Published in final edited form as:
Eur J Immunol. 2011 December ; 41(12): 3423–3435. doi:10.1002/eji.201141759.

DOCK8 is essential for T-cell survival and the maintenance of CD8+ T-cell memory

Teresa Lambe[1,*], **Greg Crawford**[1,*], **Andy L. Johnson**[1], **Tanya L. Crockford**[1], **Tiphaine Bouriez-Jones**[1], **Aisling M. Smyth**[1], **Trung H.M. Pham**, **Qian Zhang**[2], **Alexandra F. Freeman**[3], **Jason G. Cyster**[4], **Helen C. Su**[2], and **Richard J. Cornall**[1,*]

[1]Nuffield Department of Medicine, Henry Wellcome Building of Molecular Physiology, Oxford University, Oxford OX3 7BN, UK

[2]Laboratory of Host Defenses, National Institute of Allergy and Infectious Diseases, NIH, Bethesda, MD, USA

[3]Laboratory of Clinical Infectious Diseases, National Institute of Allergy and Infectious Diseases, NIH, Bethesda, MD, USA

[4]Howard Hughes Medical Institute and Department of Microbiology and Immunology, UCSF, U.S.A.

Abstract

Deficiency in the guanine nucleotide exchange factor DOCK8 causes a human immunodeficiency syndrome associated with recurrent sinopulmonary and viral infections. We have recently identified a DOCK8-deficient mouse strain, carrying an ethylnitrosourea-induced splice-site mutation that shows a failure to mature a humoral immune response due to the loss of germinal centre B cells. In this study we turned to T-cell immunity to investigate further the human immunodeficiency syndrome and its association with decreased peripheral $CD4^+$ and $CD8^+$ T cells. Characterisation of the DOCK8-deficient mouse revealed T-cell lymphopenia, with increased T-cell turnover and decreased survival. Egress of mature $CD4^+$ thymocytes was reduced with increased migration of these cells to the chemokine CXCL12. However, despite the two-fold reduction in peripheral naïve T cells, the DOCK8-deficient mice generated a normal primary $CD8^+$ immune response and were able to survive acute influenza virus infection. The limiting effect of DOCK8 was in the normal survival of $CD8^+$ memory T cells after infection. These findings help to explain why DOCK8-deficient patients are susceptible to recurrent infections and provide new insights into how T-cell memory is sustained.

Keywords

Human; Rodent; Immunodeficiency; T cells; Memory

Introduction

The recent discovery of the human DOCK8 immunodeficiency syndrome has highlighted DOCK8 and the DOCK super-family of guanine nucleotide exchange factors as important mediators of normal immune function [1-4]. As a group, members of the DOCK family

Address correspondence to Richard Cornall, H.W.B.M.P, Roosevelt Drive, Oxford, OX3 7BN, UK. Phone: (44)-1865 287790, Fax: (44)-1865 287787; richard.cornall@ndm.ox.ac.uk.
*Joint first authors

Conflicts of Interest The authors declare no financial or commercial conflict of interest.

interact with Rho GTPases to regulate pathways that are typically involved in the cytoskeletal rearrangements required to maintain cell structure, migration and adhesion; but the details of their individual functions are largely unknown [3, 5]. The DHR1 (CZH1) domain in DOCK proteins has been shown to bind phosphatidylinositol (3,4,5)-triphosphate (PIP3) generated by PI3K activity, thus promoting localized membrane binding and activation of DOCK proteins [3], while the DHR2 (CZH2) domain binds to Rho-family GTPase proteins (Rac, Cdc42) and provides catalytic GEF activity to stimulate these proteins and promote integrin reorganization and adhesion, lamellipodia formation, cell polarization, phagocytosis or cell fusion [3, 4]. DOCK8 had been associated with Cdc42 and found to localize to lamellipodia in fibroblasts; but its role in immune function is largely unknown [6].

Recently described patients who are deficient in DOCK8 fail to mount an effective immune response and are susceptible to recurrent sinopulmonary and viral infections, hyper-IgE, atopy and allergic disease and some types of cutaneous cancer and lymphoma [1, 2]. The majority of patients have low absolute counts of both $CD4^+$ and $CD8^+$ T cells in the blood and impaired proliferation of the cells following mitogen stimulation with a modest decrease in the production of the anti-viral cytokines IFN-γ and TNF-α. These facts suggest a defect in T-cell immunity; yet the nature of any T-cell dysfunction in the absence of DOCK8 remains to be defined.

DOCK8 was also recently identified as being critical for a functional B-cell response in a screen for *N*-ethyl-*N*-nitrosourea (ENU) induced mutations that cripple the maturation, but not initiation, of antibody production [7]. Two independent strains (CPM and pri) were found to have loss-of-function DOCK8 mutations causing the cell intrinsic loss of germinal center (GC) B cells during affinity maturation [7]. Further analysis showed that naïve DOCK8-deficient cells are unable to recruit the intercellular adhesion molecule ICAM-1 into the B-cell immune synapse during recognition of membrane-bound antigen. This deficit may explain the impact on GC B-cell survival, which depends on binding antigen and integrin ligands on follicular dendritic cells [8].

In this study we use the DOCK8-deficient mice to gain insights into the clinical and laboratory findings of DOCK8 immunodeficiency in humans, extending our study of B-cell immunity to examine the T-cell compartment. Like humans, DOCK8-deficient mice have decreased peripheral T cells. Although development of T cells is normal, mature single positive $CD4^+$ T cells accumulate in the thymus and show increased chemotaxis to CXCL12; peripheral $CD4^+$ and $CD8^+$ T cells show increased turnover and decreased survival. Despite these defects, DOCK8-deficient animals are able to generate a comparatively normal primary T-cell response to immunisation with Modified Vaccinia Ankara (MVA) or infection with influenza. However, the memory recall response is significantly reduced due to decreased survival of memory $CD8^+$ T cells. These results may explain the relentless pattern of recurrent infection in the human DOCK8 immunodeficiency syndrome, and may provide clues to how T-cell memory is generated and sustained.

Results

T-cell lymphopenia in DOCK8-deficient humans and mice

To establish the effects of DOCK8 deficiency on T cells in humans we counted naïve CD45RA and activated effector/memory CD45RO $CD4^+$ and $CD8^+$ T cells in the blood of patients of different ages. This showed a reduction in both naïve and effector/memory numbers, although the latter were less affected and even normal in some patients (Figure 1A). However, it is difficult to interpret the significance of these results or establish the causes of immunodeficiency when T-cell phenotype is continually altered by persistent

infection and antigenic stimulation. Therefore, we turned to a recently described ENU mutant, CPM, carrying homozygous null alleles of DOCK8 [7]. The CPM splice site mutation truncates the native protein, removing the DHR2 GEF domain. To investigate the effects of DOCK8 deficiency on T cells, we enumerated $CD4^+$ and $CD8^+$ T cells in the secondary lymph organs of DOCK8-deficient CPM mice (Figure 1B-E).

Examination of spleen, mesenteric lymph nodes (MLN) and peripheral lymph nodes (PLNs) revealed a 2-3 fold reduction in $CD4^+$ and $CD8^+$ T cells compared with WT C57BL/6 controls (Figure 1B-D). Total $CD4^+$ and $CD8^+$ T cells were reduced in the blood as a fraction of total lymphocytes (Figure 1E, upper panel) and in absolute terms, where $CD4^+$ counts were 5.28×10^5/ml (± SE 0.541) in CPM versus 14.53×10^5/ml (± SE 2.132) in WT ($p = 0.014$), and $CD8^+$ 3.32×10^5/ml (± SE 0.240) in CPM versus 5.62×10^5/ml (± SE 0.404) in WT ($p = 0.008$). In contrast, the absolute number of activated $CD44^{hi}/CD62L^{lo}$ $CD4^+$ and $CD8^+$ T cells was more similar in the spleen and PLNs and 1.5-2 fold higher in the MLN of CPM mice compared with that of WT (Figure 1B-D). The increased fraction of activated $CD44^{hi}$ $CD8^+$ and $CD4^+$ T cells was also seen in the blood of CPM mice (Figure 1E, lower panel and data not shown). We conclude that DOCK8 deficiency in mice leads to peripheral T-cell lymphopenia predominantly affecting the number of naïve rather than activated cells, reminiscent of the phenotype in the human immunodeficiency syndrome.

Normal activation and differentiation of peripheral DOCK8-deficient T cells

Studies in DOCK8-deficient patients have demonstrated decreased T cell activation and proliferation, but are inconclusive regarding function due to the difficulty in isolating naïve T cells from the peripheral blood [1, 2]. Therefore we analyzed the response of DOCK8-deficient T cells from CPM to antigenic stimulation to see if loss was associated with a defect in activation or differentiation. Overnight activation of naïve DOCK8-deficient mouse T cells with anti-CD3 showed no difference in the upregulation of CD69 and CD25 on CPM and WT $CD4^+$ and $CD8^+$ T cells (Figure 2A); moreover, when stimulated with anti-CD3 and anti-CD28 in vitro, CPM and WT $CD4^+$ and $CD8^+$ T cells underwent the same number of divisions, after five days, in culture (Figure 2B). These findings show that DOCK8-deficient T cells from uninfected mice are able to respond normally to antigenic stimulation and proliferate freely in vitro.

