DISCUSSION
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1. Differential signals are required for the generation of primary versus secondary T cell response

2. Elevated intracellular cAMP levels increase memory T cell frequency by inhibiting activation induced cell death
Complete activation of T cells has two functional outcomes; one it generates short term effector cells which multiply, differentiate and secrete cytokines to provide B cell help or kill their target cells and second it generates cells which survive for days and weeks as antigen primed memory cells which multiply and carry out effector functions only when they get re-exposed to the same antigen. Pathways which direct these changes are not clearly understood. It is still debatable whether similar or different signals direct these changes.

The aim of this study was to find out signals affecting secondary or memory T cell development. An allore cognition system was used, where primary proliferation was used as a read out for effector T cell response, and secondary proliferative response as a read out for memory T cell response. Conditions for primary and secondary proliferative responses were optimized first and later modified. In this approach, signaling pathways were modified during priming using pharmacological agents known to affect pathways in T cell activation and their effects on secondary T cell development were analysed. Two types of agents were used which modulate different signaling pathways in T cells; one of the pathways is calcium-calcineurin dependent and the other is cAMP dependent pathway. Both pathways result in the inhibition of T cell proliferation. In another approach we have used cell proliferation inhibitor to block T cell replication and looked for its role in memory T cell commitment.
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1. Differential signals are required for the generation of primary versus secondary T cell response.

First question we have tried to address is whether there is any difference in signaling requirements for the generation of primary and secondary T cells or not. T cell activation requires sufficient levels of co-stimulatory signals. We have altered co-stimulatory activity of APCs by paraformaldehyde (PFA) fixation and used them for generating primary proliferative response as well as for priming T cells. We have titrated concentration of PFA to provide differential signals during priming. Most of the instances quoted in the literature use 0.1% to 0.3% PFA to fix the APCs. At these concentrations cells are not viable (as measured by Trypan Blue exclusion). We have titrated the concentration of PFA required to bring down primary allo-response. In various responder-stimulator pairs minimum dose required to bring down the primary proliferative response close to background level varied from 0.003% to 0.3% (data not shown). Using 3 to 10 fold lower concentrations of PFA as mentioned in literature, resulted in a decrease in the response as shown in figure 1A and 2A. However, T cells primed with PBMCs treated with graded doses of PFA showed a completely different pattern as far as secondary or recall response was concerned. APCs fixed with lower concentrations of PFA induce priming comparable to or better than that of unfixed cells in responder T cells as reflected in proliferative response upon second stimulation (Fig 1B, 2B). Higher concentration of PFA (0.3%) when used for fixing APCs, blocks signals to generate primary proliferation (Fig 1A, 2A). It also diminishes T cell priming ability of APCs and hence T cells respond very poorly to second stimulus, thus showing a state of non-responsiveness (Fig 1B, 2B) or anergy as reported earlier (Schwartz
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1990). One way of interpreting results obtained with lower concentrations of PFA is that such APCs provide relatively different signals for primary response but their priming potential is unaltered.

Results in Figure 1 and 2 also suggest that decrease in a primary proliferative response, taken as an indicator of effector T cell numbers generated, is not necessarily accompanied by a similar decrease in the secondary proliferative response. Since T cell activation is expected to result in cell replication and generation of effector and memory T cell, the dichotomy observed with fixation of APCs with lower PFA concentrations was interesting. However, further decrease in the primary proliferative response brought about by increasing PFA concentration decreases recall response as well. Hence we tried another approach to control T cell proliferation directly. We have used aphidicolin, a DNA polymerase inhibitor as a DNA replication blocker (Pedrali and Spadari 1979, Ikegami et al 1979). Aphidicolin inhibits cell proliferation in a reversible fashion. We have used this drug during priming to inhibit clonal expansion and have looked for its effect on secondary T cell response. Figure 4 shows that inhibitory effects of aphidicolin on primary proliferative response are not reflected in the recall response. Inspite of lower effector numbers generated during primary response in the presence of aphidicolin, the recall response is equal to that seen when T cells were primed in the absence of aphidicolin. This suggests that clonal expansion is not essential for the commitment of naive T cells to memory T cells.

