Regulation of CD8 T cell memory by ADAP

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ABSTRACT

During acute infections, naïve antigen-specific CD8 T cells are activated and differentiate into effector T cells, the majority of which undergo contraction after pathogen clearance. A small population of CD8 T cells survives the contraction phase and persists as memory, to protect against future infections. Memory CD8 T cells are heterogeneous and can be found in secondary lymphoid organs (SLOs), blood and non-lymphoid tissues (NLTs). Here I demonstrate the adaptor protein ADAP enhances the formation of memory CD8 T cells in both SLOs and NLTs after pathogen challenge. ADAP-deficient memory CD8 T cells in SLOs proliferate robustly to a systemic secondary challenge. Additionally, ADAP-deficient resident memory CD8 T cells are functional in response to local peptide challenge, but only when in the presence of wild-type antigen-specific T cells. In the absence of an infection, memory-like or memory phenotype (MP) CD8 T cells can arise from homeostatic cytokine exposure during lymphopenia. In contrast to the role of ADAP after pathogen challenge, I have identified a negative regulatory role for ADAP in the formation of MP CD8 T cells in the steady state. Naïve ADAP-deficient CD8 T cells are hyperresponsive to lymphopenia *in vivo* and exhibit enhanced activation of STAT5 and homeostatic antigen-independent proliferation *in vitro* in response to IL-15. My results indicate that ADAP dampens naïve CD8 T cell responses to lymphopenia and IL-15, and demonstrates a novel antigen-independent function for ADAP in the suppression of MP CD8 T cell generation. These findings contribute to our knowledge of the generation of different memory CD8 T cell populations, and we hope to augment vaccine efficacy and better understand the formation and maintenance of memory CD8 T cells.

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INTRODUCTION

The adaptive immune system protects the body against specific pathogens. The two main arms of the adaptive immune system are T cells and B cells. T cells undergo a rigorous selection process in the thymus before entering the periphery to protect the host. T cells exit the thymus in a naïve state, and cannot perform protective functions until they are licensed by a professional antigen presenting cell (APC), such as a dendritic cell (DC). A naïve CD8 T cell must receive three signals before gaining effector functions: 1) TCR stimulation by cognate antigen (Ag) displayed on MHC-I; 2) costimulation, and 3) an inflammatory cue, such as IL-12 or type I interferon (IFN) [1, 2]. After stimulation, Ag specific T cells undergo a robust expansion, culminating at the peak of the response which often coincides with clearance of the pathogen [3]. A rapid contraction phase ensues, wherein all but 5-10% of the peak population dies [3]. The remaining population is maintained in the host as memory to protect against further infections.

A diverse population of memory cells exists after response to a pathogen [3]. The host generates memory cells with different survival and functional capabilities, balancing the long term survival of responding cells against the potential to proliferate and rapidly respond to a secondary infection [3]. A pivotal study created a paradigm for the field of memory T cells [4]. Central memory (T_{CM}) CD8 T cells with high longevity and proliferation potentials, patrol secondary lymphoid organs (SLOs), ready to expand in number to fight off invaders [3, 4]. Effector memory (T_{EM}) CD8 T cells with cytolytic

activity and cytokine production capacity, patrol non-lymphoid tissues and quickly kill pathogens and alert the rest of the immune system [3, 4]. While the T_{CM}-T_{EM} paradigm has assisted the field in understanding the localization and function of memory CD8 T cells, there are limits to this model, as a third population of resident memory (T_{RM}) CD8 T cells remains in one organ, protecting against the initial entry of pathogens [5, 6]. T_{RM} cells are transciptionally distinct from T_{CM} and T_{EM} cells [6]. A memory-like population can be generated when naïve CD8 T cells bypass the traditional three signal requirement in the absence of infection in instances of low T cell numbers [7]. These memory phenotype (MP) CD8 T cells can protect a neonatal host before the adaptive immune system has generated an immune response [8, 9].

Many molecules are known to influence and regulate the CD8 T cell immune response and dictate what type of memory cells will be generated. This introduction will summarize our current knowledge concerning memory CD8 T cell populations and the emerging role of adhesion and degranulation-promoting adaptor protein (ADAP) as a positive and negative regulator of memory generation.

MEMORY PHENOTYPE CD8 T CELLS

Memory phenotype (MP) CD8 T cells are memory-like CD8 T cells that can be generated by naïve CD8 T cells in the absence of an infection [10]. These cells have similar functions as bona fide memory cells [7], and are thought to protect the host early during an infection before the adaptive immune response can be mounted [7, 11, 12]. MP CD8 T cells can be generated in the thymus [13], or by extra-thymic means through: 1) exposure to the homeostatic cytokines IL-7 and IL-15; 2) self-reactivity; and 3) absence of negative signals [7].

Thymic generation of memory phenotype CD8 T cells

During T cell development in the thymus, $CD4^+$ $CD8^+$ double positive (DP) thymocytes are tested for their ability to bind self-peptide MHC ligands [14]. Thymocytes with high affinity for self-peptide MHC ligands are eliminated by negative selection [14]. Thymocytes that have negligible affinity for self-peptide MHC ligands undergo 'death by neglect' [14]. Only a small percentage of thymocytes with weak affinity for self-peptide MHC initiate weak TCR signaling and survive by positive selection [14]. Positively selecting DP thymocytes are MHC-restricted and downregulate either CD4 or CD8 to become CD4 or CD8 SP thymocytes which can finish maturation and egress to the periphery.

A small percentage of DP thymocytes do not develop into CD4 or CD8 SP thymocytes, these cells instead develop into an innate T cell subset [15]. Innate T cells are T cells that develop in the thymus, but do not recognize classical MHC-I or MHC-II molecules [15]. Innate T cells develop with activated or memory phenotype characteristics, such as the capacity for rapid IFN-γ production [15]. Examples of innate T cells are: CD1-specific $\alpha\beta$ invariant natural killer T (iNKT) cells, $\gamma\delta$ lineage $NK1.1^+$ NKT cells and $CD8\alpha\alpha^+$ intra-epithelial T cells [13, 15]. In some instances, an overabundance of innate T cells develop and can induce CD8 SP thymocytes to become MP CD8 T cells.

Thymic generation of MP CD8 T cells was initially discovered in mice that lack expression of the Tec family kinase, inducible T cell kinase (Itk) [13, 15]. Itk-deficient CD8 SP thymocytes exhibited memory-like T cell functions, such as the rapid production of IFN-γ after TCR stimulation and expressed surface molecules consistent with memory cells, $CD44^{hi}$ $CD122^{hi}$ [13, 15]. Later studies identified MP CD8 SP thymocyte

generation in the absence of the transcription factor Krüppel-like factor 2 (KLF2) [16]. Experiments using mixed bone marrow chimeras (BMCs) determined that the development of thymic MP CD8 T cells was not due to the lack of KLF2 in the developing CD8 SP thymocytes, but to the environment generated by a distinct population of KLF2-deficient thymocytes [16]. The MP CD8 T cell phenotype was traced to an expanded population of promyelocytic leukemia zinc finger protein (PLZF) expressing thymic NKT cells in the absence of KLF2 [16, 17]. PLZF is expressed by NKT2 and NKT17 subsets of iNKT cells [18]. $PLZF^+$ NKT2 cells produce IL-4, which at higher concentrations can induce CD8 SP thymocytes to convert to MP CD8 T cells [16-18]. Thus, in the absence of KLF2 or Itk, greater frequencies of $PLZF^+$ NKT cells result in a higher concentration of IL-4 in the thymus, resulting in the generation of MP CD8 T cells [13].

The exact mechanism of how Itk- and KLF2-deficiencies result in the accumulation of thymic PLZF+ NKT cells is still unclear [13]. It has been proposed that reduced pre-TCR signaling in Itk-deficient $CD4^-$ $CD8^-$ double negative (DN) thymocytes may promote selection of $\gamma\delta$ NKT cells over selection of conventional $\alpha\beta$ T cells [13, 15]. TCR signaling induced by non-classical MHC molecules may be attenuated in the absence of Itk, allowing for the positive selection of $\gamma\delta$ NKT cells that would have been negatively selected in the presence of Itk [13, 15]. Not surprisingly, mice with a mutation in Src homology 2 domain-containing leukocyte phosphoprotein of 76 kDa (SLP-76:Y145F) phenocopy Itk-deficient mice, as Itk binds Y145 on SLP-76 [13]. In contrast, other mouse models lacking TCR signaling molecules, such as guanine nucleotide exchange factor (GEF) Vav1 [19] and non-catalytic region of tyrosine kinase (Nck) [20], increased frequencies of $\gamma\delta$ NKT, iNKT, or MP CD8 T cells have not yet been identified. Additionally, KLF2 can suppress c-Myc expression, which is required for iNKT

proliferation [13]. In the absence of KLF2, $PLZF^{+}$ iNKT cells may have increased survival or proliferation capacities [13]. It is likely that additional proteins that regulate TCR signaling, T cell proliferation and survival will be found to regulate innate T cell and CD8 MP development.

Extrathymic generation of memory phenotype CD8 T cells

CD4 and CD8 SP T cells that have survived thymic development exit the thymus and circulate in the blood, scanning SLOs for cognate antigen [21]. T cells that have recently exited the thymus are termed recent thymic emigrants (RTEs) and compete with existing T cells in SLOs for survival signals [21]. Naïve CD8 T cells require self-peptide MHC-I and IL-7 signaling to survive, and additional IL-15 signaling is needed for optimal survival [22]. RTEs and naïve T CD8 cells must also compete with memory T cells for survival signals. Unlike naïve CD8 T cells, memory CD8 T cells do not require MHC-I signals for survival, but do require IL-7 or IL-15 signaling [23].

During the neonatal period, RTEs that enter the periphery have little competition for self-peptide MHC-I or IL-7 [10]. Under conditions of low T cell numbers, there is an increased availability of self-peptide MHC-I, IL-7 and IL-15, inducing naïve T cell proliferation, expansion and differentiation into MP CD8 T cells [10]. This phenomenon is termed lymphopenia induced proliferation (LIP) [12, 14]. Neonatal lymphopenia is the primary generator of MP CD8 T cells [8]. In addition to neonatal lymphopenia, acute lymphopenic environments can result after chemotherapy, late-stage HIV infection and exposure to radiation [11].

Thymic education, rendering T cells tolerant to self-antigens, largely prevents naïve T cell reactivity to self-tissues, though tonic self-MHC signaling is required for

naive T cell survival [14]. In a healthy adult unimmunized mouse, 10-30% of foreign Ag-specific CD8 T cells are MP CD8 T cells [8, 9]. This same frequency of MP CD8 T cells can be found in germ free mice [8]. TCR-transgenic (TCR-Tg) lines with a fixed TCR on a recombination activating gene (RAG) deficient background, which prevents TCR rearrangement, also demonstrate MP CD8 T cells [9]. These results indicate the majority of MP CD8 T cells are generated from self-peptide MHC-I interactions and not environmental antigens. Mutations in molecules involved in the positive regulation of TCR signaling, such as Vav1 [19] and Nck [20] decrease the survival of naïve T cells [7]. Thus, a low level of signaling in response to self-peptide MHC-I must be maintained for naïve T cell survival. In some instances, likely due to local increases in homeostatic cytokines, a small population of cells convert to MP CD8 T cells.

A process of TCR 'tuning' modulates the intensity of TCR signaling to prevent naïve T cells from being activated without proper costimulatory signaling [7]. This process occurs during thymic development [7] and continues as RTEs mature in the periphery [24]. TCR tuning involves the upregulation of molecules that limit TCR responsiveness [7], such as cbl-b [25], PTPN2 [26] and PTPN22 [27]. Cbl-b negatively regulates Vav1 phosphorylation and TCR signaling [25]. In the absence of cbl-b, T cells are more self-reactive and more sensitive to TCR stimulation, resulting in autoimmunity [25]. PTPN2 is also a phosphatase, which dephosphorylates Src family protein tyrosine kinases (SFK) Lck and Fyn. SFK initiate TCR signaling cascades [28]. Mutations in PTPN2 can lead to widespread inflammation and autoimmunity [28]. In the absence of PTPN2 naïve CD8 T cells undergo greater LIP, but IL-7 and IL-15 signaling is not altered [26]. Instead, increased LIP is due to altered TCR signaling in the absence of PTPN2 [26, 28]. A similar mechanism occurs in mice lacking the tyrosine phosphatase PTPN22, where T cells are more responsive to weak agonist peptide ligands [27]. Greater T-APC

adhesion and inflammatory cytokine production occurs in the absence of PTPN22 with weak agonist ligands [27]. In addition, mutations in PTPN22 increase the risk for development of autoimmune diseases [27]. Finally, studies under lymphopenic conditions have revealed higher affinity TCR-Tg cells undergo more robust proliferation than lower affinity TCR-Tgs [29]. Thus, negatively regulating tonic TCR signaling maintains self-peptide MHC-I tolerance and T cell homeostasis without allowing for T cell-mediated autoimmunity.

Alterations in cytokine signaling pathways can also result in increased MP CD8 T cell generation. The latter occurs in mice that lack suppressor of cytokine signaling-1 (SOCS-1), where both naïve and MP CD8 T cells are hyperresponsive to IL-15, resulting in robust proliferation, MP generation and neonatal mortality [30, 31]. The SOCS-1 protein binds directly to JAKs to inhibit tyrosine-kinase activity and promotes ubiquitin-mediated degradation, resulting in attenuated IL-15 signaling [32]. A similar mechanism occurs in the absence of tumor growth factor-β receptor II (TGF-βRII) signaling [33]. Deletion of TGF-βRII late in T cell development results in greater frequencies of MP CD8 T cells and increased LIP after transfer of naïve CD8 T cells into lymphopenic hosts [33]. Additionally, TGF-βRII deficient naïve CD8 T cells are more responsive to weak antigens, indicating a role for TGF-β signaling in maintaining tolerance to TCR signaling in response to self-peptide MHC-I [33]. Cytokine signaling is tightly regulated for T cell survival, and alterations in that balance can be lethal for the host.

Memory phenotype CD8 T cell generation is negatively regulated by ADAP

T cell homeostasis is carefully balanced in a healthy host to maintain a diverse T cell repertoire against potential foreign pathogens without using up essential host

resources. While we know of a few factors that promote the thymic and extra-thymic generation of MP CD8 T cells, additional molecular regulators that control the reactivity to MHC-I and homeostatic cytokines for MP generation have yet to be identified. Work completed for my thesis identified ADAP as a negative regulator of naïve CD8 T cell responses to weak agonist peptides and IL-15; this work will be discussed in detail in Chapter 2. My findings support the suppression of an extra-thymic generation of MP CD8 T cells by ADAP, primarily occurring in the neonatal period of lymphopenia, due to an suppression of IL-15 signaling.

ADHESION AND DEGRANULATION-PROMOTING ADAPTOR PROTEIN (ADAP)

Adaptor proteins lack enzymatic function and organize molecular signaling complexes via-protein binding domains to elicit downstream effects. ADAP is a hematopoietic-restricted cytosolic molecule that has been characterized in multiple signaling pathways. ADAP was originally identified though its association with SLP-76 [34] and Fyn [35]. In addition to these associations ADAP can bind numerous partners with roles in hematopoietic cell signaling, including: Src kinase-associated phosphoprotein of 55 kDa (SKAP55) [36], caspase recruitment domain (CARD) membrane-associated guanylate kinase (MAGUK) protein 1 (CARMA-1) [36], transforming growth factor-β (TGF-β)-activated protein kinase (TAK-1) [36], Wiscott-Aldrich syndrome protein (WASp) [37, 38] and Nck [39]. In primary T cells, ADAP is a positive regulator of T cell signaling following Ab-mediated TCR stimulation or cognate-peptide MHC-II stimulation [40]. ADAP promotes T-APC interactions and T cell activation [36].

Hematopoietic expression pattern of ADAP

The expression of ADAP is restricted to cells of hematopoietic origin. ADAP mRNA been detected in T cells, natural killer (NK) cells and myeloid cells, including macrophages and DCs [35, 41, 42] . The earliest progenitor cell with verified ADAP expression is the common lymphoid progenitor (CLP) [41]. In the bone marrow ADAP is expressed in pre-pro B cells and pro-B cells, but is not expressed in pre-B cells or mature B cells [35, 41]. Both myeloid and erythroid lineage cells express ADAP in the bone marrow, but mature red blood cells do not have detectable levels of ADAP [41]. In contrast, platelets do express ADAP [43].

The functions of ADAP have been most thoroughly studied in T cells. Upon entry into the thymus, T cell progenitors express detectable levels of ADAP [41]. ADAP is dynamically regulated throughout T cell development [41] and expression is required for both positive and negative selection [44]. In addition to DN, DP, CD4 SP and CD8 SP thymocytes, ADAP is also expressed in unconventional thymocytes: NKT, CD8$\alpha\alpha$ and $\gamma\delta$ TCR T cells [41]. Whereas ADAP is required for conventional $\alpha\beta$ TCR T cell development, ADAP is not required for the development of unconventional thymocytes [41, 44]. ADAP is expressed in naïve mature CD4 and CD8 T cells in SLOs, and is upregulated with TCR-signaling [41].

ADAP couples TCR signaling to integrin activation

Naïve T cells circulate throughout the body, visiting SLOs to receive survival signals and increase the probability of contacting cognate antigen. Upon cognate antigen stimulation, T cells rapidly activate $\alpha_L\beta_2$ integrins to bind intercellular adhesion molecule 1 (ICAM-1) on the surface of the antigen-presenting cell (APC). Integrin-ICAM

interactions promote stable T-APC interaction [45]. Integrins are heterodimeric membrane spanning proteins that facilitate cell-extracellular matrix (ECM) and cell-cell interactions [46, 47]. TCR-mediated integrin activation is dependent on the transmission of TCR-proximal signaling events to larger signaling complexes that assemble at the intracellular tails of integrins [45]. Binding of signaling complexes to $\alpha_L\beta_2$ integrin tails induces a conformational change that increases the affinity of the integrin for its ligand, ICAM-1, termed “inside-out” signaling [46]. The site of interaction between the T cell and APC is termed the immunological synapse (IS) [47]. The center of the IS contains TCR molecules, while the periphery is rich in activated $\alpha_L\beta_2$ integrins [47]. The arrangement of the IS requires both integrin activation and cytoskeletal rearrangement [48]. Activated integrins stabilize the adhesion between the T cell and APC by connecting to the actin cytoskeleton [45].

ADAP enhances T-APC interactions by coupling TCR stimulation to the activation of integrins via the interaction of ADAP with SKAP55 [36]. After TCR stimulation, multiple tyrosine residues on ADAP are phosphorylated by Fyn, creating binding sites for various ADAP-binding proteins, such as SLP-76 and Nck [36]. The majority (70%) of ADAP is constitutively associated with SKAP55 [43]. A second pool of ADAP, not associated with SKAP55, can be induced to interact with CARMA-1 and TAK-1 after TCR signaling [36]. Early steps of TCR-mediated inside-out signaling involve the binding of SLP-76 to the ADAP-SKAP55 signaling module [36]. After TCR stimulation, signaling complexes containing SLP-76 form on the inner leaflet of the plasma membrane [45]. ADAP is recruited to these signaling complexes after being phosphorylated by Fyn, which creates binding sites for SLP-76 [36]. The R131 residue in the PH domain of SKAP55 is required for the association of SKAP55 with the inner

leaflet of the plasma membrane, positioning the ADAP-SKAP55-signaling module near integrin tails [49].

Later steps for TCR-mediated inside-out signaling to integrins involve the association of signaling complexes to integrin cytoplasmic domains and integrin activation [45]. The small GTPase Rap1 regulates integrin function [45]. Rap1 is recruited to integrin tails by protein kinase D (PKD), regulator for cell adhesion and polarization enriched in lymphoid tissues (RapL) and Rap1 interacting adapter molecule (RIAM) [36, 45]. SKAP55 binds directly to RapL [50], and constitutively associates with RIAM [51]. Both ADAP-deficient and SKAP55-deficient naïve mature T cells demonstrate T-APC conjugate defects and reduced recruitment of the ADAP-SKAP55-signaling module to integrin tails *in vitro* [36, 52]. In addition, ADAP-deficient CD4 T cells exhibit reduced T-DC contact stability and duration *in vivo* [53]. Thus, the ADAP-SKAP55 signaling module facilitates the formation of signaling complexes at the tails of integrins after TCR-stimulation, resulting in stable, long-lived T-APC interactions.

Connection of integrin activation to the reorganization of the actin cytoskeleton further stabilizes T-APC adhesion [45]. Activated integrins are coupled to the actin cytoskeleton through talin. The cytoplasmic tail of β_2 integrin directly interacts with N-terminus of talin, while the C-terminus of talin can bind actin [45]. The interaction between talin and integrin tails is regulated by Rap1 [45]. As discussed earlier, the ADAP-SKAP55 signaling module is required for optimal Rap1 recruitment to integrin tails [50, 51, 54], thus linking ADAP to actin rearrangement.

Dynamic changes in the actin cytoskeleton also occur after TCR stimulation. Actin reorganization is initiated by nucleation factors including actin-related protein 2/3 (Arp2/3) activating protein [55]. The Arp2/3 complex can initiate new branches of actin from preexisting filaments [55]. After TCR stimulation phosphorylated SLP-76 binds

Vav1 and Nck [55]. Vav1 activates Rho family GTPases, Cdc42 and Rac1, which together with WASp, promote Arp2/3 actin nucleation [55]. WASp is recruited by the joint action of Nck and ADAP [37]. The larger molecular complex formation between SLP-76, Nck, ADAP and WASp is required for regulating actin rearrangement, and localizes at the T-APC interface [37, 38]. This role for ADAP is further supported by work in macrophages. ADAP colocalizes with WASp, Nck and SLP-76 at actin rich sites of phagocytosis in macrophages [56]. These findings are in contrast to reports where actin polymerization was normal after TCR stimulation [57, 58]. The involvement of ADAP in directly regulating the actin cytoskeleton after TCR stimulation remains controversial.

Chemokine signaling to integrin inside-out activation is dependent on ADAP

Only select cells of the immune system have the capacity to enter SLOs. High endothelial venules (HEVs) are a specialized vascular endothelium present in lymph node paracortical regions through which naïve T cells enter SLOs [5, 46]. The entry of T cells into SLOs is facilitated by the interaction of T cell surface molecules with HEV ligands and occurs in four major steps: 1) rolling; 2) activation; 3) firm adhesion; and 4) transmigration [46]. First, CD62L on the surface of naïve T cells interacts with peripheral node addressins (PNAds) on the surface of HEVs, causing the T cell to roll along the vascular endothelium [5, 46]. Rolling along the HEV increases the probability that the T cell CC-chemokine receptor 7 (CCR7) will encounter CC-chemokine ligand 21 (CCL21) displayed on the HEV [46]. CCR7 stimulation will initiate a signaling cascade in the T cell, activate $\alpha_L\beta_2$ integrin and facilitate $\alpha_L\beta_2$ binding to HEV expressed ICAM-1 [46] . Active $\alpha_L\beta_2$ integrin adhesion initiates firm adhesion of the T cell to the surface of the

HEV [5, 46]. After stopping, the T cell transmigrates thorough the endothelium into the paracortial region of lymph node [5, 46].

