RESULTS
Results

1. Role of PDE inhibitor PF in T cell memory responses 64
   1.1 Allogenic recognition system of T cell activation 64
      1.1.1 Effects of modulation of cAMP levels on T cell priming 64
   1.2 Effect of PF on T cell priming in vivo 65
      1.2.1 Priming with protein antigen 65
      1.2.2 Allo-priming in vivo 65
   1.3 Effect of PF in post activation cell death in vivo 66
   1.4 Effect of PF on activation marker upregulation 68
   1.5 Effect of PF on post activation death in vitro 69

2. Role of reactive nitrogen intermediates (RNI) in T cell memory responses 70
   2.1 Effect of RNI on antigen specific T cell frequency 70
   2.2 Effect of RNI on memory CD4 T cell generation in vivo 71
   2.3 Effect of iNOS+/+ antigen presenting cells on stimulation of WT or iNOS−/− T cells 71
   2.4 Effect of iNOS+/+ environment on survival of iNOS−/− T cells 72
   2.5 Effect of RNI on memory CD4 T cell generation in vitro 73
   2.6 Effect of RNI on T cell death 74
      2.6.1 Effect of RNI on death of mouse T cells 74
      2.6.2 Effect of RNI on death of human T cells 75
   2.7 Effect of iNOS or peroxynitrite scavengers on primary T cell responses 76

3. Effect of Aging on T cells 77
   3.1 Phenotypic characterisation of aged C57BL/6 and c-Rel−/− mice 78
   3.2 Effect on T cell proliferation 78
   3.3 Effect on secondary T cell responses 79
   3.4 Alteration of T_{CM} / T_{EM} ratio 79
   3.5 Effect of aging on proliferation of naïve T cells (CD44lo) 81
   3.6 Scoring death in activated CD4 and CD8 T cells 84
   3.7 Effect of aging on PAD of T cells 85
   3.8 Effect of aging on memory T cell generation in vivo 86

4. T cells as antigen presenting cells 87
   4.1 T cell as stimulators in allogenic mode of stimulation 87
   4.2 T cells as APCs for presenting nominal protein antigen to responding T cells 89
1. Role of PDE inhibitor, PF in T cell memory responses:

1.1 Allogenic recognition system of T cell activation:

The allore cognition system of specific activation of T cells has the advantage of giving 1000-10,000 fold higher frequencies than the nominal antigen specific cells. Due to this, both primary and secondary T cells responses can be measured in vitro. C57BL/6 (H-2\textsuperscript{b}) splenocytes were used as responders and stimulated using PFA-fixed, $\gamma$-irradiated BALB/c (H-2\textsuperscript{d}) splenocytes as APCs. For primary proliferation, $3 \times 10^5$ responder cells were incubated with titrating numbers of stimulator cells for 72h and $^3$H-thymidine incorporation during the last 12-16h of an 84-90h assay was used to estimate the primary response. For estimating secondary proliferative response, responder and stimulator cells were incubated at a ratio of 3:1 for 72h. At 72h, live cells were separated (as described in section 3 methods), washed and rested for 24-36h. Following rest, the cells were restimulated with titrating numbers of fresh irradiated BALB/c stimulators for 48-72h and proliferation was estimated using thymidine incorporation during last 12-14h of the assay.

We have used this model to address some of the questions based on earlier findings in the laboratory.

1.1.1 Effects of modulation of cAMP levels on T cell priming:

Earlier findings in the lab were confirmed using pentoxifylline (PF), a pan-phosphodiesterase (PDE) inhibitor. Splenic cells from C57BL/6 (H-2\textsuperscript{b}) mice were stimulated in vitro with titrating numbers of irradiated splenocytes from BALB/c (H-2\textsuperscript{d}) mice in presence or absence of PF (360 $\mu$M) as described above. Priming done in presence of PF inhibited the primary proliferative response of T cells (Fig. 1A). Cells primed in presence of the same dose of PF, when restimulated with titrating numbers of irradiated BALB/c (H-2\textsuperscript{d}) splenocytes showed enhanced secondary T cell response as compared to cells primed in absence of PF (Fig. 1B).
Figure 1: Effect of PF on T cell allogenic response.
Panel A shows primary allogeneic response of T cells in absence or presence of 360 µM PF. Panel B shows secondary response of T cells allogeneic in absence or presence of 360µM PF. Data are represented as mean cpm of triplicate culture ± SE. Background cpm<5000. This experiment is representative of three independent experiments (Nil indicates cultures without any pharmacological agent).
1.2 Effect of PF on T cell priming in vivo:

1.2.1 Priming with protein antigen:

It has also been shown in lab that mice immunized with nominal antigens such as ovalbumin (OVA) under the cover of PF show enhanced persistence of memory T cells as compared to mice immunized without giving PF.

We examined whether PF cover in immunized mice enhanced the frequency and/or the clonal burst size of Ag-specific T cells. C57BL/6 mice (3 mice/group) were immunized s.c. with maleyl ovalbumin (mOVA, 100 μg/mouse) on CFA with or without PF cover (2 mg/mouse/day, from day -1 to day +5 of immunization) (as described in materials and methods). 7, 21 and 45 days later, mice were euthanised, draining lymph nodes were harvested and limiting dilution assay was performed as described in section 8 in methods. Titrating numbers of lymph node cells were restimulated with mOVA (1 mg/ml) to estimate the frequencies and clonal burst size of T cells. As early as day 7 post immunization there was no significant difference in frequency of antigen specific cells between two groups, but results show that by day 21 and day 45, PF treatment significantly increased the clonal frequency of mOVA specific T cells (Fig. 2A). In order to know whether enhanced memory response seen in PF treated group was due to increased potential of every primed cell to undergo replication, data obtained from LDA done on day 45 were re-analysed to calculate clonal burst size. Based on the number of positive wells in each groups, only those groups that showed less than 37% of the positive wells were selected to calculate the burst size of memory cells. The average cpm of the positive wells compared to the average cpm of negative wells gave the estimate. Fig. 2B shows that PF treatment did not affect the clonal burst size of antigen specific memory T cells.

1.2.2 Allopriming in vivo:
Figure 2: PF enhances memory T cell frequencies rather than their clonal burst size.
Panel A shows frequencies of antigen specific T cells from mOVA immune mice as calculated by LDA on indicated days post immunization. Panel B shows clonal burst sizes of the memory T cells as calculated from LDA done on day 45. $^3$H thymidine incorporation in non responding wells is also shown for comparison. Panel A, data are represented as mean frequency of three mice/group±SE. Panel B, data are represented as mean cpm of triplicate cultures±SE. This experiment is representative of three independent experiments.
Thyl.1 congenic mice were immunized s.c. with 30 million γ-irradiated BALB/c spleen cells in absence or presence of PF treatment (2 mg/mouse/day from day -1 to day +5 of immunization and three mice per group). Seven weeks later, draining lymph node cells were harvested and labelled with CFSE and either kept in culture without further stimulation or stimulated with γ-irradiated BALB/c spleen cells as APCs. After 3 days in culture, cells were stained for Thy-1.1 and CD4 and flow cytometric analysis was done. Fig. 3 shows contour plots of cell size vs. CFSE, on gated Thy 1.1-CD4 responder T cells. Panels A-C are unstimulated cultures and panels D-F are cultures restimulated *in vitro* with MHC mismatch BALB/c APCs from different group of mice as indicated. Increase in cell size is an indicative of cells getting activated, therefore to examine activation induced T cell proliferation, we looked at the frequency of proliferating cells in the large sized cells (gate in panels A-F). Stimulated CD4 T cells (panels D-F) showed substantial increase in cell size as compared to the unstimulated cells (panels A-C). Greater frequencies of CD4 T cells, from mice immunised under the cover of PF, underwent proliferation during restimulation (panel F) as compared to CD4 T cells from mice immunised under the cover of saline (panel E). As described in section 5 in methods, using proliferation platform, the frequencies of CD4 T cells undergoing various numbers of divisions were estimated. It could be observed that, while the frequencies of responding cells were higher in PF-treated mice at every cell division, CD4 T cells from mice immunized under PF cover have undergone clonal division at same rate as the CD4 T cells from mice immunized without PF cover (Fig. 3G). Thus, the frequency, but not the clonal burst size, of antigen specific proliferation-competent CD4 T cells increases if the mice are treated with PF at the time of immunization.

1.3 Effect of PF in post activation cell death in vivo:
Figure 3: PF increases frequency of antigen specific CD4 T cells, but does not affect the kinetics of response.

*In vitro* allo-proliferative response of mice allo-immunised 7 weeks earlier. Fig. shows two color contour plots of cell size vs. CFSE on gated Thy1.1-CD4 responder T cells. Panel A1 shows the representative Thy1.1-CD4 gate used for the analysis. Panels A-C are unstimulated cultures and panels D-F are cultures restimulated *in vitro* from different group of mice as indicated. Panel G shows the frequencies of CD4 cells undergoing various numbers of divisions as plotted for the stimulated cultures shown in panels E and F. This experiment is representative of three independent experiments.
We next tested the effect of PF on T cell activation and post activation death in vivo, using the well-characterized model of superantigen-mediated activation and deletion of T cells bearing certain TCR-Vβ elements (Webb SR and Sprent J 1990), in the MHC-matched (H-2K) mls-disparate mouse strains CBA/J (mls-stimulator) and CBA/CaJ (mls-responder). We transferred mls-responder T cells into mls-stimulator recipients intravenously, with or without PF treatment from day -1 to the day of analysis. 12 days post cell transfer, mice were euthanised and splenic cells were stained to look at TCR-Vβ6-bearing CD4 cells. Fig. 4, panels A-D, show contour plots (size vs. TCR-Vβ6) on gated CD4 T cells. Gate in panels A-D indicate frequency of TCR-Vβ6 bearing CD4 T cells. Panel A and B are control CBA/J and CBA/CaJ mice respectively. Panels C and D show cell transfer without and with PF cover respectively. We found that the frequency of TCR-Vβ6-bearing CD4 T cells was higher in PF-treated mice than in untreated mice as late as day 12 post cell transfer (Fig. 4). The effect of PF on proliferation and loss of TCR-Vβ6-bearing cells was further examined by marking the input cells from mls-responder mice with the fluorescent dye CFSE before being transferred into mls-stimulator recipients as before. Fig. 5 panels A-E and G-H show two color dot plots, of TCR-Vβ6 vs. CFSE on gated CD4 cells. Gate in panels A-E and G, H indicate frequency of TCR-Vβ6 bearing CD4 T cells. Panel A and B are control CBAJ and CBA/CaJ mice respectively. Panel C shows syngenic transfer of cells from CBA/CaJ to CBA/CaJ. Since, there was no mls mismatch between the donor and recipient mice, transferred TCR-Vβ6-bearing CD4 T cells from CBA/CaJ mice did not proliferate in recipient CBA/CaJ mice. Small square gate with dashed border in panel C indicate CFSE high non-proliferating TCR-Vβ6-bearing CD4 T cells from donor mice. The rectangular gate with dashed border indicates non-TCR-Vβ6-CD4 T cells from donor mice. These were the internal TCR-Vβ6 specificity controls, and were not stimulated by the superantigen and hence remained CFSE high. By 48h post transfer, TCR-Vβ6-bearing CD4 cells had
Figure 4: PF protects superantigen-responsive CD4 T cells from activation induced deletion in vivo.

