Title
The impact of CD4 on selection of the T cell receptor repertoire

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Publication Date
2000

Peer reviewed|Thesis/dissertation

THE IMPACT OF CD4 ON SELECTION OF THE T CELL RECEPTOR REPERTOIRE

by

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DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

IMMUNOLOGY

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA SAN FRANCISCO

Date University Librarian

Degree Conferred: .....

ACKNOWLEDGEMENTS

First and foremost, I would like to thank my thesis advisor Dr. Nigel Killeen for his support and guidance in the past five years. He has been a mentor in the truest sense, not only in science, but also in career and in life. This work would not have been accomplished without his dedication to science and teaching, his optimism and encouragement.

I owe as much of my degree to Nigel as to the members of the Killeen lab, past and present. Susanne, Linda, Bryan, Kurt, Julie, Joanne, Sean, Jay, Arin, Claudia, Fernando, Leo, John, Lingling, and Dongji, thank you all for your help and support. I cannot think of a better, nicer or livelier group of people to work with.

I would like to thank my thesis committee members Dr. Arthur Weiss and Dr. Jason Cyster for valuable advice and critical reading of this thesis. I also would like to thank Dr. Richard Locksley and our collaborators Dr. Nicolas Gleichenhaus and Dr. to be Laurent Malherbe in France for providing extremely valuable reagent and helpful discussion.

My dear friends, Yanan, Hailing, Kuanhong, Walter, Jade, Stephanie, Jen, Zheng, and Wayne, thank you for making my past six years in San Francisco a blast.

And thank you, Rickmer, for not being here distracting me while I wrote my thesis :)

Last but definitely not the least, I would like to thank my parents for showing me this wonderful world of biology and for letting me be who I am and live my life the way I choose to.

Thank you all.

THE IMPACT OF CD4 ON SELECTION OF THE T CELL RECEPTOR REPERTOIRE

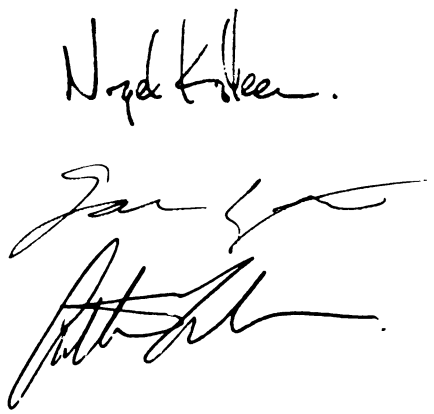
ABSTRACT

CD4 is a 55 Kd glycoprotein expressed on the majority of thymocytes and the helper lineage of αβ T cells. Its primary function is to bind directly to Major Histocompatibility Complex (MHC) Class II molecules and to assist in antigen-specific signaling from T cell receptors (TCRs). Without CD4, such signaling is impaired, helper lineage T cells fail to develop in normal numbers and immune responses are therefore compromised.

The work described in this thesis was directed toward obtaining a better understanding of how CD4 improves antigen recognition and potentiates the development and responses of T helper cells. We have focused primarily on determining the properties that T cell receptors must exhibit in order for them to function effectively without the contribution of CD4. In part, we sought to determine whether these properties would be a useful indirect measure of the normal function that CD4 performs.

T cell receptors expressed by CD4-deficient T helper cells were examined at the population level and also in isolation. We compared their sequences, their capacity to confer antigen responsiveness on T helper cells and various aspects of their ligand binding properties. We also generated transgenic mice expressing these TCRs and examined their capacity to promote T helper cell development in the presence or absence of CD4. The results of these studies clarify a modest, but still demonstrably essential effect of CD4 on TCR function during repertoire selection.

In a separate line of inquiry, we attempted to develop improved systems for identifying and studying memory T cells. We employed a genetic approach to label these cells using the Cre recombinase. Memory cells are defined by their prior involvement in immune responses - we therefore expressed the Cre recombinase selectively during T cell activation by making mice that had the *cre* gene inserted directly into the *Ox40* locus. *LoxP*-containing reporters that inform on prior Cre activity were engineered and expressed in transgenic mice, so that memory cells in *Ox40-cre* mice could be irreversibly labeled. Although one version of the system failed to perform successfully, the feasibility of the system was eventually demonstrated and it can now be used to study the properties of memory T cells.



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INTRODUCTION

Although many T cells can develop extrathymically, the thymus is the major source of the conventional T lymphocytes found in secondary lymphoid tissues (1). Two major lineages of cells comprise the T cell population and these are distinguished by the type of T cell receptor (TCR) they express: αβ T cells that express an αβ TCR and γδ T cells that express a γδ TCR. In juvenile and adult mice and humans, αβ T cells account for more than 95% of the cells in thymus. αβ T cells themselves fall into two lineages that can be readily distinguished from one another by their differential expression of the CD4 and CD8 coreceptor molecules. The majority of $CD4^+$ T cells recognize antigenic peptides presented by MHC Class II molecules (2-5). Upon activation, $CD4^+$ T helper cells secrete cytokines (6-8) and express cell surface molecules such as CD40 ligand (9) that are important for the initiation and amplification of immune responses. $CD8^+$ T cells recognize antigenic peptides presented by MHC Class I molecules (10, 11). These cells have cytotoxic activity that is important for the control and elimination of tumors and virally infected cells (12).

Thymocyte Development

The maturation of T cells in the thymus is a tightly regulated multi-step process. One of the first major steps begins when $CD4^-CD8^-$ double negative (DN) precursors rearrange their TCRβ loci allowing them to express a cell surface TCRβ chain as part of a pre-TCR complex. Signals from the pre-TCR cause the DN cells to proliferate and differentiate into $CD4^+CD8^+$ double positive (DP) cells. These cells then undergo TCRα locus rearrangement allowing for expression of an αβ TCR complex. Ligand-dependent

signals from this complex then determine whether the cell will continue to live or will instead die by apoptosis in processes termed positive and negative selection.

Positive selection rescues from cell death thymocytes bearing receptors that can signal productively in response to binding of thymic peptide/MHC ligands (13-15). Negative selection eliminates potentially autoreactive thymocytes (16, 17). Only about 5% of the DP thymocytes survive the selections to become mature $CD4^+$ or $CD8^+$ single-positive thymocytes (reviewed in (18)). The resulting TCR repertoire that emerges from the thymus is self-tolerant yet retains the ability to recognize foreign antigens in the context of self-MHC.

There are two theories concerning what determines the outcome of positive and negative selection. The avidity hypothesis states that the fate of immature thymocytes during positive and negative selection is determined by the avidity of interaction between the TCRs expressed by developing thymocytes and the peptide/MHC ligands on thymic stromal cells. Thymocytes with low avidity interactions fail positive selection and die by neglect, while thymocytes with high avidity interactions are subject to negative selection and die by programmed cell death. Only thymocytes with intermediate avidity interactions survive and develop into mature single positive cells. The avidity between a TCR and its peptide/MHC ligand is determined by both the affinity of the TCR:peptide/MHC interaction and the number of such interactions (19-22).

In contrast, the differential signaling hypothesis states that signals for positive and negative selection differ in nature (reviewed in (13),(23)). Supporting evidence for this hypothesis came from studies using altered peptide ligands. Altered peptide ligands

are variant, sometimes antagonistic, forms of agonistic peptides. Several studies showed that antagonistic peptides can induce positive selection (24-26), while agonistic peptides caused negative selection (26). These observations suggest that thymocytes may be positively selected by a peptide/MHC complex that delivers only a partial signal, while they may be negatively selected by a ligand that delivers an activating signal.

Recent studies demonstrated that positively and negatively selecting ligands differ in the kinetics of binding to TCRs (27, 28). Thus, peptide/MHC ligands that bind TCRs with a relatively slow dissociation rate lead to the negative selection of the thymocytes, while selecting ligands that bind TCRs with faster dissociation kinetics lead to positive selection.

The CD4 Coreceptor

CD4 is a 55kD type I transmembrane glycoprotein expressed on the helper subset of αβ T cells and immature double-positive thmocytes. In humans, CD4 is also expressed on macrophages and a subset of dendritic cells. The extracellular domain of CD4 is composed of four immunoglobulin-like domains (29) while its cytoplasmic domain is approximately 40 amino acids in length. The CD4 coreceptor enhances antigen responsiveness in $CD4^+$ T cells through its capacity to coordinate two distinct physical interactions. One is a very weak extracellular engagement between the amino-terminal domain of CD4 and the α2/β2 domains of MHC class II molecules (30-32). The other is a stronger, zinc-dependent interaction between cysteine-containing motifs in the cytoplasmic tail of CD4 and the amino-terminus of the *src*-related tyrosine kinase $p56^{lck}$ (33-35). Together, these two interactions are critical for the 'coreceptor' function

of CD4 – i.e., its capacity to potentiate MHC class II-restricted T helper cell responses. Thus, disturbing either interaction can block or significantly diminish both MHC class II-restricted T cell responses (36-38) and the development of the T helper lineage in the thymus (39-42).

Although the importance of its two binding interactions can be readily demonstrated, the details of how they allow CD4 to augment TCR signal transduction are incompletely understood. CD4 and the TCR should both be able to bind MHC class II molecules simultaneously because the binding surfaces they engage are not overlapping (43). Thus, CD4 could stabilize the binding between a TCR and its ligand and thereby prolong an otherwise short-lived association as has been demonstrated for CD8 (44). This prolongation might in turn be essential for the initiation of productive TCR signaling and the assembly of the immunological synapse (45). Interestingly, both CD4 (29, 46) and MHC Class II molecules (47) have some potential to form dimers as observed in crystal lattices, in solution (29) and on the cell surface (48). It is possible, therefore, that one of CD4's key contributions to TCR signaling is to promote the assembly of higher order structures in which TCR-engaged MHC molecules would initially be linked together by dimers of CD4 molecules (46). Support for this notion comes from the dominant-interfering function exhibited by a mutation at residue 43 of the extracellular domain of CD4 (49), and from the results of mutagenesis and inhibitor experiments (31, 50, 51). Thus, a plausible mechanism by which CD4 enhances T cell responses involves an initial role in stabilizing transient TCR:peptide/MHC interactions followed by an ensuing role in promoting the assembly of higher-order multimeric complexes at the interface between a T cell and its target (31, 43, 46, 49).

A complimentary mechanism by which CD4 can potentiate antigen recognition relies on its capacity to recruit the cytoplasmic protein tyrosine kinase $p56^{lck}$ into a nascent immunological synapse ((36, 52-54), reviewed in (55)). CD4 shares this *lck*-binding property with CD8, a molecule of remarkably divergent overall structure that nonetheless has an analogous capacity to augment the responsiveness of MHC Class I-restricted T cells through a direct binding interaction with MHC class I molecules (56, 57). Increasing the density of *lck* molecules at the site of TCR engagement is an attractive contributory explanation for the amplification function that both CD4 and CD8 perform during antigen recognition ((52), reviewed in (55, 58)).

CD4 in T Cell Development

Both CD4 and CD8 coreceptor molecules play a crucial role in thymic selection and the shaping of the TCR repertoire. Reduced coreceptor expression renders certain TCRs unable to meet the threshold for positive selection (59-61), while forced coreceptor overexpression leads to negative selection of some TCRs (62-64). For instance, in the absence of CD8, very few CD8 lineage cells are selected (65), and both positive and negative selection of MHC Class I-restricted transgenic TCRs in the absence of CD8 are impaired (59). On the other hand, overexpression of CD8 leads either to improved selection (66) or to deletion of transgenic TCRs in a CD8 dose-dependent manner (62, 63). Similar effects on selection have been observed for CD4. Loss of CD4 leads to a dramatic decrease in the number of CD4 lineage cells that are selected (67, 68). Positive selection of MHC II-restricted transgenic TCRs is impaired when CD4 expression is reduced (61) or absent (60). Overexpression of CD4 leads

either to enhanced selection or to deletion of MHC Class II-restricted transgenic TCRs (64, 69). These observations emphasize the role of coreceptor molecules in thymic selection.

Whereas the development of MHC Class I-restricted 'CD8 lineage' T cells is crucially dependent on CD8 expression (65), the CD4 lineage of MHC Class II-restricted T cells is clearly less dependent on CD4 expression (67, 68). Thus, in the absence of CD4, there is a substantial population of T helper cells that completes development in the thymus and emigrates to secondary lymphoid tissue where it can engage in immune responses. These CD4-deficient T cells account for protective immunity against protozoal and viral challenges and they also direct the elaboration of diverse T cell-dependent antibody responses (68, 70, 71). Interestingly, although these cells have the capacity to differentiate into Th1 effector cells, they are significantly compromised in Th2 differentiation in multiple experimental settings (72, 73).

Objectives

A main goal for this thesis was to determine the impact of CD4 on thymic selection of T helper cells. To do so, we focused on examining the properties of TCRs that are expressed by T helper cells that develop in the absence of CD4. We have attempted to determine whether CD4-independent TCRs can be distinguished from CD4-dependent TCRs by the manner in which they bind to peptide/MHC Class II ligands. It seemed possible that the absence of CD4 might promote selection for TCRs that bind more avidly to their ligands and therefore would not require the coreceptor activity of CD4 for antigen-specific signaling. From the outset, a measure of the type of

compensation required to overcome CD4 deficiency appears as an indirect, but nonetheless useful, measure of the normal function that CD4 performs during thymocyte selection.

Summary

In the first series of experiments, we examined the diversity of the TCR repertoire selected in the absence of CD4. We first determined the usage of TCR Vα and Vβ elements in peripheral T cells from naïve wildtype and CD4-deficient mice using specific antibodies. Next, we generated antigen-specific T cell hybridomas and determined the sequence of the CDR3 of their TCRs. Although diverse, the TCR repertoire selected without CD4 was subtly distinct from the repertoire selected in the presence of CD4. In the second series of experiments, we examined the ligand-binding properties of CD4-dependent and CD4-independent antigen-specific TCRs. Antigen stimulation assays, peptide/MHC multimer staining experiments, T cell hybridom:antigen presenting cell (APC) conjugation assays and surface plasmon resonance were used. From these experiments, we found that TCRs derived from $CD4^{-/-}$ mice conferred greater responsiveness to antigen stimulation than TCRs derived from wildtype mice. They were also more effective at promoting conjugate formation between T cell hybridomas and APCs. Selected antigen-specific TCRs derived from $CD4^{-/-}$ mice were expressed as transgenes in mice to determine whether they could be selected in the presence and absence of CD4. We found that TCRs that were originally selected in the absence of CD4 could be efficiently selected in the presence of CD4 but were surprisingly variable in their capacity to be selected without CD4.

We conclude that TCRs selected in the absence of CD4 are enriched for those that make avid interactions with their peptide/MHC Class II ligands. However, the binding properties of the TCRs are not so distinctive as to prevent their inclusion within a normal CD4-expressing repertoire. Therefore, the TCR repertoires selected in the absence and the presence of CD4 appear to be overlapping. Our findings suggest that the contribution of CD4 in terms of TCR:peptide/MHC binding during thymic selection is moderate. This moderate contribution might be predicted by the very weak affinity of CD4 for MHC Class II. Nonetheless, the contribution that CD4 makes is clearly highly significant as T helper cell development is substantially compromised when it is not expressed. It remains to be established how thymocyte development can be so tightly regulated by a molecule that has such a markedly weak impact on TCR:ligand recognition.

MATERIALS AND METHODS

Mice

CD4-deficient ($CD4^{-/-}$) mice (68) were back-crossed onto the C57BL/6 background ($H\text{-}2^b$) for 13 generations. C57BL/6 $CD4^{-/-}$ mice were subsequently crossed to B10.D2 mice and then intercrossed to produce homozygous $H\text{-}2^d$ offspring. The CD4-Tva (the cell surface receptor for avian leukosis virus) transgenic mice were generated from B6/CBA F2 eggs by pronuclear injection of a modified form of transgene 'b' (74) in which the human CD2 cDNA was replaced with a Tva cDNA (75). Transgenic founders and their offspring were screened for the presence of the transgene by Southern blot and/or FACS analysis. Founders were bred onto the C57BL/6 background and then crossed with $H\text{-}2^d$ $CD4^{-/-}$ mice to generate $CD4^{-/-}$ Tva transgenic mice. $TCR\alpha^{-/-}$ ($C\alpha^{-/-}$) mice were purchased from Jackson Laboratories. All mice were housed in the Parnassus Heights pathogen-free barrier facility at UCSF.

Antibodies, LACK/I-A^d Fc Protein and Flow Cytometry

PE-conjugated anti-CD3 (145-2C11) and Biotinylated anti-CD3 (145-2C11), -CD24 (HSA) (M1/69), -Vα2 (B20.1), -Vα11 (RR8-1), -Vβ2 (B20.6), -Vβ3 (KJ25), -Vβ6 (RR4-7), -Vβ7 (TR310), -Vβ8.1+8.2 (MR5-2), -Vβ11 (RR3-15), -Vβ13 (MR12-3) and -Vβ14 (14-2) antibodies were obtained from Pharmingen Inc. Biotinylated anti-Vβ10 (CTVB10b), -Vβ12 (CTVB21b) and various conjugates of anti-Thy-1.2, -CD4, -CD8, -B220, –Mac-1 antibodies, and secondary reagents Streptavidin-Tricolor and -PE were purchased from Caltag Laboratories. The following antibodies were purified from tissue culture supernatant and biotinylated according to standard protocol (76): anti-

Vα3.2 (RRB-16), -Vβ4 (KT4), -Vβ5.1+5.2 (MR9-4), -Vβ8.2 (F23.2), -Vβ8.1+8.2+8.3 (F23.1), and -Vβ9 (MR10-2).

Single cell suspensions of lymphocytes (0.2-1x10^6 cells) were incubated for 30min on ice in a final volume of 25-50μl of FACS buffer (PBS with 0.3% BSA and 0.01% NaN_3) containing diluted antibodies. The cells were washed once in FACS buffer, stained with secondary reagents as necessary, and then analyzed using a FACScan flow cytometer (Becton Dickinson) and CellQuest software.

Expression of the Tva protein was detected using a viral envelope-rabbit IgG fusion protein, SuA-rabbit IgG (77), and a donkey anti-rabbit Ig-FITC secondary antibody (Jackson Laboratories).

For TCR repertoire analysis, pooled lymph node cells were stained with FITC-anti-Thy1.2, TC-anti-CD8, TC-anti-B220, TC-anti-Mac-1 and biotinylated anti-Vα or anti-Vβ antibodies. Streptavidin-PE was used as a secondary reagent.

LACK/I-A^d Fc-expressing LMS2 Drosophila cells (78) were grown in Drosophila SFM media (Gibco). Tissue culture supernatant was purified over an anti-I-A^d (M5/114) affinity column followed by a Mono-Q ion-exchange column (Pharmacia). To stain 2x10^5 hybridoma cells or primary T cell, 4μg of purified LACK/I-A^d Fc protein was mixed with 1μl of 0.5mg/ml Protein A-Alexa 488 (Molecular Probes) at room temperature for 30min. 0.5μl of normal mouse serum and 0.5μl of normal rat serum were added to the above mixture immediately before staining. Hybridoma cells were stained at 37°C for 1-3 hours and primary T cells were stained on ice for 1 hour with intermittent mixing.

$LACK_{156-173}$-Specific T Cell Hybridomas

Wild-type ($CD4^{+/+}$) and CD4-deficient ($CD4^{-/-}$) H-2^d mice were immunized in their footpads with 30µl of 0.5mg/ml recombinant LACK protein in Complete Freund's Adjuvant. Popliteal lymph nodes were harvested 10 days after immunization. Lymph node cells were activated *in vitro* with irradiated (2000rad) syngeneic spleen cells and 20µg/ml of $LACK_{156-173}$ peptide (ICFSPSLEHPIVVSGSWD). 3-4 days later, activated cells were fused to BW5147 cells to generate T cell hybridomas following a standard protocol (79). The resulting hybridomas were screened for CD3ε and CD4 expression by FACS analysis, and tested for $LACK_{156-173}$-specificity by IL-2 secretion.

CD4-loss variants of wild-type hybridomas were generated by depletion with anti-mouse CD4 magnetic beads (Dynal). CD4-expressing variants of hybridomas derived from CD4-deficient mice were generated by infection with an ecotropic retrovirus carrying a CD4 cDNA. The retroviral particles were generated by co-transfection of 293-T cells with SVΨ-E-MLV and pBabe-PURO-CD4 plasmids as previously described (36). The $CD4^+$ and $CD4^-$ variants of $CD4^{+/+}$-derived (designated WT and WT-4 respectively) and $CD4^{-/-}$-derived hybridomas (designated KO+4 and KO respectively) were sorted for equivalent surface TCR and CD4 expression using a FACS Vantage sorter (Becton Dickinson).

$1x10^5$ hybridoma cells were stimulated with 0-15µg/ml of $LACK_{156-173}$ peptide or an irrelevant peptide $OVA_{323-336}$ (ISQAVHAAHAEINE) in flat-bottom 96-well plates. Either $1x10^5$ syngeneic irradiated (2000rad) spleen cells, or $1x10^5$ I-A^d-transfected L cells (44/14.B5) (30) were used as antigen-presenting cells. Alternatively, $1x10^5$

hybridoma cells were stimulated with plate-bound purified LACK/I-A^d Fc protein at 0.5-200ng/well in flat-bottom 96-well plates. Tissue culture supernatant was removed 24 hours after stimulation and IL-2 content was determined by sandwich ELISA using anti-mouse IL-2 antibodies (Pharmingen).

TCR Sequence Analysis

Total RNA was isolated from individual hybridomas and converted into cDNA using SuperScript II reverse transcriptase and random hexamers (Gibco). To obtain TCRβ sequences, PCR amplification was performed on hybridoma cDNA using a Vβ4 primer 5'-GCC TCA AGT CGC TTC CAA CCT C-3' and a Cβ2 primer 5'-ATT GCT CTC CTT GTA GGC CTG AGG-3'. PCR products were gel-purified and sequenced using a nested Vβ4 primer 5'-AGA CCT TCA GAT CAC AGC TC-3'. To obtain TCRα sequences, multiple PCR amplifications were performed on each hybridoma cDNA sample using a Cα primer NJ108 5'-GGC CCC ATT GCT CTT GGA ATC-3' and one of the Vα primers:

Vα	Sequence
1	CAG CAG AGC CCA GAA TCC CTC
2	ACC TTC TTC AAT AAA AGG GAG AAA AAG CTC
3	CTC AAG TAC TAT TCC GGA GAC CCA GTG GTT
4	GGA AGC AGC AGA GGT TTT GAA GCT ACA TAC
5	AAG GTT TTC TCA AGT ACG GAA ATA AAC GAA
6	AGT ATG GCT TTC CTG GCT ATT GCC TCT GAC
7	CGA CAA ACG TCT TCT TCT ACT GCA AAA GAG
8	ACA GAC AAC AAG AGG ACC GAG CAC CAA GGG
9	CAA AGA GCT GCG ACG TTC CTT
10	CTG ACA TCC ACC ACA GTC ACT AAG GAA CGT
11	AAT GGG AGG TTA AAG TCA ACA TTC AAT TCT
12	GTG GCA TCT CTG TTT ATC TCT GCT GAC CGG
13	CGT TCA AAT ATG GAA AGA AAG CAG ACC CAA
14	CTG GTT GAC CAA AAA GAC AAA ACG TCA AAT

PCR products were further amplified with the same Vα primer and a nested Cα primer NJ109 5'-CGG CAC ATT GAT TTG GGA GTC-3'. PCR products from the second around of amplification were gel purified and sequenced with a second nested Vα primer NJ 110 5'-CAG GCA GAG GGT GCT GTC C-3'.

