IL-2 has been suggested to be one of key cytokines that mediates CD8 T-cell memory (Schluns and Lefrancois, 2003). We set up an experiment to examine the role of this molecule in CD4 T-cell memory. Naïve CD4 T-cells, obtained from OT2/IL-2⁻/RAG⁻ mice, were co-cultured with APCs (wild-type B6 spleen cells) and peptide antigen (OVA_{p323-339}, 1 µg/mL) for 3 days. Exogenous recombinant mouse IL-2 (rmIL-2, 10 ng/mL) was supplemented in the culture medium (no supplementation of rmIL-2 as a control). FACS analysis showed that approximately 36% and 46% of the total CD4 T-cells were effector OT2 T-cells in presence and absence of IL-2 treatment, respectively, as compared to 32% of OT2 effectors generated from naïve OT2/RAG⁻ T cells (Figure 6-2A).

The activated CD4 T-cells were then washed with PBS buffer and transferred into IL-2⁻/RAG⁻ mice. We chose IL-2⁻/RAG⁻ mice as the recipients because these mice do not have endogenous T-cells and provide a clean background to identify the presence of memory OT2/IL-2⁻ T-cells. The lack of endogenous IL-2 in these recipients allows the study of an IL-2 requirement for memory development. In our typical transfer experiment, one mouse received approximate 10×10^6 cells collected from the day 3 culture, including 2.5×10^6 OT2/IL-2⁻/RAG⁻ effector T-cells and 0.5×10^6 OVA_p non-specific naïve T-cells. These naïve T-cells originated from B6 spleen cells when the spleen cells were added into the culture as APCs. We did not adopt the protocol of only transferring the purified OT2 effectors because: 1) we are able to trace the OT2

cells by antibody staining and FACS analysis; 2) we believe that our protocol mimics more closely the physiological environment of memory T-cell development. One potential concern is that these transferred non-OT2/IL-2⁻ cells could produce IL-2 in the recipient, contradicting our design of an IL-2 free environment. However, in our experiment no IL-2 was detected during both the primary and memory response (Figure 2C), indicating that this type of IL-2 interference was negligible. This can be understood by our finding that these non-OT2/IL-2⁻ T-cells retained their naïve phenotype during the course of these experiments. Huston and co-workers used a similar transfer protocol for study of the class II-independent generation of CD4 memory T-cells and also found that the contaminating APCs were unable to elicit the necessary signals to promote MHC-dependent survival (Swain et al., 1999).

Fourteen weeks later, spleen cells from the host mice were harvested and the existence of long-lived memory CD4 T-cells was determined. FACS plots showed the presence of OT2 cells (~2% of total CD4 T-cells) in the mice that received OT2/IL-2⁻ effectors treated with rIL-2 during the primary response; without rIL-2 treatment, there were no detectable OT2 cells (Figure 6-2, A and B). As the control experiments, OT2 effectors were transferred into either RAG- (data not shown) or RAG-/IL-2⁻ mice (Figure 6-2, A and B); approximately 5% of memory OT2 cells could be recovered. Nevertheless, these OT2 cells clearly were of the memory phenotype, and IL-2 treatment during the primary response was necessary to generate them. Considering that these cells survived in IL-2

deficient recipient mice after transfer, it would appear that IL-2 is not a necessary cytokine to maintain CD4 T-cell memory.

To examine whether these cells can provide memory functions, we tested their ability to mount a secondary response. Spleen cells harvested from adoptive hosts were stimulated with APCs and different concentrations of OVA_p, with or without addition of rmlIL-2. Spleen cells from OT2 transgenic mice displayed a typical primary anti-OVA_p response (weak and slow). The response of OT2/IL-2⁻ naïve CD4 T-cells was weaker than that of OT2/IL-2⁺ naïve T-cells, but recovered to the same level when exogenous rmlIL-2 was provided (Figure 6-2C, compare naïve OT2/IL-2⁻ and naïve OT2). The adoptively transferred cells differed in their behavior depending upon whether they were treated with IL-2 or not. When the transferred effectors were treated with rmlIL-2 during the primary response, spleen cells exhibiting memory OT2 phenotype mounted a typical secondary response with effector cytokine IL-4 production (strong and fast) (Figure 6-2C). When exogenous rmlIL-2 was added, the magnitude of the response was greatly enhanced. For the production of another effector cytokine IFN- γ , these cells showed a slightly better response than that of naïve OT2 cells; the memory characteristic of IFN- γ production became obvious when exogenous rmlIL-2 was provided in the culture. It is known (Yang et al., 2002b) that IL-4 production is less dependent on an IL-2 signal than IFN- γ products, so the addition of rmlIL-2 to memory OT2/IL-2⁻/RAG⁻ cells significantly enhanced the production of IFN- γ .

In contrast to the cells treated with IL-2 during the primary response, spleen cells not treated with IL-2 contained no detectable memory OT2/IL-2⁻ cells (Figure 6-2, A and B), and showed no response to OVA_p stimulation, with or without addition of rmIL-2 (Figure 6-2C), confirming the lack of memory cells. We also performed proliferation assays and found that the memory OT2/IL-2⁻ T-cells proliferated more extensively than naïve OT2/IL-2⁻ T-cells upon antigenic stimulation *in vitro* (data not shown).

In support of our *in vitro* results, we tested the ability of these memory cells to mount the recall responses by challenging the mice with OVA_p *in vivo*, and we observed the characteristic memory T-cell response (data not shown). Thus, CD4 OT2 T-cells with the memory phenotype exhibited memory function both *in vitro* and *in vivo*.

Development of a Lentiviral Gene Delivery Method as a Tool to Study IL-2 Signaling in CD4 T-cells

A key goal of our work has been to identify the components of the IL-2-driven signaling pathway that mediates the generation and maintenance of memory CD4 T-cells. Having shown above that we could generate quiescent and naïve populations of CD4 cells with a known antigen specificity, we now needed a method to insert into them genes encoding proteins of interest. We had been working with lentivirus vectors (An et al., 2003; Brown and Baltimore, 2003; Lois et al., 2002b; Qin et al., 2003; Rubinson et al., 2003) and therefore explored their potential to serve our purposes. We used the FUGW vector (Lois

et al., 2002b) carrying the human ubiquitin-C promoter driving a GFP reporter gene (Figure 6-3A). We made the viruses with an ecotropic envelope since we only wanted to infect mouse cells. This provided us with a margin of safety because such virus cannot infect human cells.

When we infected naïve, quiescent T-cells, without additional treatment we did not observe GFP expression, suggesting that quiescent cells, although they could be infected by the lentivirus, could not express the delivered gene (Yang and Baltimore, 2003). Upon the treatment with anti-CD3 antibodies to activate the T-cells, GFP was expressed as shown by FACS analysis (Figure 6-3B). As early as 18 hr. post-infection, both CD4 and CD8 T-cells began expressing GFP. We used DRAQ5, a cell membrane permeable dye capable of staining DNA, to monitor cell division and found that GFP expression did not require cell division, only cell activation (Figure 6-3C). Staining for the T-cell surface activation marker CD69 confirmed that T-cells expressing GFP were indeed activated after treatment with anti-CD3 antibodies (Figure 6-3D). The level of GFP expression also correlated with the dosage of anti-CD3 used for the activation; a concentration of 0.1 $\mu\text{g}/\text{mL}$ was enough to achieve the highest GFP expression. These results show that lentivirus-mediated gene transfer can deliver a gene to naïve T-cells and the expression of gene requires activation of the T-cell but not cell division.