Cell proliferation and differentiation usually do not take place simultaneously. It is common knowledge that poorly differentiated cells multiply very rapidly whether they are cancerous cells or early haemopoietic precursors. As cells get highly differentiated their
replication potential becomes more restricted; in neuronal cells this reaches an extreme state where multiplication is practically impossible. In lymphoid cells, especially in B cells as well as plasma cells this dichotomy is clearly documented. However, plasma cells which are more differentiated cells with specialized function do not multiply. However, a single B cell can produce many B cells with further potential to multiply. Each of these in turn can give rise to many more B cells and plasma cells. The role of B cell multiplication in differentiation is well illustrated in the study by George and Cebra (George and Cebra 1991). Addition of aphidicolin to B cell cultures results in increased numbers of isotype switched, IgA secreting cells as compared to controls in their study. It is possible that effector versus memory T cell commitment pathways are also governed by these rules. If the primary stimulus results in strong effector response a relatively weaker memory response may develop or a weak effector response may actually give rise to a better memory response.

In earlier two experimental systems priming signals were manipulated either by inhibiting proliferation with aphidicolin or by inhibiting co-stimulatory activity of APCs using PFA fixation. In both these cases primary proliferation was decreased but signals for inducing priming were not reduced. In fact this manipulation enhanced the ability of primed T cells to respond to recall stimuli. Priming in presence of PFA-fixed APCs brings about results similar to those seen with aphidicolin, though T cell proliferation is not directly affected. Since PFA affects membrane fluidity, signaling through various cell surface molecules might be modified resulting in decreased primary and enhanced secondary response. CD28-B7 interaction is needed for development of normal T cell response following activation, absence of which causes anergy (Schwartz 1990). We have shown that PFA
fixation of APCs (0.003% or 0.01%) at least partially disrupts co-stimulatory signals and can be cured by adding anti-CD28 Abs (Figure 3). Figure 1 and 2 suggest that PFA fixed cells are capable of inducing priming signals to T cells for the development of memory response. This means that level of co-stimulatory activity might regulate responding T cell commitment to memory versus anergic pathways. However, restoring the ability of PFA fixed cells to elicit primary proliferative response did not modulate the secondary T cell commitment reproducibly (data not shown), suggesting that signaling through CD28 may not be a major factor responsible for effector versus memory T cell commitment.

This leaves the possibility that there are signal transduction pathways that might be differentially regulating effector versus memory T cell commitment. Since IL-2 is one of the major growth factor responsible for T cell proliferation, we have used two drugs, cyclosporin A (CsA) which inhibits calcium-dependent pathways (Flanagan et al 1991) and pentoxifylline which modulates cAMP dependent pathways (Rott et al 1993). Figure 5 shows that similar to PFA and aphidicolin dose responses, increasing doses of CsA bring about a decrease in the primary proliferative response. Only the lowest dose of CsA used (0.1 μg/ml) can prime the responding T cells efficiently to bring about enhancement in the recall response. Higher doses of CsA (1 and 10 μg/ml) fail to do so. Effect of pentoxifylline on primary proliferative response is similar to CsA, i.e. higher doses (from 10 μg/ml to 100 μg/ml) tested in different experiments bring about more inhibition in the response (Fig 6A). However, the effect of increasing doses of pentoxifylline on T cell priming is remarkably different from that observed with CsA. More the inhibition in the primary proliferative response, higher is the priming potential resulting in better secondary response (Fig 6B and 7B) in the dose range tested. These data suggest that although both
CsA and pentoxifylline inhibit IL-2 synthesis, their mechanism of action on T cell priming may be different, and that IL-2 may not be directly involved in the effect observed.

IL-2 is a T cell growth factor and promotes cell multiplication. Results in figures 6B and 7B show that blocking IL-2 production is enhances the potential of priming cells to mount a recall response. By adding aphidicolin to this system, we further inhibited cell replication during priming and could show that the secondary T cell commitment is enhanced further (Figure 9). Thus, it appears that cellular-proliferation and differentiation to memory T cell phenotype are the alternative pathways for an activating T cell. It is possible that one pathway might be selected at the cost of the other in this case as well, similar to what observed in various progenitor cells during development.