CBA/J mice were given CBA/CaJ spleen cells i.v. (+CBA/CaJ) and were treated with either saline (panel C) or PF (panel D), daily until analysis. 12 days post injection, splenic cells from control CBA/J (panel A), CBA/CaJ (panel B) mice and different recipient mice were stained for two color flow-cytometry. Shown are contour plots, of TCR-Vβ6 vs. cell size on gated CD4 spleen cells. The proportion of the TCR-Vβ6-bearing cells as a percentage of the total CD4 T cells is shown in each plot. Panel A1 shows the representative CD4 gate used for the analysis. This experiment is representative of two independent experiments.
proliferated well, and PF treatment made no difference in the number of cell divisions they went through by this time. Panels D and E, show cell proliferation 48h post transfer, without and with PF cover respectively.

By 72h post transfer, CFSE-labelled TCR-Vβ6-bearing cells had proliferated to the point of losing the fluorescein label irrespective of PF treatment (Fig. 5G-H). However the non-TCR-Vβ6-CD4 T cells, as indicated by the gate with dashed border in panel H, did not proliferate and hence were CFSE high even till 72h. This suggested that the stimulus was specific for TCR-Vβ6-CD4 T cells. Frequency of CD4 T cells that underwent proliferation was higher with PF treatment (panel H) as compared to those without PF treatment (Panel G). Using proliferation platform, data in panels D and E, were re-analysed. Our results showed that, after 48h of *in vivo* stimulation, frequency of CD4 cells in each cell division was same with and without PF treatment. Thus, while PF inhibited TCR-Vβ6 bearing CD4 T cell loss *in vivo*, it did not appear to slow down their activation-induced proliferation to any significant degree.

1.4 Effect of PF on activation marker upregulation:

We next examined the effect of PF on activation marker upregulation in mls system *in vitro*. CBA/CaJ responder cells were stimulated with CBA/J stimulators mixed in a ratio of 1:1 with or without PF (360 μM). Fig. 6 shows histograms for activation markers such as CD25 and CD69 on gated TCR-Vβ6-CD4 T cells, 6h (Panels A, C, E, G, I, K) and 30h (Panels B, D, F, H, J, L) post stimulation. Panels A-D show unstimulated cells, panels E-H and panels I-L show, cells stimulated in absence or presence of PF respectively. Our results show that, upon stimulation, there was upregulation of CD25 and CD69 activation markers over the unstimulated cells. The proportionate positivity for activation markers increased from 6h to
Figure 5: PF does not inhibit proliferation of CD4 T cells in vivo.

CBA/CaJ or CBA J mice were given CFSE labelled spleen cells i.v. and were treated with either saline or PF from day -1 to the day of analysis. Splenic cells from control CBA/J (panel A), CBA/CaJ (panel B), syngenic transfer (panel C) and recipient mice were stained for flow cytometry at 48h (panels D and E) and 72h (panels G and H) post cell transfer. Data are shown as two color dot plots, for TCR-Vβ6 vs. CFSE fluorescence, on gated CD4 T cells. The number of cell divisions undergone by the TCR-Vβ6-bearing CD4 cells were calculated and are plotted as shown for PF or saline treated mice in Panel F. Panel A1 shows one of the representative plot with CD4 gate used for the analysis. This experiment is representative of two independent experiments. Gates with dashed borders are explained in text.
Figure 6: PF does not inhibit upregulation of early activation markers in CD4 T cells in vitro.
Spleen cells from CBA/CaJ mice were stimulated with CBA/J splenocytes (panels E-L) in absence (panels E-H) or presence (panels I-L) of 360 μM PF. After 6 or 30 h of stimulation, as indicated, cells were stained for CD4 and CD25/CD69 and analysed by flow cytometry. Fig 6 shows histograms for CD25 and CD69 staining on gated TCR-Vβ6-CD4 T cells. Panel A1 shows the representative TCR-Vβ6-CD4 gate used for the analysis and panels A-D show staining profiles on unstimulated cells. This experiment is representative of two independent experiments.
30h. There was no significant difference in kinetics of activation marker upregulation in absence or presence of PF (Fig. 6).

1.5 Effect of PF on post activation death in vitro:

A direct measurement of post activation death was next undertaken. Lymph node cells from C57BL/6 mice were stimulated in vitro with anti CD3ε mAb in the presence or absence of PF (360 μM). The cultured cells were first stained for CD4 and then were permeabilised with 70% ethanol. Permeabilised samples were stained with propidium iodide (5 μM) and analysed immediately by flow cytometry. Propidium iodide (PI) binds DNA and gives an estimate of DNA in resting, dividing and dying cells. Live cells show diploid (2n) amount of DNA, apoptotic cells have hypodiploid (less than 2n) DNA and dividing cell show tetraploid amount of DNA (4n). Fig. 7 shows histograms for PI staining on gated CD4 cells. Panels A-C and D-F show samples analysed after 48 and 96h of anti CD3ε stimulation respectively. Our results showed that, a large proportion of cells were proliferating by 48h (panel B). In presence of PF there was a slight inhibition of proliferation (panel C). This was in contrast to the in vivo data (Fig. 5F), where PF did not have any effect on CD4 T cell proliferation. By 96h, there was significant death in CD4 T cells stimulated without PF (panel E). However, in presence of PF, the death induction was substantially reduced from 45.7% to 7.66% (panel F). Thus Fig. 5 shows that, while anti CD3ε mAb induced substantial apoptosis in CD4 T cells, the presence of PF significantly inhibited the apoptosis.

Published literature highlights the role of reactive oxygen species in the death of T cells (Bidere N and Senik A 2001). Earlier work from lab had shown that peroxynitrite scavengers, which intersect both ROS and RNI pathways in the cell, prevent apoptosis in stimulated human T cells. In vitro priming experiments with blockers of RNI pathway such
Figure 7: PF prevents post activation death in CD4 T cells.
C57BL/6 splenic cells were stimulated with anti CD3ε mAb for 48h (panels B and C) or 96h (panels E and F) in absence (panels B and E) or presence (panels C and F) of PF as indicated. Fig shows histogram for PI staining on gated CD4 T cells. Each plot depicts Region P: Hypodiploid phase (= Death, DNA<2n, Region Q: Diploid phase (DNA=2n), Region R: Mitotic phase (= Proliferation, DNA=4n). Panel A1 shows the representative CD4 gate used for the analysis. This experiment is representative of four independent experiments.
as MnTBAP, AG, L-NMMA have been shown to enhance mouse T cell memory responses (Vig M et al. 2004). Thus, RNI blockers seem to mimic the role of PF in T cell memory commitment. On this background we undertook studies dissecting the role of RNI in T cell death and memory.

2. Role of reactive nitrogen intermediates (RNI) in T cell memory responses:

We have used pharmacological as well as genetic approaches for this work. Pharmacological modulators such as MnTBAP, AG, L-NMMA were used in T cell proliferation and activation assays.

In cells nitric oxide (NO) is mainly produced by inducible nitric oxide synthase (iNOS) enzyme. Nitric oxide upon reacting with reactive oxygen species forms reactive nitrogen intermediates such as peroxynitrites, which damage mitochondria. We have used iNos-/- mouse (which lacks inos gene) as a genetic model to study role of reactive nitrogen intermediates in T cell death and memory commitment.

2.1 Effect of RNI on antigen specific T cell frequency:

WT (C57BL/6) or iNOS-/- mice were immunized with protein immunogen, maleylated ovalbumin (mOVA, 100 μg/mouse), on alum. Six weeks after immunization, the T cell-proliferative responses recalled in culture with mOVA (titrating doses) were found to be 100 fold greater in the iNOS-/- mice than in WT counterparts (Fig. 8A). T cells from unimmunised WT or iNOS-/- mice showed very less proliferation. As described earlier in section 1.2.1, limiting-dilution assays were done to find out the frequency of mOVA specific T cells. Antigen dose used in LDA was 1 mg/ml mOVA. Results showed that the clonal frequency of mOVA specific T cells was 2.5 to 3 fold greater in iNOS-/- mice than in WT mice (Fig. 8B). However, the extent of proliferation in mOVA reactive wells at clonal
Figure 8: Higher frequencies of proliferation competent memory CD4 T cells in the absence of iNOS.

T cell memory responses were measured 6 weeks after immunization with mOVA, as bulk proliferative responses from C57BL/6 WT and iNOS-/- mice (panel A), limiting-dilution analysis-based frequency (panel B), and clonal burst size as measured by in vitro proliferative response to mOVA in wells scoring positive or negative at clonal frequencies (panel C). Data in panel A are represented as mean cpm for triplicate cultures±SE, background cpm<1000. Data in panel B-C are from three mice per group (mean±SE). This experiment is representative of three independent experiments. UI indicates 'unimmunised' group and 'IMM' indicates immunised groups.
frequency, indicating the clonal burst size (calculated as described in section 1.2.1 Results) of mOVA specific T cells, did not differ between WT and iNOS$^{-/-}$ mice (Fig. 8C).