We then tested the use of lentivirus-mediated gene transfer to dissect cytokine-mediated T-cell signaling. IL-2 triggers a signaling pathway that promotes proliferation by activating the STAT5 transcriptional factor (Antov et al.,

2003; Fujii et al., 1995; Kelly et al., 2002; Moriggl et al., 1999; Teglund et al., 1998; Vig et al., 2003). IL-2 can also activate the protein kinase AKT, which is known to transduce an antiapoptotic signal to promote cell survival (Ahmed et al., 1997; Dudek et al., 1997; Jones et al., 2000; Kane et al., 2001; Kelly et al., 2002; Van Parijs et al., 1999; Vig et al., 2003). We confirmed the role of IL-2 in enhancing the activation of STAT5 and AKT molecules in vitro by intracellular staining of phosphorylated STAT5 (pSTAT5) and AKT (pAKT) in activated OT2/RAG⁻/IL-2⁻ cells (Figure 6-4D); treatment of exogenous IL-2 to IL-2 deficient OT2 cells could upregulated the pSTAT5 and pAKT to the same level that normal OT2 would achieve upon antigenic stimulation (Figure 6-4D).

We used the lentivirus-based method to express the active form of either STAT5 or AKT gene in naïve CD4 lymphocytes, and their functional consequences were assayed. We introduced a consistently active form of STAT5 (designated as STAT5*, (Antov et al., 2003; Onishi et al., 1998b)) or AKT (designated as AKT*, (Kelly et al., 2002; Zhou et al., 1997b)) together with GFP into FUGW. GFP was used as a marker to track the infected cells. To accomplish the co-expression of two genes, an internal ribosome entry site (IRES) gene was placed upstream of GFP. The resultant vector was designated FUIGW (Figure 6-4A).

The titer of virus derived from FUIGW was slightly lower than virus produced from FUGW, indicating that the IRES could affect production of lentivirus (Yang and Baltimore, 2003). Nevertheless this configuration (FUIGW) can prepare virus with high enough titer for the functional expression of both

STAT5^{*}/AKT^{*} and GFP (Yang and Baltimore, 2003). We tested the efficacy of these engineered vectors for infecting naïve CD4 T-cells. Figure 6-4B shows the FACS plots from a representative experiment at day 3 post-vector infection. Quantitative FACS analysis showed that CD4 cells from both control mice (C57BL/6) and IL-2 deficient mice (OT2/IL-2⁻/RAG⁻) infected with FUIGW lentiviruses had over 20% GFP positive cells. STAT5^{*} infected CD4 cells had 30~38% GFP⁺. Similarly, over 22% of CD4 cells from both mice were positively infected by AKT^{*} (Figure 6-4B). These lentiviral vectors exhibited similar infection efficiency towards naïve CD4 cells from both wild-type and IL-2⁻ mice. Thus, a lentivector containing an IRES unit can simultaneously deliver a gene of interest and a GFP reporter to naïve CD4 T-cells.

We then tested whether STAT5 or AKT could promote the proliferation and survival of CD4 T-cells from OT2/IL-2⁻/RAG⁻ mice by expressing their constitutively active forms using the lentivirus-based gene delivery approach. In these experiments, we determined the percentage of cells that were viable by flow cytometry; viable cells were distinguished from dead cells on the basis of their forward- and side-scatter characteristics. For the CD4 T-cells from wild-type mice, expression of AKT^{*} led to the increased survival and expansion of anti-CD3 pulsed T-cells 3 day post-infection (data not shown). When STAT5^{*} was expressed in IL-2⁻ CD4 T-cells, we did not observe a change in cell survival (data not shown), but did observe a significant increase of cell proliferation, indicating that STAT5^{*} enhanced T-cell proliferation (Figure 6-4C).

Nevertheless this series of experiments told us that in absence of IL-2, activation of STAT5 could enhance CD4 cell proliferation; activation of AKT can prolong CD4 T-cell survival, consistent with previous reports on the function of these two genes. Thus, the lentivector approach is appropriate for our intended experiments.

Activation of the AKT Signaling Pathway During Primary Response Cannot Rescue CD4 T-cell Memory Failure Caused by IL-2 Deficiency

Over the past years, evidence has appeared in the literature suggesting that AKT is one of important downstream mediators of the IL-2 survival signal (Ahmed et al., 1997; Dudek et al., 1997; Jones et al., 2000; Kane et al., 2001; Kelly et al., 2002; Van Parijs et al., 1999; Yao and Cooper, 1995). First, AKT is frequently expressed in T-cells and can be activated in response to IL-2 stimulation (Ahmed et al., 1997; Kane et al., 2001; Kelly et al., 2002; Van Parijs et al., 1999). Second, AKT has been shown to provide an antiapoptotic signal to T-cells (Jones et al., 2000; Kelly et al., 2002). Third, T-cell survival signals are attenuated once IL-2 receptor is mutated so as to be unable to activate AKT (Ahmed et al., 1997; Van Parijs et al., 1999). Most importantly, Van Parijs and coworkers showed that IL-2 can promote CD4 T-cell survival by activating AKT (Kelly et al., 2002). Considering that memory cells are long-lived cells after activation, we decided to test whether the AKT pathway might have some role in CD4 T-cell memory.

As described in previous section, an IL-2 signal was required for production of CD4 memory T-cells; memory failure due to IL-2 dependency could be rescued by addition of exogenous rIL-2 during the primary response. We want to examine whether such a failure could be rescued by the activation of the AKT pathway during the primary response. In previous section, we have shown that lentivector FUIGW-AKT* encoding a constitutively active form of AKT can infect naïve CD4 T cells, and activate AKT pathway to promote T-cell survival. Here we examined these results more rigorously by analyzing the intracellular expression level of pAKT and surface expression of activation markers (Figure 6-5, A and B). Transient expression of AKT* significantly elevated the intracellular pAKT, as shown in Figure 6-5A. FACS analysis showed that the majority of OT2/IL-2⁻ cells were GFP positive. Among these GFP⁺ cells, 20% of them were GFP^{high} and 80% of them were GFP^{low} (Figure 6-5B, left). The difference of GFP expression level could be due to the different viral integration numbers and sites, representing the different cellular levels of pAKT. The activation markers (CD25, CD69, CD62L and CD44) were presented as GFP vs. marker dotplots; GFP positive cells (equivalent of OT2/IL-2⁻ cells) showed the activation of T cell characteristics of CD25^{high}, CD69^{high}, CD62L^{low} and CD44^{high} (Figure 6-5B).

OT2/IL-2⁻ effectors expressing AKT* were adoptively transferred into IL-2⁻/RAG⁻ host mice (the transfer protocol was the same as that described previously). Four weeks later, spleen and lymph node (LN) cells from the adoptive hosts were analyzed. Unexpectedly, no detectable OT2/IL-2⁻ memory T-cells were observed, as evidenced by the absence of GFP positive cells (Figure

6-5C) and confirmed by OT2 TCR staining (data not shown). This investigation led us to conclude that activation of AKT pathway during a primary response cannot rescue CD4 T-cell memory failure caused by IL-2 deficiency.

Activation of the STAT5 Signaling Pathway During Primary Response Is Able to Rescue CD4 T-cell Memory Generation Caused by IL-2 Deficiency

In response to IL-2, Janus kinases (JAK1 and JAK3) are activated for phosphorylation of those tyrosine residues on the IL-2 receptor β and γ chains that serve as binding sites for STAT5. STAT5 is then phosphorylated by JAKs. The phosphorylated STAT5, dissociates from the IL-2 receptor, dimerizes and translocates into the nucleus to regulate the transcription of target genes, including IL-2R α , β -casein, CIS, etc (Lin and Leonard, 2000; Matsumoto et al., 1997; Matsumura et al., 1997; Matsumura et al., 1999; Mui et al., 1996; Ward et al., 2000). In a recent experiment we showed that STAT5 activation was required to generate the normal numbers of T regulatory cells and prevent the development of autoimmunity (Antov et al., 2003). We hypothesized that STAT5 might also be involved in development of CD4 T-cell memory.

