Results
Results

3.1. CD8 T cell responses to APC-targeted exogenous antigen
   3.1.1. In vitro CD8 T cell responses
   3.1.2. In vivo CD8 T cell responses 42

3.2. Role of intracellular cAMP dependent pathways in T cell memory and death
   3.2.1. Role of PF in in vivo CD8 T cell responses
   3.2.2. Role of PF in post activation death 45

3.3. Role of reactive nitrogen intermediates in determining CD8 T cell memory and death
   3.3.1. In vivo memory CD8 T cell frequencies in the absence of iNOS
   3.3.2. T cell specific absence of iNOS responsible for enhanced in vivo memory
   3.3.3. Effect of absence of iNOS on TSWD in CD8 T cell blasts
         3.3.3.1. TSWD in iNOS- CD8 T cell blasts
         3.3.3.2. Pharmacological inhibition of iNOS activity during induction of TSWD
         3.3.4. Role of iNOS in AICD of CD8 T cell blasts
         3.3.5. Role of Reactive Nitrogen Intermediates in primary T cell responses
         3.3.5.1. In vitro responses
         3.3.5.2. In vivo responses 48

3.3.4. Role of AIF in T Cell Death, Development and Memory Commitment
   3.4.1. Role of AIF in post activation T cell death
          3.4.1.1. Absence of AIF in T cells confers protection from TSWD 51

42 43 45 49 50 50 50 51 52 52 53 54 54 55
3.4.1.2 Effect of MnTBAp and aminoguanidine in T cell TSWD

3.4.1.3 Autophagic death component in TSWD of T cell blasts

3.4.1.4 Loss of Bcl-xl in T cell TSWD

3.4.1.5 Cytochrome C release delayed in the absence of AIF

3.4.1.6 Absence of AIF from T cells leads to enhanced AICD

3.4.1.7 Role of superoxides in AIF associated AICD

3.4.1.8 Role of Fas-Fasl expression in AIF associated AICD

3.4.2 Role of AIF in generation of T cell memory

3.4.2.1 Higher frequencies of peripheral memory T cells (CD44^hi) in Hq mice

3.4.2.2 Reduced peripheral T cell frequencies in Hq mice

3.4.2.3 Lower numbers of naive T cells in Hq mice

3.4.2.4 B cell and macrophage frequencies and numbers unaffected in Hq mice

3.4.2.5 Ex vivo T cells from Hq mice are protected from TSWD

3.4.2.6 Functional T cell and B cell responses unaffected by AIF absence

3.4.3 Role of AIF in T cell development

3.4.3.1 Hypocellular thymus in Hq mice

3.4.3.2 AIF hypomorphism in T lineage cells leads to compromised development
3.1. CD8 T cell responses to APC-targeted exogenous antigen

Maleylating protein antigens target them to scavenger receptors (SRs) on APCs. It has been shown that SR targeted exogenous protein antigens are better presented on MHC-I in vitro (Bansal et al., 1999). Whether this enhanced presentation in vitro leads to a better CD8 T cell priming is one of the questions to be asked, as APC-exogenous vaccine proteins normally induce poor CD8 immune responses unless adjuvants like CFA are used to ensure high degree of co-stimulation. CD8 T cell responses in vitro have been tested using OT-I TCR transgenic mice that recognise an epitope of OVA in association with K\(^b\). The hypothesis that maleyl-protein immunisation would allow CD8 T cell responses to be mounted even with a weak adjuvant such as alum has also been tested using C57BL/6 mice that have a polyclonal repertoire of T cells.

3.1.1 In vitro CD8 T cell responses

Spleen cells from OT-I TCR transgenic mice or C57BL/6 mice were cultured at 3 \(10^6\)/ml density with titrating doses of ovalbumin (OVA) and maleyl OVA (mOVA). CD8 T cell responses to these antigens were scored as proliferation estimated at 72-84 hrs by \(^{3}H\) thymidine incorporation. Fig 1 shows proliferative responses of OT-I TCR transgenic and C57BL/6 spleen cells to mOVA and OVA represented as counts per minute (cpm). The CD8 T cell response to both mOVA and OVA was seen in spleen cells from the transgenic mice but the response seen in spleen cells from C57BL/6 mice was very low. The transgenic cells
Spleen cells from OVA specific OT-I TCR transgenic mice and C57BL/6 (WT) mice were stimulated with titrating doses of mOVA and OVA. Proliferation was scored at 72 hrs by [3H] incorporation and is represented as mean ± SE cpm of triplicate cultures. Background cpm were below 1000 for both OT-I and WT cultures.
responded better to mOVA than OVA suggesting enhanced presentation of the exogenous antigen through SRs.

3.1.2 In vivo CD8 T cell responses

Having tested the efficiency of SR mediated exogenous antigen presentation in vitro, in vivo immunogenicity was assayed in C57BL/6 mice immunised subcutaneously with mOVA or OVA with either Complete Freund's Adjuvant (CFA) or alum. C57BL/6 mice were immunised with mOVA or OVA (100 μg/mouse) respectively in CFA. C57BL/6 mice immunised with PBS in CFA served as immunisation controls. Another set of C57BL/6 mice were immunised with mOVA and OVA (300 μg/mouse) respectively on alum adjuvant while mice immunised with PBS in alum served as immunisation controls. Seven days post immunisation, cells from draining lymph nodes of these mice were restimulated in vitro with either mOVA or OVA (100 μg/ml). Cells cultured without any antigen served as controls. Intracellular cytokine staining was done 60 hrs post restimulation to determine frequencies of CD8 T cells expressing interferon-gamma (IFN-γ) in response to antigenic restimulation by flow cytometry. Fig 2 shows the CD8 T cell responses of mice immunised with mOVA or OVA on CFA, and recalled with either mOVA or OVA. Frequencies of IFN-γ expressing CD8 T cells generated following mOVA immunisation on CFA were higher than following OVA immunisation. However, both mOVA and OVA on CFA generated detectable frequencies of antigen specific CD8 T cells. When alum was used as an adjuvant instead of CFA, only mOVA immunisation yielded detectable frequencies of IFN-γ expressing CD8 T cells as shown in Fig 3. Fig 4 is a
Recalled with

<table>
<thead>
<tr>
<th>None</th>
<th>mOVA</th>
<th>OVA</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.059</td>
<td>0.013</td>
<td>0.066</td>
</tr>
</tbody>
</table>

Fig 2: CD8 T cells show better in vivo response to mOVA-CFA than to OVA-CFA

WT mice were immunised with PBS on CFA (panel A), OVA (panel B) on CFA and mOVA on CFA (panel C). Seven days later immune CD8 T cell responses were scored by restimulation with mOVA (100 μg/mL) and OVA (100 μg/mL) respectively for 60 hrs followed by flow cytometric analysis of IFN-γ expressing CD8 T cells. The unstimulated cultures served as controls. Data are representative of three mice per group and two independent experiments.
Fig 3: CD8 T cells show better in vivo response to mOVA-Alum than to OVA-Alum

