Negative Regulation of Memory Phenotype 
CD8 T Cell Conversion by Adhesion and 
Degranulation–Promoting Adapter Protein

Jessica K. Fiege, Brandon J. Burbach and Yoji Shimizu

J Immunol 2015; 195:3119-3128; Prepublished online 28
August 2015;
doi: 10.4049/jimmunol.1402670
http://www.jimmunol.org/content/195/7/3119

Supplementary Material

http://www.jimmunol.org/content/suppl/2015/08/28/jimmunol.140267
0.DCSupplemental

Why The JI?

• Rapid Reviews! 30 days* from submission to initial decision
• No Triage! Every submission reviewed by practicing scientists
• Speedy Publication! 4 weeks from acceptance to publication

*average

References

This article cites 29 articles, 16 of which you can access for free at:
http://www.jimmunol.org/content/195/7/3119.full#ref-list-1

Subscription

Information about subscribing to The Journal of Immunology is online at:
http://jimmunol.org/subscription

Permissions

Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts

Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Negative Regulation of Memory Phenotype CD8 T Cell Conversion by Adhesion and Degranulation–Promoting Adapter Protein

Jessica K. Fiege, Brandon J. Burbach, and Yoji Shimizu

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 class 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 naive CD8 T cells to MP CD8 T cells during acute lymphopenia. We have identified a novel negative regulatory role for adhesion and degranulation–promoting adapter protein (ADAP) in CD8 T cell function. We show that in the absence of ADAP, naive CD8 T cells exhibit a diminished response to stimulatory Ag, but an enhanced response to weak agonist-altered peptide ligands. ADAP-deficient mice exhibit more MP CD8 T cells that occur following thymic emigration and are largely T cell intrinsic. Naive ADAP-deficient CD8 T cells are hyperresponsive to lymphopenia in vivo and exhibit enhanced activation of STAT5 and homeostatic Ag-independent proliferation in response to IL-15. Our results indicate that ADAP dampens naive CD8 T cell responses to lymphopenia and IL-15, and they demonstrate a novel Ag-independent function for ADAP in the suppression of MP CD8 T cell generation. The Journal of Immunology, 2015, 195: 3119–3128.

Carefully balanced in a healthy host, T cell homeostasis maintains a diverse T cell repertoire against potential foreign pathogens. Utilizing both self-peptide MHC class I (MHC-I) and IL-7 signaling pathways, naive CD8 T cells compete for space with each other and a steady emigration of newly developed T cells out of the thymus (1). 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) (2). This period of neonatal lymphopenia is the primary generator of MP T cells, which are predominately foreign Ag inexperienced and are maintained long into adulthood (3). MP T cells have similar functional capabilities as foreign Ag–induced memory cells, but they do not require prior Ag experience (4). 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 (1). Furthermore, allowing naive 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 (1, 4, 5).

The molecular factors that drive naive T cell homeostasis and permit the generation of MP T cells from the naive, Ag-inexperienced pool are only partially understood. Although joint signaling by IL-7 and self-peptide MHC-I are thought to be the main drivers of naive T cell homeostasis, optimal survival of naive T cells is dependent on additional signaling from IL-15 (6). IL-15 signaling in naive T cells drives the expression of the antiapoptotic protein Bcl-2, but it does not trigger proliferation, except in extreme situations, such as in the absence of CD122 (6, 7). Indeed, disruption of IL-15 signaling in mice lacking suppressor of cytokine signaling (SOCS)-1 results in altered T cell homeostasis (8). Both naive 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 (8). However, whereas 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.