In previous section, we described the successful approach of using a lentiviral vector, FUIGW-STAT5*, which expresses a constitutively active form of STAT5 to activate STAT5 pathway, and observed enhanced proliferation of infected CD4 T-cells upon stimulation by OVAp and APCs (Figure 6-4C). In addition, we performed FACS analysis of these activated OT2/IL-2⁻ T-cells to determine whether the infected cells exhibited the characteristics of CD4 effector

T-cells. High transfection efficiency was achieved, as indicated by nearly 100% correlation between GFP⁺ and OT2⁺ cells (Figure 6-6A). We also observed two populations of GFP positive cells: GFP^{high} (26%) and GFP^{low} (74%). Without an IL-2 signal during primary stimulation, GFP⁺ cells (OT2/IL-2⁻ cells constitutively expressing the active form of STAT5) were able to become typical effector T-cells, as shown by the surface activation markers (CD25^{high}, CD69^{high}, CD62L^{low} and CD44^{high}). In previous experiments, we found that the IL-2 signal is not required to activate naïve CD4 T-cells (Figure 6-1B). We also found that the constitutive activation of STAT5 can promote CD4 T-cell proliferation (Figure 6-4C). Here we observed the normal pattern of surface activation markers on the effector OT2/IL-2⁻ T-cells expressing STAT5*. In addition, GFP could be used to conveniently monitor OT2/IL-2⁻ T-cells expressing STAT5*.

We investigated the potential role of the STAT5 pathway in the generation and persistence of memory CD4 T-cells. A large population of OVAp-specific OT2/IL-2⁻ effector T-cells expressing STAT5* was generated by lentiviral infection of naïve OT2/IL-2⁻ CD4 T-cells and subsequent stimulation with OVAp and APCs (no addition of rIL-2). Using the same protocol as described previously, these cells were then transferred into IL-2⁻/RAG⁻ mice, and the population of OT2/IL-2⁻ CD4 memory T-cells was counted 4 weeks later. Interestingly, a significant population of T-cells (approximate 1.7×10^6 from both spleen and lymph nodes per mouse) was GFP⁺ (Figure 6-6B). When we gated on the GFP⁺ cells, all of them were apparently CD4 positive and OT2 TCR positive (Figure 6-6B). Examination of the surface activation markers on these

GFP⁺ cells indicated that they had memory characteristics: CD69^{low}, CD62L^{low/high} and CD44^{high} (Figure 6-6C). It is known that memory T-cells show a low expression of CD25. The high expression of CD25 in our memory T-cells was due to the constitutive expression of STAT5*, which turned on its target downstream CD25 gene.

In support of our FACS results, the memory CD4 T-cells expressing STAT5* (indicated as GFP+) from adoptive hosts produced a higher level of cytokine (Figure 6-6D top panel) and proliferated intensively (data not shown) upon *in vitro* antigenic stimulation with APCs and various concentrations of OVAp. They also showed a more sensitive response to the antigen, as indicated by their ability to mount a response at a low concentration of OVAp (Figure 6-6D, top panel). In the time course experiment, the memory cells responded with greater regularity and strength than control cells (Figure 6-6D, bottom panels).

Our data clearly shows that activation of the STAT5 pathway can rescue the failure of CD4 T-cells to develop into memory cells as caused by IL-2 deficiency, indicating that the STAT5 signaling pathway has a key role in CD4 memory generation. To our knowledge, this is the first time that CD4 T-cell memory has been linked to a specific signaling pathway.

4. Discussion

Starting with the naïve, IL-2 deficient cells and using the lentivector approach, we did a series of experiments to investigate the roles of IL-2 signaling in CD4 T-cell memory generation and maintenance. We first found that when

transferred into a RAG⁻, IL-2⁻ mouse, strongly activated T-cells that lacked IL-2 generated no memory. We then examined conditions that might allow for memory generation. The results are summarized in Figure 6-7. We found that 1) An IL-2 signal can provide the conditions for generating CD4 T-cell memory, but IL-2 is not required for memory maintenance; 2) Activation of the AKT signaling pathway during a primary response cannot rescue the failure of CD4 T-cell memory generation caused by IL-2 deficiency; 3) Activation of the STAT5 signaling pathway during a primary response is able to rescue CD4 T-cell memory generation.

These studies have led us to recognize the importance of the STAT5 pathway in the development of CD4 T-cell memory. We therefore propose that memory is controlled by multiple paths to STAT 5 activation leading to the "Paths to STAT5" memory model as one possible mechanism determining CD4 T-cell memory generation and maintenance (Figure 6-8). According to this model, a CD4 T-cell immune response starts with interaction between CD4 T-cell receptors (TCRs) and antigen-loaded MHC II molecules presented by professional APCs (Janeway et al., 2001). This will activate the CD4 T-cell to synthesize IL-2. The IL-2 will bind to the low affinity receptors that pre-exist on the CD4 cell surface; they are composed of IL-2 receptor (IL-2R) α and common γ chains. Binding of IL-2 should cause heterodimerization of the receptor complexes (Lin and Leonard, 2000; Nakamura et al., 1994; Nelson et al., 1994), recruiting and activating the JAK kinases (JAK1 and JAK3) (Ascherman et al., 1997; Johnston et al., 1995; Johnston et al., 1994; Lin and Leonard, 2000;

Witthuhn et al., 1994; Zhu et al., 1998). The JAKs will then phosphorylate tyrosine residues on the IL-2R β and γ chains as well as themselves to trigger the activation of STAT5 (Lin and Leonard, 2000). Active STAT5 will stimulate transcription of the IL-2R α gene, and the new IL-2R α will come to the cell surface and combine with the pre-existing chains, converting the low affinity receptor into a high affinity IL-2R, thus making the cell more responsive to IL-2 and enhancing STAT5 signaling. This constitutes a self-re-enforcing STAT5 activation loop, contributing to the generation of effector cells and the precursors of memory CD4 T-cells.

The hallmark of memory is that it is maintained long after the generation of antigen has ceased and all antigens have been mopped up. The withdrawal of antigen should stop TCR signaling and therefore end the synthesis of IL-2. To keep STAT5 active, another interaction must occur.

We suggest an alternative interaction between CD4 T-cells and APCs to sustain STAT5 activation and thus maintain memory. We suggest that the committed CD4 memory precursors with high STAT5 activation turn on the expression of one or more genes that we simply denote as X. X is a surface-bound ligand for a receptor on APCs that activates a pathway to synthesis of IL-15 or/and IL-7. The IL-15 or/and IL-7 then binds to its receptor on the CD4 T-cells, maintaining the activation of STAT5. Protein X can be a membrane-bound molecule because then binding of X to its counterpart receptor on the APCs would bring CD4 T-cells in close contact with APCs. This configuration permits specific delivery of IL-15 or/and IL-7 to the committed memory CD4 T-cells and

achieves specificity of memory maintenance. This constitutes a totally self-sustaining STAT5 activation loop, which could regulate memory maintenance. The central role of STAT5 in this memory model is manifest in two key aspects: 1) during memory generation, the STAT5 signal is important for the commitment to the memory cell fate; 2) the ability to sustain STAT5 activation determines the success of memory maintenance.