To explore T cell differentiation we analyzed the cytokines produced by activated/memory $CD45RO^+$ $CD4^+$ T cells from the blood of DOCK8-deficient patients, parents and controls stimulated *ex vivo* with PMA and ionomycin. A higher proportion of activated cells secreting TH2 cytokines IL4 or IL13 were observed in 3/5 and 4/5 DOCK8-deficient patients respectively, a finding that may be linked to the phenotype of hyper-IgE in DOCK8 immunodeficiency whilst, the number of cells secreting IFN-γ was variable (Suppl Figure 1A). However, the degree to which these phenotypes reflect ongoing infection, over-stimulation/exhaustion or other secondary effects caused by the immunodeficient state was again uncertain. Therefore, we asked if CPM $CD4^+$ T cells could differentiate normally under TH1 or TH2 polarising conditions in vitro. Under these conditions, Tbet and GATA3 were induced appropriately (Suppl Figure 1B). IFN-γ production by TH1 cells was equivalent, and only the proportion of IL4 secreting cells was higher in CPM mice under TH2 conditions (Suppl Figure 1C and 1D). It is possible that DOCK8 and integrin dependent effects on the signalling threshold of the TCR or other receptors, or small differences in the basal and induced levels of T-bet and GATA-3, might contribute to such a bias in humans and mice. Overall, however, the differentiation of effector cell subsets is not intrinsically unimpaired and therefore unlikely to contribute to the state of immunodeficiency.

T cell development in DOCK8 deficiency

We next asked if abnormal thymic development might explain the reduction in peripheral T cells. We found no difference in the numbers of developing T cells at the DN or DP stage (Figure 3A) or in the number of mature $CD8^+$ T cells (Figure 3A and 3B); and no consistent differences were seen in CD3, CD5, TCRβ, CD44 or CD25, staining (data not shown). The sole abnormality observed was an accumulation of mature single positive $CD4^+$ T cells, particularly the most mature $CD4^+CD62L^{hi}CD69^{lo}$ population (Figure 3A, right panel, and 3B). On its own this may contribute to the peripheral lymphopenia, but cannot explain the deficiency of $CD8^+$ T cells. Labelling CPM and WT mice with BrdU showed no difference in the absolute number of $BrdU^+$ single-positive $CD4^+$ T cells generated over a 40hr period ($1.00 \times 10^6 \pm$ SE 0.14 in CPM vs. $1.33 \times 10^6 \pm$ SE 0.18 in WT, p=0.1674; unpaired t-test), implying that accumulation of mature DOCK8-deficient $CD4^+$ T cells is due to delayed egress.

T-cell lymphopenia in DOCK8-deficiency is an intrinsic defect

To examine if T-cell lymphopenia in DOCK8 deficiency was cell intrinsic we reconstituted lethally irradiated CD45.1 allotype-marked recipients with 50:50 or 20:80 mixtures of WT (CD45.1) and CPM or WT (CD45.2) bone marrow. Whilst the 50:50 WT:CPM bone marrow generated the expected 1:1 ratio of follicular B cells it failed to reconstitute normal numbers of DOCK8-deficient T cells due to a block in T cell development prior to the DN stage, which was not previously seen in the absence of competition (data not shown). The cause of this competitive disadvantage in establishing early thymic development is unknown; but it is not limiting in non-chimeras where the number of cells at the DN stages of thymic development are similar in CPM and WT mice (Figure 4A). Analysis of 20:80 WT:CPM mixed chimeras showed the same competitive failure to reconstitute thymic development relative to the B cell lineage but no effect of DOCK8 in the transition from DN to DP and mature single-positive cells (Figure 4A & data not shown). However, naïve peripheral T cell numbers were reduced 2-3 fold in CPM compared with WT mice (Figure 4A), which suggests a cell intrinsic defect in peripheral $CD4^+$ and $CD8^+$ survival underlying the immunodeficiency. The absence of $CD4^+$ cell accumulation in the thymus of mixed chimeras also supports the idea that this is not the principal cause of peripheral lymphopenia (Figure 4A). Moreover, the absence of greater numbers of peripheral CPM $CD44^{hi}$ T cells in the mixed chimeras (Figure 4B) implies this is due to homeostatic proliferation in unmanipulated DOCK8-deficient animals, where T cell numbers are low.

Migration and survival of early naïve T cells in the absence of DOCK8

B and T cells from DOCK2-deficient mice fail to migrate to a variety of chemoattractants, resulting in the loss of T cell thymic egress in response to S1P, and peripheral lymphopenia [9, 10], therefore we considered whether similar effects could contribute to immunodeficiency in the absence of DOCK8. To assess chemotaxis, we mixed equivalent numbers of mature allotype-marked thymic T cells from CPM and WT and assessed their ability to migrate in response to constitutively expressed cytokines CXCL12, CCL19/21 and S1P. In contrast to DOCK2-deficient mice, the migration of thymic and peripheral T cells to these chemokines was normal, with the exception of a consistent increase in the migration of mature DOCK8-deficient thymic $CD4^+CD62L^{hi}$T cells towards CXCL12, (Figure 5A; p=0.0224 by ANOVA, combining data from 3 experiments). As CXCL12 signalling via CXCR4 is important in lymph node entry [11], we transferred 50:50 mixtures of mature thymic CPM & WT T cells into WT recipients and *plt/plt* recipients, which lack expression of the other main T cell attractants CCL21 and CCL19 [11]. In these experiments, the proportion of thymic CPM $CD4^+$ $CD62L^{hi}$ T cells in peripheral lymphoid organs after 90 mins was increased in comparison to WT cells (Figure 5B). These findings are consistent

with an increased chemotactic response to CXCL12 but also confirm that entry and localisation of cells to peripheral lymphoid organs is normal in unimmunised mice.

Twenty-four hours after transfer, however, the proportions of mature thymic CPM $CD4^+$ and $CD8^+$ T cells were reduced relative to WT (Figure 5C), suggesting that DOCK8 deficiency causes these emigrants to survive less well after localisation to secondary lymphoid organs. We found that expression of the survival factor Bcl-2 is reduced in CPM single positive $CD4^+$ and $CD8^+$ thymocytes, suggesting that DOCK8-dependent activation of anti-apoptotic pathways may contribute to survival at this stage in T cell development (Figure 5D).

Decreased survival of peripheral DOCK8-deficient T cells

Together, the above results suggested that defects in cell survival were the principal reason for the loss of mature T cell populations in DOCK8 immunodeficiency. To evaluate this further, CPM and WT mice were fed BrdU for 15 days and the survival of labelled cells was followed for a further 30 days. In the first phase, BrdU labelling of $CD4^+$ and $CD8^+$ T cells was equivalent or more rapid in the spleen and lymph nodes of CPM compared with WT (Figure 6A-D). This is consistent with a higher level of homeostatic proliferation and the majority of newly labelled cells were $CD44^{hi}$ (data not shown). More importantly, during the washout phase, DOCK8-deficient $CD4^+$ and $CD8^+$ T cells disappeared much faster in all tissues, confirming a survival defect in mature T cells (Figure 6A-D).

Loss of memory T cells in the absence of DOCK8

During a normal immune response to pathogens in vivo, the activation, proliferation and differentiation of T cells leads to short-term effector cells and long-lived memory cells, which survive after contraction of the effector population and act to curtail recurrent infection. The in vitro experiments, we have described, suggest that the primary effect of DOCK8 might not be on the generation of short-term effector cells but rather the survival of long-lived memory cells. To investigate these possibilities we compared the response of CPM and WT mice to vaccination with replication deficient Modified Vaccinia Ankara virus (MVA). After 9 days there was no significant difference between CPM and WT in IFN-γ, IL2 or TNF-α production by antigen-specific $CD8^+$ T cell directed against MVA (Figure 7A and data not shown) or IFN-γ ELISpot (data not shown), either as percentage of $CD8^+$ T cells or in absolute terms. In three separate experiments, this analysis showed no difference between CPM and WT confirming that the initial phase of the T-cell response is not functionally limited by the lymphopenia in pre-immune animals. Anti-MVA specific $CD8^+$ T cell numbers also remained similar after the contraction phase (Figure 7A); therefore, there was no premature collapse of the effector T-cell population in the absence of DOCK8. However, 42 days after the primary immunisation, during the phase of long-lived memory cell survival, the number of antigen-specific cells was significantly reduced (Figure 7A). The loss of surviving T cells was not due to a block in memory cell differentiation as numbers of memory precursor cells (MPECS), defined as $KLRG1^{low}$ $CD127^{hi}$ ($IL7R^{hi}$), were unaltered (Figure 7B). Furthermore, expression levels of memory cell markers $CD127^+$, $CD62L^+$, $KLRG1^+$ and Bcl-2 were similar on MVA-specific WT and CPM T cells at day 9, 14, 42 and after rechallenge supporting this conclusion (Figure 7C and data not shown). After 55 days, the response to rechallenge with MVA in vivo was reduced in DOCK8-deficient mice compared with WT (Figure 7A). However, the fold-increase in the secondary response was proportionate to the number of surviving memory cells (Figure 7A), once again suggesting that it is the survival of memory cells rather than the generation of the secondary effector response that is limiting for $CD8^+$ T-cell immunity in the absence of DOCK8.

