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TRAF2 regulates peripheral CD8$^+$ T-cell and NKT-cell homeostasis by modulating sensitivity to IL-15.

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ABSTRACT

In this study, a critical and novel role for TNFR-associated factor 2 (TRAF2) is elucidated for peripheral $CD8^+$ T-cell and NKT-cell homeostasis. Mice deficient in TRAF2 only in their T cells (TRAF2TKO) show ~40% reduction in effector-memory and ~50% reduction in naïve $CD8^+$ T-cell subsets. IL-15-dependent populations were reduced further, as TRAF2TKO mice displayed a marked ~70% reduction in central-memory $CD8^+CD44^{hi}CD122^+$ T cells and ~80% decrease in NKT cells. TRAF2TKO $CD8^+CD44^{hi}$ T cells exhibited impaired dose-dependent proliferation to exogenous IL-15. In contrast, TRAF2TKO $CD8^+$ T cells proliferated normally to anti-CD3 and TRAF2TKO $CD8^+CD44^{hi}$ T cells exhibited normal proliferation to exogenous IL-2. TRAF2TKO $CD8^+$ T cells expressed normal levels of IL-15 associated receptors and possessed functional IL-15 mediated STAT5 phosphorylation, however TRAF2 deletion caused increased AKT activation. Loss of $CD8^+CD44^{hi}CD122^+$ and NKT cells was mechanistically linked to an inability to respond to IL-15. The reduced $CD8^+CD44^{hi}CD122^+$ T-cell and NKT-cell populations in TRAF2TKO mice were rescued in the presence of high dose IL-15 by IL-15/IL-15Rα complex administration. These studies demonstrate a critical role for TRAF2 in the maintenance of peripheral $CD8^+$ $CD44^{hi}CD122^+$ T-cell and NKT-cell homeostasis by modulating sensitivity to T-cell intrinsic growth factors such as IL-15.

INTRODUCTION

TNF-receptor associated factor 2 (TRAF2) belongs to a family of intracellular signalling molecules (TRAF1-7) which integrate downstream signalling cascades for TNF family receptors [reviewed in [1]]. Global TRAF2 deletion results in embryonic and early postnatal lethality [2], which is rescued through the expression of dominant negative mutant TRAF2 (TRAF2.DN) [3]. TRAF2.DN mice have normal thymocyte and peripheral T-cell numbers but display a 3-fold increase in B-cell numbers [3] highlighting a role for TRAF2 in peripheral lymphocyte homeostasis. However, despite TRAF2.DN mice harbouring normal T-cell numbers, a potential T-cell phenotype in TRAF2.DN mice may be confounded by the extensive splenic hypertrophy and B-cell hyperplasia [3]. Further, the TRAF2.DN protein contains N- and C- terminus binding regions that may trigger the activation of parallel signalling pathways potentially masking any T-cell phenotype resulting from loss of functional TRAF2. Other studies in TRAF2.DN mice demonstrate impaired secondary viral recall responses with reduced cytotoxic $CD8^+$ effector T-cell numbers [4] however whether the decrease in viral specific $CD8^+$ T cells reflects a reduction of steady state effector-memory or central-memory $CD8^+$ T-cell numbers is not known.

The notion that TRAF family members can modulate T-cell homeostasis has recently come to light. T cell specific deletion of TRAF6 results in increased peripheral $CD4^+$ T cells [5]. Studies in T-cell specific TRAF3 knockout mice highlight a reduction in CD44 expression on $CD8^+$ T cells [6, 7] possibly reflecting reduced numbers of central-memory or effector-memory $CD8^+$ T cells however the cellular source of reduced CD44 expression has not been demonstrated. In addition, TRAF3 T-cell knockout mice exhibit impaired thymic

development of NKT cells [7] and enhanced peripheral regulatory T cells [6] due to dysregulated thymic development [8]. The reduction of NKT cells in TRAF3 T-cell knockout mice is IL-15 dependent, as TRAF3 knockout NKT cells failed to respond to IL-15 and expressed reduced levels of the IL-2R beta chain required to mediate IL-15 signalling [7, 9]. Studies in melanoma cell lines indicate that TRAF2 associates with IL-15Rα [10, 11] suggesting a requirement for TRAF2 in IL-15 signalling. However, a requirement for TRAF2 in IL-15 signalling in T cells has not been demonstrated. Further, as TRAF3 interacts with TRAF2 to form part of a larger E3-ligase complex to regulate the non-canonical NF-κB signalling pathway [12], it is possible that mice with a T-cell specific deletion of TRAF2 share an overlapping T-cell phenotype with mice lacking TRAF3 in T cells. Given that NKT cell and central-memory $CD8^+$ T cells are highly dependent on IL-15 for their maintenance and survival [13-16], we hypothesise that loss of TRAF2 from T cells would impact on the homeostatic maintenance of NKT cells and central-memory $CD8^+$ T cells. To date, the consequences of deleting TRAF2 from T cells on the homeostasis of specific T-cell subsets has not been defined.

RESULTS

Reduced $CD8^+$ T-cell subsets in TRAF2TKO mice.

To determine how peripheral T-cell populations were affected by the T-cell specific deletion of TRAF2, $CD4^+$ and $CD8^+$ T-cell frequencies of floxed control and TRAF2TKO mice were assessed by flow cytometry and the calculated cell numbers were determined. Whereas no difference was observed in splenic $CD4^+$ T-cell numbers, TRAF2TKO mice exhibited a ~50% reduction in splenic $CD8^+$ T-cell numbers compared to floxed controls (Fig 1 A and B). The reduction in peripheral $CD8^+$ T cells was not due to impaired thymic cellularity as this was identical to floxed controls (i.e., $35.6x10^6$ versus $35.52x10^6$; $p = 0.7852$). This also rules out any influence of the LCKcre on thymic cellularity as has been reported for other Cre lines [17]. The composition of the peripheral $CD8^+$ T-cell pool was assessed by flow cytometry to determine whether all or specific $CD8^+$ T-cell subsets were reduced in TRAF2TKO mice. The expression of cell surface markers CD44 and CD62L expression on $CD8^+$ T cells was used to distinguish between $CD44^{lo}CD62L^{hi}$ (naïve), $CD44^+CD62L^{lo}$ (effector-memory), and $CD44^{hi}CD62L^{hi}$ (central-memory) T-cell subsets (Fig. 1 C). Compared to floxed controls, TRAF2 deletion resulted in ~50% reduction in naïve and ~40% reduction in effector-memory $CD8^+$ T-cell numbers (Fig. 1 D and E; $p = 0.0119$ and 0.0473 respectively) and a ~70% reduction in central-memory $CD8^+$ T cells within the spleen (Fig. 1 F; $p = 0.0006$).

Analysis of central memory cells in TRAF2TKO mice.

Central-memory T cells co-express CD122 and CCR7 [18, 19] and together with CD44 provide a means to identify central-memory $CD8^+$ T cells. Analysis of CD122 and CCR7 expression on $CD8^+$ T cells revealed that TRAF2TKO mice exhibited a ~35% reduction in the percentages of $CD44^{hi}CD122^+$ and $CD122^+CCR7^+$ $CD8^+$ T cells compared to floxed controls (Fig. 1 G and I). This reduction related to a ~70% decrease in the absolute numbers of $CD44^{hi}CD122^+$ and $CD122^+CCR7^+$ $CD8^+$ T cells in TRAF2TKO mice compared to floxed control mice (Fig. 1 H and J; $p = 0.0008$ and $p = 0.0036$ respectively). Further, reduced frequencies of $CD8^+CD44^{hi}CD62L^{hi}$ and $CD8^+CD44^{hi}CD122^+$ T cells were observed in the bone marrow (Supporting Information Fig. 1), a survival niche for memory $CD8^+$ T cells [20]. Therefore, specific deletion of TRAF2 from T cells results in reduced proportions of peripheral $CD8^+$ T-cell subsets, predominantly observed in the central-memory $CD8^+$ T-cell compartment.