Conjugate Formation Assay

$LACK_{156-173}$-specific T cell hybridomas were labeled with 667nM of carboxyfluorescein diacetate succinimidyl diester (CFSE) (Molecular Probes) 16 hours before analysis (80). At the same time, 2PK-3 cells were labeled with 4μM PKH26 (Sigma) according to the manufacturer's instructions. The 2PK-3 cells were then loaded overnight with various concentrations of $LACK_{156-173}$ peptide in complete DMEM supplemented with 25mM HEPES. 20μl of the $LACK_{156-173}$-specific hybridoma cells (10^8 cells/ml) were mixed with 20μl of loaded 2PK-3 cells (10^8 cells/ml) and incubated at 37°C for varying amounts of time. At the end of the incubation period, the cell mixture was vortexed on a Fisher Vortex Genie 2 (Fisher Scientific) at high speed for 10 seconds to disrupt non-specific conjugation, transferred to 0.5ml of FACS buffer and analyzed on a FACScan flow cytometer (Becton Dickinson) using CellQuest software.

Generation of $LACK_{156-173}$-Specific TCR Transgenic Mice

Selected $LACK_{156-173}$-specific TCR cDNAs were cloned from $CD4^{+/+}$-derived (WT) and $CD4^{-/-}$-derived (KO) hybridomas using a PCR strategy. The TCRα chains were amplified with a Vα5 primer 5'-CCG GAA TTC GCC GCC ATG AAG ACG GTG

ACT GGA CC-3' and a Cα primer 5'-CCG GAA TTC TCA ACT GGA CCA CAG CCT CAG-3', and then subcloned into a CD2 expression vector (81). The TCRβ chains were amplified with a Vβ4 primer 5'-CG GGA TCC GCC GCC ATG GGC TCC ATT TTC CTC AGT TG-3' and a Cβ2 primer 5'-CGG GAT CCT CAG GAA TTT TTT TTC TTG ACC-3', and then subcloned into a CD4 expression vector that lacked the intronic transcriptional silencer element (construct i (74)). Transgenic mice expressing the WT15, KO15 or KO23 TCRs were generated by pronuclear co-injection of CD2-TCRα and CD4-TCRβ constructs into B6/DBA/2 F2 eggs. Transgenic founders were identified by Southern blot and FACS analysis, and then crossed onto $CD4^{+/+}C\alpha^{-/-}H\text{-}2^{d}$, $CD4^{+/-}C\alpha^{-/-}H\text{-}2^{d}$ or $CD4^{-/-}C\alpha^{-/-}H\text{-}2^{d}$ backgrounds.

In Vitro Stimulation of TCR Transgenic T Cells

Lymph node cells were harvested from $CD4^{+/-}C\alpha^{-/-}$ TCR transgenic mice or $CD4^{+/-}C\alpha^{+/-}$ non-transgenic control mice and incubated with anti-mouse CD4 (L3T4) MicroBeads (Miltenyi Biotec). $CD4^{+}$ T cells were then purified using MACS Separation Columns (Miltenyi Biotec). The purity of the resulting populations was assayed by flow cytometry and was typically around 90%. $1x10^{5}$ purified $CD4^{+}$ TCR transgenic T cells were incubated in flat-bottom 96-well plates at 37°C for 24 hours with $2.5x10^{5}$ irradiated (2000rad) $H\text{-}2^{d}$ $C\alpha^{-/-}$ splenocytes in the presence of varying concentrations of the $LACK_{156\text{-}173}$ peptide. A sandwich ELISA (PharMingen) was used to quantify IL-2 present in the culture medium after 24 hours.

Transient Transfection into Jurkat Cells

PCR amplified TCRα and β chains were subcloned into a pEF-BOS expression vector (82). $10x10^6$ Jurkat cells were electroporated with 10μg of EF-BOS-TCRα and 10μg of EF-BOS-TCRβ using a Bio-Rad Gene Pulser (Bio-Rad), 240V, 960μF. Transfected cells were cultured in 10ml complete RPMI medium (with 10% FCS, L-glutamine, non-essential amino acids, penicillin/streptomycin) and stained for CD3 expression 24 hours after transfection. $1x10^5$ of the transfected cells were stimulated with either 4μg/ml of soluble anti-human CD3ε antibody or 20μg/ml of $LACK_{156-173}$ peptide presented by I-A^d-expressing L cells. Cells were harvested 24 hours post-stimulation and stained with anti-Vβ4, -mouse CD4 and -human CD69 (Becton Dickinson) antibodies.

Expression of Soluble $LACK_{156-173}$-Specific TCRs

Selected $LACK_{156-173}$-Specific TCRs were amplified from EF-BOS-TCRα and EF-BOS-TCRβ constructs using the following primers that had been engineered to optimize protein expression in *E. coli*:

Vα5-1	5'-GAC GTT CAT ATG GT**T** **TCA** AGA GG**T** GAG CAG GTG GAG C-3'
Vα5-2	5'-ATC GAC CAT ATG GT**A** AG**T** AGA GG**A** GAG CAG GTG GAG C-3'
Cα	5'-TTT GAA GAT ATC TTG GCA GGT GAA GCT TG-3'
Vβ4-1	5'-TAC GAT CAT ATG GGT CCA GT**T** GA**T** CCG AAA ATT ATC CAG AAA CC-3'
Vβ4-2	5'-GTC TAC CAT ATG GGT CC**T** GT**A** GA**T** CCG AAA ATT ATC CAG AAA CC-3'
Cβ	5'-CAG CTC CAC GTG GTC AGG GAA GAA GCC-3'

The amplified TCRα and β chains were subcloned into pRSETb expression vector (Invitrogen). The expression constructs were transformed into *E. coli* strain BL21(DE3), TCRα and β chains were purified from the inclusion body and refolded *in vitro* (83).

Surface Plasmon Resonance

Binding studies were carried out at 5-25°C using the BIAcore (BIAcore AB) as described (84).

Gel Filtration

G4000 column (TosoHaas) was equilibrated with PBS at 4°C. 50μl of purified LACK/I-A^d Fc (1.5mg/ml), LACK/I-A^d Fc-Protein A-Alexa 488 conjugate (1.25mg/ml total protein) or molecular weight marker (15mg/ml) was loaded onto the column and run at 0.5ml/min. Absorption at 239nm and 280nm was recorded.

CHAPTER ONE: TCR REPERTOIRE DIVERSITY IN CD4 LINEAGE T CELLS SELECTED WITHOUT CD4

Helper T cell development and function is severely but not completely diminished in mice that lack expression of CD4 (67, 68, 70, 72, 73, 85). Residual helper T cell function in $CD4^{-/-}$ mice can be attributed to a small population of peripheral MHC Class II-restricted lymph node cells that are $CD3^+$ and $CD4^-CD8^-$ (67, 70, 85). These cells can be assigned to the CD4 lineage on the basis of their functional properties and their expression of reporter transgenes or targeted insertions controlled by CD4 regulatory elements (86-88). We took two different approaches to compare the diversity of TCR repertoire selected in the presence or the absence of CD4. In the first approach, we determined Vα and Vβ domain usage by peripheral T cells from wildtype or CD4-deficient mice by antibody staining. In the second approach, we examined CDR3 diversity of antigen-specific TCRs derived from wildtype or CD4-deficient mice.

Marking CD4 Lineage T Cells with a Transgenic Reporter

We marked the CD4 lineage cells using a reporter transgene comprised of the chicken gene for Tva (encodes the cell surface receptor for avian leukosis virus) under the control of the murine CD4 promoter, enhancer and silencer elements (74, 75, 77, 89) (Figure 1A). In $CD4^{+/+}$ Tva transgenic mice, the reporter was coexpressed with CD4 in both peripheral T cells (Figure 1B) and thymocytes (data not shown), indicating that the expression of Tva is a reliable indicator of CD4 promoter activity. In $CD4^{-/-}$ Tva transgenic mice, about 2/3 of the $CD4^-CD8^-$ double negative (DN) peripheral T cells expressed Tva on their surfaces and could therefore be assigned to the CD4 lineage

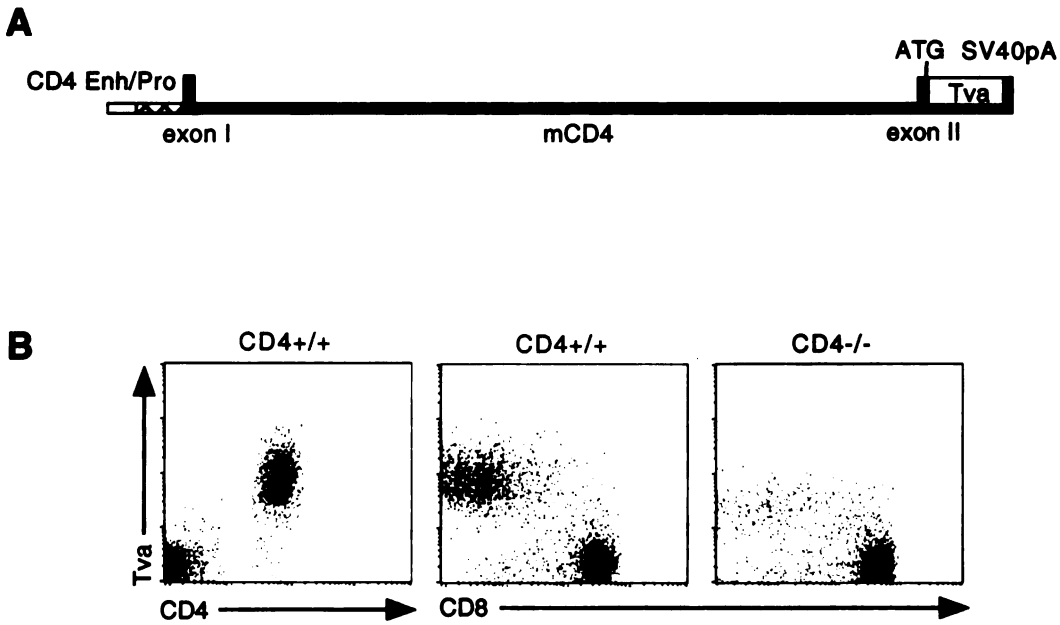

Figure 1. Marking CD4 lineage T cells with the Tva reporter gene. (A) A schematic of the transgene construct. (B) Tva expression on CD4 lineage T cells. Lymph node cells were stained with SuA-rabbit IgG fusion protein followed by FITC-anti-rabbit IgG secondary reagent and anti-CD3, -CD4, -CD8 antibodies. Gated T cells are shown.

(Figure 1B). This proportion of CD4 lineage cells in $CD4^{-/-}$ mice was similar to that observed with other reporters (86, 88). Interestingly, the level of TVA expression on CD4 lineage T cells from $CD4^{-/-}$ mice was lower than that on $CD4^+$ T cells from CD4-expressing mice. The reason for this is unclear, but it could in part be a consequence of the enrichment for memory T cells in $CD4^{-/-}$ mice (90) and the potential for differential regulation of CD4 in memory versus naïve T cells (91).

TCR Repertoire Diversity in CD4-Deficient Mice

To survey the T cell receptor (TCR) repertoire of T helper cells selected in the absence of CD4, we initially used a panel of monoclonal antibodies specific for individual Vα or Vβ domains. CD4 lineage T cells were identified by their lack of CD8 expression and their expression of either Thy-1 or the Tva reporter. As shown in Figure 2A and 2C, a diverse TCR repertoire was expressed by CD4-deficient CD4 lineage T cells selected on both $H\text{-}2^d$ and $H\text{-}2^b$ backgrounds. $CD4^+$ T cells from $CD4^{+/+}$ and $CD4^{+/-}$ mice displayed an indistinguishable TCR repertoire in this assay (Figure 2A). Nevertheless, despite the apparent diversity of the TCR repertoire, there were some notable differences between the usage of certain Vα and Vβ elements by cells selected in the presence or absence of CD4. Specifically, the populations differed in their relative usage of Vα3.2, Vβ5, and Vβ12 in $H\text{-}2^d$ mice, and the usage of Vα3.2, Vβ3, Vβ5, and Vβ10 in $H\text{-}2^b$ mice. The observed differences in the TCR repertoire were specific to the CD4 lineage as they were not observed in $CD8^+$ T cells from $CD4^{+/+}$ and $CD4^{-/-}$ mice (Figure 2B and 2D).

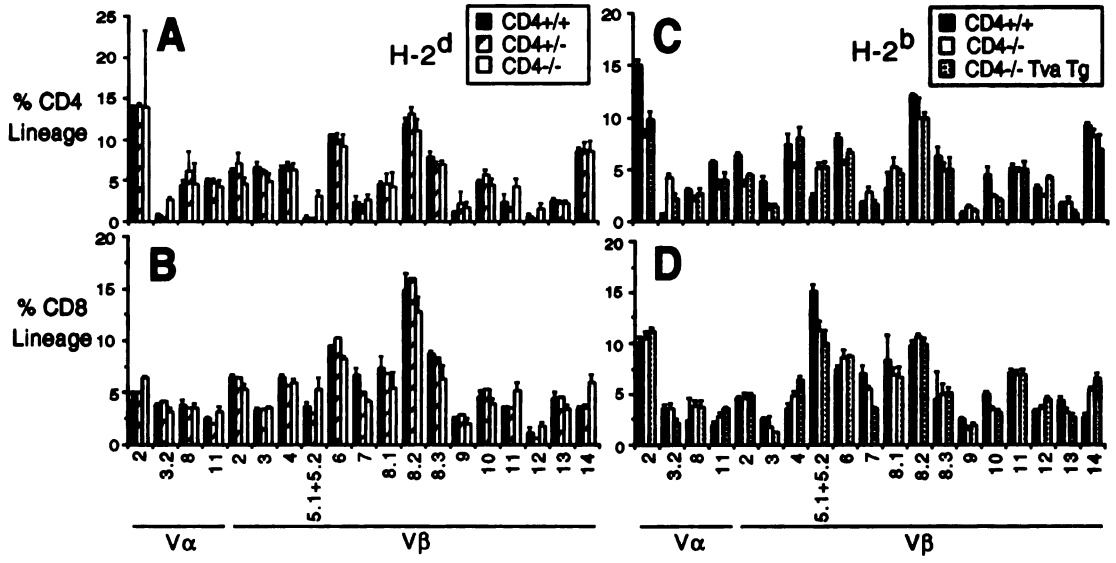

Figure 2. T cell receptor repertoire expressed by CD4 and CD8 lineage T cells selected in the presence or absence of CD4. (A) CD4 lineage T cells from H-2^d mice. (B) $CD8^+$ T cells from H-2^d mice. (C) CD4 lineage T cells from H-2^b mice. (D) $CD8^+$ T cells from H-2^b mice.

Diversity of Antigen-Specific TCRs from CD4-Deficient Mice

As an alternative approach for studying the TCR repertoire of CD4-deficient T helper cells, we generated a panel of antigen-specific T cell hybridomas from $CD4^{+/+}$ and $CD4^{-/-}$ mice. As a model antigen, we immunized H-2^d mice with recombinant *Leishmania Major* LACK protein. We then restimulated T cells from the draining lymph nodes of the mice with the immunodominant I-A^d-restricted peptide corresponding to residues 156-173 in the native protein (92). Hybridomas derived from wild-type (WT) but not CD4-deficient (KO) mice expressed CD4 on their surfaces (data not shown). $LACK_{156-173}$-reactive T cell hybridomas from both wild-type and CD4-deficient mice all expressed Vβ4 as determined by FACS analysis (data not shown). RT-PCR and sequence analysis revealed extensive CDR3 diversity in TCRβ chains expressed by both types of T cell hybridomas (Table 1). As previously observed, the majority (13/15) of $LACK_{156-173}$-specific Vβ4 chains from wild-type hybridomas had either a glutamate (E) or aspartate (D) residue at their V-D junctions (92). These residues are likely to participate in a direct interaction with histidine (H) at position 164 in the $LACK_{156-173}$ peptide, and are therefore crucial for the antigen specificity of the TCRs (79). Hybridomas from CD4-deficient mice showed a similarly high frequency (21/22) of acidic residues at the V-D junctions of their TCRs, suggesting that their mechanism of engaging the $LACK_{156-173}$/I-A^d complex was likely to be similar to that of wild-type TCRs.

In contrast to the dominant selection for Vβ4, RT-PCR and sequence analysis revealed considerable variability in Vα domain usage in hybridomas derived from both

	Clone	Jβ	Junctional Sequences		
$CD4^{+/+}$-Derived Hybridomas	WT1	2.5	CASS	QDRVNQDTGY	FGPG
	WT2	2.3	CASS	QDRTVYGAETLY	FGSG
	WT3	2.4	CASS	QEMGLGFSQNTLY	FGAG
	WT5	2.4	CASS	QSSQNTLY	FGAG
	WT6	1.5	CASS	QDGGVGNQAPL	FGEG
	WT7	2.3	CASS	QDSGHPAETLY	FGSG
	WT9	2.5	CASS	QDRWGGATQY	FGPG
	WT10	1.2	CASS	QEAGKWGTQY	FGSG
	WT11	1.6	CASS	QDRVGSYNSPLY	FAAG
	WT14	2.5	CASS	QDWVNQDTQY	FGPG
	WT15	2.5	CASS	QEWVNQDTQY	FGPG
	WT16	1.1	CASS	QEQGNTEVF	FGKG
	WT17	1.3	CASS	QPGGNTLY	FGEG
	WT18	1.6	CASS	QEWNSYNSPLY	FAAG
	WT19	1.2	CASS	QDLGSDYT	FGSG
$CD4^{-/-}$-Derived Hybridomas	KO1	2.1	CASS	QEGAAEQF	FGPG
	KO2	2.4	CASS	QEQASQNTLY	FGAG
	KO3	2.2	CASS	QELPTNTGQLY	FGEG
	KO5		CASS	QEGAPGQLY	FGEG
	KO6	1.6	CASS	QDWGGGNSDYT	FGSG
	KO7	2.2	CASS	QELGSNTGQLY	FGEG
	KO8	2.4	CASS	QDRGGENTLY	FGAG
	KO9	2.7	CASS	QETGRS	FGPG
	KO10	2.5	CASS	QELVAQDTQY	FGPG
	KO11	2.4	CASS	QDGTGSTLY	FGAG
	KO12	2.4	CASS	QDWASQNTLY	FGAG
	KO15	2.5	CASS	QENVNQDTQY	FGPG
	KO16	2.2	CASS	QDRLGNTGQLY	FGEG
	KO17	2.2	CASS	QDTSTGQLY	FGEG
	KO18	2.4	CASS	QDAGSQNTLY	FGAG
	KO20	1.1	CASS	QDRGGVF	FGKG
	KO21	2.1	CASS	QESGGYAEQF	FGPG
	KO22	2.4	CASS	QDGLSQNTLY	FGAG
	KO23	2.4	CASS	PQGFQNTLY	FGAG
	KO25	1.6	CASS	QEGQYNSPLY	FAAG
	KO27	1.3	CASS	QDRSSGNTLY	FGEG
	KO28	1.1	CASS	QEGANTEVF	FGKG

Table 1. CDR3 sequences of TCRβ chains used by $LACK_{156-173}$-specific hybridomas. Non-consensus residues are highlighted.

wild-type and CD4-deficient mice (Table 2). Both types of hybridomas exhibited extensive CDR3 diversity at the V-J junctions of their TCRα chains.

Summary

The above-described study of the usage of TCR Vα and Vβ elements by peripheral T cells suggested that TCR repertoire selected in the absence of CD4 was both diverse and subtly distinct from the TCR repertoire selected in the presence of CD4. Sequence analysis of the CDR3 region of antigen-specific TCRs further demonstrated the diversity of TCRs selected in the presence and the absence of CD4. We did not observe any preferential usage of a certain CDR3 sequence in either the wide-type or the CD4-deficient hybridomas. This study was clearly limited, however, by the relatively small sample size and also by the emphasis on only one antigenic peptide. It has been shown that properties of T cells may not be fully revealed by studying the response to a single antigen (93). Thus, it seems possible that additional experiments employing other antigenic peptides and examining larger numbers of TCRs might reveal evidence of an impact of CD4 on selection of antigen-specific TCRs.

Despite the limitations of the repertoire survey, we considered it useful to examine the properties of the CD4-independent TCRs in more detail using a variety of approaches. The results of these studies are described in the next chapter.

	Clone	Vα	Jα		CDR3 Sequence	
	WT1	5		CA	VKDNAGAKLTFGGGTRLTVRP	DIQN
	WT2	3				
	WT5	10	33			
	WT6	13	11-2-C			
	WT9					
$CD4^{+/+}$-	WT10	5	33	CA	VSFVNTGNYKYVFGAGTRLKVIA	HIQN
Derived	WT12	8	TA80	CA	LSDRDTEGADRLTFGKGTQLII	
Hybridomas		10				
	WT13	8	34			
	WT14	5		CA	VIDNAGAKLTFGGGTRLTVRP	DIQN
	WT15	5				
		8		CA	LSDPDNNNAPRFGAGTKLSVKP	NIQN
	WT16	5		CA	VRNYNQGKLIFGQGTKLSIKP	NIQN
	WT17	8	TA72	CA	LSEDMGYKLTF	
	WT19	novel		CA	VRGNMGYKLTFGTGTSLLVDP	NIQN
	KO1	1				
	KO2	novel	112-2	CA	INNYAQGLTFGLGTRVSVFP	YIQN
	KO4	8		CA	LSGINTGNYKY...	
	KO5	1				
		5	11-2-C			
$CD4^{-/-}$-	KO6	5	47	CA	VATGGNNKLTFGQGTVLSVIP	DIQN
Derived		8				
Hybridomas	KO9	3				
		4				
	KO15	5	21	CA	VSTNTGKLTFGDGTVLTVKP	NIQN
	KO20	3	11			
	KO21	1				
		4				
	KO23	5	23	CA	LHTNAYKVIFGKGTHLHVLP	NIQN
	KO25	8	TA65	CA	LSEDTGYQNFYFGKGTV	
	KO27	8		CA	LSNYNAPLLR...	

Table 2. Vα usage and CDR3 sequences of $LACK_{156-173}$-specific hybridomas.

