

GADS is required for TCR-mediated calcium influx and cytokine release, but not cellular adhesion, in human T cells



Mahmood Y. Bilal^a, Elizabeth Y. Zhang^c, Brittney Dinkel^b, Daimon Hardy^b, Thomas M. Yankee^c, Jon C.D. Houtman^{a,b,*}

^a Interdisciplinary Graduate Program in Immunology, University of Iowa, Iowa City, IA 52242, United States

^b Department of Microbiology, Carver College of Medicine, University of Iowa, Iowa City, IA 52242, United States

^c Department of Microbiology, Molecular Genetics and Immunology, University of Kansas Medical Center, Kansas City, KS 66160, United States

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ABSTRACT

GRB2 related adaptor protein downstream of Shc (GADS) is a member of the GRB2 family of adaptors and is critical for TCR-induced signaling. The current model is that GADS recruits SLP-76 to the LAT complex, which facilitates the phosphorylation of SLP-76, the activation of PLC- γ 1, T cell adhesion and cytokine production. However, this model is largely based on studies of disruption of the GADS/SLP-76 interaction and murine T cell differentiation in GADS deficient mice. The role of GADS in mediating TCR-induced signals in human CD4⁺ T cells has not been thoroughly investigated. In this study, we have suppressed the expression of GADS in human CD4⁺ HuT78 T cells. GADS deficient HuT78 T cells displayed similar levels of TCR-induced SLP-76 and PLC- γ 1 phosphorylation but exhibited substantial decrease in TCR-induced IL-2 and IFN- γ release. The defect in cytokine production occurred because of impaired calcium mobilization due to reduced recruitment of SLP-76 and PLC- γ 1 to the LAT complex. Surprisingly, both GADS deficient HuT78 and GADS deficient primary murine CD8⁺ T cells had similar TCR-induced adhesion when compared to control T cells. Overall, our results show that GADS is required for calcium influx and cytokine production, but not cellular adhesion, in human CD4⁺ T cells, suggesting that the current model for T cell regulation by GADS is incomplete.

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1. Introduction

The adaptor protein GADS is a hematopoietic-specific homolog of growth factor receptor bound-protein 2 (GRB2), both of which contain a central SH2 domain flanked by two SH3 domains [1]. The major structural difference is that GADS contains an extended linker between the SH2 domain and the C-terminal SH3 domain. The homologous SH2 regions of GADS and GRB2 allow direct binding of both proteins to the same phosphorylated tyrosine residues at linker for activation of T cells (LAT). The SH3 domains of GADS and GRB2 facilitate the recruitment of different proline-rich ligands to LAT. The most studied ligand for GADS is SH2 domain-containing leukocyte protein of 76 kDa (SLP-76), a vital component in T cell receptor (TCR)-mediated signal transduction [2–8].

Activation of human CD4⁺ T cells requires a primary signal received by the TCR from peptide antigen bound to major histocompatibility complexes (pMHC) on antigen presenting cells. Upon TCR activation, activated lymphocyte-specific protein tyrosine kinase (LCK) phosphorylates zeta chain associated protein kinase 70 kDa (ZAP-70). ZAP-70 mediates the phosphorylation of LAT thereby allowing GRB2 and

GADS to recruit critical ligands that drive the formation of the LAT signalosome [5,9]. In T cells, GADS/SLP-76-mediated complexes at LAT lead to the activation of several pathways including cytoskeletal rearrangement and adhesion, transcription, calcium signaling and cellular proliferation [5,8–12].

The current model is that the recruitment of GADS/SLP-76 complex to LAT facilitates the binding of VAV1 and interleukin-2-inducible T-cell kinase (ITK), which is important for the activation, and recruitment of phospholipase- γ 1 (PLC- γ 1) to the LAT complex [13–16]. The recruitment of enzymatically active PLC- γ 1 to the cellular membrane through the binding of Y132 at LAT catalyzes the formation of inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG) from phosphatidylinositol 4,5-bisphosphate (PIP2). Increased concentration of IP3 and DAG induced by the GADS/SLP-76 complexes enhances calcium influx and activation of protein kinase C (PKC θ), resulting in increased T cell functions such as cytokine release [10,13,17–19].