Persistence of TRAF2TKO $CD8^+$ T cells in lymphopenic environments

The reduced numbers of $CD8^+$ T cells in TRAF2TKO mice may be due to a cell intrinsic impairment in their ability to respond to homeostatic signals. To test this, MACS-purified $CD8^+$ T cells from floxed control or TRAF2TKO mice were CFSE-labeled and transferred into lymphopenic $RAG^{-/-}$ mice. Analysis of pooled lymph nodes from $RAG^{-/-}$ mice receiving TRAF2TKO $CD8^+$ T cells revealed a reduced frequency of $CD8^+$ T cells compared to $RAG^{-/-}$ mice receiving floxed control cells (Fig. 2 A). The reduction in TRAF2TKO $CD8^+$ T cells was due to a reduction in the absolute number of TRAF2TKO $CD8^+$ T cells (Fig. 2 B; $p = 0.015$). The impaired response of TRAF2TKO $CD8^+$ T cells to lymphopenic signals was not due to decreased T-cell proliferation or activation since both floxed control and TRAF2TKO

$CD8^+$ T cells underwent a similar number of cellular divisions as indicated by CFSE-dilution and showed a similar pattern of CD44 upregulation (Fig. 2 C).

To address whether TRAF2TKO $CD8^+$ $CD44^{hi}CD122^+$ T cells could respond to homeostatic cytokines in a different setting, FACS-purified $CD8^+CD44^{hi}$ $CD122^+$ T cells from CD45.1 congenic C57BL/6 and TRAF2TKO (CD45.2 congenic) mice were CFSE-labeled and transferred into irradiated C57BL/6 mice. Analysis of the spleen on day 4 after cell transfer indicated reduced numbers of recovered TRAF2TKO $CD8^+CD44^{hi}CD122^+$ T cells compared to wildtype $CD45.1^+$ control T cells (Fig. 2 D; $p = 0.0178$). These data demonstrate that TRAF2 is necessary for the homeostatic expansion of $CD8^+CD44^{hi}CD122^+$ T cells and reflect possible impaired responses to cytokines known to maintain $CD8^+CD44^{hi}$ T cells such as IL-7 and IL-15 [21].

Persistence of TRAF2TKO $CD8^+$ T cells in non-lymphopenic environments

The impaired lymphopenic-induced persistence of $CD8^+$ T cells was most evident in a lymphopenic environment as the recovery of floxed or TRAF2TKO $CD8^+$ T cells (both $CD45.2^+$ congenic) transferred into non-irradiated $CD45.1^+$ congenic C57BL/6 mice was comparable with controls (Fig. 2 E). Further, the steady-state turnover of $CD8^+$ T cells, as measured by the incorporation of the thymidine analogue BrdU, was similar between floxed and TRAF2TKO mice (Fig. 2 F). Additionally, in the steady-state TRAF2TKO $CD8^+CD44^{hi}CD122^+$ T cells showed normal expression of the pro-survival factor Bcl-2 (Fig. 2 G). Therefore, under lymphopenic conditions where T-cell homeostatic factors are abundant, TRAF2-deficient $CD8^+$ T cells can proliferate and express CD44, but cannot expand or persist to the extent of floxed control $CD8^+$ T cells.

Responses of TRAF2TKO T cells to IL-2, IL-7, and IL-15

The homeostatic maintenance and survival of T cells relies on signals from the T cell receptor and cytokines including IL-2, IL-7, and IL-15 [reviewed in [22]]. To determine whether TRAF2 was required for optimal proliferation induced by homeostatic cytokines, total T cells were stimulated in vitro with IL-2, IL-7, or IL-15 (1000 ng/ml). The majority of floxed and TRAF2TKO $CD8^+$ T cells showed a robust proliferative response to IL-2 or IL-15 alone but proliferated only weakly to IL-7 alone (Fig. 3 A). We observed a trend for reduced proliferation of TRAF2TKO $CD8^+$ T cells in response to IL-15, thus we investigated whether this would exacerbated at lower concentrations ($\leq$ 100 ng/ml). Floxed control and TRAF2TKO T cells were stimulated in vitro for 72 hours with 1 – 100 ng/ml of IL-15. Compared to floxed control cells, TRAF2TKO $CD8^+$ T cells proliferated ~20% less in response to 100 ng/ml of IL-15 (Fig. 3 B and C; $p = 0.0008$). Therefore, TRAF2TKO $CD8^+$ T cells exhibit a dose-dependent impaired response to the proliferative stimulus of IL-15.

TRAF2TKO T cells show impaired responses to homeostatic cytokines

Some data show that $CD8^+CD44^{hi}$ T cells specifically proliferate in response to exogenous IL-2 and IL-15 [19, 23]. To determine whether the proliferative defect of TRAF2TKO $CD8^+$ T cells was specific to IL-15, FACS-sorted $CD8^+CD44^{hi}$ T cells from floxed control or TRAF2TKO mice were CFSE-labeled and stimulated in vitro with 50 ng/ml and 100 ng/ml of IL-15. Compared to control $CD8^+$ T cells, TRAF2TKO $CD8^+CD44^{hi}$ T cells showed a reduced proliferative response; being 20 and 50% less than controls for 100 ng/ml and 50 ng/ml of IL-15 respectively (Fig. 3 D and E). The proliferative defect of TRAF2TKO $CD8^+CD44^{hi}$ T cells in response to IL-15 was specific, as IL-2 induced proliferation was comparable between floxed control and TRAF2TKO $CD8^+CD44^{hi}$ T cells (Fig. 3 F). Further, TRAF2TKO $CD8^+$ T cells exhibited comparable expression of T cell activation markers CD25, CD44, and CD69 (Fig. 3 G) and proliferation (Fig. 3 H) following anti-CD3 stimulation. These data indicate that TRAF2-deficient $CD8^+CD44^{hi}$ T cells possess a selective impairment in IL-15 mediated responses.

TRAF2TKO mice have reduced numbers of NKT cells

The peripheral maintenance of NKT cells is dependent on IL-15 [13, 16]. Since TRAF2TKO $CD8^+CD44^{hi}$ T cells exhibited sub-optimal proliferation to IL-15, we hypothesised that NKT-cell numbers would also be reduced in the absence of TRAF2. Indeed, TRAF2TKO mice exhibited a ~7-fold reduction (e.g., ~25% versus ~3% in the liver) in peripheral NKT cells ($TCR\beta^+\alpha GalCer^+$ or $CD3^+\alpha GalCer^+$) compared to floxed controls (Fig. 4 A), a reduction which was also observed in the calculated cell numbers (Fig. 4 B; $p = 0.0037$). Thus, T cell intrinsic TRAF2 is required to maintain IL-15 dependent cell populations; TRAF2 deletion results in reduced numbers of $CD8^+CD44^{hi}$ and NKT cells in the periphery.

Normal expression of IL-15-associated receptors in TRAF2TKO mice.

The IL-15Rα subunit plays a key role in the maintenance of $CD8^+CD44^{hi}CD122^+$ and NKT cells [16]. Therefore, the reduced responsiveness of TRAF2TKO $CD8^+CD44^{hi}CD122^+$ T cells and reduced NKT cells in the periphery may be due to reduced expression of IL-15Rα. Flow cytometric analysis indicated that floxed control and TRAF2TKO $CD8^+$ T cells exhibited comparable basal expression of IL-15Rα (Fig. 5 A) and could upregulate IL-15Rα expression to a similar extent after a stimulus with anti-CD3 in vitro (Fig. 5 B). IL-15 signals through a trimeric receptor composed of IL-15Rα, IL-2Rβ (CD122), and the IL-2 common gamma chain (IL-2cγ; CD132) [24]. Flow cytometric analysis of TRAF2TKO $CD8^+$ T cells indicated that basal IL-2cγ expression was comparable to controls (Fig. 5 C). Further, despite TRAF2TKO mice harboring reduced numbers of $CD8^+CD44^{hi}CD122^+$ T cells, IL-2Rβ expression on $CD8^+CD44^{hi}CD122^+$ T cells were similar between TRAF2TKO and floxed control mice (Fig. 5 D). Therefore, TRAF2TKO $CD8^+$ T cells exhibit normal expression of

IL-15-associated receptors suggesting that downstream IL-15 signalling components may be disturbed.

