Fate of an activated T cell—survival versus death
Review of Literature
3.1 Background

3.1.1 T cell activation

3.1.1.1 Anatomy of T cell stimulation

Naïve mature CD4 T cells exiting the thymus traffic through the peripheral blood system and various secondary lymphoid tissues (Scollay, R. G. et al. 1980.; Berzins, S. P. et al. 1998.). The secondary lymphoid tissues are the spleen, lymph nodes, organised lymphoid tissues associated with mucosal surfaces such as tonsils, Peyer’s patches, gut-associated lymphoid tissues and bronchial-associated lymphoid tissues. Lymphocytes, antigen presenting cells are also localised in these anatomically defined tissues. These secondary lymphoid organs are located at the sites where the foreign antigens entering the body through the skin or mucosa are trapped and concentrated and therefore are the sites for the antigen encounter by T cells (Goodnow, C. C. 1997.; Zinkernagel, R. M. et al. 1997.). The antigen presenting cells from the regional lymphoid tissues after capturing and processing the antigen migrate to these secondary lymphoid organs where they present the antigen in the form of short antigenic peptides loaded onto MHC class II molecules on their cell surface (Steinman, R. M. et al. 1997.; Kundig, T. M. et al. 1995). In addition to peptide-MHC complex: TCR interaction (signal 1), accessory/adhesive and costimulatory interactions (signal 2), which synergize with the TCR signaling, are also required to transduce full activating signals to the T cell (Chambers, C. A., and J. P. Allison. 1997.). A naïve T cell receiving only signal 1 in the absence of signal 2 is rendered anergic or non-responsive (Schwartz, R. H. 1990.).
3.1.1.2 The immunological synapse in T cell activation

The plasma membrane of T cells is compartmentalised into specialised membrane microdomains called "lipid rafts". These detergent resistant lipid rafts are enriched in glycosphingolipids, cholesterol, saturated phospholipids, and a subset of cellular proteins such as Src family members, small G proteins, PI-3 kinase, LAT, (Hope, H. R., and L. J. Pike. 1996.; Harder, T., and K. Simons. 1997.; Zhang, W. et al. 1998.) and are present as laterally associated membrane structures within the otherwise glycerophospholipid-rich plasma membrane (Simons, K., and E. Ikonen. 1997.).

Recognition of peptide-MHC complexes on the APC surface by TCR on T cells triggers a cascade of membrane proximal and intracellular events which culminate in T cell activation. The initial event occurring post-recognition is the oligomerisation of TCR, also known as TCR capping, and the coreceptor CD4, in the plasma membrane of T cell, at the site of contact with the APC bearing specific ligand (Kupfer, A., and S. J. Singer. 1989.; Monks et al, 1998.; Anderson, P. et al. 1988.). Engagement of TCR with the peptide-MHC complex in addition to accessory/costimulatory interactions results in the induction of recruitment of lipid rafts to the TCR contact site and formation of an immunological synapse that facilitates sustained TCR engagement and signal transduction (Moran, M., and M. C. Miceli. 1998.; Viola, A. et al. 1999.). Sustained T cell engagement and formation of an immunological synapse are critical events for T cell activation since the interaction of a T cell with an APC is impeded by various factors such as the small size of the MHC and TCR molecules, large abundant glycoproteins CD43 and CD45 that impose a steric barrier to this interaction, (van der Merwe, P. A. et al. 1995.; Shaw, A. S., and M. L. Dustin. 1997.), low affinity of TCR for peptide-MHC complex (Lyons, D. S. et
al., 1996.; Kersh, G. J. et al., 1998.; Alam, S. M. et al., 1996.), low number of peptide-MHC complexes on the APC surface (Wang, W. et al., 1997.), the opposite direction of movement of the T cell with respect to the APC (Springer, T. A., 1997.). Clustering of TCR in the immunological synapse results in prolonged TCR interaction with the otherwise low affinity, less abundant peptide-MHC complexes and clustering and concentration of various key TCR signal transducing adaptor molecules such as lck, lyn, LAT and GPI-linked CD48, (Xavier, R. et al., 1998.; Cerny, J. et al., 1996.), adhesion molecules such as LFA-1 (Monks, C. R. F. et al., 1998.), increased tyrosine phosphorylation of TCR-ζ chain (Janes P. W. et al., 1999.; Xavier R. et al., 1998.; Montixi, C. et al., 1998.), recruitment of the kinase ZAP-70 to the TCR, (Kersh G. J. et al., 1998.) respectively. Also the putative negative regulators of TCR engagement and activation, such as CD43 and CD45 are preferentially excluded from the raft microdomains (Rodgers, W., and J. K. Rose. 1996.; Janes, P. W. et al., 1999.), thereby optimising the conditions for T cell activation. Disruption of the structural integrity of the lipid rafts by using cholesterol depleting agents such as β-cyclodextrin, nystatin or filipin inhibits T cell activation as seen by reduced TCR-induced protein tyrosine phosphorylation and Ca²⁺ flux (Monks, C. R. F. et al., 1998.; Xavier, R. et al., 1998.; Cerny, J. et al., 1996.; Montixi, C. et al., 1998.).

The actin cytoskeleton also plays a major role in T cell activation. Scaffolding of actin filaments results in the clustering of various signaling complexes. Filamentous actin (Ryser, J. E. et al., 1982.) and the actin-binding protein talin accumulate at the T cell:APC interface (Kupfer, A., and S. J. Singer, 1989.). Talin is polarised in the zone of contact with the APC upon integrin engagement (Sedwick, C. E. et al., 1999.). Thus, by an actin-
dependent mechanism T cells get a prolonged time for engagement with sufficient number of TCRs (Valitutti, S. et al. 1995.; Delon, J. et al. 1998.). Various other intracellular adaptor molecules such as Vav, SLP-76, PKC-θ, Cbl-b have been implicated as regulators of cytoskeletal rearrangement and T cell activation. (Fischer, K. D. et al. 1998.; Holsinger, L. J. et al. 1998.; Bubeck Wardenberg, J. et al. 1998.; Villa'ba, M. et al. 2000.) Overexpression of Vav in T cells results in increased basal activation of interleukin-2 and enhanced response to TCR signaling (Wu, J. et al. 1995.). Similar studies with SLP-76 overexpression also demonstrate an enhanced response to TCR engagement (Motto, D. G. et al. 1996.). Lymphocytes from mice deficient in Vav-1 fail to form TCR caps and radiating actin polymers from the cap. (Holsinger et al. 1998.; O'Rourke et al. 1998.; Turnet et al. 1997). Moreover, Ca2+ mobilisation is also impaired in Vav-1-deficient T lymphocytes. SLP-76 deficient T cells also exhibit a marked reduction in PLCγ, tyrosine phosphorylation, Ca2+ flux and ERK activation following TCR ligation. Cbl-b, member of Cbl family molecules, has been reported to play a negative role in TCR clustering and induction of raft aggregation in T cells upon stimulation (Krawczyk, C. et al. 2000.). Mice deficient in Cbl-b show TCR stimulation independent of CD28 costimulation and Cbl-b/-CD28-/- show normal IL-2 production and T cell proliferation (Bachmaier, K. et al. 2000.). Loss of Cbl-b in T cells makes antigen induced TCR clustering, membrane raft aggregation and sustained tyrosine phosphorylation independent of CD28 costimulation. Moreover, absence of Cbl-b in Vav-1 -/- mice rescues the functional defects in T cell activation and causes spontaneous autoimmunity, thereby indicating the role of receptor clustering, aggregation of lipid rafts and downstream signaling pathways in the activation of T cells.
3.1.1.3 Relevance of cAMP mediated signaling in T cell activation