WT mice were immunised with PBS on Alum alone (panel A), OVA on Alum (panel B) and mOVA on Alum (panel C). Seven days later immune CD8 T cell responses were scored by restimulation with mOVA (300 μg/mL) and OVA (300 μg/mL) respectively for 60 hrs followed by flow cytometric analysis of IFN-γ expressing CD8 T cells. The unstimulated cultures served as controls. Data are representative of three mice per group and two independent experiments.
**Fig 4: Frequencies of mOVA specific IFN-γ+CD8 T cells higher**

Statistical representation of frequencies of IFN-γ expressing CD8 T cells from PBS, OVA and mOVA immunised mice. Panel A represents IFN-γ expressing CD8 T cell frequencies when antigen was given on CFA while panel B represents IFN-γ expressing CD8 T cell frequencies when antigen was given on alum adjuvant. Data are plotted as mean ± SE from three mice per group. Data are representative of two independent experiments.
statistical representation of detectable frequencies of antigen specific CD8 T cells expressing IFN-γ following immunisation.
3.2. Role of intracellular cAMP dependent pathways in T cell memory and death

3.2.1. Role of PF in in vivo CD8 T cell responses

Previous work using an *in vitro* human allo-recognition system has shown that presence of pentoxifylline (PF), a pan-phosphodiesterase inhibitor that elevates cAMP levels, during T cell priming, results in an enhanced CD4 T cell memory response. Such an effect of PF was tested for CD8 T cells in an *in vivo* allo-immunisation model. Thy-1.1 congenic C57BL/6 mice were treated with either saline or PF (2 mg/mouse/day) from day-2 to +5 followed by immunisation with gamma-irradiated BALB/c spleen cells subcutaneously. Seven weeks later, lymph node cells from the immunised mice were labeled with CFSE, and cultured with or without gamma-irradiated BALB/c spleen cells. The frequency of Thy-1.1-bearing responder CD8 T cells undergoing proliferation was scored flow cytometrically at different times of restimulation. The two color flow cytometry plot (Fig 5A) shows that the frequencies of activated (large) CD8 T cells undergoing division and CFSE dilution were greater if the immunised mice were PF-treated. Fig 5B shows the proliferation analysis for CFSE-diluting responding CD8 T cells from the above flow cytometric plot. The frequency of responding CD8 T cells were higher in the PF treated mice at each cell division, but the rate of progression through successive cell divisions was comparable between the PF treated and untreated mice (Fig 5B).

3.2.2. Role of PF in post activation death

Previous results have also shown that PF protects T cells from anti-CD3 mediated death. On that background, the ability of PF to prevent different kinds of
**Fig 5: Presence of PF at the time of immunisation, enhances memory CD8 T cell frequencies**

B6.Thy-1.1 congenic mice were immunised with irradiated BALB/c spleen cells s.c. in the presence of either PF or saline. Seven weeks later lymph node cells from these mice were CFSE labeled and restimulated with or without irradiated BALB/c splenic APCs for 96 hrs. Flow cytometric analysis of CFSE dilution by responding Thy-1.1*CD8 T cells from unstimulated and stimulated cultures is represented as two color plot for cell size (FSC) vs CFSE (panel A). Frequencies (panel B) of responding CD8 T cells from panel A undergoing various numbers of divisions are plotted as mean ± SE from three mice per group. Data are representative of three independent experiments.
Post activation death (PAD) of CD8 T cells was tested. T cell blasts were generated, either in the presence (WT+PF) or in the absence of PF (WT) (100 μg/ml). Trophic signal withdrawal death (TSWD) was induced in these T cell blasts (Fig 6A) by culturing them without IL-2 either in the presence (-IL2+PF) or absence of PF (-IL2) (100 μg/ml). Cultures that received 5 IU/mL of IL-2 (+IL2) served as negative controls for TSWD. In a separate assay, activation induced cell death (AICD) was induced in these T cell blasts (Fig 6B) in the presence (+aCD3+PF) or absence (+aCD3) of PF. T cell blasts cultured without plate bound anti-CD3 stimulus (-aCD3) served as controls for AICD. Cells were stained with anti-CD8 and the DNA binding dye Hoechst 33342 to score for cell death. Samples were examined by fluorescence microscopy to count the frequencies of CD8 T cells showing apoptotic nuclei characterized by appearance of condensed and/or fragmented nuclei. Fig 6A shows that CD8 T cell blasts generated in the presence of PF showed less TSWD as compared to the group generated without PF. The presence of PF during the induction of TSWD also conferred protection (Fig 6A). Similarly, Fig 6B shows that CD8 T cell blasts generated in the presence of PF showed less AICD as compared to CD8 T cell blasts generated in the absence of PF. However, the presence of PF only during AICD induction also led to protection.

Together these results suggest that presence of PF during CD8 T cell priming, which results in increased frequencies of memory CD8 T cells, protects activated T cells from the two major death pathways known to function in T cells post activation. Of these two, the TSWD pathway has been associated with increased survival of memory T cells while the AICD pathway, triggered in conditions where
Fig 6: Presence of PF protects CD8 T cells from TSWD and AICD

C57BL/6 (WT) T cell blasts were generated in the absence or presence of PF (100 μg/mL). TSWD and AICD was induced in these T cell blasts either in the presence or absence of PF. Panel A shows frequencies of CD8 T cells undergoing TSWD after 24 hrs of culture while panel B shows frequencies of CD8 T cells undergoing AICD after 48 hrs of culture. Death in CD8 T cell blasts was scored by staining for surface CD8 and nuclear morphology. Data have been plotted as the mean ± SE of triplicate samples and are representative of three independent experiments.
T cells are stimulated repeatedly with an antigen, has been reported to be irrelevant for memory compartment.

The analysis of the role of nitric oxide in CD8 T cell death and memory, presented in the next section provides further insights into the roles of these two death pathways in controlling memory.
3.3. Role of reactive nitrogen intermediates in determining CD8 T cell memory and death

3.3.1. In vivo memory CD8 T cell frequencies in the absence of iNOS

WT or iNOS<sup>−/−</sup> mice were immunised with an MHC-I restricted OVA peptide (OVA-I) (10 µg/mouse) on CFA subcutaneously. Control mice were immunised with PBS in CFA. Six weeks later, draining lymph node cells from these mice were restimulated with OVA-I peptide (3 µg/mL). The unstimulated cultures served as controls. After 72 hrs of restimulation, cultures were harvested and stained for CD8, CD44 (an activation marker that is upregulated post activation on effector/memory T cells), and intracellular interferon-gamma (IFN-γ), to determine frequencies of memory CD8 T cells. Fig 7A shows intracellular IFN-γ staining on gated CD8 T cells. CD8 T cells from control mice did not show any significant frequency of IFN-γ expression but peptide immunised WT mice showed small yet significant frequencies of IFN-γ expressing CD8 T cells. These IFN-γ expressing CD8 T cells were CD44<sup>hi</sup>. Their number was higher in cultures from immunised iNOS<sup>−/−</sup> mice than the WT mice, showing enhanced persistence of memory CD8 T cells in the absence of iNOS. Fig 7B is a statistical representation of detectable frequencies of memory CD8 T cells expressing IFN-γ following immunisation. Since iNOS<sup>−/−</sup> mice showed enhanced persistence of memory CD8 T cells, their peripheral lymphoid organs would be expected to reflect this. In order to address this issue, memory-phenotype CD8 T cell frequencies and numbers were analysed in inguinal lymph nodes of naive unimmunised WT and iNOS<sup>−/−</sup> mice by staining lymph node cells for CD8 and CD44 or CD62L since memory T cells are
Fig 7: Higher frequencies of cytokine-secreting CD8 memory T cells in iNOS−/− mice