IL-15 signalling in TRAF2TKO $CD8^+CD44^{hi}CD122^+$ T cells.

IL-15 signalling incorporates the JAK/STAT pathways activating STAT5 [25]. Further, TRAF2 has been shown to associate with the cytoplasmic tail of IL-15Rα during IL-15 signal transduction in a melanoma cell line [10, 11] suggesting that TRAF2 may be important for IL-15 signalling in T cells. Western blot analysis showed that IL-15 triggered robust STAT5 phosphorylation in TRAF2TKO thymocytes 10 minutes post stimulation (Fig. 5 E) followed by a rapid return to baseline when compared to floxed control thymocytes (Fig. 5 E and F). To determine signalling in $CD8^+CD44^{hi}$ T cells, splenic lymphocytes from floxed control and TRAF2TKO mice were stimulated with IL-15 and intracellular STAT5 phosphorylation was analysed by flow cytometry. In this case the magnitude of STAT5 phosphorylation in TRAF2TKO $CD8^+CD44^{hi}$ T cells compared to floxed control cells (Fig. 5 G).

IL-15 does not trigger the canonical NF-κB pathway in T cells.

IL-15 may signal through an alternative pathway such as the canonical NF-κB pathway, as demonstrated previously in cerebral endothelial cells [26] and human neutrophils [27]. However, IL-15 stimulation did not trigger IκBα degradation, indicative of canonical NF-κB activation, in either floxed control or TRAF2TKO thymocytes (Fig. 5 E).

TRAF2TKO T cells exhibit altered AKT phosphorylation.

Recently, IL-15 has been shown to signal via IL-15Rα in the absence of IL-15Rβ or IL-15Rγc [28]. However, IL-15Rβ and IL-15Rγc independent signalling did not activate STAT5 but rather the phosphatidylinositol-3-kinase-AKT (AKT) pathway suggesting an alternative mechanism for the reduced IL-15 responsiveness of TRAF2TKO cells in vitro. To address this, thymocytes from floxed and TRAF2TKO mice were stimulated with IL-15 for 0 – 20 mins and assessed for the activation of AKT. IL-15 triggered AKT activation in both floxed control and TRAF2TKO thymocytes (Fig. 6 A). TRAF2TKO thymocytes exhibited increased AKT pathway activation in the steady state, and higher AKT phosphorylation was maintained following IL-15 stimulation (Fig. 6 A and B). In summary, these signalling data indicate that very early IL-15 signalling events via JAK/STAT pathways are largely intact in the absence of TRAF2 and that for T cells canonical NF-κB is not activated by IL-15, but AKT is hyper-responsive in the absence of TRAF2.

Rescue of TRAF2TKO $CD8^+CD44^{hi}CD122^+$ T-cell and NK T-cell numbers with exogenous IL-15.

TRAF2TKO mice exhibit reduced numbers of $CD8^+CD44^{hi}$ and NKT cells. Since there was a reduced response to IL-15 by TRAF2TKO T cells in vitro, and both $CD8^+CD44^{hi}$ and NKT cells depend on IL-15 for their survival [15, 16], we tested whether exogenous IL-15 would rescue these T-cell subsets. In vivo administration of a complex composed of both IL-15 and IL-15Rα in soluble form increases the biological activity of IL-15 and expands both $CD8^+CD44^{hi}$ T cells and NK cells [29]. Administration of IL-15/IL-15Rα complex into TRAF2TKO mice resulted in a 3-fold increase in the frequency of NKT cells (Fig. 7 A) and a 6-fold increase in the frequency of $CD8^+CD44^{hi}CD122^+$ T cells (Fig. 7 B) with respect to untreated mice. In contrast, IL-15/IL-15Rα complex administration in floxed control mice resulted in a 2-fold increase in the frequency of NKT cells (Fig. 7 A) and a 3-fold increase in $CD8^+CD44^{hi}CD122^+$ T cells (Fig. 7 B) compared to untreated mice. The increase in both

peripheral NKT cells and $CD8^+CD44^{hi}CD122^+$ T cells was also observed in the calculated cell numbers (Fig. 7 C and D). Whilst the relative fold-change of NKT cells and $CD8^+CD44^{hi}CD122^+$ T cells in TRAF2TKO mice after treatment with IL-15/IL-15Rα complex was higher compared to floxed controls, this was not statistically significant (Fig. 7 E and F). These data indicate that administration of highly biologically active IL-15 can rescue $CD8^+CD44^{hi}CD122^+$ and NKT-cell populations in TRAF2TKO mice.

Reduced IL-15-dependent T-cell populations in TRAF3TKO mice.

A recent study investigating TRAF3 T-cell deficiency driven by a CD4-CRE showed that these mice harbored reduced numbers of NKT cells with impaired IL-15-induced signalling [7]. Further, CD44 levels on $CD8^+$ T cells are reduced in TRAF3 T-cell deleted mice [6], possibly due a reduction in $CD8^+CD44^{hi}CD122^+$ T cells. However, the effect of TRAF3 deletion on the frequency and number of memory $CD8^+$ T cell sub-populations was not presented in either study [6, 7]. Given that we have shown that TRAF2TKO mice exhibited reduced peripheral T cells, similar to TRAF3 T-cell deleted mice [7], and that TRAF3 interacts with TRAF2 to mediate signal transduction pathways, we investigated whether the TRAF2TKO central-memory $CD8^+$ memory T-cell phenotype would also be affected by loss of TRAF3. Phenotypic analysis of independently generated T-cell specific TRAF3 knockout mice (TRAF3TKO) revealed a ~60-70% decrease in the frequency and absolute cell number of peripheral $CD8^+CD44^{hi}CD122^+$ T cells compared to floxed controls (Fig. 8 A and B). Further to this, TRAF3TKO mice exhibited a ~45% reduction in the frequency and ~60% reduction in the number of hepatic NKT cells compared to floxed controls (Fig. 8 C and D), consistent with previous findings [7]. Thus loss of either TRAF2 or TRAF3 from T cells results dysregulated $CD8^+CD44^{hi}CD122^+$ and NKT-cell homeostasis.

Discussion

The cytokine IL-15 has been established as a T-cell growth factor that promotes the proliferation and survival of $CD8^{+}CD44^{hi}$ T cells [19, 30]. The essential requirement for IL-15 by $CD8^{+}CD44^{hi}$ T cells is highlighted in IL-$15^{-/-}$ and IL-$15R\alpha^{-/-}$ mice, which harbor specific reductions in $CD8^{+}CD44^{hi}$ T cells and NKT cells [15, 16]. Our study has uncovered a selective role for TRAF2 in the homeostatic maintenance of $CD8^{+}CD44^{hi}$ T-cell and NKT T-cell subsets, indicating a potential relationship between TRAF2 and IL-15 at the molecular level. Indeed, studies with melanoma cell lines show co-localization of TRAF2 with IL-15Rα following IL-15 ligation, whereas treatment with blocking anti-IL-15 mAbs resulted in TRAF2/IL-15Rα dissociation [11]. In this present study early IL-15 mediated STAT5 phosphorylation was intact in TRAF2TKO T cells, but TRAF2TKO T cells exhibited a more rapid decline in STAT5 phosphorylation through time following IL-15 ligation. Such a difference may result in the reduced proliferation of $CD8^{+}CD44^{hi}$ T cells in response to IL-15, given the particular sensitivity of $CD8^{+}CD44^{hi}$ cells to IL-15 [19]. Also, administration of an IL-15/IL-15Rα complex in vivo, which effectively concentrates IL-15 [29], was able to boost TRAF2TKO $CD8^{+}CD44^{hi}$ T-cell and NKT-cell numbers supporting the interpretation that TRAF2 plays a role in the vigour of the IL-15 signalling response. It is of interest that responses to IL-2 are maintained in TRAF2TKO T cells indicating the specific requirement of TRAF2 for IL-15. Therefore, loss of TRAF2 disturbs IL-15-dependent signalling in $CD8^{+}$ T cells and subsequently the homeostatic maintenance of $CD8^{+}$ $CD44^{hi}$ T cells and NKT cells.

