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Gads Regulates the Expansion Phase of CD8+ T Cell-Mediated Immunity

Elizabeth Yan Zhang, Brooks L. Parker, and Thomas M. Yankee

The Gads adaptor protein is critical for TCR-mediated Ca2+ mobilization. We investigated the effect of Gads deficiency on the proliferation of CD8+ T cells following peptide stimulation and in the context of infection with an intracellular pathogen. We stimulated CD8+ T cells from Gads+/+ OT-I and Gads−/− OT-I mice with cognate Ag (SIINFEKL) or altered peptide ligand. In vitro experiments revealed that Gads was required for optimal proliferation of CD8+ T cells. This defect was most evident at the early time points of proliferation and when low doses of Ag were used as stimuli. Cell cycle analysis demonstrated that Gads−/− CD8+ T cells had impaired TCR-mediated exit from the G0 phase of the cell cycle. Furthermore, Gads−/− CD8+ T cells had delayed expression of c-myc and CD69 upon the stimulation with SIINFEKL. We then investigated how Gads deficiency would affect proliferation and when low doses of Ag were used as stimuli. Cell cycle analysis demonstrated that Gads−/− CD8+ T cells had impaired TCR-mediated exit from the G0 phase of the cell cycle. Furthermore, Gads−/− CD8+ T cells had delayed expression of c-myc and CD69 upon the stimulation with SIINFEKL. We then investigated how Gads deficiency would impact CD8+ T cell-mediated immunity in the context of infection with an intracellular pathogen. At early time points, Gads+/+ and Gads−/− CD8+ T cells proliferated to a similar extent, despite the fact that expression of CD69 and CD25 was reduced in the absence of Gads. After 5 d postinfection, Gads was required to sustain the expansion phase of the immune response; the peak response of Gads−/− CD8+ T cells was significantly lower than for Gads+/+ cells. However, Gads was not required for the differentiation of naive CD8+ T cells into memory cells. We conclude that the primary function of Gads is to regulate the sensitivity of the TCR to Ag ligation. The Journal of Immunology, 2011, 186: 4579–4589.

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CD8+ T cells represent the branch of the adaptive immune system responsible for recognizing and killing cells infected with intracellular pathogens. For CD8+ T cells to fulfill this function, the TCR on the CD8+ T cells must recognize foreign peptides presented on MHC class I. When the TCR binds peptide–MHC complexes, signals are transmitted to the CD8+ T cells that induce activation and proliferation, which precedes differentiation into effector or memory cells. Like with CD4+ T cells (1), proliferation of CD8+ T cells is required for the differentiation of CD8+ T cells into effector and memory cells (2–7). Thus, to fully understand the differentiation program of CD8+ T cells, we must first understand how proliferation is initiated.

The interaction of the TCR complex with a peptide–MHC complex leads to the recruitment and activation of Src and Syk/ZAP-70 families of protein tyrosine kinases (8, 9). This kinase activity results in the phosphorylation of the membrane-bound adaptor protein LAT and the recruitment of the SLP-76 adaptor protein. Gads, a member of the Grb2 family of adaptor proteins, bridges LAT and SLP-76, enabling the recruitment of SLP-76 to LAT (10–14). The Src homology 2 domain of Gads binds phosphorylated LAT, and the C-terminal Src homology 3 domain of Gads constitutively binds SLP-76. The formation of the LAT–Gads–SLP-76 complex leads to the activation of phospholipase Cγ1 and calcium mobilization. Consistent with this model, TCR-mediated calcium influx in Gads-deficient T cells was markedly impaired (15, 16). However, when Gads−/− T cells were stimulated with high doses of anti-CD3ε, there was detectable calcium mobilization (16), suggesting that Gads might regulate the signaling threshold through the TCR.

To examine the function of Gads in T cells, Gads-deficient mouse lines were generated (15, 16). Gads−/− mice had defects in T cell development at stages that correspond to the expression of TCRβ and TCRα. During the CD4+CD8− double-negative (DN) stage of T cell development, Gads is required for the survival of thymocytes expressing TCRβ (17). Later, when TCRα is expressed, Gads is required for positive and negative selection of CD4+CD8− double-positive thymocytes (18). Although the locations of these blocks are consistent with a role for Gads in regulating TCR-mediated signal transduction, the fact that the blocks are not complete indicates that Gads expression is not an absolute requirement for TCR-mediated signal transduction. Rather, Gads may regulate a subset of signaling pathways or the intensity of signals through all pathways. Further, the function of Gads may change during T cell development and activation.