2.2 Effect of RNI on memory CD4 T cell generation in vivo:

To specifically look at the effect of RNI on CD4 T cells, WT or iNOS$^{-/-}$ mice (three mice/group) were immunized in the foot pad with OVAII (ISQAVHAAHAINEAGR) peptide in CFA (10 μg/mouse). OVAII peptide is derived from ovalbumin protein and is presented by I-A$^{b}$ MHC class II molecules. Control mice were immunized with CFA alone. Six weeks later draining lymph nodes were harvested, CFSE labelled and restimulated in vitro with OVAII peptide (3 μg/ml). After 96h of in vitro stimulation samples were analysed flow-cytometrically for CD4 and CFSE. Fig. 9 shows contour plots of CFSE vs. cell size on gated CD4 T cells. As compared to the unstimulated groups (panels A and C) there was an increase in cell size (indicative of T cell activation) in the stimulated groups (panels B and D). Gate in panels A-D indicate frequency of cells that have increased in cell size and are undergoing proliferation. Results, as measured by CFSE dilution, showed that upon in vitro restimulation, greater frequencies of antigen specific CD4 T cells from iNOS$^{-/-}$ mice (Panel D) underwent proliferation as compared to those from WT mice (Panels B). Data processed on Flowjo proliferation platform (described in section 5 methods) showed that greater frequencies of antigen specific CD4 T cells from iNOS$^{-/-}$ mice were there in each cell division as compared to those from WT mice, however total number of cell divisions, these stimulated cells were undergoing, was similar in both the groups (Fig. 9E). Control mice did not show significant proliferation over background.

2.3 Effect of iNOS$^{+/+}$ antigen presenting cells on stimulation of WT or iNOS$^{-/-}$ T cells:
Figure 9: Higher frequencies of antigen specific CD4 T cells undergoing proliferation in iNOS\textsuperscript{−/−} mice. Lymph node cells from C57BL/6 or iNOS\textsuperscript{−/−} mice immunized 6 weeks earlier with OVA\textsubscript{II} peptide, were labelled with CFSE, cultured with or without peptide for 72h, and stained. The degree of CFSE dilution is shown as a contour plot of CFSE vs. cell size on gated CD4 cells from WT (panels A and B) or iNOS\textsuperscript{−/−} mice (panels C and D) cultures. The frequencies of CD4 cells undergoing various numbers of divisions are plotted for the experiment done in B and D (panel E). Data in panel E is represented as mean CD4 frequency of triplicate cultures±SE. Panel A1 shows the representative CD4 gate used for the analysis. This experiment is representative of three independent experiments.
Results

We were interested in looking at whether the enhanced persistence of immune memory depends on the absence of iNOS from T cells or from APCs. To look at the issue, WT or iNOS−/− mice were immunised subcutaneously with 20 million MHC-mismatched APCs from iNOS+/+ BALB/c mice, and CD4 T cell responses were measured 7 weeks later. For this cells were harvested from draining lymph nodes and responder cells were labelled with CFSE prior to stimulation with BALB/c APCs in culture. After 72h of in vitro stimulation, the frequency of CD4 T cells undergoing proliferation as indicated by dilution of fluorescein label was estimated by flow cytometric analysis. To distinguish responders from stimulators, antibody secreted by Y3 hybridoma cell line was used. This antibody binds to MHC-I molecules from all mice strains except for BALB/c mice (H-2d). Hence, only responders (H-2Kb) will stain positive with Y3 culture supernatant. Fig. 10 shows contour plots of CFSE vs. cell size on gated H2Kb positive CD4 T cells. As compared to the unstimulated groups there was an increase in cell size (indicative of T cell activation) in the stimulated groups. Gate in panels A-D indicate frequency of cells that have increased in cell size and are undergoing proliferation. Upon in vitro restimulation greater frequencies of CD4 T cells from iNOS−/− mice (Fig. 10D) underwent proliferation, than did cells from WT mice (Fig. 10B). However, the responding iNOS−/− CD4 T cells on an average had not undergone greater numbers of proliferation cycles as compared with the responding WT cells (Fig. 10E).

2.4 Effect of iNOS+/+ environment on survival of iNOS−/− T cells:

We extended the above observation by transferring WT or iNOS−/− splenic cells into iNOS+/+ environment. B6.PL-Thy1b/Cy congenic mice carrying the Thy-1.1 allotype were lightly g-irradiated (400 rads) and were intravenously given 20x10⁶ B6.PL-Thy1b/Cy splenic cells together with 20x10⁶ Thy-1.2 splenic cells from either WT or iNOS−/− mice. In Thy-1-congenic iNOS+/+ B6.PL-Thy1b/Cy mice, donor WT or iNOS−/− C56BL/6 Thy-1.2 T cells
Figure 10: Enhanced persistence of memory in iNOS−/− mice is due to absence of iNOS in CD4 T cells.

Responses of allo-primed WT or iNOS−/− T cells to irradiated BALB/c spleen APCs 7 weeks after immunization, measured by CFSE dilution 72h after in vitro restimulation with or without allo-stimulator APCs. The degree of CFSE dilution is shown as contour plot of CFSE vs. cell size on gated H-2 Kb positive CD4 cells from WT (panels A and B) or iNOS−/− mice (panels C and D) cultures. The frequencies of CD4 cells undergoing various numbers of divisions are shown for the WT or iNOS−/− CD4 T cells (panel E). Panel A1 shows the representative H-2 Kb-CD4 gate used for the analysis. This experiment is representative of three independent experiments.
can be distinguished from endogenous Thy-1.1 T cells. 24h post cell transfer, the recipient mice were immunized with 300 μg/mouse mOVA in CFA. 42 days after immunization, draining lymph node cells were cultured in absence or presence of mOVA (100 μg/ml) for 24h, and were stained for CD4, Thy 1.2 and intracellular IFN-γ (described in section 11.1 methods). Antigen specific CD4 T cells of WT or iNOS⁻/⁻ donor origin were thus enumerated flow-cytometrically in the appropriate recipient mice. Fig. 11, panels A-B (WT donors), panels C-D (iNOS⁻/⁻ donors) are contour plots, showing cell size vs. IFN-γ on gated Thy1.2-CD4 donor T cells. Gate in panels A-D indicate frequency of donor CD4 T cells that are IFN-γ positive. Panel 11E shows frequency of IFN-γ expressing donor CD4 T cells as calculated from three recipient mice/group. The cells synthesising IFN-γ were bigger in size, suggesting that IFN-γ was secreted by activated CD4 T cells. There were significantly higher frequencies of mOVA-specific donor Thy-1.2-CD4 T cells persisting in recipients given iNOS⁻/⁻ cells than in those given WT cells. Although not shown here, the, frequencies of endogenous mOVA specific CD4 T cells were comparable in both sets of recipient mice.

2.5 Effect of RNI on memory CD4 T cell generation in vitro:
T cells from WT or iNOS⁻/⁻ mice were stimulated in vitro with MHC-mismatched APCs (methodology described in section 1.1) from iNOS⁺/+ BALB/c mice. T cell secondary alloproliferative responses were measured in a restimulation assay against the titrating numbers of stimulator APCs, and the enhancement of proliferative responses in primed versus unprimed responder cells was taken as a quantitative estimate of effective priming. APCs used in priming were lightly fixed with paraformaldehyde, rendering them metabolically inactive but still capable of effective priming, in order to ensure that the effects of pharmacological agents, if any, would be due to their effects on metabolically active
Figure 11: iNOS⁻/⁻ CD4 T cells show enhanced persistence even in iNOS⁺/⁺ environment.

T cell memory responses in donor WT or iNOS⁻/⁻ T cells 42 days after mOVA immunization of Thy-1-congenic recipients given either WT or iNOS⁻/⁻ Thy 1.2 cells prior to immunization. Immune response were scored by restimulation with mOVA followed by flow cytometric analysis of the donor Thy 1.2-CD4 cells for expression of intracellular IFN-γ. The contour plots show, cell size vs. IFN-γ, on gated Thy 1.2-CD4 WT (panel A and B) or iNOS⁻/⁻ (panels C and D) donor cells. The frequencies of IFN-γ expressing mOVA specific CD4 T cells from WT or iNOS⁻/⁻ donors are shown as mean±SE (panel E). Panel A1 shows the representative Thy1.2-CD4 gate used for the analysis. The experiment is representative of two independent experiments.
Results

responder T cells. WT and iNOS\textsuperscript{+/+} T cells mounted comparable primary response (Fig. 12A). However, iNOS\textsuperscript{−/−} T cells mounted a far better secondary proliferative response than WT T cells (Fig. 12B).

We also tested the effects of pharmacological, rather than genetic, inhibition of iNOS activity in this system. Presence of iNOS inhibitor aminoguanidine (AG, 100 \(\mu\)g/ml) during T cell priming had no effect on the primary allo-response of T cells (Fig. 12C). However, T cells allo-primed in presence of AG showed substantially enhanced secondary T cell response as compared to the response seen in T cells primed in absence of AG (Fig. 12D).

2.6 Effect of RNI on T cell death:

As described in review of literature, after clearing the antigen, T cells undergo post activation death (PAD). Two major pathways of PAD that are extensively described in literature are: Activation induced cell death (AICD) and Trophic signal withdrawal death (TSWD).

2.6.1 Effect of RNI on death of mouse T cells:

We tested in vitro whether activated T cells in absence of RNI were protected from AICD or TSWD. T cell blasts were generated by anti CD3\textgreek{e} mAb (100 ng/ml) activation of splenic cells from WT mice (described in detail in section 9 methods) and were restimulated with plate bound anti CD3\textgreek{e} mAb (10 \(\mu\)g/ml) and IL-2 (5 U/ml), either in absence or presence of MnTBAP (100 \(\mu\)M), to induce AICD. Alternatively, after anti CD3\textgreek{e} mAb activation cells were cultured with or without IL-2 (5 U/ml), in absence or presence of MnTBAP to induce TSWD. Cells were stained for CD4 after 48h in case of AICD and after 12h in case of TSWD. Additional staining with DNA binding dye Hoechst-33342 helped determine the
Figure 12: Mouse T cells show enhanced secondary response in the absence or inhibition of iNOS in vitro.

