Results .......
4.1 Role of AIF in T cell development and function

AIF has been reported to have pro-apoptotic function during early embryogenesis (Joza et al., 2001) and an anti-apoptotic function in neuronal tissues, (Klein et al., 2002) where it acts as a ROS scavenger. In Hq mouse, which carries a proviral insertion in AIF promoter region, it leads to ~80% reduction in AIF expression causing various pathological outcomes, particularly in the neuronal tissues. In human T cells, AIF release from mitochondria on activation has been reported in response to various death inducers (Dumont et al. 2000). This death has also been shown to be independent of extrinsic apoptotic pathway suggesting, a possible role of AIF in neglect induced death. Both AIF mediated and mitochondria mediated death pathways have been reported to be caspase independent in some instances (Bidere et al., 2001). TSWD/NID pathway has also been reported to play a role in thymic development, suggesting a possible role of AIF in T cell development, peripheral homeostasis and memory. (Rathmell et al., 2002 Hildeman et al., 2002). Therefore, AIF may have a role to play in regulation of cell death and memory cell survival.

We tested this possibility by using in vivo and in vitro experiments on Hq mice that are hypomorphic for expression of AIF.

4.2 Hq T cell blast lack or are hypomorhic for AIF expression

Splenocytes from WT and Hq mice were isolated from spleen followed by culture in presence of anti CD3 for two days. On day two, cells were harvested and put back to culture in presence of recombinant human IL-2 for another two days. On day four, we get about 99 % pure T cell blasts. Expression of AIF was tested on T cell blasts (TCBs) from AIF hypomorphic mice (Hq) and WT littermate controls. (Fig 1) is a western blot for AIF
**Fig 1: Lower levels of Aif expression in T cell blast from Hq mouse**

Splenocytes from Hq mouse and WT mouse were put in culture for 2 days with anti-CD3 stimulation followed by rest in presence of IL-2 containing medium. On day 4, we get T cell blasts from which cell lysates were prepared followed by western blot analysis for AIF expression. Constitutively expressed p38MAPK was used as the loading control.
Fig 2: Absence of AIF leads to enhanced AICD in T cell blasts
Plate bound anti-CD3 (+aCD3) was used to induce AICD in WT and Hq T cell blasts in presence of recombinant human IL-2. Cells cultured in presence of recombinant human IL-2 with no aCD3 stimulation was taken as control. Death was scored at different time points by staining for nuclear morphology by Hoechst33342 dye (panel A) or by percentage trypan blue positivity (panel B) for both CD4 and CD8 cells.
expression on cell lysates of T cell blasts from WT and Hq mice. WT T cell blasts show a much more prominent AIF band (67 KDa) than Hq T cell blasts, thus confirming reduced expression of AIF in T cell blast from Hq mice. Constitutive expression of p38MAPK was used as reference for equal loading of cell lysates.

4.3 Enhanced AICD in AIF hypomorphic T cell blasts

WT T cell blasts on day 4 were stimulated with anti-CD3 to induce AICD in presence of recombinant human IL-2. Cells cultured in presence of recombinant human IL-2 without any anti-CD3 stimulation were taken as control (Fig2). Both CD4 and CD8 cells of Hq T cell blasts showed higher cell death compared to T cell blasts from WT littermate controls by both nuclear morphology (apoptotic nuclei) by Hoechst33342 (Panel A) and by trypan blue positive (Panel B) cell death counts at various time points (24, 48 and 60 hrs). These data suggest that AIF plays an anti-apoptotic role in AICD. In the IL-2 control cultures, there was no difference in the cell death pattern between WT and Hq T cell blasts.

4.4 T cell blasts from Hq mice were protected from NID/TSWD

For NID, live T cell blasts were cultured in absence of IL-2, to induce NID due to absence of any cytokine survival signal as well as absence of TCR stimulus, for both WT and Hq T cell blasts. T cells blasts cultured in presence of IL-2 were taken as control. Cells harvested at 24, 48, 60 h were taken and staining for nuclear morphology was done to score for apoptotic nuclei using Hoechst33342. Death was also scored using trypan blue positivity count for both CD4 and CD8 cells. (Fig3) shows lower cell death in case
Fig 3: T cell blasts protected from cell death by neglect in absence of AIF
NID/TSWD in cell culture in absence of TCR stimulus and absence of IL-2 signal was
induced in WT and Hq T cell blasts. IL-2 + cultures served as controls. Death was
scored for both CD4 and CD8 using nuclear morphology (Panel A) using
Hoechst33342 and percentage trypan positivity (Panel B). At all time points AIF
hypomorphic T cell blasts showed lower cell death.
**Fig 4:** *Higher caspase 9 activation in T cell blasts from WT mice compared to Hq mice*

T cell blasts were cultured in absence of IL-2 and TCR stimulus and caspase-9 activation was analyzed on live gated T cell blasts at 12 hrs. Hq show lower caspase-9 activation suggesting the role of intrinsic pathway in NID and pro-apoptotic role played by AIF.
of *Hq* T cell blasts, in both CD4 and CD8 by Hoechst33342 (Panel A) and percentage trypan blue positivity (Panel B). This is indicative of pro-apoptotic role of AIF in NID/TSWD. IL-2 control shows no difference between cell death profiles of the two strains.