Gads−/− mice had few mature peripheral T cells (16). However, within the peripheral T cell population, CD8+ T cells were more dependent on Gads expression for survival and homeostasis than CD8+ T cells. This conclusion must be tempered by the observation that nearly all T cells in Gads−/− mice were of a memory-like phenotype. The signaling pathways required for the activation of memory T cells are different from those required for the activation of naive T cells (19–21).

During our analysis of the function of Gads in T cell development, we found that crossing Gads−/− mice with mice expressing an MHC class I-restricted transgenic TCR could rescue the production of naive CD8+ T cells (18). These transgenic TCR-expressing Gads−/− mouse lines enable us to examine the function of Gads during the activation of naive CD8+ T cells. We present
data from studies in which cells were stimulated with peptide Ag. We analyzed the kinetics of cell cycle entry and expression of activation markers in this context. We then examined how Gads regulates the expansion and memory phases of CD8+ T cell-mediated immune responses against a live intracellular pathogen.

Materials and Methods

Mice

C57BL/6 Gads−/− mice and Gads+/− OT-I mice were described previously (16, 18) and backcrossed onto the CD45.1+ C57BL/6 genetic background. To generate CD45.1+CD45.2+ OT-I mice, CD45.1+ OT-I mice were bred with CD45.2+ wild-type C57BL/6 mice. All mice were housed under specific pathogen-free conditions, and all experiments were performed in compliance with the University of Kansas Medical Center Institutional Animal Care and Use Committee. Mice were used between the ages of 6 and 10 wk at the start of the experiments.

Abs, cell labeling, and flow cytometry

Anti-CD8α–FITC, anti-CD8α–Alexa Fluor 647, anti-CD44–PE-Cy7, anti-CD44–Horizon V450, anti-CD45.1–PE, anti-CD45.1–allophycocyanin-Cy7, anti-CD45.2–PE-Cy5.5, anti-CD45.2–FITC, anti-CD62L–PE-Cy7, anti-CD122–PE, anti-TCRVγv2–FITC, anti-CD25–allophycocyanin-Cy7, anti-CD127–allophycocyanin-e-Fluor 780, and anti-killer cell lectin-like receptor G1 (KLRG1)–PE were purchased from BD Biosciences (San Jose, CA), eBioscience (San Diego, CA), or Biolegend (San Diego, CA).

Surface labeling of cells was performed as described previously (18). Briefly, single-cell suspensions were prepared and labeled in staining buffer (PBS containing 2% FetalClone I bovine serum product [HyClone Laboratories, Logan, UT]) before fixing with 1% paraformaldehyde in PBS overnight or at least 1 h at room temperature.

For CFSE labeling, cells were labeled with CFSE as described previously (16). Briefly, the cell concentration was adjusted to 2 × 106 cells/ml, and an equal volume of 10 μM CFSE (Invitrogen, Carlsbad, CA) was added. Cells were incubated for 10 min at 37°C, and the reaction was quenched with cell culture media. For DAPI labeling, cells were harvested after stimulation, labeled with anti-CD8, and fixed in 1% paraformaldehyde. Then, cells were washed twice with staining buffer, incubated with 1 ml 1 μg/ml DAPI (Invitrogen, Carlsbad, CA) in 0.3% Tween-20 in staining buffer for 30 min at room temperature, and analyzed immediately by flow cytometry. For pyronin Y (PY) staining, cells were pelleted after DAPI staining, and 80 μl the supernatant was aspirated. Twenty microliters 25 μg/ml PY (Polyscience, Warrington, PA) in staining buffer containing 0.1% Tween-20 was added, and cells were incubated for 60 min at room temperature and analyzed by flow cytometry. For IFN-γ assays, splenocytes were restimulated in culture with SIINFEKEL 1 nM in the presence of brefeldin A. After fixation with 1% paraformaldehyde, cells were permeabilized with 0.3% Tween-20 and labeled with anti-CD8, anti-CD45.1, anti-CD45.2, and anti–IFN-γ.

Statistics

All data are presented as mean ± SD and were analyzed using two-tailed Student’s t tests.

Results

Most CD8+ T cells in Gads−/− OT-I mice have a naive phenotype

Our previous analysis of Gads−/− mice revealed that nearly all peripheral CD8+ T cells in Gads−/− mice were of a memory-like phenotype (16), but crossing Gads−/− mice with mice expressing the MHC class I-restricted TCR OT-I partially restored T cell development (18). On average, spleens from Gads+/− OT-I mice contained 13 ± 2.5 × 106 CD8+ cells, and spleens from Gads−/− OT-I mice contained 2.2 ± 0.80 × 106 CD8+ cells.