To investigate further the nature of DOCK8 deficiency in the maintenance $CD8^+$ memory T cells, we repeated our MVA immunization protocol with chimeric mice reconstituted as before with 20:80 mixtures of WT (CD45.1) and CPM or WT (CD45.2) bone marrow. In this competitive setting, DOCK8 deficiency resulted in reduced primary $CD8^+$ responses to MVA relative to WT cells (Figure 7D). The loss of DOCK8 $CD8^+$ response in this competitive situation is consistent with an underlying defect in survival, which is normally compensated for by increased proliferation in non-chimeras. The greatest effect of competition is on the $KLRG1^{hi}$ short-lived effectors, which are turning over most rapidly (Fig 7D middle panel). During the maintenance phase of memory, day 42, DOCK8 responders were further affected when compared with day 9 MPEC. Together these data support an intrinsic role for DOCK8 in the maintenance of $CD8^+$ T cell memory. The WT and DOCK8 $CD8^+$ memory T cells expressed similar levels of markers such as $CD127^+$ (IL7R) and KLRG1 as well as the survival factor Bcl2 (Figure 7E).

We also examined T-cell responsiveness in DOCK8-deficient cells during pathogenic infection by challenging CPM and WT mice with mouse adapted influenza strain x31(A/HK-x31 (H3N2)). Using daily weight loss as a clinical parameter we found no significant difference in disease severity or the ability to survive the acute infection through the nadir of infection at day 6 to recovery by day 10 (Figure 8A). 11 of 12 WT mice survived the challenge, while 9 of 12 CPM mice survived. Seven days post immunization, there was no significant difference between WT and CPM in IFN-γ production and numbers of $LAMP1^{hi}$ $CD8^+$ T cells to T-cell restricted epitopes with similar expression profiles of $CD127^+$, KLRG1 and $CD62L^+$ (Figure 8B and data not shown). Numbers of MPECS were also produced to normal levels (data not shown). These findings again indicate that the immunodeficiency in the absence of DOCK8 is not due to an inability to mount an effective response to acute infection. To assess the memory response we bled mice 56 days after primary infection and counted $LAMP\text{-}1^{hi}$ $CD8^+$ T cells responsive to conserved internal 'flu T-cell restricted epitopes (Figure 8B). Whilst WT mice produced a robust response to x31, memory T cells were not sustained above background in CPM mice; these data were substantiated by blood ELISpot (data not shown). Together these findings show that the functions of DOCK8 are not limiting during primary T-cell immune response against viral infection but are critical for maintenance of memory to vaccination and viral infection.

Discussion

The results presented here contribute to our understanding of how deficiency in DOCK8 can increase susceptibility to disease, particularly viral infection [1, 2]. Our findings show that DOCK8 operates at multiple steps in cellular immunity, but its main effects are on T-cell survival, and it is most limiting in the survival of memory T cells. The inability to generate high affinity antibodies to pathogens due to a failure to sustain GC B cells, shown previously, and the absence of T-cell memory shown here may explain the increased susceptibility of human DOCK8 immunodeficiency syndrome patients to recurrent infections [1, 2, 7].

The relative paucity of peripheral T cells in DOCK8-deficient mice is not due to defective thymic development. Although DOCK8-deficient T cells have a selective disadvantage in populating early thymic development relative to WT cells in mixed chimeras this is not limiting in natural mutants or single bone marrow chimeras. B-cell development is normal in the mixed chimeras and so competitive effects on the early thymus might be due to the defective survival, proliferation and migration of T cell precursors. However, at terminal thymic development, DOCK8-deficient single positive $CD4^+$ T cells accumulate in the thymus and these $CD4^+$ cells show increased migration to SDF-1/CXCL12. Emigration from the thymus is mediated primarily by trafficking to S1P, and whilst CXCL12-dependent

signals have been suggested to play a role in thymic egress it remains to be formally demonstrated in physiological circumstances [12, 13].

The discovery that mature naive thymic $CD4^+$ T cells show increased migration to CXCL12 does raise the interesting possibility of a connection between DOCK8 and the human WHIM syndrome, which is a combined immunodeficiency defined by warts, hypogammaglobulinemia, immunodeficiency, and myelokathexis causing neutropenia [14]. WHIM and DOCK8-deficient patients share a similar spectrum of recurrent respiratory and cutaneous viral infections [14] although not all clinical features are comparable. T cells from patients with the WHIM syndrome also show augmented T-cell responses to CXCL12, and a substantial number of these patients have mutations in the CXCR4 chemokine receptor gene [14, 15]. DOCK8 may, therefore, be a regulator of CXCR4 signalling and speculatively, WHIM patients with no obvious abnormality in CXCR4 dependent signalling may have mutations in DOCK8-dependent signalling pathways [15, 16].

In theory, the decreased number of naïve T cells in the peripheral T-cell compartment could be due to defects in thymic egress, differentiation and proliferation or the increased death of cells. However, our data suggest that decreased survival is the principal reason for the loss of T cells in DOCK8-deficient animals. The increased turnover of DOCK8-deficient T cells in vivo and the increase in activated or memory-like ($CD44^{hi}/CD62L^{lo}$) T cells is likely to be caused by lymphopenia-induced proliferation, which occurs when T cells in lymphopenic hosts divide and irreversibly acquire a ‘memory-like’ phenotype [17]. The increase in $CD44^{hi}/CD62L^{lo}$ T cells is not present in mixed chimeras where cell numbers are normal, and so, in this case, the loss of naive peripheral DOCK8 T cells must be due to an intrinsic defect in survival rather than forced differentiation and division. More importantly, lymphopenia affecting the primary repertoire does not seem to be limiting in the primary response to vaccination or infectious challenge, when proliferation can presumably compensate for the reduction in T cells, whereas the most striking effect of DOCK8 deficiency is on the survival of memory $CD8^+$ T cells.

The $CD8^+$ T cell immune response can be divided into three phases [18]: the first phase is of growth and differentiation of naïve $CD8^+$ T cells into effector cells with lytic activity and cytokine production; the second is a phase of 10-20 fold contraction leading to a smaller population of long lived memory cells, which arise by the differentiation of effectors [19, 20]; and the third, a phase in which the memory cells are maintained in the host [20, 21]. Long-lived $CD44^+CD62L^{hi}CD127^+$ central memory T cells (T_{CM}) arise after 5 days following a typical infection and survive within secondary lymphoid organs where they can respond swiftly in a recall response [20-23] The generation of the memory cells and the expansion of the immune response depend on TCR signalling as well as costimulatory molecules including CD28, CD40, 4-1BB, CD27, ICOS, and/or OX40, IL2 and inflammatory cytokines including IL-12 and IFN-α [24-29]. The normal response following MVA vaccination or influenza infection and the initial generation of memory cells suggests that DOCK8 is not limiting downstream of these pathways. The long-term survival of memory $CD8^+$ T cells does not require the presence of antigen but is dependent on cytokines, particularly IL-15 and IL-7 [30-33]. However, the absence of defects in lymphopenia-induced homeostatic expansion and NK cell development (Greg Crawford, unpublished observations) which depend on IL-7 and IL-15 [17] argues against a deficiency in cytokine signalling as the cause of $CD8^+$ T-cell memory failure in the absence of DOCK8.

Since DOCK8 mutant B cells are unable to cluster ICAM-1 following antigen-receptor signalling it is tempting to speculate that similar defects in integrin-dependent signalling might affect the memory T-cell response. Although ICAM-1-dependent interactions

between T cells and DCs play a role in the formation of $CD8^+$ memory T cells [34, 35], the absence of ICAM-1/LFA-1 interactions has already been described as having its greatest effect on the survival of memory cells [36-38]. It is interesting to speculate that a dependence on external signals from within the cellular niche might also account for the low Bcl-2 expression in DOCK8-deficient single positive thymocytes and their decreased survival on transfer into the periphery.

How DOCK8-dependent reorganization of the cytoskeleton could affect lymphocyte survival is also not yet clear. However, a decreased memory response to viral infections is also reminiscent of Wiskott-Aldrich Syndrome (WAS), which is an X-linked, immunodeficiency syndrome principally caused by mutations in the WAS protein (WASP) gene [39]. WASP regulates the actin cytoskeleton in a variety of immunological functions including cell migration and the formation and the preservation of T-cell memory [40]. WASP and WASP-family verprolin-homologous (WAVE) proteins are fundamental actin-cytoskeleton reorganizers found in eukaryotes. The conserved function across species is to receive upstream signals from Rho-family GTPases and then activate the actin-related protein (ARP) 2/3 complex, leading to rapid actin polymerization [41, 42]. There are a number of striking similarities between WAS and DOCK8-deficiency including peripheral T-cell lymphopenia, a lack of marginal zone B cells and poor immunological synapse formation in WASP-deficient B cells as a consequence of impaired BCR and integrin signalling [43, 44]. Cdc42, the probable target of DOCK8, is an important regulator of WASP function [45]. Therefore it is an intriguing possibility that DOCK8 augments the formation of active Cdc42, at the membrane interface thereby signalling through WASP. Further investigation of such pathways and their relationship to the human immunodeficiency syndromes will provide important insights into how T-cell memory is sustained.

Materials and Methods

Human studies, Mouse Strains and Procedures

Human studies were approved by the institutional review board of the National Institute of Allergy and Infectious Diseases in accordance to Declaration of Helsinki principles. Mouse experiments were performed under UK Home Office License or in accordance UCSF Institutional Animal Care and User Committee approved protocols. CPM mice were generated by ENU-mutagenesis [7]; C57BL/6 *plt/plt* mice were used for transient transfer experiments [46]. Bone marrow chimeras, C57BL/6 ($CD45.1^+$) mice were irradiated with 2 fractions of 4.5 Gy spaced by 3 hours and reconstituted with $5\text{-}10\times10^6$ bone marrow cells, comprising 50:50 or 20:80 mixtures of C57BL/6 ($CD45.1^+$) bone marrow and either CPM or C57BL/6 ($CD45.2^+$) cells and allowed to reconstitute for 8–10 weeks.