Activation of T cells via TCR/CD3 complex or its cognate ligand and CD28 results in induction of adenylate cyclase which in turn hydrolyses adenosine triphosphate (ATP) to cyclic adenosine monophosphate (cAMP) (Schultz, L. A. et al. 1988.). cAMP is catalytically hydrolysed to its biologically inactive form adenosine 5’-monophosphate by a family of enzymes, phosphodiesterases (PDEs). Increasing cAMP levels by either cAMP analogues such as dibutryl cAMP or cAMP elevating drugs such as theophylline, pentoxifylline (PF) (Houslay, M. D., and Milligan, G. 1997.) or PDE inhibitors such as rolipram have been shown to inhibit T cell activation, proliferation and cytokine production (Scordiamaglia, A. et al. 1988.; Mary, D. C. et al. 1987.; Anastassiou, E. D. et al. 1992.; Chen, D. and E. V. Rothenberg. 1994.; Katamura, K. et al. 1995.). PF has been shown to block anti-CD3 induced c-Rel (a transcription factor) in T cells (Wang, W. et al. 1997.). It inhibits the production of cytokines such as TNF-α, IFN-γ and granulocyte-macrophage colony stimulating factor from HIV specific CD8 T cells (Heinkelein, M. et al. 1995.). There are 11 broad families of PDEs of which three cAMP specific PDEs have been reported to be present in T cells- PDE3, PDE4 and PDE7 (Giembycz, M. A. et al. 1996.; Seybold, J. et al. 1998.). In a HIV infection model, high levels of PDE4 have been detected in memory CD4 T cells and selectively blocking PDE4 activity abolishes IL-2Rα chain and c-Myc expression. However, it does not inhibit DNA synthesis although the nuclear entry of HIV DNA is blocked (Sun, Y. et al. 2000.). PDE7 has also been reported to be induced as a consequence of CD3 and CD28 ligation. Increased PDE7 activity correlates with decreased levels of cAMP, increased levels of IL-2 expression and increased proliferation. Inhibiting expression of PDE7 by PDE7 antisense oligonucleotides
have been shown to inhibit T cell proliferation (Li, L. et al. 1999.). Inhibiting PDE3 inhibits human T cell proliferation (Moodley, I. et al. 1995.). PDE inhibitors have also been reported to attenuate T cell responsiveness in EAE in rats and in patients with autoimmune diseases such as multiple sclerosis (Rieckman, P. et al. 1996.). cAMP executes its effects by activating cAMP dependent protein kinase A (PKAs). There are two type of PKAs. Type I PKA is localised to the cytoplasm and gets recruited to the TCR-CD3 complex upon T cell activation (Skalhegg, B. S. et al. 1994.). The inhibitory effects of cAMP are mediated by activation of type I PKA. Therefore, blocking type I PKA could reverse the inhibition induced by antisense oligonucleotides to PDE7 (Li, L. et al. 1999.). Type I PKA antagonists have been shown to restore immune responses of T cells from HIV-infected patients (Aandahl, E. M. et al. 1998.). Type II PKA is predominantly associated with the cytosolic side of the plasma membrane (Skalhegg, B. S. et al. 1992.) and its activation does not mediate the inhibitory effects of cAMP.

Thus, in the T cell, adenylate cyclase-cAMP-protein kinase A pathway conveys the inhibitor signals resulting in dampening of T cell effector functions; however, its role in the generation of memory T cells is still not well understood.

3.1.2 Effector T cell generation

3.1.2.1 Life span of effector T cells

An activated T cell undergoes a phase of marked expansion, the extent of expansion reflecting the concentration of antigen and the affinity of the responding T cells. This initial proliferative phase of the T cell activation occurs in response to the antigen and engagement of IL-2 with its receptor. IL-2:IL-2R interaction results in the initiation of cell
cycle progression. The proliferating cells acquire various effector functions and are released into the circulation. The effector cells express various homing/adhesion markers which enable them to penetrate the walls of blood vessels and percolate through various tissues. The duration of the primary T cell response depends on the nature of antigen. In case of infection with micro-organisms or antigens administered with strong adjuvants, it may continue for weeks or months since the immune system is exposed to the antigen for prolonged periods. However, as the primary response progresses, there is a clearance of the antigen and the bulk of the effector cells is eliminated. Reports wherein superantigens have been used to evoke primary T cell responses, have demonstrated an initial proliferative phase followed by progressive disappearance of the effector cells (Herman, A. et al. 1991.). This elimination is by a combination of irreversible homing to the gut and cell death in the spleen (Webb, S. R. et al. 1994.). Studies with TCR transgenic mice have also shown the rapid disappearance of effector cells generated in primary immune response against a wide variety of antigens such as male histocompatibility HY antigen (Rocha, B., and H. von Boehmer. 1991.), lymphocyte choriomeningitis virus (LCMV) (Moskophidis, D. et al. 1993.) and ovalbumin. This cell death could be because of exhaustive differentiation, prolonged TCR signaling causing activation of intracellular pathways leading to apoptosis (Webb, S. et al. 1990.) since superantigen-induced apoptosis is reduced in Bcl-2 transgenic mice (Strasser, A. et al. 1991.). It could also be because of depletion of cytokines such as IL-2 from the microenvironment (Kirkberg, J. et al. 1993.). However, the cause of the disappearance of effector T cells is unknown and it is uncertain whether T lymphocytes have a predetermined life span.
Apoptosis is an orderly process of programmed cell death which utilises various signaling cascades and culminates in several morphological changes (Duvall, E. and A. H. Wyllie. 1986.). Dying cells lose contact with the neighbouring cells and the morphological hallmarks of apoptotic cells are the condensation of both nucleus and the cytoplasm, causing the cell to shrink in size. Mitochondria release cytochrome c in the cytoplasm (Kluck, R. M. et al. 1997.; Yang, J. et al. 1997.). The nuclear envelope and the nucleolus break apart as the chromatin condenses and there is generation of 180 bp fragments of DNA (Skalka, M. et al. 1976.; Wyllie, A. H. 1980.; Wyllie, A. H. et al. 1984.). The plasma membrane also begins to bleb which divide the cells into smaller apoptotic bodies that contain condensed or morphologically normal organelles. The downstream events are the reduction of the mitochondrial membrane potential, intracellular acidification, generation of reactive oxygen species (ROS), presentation of phosphatidylserine residues at the cell surface, production of ceramide, proteolysis of distinct proteins (Wyllie, A. H. et al. 1984.; Hockenbery et al. 1993.; Lazebnik et al. 1994.; Martin et al. 1995.; Gottlieb et al. 1996.; Zamzami et al. 1996a).