To more fully characterize the peripheral CD8+ T cells in Gads+/− OT-I and Gads−/− OT-I mice, we compared CD44, CD62L, CD122, and TCR expression in Gads+/−, Gads−/− OT-I, and Gads+/− OT-I mice (Fig. 1). Despite the reduced numbers of CD8+ T cells in Gads−/− OT-I mice, as compared with Gads+/− OT-I mice, most CD8+ T cells in each mouse line were CD44hiCD62L+CD122+. On average, 78 ± 5.3% of Gads−/− OT-I CD8+ cells were CD44hiCD62LhiCD122hi, as compared with 90 ± 3.8% of Gads+/− OT-I CD8+ cells (p < 0.001; n = 12 mice of each genotype). In addition, naive CD8+ T cells from Gads+/− OT-I and Gads−/− OT-I mice were CD122+ (Fig 1B) and had comparable surface TCR levels (Fig 1C). Thus, although the number of naive CD8+ T cells in Gads−/− OT-I mice was lower than in Gads+/− OT-I mice, we are able to use this model to obtain a sufficient number of naive T cells to examine the function of Gads in naive CD8+ T cells.

Splenocytes from Gads+/− OT-I or Gads−/− OT-I mice were isolated and stimulated with 1 nM SIINFEKEL. At various time points, CD8+ T cells were isolated using anti-mouse CD8a Magnetic Particles–DM (BD Pharmingen) and positive selection. Lysates were prepared, separated by SDS-PAGE, and transferred to polyvinylidene difluoride membrane. Membranes were probed with anti-κ- and anti-β-actin (Cell Signaling Technology, Danvers, MA).

Recombinant Listeria monocytogenes-expressing OVA infections

Splenocytes were harvested from CD45.1+CD45.2+ Gads+/− OT-I or CD45.1+CD45.2− Gads−/− OT-I mice, and naive CD8+ (CD8+CD44hi) T cells were FACS-purified using a BD FACSaria (BD Biosciences). A total of 106 naive CD8+ T cells were adoptively transferred into CD45.2+ congenic mice. For competition assays, 5 × 104 cells of each genotype were injected. rL. monocytogenes-expressing OVA (rLM-OVA) (generous gift of Dr. Leo LeFrançois, University of Connecticut Health Center) was cultured as previously reported (22), and 2 × 105 CFU rLM-OVA or vehicle control was injected i.v. into each mouse. At the indicated time points, blood or splenocytes were harvested, labeled with anti-CD45.1, anti-CD45.2, anti-CD8, anti-KLRG1, and anti-CD127 and analyzed by flow cytometry. RBCs from blood samples were lysed with ACK lysis buffer prior to staining. For challenge assays, mice were reinjected i.v. 60 d postinfection with 104 CFU rLM-OVA. Blood and splenocytes were harvested and analyzed 4 d postchallenge.

For short-term activation assays, 2 × 106 CD8+ T cells were positively selected from Gads+/− OT-I and Gads−/− OT-I mice using anti-CD8a Magnetic Particles–DM (BD Pharmingen) and injected into congenic wild-type mice. The following day, mice were infected with 2 × 105 CFU rLMOV A, SIINFEKEL, or vehicle control. Twenty-four hours postinfection, splenocytes were harvested and labeled with anti-CD8, anti-CD45.1, anti-CD45.2, and anti-CD69.

Immunoblot assay

Splenocytes from Gads+/− OT-I or Gads−/− OT-I mice were isolated and stimulated with 1 nM SIINFEKEL. At various time points, CD8+ T cells were isolated using anti-mouse CD8a Magnetic Particles–DM (BD Pharmingen) and positive selection. Lysates were prepared, separated by SDS-PAGE, and transferred to polyvinylidene difluoride membrane. Membranes were probed with anti-κ- and anti-β-actin (Cell Signaling Technology, Danvers, MA).
Gads regulates proliferation of CD8+ T cells

To test whether Gads could regulate proliferation of CD8+ T cells, CD8+ T cells from Gads+/+ OT-I or Gads−/− OT-I mice were injected into congenic wild-type hosts. The following day, recipient mice were injected i.v. with SIINFEKL peptide. After 3 d, fewer Gads−/− cells divided than Gads+/+ cells, and Gads−/− cells that divided did not divide as extensively as Gads+/+ cells (Fig. 2A), suggesting that Gads regulates proliferation of CD8+ T cells.