CHAPTER TWO: CHARACTERIZATION OF ANTIGEN-SPECIFIC TCRs SELECTED IN THE ABSENCE OF CD4

The repertoire analysis at the level of Vα and Vβ usage indicated that there might be differences in CD4-dependent versus CD4-independent repertoire. Given the function of CD4, it seems reasonable that these differences could reflect differences in the way that CD4-indepdent and –dependent TCRs engage their ligands. Thus, it seemed possible that the absence of CD4 might promote selection for TCRs that bind more avidly to their ligands and therefore would not require the coreceptor activity of CD4 for antigen-specific signaling. The immunization experiment described in the previous chapter gave us access to antigen-specific TCRs that we could study in more detail, the assumption being that the limited number of TCRs we obtained could be taken as representative of the CD4-dependent and CD4-independent TCR repertoires. What follows is a description of several experiments to provide information on the antigen binding properties of these CD4-independent $LACK_{156-173}$-specific TCRs.

Antigen Responsiveness

The relative antigen responsiveness of hybridomas derived from wild-type and $CD4^{-/-}$ mice (designated WT and KO respectively) was evaluated by stimulating the hybridomas with LACK/I-A^d. The antigen was exposed to the T cell hybridomas either as a peptide/MHC complex on APCs or as a plate-bound, purified LACK/I-A^d Fc fusion protein. This latter fusion protein is a dimer of a truncated I-A^d α chain (linked at its carboxy-terminus to an acidic leucine zipper and the Fc portion of mouse IgG2a) and a truncated I-A^d β chain (fused at its amino-terminus to the $LACK_{156-173}$ peptide and at its

carboxy-terminus to a basic leucine zipper) (78). $LACK_{156-173}$-specific WT and KO T cell hybridomas exhibited a wide and overlapping range of responsiveness to the fusion protein as measured by IL-2 production (Figure 3A). Similar dose response curves were obtained regardless of whether the T cell hybridomas were stimulated with the fusion protein or with $LACK_{156-173}$-pulsed I-A^d-expressing spleen or L cells (30) (data not shown).

To isolate the effect of CD4 expression on the reactivity of the T cell hybridomas in the assays, we generated CD4-negative variants of WT hybridomas (WT-4) by magnetic bead depletion, and CD4-positive variants of KO hybridomas (KO+4) by retroviral gene transfer (Figure 4). Sorted populations showing equivalent CD4 and TCR expression were then tested for their capacity to make IL-2 when stimulated with plate-bound LACK/I-A^d Fc fusion protein (Figure 3B) or I-A^d-transfected L cells loaded with $LACK_{156-173}$ peptide (data not shown). CD4 potentiated the responsiveness of both KO and WT hybridomas to a similar extent as shown by a 50-70% decrease in the dose of peptide required to achieve half-maximum IL-2 secretion (compare WT-4 vs. WT, or KO vs. KO+4 in Figure 3B). Interestingly, T cell hybridomas bearing the KO TCRs were typically more responsive than their wild-type counterparts when they were matched for CD4 expression (compare WT-4 vs. KO, p=0.003, or WT vs. KO+4, p=0.044, in Figure 3B). These data would be consistent with the hypothesis that the absence of CD4 selects for T cells bearing distinctive T cell receptors that might differ from WT TCRs in the kinetics with which they engage antigenic peptide/MHC complexes.

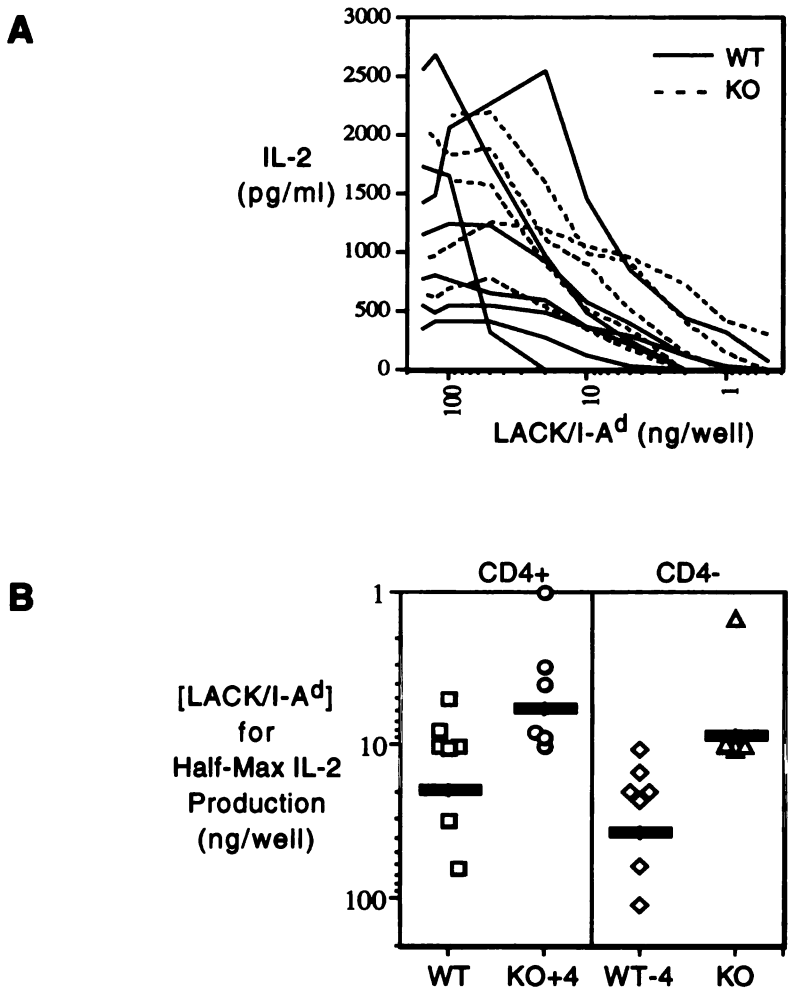

Figure 3. Antigen response of $LACK_{156-173}$-specific hybridomas. (A) IL-2 production by hybridomas stimulated with plate-bound LACK/I-A^d Fc protein. Hybridomas were FACS-sorted for comparable TCR expression. (B) LACK/I-A^d Fc dose required for half-maximum IL-2 production. Hybridomas had been sorted for comparable TCR and CD4 expression. WT: CD4-expressing hybridomas derived from $CD4^{+/+}$ mice; WT-4: CD4-deficient variants of WT; KO: CD4 null hybridomas derived from $CD4^{-/-}$ mice; KO+4: CD4-expressing variants of KO.

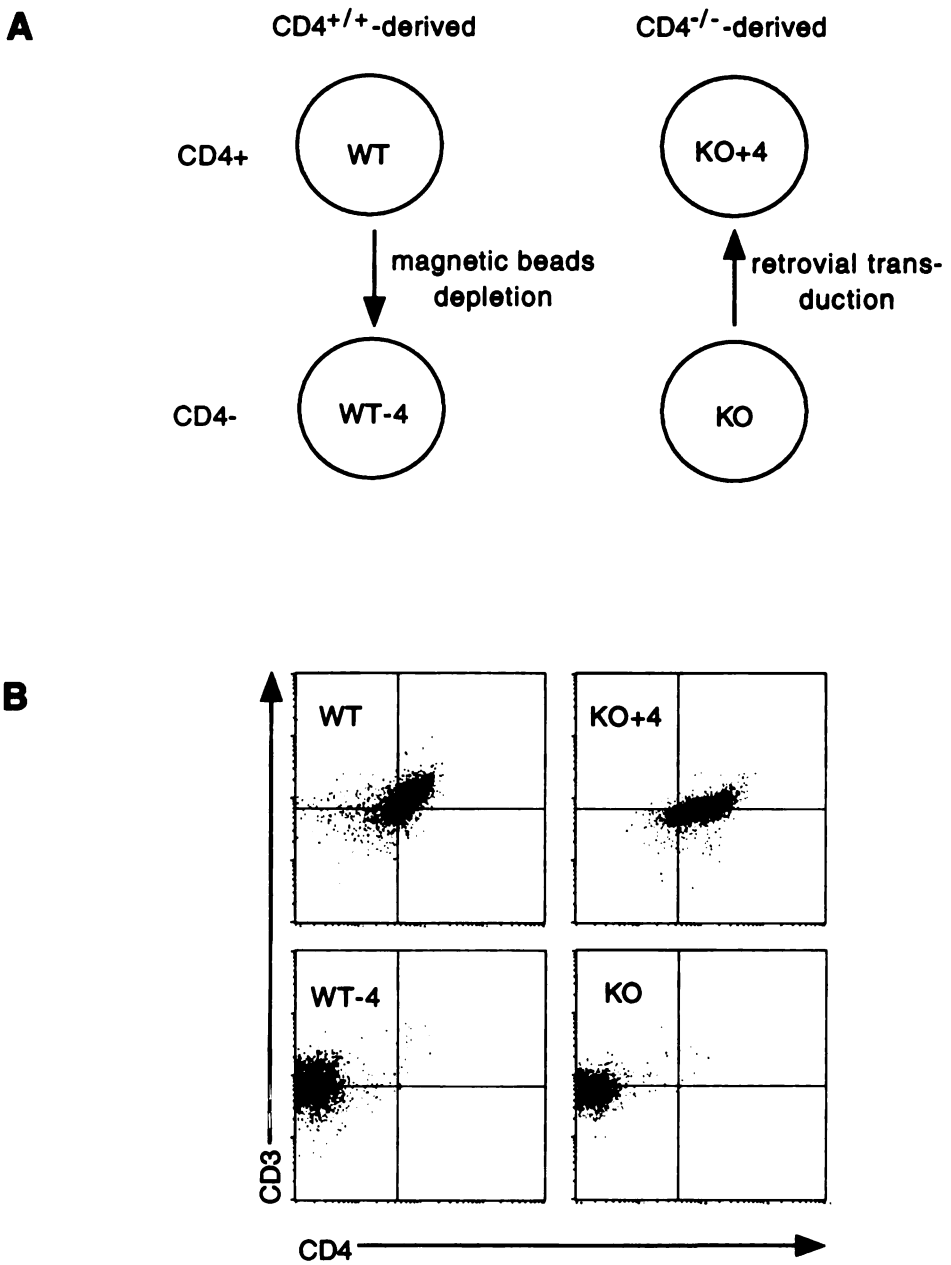

Figure 4. Generating hybridoma variants with matching TCR and CD4 levels. (A) A schematic of the strategy. (B) Surface TCR and CD4 expression of selected hybridoma variants after FACS sorting.

Response to Analogous Peptide

To examine the potential significance of the histidine residue at position 164 in $LACK_{156-173}$ peptide in more detail, we tested the panel of T cell hybridomas for reactivity against a variant peptide with an alanine substitution at this site (LACK A). This change largely eliminated the reactivity of CD4-deficient hybridomas (KO), but had a much milder effect on the reactivity of the CD4-expressing hybridomas from wild-type mice (WT, Table 3). All but one of the CD4-deficient hybridomas failed to respond to the mutant peptide, whereas the majority (6 out of 7) of the wild-type hybridomas showed significant reactivity. These observations are potentially consistent with the absence of CD4 providing a selection for T cell receptors that engage their ligands in a qualitatively distinct fashion from those selected in the presence of CD4. Further support for this view came from an analysis of variants of the T cell hybridomas selected for either loss of CD4 expression (WT-4) or gain of CD4 expression (KO+4). Whereas the gain of CD4 rescued reactivity in the latter case, the loss of CD4 did not abrogate reactivity in the former case. Thus, CD4-deficiency appeared to have a differential effect on reactivity to the alanine-substituted peptide, dependent on whether the T cell hybridomas had been selected in the presence or absence of CD4.

LACK/I-A^d Multimer Staining

Alexa 488-Protein A-LACK/I-A^d Fc is a multimeric staining reagent

As one approach for comparing the kinetic properties of WT and KO $LACK_{156-173}$-specific TCRs, we employed the LACK/I-A^d Fc fusion protein in a variation of a flow cytometric assay previously used to inform on TCR binding properties (94, 95). The

	$LACK_{156-173}$	LACK A		$LACK_{156-173}$	LACK A
WT5	+++	++++	KO5	++	-
WT6	+	+++	KO6	+++	-
WT9	++	++++	KO15	+++	-
WT10	++++	++++	KO20	++	-
WT15	+++	-	KO21	++	++++
WT17	+++	++++	KO23	+++	-
WT19	+	+++			
WT5-4	++	+++	KO5+4	+++	++
WT6-4	-	+++	KO6+4	++++	++
WT9-4	+	++	KO15+4	++++	++
WT10-4	+++	++	KO20+4	++	-
WT15-4	++	-	KO21+4	+++	+++
WT17-4	++	-	KO23+4	++++	+++
WT19-4	-	+			

Table 3. Antigen response of selected $LACK_{156-173}$-specific hybridomas to an altered peptide LACK A. Antigen response was measured by IL-2 production. Peptide dose required for half-maximum IL-2 production is represented by ++++, 0.1-1μg/ml; +++, 1-2μg/ml; ++, 2-5μg/ml; +, 5-10μg/ml; -, >10μg/ml.

staining reagent was generated by conjugating purified LACK/I-A^d Fc protein with fluorescently labeled Protein A (Alexa 488-Protein A). Purified LACK/I-A^d Fc fusion protein is in dimeric form (approximately 160kD) as determined by gel filtration analysis (Figure 5A). The dimerization is likely to be mediated by the Fc portion of the fusion protein. Even though there are three Fc binding sites on Alexa 488-Protein A, only two of them can be occupied simultaneously due to steric hindrance (96-98). Therefore, the staining reagent should be a dimer of LACK/I-A^d Fc dimers, i.e. tetrameric with regard to TCR binding. Indeed, gel filtration analysis of Alexa 488-Protein A-LACK/I-A^d Fc conjugates (Alexa-LACK) yielded a peak at the appropriate size for a tetramer (approximately 365kD) (Figure 5B). However, at least half of the material in the Alexa-LACK reagent was found in larger complexes. Furthermore, the fraction of high molecular weight complexes increased over time (Figure 5C). The exact molecular weight or the composition of the high molecular weight complexes could not be accurately determined.

Alexa-LACK stains $LACK_{156-173}$-specific hybridomas

Alexa-LACK stained $LACK_{156-173}$-specific, but not other T cell hybridomas in a dose- and temperature-dependent fashion (Figure 6A, B). Like other soluble peptide/MHC class II reagents, the binding of Alexa-LACK to hybridoma cells was not CD4-dependent, as it was not inhibited by the presence of anti-CD4 antibody (Figure 6C) (94). These properties indicated that Alexa-LACK could be used as a specific probe to study the ligand-binding properties of $LACK_{156-173}$-specific T cell receptors.

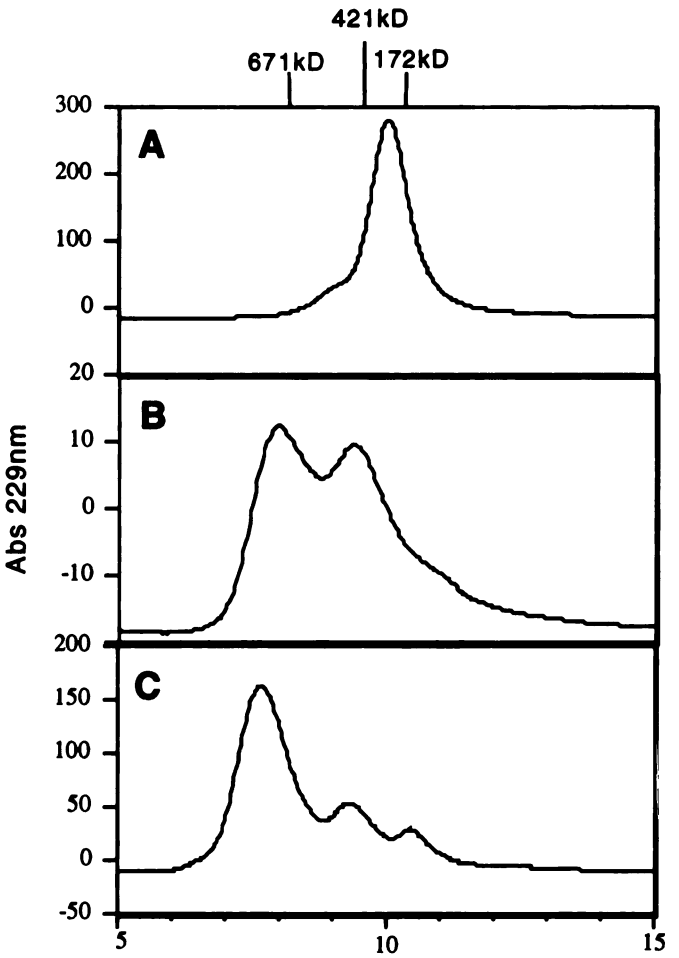

Figure 5. Gel filtration analysis of purified LACK/I-A^d Fc fusion protein and Alexa 488-Protein A-LACK/I-A^d Fc conjugate. (A) Purified LACK/I-A^d Fc fusion protein. (B) Alexa 488-Protein A-LACK/I-A^d Fc conjugate. 0.5μg of Alexa 488-Protein A was mixed with 4μg of LACK/I-A^d Fc at room temperature for 30min, then kept on ice for 3 hours before loading onto the column. (C) The same conjugate preparation after storage at 4°C for a week.

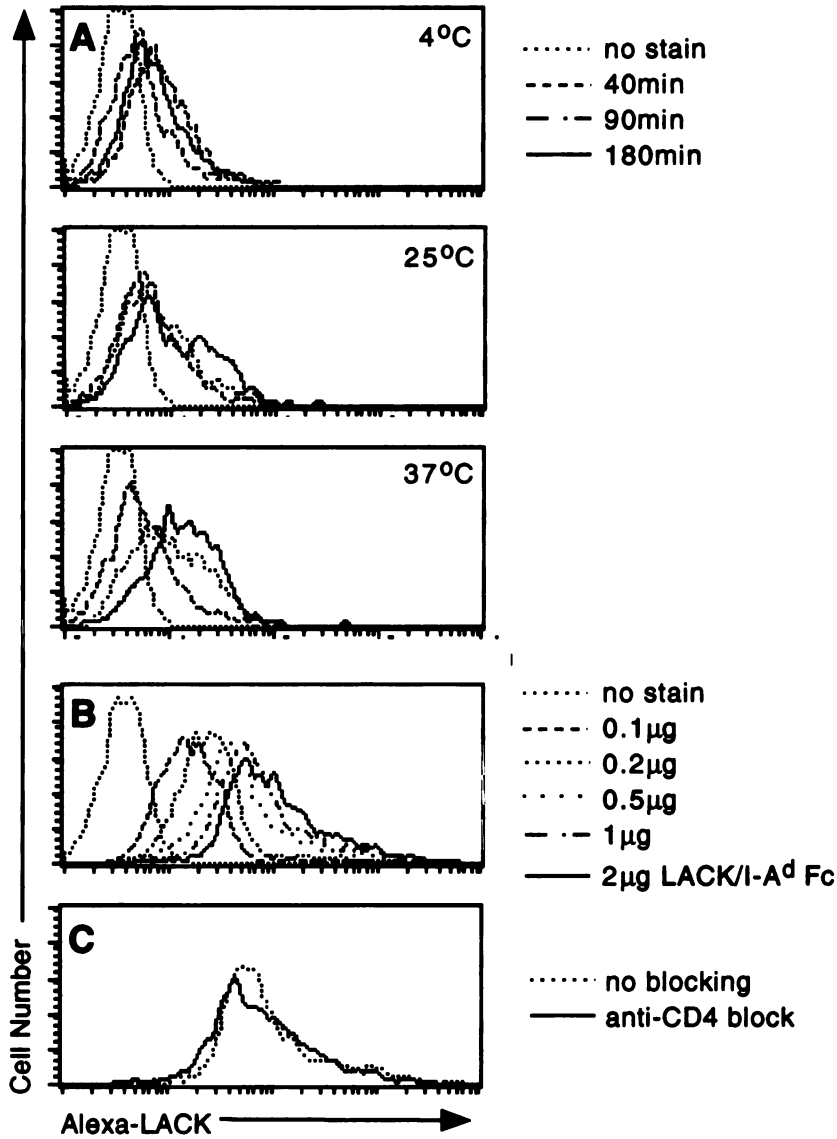

Figure 6. Characterizing Alexa 488-Protein A-LACK/I-A^d Fc (Alexa-LACK) staining reagent. (A) Alexa-LACK staining of $LACK_{156-173}$-specific hybridomas is temperature- and time-dependent. $2.5x10^5$ KO15 hybridoma cells were stained with 8μg of LACK/I-A^d pre-conjugated with 0.55μg of Alexa 488-protein A. (B) Alexa-LACK staining is dose-dependent. KO15+4 hybridoma cells were stained at 37°C for 3 hours. (C) Alexa-LACK staining is CD4-independent. KO15+4 cells were either stained with Alexa-LACK directly or pre-blocked with saturating amount of anti-CD4 antibody (GK1.5) and then stained with Alexa-LACK.

Alexa-LACK staining intensity was variable across the panel of T cell hybridomas even after accommodating for differences in the level of TCR expression (examples shown in Figure 7A). As shown in Figure 7B, KO hybridomas exhibited a similar degree of staining with Alexa-LACK as WT hybridomas suggesting that their TCRs were not significantly different in affinity towards the LACK/I-A^d complex (94). However, KO hybridomas did exhibit a noticeably greater range of staining intensity than WT hybridomas and there was also a trend toward brighter staining in the KO group.

We also tried to study the dissociation kinetics of TCR:LACK/I-A^d binding because the dissociate rate has been demonstrated to determine the physiological outcome of a TCR:peptide/MHC interaction (99-101). However, we were unable to obtain the dissociation rates with the flow cytometric approach because we could not detect significant dissociation of the multimeric Alexa-LACK reagent even in the presence of competing anti-I-A^d antibody (data not shown). The absence of Alexa-LACK dissociation is likely to be due to its multimeric structure as demonstrated by gel filtration analysis described above. Ongoing experiments with a less multimeric form of LACK/I-A^d are expected to resolve this issue (unpublished data, L. Malherbe and N. Glaichenhaus, Valbonne, France).