CD8 T cell memory responses at six weeks post immunisation of C57BL/6 (WT) and iNOS−/− mice with OVA-I peptide (+peptide) or with adjuvant alone (-peptide) were analysed by restimulation with (+) or without (-) OVA-I peptide for 72 hrs. This was followed by flow cytometric analysis of CD8 cells for expression of CD44 versus intracellular IFN-γ (panel A). The frequencies of IFN-γ expressing CD8 T cells are shown as mean ± SE from three mice per group (panel B).
CD44^{hi} CD62L^{lo}. It was observed that absence of iNOS resulted in enhanced frequencies (Fig 8A) and numbers (Fig 8B) of memory CD8 T cells in the peripheral lymph nodes.

### 3.3.2 T cell specific absence of iNOS responsible for enhanced in vivo memory

The iNOS^{+/-} mice that were used in the experiments above had iNOS inactivated from cells of all lineages including B cells, T cells and APCs. Therefore, the enhanced CD8 T cell memory that was observed could be because of absence of iNOS in either T cells or APCs. To address this issue, chimeric mice were generated where splenic cells from WT or iNOS^{-/-} mice, which express Thy-1.2 allele, were transferred intravenously (i.v) to sublethally irradiated Thy-1-congenic iNOS^{+/+} B6.PL-Thy1^{a}/Cy mice that use the Thy-1.1 allele, allowing a distinction to be made between the donor and endogenous T cell populations by staining for Thy-1.2 allele. Following transfer, the recipient mice were immunised subcutaneously with mOVA (100 μg/mouse) in CFA, which would allow estimation of CD8 T cell response, as mOVA is presented efficiently on MHC class I and is capable of priming CD8 T cells in vivo (see section 3.1 above). Six weeks post immunisation, cells from draining lymph nodes were restimulated with mOVA (100 μg/ml) for 24 hrs following which they were stained for Thy-1.2, CD8 and intracellular interferon-gamma (IFN-γ). Flow cytometric analysis (Fig 9) revealed that frequencies of antigen specific donor Thy-1.2 CD8 T cells persisting in an iNOS sufficient environment were higher when the donor T cells were from the iNOS^{+/} mice, suggesting that the enhanced memory observed in the iNOS^{+/-} mice is an effect specific to iNOS^{+/-} T cells. Antigen specific frequencies of
Fig 8: Higher numbers and frequencies of CD8 memory-phenotype T cells in lymph nodes of iNOS⁻/- mice

Inguinal lymph node cells from WT and iNOS⁻/- mice were stained flow cytometrically for CD8 and the activation/memory markers CD44 and CD62L. Frequencies (panel A) and absolute number (panel B) of memory CD8 T cells are shown as mean ± SE for three mice per group. Data has been compared using the student's t test for panel A (*p<0.02; **p<0.001) and panel B (*p<0.02; **p<0.001).
WT and iNOS−/− chimeric mice were immunised with mOVA. Six weeks later donor WT and iNOS−/− CD8 T cell memory responses were scored by re-stimulation with mOVA followed by flow cytometric analysis of the IFN-γ expression by Thy-1.2 CD8 T cells. The frequencies of IFN-γ expressing WT and iNOS−/− donor Thy-1.2*CD8 T cells as well as endogenous Thy-1.1*CD8 T cells are shown. Data are shown as mean ± SE and are representative of two independent experiments. Data has been compared using the student's t test (*p<0.007).

**Fig 9: Absence of iNOS in T cells leads to enhanced persistence of CD8 immune memory in vivo**
endogenous CD8 T cells were comparable between the two sets of chimera as well as to the donor CD8 T cells of WT origin (Fig 9).

3.3.3. Effect of absence of iNOS on TSWD in CD8 T cell blasts

3.3.3.1 TSWD in iNOS−/− CD8 T cell blasts

T cell blasts generated from WT, CD95-deficient B6.1pr and iNOS−/− mice were cultured without IL-2 (-IL2) to induce TSWD. The IL-2 containing cultures (+IL2) served as controls. Cells were harvested 24 hrs later and stained for CD8 as a surface marker and the DNA binding dye Hoechst 33342 to score for cell death. Samples were examined by fluorescence microscopy to count number of cells showing apoptosis characterized by appearance of condensed or fragmented nuclei. Fig 10 shows that CD8 T cell blasts from WT and CD95-deficient B6.1pr mice showed significant death in the absence of IL-2 whereas iNOS−/− CD8 T cell blasts were protected. The above data show that absence of iNOS in CD8 T cells protects them from a death pathway that functions independently of the Fas-FasL (CD95-CD95L) pathway.

3.3.3.2 Pharmacological inhibition of iNOS activity during induction of TSWD

In the above experiments, iNOS expression was absent in T cells at all times and therefore the differences that were seen in the extent of death could be because of differences at the time of T cell blast generation. Whether preventing the synthesis/activity of iNOS at the time of death induction has the same effect as
*Fig 10: Activated iNOS−/− T cells are protected from TSWD*

T cell blasts from WT, iNOS−/− and B6.lpr mice were cultured in the absence of IL-2 (-IL2) to induce TSWD. The control cultures received 5 IU/mL of IL-2 (+IL-2). Death in CD8 T cell blasts was scored by staining for surface CD8 and nuclear morphology at 24 hrs. Data have been plotted as the mean ± SE of triplicate samples and are representative of three independent experiments.
complete absence of iNOS from T cells was next tested. For this Mn (III) tetrakis (5,10,15,20-benzoic acid) porphyrin [MnTBAP] which is an SOD mimic as well as a peroxynitrite scavenger, and iNOS inhibitors such as aminoguanidine (AG), and \( \text{N}^2 \)-monomethyl-L-arginine (L-NMMA), were added to WT T cell blast cultures undergoing TSWD in the absence of IL-2. WT CD8 T cell blasts showed protection from TSWD in the presence of MnTBAP (Fig 11A) and the iNOS inhibitors AG and L-NMMA (Fig 11B). Death in CD8 T cell blasts from iNOS-/- mice is also shown for comparison.