Flow Cytometry

Human PBL and mouse lymphocytes were isolated and stained as previously described [1, 7]. Antibodies were from B.D. Pharmingen or eBioscience® unless otherwise stated; FITC-conjugated antibodies were against: CD24, BrdU, CD8α, CD62L and CD44 (Caltag). Phycoerythrin-(PE) conjugated antibodies were against: CD5, CD3, CD69, FoxP3, CD62L, CD45.1, CD25 and CD4 (Caltag). PE-Cy7 conjugated antibodies were against B220, CD69, CD8α, CD4 and CD45.1. PE-Cy5.5 conjugated antibodies were against CD8α. Allophycocyanin-(APC) conjugated antibodies were against: CD44 and CD4 (Caltag). APC-Cy7 conjugated antibodies were against CD4. APC-Alexa-Fluor® 750 conjugated antibodies were against: CD45.1. Tricolour-(TC) conjugated CD4 and CD8α (Caltag). BD TruCOUNT tubes were used to enumerate the leukocyte count as per manufacturer's

instructions. Data was collected on FACSCanto^{TM} or LSRII machines (B.D.) and analysed with FlowJo (Tree Star, Inc.) or FACSDiva (B.D.).

T cell chemotaxis, activation, proliferation

Chemotaxis assays were performed as described [47]. To assess T cell activation, splenocytes were plated in 96 well plates coated with anti-CD3 antibody at 10μg/ml (145-2C11) and 10μg/ml anti-CD28 ascites (37.51) in PBS. Cells were incubated at 37°C overnight in RPMI containing 10% FCS. To assess proliferation, CD45.2 WT or CPM lymphocytes were mixed with CD45.1 WT cells to give 2×10^6 cells/ml and labeled for 8 min at RT with an equivalent volume of CFSE (final 2μM). The reaction was quenched by the addition of FCS and washed 3 times with RPMI-1640 containing 10% FCS. 2×10^5 CFSE labeled cells were cultured on anti-CD3 and CD28 coated plates for 3 days.

Transient transfers

In transient transfer experiments, ~$1\text{-}2\times10^7$ cells per ml were labelled with either 3.3 or 10μM carboxyfluorescein-diacetate –succinimidyl-ester (C F S E ; Invitrogen / Molecular Probes) o r 1 0μ M 5-(and-6)-(((4-chloromethyl)benzoyl)amino)-tetramethylrhodamine (CMTMR, Invitrogen/Molecular Probes) in RPMI-1640 containing 2% FCS for 10 to 20 minutes at 37°C then washed by centrifugation through FCS. For cell transfers, recipient mice received ~$1\text{-}2\times10^7$cells by i.v., normalised to account for T-cell number. Recipients were sacrificed at designated times.

BrdU Incorporation

BrdU (Sigma/ B.D.) was given at 0.25mg/ml in drinking water and supplemented with 1% glucose (Sigma), for upto 15 days. Cells were fixed in 0.5% paraformaldehyde, permeabilised in 3M HCl/0.5% Tween20 (for 20-min at RT), neutralised with 0.1M Borax (Sigma), washed, and stained with FITC-conjugated anti-BrdU 3D4 (B.D. Pharmingen).

Immunisation and influenza challenge experiments

For MVA immunisation or influenza challenge, mice were anaesthetised and immunised intradermally with MVA expressing green fluorescent protein at a dose of 1×10^6 PFU/mouse (2×10^7 PFU/ml) [48] or infected intranasally with 25μl of Influenza X-31 (A/Aichi/68; H3N2) at a dose of 512 HAU/ml. Mice were weighed daily as an indicator of health. Animals reaching 80% of original body weight were humanely culled. Antigen-specific cells in the draining PLNs (MVA) were identified by intracellular staining for IFN-γ (early response) or LAMP-1 (memory) after stimulation with anti-CD3 or epitopes from MVA B8R epitope (sequence TSYFKESV) or a 1μg/well of pooled 20mer peptides spanning conserved internal nucleoprotein and matrix 1 protein epitopes.

Statistical Data Analysis

Statistical comparisons were performed with Prism 4.0 Software (GraphPad Inc.), using ANOVA or two-tailed paired t-tests for comparisons.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank the staff of the Oxford Biomedical Services Unit for animal husbandry and Dr Helen Chapel, Prof. Sarah Gilbert, Prof. Adrian Hill and Dr Ronald Schwartz, for advice and reagents. This work was supported by the

Medical Research Council, NIHR Biomedical Research Centre Programme, and the Intramural Research Program of the NIH, NIAID.

Non-standard abbreviations

ARP	actin-related protein
B6	C57BL/6
DOCK	Dedicator of cytokinesis
ENU	*N*-ethyl-*N*-nitrosourea
MLN	mesenteric lymph nodes
MVA	Modified Vaccinia Ankara virus
PIP3	phosphatidylinositol (3,4,5)-triphosphate (PLNs)
PLNs	peripheral lymph nodes
SDF-1	stromal cell-derived factor-1
T_{CM}	central memory T cells
WAS	Wiskott-Aldrich Syndrome
WASP	WAS protein
WAVE	WASP-family verprolin-homologous

References

1. Zhang Q, Davis JC, Lamborn IT, Freeman AF, Jing H, Favreau AJ, Matthews HF, et al. Combined immunodeficiency associated with DOCK8 mutations. N Engl J Med. 2009; 361:2046–2055. [PubMed: 19776401]
2. Engelhardt KR, McGhee S, Winkler S, Sassi A, Woellner C, Lopez-Herrera G, Chen A, et al. Large deletions and point mutations involving the dedicator of cytokinesis 8 (DOCK8) in the autosomal-recessive form of hyper-IgE syndrome. J Allergy Clin Immunol. 2009; 124:1289–1302. [PubMed: 20004785]
3. Cote JF, Vuori K. GEF what? Dock180 and related proteins help Rac to polarize cells in new ways. Trends Cell Biol. 2007; 17:383–393. [PubMed: 17765544]
4. Meller N, Merlot S, Guda C. CZH proteins: a new family of Rho-GEFs. J Cell Sci. 2005; 118:4937–4946. [PubMed: 16254241]
5. Reif K, Cyster J. The CDM protein DOCK2 in lymphocyte migration. Trends Cell Biol. 2002; 12:368–373. [PubMed: 12191913]
6. Ruusala A, Aspenstrom P. Isolation and characterisation of DOCK8, a member of the DOCK180-related regulators of cell morphology. FEBS Lett. 2004; 572:159–166. [PubMed: 15304341]
7. Randall KL, Lambe T, Johnson AL, Treanor B, Kucharska E, Domaschenz H, Whittle B, et al. Dock8 mutations cripple B cell immunological synapses, germinal centers and long-lived antibody production. Nat Immunol. 2009; 10:1283–1291. [PubMed: 19898472]
8. Koopman G, Keehnen RM, Lindhout E, Newman W, Shimizu Y, van Seventer GA, de Groot C, et al. Adhesion through the LFA-1 (CD11a/CD18)-ICAM-1 (CD54) and the VLA-4 (CD49d)-VCAM-1 (CD106) pathways prevents apoptosis of germinal center B cells. J Immunol. 1994; 152:3760–3767. [PubMed: 7511659]
9. Fukui Y, Hashimoto O, Sanui T, Oono T, Koga H, Abe M, Inayoshi A, et al. Haematopoietic cell-specific CDM family protein DOCK2 is essential for lymphocyte migration. Nature. 2001; 412:826–831. [PubMed: 11518968]
10. Nombela-Arrieta C, Mempel TR, Soriano SF, Mazo I, Wymann MP, Hirsch E, Martinez AC, et al. A central role for DOCK2 during interstitial lymphocyte motility and sphingosine-1-phosphate-mediated egress. J Exp Med. 2007; 204:497–510. [PubMed: 17325199]