Adhesion and degranulation–promoting adapter protein (ADAP) is a multifunctional adapter 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 (9). 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 (10). ADAP is required for optimal positive and negative selection during conventional CD4 and CD8 T cell development, but it is dispensable for the development of unconventional thymocytes, including NKT cells (10, 11). ADAP is localized to the cytosol, where a fraction is

Department of Laboratory Medicine and Pathology, Center for Immunology, Masonic Cancer Center, University of Minnesota Medical School, Minneapolis, MN 55455
ORCID: 0000-0001-9760-0288 (Y.S.).
Received for publication October 21, 2014. Accepted for publication July 28, 2015.
This work was supported by National Institutes of Health Grant R01 AI038474 (to Y.S.). Y.S. is also supported by the Harry Kay Chair in Biomedical Research at the University of Minnesota.
Address correspondence and reprint requests to Dr. Yoji Shimizu, University of Minnesota Medical School, Campus Code 2641, 2101 6th Street SE, Minneapolis, MN 55441. E-mail address: shimi002@umn.edu
The online version of this article contains supplemental material.
Abbreviations used in this article: ADAP, adhesion and degranulation–promoting adapter protein; APL, altered peptide ligand; B6, C57BL/6; BMC, bone marrow chimera; gMFI, geometric mean fluorescence intensity; IkB, inducible T cell kinase; LIP, lymphopenia-induced proliferation; MHC-I, MHC class I; MP, memory phenotype; N4, SIIFEKL peptide; 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, suppressor of cytokine signaling; SP, single-positive; Tg, transgenic; WT, wild-type.
Copyright © 2015 by The American Association of Immunologists, Inc. 0022-1767/15/$25.00
www.jimmunol.org/cgi/doi/10.4049/jimmunol.1402670
Copyright 2015 by The American Association of Immunologists, Inc. 0022-1767/15/$25.00

Downloaded from http://www.jimmunol.org/ by guest on October 30, 2017
constitutively associated with Src kinase–associated phosphoprotein of 55 kDa (SKAP55) (12). The ADAP-SKAP55 signaling module is critical for TCR-mediated activation of integrin-mediated adhesion with APCs (12, 13). A second pool of ADAP is not associated with SKAP55, but activates NF-kB and JNK in a TCR-inducible manner (12, 14–16).

Analysis of ADAP function utilizing primary T cells following Ab-mediated TCR stimulation or mature naive CD4 T cells following cognate peptide–MHC class II stimulation has demonstrated that ADAP is a positive regulator of T cell signaling that enhances T cell sensitivity to Ag. In contrast, the function 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 (WT) and ADAP−/− mice were generated as previously described (17). 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) (18). All mice were harvested between 8 and 12 wk of age, unless otherwise specified. B6 CD45.1, B6 CD45.2, and Rag1−/− mice were provided by Dr. M. Farrar and Dr. D. Masopust (University of Minnesota). IL−15−/− recipient mice were provided by Dr. M. Farrar and Dr. D. Masopust (University of Minnesota). 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 Abs, and then washed twice before multiparameter flow cytometric detection on a BD LSRFortessa (Becton Dickinson, San Jose, CA). Directly conjugated fluorescent Abs 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 Ra3-6B2) (BioLegend); CD44 (clone IM7), CD122 (clone TM-β 1), CD124 (clone MIL4-M1), and pSTAT5 pY694 (clone 47) (BD Biosciences); and TCR-β (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 (12). Briefly, cells from peripheral lymph nodes (pLNs) were harvested from WT 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. WT 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+Vou2+ T cells, either CD44+ or CD44+, that coexpress B220 and Cell Tracker Orange.

Mixed bone marrow chimeras

Mixed bone marrow chimeras (BMCs) were generated by mixing T cell–depleted bone marrow preparations from CD45.1/2 (WT) and CD45.2 (ADAP−/−) mice at indicated ratios and injecting 4 × 106 to 10 × 106 total cells into lethally irradiated (1100 rad) CD45.1 animals (19). All chimeras were analyzed 8–12 wk after transplant.

Naïve CD8 T cell transfer

Lymph nodes were harvested from CD45.2 (WT) and CD45.1/2 (ADAP−/−) mice, and CD8 mature naive T cells were isolated by negative magnetic bead enrichment similar to previously described methods (20). Briefly, single-cell suspensions were incubated with the following FITC-conjugated Abs: CD4, B220, F4/80, CD16/32, I-Ab, Ter119, and CD44. After washing, cells were incubated with anti-FITC microbeads and passed over LS columns on magnets according to manufacturer’s instructions (Miltenyi Biotec, Auburn, CA) to capture non-CD8 cells. Purity of the flow-through fraction was >95% CD8+CD44+. Purified cells were cotransferred into CD45.1 recipients at a 1:1 ratio. Recipient mice were analyzed 1 and 14 d after transfer. For Rag1−/− recipient experiments, CD45.1 (WT) and CD45.1/2 (ADAP−/−) OT-I mice were used as donors. Cells were purified as described above and 2 × 106 naïve CD8 T cells were cotransferred into B6 or Rag1−/− CD45.2 mice at a 1:1 ratio. Recipient mice were analyzed 7 d after transfer.

