Discussion
Interaction of the TCR with its cognate peptide-MHC initiates a tyrosine phosphorylation cascade that triggers multiple branching signaling pathways. The signals downstream of the TCR/CD3 complex are modified by concomitant or sequential signals from the other recruited surface receptor-ligand pairs. Cell surface adhesion molecules increase the avidity of the T cell-APC interaction and co-stimulatory molecules amplify or counteract signals provided by the T cell receptor complex. These complex signal interactions either intersect or compete with each other and thus regulate the outcome of T cell stimulation.

Following TCR engagement, proximal intracellular events including tyrosine phosphorylation, calcium mobilization and inositol phospholipid generation occur within 30 seconds to 5 min. The early signals however, are not sufficient to trigger complex functions such as T cell proliferation. In order for T cells to commit to proliferation, they require sustained TCR signaling that maintain the immunological synapse for hours (Huppa et al., 2003). Resting T cells that receive such productive signaling showed increase in cell size, enhanced biosynthetic activity, expression of many gene products necessary for DNA replication, upregulation of activation markers such as CD25 and CD69, IL-2 secretion and proliferation.

The contact time required for T cells to commit to proliferation varies between CD4 and CD8 T cells and depends largely on the amount of antigen and the co-stimulatory signals available. Under conditions of high antigen availability and presence of co-stimulatory APCs, the time of commitment of naïve CD4 T cell is about 15-20 h (Iezzi et al., 1998). On the other hand, naïve CD8 T cells achieve commitment more rapidly, and need only 2-4 h of contact with the APC (Cai et al.,
The extent of CD8 T cell proliferation is not determined by the continuous presence of antigen and reports suggest that following the initial contact phase, CD8 T cells undergo autonomous expansion in an antigen independent manner (Mercado et al., 2000). CD4 T cell proliferation on the other hand is dependent on presence of antigen at the early stages and is cytokine driven in the later stages (Jelley-Gibbs et al., 2000).

Each T cell-APC interaction does not necessarily result in productive TCR signaling. T cells that receive TCR signals in the absence of co-stimulation enter a state of unresponsiveness that can be broken by exogenous IL-2 (Appleman and Boussiotis, 2003). On the other hand, T cells that fail to divide after activation acquire a hyporesponsive state that is refractory to both TCR/CD28-mediated and IL-2R-mediated proliferative signals (Wells et al., 1997).

Depending on the strength of signal or the presence of polarizing cytokines at the time of priming, T cells commit to differentiate into T_{H1} or T_{H2} polarized effectors (Constant and Bottomly, 1997; Rogers and Croft, 1999). Prior division history of individual activated T cells also induces distinct secondary response patterns (Itoh and Germain, 1997; Bucy et al., 1994). Cells that undergo more rounds of division show greater IL-2 production and proliferation in response to restimulation. Although many signals that modulate differentiation have been reported, it is not clear how these signals delivered early during a primary T cell response influence relatively late events such as proliferation or even later events such as effector or long-lived memory generation.

To determine the precise relationship between signal strength and cell fate, \textit{in vitro} priming systems have been used where strength of stimulation and co-
stimulation can be controlled. One receptor-ligand pair that influences T cell responses at various levels is ICAM-1/LFA-1. This interaction is a major player in the migration of leukocytes towards sites of infection. Its importance in recruiting neutrophils to inflamed tissues was demonstrated in mutant mice lacking ICAM-1 that show resistance to the lethal effects of high doses of LPS due to lower neutrophil infiltration in the liver (Xu et al., 1994). The adhesive function of ICAM-1/LFA-1 is crucial for most immune cell interactions, especially at the immunological synapse between T cell and APC. ICAM-1/LFA-1 interaction facilitates synapse formation and the high avidity binding helps stabilize the synapse (Dustin et al., 1998). In addition, ICAM-1/LFA-1 also delivers co-stimulatory signals inducing T cell activation and proliferation. ICAM-1 and LFA-1 are expressed on T cells as well as APC and both sets of interactions - ICAM-1 on T cells interacting with LFA-1 on APCs, and LFA-1 on T cells engaging ICAM-1 on APCs can provide co-stimulation. In an earlier study LFA-1 on T cells was reported to influence in vivo antigen specific T cell responses (Kandula and Abraham, 2004). However, in this system LFA-1 (CD11a/CD18) interaction with all its ligands e.g. ICAM-1, ICAM-2 and ICAM-3 gets abrogated in CD18 null mice. The present study made use of mice lacking ICAM-1 and had undertaken specific analysis of the role of signaling through LFA-1 or ICAM-1 on T cells in early and late T cell responses. The use of ICAM-1 null mice allowed the study of activation events, following polyclonal stimulation, where either T cells or APC from the KO mice could be specifically used for ex vivo cultures. ICAM-1 null mice further allowed analysis of long term antigen specific responses in vivo, following immunizations. The absence of ICAM-1 can influence the ability of T cells to
traffic in vivo. The possibility of poor in vivo trafficking of cells affecting T cell priming was ruled out as earlier reports have demonstrated that migration of T cells to site of infection was similar between WT and ICAM-1 null mice (Zhang et al., 2003). The present study shows that lack of ICAM-1/LFA-1 interaction affects very early and late events in T cell activation and differentiation and that other ligands of LFA-1 as well as other co-stimulatory molecules that are normally expressed in ICAM-1 deficient mice cannot fully compensate for the lack of ICAM-1.