Panel A shows primary allo-proliferative response of WT or iNOS−/− splenocytes stimulated with BALB/c APCs. Panel B shows secondary proliferative response of allo-primed cells upon restimulation with BALB/c APCs. Panel C shows primary allo-proliferative response of WT spleen cells in absence or presence of aminoguanidine, 100 μg/ml. Panel D shows secondary proliferative response of WT spleen cells allo-primed in absence or presence of aminoguanidine. Data are represented as mean cpm of triplicate cultures±SE. Background cpm<2000. This experiment is representative of four independent experiments.
proportions of apoptotic nuclei in CD4 T cells by microscopy. Fig. 13A-B, is representative photograph showing the nuclear morphology of live and dead CD4 T cell blasts, that were restimulated with anti CD3 to induce AICD and stained for CD4 and Hoechst-33342 after 48h of restimulation. Panel A shows Hoechst-33342 staining on the cells. Live cells showed big nucleus with circular morphology, while dead cells showed shrunken nucleus with apoptotic blebs in the nuclear membrane. Panel B shows CD4-Hoechst-33342 double stain on live or dead T cell blasts as indicated.

When T cell blasts were stimulated in presence of MnTBAP, CD4 T cells showed very little protection from AICD (Fig. 13C), although the protection from TSWD was substantial in its presence (Fig. 13D).

Second cycle of signalling of T cells through anti CD3 activation resulted in more than 80% cells dying at the end of 48h (panel C). CD4 T cells which did not receive the second signal but only received IL-2, survived better with only ~15% cells showing apoptotic nuclei. Addition of MnTBAP during anti CD3 restimulation, only marginally offered protection from AICD (panel C).

CD4 T cells that received no IL-2 during the second cycle died a rapid death. At the end of 12h of withdrawal of growth factor (IL-2, here) more than 50% cells were dead (panel D). Addition of IL-2 resulted in only background death (~10%). Presence of MnTBAP in the cultures, during second cycle, offered a significant protection to CD4 T cells from TSWD.

2.6.2 Effect on RNI on death of human T cells:

We extended these findings to human T cells. To confirm the effect of MnTBAP on PAD of human T cells, human PBMCs were activated with anti-human CD3 mAb and IL-2. These activated T cell blasts were cultured in medium devoid of IL-2 for induction of TSWD, in absence or presence of AG (100 μg/ml), L-NMMA (300 μM), or MnTBAP (100 μM). After 48h, cells were stained for CD4 and CD8 cells. To determine the proportion of apoptotic
Fig 13: Nuclear morphology of live and dead CD4 T cells:
Panel A shows picture of T cell blasts stained with DNA binding dye Hoechst-33342. The cell at right (→) is a dying cell with shrunken nucleus and apoptotic blebs. The cell at bottom left is a live cell with a comparatively bigger and circular nucleus.
Panel B shows T cell blasts stained for CD4-fluorescein and Hoechst-33342. Right side of the panel shows a dead cell (←) and a live cell is shown at the bottom left. Picture captured with TE2000-U; Nikon fluorescent microscope.
Figure 13: Peroxynitrite scavenger MnTBAP, protects mouse CD4 T cells from TSWD substantially.
Panel A shows AICD in T cell blasts from WT mice after 48h of restimulation with plate bound anti CD3, in absence or presence of MnTBAP. Panel B shows TSWD in T cell blasts from WT mice after 12 h of IL-2 withdrawal in absence or presence of MnTBAP as indicated. Data are shown as mean % frequency (of apoptotic CD4 T cells) of triplicate cultures±SE. This experiment is representative of four independent experiments.
nuclei, surface stained cells were additionally stained with Hoechst-33342 and samples were 
analysed by microscopy.

At the end of 48h of withdrawal of growth factor (IL-2) more than 35% CD4 cells were 
dead (Fig. 14A). Addition of IL-2 resulted in only background death (≈10%). Presence of 
MnTBAP, AG or L-NMMA in the activated T cell cultures, during second cycle, offered a 
significant protection to CD4 T cells from TSWD.

More than 45% CD8 cells were dead by 48h (panel B). Addition of IL-2 resulted in only 
background death (≈10%) (Fig. 14B). The presence of AG, L-NMMA, or MnTBAP during 
TSWD induction substantially reduced the proportion of CD8 T cells undergoing TSWD.

2.7 Effect of iNOS or peroxynitrite scavengers on primary T cell responses:

The data shown so far demonstrate the enhanced persistence of primed T cells in the 
presence of peroxynitrite scavengers or absence of iNOS. Data indicate that this enhanced 
survival is due to protection of T cells from death by neglect or TSWD, in absence of RNI.

At this point it was necessary to examine whether primary T cell activation was enhanced in 
the absence of iNOS or peroxynitrite, resulting in generation of greater numbers of primed 
T cells, which could provide a mechanism for enhanced persistence of T cell memory 
independent of effects on T cell death pathways.

To address this, T cells from WT or iNOS−/− mice were stimulated with titrating 
concentration of anti CD3ε mAb (Fig. 15A-C). In another set of experiment T cells from 
WT mice were stimulated with titrating concentration of anti CD3ε mAb in absence or 
presence of MnTBAP (100 µM) (Fig. 15D-F). In order to ensure that T cells in presence or 
absence of iNOS or MnTBAP showed no difference in the kinetics of proliferation during 
anti CD3 mediated proliferation, the comparison was made between the groups at multiple 
time points over 72h. There was possibility that T cells in absence of RNI might show an
Figure 14: Depletion of peroxynitrite and iNOS inhibition blocks TSWD in human CD4 and CD8 T cells.
To induce TSWD, T cell blasts from human PBMCs were put in culture for 48h, with or without IL-2 in medium alone or with MnTBAP, AG or L-NMMA as indicated. Data are shown as mean % frequency of apoptotic CD4 (panel A) and CD8 (panel B) T cells from triplicate cultures±SE. This experiment is representative of two independent experiments.
initial slow proliferation and catch up with time. Therefore, time kinetics of T cell proliferation was examined. Cultures were pulsed with tritiated thymidine at various time points (24, 48 and 72 h as indicated in Fig. 15). Our results showed that T cells from WT or iNOS−/− mice showed similar T cell proliferation kinetics (panels A-C). Also the WT cells stimulated in absence or presence of MnTBAP, showed no difference in T cell proliferation profile at all time points studied (panels D-F).

Since in vitro experiments showed that there was no difference in primary T cell activation in absence of RNI, we further examined the effect of absence of iNOS on early immune response invoked by in vivo immunization with nominal protein antigen. WT or iNOS−/− mice were immunized subcutaneously with mOVA (100 μg/mouse) in CFA and proliferative response to titrating concentrations of mOVA in culture was estimated 1 week later. T cells from mice immunised with mOVA showed substantial proliferative response as compared to those from unimmunised mice.

Our results showed that absence of iNOS did not lead to any increase in the magnitude of immune response measured early during immunization (Fig. 16), in contrast to the differences revealed at later time points (Fig. 8A).

3. Effect of Aging on T cells:

Deterioration of the immune system with aging ("immunosenescence") is believed to contribute to morbidity and mortality in man due to the greater incidence of infections, as well as possibly autoimmune phenomenon and cancer. Dysregulation of T cell function is thought to play a critical part in these processes. There are reports suggesting that with age, there is rise in levels of free oxygen and nitrogen radicals, which damage the mitochondria and hence make cells more susceptible to death. Our data show that RNI play important role in death pathways (TSWD) in activated T cells. Therefore, we tried to examine the defects
Figure 15: Primary T cell responses are not enhanced either by the absence of iNOS or by peroxynitrite scavenging.

Mouse splenic cells were stimulated with titrating concentrations of anti CD3ε mAb, and T cell proliferation was assayed at the times indicated. Panels A-C show proliferative response of WT or iNOS−/− mice. Panels D-F show proliferative response of WT cells in absence or presence of MnTBAP. Data are shown as mean cpm of triplicate cultures±SE. Background cpm<3500. This experiment is representative of three independent experiments.
Figure 16: Absence of iNOS does not have any effect on early T cell immune responses.

WT or iNOS<sup>-/-</sup> mice were immunized with mOVA and the proliferative response was measured by <em>in vitro</em> restimulation of draining lymph node cells, 1 week after immunization. Data are shown as mean cpm of triplicate cultures±SE. Background cpm< 3000. The experiment is representative of three independent experiments. UI and IMM in fig indicate, unimmunised and immunised mice respectively.
in T cell response and the role played by TSWD in poor memory response seen in aged mice.

To study the effect of aging on T cell death and memory, young C57BL/6 mice (6-8 weeks old) and aged C57BL/6 mice (18-19 months old) were used. There are reports, which suggest that the number and/or affinity of IL-2Rα expressed by activated T lymphocytes declines with age (Linton PJ et al. 1996). c-Rel is a lymphoid-specific member of the NF-kB/Rel family of transcriptional factors. In c-Rel−/− mice there is an impaired production of cytokines IL-2, IL-3 and granulocyte macrophage colony stimulating factor (Kontgen F et al. 1995). Based on these similarities in IL-2-IL-2R mediated signalling, in most of the experiments, along with aged mice, c-Rel−/− mice were also used.

3.1 Phenotypic characterisation of aged C57BL/6 and c-Rel−/− mice:
Splenic and lymph node cells from young and aged C57BL/6 and c-Rel−/− mice were stained for surface markers. In comparison to young mice, aged mice showed slightly reduced frequency of CD4 and CD8 T cells (Fig. 17A and B), substantially reduced frequencies of B cells (Fig. 17D and E, shown as B220 positive cells) and increased frequencies of macrophages (Fig. 17G and H, shown as CD11b positive cells) whereas c-Rel−/− mice showed a decreased frequency of CD4 and CD8 T cells (Fig. 17C), and comparable frequency of B cell (Fig. 17G) and macrophages (Fig. 17I) when compared to WT mice.