Most of the individual signaling events occurring in the loop for memory generation have been well documented. Our results enable us to have individual pieces assembled and constitute a signaling pathway to regulate the generation of CD4 T-cell memory. Although the loop of memory maintenance in our model is largely unknown, much of evidence from the literature and our own experiments suggest that the STAT5 pathway may play a role in maintenance of CD4 T-cell memory. In the experiment that we generated memory IL-2 deficient T-cells, we found that not only did introduction of STAT5* into these T-cells restore the memory T-cell pool but also the pool was maintained at a larger size (~10-fold difference). Farrar and coworkers made transgenic mice constitutively expressing an active form of STAT5 and also found that these mice maintain an increased T-cell memory pool (Burchill et al., 2003).

It was shown that the turnover and survival of memory CD4 T-cells does not require the expression of MHC II molecules (Swain et al., 1999). This suggests that memory maintenance does not require antigen presentation and suggests that cytokines are probably the determinants of CD4 T-cell memory maintenance. In previous studies, IL-15 and IL-7 have been shown to play key

roles in CD8 memory T-cell proliferation and survival (Becker et al., 2002; Goldrath et al., 2002; Kanai et al., 1996; Ku et al., 2000; Schluns and Lefrancois, 2003; Schluns et al., 2002). In contrast to CD8 T-cells, the role of cytokines in maintaining CD4 T-cell memory remains controversial (Schluns and Lefrancois, 2003; Tan et al., 2002; Wu et al., 2002). However, recent data do indicate that IL-15 and IL-7 might be involved in regulating proliferation and survival of memory CD4 T-cells (Geginat et al., 2003; Kanegane and Tosato, 1996; Kondrack et al., 2003a; Kondrack et al., 2003b). Receptors for these two cytokines belong to the common γ chain receptor family, which also includes IL-2R, and share the STAT5 signaling pathway (Janeway et al., 2001).

In our “Paths to STAT5” model, cross talk between CD4 T-cells and the nearby APCs is a crucial aspect. In absence of autocrine IL-2, we think that memory can be maintained by cross talk between the activated CD4 T-cells that are memory precursors and APCs. This cross talk is mediated by a protein or proteins yet to be determined (designated as X in the model). We suggest that CD40L/CD40 (Borrow et al., 1996; Diehl et al., 1999; Grewal et al., 1996; Grewal et al., 1995; Schoenberger et al., 1998; Soong et al., 1996; Vanessen et al., 1995) and RANKL/RANK (receptor activator of NF- κ B ligand, also called TRANCE/ODF/OPGL) (Bachmann et al., 1999; Green and Flavell, 1999; Josien et al., 1999; Kim et al., 2000; Lum et al., 1999; Wong et al., 1999; Wong et al., 1997a; Wong et al., 1998; Wong et al., 1997b) are good candidate systems to carry out this task. They are members of the TNF superfamily, frequently expressed on immune cells, and have critical roles in mediating immune

responses (Mackay and Kalled, 2002). CD40L is predominately expressed on activated CD4 T-cells. Its receptor, CD40, is constitutively expressed in immature DCs. CD40 ligation induces maturation of DCs (Caux et al., 1994; Mackey et al., 1998), and production of many cytokines (e.g., IL-1, IL-6, TNF- α , IL-15, etc.) (Cella et al., 1996; Koch et al., 1996; Kuniyoshi et al., 1999), as well as increasing DC survival (Cella et al., 1996). IL-15 was found to upregulate CD40L expression in CD4 T-cells, probably through activating the STAT5 pathway (BulfonePaus et al., 1997).

RANKL was discovered as a TNF family member (Anderson et al., 1997; Wong et al., 1997b). RANKL on T-cells, and its receptor RANK on DCs, shares sequence, structural, and functional homology with their CD40L and CD40 counterparts (Anderson et al., 1997; Wong et al., 1997b). Choi and coworkers recently have shown that RANKL is highly expressed on memory CD4 T-cells (Figure 10) (Josien et al., 1999), attracting our attention to RANKL as candidate X. In their experiments to examine the role of RANKL in the regulation of T-cell responses, CD40L was found to be ubiquitously expressed in all CD4 T-cell subsets. In contrast, RANKL was only expressed on effector and memory CD4 T-cells, suggesting RANKL may play a specific role in CD4 T-cell memory. In DCs, it was shown that RANKL induces the expression of cytokines (e.g., IL-15) and promotes DC survival (Josien et al., 1999). It was also shown that RANK shares the same TRAF2 signaling pathway as CD40 (Anderson et al., 1997; Wong et al., 1997b). Horseman and coworkers demonstrated that RANKL expression in mammary epithelial cells is induced by STAT5 (Anderson et al.,

1997). More interestingly, Tindle and coworkers over-expressed RANK in DCs and the resulting DCs exhibited an enhanced ability to generate memory T-cell responses (Wiethe et al., 2003).

Although there is accumulating evidence to support this memory model, significant experiments are required to further test and establish it. This memory model is significant both for the fundamental study of T-cell memory and the practical design of vaccines. This model indicates a central role for the STAT5 signaling pathway, which would be the first specific pathway shown to regulate the generation and maintenance of CD4 memory T-cells. The model suggests that activation of STAT5 is going to present a molecular signature, which will allow identification, quantitation and isolation of memory precursors and the performance of molecular, genetic and biochemical analysis. This model can also provide a molecular picture of how CD4 T-cells help generation of CD8 memory T-cells. The model could guide production of new vaccine adjuvants from the information we will generate. In particular, if IL-2, IL-7 and/or IL-15 are critical, it may be important to include them, or molecules that can generate them, in a vaccine.