The initial studies focused on very early activation events in the absence of ICAM-1. To evaluate TCR downmodulation, following antigen driven signaling through the TCR, T cells from TCR-transgenic mice were used. This study finds that MHC I restricted TCR-transgenic T cells specific for LCMV peptide rapidly downregulate surface TCR when stimulated with peptide pulsed APCs (Fig 10). However, when ICAM-1 null APCs were used, TCR downregulation was poor at low doses of stimulating peptide. At the higher doses of peptide both WT and KO APCs could induce equivalent TCR downregulation. These results are consistent with earlier reports showing ICAM-1 to be important for the loss of surface TCR at limiting doses of antigen (Cai et al., 1997; Bachmann et al., 1997).

The next step was to look at the role of ICAM-1/LFA-1 on T cell activation by monitoring expression of CD25, CD69, CD44, CD27, CD134, CD122 and CD127 on splenocyte cultures activated with anti-CD3. CD69 gets upregulated very early on activated T cells and stays on transiently (Testi et al., 1994). The expression of CD44 increases after activation enabling primary adhesion or rolling of activated T cells, allowing extravasation into inflammatory sites (DeGrendele, 1997). Members of the TNFR family, CD27 and CD134, are also upregulated on
activated T cells and CD27 facilitates T cell-T cell or T cell-APC interaction post expansion (Van Lier et al., 1987; Hintzen et al., 1994). CD134 with CD134L on DCs potentiates the proliferation and cytokine secretion by T cells (Gramaglia et al., 1998). The cytokines IL-2 and IL-7 have been shown to have integral roles in T cell proliferation, survival and memory responses (Vella et al., 1997; Grabstein et al., 1990; Schluns et al., 2000; Zhang et al., 1998). The effects of these cytokines at various stages are regulated by the differential receptor expression. Naïve T cells express IL-7Rα (CD127) and low affinity IL-2Rβ (CD122) but do not express the high-affinity receptor IL-2Rα (CD25). CD25 expression on the surface increases after T-cell activation and facilitates cell proliferation (Smith, 1988; Janeway and Bottomly, 1994). CD122 gets upregulated after activation and its expression on memory T cells facilitates their maintenance in vivo (Kanegane and Tosato, 1996). T cell activation results in downregulation of CD127 accompanied by a decrease in the level of BCL-2, which might promote contraction of immune responses in vivo (Schluns et al., 2000). However, expression of CD127 is slowly regained throughout the contraction phase, which is followed by an upregulation of expression of BCL-2. These differentiation markers therefore allow study of the activation status of T cells.

This study finds that CD69 upregulation was poor on transgenic T cells when peptide pulsed ICAM-1 null APCs were used to stimulate antigen specific activation (Fig 10). It was seen that when stimulated with soluble anti-CD3 in culture, CD4 and CD8 T cells from ICAM-1 null mice show poor upregulation of early activation markers CD69, CD25, CD44, CD27, CD134 and CD122 and poor downregulation of CD127 (Fig 2 & Fig 3). In parallel cultures, CD4 and CD8 T
cells from ICAM-1 null mice show poor proliferation, as compared to WT T cells, in response to anti-CD3 (Fig 4). Since only a fraction of ICAM-1 null T cells upregulate the IL-2 receptors, it suggests that majority of the KO T cells might not be responsive to the proliferation inducing effects of IL-2. Similar to earlier reports, CD8 T cells proliferate more extensively as compared to CD4 T cells in both WT and KO populations (Gudmundsdottir et al., 1999; Foulds et al., 2002). The defect in proliferation of KO T cells was more pronounced at lower doses of anti-CD3. Similar dose dependent requirement for ICAM-1 was observed earlier in TCR downregulation and CD69 upregulation studies (Fig 10). These results are in keeping with earlier reports where in vitro T cell proliferation experiments using LFA-1 deficient TCR-transgenic T cells showed a similar dependence on peptide dose (Bachmann et al., 1997). These data indicate that ICAM-1/LFA-1 interaction may be crucial in determining productive T cell activation under limiting antigen concentration.