3.2 Effect on T cell proliferation:
Splenic cells from young and aged C57BL/6 mice were stimulated with titrating concentrations of anti CD3e mAb in absence or presence of IL-2 (1 U/ml). T cells from aged mice proliferated less as compared to T cells from young mice and this defect was rescued in presence of IL-2 (Fig. 18A). Similarly, T cells from c-Rel−/− mice, showed less
Figure 17: Phenotypic characterization of young and aged C57BL/6 and c-Rel−/− mice.

Splenic cells from young and aged C57BL/6 and c-Rel−/− mice were stained for CD4 and CD8 (panels A-C, shown as two color contours), B220 (panels D-F, shown as histogram) and CD11b (panels G-H, shown as histogram). The experiment is representative of seven independent experiments.
proliferation as compared to WT mice and that was also rescued by exogenous IL-2 feeding (Fig. 18B).

3.3 Effect on secondary T cell responses:
As described in section 1.1, young and aged C57BL/6 and c-Rel<sup>−/−</sup> mice were stimulated with MHC mismatched APCs from BALB/c mice. We found that aged and young mice show comparable primary allo-proliferative response (Fig. 19A). To look at secondary response, alloprimed T cells from young and aged mice were restimulated with titrating numbers of MHC mismatched APCs from BALB/c mice. T cells from aged mice showed substantially compromised secondary response as compared to those from young mice (Fig. 19B).

T cells from c-Rel<sup>−/−</sup> mice when stimulated with MHC mismatched APCs from BALB/c mice showed less primary alloresponse as compared to those from young mice (Fig. 20A). Also the alloprimed T cells from c-Rel<sup>−/−</sup> mice, showed a decrease in the secondary response when compared to primed T cells from WT mice (Fig. 20B).

3.4 Alteration of T<sub>CM</sub> / T<sub>EM</sub> ratio:
Further, we looked at the various memory markers expressed on T cells from young, aged and c-Rel<sup>−/−</sup> mice. For this splenic and lymph node cells from young and aged C57BL/6 and splenic cells from c-Rel<sup>−/−</sup> mice were stained for CD4/CD8, CD44 and CD62L.

Fig. 21A-D, shows two color contour plots of CD44 vs. CD62L on gated CD4 (Fig. 21A and B) and CD8 (Fig. 21C and D) splenic T cells from young (panels A and C) and aged (panels B and D) mice. Memory T cells are characterised by their CD44<sup>hi</sup> phenotype. On an average 70-80% of splenic CD4 T cells from aged mice were CD44<sup>hi</sup> (panel B) as compared to 35-50% of CD4-CD44<sup>hi</sup> T cells from young mice as depicted in panel B and A as representative plots respectively. In CD8 T cell compartment the difference in CD44<sup>hi</sup>
Figure 18: C57BL/6 Aged and c-Rel"/- mice show compromised T cell proliferation as compared to young C57BL/6 mice.

Splenic cells from young and aged C57BL/6 (panel A) and WT and c-Rel"/- mice (panel B) were cultured in presence or absence of anti CD3 mAb with or without IL-2 for 48h. Background cpm<4500. Data are shown as mean cpm of triplicate cultures ± SE. The experiment is representative of three independent experiments.
Figure 19: T cells from C57BL/6 Aged mice show decreased secondary T cell response as compared to those from young C57BL/6 mice. Panel A shows primary allo-proliferative response of splenic cells from young and aged C57BL/6 mice stimulated with BALB/c spleen cells. Panel B shows secondary response of young and aged unprimed or allo-primed splenic cells. Data are shown as mean cpm of triplicate cultures±SE. Background cpm<2500. The experiment is representative of three independent experiments.
Figure 20: T cells from c-Rel<sup>−/−</sup> mice show decreased secondary T cell response as compared to those from WT C57BL/6 mice. Panel A shows primary allo-proliferative response of splenic cells from WT C57BL/6 and c-Rel<sup>−/−</sup> mice stimulated with BALB/c spleen cells. Panel B shows secondary response of unprimed or allo-primed splenic cells from WT and c-Rel<sup>−/−</sup> mice. Data are shown as mean cpm of triplicate cultures±SE. Background cpm< 1000. The experiment is a representative of three independent experiments.
Results

frequency, between young and aged mice was equally dramatic. Aged mice showed a
frequency of 70-80% CD8-CD44hi cells (as represented in panel D) as compared to 30-40%
CD8-CD44hi cells in young mice (as represented in panel C). Thus, aged mice showed an
increase in CD4/CD8-CD44hi population as compared to young mice.

Depending on the CD62L profile, memory T cells are further divided into effector and
central memory subsets. Effector memory T cells (T_{EM}) are CD44hi CD62Llo whereas central
memory T cells (T_{CM}) are CD44hi CD62Lhi. We found that, most of the CD44hi cells in aged
mice were CD62Llo, results in decreased frequency of CD44hi CD62Lhi population and
hence decreased T_{CM} to T_{EM} ratio in aged mice as compared to young mice. As depicted in
Fig. 21B, in aged mice, only 10% from a total of ~82% of CD4-CD44hi cells were CD62Lhi
(panel B) as compared to young mice, in which 14% from a total of ~50% of CD4-CD44hi
cells were CD62Lhi (panel A). Splenic CD8+-CD44+ compartments from young (panel C)
and aged mice (panel D) also showed similar variations in CD62Lhi population as those seen
in CD4+-CD44+ compartment.

Fig. 21-Table I shows the calculated T_{CM}/T_{EM} ratio for splenic CD4 and CD8 T cells from
young and aged mice based on the representative plots shown in Fig. 21A-D. It showed that
the difference in T_{CM}/T_{EM} ratio for CD4 and CD8 T cells, between young and aged mice
was statistically significant.

We did similar stainings as described above on lymph node cells from young and aged
mice. Fig. 22A-D, shows two color contour plots of CD44 vs. CD62L on gated CD4 (Fig.
22 A and B) and CD8 (Fig. 22 C and D) lymph node T cells from young (panels A and C)
and aged (panels B and D) mice. We found that frequencies of CD4-CD44hi (panel A and B)
and CD8-CD44hi (panel C and D) T cells were higher in lymph node cells of aged mice
(panel B and D) as compared to those from lymph node cells from young mice (panel A and
C).
Table 1

<table>
<thead>
<tr>
<th>Mice</th>
<th>n</th>
<th>CD4 T_{CM}/T_{EM}</th>
<th>P value</th>
<th>CD8 T_{CM}/T_{EM}</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Young</td>
<td>8</td>
<td>0.335 ± 0.0399</td>
<td>&lt; 0.02</td>
<td>0.98 ± 0.03</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Aged</td>
<td>7</td>
<td>0.0233 ± 0.0084</td>
<td></td>
<td>0.029 ± 0.002</td>
<td></td>
</tr>
</tbody>
</table>

Figure 21: CD4 and CD8 splenic T cells from aged mice show lower T_{CM}/T_{EM} ratio as compared to young mice.

Splenic cells from young and aged C57BL/6 mice were stained for CD4/CD8, CD44 and CD62L to look at the ratio of T_{CM} to T_{EM}. Fig. shows two color contour plots of CD44 vs. CD62L staining on gated CD4 (panels A-B) and CD8 (panels C-D) T cells. Region 1 depicts T_{EM} compartment and region 2 depicts T_{CM}. Panels A1 and A2 show the representative CD4 and CD8 gates respectively used for the analysis. This experiment is representative of eight independent experiments. Table 1 shows the T_{CM}/T_{EM} ratios in CD4 and CD8 T cell compartment of young and aged mice.
On analysing CD62L profile on lymph node CD4 and CD8 T cells from young and aged mice, we found that aged mice showed decreased frequency of CD44<sup>hi</sup>CD62L<sup>hi</sup> in both CD4 (panel B) and CD8 (panel D) T cells, as compared to frequency of CD44<sup>hi</sup>CD62L<sup>hi</sup> in CD4 (panel A) and CD8 (panel C) T cells from young mice. Therefore, the T<sub>CM</sub> to T<sub>EM</sub> ratio in CD4 (Fig. 22B) and CD8 (Fig. 22D) T cells from lymph nodes of aged mice was less as compared to the ratio in CD4 (Fig. 22A) and CD8 (Fig. 22C) T cells from lymph nodes of young mice.

Fig. 22- Table II shows the calculated T<sub>CM</sub>/T<sub>EM</sub> ratios for CD4 and CD8 T cell compartment from lymph nodes of young and aged mice, based on the representative plots shown in Fig. 22A-D. It showed that the difference in T<sub>CM</sub>/T<sub>EM</sub> ratios for CD4 and CD8 T cell compartment between young and aged mice was statistically significant.

Similar stainings for memory markers was done on splenic cells from WT and c-Rel<sup>−/−</sup> mice. Fig. 23A-D, shows two color contour plots of CD44 vs. CD62L on gated CD4 (Fig. 23A and B) and CD8 (Fig. 23C and D) splenic T cells from WT (panels A and C) and c-Rel<sup>−/−</sup> (panels B and D) mice. Unlike T cells from aged mice, T cells from c-Rel<sup>−/−</sup> mice did not show any substantial increase in CD44<sup>hi</sup> frequency in either CD4 (panel B) or CD8 (panel D) T cell compartments when compared to CD44<sup>hi</sup> frequency in CD4 (panel A) and CD8 (panel C) T cell compartment of WT mice.