TRAF2 integrates TNF signalling pathways including the canonical NF-κB pathway [2, 3]. Thus it is possible that IL-15 may signal through the canonical NF-κB pathway for

$CD8^+CD44^{hi}$ T cells, as previously reported for cerebral endothelial cells [26]. However, we did not observe canonical NF-κB signalling in T cells in response to IL-15. Mice with severe mutations in the canonical NF-κB pathway present with reduced peripheral Tregs [31] whereas Treg numbers in TRAF2TKO mice are within the normal range of littermate control mice (JEV & STG, manuscript in preparation). Further data to support a role for TRAF2 independent of the canonical NF-κB pathway is the fact that TRAF3TKO mice phenocopy TRAF2TKO with regards to numbers of $CD8^+CD44^{hi}$ T cells and NKT cells yet TRAF3 is redundant for canonical NF-κB signalling [32]. Therefore it is unlikely that impaired canonical NF-κB signalling would contribute towards the reduced $CD8^+CD44^{hi}$ T-cell and NKT-cell phenotype in TRAF2TKO mice.

IL-15 can also activate AKT and SYK pathways via the IL-15Rα [28, 33]. Whilst SYK activation has been shown in IL-15-stimulated human neutrophils [33], we failed to observe induction of this pathway in T cells (data not shown). However, TRAF2TKO T cells showed increased activation of the AKT pathway both in the steady state and following stimulation with IL-15. Increased AKT activity has been linked to a reduced persistence of long-term memory $CD8^+$ T cells in the case of human immunodeficiency caused by a gain of function mutation in the *PIK3CD* gene which encodes the PI(3)K subunit p100δ [34]. This observation would support further studies exploring the link between IL-15, TRAF2, and AKT signalling in the homeostatic maintenance of $CD8^+$ T cells.

TRAF2 interacts with TRAF3 to form part of an E3-ligase complex that regulates the non-canonical NF-κB signalling pathway [12], whereby loss of either TRAF2 or TRAF3 results in cell intrinsic activation of non-canonical NF-κB signalling [35, 36]. As demonstrated here, and by others [36, 37], T-cell specific TRAF3 deficiency results in decreased numbers of $CD8^+CD44^{hi}$ T cells and NKT cells. Further to this, IL-15 stimulated STAT5 phosphorylation

is reduced in TRAF3-deficient NKT cells [7]. Thus, the molecular interconnectivity between TRAF2 and TRAF3 raises the possibility that loss of TRAF2 may have triggered non-canonical NF-κB signalling in T cells impairing the homeostatic maintenance of $CD8^{+}CD44^{hi}$ T cells. However, TRAF3 T-cell deficient mice harbour increased Treg cells [6], a specific phenotype we do not observe for TRAF2TKO mice. Also, direct activation of the non-canonical NF-κB signalling pathway by deletion of the E3 ligase BIRC2 [38] or via administration of small molecule antagonists [39] does not alter $CD8^{+}$ T-cell sub-populations. Collectively these data indicate that the non-canonical NF-κB signalling pathway is unlikely to play a dominant role in the TRAF2TKO $CD8^{+}$ T-cell phenotype. One possible explanation for the phenotypic overlap is that TRAF2 and TRAF3 influence an alternative T cell signalling pathway. Recent studies demonstrate that TRAF3 mediates CD40L-dependent activation of AKT in B cells [40]. As TRAF2 interacts with TRAF3 to regulate the non-canonical NF-κB pathway it is possible that TRAF2 modulates AKT activation through interactions with TRAF3.

TRAF2 is emerging as an important control point for lymphocyte homeostasis functioning in a select cell type specific manner. B-cell specific deletion of TRAF2 results in a striking expansion of B cells, specifically marginal zone B cells [36, 41]. Targeting a TRAF2 negative mutation to T cells and B cells as in TRAF2.DN mice revealed gross splenomegaly as a result of B-cell expansion [3], however T-cell numbers were unaltered demonstrating TRAF2 clearly has markedly different roles in B and T cells. TRAF2 was first described through binding to the TNF receptor [42] but is now recognised to play a key role in multiple signalling networks including canonical and non-canonical NF-κB signalling [41, 43], the JNK pathway [2], endoplasmic reticulum (ER) stress [44], and cell death pathways [45]. Here we demonstrate a critical role for TRAF2 in the regulation of T-cell growth factor signalling, highlighting a novel interaction between TRAF2 and the IL-15 and AKT signalling pathways. Given that these latter signalling data were produced in thymocytes and our findings support further analysis of AKT signalling in peripheral T-cell sub-populations. TRAF2 is essential for the maintenance of $CD8^{+}CD44^{hi}$ T-cell and NKT-cell steady-state homeostasis by modulating sensitivity to T-cell intrinsic growth factors such as IL-15.

MATERIALS AND METHODS

Mice

TRAF2$^{lox/lox}$ and TRAF3$^{lox/lox}$ mice are on a C57BL/6 background and were previously described [36, 41]. T-cell specific deletion of TRAF2 or TRAF3 was achieved by crossing TRAF2$^{lox/lox}$ or TRAF3$^{lox/lox}$ with LCKcre mice (Jackson Laboratory). C57BL/6 mice were from the Australian BioResources (Moss Vale, New South Wales). CD45.1 congenic mice (B6.SJL/*Ptprc*^{a}) and RAG$^{-/-}$ mice were from the Animal Resources Centre (Canning Vale, Western Australia). Male and female mice aged between 6 –30 weeks were used. The Garvan/St Vincent's Hospital Animal Ethics Committee approved all protocols for animal experiments.

Western blot

Protein lysates were prepared using RIPA buffer (25mM HEPES/250mM NaCl/2mM EDTA/0.1% SDS/0.5% sodium deoxycholate/1% Nonidet P-40/10mM NaF; PhosSTOP, Complete Mini EDTA-free (Roche)). Protein expression was assessed by SDS-page (10%) denaturing gel and the following antibodies; Santa Cruz Biotechnology: anti-TRAF2 (C-20); Sigma-Aldrich: anti-β-actin (AC15); Cell Signalling: anti-phospho-STAT5 (Y694), anti-STAT5, anti-phospho-Akt (Ser473), Pan Akt (40D4), anti-IκBα (9242). GE Healthcare: anti-rabbit IgG-horseradish peroxidase; Thermo Scientific: anti-mouse IgG-horseradish peroxidase. Densitometry analysis was performed using ImageJ software (National Institutes of Health, Maryland USA).

$CD8^+$ T-cell purification

Lymphocyte suspensions were prepared from spleen and pooled lymph nodes (axillary, brachial, cervical, inguinal, and mesenteric) as per standard protocols and enriched for $CD3^+$ T cells using the Pan T cell Isolation Kit II or $CD8^+$ T cells using the $CD8^+$ T cell Isolation Kit (both Miltenyi Biotec). For $CD8^+$ T cell subset purification, enriched T cells were labelled with fluorochrome-labelled antibodies against CD44, CD62L, CD122, CD8, and CD4 for cell sorting into $CD8^+CD44^{hi}CD122^+$ and $CD8^+CD44^{lo}CD122^-$ populations. Cell sorting was performed using a FACSAria (BD Biosciences) with the purity of sorted populations being >98%.

$CD8^+$ T-cell adoptive transfer.

Purified $CD8^+$ T cells resuspended in PBS containing 0.1% FCS at a concentration of $2x10^7$ cells/ml and incubated with 2.5 µM CFSE (Invitrogen) for 7 mins at 37°C. Labelled cells were washed twice in PBS containing 10 % FCS and resuspended in PBS at $10x10^6$ cells/ml. $RAG^{-/-}$ mice received an intravenous injection of $2x10^6$ cells. For adoptive transfer of $CD8^+CD44^{hi}CD122^+$ T cell populations, C57BL/6 mice were irradiated with 500 cGy using an X-RAD 320 Biologic Irradiator (Precision X-Ray). The following day, irradiated C57BL/6 mice received $1x10^5$ CFSE-labelled $CD8^+CD44^{hi}CD122^+$ wildtype (CD45.1 congenic) and TRAF2TKO (CD45.2 congenic) T cells at a 1:1 ratio.