Naïve CD8 T cell transfer into lymphopenic hosts

Lymph nodes were harvested from CD45.2 (WT) and CD45.2 (ADAP−/−) mice and CD8 mature naive 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 × 106 cells/ml. CFSE di-luted in PBS was added 1:1 to the cells (2.5 μM final concentration) and incubated at 25°C for 5 min. Cells were quenched by washing twice with PBS with 2% FBS. Cells were transferred into separate subthermally irradiated (550 rad) CD45.1 animals (21). Recipient mice were analyzed 10 d after transfer.

Detection of intracellular p-STAT5

Cytokine stimulation and detection of p-STAT5 were performed as previously described (22). Briefly, splenocytes from polyclonal WT and ADAP−/− mice were rested for 2 h at 37°C in complete T cell medium at 2 × 106 cells/ml. Recombinant mouse IL-7 (Miltenyi Biotec) or IL-15 (R&D Systems) (1 ng/ml, or 0.1–300 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 ice-cold methanol added to the cells while vortexing. After incubation for 15 min at 4°C, cells were washed twice and stained for surface and intracellular Ags for 30 min at room temperature.

In vitro cytokine culture

Cytokine culture was performed as previously described (22). Lymph nodes were harvested from CD45.1/2 (WT) and CD45.2 (ADAP−/−) OT-I mice and naive 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 pulsed at 2 × 106 cells/ml and rested overnight (∼16 h) 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 d.

Statistical analysis

GraphPad Prism (version 5.03, GraphPad Software, La Jolla, CA) was used to determine statistical significance using a Student unpaired two-tailed t test. A p value <0.05 was considered statistically significant. For p-STAT5 mean fluorescence intensity (gMFI) analysis, statistics were performed using a normalized value of 100. A nonlinear curve was generated for the dose response to IL-15 to determine log EC50s. For CFSE gMFI analysis, statistics were performed using a normalized value of 1.

Results

ADAP controls sensitivity to Ag

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 (9). We assessed the ability of WT and ADAP-deficient CD8+ OT-I TCR-transgenic (Tg) T cells, which recognize SIINFEKL (N4) peptide in the context of MHC-I H-2Kb with high affinity (23), to form stable contacts with peptide-pulsed APCs by flow cytometry. Both WT and ADAP-
deficient naive OT-I T cells exhibited minimal adhesion to APCs in the absence of peptide Ag (Fig. 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 Ag doses (Fig. 1A, left). This defect diminished with the addition of higher concentrations of N4 peptide. Thus, similar to our previous observations with CD4 T cells, naive CD8 T cells require ADAP for maximal T cell–APC interactions (24).

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 naive CD8 T cells, which allows for the analysis of a single TCR over a range of agonist strengths (23). Overall conjugate efficiency of WT naive CD8 OT-I T cells was reduced with intermediate and weak agonist APLs SIIQFEKL (Q4) and SIITFEKL (T4), respectively (23, 25). 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 cell–APC interactions with the intermediate strength agonist APL Q4 were equivalent between WT and ADAP-deficient OT-I T cells (Fig. 1A, center). Even more striking, ADAP-deficient naive OT-I T cells exhibited enhanced interaction with APCs pulsed with T4 APL when compared with WT controls (Fig. 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.