Apoptosis of T cells can be classified into two categories: antigen induced (active T cell death) and cytokine withdrawal (passive T cell death). Activated T cells undergo massive proliferation; however, when the cycling T cells are strongly stimulated by the antigen and the TCR is repeatedly engaged, antigen-induced apoptosis occurs (Russell, J. H. et al. 1991.). But, if there is no antigen, the surface expression of IL-2 and IL-2 receptor declines and T cell death occurs by lymphokine depletion, referred as passive cell death (Lenardo, M. J. 1991.).
There are also molecular differences between active and passive forms of T cell death. Active T cell death requires TCR stimulation, involves FasL and TNF (Brunner, T. et al. 1995.; Ju, S. T. et al. 1995.; Sytwu, H. K. et al. 1996.), is independent of new protein synthesis (Itoh, N. et al. 1991.; Fisher, G. H. et al. 1995.), is partially inhibited by Bcl-2 and can occur in the presence of growth promoting cytokines such as IL-2, IL-4, IL-7 and IL-15. Marked defects in T cell apoptosis, lymphoproliferation is seen in lpr and gld mice which have genetic defects in Fas and FasL respectively (Russell, J. H. and R. Wang. 1993.; Russell, J. H. et al. 1993.). Fas deficient T cells show a reduced death which can be further blocked by inhibiting TNF (Zheng, L. et al. 1995.; Tucek-Szabo, C. L. et al. 1996.).

3.1.2.3 Mediators of apoptotic T cell death

T cell apoptosis takes place indirectly by the antigen induced expression of death cytokines, mainly FasL/APO-1L and tumor necrosis factor (TNF). Both FasL and TNF are present as cell-membrane associated forms. In resting T cells, they are poorly expressed upon TCR engagement while in IL-2 stimulated cycling T cells they are highly induced (Zheng, L. et al. 1998.). Engagement of FasL or TNF initiates the death pathway by causing association of specific cytoplasmic signaling proteins on the death domain in the cytoplasmic tails of Fas or TNFR. Fas recruits FADD (Fas associated death domain)/MORT1 (mediator of receptor induced toxicity) and TNFR1 recruits TRADD (TNFR1-associated death domain) which in turn recruits FADD(MORT1) or RIP through their respective death domains (Chinnaiyan, A. M. et al. 1995.; Hsu, H. et al. 1995.; Boldin, M. P. et al. 1995.). Clustering of these signal transducers result in the recruitment of FLICE/MACH (FADD-like IL-1β-converting enzyme/MORT1-associated CED-3 homolog (Muzio, M. et al. 1996.; Boldin, M. P. et al. 1996.) to FADD or RAIDD-ICH-1 to RIP (Duan, H and V. M. Dixit. 1997.). FADD/MORT1 exists as a complex with pro-caspase-8 in the cytoplasm which gets recruited to the cytoplasmic death domain of Fas upon activation. Caspases are cystinyl-aspartate-requiring proteinases and are present as inactive precursors. They cleave proteins at specific substrate sites downstream of aspartate residues and are the executioners of cell death (Nicholson, D. W. and N. A. Thornberry. 1997.; Miller, D. K. et al. 1997.). The events post aggregation of FADD and pro-caspase-8 involves proteolytic activation of pro-caspase-8 to caspase-8 (FLICE) and release of the active enzyme. Active caspase-8 then initiates a chain of downstream proteolytic events including activation of other caspases which result in apoptosis.
TRADD can also bind to death domain containing protein called RIP. RIP is a serine-threonine kinase, which in association with TRAF1/2, can induce NF-κB. Activation of NF-κB results in the transcription of anti-apoptotic proteins leading to cell survival (Stanger, B. Z. et al. 1995.; Hsu, H. et al. 1996.; Ting, A. T. et al. 1996.; Kelliher, M. A. et al. 1998.). However, RIP can bind to RAIDD which in turn can recruit caspase-2 thereby promoting apoptosis (Duan, H and V. M. Dixit. 1997.).

Interaction of CD95 with its ligand also triggers the cleavage of substrates in the mitochondrial membrane. Damage of the outer mitochondrial membrane and the resultant inner membrane depolarization leads to release of cytochrome c, a soluble component of the respiratory chain that is normally retained in the space between outer and inner mitochondrial membrane. Cytochrome c binds to Apaf-1 (apoptotic protease activating factor) in the cytoplasm (Liu, X. et al. 1996.; Zou, H. et al. 1997.). Complexes of cytochrome c and Apaf-1 in the cytoplasm trigger the activation of pro-caspase-9 in assemblies called as apoptosomes. The active form of caspase 9 then leads to activation of pro-caspase-3 and the effector caspase-3 activates proteolytically DFF (DNA fragmentation factor)/CAD (caspase-activated DNase) and thereby triggers endonuclease activity (Liu, X. et al. 1997.; Enari, M. et al. 1998.; Sakahira, H. et al. 1998.). Alternatively, the apoptosis inducing factor (AIF) can also induce DNA degradation and activate caspase-3 independent of cytochrome c (Zamzami, N. et al. 1996b.; Susin, S. et al. 1996.; Susin, S. et al. 1997.; Susin, S. et al. 1999.).

Bcl family of proteins play a major role in inhibition of apoptosis. Bcl-2 and Bcl-x are cytoplasmic membrane-bound apoptosis-inhibitory proteins (Boise, L. H. et al. 1995.; Tamura, A. et al. 1996.). Reports suggest that the inhibition of apoptosis by Bcl-2/Bcl-x
occurs by blocking the release of cytochrome c by its binding to the mitochondrial
membrane, to Apaf-1 or both as well as by having inhibitory effects on the active caspase
complex (Reed, J. C. 1997.; Rosse, T. et al. 1998.).

Apoptosis is hence, a complex network of signaling cascades and the cross-talk between
different pathways determine either cell death or cell survival.

3.1.3 Memory T cells

3.1.3.1 Generation of memory T cells

During the late stages of a primary T cell response, there is an extensive elimination of
effector cells. The destruction of these short-live cells is essential since they are not useful
after the pathogen has been cleared from the host. Moreover, allowing effector cells to
survive would skew the repertoire to previously encountered antigens thereby
compromising primary responses to new pathogens. However, the elimination is not
complete; complete elimination would be counterproductive for the host since it would
lead to tolerance rather than immunity. Therefore, a small fraction of the cells stimulated in
the primary response somehow do not die but survive for prolonged periods. These cells
are memory cells. Hence, the generation of memory cells is a finely-tuned phenomenon
whereby a small proportion of cells that participated in the primary response are instructed
to survive and differentiate to long-live memory cells rather than die. However, the
mechanism responsible for generating memory cells by their ability to avoid death is still
poorly understood and highly controversial. Various reports have suggested the role of
upregulation of anti-apoptotic molecules such as Bcl-2, Bcl-xL and related family
members combined with the down-regulation of pro-apoptotic molecules such as Bad,
Bax and Fas (Cory, S. 1995.). Thus, the fate of the cells activated during a primary response is dictated by the relative expression of pro- and anti-apoptotic genes.