11. Okada T, Ngo VN, Ekland EH, Forster R, Lipp M, Littman DR, Cyster JG. Chemokine requirements for B cell entry to lymph nodes and Peyer's patches. J Exp Med. 2002; 196:65–75. [PubMed: 12093871]
12. Matloubian M, Lo CG, Cinamon G, Lesneski MJ, Xu Y, Brinkmann V, Allende ML, et al. Lymphocyte egress from thymus and peripheral lymphoid organs is dependent on S1P receptor 1. Nature. 2004; 427:355–360. [PubMed: 14737169]
13. Poznansky MC, Olszak IT, Foxall R, Evans RH, Luster AD, Scadden DT. Active movement of T cells away from a chemokine. Nat Med. 2000; 6:543–548. [PubMed: 10802710]
14. Diaz GA, Gulino AV. WHIM syndrome: a defect in CXCR4 signaling. Curr Allergy Asthma Rep. 2005; 5:350–355. [PubMed: 16091205]
15. Diaz GA. CXCR4 mutations in WHIM syndrome: a misguided immune system? Immunol Rev. 2005; 203:235–243. [PubMed: 15661033]
16. Gulino AV, Moratto D, Sozzani S, Cavadini P, Otero K, Tassone L, Imberti L, et al. Altered leukocyte response to CXCL12 in patients with warts hypogammaglobulinemia, infections, myelokathexis (WHIM) syndrome. Blood. 2004; 104:444–452. [PubMed: 15026312]
17. Surh CD, Sprent J. Homeostasis of naive and memory T cells. Immunity. 2008; 29:848–862. [PubMed: 19100699]
18. Schluns KS, Lefrancois L. Cytokine control of memory T-cell development and survival. Nat Rev Immunol. 2003; 3:269–279. [PubMed: 12669018]
19. Kaech SM, Wherry EJ. Heterogeneity and cell-fate decisions in effector and memory CD8+ T cell differentiation during viral infection. Immunity. 2007; 27:393–405. [PubMed: 17892848]
20. Obar JJ, Lefrancois L. Memory CD8+ T cell differentiation. Ann N Y Acad Sci. 2010; 1183:251–266. [PubMed: 20146720]
21. Lefrancois L, Obar JJ. Once a killer, always a killer: from cytotoxic T cell to memory cell. Immunol Rev. 2010; 235:206–218. [PubMed: 20536565]
22. Kaech SM, Tan JT, Wherry EJ, Konieczny BT, Surh CD, Ahmed R. Selective expression of the interleukin 7 receptor identifies effector CD8 T cells that give rise to long-lived memory cells. Nat Immunol. 2003; 4:1191–1198. [PubMed: 14625547]
23. Obar JJ, Lefrancois L. Early signals during CD8(+) T cell priming regulate the generation of central memory cells. J Immunol. 2010; 185:263–272. [PubMed: 20519649]
24. Mescher MF, Curtsinger JM, Agarwal P, Casey KA, Gerner M, Hammerbeck CD, Popescu F, et al. Signals required for programming effector and memory development by CD8+ T cells. Immunol Rev. 2006; 211:81–92. [PubMed: 16824119]
25. Xiao Z, Casey KA, Jameson SC, Curtsinger JM, Mescher MF. Programming for CD8 T cell memory development requires IL-12 or type I IFN. J Immunol. 2009; 182:2786–2794. [PubMed: 19234173]
26. Jelley-Gibbs DM, Lepak NM, Yen M, Swain SL. Two distinct stages in the transition from naive CD4 T cells to effectors, early antigen-dependent and late cytokine-driven expansion and differentiation. J Immunol. 2000; 165:5017–5026. [PubMed: 11046030]
27. Weinberg AD, Evans DE, Thalhofer C, Shi T, Prell RA. The generation of T cell memory: a review describing the molecular and cellular events following OX40 (CD134) engagement. J Leukoc Biol. 2004; 75:962–972. [PubMed: 14996827]
28. Dai Z, Konieczny BT, Lakkis FG. The dual role of IL-2 in the generation and maintenance of CD8+ memory T cells. J Immunol. 2000; 165:3031–3036. [PubMed: 10975812]
29. Bachmann MF, Wolint P, Walton S, Schwarz K, Oxenius A. Differential role of IL-2R signaling for CD8+ T cell responses in acute and chronic viral infections. Eur J Immunol. 2007; 37:1502–1512. [PubMed: 17492805]
30. Murali-Krishna K, Lau LL, Sambhara S, Lemonnier F, Altman J, Ahmed R. Persistence of memory CD8 T cells in MHC class I-deficient mice. Science. 1999; 286:1377–1381. [PubMed: 10558996]
31. Leignadier J, Hardy MP, Cloutier M, Rooney J, Labrecque N. Memory T-lymphocyte survival does not require T-cell receptor expression. Proc Natl Acad Sci U S A. 2008; 105:20440–20445. [PubMed: 19074272]

32. Schluns KS, Kieper WC, Jameson SC, Lefrancois L. Interleukin-7 mediates the homeostasis of naive and memory CD8 T cells in vivo. Nat Immunol. 2000; 1:426–432. [PubMed: 11062503]
33. Berard M, Brandt K, Bulfone-Paus S, Tough DF. IL-15 promotes the survival of naive and memory phenotype CD8+ T cells. J Immunol. 2003; 170:5018–5026. [PubMed: 12734346]
34. Scholer A, Hugues S, Boissonnas A, Fetler L, Amigorena S. Intercellular adhesion molecule-1-dependent stable interactions between T cells and dendritic cells determine CD8+ T cell memory. Immunity. 2008; 28:258–270. [PubMed: 18275834]
35. Camacho SA, Heath WR, Carbone FR, Sarvetnick N, LeBon A, Karlsson L, Peterson PA, et al. A key role for ICAM-1 in generating effector cells mediating inflammatory responses. Nat Immunol. 2001; 2:523–529. [PubMed: 11376339]
36. Parameswaran N, Suresh R, Bal V, Rath S, George A. Lack of ICAM-1 on APCs during T cell priming leads to poor generation of central memory cells. J Immunol. 2005; 175:2201–2211. [PubMed: 16081787]
37. Ye Z, Shi M, Xu S, Xiang J. LFA-1 defect-induced effector/memory CD8+ T cell apoptosis is mediated via Bcl-2/Caspase pathways and associated with downregulation of CD27 and IL-15R. Mol Immunol. 2010; 47:2411–2421. [PubMed: 20569988]
38. Hänninen A, Maksimow M, Alam C, Morgan DJ, Jalkanen S. Ly6C supports preferential homing of central memory CD8+ T cells into lymph nodes. European Journal of Immunology. 2011; 41:634–644. [PubMed: 21308682]
39. Ochs HD, Thrasher AJ. The Wiskott-Aldrich syndrome. J Allergy Clin Immunol. 2006; 117:725–738. quiz 739. [PubMed: 16630926]
40. Lutskiy MI, Sasahara Y, Kenney DM, Rosen FS, Remold-O'Donnell E. Wiskott-Aldrich syndrome in a female. Blood. 2002; 100:2763–2768. [PubMed: 12351383]
41. Kurisu S, Takenawa T. The WASP and WAVE family proteins. Genome Biol. 2009; 10:226. [PubMed: 19589182]
42. Ramesh N, Geha R. Recent advances in the biology of WASP and WIP. Immunol Res. 2009; 44:99–111. [PubMed: 19018480]
43. Meyer-Bahlburg A, Becker-Herman S, Humblet-Baron S, Khim S, Weber M, Bouma G, Thrasher AJ, et al. Wiskott-Aldrich syndrome protein deficiency in B cells results in impaired peripheral homeostasis. Blood. 2008; 112:4158–4169. [PubMed: 18687984]
44. Zhang H, Schaff UY, Green CE, Chen H, Sarantos MR, Hu Y, Wara D, et al. Impaired integrin-dependent function in Wiskott-Aldrich syndrome protein-deficient murine and human neutrophils. Immunity. 2006; 25:285–295. [PubMed: 16901726]
45. Torres E, Rosen MK. Contingent phosphorylation/dephosphorylation provides a mechanism of molecular memory in WASP. Mol Cell. 2003; 11:1215–1227. [PubMed: 12769846]
46. Okada T, Cyster JG. CC chemokine receptor 7 contributes to Gi-dependent T cell motility in the lymph node. J Immunol. 2007; 178:2973–2978. [PubMed: 17312142]
47. Pham TH, Okada T, Matloubian M, Lo CG, Cyster JG. S1P1 receptor signaling overrides retention mediated by G alpha i-coupled receptors to promote T cell egress. Immunity. 2008; 28:122–133. [PubMed: 18164221]
48. Schneider J, Gilbert SC, Blanchard TJ, Hanke T, Robson KJ, Hannan CM, Becker M, et al. Enhanced immunogenicity for CD8+ T cell induction and complete protective efficacy of malaria DNA vaccination by boosting with modified vaccinia virus Ankara. Nat Med. 1998; 4:397–402. [PubMed: 9546783]