Although there was not much difference in CD4/CD8-CD44<sup>hi</sup> frequency between WT and c-Rel<sup>−/−</sup> mice, there was a difference in proportions of CD44<sup>hi</sup> cells that were CD62L<sup>hi</sup> in WT and c-Rel<sup>−/−</sup> mice. Frequencies of CD4-CD44<sup>hi</sup> (panel B) and CD8-CD44<sup>hi</sup> (panel D) T cells which were CD62L<sup>hi</sup> were less in c-Rel<sup>−/−</sup> mice as compared to frequencies of CD4-CD44<sup>hi</sup> (panel A) and CD8-CD44<sup>hi</sup> (panel C) T cells from WT mice and hence the T<sub>CM</sub>/T<sub>EM</sub> ratio in c-Rel<sup>−/−</sup> mice was less as compared to the ratio in WT mice. Fig. 23- Table III shows calculated T<sub>CM</sub>/T<sub>EM</sub> ratios from CD4 and CD8 compartments of WT and c-Rel<sup>−/−</sup> mice, based on the representative plots shown in Fig. 23A-D. The trend of T<sub>CM</sub>/T<sub>EM</sub> ratios from splenic
Figure 22: CD4 and CD8 lymph node T cells from aged mice show lower $\frac{T_{CM}}{T_{EM}}$ ratio as compared to young mice. Lymph node cells from young and aged C57BL/6 mice were stained for CD4/CD8, CD44 and CD62L to look at the ratio of $T_{CM}$ to $T_{EM}$. Fig. shows two color contour plots of CD44 vs. CD62L staining on gated CD4 (panels A-B) and CD8 (panels C-D) T cells. Region 1 depicts $T_{EM}$ compartment and region 2 depicts $T_{CM}$. Panels A1 and A2 show the representative CD4 and CD8 gates respectively used for the analysis. This experiment is representative of eight independent experiments. Table 2 shows the $\frac{T_{CM}}{T_{EM}}$ ratios in CD4 and CD8 T cell compartment of young and aged mice.
Figure 23: Splenic CD4 and CD8 T cells from c-Rel−/− mice show marginal decrease in $T_{CM}/T_{EM}$ ratio as compared to those from C57BL/6 WT mice.

Splenic cells from young and c-Rel−/− mice were stained for CD4/CD8, CD44 and CD62L to look at the ratio of $T_{CM}$ to $T_{EM}$. Region 1 depicts $T_{EM}$ compartment and region 2 depicts $T_{CM}$. Fig. shows two color contour plots of CD44 vs. CD62L staining on gated CD4 (panels A-B) and CD8 (panels C-D) T cells. Panels A1 and A2 show the representative CD4 and CD8 gates respectively used in the analysis. This experiment is representative of two independent experiments. Table 1 shows the $T_{CM}/T_{EM}$ ratios in CD4 and CD8 T cell compartment of WT and c-Rel−/− mice.

Table 3

<table>
<thead>
<tr>
<th>Mice</th>
<th>n</th>
<th>CD4 $T_{CM}/T_{EM}$</th>
<th>CD8 $T_{CM}/T_{EM}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>6</td>
<td>0.59 ± 0.021</td>
<td>2.0 ± 0.03</td>
</tr>
<tr>
<td>c-Rel−/−</td>
<td>3</td>
<td>0.30 ± 0.006</td>
<td>1.2 ± 0.023</td>
</tr>
</tbody>
</table>
CD4 and CD8 T cell compartments of c-Rel\textsuperscript{−/−} mice appeared similar to what was seen in aged mice. However, the availability of c-Rel\textsuperscript{−/−} mice was restricted for any meaningful statistical analysis.

3.5 Effect of aging on proliferation of naïve T cells (CD44\textsuperscript{lo}):

As could be seen from memory marker staining, the proportions of naïve (CD44\textsuperscript{lo}) and activated (CD44\textsuperscript{hi}) T cells vary between young and aged C57BL/6 mice and c-Rel\textsuperscript{−/−} mice. In order to compare activation and proliferation kinetics of naïve T cells from young and aged C56BL/6 mice and c-Rel\textsuperscript{−/−} mice, we purified CD44\textsuperscript{lo} cells from young, aged and c-Rel\textsuperscript{−/−} mice. LS columns (MACS positive separation columns, described in section 10 methods) were used to purify CD44\textsuperscript{lo} cells. For purification of naïve (CD44\textsuperscript{lo}) T cells, non-saturating amount of anti CD44 antibody coupled to biotin was added to the splenocytes. The amount optimal for purification was decided after chequer board titration. This amount was enough to bind to most T cells expressing CD44 on their surface, however, it was not enough to bind to all non-T-CD44 expressing cells such as B cells and macrophages. As a result the CD44 levels expressed on various subpopulations in the flow-through fraction (column-non bound) showed heterogeneity in CD44 expression (Fig. 24, gate with thick border in panel A4).

Fig. 24 panels A1 and A2 are representative plots showing CD4 proportions in non purified and purified fractions respectively. Panels A3 and A4 are representative plots showing CD44 and CD4 profile of non-purified and purified fractions respectively. Upon purification the frequency of CD4-CD44\textsuperscript{hi} population reduced from 6% to less than 0.5% (gate with dashed border in panels A3 and A4 respectively). There was substantial removal of CD44\textsuperscript{hi} population from non-T cell compartment also (gate with thick border in panels A3 and A4). However, non-T cells with intermediate CD44 expression were still there.
Figure 24: Purification of CD44lo cells.
CD44lo splenic cells were purified from young and aged C57BL/6 and c-Rel−/− mice using LS (MACS positive separation) columns. Samples were stained for CD4 and CD44 and analysed by flow cytometry to check the purity. Panels A-C and panels D-F show two color contours of CD44 vs. CD4 on gated CD4 T cells, for the samples before and after purification respectively. Panel A1 shows the representative CD4 gate used in samples A-C and panel A2 shows the representative CD4 gate used in samples D-F. Panel A3 shows CD44 vs. CD4 profile of sample before purification, with gates on CD4-CD44hi population and nonCD4-CD44hi population. CD4-CD44hi population reduces from 6.57% to less than 1% and nonCD4-CD44hi population reduces from 59.5% to 6.95% (panel A4). This experiment is representative of eight independent experiments.
Fig. 24 A-F, shows two color contour plot of CD44 vs. CD4 on gated CD4 cells. Upon purification the frequency of CD4-CD44^hi T cells reduced from ~20-25% (gate in panels A-C) to less than 2% (gate in panels D-F) in young, aged and c-Rel^- mice as indicated.

Purified CD44^lo cells were CFSE labelled and stimulated with 100ng/ml of anti CD3e mAb. After 48 and 72h of stimulation, in absence or presence of IL-2 (1 U/ml), samples were analysed by flow cytometry to look at CD44 upregulation and proliferation of CD4 and CD8 T cells.

Fig. 25 and 26 show two color contour plots, of CD44 vs. CFSE, on gated CD4 (Fig. 25) and CD8 (Fig. 26) T cells, stimulated for 48h. Plots show two gates, the larger gate indicates total frequency of CD4/CD8 cells that are CD44^hi and smaller gate indicates the frequency of CD4 cells that are undergoing proliferation. After 48h of anti CD3 stimulation, there was upregulation of CD44 in stimulated groups as compared to the unstimulated groups. CD4/CD8 T cells from young mice (panel B, bigger gate) showed marginal increase in the CD44 expression as compared to aged (panel E, bigger gate) and c-Rel^- mice (panel F, bigger gate). CD4/CD8-CD44^hi T cells from young mice had started proliferating (CFSE dilution) (panel B, small gate) by this time, but no significant proliferation could be seen in CD4/CD8-CD44^hi T cells from aged and c-Rel^- mice (small gate in panels E and H respectively). At this point exogenous IL-2 feeding did not have substantial eff ect on CD44 upregulation or proliferation of CD4/CD8 T cells from young, aged and c-Rel^- mice (panels C, F and I respectively). After 72h of stimulation, samples were analysed in a similar way as described for 48h time point. Fig. 27 and Fig. 28 show two color contour plots, of CD44 vs. CFSE, on gated CD4 (Fig. 27) and CD8 (Fig. 28) T cells, stimulated for 72h.

After 72h of anti CD3 stimulation, there was substantial upregulation of CD44 in stimulated groups as compared to the unstimulated groups. Majority of the CD4/CD8 T cells from young mice had upregulated CD44 by this time (panel B, bigger gate). By 72h, CD4/CD8 T
Figure 25: CD4 T cells from aged and c-Rel<sup>−/−</sup> mice show defect in activation and proliferative response, at 48h of anti CD3 stimulation. CD44<sup>lo</sup> cells from young (panels A-C) and aged (panels D-F) C57BL/6 and c-Rel<sup>−/−</sup> mice (panels G-I) were CFSE labelled and cultured with (panels B,C, E, F, H and I) or without (A, D and G) anti CD3 mAb in absence or presence (panels C, F and I) of IL-2. After 48h of stimulation, cells were stained for CD4 and CD44 and analysed by flow cytometry to look at dilution of CFSE dye. Fig. shows two color contour plots of CD44 vs. CFSE on gated CD4 T cells. Panel A1 shows the representative CD4 gate used in the analysis. Data are representative of six independent experiments.
Figure 26: CD8 T cells from aged and c Rel−/− mice show defect in activation and proliferative response at 48h of anti CD3 stimulation.