3.3.4 Role of iNOS in AICD of CD8 T cell blasts

Post activation death can also involve another death pathway that is triggered by death receptors such as TNF-R1, CD95/Fas, TRAIL-R1, or TRAIL-R2 and is commonly referred to as Activation Induced Cell Death (AICD). Earlier data using an in vitro human allore cognition system show that blocking CD95-CD95L interaction does not lead to any enhancement in T cell memory, although it does protect from anti-CD3 mediated AICD, suggesting that AICD does not play a role in modulation of T cell memory. Data presented here so far shows that absence of iNOS in CD8 T cells results in their protection from TSWD and enhanced in vivo memory. The effect of absence of iNOS expression on CD8 T cell blasts undergoing AICD was therefore tested next.

T cell blasts from WT, iNOS-/- and CD95-deficient B6.lpr mice were restimulated with plate bound anti-CD3 in the presence of IL-2 for 48 hrs after which cells were harvested and stained for CD8 and apoptotic nuclei by fluorescence microscopy. Fig 12 shows that iNOS-/- CD8 T cell blasts underwent AICD to
**Fig 11: Mouse T cells show better protection from TSWD following peroxynitrite depletion or iNOS inhibition**

TSWD was induced in WT T cell blasts either in the presence (panel A) of MnTBAP (100 μM) (-IL2+Mn) or in the presence of (panel B) iNOS inhibitors AG (-IL2+AG;100 μg/mL) and L-NMMA (-IL2+LNMMA;300 μM). Apoptotic CD8 T cells were scored at 24 hrs by staining for both surface CD8 and for nuclear morphology. Data are represented as mean ± SE for triplicate samples and represent two independent experiments.
**Fig 12: Absence of iNOS does not protect from AICD**

T cell blasts from WT, iNOS\(^{-/-}\) and B6.lpr mice were stimulated with plate bound anti-CD3 (+aCD3) to induce AICD. T cell blasts cultured in the absence of anti-CD3 (-aCD3) stimulus served as controls. CD8 T cell blasts were analysed for death by staining for nuclear morphology at 48 hrs. Data have been plotted as the mean ± SE of triplicate samples and are representative of three independent experiments.
almost the same extent as WT CD8 T cell blasts, while CD8 T cell blasts from CD95-deficient B6.lpr mice were protected from AICD.

3.3.5 Role of reactive nitrogen intermediates in primary T cell responses

3.3.5.1. In vitro responses

Up to this point, the data presented show that the enhanced CD8 T cell memory seen in the absence of iNOS is because of their resistance to TSWD. However, an additional explanation for the enhanced memory seen in iNOS⁺ CD8 T cells could also be that absence of iNOS may result in enhanced primary activation, which would in turn result in generation of greater numbers of primed effectors contributing to the memory pool independent of any death pathways. To test this possibility, spleen cells from WT and iNOS⁻/⁻ mice were stimulated with titrating doses of anti-CD3 and the cultures were pulsed at different time points with [³H] thymidine to estimate the extent of primary proliferation. There were no differences in the proliferation profiles of T cells from WT and iNOS⁺/⁻ mice at 24 (Fig 13D), 48 (Fig 13E) and 72 hrs (Fig 13F). Further, when MnTBAP was added to the WT cultures, the proliferation profiles remained unchanged at 24 (Fig 13A), 48 (Fig 13B) and 72 hrs (Fig 13C). In order to further address the issue of iNOS and/or peroxynitrite of APC origin possibly affecting primary proliferation, spleen cells from WT and iNOS⁻/⁻ mice were allo-stimulated with iNOS⁺/⁺ gamma-irradiated BALB/c spleen cells. Again, there was no difference in the proliferation profiles in the absence or presence of MnTBAP (Fig 14A,B&C) as well as between WT and iNOS⁻/⁻ T cells (Fig 14D,E&F).
Fig 13: Primary T cell responses to anti-CD3 unaffected by absence of iNOS or by peroxynitrite scavenging

Proliferative response for iNOS^- spleen cells and WT spleen cells stimulated with titrating concentrations of anti-CD3 in the absence or presence of MnTBAP (100 μM) was estimated at the indicated time points. The proliferation profile was similar for WT spleen cells cultured with and without MnTBAP (panel A,B & C). Also WT and iNOS^+ spleen cells showed similar extent of proliferation (panel D,E & F).
Fig 14: Primary allo-T cell responses unaffected by absence of iNOS or by peroxynitrite scavenging

Allo-proliferative responses for iNOS−/− spleen cells and WT spleen cells against titrating numbers of irradiated BALB/c spleen cells in the absence or presence of MnTBAP(100 μM) were estimated at the indicated time points. The proliferation profile was comparable for WT spleen cells allo-stimulated with and without MnTBAP (panel A,B & C). Proliferative responses for WT and iNOS−/− cells (panel D,E & F) when compared were also similar at the indicated time points.
3.3.5.2. In vivo responses

WT and iNOS−/−mice were immunised with mOVA on CFA subcutaneously, and an additional set of immunised WT mice received MnTBAP from day-2 to day+5 (3 mg/kg). Seven days post-immunisation, lymph node cells from these mice were restimulated with titrating doses of mOVA followed by an estimate of IFN-γ at 72 hrs in the culture supernatants by sandwich Enzyme Linked Immunosorbent Assays (ELISA). Cytokine levels were significant and similar in the three immunised groups and above the control immunised group (Fig 15), confirming that the enhanced persistence of memory T cells seen in the absence of iNOS is due to their resistance to TSWD and not because of greater number of effectors generated during primary activation.
**Fig 15:** Early in vivo T cell responses are unaffected by absence of iNOS or by peroxynitrite depletion

WT mice were immunised with mOVA and treated with saline or MnTBAP (60 μg/mouse) daily from day -2 to day +5 of immunisation. iNOS−/− mice were also immunised with mOVA. One week later, primed T cell responses were measured by estimation of IFN-γ cytokine by ELISA. Each point has been plotted as mean ± SE of three mice per group.
3.4. **Role of AIF in T cell death, development and memory commitment**

AIF has been reported to have a pro-apoptotic role during early embryogenesis (Joza et al., 2001) and an anti-apoptotic role in neurons (Klein et al., 2002) where it acts as scavenger of reactive oxygen intermediates (ROI), in Harlequin (Hq) mice which carry a proviral insertion in the AIF gene resulting in ~80% reduction of AIF expression (Klein et al., 2002). AIF release from mitochondria in activated human T lymphocytes has been shown to occur in response to death agents that trigger the mitochondrial death pathway (Dumont et al., 2000) independent of death receptors suggesting that it has a role in mitochondrial death by neglect pathway (TSWD). Both AIF mediated and mitochondrial mediated death pathways have been reported to be caspase independent in some instances (Bidere et al., 2001). The TSWD pathway has also been reported to play a role in thymic development, peripheral T cell homeostasis and memory (Rathmell et al., 2002; Hildeman et al., 2002). Therefore, the hypothesis that AIF may have a role to play in the regulation of cell death and memory cell survival in the T cell lineage was tested using the natural AIF mutant Hq mice that are hypomorphic for AIF expression.