Increased MP CD8 T cells in the absence of ADAP

Even in the absence of immunization, ~10–20% of CD8 peripheral T cells in WT C57BL/6 mice housed in specific pathogen-free conditions express elevated levels of the activation marker CD44 (Fig. 2A) and CD122 (Fig. 2B), a phenotype characteristic of memory CD8 T cells (3). In our analysis of ADAP-deficient mice, we consistently observed a 2-fold increase in the frequency of CD44hiCD122hi MP CD8 T cells in ADAP-deficient mice compared with littermate controls (Fig. 2B, 2C). Henceforth, we define naive CD8 T cells as CD44loCD122lo and MP CD8 T cells as CD44hiCD122hi. When compared with WT mice, the total number of MP CD8 T cells in the spleen of ADAP-deficient mice was also elevated (Fig. 2D). Thus, ADAP-deficient mice demonstrate an increased CD8 MP T cell population in the steady-state.

We next assessed the adhesion of WT and ADAP-deficient CD44hi MP CD8 OT-I T cells with APCs pulsed with the N4, Q4, or T4 peptides. With all three ligands, we observed a higher level of conjugate formation by MP CD8 OT-I T cells compared with naive CD8 OT-I T cells (Fig. 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 naive CD8 T cells (Fig. 1B). These results indicate an enhanced ability of MP CD8 T cells to interact with APCs pulsed with agonist peptide ligands compared with naive CD8 T cells, and a requirement for ADAP in regulating Ag sensitivity of MP CD8 T cells.

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) (26). 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 single-positive (SP) thymocytes in WT and ADAP-deficient mice (Fig. 3A). Total numbers of mature naive CD8 SP thymocytes were also unaffected by the loss of ADAP (Fig. 3A). 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 (26). We observed a statistically significant increase in the percentage, but not in the number, of thymic CD1d-αGalCer tetramer+ NKT cells and γδ NKT cells in ADAP-deficient mice (Supplemental Fig. 1A–C). However, this was not associated with elevated levels of serum IgE in aged ADAP-deficient mice (Supplemental Fig. 1D). Thus, increases in MP CD8 T cells in the periphery of ADAP-deficient mice are not due to elevated generation of MP CD8 T cells in the thymus.

**FIGURE 1.** ADAP modulates T cell–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 cell/APC conjugate formation of naive CD44lo 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 is shown. Results are averages from duplicate or quadruplicate wells from independent experiments (±SEM): seven experiments (N4), four experiments (Q4), and three experiments (T4). *p < 0.05, **p < 0.01.
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. RTEs and mature CD8 T cells can be differentiated by the expression of QA2 and CD24 (27). RTEs that have emigrated from the thymus within a week are QA2loCD24hi, whereas T cells that have emigrated between 1 and 2 wk prior have upregulated QA2 while maintaining high levels of CD24 (27). Mature cells, which emigrated at least 2 wk prior, are QA2hiCD24lo (27). Comparison of RTE, 1–2 wk emigrated, and mature populations from WT and ADAP-deficient unimmunized adult mice demonstrate a similar frequency and number of these populations, indicating similar emigration and maturation of RTEs (Fig. 3B, 3C). We also assessed the frequency of MP CD8 T cells among peripheral CD8 populations. RTEs are predominately CD122lo, whereas 1–2 wk after 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 wk after egress CD8 T cells than WT (Fig. 3D). This difference is maintained in the mature population, which constitutes most of the CD8 T cells in the periphery of an adult mouse.

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 (11). Altered T cell development could modify the T cell repertoire of ADAP-deficient CD8 T cells, resulting in naive CD8 T cells with a higher propensity to become MP. To
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 BMCs. Bone marrow from a WT 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 WT hosts (CD45.1). Spleens and thymi were harvested 8–12 wk after transfer of donor marrow. In this environment, where most of the bone marrow-derived cells are WT, we observed that the frequency of ADAP-deficient MP CD8 T cells was 1.5-fold greater than WT (Fig. 4E, 4F), 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 having the majority of developing thymocytes lacking ADAP on the minority of WT CD8 T cells. In an ADAP-deficient thymocyte majority situation, we observed an increased frequency of WT MP CD8 T cells when compared with normal WT mice (Figs. 2B, 2C, 4G, 4H). Similar to what we observed with mixed BMCs with a majority of WT cells, there was a higher percentage of ADAP-deficient MP CD8 T cells when compared with WT CD8 T cells, although the difference was not as pronounced as what we observed in unmanipulated mice or BMCs with a majority of WT cells (Fig. 4G, 4H). These results suggest that there may also