CD44lo cells from young (panels A-C) and aged (panels D-F) C57BL/6 and c-Rel−/− mice (panels G-I) were CFSE labelled and cultured with (B,C, E, F, H and I) or without (A,D and G) anti CD3 mAb in absence or presence (panels C, F and I) of IL-2. After 48h of stimulation, cells were stained for CD8 and CD44 and analysed by flow cytometry to look at dilution of CFSE dye. Fig. shows two color contour plots of CD44 vs. CFSE on gated CD8 T cells. Panel A1 shows the representative CD8 gate used in the analysis. Data are representative of six independent experiments.
Figure 27: CD4 T cells from aged and c-Rel-/- mice show delayed activation and proliferative response at 72h of anti CD3 stimulation. CD44lo cells from young (panels A-C) and aged (panels D-F) C57BL/6 and c-Rel-/- mice (panels G-I) were CFSE labelled and cultured with (panels B,C, E, F, H and I) or without (A, D and G) anti CD3 mAb in absence or presence (panels C, F and I) of IL-2. After 72h of stimulation, cells were stained for CD4 and CD44 and analysed by flow cytometry to look at dilution of CFSE dye. Fig. shows two color contour plots of CD44 vs. CFSE on gated CD4 T cells. Panel A1 shows the representative CD4 gate used in the analysis. Data are representative of six independent experiments.
Figure 28: CD8 T cells from aged and c-Rel⁻/⁻ mice show delayed activation and proliferative response at 72h of anti CD3 stimulation. CD44lo cells from young (panels A-C) and aged (panels D-F) C57BL/6 and c-Rel⁻/⁻ mice (panels G-I) were CFSE labelled and cultured with (panels B,C, E, F, H and I) or without (A, D and G) anti CD3 mAb in absence or presence (panels C, F and I) of IL-2. After 72h of stimulation, cells were stained for CD4 and CD44 and analysed by flow cytometry to look at dilution of CFSE dye. Fig. shows two color contour plots of CD44 vs. CFSE on gated CD4 T cells. Panel A1 shows the representative CD4 gate used in the analysis. Data are representative of six independent experiments.
cells from aged and c-Rel$^{−/−}$ mice (bigger gate in panels E and H respectively) also upregulated CD44, although the frequency of CD4/CD8-CD44$^{hi}$ population was substantially lower in aged and c-Rel$^{−/−}$ mice as compared to young mice. Majority of CD4/CD8-CD44$^{hi}$ T cells from young mice had undergone cell division by this time (panel B, small gate). Significant proportions of CD4/CD8-CD44$^{hi}$ T cells from aged (panel E, small gate) and c-Rel$^{−/−}$ (panel H, small gate) mice had also undergone cell division, but the proportion was much lower than that from young mice (panels B). Exogenous IL-2 feeding, resulted in enhanced CD44 upregulation and T cell proliferation in aged and c-Rel$^{−/−}$ mice (panels F and I respectively), but CD44 upregulation and T cell proliferation in aged and c-Rel$^{−/−}$ mice never reached to the level as was seen in young mice.

Fig. 29 shows the processed data obtained from the contour plots shown in Fig. 27 and 28. Using Flowjo proliferation platform, frequency of CD4 and CD8 T cells in each cell division was calculated. Based on the absolute number of total cells obtained after 72h of stimulation in all the groups and using the frequency data obtained from the analysis, we calculated the absolute number of CD4 (Fig. 29A and B) and CD8 (Fig. 29C and D) T cells from young, aged and c-Rel$^{−/−}$ mice, as indicated, in each cell division.

As seen together from Figs. 25-29, aged C57BL/6 and c-Rel$^{−/−}$ mice showed a delayed kinetics of proliferation and CD44 upregulation in both CD4 and CD8 compartments as compared to young WT mice. This defect was at least partially rescued by feeding the cultures with exogenous IL-2, in both CD4 and CD8 compartments. Although this rescue was not very evident at 48h but at 72h (Figs. 27 and 28), the rescue was substantial.

3.6 Scoring death in activated CD4 and CD8 T cells:

As described earlier, purified CD44$^{lo}$ cells from young and aged C57BL/6 and c-Rel$^{−/−}$ mice were CFSE labelled and stimulated with 100 ng/ml of anti CD3ε mAb. After 108h of stimulation, cells were stained with CD4/CD8, CD44 and annexin-V. In early stages of
Figure 29: Poor frequencies of proliferating CD4 and CD8 T cells from aged and c-Rel−/− mice as compared to those from young mice.
Panels showing processed data from Figs. 27 and 28. Using Flowjo proliferation platform, frequency of CD4 (panels A and B) and CD8 (panels C and D) T cells in different cell divisions was calculated. CD4 and CD8 frequencies were calculated using data from Fig 27 and Fig 28 respectively.
apoptosis cells flip their membrane and phosphatidyl serine (PS) is exposed towards the exterior of the cell. Annexin-V binds this PS and apoptosis can be scored by flowcytometrically by fluorochrome conjugated Annexin-V.

By 108h of anti CD3 stimulation majority of CD4/CD8 cells had upregulated CD44. Fig. 30 shows histograms for annexin-V staining on gated CD4-CD44\textsuperscript{hi} (panels A-C) and CD8-CD44\textsuperscript{hi} (panels D-F) T cells from young, aged and c-Rel\textsuperscript{-/-} mice as indicated. We found that though the proportion of T cells upregulating CD44 in various groups were different, the proportion of CD4 and CD8 T cells undergoing apoptosis, among CD44\textsuperscript{hi} population as indicated by annexin-V positivity, was comparable in young (panels A and D for CD4 and CD8 respectively), aged (panels B and E for CD4 and CD8 respectively) and c-Rel\textsuperscript{-/-} mice (panels C and F for CD4 and CD8 respectively).

3.7 Effect of aging on PAD of T cells:

As described in section 9 methods, AICD and TSWD was induced in T cell blasts from young and aged C57BL/6 and c-Rel\textsuperscript{-/-} mice. Samples were stained for CD4/CD8 along with DNA binding Hoechst-33342 dye and scored for apoptotic nuclei using fluorescence microscope.

After 36h of restimulation with anti CD3 mAb, there was substantial death induction in CD4 and CD8 T cells from both young and aged mice, when compared to the unstimulated groups (Fig. 31A). However, there was no difference in the frequency of CD4 and CD8 T cells undergoing AICD between young and aged mice.

In another set of experiment to induce TSWD, T cell blasts from young and aged mice were cultured in absence or presence of IL-2 (5 U/ml). After 12h of growth factor (IL-2) withdrawal there was substantial death induction in CD4 and CD8 T cells from both young and aged mice, when compared to death induction in groups cultured with IL-2 (Fig. 31B).
Figure 30: Activated T cells from C57BL/6 aged and c-Rel^-/- mice show equivalent death as compared to those from young C57BL/6 mice.

CD44lo cells from young and aged C57BL/6 and c-Rel^-/- mice were stimulated with anti CD3 mAb. After 108h of stimulation, cells were stained for CD4/CD8, CD44 and Annexin-V and analysed by flow cytometry. Panels A-C show annexin-V staining on gated CD4-CD44^hi population. Panels D-F show annexin-V staining on gated CD8-CD44^hi population. Panels A1 and A2 show the representative CD4-CD44^hi and CD8-CD44^hi gates respectively, used in the analysis. Data are representative of two independent experiments.
Figure 31: Activated T cells from aged mice are not more susceptible to AICD or TSWD as compared to those from young mice.
Panel A shows frequency of apoptotic CD4 and CD8 T cell nuclei undergoing AICD, from young and aged C57BL/6 mice after 36h of restimulation with plate bound anti CD3. Panel B shows frequency of apoptotic CD4 and CD8 nuclei undergoing TSWD, from young and aged C57BL/6 mice after 12h of IL-2 withdrawal. This experiment is representative of four independent experiments.
However, there was no difference in the frequency of CD4 and CD8 T cells undergoing TSWD between young and aged mice.

T cell blasts from WT and c-Rel<sup>−/−</sup> mice were re-stimulated with anti CD3 mAb for 48h to induce AICD. After 48h cells were stained for CD4 and CD8 followed by staining with DNA binding dye Hoechst-33342. As compared to the unstimulated groups substantial death was induced in the groups stimulated with anti CD3 mAb. However, there was no significant difference in the frequency of apoptotic nuclei in stimulated CD4 and CD8 T cell blasts from WT and c-Rel<sup>−/−</sup> mice (Fig. 32A).

To induce TSWD in T cell blasts from WT and c-Rel<sup>−/−</sup> mice, cells were cultured in absence or presence of IL-2 (5 U/ml) for 12h and then stained for CD4 and CD8. To look at nuclear morphology cells were additionally stained with DNA binding dye Hoechst-33342. IL-2 withdrawal resulted in significant death induction in CD4 and CD8 T cells, as compared to the groups cultured in presence of IL-2. There was no significant difference in the frequency of apoptotic nuclei in CD4 and CD8 T cell blasts from WT and c-Rel<sup>−/−</sup> mice (Fig. 32B).

3.8 Effect of aging on memory T cell generation in vivo:

Young and aged C57BL/6 mice were immunized with mOVA on CFA (100 μg/mouse) subcutaneously. Day 7, Day 21 and day 45 post immunization, draining lymph nodes were harvested and cells were restimulated with titrating doses of mOVA. Proliferation profile was looked at, by pulsing the plates with tritiated thymidine in last 12h of the 84h assay. Culture supernatants were collected before pulsing the plates to look at IFN-γ and IL-4 cytokines using cytokine ELISA kit (described in section 11.2 methods).

At day 7 post immunization, significant proliferation was seen in T cells from immunised mice as compared to those from unimmunised mice. However, there was no significant difference in proliferation profile of T cells from immunised young and aged C57BL/6 mice.
Figure 32: Activated T cells from c-Rel \(-/-\) mice are not more susceptible to AICD or TSWD as compared to those from WT mice.

Panel A shows frequency of apoptotic CD4 and CD8 nuclei undergoing AICD, from C57BL/6 WT and c-Rel\(-/-\) mice after 48h of restimulation with plate bound anti CD3. Panel B shows frequency of apoptotic CD4 and CD8 nuclei undergoing TSWD after 12h of IL-2 withdrawal, from C57BL/6 WT and c-Rel\(-/-\) mice. This experiment is representative of four independent experiments.
(Fig. 33A). However, T cells from immunised aged mice showed decreased IFN-γ cytokine secretion as compared to those from immunised young mice (Fig. 33B).

At day 21 post immunization, significant proliferation was seen in T cells from immunised mice as compared to those from unimmunised mice. However, as seen on day 7, there was no significant difference in proliferation profile of T cells from immunised young and aged C57BL/6 mice (Fig. 34A), even on day 21. T cells from immunised aged mice showed substantial decrease in IFN-γ cytokine secretion as compared to those from immunised young mice (Fig. 34B).

At day 45 post immunization, T cells from immunised aged mice showed significantly reduced T cell proliferation (Fig. 35A) as well as IFN-γ cytokine levels (Fig. 35B) as compared to the T cells from immunised young mice.

IL-4 cytokine could not be detected in the supernatants collected at any point of time, suggesting that the levels secreted were below 10 pg/ml.

Thus, the preliminary data obtained from in vivo immunization of aged and young mice with nominal antigen, suggested that in comparison to T cells from young mice, T cells from aged mice show compromised memory response, in terms of T cell proliferation and IFN-γ secretion, when recalled with antigen in vitro.