Alexa-LACK stains primary T cells

To compare the binding properties of $LACK_{156-173}$-specific TCRs at the population level without the selection introduced in generating hybridomas, we chose to look at primary T cells directly. Lymphocytes were harvested from the draining lymph

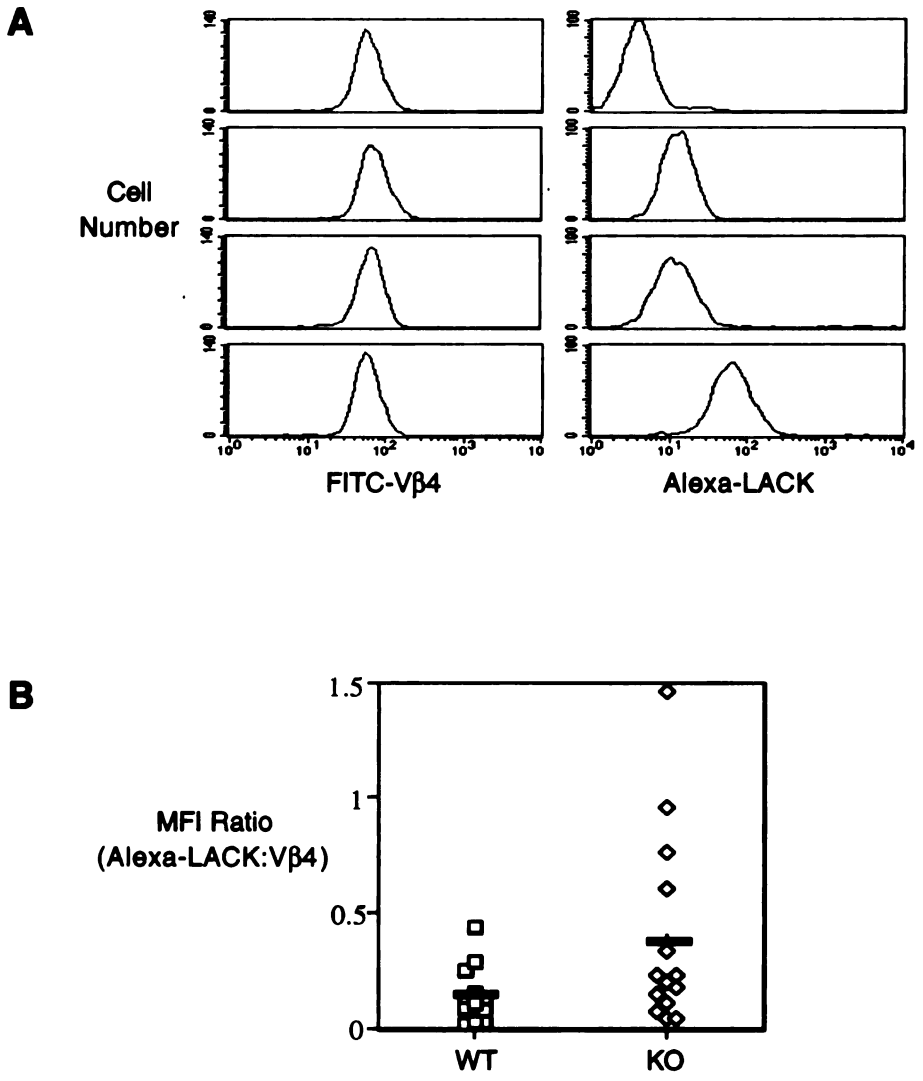

Figure 7. Staining of $LACK_{156-173}$-specific hybridomas with Alexa 488-Protein A-LACK/I-A^d Fc. (A) Vβ4 and Alexa-LACK staining on representative WT (top two panels) and KO (bottom two panels) hybridomas. Vβ4 staining, 4°C, 25min. Alexa-LACK staining, 37°C, 1hr. (B) Specific Alexa-LACK staining of WT and KO hybridomas: mean fluorescence intensity of Alexa-LACK/mean fluorescence intensity of FITC-Vβ4.

nodes of either wildtype or $CD4^{-/-}$ mice that had been previously immunized with the LACK protein. The cells were stained with Alexa-LACK and analyzed by FACS. A small population (1-2%) of CD4 lineage T cells from LACK-immunized wild-type mice showed positive staining for Alexa-LACK (Figure 8B). This population could be further expanded *in vitro* when stimulated with the LACK protein presented by syngeneic splenocytes (Figure 8C). A similar population could also be detected in CD4-deficient mice immunized with the LACK protein. Currently, experiments are underway to compare the Alexa-LACK staining intensity of $LACK_{156-173}$-specific primary T cells from wild-type and CD4-deficient mice.

Conjugate Formation

To examine further the kinetic properties of $LACK_{156-173}$-specific TCRs, we established a flow-cytometric conjugation assay using fluorescently labeled T cell hybridomas and antigen presenting cells. $LACK_{156-173}$-specific hybridomas that had been sorted for comparable surface TCR and CD4 expression were labeled with the green fluorescent dye carboxyfluorescein diacetate succinimidyl diester (CFSE). As antigen presenting cells, we used the B cell lymphoma 2PK-3 labeled with the red fluorescent dye PKH26 and loaded with the $LACK_{156-173}$ peptide. The labeled hybridomas and 2PK-3 cells were mixed at 37°C and the formation of conjugates was followed over time by FACS analysis (Figure 9A). Conjugate formation between $LACK_{156-173}$-specific hybridomas and 2PK-3 cells was peptide-specific, peptide dose- and time-dependent (Figure 9A and 9B). Conjugation was also potentiated by the presence of CD4, as CD4-

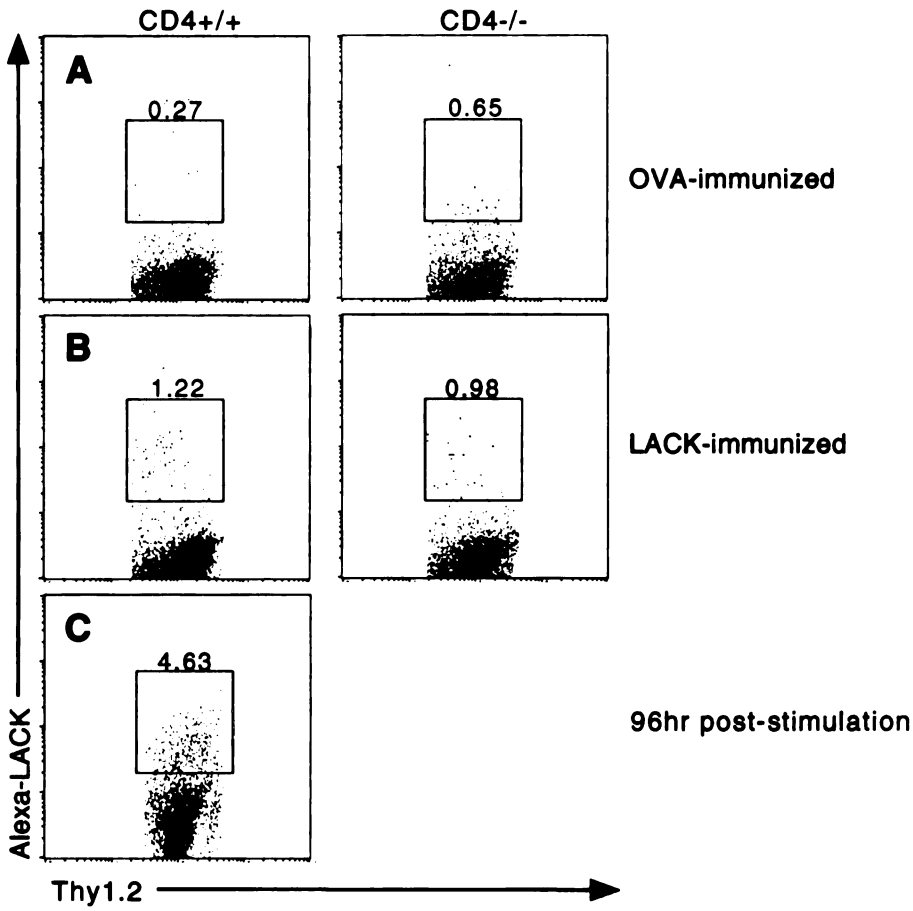

Figure 8. Staining primary T cells with Alexa-LACK. Lymphocytes from the draining lymph nodes of mice immunized with either (A) OVA or (B) LACK protein were stained with Alexa-LACK, anti-Thy1.2 and anti-CD8. Gated CD4 lineage T cells (Thy1.2$^+$ CD8$^-$) are shown. The numbers represent the percentage of CD4 lineage T cells that are LACK-specific. (C) Lymphocytes from LACK-immunized mice were stimulated *in vitro* with LACK protein and stained with Alexa-LACK 96hr later.

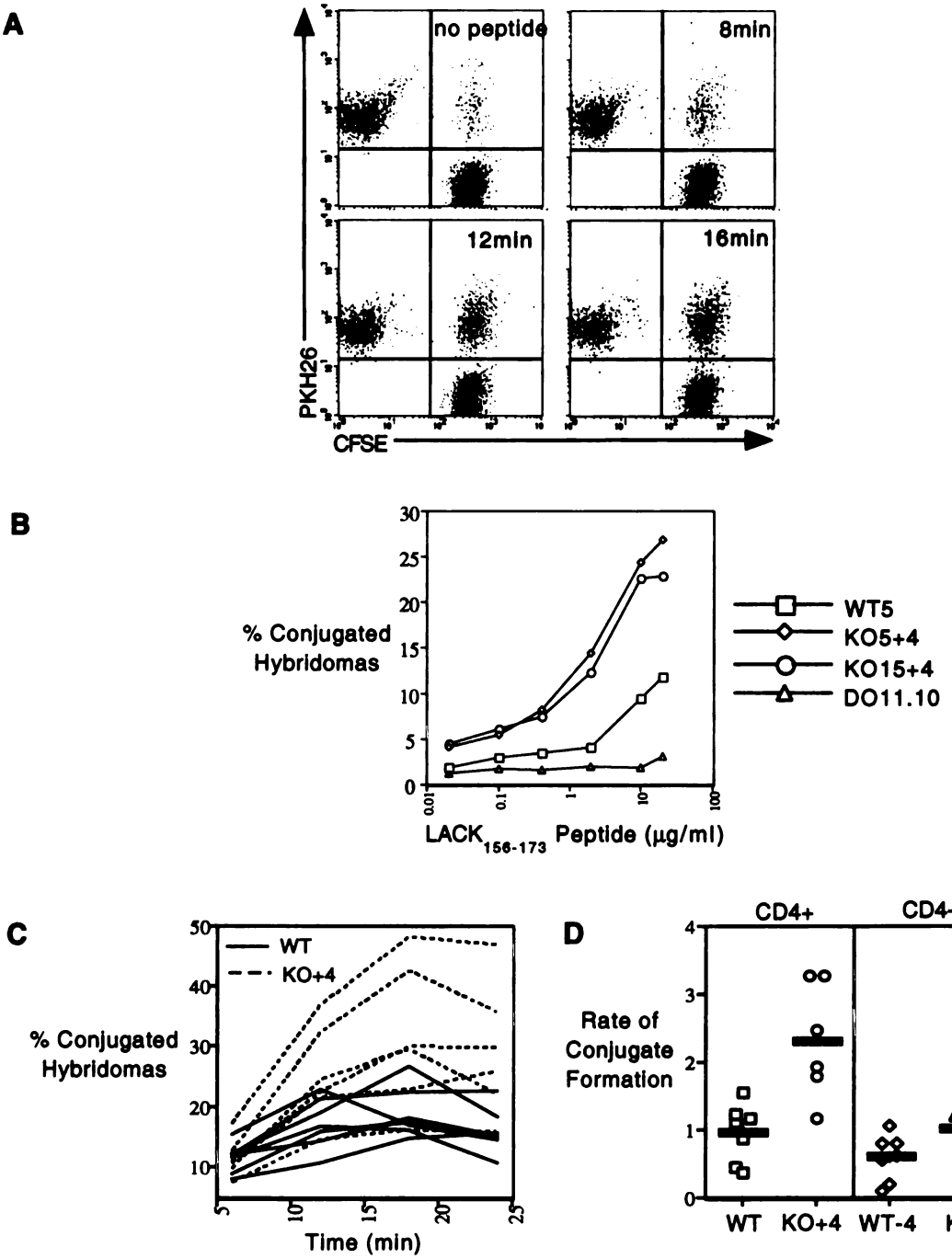

Figure 9. Conjugate formation between $LACK_{156-173}$-specific hybridomas and antigen presenting cells 2PK-3. (A) Conjugate formation over time. 10μg/ml of $LACK_{156-173}$ peptide. (B) Conjugate formation is antigen dose-dependent. 16min. (C) Kinetics of conjugate formation. 10μg/ml of $LACK_{156-173}$ peptide. (D) Rate of conjugate formation calculated from (C) and presented as % conjugated hybridomas/min.

expressing hybridomas formed conjugates more rapidly than their CD4-deficient derivatives/parents (compare KO vs. KO+4, p=0.025, in Figure 9D). Despite this potentiating effect of CD4, KO hybridomas formed conjugates at rates that were comparable to those of WT CD4-expressing hybridomas (Figure 9D). Furthermore, the acquisition of CD4 by KO hybridomas markedly enhanced their conjugation rate above that of CD4-expressing WT hybridomas (Figure 9C and 9D). Cumulatively, the data suggest that $CD4^{-/-}$-derived TCRs are more effective than $CD4^{+/+}$-derived TCRs at driving conjugation between a T cell and its target. Thus, these data provide support for the hypothesis that the absence of CD4 selects for TCRs with distinctive kinetic properties.

Surface Plasmon Resonance

Surface plasmon resonance studies are likely to provide the most quantitative data concerning the antigen binding properties of $LACK_{156-173}$-specific TCRs. We therefore cloned several TCRs from $LACK_{156-173}$-specific WT and KO hybridomas and expressed them in *E. coli* according to recently developed methodology (83). The binding kinetics between immobilized TCRs and the soluble LACK/I-A^d Fc fusion protein were then measured using the BIAcore biosensor machine. To date, we have acquired only preliminary data suggesting at best a moderate difference between the WT and KO TCRs. Thus, a comparison between $LACK_{156-173}$-specfic TCRs matched for similar affinity showed roughly a 2-3 fold slower off-rate for the KO TCRs compared to WT TCRs (unpublished data, S. Bell).

Summary

Cumulatively, the antigen response, tetramer staining, conjugate formation and preliminary surface plasmon resonance studies suggested that if there are differences in the ligand-binding properties of WT and KO TCRs, these are likely to be modest. Antigen stimulation studies on a subpanel of hybridomas showed that the KO hybridomas responded more vigorously to antigen stimulation than WT hybridomas on average. However, the ranges of responsiveness exhibited by WT and KO hybridomas overlapped with one another. Staining studies with the peptide/MHC multimer failed to reveal statistically significant differences in ligand-binding affinities of KO and WT TCRs. Nonetheless, KO TCRs exhibited a greater range of affinities than WT TCRs and there was a trend toward tighter binding that might be substantiated by analysis of a larger sample size. Finally, the KO TCRs were shown to be more effective at mediating conjugation between T cell hybridomas and APCs in the conjugate formation assay.

To evaluate the physiological significance of any differences between KO and WT TCRs in their ligand-binding kinetics, we chose to clone and express selected $LACK_{156\text{-}173}$-specific TCRs in transgenic mice. A major goal here was to determine whether the moderate differences observed between WT and KO TCRs were significant enough to lead to different fates during their thymic selection. In particular, we were interested in testing whether the KO TCRs would cause deletion of CD4 lineage T cells when expressed in the presence CD4. Results from such transgenic experiments should provide information on the extent of the impact of CD4 in thymic selection. A large contribution of CD4 would predict that TCRs initially selected in the absence of CD4

would be negatively selected in the presence of CD4. In contrast, a small contribution would predict that the CD4-independent TCRs could be positively selected in the presence of CD4.

CHAPTER THREE: SELECTION OF $LACK_{156-173}$-SPECIFIC TCRs IN TRANSGENIC MICE

We next attempted to determine whether the kinetic properties of the KO TCRs might be sufficiently distinctive as to preclude their inclusion in a normal repertoire selected in the presence of CD4. Thus, it seemed possible that the absence of CD4 might impose selection for a type of TCR that would normally be excluded from the TCR repertoire because of overly strong interactions with thymic peptide/MHC class II ligands. We therefore generated transgenic mice expressing the KO TCRs.

Cloning $LACK_{156-173}$-specific TCRs

As discussed in Chapter One, all of the $LACK_{156-173}$-specific TCRs utilize the Vβ4 domain and a large number of them use the Vα5 domain. Therefore, we used Vα5 and Cα primers, and Vβ4 and Cβ primers to amplify the TCRα and β chains by PCR from selected WT and KO hybridomas. The TCRα and β chains obtained were sequenced and subcloned into modified CD2 (81) and CD4 (74) expression vectors and these were used to create transgenic mice. In addition, the TCRα and β chains were subcloned into the pEF-BOS expression vector (82).

Expression of the cloned TCRα and β chains was tested by transfecting β-deficient Jurkat cells with EF-BOS-TCRα and EF-BOS-TCRβ constructs. β-deficient Jurkat cells lacked surface expression of CD3, but the TCR could be reconstituted on the cell surface by the introduction of constructs expressing TCRβ chains. The addition of an exogenous TCRα further enhanced the surface expression of CD3 because the endogenous α chain appears to be expressed at limiting levels in the cells (Figure 10A).

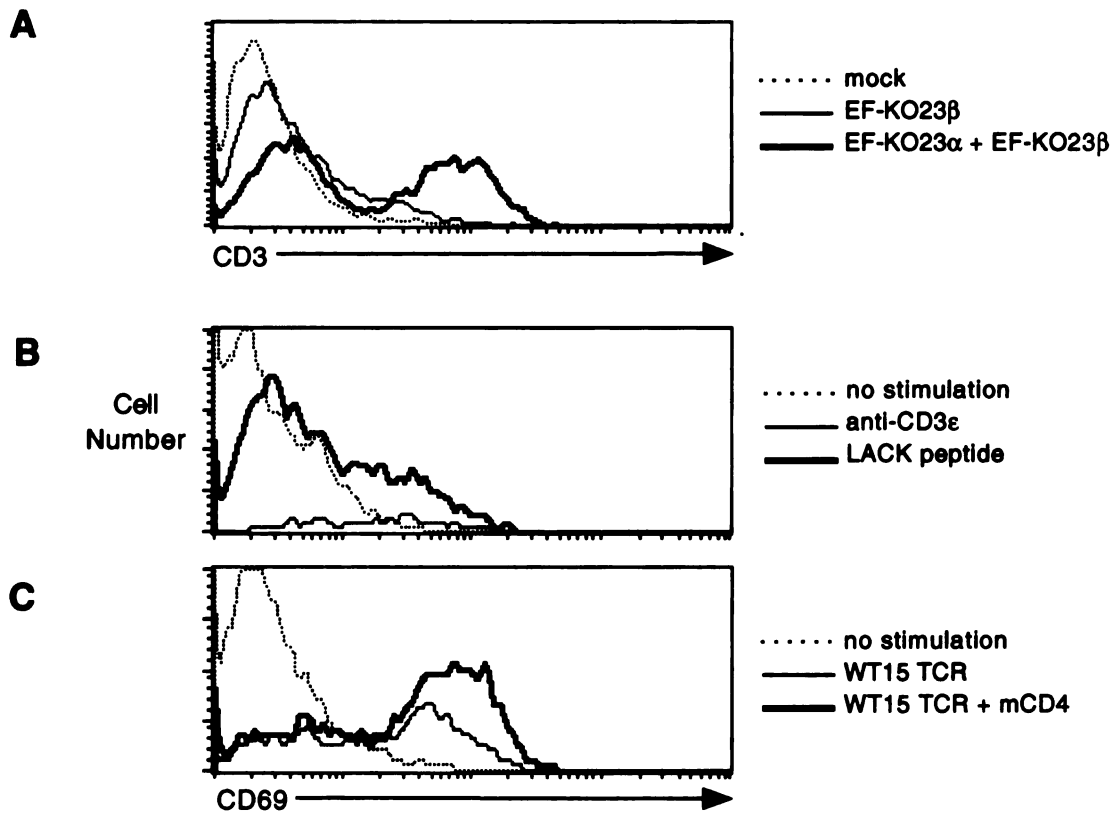

Figure 10. Expression and antigen-specificity of cloned TCRs. (A) Expression of KO23 TCR in transiently transfected β-deficient Jurkat cells. (B) CD69 upregulation on transfected Jurkat cells upon antigen stimulation. KO23-expressing Jurkat cells were stimulated with either 4μg/ml of soluble anti-CD3 antibody or 20μg/ml of $LACK_{156-173}$ peptide presented by I-A^d-expressing L cells. (C) Co-transfection of mouse CD4 enhances antigen response.

To confirm the antigen specificity of the cloned TCRs, transiently transfected Jurkat cells were stimulated with $LACK_{156-173}$ peptide presented by I-A^d-expressing L cells. Jurkat cell activation was then monitored by induction of surface CD69 expression (Figure 10B). Co-transfection of mouse CD4 into Jurkat cells typically enhanced their antigen responsiveness (Figure 10C). 8 of 13 cloned TCRs were shown to be $LACK_{156-173}$-specific in this manner. 2 of the 8 TCRs showed detectable response to antigen stimulation only in the presence of mouse CD4.

Characterization of $LACK_{156-173}$-Specific TCR Transgenic Mice

Two of the cloned KO TCRs were used to create transgenic mice KO15, and KO23. Multiple transgenic founders were obtained for both TCRs and several of these were bred to create TCR transgenic H-2^d $CD4^{-/-}$ and $CD4^{+/-}$ control mice.

Selection of transgenic TCRs

In the presence of CD4, both transgenic TCRs allowed for efficient development of CD4 lineage cells (Figures 11A and 12A). The majority of mature CD4 lineage cells in the thymuses and periphery of these mice expressed both chains of the transgenic TCRs on their surfaces, as shown by staining with the Alexa-LACK reagent. Complimentary analysis of $TCR\alpha^{-/-}$ mice showed that the transgenic TCR heterodimers could allow for positive selection of $CD4^+$ T cells without the involvement of endogenous
TCRα chains (Figures 11B and 12B). Thus, despite their distinctive *in vitro* properties, the KO TCRs could be selected into the T helper cell repertoire in the presence of CD4.