address this possibility, we assessed the frequency of MP CD8 T cells in WT and ADAP-deficient, Rag2-deficient, P14 TCR-Tg mice. The use of P14 TCR-Tg mice allowed us to compare WT and ADAP-deficient CD8 T cells with the same TCR, eliminating any differences that may have arisen in the T cell repertoire due to differences in positive and negative selection in the absence of ADAP. The frequency of peripheral MP CD8 T cells was increased in ADAP−/−Rag2−/− P14-Tg mice compared with WT mice (Fig. 4A, 4B). These results support a TCR-independent role for increased MP CD8 T cells in the absence of ADAP.

We also used the Nur77-GFP reporter mouse to determine whether WT and ADAP-deficient CD8 T cells had similar tonic signaling (18). In the absence of ADAP, defects in negative selection may allow for the development of CD8 T cells with higher self-reactive TCRs than WT. After thymic egress, these self-reactive naive CD8 T cells could come into contact with self-antigen, proliferate, and gain memory-like characteristics. This might explain why more CD8 T cells become MP in the absence of ADAP. The Nur77-GFP reporter mouse allows us to measure the tonic TCR signaling and determine whether ADAP-deficient CD8 T cells are more self-reactive. The gMFI of Nur77-GFP was similar in WT and ADAP-deficient naive or MP CD8 T cells isolated from unmanipulated mice (Fig. 4C, 4D). These results suggest that naive and MP CD8 T cells in the absence of ADAP are not more self-reactive than WT CD8 T cells.
both WT and ADAP-deficient CD8 T cells retained a CD44lo
in which RTEs mature and can convert to MP. The majority of
conversion of donor naive CD8 T cells to MP, a time frame
adult WT hosts. Two weeks after transfer, we assessed the
and ADAP-deficient pLNs and co-transferred these cells into
mixed at a 1:1 ratio, and transferred into CD45.1 recipient mice (1 × 10^5
cells/mouse). (A) CD44 and CD122 staining of transferred cells from re-
cipient spleens. Numbers represent the percentage of MP CD8 T cells. (B) Percentage of CD44hiCD122hi donor cells. The results in (B) are compiled from two independent experiments, each with four mice (±SEM). be an additional role for T cell–extrinsic factors in ADAP-
dependent generation of MP CD8 T cells.

Mature naive CD8 T cells do not convert to MP in the absence
of ADAP
To determine whether mature naive ADAP-deficient CD8 T
cells have a higher propensity to convert to MP in the steady-
state, we isolated CD44loCD122lo naive CD8 T cells from WT
and ADAP-deficient pLNs and cotransferred these cells into
adult WT hosts. Two weeks after transfer, we assessed the
conversion of donor naive CD8 T cells to MP, a time frame
in which RTEs mature and can convert to MP. The majority of
both WT and ADAP-deficient CD8 T cells retained a CD44lo
CD122hi naive phenotype (Fig. 5), with minimal conversion
to MP. These findings suggest that naive ADAP-deficient T
cells do not have a higher propensity to convert from naive to
MP in a WT host.

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 in-
volves the response of naive CD8 T cells to the lymphopenic
environment during the neonatal period (2). The highest fre-
cquency of MP CD8 T cells in a WT mouse occurs at 3 wk
(~30%) and decreases to adult levels (~20%) by 5 wk of age (3).
When compared with WT littermates, the frequency of MP CD8
T cells in 3-wk-old ADAP-deficient mice was 2-fold higher
(Fig. 6A, 6B). Similar to published accounts, we found that the
frequency of WT MP CD8 T cells was highest at 3 wk and
reached adult frequencies by 5 wk (3). 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.