The next attempt was to identify specifically whether the observed defect in proliferation was due to lack of signals via ICAM-1 on T cells or lack of signaling via LFA-1 on T cells. Co-culture studies showed that ICAM-1 deficient T cells cultured with WT and KO APCs get activated and proliferate similar to the WT T cells that are cultured alone (Fig 8 & Fig 9). However, activation and proliferation of WT T cells in the same co-culture was slightly compromised. This suggested that presence of ICAM-1 on APC was crucial, which was confirmed when different APCs from ICAM-1 deficient mice were shown to be poor at inducing proliferation of allogenic responder T cells in vitro (Fig 11). This study also demonstrated that WT and KO T cells transferred into irradiated ICAM-1 sufficient, allogenic
recipients showed equivalent proliferation within the host. Together these results indicate a specific role for ICAM-1 on APCs and not on T cells.

There is evidence to indicate that the extent of primary clonal expansion of T cells correlates with the level of memory generated. Immunization of mice with heat killed *Listeria monocytogenes* resulted in fewer divisions of antigen-specific cells during primary expansion and generated poor protective immunity. In contrast live immunization induced much better expansion during priming and generated long-lived protective immunity (Lauvau et al., 2001). Another report that supports this notion shows that co-stimulatory receptor, OX-40, sustains IL-2 production, clonal expansion and survival of activated T cells during the primary response, and results in accumulation of greater numbers of memory cells with time (Gramaglia et al., 2000). The *in vitro* proliferation defect associated with lack of ICAM-1/LFA-1 interaction makes it likely that *in vivo* clonal expansion following immunization also might be restricted and might therefore lead to poor generation of T cell memory in ICAM-1 deficient mice. In this study, both WT and ICAM-1 null mice generated equivalent proliferation and cytokine response to recall antigen early after OVA protein immunization (*Fig 12 & Fig 13*). However, the CD4-dominant recall proliferative response in KO mice was negligible at later times. On the other hand, cytokine responses were similar in WT and KO mice (*Fig 13*). OVA immunized ICAM-1 null mice showed good effector cytokine response even at later times. However, by the later time proliferation competence during recall declined. This suggests that T cells may not follow the linear differentiation model proposed for memory generation. The rapid decline in the recall proliferative response could be due to decreased survival of activated T cells in mice lacking ICAM-1. Previously it
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has been reported that signals mediated by ICAM-1 on APCs interacting with LFA-1 on T cells results in fewer cell divisions and more apoptosis (Kohlmeier et al., 2003). Consistent with these reports, it was observed that activated T cells from ICAM-1 null mice were not more susceptible to post activation death and, if anything, survival of KO T cells was slightly better (Fig 16 - Fig 19).

This study also showed another interesting finding. It was observed that anti-CD3 driven proliferation of WT and KO T cells, cultured at high cell densities, were equivalent. While with decrease in cell density, proliferation of WT T cells declined proportionately, however, proliferative response of KO T cells declined drastically (Fig 5). Increasing the duration of stimulation enhanced the response of KO T cells even at slightly lower cell density. Additionally, providing exogenous IL-2 or co-stimulation via CD28 reversed the defect in proliferation of KO T cells seen at low cell density (Fig 6). Increasing the number of T cells cultured along with a fixed number of KO APCs also improved the proliferation of T cells indicating that presence of ICAM-1 on APCs became crucial at lower responder T cell numbers. At high cell densities in culture, due to crowding, duration or stability of the otherwise weak T cell-APC interactions may increase sufficiently to result in equivalent proliferation of WT and KO T cells. These results point towards a significant role for ICAM-1/LFA-1 interaction in T cell responses under conditions of limited IL-2 or co-stimulation. In vivo, more than one antigen-specific T cell may crowd around an APC and get activated simultaneously and further the chances of productive TCR engagements. However, for antigens that invoke small numbers of responding T cells, recall responses might be compromised in mice lacking ICAM-1.
To test this notion, ICAM-1 null mice were immunized with peptide antigen, that recruits very few antigen-specific responding T cells, and in vivo responses to antigen were studied. Both proliferation and cytokine production in response to recall antigen was negligible (Fig 14 & Fig 15). In contrast to OVA immunization, ICAM-1 null mice responded poorly to OVA peptide immunization. As OVA protein and OVA peptide immunizations were done as CFA emulsions, the presence of inflammatory mediators and non specific T cell activation signals are likely to be similar in both situations. The explanation for the difference in response is that larger numbers of T cells responding to the various OVA epitopes might augment the responses of individual responders. Normally in vivo, antigen specific T cells would be very few and productive T cell-APC interactions would decide the eventual magnitude of the response.