### 4.5 Protection seen in Hq T cell blasts in NID is due to absence of AIF

*Hq* T cell blasts when cultured for NID/TSWD show lower frequency of death compared to WT T cell blasts, suggesting important role played by AIF in NID. Thus caspase-9 activation which is known to be playing role in death by NID was examined by flow cytometry. It was found that *Hq* T cell blasts showed less caspase-9 activation, confirming that not only NID is dependent on intrinsic pathway for cell death but also that the release of AIF was a critical factor in NID/TSWD. Absence of AIF also delays caspase-9 release (Fig4). Therefore AIF release is important factor in NID which further aids caspase-9 release into the cytosol. The damage in mitochondria in absence of AIF therefore is incomplete and this may delay neglect induced death in *Hq* T cell blast.
Results

4.6 No difference in death between Hq and WT by other mitochondrial and endoplasmic reticulum stress pathways

To check the possibility if all intrinsic death pathway are dependent on AIF release, we induced death in T cell blasts, using various reagents that mediate death by intrinsic pathway, like etoposide (Fig 5 A), staurosporine (Fig 5 B), and γ-irradiation (Fig 6 A). As these reagents are known to cause mitochondrial stress leading to caspase-9 mediated cell death, we wanted to analyze if death in these pathways was also dependent on AIF release and if Hq T cell blasts are protected on death induction by these reagents as well. There was no difference in the death pattern as well as its kinetics between WT and Hq T cell blast death using other mitochondria mediated death inducers. This suggests that AIF is not essential for all mitochondria mediated death pathways, but is essential in certain cases like NID (Fig 3).

Another death pathway known is the ER stress mediated pathway, in which due to extensive calcium release or damage, caspase-12 gets activated followed by further damage to mitochondria leading to caspase-9 activation. We checked the possibility if endoplasmic reticulum stress mediated death is differentially affected in case of presence or absence of AIF, by inducing endoplasmic reticulum stress using thapsigargin (Fig 6B). There was no difference in endoplasmic reticulum mediated stress response between WT and Hq T cell blasts, thus suggesting that endoplasmic reticulum stress mediated death is independent of AIF.
Fig 5: No difference between WT and Hq death pattern in other mitochondrial damage pathways

T cell blasts were given death stimulus by other reagents like Etoposide (Panel A) and staurosporine (Panel B) and death was scored at various time points by frequency of trypan blue positivity. No difference was found between WT and Hq T cell blast cell death by trypan blue positivity.
Fig 6: No difference between WT and Hq death pattern in other mitochondrial damage and ER damage pathways

T cell blasts were given death stimulus by γ-irradiation (Panel A) and thapsigargin (Panel B) to induce death via mitochondrial and ER stress pathway respectively. Death was scored by percentage trypan blue positivity at various time points. No difference was found in death between WT and Hq T cell blasts.
4.7 Higher susceptibility towards death in hypomorphic AIF expressing cells by ROS and superoxide mediated stress

T cell blasts from Hq and WT mice were treated with variable concentration of DMNQ (2,3-dimethoxy-1,4-nepthoquinone) (Fig 7A) and death was scored by percentage trypan blue positivity at various time points. DMNQ is known to cause cell death by production of superoxides in the mitochondria. Hq T cell blast were seen to be more susceptible to cell death caused by treatment of DMNQ suggesting higher sensitivity to death mediated by superoxides which is generated by cell through electron transport chain, suggesting role played by AIF in quenching ROS in the mitochondria.

On treatment with H$_2$O$_2$ (Fig 7B) in the culture medium, again Hq T cell blasts were seen to be more susceptible to cell death suggesting higher sensitivity of AIF hypomorphic cells to extrinsic peroxide present in the medium. Therefore, in AIF hypomorphic state, T cell blasts are more susceptible to death mediated by ROS, both extrinsic and intrinsic, further indicating a role of AIF in protection by acting as a scavenger of reactive oxygen species.
Fig 7: Hg T cell blasts are more sensitive to ROS mediated stress
T cell blasts were given stress by DMNQ (Panel A) and H2O2 (Panel B) to cause ROS mediated stress. At all time points Hg cells show more death suggesting that in absence of AIF, cells are more susceptible to ROS and superoxide mediated cell death.
Fig 8: Excessive death in response to AICD prevented on EUK-134 treatment

T cell blasts on day 4 were given activation stimulus with plate coated anti-CD3 in presence of IL-2 to induce AICD both in presence and absence of EUK-134 and death was scored by percent trypan blue positivity (Panel A) and percentage apoptotic nuclei (Panel B). EUK-134 was found to protect Hq T cell blasts from excess cell death, suggesting role of ROS species in Hq T cell blast cell death. IL-2 control showed no difference in frequency of cell death.
4.8 Protection of AIF hypomorphic cells from cell death in presence of superoxide quencher

Since, T cell blasts of AIF hypomorphic Hq mice were more susceptible to AICD and also to ROS mediated cell death, we looked at the possible role of ROS in higher AICD as it is already being reported that ROS is a major factor in T cell death during AICD. We induced AICD in WT and Hq cells in presence and absence of superoxide quencher EUK-134 (it is a synthetic manganese porphyrin complex with an ability to quench ROS and has a SOD activity) to see if Hq T cell blasts are protected from higher cell death due to AIF hypomorphism (Fig 8). T cell blasts were given plate coated anti-CD3 stimulus in presence of IL-2 and EUK-134 was added in medium. Cell death was scored by percentage trypan blue positivity (Panel A) and percentage apoptotic nuclei counting (Panel B). Hq T cell blast were seen to be completely protected from T cell death and there was no difference in Cell death by AICD between WT and Hq T cell blast in presence of EUK-134 (Fig 8), suggesting that the excess T cell death observed in Hq cells was mediated by ROS which gets quenched in presence of EUK-134.