3.4.1 **Role of AIF in post activation T cell death**

AIF expression was tested in T cell blasts (TCBs) from the AIF hypomorphic Hq mice and their WT counterparts. **Fig 16** is a Western blot analysis of AIF expression in lysates of T cell blasts from WT and Hq mice. WT T cell blasts expressed much more AIF than Hq TCBs, confirming reduced expression of AIF.
Whole cell lysates were prepared from WT and Hq T cell blast cultures followed by Western blot analysis of AIF expression. Constitutively expressed p38MAPK was used as the reference control.

**Fig 16: Hq T cells lack AIF expression**
in T cells from these mice. Constitutively expressed p38MAPK was used as a reference control.

3.4.1.1. Absence of AIF in T cells confers protection from TSWD

Live resting T cell blasts from WT and Hq spleen cells were cultured without IL-2 (-IL2) to induce TSWD. IL-2 containing cultures (+IL2; 5 IU/mL) served as controls. Cells were harvested at 24 hrs, 48 hrs and 60 hrs and stained for CD8/CD4 and the DNA binding dye Hoechst 33342. Death was scored by apoptotic nuclear morphology visualised using a fluorescence microscope. Fig 17A shows that the Hq CD8 T cell blasts showed little death by TSWD as compared to WT CD8 T cell blasts, at all time points. Similar differences in apoptotic death were seen for CD4 T cell blasts (Fig 17B). Differences in TSWD between WT and Hq T cell blasts were also observed when death was scored by Trypan blue exclusion (Fig 17C) in total T cell blasts suggesting that AIF has a pro-apoptotic role in T cell TSWD. Since 99% of the total T cell blasts population is T cells, death scored by Trypan blue dye is specific to T cells only.

3.4.1.2 Effect of MnTBAP and aminoguanidine in T cell TSWD

Earlier data have shown that MnTBAP, which is an SOD mimic as well as a peroxynitrite scavenger, protects WT T cell blasts from TSWD. Similar protection from TSWD is seen in WT T cell blasts when inducible nitric oxide synthase (iNOS) inhibitors are used (see section 3.3.3.2). These findings were confirmed,
Fig 17: T cell blasts protected from TSWD in the absence of AIF

TSWD in the absence of IL-2 (-IL2) was induced in WT and Hq T cell blasts (TCBs). The IL-2 containing (+IL2) cultures (5 IU/mL) served as controls. Death in CD8 (panel A) and CD4 (panel B) T cell blasts was scored by staining for nuclear morphology using Hoechst 33342 dye at different time points. Death was also scored by exclusion of Trypan blue dye (panel C). Each point is plotted as the mean ± SE of triplicate samples. Data are representative of five independent experiments.
since T cell blasts when treated with MnTBAP (100 μM) or AG (100 μg/mL), showed protection from TSWD. However, these inhibitors did not protect from the residual level of death seen in Hq T cell blasts at any time point, or in WT T cell blasts at late time points (50-60hrs) when death was scored as apoptotic nuclei for CD8 (Fig 18A) and CD4 TCBs (Fig 18B). Similar trends were observed when cell death was scored by Trypan blue exclusion (Fig 18C).

3.4.1.3 Autophagic death component in TSWD of T cell blasts

The protection conferred by MnTBAP and AG at late time points was not complete in both WT and Hq T cell blasts suggesting the possibility of another slow death pathway that may be functioning independent of RNl. The autophagic death pathway involves formation of vacuoles in response to stress that are degraded by lysosomal proteases such as cathepsins. These proteases trigger death through both mitochondrial dependent and independent pathways. TSWD was induced in WT TCBs, either in the presence of AG alone (100 μg/mL), or in the presence of 3-Methyl adenine (3-MA) an autophagy inhibitor (10 μM) alone or both AG and 3-MA together. At 50-60hrs, cells were sampled for Trypan blue exclusion and apoptotic damage. Fig 19 shows that total T cell blasts of WT origin (Fig 19A) were protected in the presence of both AG and 3-MA. Similar trends were seen with Trypan blue dye exclusion (Fig 19B). These data suggest that an autophagic component may also operate in TSWD of T cell blasts.

3.4.1.4 Loss of Bcl-xl in T cell TSWD
Fig 18: Superoxide scavenging and iNOS inhibition protect from AIF associated TSWD

MnTBAP (100 μM) and AG (100 μg/ml) were added to WT and Hq T cell blast cultures in the absence of IL-2 (-IL2). Staining for nuclear morphology in both CD8 (panel A) and CD4 (panel B) T cell cultures was done to identify apoptotic cells at different time points. Death in total T cell blasts was scored by exclusion of Trypan blue dye (panel C). Each point is plotted as the mean ± SE of triplicate samples. Data are representative of three independent experiments.
Fig 19: Autophagic death component in TSWD
MnTBAP (-IL2+Mn), AG (-IL2+AG), AG+3-MA (-IL2+AG+3-MA) and 3-MA (-IL2+3-MA) alone were added to WT T cell blast cultures in the absence of IL-2. Death in total T cell blasts was scored either by staining for nuclear morphology (panel A) or exclusion of Trypan blue dye (panel B) at 70 hrs. Each point is plotted as the mean ± SE of triplicate samples. Data are representative of two independent experiments.
TSWD in T cell blasts is prevented by enhanced levels of anti-apoptotic members of Bcl-2 family of proteins, particularly Bcl-2 and Bcl-xL (Shimizu et al., 2000). Mitochondrial release of apoptogenic proteins is controlled by members of the Bcl-2 family. Therefore, it was tested whether Bcl-xL and Bcl-2 levels were affected following AIF dependent death in TSWD. Fig 20 shows a Western blot analysis of Bcl-xL expression in WT and Hq T cell blasts in the presence or absence of IL-2 after 6 hrs (Fig 20A) and 12 hrs (Fig 20B) in culture. Both WT and Hq T cell blasts cultured in the absence of IL-2 (-IL2) showed reduction in Bcl-xL levels as compared to their respective controls. However the loss of Bcl-xL was notably slower in Hq TCBs.

3.4.1.5 Cytochrome c release delayed in the absence of AIF

Loss of mitochondrial membrane integrity results in release of its apoptogenic components such as AIF and Cyt c into the cytosol. Both WT and Hq TCBs showed a loss in anti-apoptotic Bcl-xL levels, but this loss was delayed in the Hq TCBs. It was therefore important to test whether this reduced loss of Bcl-xL levels translated into differences in release of other mitochondrial resident apoptogenic factors. Since T cell blasts from Hq mice are expected to be deficient only in AIF but sufficient in other mitochondrial components known to trigger programmed cell death such as Cyt c, leakage of Cyt c during induction of TSWD was tested. WT and Hq T cell blasts undergoing TSWD were subjected to sub-cellular fractionation to yield cytosolic and mitochondrial protein extracts separately. As seen in Fig 21, Cyt c release into the cytosol at both 6 hrs and 12 hrs post induction of TSWD (-IL2), was compromised in the absence of AIF. WT T cell
Whole cell lysates of WT and Hq T cell blasts were analysed for Bcl-xL levels at 6 hrs (panel A) and 12 hrs (panel B) post TSWD induction by Western blot analysis. Constitutively expressed p38MAPK was used as the reference control.
## Cytosol Mitochondria