The poor in vitro proliferation and short-lived recall responses suggested that proliferation competent memory might be compromised in the absence of ICAM-1. The Salmonella typhimurium (Stm) infection model allowed the examination of both short term effectors, identified as IFN-γ secreting cells, and proliferation competent memory, evaluated as the ability to protect against a challenge infection at later times post immunization (Thatte et al., 1995; George, 1996). Short term effector responses were similar in Stm immunized WT and KO mice at all times (Fig 20). There was no difference in the systemic bacterial burden at early times after primary infection in WT and ICAM-1 null mice. A secondary challenge infection was cleared equally efficiently by Stm immunized WT and KO mice. However, the ability to clear a secondary infection at later times was compromised in mice lacking ICAM-1 (Fig 21). These results are consistent with a
previous report where ICAM-1 deficient mice immunized with an attenuated strain of Stm and later challenged with a virulent strain showed more mortality than the WT mice (Clare et al., 2003). The antibacterial immunity, at later times post immunization, depends mostly on the ability of persisting antigen specific memory CD4 T cells to proliferate and generate effector cells that would control the infection. The poor immunity to infection seen in mice lacking ICAM-1 indicated that the proliferation competent central memory population may be affected. The bacterial clearance ability at early times might involve the function of both effector cells as well as memory cells.

In order to further characterize the memory population, surface levels of memory markers were studied. Activated T cells can be distinguished from naïve T cells by high expression levels of CD44 and low levels of CD62L. This CD44+ population either continues to be CD62L− (effector memory; T_{EM}) or slowly regains CD62L expression (central memory; T_{CM}). Phenotypic characterization was done for the memory subsets, T_{EM} and T_{CM}, generated early after initiation of T cell responses. TCR-transgenic T cells were activated in vitro with peptide presented by either WT or ICAM-1 null APCs. The phenotypic markers used to identify T_{EM} do not differentiate between recently generated effectors and long-lived T_{EM} cells. Therefore it is difficult to make out the proportions of effectors versus the T_{EM} cells.

It was observed that T cells primed on APCs that lack ICAM-1 generated fewer cells of the T_{CM} phenotype (Fig 22).

It could therefore be expected that ICAM-1 null mice responding to environmental stimuli would accumulate in vivo, a memory pool that is compromised in T_{CM} subset. Characterization of the memory pool, using surface
markers, in the lymphoid organs showed similar numbers of cells that exhibit memory phenotype and significantly reduced numbers of \( T_{\text{CM}} \) cells (Fig 23 & Fig 24). As seen for OVA immunization, these experiments suggest that T cells follow the divergent model for memory generation where naïve T cell directly differentiates into effector or memory cells.

This study therefore puts together a model explaining the role of ICAM-1/LFA-1 interactions in long term differentiation events. ICAM-1 on APCs interacts with LFA-1 on T cells and would influence the priming event by stabilizing the T cell-APC synapse and providing co-stimulatory signals. As suggested by earlier \textit{in vitro} experiments the absence of ICAM-1 during priming would result in low IL-2 production thereby affecting proliferation of these cells. This study clearly demonstrates that in the absence of ICAM-1, T cells proliferate poorly and that signals provided by ICAM-1 on APC becomes crucial under conditions of low responder T cell numbers, low IL-2 and low co-stimulation. The fewer cell divisions observed probably could modify T cell memory lineage commitment. In other words, signaling via LFA-1 on T cells is important for the generation of the proliferation competent central memory T cells.