5-bromo-2'-deoxyuridine (BrdU) incorporation

Mice received 200µl BrdU (10 mg/ml; Sigma) i.p. five times at 12-hour intervals. Splenocytes were isolated 12 hours after the final injection and BrdU incorporation assessed by flow cytometry.

Flow cytometry

Lymphocyte subpopulations were analysed on a FACSCanto using the following markers; BD Pharmingen: CD3 (145-2C11), CD4 (GK1.1), CD8 (53-6.7), CD25 (PC61), CD44 (IM7), CD45.1 (A20), CD45.2 (104), CD62L (MEL-14), CD69 (H1.2F3), CD122 (TM-β1), CD132, NK1.1 (PK136), TCRβ (H57-597), Fc block CD16/CD32 (2.4G2), pSTAT5 (pY9694); eBiosciences: CCR7 (4B12), CD3 (145-2C11), CD4 (RM4-5), CD8 (53-6.7), CD62L (MEL-14); BioLegend: Bcl-2 (BCL/10C4). CD1d-αGalCer tetramer was provided by Dr Dale Godfrey. Intracellular detection of pSTAT5 was performed using BD Cytofix Fixation Buffer and BD Phosflow PermIII Buffer (both BD Bioscienes) according to manufacturers' instructions. Data was analysed using FlowJo (TreeStar, Ashland, OR). Light scatter gating was performed on all samples to include live lymphocytes and exclude doublet cells, dead cells and debris unless specified otherwise.

T-cell proliferation assays

Purified Total T cells (Pan T cell Isolation Kit II, Miltenyi Biotec) or $CD8^+$ T cells ($CD8^+$ T cell isolation kit, Miltenyi Biotec) were stained with 0.5 µM CFSE (Sigma) and seeded at $2x10^5$ cells/well. For anti-CD3 stimulation, round bottom 96-well culture plates (Greiner Bio-One) were pre-coated with anti-CD3 (0.1, 0.3, 1 and 3 µg/ml; BD Biosciences). For cytokine stimulation, T cells were stimulated with recombinant murine IL-2, IL-7, or IL-15 (10-1000 ng/ml; Peprotech, Rocky Hill, MJ).

Administration of IL-15/IL-15Rα complex

IL-15/IL-15Rα complex consisting of 1.5 μg recombinant mouse IL-15 (Peprotech) with 7 μg Recombinant Mouse IL-15Rα Fc (R&D systems, Minneapolis, MN) per mouse was prepared as previously described [29] and administered i.p (days 1 and 2) with FACS analysis of lymphocytes performed on day 5.

Statistical analysis

Bonferonni multiple comparison test or unpaired t test (Mann-Whitney) were performed using GraphPad Prism version 6.0b for Macintosh (GraphPad Software). Results were statistically significant when the *p* value was less than 0.05.

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CONFLICT OF INTEREST DISCLOSURE

The authors declare no commercial or financial conflict of interest.

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Figure 1. Reduced CD8$^+$ T-cell subsets in TRAF2TKO mice.
Spleens from floxed control mice and TRAF2TKO mice were analysed by flow cytometry for T-cell subsets. (A) Representative plots of splenic CD4$^+$ and CD8$^+$ T cells. Number indicates median value. (B) Total cell numbers from (A) are shown; each symbol represents an individual mouse and the bars represent the median (*n* = 13-17 mice). Black/filled symbols, floxed control mice; white/open symbols, TRAF2TKO mice. (C) Representative plots of splenic CD8$^+$ T-cell subsets; CD44^{lo}CD62L^{hi} (naïve), CD44$^+$CD62L^{lo} (effector-memory), and CD44^{hi}CD62L^{hi} (central-memory) in floxed control mice and TRAF2TKO mice. (D-F) Total numbers of CD8$^+$ (D) naïve, (E) effector-memory and (F) central-memory T cells from (C) are shown (*n* = 12-14 mice). (G and H) The number of splenic CD8$^+$CD44^{hi}CD122$^+$ T cells in floxed control mice and TRAF2TKO mice are shown as (G) representative plots and (H) calculated cell numbers (*n* = 6-8 mice). (I and J) The number of splenic CD8$^+$CD122$^+$CCR7$^+$ T cells are shown as (I) representative plots and (J) calculated cell numbers (*n* = 4-5 mice). Data shown are pooled from 2-5 experiments performed. Statistical significance determined by unpaired t-test.

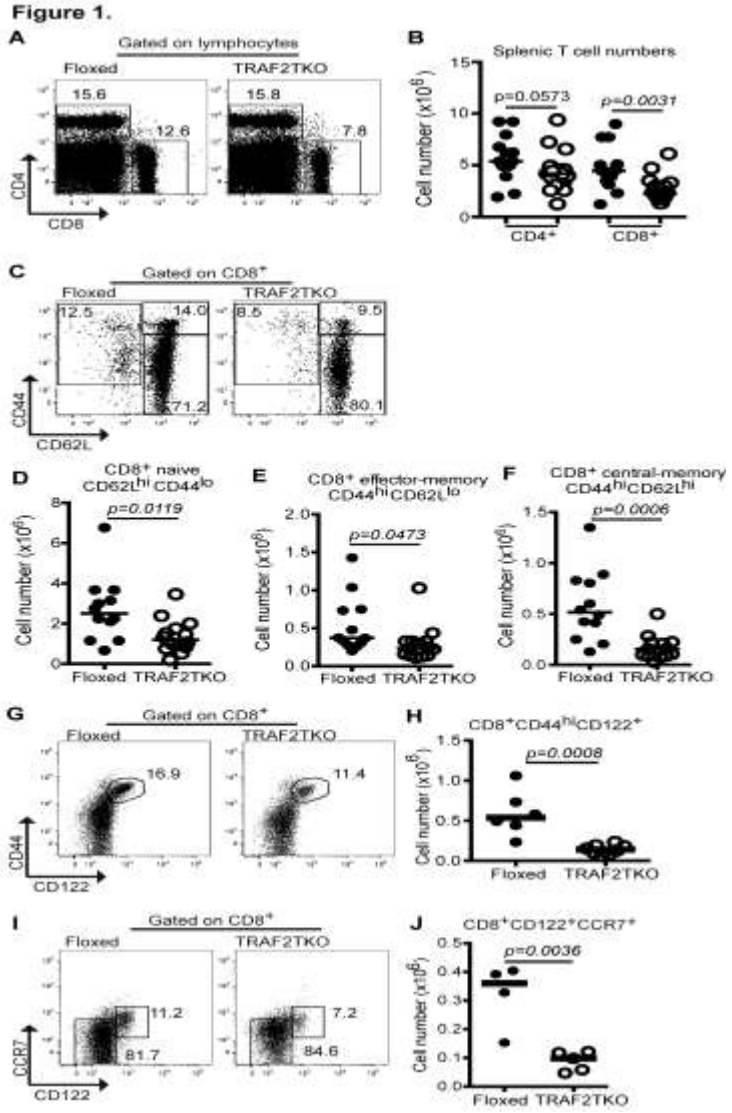

Figure 2. Impaired lymphopenia-induced proliferation of TRAF2TKO CD8$^+$ T cells. CD8$^+$ T cells from floxed control or TRAF2TKO mice were CFSE-labeled and transferred into lymphopenic RAG$^{-/-}$, irradiated C57BL/6, or non-irradiated C57BL/6 mice. (A-C) Lymph nodes from RAG$^{-/-}$ recipients were pooled and evaluated by flow cytometry. (A) Representative plots and (B) calculated cell numbers of floxed control and TRAF2TKO CD8$^+$ T cells recovered on day 7 from pooled lymph nodes of RAG$^{-/-}$ mice are shown as mean of *n*=3 mice. (C) A representative example of CD44 expression on proliferating (CFSE-diluted) CD8$^+$ T cells is shown. (D) Splenic CD8$^+$CD44^{hi}CD122$^+$ recovered from irradiated C57BL/6 recipients on Day 4 following transfer of WT CD45.1$^+$ and TRAF2TKO CD45.2$^+$ FACS-sorted CD8$^+$CD44^{hi}CD122$^+$; *n*=5 mice (E) Representative plots of floxed control or TRAF2TKO T cells transferred (top left) and recovered (bottom left) from non-irradiated C57BL/6 CD45.1 mice. The cumulative CD8+ T cell frequency (top right) and cell numbers (bottom right) are shown. Data are shown as mean±SEM of *n*=5 mice. (F) Representative plots (left) and cumulative data (right) of BrdU incorporation in CD8$^+$ T cells; *n*=7 mice. (G) Representative histogram (left) and cumulative mean fluorescence intensity (MFI, right) of Bcl-2 expression in CD8$^+$CD44^{hi}CD122$^+$ T cells. Data are shown as mean + SEM of *n* = 6-7 mice. Data shown are pooled from 2 independent experiments. Statistical significance determined by unpaired t-test.