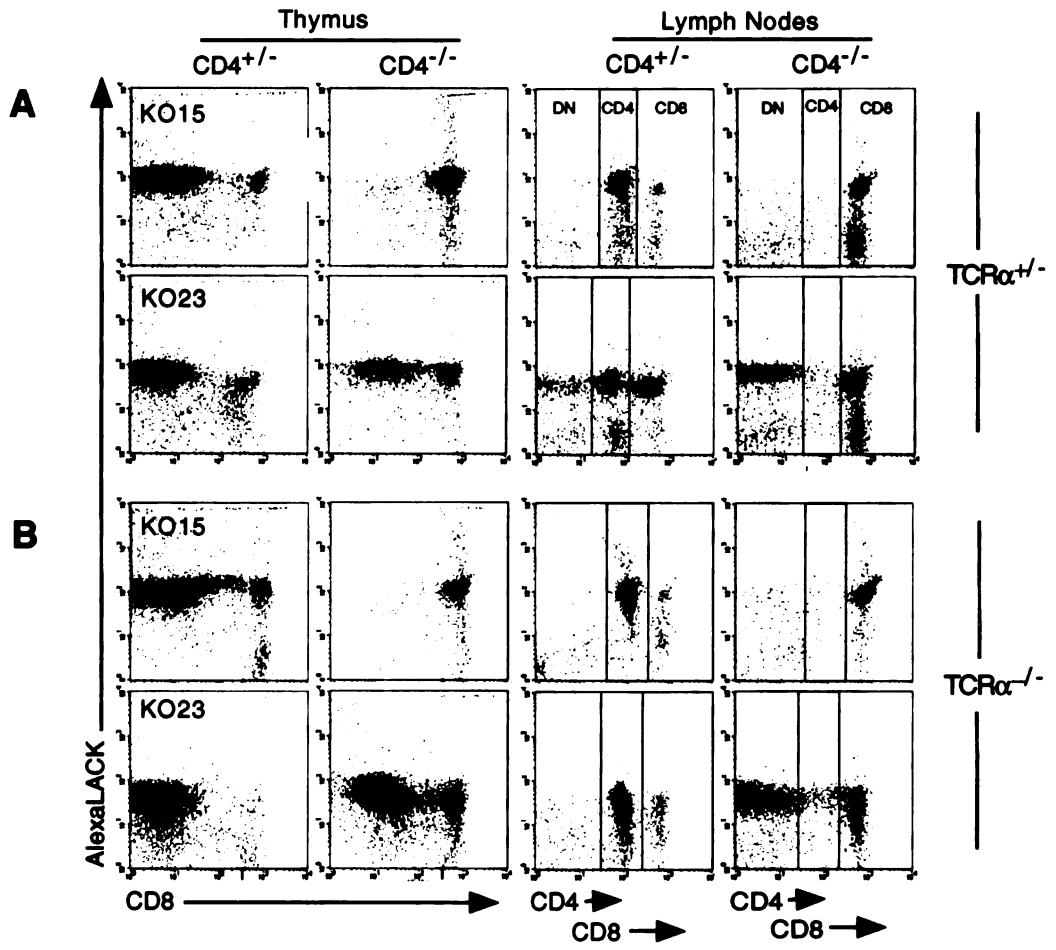

Figure 11. $LACK_{156-173}$-specific TCR transgenic mice. (A) Thymuses and lymph nodes from Cα+ TCR Tg mice. Total thymocytes were stained with Alexa-LACK, PE-CD8α and biotin-HSA followed by Streptavidin-TC. HSA^{lo} mature single positive cells are shown. Pooled mesenteric and inguinal lymph node cells were stained with Alexa-LACK, PE-CD4, PE-CD8α, TC-B220 and TC-Mac-1. $B220^{-}Mac\text{-}1^{-}$ T cells are shown. Cells with low PE staining are CD4+ cells, those with high PE staining are CD8+ cells. PE negative cells are double negative (DN) T cells which may include CD4 lineage cells. Each plot is a representative of 3-5 mice analyzed.

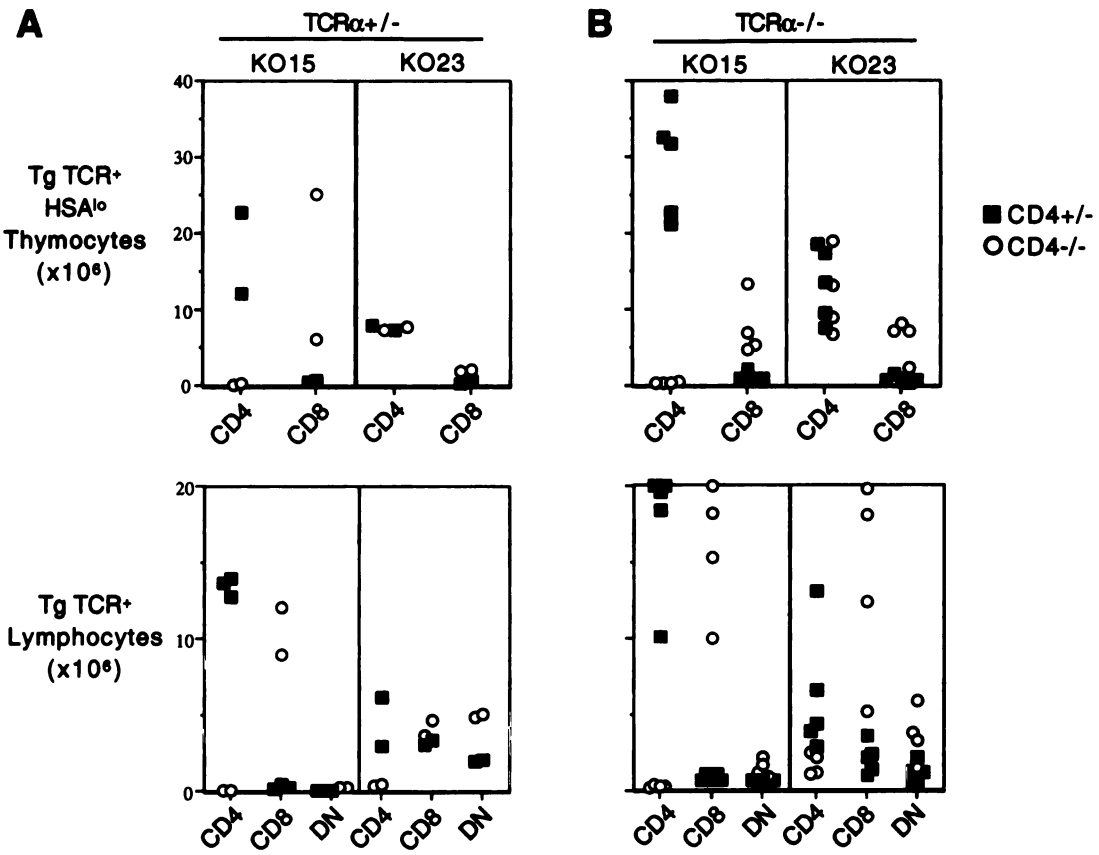

Figure 12. Number of $LACK_{156-173}$-specific TCR transgenic cells in the thymuses and lymph nodes (mesenteric and inguinal) of (A) TCR$\alpha^{+/-}$ and (B) TCR$\alpha^{-/-}$ TCR Tg mice.

In the absence of CD4, we found that KO15 TCR was inefficient at promoting selection of CD4 lineage T cells (Figure 11 and 12). Thus, there were very few $CD8^-$ mature CD4 lineage cells in the thymuses and periphery of transgenic mice expressing the KO15 TCR and most of their peripheral T cells were $CD8^+$. By contrast, the KO23 TCR allowed for more efficient CD4-independent selection of CD4 lineage cells than KO15, with the frequency of thymic CD4 lineage cells approaching that observed in the presence of CD4. To confirm that the $CD8^-$ population of T cells in the KO23 TCR transgenic mice included cells of the CD4 lineage, we analyzed the methylation status of the CD8 gene. This analysis showed that the CD8 gene was substantially demethylated in the $CD4^-CD8^-$ T cells in $CD4^{-/-}$ KO23 TCR transgenic mice (data not shown). Such data are consistent with the DN T cells being CD4 lineage cells that were derived from the equivalent of the $CD4^+CD8^+$ double-positive stage of thymocyte development (102). Thus, the KO23 TCR could allow for positive selection of CD4 lineage T cells in the absence of CD4.

CD8 lineage T cells were present in the both $TCR\alpha^+$ KO15 and KO23 TCR transgenic mice. The majority of these cells stained brightly with the Alexa-LACK reagent (Figure 11A). Positive selection of CD8 lineage cells mediated by the KO15 and KO23 transgenic heterodimers could be confirmed by the analysis of $TCR\alpha^{-/-}CD4^{-/-}$ TCR transgenic mice in which there were also abundant Alexa-LACK-staining $CD8^+$ cells in both the thymus and periphery (Figure 11B). Furthermore, the selection of KO23-bearing CD8 lineage cells was MHC class I-independent as demonstrated by the persistence of these cells in mice lacking β_2m expression (Figure 13). Cumulatively, the data indicate that the KO23 TCR could allow for positive selection of thymocytes into

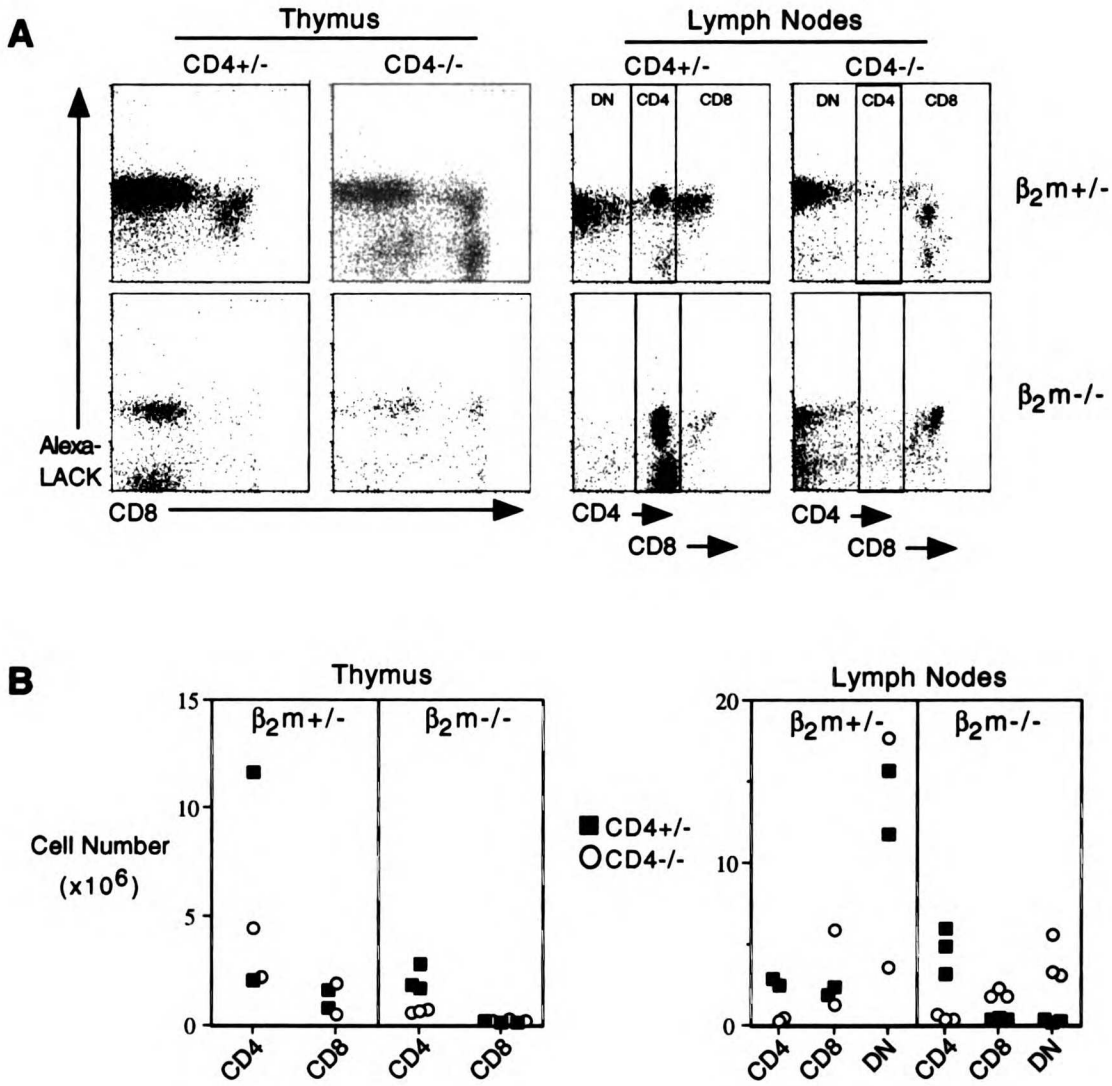

Figure 13. Selection of KO23 TCR on CD8 lineage T cells is MHC I-independent. (A) Expression of transgenic TCR KO23 on HSA^{lo} mature thymocytes and peripheral T cells from $TCR\alpha^+$ $H\text{-}2^d$ mice in the presence and absence of β_2m. (B) Number of KO23 TCR^+ mature thymocytes and peripheral T cells (mesenteric and inguinal lymph nodes).

either the CD4 or CD8 lineage in the absence of CD4. In contrast, the KO15 TCR was not effective in promoting CD4-independent selection of CD4 lineage cells, but it could allow for selection of CD8 lineage T cells.

Antigen responsiveness of TCR transgenic T cells

To confirm the antigen specificity of the transgenic TCRs, $CD4^+$ T cells were purified from $CD4^{+/-}$ $TCR\alpha^{-/-}$ TCR Tg mice and stimulated *in vitro* with the $LACK_{156-173}$ peptide presented by syngeneic splenocytes. KO23-bearing transgenic T cells responded to antigen stimulation better than KO15-bearing transgenic cells (Figure 14A), reproducing the relative $LACK_{156-173}$ reactivity of the parental hybridomas (Figure 3 and data not shown).

Conjugate formation of TCR transgenic T cells

In addition to their antigen responsiveness, we also examined the conjugation rate with APC exhibited by the transgenic T cells. Purified $CD4^+$ transgenic T cells were shown to form conjugates with $LACK_{156-173}$ -presenting 2PK-3 cells in an antigen dose- and time-dependent manner (data not shown). KO23 T cells formed conjugates at a slower rate than KO15 T cells (Figure 14B), which contradicted the observation made with parental hybridomas (Figure 9 and data not shown). However, the KO23 TCR was also expressed at lower levels (approximately 2 fold) on T cells compared to KO15 TCR (data not shown), probably because of more active TCR signaling in response to binding of endogenous MHC ligands. This lower level of surface TCR expression could explain the slower rate at which KO23 T cells formed conjugates compared to KO15 T cells.

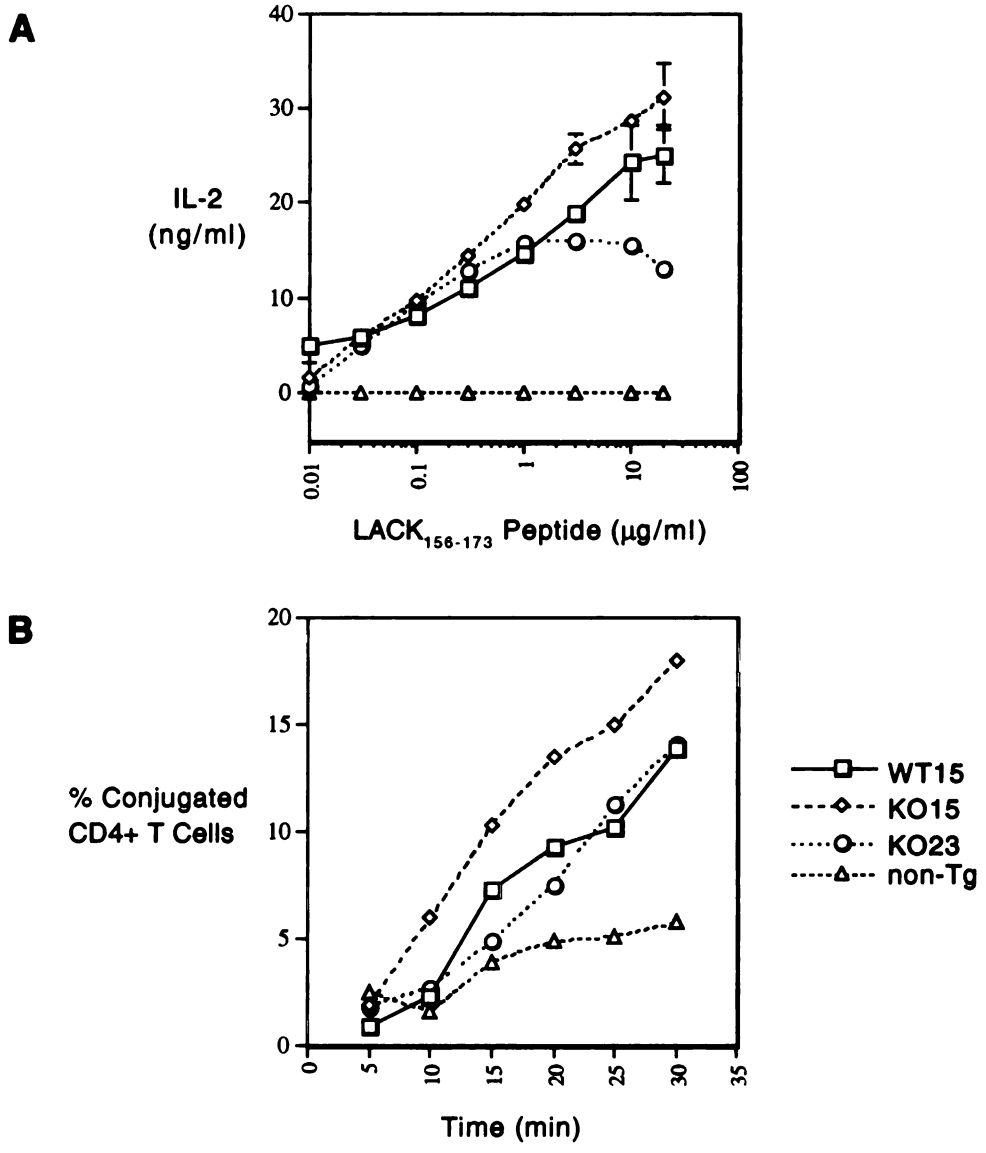

Figure 14. Characterization of CD4+ TCR$\alpha^{-/-}$ TCR Tg cells. (A) IL-2 production from *In vitro* stimulation with $LACK_{156-173}$ peptide presented by syngeneic splenocytes. (B) Conjugate formation with antigen presenting cells 2PK-3. 10μg/ml $LACK_{156-173}$ peptide.

Summary

The expression of two KO TCRs in transgenic mice showed that both receptors could be selected in the presence of CD4. Thus, TCRs selected in the absence of CD4 could be included in a repertoire selected in the presence of CD4. Therefore, even though there may be moderate differences between the ligand-binding properties of CD4-depedent and CD4-independent TCRs, the two repertoires can apparently overlap.

In the absence of CD4, KO23 TCR but not KO15 TCR could be positively selected on CD4 lineage T cells. It is paradoxical that a TCR that was initially selected in a CD4-independent manner could not be selected in the absence of CD4 in a transgenic system. One possible explanation is that the KO15 TCR was at the low end of the affinity spectrum and was inefficiently selected in $CD4^{-/-}$ mice. Immunization with the LACK protein preferentially expanded the KO15-expressing T cells in the periphery, and this allowed for the generation of hybridoma cells bearing the TCR. However, KO15 TCR failed to be efficiently selected in the absence of CD4 in transgenic mice due to its low affinity for thymic selecting ligands. Alternatively, it is formally possible that the T cell from which KO15 hybridoma was derived expressed two TCRα chains due to incomplete allelic exclusion. One of the $\alpha\beta$ TCRs on the cell surface had the appropriate affinity for the thymic selecting ligand and allowed for selection of the T cell in the absence of CD4, while the other $\alpha\beta$ TCR, KO15, was LACK-specific and CD4-dependent.

Both KO15 and KO23 TCR could be selected on $CD8^+$ T cells regardless of the expression of CD4. Furthermore, the selection of KO23-bearing $CD8^+$ T cells was MHC Class I-independent. Whether the selection of KO15-expressing $CD8^+$ T cells depends

on MHC Class I molecule was not tested. The expression of MHC Class II-restricted TCRs on $CD8^+$ T cells has been observed before and it was explained in a signal strength model in which the CD4 versus CD8 lineage choice is determined by the intensity of signals delivered to $CD4^+CD8^+$ double-positive thymocytes (60). A strong signal promotes a CD4 fate, whereas a weak signal promotes CD8 fate.

CHAPTER FOUR: DISCUSSION

Diversity of TCR Repertoire Selected in the absence of CD4

The TCR repertoire analysis described above allows for two straightforward conclusions to be made. First, the TCR repertoire selected in the absence of CD4 is diverse and second, there is evidence that it is different from the repertoire selected in the presence of CD4. Thus, the antibody staining data reveal many similarities but also clear differences in the representation of specific Vβ and Vα elements in the CD4-deficient versus CD4-expressing populations. Our observation is consistent with previous findings that the $CD4^+$ T cell repertoire from mice with compromised CD4-MHC Class II interaction was diverse and slightly different from the wildtype repertoire (41, 61). An analysis of the CDR3 sequences of $LACK_{156-173}$-reactive CD4-deficient T cell hybridomas also supports the two conclusions, as it shows diversity coupled with perhaps a slightly enhanced selection for usage of an acidic residue at a likely peptide contact site.

While it is clear that CD4-deficient mice have a large deficit of T helper cells, the repertoire analysis indicates that there is still substantial potential for the development of a diverse CD4 lineage without involvement of the coreceptor. BrdU labeling studies have established that about 15-20% of the normal number of cells transit from the $TCR^{lo}CD69^-CD8^+$ (double-positive) to the $TCR^{hi}CD69^+CD8^-$ (single-positive) stage of development in the CD4-deficient thymus (90). From this figure, and the lower than normal frequency of peripheral T helper cells, we can infer that the loss of CD4 imposes an atypical selection for a subset of the T helper cell repertoire.

Kinetic Properties of TCRs Selected in the Absence of CD4

By analogy to the demonstrated effect of CD8, it seems likely that the loss of CD4 would affect the interaction kinetics of TCRs engaging peptide/MHC class II ligands (44, 103). Thus, without CD4, there may be a selection for TCRs that bind more avidly to their ligands – i.e., TCRs that demonstrate reduced dissociation or increased association constants. If the impact of CD4 on the binding kinetics (i.e., TCR:peptide/MHC complex formation and stability) is large, then its loss might well demand an equally large compensation in binding kinetics. By contrast, if the coreceptor effect is not large, then the loss of CD4 would not impose a major penalty on the interaction kinetics and the magnitude of the compensation required for CD4-independent development would be small. Thus, the repertoire selected without CD4 would be expected to be substantially different in the former case and might favor the emergence of cells that would normally be deleted in the presence of CD4. In the latter case, where the coreceptor effect on binding kinetics is small, the CD4-independent repertoire may well be partially, or indeed wholly included within the normal CD4-expressing repertoire (perhaps at the high end of the affinity distribution). Studying the kinetic properties of TCRs used by CD4-deficient T helper cells therefore offers the potential for deriving a quantitative perspective on the developmental function of CD4.