### 3.1.3.2 Phenotype of memory T cells

The memory T cells have a distinct pattern of expression of various cell surface markers as compared to that of naïve/activated T cells. Briefly, naïve T cells are characterised by high expression of CD62L (the lymph node homing receptor), CD45RA, RB and RC and low levels of CD44, ICAM-1 (intercellular adhesion molecule, CD43, VLA-4 (very late antigen) and α4β7 integrins (Rott, L. S. et al. 1996.; Andrew, D. P. et al. 1996.; Yousefif-Etemand, R and B. Axelsson. 1996.). Upon activation, T cells downregulate CD62L and CD45RA/B/C and upregulate CD44, ICAM-1, LFA-1, CD43, α4β7 and α4β1 integrins. Some reports suggest that CD4 memory cells in mice show only limited expression of activation markers and are of CD45RB<sub>lo</sub>CD62L<sub>lo</sub>CD44<sub>hi</sub> phenotype. The downregulation of CD45RB on mouse memory cells is paralleled by upregulation of a sialylated variant of CD45RB, termed as CZ-1 (Varga, S. M and R. M. Welsh. 1996.). However, there are contradicting reports which demonstrate the phenotype reversion of memory T cells to the naïve phenotype. Mouse Vβ6 cells dividing in response to Mls-a-disparate spleen cells in vivo revert from CD45RB<sub>lo</sub>CD62L<sub>lo</sub>CD44<sub>hi</sub> to CD45RB<sub>hi</sub>CD62L<sub>hi</sub>CD44<sub>lo</sub> naïve phenotype (Hayden, K. A. et al. 1996.). Hence, the memory CD4 T cells may reacquire some of the phenotypic characteristics of naïve cells.
3.1.3.2 Life span of memory T cells

T cells with a memory phenotype represent a small population in young animals but comprise the bulk of mature T cells in old age. Thus, memory phenotype T cells represent a heterogeneous population of memory cells responding to various antigens. However, the longevity of memory cells has been a debatable issue, although it has been argued that memory cells have a prolonged life span. Under certain conditions of priming, memory can decay rapidly (Gray, D. 1993.; Mackay, C. 1993.) or it can be biphasic, implying the existence of short-lived and long-lived memory cells (Celada, F. 1971.). For the memory cells to be long-lived they would either have to exist as non-dividing cells or as cells with a slow turnover rate which constantly maintains the memory T cell pool. These studies have been made easy by the use of mice bearing transgenic TCR which can be detected by clonotype-specific antibodies. For instance, in an LCMV-specific TCR transgenic line, the turnover of Tg cells declined rapidly (as seen by BrdU labelling) at the end of the primary response. However, at later stages when the virus had been cleared, a substantial proportion of Tg cells were BrdU" and some labelled very slowly indicating that memory cell pool is a mixture of resting and cycling cells (Zimmerman, C. et al. 1996.; Tough, D. F. and J. Sprent. 1994.).

3.1.3.4 Factors regulating persistence of memory T cells

The persistence of memory T cells has been shown to be regulated by the presence of antigen and the cytokines.
3.1.3.4.1 Antigen

The role of persisting antigen in the maintenance of memory is still an unresolved issue. However, direct evidence in its favour comes from the finding that memory response decays rapidly when primed lymphocytes are adoptively transferred in the absence of antigen (Feldbush, et al. 1973.; Gray, D. et al. 1993.); conversely, cotransfer with the antigen leads to maintenance of memory. Rapid decay of memory has been observed for CD8 cells specific for HY antigen (Gray and Matzinger, 1991.), viral antigens (Oehen et al. 1992.) and for CD4 T cells reactive to keyhole limpet hemocyanin (Gray, D., and Matzinger, P. 1991.). In all these situations, absence of antigen during the transfer of primed lymphocytes resulted in the loss of memory within weeks of transfer. The requirement of persisting antigen for the maintenance of memory, however, does not imply that the memory cells engage in a chronic proliferation with the antigen. Also, how the original antigen is retained in the host for prolonged periods is still unclear.

Contradicting reports exist for the role of antigen persistence in maintenance of memory. In case of LCMV infection, LCMV specific CTL precursors taken at 3 months post priming survive for more than an year after adoptive transfer, with little or no decrease in their number where the transferred cells are completely free of infectious LCMV and LCMV antigens (Lau, L. L. et al. 1994.; Mullbacher, A. 1994.). These studies show that maintenance of late memory does not require exposure to antigen. It is also possible that certain cross-reactive environmental antigens would suffice for the low level stimulation of memory T cells (Beverley, P. C. L. 1990.), although it fails to explain the rapid decay of memory responses in cases of adoptive transfer of primed T cells in the absence of antigen.
Various bacteria and viruses have the ability to stimulate CD44\textsuperscript{hi} memory T cells in vivo by activating APCs and other cells to produce various cytokines. The type I IFNs, poly I:C which induces IFN I production, have been shown to stimulate memory T cells \textit{in vivo} and \textit{in vitro} (Tough, D. F. et al. 1996.; Dumont, F. J. and L. Z. Coker. 1986.). This intermittent non-antigen specific bystander stimulation from cytokines may act as a boost for memory cell survival and therefore, may substitute for the contact with the residual antigen. Thus, such non-TCR dependent simulation of memory T cells in conjunction with the TCR dependent stimulation by residual depots of specific antigen or cross-reactive environmental antigens leads to the long term survival of memory T cells.
Results
3.2 Results

3.2.1 Consequences of cholesterol depletion on T cell activation

T cell receptor recognition by cognate peptide-MHC complex along with non-cognate (costimulatory/adhesive) interactions results in T cell activation and differentiation to one or many of its several functional fates. The signal for T cell activation is initiated at the T cell surface by oligomerisation of TCR, coreceptor and other costimulatory molecules at the interface in specialised membrane microdomains, rafts, facing the APCs. Naive CD4 T cells produce IL-2 upon activation. IL-2 regulates the primary proliferation of T cells in an autocrine mode. Activated T cells also upregulate the expression of various cell surface molecules. In this context, the role of membrane microdomains in T cell activation has been studied by using methyl-β-cyclodextrin (MCD), which chelates cholesterol from the plasma membrane and thereby disrupts the microdomain organisation.

3.2.1.1 Anti-CD3 mediated T cell proliferation: effect of MCD

Whole splenocytes were stimulated with anti-CD3 in presence of various concentrations of MCD for 48 h. T cell proliferation was measured at 48 h. As shown in Fig 13, MCD inhibits anti-CD3 mediated proliferation in a dose dependent manner with highest inhibition at 3 mM, followed by 2 mM and no inhibition at 1 mM. The data suggest that the depletion of cholesterol from the cell membrane leads to inhibition in proliferation.

3.2.1.2 Effect of MCD on constitutive cell proliferation

To check whether MCD results in specific inhibition of induced T cell proliferation or it has an intrinsic characteristic to block both induced as well uninduced proliferation, a T
**FIG 13: Cell membrane cholesterol depletion by MCD inhibits anti-CD3 mediated T cell proliferation**

Whole splenocytes were stimulated with various dilutions of anti-CD3 alone (filled circles) or in the presence of 3 mM MCD (open squares), 2 mM MCD (filled squares) or 1 mM MCD (open triangles) for 48 h. At 48 h, the response was measured as $^3$H-thymidine incorporation. The background proliferation in absence of anti-CD3 was 2910, in presence of 3, 2 and 1 mM MCD was 1165, 1697 and 6632, respectively. The above experiment is a representative of ten independent experiments.
**FIG 14: MCD does not inhibit constitutive cell proliferation**

10⁴ cells of T cell hybridoma, 13.8, were cultured either in absence or in the presence of 3 mM MCD, 2 mM MCD or 1 mM MCD for 24 h. At 24 h, the response was measured as ³H-thymidine incorporation. The above experiment is a representative of two independent experiments.
Results
cell hybridoma, 13.8, was used which undergoes constitutive proliferation. A constant number of T cells were cultured either in absence or presence of various concentrations of MCD for 24 h. T cell proliferation was measured at 24 h. As shown in Fig 14, constitutive proliferation of a T cell hybridoma is not affected by MCD even at a concentration as high as 3 mM. Thus, MCD does not regulate constitutive cell proliferation suggesting that it interferes with signaling pathways inducing it.