Pentoxifylline is a phosphodiesterase inhibitor which increases cAMP levels inside the cell (Rott et al 1993). It is also known as c-rel inhibitor, one of the rel-family member (Wang et al 1997). It inhibits IL-2 transcription probably by inhibiting c-rel activity (Wang et al 1997). There are reports suggesting that it also blocks IL-2R transcription in human lymphocytes (Rao et al 1991), although it is still not clear for mouse cells (Wang et al 1997). CsA and FK506 inhibit IL-2 and IL-2R transcription by inhibiting NF-AT activity (Tocci et al 1989). CsA also blocks activity of rel family members (Venkataraman et al 1996). We have found in our results that CsA primed cells can give higher memory response only at lower dose. It is possible that by giving different doses of CsA we are changing the balance of different nuclear factors which decide availability of IL-2. All doses of pentoxifylline tested give higher memory T cell response at the cost of effector response (Fig 6, panel B). It is possible that graded dose of pentoxifylline might be shifting c-rel levels and changing the balance of c-rel versus NF-AT which may modulate effector
versus memory T cell commitment pathways. Bemer and colleagues have shown that presence of anti-IL-2R Abs or CsA (1 μg/ml) during priming enhances memory T cell commitment pathways although read out of T cell was B cell help rather than a recall memory assay (Bemer et al 1995). This means that memory is a function of IL-2 during priming. Our data also show that memory response is higher in the presence of IL-2 inhibitor but it is unlikely that memory function depends solely upon absence of IL-2. If that had been the case every dose of CsA should have shown higher memory response.

Pentoxifylline has been shown to affect the development of immune response in vivo. It has been shown to influence inflammatory and Th2 cytokines like IL-1β, IL-6, IL-8 and TNF-α in human PBMCs (Neuner et al 1994). It has also been known that pentoxifylline inhibits IFN-γ production, and increases IL-4 and IL-10 levels in mitogen or antigen-stimulated cultures (Rieckmann et al 1996). This suggests that pentoxifylline facilitates Th2-like cytokines by inhibiting Th1-like cytokines. Low availability of IL-2 clearly seems to trigger memory T cell commitment from our experiments. However, whether the other cytokines are present in differing amounts to influence effector versus memory outcome was a question worth asking. Accordingly we analysed culture supernatants for IFN-γ, IL-4 and IL-10. Our results show that IFN-γ levels (Fig 13) are high in T cells primed in the presence of pentoxifylline as compared to T cells primed in the absence of pentoxifylline in parallel with the proliferation results. We didn’t get any detectable levels of IL-4 and IL-10 in either of the populations (data not shown). The data did not provide conclusive answer to the role of cytokines in effector versus memory T cell commitment.

Activated T cell compartment is divided into effector and memory T cell populations. Phenotypic differences in effector and memory T cells are not clear, as distinguished
markers between two groups are not available. However, some reports suggest that there are differences in their survival time. Effector cells die after 2-10 cycles of replication while memory T cells stay in G0 phase and survive for years (Bell et al 1998). There are reports suggesting that memory T cells revert back to naive phenotype and stay as naive cell in the body (Bell et al 1998). However, more than one type of memory T cell has been described by Bunce and Bell. According to them persistence of antigen in the host decides the fate of these memory cells. Bunce and Bell have shown that there is more vigorous and rapid response generated upon restimulation by the revertant-memory phenotype population as compared to naive population (Bunce and Bell 1997). This difference can be attributed to either expansion of the antigen-specific responder cell numbers after first stimulation or to qualitative differences between naive and memory T cells. Study by Constant and colleagues have shown that there is no difference in kinetics and magnitude of the antigen-specific response of unprimed or naive and primed or memory T cells in TCR transgenic mice. They have shown that if equal number of responders are taken from naive population and primed population then there is no difference in kinetics and magnitude of the proliferative responses (Constant et al 1994). Bruno et al on the contrary, show that memory effector cells respond qualitatively better than the naive cells as per cell basis (Bruno et al 1995). Our results seem to support this notion rather than one suggested by Constant et al. When we have used different T cell proliferation inhibitors (CsA [0.1μg/ml], Pentoxifylline), we seem to get better secondary T cell response (Figs 5B, 6B and 7B). In fact cell populations which have received pentoxifylline as modulator of signals behave better in a recall response than cells which are generated in higher numbers
in the absence of pentoxifylline. Thus fewer numbers of primed (memory) cells give rise to better response in our system.