Differences in ligand-binding kinetics between CD4-dependent and CD4-independent $LACK_{156-173}$-specific TCRs are revealed in a number of assays. TCRs derived from CD4-deficient mice (KO) are on average more responsive than their wildtype counterparts (WT) to stimulation with the $LACK_{156-173}$ peptide presented by I-A^d. Furthermore, there may prove to be differences in the antigen specificity of the KO

and WT TCRs as revealed by the different responsiveness exhibited by the panel of hybridomas to the mutant LACK peptide. A peptide/MHC II multimeric staining reagent binds WT and KO TCRs to a similar degree, suggesting KO and WT TCRs have similar affinity towards LACK/I-A^d ligand on average. However, an examination of a larger sample size may eventually change this conclusion and reveal a modest effect of CD4 as suggested by the observed trend towards brighter staining of KO hybridomas with the multimeric staining reagent. KO TCRs are more efficient than WT TCRs at mediating rapid conjugate formation between T cell hybridomas and antigen presenting cells, suggesting a faster on-rate in TCR:peptide/MHC interaction. Preliminary surface plasmon resonance data suggest that KO TCRs may have a slower off-rate than WT TCRs in ligand-binding.

These results suggest that CD4-independent TCRs could be distinguished from CD4-dependent TCRs by the manner in which they bind to peptide/MHC II ligands. However, the difference is by no means drastic, suggesting that the degree of compensation required to overcome CD4 deficiency by KO TCRs is moderate. These findings suggest that CD4 has a limited contribution to TCR:peptide/MHC binding kinetics during thymic selection, and that the TCR repertoires selected in absence and presence of CD4 overlap in their ligand-binding properties.

Selection of CD4-Independent TCRs in Transgenic Mice

To compare the selection of CD4-dependent and -independent TCRs, we generated TCR transgenic mice. Surprisingly, both KO15 and KO23, two TCRs derived from CD4-deficient mice, can be positively selected in the presence of CD4. This

suggests that although CD4-independent TCRs may be enriched for those that make avid interactions with their peptide/MHC II ligands, their binding properties do not prevent inclusion within a normal CD4-expressing repertoire. Interestingly, KO23 but not KO15 allows the selection of CD4 lineage T cells in the absence of CD4.

It is paradoxical that a TCR that is originally selected in a CD4-independent manner can not be efficiently selected in the absence of CD4 in a transgenic system. One possible explanation is that the KO15 TCR was at the low end of the affinity spectrum that allowed for selection in the absence of CD4 and was inefficiently selected in $CD4^{-/-}$ mice. Immunization with the LACK protein preferentially expanded the KO15-expressing T cells in the periphery, and this allowed for the generation of hybridoma cells bearing the TCR. When expressed in transgenic mice lacking CD4, the KO15 TCR failed to be efficiently selected into the CD4 lineage due to its low affinity for thymic selecting ligands. Alternatively, it is formally possible that the T cell from which KO15 hybridoma was derived expressed two TCRα chains due to incomplete allelic exclusion. One of the $\alpha\beta$ TCRs on the cell surface had the appropriate affinity for the thymic selecting ligand and allowed for selection of the T cell in the absence of CD4, while the other $\alpha\beta$ TCR, KO15, was LACK-specific and CD4-dependent.

In addition to being efficiently selected on CD4 lineage T cells both in the presence and absence of CD4, the KO23 TCR also allows for the selection of $CD8^+$ T cells. The selection of KO23-bearing $CD8^+$ T cells does not require expression of MHC Class I molecules, however, selection is clearly enhanced in its presence. The expression of MHC II-restricted TCRs on $CD8^+$ T cells has been observed before although, interestingly, this selection was usually β_2m-dependent (60, 104).

Overexpression of CD4 on all thymocytes also leads to the selection of CD8 lineage T cells (which are $CD4^+CD8^+$) that express MHC II-restricted TCRs (69). In CD4-deficient mice, both KO15 and KO23 TCRs can be selected on $CD8^+$ T cells. Expression of MHC II-restricted TCRs on $CD8^+$ cells in CD4-deficient mice has been reported previously (60).

The Role of CD4 in Thymic Selection

Cumulatively, our data suggest that CD4-independent TCRs could be distinguished from CD4-dependent TCRs by the manner in which they bind to peptide/MHC ligands even though the differences are moderate. They are consistent with our hypothesis that the absence of CD4 might promote selection for TCRs that bind more avidly to their ligands and therefore would not require the coreceptor activity of CD4 for antigen-specific signaling. Previous studies raise the possibility that the relevant parameter in TCR:peptide/MHC interaction that dictates the physiological outcome is likely to be the dissociation rate (99-101). Experiments are underway to compare the dissociation rates of $LACK_{156-173}$-specific TCRs using an improved LACK/I-A^d-based staining reagent in a flow cytometric assay. In addition, surface plasmon resonance analysis should provide a direct measurement of the kinetic properties (on-rates and off-rates) of WT and KO TCRs which would allow a direct estimation of the contribution of CD4 in terms of TCR:peptide/MHC avidity in thymic selection. Independently, efforts are underway to determine the difference in ligand-binding kinetics between WT and KO TCRs specific for a photoactivatable variant of the HA peptide.

It should be noted that the relative avidity TCRs exhibit for their specific antigens may or may not parallel their avidity towards their thymic selecting ligands. Therefore, the contribution of CD4 to avidity during thymic selection can not be directly inferred from comparing the affinities of WT and KO TCRs towards their antigenic ligands. To address the role of CD4 in thymic selection more directly, efforts are underway to generate transgenic mice that express $LACK_{156-173}$/I-A^d on their thymic epithelium. The peptide/MHC transgenic mice will be bred to $LACK_{156-173}$-specific TCR transgenic mice. Selection of $LACK_{156-173}$-specific TCRs on LACK/I-A^d in the thymus can then be studied. It should be possible through this approach to determine the effect of varied TCR:peptide/MHC affinities on thymic selection and lineage commitment.

Transgenic mouse studies suggest that even though TCRs selected in the absence of CD4 may differ from those selected in the presence of CD4 in their ligand-binding properties, the differences are not significant enough to exclude them from the normal repertoire. Thus, two TCRs derived from $CD4^{-/-}$ mice could be efficiently selected in the presence of CD4 in transgenic mice. Furthermore, the KO15 TCR failed to be efficiently selected in the absence of CD4 in transgenic mice, a property that would have been predicted for a wildtype-derived TCR, but not for a KO TCR. Our data suggest that the TCR repertoires selected in the presence and the absence of CD4 can overlap. However, the degree of overlap can not be fully appreciated without generating and analyzing more TCR transgenic mice.

Efforts are also underway to study the ligand-binding properties of antigen-specific T cells directly from immunized mice using LACK/I-A^d tetrameric staining reagent. Such experiments will improve the scope of our analysis, should help confirm

our conclusions and will provide additional information concerning the degree of overlap between the CD4-dependent and CD4-independent repertoire.

Despite the evident potential for coreceptor-independent T helper cell development, it is nonetheless clear that the loss of CD4 disables key aspects of normal T helper cell differentiation and homeostasis. Thus, CD4-deficient T helper cells are manifestly impaired in their capacity to become Th2 cells (72, 73) and they also demonstrate reduced extrathymic survival (90). Although the molecular basis of these defects is presently unclear, they nonetheless reveal a necessary role for CD4 in the modulation of TCR signaling during immune responses and extrathymic selection. As such, they suggest evolved functions of CD4 that are apparently not readily supplanted by the compensations available to T helper cells that develop in CD4-deficient mice.

The benefit that the CD4 coreceptor confers on the modern vertebrate immune system can thus be assessed both in terms of its effect on the kinetic properties of the TCRs used by T helper cells and also in terms of its effect on fate-determining TCR signal transduction. With respect to the former, CD4 may well be important for setting limits on the kinetic properties of the TCR repertoire during selection. Such limits are likely to be essential for the emergence of a repertoire with the capacity to discriminate between ligands that differ from one another in relatively minor ways. With respect to the latter, CD4 has the capacity to change the amplitude and/or character of TCR signals in a straightforward fashion both through its direct association with the tyrosine kinase $p56^{lck}$ and by its potential effect on the stability of the TCR:peptide/MHC complex. Studying the consequences of CD4 deficiency and the compensations required to

CHAPTER FIVE: A GENETIC APPROACH FOR STUDYING CD4$^+$ MEMORY T CELLS

INTRODUCTION

Immunological memory refers to the phenomenon that the immune system mounts a more rapid and more vigorous response to antigens that have been previously encountered (reviewed in (105)). The existence of immunological memory has long been recognized, however, its cellular and molecular basis is not well understood. During the past two to three decades, there have been two major schools of thought regarding the nature of immunological memory. The first suggests that immunological memory is the result of an increase in the precursor frequency of antigen-specific cells after primary stimulation. The second suggests that immunological memory is carried by a population of differentiated memory cells that differ qualitatively from naïve and effector cells (reviewed in (105)). The term "memory cells" will be used loosely in this chapter to refer to cells that participate in immunological memory.

Antigen-specific T cell responses can be broken down into four phases: activation/ expansion, effector, activation-induced cell death (AICD), and memory. During the initial phase, significant (200- to 200,000-fold) expansion of antigen-specific T cells occurs in either the CD4$^+$ or the CD8$^+$ compartments (106-110). This expansion creates a large pool of effector cells that help to clear the host of the infecting pathogens. In the third stage, approximately 95% of the effector cells die by activation-induced cell death (107). The frequency of antigen-specific T cells that survive in the memory phase stays 20- to 10,000-fold above unimmunized background levels (106-108), with the size of the memory population determined in part by the initial clonal burst size (107, 111).

The increase in antigen-specific precursor frequency in the memory phase undoubtedly contributes to the increased magnitude of recall responses to antigens.

Just as there are two major models for the overall nature of immunological memory, there are also two models for how memory T cells develop from naïve precursors. The linear differentiation model predicts that memory T cells are the progeny of effector T cells that escape AICD. The decreasing potential model predicts that memory T cells are derived directly from naïve T cells and differentiate through a lineage parallel to effector cells (112). Evidence supporting the parallel differentiation of memory and effector T cells includes studies demonstrating that a weak stimulus leads to the generation of memory T cells while a strong stimulus leads to the generation of effector T cells (113, 114). Evidence for the linear differentiation model comes from a recent study that demonstrates that $CD8^+$ memory T cells are generated after strong antigenic stimulation and are the progeny of cytotoxic effector cells (115). Furthermore, a recent study suggests that a specific subset of effector cells contributes to CD8 memory (116).

Many studies suggest that there are qualitative differences between memory and naïve T cells. For example, $CD4^+$ memory cells may require less co-stimulation for activation than naïve cells (117). Recent studies demonstrate progressive clonal selection of antigen-specific $CD4^+$ T cells during primary and secondary responses, suggesting that memory cells may have greater reactivity for antigen than naïve cells on a population level (95, 109). Memory helper cells also produce different cytokines from naïve cells (118). Both $CD4^+$ and $CD8^+$ memory T cells reach their effector potentials more rapidly than naïve cells (119-124). A number of mechanisms may contribute to

these differences in the activation of memory versus naïve cells: modulation of chromatin structure of cytokine genes (125), methylation of cytokine genes (126), utilization of different signaling pathways (127), and regulation of co-receptor functions (128, 129). Memory cells also respond differently to Fas engagement from naïve cells (91). Furthermore, memory and naïve cells express different levels of adhesion molecules (130, 131), and they may have distinct recirculation pathways (124, 132). Taken together, there is good evidence that memory T cells are qualitatively distinct from naïve T cells in their capacity to respond to antigens. These qualitative differences in antigen reactivity may make a significant contribution to the accelerated course of memory responses.

Recent studies on the homeostasis of memory T cells have effectively put an end to the age-long debate on the subject of whether the maintenance of immunological memory requires persistence of antigen. Using different experimental systems, several groups convincingly demonstrated that neither $CD4^+$ nor $CD8^+$ memory T cells required antigen for survival (133-136). Moreover, contact with MHC molecules may not be required for the survival of memory T cells (133, 134). This is in stark contrast to naïve T cells, which appear to require contact with self-peptide/MHC complexes for long-term survival (134, 136-140).

The expression of a number of cell surface molecules has been correlated with the capacity of cells to respond to recall antigens (130, 131), and these cell surface molecules are frequently used to distinguish memory from naïve cells. Naïve T cells are said to be CD44 low, CD45RA/B/C high (CD45RO low), L-selectin high, VLA low,

while memory cells are thought to exhibit the opposite phenotype. Recent studies report upregulation of IL-2Rβ expression on $CD8^+$ memory cells (141, 142). However, several studies call into question the reliability of these markers for distinguishing naïve and memory cells. For instance, it has been demonstrated that $CD4^+$ T cells with a CD45RB+ or CD45RB- phenotype can interconvert *in vivo* (143). Moreover, in a TCR transgenic system, viral-specific $CD8^+$ memory cells were found to be heterogeneous, with some cells expressing memory/activation markers, while others expressing some naïve markers (110). Other studies have suggested that a fraction of memory cells revert to a resting state that is not readily distinguishable from naïve cells (144). Finally, effector cells express many of the same surface markers as memory cells. Indeed, some of the widely used "memory markers" such as CD45RO may be more readily associated with a recently-activated state rather than a memory state (144, 145). Therefore, at the present time, it is often difficult to define the memory population accurately using the available surface markers.

Given the limitations of existing markers for memory T cells, we sought to build a genetic system that would allow for high fidelity labeling of memory T cells. Cells that are labeled with this system can then be isolated, characterized and tested for memory activity. For instance, cell lineage, cell surface phenotype, cell cycle, life span, and activation requirements of the labeled cells could be examined. The genetic system has two components, a recombinase that is expressed in an activation-dependent fashion and a substrate for the recombinase that serves as the genetic marker.

For the first part of the system, the bacteriophage P1 recombinase Cre was inserted into the mouse *ox40* locus to achieve activation-dependent expression. Mouse

OX40 expression appears to be confined to the period when T cells are activated by antigens (146, 147). Of particular relevance to this project, there is no significant OX40 expression on thymocytes (148), so Cre-mediated recombination events will not occur before T cells encounter their specific-antigens in the periphery.

The substrate for the Cre recombinase is a transgene encoding a combination of two reporters. The coding sequence for the first reporter (R1) is flanked by two directly-repeated *lox P* sites and is followed by a second reporter (R2) (Figure 15A). In naïve cells, the *ox40* locus is silent, Cre therefore should not be expressed, and R1 should be expressed. Once the cells become activated by antigen exposure, Cre should be expressed from the *ox40* locus and act on the *lox P* sites, resulting in the removal of R1 and the expression of R2 (Figure 15B). Thus, this Cre-dependent reporter system should allow $CD4^+$ T cells to be divided into naïve and activated/post-activation cells by their differential surface expression of R1 and R2, respectively.

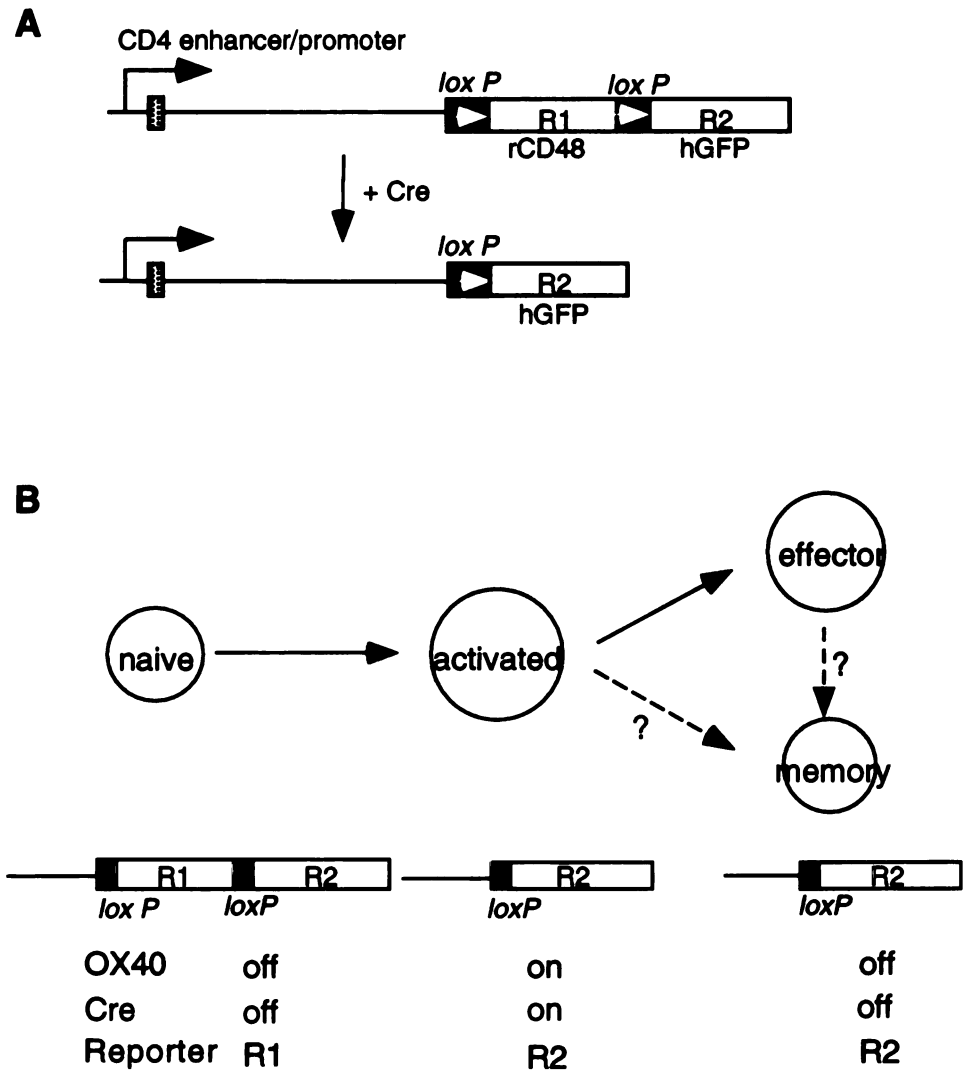

Figure 15. (A) Schematic representation of the reporter transgenes. (B) Expression of OX40, the Cre recombinase and transgenic reporters in different T cell populations..

MATERIALS AND METHODS

Generation of OX40-Cre-neo mutant mice

PCR-based mutagenesis was used to remove the translation initiation codon ATG from the first exon of the ox40 locus. Cre and FRT-neo-FRT (gift of Dr. Kevin Jones) were inserted immediately upstream of the removed ATG. The FRT-neo-FRT cassette contained a neomycin resistance gene under the control of the PGK promoter flanked by two directly-repeated FRT sites. HSV-TK cassette (gift of Drs. Kirk Thomas, Suzanne Mansour and Mario Capecchi) was inserted downstream of the 3' homology arm of the ox40 locus.

$20x10^6$ JM-1 (129) ES cells were transfected with 20μg of the linearized targeting construct by electroporation using a Bio-Rad Gene Pulser (Bio-Rad), 500μF, 250V. The cells were plated on primary embryonic feeder cells; G418 (200mg/ml) and gancyclovir (2μM) were then applied 36 hours after electroporation. 10 days later, individual G418- and gancyclovir-resistant clones were picked to 96-well plates containing feeder cells. The clones were expanded and then split in half so that a portion of each could be frozen in 96-well plates, and the remaining portion could be expanded for DNA extraction and Southern blot analysis. A probe on the 5' side of the targeted region was used on an EcoRV digest of the genomic DNA to identify targeted clones. The structure of the mutant allele was subsequently confirmed using a 3' probe on the same digest. Approximately 30% of neo- and gancyclovir-resistant colonies were mutant at the ox40 locus. Chimeric mice were produced by injection of mutant ES cells into 3.5-day-old blastocysts. The chimeric male mice were bred with C57BL/6 female mice, and germline transmission of the mutant allele was confirmed by Southern blot

analysis of tail DNA. Mice were maintained by the Animal Care facility in the UCSF Parnassus Heights Barrier Facility.

In Vitro activation of lymphocytes

Lymph node cells were harvested and resuspended at $2x10^6$ cells/ml in complete DMEM media (10% FCS, L-glutamine, non-essential amino acids, penicillin/streptomycin, 25mM HEPES) and activated with 10ng/ml of PMA plus 500ng/ml of ionomycin. Activated cells were harvested 24, 48, 72, and 96 hours after activation for various assays.

Western blot analysis of Cre

$10x10^6$ lymphocytes were lysed in 200µl of 1% NP-40 lysis buffer (10mM Tris, 150mM NaCl, 2mM EDTA, 1mM PMSF, 10µg/ml Leupeptin, 10µg/ml aprotinin). The whole cell lysate was either separated by SDS-PAGE or immunoprecipitated with the anti-Cre mAb 7.23 (BAbCO) in conjuncture with anti-mIgG-agarose beads (Sigma). Immunoprecipitated material was separated by SDS-PAGE and subsequently transferred onto a nitrocellulose membrane. The membrane was blotted with a rabbit anti-Cre anti-sera (B4197), and detected with HRP-conjugated anti rabbit antibody using ECL reagents (Amersham).

Intracellular staining of Cre

Cells were resuspended at $2\text{-}5x10^6$ cells/ml in PBS and fixed with the addition of an equal volume of 4% paraformaldehyde in PBS. The fixed cells were washed,

permeabilized in 0.5% saponin (Sigma) solution (PBS, 0.3% BSA, 0.01% NaN3), and stained with anti-Cre mAb 7.23 (BAbCO). 7% normal goat serum was used as a blocking reagent to reduce background. FITC-goat-anti-mouse IgG (Pharmingen) was used as a secondary reagent. Cells were stained at the same time with different combinations of anti-CD4, CD8 (CALTAG), CD3ε, CD25, CD69 (Pharmingen) antibodies. In some cases, cells were also stained for OX40 expression using an OX40L-humanIgG fusion protein (MSL-huIgG, gift of Dr. Wayne Godfrey) followed by PE-anti-huIg (American Qualex).

RT-PCR

Total RNA was prepared from naïve or activated thymocytes, splenocytes and lymphocytes using TRI Reagent (Molecular Research Center, Inc.). RNA was converted to cDNA using SuperScript II reverse transcriptase (Gibco BRL). 5' primer CER5 5'-cgggctgccacgaccaagtg-3' and 3' primer CRE3 5'-ggcagatggcgcggcaacac-3' were used to amplify Cre cDNA in a PCR assay. Actin primers were used as controls: 5'-gtttgagaccttcaacacccc-3' (sense) and 5'-gtggccatctcctgctcgaagtc-3' (anti-sense).

Reporter transgenic mice

Plasmids encoding rat CD48 and the T33E mutant of rat CD48 (deficient in CD2 binding) were obtained from Dr. Anton van der Merwe (University of Oxford). CDw52 cDNA was obtained by RT-PCR from Jurkat RNA using the following primers: 5' primer 5'-caucaucaucaugtcgacagccacgaagatcctac-3' and 3' primer 5'-cuacuacuacuagtcgacgtgtcacctcaactgaag-3'. hGFP cDNA was obtained from Clonetech.