4.9 Higher cell death in Hq T cell blast in response to Fas induced death pathway

Since it has been shown previously that Hq cells have upregulation of FasL and Jurkat cells when co-cultured with Hq T cell blasts show higher death, there was a distinct possibility that Hq T cell blasts may be more susceptible to Fas (CD95) mediated death through extrinsic pathway. Therefore, T cell blasts from Hq and WT mice were given stimulus by titrated concentrations of FasL-Ig to induce cell death via Fas mediated death pathway. Hq T cell blasts showed consistently higher death (Fig 9 and 10) compared to
Fig 9: Hq T cell blasts show higher Fas (CD95) mediated death through extrinsic pathway

T cell blasts on day 4 were given a stimulus by titrated concentrations of FasL-Ig to induce death by extrinsic pathway and total death was scored by percentage trypan blue positivity (panel A) or by percentage apoptotic nuclei by Hoechst33342 staining (panel B). Hq T cell blast show higher cell death compared to WT T cell blasts. T cell blasts with IL-2 were taken as control.
Fig 10: Hq T cell blasts (CD4 and CD8) show higher Fas (CD95) mediated death through extrinsic pathway

T cell blasts on day 4 were given a stimulus by titrated concentrations of FasL-Ig to induce death by extrinsic pathway and CD4 and CD8 death was scored by percentage apoptotic nuclei by Hoechst33342 staining at 24 h (panel A) and 48 h (panel B). Hq T cell blast show higher cell death compared to WT T cell blasts. T cell blasts with IL-2 were taken as control.
Fig 11: Higher TNF-α level induced in AIF hypomorphic T cell blasts in response to aCD3 stimulus

On activation with plate coated anti-CD3, Hq T cell blasts show higher production of TNF-α by ELISA assay, suggesting TNF-α to be one of the factor playing its role in causing higher cell death in Hq T cell blasts when compared to WT T cell blasts.
Results

wild type T cell blasts suggesting higher sensitivity of Hq T cells in response to Fas mediated cell death pathway.

4.10 Higher levels of TNF-α release by Hq T cell blast on α-CD3 stimulus

T cell blasts on day four were re-plated on plate coated with αCD3 in presence of IL-2 and at 24 hrs, culture supernatant was collected and TNF-α was measured by ELISA to see, if presence of TNF-α could be one of the factor to cause higher death signal in Hq T cell blasts. We found that Hq T cell blasts released significantly higher levels of TNF-α in the culture supernatant when compared to WT T cell blasts (Fig 11).

4.11 Higher caspase 8 activation in Hq T cell blast in response to AICD stimulus

Since multiple factors as stated above like Fas-FasL interaction, ROS, and TNF-α seem to be involved in AICD mediated death and are more favorable in the Hq T cell blasts and all these stimuli point to the activation of extrinsic pathway of cell death, we examined if there is higher activation of intermediates of the extrinsic pathway in Hq T cell blasts. T cell blasts were stimulated for AICD by Plate coated αCD3 in presence of IL-2, and at 12 h caspase-8 activation was analyzed (which is the initiator caspase that gets activated in extrinsic death pathway). We found that, on live gated T cell blasts, caspase-8 activation was higher in Hq T cell blasts as compared to WT T cell blasts (Fig 12). Suggesting that ROS, TNF-α, and Fas-FasL death pathways may be simultaneously
Fig 12: Higher caspase-8 activation in Hq T cell blasts in response to AICD

AICD was induced in WT and Hq T cell blasts by plate coated anti-CD3 in presence of IL-2 and at 12 h, caspase-8 activation was measured on live gated T cell blasts. Hq T cell blasts showed higher caspase-8 activation. IL-2 control cultures showed no difference in caspase-8 activation between Hq and WT T cell blasts.
Results

driving higher level of cell death by AICD in Hq T cell blasts. No difference was found in IL-2 control cultures.

4.12 Higher mitochondrial membrane potential in Hq hematopoietic cells

AIF being a mitochondrial protein, and with a suggestive role in electron transport chain, it may be playing some role in the metabolic status for the cell, apart from having known death functions in the cell. To check if hypomorphic state of AIF in cells leads to any change in the mitochondrial status in terms of membrane polarization, we looked at mitochondrial membrane potential of cells by Mitotracker Red dye staining in various lymphoid lineage cells. We found that across the entire lymphoid lineage, Hq cells were showing higher Mitochondrial membrane potential compared to WT cells. CD8 and CD4 cells showed higher alteration in the membrane potential compared to B cells and macrophages (Fig 13 and 14). Quantification was done using both flow-cytometry (Fig 13) and using confocal microscopy (Fig 14). The difference observed can be both due to change in polarization or due to difference in the net mitochondrial mass between the two strains.
**Fig 13: Higher mitochondrial membrane potential in Hq lymphoid lineage cells**

*Ex vivo* cells from spleen were taken and stained with mitochondrial membrane potential measuring dye, Mitotracker Red. Cells were then stained with cell surface receptor antibodies to determine their lineage and mitochondrial membrane potential was then measured by flow cytometry. Hq lymphoid cells had higher membrane potential when compared to WT cells. The difference in mean fluorescence intensity in CD4 and CD8 T cells was higher (*Panel A and panel B*) than in B cells and macrophages (*panel C and D*).
Fig 14: Higher mitochondrial membrane potential in lymphoid lineage cells

Ex vivo cells from spleen were taken and stained with mitochondrial membrane potential measuring dye, Mitotracker Red. Cells were then stained with cell surface receptor antibodies to determine their lineage and mitochondrial membrane potential was then measured by confocal microscopy. Hq lymphoid cells had higher membrane potential when compared to WT cells. The difference in mean fluorescence intensity in CD4 and CD8 T cells were higher when comparison was done between WT and Hq cells. Data has been compared using the student's t test (*p<0.005).
4.13 Hq mice show lower CD4 and CD8 T cell frequency and cell numbers in the periphery

Total cell yields were found to be marginally lower in Hq mice in both spleen and lymph nodes (Fig 15B), but analysis of CD8 and CD4 cells in spleen and lymph nodes show lower frequency (fig 15 A and D) and absolute cell numbers (Fig 15 C and E) in Hq mice. This suggested that difference in AICD and NID in the periphery as seen in previous figures may be affecting and determining the peripheral T cell compartment in the Hq mice thus causing changes that ultimately lead to altered peripheral T cell frequency and numbers. Another important factor as discussed in later figure that leads to altered T cell compartment is defective thymic development leading to lower number of CD4 and CD8 T cell being released into the periphery. Therefore these data indicate that T cells were either generated more slowly and/or lost faster in Hq mice.