3.2.1.3 Effect of MCD on non-TCR-mediated T cell proliferation
T cells upon activation upregulate various cell surface activation molecules of which CD25 (IL-2R alpha chain) is one. The autocrine signaling for IL-2 is mediated by signaling through CD25. Since MCD inhibits anti-CD3 mediated proliferation, it was necessary to ask if this effect is TCR-specific or if there is a defect in IL-2R mediated signaling as well. To check this possibility, splenocytes were stimulated with IL-2 in presence of various concentration of MCD. Fig 15 shows that there is a dose dependent inhibition of IL-2 mediated proliferation in presence of MCD thereby suggesting that cholesterol depletion from the cell membrane results in impaired IL-2-IL-2R mediated T cell proliferation.

Clearly, induced proliferation of T cells is inhibited by MCD. But T cell proliferation is a late event in T cell activation. Therefore, other events were tested for their sensitivity to MCD. To check the effect on IL-2 induction, an ovalbumin specific, an IA-b restricted T cell hybridoma, 13.8, which has a β-galactosidase reporter gene under the control of a nuclear factor of activated T cells (NFAT) enhancer element of the IL-2 gene was used. This enhancer element regulates the expression of β-galactosidase gene in activated T
**FIG 15: MCD inhibits IL-2 mediated T cell proliferation**

Splenocytes were stimulated with titrating concentrations of rhIL-2 alone (filled circles) or in the presence of 3 mM MCD (open squares), 2 mM MCD (filled squares), 1 mM MCD (open triangles) for 48 h. At 48 h, the response was measured as 3H-thymidine incorporation. The background proliferation in absence of rhIL-2 was 2871 and in the presence of 3, 2, 1 mM was 595, 2334 and 5835, respectively. The above experiment is a representative of four independent experiments.
cells. In this T cell hybridoma, β-galactosidase activity is specifically induced in response to peptide-MHC class II complexes and hence can be used to measure ligand induced T cell activation. The hybridoma was stimulated with titrating concentrations of maleyl-OA in presence of C57BL/6J splenocytes as APCs for 24 h. At 24 h, β-galactosidase activity, which would represent IL-2 gene induction, was assayed by cell ELISA (described in materials and methods). As shown in Fig 16, there is a dose dependent inhibition of β-galactosidase induction in the presence of MCD as compared to the cells stimulated in absence of MCD. The data, therefore, suggests that the IL-2 induction is also inhibited by MCD.

3.2.1.4 Role of exogenous IL-2

The data so far suggest that there is reduced IL-2 production. It is therefore formally possible that the reduced IL-2 production is responsible for inhibition of T cell proliferation. In order to check this possibility, splenocytes were stimulated with anti-CD3 in presence of MCD and titrating concentrations of IL-2. Fig 17 shows that the addition of exogenous IL-2 does not overcome the block in anti-CD3 mediated T cell proliferation induced in the presence MCD. However, cells stimulated with anti-CD3 in presence of IL-2 without MCD showed an increase in the proliferation. Hence, the inhibition seen in case of anti-CD3 mediated stimulation of T cells in presence of MCD is not due to limited production of IL-2.
**FIG 16: MCD inhibits production of IL-2**

T cell hybridoma, 13.8, was stimulated with titrating concentrations of maleyl-OA either in absence (filled circles) or in the presence of 3 mM MCD (open squares), 2 mM MCD (filled squares) or 1 mM MCD (open triangles) for 24 h. 10⁵ splenocytes from naive C57BL/6J strain of mice were added as APCs. At 24 h, cells were washed and β-galactosidase activity was measured using ONPG as the substrate. After 2-3 h of substrate addition, absorbance was read at 405 nm. The background absorbance in absence of maleyl-OA was 0.149 and in presence of 3, 2, 1 mM MCD was 0.144, 0.167 and 0.161, respectively. The above experiment is a representative of three independent experiments.
**FIG 17: Exogenous IL-2 does not rescue the MCD induced block in anti-CD3 mediated T cell proliferation**

Splenocytes were stimulated with anti-CD3 alone (filled circles) or in the presence of 3 mM MCD (open squares), 2 mM MCD (filled squares), 1 mM MCD (open triangles) along with titrating concentrations of rhIL-2 for 48 h. At 48 h, the response was measured as $^3$H-thymidine incorporation. The proliferation induced by anti-CD3 alone was 100657 and in the presence of 3, 2, 1 mM was 405, 2317, 41401, respectively. The above experiment is a representative of three independent experiments.
3.2.1.5 Induction of activation markers

To test whether cholesterol depletion from the membrane has an effect on the induction/expression of various activation markers, splenocytes were stimulated with anti-CD3 in presence of various concentrations of MCD for 48 h. At 48 h, viable cells were stained for CD44, CD25, CD69 and CD95. As shown in Fig 18, the upregulation of CD44 is equivalent on the cells that were stimulated either in absence or presence of 3 and 2 mM of MCD, which inhibit T cell proliferation. Induction of CD25, CD69 and CD95 is also equivalent in absence and presence of MCD. Thus the data collectively suggests that cholesterol rich membrane microdomains have a differential role in cell proliferation versus upregulation of activation markers.

3.2.1.6 Acquisition of activation markers and cell division

In order to confirm that activation markers were acquired by T cells in the absence of cell division under the influence of MCD, splenocytes were labelled with CFSE and then stimulated with anti-CD3 for 48 h. At 48 h, viable cells were analysed for the dilution of CFSE, which reflects the cell division. The cells were also stained for CD44 (Fig 19) and CD69 (Fig 20). As shown in Fig 19, cells stimulated with anti-CD3 in the presence of MCD show a block in cell division (Fig 19B and 19C). There is a decreased percentage of cells entering second cell division as compared to the cells stimulated with anti-CD3 alone. However, cells stimulated with anti-CD3 antibody upregulate CD44 even in the absence of cell division as compared to unstimulated cells (Fig 19D and 19E). This upregulation is not affected in the presence of MCD (Fig 19F) and is comparable on the cells in the first
FIG 18: Induction of activation markers in presence of MCD
Splenocytes were either unstimulated (shaded area) or stimulated with anti-CD3 alone (thick line) or in the presence of 2 mM MCD (thin black line), 1 mM MCD (thin grey line) for 24 h. AR 24 h, viable cells were stained for T cells and various activation markers. The figure shows the expression of CD69 (panel A), CD44 (panel B) and CD25 (panel C) on gated T cells. The above experiment is a representative of six independent experiments.
FIG 19: Induction of CD44 is independent of cell division

CFSE labelled splenocytes were either unstimulated (-; panels A, D) or stimulated with anti-CD3 alone (panels B, E, G and I) or in the presence of MCD, 2mM (panels C, F, H and J), for 48 h. At 48 h, viable cells were stained for CD44. Panels A-C show CFSE staining on gated T cells. Panels D-J show CD44 staining on cells gated on cell division 0 (panels D-E), 1 (panels G-H) and 2 (panels I-J). The above experiment is a representative of three independent experiments.
Results

(Fig 19G and 19H) and second cell division (Fig 19I and 19J) stimulated in absence or presence of MCD.