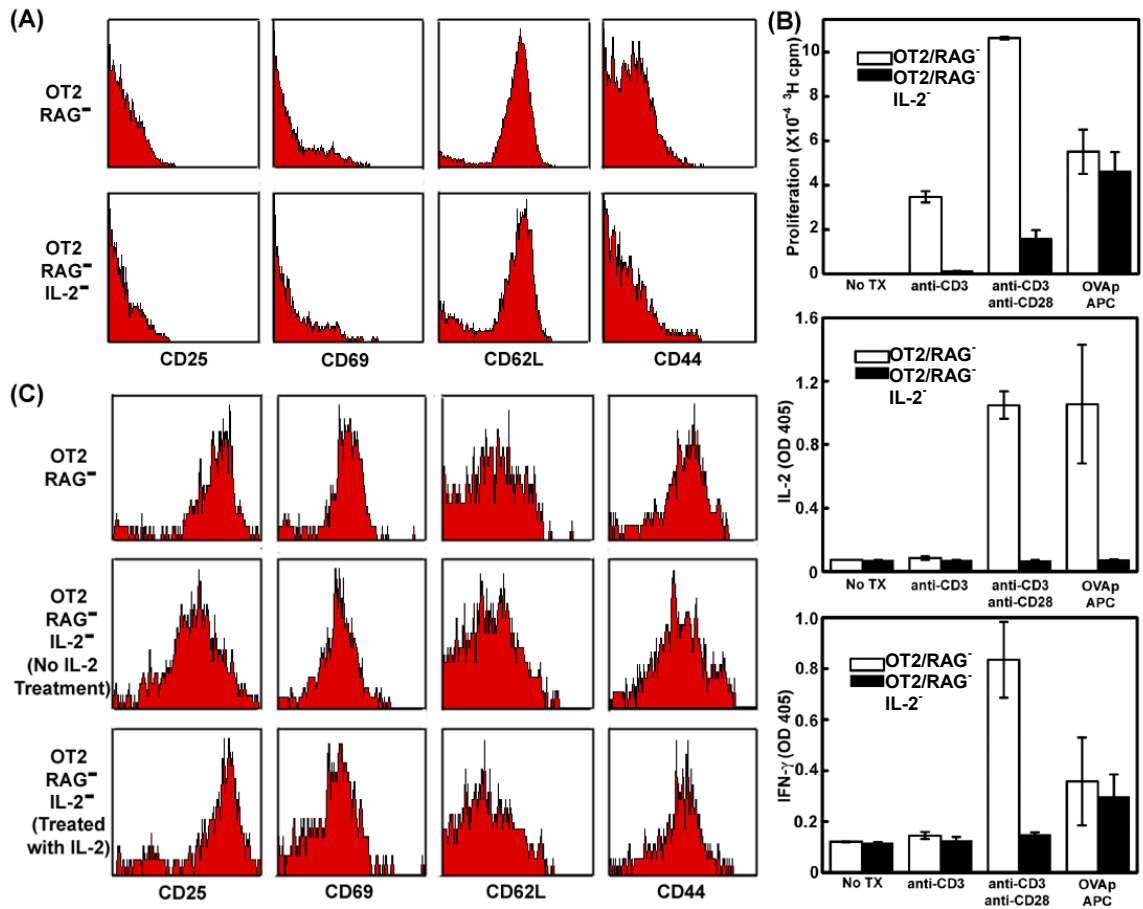


Figure 6-1

Figure 6-1: Generation and activation of OT2 CD4 T cells with IL-2 deficiency.

(A): Results from investigation of surface marker (CD25, CD69, CD62L and CD44) staining of the unchallenged OT2/RAG⁻ and OT2/RAG⁻/IL-2⁻ animals. (B): Functional responses of IL-2 deficient OT2 T cells upon different stimuli. Spleen cells obtained from either OT2/RAG⁻ or OT2/RAG⁻/IL-2⁻ mice were supplemented with anti-CD3, anti-CD3 and anti-CD28, or antigenic peptide (OVA_p) loaded APCs. After 72 hours, proliferation was assayed by 3H-thymidine incorporation and production of IL-2 and IFN- γ was assayed by ELISA. Data was normalized for the number of OT2 CD4 T cells presented in cell cultures. No treatment (No TX) was included as a control. (C): Cell surface marker staining (CD25, CD69, CD62L and CD44) of appropriately activated OT2 CD4 T cells. The top row was the results from OT2/RAG⁻ spleen cells stimulated by OVA_p-loaded APC. The middle and bottom row were results from OT2/RAG⁻/IL-2⁻ spleen cells stimulated by OVA_p-loaded APCs in the absence (no IL-2 treatment) or in the presence (treated with IL-2) of exogenous IL-2.

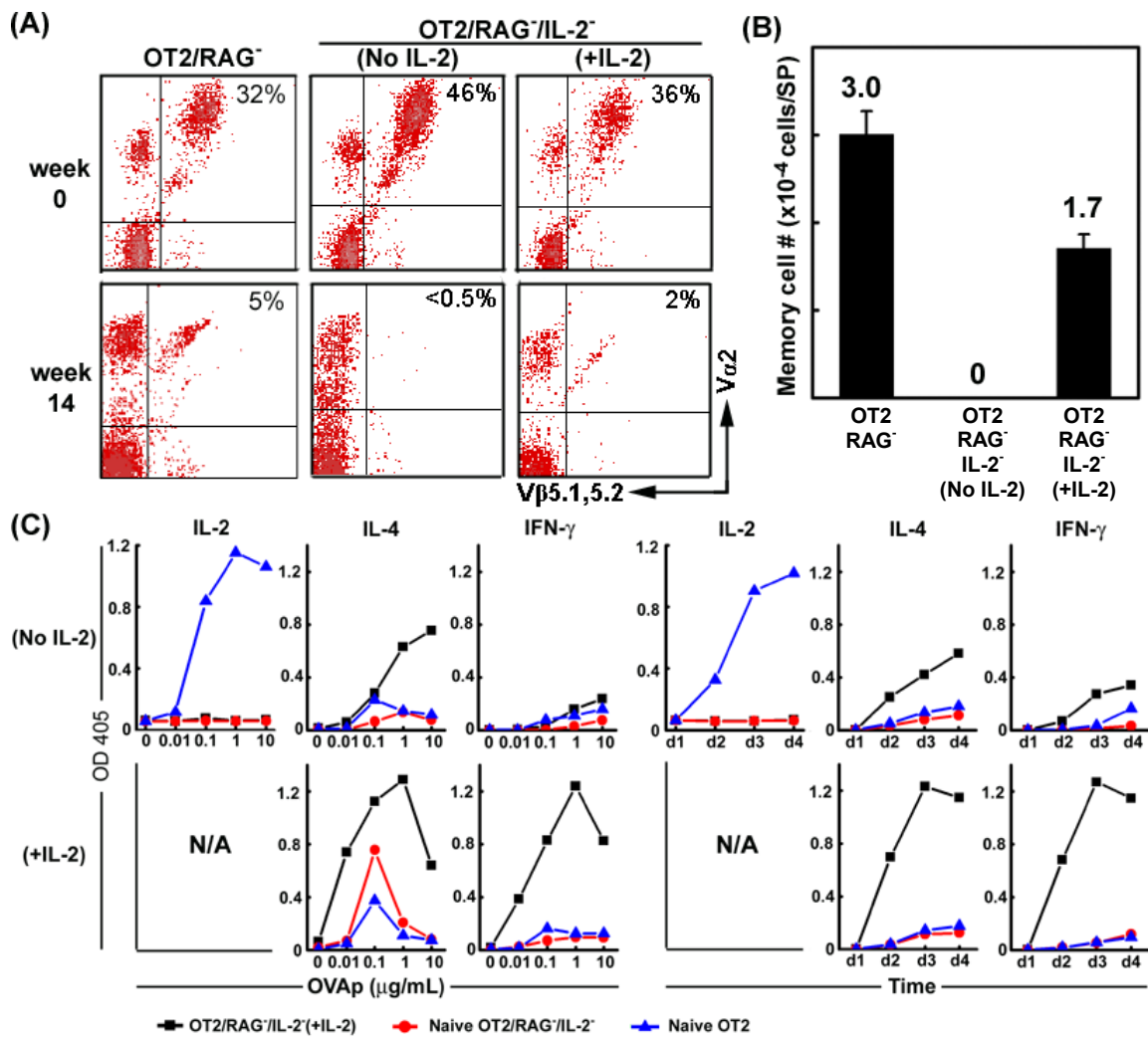


Figure 6-2

Figure 6-2: Analysis of IL-2 requirement on CD4 T cell memory generation. (A): Identification of OT2 CD4 memory T cells by FAC staining. OT2/RAG⁻/IL-2⁻ T cells were activated by OVAp-loaded APCs in absence (no IL-2) or presence (+IL-2) of exogenous IL-2. Cells from day 3 cultures were adoptively transferred into RAG⁻/IL-2⁻ recipients. The presence of memory cells was analyzed after 14 weeks. OT2 memory cells were identified by V α 2⁺ and V β 5.1,5.2⁺. Control experiment was performed for OT2/RAG⁻ T cells. (B): The recovered cell numbers of OT2 memory T cells for the different conditions as described in (A). (C): Dosage and time course responses of generated memory OT2 T cells. The recovered memory cells (OT2/RAG⁻/IL-2⁻, ■) were assayed for their ability to produce effector cytokines (IL-2, IL-4 and IFN-g) upon exposure to different concentrations of OVAp antigen in absence (no IL-2) or presence (+IL-2) of exogenous IL-2. At concentration of 1 μ g/mL, time course responses were monitored from day 1 to day4. Naïve OT2/RAG⁻/IL-2⁻ (●) and naïve OT2 (▲) T cells were included as control for all the experiments.