ADAP dampens naive CD8 T cell responses to lymphopenia
To further investigate the role of lymphopenia in ADAP-deficient
conversion of naive CD8 T cells to MP, CD44loCD122hi naive
CD8 T cells were labeled with CFSE and transferred into suble-
thally irradiated age-matched recipients. After 10 d, we assessed
the conversion of WT and ADAP-deficient naive CD8 T cells to
MP, as well as lymphopenia-induced proliferation (LIP). We
found that a higher percentage of ADAP-deficient naive CD8
cells converted to MP than did their WT counterparts in a lympho-
openic environment (Fig. 7A, 7B). Additionally, a higher per-
centage of ADAP-deficient CD8 T cells had undergone more than
four divisions compared with WT CD8 T cells (Fig. 7C, 7D). These results suggest that CD44loCD122hi naive ADAP-deficient
CD8 T cells are more sensitive to lymphopenic signals in vivo.
To be sure that ADAP-deficient CD8 T cells were responding to
lymphopenic signals and not the cytokine storm that results
from sublethally irradiation of WT recipient mice, we also assessed
the conversion of naive ADAP-deficient CD8 T cells to MP in Rag1−/− hosts. Similar to what we observed with recipient mice
that were sublethally irradiated, a higher percentage of ADAP-
deficient naive CD8 T cells compared with WT naive CD8
T cells converted to MP and underwent homeostatic proliferation
in Rag1−/− hosts (Fig. 7E–J). These findings suggest that the enhanced response of ADAP-deficient CD8 T cells is due to
the lymphopenic environment and not due to a cytokine storm.

IL-15-mediated induction of MP is suppressed by ADAP
LIP of naive CD8 T cells is driven by IL-7 and self-peptide–MHC
complexes, whereas MP CD8 T cells can use IL-7 or IL-15 and
are MHC-I–independent (1, 27). To determine a mechanism by
which ADAP-deficient CD8 T cells are more responsive to lym-
phopenia, we assessed the ability of naive CD8 T cells to trans-
duce IL-7 and IL-15 signals. Bulk splenocytes from WT or
ADAP-deficient mice were stimulated with IL-7 or IL-15 for
15 min and stained for intracellular p-STAT5. Both WT and
ADAP-deficient naive CD8 T cells expressed low levels of
p-STAT5 in the absence of cytokine stimulation (Fig. 8A). An
increase in p-STAT5 after the addition of IL-7 was compara-
bly between WT and ADAP-deficient naive CD8 T cells (Fig 8A,
8B). Interestingly, p-STAT5 induction after IL-15 treatment was

FIGURE 5. ADAP-deficient mature naive CD8 T cells do not more readily convert to MP in the steady-state. Naive 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 × 10^5
cells/mouse). (A) CD44 and CD122 staining of transferred cells from re-
cipient spleens. Numbers represent the percentage of MP CD8 T cells. (B) Percentage of CD44hiCD122hi donor cells. The results in (B) are compiled from two independent experiments, each with four mice (±SEM).

FIGURE 6. Neonatal lymphopenic generation of MP CD8 T cells is greater in the absence of ADAP. Cells from the spleen of 3-wk-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−βQA2hiCD24lo MP cells. The results in (B) are compiled from three independent experiments (±SEM), with at least three mice in each group. ***p < 0.001.
20% greater in ADAP-deficient naive CD8 T cells (Fig. 8A, 8B). Dose response experiments also demonstrate a more robust response of ADAP-deficient naive and MP CD8 T cells to IL-15 (Fig. 8C). Wild-type naive CD8 T cells responded to IL-15 with a log EC50 of $6.3 \times 10^{-21}$, whereas ADAP-deficient naive CD8 T cells responded with a log EC50 of $2.1 \times 10^{-10}$. ADAP-deficient MP CD8 T cells also had an enhanced response to IL-15 (log EC50 at $2.4 \times 10^{-10}$) compared with WT MP CD8 T cells ($9.4 \times 10^{-11}$). These results suggest that in the absence of ADAP, both naive and MP CD8 T cells exhibit an enhanced response to IL-15 signals.