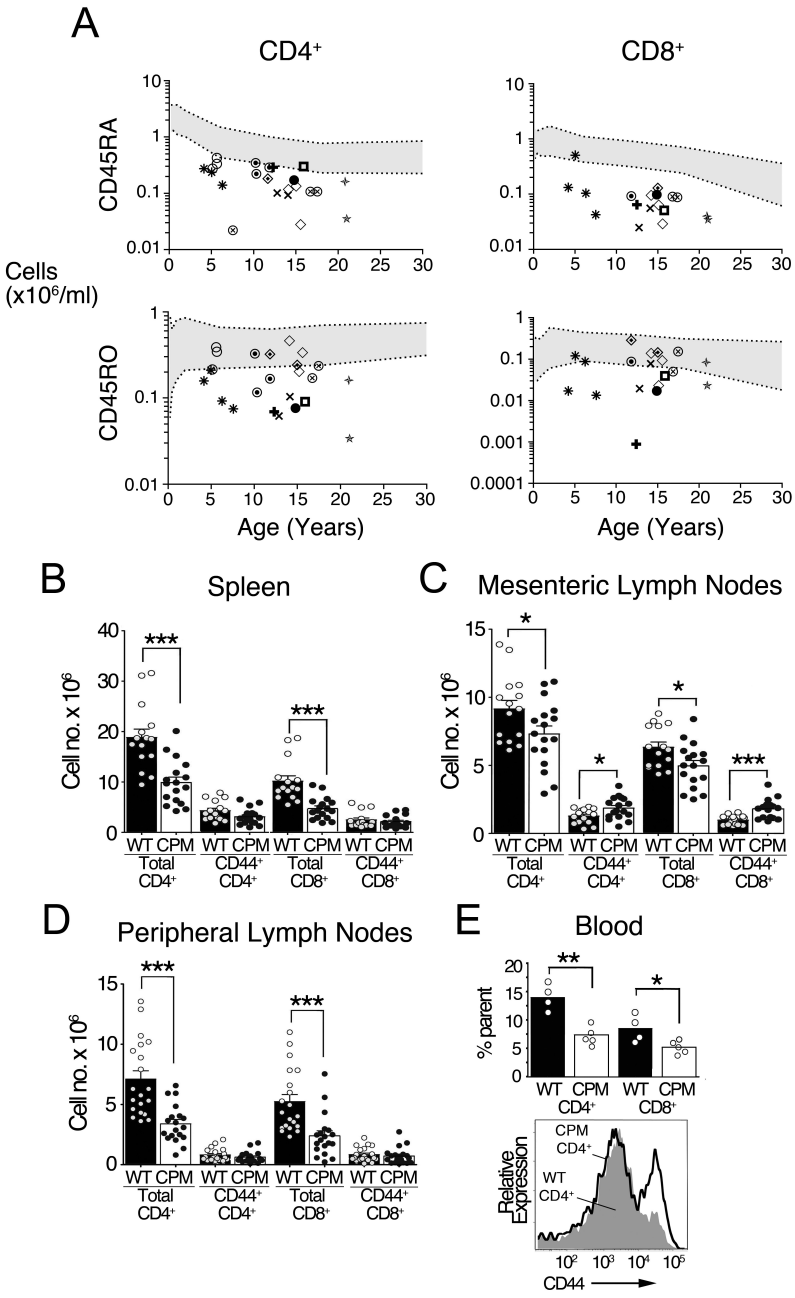

Figure 1. T-cell lymphopenia in DOCK8-deficient patients and mice

(A) Absolute numbers of total naïve (CD45RA$^+$) and effector/memory (CD45RO$^+$) CD4$^+$ and CD8$^+$ T cells in the blood of DOCK8-deficient patients. Unique symbols represent individual patients over time; grey bars show the normal 10-90 percentiles for age. (B-D) Absolute numbers of total CD4$^+$ and CD8$^+$ T-cells and activated CD44$^+$subsets in the peripheral lymphoid organs of WT and CPM mice. Dots represent individual animals aged 9-14 weeks. Bars show arithmetic means and SE. . (E) The percentage of CD4$^+$ and CD8$^+$ T cells in the blood of WT and CPM mice was calculated as a fraction of total lymphocytes (top). Histograms show typical surface CD44 expression on CD4$^+$ lymphocytes in the blood

of WT (filled histogram) and CPM mice (solid line histogram) (bottom). (B-E) *p<0.05, **p<0.01, ***p<0.001, t-test.

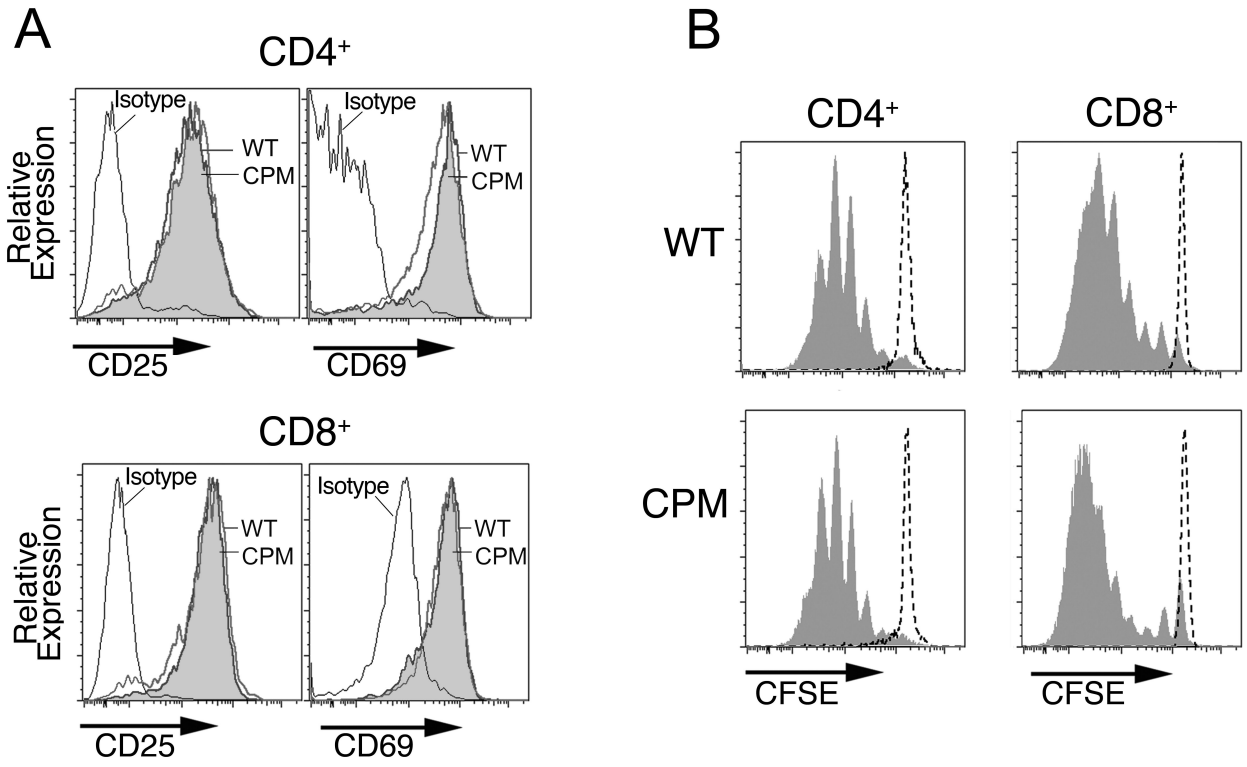

Figure 2. Normal T-cell activation and proliferation in DOCK8-deficient mice

(A) Histograms showing CD25 and CD69 expression on CPM and WT $CD4^+$ (top) and $CD8^+$ (bottom) splenocytes, stimulated overnight with plate-bound anti-CD3 (10ug/ml), and unstimulated controls (filled histograms). Analysis was gated on cells with an intact cell membrane using the LIVE/DEAD® Cell Stain, and representative of 3 experiments, with at least 3 animals. (B) Proliferation of WT (top) or CPM (bottom) $CD4^+$ or $CD8^+$ T cells (filled histograms), stimulated with 10μg/ml plate-bound anti-CD3 and 10μg/ml soluble anti-CD28 for 5 days, compared with unstimulated controls (broken line). Analysis gated as in (A). (A, B) Data are representative of at least five replicates using separate mice.

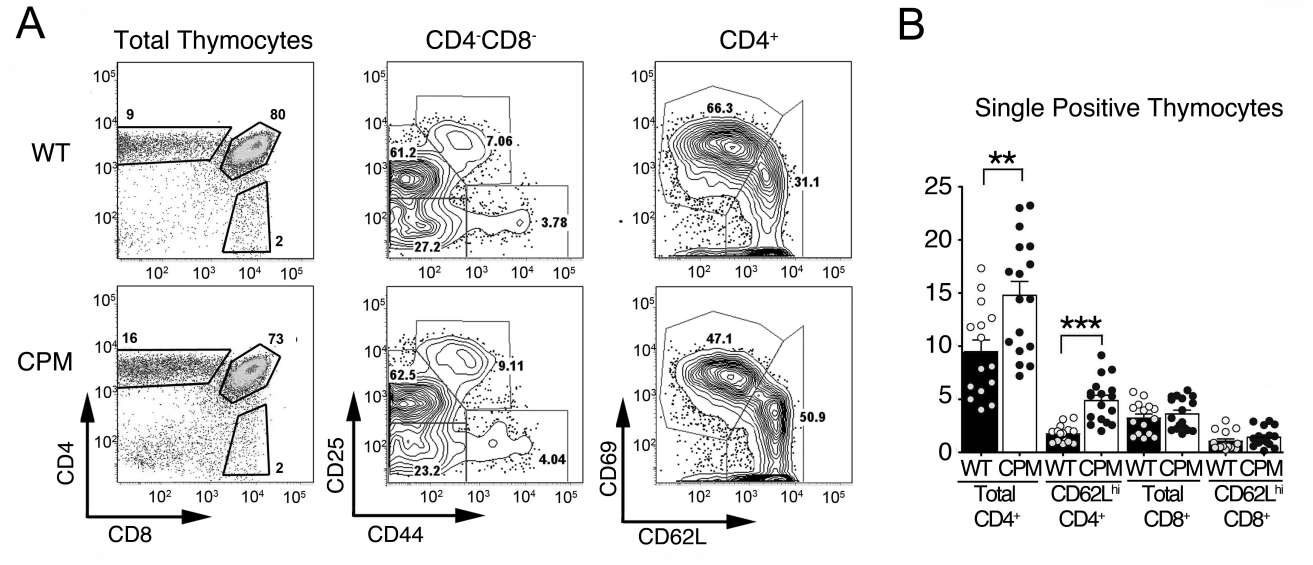

Figure 3. T-cell development and accumulation of CD4$^+$ thymocytes in DOCK8-deficient mice

(A) Representative FACS plots showing T-cell development in the thymus of WT and CPM mice: left, total thymocytes; middle, CD4$^-$CD8$^-$ double-negative cells; and right, single positive CD4$^+$ T cells. Numbers show the percentage of gated cells of parent populations. (B) Absolute number of total CD4$^+$ and CD8$^+$ single positive T cells, mature CD4$^+$ and CD8$^+$ CD62L^{hi} subsets in the thymus of WT and CPM mice. Dots represent individual animals aged 9-14 weeks. Bars show arithmetic means and SE. **p<0.01, ***p<0.001, t-test. Data are representative of at least five experiments.