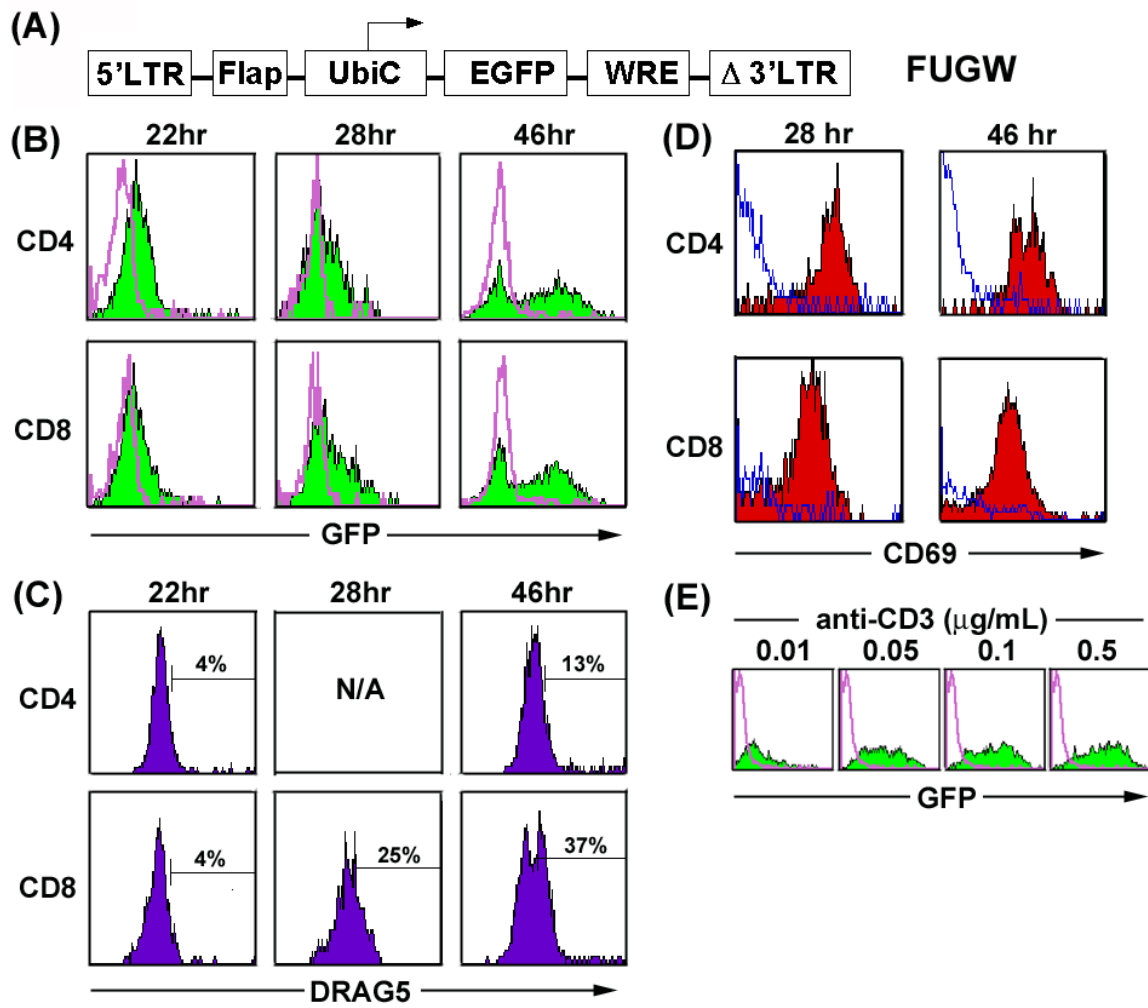


Figure 6-3

Figure 6-3: Evaluation of the potential of using FUGW lentiviral vector to genetically modify naïve T cells. (A) Schematic diagram of the GFP expressing lentiviral vector FUGW. Only the relevant portions of vector were shown. LTR, long terminal repeat; Flap, the central DNA flap; UbiC, the human ubiquitin-C internal promoter; EGFP, enhanced green fluorescent protein; WRE, the woodchuck hepatitis virus posttranscriptional regulatory element. (B): Time course of lentivirus-mediated GFP expression was monitored at times of 22 hr, 28 hr and 46 hr after infection. Unfilled curve, no infection; filled curve, infected with FUGW). (C): Time course of CD4 and CD8 T cell cycling after lentiviral infection was monitored at times of 22 hr, 28 hr and 46 hr. DRAG5 was used to stain DNA of corresponding cells. (D): Changes of surface marker expression (CD69) from times of 28 hr to 46 hr after FUGW infection and treatment of anti-CD3. Unfilled curve, no treatment of anti-CD3; filled curve, treated with anti-CD3). (E): The level of GFP expression in response to different concentrations of anti-CD3 treatment.