The Tva cDNA was provided by Dr. Kurt Zingler. All reporters were subcloned into the pNB1 vector, which contained two directly-repeated *lox P* sites embedded in the multiple cloning site of pBlueScript KS^+. lox-reporter-lox cassettes were excised and subcloned into both the EF-BOS expression vector (82) and the CD4 enhancer/promoter vector (74) upstream of a second reporter.

Transgene constructs were linearized, purified by electroelution and injected into B6/CBA embryos. Transgenic founders were identified by Southern blot analysis of tail DNA, and analyzed by FACS to determine reporter expression on peripheral blood lymphocytes. Transgenic founders were bred with C57BL/6 mice, and the transgenic offspring were subsequently bred with OX40-Cre-neo or OX40-Cre mutant mice. Double mutant mice were identified by Southern blot analysis and analyzed by FACS staining.

Transient transfection

$10x10^6$ Jurkat or EL-4 cells were transfected with either 10μg of EF-BOS-reporter construct alone or 10μg of EF-BOS-reporter plus 10μg of EF-BOS-Cre. The cells were transfected by electroporation using a Bio-Rad Gene Pulser (Bio-Rad), 240V, 960μF. Transfected cells were cultured in 10ml of complete RPMI or DMEM media. Cells were harvested and stained for reporter expression 24, 48 or 72 hours after transfection.

Purified OX45 antibody (gift of Dr. Anton van der Merwe) was used to detect rCD48 and rCD48T33E. FITC-anti-mIgG (Pharmingen) was used as a secondary reagent. Purified anti-hCD52 (BioSource) was used to detect CDw52. Biotin-anti-rat

IgM (Pharmingen) was used as secondary reagent and Strepavidin-PE (Caltag) was used as tertiary reagent. SuA-rabbit IgG fusion protein was used to detect TVA (75, 77). FITC-anti-rabbit IgG (Jackson Laboratory) was used as a secondary reagent.

MLR

Lymph node cells were harvested from OX40-Cre/reporter double-mutant mice (H-2^b). $30x10^6$ cells were mixed with an equal number of irradiated spleen cells from DBA/2 mice (H-2^d) in 20ml of complete DMEM media. Cells were harvested on day 8 and day14 and analyzed for surface expression of the transgenic reporters. Responder cells were identified by the absence of H-$2K^d$.

RESULTS

Expression of the Cre Recombinase in Activated T Cells

Generation of OX40-Cre-neo Mice

The open reading frame encoding the Cre recombinase was inserted directly into the OX40 locus immediately upstream of the translation initiation codon in exon 1 (Figure 16A). Two types of constructs were built, (a) OX40-Cre-neo and (b) OX40-neo-Cre, and they were transfected into JM-1 (129) ES cells by electroporation. The resulting ES cell colonies were screened by Southern blot analysis. Multiple digests and probes were used to confirm the expected structure of the targeted locus. The correctly targeted clones were injected into C57BL/6 blastocysts to generate chimeric mice. B6/129 chimeric males were bred to C57BL/6 female mice to achieve germline transmission of the mutant OX40-Cre allele. Offspring bearing the targeted OX40 locus were identified by Southern blot analysis of tail DNA (Figure 16B).

Expression of the Cre Recombinase in OX40-Cre-neo Mice

To test for activation-induced expression of the Cre recombinase from the OX40 locus, lymph node cells were isolated from OX40-Cre-neo mice and activated *in vitro* with either PMA plus ionomycin or anti-CD3ε antibody. The activated lymphocytes were examined for surface expression of OX40 and co-expression of Cre by intracellular staining (Figure 17A). Cre expression in these activated lymphocytes was further assessed by immunoprecipitation and Western blot analysis (Figure 17B). Results from these analyses demonstrated constitutive Cre expression from the targeted OX40 locus regardless of the activation status of the lymphocytes. This pattern of expression is

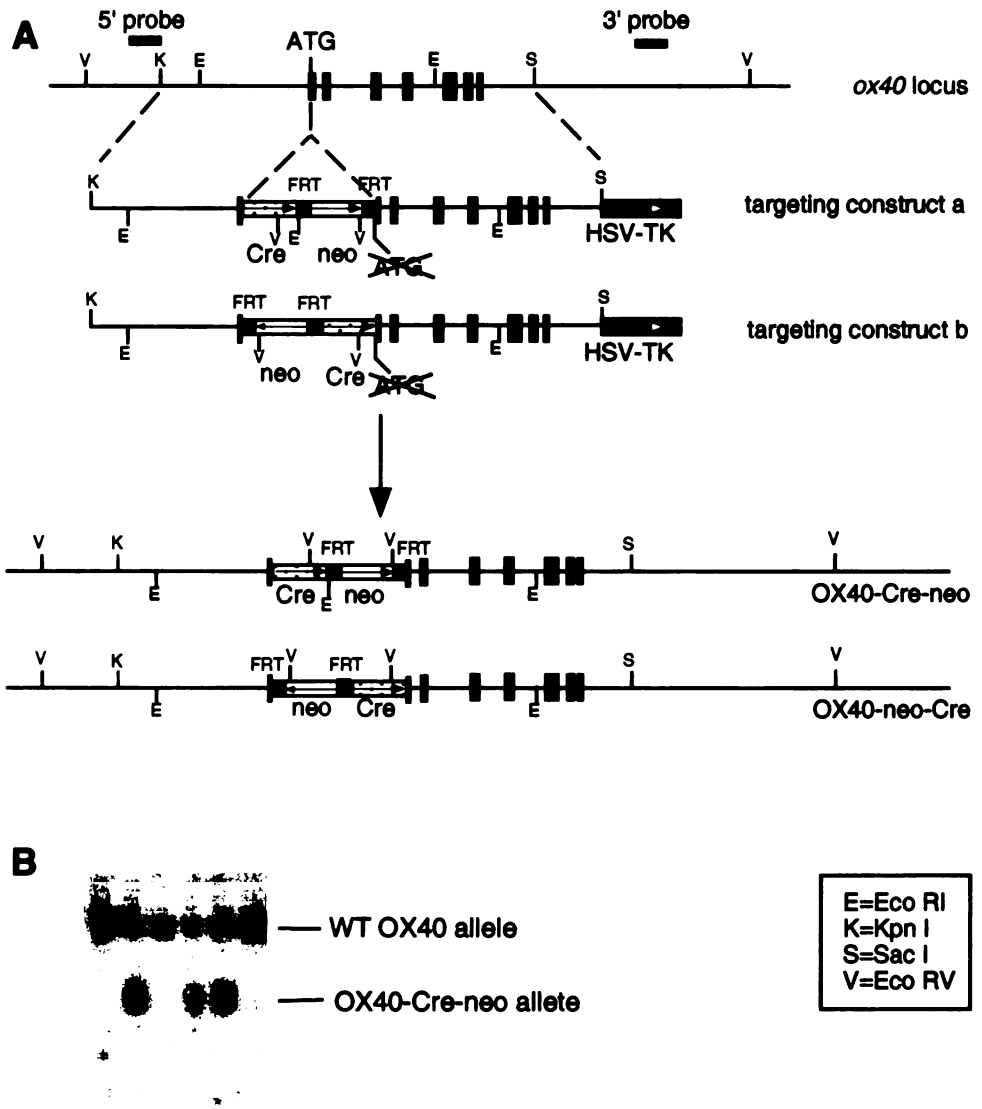

Figure 16. (A) Targeting the Cre recombinase into the mouse *ox40* locus via homologous recombination. (B) Southern blot analysis of of tail DNA from wildtype and OX40-Cre-neo mutant mice. Tail DNA was digested with EcoRV and probed with the ox40 5' probe indicated in (A).

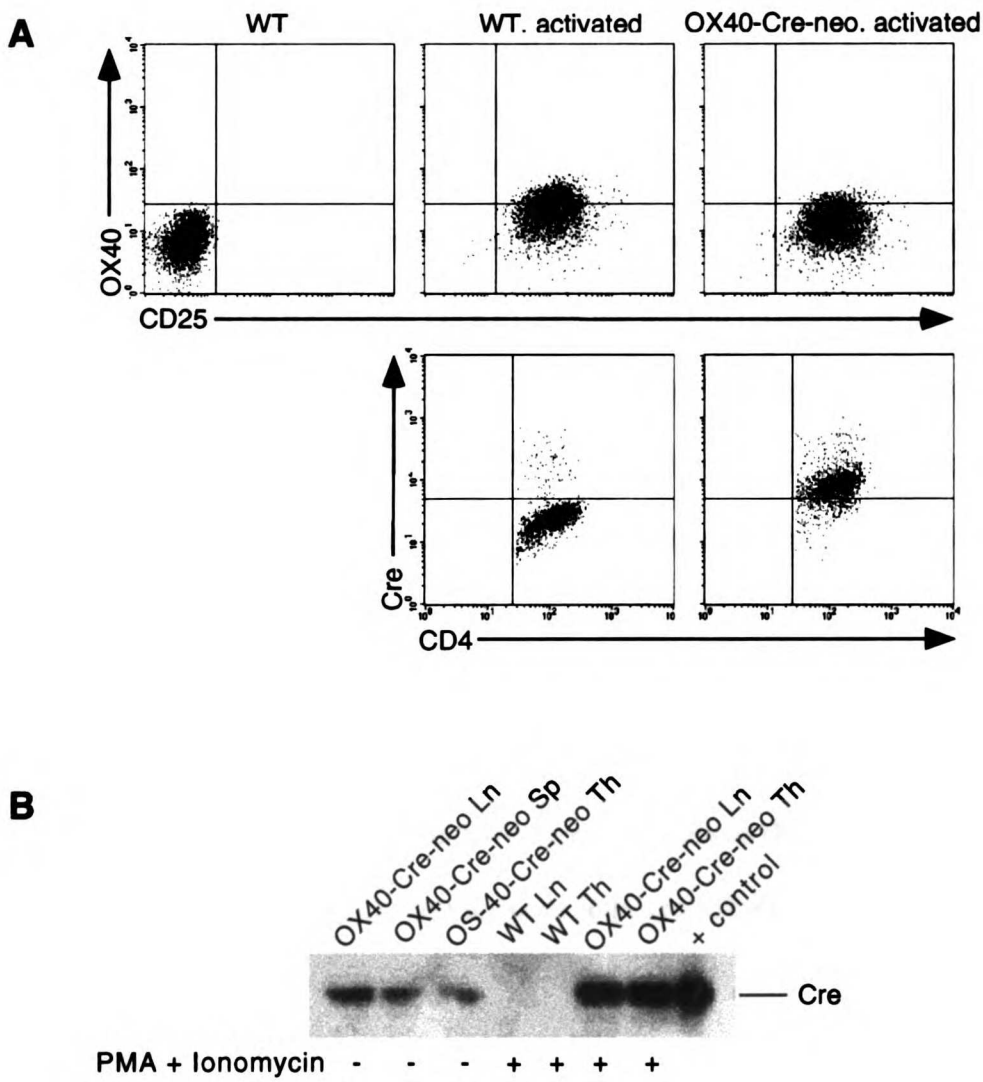

Figure 17. *In vitro* activation of lymphocytes from OX40-Cre-neo and wildtype (WT) control mice. (A) Intracellular FACS analysis of unactivated and activated lymph node cells. Lymphocytes were activated with 10ng/ml PMA and 500ng/ml ionomycin for 3 days. (B) Cre expression in unactivated and activated lymph node cells (Ln), splenocytes (Sp) and thymocytes (Th). Whole cell lysate was immunoprecipitated with a mouse anti-Cre mAb 7.23 and detected with a rabbit anti-Cre sera B4197.

obviously distinct from that of the native OX40 gene and probably caused by the presence of the PGK-neo^r cassette within the locus.

Expression of the Cre Recombinase in OX40-Cre Mice

To eliminate the effect of the PGK-neo^r cassette on Cre expression, the OX40-Cre-neo mice were bred to transgenic mice expressing the Flp recombinase under the control of the human actin promoter (hAct-Flp) (gift of Dr. Gail Martin) (Figure 18A). Southern blot analysis of tail DNA confirmed that the PGK-neo^r cassette had been removed in mice carrying both mutations (Figure 18B). The neo-deleted OX40-Cre allele was then separated from the hAct-Flp transgene through breeding. Lymph node cells were isolated from OX40-Cre mice and activated *in vitro* with PMA plus ionomycin. No Cre expression was detected in either unactivated or activated lymphocytes by immunoprecipitation and Western blot analysis (Figure 18C). RT-PCR analysis also failed to detect any Cre transcript in activated OX40-Cre lymphocytes (data not shown).

Two possible explanations for the absence of Cre expression from the mutant allele include aberrant methylation of the mutant locus due to the Cre insertion and inappropriate splicing of the composite allele (149). To circumvent the problem, we designed a new targeting construct in which the Cre coding sequence was inserted into exon 3 of the OX40 locus instead of exon 1. To allow for Cre expression in this context, the Cre coding sequences was preceded by an IRES (internal ribosomal entry sequence) element and followed by a SV40 poly A element (Figure 19). Cre expression from this revised OX40-Cre mutant allele will be discussed later in the chapter.

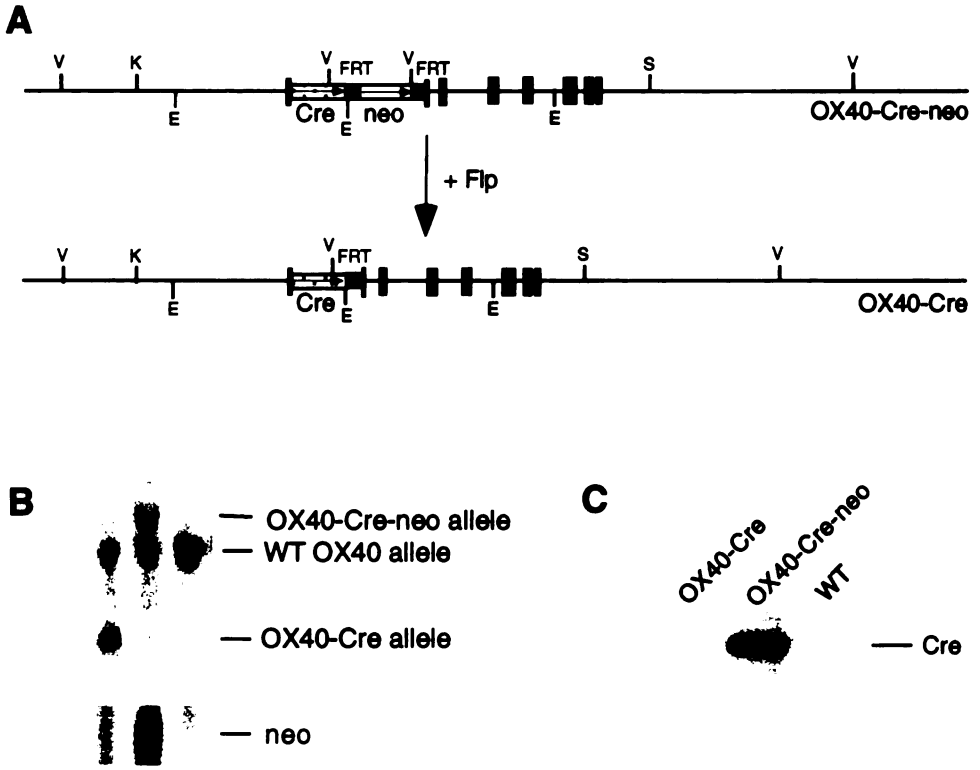

Figure 18. Removal of PGK-neo^r cassette by Flp-mediated recombination. (A) OX40-Cre-neo targeted allele and the recombination product OX40-Cre. (B) Southern blot analysis of tail DNA from OX40-Cre, OX40-Cre-neo and wildtype mice. Top, EcoRI digest probed with an OX40 cDNA probe. Bottom, EcoRI digest probed with a neo probe. (C) Cre expression in activated lymphocytes. Lymph node cells were activated *in vitro* with 10ng/ml PMA plus 500ng/ml ionomycin for 3 days. Whole cell lysate was precipitated with a mouse anti-Cre mAb 7.23 and detected with a rabbit anti-Cre sera B4197.

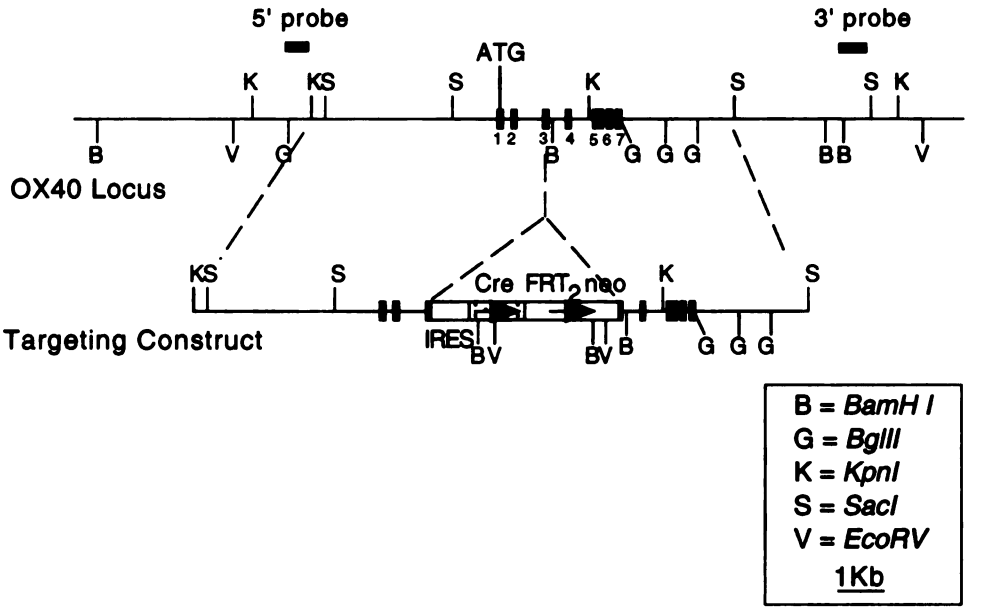

Figure 19. A revised targeting strategy for expressing the Cre recombinase from the *ox40* locus.

Reporter Transgenic Mice

Generating Reporter Constructs

The substrate for use with OX40-Cre was a transgene encoding a combination of two reporters under the transcriptional regulation of the murine CD4 promoter and enhancer elements (74). Four molecules were selected as reporters: i) rCD48T33E (rCD48*), a mutated version of rat CD48 that does not bind its ligand rat CD2 (150). ii) CD52, a small GPI-linked human protein that is heavily glycosylated and is recognized by a monoclonal antibody CAMPATH-1 (an IgM antibody that is an effective reagent for *in vivo* depletion experiments) (151). iii) hGFP, a human codon-optimized variant of GFP. Expression of GFP is unlikely to interfere with normal T cell development and activation, and cells expressing hGFP can be conveniently isolated by FACS sorting. iv) Tva, the receptor for subgroup A Rous sarcoma virus (75, 77). Expression of Tva on the cell surface permits delivery of genes via an Avian Leukosis retroviral vector (152).

The reporter constructs were tested in Jurkat (human) and EL-4 (mouse) cells by transient transfection. R1 (rCD48 in this case) but not R2 (hGFP) expression was detected by FACS staining in Jurkat cells transfected with the reporter construct alone (Figure 20B). In cells co-transfected with the reporter construct and a Cre-expressing vector EF-BOS-Cre, expression of R2 (hGFP) was detected, and expression of R1 (rCD48) was slightly diminished but not completely extinguished. In these short-term assays, the persistence of R1 expression in the presence of Cre was likely to be due to the persistence of R1 protein synthesized prior to the loss of R1 coding sequence mediated by the Cre recombinase. Similar results were obtained with all of the reporter

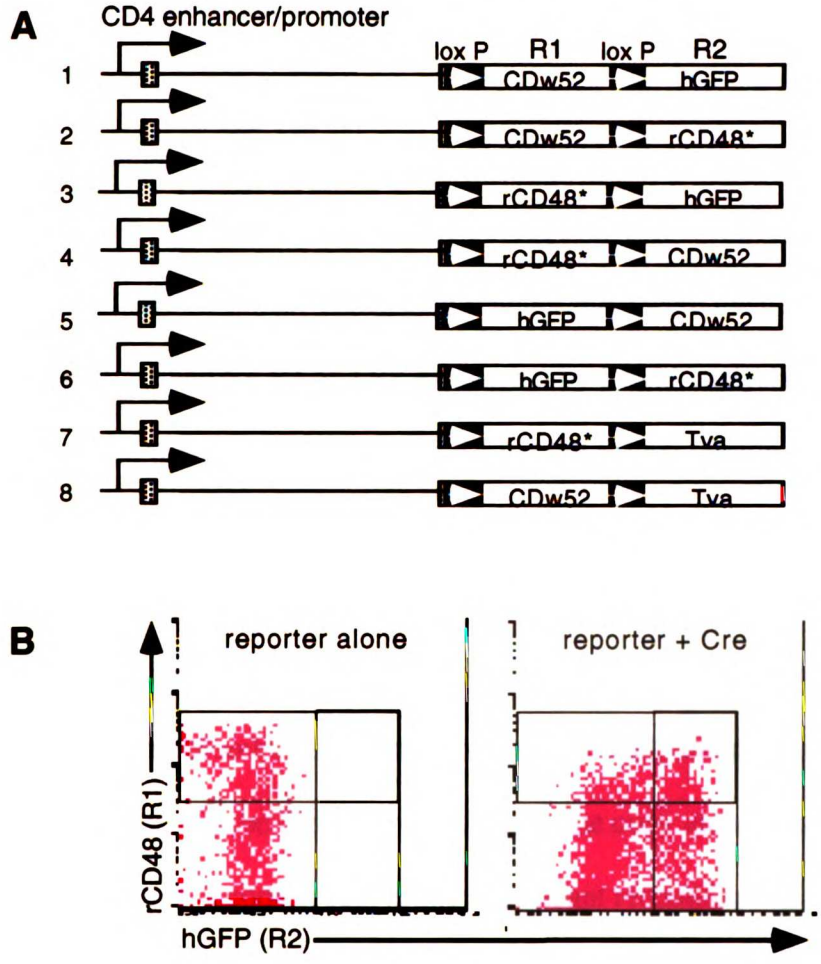

Figure 20. (A) Schematic representation of the reporter transgene constructs. (B) *In vitro* recombination of a transgenic reporter mediated by Cre. Jurkat cells were either transfected with the reporter EF-BOS-lox-rCD48*-lox-hGFP alone or co-transfected with the reporter and EF-BOS-Cre. Cells were harvested 24 hours post-transfection and analyzed by FACS.

constructs (data not shown). These transient transfection assays demonstrated that the Cre-lox system functioned as expected in the above cell lines.