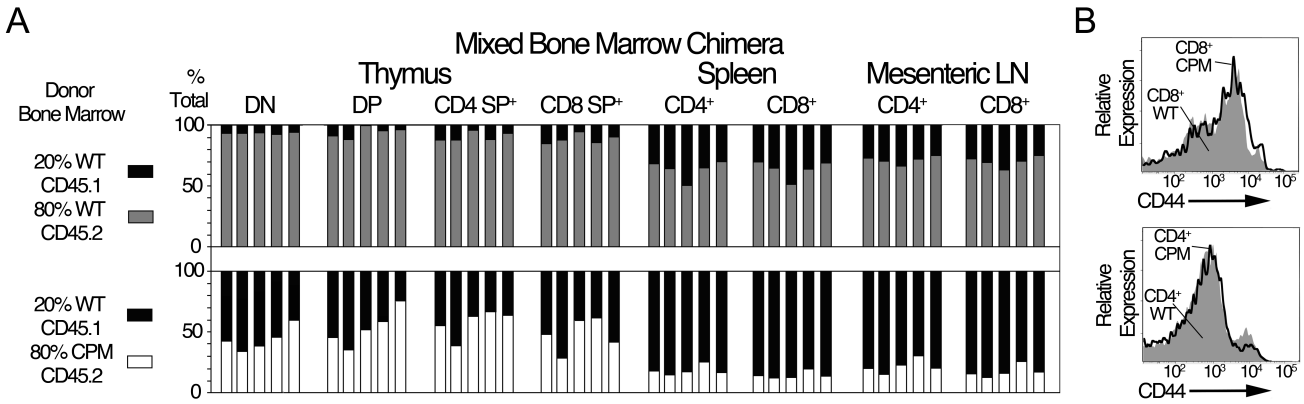

Figure 4. The T-cell lymphopenia in DOCK8 deficiency is cell-intrinsic

(A) The percentage of CD45.1 and CD45.2 T cells in thymus, spleen and MLN of mice reconstituted with an 20:80 mixture of CD45.1 WT and CD45.2 WT T cells (top) or an 20:80 mixture of CD45.1 WT and CD45.2 CPM T cells (bottom). Bars represent individual chimeric mice. (B) Histograms showing typical surface CD44 expression on WT (filled) and CPM (solid black line) $CD4^+$ (top) and $CD8^+$ (bottom) T cells from mixed chimeras. Data are representative of at least three experiments.

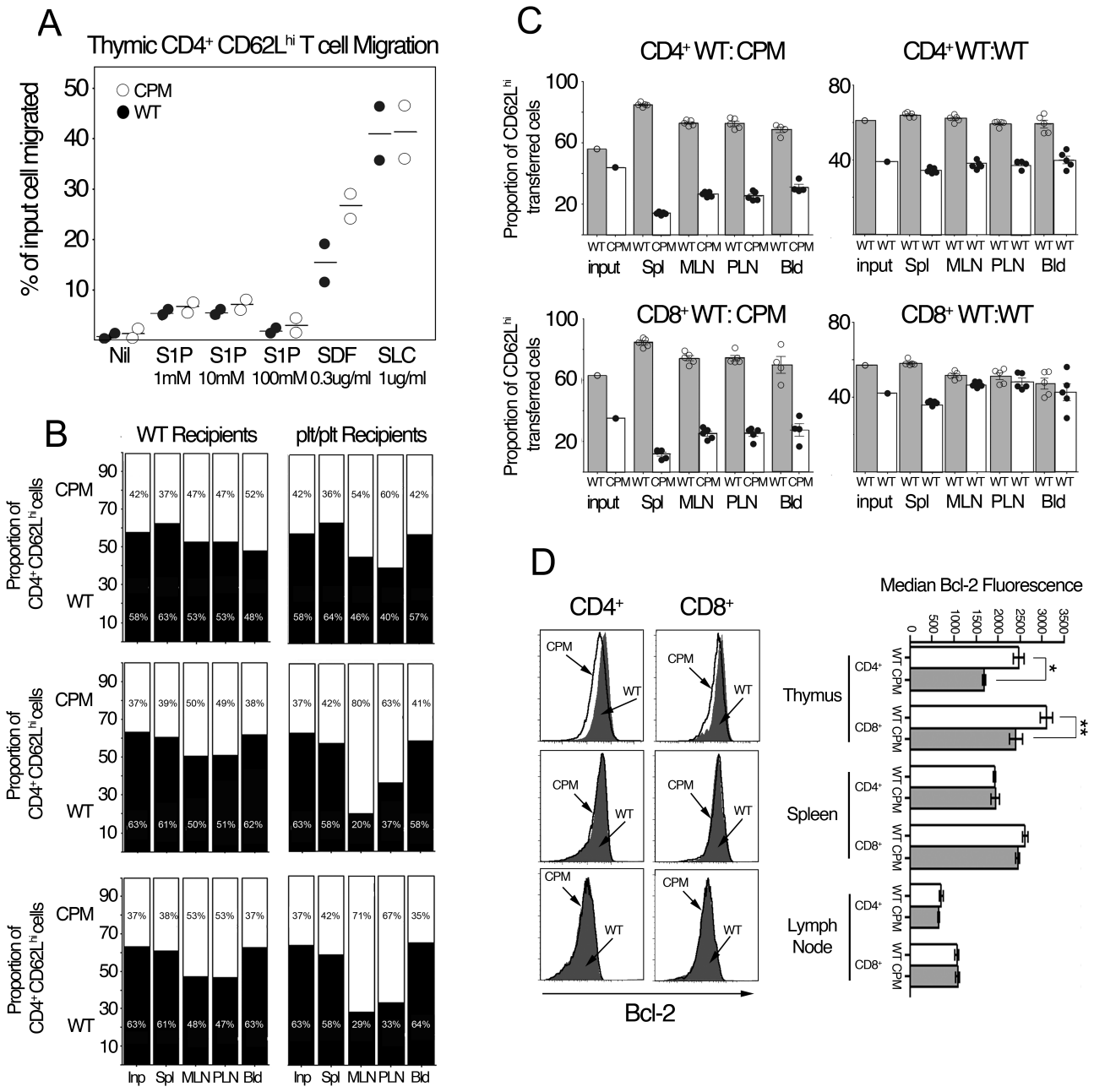

Figure 5. The migration and survival of early naïve T cells in the absence of DOCK8

(A) Transwell migration of mixed CD4^{+}CD62L^{hi} thymocytes from CPM (CD45.2) and WT (CD45.1) in response to various chemoattractants at indicated concentrations. Data are presented as mean of n=2 and are representative of at least 3 experiments. (B) Co-transfer for 90 min of 1×10^{6} fluorescently labelled thymocytes from CPM (CD45.2) and WT (CD45.1) into WT or *plt/plt* recipient mice. Data show proportions of CD4^{+}CD62L^{hi} cells at input (inp), and after transfer in spleen (spl), MLN, PLNs and blood: each bar represents an individual mouse. (C) Co-transfer for 24hr of fluorescently labelled thymocytes from WT or CPM (CD45.2) and WT (CD45.1) mice into WT recipients, adjusted so approximately equal numbers of mature (CD62L^{hi}) single positive T cells were transferred. Data show

proportions of $CD4^+CD62L^{hi}$ cells at input (inp), and after transfer in spleen (spl), MLN, PLNs and blood (Bld). Data are presented as mean + SEM of n=4-5 and are representative of at least three experiments. (**D)** Intracellular Bcl-2 expression on total $CD4^+$ and $CD8^+$ T cells in the thymus, spleen and lymph nodes of WT and CPM mice shown as representative histograms (left) and median fluorescence (right), where n=4-5, columns are means, and bars are SE. * $p<0.05$, ** $p<0.001$, t-test.