The ADAP-SKAP55-signaling module has also been implicated in chemokine-integrin inside-out signaling of T cells. Separate pools of the ADAP-SKAP55-signaling module bind to α_L and β_2 integrins [52]. One complex contains the ADAP-SKAP55-signaling module bound with RIAM, Rap1 and Talin and is associated with the cytoplasmic tail of β_2 integrin [52]. The second ADAP-SKAP55-signaling module associates with RAPL and Rap1 and is bound to α_L [52]. Reduced T cell adhesion to ICAM-1 was observed after CCL21 stimulation in the absence of ADAP [52]. Thus, ADAP is involved in chemokine to integrin inside-out signaling.

Additional inside-out signaling pathways mediated by ADAP

Platelets can perform inside-out signaling via pathways other than those initiated by the TCR and chemokine stimulation [59]. Platelet GP Ib-IX-V, a complex of 4 transmembrane polypeptides, can initiate signaling after binding von Willebrand factor (VWF) and lead to the activation of the integrin $\alpha_{IIb}\beta_3$ [59]. GP Ib-IX-V is required for the capture of platelets on the vessel wall under shear stress [59]. Downstream signaling of GP Ib-IX-V is required for stable platelet adhesion and aggregation [59]. Tyrosine phosphorylation of ADAP occurs after GP Ib-IX-V signaling [60]. ADAP is required for GP Ib-IX-V mediated inside-out $\alpha_{IIb}\beta_3$ activation, and in the absence of ADAP platelets demonstrate less stable adherence under shear flow, increased rebleeding [59] and abnormal thrombus formation [61]. ADAP is also required for platelet responses to collagen-mediated inside-out signaling to $\alpha_2\beta_1$ [62]. Thus, ADAP is required for inside-out signaling in platelets.

Outside-in signaling mediated via ADAP

Integrins can also act like signaling receptors and upon binding ligand, intracellular signaling from the integrin can influence cellular functions [47]. Signaling through $\alpha_L\beta_2$ induces mitogen-activated protein kinase (MAPK) signaling and can also initiate actin rearrangement [48]. ADAP-deficient T cells have reduced actin rearrangement after $\alpha_L\beta_2$ stimulation [48]. In addition, ADAP is phosphorylated on tyrosine residue(s) after $\alpha_L\beta_2$ stimulation and is found associated with $\alpha_L\beta_2$ integrins [48]. The molecular complexes initiated after TCR-mediated and $\alpha_L\beta_2$-mediated actin polymerization contain different proteins, which may explain the differential requirement for ADAP [45]. DCs demonstrate reduced cytokine production and increased actin polymerization after stimulation of CD11c in the absence of ADAP [63]. Additionally, platelets can perform outside-in signaling. Shear forces act as physical cues for $\alpha_{IIb}\beta_3$ outside-in activation mediated by fibrinogen binding [61]. ADAP organizes the localization of proteins after $\alpha_{IIb}\beta_3$ mediated outside-in signaling for actin cytoskeleton remodeling [61]. Thus, in T cells, DCs and platelets, ADAP is involved in outside-in signaling.

ADAP couples TCR signaling to T cell activation, proliferation and cell survival

The pool of ADAP not bound to SKAP55 is available to regulate TCR-mediated activation of the transcription factor NF-κB [49, 64]. After TCR-stimulation both nuclear NF-κB translocation and transcription of NF-κB target genes are required for T cell activation, proliferation and survival [65]. NF-κB is held in the cytoplasm by IκBα, which

must be phosphorylated and degraded for NF-κB translocation to the nucleus [65]. The IκB kinase (IKK) complex regulates IκBα degradation [65]. IKK is a trimeric complex consisting of IKKα, IKKβ and IKKγ [65]. The polyubiquitination of IKKγ and phosphorylation of IKKβ are required for IKK activation [65]. The IKK complex is inactive in the absence of TCR signaling [65]. After TCR signaling protein kinase C-θ (PKCθ) phosphorylates CARMA-1 [65]. Phosphorylated CARMA-1 recruits B cell CLL lymphoma 10 (Bcl10) and mucosa-associated lymphoid tissue lymphoma translocation gene 1 (MALT1) forming the CARMA-1-Bcl-10-MALT-1 (CBM) complex [65]. The CBM complex interacts with the ubiquitin E3 ligase TRAF6, resulting in the polyubiquitination of IKKγ [65]. The formation of the CBM complex is also dependent on the interaction between ADAP and CARMA-1 [64, 66]. In addition to regulating IKKγ polyubiquitination, ADAP is also required for phosphorylation of IKKβ, via the interaction between ADAP and TAK-1 [66]. Thus, ADAP is required for both IKKγ polyubiquitination and phosphorylation of IKKβ resulting in NF-κB translocation to the nucleus.

In addition to activating NF-κB, the interactions between ADAP and its binding partners CARMA-1 and TAK-1 are required for T cell entry into the cell cycle after TCR signaling [67]. Resting naïve mature T cells are in a quiescent state, which is maintained until TCR-mediated signaling results in the accumulation of cyclin and cyclin-dependent kinases (CDKs) [36]. Cdk4 and Cdk6 are activated and induce cyclin E, which activates Cdk2 [36]. The activation of Cdk2 is vital for transition from G_1 to M phases of the cell cycle [36]. The interaction of ADAP with CARMA-1 and TAK-1 is required for the expression of cyclin E [67]. In addition, the interaction between ADAP and CARMA-1 is required for Cdk2 induction, via c-Jun kinase (JNK) activation [67].

Thus, ADAP regulates the transition of quiescent naïve T cells to proliferating T cells after TCR signaling.

ADAP-CBM-TAK-1 signalosome regulates cytokine secretion of NK cells

NK cells, a special innate lymphoid cell subset, also develop from the CLP, but do not express TCRs. NK signaling occurs through receptors with similar characteristics to the TCR. NK cells elicit effector functions by directly killing targets or releasing cytokines [68]. Initial studies did not find a requirement for ADAP in NK responses for cytotoxicity or cytokine secretion after stimulation through FcγRIIIA, Ly49D or 2B4 [42]. This finding was unexpected, due to the known role of ADAP in positively regulating TCR-mediated integrin inside-out signaling. A later study assessed additional cytokine secretion stimulatory pathways and discovered that ADAP, in association with the CMB complex and TAK-1, was required for cytokine and chemokine production after NKG2D or CD137 stimulation [68]. Future studies will be needed to determine if the ADAP-CBM-TAK-1 signalosome is functional in other subsets, such as CD8 cytotoxic T lymphocytes (CTLs) and NKT cells.

Increased graft survival in the absence of ADAP

To date, the majority of work on ADAP has been performed in naïve mature CD4 T cells, but a few reports have assessed the function of ADAP in CD8 T cells. As ADAP is involved in TCR-mediated integrin activation, and integrins have been implicated in graft rejection, Tian *et al* assessed the role of ADAP in allogeneic CD8-dependent graft responses [69]. ADAP was required for CD8 T cell proliferation in response to allogeneic stimulation, which correlated with reduced immune cell infiltration into grafts.

Grafts survived longer in the absence of ADAP, but ultimately graft rejection still occurred [69, 70]. In contrast, responding CD8 T cells had robust CTL response both *in vitro* and *in vivo*, which is similar to findings in NK cells [42, 68-70]. Thus, ADAP is required for CD8 CTL proliferation, and possibly trafficking, but not cytotoxicity.

CD8 T cell immune response and ADAP

As mentioned, only a few studies have assessed the role of ADAP in CD8 T cells, specifically in response to allogeneic grafts. To my knowledge, the role of ADAP in CD8 T cell responses to pathogen has not been elucidated. As ADAP positively regulates TCR signaling to integrins, transcriptional activation, and entry into the cell cycle in CD4 T cells, and cytokine production in NK cells, I expected to find similar requirements for ADAP in CD8 T cells.

In response to a pathogen, antigen specific CD8 T cells undergo robust proliferation, and gain effector functions to combat the infection. After pathogen clearance a small percentage of antigen specific CD8 T cells survive to protect the host against future infections. These memory T cells are located in SLOs, circulating through various NLTs, and some remain resident in one tissue. Many factors have been identified that promote CD8 T cell proliferation, effector function, survival during contraction and formation and maintenance of memory. I propose that ADAP is a molecular regulator uniquely involved in the balance between subsets of memory CD8 T cells. Work completed for my thesis identified ADAP as a positive regulator, not of proliferation and effector T cell functions, but of the generation/maintenance of T_{RM} cells, and will be discussed in detail in Chapter 3. My findings support an increase proportion of T_{CM} in the absence of ADAP, which protects against systemic infections, but reduced

T_{RM} at the front lines, which leaves the host susceptible to local secondary infections. The next portion of this chapter will address the highlights of CD8 T cell responses to an infection.

ADAPTIVE IMMUNE RESPONSE

Naïve CD8 T cells patrol SLOs for DCs presenting cognate antigen. During an infection DCs pick up antigen and traffic to the draining lymph node. Upon contact of the antigen-specific T cell and the DC, the antigen-specific T cell is activated, undergoes fast and robust proliferation resulting in a large clonal population of differentiated cells [71]. The majority of these antigen-specific cells have effector capabilities and can specifically kill infected cells, and are termed cytotoxic T lymphocytes (CTLs) [71]. In an acute infection after the pathogen is eliminated, the majority (90-95%) of antigen-specific CD8 T cells die during the contraction phase [3]. The small population of antigen-specific CD8 T cells that survives the contraction phase are termed memory cells. Memory cells have the capacity to survive long term in the host and protect against future infections [3]. A great diversity exists among memory cells, and is partially explained by the T_{CM}-T_{EM} paradigm [4, 5]. CD8 T_{CM} T cells circulate among SLOs, blood and lymph, and generally have high proliferative capacities [3]. CD8 T_{EM} T cells circulate among non-lymphoid tissues (NLTs), blood and lymph, and can quickly demonstrate effector capabilities [3]. Subsets that are resident in a specific NLT are termed CD8 T_{RM} cells, and exist on the front lines of pathogen entry to alert the immune system and elicit effector functions [3, 5].

Naïve T cell activation

When a naïve T cell contacts an Ag-laden DC, signaling events are triggered downstream of the TCR culminating in increased adhesion of the T cell to the DC and initiation of transcriptional pathways [36, 45]. The inability to make long lasting T-APC interactions prevents effective priming [72]. Decreased T-APC interactions occur in the absence of DC expressed ICAM-1 [72]. CD8 T cells activated under these conditions do not produce optimal amounts of effector cytokines or persist as memory cells [72]. For complete CD8 T cell activation, the naïve T cell must also receive costimulation signals and a cytokine cue, such as IL-12 or type I IFN [1, 2]. After prolonged contact between the naïve T cell and DC, wherein sufficient stimulation through the TCR and costimulatory molecules has occurred, the T cell undergoes robust proliferation and differentiates, gaining effector functions [71].

CD8 CTL effector functions

CD8 CTLs mediate their effects through the production and secretion of effector cytokines and specific killing [73]. IFN-γ and tumor-necrosis factor-α (TNF-α) are the main effector cytokines produced by CD8 CTLs [74]. In the absence of the receptor for IFN-γ (IFN-γR), mice are more susceptible to viral and intracellular infections, with pathogens such as vaccinia virus and *Listeria monocytogenes* [74]. However, the exact mechanism whereby CD8 CTL secreted IFN-γ combats infection is unclear, as IFN-γ is a pleiotropic cytokine. The primary function of CD8 CTLs is targeted lysis, and perforin-dependent granule release is the major pathway by which effector CD8 CTLs kill infected cells [73, 75]. Antigen recognition of an infected cell by a CD8 CTL induces a polarized release of cytolytic granules containing granzymes and perforin towards the

target cell [73]. Perforin molecules facilitate the entry of granzymes into the cytosol of the target cell, wherein the granzymes activate cellular apoptotic pathways [73]. CD8 CTL functions are vital for clearance of multiple viral and intracellular pathogenic infections [73].

The gain of effector functions is dependent on the inflammatory cytokine milieu during priming [1, 2]. IL-12 programming drives effector CD8 differentiation via upregulation of T-bet [76], which drives the expression of effector molecules IFN-γ and Granzyme B [1, 74]. Priming is most efficient with both IL-12 and IL-2, instead of IL-12 alone [1]. IL-2 signaling drives Blimp-1 expression, which is needed for optimal differentiation of effector cells [71]. In the absence of IL-2 signaling, CD8 T cells are less cytolytic [77]. Thus, optimal effector T cell differentiation is dependent on the initial cytokine milieu during T-APC contacts.

As most infections are not initially systemic and enter through a mucosal surface, CD8 CTLs must traffic to the site of infection to elicit effector functions [5]. After activation most CD8 T cells downregulate SLO homing molecules [5] and upregulate various integrin pairs, including $\alpha_4\beta_1$, and $\alpha_4\beta_7$ [46], and chemokine receptors to facilitate entry into NLTs [5]. The integrin $\alpha_4\beta_7$ binds to vascular cell adhesion molecule 1 (VCAM-1), which is required for migration to the bone marrow and brain [46]. The integrin $\alpha_4\beta_7$ binds to mucosal vascular addressing cell adhesion molecule 1 (MAdCAM-1), which is expressed in venules of the large and small intestine, including HEVs of the mesenteric lymph node [46]. Effector CTLs that lack $\alpha_4\beta_7$ do not effectively migrate to the gut [46]. Once in a tissue, additional signals from the tissue microenvironment can induce changes on the effector T cell. In the gut, CD8 T cells upregulate effector molecules and integrins such as $\alpha_E\beta_7$, which aids in tissue retention, [46].

Contraction after the peak of the immune response

The robust expansion of naïve T cells produces a large number of cells, the majority of which will die during the contraction phase [3]. Why some cells undergo contraction and others survive is still not well understood. Death during contraction has been shown to be independent of the actions of caspases [78]. Additionally, the forced expression of anti-apoptotic molecules Bcl-x_L or Bcl-2 cannot prevent antigen-specific CD8 T cell death after pathogen challenge [79]. Instead, the main pathway that effector cells die is through the pro-apoptotic molecule Bim [3]. Antigen-specific CD8 T cells lacking Bim do not undergo contraction after pathogen challenge [80]. Inhibitor of DNA binding (Id) family of transcriptional regulators prevents E protein transcription factors from binding to DNA [81]. E proteins can directly regulate the Bim locus [81], and Bim mRNA levels are increased in the absence of Id2 effector T cells [82]. Additionally, in the absence of Id2 fewer effector CD8 T cells survive and reduced memory populations are formed after infection [81, 82]. Future studies will be needed to elucidate the requirements for survival during contraction.

CD8 T cell differentiation: Balance of generating effector and memory cells

An ideal immune response produces antigen-specific CD8 effector CTLs to combat the initial infection and maintains a smaller population long term as memory to prevent future infections. The quality and duration of signals from the TCR and cytokines can affect the differentiation of activated T cells [3].

TCR signal strength impacts expansion, contraction and memory T cell formation. Strong agonist ligands induced greater proliferation than weak agonist

ligands [83]. Weak agonist ligand stimulated antigen-specific CD8 T cells exited the splenic white pulp and entered the blood sooner than cells stimulated with strong agonists [83]. In addition, weak agonist stimulated CD8 T cells entered the contraction phase sooner than strong agonist induced cells [83]. All agonists regardless of strength induced functional CD8 memory T cells, though weak agonist ligands induced a smaller memory population [83].

Altering TCR signaling can affect the balance of CTL and memory CD8 T cell generation. Conditional knock-in (cKI) tyrosine (Y) to phenylalanine (F) mutations in SLP-76 that disrupt Itk (Y145) or Nck and Vav1 (Y112/128) binding after TCR-stimulation, altered the phosphorylation of ERK1/2 and production of effector cytokines, but not the expansion or contraction kinetics after lymphocytic choriomeningitis virus (LCMV) challenge [84]. SLP-76 cKI mutants also produce greater frequencies of cells with high expression of IL-7Rα during contraction [84]. Additionally, mutations in the TCR β transmembrane domain (βTMD) altered NF-κB activation, but did not affect expansion after *Listeria monocytogenes* infection [85]. CD8 T cells with mutations in βTMD continued to contract after wild-type cells had stabilized, resulting in an inability to form memory [85]. These studies indicate that altering TCR signaling influences the balance between producing CTLs to combat infection and producing long-lived memory cells to prevent future infections.

At the peak of the response the antigen-specific CD8 T cells have differentiated into a heterogeneous population [71]. Two main populations have been identified as short-lived effector cells (SLECs) and memory precursor effector cells (MPECs), based on the exclusive expression of killer cell lectin-like receptor G1 (KLRG1) or IL-7 receptor alpha-chain (IL-7Rα), CD127, respectively [76, 86]. Cells with higher expression of IL-

7Rα are more likely to survive the contraction phase [76]. IL-7 signaling is required for optimal survival of activated CD8 T cells to memory [87], but forced expression of IL-7Rα will not save cells from contraction [3]. As discussed earlier, IL-12 signaling promotes the expression of T-bet [76]. High levels of T-bet promote terminal effector CD8 T differentiation, and the downregulation of IL-7Rα [76, 86]. Low levels of T-bet can drive the expression of the IL-2 and IL-15 cytokine receptors [76, 86] . Pathogens vary in their ability to promote SLEC/MPEC populations, based on the inflammatory milleu [88]. Thus, the signals T cells receive during the immune response can influence the generation of effector and memory cells.

Memory CD8 T cell heterogeneity

After pathogen clearance, the cells that survive contraction are considered to be memory. Memory CD8 T cells are a multifunctional heterogeneous population. The most common way of phenotyping memory cells is based on the expression of lymph-node homing receptors CD62L and CCR7, using the T_{CM}-T_{EM} paradigm [4]. While the T_{CM}-T_{EM} paradigm is useful, it does not account for all memory CD8 T cells. T_{CM} cells express both CD62L and CCR7, while T_{EM} cells express lower levels of both molecules [4]. The expression of these molecules largely defines the localization of these populations. T_{CM} cells are predominately in SLOs and blood, while T_{EM} cells can be found in the blood and in NLTs [3]. In response to TCR or homeostatic cytokine stimulation, T_{CM} cells have greater proliferative capacities than T_{EM} cells [89, 90]. T_{EM} cells are more likely to express integrin ligands and chemokine receptors not required for SLO homing [91] and be present in non-lymphoid organs [90, 92]. In the steady state, T_{EM} cells express the effector molecule perforin, while T_{CM} cells do not, indicating T_{EM}

cells have greater cytolytic potential [4]. Indeed, memory CD8 T cells from SLOs do not exhibit immediate killing function, while memory CD8 cells from NLTs can specifically kill targets *ex vivo* [92].

There is much debate in the field as to what precursor cells generate memory cells. There is evidence that the memory precursors for both T_{CM} and T_{EM} cells are IL-7Rα^{hi} cells that are present at the peak of the infection [87, 93]. Although, IL-7Rα^{lo} cells can persist as memory, the rate is much lower than for IL-7Rα^{hi} cells [76, 87, 88, 93]. T_{RM} cells are generated via different pathways, as will be discussed below. In addition, the expression of various markers can change over time, and in response to secondary pathogen challenge [87, 90, 94, 95]. As a whole, memory CD8 T cells can proliferate, circulate through SLOs, blood and NLTs, and immediately elicit effector functions, but not all memory cells have all of these functions.

Resident memory T cells

During the initial immune response effector CD8 T cells can enter NLTs [6, 92, 96, 97]. Local tissue factors can promote activated CD8 T cells to differentiate into T_{RM} cells [6]. T_{RM} cells do not circulate through the blood and lymph but remain in one NLT [96, 98]. Residence in the skin is dependent on the trafficking of activated KLRG1$^-$ CD8 T cells from the blood [6]. When activated CD8 T cells enter NLTs, the transcription factor KLF2 is downregulated [96]. KLF2 downregulation can be mediated by various cytokines, such as TGF-β and IL-33 [6]. KLF2 promotes the expression of lymph-node homing molecules CD62L and the receptor for sphingosine 1-phosphate (SIP) ($S1PR_1$) [96]. $S1PR_1$ and CD69 interact on the surface of a T cell, and this interaction promotes the internalization of $S1PR_1$ [99, 100]. Decreased expression of KLF2 by KLRG1$^-$ CD8 T

cells in the dermis leads to increased expression of CD69 [6]. $CD69^+$ CD8 T cells migrate from the dermis to the epidermis in response to a chemokine gradient [6]. TGF-β signaling induces the upregulation of CD103 to maintain CD8 T cells in the epidermis [6]. Additionally IL-15 signaling is needed for the development and survival of CD8 T_{RM} cells in the skin [6]. The combination of these signaling pathways in the local tissue environment induces a unique population of CD8 T cells, transcriptionally distinct from T_{CM} and T_{EM} cells [6]. A similar change occurs in other non-lymphoid tissues, such as in the gut [97].

CD8 T_{RM} cells combat a local challenge by recruiting and activating both innate and adaptive immune cells at the site of infection [98, 101]. T_{RM} cells secrete IFN-γ upon local cognate peptide stimulation to recruit circulating memory cells from the blood and other sites [98]. IFN-γ induces upregulation of VCAM-1, which $\alpha_4\beta_1$ integrin expressing memory T cells and B cells adhere to for tissue entry [46, 101]. T_{RM} cells also induce the expression of the chemokine ligand CXCL9 on local vasculature to facilitate entry of immune cells [98]. Reactivated T_{RM} cells also secrete TNF-α for maturation of DCs and IL-2 to induce Granzyme B expression on NK cells and bystander CD8 memory cells [101]. All of these actions mediated by T_{RM} cells function to create an antiviral state at the site of pathogen entry [101].

CONCLUSIONS

While much is known about the generation and survival of naïve, effector, memory and memory phenotype CD8 T cells, many questions still remain. Work in this thesis has aimed to address some of these questions. I hypothesize that ADAP is vital for all life stages of CD8 T cells.

Do alterations in thymic development in the absence of ADAP affect CD8 T cell homeostasis? In Chapter 2, I will explore the balance between naïve and memory T cell subsets. In the steady state the majority of CD8 T cells in the periphery are naïve. As ADAP positively regulates T-APC interactions and differentiation after TCR stimulation, I hypothesized that ADAP would positively regulate naïve CD8 T cell survival and generation of MP CD8 T cells. Unexpectedly, once in the periphery ADAP negatively regulates the conversion of naïve CD8 T cells to MP CD8 cells. Increased MP CD8 T cell generation in the absence of ADAP is not due to alterations in the repertoire of ADAP-deficient mice, or increased lymphopenia, as may be expected by reduced conventional αβ T cell development. Instead, a novel role for ADAP has been identified in the suppression of IL-15 signaling.

How does loss of an adaptor protein known to positively regulate T-APC interactions, T cell proliferation and differentiation effect CD8 T cell responses to a pathogen? In Chapter 3, I will address the responses of naïve CD8 T cells to pathogen in the absence of ADAP. I hypothesized that ADAP would positively regulate optimal naïve CD8 T cell proliferation and differentiation in response to pathogen challenge. In contrast, ADAP is not required for naïve CD8 T cell proliferation, differentiation, or acquisition of effector CTL functions.

What are the generation and survival requirements of pathogen-generated memory CD8 T cell subsets localized in SLOs and NLTs and MP CD8 T cells? CD8 T cells undergo greater contraction in the absence of ADAP and reduced numbers of ADAP-deficient CD8 T cells survive to the stable memory phase. While T_{CM} cells are proliferation competent to systemic secondary infection in the absence of ADAP, reduced numbers of T_{RM} cells leave the host susceptible to local secondary infection. In contrast to memory CD8 T cell populations generated after pathogen challenge, the

generation of MP CD8 T cells in the steady state is suppressed by ADAP. These results indicate unique requirements for ADAP in memory CD8 T cell populations, dependent on their localization and route of generation.