To examine the defect in proliferation more closely, we stimulated Gads+/+ and Gads−/− CD8+ T cells in vitro for 3 d with varying doses of SIINFEKL or the altered peptide ligand (APL) SAINFEKL, called A2. A2 binds the MHC and TCR with comparable affinity and kinetics as SIINFEKL (23, 24), but has been shown to be less efficient as SIINFEKL in inducing proliferation in an in vitro thymidine incorporation assay (25). Using a CFSE-based assay, we found that nearly all Gads+/+ CD8+ cells proliferated after stimulation with SIINFEKL or A2 (Fig. 2B), whereas Gads−/− CD8+ T cells failed to complete as many cell divisions as Gads+/+ cells. When the concentration of peptide was reduced, the percentage of Gads−/− cells that divided declined. This dose effect was more evident when cells were stimulated with A2 than SIINFEKL.

Regardless of the stimulating conditions, Gads−/− CD8+ T cells failed to complete as many cell divisions as Gads+/+ cells. When

**FIGURE 1.** OT-I expression in Gads−/− mice restores the production of naive CD8+ T cells. Lymphocytes from Gads+/+, Gads−/−, Gads+/+ OT-I, and Gads−/− OT-I mice were labeled with anti-CD8, anti-CD44, anti-CD62L, anti-CD122, and anti-TCRα2. A, Expression of CD44 and CD62L was analyzed on CD8+ cells. Shown are the percentages of CD8+ cells that were CD44hiCD62Lhi, CD44hiCD62Llo, and CD44hiCD62Llo. B, CD44 and CD122 expression was analyzed on CD8+ cells. Shown are the percentages of CD8+CD44hi cells that expressed CD122. C, TCR expression on naive CD8+ cells from Gads+/+ OT-I (shaded histogram) and Gads−/− OT-I (dark line) mice is shown. The negative control is shown by dotted line.

**FIGURE 2.** Gads regulates proliferation of CD8+ T cells in vivo and in vitro. A, CFSE-labeled splenocytes from Gads+/+ OT-I and Gads−/− OT-I mice were adoptively transferred into congenic hosts. Then, mice were injected with the indicated quantity of SIINFEKL. Proliferation was measured by flow cytometry 3 d later. Shown are the percentages of CD8+ cells that proliferated. Bottom panel, Shown are the means ± SD of the percentages of CD8+ cells that proliferated. n = 4. **p < 0.01. B, CFSE-labeled splenocytes from Gads+/+ OT-I and Gads−/− OT-I mice were cultured for 3 d with the indicated concentrations of SIINFEKL or A2. Overlays show proliferation of Gads+/+ CD8+ T cells (filled histograms) and Gads−/− CD8+ T cells (open histograms). Left bottom panel, Shown are the means ± SD of the percentages of cells that proliferated. n = 3. Right bottom panel, Shown are the means ± SD of the percentages of cells that completed more than five rounds of cell division. n = 3. *p < 0.05, **p < 0.01.
data were gated on those cells that divided more than five times, fewer Gads−/− cells were detected, even under conditions in which nearly all Gads+/+ cells divided (Fig. 2B).

These data suggest that Gads regulates the sensitivity of the TCR to ligand and regulates the kinetics of cell proliferation. However, these in vitro assays could reflect differences in Ag presentation. To test this possibility, we purified CD8+ T cells from Gads+/+ OT-I and Gads−/− OT-I mice and stimulated the cells with wild-type peritoneal macrophages loaded with peptide. This experiment resulted in data similar to that shown in Fig. 2B (data not shown).

Gads regulates cell cycle entry

To examine the function of Gads in regulating the onset of proliferation, we analyzed the progression through the S phase of the cell cycle and the completion of the first cell cycle after peptide stimulation. After 28 h of stimulation with 1 nM SIINFEKL, 29 ± 4.3% of Gads+/+ cells entered the S phase of the cell cycle, as compared with 9.7 ± 3.2% of Gads−/− cells (p < 0.001; n = 4) (Fig. 3A). The percentage of Gads−/− cells entering S phase after stimulation with 1 nM SIINFEKL was statistically identical to the percentage of Gads+/+ cells entering S phase after stimulation with 0.1 nM SIINFEKL (9.7 ± 3.2% versus 13 ± 6.3%; p = 0.34; n = 4). We also analyzed cells after 32 and 36 h and found similar trends (Fig. 3B, 3C); fewer Gads−/− cells than Gads+/+ cells entered the S phase of the cell cycle, and the percentage of Gads−/− cells entering the cell cycle after stimulation with 1 nM SIINFEKL was comparable to the percentage of Gads+/+ cells entering the cell cycle after stimulation with 0.1 nM SIINFEKL. These experiments were repeated using A2 peptide, and identical trends were seen (Fig. 3).