To determine whether 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 WT and ADAP-deficient mature naive OT-I CD8 T cells in IL-7 or IL-15. After 5 d 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 signif-
FIGURE 8. Mature naive CD8 T cells are more responsive to IL-15 in the absence of ADAP. (A–C) Bulk splenocytes were obtained from WT and ADAP<sup>−/−</sup> adult mice and stained for intracellular p-STAT5. (A) Histograms of WT (black) or ADAP<sup>−/−</sup> (gray shaded) p-STAT5 staining on CD8<sup>+</sup> CD122<sup>lo</sup> cells after addition of 1 ng/ml IL-7 or IL-15 for 15 min. (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<sup>+</sup>CD122<sup>lo</sup> cells. (C) Dose response to IL-15 (0.1–300 ng/ml) for 15 min in CD8<sup>+</sup>CD122<sup>lo</sup> cells (top) and CD8<sup>+</sup>CD122<sup>hi</sup> cells (bottom) log EC<sub>50</sub> for CD8<sup>+</sup>CD122<sup>lo</sup> cells was 6.3 ± 10<sup>−10</sup> (WT) and 2.0 ± 10<sup>−10</sup> (ADAP<sup>−/−</sup>). For CD8<sup>+</sup>CD122<sup>hi</sup> cells, the log EC<sub>50</sub> was 2.4 ± 10<sup>−10</sup> (WT) and 9.4 ± 10<sup>−11</sup> (ADAP<sup>−/−</sup>). (D–G) Naive CD8 T cells were isolated from WT (CD45.1/2) or ADAP<sup>−/−</sup> (CD45.2) OT-I adult mice, mixed at a 1:1 ratio, and labeled with CFSE. WT and ADAP<sup>−/−</sup> cells were cocultured in the presence of 10 ng/ml IL-7 or IL-15 for 5 d. (D) CFSE staining of WT and ADAP<sup>−/−</sup> CD8 T cells at day 0 or 5 after addition of cytokine. Gate indicates cells that have undergone one or more divisions. (E) Percentage of donor cells that have undergone one or more divisions. (F) CD122 staining of WT and ADAP<sup>−/−</sup> CD8 T cells at day 5 after addition of cytokine. Gate indicates cells that are CD122<sup>hi</sup>. (G) Percentage of CD8 T cells with expression of CD122<sup>hi</sup>. (H) Naive OT-I CD8 T cells from pLNs were isolated from WT (CD45.1) and ADAP<sup>−/−</sup> (CD45.1/2) adult mice, labeled with CFSE, and cotransferred into untreated or sublethally irradiated CD45.2 or IL-15<sup>−/−</sup> CD45.2 recipients. Recipient spleens were harvested 7 d after transfer. Ratio of WT/ADAP<sup>−/−</sup> CFSE gMFI in untreated or sublethally irradiated WT or IL-15<sup>−/−</sup> recipient spleens is shown. The results in (B), (C), (E), (G), and (H) are compiled from three independent experiments (±SEM). *p < 0.05, **p < 0.01, ***p < 0.001.
ically altered when compared with WT (Fig. 8D, 8E). In contrast, IL-15 stimulation induced proliferation of ADAP-deficient CD8 T cells, but not WT CD8 T cells. Additionally, stimulation with IL-7 increased CD122 expression in both WT and ADAP-deficient CD8 T cells, but to a greater degree in the absence of ADAP (Fig. 8F, 8G). This difference in CD122 upregulation was even more striking with the addition of IL-15. To determine whether IL-15 signaling in vivo is necessary for greater proliferation of ADAP-deficient naive CD8 T cells, we cotransferred CFSE-labeled WT and ADAP-deficient naive CD8 T cells into untreated or sublethally irradiated WT or IL-15−/− hosts. After 7 d, we assessed the CFSE gMFI of WT and ADAP-deficient CD8 T cells. As observed in previous experiments, ADAP-deficient CD8 T cells had undergone more divisions than did their WT counterparts in a lymphopenic host. In contrast, in the absence of IL-15 signals, WT and ADAP-deficient CD8 T cells proliferated equally in a lymphopenic environment. We conclude that expression of ADAP dampens the ability of naive CD8 T cells to respond to IL-15 signals.