Are the requirements for MP and effector CD8 T cell trafficking to allogeneic grafts and non-lymphoid tissues different? In Chapter 4, I will discuss the similarities and differences between pathogen- and allogeneic graft-generated effector CD8 T cells and MP CD8 cells. In the absence of ADAP, effector CD8 T cells can traffic to NLTs, but generate reduced numbers T_{RM} cells, as compared to wild-type. In contrast, allogeneic graft-generated effector CD8 T cells in ADAP-deficient hosts demonstrate reduced trafficking [69, 70]. MP CD8 T cells promote allogeneic graft rejection [102], but ADAP-deficient hosts demonstrate delayed allogeneic graft rejection compared with wild-type littermates [69, 70]. These results may indicate differences between stimulation conditions, or indicate altered repertoire generation in the absence of ADAP. In Chapter 4, I will also discuss the implications for ADAP in memory CD8 T cells, and how this may affect the field and disease.

FOOTNOTES

[1]The following abbreviations were used in this Chapter: Ab, antibody; ADAP, adhesion and degranulation-promoting adaptor protein; Ag, antigen; APC, antigen presenting cell; Arp2/3, actin-related protein 2/3; Bcl10, B cell CLL lymphoma 10; BMC, bone marrow chimera; βTMD ,TCR β transmembrane domain; CARMA-1, caspase recruitment domain (CARD) membrane-associated guanylate kinase (MAGUK) protein-1; CBM, CARMA-1-Bcl-10-MALT-1; CCL21, CC-chemokine ligand 21; CCR7, CC-chemokine receptor 7; CDK, cyclin-dependent kinase; cKI, conditional knock-in; CLP, common

lymphoid progenitor; CTL, cytotoxic T lymphocyte; DC, dendritic cell; DN, $CD4^+$ $CD8^+$ double negative; DP, $CD4^+$ $CD8^+$ double positive; ECM, extracellular matrix; ICAM1, intercellular adhesion molecule 1; HEV, high endothelial venule; Id, Inhibitor of DNA binding; IFN, interferon; IFN-γR, interferon γ receptor; IL, interleukin; IL-7Rα, interferon receptor α; iNKT, invariant natural killer T; IS, immunological synapse; Itk, inducible T cell kinase; JNK, c-Jun kinase; KLF2, Krüppel-like factor 2; LIP, lymphopenia induced proliferation; MAdCAM-1, mucosal vascular addressing cell adhesion molecule 1; MALT1, mucosa-associated lymphoid tissue lymphoma translocation gene 1; MAPK, mitogen-activated protein kinase; MHC, major histocompatability complex; MP, memory phenotype; MPEC, memory precursor effector cells; Nck, non-catalytic region of tyrosine kinase; NK, natural killer; NKT, natural killer T; PKCθ, protein kinase C-θ; PKD, protein kinase D; PLZF, promyelocytic leukemia zinc finger protein; RAG, recombination activating gene; RapL, regulator for cell adhesion and polarization enriched in lymphoid tissues; RIAM, Rap1 interacting adapter molecule; RTE, recent thymic emigrant; $S1PR_1$, sphingosine 1-phosphate (SIP); SFK, src family protein tyrosine kinase; SKAP55, Src kinase-associated phosphoprotein of 55 kDa; SLEC, short lived effector cells; SLO, secondary lymphoid organ; SLP-76 Src homology 2 domain-containing leukocyte phosphoprotein of 76 kDa; SOCS-1, suppressor of cytokine signaling-1; SP, single positive; TAK-1, transforming growth factor-β (TGF-β)-activated protein kinase; T_{CM}, central memory; TCR, T cell receptor; TCR-tg, TCR-transgenic; T_{EM}, effector memory; TGF-β, tumor growth factor-β; TGF-βRII, tumor growth factor-β receptor II; TNF-α; tumor necrosis factor-α; T_{RM} Resident memory; VCAM-1, vascular cell adhesion molecule 1; VWF, von Willebrand factor; WASp, Wiscott-Aldrich syndrome protein

CHAPTER 2: Negative regulation of memory phenotype CD8 T cell conversion by ADAP

ABSTRACT

The maintenance of T cell repertoire diversity involves the entry of newly developed T cells, as well as the maintenance of memory T cells generated from previous infections. This balance depends on competition for a limited amount of homeostatic cytokines and interaction with self-peptide MHC-I. In the absence of prior infection, memory-like or memory phenotype (MP) CD8 T cells can arise from homeostatic cytokine exposure during neonatal lymphopenia. Aside from downstream cytokine signaling, little is known about the regulation of the conversion of naïve CD8 T cells to MP CD8 T cells during acute lymphopenia. We have identified a novel negative regulatory role for the adapter protein ADAP in CD8 T cell function. We show that in the absence of ADAP, naïve CD8 T cells exhibit a diminished response to stimulatory Ag, but an enhanced response to weak agonist altered peptide ligands. ADAP-deficient mice exhibit an increased number of MP CD8 T cells that occurs following thymic emigration and that is largely T cell intrinsic. Naïve ADAP-deficient CD8 T cells are hyperresponsive to lymphopenia *in vivo* and exhibit enhanced activation of STAT5 and homeostatic antigen-independent proliferation *in vitro* in response to IL-15. Our results indicate that ADAP dampens naïve CD8 T cell responses to lymphopenia and IL-15, and demonstrates a novel antigen-independent function for ADAP in the suppression of MP CD8 T cell generation.

INTRODUCTION

T cell homeostasis is carefully balanced in a healthy host to maintain a diverse T cell repertoire against potential foreign pathogens. Utilizing both self-peptide MHC-I and IL-7 signaling pathways, naïve CD8 T cells compete for space with each other and a steady emigration of newly developed T cells out of the thymus [11]. Early in life, while the T cell pool is developing, increased availability of homeostatic cytokines in the secondary lymphoid organs can induce some recent thymic emigrants (RTEs) to gradually proliferate and differentiate into memory-like T cells, termed memory phenotype (MP) [10]. This period of neonatal lymphopenia is the primary generator of MP T cells, which are predominately foreign antigen-inexperienced and are maintained long into adulthood [8]. MP T cells have similar functional capabilities as foreign-antigen induced memory cells, but do not require prior antigen experience [7]. These cells can also be generated after exposure to an acute lymphopenic environment, which is of clinical relevance, as chemotherapy, late-stage HIV infection and exposure to radiation can render the host lymphopenic [11]. Furthermore, allowing naïve T cells to become MP in the absence of an infection is proposed to help protect the neonate from infections, although there is a risk of promoting the survival of self-reactive T cells in this process [7, 11, 12]

The molecular factors that drive naïve T cell homeostasis and permit the generation of MP T cells from the naïve, antigen-inexperienced pool are only partially understood. While joint signaling by IL-7 and self-peptide MHC-I are thought to be the main drivers of naïve T cell homeostasis, optimal survival of naïve T cells is dependent on additional signaling from IL-15 [22]. IL-15 signaling in naïve T cells drives the expression of the anti-apoptotic protein Bcl-2, but does not trigger proliferation, except in

extreme situations, such as in the absence of CD122 [22, 103]. Indeed, disruption of IL-15 signaling in mice lacking suppressor of cytokine signaling-1 (SOCS-1) results in altered T cell homeostasis [30]. Both naïve and MP CD8 T cells are hyperresponsive to IL-15 in the absence of SOCS-1, leading to robust proliferation, MP generation and neonatal mortality [30]. However, while IL-15 can drive MP, additional molecular regulators that control the reactivity to MHC-I and homeostatic cytokines for MP generation have yet to be identified.

ADAP is a multifunctional adaptor protein that coordinates the formation of signaling complexes that promote TCR-mediated activation of integrins, as well as activation of the NF-κB and JNK signaling pathways [36]. The expression of ADAP is restricted to cells of hematopoietic origin, including conventional CD4 and CD8 T cells and unconventional thymocytes, but is not expressed in B cell lineage cells after the Pro-B stage [41]. ADAP is required for optimal positive and negative selection during conventional CD4 and CD8 T cell development, but dispensable for the development of unconventional thymocytes, including natural killer T (NKT) cells [41, 44]. ADAP is localized to the cytosol, where a fraction is constitutively associated with Src kinase-associated phosphoprotein of 55 kDa (SKAP55) [49]. The ADAP-SKAP55 signaling module is critical for TCR-mediated activation of integrin-mediated adhesion with APCs [49, 53]. A second pool of ADAP is not associated with SKAP55, but activates NF-κB and JNK in a TCR-inducible manner [49, 64, 66, 67].

Analysis of ADAP function utilizing primary T cells following Ab-mediated TCR stimulation or mature naïve CD4 T cells following cognate-peptide MHC-II stimulation has demonstrated that ADAP is a positive regulator of T cell signaling that enhances T cell sensitivity to antigen. In contrast, the function of ADAP in CD8 T cells and in self-

peptide MHC-I interactions has been largely unexplored. We sought to understand the role of ADAP in the homeostasis of CD8 T cells. Our findings reveal an unexpected negative role for ADAP in the generation of MP CD8 T cells and IL-15 signaling.

MATERIALS AND METHODS

Mice

C57BL/6 (B6) wild-type and ADAP$^{-/-}$ mice were generated as previously described [58]. P14 ADAP$^{-/-}$ Rag2$^{-/-}$ mice were generated by crossing ADAP$^{-/-}$ mice with P14 Rag2$^{-/-}$ mice (provided by Dr. S. Jameson, University of Minnesota). OT-I ADAP$^{-/-}$ mice were generated by crossing ADAP$^{-/-}$ mice with OT-I mice (The Jackson Laboratory, Bar Harbor, ME). Nur77-GFP ADAP$^{-/-}$ mice were generated by crossing ADAP$^{-/-}$ mice with Nur77-GFP mice (provided by Dr. K. Hogquist and Dr. M. Farrar, University of Minnesota) [104]. All mice were harvested between 8-12 weeks of age, unless otherwise specified. B6 CD45.1 recipient mice were purchased from The Jackson Laboratory. Mice were housed in specific pathogen-free facilities at the University of Minnesota. All experimental protocols involving the use of mice were approved by the Institutional Animal Care and Use Committee at the University of Minnesota.

Flow cytometry and reagents

Cell surface staining for flow cytometry was performed with ice-cold HBSS supplemented with 2% bovine serum (FACS buffer). Single cell suspensions were washed with FACS buffer, stained with surface marker antibodies, then washed twice before multi-parameter flow cytometric detection on a BD LSRFortessa (Becton Dickinson, San Jose, CA, USA). Directly conjugated fluorescent antibodies used include: CD4 (clone GK1.5), CD8α (clone 53-6.7), CD45.1 (clone A20), CD45.2 (clone 104), and B220 (clone RA3-6B2) (Tonbo); CD16/32 (clone 93), CD24 (clone M1/69),

CD127 (clone SB/199), I-A^b (clone 25-9-17), Ter-119 (clone TER-119), and Vα2 (clone B20.1) (Biolegend); CD44 (clone IM7), CD122 (clone TM-Beta 1), CD124 (clone MIL4R-M1) and pSTAT5 pY694 (clone 47) (BD Biosciences); TCR-beta (clone H57-597), QA2 (clone 69H1-9-9) and F4/80 (clone BM8) (eBioscience). Cell cultures were maintained in complete T cell media: RPMI 1640 supplemented with 10% FCS, 4 mM L-glutamine, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 100 U/ml penicillin and streptomycin, 10 mM HEPES, and 5 mM 2-ME (RP-10).

Conjugate assay

Flow cytometry-based conjugate assays were performed as previously described [49]. Briefly, cells from pLN were harvested from wild-type and $ADAP^{-/-}$ OT-I adult mice and stained with FITC-conjugated anti-CD44. Polyclonal B6 splenocytes were harvested and stained with Cell Tracker Orange (Molecular Probes), then left unpulsed, or pulsed with N4, Q4 or T4 peptides for 30 min. at 37°C at the indicated concentrations. Wild-type or $ADAP^{-/-}$ OT-I T cells were mixed at a 1:1 ratio with labeled, pulsed splenocytes in a 96-well round bottom plate, pelleted and incubated at 37°C for 10 min. The cells were then vigorously mixed in a plate shaker for 20 s., fixed for 30 min. in 1% paraformaldehyde, and stained for flow cytometry. Conjugates were defined as $CD8^+$ $Vα2^+$ T cells, either $CD44^{lo}$ or $CD44^{hi}$, that co-stain with B22O and Cell Tracker Orange.

Mixed bone marrow chimeras

Mixed bone marrow chimeras were generated by mixing T cell-depleted bone marrow preparations from CD45.1/2 (wild-type) and CD45.2 ($ADAP^{-/-}$) mice at indicated

ratios and injecting 4 x 10^6 to 10 x 10^6 total cells into lethally irradiated (1100 rads) CD45.1 animals [16]. All chimeras were analyzed 8-12 weeks after transplant.

Naive CD8 T cell transfer

Lymph nodes were harvested from CD45.2 (wild-type) and CD45.1/2 (ADAP$^{-/-}$) mice and CD8 mature naïve T cells were isolated by negative magnetic bead enrichment similar to previously described methods [105]. Briefly, single-cell suspensions were incubated with the following FITC-conjugated antibodies: CD4, B220, F4/80, CD16/32, I-A^b, Ter119, and CD44. After washing, cells were incubated with anti-FITC microbeads and passed over LS columns on magnets according to manufacturer's instructions (Miltentyi Biotec, Auburn, CA) to capture non-CD8 cells. Purity of the flow-through fraction was > 95% CD8$^+$ CD44^{lo}. Purified cells were co-transferred into CD45.1 recipients at 1:1 ratio. Recipient mice were analyzed 1 and 14 days after transfer.

Naïve CD8 T cell transfer into lymphopenic hosts

Lymph nodes were harvested from CD45.2 (wild-type) and CD45.2 (ADAP$^{-/-}$) mice and CD8 mature naïve T cells were isolated by negative magnetic bead enrichment as described above. Cells were labeled with the intravital dye CFSE (Invitrogen) for cell proliferation. Briefly, cells were resuspended in PBS with 5% FBS at 2 x 10^7 cells/mL. CFSE diluted in PBS was added 1:1 to the cells (2.5 uM final concentration) and incubated at 25ºC for 5 min. Cells were quenched by washing twice with PBS with 2% FBS. Cells were transferred into separate sub-lethally irradiated (550 rads) CD45.1 animals [23]. Recipient mice were analyzed 10 days after transfer.

Detection of intracellular phospho-STAT5

Cytokine stimulation and detection of pSTAT5 were performed as previously described [106]. Briefly, splenocytes from polyclonal wild-type and ADAP$^{-/-}$ mice were rested for 2 hours at 37ºC in complete T cell medium at 2 x 10^6 cells/ml. Recombinant mouse IL-7 (Miltenyi Biotech) or IL-15 (R&D Systems) (1 ng/ml) was added to culture media for 15 min. Cells were fixed by the addition of paraformaldehyde (to 1.6%) for 10 min at room temperature, washed and then permeabilized with 1 ml of ice-cold methanol added to the cells while vortexing. After incubation for 15 min at 4ºC, cells were washed twice and stained for surface and intracellular antigens for 30 min at room temperature.

In vitro cytokine culture

Cytokine culture was performed as previously described [106]. Lymph nodes were harvested from CD45.1/2 (wild-type) and CD45.2 (ADAP$^{-/-}$) OT-I mice and naïve CD8 mature T cells were isolated by negative magnetic bead enrichment as described above. Cells were mixed at a 1:1 ratio and labeled with CFSE, as described above. Cells were then plated at 2 x 10^5 cells/ml and rested overnight (~16h) at 37°C in complete T cell medium without cytokine. IL-7 or IL-15 (10 ng/ml) was then added to the cultures for 5 days.

Statistics

Graphpad Prism (version 5.03, Graphpad Software, La Jolla, CA) was used to determine statistical significance using Student's unpaired two-tailed *t* tests. The *p* value

cutoffs and notation were used as follows: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. For pSTAT5 gMFI analysis, statistics were performed using a normalized value = 100.

RESULTS

ADAP controls sensitivity to antigen

Initially we sought to confirm ADAP as a positive regulator of CD8 T cell interactions with Ag-pulsed APCs, as has been documented for CD4 T cells [36]. We assessed the ability of wild-type and ADAP-deficient $CD8^+$ OT-I TCR-Tg T cells, which recognize SIINFEKL (N4) peptide in the context of MHC-I H-$2K^b$ with high affinity [107], to form stable contacts with peptide-pulsed APCs by flow cytometry. Both wild-type and ADAP-deficient naïve OT-I T cells exhibited minimal adhesion to APCs in the absence of peptide Ag (Fig. 2-1A, left). Addition of Ag resulted in a dose-dependent increase in CD8 T cell-APC conjugates, but we consistently observed reduced CD8 T cell-APC contacts in the absence of ADAP at low antigen doses (Fig. 2-1A, left). This defect diminished with the addition of higher concentrations of N4 peptide. Thus, similar to our previous observations with CD4 T cells, naïve CD8 T cells require ADAP for maximal T-APC interactions [40].

As ADAP positively regulates TCR signaling, we hypothesized that intermediate and weak agonist interactions would be more dependent on ADAP. Altered peptide ligands (APLs) have been developed for the OT-I TCR that trigger less potent responses of mature naïve CD8 T cells, which allows for the analysis of a single TCR over a range of agonist strengths [107]. Overall conjugate efficiency of wild-type naïve CD8 OT-I T cells was reduced with intermediate and weak agonist APLs SIIQFEKL (Q4) and SIITFEKL (T4), respectively [107, 108]. Unexpectedly, in contrast to our observation with N4, where ADAP was required for maximal conjugate efficiency at a given peptide dose, we found that T-APC interactions with the intermediate strength agonist APL Q4 were

equivalent between wild-type and ADAP-deficient OT-I T cells (Fig. 2-1A, center). Even more striking, ADAP-deficient naïve OT-I T cells exhibited enhanced interaction with APCs pulsed with T4 APL when compared to wild-type controls (Fig. 2-1A, right). Thus, ADAP appears to modulate CD8 T cells interactions with Ag-pulsed APCs, both by boosting strong agonist interactions, and by minimizing weak agonist interactions.

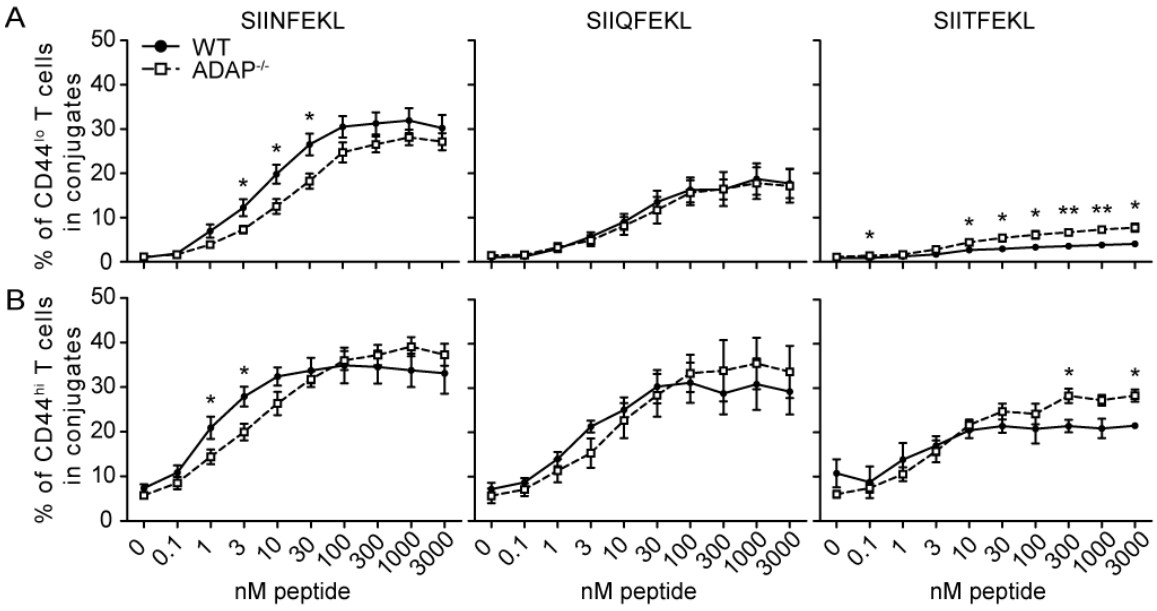

Figure 2-1: ADAP modulates T-APC interactions across multiple TCR affinities.

Conjugate assays between WT or ADAP$^{-/-}$ OT-I T cells and B6 splenocytes were performed as described in *Materials and Methods*. T:APC conjugate formation of naïve CD44^{lo} OT-I T cells (**A**) and MP CD44hi OT-I T cells (**B**) with B6 splenocytes pulsed with the indicated concentrations of N4 (left), Q4 (center) or T4 (right) peptides. Results are averages from duplicate or quadruplicate wells from independent experiments (± SEM): seven experiments (N4), four experiments (Q4), and three experiments (T4).

Increased memory phenotype CD8 T cells in the absence of ADAP

Even in the absence of immunization, approximately 10-20% of CD8 peripheral T cells in wild-type C57BL/6 mice housed in SPF conditions express elevated levels of the activation marker CD44 (Fig. 2-2A) and CD122 (Fig. 2-2B), a phenotype characteristic of memory CD8 T cells [8]. In our analysis of ADAP-deficient mice, we consistently observed a two-fold increase in the frequency of $CD44^{hi}$ $CD122^{hi}$ MP CD8 T cells in ADAP-deficient mice compared to littermate controls (Figs. 2-2B and C). From here on, we define naïve CD8 T cells as $CD44^{lo}$ $CD122^{lo}$ and MP CD8 T cells as $CD44^{hi}$ $CD122^{hi}$. When compared to wild-type mice, the total number of MP CD8 T cells in the spleen of ADAP-deficient mice was also elevated (Fig. 2-2D). Thus, ADAP-deficient mice demonstrate an increased CD8 MP T cell population in the steady state.

We next assessed the adhesion of wild-type and ADAP-deficient $CD44^{hi}$ MP CD8 OT-I T cells with APCs pulsed with the N4, Q4 or T4 peptides. With all 3 ligands, we observed a higher level of conjugate formation by MP CD8 OT-I T cells compared to naïve CD8 OT-I T cells (Fig. 2-1). The pattern of promoting strong agonist interactions and suppressing weak agonist interactions was maintained in MP CD8 T cells in the absence of ADAP, although to a lesser degree than we observed with naïve CD8 T cells (Fig. 2-1B). These results indicate an enhanced ability of MP CD8 T cells to interact with APCs pulsed with agonist peptide ligands compared to naïve CD8 T cells, and a requirement for ADAP in regulating Ag sensitivity of MP CD8 T cells.