Similarly, Fig 20E and 20F show acquisition of CD69 upon activation prior to cell division which is not affected by MCD (Fig 20F) and its expression on subsequent cell divisions (Fig 20G-J) is also not affected by the presence of MCD during stimulation. Thus, acquisition of activation markers can be signalled independently of cell division.

3.2.2 Role of intracellular cAMP dependent pathways in T cell memory and death

It has been already shown in the laboratory that pentoxifylline (PF) enhances secondary responses in vivo. PF can inhibit all phosphodiesterases (PDEs) and therefore can increase intracellular cAMP. cAMP can mediate its effects through cAMP dependent protein kinase A (PKA), although PKA-independent effects are also reported. Thus, increasing intracellular cAMP could result in activation of downstream cAMP signaling pathways and hence the inhibitors of PDEs act as PKA agonists. However, there could be entirely PDE/PKA-independent pathways involved. Therefore, the role of cAMP dependent PKA signaling pathways in T cell memory have been studied using pharmacological modulators, in the context of the already reported effects of PF.

3.2.2.1 Role of increased cAMP concentrations

To see whether the enhancement of secondary responses could be caused by an increase in intracellular cAMP, mice were immunised with maleyl-CA in the presence of either PF or dbcAMP (described in materials and methods). dbcAMP is a cell permeable analogue of
**FIG 20: Induction of CD69 is independent of cell division**

CFSE labelled splenocytes were either unstimulated (-; panels A,D) or stimulated with anti-CD3 alone (panels B, E, G and I) or in the presence of MCD, 2mM (panels C, F, H and J), for 48 h. At 48 h, viable cells were stained for CD69. Panels A-C show CFSE staining on gated T cells. Panels D-J show CD44 staining on cells gated on cell division 0 (panels D-E), 1 (panels G-H) and 2 (panels I-J). The above experiment is a representative of three independent experiments.
cAMP. On day 42 post immunisation, splenocytes were harvested and restimulated with
titrating concentrations of maleyl-CA for 72 h. Fig 21 shows that like PF, dbcAMP also
enhances antigen specific secondary responses in vivo.

There are seven isoforms of PDEs. PF inhibits all the isoforms of PDEs. However, PDE3,
4 and 7 have been shown to be expressed in T cells. PDE4 is markedly induced in T cells
upon activation. To examine if PDE4 inhibition alone was sufficient to give enhanced T
cell memory, an isoform specific inhibitor was used. Rolipram inhibits PDE4. Mice were
immunised with maleyl-CA in presence of either PF or rolipram. Day 42 post
immunisation, splenocytes were restimulated in vitro with titrating concentrations of
maleyl-CA for 72 h. As shown in Fig 21, inhibiting only PDE4 by rolipram also results in
enhancement in secondary T cell response. The data therefore suggest that cAMP
mediated signaling pathways are involved in enhancement of peripheral secondary T cell
responses and that PDE4 is a key regulator of this pathway.

3.2.2.2 Role of protein kinase A

The data so far suggest that increasing cAMP concentrations leads to an enhancement in T
cell memory responses. If this was mediated by PKA, PKA antagonists would be expected
to block this effect. To test this, Rp-8Br-cAMP, which inhibits all PKA isoforms, was
used in a similar experimental system as described above.

Mice were immunised with maleyl-CA in the presence of PF or PF and Rp-8Br-cAMP.
Day 21 post immunisation, splenocytes were harvested and restimulated in vitro with
titrating concentrations of maleyl-CA. PF treatment shows enhancement of memory T cell
responses, as shown in Fig 22. However, mice that were immunised in the presence of
FIG 21: Enhancement of memory T cell response by dbcAMP and PDE4 inhibitor, rolipram

Mice were immunised with maleyl-CA (300 μg/ml) in saline (open circles) or in the presence of PF (filled circles), dbcAMP (open squares), rolipram (filled triangles). Day 42 post immunisation, splenocytes were harvested and restimulated in vitro with titrating concentrations of maleyl-CA for 72 h. At 72 h, the proliferation was measured as ^{3}H-thymidine incorporation. The background proliferation was 5983 (saline), 7787 (PF), 8259 (dbcAMP) and 12696 (rolipram). The above experiment is a representative of five independent experiments.
FIG 22: PKA antagonists block the enhancement of memory T cell response mediated by PDE inhibitor, PF
Mice were immunised with maleyl-CA (300 µg per mouse) in saline (open circles) or in the presence of PF (filled circles), Rp-8Br-cAMP/PF (filled triangles). Day 21 post immunisation, splenocytes were harvested and restimulated with titrating concentrations of maleyl-CA for 72 h. At 72 h, the proliferation response was measured as \(^{3}\)H-thymidine incorporation. The background proliferation was 1762 (saline), 1607 (PF) and 1084 (Rp-8Br-cAMP/PF). The above experiment is a representative of two independent experiments.
both PF and Rp-8Br-cAMP, the enhancement in the response brought about by PF is significantly decreased. The data, therefore, suggest that better memory generated by PF is a result of signaling through cAMP dependent PKA mediated pathways.

3.2.2.3 Proliferation of superantigen reactive T cells

The data collectively in Fig 21 and 22 show that activating a cAMP dependent PKA pathway during priming of T cells results in better memory generation. So how does PF enhance memory? To address this issue, it is important to be able to track antigen specific T cells which is not possible in a normal mouse. For this, a well documented activation-deletion model using Mls superantigen based differences to identify reactive T cells was used.

CBA/J and CBA/CaJ strains are haplotype matched but Mls disparate strains. CBA/J strain of mice (Mls stimulators) expresses the MMTV antigen V-sag-7 and therefore deletes reactive Vβ6 bearing T cells. However, the CBA/CaJ strain of mice (Mls responders) does not show any such deletion. When splenocytes from responder mice are transferred into stimulator mice, the TCR-Vβ6-bearing T cells from the responders undergo massive expansion followed by deletion via activation induced cell death (AICD). Using this model, earlier data in the laboratory show that the responders persist for a longer time when they are transferred in the presence of PF. This experimental model was used to address the in vivo effects of PF on T cell activation and its subsequent outcomes. CFSE labelled splenocytes from responder mice were transferred retro-orbitally into the stimulator mice either in absence or presence of PF. At various time periods post transfer, splenocytes were harvested and analysed for the presence of TCR-Vβ6 bearing T cells. At
Results
day 1 post transfer (Fig 23C and D), there are few CFSE bright TCR-Vβ6 bearing T cells which are the cells that have not divided so far as well as some CFSE negative TCR-Vβ6 bearing T cells that have undergone many cell divisions and have lost CFSE completely. This profile is similar when the cells are transferred either in absence (Fig 23C) or presence (Fig 23D) of PF. The proliferation, however, becomes more prominent at day 2 post transfer. There is a massive cell expansion in vivo when the cells are transferred in absence of PF (Fig 24A) and this expansion is not affected even if the responders were treated with PF (Fig 24B). As a control, CFSE labelled splenocytes from the responder mice were transferred to naïve syngenic mice. The transferred cells do not divide in the recipients at day 1 (Fig 23E and F) as well as at day 2 (Fig 24C and D) and PF does not have any effect. These recipients also show CFSE negative TCR-Vβ6 bearing T cells which represent the endogenous population in the responder strain of mice. The data therefore suggest that PF by itself does not have any effect on in vivo expansion of Vβ6 bearing T cells.