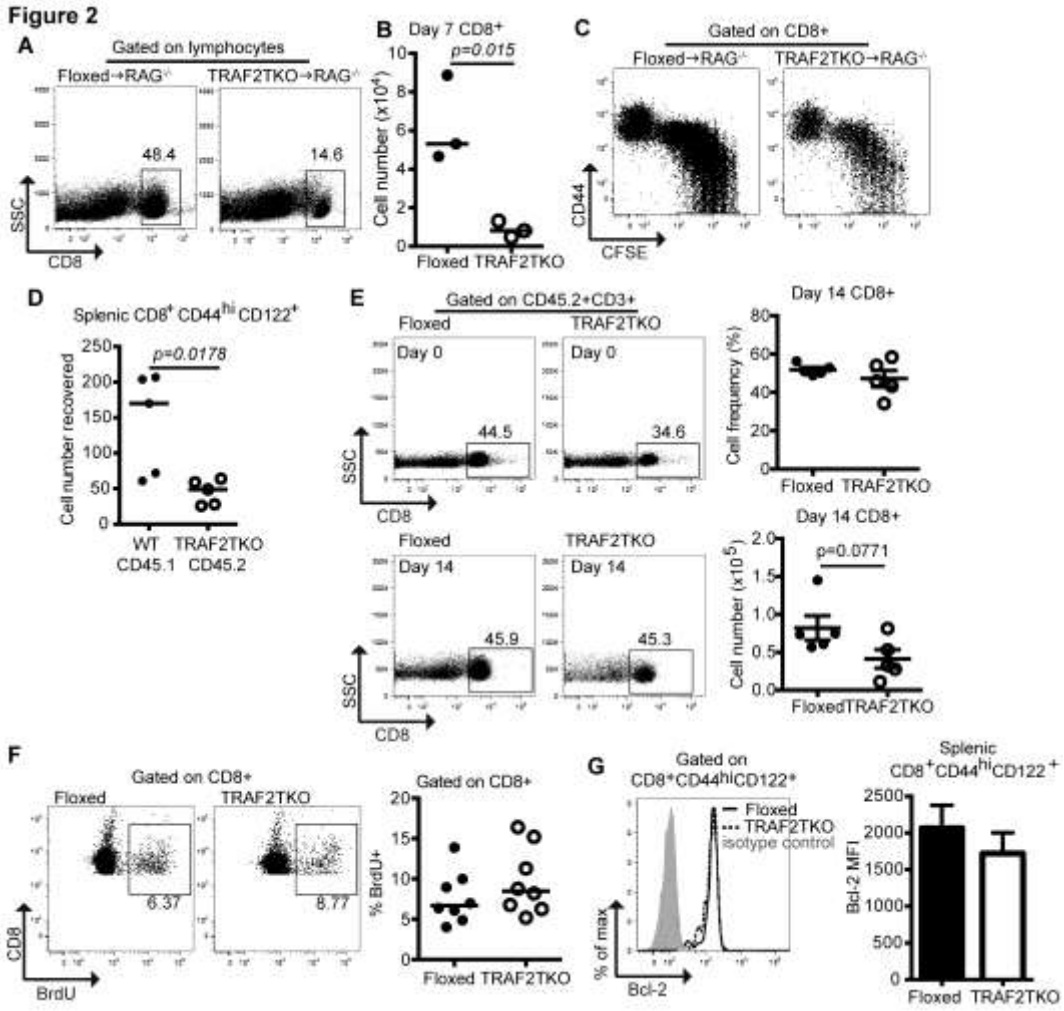

Figure 3. Cell intrinsic defects in IL-15 responses in TRAF2-deficient CD8$^+$ T cells. Total T cells or purified CD8$^+$ T cells were stimulated with IL-2, IL-7, or IL-15 in vitro. (A-C) Cumulative proliferation of floxed control and TRAF2TKO T cells following stimulation with (A) 1000 ng/ml IL-2, IL-7, or IL-15 and (B) 1-100 ng/ml IL-15. (C) Representative histogram of proliferation (CFSE dilution) from (B) is shown. (D–F) FACS-sorted CD8$^+$CD44^{hi} T cells were stimulated with (D, E) IL-15 or (F) IL-2 in vitro. (D) Cumulative proliferation and (E) representative CFSE histogram after stimulation with 50 and 100 ng/ml IL-15. (F) Cumulative proliferation after stimulation with 50 and 100 ng/ml IL-2. (G, H) MACS-purified floxed control and TRAF2TKO T cells were stimulated with anti-CD3 and analysed by flow cytometry for (G) CD25, CD44, and CD69 expression and (H) proliferation based on CFSE dilution. Data are shown as mean±SEM of pooled T cells from 3-5 mice evaluated in 2-3 experiments. Statistical significance determined by Sidak-Bonferonni multiple comparisons test.