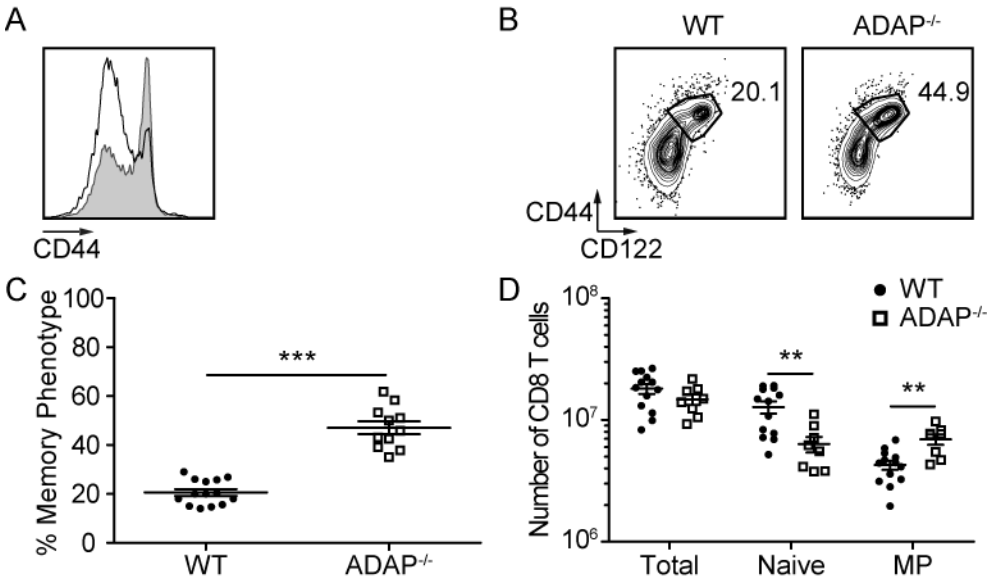

Figure 2-2: Increased memory phenotype CD8 T cells in the absence of ADAP.

Cells from spleens were obtained from WT and ADAP$^{-/-}$ adult mice (aged 8-12 weeks), and the expression of CD44 and CD122 was analyzed on CD8^{+} CD4^{-} TCR-β^{+} cells. (**A**) CD44 staining from WT (black) and ADAP$^{-/-}$ (grey shaded) cells. (**B**) Contour plots of bulk CD8 T cells. Numbers represent the percentage of cells with the phenotype of memory (CD44^{hi} CD122^{hi}). (**C**) The percentage of CD44^{hi} CD122^{hi} cells in the spleens of WT (black circles) and ADAP$^{-/-}$ (open squares) mice. (**D**) Absolute number of total CD8^{+} CD4^{-} TCR-β^{+}, CD44^{lo} CD122^{lo}, and CD44^{hi} CD122^{hi} cells from the spleen. The results (C and D) are compiled from at least five independent experiments (± SEM), with at least two mice per experiment.

Loss of ADAP does not enhance MP CD8 T cells in the thymus

One mechanism for the development of MP CD8 T cells in the periphery involves the response of mature CD8^{+}CD4^{-} thymocytes to IL-4 produced by thymic NKT cells expressing promyelocytic leukemia zinc finger protein (PLZF) [13]. When we analyzed ADAP-deficient mice, we did not observe changes in the thymus indicative of ADAP-dependent generation of CD8 MP T cells via PLZF^{+} NKT cells. First, there were comparable numbers of MP CD8 T cells among mature CD8 SP thymocytes in wild-type and ADAP-deficient mice (Fig. 2-4A). Total numbers of mature naïve CD8 SP

thymocytes were also unaffected by the loss of ADAP (Fig. 2-4A). Second, increases in MP CD8 thymocytes in other mouse models are associated with an increased percentage of IL-4-producing PLZF$^+$ NKT cells in the thymus and elevated levels of serum IgE [13]. A subset of invariant NKT (iNKT) cells can bind CD1d-αGalCer tetramer$^+$, expresses PLZF can produce IL-4 [18]. Additionally some γδ$^+$ NKT cells can also express PLZF and produce IL-4, bind GL3 [16, 17]. We observed a statistically significant increase in the percentage of thymic CD1d-αGalCer tetramer$^+$ NKT cells and γδ$^+$ NKT cells in ADAP-deficient mice (Fig. 2-3A and B). However, this was not associated with elevated levels of serum IgE in aged ADAP-deficient mice (Fig. 2-3C). Thus, increases in MP CD8 T cells in the periphery of ADAP-deficient mice are likely not due to elevated generation of MP CD8 T cells in the thymus.

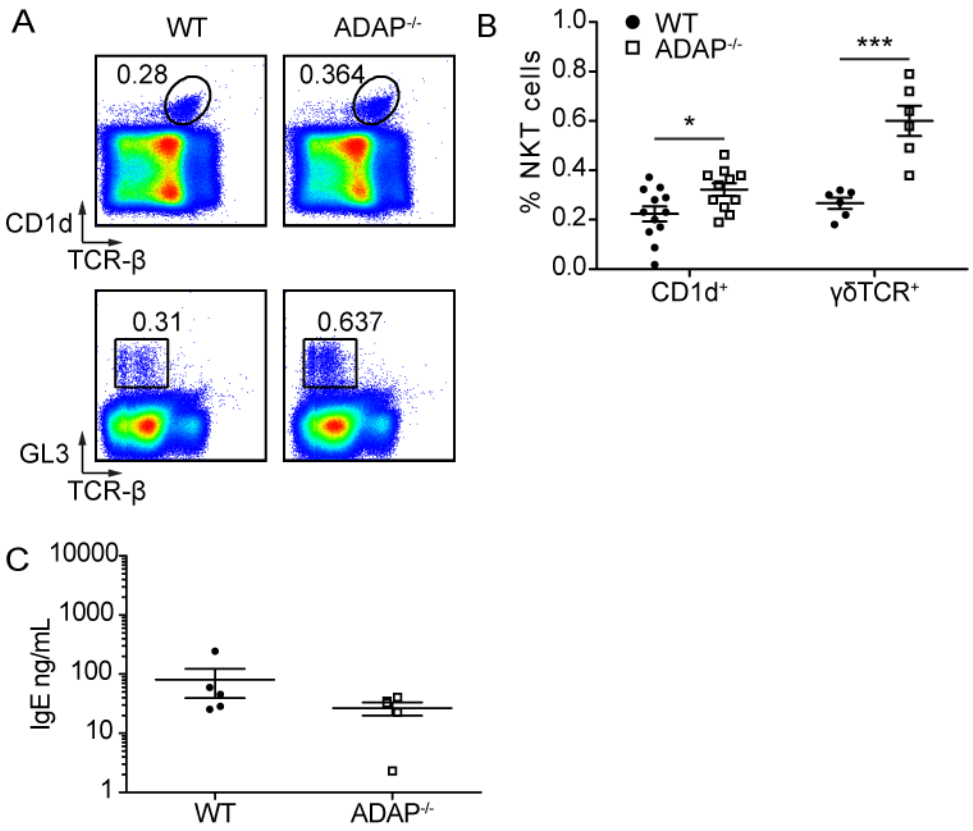

Figure 2-3: Loss of ADAP does not enhance NKT mediated generation of CD8 MP T cells in the thymus.

Cells from thymocytes were obtained from WT and ADAP-/- mice and stained for NKT cells (**A**) NKT staining from WT (left) and ADAP-/- (right) thymi. Numbers represent the percentage of cells NKT cells either CD1d-αGalCer tetramer^{+} TCR-β^{int} (top) or GL3^{+} TCR-β^{lo}(bottom). (**B**) The percentage of NKT cells of total thymocytes in WT (black circles) or ADAP-/- (open squares) mice. (**C**) Serum IgE from the blood of aged (25-40 week) mice. The results (B) are compiled from at least three independent experiments (± SEM).

Higher conversion of recent CD8 thymic emigrants to MP in the absence of ADAP

We next analyzed the maturation of CD8 T cells after emigration from the thymus in adult mice. Recent thymic emigrants (RTEs) and mature CD8 T cells can be differentiated by the expression of QA2 and CD24 [24]. RTEs that have emigrated from

the thymus within a week are QA2^{lo} CD24^{hi}, while T cells that have emigrated between 1-2 weeks prior have upregulated QA2 while maintaining high levels of CD24 [24]. Mature T cells, which emigrated at least 2 weeks prior, are QA2^{hi} CD24^{lo} [24]. Comparison of RTE, 1-2 week emigrated, and mature populations from wild-type and ADAP-deficient unimmunized adult mice demonstrate a similar frequency and number of these populations, indicating similar emigration and maturation of RTEs (Fig. 2-4B and C). We also assessed the frequency of MP CD8 T cells among peripheral CD8 populations. RTEs are predominately CD122^{lo}, while 1-2 week post-egress CD8 T cells exhibit a large population of MP cells. There is a significantly greater frequency of MP CD8 T cells among ADAP-deficient 1-2 week post egress CD8 T cells than wild-type (Fig. 2-4D). This difference is maintained in the mature population, which constitutes the majority of the CD8 T cells in the periphery of an adult mouse.

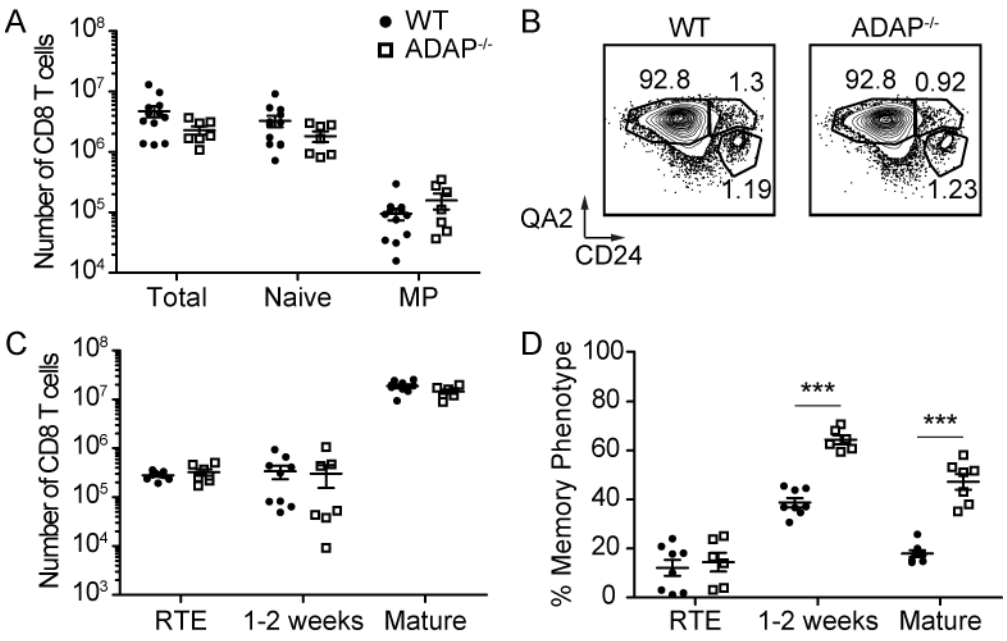

Figure 2-4: Memory phenotype in the absence of ADAP occurs after CD8 T cells exit the thymus.

Cells from thymus (**A**) or spleen (**B-D**) were obtained from WT and ADAP$^{-/-}$ unimmunized 8-12 week old adult mice and the expression of surface molecules was analyzed on CD8^{+} CD4^{-} TCR-β^{+} cells. (**A**) Absolute number of total CD8SP TCR-β^{hi}, CD44^{lo} CD122^{lo}, and CD44^{hi} CD122^{hi} cells from the thymus. (**B**) CD24 and QA2 staining from WT and ADAP$^{-/-}$ spleens. Numbers represent the percentage of RTE, 1-2 week post egress and mature CD8 T cells. Recent thymic emigrants (RTEs) are defined as CD24^{hi} Qa2^{lo}. Cells that have emigrated between 1-2 weeks prior are defined as CD24^{hi} Qa2^{hi}. Mature cells are defined as CD24^{lo} Qa2^{hi}. (**C**) Absolute number of RTE, 1-2 week post egress and mature CD8 T cells. (**D**) The percentage of MP from RTE, 1-2 week post egress and mature CD8 T cell populations. The results (A) are compiled from at least five independent experiments (± SEM) with at least two mice per experiment, and (C and D) are compiled from at least three independent experiments with at least three mice per experiment.

Alterations in the CD8 T cell repertoire are not driving ADAP-dependent CD8 MP conversion

Previous studies have demonstrated a requirement for ADAP in both positive and negative selection during T cell development in the thymus [44]. Altered T cell

development could modify the T cell repertoire of ADAP-deficient CD8 T cells, resulting in naïve CD8 T cells with a higher propensity to become MP. To address this possibility, we assessed the frequency of MP CD8 T cells in wild-type and ADAP-deficient, RAG2-deficient, P14 TCR-Tg mice. The frequency of peripheral MP CD8 T cells was increased in ADAP$^{-/-}$ RAG2$^{-/-}$ P14-Tg mice compared to wild-type mice (Fig. 2-5A and 4B). These results support a TCR-independent role for increased MP CD8 T cells in the absence of ADAP. We also utilized the Nur77-GFP reporter mouse to determine if wild-type and ADAP-deficient CD8 T cells had similar tonic signaling [104]. The geometric mean fluorescence intensity (gMFI) of Nur77-GFP was similar in wild-type and ADAP-deficient naïve or MP CD8 T cells isolated from unmanipulated mice (Fig. 2-5C and D). These results suggest that naïve and MP CD8 T cells in the absence of ADAP are not more self-reactive than wild-type CD8 T cells.

Loss of ADAP induces a CD8 T cell intrinsic increase in MP CD8 T cells

To directly test whether the increased MP observed in ADAP-deficient T cells is cell intrinsic, we created mixed bone marrow chimeras (BMCs). Bone marrow from a wild-type donor (CD45.1/2) was mixed with a minority of ADAP-deficient bone marrow (CD45.2) at a 90:10 ratio and transferred into lethally irradiated wild-type hosts (CD45.1). Spleens and thymi were harvested 8-12 weeks after transfer of donor marrow. In this environment, where the majority of the bone marrow-derived cells are wild-type, we observed that the frequency of ADAP-deficient MP CD8 T cells was 1.5 fold greater than wild-type (Fig. 2-5E and F), similar to what we observed in

unmanipulated ADAP-deficient mice. This result suggests a T cell intrinsic mechanism for ADAP-dependent generation of MP CD8 T cells.

We also generated mixed BMCs with a majority of ADAP-deficient bone marrow to test the effect of the ADAP-deficient environment on wild-type CD8 T cells. In this environment, we observed an increased frequency of wild-type MP CD8 T cells when compared to normal wild-type mice (Fig. 2-5G and H, Fig. 2-2B and C). Similar to what we observed with mixed BMCs with a majority of wild-type cells, there was a higher percentage of ADAP-deficient MP CD8 T cells when compared to wild-type CD8 T cells although the difference was not as pronounced as what we observed in unmanipulated mice or BMCs with a majority of wild-type cells (Fig. 2-5G and H). These results suggest that there may also be an additional role for T cell extrinsic factors in ADAP-dependent generation of MP CD8 T cells.

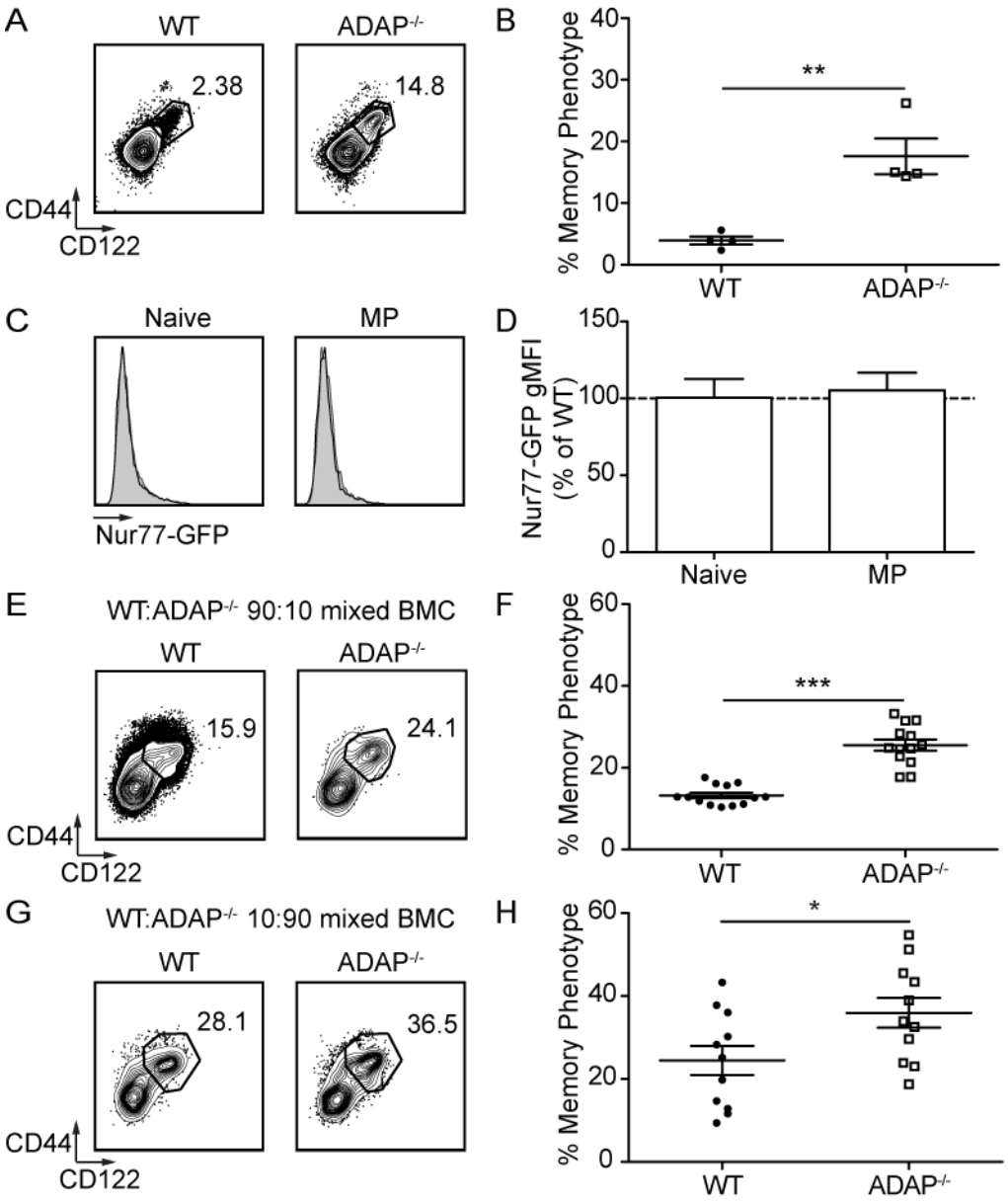

Figure 2-5: Memory phenotype in the absence of ADAP is not due to CD8 T cell self-reactivity, and is specific to ADAP-deficient CD8 T cells.

CD44 and CD122 staining of spleens from WT and ADAP$^{-/-}$ RAG2$^{-/-}$ P14-Tg mice. Numbers represent the percentage of MP cells. Analyzed cells are CD8^{+} CD4^{-} Vα2^{+} cells. (**B**) Percentage of MP WT and ADAP$^{-/-}$ RAG2$^{-/-}$ P14-Tg T cells. (**C)** Spleens from littermate polyclonal WT and ADAP$^{-/-}$ mice expressing the Nur77-GFP reporter were harvested and the expression of Nur77-GFP was analyzed by flow cytometry in CD8^{+}CD44^{lo}/CD122^{lo} (Naïve) and CD8+CD44^{hi}/CD122^{hi} (MP) cells. Representative

histograms depict Nur77-GFP expression for WT (black line) overlaid with ADAP$^{-/-}$ (grey shaded). **(D**) Quantification of Nur77-GFP fluorescence of naïve and MP CD8^{+} T cells from 4 separate littermate pairs of WT and ADAP$^{-/-}$ mice analyzed in two independent experiments. Values are normalized to Nur77-GFP expression observed in WT mice. (**E-H**) WT (CD45.1/2) and ADAP$^{-/-}$ (CD45.2) bone marrow cells at the indicated ratios were transferred into lethally irradiated CD45.1 recipients. Mixed bone marrow chimeras were harvested 8-12 weeks after transfer of donor marrow. (**E-F**) WT: ADAP-/-, 90:10 ratio, (**G-H**) WT: ADAP-/-, 10:90 ratio. (**E** and **G**) CD44 and CD122 staining of CD8^{+} CD4^{-} TCR-β^{+} donor cells from recipient spleens. Numbers represent the percentage of MP cells. (**F** and **H**) Percentage of CD44^{hi} CD122^{hi} donor cells. The results (B) are compiled from three independent experiments (± SEM) with at least two mice per experiment, (D) is compiled from three independent experiments, each with at least one littermate pair, and (F and G) are compiled from three independent experiments, each with at least 4 mice.

Mature naïve CD8 T cells do not convert to MP in the absence of ADAP

To determine if mature naïve ADAP-deficient CD8 T cells have a higher propensity to convert to MP in the steady state, we isolated CD44^{lo} CD122^{lo} naïve CD8 T cells from wild-type and ADAP-deficient pLNs, and co-transferred these cells into adult wild-type hosts. Two weeks after transfer, we assessed the conversion of donor naïve CD8 T cells to MP, a time frame in which RTEs mature and can convert to MP. The majority of both wild-type and ADAP-deficient CD8 T cells retained a CD44^{lo} CD122^{lo} naïve phenotype (Fig. 2-6A and B), with minimal conversion to MP. These findings suggest that naïve ADAP-deficient T cells do not have a higher propensity to convert from naïve to MP in a wild-type host.

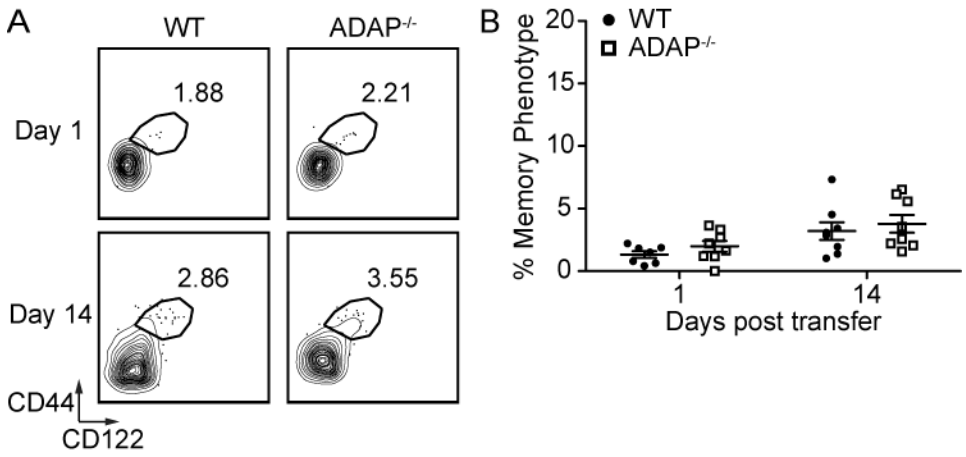

Figure 2-6: ADAP-deficient mature naïve CD8 T cells do not more readily convert to memory phenotype in the steady state.

Naïve CD8 T cells from pLNs were isolated from WT (CD45.2) and ADAP$^{-/-}$ (CD45.1/2) adult mice, mixed at a 1:1 ratio and transferred into CD45.1 recipient mice (1 x 10^5 cells/mice). (**A**) CD44 and CD122 staining of transferred cells from recipient spleens. Numbers represent the percentage of MP CD8 T cells. (**B**) Percentage of CD44^{hi} CD122^{hi} donor cells. The results (B) are compiled from two independent experiments, each with four mice (± SEM).