4.14 B cell and macrophage peripheral compartment unaffected in Hq mice

Hq T cells showed defect in T cell compartment. Thus it was important to find if hypomorphic state of AIF lead to defect in only T cell compartment or it was a general lymphoid lineage defect. This was tested by examining B cells and macrophages in the periphery of Hq mice. Analysis of staining profile of B cells and macrophages in spleen (Fig 16 A and B) and lymph nodes (Fig 16 C and D) showed that frequency of B cells and macrophages (Fig 16 A and C) was higher in Hq mice but, when absolute cell counts of spleen and lymph nodes was done, we found that there was no difference (Fig 16 B
**Fig 15: Reduced frequency and number of splenic and lymph node CD4 and CD8 T cells in AIF hypomorphic mice**

Representative staining profile of Spleen and Lymph nodes for cell frequency and cell numbers. CD4 and CD8 cell percentage are altered in Hq mice. Cell frequency and cell numbers are reduced in Hq in both spleen (A) and (C) and in lymph nodes (B) and (D). Total cell counts are also lower (B) in Hq mice. All data are shown as mean±SE (n=3-5)
Fig 16: B cell and macrophage compartment in Hq mice are unaffected
Representative staining profile of spleen (panel A and B) and lymph nodes (panel C and D). Cell frequency and absolute cell numbers of B cells and macrophages were calculated. B cell and macrophage compartment are unaffected in Hq mice when compared to WT mice. Though cell percentages seem to be increased (A and C) the actual cell numbers are unaltered (B and D). All data are shown as mean±SE (n=3-5)
and D) in the absolute cell numbers, thus suggesting that absence of AIF in B cells and macrophages does not alter their peripheral compartment in *Hq* mice. It was however noteworthy that, due to lower cell frequency of T cells, the frequency of B cells and macrophages was increased in *Hq* mice in both spleen (Fig 16 A) and lymph nodes (Fig 16C).
4.15 Higher activated T cell frequency in Hq mice

CD44 analysis on gated CD4 and CD8 cells in Hq periphery (spleen and lymph nodes) showed higher frequency of CD44$^{hi}$ T cells. In terms of frequency of CD44$^{hi}$ cells in spleen (Fig 17 A) and lymph node (Fig 17 B) Hq mice consistently showed higher % of CD44$^{hi}$ cells. Thus percentage of naïve (CD44$^{lo}$) T cells in the periphery of Hq mice was substantially lower in Hq mice as compared to WT mice (Fig 17 A and B). In terms of absolute cell counts, there was no difference in the number of CD44$^{hi}$ CD4 and CD8 cells (Fig 17 C) but the actual number of naïve T (CD44$^{lo}$) cells was substantially lower. This supports the prediction that T cells, after being protected from NID show enhanced accumulation of memory T cells. Lower cell counts for naïve CD4 and CD8 cells also is an indicative of a possibility for lower naïve cell production during thymic development in thymus as discussed later.

4.16 Altered CD4 and CD8 memory compartment in Hq mice

Characterization of memory compartment was also done using CD44 and CD62L markers on CD4 and CD8 gated cells in the periphery. Hq mice show a substantially lower frequency of CD44$^{low}$CD62L$^{hi}$ naïve T cells. Both central memory compartment CD44$^{hi}$CD62L$^{hi}$ and effector memory compartment CD44$^{hi}$CD62L$^{low}$ cells were of significantly higher frequency in Hq mice (Fig 18 A and B). This again can be indicative of lower cell death by neglect seen in vitro culture by AIF hypomorphic cells. Once activated the cells in the periphery may escape death by neglect resulting in higher accumulation of memory cells in the peripheral compartment of Hq mice.
Activation profile on T cells in the periphery

**Fig 17: Higher activation profile of CD4 and CD8 T cells in Hq mice**

CD44 expression profile of CD8 and CD4 gated cells from spleen (panel A) and Lymph nodes (Panel B) in Hq and WT mice are shown. Frequency of CD4 and CD8 cells showing high CD44 (A and B) was calculated and Hq T cell compartment shows higher activation profile. In terms of absolute cell counts (Panel C) the naïve CD4 and CD8 (Panel C) is significantly reduced. CD44hi Activated T cell compartment is unaffected (Panel C) in terms of actual cell numbers. All data are shown as mean±SE (n=3-5)
Fig 18: Altered memory compartment of CD4 and CD8 T cells in Hq mice. CD44 versus CD62L expression profile of peripheral T cells on CD4 (Panel A) and CD8 (Panel B) in the spleen. In both T cell types the central and effector memory frequency population is substantially higher in Hq mice. Frequency of naïve population CD62LhiCD44lo is radically reduced in Hq mice. All data are shown as mean±SE (n=3-5)
Results

4.17 AIF hypomorphism does not lead to differential rate of proliferation by T cells

One of the important factors apart from death, that lead to differences in the frequency of T cells in the periphery, may be differential rate of proliferation of T cells post activation. To check this as a possibility, CD4 and CD8 cells were given aCD3 stimulus in vitro and rate of proliferation was checked by tritiated thymidine incorporation (Fig 19) and by CFSE (Carboxy fluorescein succinimidyl ester) dilution (Fig 20). Purified CFSE labelled T cells were given plate coated anti-CD3 stimulus, and no significant difference was found in the proliferation rates of CD4 (Fig 20) and CD8 cells (Fig 20) between Hq T cells and WT T cell proliferation (though Hq cell proliferation was slightly lower), suggesting differential rate of death may not be a major factor for some of the differences seen in the T cell peripheral compartment.