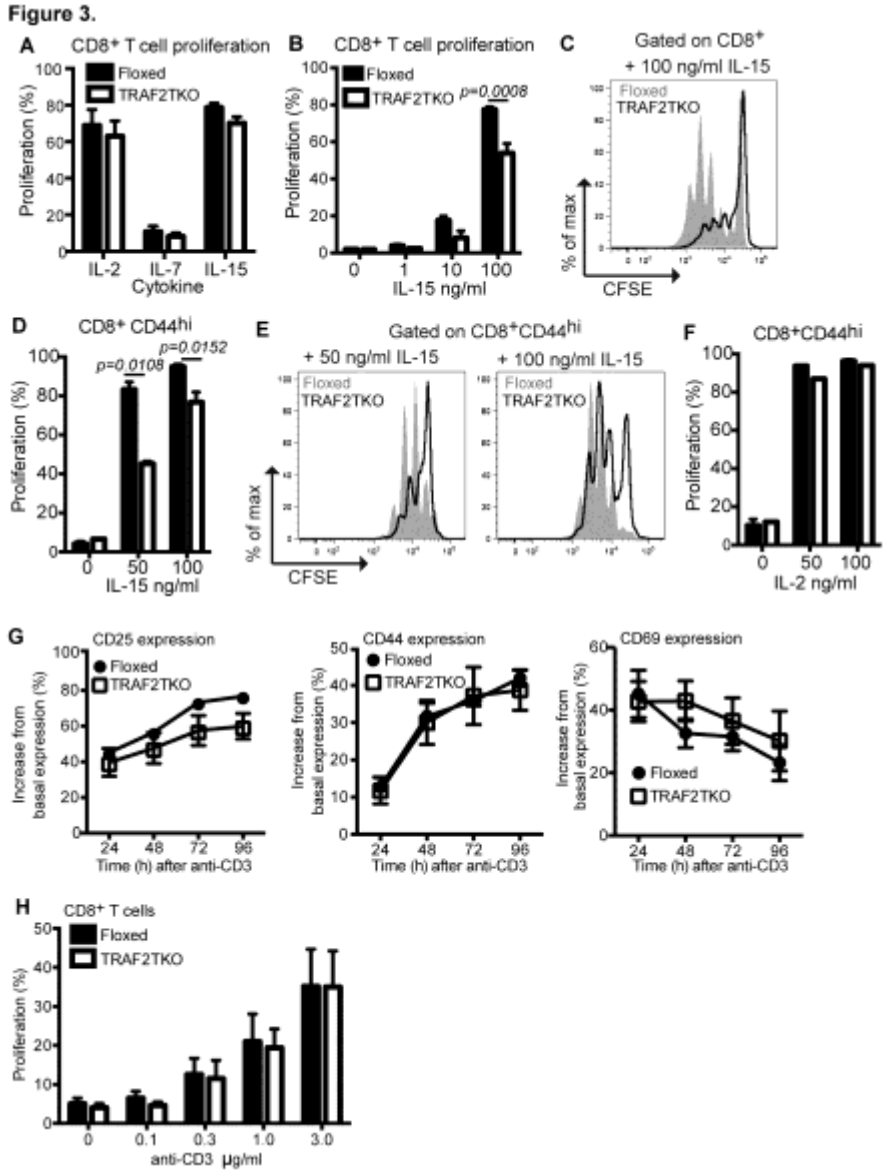

Figure 4. Reduced peripheral NKT cells in TRAF2TKO mice.
Livers from floxed control mice and TRAF2TKO mice were analysed by flow cytometry for NKT cells. (A) Representative plots of liver CD1d-αGalcer^{+} TCRβ^{+} NKT cells. Number indicates median value. (B) Total cell numbers from (A) are shown; each symbol represents an individual mouse and the bars represent the median (n = 6-7 mice). Black/filled symbols, floxed control mice; white/open symbols, TRAF2TKO mice. Data shown are pooled from 2 individual experiments. Statistical significance determined by unpaired t-test.

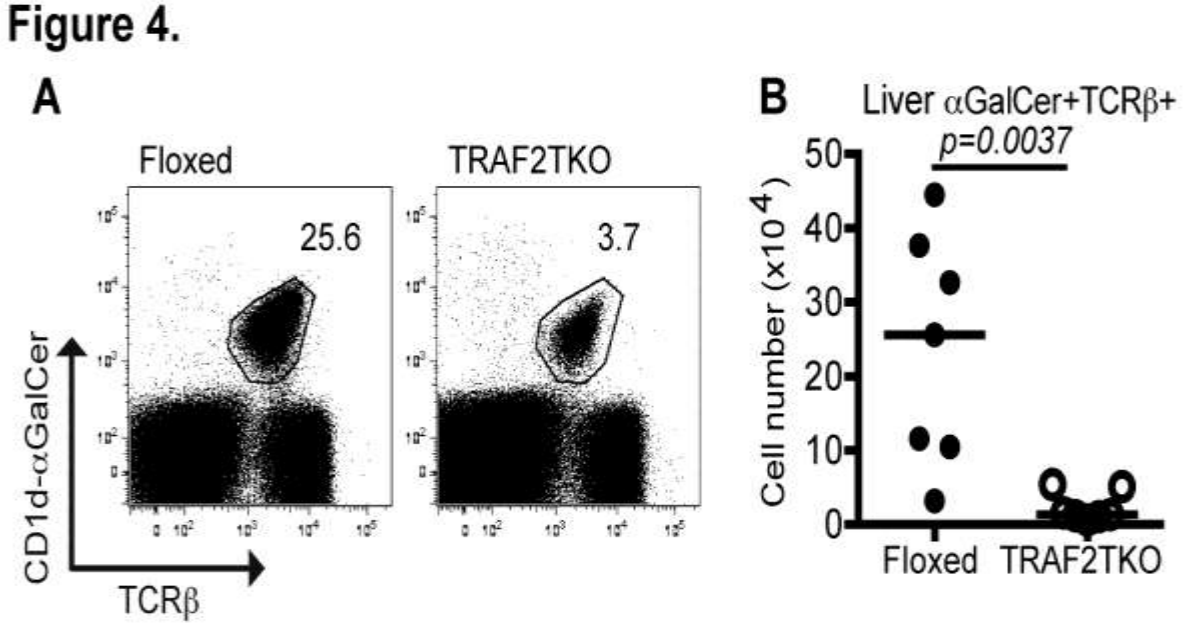

Figure 5. Intact IL-15 signalling components in TRAF2TKO mice. Flow cytometry and western blot analysis of IL-15-associated receptor expression and signalling in floxed control and TRAF2TKO T cells. (A, B) Representative plots of IL-15Rα expression on (A) unstimulated $CD8^+$ T cells and (B) anti-CD3 stimulated $CD8^+$ T cells. (C, D) Representative plots of the basal expression of (C) IL-2Rcγ/CD132 and (D) IL-2Rβ/CD122 on floxed control and TRAF2TKO T cells. Filled histogram, isotype control; blue-line histogram, floxed controls; red-line histogram, TRAF2TKO. Data shown are representative of cellular samples from *n*=4-7 mice pooled from 3 individual experiments. (E, F) Floxed control and TRAF2TKO thymocytes were stimulated with 50 ng/ml IL-15. (E) Expression of TRAF2, pSTAT5, total STAT5, IκBα, and β-actin was assessed by western blot. Data shown are from a single experiment representative of 3 independent experiments performed; *n*=3 mice. (F) Densitometry analysis of pSTAT5-to-total STAT5 protein expression as a fold change from floxed 0 mins. Data are shown as mean±SEM. (G) Floxed control and TRAF2TKO splenocytes were stimulated with 50 ng/ml for 15 min and pSTAT5 expression in $CD8^+CD44^+$ T cells was assessed by flow cytometry. Representative plots (left) and calculated mean fluorescence intensity (MFI, right) of pSTAT5 displayed as a fold change from unstimulated cells. Filled histogram, unstimulated; black-line histogram, IL-15 stimulated. Data are shown as mean±SD from *n*=5 mice pooled from 2 individual experiments. Statistical significance determined by Sidak-Bonferonni multiple comparisons test.

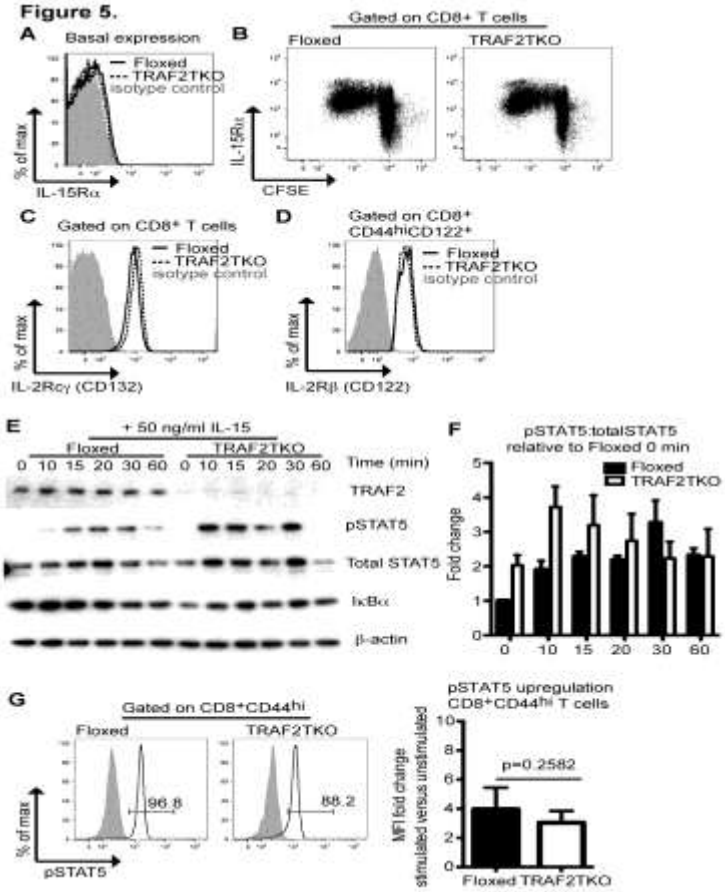

Figure 6. IL-15 effects on AKT signalling. Floxed control and TRAF2TKO thymocytes were stimulated with 50 ng/ml IL-15 for 0-20 mins and AKT signalling was assessed by western blot. (A) Expression of TRAF2, pAKT, total AKT, and β-actin (loading control). Data are from a single experiment representative of 3 independent experiments performed. (B) Densitometry analysis of pAKT-to-total AKT protein expression expressed as a fold change from Floxed 0 min. Data is shown as mean±SEM; n=3 mice evaluated from 3 independent experiments. Statistical significance determined by Sidak-Bonferonni multiple comparisons test.