3.2.2.4 Induction of activation markers on superantigen reactive T cells
Induction/upregulation of various activation markers is another key event of T cell activation. Although, PF does not affect the in vivo expansion (Fig 23 and 24), it was necessary to ask whether other events of T cell activation are modulated by PF. In a similar experimental procedure described above, at various time periods post transfer, the expression of activation markers on TCR-Vβ6 bearing T cells was analysed. The expression of the activation markers on the TCR-Vβ6 bearing T cells when the cells were transferred into naïve syngenic mice was taken as the basal expression on unactivated T
FIG 23: Profile of superantigen reactive T cells at day 1 post transfer
CFSE labelled splenocytes (30 million per mouse) from responder mice (CBA/CaJ) were transferred retro-orbitally into the stimulator mice (CBA/J) either in absence (panel C) or presence of PF (panel D). As a control, cells were also transferred to naive syngenic responder mice in absence (panel E) or presence of PF (panel F). Panels A and B show the profile of V\(\beta\)6 bearing T cells in normal stimulator and responder mice respectively. Day 1 post transfer, splenocytes from recipient mice were harvested and stained for V\(\beta\)6 bearing T cells. The above experiment is a representative of five independent experiments.
FIG 24: *In vivo expansion of superantigen reactive T cells at day 2 post transfer*

CFSE labelled splenocytes (30 million per mouse) from responder mice (CBA/CaJ) were transferred retro-orbitally into the stimulator mice (CBA/J) either in absence (panel A) or presence of PF (panel B). As a control, cells were also transferred to naive syngenic responder mice in absence (panel C) or presence of PF (panel D). Day 2 post transfer, splenocytes from recipient mice were harvested and stained for Vβ6 bearing T cells. The above experiment is a representative of five independent experiments.
cells. As shown in Fig 25, there is a significant upregulation of CD69 (Fig 25A), CD44 (Fig 25B), CD95 (Fig 25C) and CD122 (Fig 25D) on the TCR-Vβ6 bearing T cells when splenocytes from the responder mice are transferred to the stimulator mice. This upregulation is not affected when the transfer is done in the presence of PF. There is a marked upregulation of CD25 (Fig 26A) as well as downregulation of CD62L (Fig 26B) which is comparable between the PF treated and non-treated groups. The data therefore suggest that the cells get activated in the presence of PF as seen by normal expansion in vivo and expression of various activation markers and their persistence is probably due to specific reduction in AICD.

3.2.2.5 Persistence of DO11.10 TCR Tg T cells

The above used model makes use of superantigen reactive T cells. The mechanisms by which superantigen activate T cells are still not clearly understood. Therefore, a TCR transgenic system was used wherein the mice bear TCR alpha and beta transgenes specific for a peptide epitope of an antigen. DO11.10 TCR transgenic mice express a TCR that recognises ovalbumin peptide 323-339 in the context of MHC class II of I-Ad haplotype. Splenocytes from transgenic mice were transferred into naïve non-transgenic mice. Twenty-four h later, the recipients were immunised with maleyl-OA in the absence or presence of PF. At day 4 post immunisation, splenocytes were harvested and restimulated in vitro with titrating concentrations of maleyl-OA. Fig 27 shows that the splenocytes from mice receiving Tg cells in the presence of PF showed a higher proliferation than those which received in the absence of PF. The data therefore, show that PF results in an enhanced secondary response of TCR transgenic T cells even at early time points.
FIG 25: In vivo induction of CD44, CD69, CD95L and CD122 on superantigen reactive T cells

CFSE labelled nylon wool enriched T cells (20 million per mouse) from the responder mice were transferred retro-orbitally into the stimulator mice in either absence (thin black line) or presence (thick black line) of PF. As a control, cells were also transferred into syngenic naive responder mice (grey line). At day 2 post transfer, splenocytes were harvested and stained for Vβ6 with various activation markers. The data above shows expression of CD44 (panel A), CD69 (panel B), CD95L (panel C) and CD122 (panel D) on gated Vβ6 bearing T cells. The shaded area represents the isotype control. The above experiment is a representative of five independent experiments.
FIG 26: In vivo expression of CD62L and CD25 on activated superantigen reactive T cells

CFSE labelled splenocytes (30 million per mouse) from the responder mice were transferred retro-orbitally into the stimulator mice either in absence (thin black line) or presence (thick black line) of PF. As a control, cells were also transferred into syngenic naive responder mice (grey line). Day 2 post transfer, splenocytes from the recipients were harvested and stained for Vβ6 and the activation markers: CD62L (panel A) and CD25 (panel B). The data shows the expression of CD62L and CD25 on gated Vβ6 bearing T cells. The shaded area represents the isotype control. The above experiment is a representative of four independent experiments.
FIG 27: Proliferation of DO11-10 TCR Tg T cells
Splenocytes (50 million per mouse) from DO11.10 TCR Tg mice were transferred retro-orbitally to naive non-Tg mice. 24 h later, the recipients were immunised with maleyl-OA (300 µg per mouse; i.p) either in absence (open symbols) or in the presence (filled symbols) of PF. At day 4 post immunisation, splenocytes from the recipients were harvested and restimulated with titrating concentrations of maleyl-OA for 48 h. At 48 h, the response was measured as $^3$H-thymidine incorporation. The response for two mice in each group is shown in the figure. The above experiment has been done only once.
Discussion
3.3 Discussion

Recognition of peptide-MHC class II complexes by the T cell receptor on T cells initiates the early activation events. Post recognition, TCR along with its co-receptor and other costimulatory molecules gets recruited to the specialised membrane microdomains, rafts, which in turn results in concentration of various signaling adaptor molecules at these sites. Disruption of this organised structure of the plasma membrane of T cells has been shown to result in reduced TCR-induced tyrosine phosphorylation and Ca^{2+} flux (Monks, C. R. F. et al. 1998.; Xavier, R. et al. 1998.). However, the approaches so far used have either concentrated on the separation of detergent insoluble membrane fractions (which constitute the rafts) and analysing them for their association with signaling molecules under the conditions of raft disruption (Xavier, R. et al. 1998.) or looking at the cell surface and the related changes upon TCR interaction with its ligand (Kupfer, A., and S. J. Singer. 1989.).

In this study, role of rafts in T cell activation has been analysed directly by studying parameters such as proliferation, cell division and acquisition of activation markers. T cells stimulated with anti-CD3 in the presence of MCD, a cholesterol chelating agent that disrupts rafts by depleting membrane cholesterol, show a decreased proliferation in a dose dependent manner (Fig 13). This effect on proliferation is not seen with T cell hybridoma (Fig 14), which undergoes constitutive proliferation. On the other hand, IL-2 mediated proliferation is also inhibited by MCD (Fig 15). The effect of MCD on IL-2 production, an ovalbumin (OA) specific T cell line carrying β-galactosidase gene under the control of the minimal NF-AT-binding motif from the IL-2 promoter was used. This T cell line upon activation, transcribe β-galactosidase gene and the activation is measured as induced enzymatic activity. This T cell line, stimulated with OA along with syngenic APCs in the presence of 2 mM MCD, shows reduced induction of β-galactosidase activity (Fig 16), indicating reduced induction of the IL-
Discussion

It was possible that the reduced T cell proliferation is also in part due to decreased production of IL-2 and that the block in proliferation could be overcome by addition of exogenous IL-2. However, Fig 17 shows that exogenous IL-2 fails to overcome the block in anti-CD3 mediated proliferation induce by MCD. It is therefore likely from these data that MCD interferes with the signal transduction for receptor mediated proliferation which may be minimally transduced as a result of which the cells fail to proliferate.