4.18 AIF hypomorphism leads to T cell lineage developmental block

Since AIF is an important molecule in the apoptotic pathway and thymic development is a primary source of T cells in the periphery, looking at altered peripheral compartment in Hq mice, we wanted to see if there is any defect in the thymic development of Hq mice. Ex vivo staining showed that AIF hypomorphism in Hq mice, led to reduced cellularity of the thymus (Fig 21 B). Dual color staining for CD4 and CD8 showed lower CD4^+CD8^+ cell frequency. Frequency of CD4 and CD8 single positive (SP) cells was also lower (Fig 21 A). The frequency of CD4^{low}CD8^{low} double negative (DN) population was significantly and consistently higher in Hq mice when compared to WT mice (Fig 21A).
Purified T cells were given stimulation with titrating doses of antiCD3. After 48 hrs thymidine (radioactive) was added to the medium and at 60 hrs thymidine incorporation was accessed to quantify proliferation of T cells. No major difference was found in the proliferation of WT and Hq T cells. Background proliferation was less than 1000 cpm.
Fig 20: T cells proliferation response not affected in AIF hypomorphic state

Purified CFSE labeled splenocytes were given stimulus by plate coated anti-CD3 and at 48 h samples were stained for CD4 and CD8 and rate of proliferation was accessed using dilution of CFSE. Cells without any anti-CD3 stimulus was used as control. Control samples show no CFSE dilution. There was no difference in CFSE dilution profile between WT and Hq CD4 T cells.
Results

In terms of absolute cell numbers the total cellularity of thymus in 4 weeks old *Hq* mice was lower when compared to WT mice (Fig 21 B). The double negative thymocyte numbers were not altered, but the absolute cell numbers of Double positive, and single positive thymic cell were significantly lower in *Hq* mice (Fig 21 B). These data suggest that developmental transition from DN to DP stage may be aberrantly blocked leading to total hypocellularity and smaller DP and SP populations in *Hq* mice.

4.19 Developmental block in AIF hypomorphic thymocytes is in Double Negative-3 (CD44\textsuperscript{low}CD25\textsuperscript{high}) stage.

For further characterization of the DN block, we stained the Thymic cells with a mix of antibodies (CD3, CD4, CD8, CD11b, CD11c, Gr-1, Nk1.1, γδ-TCR, CD45R, CD19) followed by further characterization of the double negative cells on the basis of CD44 versus CD25 markers. The double negative cells have four developmental stages. The frequency of DN3 (CD44\textsuperscript{low}CD25\textsuperscript{high}) population (Fig22 A) was substantially higher in *Hq* mice, while frequency and absolute cell numbers of DN4 cells were lower in *Hq* mice when compared to WT mice (Fig 22 A and B). This suggested that the block leading to thymic recession in *Hq* thymus is due to defect in the DN3 to DN4 transition. DN1 and DN2 absolute cell numbers were also variable in *Hq* thymus, but this did not seem to cause defect in absolute cell numbers of DN3 population (Fig 22 B).
Fig 21: Thymus hypocellularity and double negative block in Hq mice
Thymus from WT and Hq were isolated from mice and cells were stained for CD4 and CD8 as shown in flow cytometry plot (panel A). Absolute number of each thymic subsets and total thymic cellularity was calculated (panel B). Hq thymus was found to be hypocellular and there seems to be a block at Double negative stage, A higher frequency of double negative cells CD8-CD4- was seen in Hq mice, though the absolute cell counts of double negative cells are similar. The frequency and absolute cell numbers of double positive and single positive thymic cells is also lower in Hq mouse when compared to WT mouse. All data are shown as mean±SE (n=3-5) (*p<0.05)
Fig 22: DN3 to DN4 transition block in hypocellular Hq mice thymus

Thymus from WT and Hq mice were stained for double negative antibody mix (CD4, CD8, CD11b, CD11C, Gr-1, γδ-TCR, NK1.1, CD3, CD45R/B220, CD19) and the negative population was plotted as CD25 versus CD44, to differentiate the population into double negative early thymic developmental stages. Frequency of DN3 population CD44lowCD25high (Panel A) was found to be substantially higher in Hq mice. In terms of absolute cell numbers, the DN4 population (CD44lowCD25low) was substantially lower (Panel B). No difference was found in absolute cell numbers of DN3 population, suggesting, a block in DN3 to DN4 transition. All data are shown as mean±SE (n=3-5) (*p< 0.005)
4.20 No difference in cell size of WT and Hq DN3 population

Since there seems to be a block in DN3 to DN4 transition in Hq mice, there is a possibility of WT DN3 population dividing at a higher rate, thus suggesting that DN3 cells in Hq mice should be smaller in size. To look at this as a possibility, we gated the DN3 population and looked at cell size of this population based on forward scatter (Since dividing cells show higher forward scatter. Surprisingly, Hq mice did not show any difference in frequency of High forward scatter cells (Fig 23) suggesting, that the cells may be dividing well in Hq mice but still in absence of some survival stimulus or due to presence of some death stimulus may be dying before their transition to DN4 population.