Neonatal lymphopenic generation of MP CD8 T cells is increased in the absence of ADAP

A second mechanism for development of MP CD8 T cells involves the response of naïve CD8 T cells to the lymphopenic environment during the neonatal period [10]. The highest frequency of MP CD8 T cells in a wild-type mouse occurs at 3 weeks (approximately 30%) and decreases to adult levels (approximately 20%) by 5 weeks of age [8]. When compared to wild-type littermates, the frequency of MP CD8 T cells in 3 week old ADAP-deficient mice was 2 fold higher (Fig. 2-7A and B). Similar to published accounts, we found that the frequency of wild-type MP CD8 T cells was highest at 3 weeks and reached adult frequencies by 5 weeks [8]. At all ages, we observed a greater

frequency of MP CD8 T cells in ADAP-deficient mice (data not shown). These results support an early and consistent increased generation of MP CD8 T cells in the absence of ADAP.

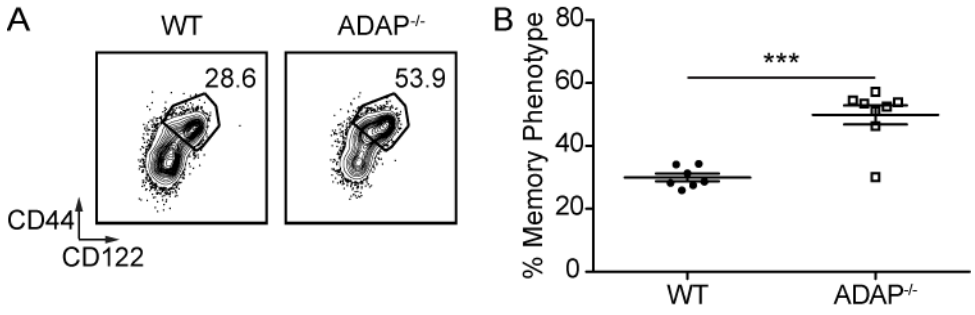

Figure 2-7: Neonatal lymphopenic generation of memory phenotype CD8 T cells is greater in the absence of ADAP.

Cells from the spleen of 3 week old WT or ADAP$^{-/-}$ mice were harvested and analyzed for MP. (**A**) CD44 and CD122 staining. Numbers represent the percentage of MP CD8 T cells. (**B**) Percentage of CD8^{+} TCR-beta^{+} QA2^{hi} CD24^{lo} MP cells. The results (B) are compiled from two independent experiments (± SEM), with at least three mice in each group.

ADAP dampens naïve CD8 T cell responses to lymphopenia

To further investigate the role of lymphopenia in ADAP-deficient conversion of naïve CD8 T cells to MP, CD44^{lo} CD122^{lo} naïve CD8 T cells were labeled with CFSE and transferred into sub-lethally irradiated age-matched recipients. After 10 days, we assessed the conversion of wild-type and ADAP-deficient naïve CD8 T cells to MP, as well as lymphopenia induced proliferation (LIP). We found that a higher percentage of ADAP-deficient naïve CD8 cells converted to MP than their wild-type counterparts in a lymphopenic environment (Fig. 2-8A and B). In addition, a higher percentage of ADAP-deficient CD8 T cells had undergone greater than 4 divisions compared to wild-type CD8

T cells (Fig. 2-8C and D). These results suggest that CD44^{lo} CD122^{lo} naïve ADAP-deficient CD8 T cells are more sensitive to lymphopenic signals *in vivo*.

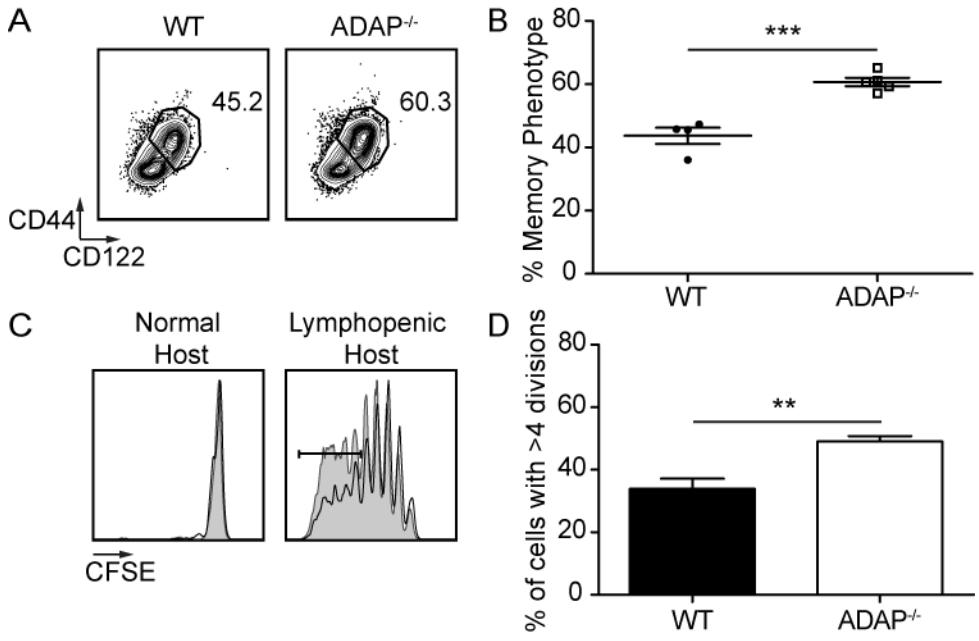

Figure 2-8: Mature naïve CD8 T cells convert to memory phenotype in the absence of ADAP in response to lymphopenic signals.

Naïve CD8 T cells from pLNs were isolated from WT (CD45.2) and ADAP$^{-/-}$ (CD45.2) adult mice, labeled with CFSE, and separately transferred into normal or sub-lethally irradiated CD45.1 recipients. Recipient spleens were harvested 10 days after transfer. (**A**) CD44 and CD122 staining of transferred cells from lymphopenic spleens. Numbers represent the percentage of MP CD8 T cells. (**B**) Percentage of CD44^{hi} CD122^{hi} donor cells from lymphopenic recipient spleens. (**C**) CFSE staining from WT (black) or ADAP$^{-/-}$ (grey shaded) donor cells. (**D**) Percentage of donor cells that had undergone >4 cell divisions. The results (B and D) are representative of three independent experiments (± SEM), with at least four mice of each condition.

IL-15 mediated induction of memory phenotype is suppressed by ADAP

LIP of naïve CD8 T cells is driven by IL-7 and self-peptide-MHC complexes, while MP CD8 T cells can utilize IL-7 or IL-15 and are MHC-I independent [11, 24]. To

determine a mechanism by which ADAP-deficient CD8 T cells are more responsive to lymphopenia, we assessed the ability of naïve CD8 T cells to transduce IL-7 and IL-15 signals. Bulk splenocytes from wild-type or ADAP-deficient mice were stimulated with IL-7 or IL-15 for 15 minutes and stained for intracellular phospho-STAT5. Both wild-type and ADAP-deficient naïve CD8 T cells expressed low levels of pSTAT5 in the absence of cytokine stimulation (Fig. 2-9A). An increase in pSTAT5 after the addition of IL-7 was comparable between wild-type and ADAP-deficient naïve CD8 T cells (Fig. 2-9A and B). Interestingly, pSTAT5 induction after IL-15 treatment was 20% greater in ADAP-deficient naïve CD8 T cells (Fig. 2-9A and B). These results suggest that in the absence of ADAP, naïve CD8 T cells exhibit an enhanced response to IL-15 signals.

To determine if this difference in IL-15 signaling is associated with functional differences in the response of ADAP-deficient CD8 T cells to IL-15, we cultured CFSE-labeled wild-type and ADAP-deficient mature naïve OT-I CD8 T cells in IL-7 or IL-15. After 5 days in culture, we assessed cytokine induced proliferation and MP conversion. The addition of IL-7 induced minimal proliferation of ADAP-deficient CD8 T cells, which was not significantly altered when compared to wild-type (Fig. 2-9C and D). In contrast, IL-15 stimulation induced proliferation of ADAP-deficient CD8 T cells, but not wild-type CD8 T cells. In addition, stimulation with IL-7 increased CD122 expression in both wild-type and ADAP-deficient CD8 T cells, but to a greater degree in the absence of ADAP (Fig. 2-9E and F). This difference in CD122 upregulation was even more striking with the addition of IL-15. We conclude that expression of ADAP dampens the ability of naïve CD8 T cells to respond to IL-15 signals.

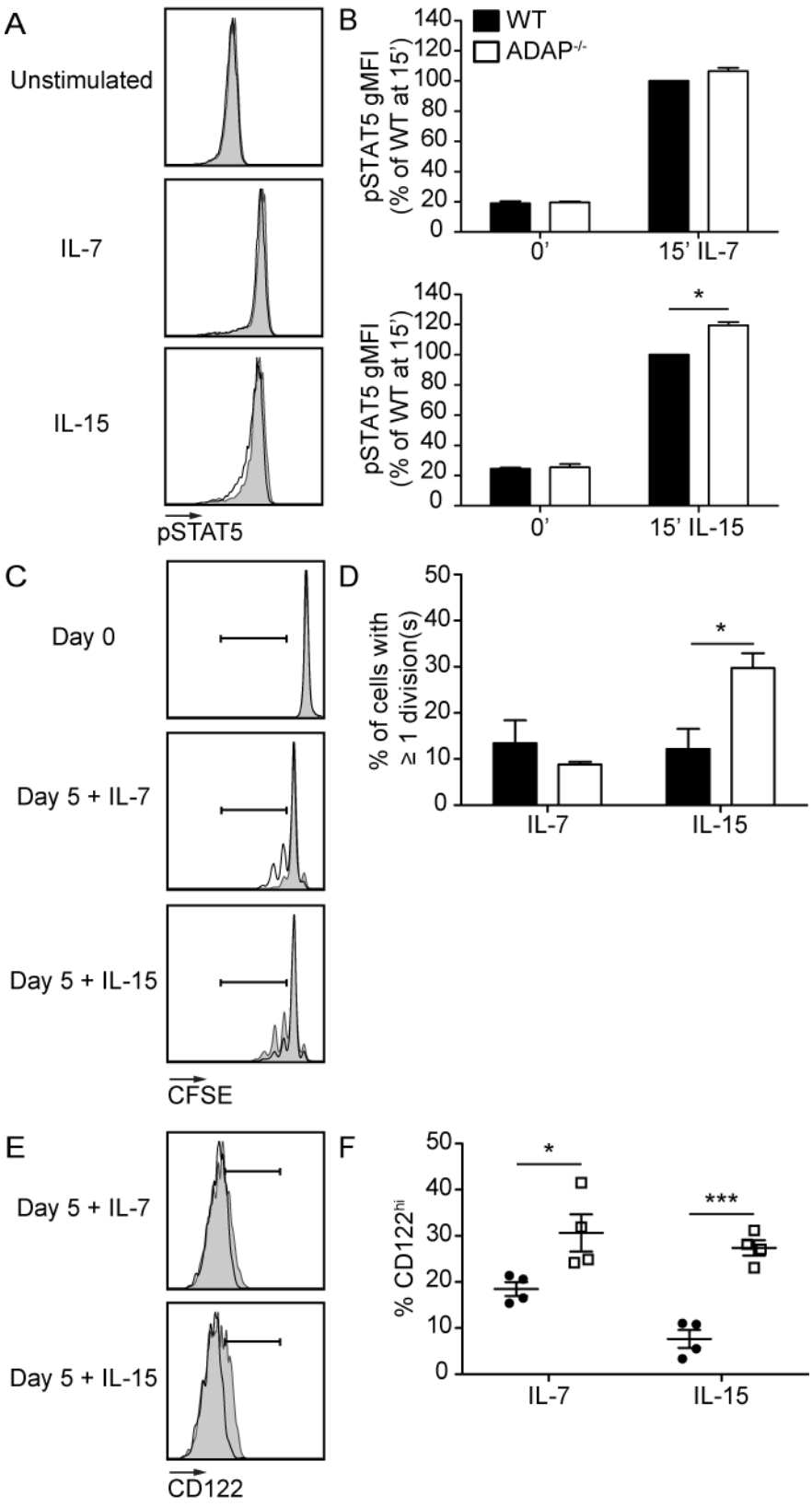
A
Unstimulated
IL-7
IL-15
pSTAT5
B
WT
ADAP-/-
pSTAT5 gMFI (% of WT at 15')
0'
15' IL-7
15' IL-15
*
C
Day 0
Day 5 + IL-7
Day 5 + IL-15
CFSE
D
% of cells with ≥ 1 division(s)
IL-7
IL-15
E
Day 5 + IL-7
Day 5 + IL-15
CD122
F
% CD122hi
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Figure 2-9: Mature naïve CD8 T cells are more responsive to IL-15 in the absence of ADAP.

(**A-B**) Bulk splenocytes were obtained from WT and ADAP$^{-/-}$ adult mice and stained for intracellular pSTAT5. (**A**) Histograms of WT (black) or ADAP$^{-/-}$ (grey shaded) pSTAT5 staining on CD8^{+} CD122^{lo} cells after addition of 1 ng/mL of IL-7 or IL-15 for 15 minutes. (**B**) STAT5 phosphorylation was measured by flow cytometry and quantified as the population gMFI staining normalized to WT IL-7 or IL-15 stimulated samples from CD8^{+} CD122^{lo} cells. (**C-F**) Naïve CD8 T cells were isolated from WT (CD45.1/2) or ADAP$^{-/-}$ (CD45.2) OT-I adult mice, mixed at a 1:1 ratio, and labeled with CFSE. WT and ADAP$^{-/-}$ cells were co-cultured in the presence of 10 ng/mL of IL-7 or IL-15 for 5 days. (**C**) CFSE staining of WT and ADAP$^{-/-}$ CD8 T cells at day 0 or 5 after addition of cytokine. Gate indicates cells that have undergone 1 or more divisions. (**D**) Percentage of donor cells that have undergone one or more divisions. (**E**) CD122 staining of WT and ADAP$^{-/-}$ CD8 T cells at day 5 after addition of cytokine. Gate indicates cells that are CD122^{hi}. (**F**) Percentage of CD8 T cells with expression of CD122^{hi}. The results (B, D and F) are compiled from three independent experiments ($\pm$ SEM). Statistical analysis was performed using a Student's *t* test. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$

DISCUSSION

In this study, we have revealed a novel negative regulatory role for the adapter protein ADAP in naïve CD8 responses to weak agonist peptides and IL-15. This work focused on the thymic and extra-thymic pathways that contribute to the generation of antigen-independent MP CD8 T cells [8, 13]. Thymic-based MP CD8 T cell generation pathways can be traced to alterations in unconventional thymocyte development that result in an increase in NKT cells expressing IL-4, a cytokine that can drive the conversion of mature CD8 SP thymocytes to MP at high concentrations [13]. In the periphery, the prime drivers of peripheral mature naïve CD8 T cell conversion to MP are: 1) self-reactivity; 2) exposure to the homeostatic cytokines IL-7 and IL-15; and 3) absence of negative signals [7]. Our findings support an extra-thymic generation of MP CD8 T cells in the absence of ADAP, due to an enhancement of IL-15 signaling.

After our initial observation that ADAP-deficient CD8 T cells had greater frequencies of MP CD8 T cells, we investigated thymic-based MP CD8 T cell generation. Both inducible T cell kinase (Itk)-deficient mice and mice with a mutation in Src homology 2 domain-containing leukocyte phosphoprotein of 76 kDa (SLP-76:Y145F) demonstrate IL-4^+ NKT cell-mediated CD8 MP generation [13]. Itk inducibly binds SLP76-pY145 after TCR signaling, which may explain why the SLP-76:Y145F mutation phenocopies Itk-deficient animals [13]. Although ADAP also inducibly associates with SLP-76 [36], we did not find evidence that the increased frequency of MP CD8 T cells in ADAP-deficient mice was due to an IL-4-dependent mechanism in the thymus. Our results are also consistent with the findings that the significant developmental defects in conventional T cells and NKT cells in SLP-76:Y145F and Itk-deficient mice are not

observed in ADAP-deficient mice. Our findings do not support a thymic pathway generating MP CD8 T cells in the absence of ADAP.

A role for ADAP in regulating T cell responses to self peptides might be predicted, since ADAP is required for the optimal response of naïve CD4 T cells to Ag both *in vitro* and *in vivo* [40, 53], and TCR signaling has been proposed to be moderately attenuated in ADAP-deficient thymocytes [44]. Similar to naïve CD4 T cells, we have observed that ADAP positively regulates the interaction of naïve CD8 OT-I T cells with APCs pulsed with the strong agonist N4 peptide. In contrast, the loss of ADAP enhanced the weak agonist interaction of OT-I T cells to APCs pulsed with the T4 APL. The enhanced adhesion of ADAP-deficient CD8 T cells to APCs pulsed with the weak agonist T4 APL is unusual and indicates that ADAP can play a negative regulatory role in TCR-dependent responses under certain conditions. This result suggested the possibility that the loss of ADAP might enhance the self-reactivity of naïve CD8 T cells, leading to increased MP CD8 T cell generation. The enhanced LIP of ADAP-deficient naïve CD8 T cells *in vivo* is consistent with this possibility. However, similar to what was observed in polyclonal mice, we found an increased percentage of MP CD8 T cells in P14 TCR-Tg mice. The P14 TCR-Tg is on RAG2$^{-/-}$ background, which prevents endogenous receptor rearrangement, ensuring TCR specificity. This result suggests that ADAP-deficient CD8 T cells do not convert to MP because of an altered TCR-repertoire with self-reactive T cells. Additionally, measuring tonic TCR signaling in naïve or MP CD8 T cells with the Nur77-GFP reporter system indicates similar self-reactivity between wild-type and ADAP-deficient CD8 T cells. Thus, ADAP negatively regulates the response of naïve CD8 T cells to weak agonist APLs, but the enhanced frequency of

MP CD8 T cells in ADAP-deficient mice does not appear to be due to altered self-reactivity of ADAP-deficient naïve CD8 T cells.

Altered thymocyte development could result in decreased thymic output, thus enhancing MP conversion of peripheral mature naïve CD8 T cells by increasing the exposure of these T cells to homeostatic cytokines during neonatal development [7]. We failed to detect defects in the development of mature CD8 SP T cells or in CD8 thymic output in the absence of ADAP. We do recognize that by qualifying RTEs only by $QA2^{lo}CD24^{hi}$ cells, we are excluding a large percentage of cells that could be identified in other systems [21]. When we assessed mature naïve CD8 T cell conversion to MP in neonatal mice, we observed a greater frequency of MP CD8 T cells in the absence of ADAP. Together these results suggest that the enhanced conversion of neonatal ADAP-deficient CD8 T cells to MP is not likely due to thymic development or export defects. We performed mixed BMC experiments to rule out the possibility that the enhanced frequency of ADAP-deficient MP CD8 T cells is due to lymphopenic conditions in ADAP-deficient mice that might result in greater exposure of naïve CD8 T cells to homeostatic cytokines such as IL-7 and IL-15. These experiments support a T cell intrinsic response of ADAP-deficient CD8 T cells to lymphopenia, as 1.5 fold greater frequency of MP CD8 T cells was observed in ADAP-deficient CD8 T cells, compared to wild-type CD8 T cells, regardless of the majority population. Taken together, these results suggest that the conversion of naïve mature CD8 T cells to MP CD8 T cells in the absence of ADAP is not likely due to increased exposure to homeostatic cytokines.

We were surprised to discover our results support a unique negative regulatory role for ADAP in naïve CD8 T cell responses to the homeostatic cytokine IL-15. This is a previously unreported function for ADAP independent of TCR signaling. It is interesting

to note that while ADAP-deficient CD8 T cells exhibit enhanced responsiveness to IL-15, the response of ADAP-deficient CD8 T cells to IL-7 is similar to what we observed with wild-type CD8 T cells. This suggests the possibility that ADAP plays a particularly important role in regulating CD8 T cell survival, since the optimal survival of naïve T cells is dependent on an IL-15 signal [22]. The SOCS-1 protein, which binds directly to JAKs to inhibit the tyrosine-kinase activity and promotes ubiquitin-mediated degradation [32], has also been implicated in negatively regulating T cell homeostasis. SOCS-1-deficient mice have severe neonatal lethality due to overproduction of type II IFN [31]. Similar to ADAP-deficient mice, SOCS-1-deficient mice have increased LIP and MP CD8 T cell conversion that is not linked to TCR affinity, but associated with an enhancement of IL-15 signaling [30, 31]. As SOCS-1-deficient mice succumb to neonatal mortality [32], a phenotype that is not observed in ADAP-deficient mice, the molecular link between the enhanced responsiveness of SOCS-1-deficient CD8 T cells and ADAP-deficient CD8 T cells will require further investigation.

Our studies have revealed a novel negative regulatory role for ADAP in the conversion of naïve CD8 T cells to MP in the steady state and the response of naïve CD8 T cells to IL-15. ADAP is therefore a signaling protein involved in both the positive regulation of TCR responses to foreign antigens, and the negative regulation of naive T cell responses to homeostatic cytokines. Our results indicate that ADAP is a critical mediator of the body's need to balance the maintenance of potentially useful T cells against genuine memory T cells from prior infections. We propose that ADAP functions to license pathogen-reactive T cells for activation during an immune response, while limiting self-reactive cells from attaining space in the T cell pool. By promoting strong agonist peptide MHC-I interactions, ADAP enhances activation of naïve T cells with

foreign-peptide MHC-I specificity. In contrast, ADAP dampens weak agonist interactions and prevents generation of self-reactive cells. In addition, suppressing naïve IL-15 signaling could decrease the generation of MP CD8 T cells during instances of acute lymphopenia, which can occur after an infection [11]. These findings reinforce the importance of future studies to understand how T cell homeostasis is maintained throughout life, from birth, during infections and into old age.

FOOTNOTES

[1]This work is currently under revision for submission to *The Journal of Immunology*

[2]Abbreviations used in this Chapter: ADAP, adhesion and degranulation promoting adapter protein; BMC, bone marrow chimera; gMFI, geometric mean fluorescence intensity; Itk, inducible T cell kinase; iNKT, invariant natural killer T; LIP, lymphopenia induced proliferation; MP, memory phenotype; N4, SIINFEKL peptide, NKT cells, natural killer T cells; PLZF, promyelocytic leukemia zinc finger protein; pLN, peripheral lymph node; Q4, SIIQFEKL peptide; RTE, recent thymic emigrant; SKAP55, Src kinase-associated phosphoprotein of 55 kDa; SLP-76, Src homology 2 domain-containing leukocyte phosphoprotein of 76 kDa; SOCS-1, suppressor of cytokine signaling-1; T4, SIITFEKL peptide.

CHAPTER 3: The adaptor protein ADAP promotes resident memory CD8 T cells

ABSTRACT

During acute infections, naïve antigen-specific CD8 T cells are activated and differentiate into effector T cells, the majority of which undergo contraction after pathogen clearance. A small population of CD8 T cells survives the contraction phase and persists as memory, to protect against future infections. Memory CD8 T cells are heterogeneous and can be found in secondary lymphoid organs, blood and non-lymphoid tissues. We demonstrate the adaptor protein ADAP enhances the formation of memory CD8 T cells in non-lymphoid tissues. We show that in the absence of ADAP, reduced numbers of $KLRG1^-$ CD8 resident memory T cell precursors are present in non-lymphoid tissues at the peak of the immune response and total resident memory T cell numbers are reduced at memory time points. ADAP-deficient resident memory T cells are functional in response to local peptide challenge, but only when in the presence of wild-type antigen-specific T cells. In contrast, greater frequencies of ADAP-deficient $CD127^+$ CD8 memory precursors are present in secondary lymphoid organs during the contraction phase, but total memory CD8 T cell numbers are reduced at memory time points. Memory CD8 T cells in secondary lymphoid organs proliferate robustly to a systemic secondary challenge in the absence of ADAP. Our results indicate that ADAP positively regulates the formation of resident memory CD8 T cells after acute pathogen challenge.