Discussion

In this study, we have revealed a novel negative regulatory role for the adapter protein ADAP in naive CD8 responses to weak agonist peptides and IL-15. This work focused on the thymic and extrathymic pathways that contribute to the generation of Ag-independent MP CD8 T cells (3, 26). 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 (26). In the periphery, the prime drivers of peripheral mature naive 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 (4). Our findings support an extrathymic 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 (26). Itk inducibly binds SLP-76;pY145 after TCR signaling, which may explain why the SLP-76;Y145F mutation phenocopies Itk-deficient animals (26). Although ADAP also inducibly associates with SLP-76 (9), 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, because ADAP is required for the optimal response of naive CD4 T cells to Ag both in vitro and in vivo (13, 24), and TCR signaling has been proposed to be moderately attenuated in ADAP-deficient thymocytes (11). Similar to naive CD4 T cells, we have observed that ADAP positively regulates the interaction of naive 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 naive CD8 T cells, leading to increased MP CD8 T cell generation. The enhanced LIP of ADAP-deficient naive 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 transgene 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 naive or MP CD8 T cells with the Nur77-GFP reporter system indicates similar self-reactivity between WT and ADAP-deficient CD8 T cells. Thus, ADAP negatively regulates the response of naive CD8 T cells to weak agonists 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 naive CD8 T cells.

Altered thymocyte development could result in decreased thymic output, thus enhancing MP conversion of peripheral mature naive CD8 T cells by increasing the exposure of these T cells to homeostatic cytokines during neonatal development (4). We failed to detect defects in the development of mature CD8 SP T cells or in CD8 thymic output in the absence of ADAP, which have been reported in a previous study (11). We are uncertain of the reasons for this discrepancy, although we note that our experiments analyzed older mice (8–12 wk) than did the previous work, which assessed mice at 6 wk of age (11). We do recognize that by qualifying RTEs only by QA2+CD24hi cells, we are excluding a large percentage of cells that could be identified in other systems (28). When we assessed mature naive CD8 T cell conversion to MP in neonatal mice, we observed a greater frequency of MP CD8 T cells in the absence of ADAP. Taken 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 naive 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 a 1.5-fold greater frequency of MP CD8 T cells was observed in ADAP-deficient CD8 T cells, compared with WT CD8 T cells, regardless of the majority population. Taken together, these results suggest that the conversion of naive 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 that our results support a unique negative regulatory role for ADAP in naive CD8 T cell responses to the homeostatic cytokine IL-15. This is a previously unreported function for ADAP independent of TCR signaling. Interestingly, whereas 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 WT CD8 T cells. This suggests the possibility that ADAP plays a particularly important role in regulating CD8 T cell survival, because the optimal survival of naive T cells is dependent on an IL-15 signal (6). The SOCS-1 protein, which binds directly to JAKs to inhibit the tyrosine kinase activity and promotes ubiquitin-mediated degradation (29), 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 (30). 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 is associated with an enhancement of IL-15 signaling (8, 30). Because SOCS-1–deficient mice succumb to neonatal mortality (29), 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 naive CD8 T cells to MP in the steady-state and the response of naive CD8 T cells to IL-15. ADAP is therefore a signaling protein involved in both the positive regulation of TCR responses to foreign Ags 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 naive T cells with foreign peptide–MHC-I specificity. In contrast, ADAP dampens weak agonist interactions and prevents generation of self-reactive cells. Additionally, suppressing naive IL-15 signaling could decrease the generation of MP CD8 T cells during instances of acute lymphopenia, which can occur after an infection (1). 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.

Acknowledgments

We thank F. Shoyama, T. Rivard, S. Jin, and K. Benson for mouse genotyping and colony maintenance. Dr. Sarah Hamilton provided assistance with LIP and pSTAT5 staining. Dr. Yu-Jung Lee provided assistance with mixed BMCs and unconventional thymocyte staining. Dr. Kristin Hogquist provided CD1d-exGαCεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεee

Disclosures

The authors have no financial conflicts of interest.

References

Supplemental Figure 1: 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+ TCR-β int (top) or GL3+ TCR-β lo (bottom). (B) The percentage or (C) number of NKT cells of total thymocytes in WT (black circles) or ADAP−/− (open squares) mice. (D) Serum IgE from the blood of aged (25-40 week) mice. The results (B) and (C) are compiled from at least three independent experiments (± SEM).