4.21 Comparable frequency of TCRβ positive DN3 cells in Hq mice

We further wanted to see if the block in DN3 subset in thymocyte was a β-selection block due to lack of TCRβ expression. Staining of DN3 gated cells for intra cellular TCRβ levels was followed by flow cytometry. Comparable frequencies and absolute cell numbers of DN3 cells were found to be TCRβ positive in both Hq and WT mice (Fig 24). This suggested that probably hypomorphic state of AIF may not be affecting β-recombination event, but after β-selection all the cells may not transit to DN4 population.
Fig 23: No difference in DN3 population cell size

Staining for DN subpopulation was done as stated in Fig 21 followed by gating of the DN developmental subsets. DN3 population (CD44lowCD25high) was gated and further examined to look for cell size of the population on the basis of forward scatter. No difference in the frequency of cells with high forward scatter was found.
**Intra cellular TCR beta staining on DN3 cells**

**Fig 24: Comparable frequency of TCRβ+ cells in Hq mice**

Thymocytes from WT and Hq mice were stained for double negative phenotype and CD44 Versus CD25. These cells were fixed followed by permeabilization and intra cellular staining for TCRβ, to look for TCRβ frequency in DN3 population. Hq DN3 cells showed comparable frequency of TCRβ+ cells when compared to WT mouse thymocytes.
4.22 No difference in Vβ lineage frequency in Hq Vs WT spleen

Hypomorphic level of AIF does not seem to cause any difference in the physiology in terms of TCR-Vβ usage frequency of CD4 and CD8 cells in the peripheral T cell compartment. When spleen cells from WT and Hq mice were stained for CD4 and CD8 as well as for various representative TCRVβ, no difference was found in the frequency of various TCRVβ profile in Hq mice when compared to WT mice (Fig 25). No difference in the TCRVβ profile between WT and Hq mice, further indicates that there is no skewing in favor or against any particular Vβ lineage and therefore the DN3 block in Hq mice was independent of β-recombination event.

4.23 DN3 block retained in TCR Vβ specific transgenic background

To further confirm that the DN3 block is independent of β-recombination, Hq mice were bred with P14 and OT-1 mouse to generate double transgenic mice which are specific for TCRVβ-lineage. In these mice since there is a recombined TCRβ in the germline, β-recombination event does not occur. In such a transgenic background like in P14 mice (Fig 26) and OT-1 mice (Fig 27 and 28) when Hq line was bred to generate double transgenic mice, and thymic cells were stained for the various DN subsets based on CD44 versus CD25, the block in the DN3 to DN4 transition was retained. In terms of absolute cell numbers, there was no difference in DN3 cell numbers but absolute cell numbers of DN4 population was lower on a double transgenic background. This suggested that the DN3 block in Hq mice was independent of the β-recombination event.
Fig 25: No difference in Vβ lineage frequency in spleen
Spleen cells from WT and Hq cells were stained for CD4, CD8 and various representative anti TCR-Vβ antibodies. No difference was found in TCRVβ frequencies for CD4 (Panel A) as well as CD8 (Panel B) gated cells.
Fig 26: DN3 block retained in Vβ transgenic P14 mice when on AIF hypomorphic background

Hq mice were bred with P14 mice to generate P14-Hq double transgenic mice. Thymus from double transgenic mice and WT and Hq mice were taken, and stained for triple negative phenotype and CD44 vs CD25 to see frequency of DN3 and DN4 subpopulation. The DN3 block was found to be retained on a P14 transgenic background, suggesting that the DN3 block was independent of Vβ lineage. (n=2)
**Fig 27: DN3 block retained in Vβ transgenic OT-1 mice when on AIF hypomorphic background**

Hq mice were bred with OT-1 mice to generate OT-1-Hq double transgenic mice. Thymus from WT, Hq, OT-1 and OT1-Hq double transgenic mice were taken and stained for CD44 vs CD25 profile, on Thy-1.2 gated double negative thymic cells. DN3 block was retained in double transgenic mice, suggesting DN3 to DN4 transition block to be independent of Vβ lineage.
Fig 28: DN3 block retained in V$\beta$ transgenic OT-1 mice when on AIF hypomorphic background

*HQ mice were bred with OT-1 mice to generate OT-1-HQ double transgenic mice. Thymus from WT, HQ, OT-1 and OT1-HQ double transgenic mice were taken and stained for CD44 Vs CD25 profile, on Thy-1.2 gated double negative thymic cells. DN3 block was retained in double transgenic mice, suggesting DN3 to DN4 transition block to be independent of V$\beta$ lineage.
4.24 The altered phenotype of T cells in the peripheral compartment was also retained in double transgenic mice.

To further check if the altered T cell profile in the peripheral compartment reported previously in Hq mice (Fig 13), was also retained on a transgenic background, the peripheral compartment of OT-1-Hq double transgenic mouse were analyzed. These mouse were stained for frequency of CD8 cells in Spleen (Fig 29 A) and lymph nodes. Frequency and absolute cell numbers of CD8 T cells (Fig 29 B) was lower in Hq-OT-1 double transgenic mouse when compared to OT-1 transgenic mice suggesting, that AIF hypomorphism was affecting CD8 cell frequency even on OT-1 transgenic background. In terms of absolute cell numbers as well, the double transgenic mice showed lower T cell numbers compared to OT-1 mice (Panel C).

4.25 Hq mice show a DN3a to DN3b transition block

Further characterization for the DN-3 block was carried out based on a developmental marker CD27 that sub-characterizes DN-3 thymocytes into two subsets, DN3a (CD27<sup>low</sup>) and DN3b (CD27<sup>hi</sup>). When staining for CD27 on gated DN-3 cells was examined, Hq DN-3 cells showed a prominent peak for both DN3a and DN3b, where as WT thymocyte profile showed very few cells of DN3a subsets with most cells in DN3b subset (Fig30). This suggested that the actual block seen in Hq mice is in DN3a to DN3b transition. The movement of DN3a to DN3b is one of the critical events where the cell death seems to occur in Hq mice development but independent of β-recombination event.
**Fig 29: Frequency of CD8 T cells lower in the peripheral compartment when on AIF hypomorphic background**