INTRODUCTION

After pathogen entry into a host, a robust expansion of antigen specific CD8 T cells occurs, followed by the loss of 90-95% of responding cells [3]. One week after pathogen challenge, a substantial population of activated CD8 T cells migrates from SLOs into the blood and NLTs [5, 96]. At this time the majority of activated CD8 T cells express killer cell lectin-like receptor G1 (KLRG1) [6, 76, 88]. $KLRG1^-$ CD8 T cells are the predominant population that gives rise to CD8 resident memory T (T_{RM}) cells [6]. CD8 T cells expressing the receptor for IL-7 (IL-7Rα, CD127) and negative for KLRG1 are more likely to survive the contraction phase in blood and SLOs [76, 87]. The small numbers of cells remaining after contraction are maintained in the host as memory [3]. CD8 T cell memory is heterogeneous and memory CD8 T cells can be found in SLOs, blood, lymph and NLTs [3, 5]. Memory CD8 cells exhibit various functions, such as proliferation in response to TCR or homeostatic cytokine stimulation [90], cytokine production and targeted killing [3]. Once memory has stabilized after contraction, numerous groups have identified markers that correlate with various protective and proliferative capacities of memory CD8 T cells [94, 95]. Memory cells with the cell surface phenotype $KLRG1^{hi}$, $CD127^{int}$, $CD27^{lo}$, $CD62L^{lo}$ preferentially localize to the red pulp of the spleen and NLTs to provide robust protective immunity, in spite of suboptimal recall proliferation [95]. As a whole, CD8 memory T cells protect the host from secondary infection by being present in SLOs, blood and NLTs and responding to infection by proliferating and eliciting effector functions [3].

The initial interaction between the naïve T cell and antigen presenting cell can affect the formation and function of memory CD8 T cells. Naïve T cell contact with

cognate antigen initiates TCR-signaling events that culminate in increased adhesion of the T cell to the antigen presenting cell (APC) and initiation of transcriptional pathways [36, 45]. Reduced TCR signaling [84, 106], or reduced T-APC interactions [72] prevents effective priming and results in reduced cytotoxic T lymphocyte (CTL) functions and altered memory generation. These studies suggest that molecules that regulate positive signaling from the TCR to transcriptional pathways and T-APC adhesion are vital for balancing the production of terminally-differentiated effectors and memory cells.

ADAP is a cytosolic adaptor protein that coordinates the formation of signaling complexes after TCR signaling. ADAP positively regulates both T-APC interactions and transcriptional pathways [36]. A fraction of ADAP is constitutively associated with Src kinase-associated phosphoprotein of 55 kDa (SKAP55) [49]. The ADAP-SKAP55 signaling module is critical for T-APC interactions, mediated by TCR inside-out signaling to integrins [49, 53]. ADAP not associated with SKAP55, can participate in the activation of NF-κB and JNK in a TCR-inducible manner [49, 64, 66, 67]. The downstream effects of TCR-inducible interactions of ADAP with caspase recruitment domain (CARD) membrane-associated guanylate kinase (MAGUK) protein 1 (CARMA-1) [36] and, transforming growth factor-β (TGF-β)-activated protein kinase (TAK-1) [36] promote T cell entry into the cell cycle [67]. The ADAP-CBM-TAK-1 signalosome is also required for cytokine and chemokine production by NK cells after NKG2D or CD137 stimulation [68].

We and others have demonstrated defects in T-APC contacts, entry into the cell cycle, proliferation, differentiation and survival in the absence of ADAP [36]. Additionally, we have recently identified ADAP as a negative regulator of TCR-mediated integrin activation in response to weak agonist peptide ligands in naïve CD8 T cells, as

discussed in Chapter 2. ADAP also negatively regulates naïve T cell responses to IL-15, by suppressing proliferation and memory phenotype acquisition. Although, CD8 CTLs exhibit normal cytotoxicity in the absence of ADAP in response to an allogeneic graft [69, 70], little is known about the role that ADAP plays in CD8 T cells in response to an infection. To our knowledge, the function of ADAP in CD8 T cells in response to a pathogen has not been investigated. We sought to understand the role of ADAP in the generation and function of CD8 CTLs and memory CD8 T cells. Our findings reveal a role for ADAP positively regulating memory CD8 T cells in SLOs and NLTs.

MATERIALS AND METHODS

Mice

C57BL/6 (B6) wild-type and ADAP$^{-/-}$ mice were generated as previously described [58]. OT-I ADAP$^{-/-}$ mice were generated by crossing ADAP$^{-/-}$ mice with OT-I mice (The Jackson Laboratory, Bar Harbor, ME). B6 CD45.1 recipient mice were purchased from The Jackson Laboratory. Mice were housed in specific pathogen-free facilities at the University of Minnesota. All experimental protocols involving the use of mice were approved by the Institutional Animal Care and Use Committee at the University of Minnesota.

Flow cytometry and reagents

Cell surface staining for flow cytometry was performed with ice-cold HBSS supplemented with 2% bovine serum (FACS buffer). Single cell suspensions were washed with FACS buffer, stained with surface marker antibodies, then washed twice before multi-parameter flow cytometric detection on a BD LSRFortessa (Becton Dickinson, San Jose, CA, USA). For IFN-γ staining on day 7 post infection, splenocytes were harvested and single cell suspensions were incubated in complete T cell media $\pm$ 10 ng SIINFEKL peptide for 3 hours at 37°C, then washed twice with FACS buffer. Intracellular staining for IFN-γ (1:100 dilution) or Granzyme B (1:50 dilution) was performed using the BD Cytofix/Cytoperm kit (BD Biosciences). Briefly, cells were stained with surface antibodies, washed with FACS buffer, fixed with 300 µL BD Cytofix/Cytoperm, and incubated at 4°C for 30 min. Cells were washed twice with 1X

BD Perm/Wash buffer then incubated with intracellular staining antibodies for 30 min. Cells were washed twice with 1X BD Perm/Wash buffer, and resuspended in FACS buffer for flow cytometry. Directly conjugated fluorescent antibodies used include: CD4 (clone GK1.5), CD8α (clone 53-6.7), CD45.1 (clone A20), CD45.2 (clone 104), B220 (clone RA3-6B2) and KLRG1 (clone 2F1) (Tonbo); CD8-β (clone YTS156.7.7); CD16/32 (clone 93), CD69 (clone H1.2F3); CD127 (clone SB/199), I-A^b (clone 25-9-17), Ter-119 (clone TER-119), and Vα2 (clone B20.1) (Biolegend); CD43 (clone 1B11); CD44 (clone IM7) and CD103 (clone M290); (BD Biosciences); CD27 (clone LG.7F9); F4/80 (clone BM8), and IFN-γ (clone XMG1.2) (eBioscience); Granzyme B (clone GB11) (Invitrogen). Complete T cell media: RPMI 1640 supplemented with 10% FCS, 4 mM L-glutamine, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 100 U/ml penicillin and streptomycin, 10 mM HEPES, and 5 mM 2-ME (RP-10)

Negative selection of naïve OT-I T cells, adoptive transfer, pathogen challenge and local peptide challenge

Lymph nodes were harvested from wild-type (CD45.1/2) and $ADAP^{-/-}$ (CD45.2) mice and CD8 naïve mature T cells were isolated by negative magnetic bead enrichment similar to previously described methods [105]. Briefly, single-cell suspensions were incubated with the following FITC-conjugated antibodies: CD4, B220, F4/80, CD16/32, I-A^b, Ter119, and CD44. After washing, cells were incubated with anti-FITC microbeads and passed over LS columns on magnets according to manufacturer's instructions (Miltentyi Biotec, Auburn, CA) to capture non-CD8 cells. Purity of the flow-through fraction was > 95% $CD8^+$ $CD44^{lo}$. Purified cells were cotransferred into CD45.1

recipients at a 1:1 ratio, and unless noted otherwise, either 3e3 or 1e4 cells of each genotype were transferred. Mice were challenged with 500 CFU of *Listeria monocytogenes*-OVA (LM-OVA) (retro-orbital) [83] or 1e6 PFU of Vesicular Stomatitis Virus (VSV-OVA) (tail vein) [83, 98]. For secondary challenge of LM-OVA mice, animals were given 1e6 PFU of VSV-OVA. Local SIINFEKL peptide stimulation was given transcervically (t.c.) with 50μg of peptide, as described [98], and FRT was harvested 12 hours post challenge, as described below.

BrdU labeling and staining

At the start of labeling, mice were injected intraperitoneal (i.p.) with 1 mg of BrdU (Sigma) in PBS. Tissues were harvested 5 hours after injection. Single cell suspensions were stained with surface antibodies, washed with FACS buffer, fixed with 300 μL BD Cytofix/Cytoperm, and incubated at 4°C for 30 min. Cells were washed once with 1X BD Perm/Wash buffer then incubated with 2 mL of 1X BD Perm/Wash for 10 min at 4°C. Cells were washed once with 1X BD Perm/Wash buffer and incubated with 300 μL BD Cytofix/Cytoperm for 5 min at 4°C. Cells were washed once with 1X BD Perm/Wash buffer and incubated in 100 μL DNase (Sigma) in PBS at 37°C for 60 min. Cells were washed once with 1X BD Perm/Wash buffer stained with Pacific Blue Anti-BrdU (Molecular Probes) for 30 min. Cells were washed twice with 1X BD Perm/Wash buffer, and resuspended in FACS buffer for flow cytometry.

In vivo killing assay

An *in vivo* killing assay was performed similarly to a previously described study [75]. Bulk splenocytes (CD45.1/2) were used as targets. Cells were labeled with an intravital dye, CFSE (Invitrogen). Briefly, cells were resuspended in PBS with 5% FBS at 2 x 10^7 cells/mL. CFSE diluted in PBS was added 1:1 to the cells (5 μM (hi) or 0.5 μM (lo) final concentrations) and incubated at 25°C for 5 min. Cells were quenched by washing twice with PBS with 2% FBS. An aliquot of $CFSE^{lo}$ labeled cells were pulsed with 10nM of SIINFEKL peptide for 30 min at 37°C. Cells were washed twice before mixing 1:1 with $CFSE^{hi}$ labeled cells. Mixed populations of peptide pulsed or unpulsed cells were transferred into mice (CD45.1) which had received WT (CD45.2) or $ADAP^{-/-}$ (CD45.2) OT-I T cells and challenged with LM-OVA 7 days prior. After 5 hours, splenocytes were harvested and the ratio of $CFSE^{lo}$:$CFSE^{hi}$ was determined by flow cytometry. Specific killing is calculated by: (1- (control ratio/experimental ratio)) x 100 [75].

In vivo intravenous injection of anti-CD8 and lymphocyte isolation from NLTs

Mice were given an intravenous injection of anti-CD8α (5 μg) for 2.5 min, as described [109]. Mice were euthanized and organs were harvested. Single cell suspensions of lymph nodes and spleen were processed as described above. Salivary glands were processed as described previously [96]. Briefly, salivary glands were minced and incubated at 37°C in a solution of RPMI 1640/10%FCS/2 mM $MgCl_2$/2mM $CaCl_2$/HEPES/L-glutamine medium containing 100U/mL collagenase type I (Worthington Biochemical) with mixing for 45min. Small intestines were processed as described [97].

Briefly, Peyer's patches were removed; the remaining tissue was cut longitudinally and minced into 1cm pieces. Pieces were incubated in 15.4 mg/mL dithioerythritol in 10% 1X HBSS/HEPES bicarbonate for 30 min at 37°C to remove IEL. The remaining pieces were then treated with EDTA followed by collagenase treatment as described above for salivary gland. Female reproductive tract, including the uterine horns, cervix and vaginal tissue, were processed as described [110]. Briefly, the FRT was removed and minced into small pieces, then incubated in a solution of RPMI 1640/10%FCS/2 mM $MgCl_2$/2mM $CaCl_2$/HEPES/L-glutamine medium containing 100U/mL collagenase type IV (Sigma) for 1 hour at 37°C. Lymphocytes from all NLTs were purified on a 44/67% Percoll gradient (800 x *g* at 20°C for 20 min).

Statistics

Graphpad Prism (version 5.03, Graphpad Software, La Jolla, CA) was used to determine statistical significance using Student's unpaired two-tailed *t* tests. The *p* value cutoffs and notation were used as follows: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. For $ADAP^{-/-}$numbers as a percent of WT analysis, statistics were performed using a normalized value = 100

RESULTS

Altered immune response to Listeria monocytogenes in the absence of ADAP

We chose to evaluate the requirement for ADAP in response to a model pathogen, *Listeria monocytogenes* (LM). We isolated naïve OT-I CD8 T cells from wild-type and ADAP-deficient peripheral lymph nodes (pLNs) and co-transferred these cells into naïve recipients. A genetically modified LM expressing OVA (LM-OVA) was used to challenge recipient animals, generating a robust expansion of wild-type T cells (Fig. 3-1A), [83]. As ADAP is required for optimal high affinity T-APC interactions and proliferation in CD4 T cells, we expected to observe reduced responses to LM in the absence of ADAP. Interestingly, ADAP-deficient OT-I CD8 T cell expansion was not altered (Fig. 3-1A). Differences between the number of wild-type and ADAP-deficient cells were not observed until after day 8 post challenge, during the contraction phase of the immune response (Fig. 3-1A). We observed a reduced number and frequency of ADAP-deficient T cells at all times after the peak of the immune response (Fig. 3-1A). Additionally, when ADAP-deficient OT-I T cell numbers are shown as a percentage of wild-type, subtle reductions in ADAP-deficient numbers are present on days 6-8 post challenge (Fig. 3-1B). This subtle reduction in numbers is not due to reduced proliferation, as detected by BrdU incorporation on days 6-8 post infection (Fig. 3-2A and B). Thus, ADAP is not required for initial CD8 T cell responses to LM-OVA, but is required for optimal survival during contraction and formation of memory.

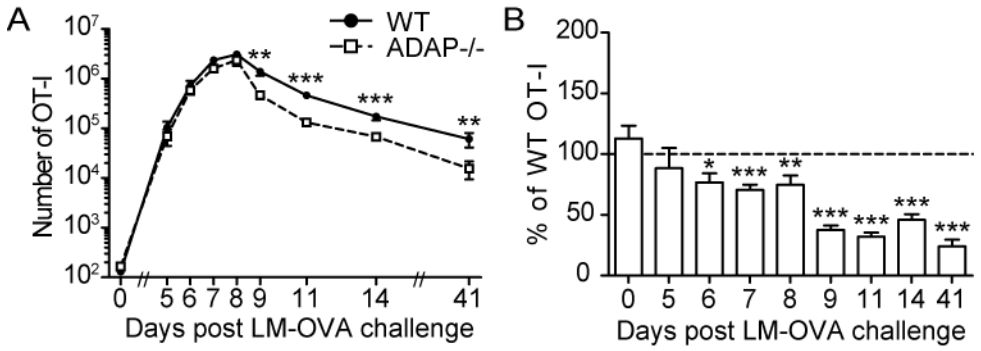

Figure 3-1: ADAP is not required for initial expansion, but is required for optimal survival to memory after LM-OVA challenge.

Naïve WT (CD45.1/2) and ADAP$^{-/-}$ (CD45.2) OT-I T cells were cotransferred into CD45.1 hosts and challenged with LM-OVA. Splenocytes were isolated and stained for cell surface markers. (**A**) Number of WT (black circles) or ADAP$^{-/-}$ (open squares) OT-I T cells in the spleen after LM-OVA challenge. (**B**) ADAP$^{-/-}$ expressed as a percentage of WT OT-I T cells. The results (A and B) are compiled from 12 independent experiments, with at least 4 mice per time point (± SEM).

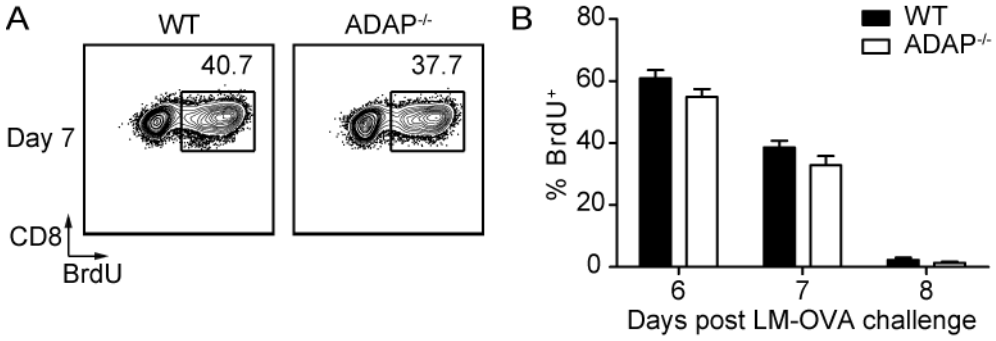

Figure 3-2: CD8 T cell proliferation is independent of ADAP.

LM-OVA challenged mice were generated as in Figure 1. Five hours prior to harvest, mice were injected with BrdU. Splenocytes were isolated and stained for cell surface markers and intracellular BrdU. (**A**) Representative BrdU staining of WT and ADAP$^{-/-}$ OT-I T cells on day 7 post challenge. Gate indicates cells that are positive for BrdU. (**B**) Percentage of BrdU^{+} WT (black bar) or ADAP$^{-/-}$ (white bar) OT-I T cells in the spleen. The results (B) are representative of 2 independent experiments with 4 mice per experiment (± SEM).

Loss of ADAP does not alter effector T cell responses

CD8 CTLs are vital for the immune response against LM [111]. CD8 CTLs produce effector cytokines IFN-γ and Granzyme B, and specifically kill antigen-expressing cells [73-75] . To test the requirement of ADAP in CTL functions, we analyzed effector functions on day 7 post LM challenge. The production of IFN-γ was similar between wild-type and ADAP-deficient cells (Fig. 3-3A and B). Similarly, Granzyme B production is normal in the absence of ADAP (Fig. 3-3C and D). To more specifically test CTL function, we performed an *in vivo* cytotoxicity assay [75]. We challenged mice with LM-OVA after single transfer of wild-type or ADAP-deficient OT-I T cells. On day 7 post challenge, SIINFEKL peptide pulsed target cells were transferred and specific killing was assessed 5 hours later. Both wild-type and ADAP-deficient OT-I T cells efficiently killed peptide-pulsed cells (Fig. 3-3E and F). Consistent with previous findings [69, 70], we conclude that ADAP is not required for CD8 CTL functions.

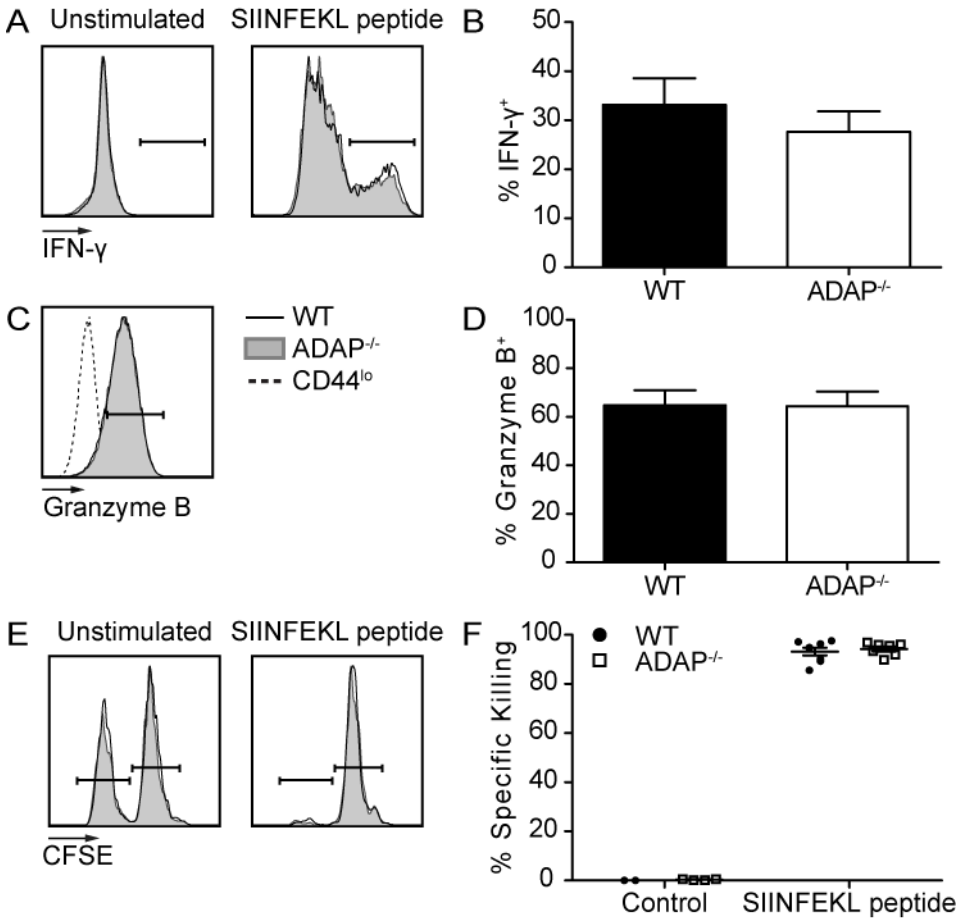

Figure 3-3: Development of effector functions in CD8 T cells is independent of ADAP.

LM-OVA challenged mice were generated as in Figure 1. Splenocytes were isolated and stained for cell surface markers and intracellular proteins on day 7-post LM-OVA challenge. (A-B) Splenocytes were stimulated *ex vivo* with 10 ng SIINFEKL peptide for 3 hours. (**A**) IFN-γ staining of unstimulated (left) and SIINFEKL stimulated (right) WT (black) and ADAP$^{-/-}$ (grey shaded) OT-I T cells. Gate indicates cells that are positive for IFN-γ. (**B**) Percentage of IFN-γ^{+} WT (black bar) or ADAP$^{-/-}$ (white bar) OT-I T cells in the spleen. (**C**) Granzyme B staining of WT or ADAP$^{-/-}$ OT-I T cells, or CD44^{lo} host cells (dashed line). (**D**) Percentage of Granzyme B^{+} WT or ADAP$^{-/-}$ OT-I T cells in the spleen. (E-F) WT (CD45.2) or ADAP$^{-/-}$ (CD45.2) OT-I T cells were transferred into separate CD45.1 hosts, and challenged with LM-OVA. On day 7-post challenge, an *in vivo* killing assay was performed as described in *Materials and Methods*. Spleens were harvested 5 hours after transfer of targets and specific killing was determined. (**E**) Representative target frequencies from hosts containing WT or ADAP$^{-/-}$ OT-I T cells. (**F**) Percentage specific killing. The results (B, D and F) are compiled from at least 2 independent experiments with 4 mice per experiment (± SEM).