<table>
<thead>
<tr>
<th></th>
<th>Cytosol</th>
<th>Mitochondria</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT</td>
<td>Hq</td>
</tr>
<tr>
<td><strong>IL-2</strong></td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

**Panel A**
- Cyt c
- COX-IV
- p38MAPK

**Panel B**
- Cyt c
- COX-IV
- p38MAPK

---

**Fig 21: Cyt c release delayed in AIF hypomorphic T cells following TSWD**

Cytosolic and mitochondrial extracts from WT and Hq T cell blasts were analysed for Cyt c release at 6 hrs (*panel A*) and 12 hrs (*panel B*) post TSWD induction. COX-IV expression (restricted to mitochondria) was used as a measure for purity of fractions while constitutively expressed p38MAPK was used as the reference control.
blasts showed significant amounts of Cyt c in the cytosol while Hq T cell blasts showed relatively lower amounts of Cyt c in cytosol especially at the earlier time point. Mitochondrial extracts from both WT and Hq T cell blasts showed substantial amounts of Cyt c. Constitutively expressed p38MAPK was used as a reference control. The purity of fractions was determined by western blot analysis of COX-IV which is associated with the respiratory chain in the mitochondrial membrane and is not released into cytosol even during induction of death.

3.4.1.6 Absence of AIF from T cells leads to enhanced AICD

WT and Hq T cell blasts were stimulated with plate bound anti-CD3 (+aCD3) to induce AICD in the presence of IL-2. Cells without anti-CD3 served as controls (-aCD3). CD8 (Fig 22A) and CD4 (Fig 22B) T cell blasts from Hq mice showed early and enhanced AICD compared to WT CD8 and CD4 T cell blasts when death was scored as apoptotic nuclei at different time points. Hq T cell blasts started dying as early as 24 hrs (Fig 22) while WT T cell blasts were still viable. At later time points that is at 48 and 60 hrs, WT T cell blasts showed significant AICD but the levels were still less than those in the Hq T cell blasts. These data suggest that AIF has an anti-apoptotic role in AICD.

3.4.1.7 Role of superoxides in AIF associated AICD

AICD was induced in WT or Hq T cell blasts in the presence of MnTBAP, which is an SOD mimic and peroxynitrite scavenger. WT CD8 (Fig 23A) and CD4 (Fig 23B) T cell blasts were protected from AICD in the presence of MnTBAP at all time points. No such protection was seen in Hq T cell blasts for both CD4 and
Plate bound anti-CD3 (+aCD3) was used to induce AICD in the WT and Hq T cell blasts. Cells cultured without anti-CD3 (-aCD3) served as controls. Apoptotic CD8 (panel A) and CD4 (panel B) T cell blasts were identified at different time points by staining for nuclear morphology using the Hoechst 33342 dye. Each point is plotted as the mean ± SE of triplicate samples. Data are representative of four independent experiments.
Plate bound anti-CD3 (+aCD3) was used to induce AICD in the WT and Hq T cell blasts. Cells without anti-CD3 (-aCD3) served as controls. MnTBAP (100 μM) was added to WT and Hq T cell blast cultures in the presence of anti-CD3 (+aCD3+Mn). Apoptotic CD8 (panel A) and CD4 (panel B) T cell blasts were identified at different time points by staining for nuclear morphology using the Hoechst 33342 dye. Each point is plotted as the mean ± SE of triplicate samples. Data are representative of four independent experiments.
CD8 (Fig 23A&B). The protection observed is due to the SOD function of MnTBAP and not because of its peroxynitrite scavenger ability, since addition of the iNOS inhibitor AG did not protect either WT or Hq T cell blasts from AICD (Fig 24). This was confirmed further by enhanced AICD seen in T cell blasts from MnSOD-/+ mice (Fig 25A) that are heterozygous for a mutation in the mitochondrial MnSOD. Death was scored by staining for nuclear morphology. TSWD was not different in T cell blasts from these mice when compared to their WT counterparts (Fig 25B). These findings suggest that SOD activity is anti-apoptotic during AICD, but only in the presence of AIF, thereby pointing to the importance of peroxide scavenging role of mitochondrial resident AIF in AICD.

3.4.1.8 Role of Fas-FasL expression in AIF associated AICD

One possible reason for the enhanced AICD seen in Hq T cell blasts might be that the absence of AIF would translate into loss of superoxide/peroxide scavenging functions, leading to an increase in reactive oxygen species (ROS) that have been reported to induce the expression of FasL (CD95L) (Devadas et al., 2002), which is a ligand for death receptor Fas (CD95). To test this possibility, WT and Hq T cell blasts were stained for surface CD95 and CD95L expression just before initiating AICD in them. Hq and WT T cell blasts expressed both CD95L and CD95, but Hq T cell blasts showed a small but consistent increase in CD95L surface expression as compared to WT T cell blasts (Fig 26A). However, the CD95 surface expression was similar in WT and Hq T cell blasts (Fig 26B).

3.4.2 Role of AIF in generation of T cell memory

- 59 -
AG (100 μg/ml) was added to WT and Hq T cell blast cultures in the presence of anti-CD3 (+aCD3+AG). Death was scored for both CD8 (panel A) and CD4 (panel B) T cell blasts by staining for nuclear morphology. Each point is plotted as the mean ± SE of triplicate samples. Data are representative of two independent experiments.
Fig 25: Enhanced AICD in mice with compromised MnSOD levels

T cell blasts from WT (MnSOD^{+/+}) and mice heterozygous for MnSOD gene (MnSOD^{--/}) were stimulated either with plate bound anti-CD3 (+aCD3) to induce AICD (panel A) or were cultured in IL-2 devoid medium (-IL2) to induce TSWD (panel B). Death in total T cell blasts was scored as apoptotic nuclei after 24 hrs and is represented as mean ± SE for three to five mice per group. Data has been compared using the student's t test (*p<0.005).
Fig 26: FasL expression enhanced in Hq T cell blasts
Flow cytometric analysis of FasL (panel A) and Fas expression (panel B) on WT (dotted line) and Hq (solid line) T cell blasts (CD8 and CD4) before AICD induction. The staining control represented as shaded histogram. Data are representative of three to four independent experiments.
Hq T cell blasts show protection from TSWD pathway, reported to be responsible for survival of long-lived memory T cells in vivo. Prolonged survival of activated T cells in vivo has also been associated with persistent levels of Bcl-xL. Since Hq T cell blasts showed these features, the memory phenotype of T cells in peripheral lymphoid organs of these mice was investigated by staining for CD4/CD8 to identify T cells and CD44 on T cells, to identify naive (CD44lo) and effector/central memory (CD44hi) T cells by flow cytometry.

3.4.2.1 Higher frequencies of peripheral memory T cells (CD44hi) in Hq mice

Analysis of CD44 expression on CD8 and CD4 T cells from spleen (Fig 27A) and inguinal lymph nodes (Fig 27B) of Hq mice showed higher frequencies of CD44hi T cells as compared to WT (Fig 27A&B). These data confirm the prediction that T cells protected from TSWD would show enhanced accumulation of the memory phenotype. CD44hi frequencies for CD8 and CD4 T cells from spleen (Fig 27C) and lymph nodes (Fig 27D) were calculated from the above staining data.