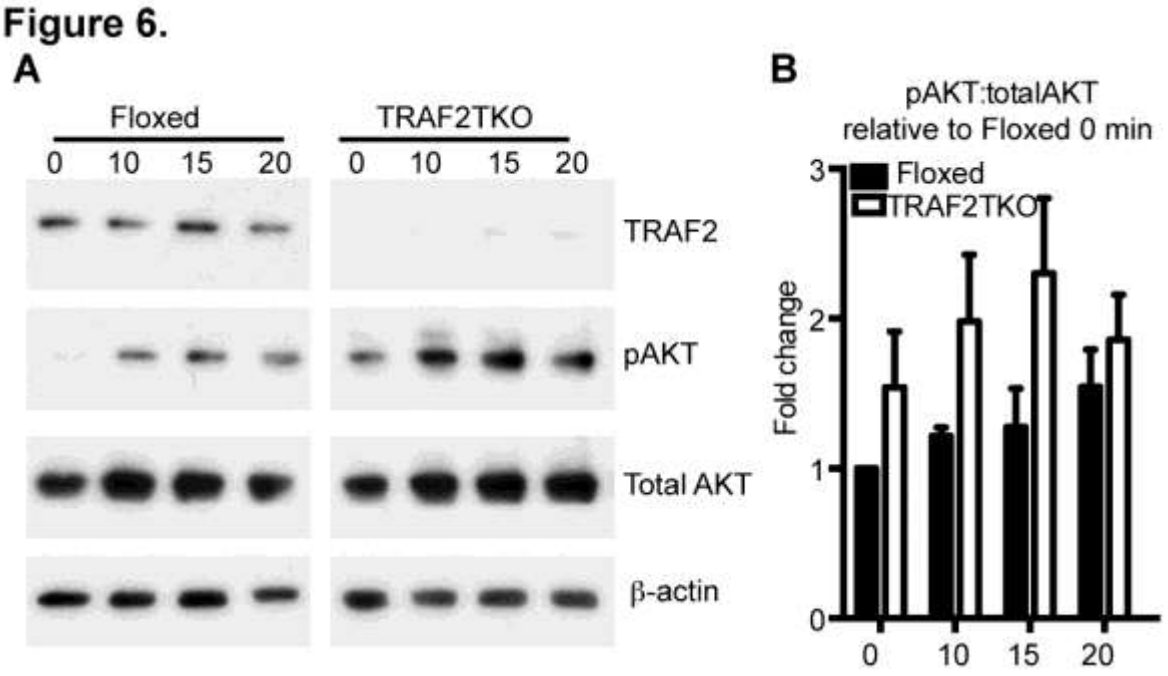

Figure 7. In vivo rescue of $CD8^+CD44^{hi}CD122^+$ and NK T cells by high dose IL-15. Floxed control and TRAF2TKO mice were treated with PBS or IL-15/IL-15Rα Fc for 2 days and peripheral $CD8^+CD44^{hi}CD122^+$ and NKT cells were assessed by flow cytometry. (A, B) Representative plots on day 5 of (A) liver NKT cells and (B) splenic $CD8^+CD44^{hi}CD122^+$ T cells from mice treated with PBS (left) or IL-15/IL-15Rα Fc (right). (C, D) Calculated numbers of (C) liver NKT cells and (D) splenic $CD8^+CD44^{hi}CD122^+$ T cells derived from (A) and (B) respectively. Each symbol represents an individual mouse and the bars represent the median; n = 3 mice evaluated over 2 independent experiments. Black/filled symbols, floxed control mice; white/open symbols, TRAF2TKO mice. (E, F) Cell number fold change of IL-15/IL-15Ra Fc-treated mice compared to PBS-treated mice, shown as (E) NKT-cell fold change in the liver and (F) $CD8^+CD44^{hi}CD122^+$ T-cell fold change in the spleen. Statistical significance determined by unpaired t-test.

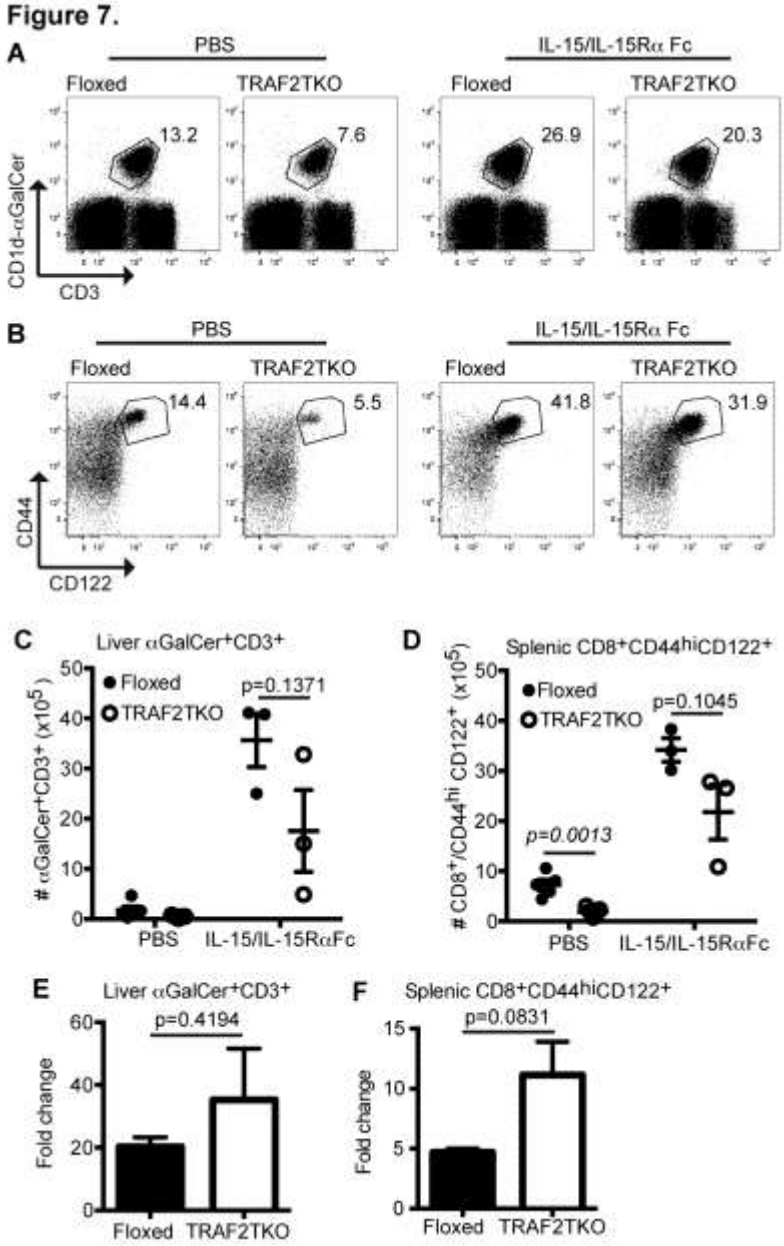

Figure 8. TRAF3TKO mice phenotypically resemble TRAF2TKO mice. Spleens and livers from floxed control mice and TRAF3TKO mice were analysed by flow cytometry for T-cell subsets. (A) Representative plots of splenic $CD8^{+}CD44^{hi}CD122^{+}$ T cells. Number indicates median value. (B) Total cell numbers from (A) are shown. (C) Representative plots of liver CD1d-αGalcer$^{+}CD3^{+}$ T cells. Number indicates median value. (D) Total cell numbers from (C) are shown. (B, D) Each symbol represents an individual mouse and the bars represent the median (*n* =3-5 mice). Data shown are pooled from 2 experiments. Black/filled symbols, floxed control mice; white/open symbols, TRAF2TKO mice. Statistical significance determined by unpaired t-test.