Altered memory generation in the absence of ADAP

During contraction, cells expressing higher levels of cytokine receptors are more likely to survive and be maintained as memory cells [112]. As reduced numbers of ADAP-deficient T cells are present at memory time points (Fig. 3-1), we hypothesized that ADAP-deficient T cells would express reduced amounts of the IL-7 cytokine receptor α-chain, CD127, post contraction. Interestingly, a greater frequency of ADAP-deficient OT-I T cells expressed CD127 on day 14 post challenge (Fig. 3-4A and B). In addition, the reduced numbers of ADAP-deficient OT-I T cells can be traced to $CD127^-$ populations (Fig. 3-4C). Of note, greater frequencies of $KLRG1^+$ $CD127^+$ cells and reduced frequencies of $KLRG1^-$ $CD127^-$ are also observed (Fig. 3-4A and B). This trend is maintained into memory time points, where decreased frequencies and numbers of $KLRG1^+$ memory cells are still reduced in the absence of ADAP (data not shown).

At memory time points the markers CD27 and CD43 are used to identify memory CD8 T cells with recall capabilities against secondary infections [94, 95]. Three main populations are observed after LM challenge: $CD27^{hi}$ $CD43^{lo}$, $CD27^{hi}$ $CD43^{hi}$, $CD27^{lo}$ $CD43^{lo}$, with the latter population mediating rapid protective immunity [95]. At one month post challenge all three populations are observed in wild-type OT-I T cells at similar frequencies as previously published data (Fig. 3-4D and E) [95]. Reduced frequencies of the $CD27^{lo}$ $CD43^{lo}$ population are observed in the absence of ADAP, with a corresponding increased in both $CD27^{hi}$ $CD43^{lo}$ and $CD27^{hi}$ $CD43^{h}$ populations (Fig. 3-4D and E). Using two subsetting systems, we have found altered frequencies of memory T cells.

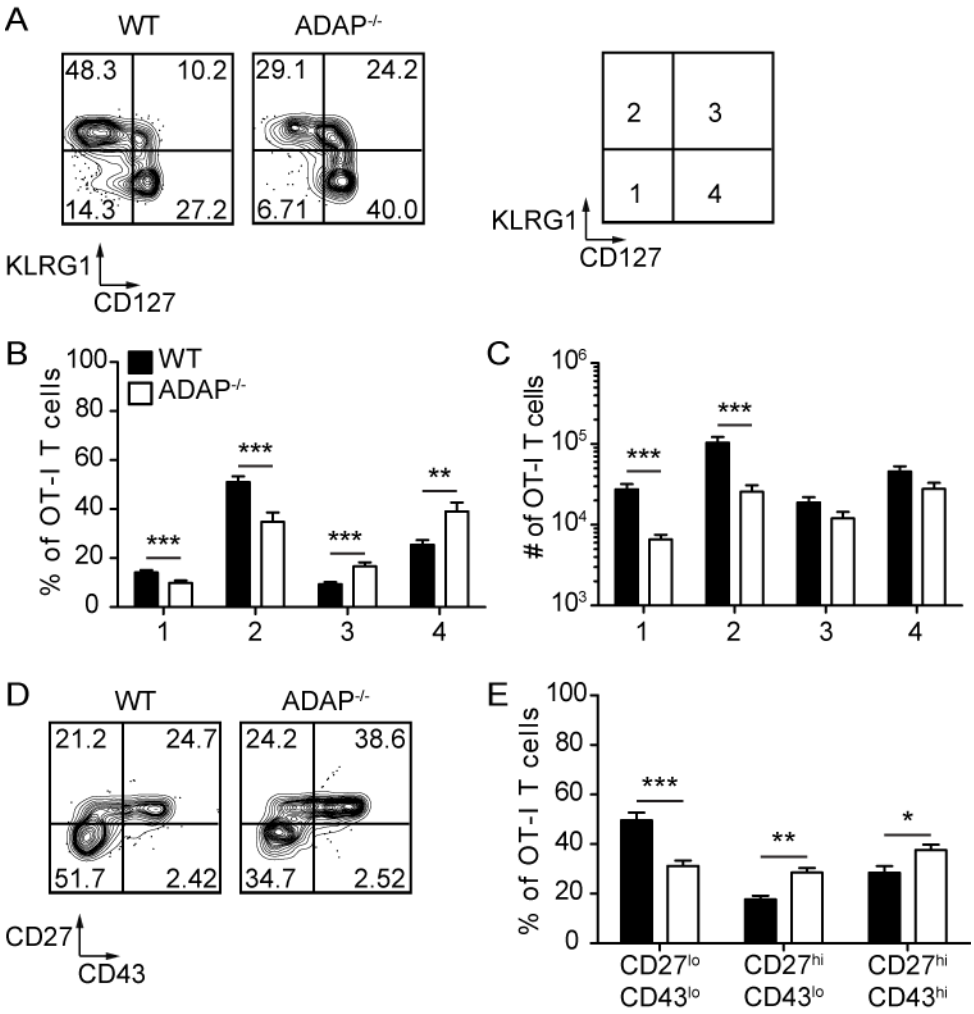

Figure 3-4: CD8 T cell effector differentiation is dependent on ADAP.

LM-OVA challenged mice were generated as in Figure 1. Splenocytes were isolated and stained for cell surface markers on day 14 (A-C) or 1 month (D-E) post challenge. (**A**) CD127 and KLRG1 staining. Numbers represent the percentage of cells in each quadrant. (**B**) Percentage or (**C**) number of WT (black bars) or ADAP$^{-/-}$ (white bars) OT-I T cells from each quadrant. (**D**) CD43 and CD27 staining. Numbers represent the percentage of cells in each quadrant. (**E**) Percentage of CD43^{lo}CD27^{lo}, CD43^{lo}CD27^{hi} and CD43^{hi}CD27^{hi} cells. The results (B and C) are compiled from 4 independent experiments, with 4 mice per experiment (± SEM), and the results (E) are compiled from 2 independent experiments with at least 3 mice per experiment (± SEM).

Loss of ADAP does not alter memory CD8 T cell expansion after systemic secondary challenge

To test the memory recall potential of ADAP-deficient OT-I T cells, memory recipient mice were challenged with Vesicular Stomatitis Virus expressing OVA (VSV-OVA). After 4 days we assessed the fold expansion of wild-type and ADAP-deficient OT-I T cells. ADAP-deficient OT-I T cells responded robustly to VSV-OVA secondary challenge (Fig. 3-5A and B). While the fold expansion is not statistically greater for ADAP-deficient OT-I T cells, there is not a defect in the proliferative response of these cells to a systemic challenge.

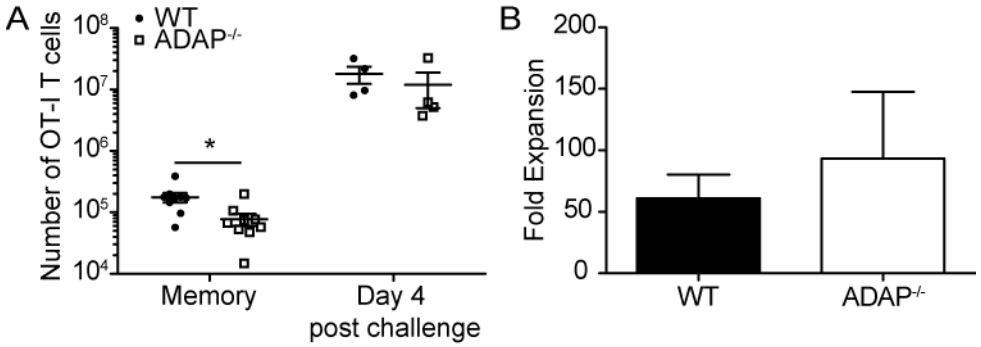

Figure 3-5: CD8 T cell secondary proliferation is independent of ADAP.

LM-OVA challenged mice were generated as in Figure 1. At day 45^+ animals were given a systemic challenge with 1e6 PFU of VSV-OVA. After 4 days spleens were harvested and stained for surface molecules. (**A**) Number of WT (black circles) or ADAP$^{-/-}$ (open squares) OT-I T cells in the spleen. (**B**) Fold expansion after VSV-OVA challenge. Data are representative of two experiments with 4 mice per experiment.

Reduced T_{RM} cell populations at memory time points in the absence of ADAP

As we observed altered memory populations in the absence of ADAP in SLOs, we next assessed memory in NLTs. Using intravascular staining techniques [109] we

identified tissue lymphocyte populations (Fig. 3-6A and B). Interestingly, the loss of ADAP-deficient CD8 T cells was not detected in the inguinal lymph node (iLN) or white pulp of the spleen, but was observed in the splenic red pulp and various NLTs (Fig. 3-6A and B). We found this of interest, as there are less $CD27^{lo}$ $CD43^{lo}$ cells in the absence of ADAP, and $CD27^{lo}$ $CD43^{lo}$ cells localize to the red pulp and non-lymphoid sites [95]. At the peak of the response, the window at which effector CD8 T cells can enter NLTs [5], we did not observe differences between wild-type and ADAP-deficient OT-I T cell numbers in NLTs (Fig. 3-6D). As $KLRG1^-$ CD8 T cells are precursors for CD8 T_{RM} [6], we assessed the frequency and number of wild-type and ADAP-deficient OT-I T cells in the spleen and female reproductive tract (FRT) at day 7 post challenge. Interestingly there were reduced numbers of $KLRG1^-$ ADAP-deficient OT-I T cells in the FRT at day 7 post challenge (Fig. 3-6F). Altered CD8 T cell differentiation may be the cause of reduced CD8 T_{RM} cell numbers in the absence of ADAP.

When we attempted to test the functionality of T_{RM} cells, the number of wild-type or ADAP-deficient antigen specific CD8 T cells was not sufficient to perform the assay (data not shown). In our hands, VSV-OVA generated greater numbers of T_{RM} cells in select tissues. Accordingly we changed our primary challenge from LM-OVA to VSV-OVA.

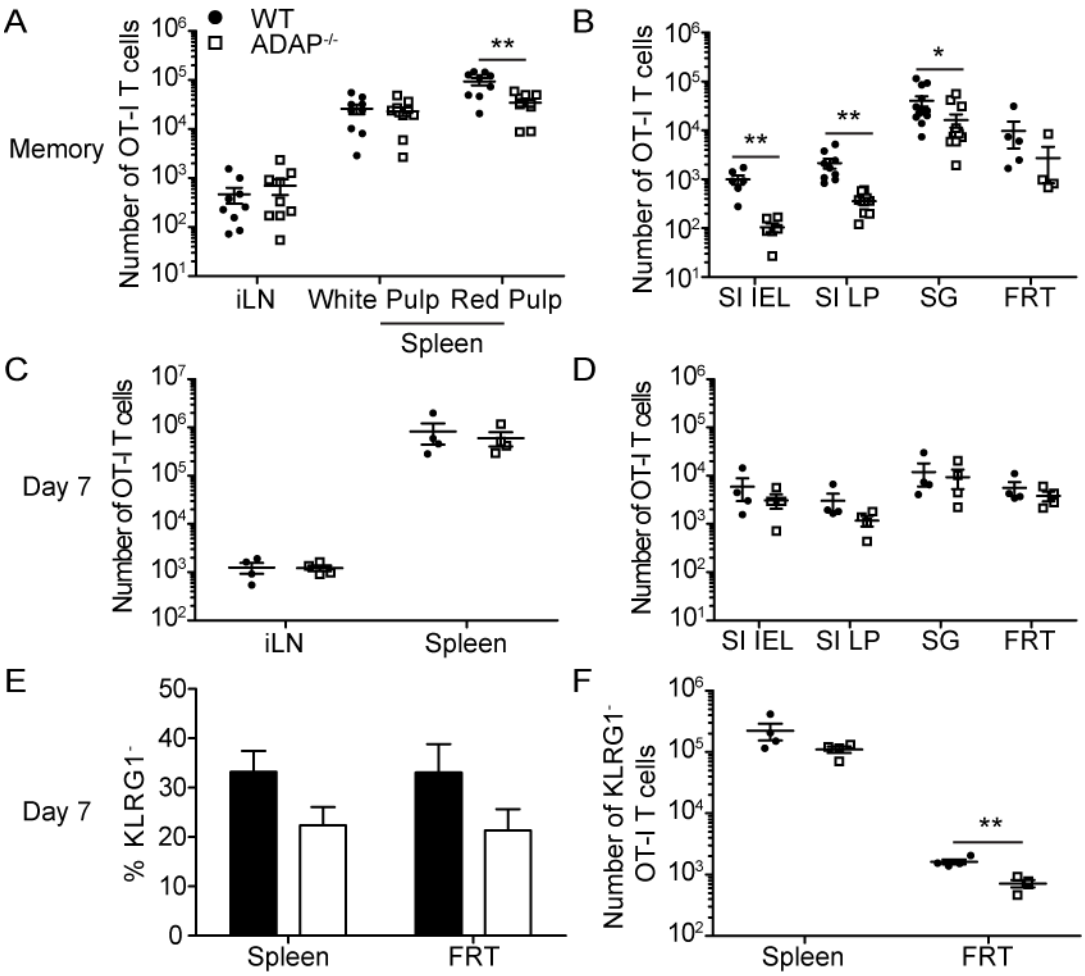

Figure 3-6: CD8 T_{RM} cells are reduced in the absence of ADAP.

LM-OVA challenged mice were generated as in Figure 1. (A-B) At day 45^{+} animals were injected with anti-CD8α mAb i.v., harvested and assessed for the number of wild-type and $ADAP^{-/-}$ OT-I T cells. (**A**) Number of WT (black circles) or $ADAP^{-/-}$ (open squares) OT-I T cells in the iLN, white pulp (WP) and red pulp (RP) of the spleen. (**B**) Number of anti-CD8α mAb negative wild-type and $ADAP^{-/-}$ OT-I T cells from the small intestine (SI) intraepithelial lymphocytes (IEL), lamina propia (LP), salivary gland (SG) and female reproductive tract (FRT). (C-D) At day 7 animals harvested and assessed for the number of wild-type and $ADAP^{-/-}$ OT-I T cells and KLRG1 expression. (**C**) Number of WT (black circles) or $ADAP^{-/-}$ (open squares) OT-I T cells in the iLN or spleen. (**D**) Number of wild-type and $ADAP^{-/-}$ OT-I T cells from NLTs. (**E**) Percentage or (**F**) number of KLRG1- wild-type and ADAP-/- OT-I T cells in the spleen or FRT. The results (A and B) are combined from at least 2 experiments with 6 mice per experiment (± SEM). The results (C-F) are one experiment with 4 mice (± SEM).

After VSV-OVA challenge ADAP-deficient OT-I T cell expansion was similar to wild-type (data not shown). Similar to our findings with LM-OVA, VSV-OVA challenge also resulted in reduced ADAP-deficient CD8 T cell numbers in the spleen at memory time points (Fig. 3-7A). At memory time points we harvested NLTs to assess the number of T_{RM} cells. Reduced numbers of ADAP-deficient OT-I T cells were found in the salivary gland (SG) and female reproductive tract (FRT) (Fig. 3-7A). A consistent marker for T_{RM} cells is CD69 [96]. A large percentage of wild-type OT-I cells expressed CD69, while reduced frequencies of ADAP-deficient cells were $CD69^{hi}$ in the FRT (Fig. 3-7B and C). These data suggest ADAP is required for the presence of T_{RM} cells at memory time points.

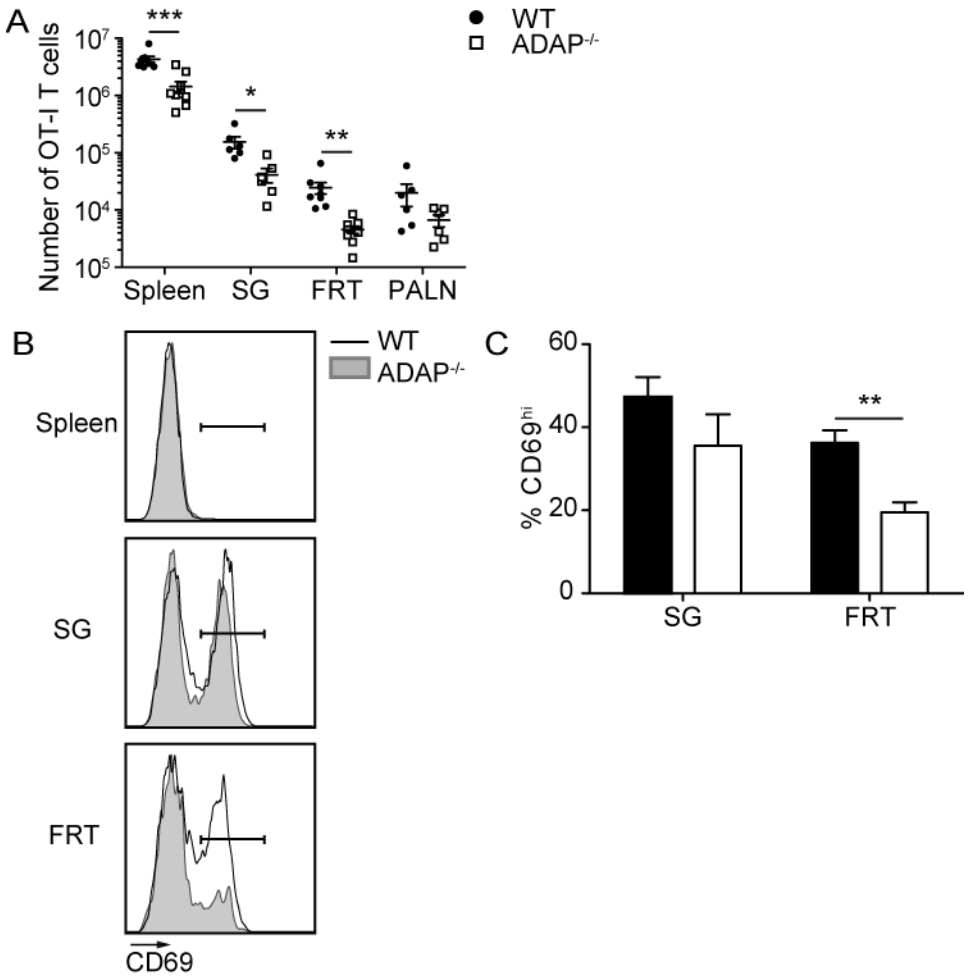

Figure 3-7: ADAP is required for optimal CD8 T_{RM} cell presence at memory time points.

Naïve WT (CD45.1/2) and ADAP$^{-/-}$ (CD45.2) OT-I T cells were cotransferred into CD45.1 hosts and challenged with VSV-OVA. Tissues were harvested and single cell suspensions were stained for cell surface markers at D33+ challenge. (**A**) Number of WT (black circles) or ADAP$^{-/-}$ (open squares) OT-I T cells in the spleen, SG, FRT or para-aortic lymph node (PALN) after VSV-OVA challenge. (**B**) CD69 staining from WT (black line) and ADAP$^{-/-}$ (grey shaded) OT-I T cells from spleen, SG and FRT. Gate represents CD69^{hi} population (**C**) Percentage of wild-type or ADAP$^{-/-}$ OT-I cells with CD69^{hi} staining. The results (A) are compiled from 3 independent experiments, with at least 4 mice per experiment ($\pm$ SEM). The results (C) are compiled from 2 independent experiments, with at least 3 mice per experiment ($\pm$ SEM).

Loss of ADAP does not alter T_{RM} cell responses with local peptide challenge

We next assessed the functionality of ADAP-deficient T_{RM} cells with a local peptide challenge in the FRT [98]. After stimulation, ADAP-deficient T_{RM} cells produced IFN-γ at the same levels as wild-type (Fig. 6A and B). Granzyme B production was also similar between wild-type and ADAP-deficient T_{RM} cells (Fig. 6C and D). Thus, ADAP is not required for effector cytokine production by T_{RM} cells after a local peptide challenge.

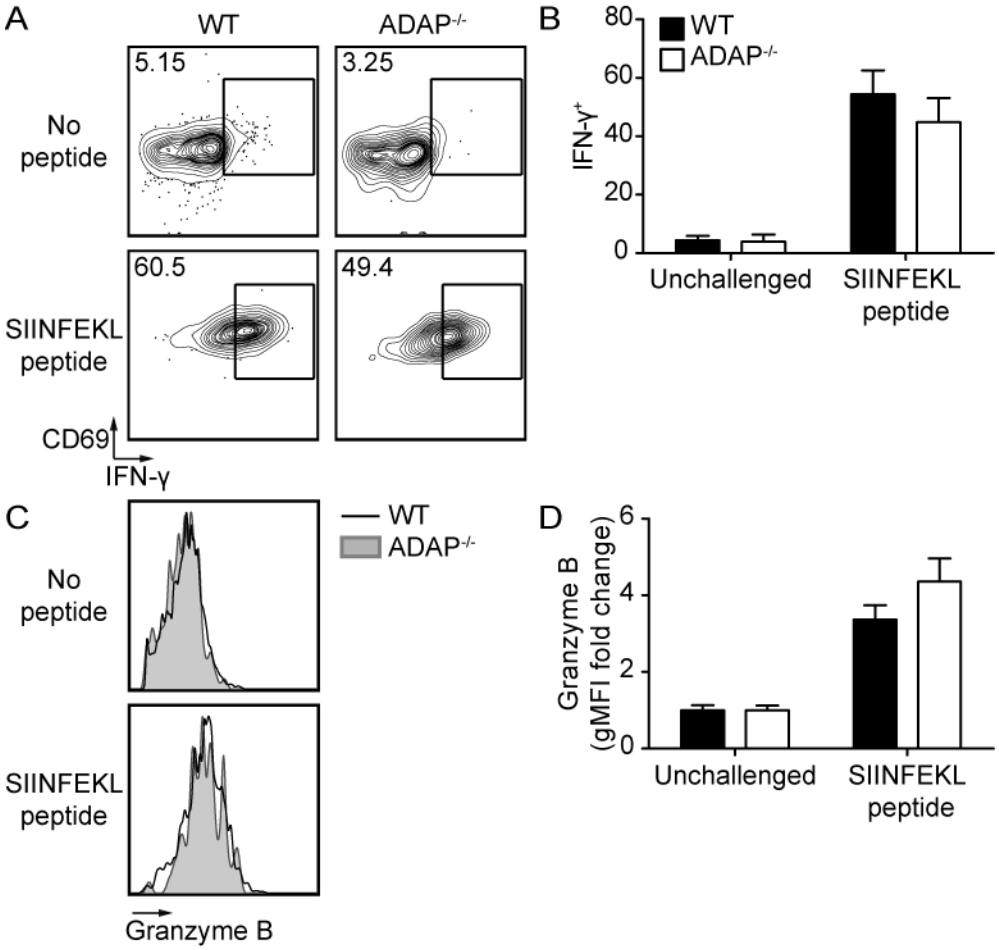

Figure 3-8: CD8 T_{RM} cell effector functions are not dependent on ADAP.

Mice were generated as in previous figures replacing pathogen with 1e6 PFU VSV-OVA. On day 30+ post infection animals were t.c. challenged with SIINFEKL peptide. At 12

hours post challenge spleen, PALN and FRT were harvested and stained for cell surface markers and intracellular cytokines. (**A**) Representative IFN-γ and CD69 staining from WT or ADAP$^{-/-}$ OT-I T cells from FRT. (**B**) Percentage of IFN-γ^{+} WT (black bars) or ADAP$^{-/-}$ (white bars) OT-I T cells in the FRT. (**C**) Representative Granzyme B staining from WT or ADAP$^{-/-}$ OT-I T cells. (**D**) Fold change in gMFI of Granzyme B^{+} WT or ADAP$^{-/-}$ OT-I T cells over unstimulated in the FRT. The results (B and D) are compiled from 3 independent experiments, with at least 3 mice per experiment (± SEM).