3.4.2.2 Reduced peripheral T cell frequencies in Hq mice

While total cell yields for spleen and lymph nodes were only marginally lower in Hq mice (Fig 28), analysis of CD8 and CD4 staining showed that Hq mice had substantially lower frequencies of CD8 T cells (Fig 29A) and CD4 T cells (Fig 29B) in the spleen as compared to WT. CD8 and CD4 T cell frequencies were lower in Hq mice (Fig 29C) as were absolute CD8 and CD4 T cell numbers (Fig
**Fig 27: Increased memory T cell frequencies in Hq mice**

CD44 expression profile of CD8 and CD4 T cells from spleen (*panel A*) and lymph node (*panel B*) cells of naïve WT and Hq mice are shown. Frequencies of CD8 as well as CD4 T cells showing high CD44 (*panel C*) expression were calculated from staining data of both spleen (*panel C*) and lymph node (*panel D*) cells. Data are shown as mean ± SE for three mice and are representative of five independent experiments. Data has been compared using the student's t test for *panel C* (*p<0.0001; **p<0.0005*) and *panel D* (*p<0.0005; **p<0.005*).
Ex vivo spleen and lymph node cell yields were compared between WT and Hq mice. Data are shown as mean ± SE for three mice and are representative of five independent experiments.
**Fig 29: Reduced frequencies and number of splenic CD8 and CD4 T cells in the absence of AIF**

Representative staining profiles of CD8 (panel A) and CD4 (panel B) T cells on spleen cells from naive WT and Hq mice are shown. Frequencies of CD8 and CD4 T cells as measured by staining from three mice are shown (panel C). Absolute numbers of CD8 and CD4 T cell are also shown (panel D). Data are shown as mean ± SE for three mice and are representative of five independent experiments. Data has been compared using the student's t test for panel C (*p<0.005; **p<0.0005) and panel D (*p<0.0001; **p<0.0005).
29D). Inguinal lymph nodes from Hq mice showed a similar reduction in peripheral CD8 (Fig 30A) and CD4 T cell (Fig 30B) frequencies (Fig 30C) and absolute numbers (Fig 30D) as compared to WT mice.

3.4.2.3 Lower numbers of naive T cells in Hq mice

Hq mice showed higher frequencies of peripheral memory T cells. However, when absolute numbers of CD44^hi (memory) and CD44^lo (naive) T cells were calculated, Hq and WT mice showed equivalent numbers of CD44^hi (memory) T cells in spleen (Fig 31A) and lymph nodes (Fig 31B). The CD44^lo (naive) T cell numbers were significantly lower in Hq spleen (Fig 31A) and lymph node (Fig 31B) indicating that Hq mice have fewer peripheral naive T cells.

3.4.2.4 B cell and macrophage frequencies and numbers unaffected in Hq mice

Hq mice showed reduced naive T cell numbers in the periphery pointing to defective T cell development. Whether this defect was specific to T cells or extended to other lymphocytic lineage cells was tested by examining B cells and macrophages. Analysis of staining profiles of B cells and macrophages in spleen (Fig 32A) and lymph nodes (Fig 32B) showed that frequencies of B cells and macrophages (Fig 32C) were not significantly different between the two strains. Absolute numbers of B cells and macrophages were again not different in spleen (Fig 32D) and lymph nodes (Fig 32E) of WT and Hq mice, suggesting that AIF has a role specific to T cells.
**Fig 30: Lower frequencies and number of lymph node CD8 and CD4 T cells in Hq mice**

Representative staining profiles of CD8 (panel A) and CD4 (panel B) T cells on lymph node cells from naïve WT and Hq mice are shown. Frequencies (panel C) of CD8 and CD4 T cells as measured by staining and absolute CD8 and CD4 T cell numbers from three mice are shown (panel D). Data are shown as mean ± SE for three mice and are representative of five independent experiments. Data has been compared using the student’s t test for panel C (*p<0.01; **p<0.01) and panel D (*p<0.01; **p<0.01).
**Fig 31: Lower numbers of naive T cells in Hq mice**

Absolute numbers of naive (CD44lo) and memory (CD44hi) CD8 and CD4 T cells present in spleen (panel A) and lymph node (panel B) of WT and Hq mice were calculated. Data are shown as mean ± SE for three mice and are representative of five independent experiments. Data has been compared using the student's t test for panel A (*p<0.0001; **p<0.0005) and panel B (*p<0.01; **p<0.005).
Fig 32: B cell and macrophage compartments are unaffected in Hq mice

Ex vivo spleen (panel A) and lymph node (panel B) cells from WT and Hq mice were stained for B220 and CD11b (Mac1) as shown in the flow cytometry plots. B220 and Mac1 frequencies (panel C) as well as absolute numbers for spleen (panel D) and lymph node (panel E) were calculated for both WT and Hq. Data are shown as mean ± SE for three mice and are representative of five independent experiments.
3.4.2.5 *Ex vivo* T cells from Hq mice are protected from TSWD

One reason for the reduced frequencies and absolute numbers of peripheral T cells in the Hq mice could be that these T cells were dying in the absence of any survival signal. This was tested in a TSWD assay where *ex vivo* spleen cells from WT and Hq mice were cultured with and without IL-2 for 24 hrs. CD8 (Fig 33A) and CD4 (Fig 33B) T cells undergoing TSWD were scored by staining for nuclear morphology. Hq CD8 and CD4 T cells showed protection from TSWD as compared to their WT counterparts.

3.4.2.6 Functional T cell and B cell responses unaffected by AIF absence

Hq mice that are hypomorphic for AIF showed increased frequencies of memory T cells in their peripheral lymphoid organs. The possibility that such an increase may in part be due to their ability to respond better to an activating stimulus was tested. To score for CD4 and CD8 specific T cell responses, spleen cells from WT and Hq mice were labeled with carboxy fluorescein-succinimidyl ester (CFSE) and cultured with titrating doses of anti-CD3. Cultures were harvested at different time points, stained for CD4/CD8 and proliferation was measured by scoring for T cells showing serial doubling dilution of the CFSE label. Fig 34 shows the proliferation analysis for responding CD8 (Fig 34A) and CD4 (Fig 34B) T cells. The frequencies of CD8 T cells diluting CFSE in response to different doses of anti-CD3 were similar between WT and Hq at different time points (Fig 34A). Similar trends were seen for CD4 T cells (Fig 34B).
Fig 33: Resting T cells from Hq mice show protection from TSWD

Ex vivo spleen cells from WT and Hq mice were cultured in the presence or absence of IL-2 for 24 hrs. Death in CD8 and CD4 T cells was scored by staining for nuclear morphology. Data are shown as mean ± SE for triplicate samples and are representative of three independent experiments.
Alf

CFSE labeled spleen cells from WT and Hq mice were stimulated with titrating doses of anti-CD3. Flow cytometric analysis of CFSE-diluting cells was done to calculate percentage of CD8 (panel A) and CD4 (panel B) T cells proliferating in response to stimulus. Total T cell proliferation of unlabeled spleen cells against anti-CD3 was also measured at 72 hrs by [3H] thymidine incorporation (panel C). Background proliferation was less than 1000 cpm. Data are representative of three experiments.