These data suggest that Gads regulates the kinetics of cell cycle entry and the sensitivity of CD8+ T cells to Ag. Alternatively, Gads could regulate survival of CD8+ T cells. To test if Gads could regulate survival, Gads+/+ and Gads−/− splenocytes were stimulated with varying doses of SIINFEKL for 48 h. Cell death was assessed by Annexin V and PI staining (Fig. 4). No differences were observed in the survival of Gads+/+ and Gads−/− cells, indicating that the primary function of Gads within the first 2 d of Ag stimulation is to regulate proliferation.

Gads regulates exit from G0 phase of cell cycle progression

To further define the stage of the cell cycle regulated by Gads, we stimulated splenocytes from Gads+/+ OT-I and Gads−/− OT-I mice with varying doses of SIINFEKL and analyzed RNA content using PY and DNA content using DAPI (Fig. 5). After 15 h of stimulation with 1 nM SIINFEKL, 27 ± 5.4% of Gads+/+ CD8+ T cells had exited the G0 phase of the cell cycle, as compared with 6.7 ± 5.2% of Gads−/− cells (p < 0.0001; n = 6). As in the previous assay, a comparable percentage of Gads−/− cells stimulated with 1 nM SIINFEKL entered the cell cycle as Gads+/+ cells stimulated...
with 0.1 nM SIINFEKL. By 21 h after stimulation, some Gads<sup>+/+</sup> cells proceeded through the G<sub>1</sub> phase and entered S phase, whereas most Gads<sup>−/−</sup> cells remained in the G<sub>0</sub> phase of the cell cycle (Fig. 5B). Because Gads is proposed to regulate phospholipase C<sub>γ1</sub> activity, we tested whether PMA 0.1 μg/ml and ionomycin 0.25 μg/ml could overcome the need for Gads. Indeed, cell cycle entry in Gads<sup>−/−</sup> cells was fully restored by PMA and ionomycin (Fig. 5). In fact, at the 15-h time point, we noted a consistent increase in the percentage of Gads<sup>−/−</sup> cells that exited the G<sub>0</sub> phase of the cell cycle, as compared with Gads<sup>+/+</sup> cells. This difference was not seen at the 21 h time point. These data indicate that Gads regulates early signaling pathways that control the exit from the G<sub>0</sub> phase of the cell cycle.

To identify the mechanism by which Gads regulates cell cycle entry, we analyzed the expression of c-myc (Fig. 6A), a regulator of quiescence in T cells (26-28). In Gads<sup>+/+</sup> cells, c-myc was expressed within 1 h of stimulation and remained expressed throughout the duration of the experiment. By contrast, no c-myc expression was detected in Gads<sup>−/−</sup> cells until 3 h after stimulation. Within 24 h of stimulation, c-myc expression in Gads<sup>+/+</sup> and Gads<sup>−/−</sup> cells was comparable, consistent with our model that Gads regulates the onset of proliferation.

**Gads regulates the expression of early activation markers**

Next, we tested whether Gads could regulate the kinetics of expression of the early activation marker CD69 (Fig. 6B). Like c-myc expression, CD69 expression was reduced on Gads<sup>−/−</sup> cells at early time points, but nearly indistinguishable at later time points, as compared with Gads<sup>+/+</sup> cells. These data indicate that Gads regulates the kinetics of activation and proliferation of CD8<sup>+</sup> T cells but is not required for the activation and proliferation of CD8<sup>+</sup> T cells.

**Gads regulates the expansion phase of CD8<sup>+</sup> T cell-mediated immunity**

To test how a delay in peptide-induced activation and proliferation could relate to an immune response against a live pathogen, we adoptively transferred naive CD8<sup>+</sup> T cells from Gads<sup>+/+</sup> OT-I or Gads<sup>−/−</sup> OT-I mice into congenic wild-type mice, infected the recipients with rLM-OVA, and analyzed the CD8<sup>+</sup> T cells derived from the donor mice. For these experiments, we generated

![Figure 4](http://www.jimmunol.org/)

**FIGURE 4.** Gads is not required for survival of CD8<sup>+</sup> T cells. Splenocytes from Gads<sup>+/+</sup> OT-I and Gads<sup>−/−</sup> OT-I mice were cultured with the indicated concentrations of SIINFEKL for 2 d. Cells were analyzed for Annexin V and PI staining. Shown are the percentages of cells that were Annexin V<sup>+</sup>PI<sup>+</sup>. Representative of three independent experiments.