Spleen and Lymph node from OT-1 and OT-1 Hq mouse were taken and stained for CD8 T cell frequency. OT-1-Hq mice has significantly lower frequency of CD8 T cells in spleen (Panel A) and Lymph node (Panel B), absolute cell counts in spleen and lymph nodes were also lower in double transgenic background (Panel C) suggesting, that AIF hypomorphism affects not only T cell development in transgenic background but also T cell frequency in the periphery.
Fig 30: Double negative 3 subset in Hq mice shows a block in DN3a to DN3b transition
Hq and WT thymus were taken and stained for DN subsets and CD27 for characterization of DN-3 population. Gated DN-3 cells in WT and Hq mice were looked for CD27 to further characterization into DN3a (CD27low) and DN3b (CD27high) cells. Hq Mice show higher frequency and cell numbers of DN3a cells compared to WT thymocytes.
4.26 **DN3 thymocytes in Hq show higher frequency of annexin v positive cells.**

Since there was a block in DN3 to DN4 transition, leading to a hypocellularity of thymus, it was necessary to characterize DN3 cell death. For this we stained WT and Hq thymic cells for DN subsets and on gated DN3 cells, looked at annexin v positive cell frequency and absolute cell numbers. As expected in the DN3 stage there was a substantially higher frequency and absolute cell numbers of annexin v positive cells in Hq mice thymocytes when compared to WT thymic DN3 cells (Fig 31 A). When we looked at forward scatter, annexin v positive cells were smaller in size which is another hallmark of dying cells due to shrinkage (Fig 31 B). When actual cell numbers of annexin v positive DN3 cells were calculated, there are nearly double annexin v positive cell in Hq compared to WT mice (Fig 31 C).

4.27 **Higher frequency of annexin v positive cells (cell death) in DN3a cells in Hq mice**

To further characterize cell death stage DN subset staining with CD27 and annexin v was done by flow cytometry. When CD27 based gating was done on DN3 cells, the difference in cell death between WT and Hq cell death was found to be contributed by higher death in DN3a subset both in terms of frequency and absolute cell numbers (Fig 33A and C). No difference was found in the cell death frequency of DN3b subset (Fig 33B) between WT and Hq mice. This further confirmed that the block was in the DN3a to DN3b transition and once the cells have moved to DN3b stage in Hq mice, they seem to be normally moving to DN4 stage without any further block or the transition is rapid enough.
Fig 31: Higher frequency of Annexin v positive DN3 cells in Hq thymocytes
Thymocytes from WT and Hq mice were stained for DN subsets and frequency of Annexin v positive cells were determined by flow cytometry on gated DN3 cells. Hq DN3 cells showed a higher frequency of annexin v positive cells compared to WT DN3 cells (Panel A). When AnnexinV was plotted with Fsc the Annexin positive cells were found to be of low forward scatter (smaller sized cells) (Panel B). Absolute cell numbers of DN3 annexin v positive cells was higher in Hq mice (Panel C).
Annexin positivity in CD27 low DN3 cells

A

WT

Hq

<FITC>- Annexin

<PE-A>: CD27

Annexin positivity in CD27 High DN3 cells

B

WT

Hq

<FITC>- Annexin

<PE-A>: CD27

C

Absolute cell no. (in million)

Annexin +ve

(*p< 0.003)

Fig 32: Higher frequency of annexin v positive cells in Hq is contributed by DN3a subset

Hq and WT thymocytes were stained for DN3a and DN3b subsets along with annexin v staining. Most of the cell death in both WT and Hq DN3 cells was in DN3a subset. The difference between the frequency of cell death between Hq and WT was due to higher cell death in Hq DN3a cells compared to WT (Panel A). There was no difference in frequency of cell death in DN3b (CD27hi) subset (Panel B). Absolute cell numbers of CD27 low DN3 annexin v positive cells (Panel C).
Fig 33: Higher frequency of cell death in DN4 and DP stages in Hq mice
Thymic cells were stained for CD4, CD8 and DN subsets and annexin v. DP cells and DN4 cells were gated and then looked for Annexin v positivity to score for cell death. Both DN4 cells (Panel A) and DP cells (Panel B) show higher annexin v positive cells in Hq mice, suggesting that along with DN3 block, a higher frequency in cell death in DN4 and DP also contribute to thymic recession.
for cell death not to be detected. These data suggest that major contribution in the DN3 block seen in the *Hq* mice was due to death in DN3a stage.

**4.28 Higher frequency of dead cells in DP and DN4 stage in Hq**

To look at the possibility that there may be other developmental stages in which there is differential cell death between WT and *Hq* mice, thymocytes were stained for various developmental stages and annexin V positive frequency was characterized. DN4 gated cells ([Fig 33 A](#)) and DP cells ([Fig 33 B](#)) showed higher frequency of cell death in AIF hypomorphic *Hq* mice. This suggests that apart from the DN3 block, higher cell death in DN4 and DP stages also contribute to the lower thymic cell counts in *Hq* mice.

**4.29 AIF hypocellularity does not lead to altered cell death frequency in single positive stage**

When gated CD4 and CD8 single positive cells were looked for annexin V positivity in WT and *Hq* T cells, there was no difference in cell death frequency in the Naïve CD4 ([Fig 34 A](#)) and CD8 ([Fig 34 B](#)) single positive thymic compartment. This suggests that after the development has happened in the thymus and single positive phenotype is attained by the thymic cells, there is no further cell death due to AIF hypomorphism in the mature SP T cells.
Fig 34: No difference in frequency of dead cell in CD4 and CD8 single positive cells between WT and Hq mice

Thymic cells were stained for CD4, CD8 and annexin v. Gated CD4 (Panel A) and CD8 (Panel B) single positive cells were analyzed for annexin v positive cell frequency. No difference was found in the frequency of SP cells between WT and Hq mouse.
Results