Fig 34: T cell proliferative responses unaffected by AIF levels
Proliferation was also assayed by culturing 50000 splenic T cells from WT and Hq, with titrating doses of anti-CD3 for 72 hrs. At the end of 72 hrs, cells were pulsed with \[^{3}H\] thymidine for 12 hrs, at the end of which proliferation was expressed as the extent of thymidine incorporation into DNA. The proliferative responses between WT and Hq T cells were almost comparable, though WT T cells proliferated slightly better (Fig 34C). B cell responses to LPS were also measured either by flow cytometry using CFSE dilution as a readout (Fig 35A), or by measuring \[^{3}H\] thymidine incorporation (Fig 35B). In both instances, B cells of Hq origin proliferated equally or marginally less than the WT B cells. Thus, enhanced frequency of T cell memory in Hq mice could not be attributed to enhanced proliferative responses.

3.4.3 Role of AIF in T cell development

3.4.3.1 Hypocellular thymus in Hq mice

*Ex vivo* staining data showed that AIF hypomorphic Hq mice persistently showed reduced naive T cell frequencies in peripheral lymphoid organs while B cell and macrophage numbers remained comparable suggesting a defect in T cell development. To address this issue the thymic phenotype of Hq mice was analysed. Total thymic cellular yields were low in the Hq mice as compared to WT (Fig 36A). Two color staining for CD4 and CD8 (Fig 36B) was done followed by calculations of absolute cell yields of each subpopulation (Fig 36C). This analysis revealed that numbers of CD4\(^{-}\)CD8\(^{-}\)double negative (DN) thymocytes were similar between WT and Hq (Fig 36C), but the Hq thymus had far fewer numbers of CD4\(^{+}\)CD8\(^{+}\) double positive (DP) cells as seen in Fig 36C. Hq thymus
CFSE labeled spleen cells from WT and Hq mice were stimulated with titrating doses of LPS. Flow cytometric analysis of CFSE diluting cells was done to calculate percentage of B220⁺ B cells proliferating in response to stimulus (panel A). B cell proliferation of unlabeled spleen cells against LPS was also measured at 72 hrs by [³H] thymidine incorporation (panel B), where background proliferation was 600 cpm. Data are representative of three experiments.

**Fig 35: B cell proliferative responses are unaffected in Hq mice**
Panel A shows thymic cell yields for WT and Hq mice. Thymus from WT and Hq mice was stained for CD4 and CD8 as shown in the flow cytometry plot (panel B). Absolute number of each thymic subset was calculated (panel C). Data are shown as mean ± SE for three mice and are representative of four independent experiments. Data has been compared using the student's t test for panel A (*p<0.05) and panel C (*p<0.5; **p<0.05; ***p<0.05; ****p<0.05).
also showed reduction in single positive (SP) CD8+ and CD4+ thymocytes (Fig 36C). These data suggest that the developmental transition from the DN to the DP stage is aberrant in Hq mice.

### 3.4.3.2 AIF hypomorphism in T lineage cells leads to compromised development

Since AIF is ubiquitously present in all cell types, it was decided to test whether the defect in thymic selection in Hq mice was due to absence of AIF in T lineage cells or in thymic stromal cells, which are also critical for T cell development. This issue was examined by the use of bone marrow chimeras. Since the Hq mouse strain is on a mixed C57BL/6xCBA/CaJ genetic background, (B6.SJL (CD45.1) x CBA/CaJ) F1 mice were lethally irradiated and reconstituted with a mixture of bone marrow cells from B6.SJL mice (CD45.1) and either WT or Hq mice (CD45.2). Seven weeks later, the frequencies of CD45.2-bearing donor CD4, CD8 and B220+ cells in various anatomical compartments were examined by flow cytometry. Analysis of splenic cells from the chimeras showed that Hq T cells reconstituted poorly as compared to WT, for both CD8 (Fig 37A) and CD4 (Fig 37B) subsets, but B cell reconstitution was not as severely affected (Fig 37C). The frequencies of donor CD45.2+ T cells were lower when the marrow was of Hq origin (Fig 37D). Reconstitution of donor CD45.2+ B cells (Fig 37D) was not as drastically affected by the Hq mutation. Similar findings were seen in the case of lymph node cells from these chimeras (Fig 38 A,B,C,D). The CD45.2+ CD45.1+ double positive cells as seen in Fig 37 and 38 are from the endogenous reconstitution of the F1 progenitor cells post-irradiation. Analysis of donor CD45.2+ frequencies in the chimeric thymus showed that the contribution of Hq
Fig 37: Defect in splenic reconstitution of AIF hypomorphic T cell lineage

Staining profile of CD45.2+ (donor) and CD45.1+ (recipient) cells post reconstitution on spleen cells from chimeric mice. The above flow cytometry plots show frequency of CD45.2+ and CD45.1+ CD8 T cells (panel A), CD4 T cells (panel B) and B cells (panel C). The CD45.2+ CD45.1+ double positive cells are from the endogenous reconstitution of the F1 progenitor cells post-irradiation. Statistical representation of donor CD45.2+ (panel D) frequencies in each subset is also shown as mean ± SD (n=2).
**Fig 38: Poor lymph node reconstitution of AIF-hypomorphic T cells**

Staining profile of CD45.2+ (donor) and CD45.1+ (recipient) cells post reconstitution on lymph node cells from chimeric mice. The above flow cytometry plots show frequency of CD45.2+ and CD45.1+ CD8 T cells (panel A), CD4 T cells (panel B) and B cells (panel C). The CD45.2+ CD45.1+ double positive cells are from the endogenous reconstitution of the F1 progenitor cells post-irradiation. Statistical representation of donor CD45.2+ (panel D) frequencies in each subset is also shown as mean ± SD (n=2).
marrow to the DN compartment was close to that of WT marrow (Fig 39A&E). However, Hq marrow contribution decreased drastically for the DP (Fig 39B&E) as well as CD8SP (Fig 39C&E) and CD4SP (Fig 39D&E) subsets. These data suggest that AIF hypomorphism in T lineage cells leads to poor thymic development during DN to DP transition.
**Fig 39: Poor reconstitution of DP and SP thymocytes in the absence of AIF**

Staining profile of CD45.2+ (donor) and CD45.1+ (recipient) cells post reconstitution on thymus from chimeric mice. The above flow cytometry plots show frequency of CD45.2+ and CD45.1+ CD8+CD4- DNs (panel A), CD8+CD4+ DP thymocytes (panel B), CD8+ SPs (panel C) and CD4+ SPs (panel D). Statistical representation of CD45.2+ (panel E) frequencies in each subset as mean ± SD from two mice per group.