![Figure 5](http://www.jimmunol.org/)

**FIGURE 5.** Gads is required for entry into the G<sub>1</sub> phase of the cell cycle. Splenocytes from Gads<sup>+/+</sup> OT-I and Gads<sup>−/−</sup> OT-I mice were cultured with the indicated concentrations of SIINFEKL, A2, or PMA and ionomycin for 15 (A) or 21 h (B). At harvest, cells were labeled with DAPI and PY. Dot plots. Shown are representative data from cells stimulated with SIINFEKL and PMA and ionomycin. The percentages of cells in the G<sub>1</sub>, S, G<sub>2</sub>, or M phase of the cell cycle are shown. Bar graphs. Shown are the means ± SD of the percentages of CD8<sup>+</sup> T cells that exited the G<sub>0</sub> phase of the cell cycle. n ≥ 3 independent experiments for each sample. *p < 0.05, **p < 0.01.
Gads REGULATES CD8+ T CELL PROLIFERATION

To determine whether Gads was required for the differentiation of naïve CD8+ T cells into memory cells, naïve CD8+ T cells from Gads+/+ OT-I and Gads−/− OT-I mice were adoptively transferred into separate congenic hosts or mixed and injected into the same host. Mice were infected with rLM-OVA and the percentage of CD8+ T cells in the blood derived from donor mice was tracked over time (Fig. 10A). As shown in the previous experiment (Fig. 7), Gads was necessary for optimal expansion of the CD8+ T cell population. However, both Gads+/+ and Gads−/− cells persisted 60 d postinfection, indicating that Gads−/− cells could differentiate into memory cells. When Gads+/+ and Gads−/− cells were injected into separate mice, the percentages of CD8+ T cells derived from the donor mice were identical after 3 wk. When in direct competition, the percentages of donor-derived CD8+ T cells that were Gads+/+ were 2.4 ± 0.46-fold greater than the percentages of donor-derived CD8+ T cells that were Gads−/− cells. Because Gads−/− cells were readily detectable 60 d postinfection, we concluded that Gads−/− cells could differentiate into memory cells.

As an additional indicator of whether Gads is required for memory T cell development, we analyzed CD127 and KLRG1 expression, markers that can identify the short-lived effector cells (KLRG1+CD127+) and memory precursor effector cells (CD127+ KLRG1−) (29–32). There were no statistically significant differences in the percentages of Gads+/+ and Gads−/− CD8+ T cells that were short-lived effector cells and memory precursor effector cells (Fig. 10B). However, more Gads−/− cells were CD127+ KLRG1+ than Gads+/+ cells. These data are consistent with a model in which Gads is not required for the differentiation of naïve CD8+ T cells into memory T cells.

We next tested whether Gads−/− memory CD8+ T cells could expand in number in response to reinfecction with rLM-OVA. More Gads−/− cells were found 4 d postchallenge than prechallenge, indicating that Gads−/− cells had differentiated into memory cells and could proliferate in response to challenge (Fig. 10C). However, Gads−/− cells did not expand in number as robustly as Gads+/+ cells, suggesting that, like in the primary immune response, Gads is required for optimal expansion of CD8+ T cells in a secondary immune response. Also, as with the primary immune response, the defect with Gads−/− cells was more apparent when Gads+/+ and Gads−/− cells were in direct competition.

Discussion

We report the first, to our knowledge, analysis of the function of Gads in peripheral naïve CD8+ T cells. We examined the activation and proliferation of T cells in response to cognate peptide Ag and found that Gads-dependent signaling pathways accelerate exit from the G0 phase of the cell cycle and enhance the sensitivity of CD8+ T cells to Ag. However, in the context of infection with a live pathogen, Gads was not required for the early expansion of CD8+ T cells, but rather, Gads was required to maintain the expansion phase of the immune response. Further, Gads was not required for the differentiation of naive CD8+ T cells into memory cells, but was required for optimal expansion of memory cells following challenge.

As we previously described, the CD8+ T cells in Gads−/− mice were nearly exclusively of a memory phenotype (16), making a thorough analysis of the function of Gads in naive CD8+ T cells impossible. Also in our previous studies, we found that the single-positive CD8+ thymocytes in Gads−/− mice were of an unusual...
phenotype; nearly half of the Gads<sup>−/−</sup> SP CD8<sup>+</sup> thymocytes expressed CD122 (18). This unusual phenotype within the SP thymocyte population was overcome by the expression of an MHC class I-restricted TCR. In Fig. 1, we demonstrate that many peripheral CD8<sup>+</sup> T cells in Gads<sup>−/−</sup> OT-I mice were naive, as seen by the expression of CD44, CD62L, and CD122. These data indicate that the OT-I system provides us an opportunity to examine the effects of Gads deficiency on the proliferation and activation of naive CD8<sup>+</sup> T cells.