Proliferation is one of the far downstream events of T cell activation. Among early events of T cell activation, the induction of various activation markers is prominent. Surprisingly, T cells stimulated with anti-CD3 in the presence of MCD, do show induction of CD69, CD25 and CD95 and upregulation of CD44. This implies that the T cells in the presence of MCD receive complete signal for some of the early events while at the same time fail to receive the signals to induce IL-2 and proliferate.

Various cytoskeletal changes are associated with T cell activation (Ryser, J. E. et al. 1982.; Kupfer, A., and S. J. Singer. 1989.; Sedwick, C. E. et al. 1999.). The block in cell proliferation could be either due to failure of complete signal transduction or the inability of the cells to divide as a result of disrupted cytoskeletal changes. CFSE labelled cells do not divide (Fig 19 and Fig 20) when stimulated with anti-CD3 in the presence of MCD; nonetheless they acquire CD44 (Fig 19) and CD69 (Fig 20) upon stimulation prior to cell division. These data suggest that cholesterol rich membrane microdomains have a differential role in cell proliferation versus upregulation of activation markers. The signal transduction for activation markers does not require organised membrane structure and the expression of activation markers is independent of cell division. These data make it possible to begin a systematic dissection of these differential controls in the future.

Post activation, T cells differentiate into either effector T cells or memory T cells. Effector cells execute their function during the primary immune response and undergo activation
induced cell death (AICD) or apoptosis. The generation of memory T cells is a controversial issue and various hypotheses have been put forward to explain it. To address some of the questions related to generation of memory T cells, a previously established system was used. Pentoxifylline (PF), a phosphodiesterase inhibitor, has been shown to enhance secondary T cell proliferative responses of human T cells primed in vitro by HLA-mismatched PBMCs (Gupta, M. et al. 1997.). This study was then extended to mouse system wherein it has been shown that treatment of mice with PF during priming results in the generation of enhanced and long-lasting secondary immune responses (Suresh, R. 2000.). Increase in intracellular cAMP has been shown to result in inhibition of primary T cell activation and proliferation (Scordiamaglia, A. et al. 1988.; Mary, D. C. et al. 1987.; Anastassiou, E. D. et al. 1992.) although its effect on the late events such as generation of memory responses has only been reported recently.

The effect seen with PF is likely to be mediated by an increase in intracellular cAMP since it is mimicked by a cell permeable analogue of cAMP, dbcAMP (Fig 21). PF inhibits all PDEs. There are 11 broad families of PDEs, of which PDE3, PDE4 and PDE7 have been reported to be present in T cells (Giembycz, M. A et al. 1996.; Seybold, J. et al. 1998.). High levels of PDE4 have been reported to be induced upon activation of T cells (Sun, Y. et al. 2000.). Interestingly, the effect of PF can be mimicked by the use of a PDE4 inhibitor, rolipram (Fig 21), suggesting that inhibiting PDE4 is sufficient for an enhanced T cell memory. Thus regulation of PDEs upon T cell activation may in turn regulate secondary T cell responses.

A majority of the effects of cAMP are mediated through activation of cAMP dependent protein kinase A (PKA) and therefore cAMP acts as a PKA agonist. In such a scenario, the effect of increased intracellular cAMP and subsequent PKA activation would be expected to be reversed by the use of PKA antagonist. This is in fact true in this system since mice primed with an antigen in the presence of PF in conjunction with PKA antagonist, Rp-8Br-
cAMP, show a reduction in the enhancement brought about by PF (Fig 22). Thus it appears that the activation of the PKA pathway during priming results in the generation of better T cell memory.

Various possible explanations can be envisaged for such a phenomenon. First, it is possible that cAMP and its downstream signaling pathway skews the differentiation pathway towards memory with or without affecting effector cell generation, or secondly, there is reduced activation induced cell death during the primary immune response, or thirdly, memory T cells generated survive better and longer.

To explore these possibilities, a well characterised model of superantigen mediated activation and AICD was used. Two mouse strains that are MHC matched (H-2^k) but differ in their Mls superantigen status were used. CBA/J strain (stimulator) of mice express the endogenous retroviral Mls-1 superantigen encoded by Mtv-7 while CBA/CaJ strain of mice (responder) does not. This superantigen causes the deletion of both CD4 and CD8 T cells bearing the appropriate TCR-Vβ, notably TCR-Vβ6. CFSE labelled splenocytes from responder strain of mice were transferred into stimulator strain of mice and the Vβ6 bearing T cells show equivalent proliferation by day 2 in absence or presence of PF (Fig 24). However, by day 7, these cells undergo proliferation to an extent that they lose their CFSE label but the frequency of Vβ6 bearing T cells is much higher in the mice that received PF during the transfer (Suresh, R. 2000.).

Acquisition of various activation markers is one of the early events of T cell activation. A day 2, these proliferating Vβ6 bearing T cells undergo normal activation as seen by upregulation of markers such as CD44, CD122, CD69 in the presence of PF (Fig 25). The expression of CD95L is of particular interest since it is associated with apoptotic cell death and its induction was not affected by PF. Induction of CD25 and downregulation of CD62L is also
unaffected by PF treatment (Fig 26). Thus on the whole, Mls-1 driven T cell activation is not inhibited by PF.

These data demonstrate that transient PF treatment during either immunisation with protein antigen or superantigen based T cell activation generates longer lasting T cell memory in vivo, probably by inhibiting AICD mediated T cell loss rather than inhibiting T cell activation per se.

To further extend the study to explain the phenomenon of induction of better T cell memory by PF, a transgenic mouse model was adopted. DO11.10 TCR transgenic (Tg) mice recognise OVA peptide in the context of I-A\textsuperscript{d}. Cells from TCR Tg mice were transferred into naive non-Tg recipients and the recipients then immunised with OA either in the presence or absence of PF. Four days post immunisation, there was an increased proliferation of the cells from the recipients when priming was done in the presence of PF (Fig 27). Since experiment was not followed till late time periods it is not possible to comment about the frequency of surviving TCR Tg T cells.

These data might seem to contradict the finding observed in the superantigen activation model although it is not the case. It is important to note that the responding T cells in the superantigen activation model are under constant stimulation and therefore, it is possible that the greater persistence of V\textbeta6 bearing T cells is due to reduced AICD. However, in the TCR Tg model, the responding T cells get a short pulse of antigenic exposure post which the differentiation to effector/memory T cells occurs. Therefore, in this system PF might act by increasing the proliferation potential of surviving memory T cells and/or by preventing AICD.

Taken together, the data in this chapter suggests, (a) that rafts have a differential role in regulating T cell activation and (b) cAMP mediated signal transduction pathway plays distinct roles in the generation of effector/memory T cells.