Absence of sufficient co-stimulatory molecules has been demonstrated to cause T cell anergy. T cell anergy affects at the level of IL-2 transcription (Fraser et al 1991). Earlier reports and our data suggest that blocking IL-2 gene expression by CsA (Fig 5) or neutralizing its functional activity by IL-2R Abs generates better memory T cells (Bemer et al 1995). Whether levels of IL-2 decide memory versus anergy commitment pathways is still controversial. In this context, we have found that pentoxifylline mediated enhancement of recall response is unaffected by the presence of exogenous IL-2 (Figure 14) though addition of IL-2 during allo-priming increases effector response. Frequency of IL-2 responsive T cells when allo-primed in the presence or absence of pentoxifylline is not different (Fig 21) suggesting that IL-2 alone may not be the only factor involved in effector versus memory T cell commitment pathways.

Pentoxifylline is a phosphodiesterase inhibitor which increases intracellular cAMP levels and hence activate cAMP dependent pathways (Rott et al 1993). It is relevant to ask what happens if we provide a direct source of cAMP to the cells. We have used a cell-permeable analogue of cAMP, dibutyryl-cAMP (dbcAMP) during priming and looked for its effect on secondary response. Figure 15 shows that, like pentoxifylline, dbcAMP inhibits primary proliferation and enhances secondary response. Earlier report using froskolin or theophylline shows that high cAMP levels inhibit IL-2 mediated T cell proliferation and facilitate differentiation of T-helper cells towards Th2 phenotype (Gajewski and Fitch 1991) but the role of cAMP in facilitating memory T cell commitment in terms of T cell proliferation is something not reported before.
CsA, cAMP and pentoxifylline inhibit IL-2 transcription by blocking at different levels in signal transduction pathway (Tocci et al 1989, Wang et al 1997). Our data suggest that CsA does not show memory enhancement at all doses as pentoxifylline or dbcAMP show despite the fact that all inhibit IL-2 transcription. We used another approach to see if CsA, dbcAMP and pentoxifylline influence direct IL-2 mediated proliferation similarly. Figure 16, 17, 18 show that both dbcAMP and pentoxifylline inhibit IL-2 and IL-4 mediated CTLLs proliferation in dose dependent manner whereas CsA has no effect. Thus dbcAMP and pentoxifylline seem to influence T cell proliferation downstream of their effect on IL-2 and IL-2R transcription, unlike CsA.

2. Elevated intracellular cAMP levels increase memory T cell frequency by inhibiting activation induced cell death

Pentoxifylline influences activity of wide range of cell types. It is known to affect lymphocytes (Wang et al 1997), macrophages (Streiter et al 1988), endothelial cells (Kovach et al 1994), muscle cell (Bellas et al 1995) and other haemopoietic and non-haemopoietic cells (Samlaska and Winfield 1994). We have shown that pentoxifylline enhances secondary T cell response. It is relevant to ask whether pentoxifylline is directly affecting T cell activity or indirectly affecting co-stimulatory function of APCs which influence T cell activation. We have blocked co-stimulatory function of APCs by PFA fixation and studied their ability to prime T cells in the presence or absence of pentoxifylline. Figure 12 shows that pentoxifylline mediated enhanced recall response is
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not changed. This rules out a major role of co-stimulatory activity of APCs in the observed effect of pentoxifylline.

There could be two possibilities of higher bulk response in secondary stimulation with pentoxifylline. One could be that the antigen-specific T cell numbers generated under the cover of pentoxifylline are higher and/or replication capacity of individual primed cell is higher. Second possibility is that there are more end stage effectors generated during priming in the absence of pentoxifylline cover and die at the end of priming before they respond to recall stimulus. We have analysed for both the possibilities. Using limiting dilution analysis method (Taswell 1981) antigen-specific precursor frequency and replication capacity of each primed T cell is checked. Figure 20 shows that the number of cells increase by 3 fold when priming is done in presence of pentoxifylline or dbcAMP. We have also calculated replication capacity of cells at clonal level (37% or less positivity). Figure 20, panel B shows that there is no change in replication capacity of cells whether they were primed with or without pentoxifylline. This suggests that pentoxifylline may be affecting at the level of conversion of terminal effectors to memory T cells rather than changing the replication potential of memory cell. It means that probably pentoxifylline helps in generating more memory cells at the cost of effector numbers.