To test the effect of Gads deficiency on the expansion of the Ag-specific CD8<sup>+</sup> T cell population, we stimulated cells with cognate Ag and an APL. We found that Gads<sup>−/−</sup> cells could not expand in number to the same extent as Gads<sup>+/+</sup> cells (Fig. 2). This impairment could be caused by a failure of Gads<sup>−/−</sup> cells to initiate proliferation, sustain proliferation, or survive. In investigating these possibilities, we found that the major function of Gads in peptide-induced proliferation is during the initiation of CD8<sup>+</sup> T cell activation and proliferation. Among proliferating cells several days after stimulation, the percentage of Gads<sup>−/−</sup> cells in the G<sub>1</sub>, G<sub>2</sub>, or M phase of the cell cycle was comparable to that of Gads<sup>+/+</sup> cells, and proliferating Gads<sup>−/−</sup> cells survived in vitro at the same rate as proliferating Gads<sup>+/+</sup> cells (data not shown). This indicates that once Gads<sup>−/−</sup> CD8<sup>+</sup> T cells begin proliferating, they progress through subsequent cell cycles normally.

A key event that is required for the onset of proliferation is expression of c-myc (33, 34). Expression of c-myc is initiated within 30 min of TCR ligation, and its expression precedes that of CD25 (35, 36). Analysis of c-myc expression during T cell development revealed that levels of c-myc expression tightly correlate with stages of T cell development in which cells proliferate.
The importance of c-myc in the proliferation of CD4\(^+\)CD8\(^-\) DN thymocytes was seen when c-myc–deficient DN thymocytes could not proliferate following pre-TCR expression (37). Further, loss of c-myc expression in CD4\(^+\) T cells resulted in a lack of TCR-induced proliferation, despite the upregulation of the activation markers CD25 and CD44 (27). These observations illustrating the importance of c-myc during cell cycle progression were supported by studies showing that c-myc expression can regulate exit from quiescence in T cells (26).

Because c-myc expression is critical for exit from quiescence and cell cycle entry, we examined c-myc expression following TCR ligation of Gads\(^{+/+}\) and Gads\(^{-/-}\) CD8\(^+\) T cells. In the absence of Gads, c-myc expression was delayed as compared with Gads\(^{+/+}\) cells (Fig. 6A).

**FIGURE 8.** Gads regulates the expression of CD69 and CD25 on CD8\(^+\) T cells in vivo. CD8\(^+\) T cells from CD45.1\(^+\) Gads\(^{-/-}\) OT-I mice, CD45.1\(^+\)CD45.2\(^+\) Gads\(^{+/+}\) OT-I mice, or both were adoptively transferred into CD45.2\(^+\) wild-type mice. Mice were injected i.v. with SIINFEKL (A) or rLM-OVA (B, C). A and B. Splenocytes were analyzed 24 h poststimulation. C. Cells were analyzed 5 d postinfection. Dot plots are representative data showing the percentages of donor-derived CD8\(^+\) T cells that were CD69\(^+\), CD25\(^+\), or CD69\(^+\)CD25\(^+\). Bar graphs. Shown are the means ± SD of the percentages of donor-derived CD8\(^+\) T cells from A and B expressing CD69 and CD25. n = 5 from two independent experiments. *p < 0.05, **p < 0.01, when comparing stimulated Gads\(^{+/+}\) and Gads\(^{-/-}\) cells.
marker CD69 was also delayed in Gads$^{-/-}$ cells (Fig. 6B). These data indicate that the function of Gads is to regulate the kinetics of CD8$^+$ T cell activation and proliferation.

The impaired onset of proliferation seen in Gads$^{-/-}$ CD8$^+$ T cells was most dramatic when the concentration of stimulating peptide was reduced or when a less potent peptide agonist, A2, was used (Fig. 2). Although TCR–SIINFEKL–MHC and TCR–A2–MHC complexes have comparable dissociation constants and $t_{1/2}$ (23, 24), A2 is a less potent agonist for CD8$^+$ T cell activation and proliferation than SIINFEKL (25, 38). The more dramatic delay in cell cycle entry seen when Gads$^{-/-}$ cells were cultured with weaker stimuli is consistent with our model that Gads regulates the signaling threshold through the TCR.