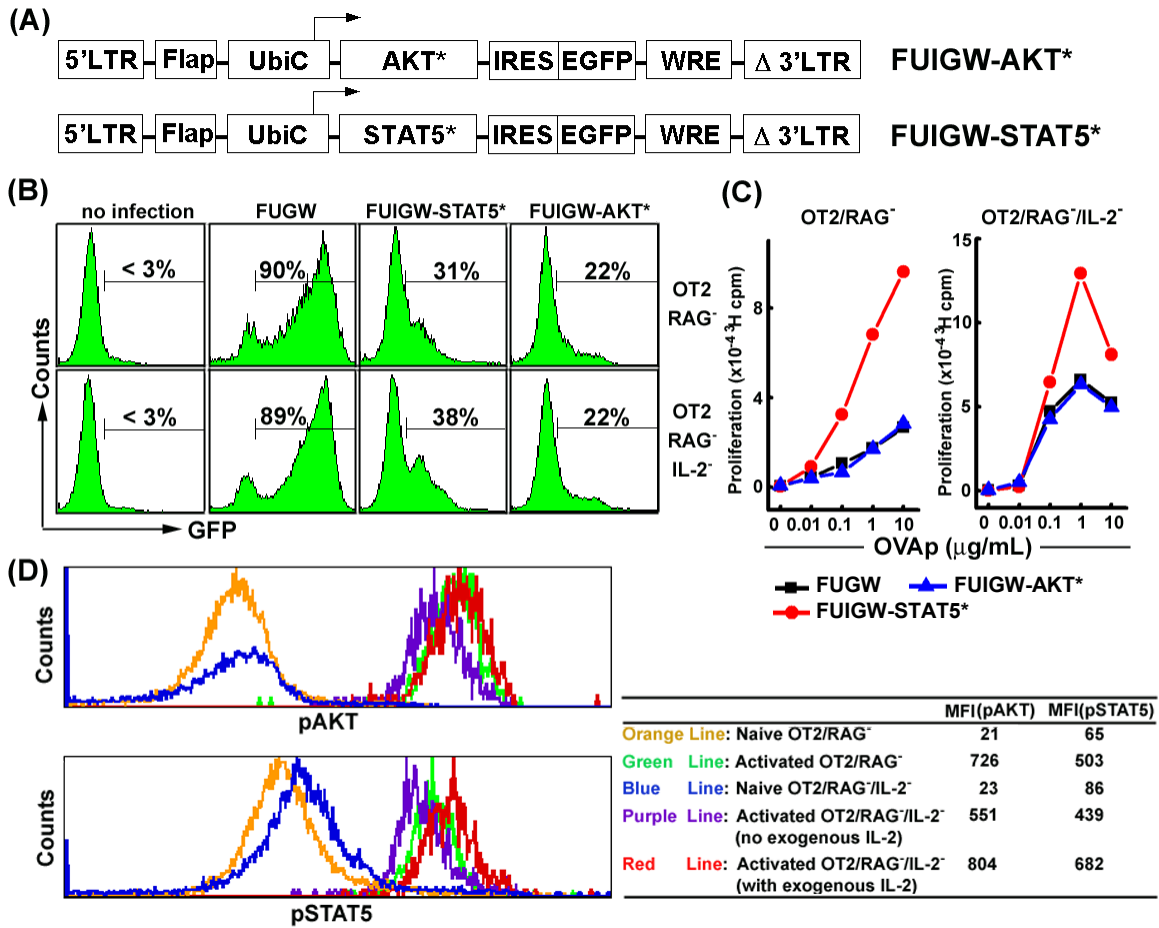


Figure 6-4

Figure 6-4: Potential of lentivirus-mediated gene transfer for study of IL-2 signaling. (A) Schematic diagram of lentivirus vector FUIGW co-expressing STAT5* and GFP (designated as FUIGW-STAT5*), or AKT* and GFP (designated as FUIGW-AKT*). Only the relevant portions of vector were shown. LTR, long terminal repeat; Flap, the central DNA flap; UbiC, the human ubiquitin-C internal promoter; EGFP, enhanced green fluorescent protein; WRE, the woodchuck hepatitis virus posttranscriptional regulatory element. (B): The efficiency of using lentiviral vectors FUIGW-STAT5* and FUIGW-AKT* to infect naïve OT2/RAG⁻ and OT2/RAG⁻/IL-2 T cells. The efficiency was estimated by the percentage of GFP positive cells. (C): Functional consequences after transient expression of STAT5* or AKT*. ³H-thymidine incorporation was used to measure the changes of proliferation capacity. Infection by FUGW was included as a control. Data was normalized for the number of GFP positive OT2 CD4 T cells presented in cell cultures. (D): Intracellular expression of phosphorylated STAT5 (pSTAT5) and AKT (pAKT) in naïve or active OT2/RAG⁻ and OT2/RAG⁻/IL-2- cells. For activated cells, treatment and no treatment of exogenous IL-2 were compared. The mean fluorescent intensity (MFI) was indicated.

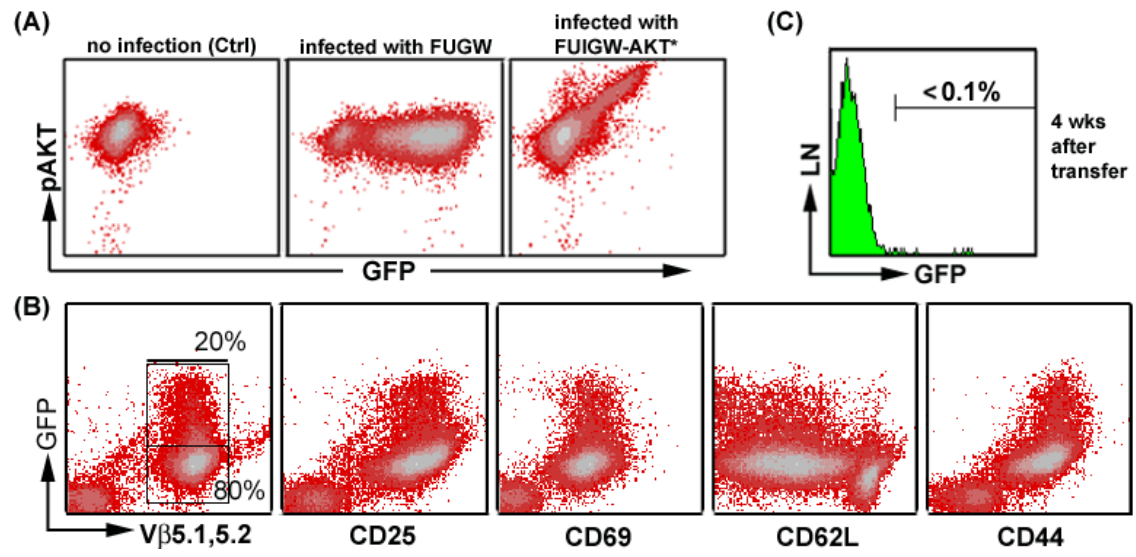


Figure 6-5

Figure 6-5: Evaluation of possibility of activation of AKT signaling pathway to rescue CD4 memory failure caused by IL-2 deficiency. (A): Intracellular expression level of phosphorylated AKT after infection with FUIGW-AKT*. No infection or infected with FUGW were included as controls. (B): Surface activation markers of effector OT2 transduced by lentivector FUIGW-AKT* and stimulated by OVAp and APCs for 3 days. (C): Identification of presence of memory OT2 cells after 4 weeks. The FUIGW-AKT*-infected effector OT2/RAG⁻/IL-2⁻ cells were adoptively transferred into RAG-/IL-2- recipients. Four weeks later, spleens and lymphonodes were analysed for the presence of GFP positive cells.