4. T cells as antigen presenting cells:

For a T cell to get optimally activated, it should receive two signals. TCR ligation by antigen provides the first signal and co-stimulatory molecules present on the surface of professional APCs provide the other signal. In the absence of the second signal, T cells undergo anergy (discussed in section 4.2, review of literature). Human T cells upon activation express MHC class II on their surface and there are previous reports showing that when activated human T cells present antigen to responding T cells, responders are
Figure 33: On day 7 post immunization, T cells from aged mice show no difference in proliferation though they produce lower amounts of IFN-γ as compared to T cells from young mice.

Young and aged C57BL/6 mice were immunized with mOVA on CFA. Day 7 post immunization draining lymphnodes were harvested and cells were restimulated in vitro with mOVA. Panel A shows proliferative response to mOVA after 72h of stimulation. Data are shown as mean cpm of triplicate culture± SE. Panel B shows IFN-γ levels, in the culture supernatants obtained from the experiment in panel A. Control mice (UI) were immunized with CFA alone. Background cpm<3000. Background IFN-γ levels<60 pg/ml in panel B. This experiment is representative of two independent experiments.
Figure 34: T cells from aged mice show less proliferation and IFN-γ production as compared to those young mice at day 21 post immunization.

Panel A shows proliferative response to mOVA on day 21 post immunization, after 72h of restimulation in vitro. Data are shown as mean cpm of triplicate cultures±SE. Panel B shows IFN-γ levels, in the culture supernatants obtained from the experiment in panel A. Control mice (UI) were immunized with CFA alone. Background cpm<5000 and background IFN-γ levels<40 pg/ml. This experiment is representative of two independent experiments.
Figure 35: Aged mice show poor memory response as measured by T cell proliferation and IFN-γ production.

Panel A shows proliferative response to mOVA on day 45 post immunization, after 7h of restimulation in vitro. Data are shown as mean cpm of triplicate cultures ±SE. Panel B shows IFN-γ levels, in the culture supernatants obtained from the experiment in panel A. Control mice (UI) were immunized with CFA alone. Background cpm< 5000 and background IFN-γ levels<250pg/ml. This experiment was done once.
anergised (Sidhu S et al. 1992). We were interested in studying whether this holds true in the mouse system as well. To examine the role of T cells as antigen presenting cells, we have used transgenic mouse strain, named JLA, expressing I-A<sup>b</sup> on T cells.

**4.1 T cell as stimulators in allogenic mode of stimulation:**

CD44<sup>lo</sup> cells were purified from splenic cells of BALB/c mice (described in section 10, methods, purity of CD4/CD8-CD44<sup>lo</sup> cells was more than 97%). These purified cells were allogenically stimulated with irradiated, MHC mismatched APCs such as, splenic cells from C57BL/6 mice, purified T cells from C57BL/6 and JLA mice, T cell blasts from C57BL/6 and JLA mice (preparation of APCs is described in section 4.3.4, methods).

Fig. 36 shows two color contour plots, T cells (CD4+CD8) vs. MHC class II, on samples collected before and after purification as indicated. Panels C and F represent T cell blasts after 4 days of *in vitro* activation by anti CD3 and IL-2. Panels A-C represent samples from C57BL/6 mice and panels D-F represent samples from JLA mice. T cell blasts (activated T cells) from C57BL/6 (Fig. 36C) and JLA mice (Fig. 36F) were more than 97% pure and T cells from JLA mice were showing MHC class II positivity (panels E and F). Generally, purification achieved for T cells from splenic cells of C57BL/6 or JLA mice as seen in panels B and F respectively, used to be in the range of 88-92%.

Along with setting up a primary T cell proliferation assay, responder cells were stimulated in bulk, in a ratio of responder:stimulator :: 3:1. These primed T cells were then restimulated with titrating numbers of irradiated splenic cells from C57BL/6 mice to look at the secondary response.

We found that the T cells (responders), primed with T cell blasts from JLA and C57BL/6 mice, showed poor primary proliferation as compared to those T cells primed with splenic cells from C57BL/6 mice (Fig. 37A).
Figure 36: Purification of T cells from C57BL/6 and JLA splenic cell population.
Splenic cells from C57BL/6 and JLA mice were purified using LS (MACS positive separation) columns. Samples collected before and after purification and the T cell blasts, were stained for CD4, CD8 and MHC Class II surface markers. Panels A-C and D-F show flowcytometric analysis of samples from C57BL/6 and JLA mice respectively. This experiment is representative of three independent experiments.
Figure 37: T cell blasts from JLA mice optimally allo-prime the responder splenic T cells.
Panel A shows primary response of BALB/cJ CD44lo splenic cells stimulated with C57BL/6 splenic APCs, C57BL/6 T cell blasts and JLA T cell blasts. Panel B shows secondary response of BALB/cJ cells (primed with indicated stimulators during priming) restimulated with C57BL/6 splenic APCs. Data are shown as mean cpm of triplicate cultures±SE. Background cpm<4000. This experiment is representative of three independent experiments.
Results from secondary allo-stimulation showed that primed responders elicit enhanced response over the unprimed cells. T cells primed with T cell blasts from JLA mice showed enhanced secondary response as compared to those responders primed with splenic cells from C57BL/6 mice. T cells primed with T cell blasts from C57BL/6 mice showed poorer secondary response (Fig. 37B).

To look at the priming abilities of naïve T cells, CD44lo BALB/c splenic cells were primed with purified T cells from JLA and C57BL/6 mice, along with the other groups as described above. Secondary response of the primed T cells was looked at by restimulation with titrating numbers of irradiated splenic cells from C57BL/6 mice. After 72h of restimulation, plates were pulsed with $^3$H thymidine for 12h. Before pulsing the plates, at 60h, culture supernatant was collected from the all the primed groups, that were restimulated with $3\times 10^4$ stimulators and IFN-γ levels were estimated using IFN-γELISA kit (described in section 11.2 methods). Fig. 38A shows secondary proliferative response of T cells primed with splenic cells from C57BL/6 mice, purified T cells from C57BL/6 and JLA mice and T cell blasts from C57BL/6 and JLA mice. Panel B shows amount of IFN-γ produced during restimulation of primed responders with $3\times 10^4$ splenic cells from C57BL/6 mice.

We found that T cells primed with purified T cells as well as T cell blasts from JLA mice showed substantially enhanced secondary proliferative response (panel A) and produced substantial amount of IFN-γ (panel B). This secondary response, both in terms of thymidine incorporation and IFN-γ secretion was even better than those responders primed with splenic cells from C57BL/6 mice. However, T cells primed with purified T cells and T cell blasts from C57BL/6 mice showed negligible secondary proliferation (panel A) and IFN-γ secretion (panel B). Unprimed responders also showed secondary response above background.
Figure 38: T cells allo-primed with naive and activated T cells (T cell blasts) from JLA mice, show enhanced secondary responsiveness. Panel A shows secondary response of primed BALB/c splenic cells (primed with indicated stimulators) when restimulated with C57BL/6 splenic cells. Data are shown as mean cpm of triplicate cultures±SE. Background cpm<15,000. Panel B shows IFN-γ produced by primed responders (primed with indicated stimulators) when restimulated with $3 \times 10^4$ C57BL/6 splenic cells. Background IFN-γ< 30 pg/ml. This experiment is representative of two independent experiments.
4.2 T cells as APCs for presenting nominal antigen to responding T cells:

For analysing capability of T-APCs to present nominal antigen, we have made use of TCR-transgenic OT-II mice. T cells from OT-II mice express Vα2/Vβ5 TCR that is specific for OVAII peptide presented by I-Ab MHC-II molecule. Splenic cells from OT-II TCR transgenic mice were stimulated with OVAII pulsed (peptide dose of 1, 3, 10 μg/ml) C57BL/6 splenic APCs, C57BL/6 purified T cells and JLA purified T cells. Purification of T cells from splenic cell suspension was done as described in section 4.3.4.1, methods and the purity used to be in range of 92-95%. All the APCs were γ-irradiated at 1000 rads. For peptide pulsing, 3-5 million cells suspended in Click's medium without FCS were incubated with peptide at 37°C for 1-2h and then washed thrice with medium containing 5% FCS.

T cells from OT-II TCR transgenic mice showed poorer primary response when primed with OVAII pulsed purified T cells from JLA and C57BL/6 mice as compared to their response when primed with OVAII pulsed splenic cells from C57BL/6 mice (Fig. 39A). We did not expect any primary response in T cells primed with OVAII pulsed purified T cells from C57BL/6 mice as T cells from C57BL/6 mice do not express MHC-II on their surface which is required for antigen presentation to T cells from OT-II TCR transgenic mice. This minimal primary response of T cells might be due to the presence of contaminating APCs from C57BL/6 mice in the purified T cell fractions, as the purity was never 100%.

To look at the secondary response, primed T cells were restimulated with titrating numbers of OVAII pulsed splenic cells from C57BL/6 mice. T cells primed with different OVAII pulsed APCs showed substantially enhanced response as compared to those primed with unpulsed splenic cells from C57BL/6 mice or unpulsed purified T cells from JLA and C57BL/6 mice (Fig. 39 B).
Figure 39: T cells from JLA mice, optimally prime splenic T cells from OT-II TCR transgenic mice.
Panel A shows primary response of OT-II TCR transgenic mice stimulated with indicated stimulators. Panel B shows secondary response of primed OT-II TCR transgenic (primed with indicated stimulators during priming) restimulated with C57BL/6 splenic cells. Data are shown as mean cpm of triplicate cultures±SE. Background cpm<1500. This experiment is representative of two independent experiments.
T cells primed with OVAII pulsed purified T cells from JLA mice showed enhanced secondary response as compared to cells primed with OVAII pulsed splenic cells from C57BL/6 mice. Cells primed with OVAII pulsed purified T cells from C57BL/6 mice showed very minimal secondary response as compared to other primed cells (Fig. 39B). Thus together the preliminary data suggested that mouse T cells expressing MHC-II, can present antigen, without inducing anergy in the responding T cell population.