Like Gads deficiency, APLs have been demonstrated to slow the kinetics of T cell activation (39–42). T cells stimulated with APLs have reduced phosphorylation of $\zeta$-chain and reduced recruitment of ZAP-70 to $\zeta$-chain than cells stimulated with cognate Ag (43, 44). The result of this decrease in ZAP-70 recruitment is less LAT and SLP-76 phosphorylation, reduced calcium mobilization, and reduced MAPK activation (40, 45). This decreased signaling when T cells are stimulated with APLs leads to reduced expression of activation markers and proliferation (42), similar to our results with Gads$^{-/-}$ cells.

To demonstrate how the impaired activation and proliferation of Gads$^{-/-}$ cells observed when cells were stimulated with peptide relates to an immune response against a live pathogen, we used the rLM-OVA infection model. In contrast to peptide-induced proliferation, there was no apparent defect in Gads$^{-/-}$ cells during the initial stages of CD8$^+$ T cell expansion following infection.
Among CD127-expressing Gads-a marker typically associated with memory precursors (29, 51). L. monocytogenes strains expressing APLs proliferated at a similar rate as cells responding to RLM-OVA during the first few days postinfection (38).

Why Gads<sup>−/−</sup> cells had impaired cell cycle entry after peptide stimulation, but not after RLM-OVA infection is unclear. A possible explanation for this phenomenon may relate to the reduced level of CD25 expression observed in Gads<sup>−/−</sup> cells (Fig. 8). The function of IL-2 and CD25 in CD8<sup>+</sup> T cells is controversial. In the context of infection with L. monocytogenes, IL-2 is required for sustained expansion of Ag-specific T cells (46). This is consistent with an in vitro role for IL-2 in driving sustained proliferation of CD8<sup>+</sup> T cells after the removal of Ag (5), but, in contrast to studies using LCMV infection, where IL-2 did not contribute to the expansion phase of the primary immune response (47–50). These observations suggest that the reduced CD25 expression seen in Gads<sup>−/−</sup> cells may result in reduced responsiveness to IL-2 and a lower peak of the expansion phase.

Consistent with a role for CD25 in Gads<sup>−/−</sup> cells, when CD8<sup>+</sup> T cells are stimulated with low doses of IL-2, more cells express CD127 than when cells are stimulated with high concentrations of IL-2 (49). In our studies, more Gads<sup>−/−</sup> cells than Gads<sup>+/+</sup> cells expressed CD127 after RLM-OVA infection (Fig. 10B). CD127 is a marker typically associated with memory precursors (29, 51). Among CD127-expressing Gads<sup>−/−</sup> cells, many also expressed KLRG1, a marker typically associated with short-lived effector cells (30–32). The function of these CD127<sup>+</sup>KLRG1<sup>+</sup> cells is unknown, but these cells may represent a transition point in the differentiation between effector and central memory cells (52).

The increased percentage of Gads<sup>−/−</sup> CD8<sup>+</sup> T cells expressing CD127 seen at the peak of the primary immune response suggested that Gads was not required for the differentiation of naive CD8<sup>+</sup> T cells into memory T cells. Indeed, when Gads<sup>+/+</sup> and Gads<sup>−/−</sup> cells were injected into separate hosts, the percentage of donor-derived T cells in the blood was identical during the memory phase of the immune response, even though the peak response was lower with Gads<sup>−/−</sup> cells (Fig. 10). This is contrast to data from experiments using APL-expressing rL. monocytogenes, in which the number of memory cells produced was more dramatically impaired than when Gads<sup>+/+</sup> and Gads<sup>−/−</sup> cells were injected into separate mice. These data suggest that multiple factors influence the expansion of Ag-specific T cells and the number of memory cells that differentiate from a T cell population. Although strength of signaling intensity may be a component, another important factor is the nature of the other T cell populations in the mouse responding to the infection. If there are competing cells with higher affinity for the Ag or cells more capable of efficient signal transduction, then these competing populations are more likely to dominate the effector and memory T cell pools. The mechanisms by which some populations dominate the immune response are unclear, but subdominant cells may cease proliferating and undergo apoptosis earlier in the immune response than dominant cells.

In conclusion, we demonstrate that Gads regulates the kinetics of cell cycle entry and expression of early activation markers following TCR ligation with a peptide agonist. In the context of infection, the initial expansion of the Ag-specific CD8<sup>+</sup> T cell population was independent of Gads. However, Gads was necessary to sustain the expansion phase. The differentiation of naive CD8<sup>+</sup> T cells into memory T cells was independent of Gads.

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Disclosures

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References