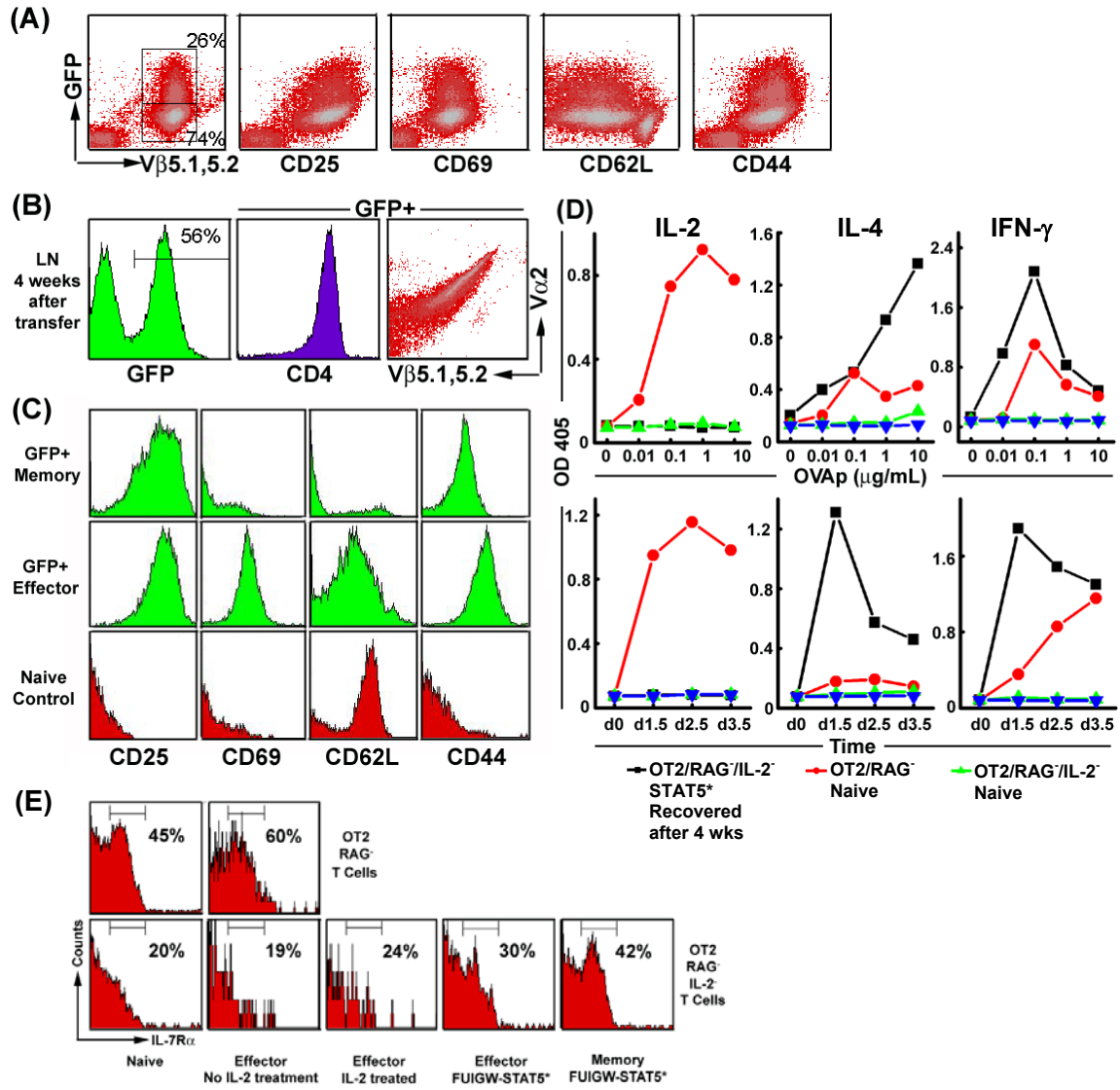


Figure 6-6

Figure 6-6: Evaluation of possibility of activation of STAT5 signaling pathway to rescue CD4 memory failure caused by IL-2 deficiency. (A): Surface activation markers of effector OT2 transduced by lentivector FUIGW-STAT5* and stimulated by OVAp and APCs for 3 days. (B): Identification of presence of memory OT2 cells. The FUIGW-STAT5*-infected effector OT2/RAG⁻/IL-2⁻ cells were adoptively transferred into RAG⁻/IL-2⁻ recipients. Four weeks later, spleens and lymphonodes were analysed by GFP gating, CD4 staining and OT2 staining. (C): The surface activation markers of these memory CD4 T cells, as compared to the corresponding naïve cells and effector cells. (D): Dosage and time course responses of memory OT2/RAG⁻/IL-2⁻ (■) rescued by activation of STAT5. Cells were isolated from spleens and stimulated with OVAp and APCs. The production of IL-2, IL-4 and IFN-g was measured by ELISA. As controls, similar assays were applied to naïve OT2/RAG⁻ (●) and naïve OT2/RAG⁻/IL-2⁻ (▲) T cells.

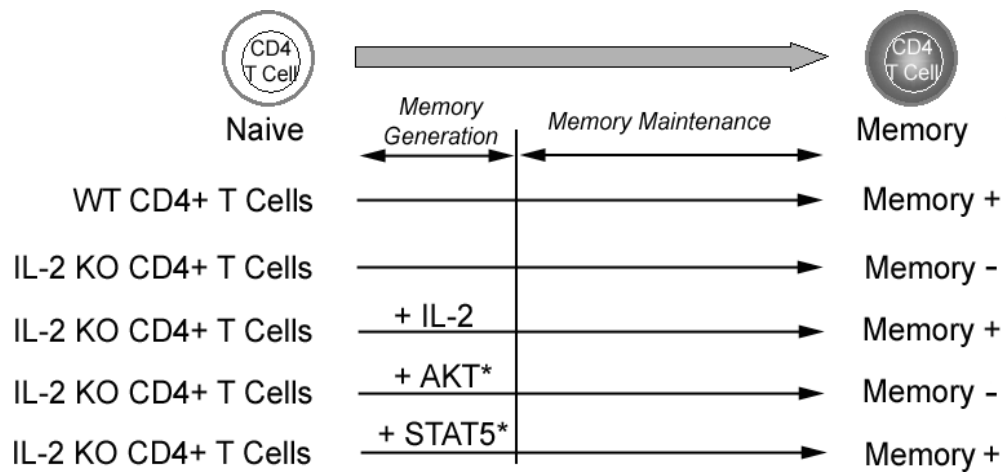


Figure 6-7: Summarized results from study of the role of IL-2 in CD4 T cell memory.

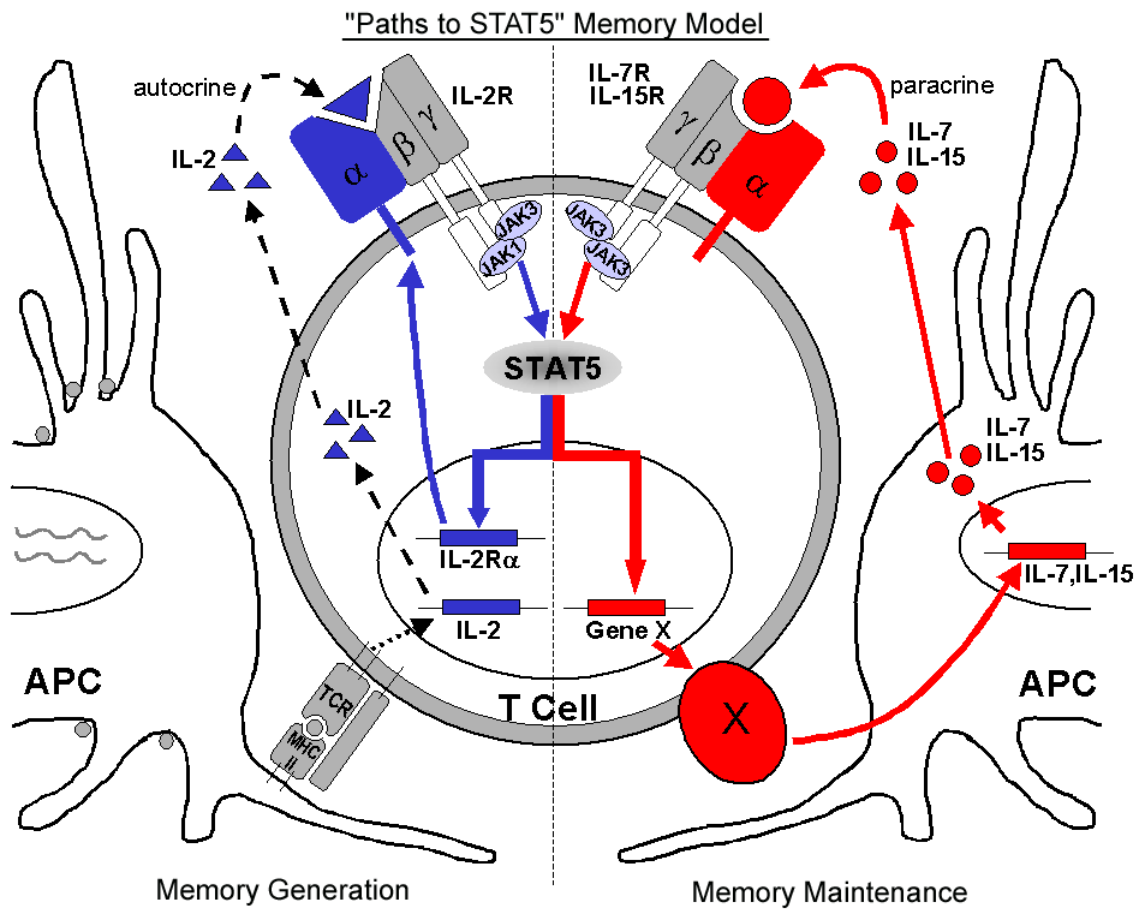


Figure 6-8: Schematic representation of the proposed "Paths to STAT5" memory model.

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