In Vivo Recombination of the Reporters Mediated by the Cre Recombinase

Selected reporter constructs were subsequently microinjected into fertilized eggs to generate transgenic mice. Transgenic founder mice were identified by Southern blot analysis and screened for R1 and R2 expression by FACS analysis. Multiple lines of transgenic mice were obtained showing R1 but not R2 expression on peripheral blood T cells as expected (Figure 21B and data not shown). The reporter transgenic mice carried high copy number of the transgenes (10 copies in most cases, data not shown).

Selected reporter transgenic mice were bred with OX40-Cre-neo mice that expressed the Cre recombinase constitutively. In double mutant mice carrying both the reporter transgene and the OX40-Cre-neo allele, the copy number of the transgenes was greatly reduced to 1-2 copies, suggesting significant recombination at the transgene locus (Figure 21A). Southern blot analysis further demonstrated the loss of R1 in OX40-Cre-neo/reporter mice (data not shown). FACS analysis of peripheral blood lymphocytes confirmed the loss of R1 expression in the double mutant mice (Figure 21B). The level of residual R1 expression varied among different transgenic lines with complete loss of R1 expression observed in some lines (data not shown). Since there were a large number of directly-repeated *lox P* sites in the transgene locus, Cre-mediated recombination might be incomplete in some cases, resulting in residual R1 expression. Unfortunately, expression of R2 could not be detected in any of the double mutant mice (Figure 21B and data not shown). The absence of R2 expression could be due to a

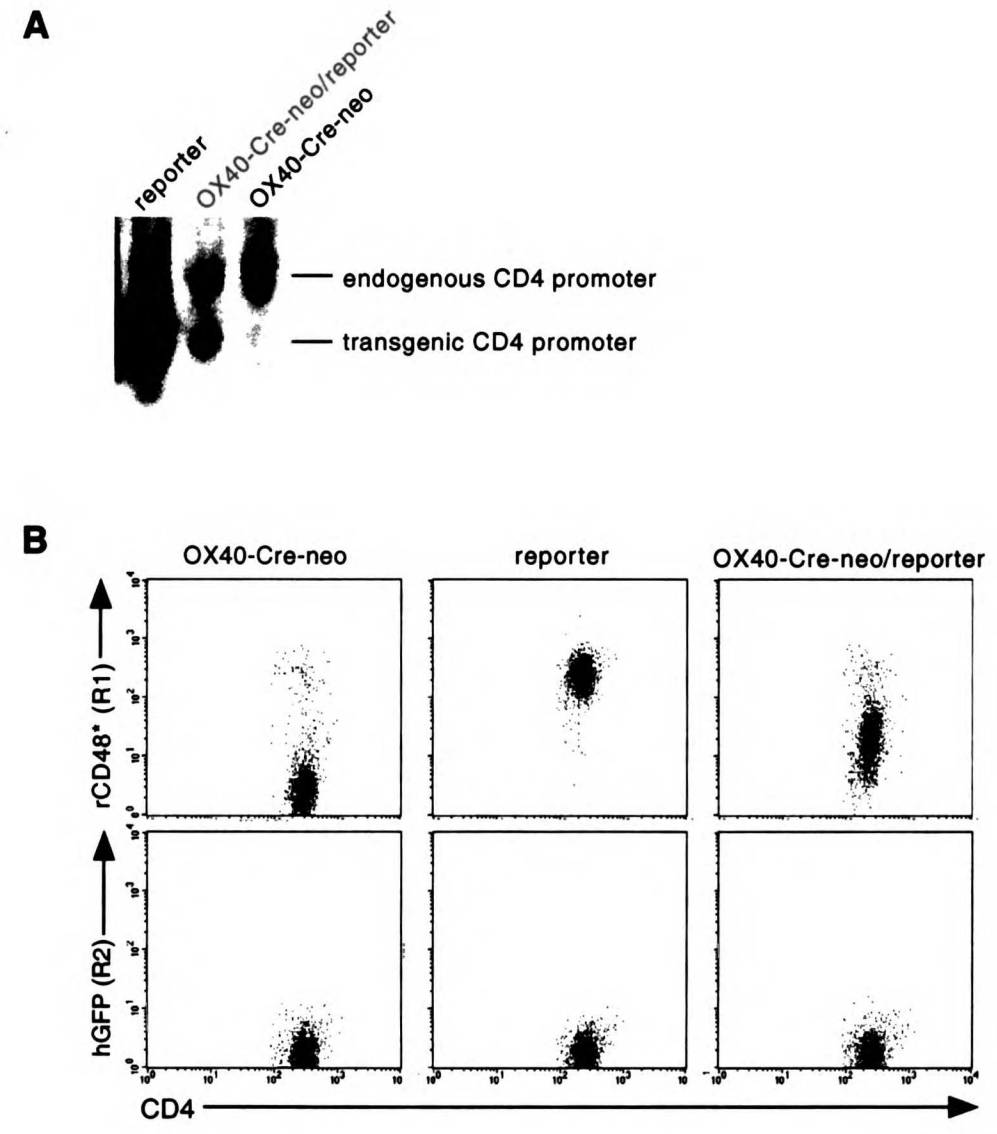

Figure 21. *In vivo* recombination of a reporter transgene CD4-lox-rCD48*-lox-hGFP mediated by the Cre recombinase. (A) Reduction in transgene copy number mediated by Cre in OX40-Cre-neo/reporter double mutant mice. Tail DNA from single and double mutant mice was digested with BamH1 and probed with a CD4 promoter probe. (B) Expression of rCD48* (R1) and hGFP (R2) on peripheral $CD4^+$ T cells from single and double mutant mice.

number of reasons. First, rearrangement at the transgene locus mediated by the Cre recombinase might not leave behind a functional R2 gene because of the structure of the transgenic array. Second, expression from one copy of R2 coding sequence left in the rearranged transgene locus might be too low to be detected. Nevertheless, despite the absence of detectable R2 expression, cells with a different history of Cre expression could be distinguished by their different R1 expression. Thus, the reporter transgenic mice could be used to label cells that had undergone Cre expression.

It seems reasonable that further screening of additional transgenic lines could allow for R2-expressing lines to be isolated. Additional approaches to improve the transgenic reporter system include electroporating reporter constructs into ES cells to allow for selection of clones with low transgene copy numbers. Alternatively, a single copy of the reporters could be directly inserted into a lymphocyte-specific gene (e.g. CD2, CD4) via homologous recombination. Reducing the number of integrated transgenes should reduce the degree of rearrangement mediated by the Cre recombinase, thus preserving the structure of the transgene locus and the expression of R2.

OX40-Cre/Reporter Double Mutant Mice

The reporter transgenics were bred with the OX40-Cre mice to generate OX40-Cre/reporter double mutant mice. The presence of both mutations was confirmed by Southern blot analysis (data not shown). FACS analysis showed no decrease in the level of R1 expression in T cells from the double mutant mice compared to mice carrying reporter transgene alone (Figure 22A). This result was predicted based on the absence of Cre protein in the OX40-Cre mice.

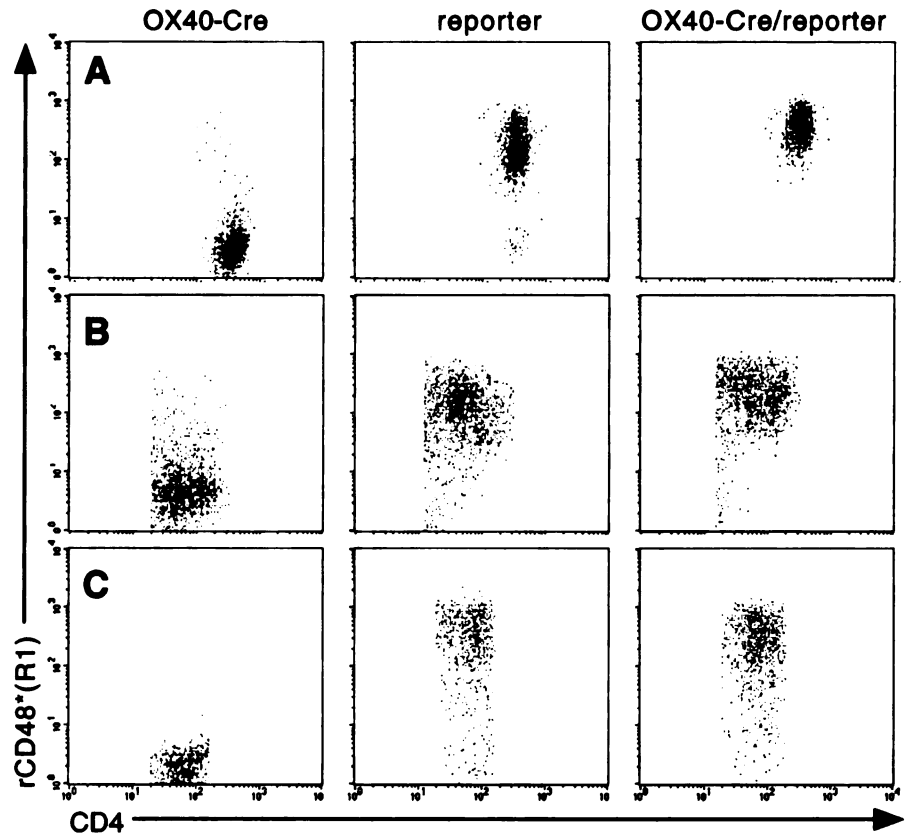

Figure 22. *In vitro* activation of lymph node cells from OX40-Cre/reporter (CD4-lox-rCD48*-lox-hGFP) double mutant mice. (A) Surface expression of rCD48* (R1) on unactivated $CD4^+$ T cells. (B) rCD48* expression on $CD4^+$ T cells 24 hours after *in vitro* activation with 10ng/ml PMA plus 500ng/ml ionomycin. (C) rCD48* expression on $CD4^+$ T cells 14 days into MLR. Lymph node cells from double mutant mice ($H\text{-}2^b$) were the responder cells. Splenocytes from DBA mice ($H\text{-}2^d$) were used as stimulator cells. Responder cells were identified by the absence of $H\text{-}2K^d$ expression.

To test further for Cre expression, lymph node cells were harvested from OX40-Cre/reporter double mutant mice and activated *in vitro* with PMA and ionomycin. R1 expression on $CD4^+$ T cells was followed by FACS analysis over a period of 4 days. R1 expression on the double mutant cells showed no significant change over time and remained comparable to R1 level on reporter transgenic cells, again suggesting the lack of Cre activity (Figure 22B). Finally, lymph node cells from OX40-Cre/reporter double mutant mice were mixed with irradiated allogeneic spleen cells in a long-term mixed lymphocyte reaction (MLR). Loss of R1 expression was not detected on $CD4^+$ T cells 14 days into MLR (Figure 22C), again confirming the absence of Cre expression/function in these activated lymphocytes.

A New OX40-Cre Allele

Mice carrying the revised OX40-Cre allele (IRES-Cre in exon 3 as mentioned above) were generated by gene targeting as before. Triple-mutant mice were generated that carried the new OX40-Cre allele, the $CD4^{null}$ allele (68) and a conditional CD4 allele (90) (Figure 23A). CD4 expression can be shut off from the conditional allele through Cre-mediated recombination. Lymphocytes were harvested from these triple-mutant mice and activated *in vitro* with PMA plus ionomycin. CD4 expression from the conditional allele was monitored by FACS analysis. While 30% of unactivated CD4 lineage T cells (defined by the presence of CD3 and the absence of CD8 expression) lacked CD4 expression, approximately 70% of them lost CD4 expression during activation, indicating activation-induced Cre expression of in these cells (Figure 23B). This Cre expression was confirmed by intracellular FACS (data not shown) and Western

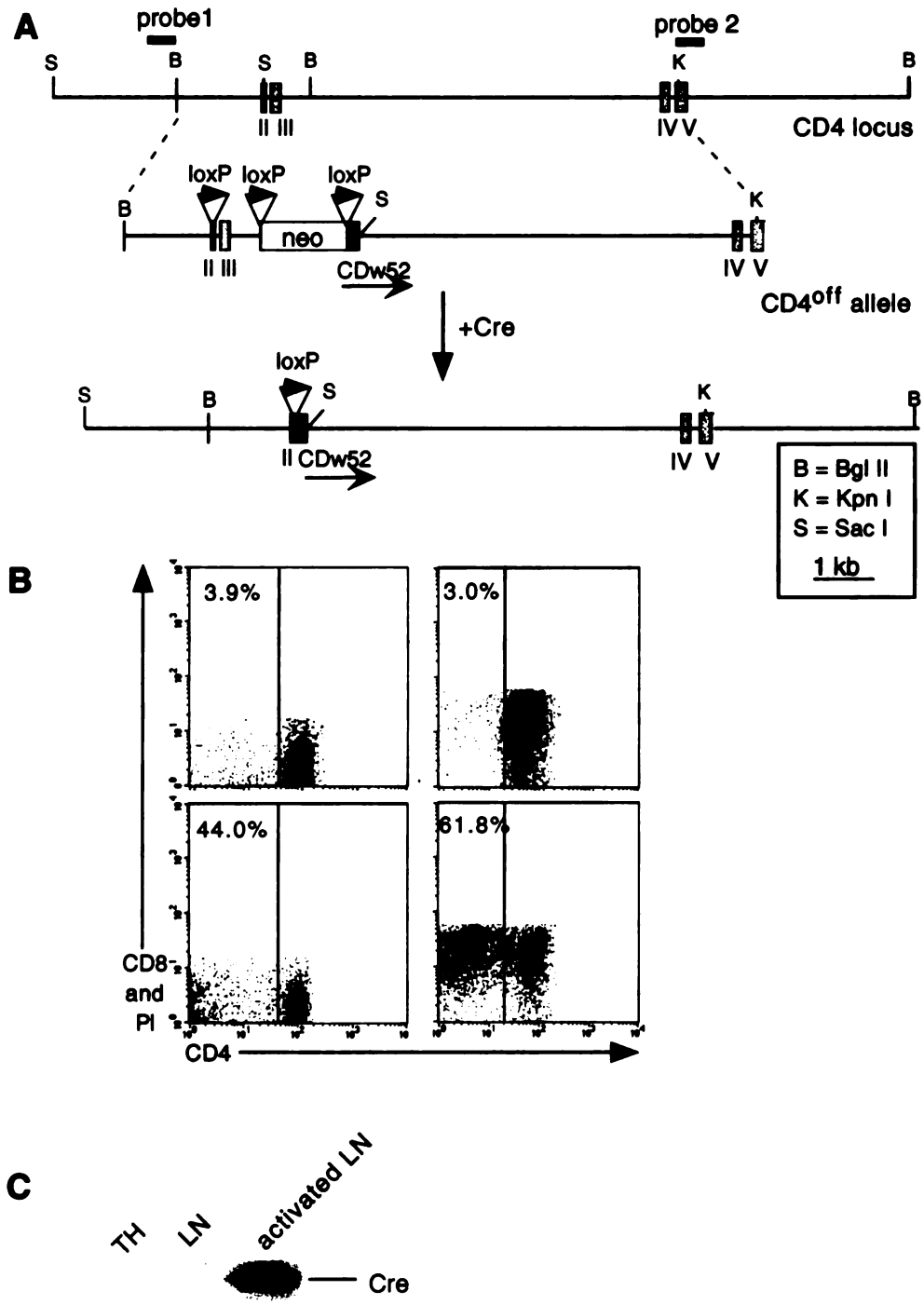

Figure 23. (A) A schematic of Cre-mediated recombination at the $CD4^{off}$ allele. (B) CD4 expression is turned off in activated CD4 lineage T cells from OX40-Cre/$CD4^{off}$/$CD4^{null}$ mice. Lymph node cells were activated *in vitro* with 10ng/ml PMA plus 500ng/ml ionomycin for 3 days. $CD3^+CD8^-$ (CD4 lineage) T cells are shown. (C) Expression of the Cre recombinase in activated lymphocytes from mice carrying the revised OX40-Cre allele.

blot analysis (Figure 23C). The CD4 lineage T cells that had lost their surface CD4 expression prior to *in vitro* activation are likely to be cells that had previously been activated *in vivo* although this interpretation is still being tested. While the precise origin and phenotype of these $CD4^{-}CD8^{-}$ T cells remains to be fully established, the revised OX40-Cre allele appears to promote activation-induced Cre expression, and thus is likely to be useful for marking $CD4^{+}$ memory T cells.

DISCUSSION

To obtain activation-induced expression of the Cre recombinase, we generated and analyzed two OX40-Cre alleles in mice. Despite early promise, the first OX40-Cre allele in which the Cre coding sequence was inserted immediately downstream of the translation initiation codon in exon 1 did not provide activation-induced Cre expression. The absence of Cre production from the allele was likely to be due to aberrant splicing of transcripts from the mutant allele. An alternative explanation is inappropriate methylation of the mutant allele caused by insertion of Cre. Evidence suggesting a methylation effect came from the observation that Cre was expressed constitutively from the OX40-Cre-neo allele and the OX40-Cre allele in offspring of OX40-Cre-neo and hAct-Flp cross. However, Cre expression was completely lost when the OX40-Cre allele was passed through the germline by breeding OX40-Cre/hAct-Flp mice to C57BL/6 mice to generate OX40-Cre single mutant mice. To circumvent these problems, we generated a second OX40-Cre allele in which the Cre coding sequence, flanked by IRES and poly A elements, was inserted into exon 3 of the OX40 gene. Preliminary analysis has shown Cre expression and function in lymphocytes activated *in*

vitro. Two pressing issues need to be addressed. First, how tightly the Cre expression is regulated. Second, whether Cre expression and function can be detected in lymphocytes activated *in vivo*. Based on the preliminary findings, the revised OX40-Cre allele is likely to allow activation-induced Cre expression and thus will be useful in marking memory T cells *in vivo*.

To provide substrates for the activation-induced Cre recombinase, we generated and analyzed multiple reporter transgenic lines. In all transgenic lines, we observed Cre-mediated loss of R1 expression as designed, however, we fail to detect any concomitant R2 expression. Even though not ideal, these transgenic reporters can be used in conjunction with activation-induced Cre to mark antigen-experienced T cells. Three strategies can be taken to generate improved lines of reporter transgenic mice. First, more transgenic founders will be generated by pronuclear injection of the existing transgene constructs and they will be screened for Cre-mediated R2 expression. Second, transgenic reporter constructs can be transfected into ES cells. Clones with low copy number of the transgenes can then be injected to generate mutant mice. Third, the reporter cassettes can be inserted directly into a lymphocyte-specific gene such as CD2 or CD4 via homologous recombination. The latter two approaches would reduce the number of transgenes in each mouse, thus minimizing the extent of genomic rearrangement at the transgene locus in the presence of Cre.

A recent study demonstrated the power of a similar genetic marking strategy in studying $CD8^+$ memory T cells (116). Using an inducible Cre under the regulation of the human granzyme B promoter and human placental alkaline phosphatase (PLAP) as

the reporter, Jacob and Baltimore demonstrated the efficacy of the genetic marking system and showed evidence for linear differentiation of $CD8^+$ memory T cells using the system.

Once well characterized, the revised OX40-Cre allele can be used in conjunction with the transgenic reporters to mark $CD4^+$ memory T cells, providing a powerful tool to study these cells. For example, the lineage and differentiation of $CD4^+$ memory cells can be followed *in vivo*, $CD4^+$ memory cells can be readily isolated and examined for their surface phenotype, activation requirements, and signaling properties.

The genetic system described above may provide a means to regulate gene expression in an activation-dependent manner. Genes can be turned on and off in activated cells (Figure 24), and the expression of the Tva molecule as R2 may be one strategy for the delivery of genes into the memory population using Avian Leukosis Virus (152). Ultimately, these experiments will facilitate an assessment of the roles played by various genes in the maintenance and re-activation of memory cells.

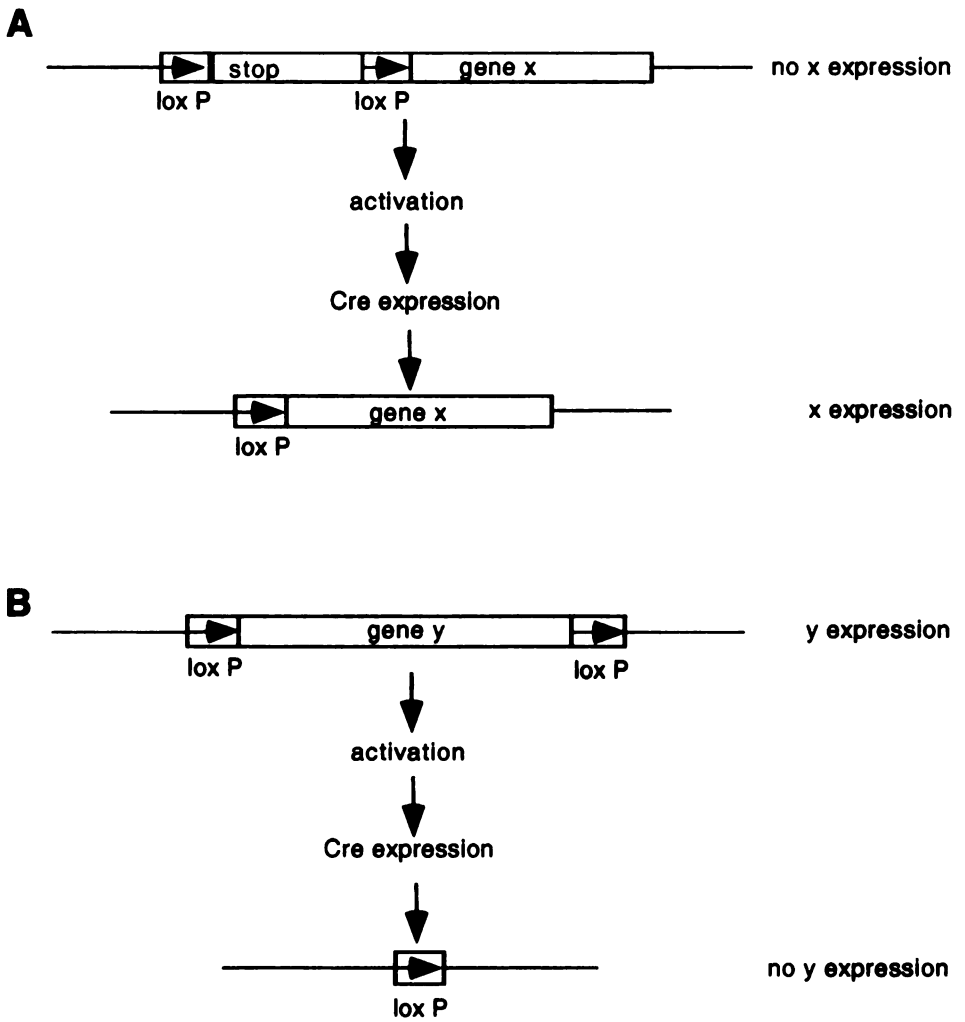

Figure 24. Manipulating gene expression in an activation-dependent fashion using the revised OX40-Cre allele. (A) Turning gene x on upon activation. (B) Turning gene y off upon activation.

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