Naive T cells are different from effector cells and memory T cells in terms of function and phenotype. Naive T cells show CD44hiCD62hiCD45Rhi phenotype (CD45RA in humans) while memory or activated cells show CD44hiCD62loCD45Rlo phenotype (CD45RO in humans) (Sprent 1994). They also have high levels of CD2, CD26, CD40L, LFA-1 (Williams et al 1996). In light of this information we have looked for various phenotypic markers which might help us in distinguishing effector T cells from memory T cells. We
have looked for markers and didn't find any significant difference in expression levels of CD45RO, CD26, CD69 (Williams et al 1996) and gp240 (Cotner et al 1983) in cells primed under the cover of pentoxifylline or in its absence, since our functional read out suggested that the numbers of memory cells responding to secondary stimulus were higher in pentoxifylline treated as compared to untreated group. Activated T cells show increased levels of high affinity IL-2R levels, which can be detected by the presence of alpha chain, which is associated with beta-chain of IL-2R. We have checked the kinetics of expression of IL-2R levels in our system. Staining profiles in Figure 22A and 22B show that the number of high affinity IL-2R\(^+\) cells increases at the end of allo-priming in CD4\(^+\)T cell population and decreases after 2 days of rest. While priming in the presence of pentoxifylline results in less IL-2R\(\alpha\) positive cells, the number of IL-2R\(\alpha\) chain positive cells increases after 2 days of rest. Relatively low numbers of IL-2R\(\alpha\) positive cells in pentoxifylline treated group may be a direct consequence of effect of pentoxifylline on IL-2R transcription (Rao et al 1991). Decay of IL-2R\(\alpha\) levels on these cells is also slower than the cells primed without pentoxifylline even after 2 days of resting period. This increase in the percentage of IL-2R\(^+\) cells is not due to proliferation as a consequence of withdrawal of pentoxifylline, which has been checked by adding aphidicolin during resting period (Fig 23). It is well documented that IL-2, by its interaction with IL-2R and subsequent intracellular signaling provides a 'survival signal' for T cells. Using IL-2R\(\alpha\)^+ mice, van Parijs and others have recently shown that signaling by IL-2 through IL-2R also contributes to activation induced cell death. Using IL-2R\(\alpha\)^+ TCR transgenic mice they have successfully shown a role for IL-2R\(\alpha\) in apoptosis (van Parijs et al 1997b). Since T cells primed in presence of pentoxifylline are showing increase in the percentage of CD25^
cells (Fig 22B) over a two day period, we investigated the role of pentoxifylline in apoptosis. Amongst various ways available to look for apoptosis, we have used propidium-iodide to stain DNA. Figure 24 shows fluorescence in CD25+ cells. The hypodiploid cell numbers, indicative of apoptosis, are much higher in T cells primed in the absence of pentoxifylline as compared to those primed in its presence observed over a 2 day period. We have also used annexin V staining of CD25+ cells and looked for DNA pattern after antigen priming (data not shown). These studies also support the findings using propidium-iodide staining, that pentoxifylline treatment retards the rate of apoptosis. Thus pentoxifylline treatment clearly delays the rate of activation induced apoptosis. van Parijs and others have shown that IL-2 potentiates Fas mediated activation induced cell death (van Parijs et al 1997b). Other studies have also shown that Fas-FasL interaction is involved in activation induced cell death (Brunner et al 1995). Hence we looked for the role of Fas in inducing apoptosis in our system. Cells primed with or without pentoxifylline were stained for the expression of Fas. Figure 25 shows that the number of Fas+ cells goes down on day 2 post-priming in both pentoxifylline treated and untreated populations. There is no major difference in the downmodulation of Fas levels between two groups over a 2 day period. Proportion of Fas+ cells to that of total IL-2R+ cells is equal in both the population on day 0 and day 2 post-priming. These data together suggest that pentoxifylline clearly slows activation induced cell death. However, the mechanism of inhibition of apoptosis is not clear. Taken together these results suggest that memory T cell frequency can be enhanced by delaying or inhibiting apoptosis.