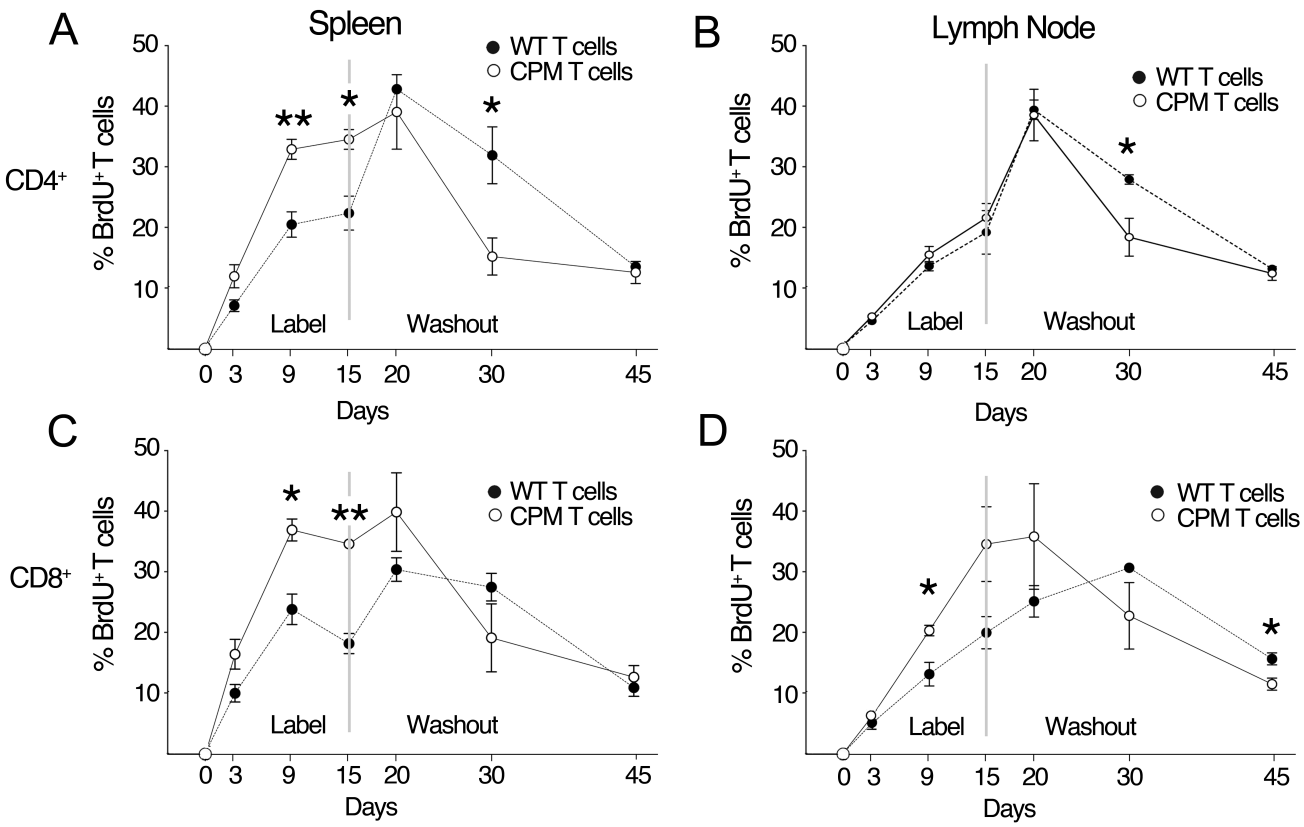

Figure 6. Decreased survival of DOCK8-deficient peripheral CD4$^+$ and CD8$^+$ T cells

(A-D) The percentage of BrdU labelled CD8$^+$ and CD4$^+$ T cells in the spleens and pooled mesenteric and peripheral lymph nodes (LNs) of CPM and WT mice fed BrdU for up to 15 days. Cohorts of 3-5 mice were analysed in each group at day 3, 9, 15, 20, 30 and 45. Data shows means and SE. * $p<0.05$, ** $p<0.01$, t-test. Data are representative of one experiment with n of 2-3 per timepoint

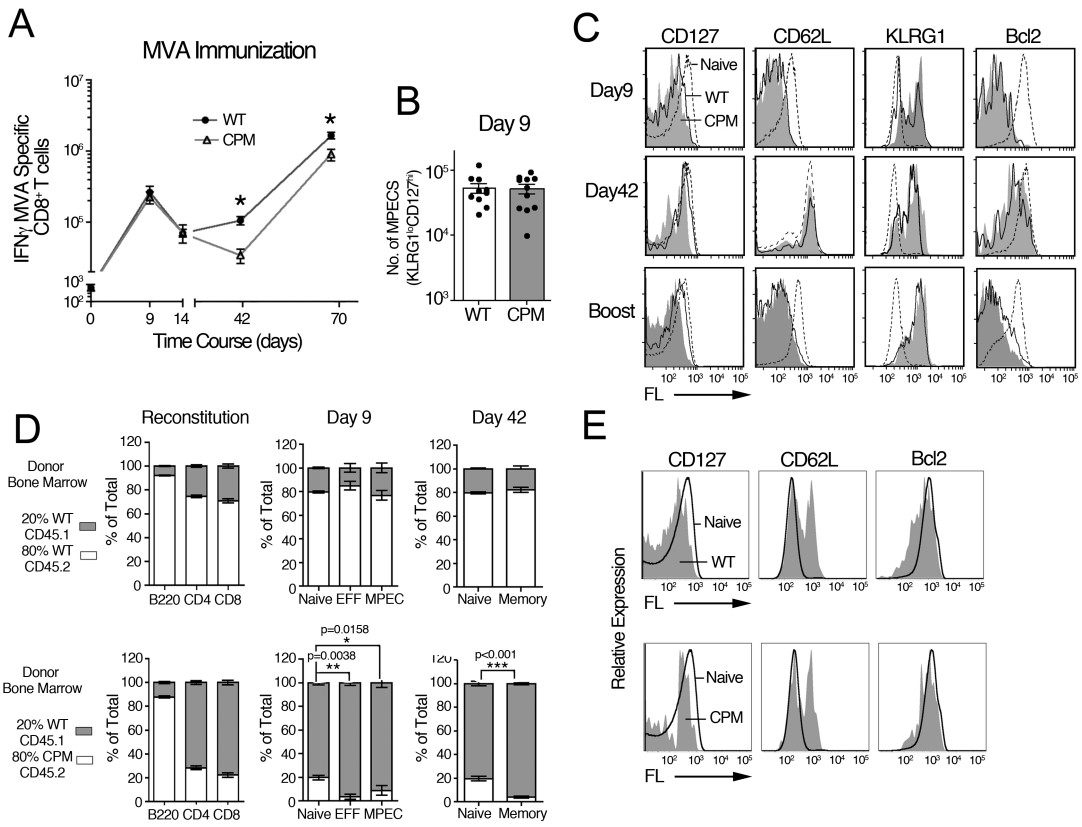

Figure 7. DOCK8-deficient mice mount normal anti-viral responses but fail to sustain memory

(A) Numbers of LIVE/DEAD® negative splenic $CD8^+$ T cells secreting IFN-γ in response to the MVA specific peptide B8R taken at various time points before and after primary and secondary immunisation of mice with intradermal MVA. WT, circles and CPM, triangles. Data show means + SEM of data combined from 3 experiments where n>4. * $p<0.05$, t-test; however there is no statistical difference in fold change between the memory response at day 42 and the boost at day 70 comparing WT (Mean 15.83 +/− S.E 1.81) and CPM (Mean 26.22 +/− S.E 4.98) (B) Numbers of memory precursor cells (defined as $KLRG1^{low}$ $CD127^{hi}$) at day 9 post-immunisation from (A). Data are presented as means and SE and are representative of three experiments with n=5 samples per time point. (C) CD127, CD62L, KLRG1 and Bcl-2 expression on B8R-responsive IFN-γ-secreting $CD8^+$ splenic T cells from WT (solid black line) and CPM (grey-filled histogram) mice at day 9 and day 42 post immunization and after rechallenge. Dotted line shows staining on naïve non-responding cells. (D) The percentage of CD45.1 and CD45.2 B8R specific $CD8^+$ cells in the spleen of chimeric mice reconstituted with an 20:80 mixture of CD45.1 WT and CD45.2 WT T cells (top) or CD45.1 WT and CD45.2 CPM T cells (bottom). Left panel shows $B220^+$, $CD4^+$ and $CD8^+$ populations in blood of recontituted animals two weeks preimmunization; right panels show B8R antigen-specific $CD8^+$ T cells from animals culled 9 days and 42 days post immunization with the percentage of 'naïve' non-responding $CD8^+$ T cells for comparison. Effector (EFF,) and memory precursor (MPEC) are defined as $KLRG1^{hi}$ $CD127^{low}$ and $KLRG1^{low}$ $CD127^{hi}$ respectively. B8R specific $CD8^+$ T cells at day 42 are defined as 'Memory'. Data are presented as means + SE of n=5 samples with statistical values determined by t-test. **(E)** Histograms showing expression of CD127, KLRG1 and Bcl2 on 'Memory' cells on CD45.2 WT (top, filled histogram) and CD45.2 CPM (bottom, filled)

compared with CD45.2 ‘Naïve’ cells (solid black line). C-E Data from one experiment with n=5 samples.

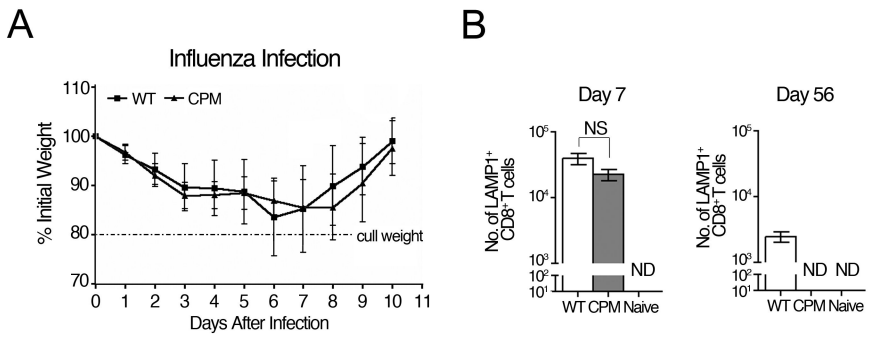

Figure 8. DOCK8-deficient mice survive live influenza challenge with loss of memory CD8$^+$ responses

(A) Weight loss and recovery in CPM and WT mice infected with influenza strain X-31 (n=12 in each group). (B) The ex vivo influenza X-31 antigen-specific response in CPM and WT mice from (A), showing numbers of LAMP1^{hi+} CD8$^+$ T cells 7 days (left) and LAMP1$^+$ at 8 weeks (right). Numbers are corrected for background levels observed in unstimulated controls, (ND, not detectable.) Data are presented as means and SE and are representative of two experiments.