4.30 AIF hypocellularity leads to oxidative stress in Hq DN3 cells

Another important factor known to cause cell death in various death pathways, as has been stated before, is ROS. Since AIF is known to be playing role of ROS quencher, we examined the possibility of higher oxidative stress in DN3 cells of WT and Hq cells. For this we measured the level of 8-Oxoguanine on gated DN3 cells. It is known that under ROS stress, the oxidation of Guanine occurs on the DNA to give rise to 8-Oxo guanine. When we looked at 8-Oxoguanine levels in WT and Hq DN3 cells (Fig 35) Hq DN3 cells showed higher 8-oxoguanine levels compared to WT cells suggesting that higher oxidative stress in Hq DN3 population is causing DNA damage and therefore may be a determining factor in higher cell death in Hq thymus leading to hypocellularity. We also measured ROS levels in various developmental sub-populations in the WT and Hq thymocytes using ROS measuring dye DCFDA (2'-7'-dichlorofluorescein diacetate) that directly measured ROS (Fig36). Nearly all the subpopulation of Hq thymocytes show higher ROS levels when compared to WT thymocytes, suggesting that ROS is a determining factor for higher death in DN3, DN4 and DP stages in Hq thymus.

4.31 DN3 block prevented Hq mice on treatment with EUK-134

When Hq mice were given a treatment of EUK-134 10mg/Kg body weight for 14 days followed by staining of thymic cells and absolute number determination of various population of mature and developmental stages. EUK-134 treatment seems to prevent cell death in the DN3 stage, as absolute cell numbers for both DP and DN4 stage increase on EUK-134 treatment (Fig 37). Thymic cellularity also increase significantly in Hq mice on
**Fig 35: Higher oxidative stress in Hq DN3 cells**

Thymocytes were stained for DN subset, followed by fixation and permeabilization. After permeabilizing the cells, cells were incubated by FITC-labeled Streptavidin, which binds to chromosomal 8-oxoguanine, that is a marker for oxidative stress. *Hq* cells were showing higher 8-oxoguanine staining.
**Fig 36: Higher oxidative stress in Hq DN3 cells**
Thymocytes from WT and Hq mice were stained for developmental sub-population followed by staining with DCFDA. The cells were then analysed using flow cytometry to look for ROS levels on gated populations. All the stages of development and mature thymocytes show higher ROS levels in Hq mice.
**Fig 37: DN3 block prevented Hq mice on treatment with EUK-134**

WT and Hq mice were given EUK-134 treatment for 14 days and thymic cellularity and various cell population were characterized by flow cytometry. DP population increase in Hq mice on EUK-134 treatment. Similarly DN4 population also increases on EUK-134 treatment (Panel C). The cell numbers of SP population also seem to increase to some extent (Panel B). Total cellularity is also recovered to certain extent on EUK-134 treatment (Panel A).
EUK-134 treatment, further confirming the hypothesis that higher ROS is a major cause of higher cell death in DN3 stage of Hq thymocyte.

4.32 DN3 block retained in Hq in vitro development culture

Since AIF is ubiquitously present in all cell types, it was decided to test whether the defect in thymic selection seen in Hq mice was due to absence of AIF in T lineage cells or in thymic stromal cells, which are also known to be critical for T cell development. This issue was examined using in vitro T cell development culture OP9-DL1 co-culture system. DN1+DN2 cells were purified, CFSE labelled and were co-cultured on OP9-DL1 feeder layers for various time. On day 3 co-cultured thymic cells were then taken out and CD25 versus CD44 profile along with CFSE dilution pattern was examined using flow cytometry. WT cells showed higher CFSE dilution compared to Hq cells (Fig 38, 39, 40). When CD25 and CD44 pattern was examined, higher frequency of WT T cells were seen to have moved in DN4 stage when compared to Hq cells which were still lagging behind in the DN3 stage (Fig 38). On day five, when cells were stained for CD4 and CD8, a higher frequency of WT cells were seen to have moved to double positive stage with few cells in SP compartment as well, but Hq cultures had very few cells in DP stage and did not have any SP cells (Fig 40). This suggests, that the thymic block seen in Hq mice was due to absence of AIF in T cells and was not a bystander effect observed due to absence of AIF in the thymic stromal cells.
Fig 38: *Harlequin cells show defect in 'in vitro' T cell development*

Purified CD44high DN1+DN2 cells were CFSE labeled and seeded on OP-9 DL-1 stromal cells in presence of Flt-3 ligand and IL-7. Cells were kept in development culture for 3 days and on Day 3 receptor profile of Thymocytes was looked at. WT thymocytes on Day 3 show higher frequency of CD44 low cells (Panel A). Frequency of DN3 cells was higher in Hq cultures where as DN4 frequency was higher in WT culture (Panel B and D). In terms of absolute cell number total, DN3 and DN4 cells are lower in Hq cultures (Panel C).
Fig 39: Hq Thymocytes after 3 days of in vitro development show less proliferation.

In vitro T cell development cultures when DN3 and DN4 cells were gated and CFSE dilution was looked at to track proliferation, WT DN3 cells (Panel A) was seen to be showing higher proliferation when compared to Hq DN3 cells (Panel B). Similarly WT DN4 cell population (Panel C) was seen to be showing higher CFSE dilution, therefore higher proliferation compared to Hq DN4 cells (Panel D).
Fig 40: Harlequin cells show defect in in vitro T cell development
Purified CD44high DN1+DN2 cells were CFSE labeled and seeded on OP-9 DL-1 stromal cells in presence of Flt-3 ligand and IL-7. Cells were kept in development culture for 5 days and on Day 3 receptor profile of Thymocytes was looked at. WT cells seem to have moved into DP and SP stage, where all Hq cells seem to be lagging behind and no SP cells are seen in the Hq culture.