Reduced numbers of ADAP-deficient CD8 T_{RM} cells alter responses to local challenge

While ADAP-deficient CD8 T_{RM} cells can produce effector cytokines after a local peptide challenge, we wanted to directly test the effector capabilities of CD8 T_{RM} cells in the absence of ADAP. As there are reduced numbers of ADAP-deficient CD8 T_{RM} cells in the FRT, there might be too few antigen-specific cells to respond to antigen stimulation. In the absence of wild-type OT-I T_{RM} cells, ADAP-deficient T_{RM} produced significantly less IFN-γ after local peptide challenge (Fig. 3-9A and B). Though not significant, a trend for less Granzyme B production occurs in OT-I T cells in the absence of ADAP (Fig. 3-9C and D). Thus, the inability of ADAP-deficient OT-I T cells to be present in NLTs at memory time points leaves the host vulnerable.

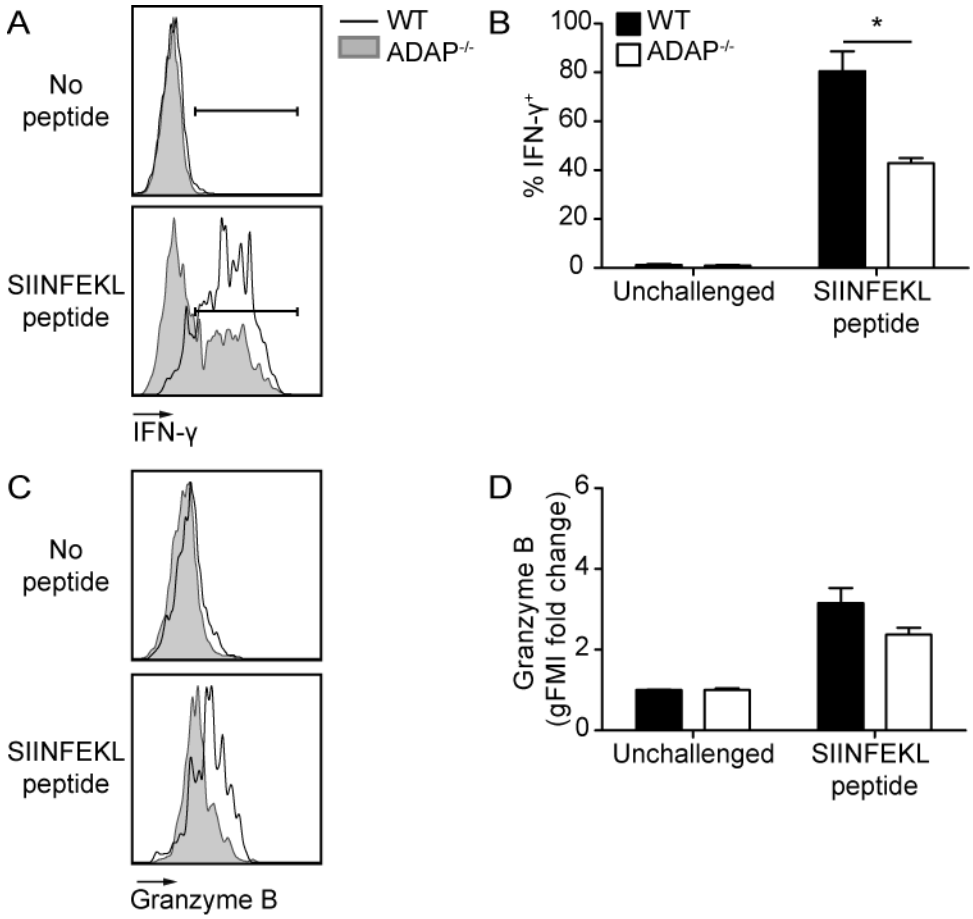

Figure 3-9: Reduced numbers of CD8 T_{RM} cells in the absence of ADAP blunts the response to local antigen challenge.

Naïve WT (CD45.2) or ADAP$^{-/-}$ (CD45.2) OT-I T cells were single transferred into CD45.1 hosts, and challenged with VSV-OVA. On day 30+ post infection animals were t.c. challenged with SIINFEKL peptide. At 12 hours post challenge FRTs were harvested and stained for cell surface markers and intracellular cytokines. (**A**) Representative IFN-γ staining from WT or ADAP$^{-/-}$ OT-I T cells. (**B**) Percentage of IFN-γ^{+} WT (black bars) or ADAP$^{-/-}$ (white bars) OT-I T cells. (**C**) Representative Granzyme B staining from WT or ADAP$^{-/-}$ OT-I T cells. (**D**) Fold change in gMFI of Granzyme B^{+} WT or ADAP$^{-/-}$ OT-I T cells over unstimulated. The results (B and D) are representative of 1 independent experiment, with 3 mice per group (± SEM).

DISCUSSION

In this study we have described a role for ADAP in CD8 T cell responses to pathogens. We focused on clonal expansion, effector CTL functions, contraction, memory generation, and the functionality of memory CD8 T cells. Sustained TCR signaling is required for optimal proliferation and clonal expansion of naïve CD8 T cells [113], while upregulation of numerous transcription factors facilitates differentiation into effector CD8 CTLs [76, 81, 82, 114]. Initial T-APC interactions during priming must be stable and long-lived for survival of activated CD8 T cells during contraction [72]. Alterations in the quality of TCR signaling can alter the balance between effector and memory CD8 T cell generation [84]. Our findings support a function for ADAP as a positive regulator of CD8 T cell contraction and memory generation, especially in the formation of memory in NLTs.

We have previously assessed the ability of ADAP-deficient CD8 T cells to interact with APCs, and observed reduced interactions with strong agonist ligands, as discussed in Chapter 2. Because of these findings, we expected to observe reduced proliferation of CD8 T cells in the absence of ADAP. In contrast, we observed normal proliferation and clonal expansion up to the peak of the immune response. These findings are in contrast to previous reports, which demonstrated a requirement for ADAP in CD4 and CD8 T cell expansion after TCR stimulation [36, 69, 70]. This could be due to inherent differences between CD4 and CD8 T cells. Previous assays of CD8 T cell proliferation were performed *in vitro* with either Ab-mediated or allogeneic DC-mediated TCR stimulation [69, 70]. These conditions may be different from the response of CD8 T cells *in vivo* in response to an intact pathogen, where the CD8 T cells receive TCR

stimulation, costimulation and inflammatory cytokine signals. Instead our findings are consistent with another model where CD8 T cells undergo sub-optimal T-APC interactions. CD8 T cells activated in the absence of ICAM-1 on DCs undergo robust proliferation, but exhibit increased contraction [72]. These results indicate CD8 T cells that receive reduced T-DC interactions and contact times do not exhibit proliferation and clonal expansion defects, but instead generate activated CD8 T cells with decreased survival potential during the contraction phase.

The differentiation of naïve CD8 T cells into effector CTLs is partially dependent on the cytokine milleu during priming [1, 2, 76, 88]. IL-12 is the major inflammatory cytokine produced in response to *Listeria monocytogenes* [111, 115]. IL-12 drives expression of the transcription factor T-bet, and the effector cytokine IFN-γ [76, 115]. Additionally, T-bet promotes the expression of IFN-γ and Granzyme B [76, 114]. As ADAP negatively regulates naïve T cell responses to the homeostatic cytokine IL-15 (Chapter 2), we expected to find greater differentiation of ADAP-deficient CTLs. In contrast, at the peak of the immune response equal production of the effector molecules IFN-γ and Granzyme B was observed between wild-type and ADAP-deficient CD8 T cells. Lack of a defect in ADAP-deficient CTL differentiation may be due to differences between IL-12 and IL-15-mediated signaling. IL-12 signaling occurs via the cell surface receptors IL-12Rβ1 and IL-12Rβ2, the former of which binds Jak2, and triggers STAT4 phosphorylation [115]. IL-15 signaling is initiated by binding of IL-15 to the IL-15Rβ and γC molecules on the cell surface, which activates JAK1 and 3, followed by activation of STAT5 [116]. Thus, unlike IL-15, IL-12 signaling is not altered in the absence of ADAP.

A role for ADAP in regulating CTL targeted killing may be predicted, since ADAP is required for TCR-mediated inside-out signaling to integrins, and integrin outside-in signaling [36, 63]. Integrins are used by NK cells for target recognition [117] and strong adhesion mediated by $\alpha_L\beta_2$ is required for target cell lysis by CD8 CTLs and NK cells [118]. In contrast, we and others have not found a role for ADAP in targeted killing by CD8 T cells [69, 70] or NK cells [42, 68]. CD8 T cells primed by ICAM-1-deficient DCs also exhibit normal targeted lysis [72]. These results indicate that ADAP does not regulate integrin signals in CTLs during targeted lysis.

After clearance of the pathogen, the majority of antigen-specific CD8 T cells die while a small percentage of cells are maintained into the memory phase [3]. At the peak of infection, activated CD8 T cells that have upregulated CD127 and downregulated KLRG1 are more likely to survive the contraction phase [76, 87]. Src homology 2 domain-containing leukocyte phosphoprotein of 76 kD (SLP-76) conditional knock-in (cKI) mutations of the tyrosine residues that mediate inducible T cell kinase (Itk), (Y145) and non-catalytic region of tyrosine kinase (Nck) and Vav1 (Y112/128) binding after TCR stimulation result in reduced proximal TCR signaling [84]. SLP-76 cKI mutations did not alter antigen-specific CD8 T cell expansion or contraction, instead promoted a greater percentage of $CD127^{hi}$ cells and reduced percentage of $KLRG1^{hi}$ cells during contraction [84]. A role for ADAP in generating a greater percentage of $CD127^{hi}$ cells could be predicted, as ADAP inducibly binds to SLP-76 after TCR stimulation, and similarly to Itk and Vav1, ADAP positively regulates TCR-mediated integrin activation [45]. Similar to SLP-76 cKI mice, a greater percentage of ADAP-deficient CD8 T cells upregulated CD127 and downregulated KLRG1, as compared to wild-type, during the contraction

phase. Whether this phenotype is due to altered TCR-mediated integrin activation or global TCR signaling defects is unknown, and warrants future investigation.

Altered differentiation of activated CD8 T cells could prevent the seeding of NLTs and generation of CD8 T_{RM} cells. As there are reduced frequencies of $KLRG1^{-}CD127^{-}$ ADAP-deficient CD8 T cells present during the contraction phase, we predicted that ADAP-deficient CD8 T cells would not generate T_{RM} cells to the same extent as wild-type. Reduced numbers of $KLRG1^{-}$ T_{RM} precursors were present in the FRT on day 7 after challenge in the absence of ADAP. The reduced potential of ADAP-deficient CD8 T cells to form T_{RM} in the FRT may also be attributed to an inability to upregulate CD69 as efficiently as wild-type cells. These results indicate that optimal formation of CD8 T_{RM} cells is dependent on ADAP.

CD8 T_{RM} cells provide rapid and near-sterilizing protection at the sites of pathogen entry [6, 98, 101]. Upon antigen recognition CD8 T_{RM} cells can specifically kill target cells, as well as recruit and activate both innate and adaptive immune cells [98, 101]. The secretion of IFN-γ by CD8 T_{RM} cells induces local inflammation, recruiting immune cells from the blood [98]. Due to the reduced numbers of ADAP-deficient CD8 T_{RM} cells and decreased CD69 expression, we expected ADAP-deficient CD8 T_{RM} cells to exhibit reduced functions in response to a local peptide challenge. Interestingly, ADAP-deficient CD8 T_{RM} cells upregulate IFN-γ and Granzyme B to wild-type levels, but only when wild-type and ADAP-deficient OT-I T cells were in the same host. Without the help of wild-type OT-I T_{RM} cells, ADAP-deficient OT-I T_{RM} cells were unable to produce similar levels of IFN-γ as wild-type cells. This may be due to the reduced number of antigen-specific CD8 T cells in the NLTs of hosts that only received ADAP-deficient OT-I T cells. CD8 T_{RM} cells in NLTs are often organized in large lymphoid-like aggregates, in

proximity to B cells, CD4 T cells, macrophages and DCs (Jason Schenkel, personal communication). It is possible that ADAP-deficient CD8 T_{RM} cannot enter, or effectively migrate in the absence of normal TCR-mediated integrin activation, or integrin-mediated signaling pathways. Futures studies will characterize the localization of ADAP-deficient CD8 T_{RM} cells in NLTs with respect to these lymphoid-like aggregates.

Our studies have revealed a unique role for ADAP in the differentiation of memory CD8 T cells and presence of CD8 T_{RM} in NLTs. Our results indicate that ADAP is required for optimal balance in the production of both effector and memory precursor CD8 T cells after pathogen challenge. Alterations in the balance between effector and memory CD8 T cells prevent optimal seeding of NLTs and may leave the host vulnerable to secondary infection. It is possible that a minimum threshold of antigen-specific CD8 T_{RM} cells must be present in NLTs for the ability of CD8 T cells to function as an alarm system for pathogen entry. These findings reinforce the importance of future studies to identify how many and what type of CD8 T cells are generated after vaccination to promote immunity both in SLOs and at the front lines of pathogen entry.

FOOTNOTES

[1]Abbreviations used in this chapter: ADAP, adhesion and degranulation promoting adapter protein; APC, antigen presenting cell; CARMA-1, caspase recruitment domain (CARD) membrane-associated guanylate kinase (MAGUK) protein; CFU, colony forming unit; CTL, cytotoxic T lymphocyte; FRT, female reproductive tract; gMFI, geometric mean fluorescence intensity; IL-7Rα, interleukin-7 receptor α; iLN, inguinal lymph node; KLRG1, killer cell lectin-like receptor G1 (KLRG1); *Listeria monocytogenes*, LM; NLTs,

non-lymphoid tissues; OVA, ovalbumin; PALN, para-aortic lymph node; pLN, peripheral lymph node; PFU, plaque forming units; SG, salivary gland; SI IEL, small intestine intraepithelial lymphocyte; SI LP, small intestine lamina propia; SKAP55, Src kinase-associated phosphoprotein of 55 kDa; SLO, secondary lymphoid organ; SLP-76, Src homology 2 domain-containing leukocyte phosphoprotein of 76 kD; TAK-1; transforming growth factor-β (TGF-β)-activated protein kinase; T_{CM} cells, central memory T cells; TCR, T cell receptor; T_{EM} cells, effector memory T cells; T_{RM} cells, resident memory T cells; VSV, vesicular stomatitis virus.

CHAPTER 4: Implications for ADAP-mediated regulation of memory CD8 T cells

CONCLUSIONS

Work in my thesis has characterized the role of adhesion and degranulation-promoting adapter protein (ADAP) in the generation of memory and memory phenotype CD8 T cells. The majority of work on ADAP prior to my thesis has been on the role of ADAP in CD4 T cells after Ab or cognate antigen stimulation of the TCR [36]. From this work we know that ADAP positively regulates TCR-mediated integrin activation, contacts between T cells and antigen presenting cells (APCs), entry into the cell cycle and early CD4 T cell differentiation [36]. Other work has documented the role of ADAP in cytokine-mediated integrin activation and outside in integrin signaling [36]. Additionally, a few reports on the role of ADAP in graft rejection have documented normal CD8 cytotoxic T lymphocyte (CTL) functions, but reduced infiltration into donor grafts [69, 70]. Work in Chapter 2 has identified ADAP as a negative regulator of MP CD8 T cell generation, by limiting IL-15 signaling. Additionally, ADAP positively regulates strong agonist T-APC interactions, but negatively regulates weak agonist interactions. In Chapter 3, I assessed the role of ADAP in CD8 T cell immune responses. ADAP was not required for naïve CD8 T cell activation or proliferation but positively regulated survival during contraction and memory generation in the blood, red pulp of the spleen and in non-lymphoid tissues (NLTs). In this Chapter, I will discuss some implications for increased MP CD8 T cell generation and decreased resident memory T (T_{RM}) cell generation in the absence of ADAP.

ADAP-deficient mice demonstrate increased graft survival

Even in the absence of an infection, a percentage of CD8 T cells exhibit memory-like properties, termed memory phenotype (MP). MP CD8 T cells can be generated in the thymus or after thymic egress [7, 13]. Increased availability of homeostatic cytokines or increased reactivity to self-peptide MHC-I ligands in secondary lymphoid organs (SLOs) can stimulate naïve CD8 T cells to differentiate into MP CD8 T cells [7]. As discussed in Chapter 2, the extrathymic generation of MP CD8 T cells in the absence of ADAP is enhanced. Naïve ADAP-deficient CD8 T cells are hyperresponsive to lymphopenia, and exhibit increased signaling downstream of IL-15. The enhanced generation of MP CD8 T cells is not due to altered self-reactivity of ADAP-deficient naïve CD8 T cells. MP CD8 T cells can protect the host early during the immune response, before naïve CD8 T cells are and exhibit effector functions [7].

While there are benefits to generating MP CD8 T cells, these cells can also cause unwanted damage. Recent work has highlighted the role of MP CD8 T cells in mediating rejection of grafts [102]. Memory CD8 T cells can quickly produce effector molecules and specifically kill target cells after TCR-stimulation [3]. Endogenous MP CD8 T cells in mice receiving cardiac allografts quickly infiltrate the donor graft and mediate inflammation and injury of the allograft [102]. Inhibition or removal of endogenous MP CD8 T cells attenuates donor graft injury and rejection [102].

While MP CD8 T cells can mediate allograft rejection and greater numbers of MP CD8 T cells can be found in the periphery of ADAP-deficient mice, mice lacking ADAP are protected from graft rejection. Two studies documented the increased graft survival of donor grafts in ADAP-deficient mice [69, 70]. The latter study assessed cardiac allografts and used the same method as Su and colleagues [70, 102]. Reduced

numbers of CD8 and CD4 T cells infiltrated cardiac allografts from ADAP-deficient hosts [70]. As I observed equal numbers of memory CD8 T cells in lymph nodes and the white pulp of the spleen after pathogen challenge (Chapter 3), MP CD8 T cells may also preferentially localize to SLOs. While I did not detect defects in the ability of ADAP-deficient effector CD8 T cells to traffic to NLTs at the peak of the immune response (Chapter 3), it is possible that ADAP-deficient MP CD8 T cells do not efficiently traffic outside of SLOs. Interestingly, wild-type T cells are preferentially associated with lymphatic vessels in SLOs of ADAP-deficient mice [119]. These results may indicate that in ADAP-deficient hosts, MP CD8 T cells cannot efficiently exit SLOs to traffic to the site of the allograft. Additionally, MP CD8 T cells from ADAP-deficient hosts may be less reactive to allografts than wild-type MP CD8 T cells. Another possibility is that ADAP-deficient MP CD8 T cells are not as functional as wild-type CD8 T cells. I have not tested the effector molecule production or specific killing functions of ADAP-deficient MP CD8 T cells. These results highlight that the increased generation of MP CD8 T cells in the absence of ADAP is not detrimental to the host, but also indicate that future studies are needed to assess the functionality of ADAP-deficient MP CD8 T cells.

Decreased risk of experimental autoimmune encephalomyelitis (EAE) in the absence of ADAP

A minority of CD8 T cells escape negative selection in the thymus and have the potential to become activated in response to self-peptide MHC-I ligands [7]. These cells are suppressed by $CD4^+$ T regulatory cells, or are functionally anergic [7]. Upon instances of acute lymphopenia, such as an infection, exposure to irradiation or

chemotherapy, these self-reactive CD8 T cells can gain memory-like functions and cause damage to the host [7]. Molecules associated with increased MP CD8 T cell generation, c-Cbl [7], PTPN2 [26], PTPN22 [27], and SOCS-1 [30, 31], are also associated with autoimmune diseases. While single nucleotide polymorphisms (SNPs) in the gene encoding ADAP have been associated with an increased risk of acquiring the autoimmune disease systemic lupus erythemathosus (SLE) [120], a CD8-mediated autoimmune disease has not been associated with ADAP. This may partially be due to a lack in altered self-reactivity in the absence of ADAP (Chapter 2), or a decreased ability to traffic to NLTs [119].

Experimental autoimmune encephalomyelitis (EAE) is an animal model used to study multiple sclerosis (MS) [119, 121]. MS is a proinflammatory demyelinating disease classically thought to be mediated by autoimmune CD4 T cells [119, 121]. Recent studies have shown that autoimmune CD8 T cells also play a role in EAE [121]. Autoreactive CD8 T cells can kill oligodendrocytes and neuronal cells after entering the spinal chord and brain [121]. As ADAP positively regulates TCR- and chemokine-mediated integrin activation [36], loss of ADAP has been proposed to protect hosts from EAE [119]. Interestingly, ADAP-deficient hosts develop less severe EAE, but this result was not due to alterations in CD4 or CD8 T cells [119]. Instead radio-resistant non-hematopoietic cell populations modulate EAE in ADAP-deficient hosts [119]. Even though greater MP CD8 T cell generation occurs in the absence of ADAP, ADAP-deficient MP CD8 T cells do not appear to be involved in EAE or graft rejection.

Immune responses and protection in the absence of ADAP

Work in my thesis has demonstrated that ADAP positively regulates CD8 T_{RM} cell generation, as discussed in Chapter 3. Reduced numbers of antigen-specific ADAP-deficient CD8 T_{RM} decrease local responses to peptide challenge. In contrast, in the absence of ADAP, normal numbers of memory CD8 T cells are present in lymph nodes and white pulp of the spleen. In response to a systemic secondary challenge ADAP-deficient memory CD8 T cells from SLOs proliferate robustly. Additionally, increased MP CD8 T cell generation occurs in the absence of ADAP (Chapter 2), and MP CD8 T cells are mainly located in the blood and SLOs. These results indicate that ADAP-deficient hosts would be able to respond to a systemic pathogen, but may not offer protection against pathogens that enter the host via NLTs, like lung, intestinal mucosa or skin. Especially considering that T cells preferentially localize to lymphatic vessels in ADAP-deficient hosts [119], it is interesting that patients with defects in ADAP have not been reported with increased mucosal infections. Possibly the robust response in SLOs in the absence of ADAP compensates for decreased pathogen protection in local sites. For example, in response to systemic *Listeria monocytogenes* or lymphocytic choriomeningitis virus infections, ADAP-deficient hosts respond similarly to wild-type hosts. Robust expansion of both antigen-specific CD4 and CD8 T cells occurs. The ability of ADAP-deficient hosts to protect against secondary systemic or local infections will need to be investigated in future studies.

ADAP: The all important adaptor protein

Work in this thesis and from previous studies have implicated ADAP in many hematopoietic cell functions. Understanding how ADAP is functioning in CD8 T cells may aid both MP CD8 T cell and memory T cell fields. The balance of naïve and MP CD8 T cells is altered in the absence of ADAP, potentially aiding in responses to pathogens without increasing the risk for generating CD8-mediated autoimmune diseases. Additionally ADAP-deficient hosts can respond robustly to systemic infection. Future studies will focus on the implications of greater MP CD8 T cell during systemic immune responses and how loss of ADAP affects local pathogen infections.

FOOTNOTES

[1]Abbreviations used in this chapter: ADAP, adhesion and degranulation promoting adapter protein; APC, antigen presenting cell; CTL, cytotoxic T lymphocyte; EAE, experimental autoimmune encephalomyelitis; MP, memory phenotype; NLTs, non-lymphoid tissues; SLO, secondary lymphoid organ; TCR, T cell receptor; T_{RM} cells, resident memory T cells.

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