TCR activation drives extensive actin polymerization needed for changes in T cell morphology, motility and adhesion; these functions are critical in mediating interactions with antigen presenting cells (APC) and subsequent T cell function [20,21]. Previous studies have suggested a role of the LAT signaling complex in driving complete cytoskeletal organization. LAT deficient Jurkat T cells have substantially reduced TCR-induced spreading and actin polymerization [22]. These cells were

* Corresponding author at: 2210 MERF, University of Iowa, Iowa City, IA, 52242.

also unable to recruit proteins associated with the actin cytoskeleton to the T cell plasma membrane such as the adaptor protein NCK [11]. Reconstitution with wild-type LAT but not LAT lacking tyrosines important for SLP-76 recruitment via GADS rescued NCK recruitment to signaling clusters [11]. Similarly, SLP-76 has been linked as a core player in stabilizing NCK and WASp protein complexes at LAT for the regulation of actin polymerization [3,11,23–25]. However, although these studies provided an insight on the role of SLP-76 in recruiting proteins that drive cytoskeletal organization, SLP-76 deficient Jurkat T cells were still able to form actin rings indicating a non-essential role or a redundancy in inducing actin polymerization from the LAT complex [11]. In addition, recent studies demonstrated that NCK and VAV1 could interact in the absence of SLP-76 and this interaction regulates actin polymerization [3,24]. Therefore, whether the GADS/SLP-76 complex is essential in regulating TCR-mediated cytoskeletal rearrangement and adhesion is unclear.

The current model for the role of GADS in T cell biology is based on studies disrupting the GADS/SLP-76 interaction and examining T cell development and function in GADS knockout (KO) murine T cells. Several studies have characterized the role of GADS by inhibiting its interaction with SLP-76, either by mutation of the GADS binding site on SLP-76 or expression of short peptides derived from this site. Expression of dominant negative version of GADS or mutant SLP-76 proteins blocked the entrance of SLP-76 into signaling clusters and subsequent LAT-mediated signaling [26–28]. The disruption of GADS/SLP-76 interaction also abolished the recruitment of SLP-76 into clusters and subsequent signaling in mast cells [18]. Similarly, examination of both CD4 + and CD8 + GADS KO populations revealed impairment in T cell development [1,10,12]. GADS KO CD4 + thymocytes exhibited reduction of PLC- γ 1 phosphorylation compared to wild-type controls but had similar levels of SLP-76 phosphorylation [10]. The impaired PLC- γ 1 activation coincided with the reduced calcium mobilization in peripheral GADS KO CD4 + and CD8 + T cells [10,12]. However, despite the reduced TCR-induced calcium influx, GADS KO CD8 + T cells were able to secrete IFN- γ in a manner similar to wild-type T cells [29].

Although the above studies suggest an essential role for GADS in T cell development and function, the role of GADS in mature human CD4 + T cell function has yet to be carefully examined. These studies are necessary since the use of SLP-76-based mutations and/or peptides could inhibit the interaction of SLP-76 with all SH3 domain proteins and not selectively target the GADS/SLP-76 interaction. It is also possible that part of the defects in TCR-mediated signaling observed in GADS KO murine T cells may stem from obstacles confronted during development or the distinct role of GADS in the function of CD4 +, CD8 +, and DN T cell subsets [12,30]. To elucidate the role of GADS in mature human CD4 + T cells, we have developed efficient shRNA viral vectors to suppress the expression of GADS. We found that GADS is dispensable for TCR-mediated actin polymerization and cellular adhesion, but is essential for driving TCR-induced calcium mobilization and cytokine production. Overall, our studies demonstrate an essential role for GADS in mediating mature human CD4 + T cell function.

2. Materials and methods

2.1. HuT78 T cell growth and stimulation

Human CD4 + HuT78 T cell lines were cultured at 37 °C and 5% CO₂ in complete IMDM media (IMDM media supplemented with 20% FBS, 50 U/mL penicillin, 50 μ g/mL streptomycin, and 2 mM L-glutamine) (Gibco). Lentiviral transduced cell lines were kept in selection with 2 μ g/mL puromycin (CAS 58-58-2, Santa Cruz). Prior to stimulations, cell lines were grown to a concentration of 2–5 $\times$ 10⁵ cells/mL, and then washed in un-supplemented RPMI 1640 (Gibco). Cells were resuspended in un-supplemented RPMI 1640 to a concentration of 5 $\times$ 10⁷ cells/mL and rested for 10 min at 37 °C. The cells were stimulated with soluble 2 μ g/mL anti-CD3 (OKT3, BioLegend) for various time

points and then lysed with the addition of 4-fold excess of hot 2 $\times$ lysis buffer (20 mM Tris pH 8.0, 2 mM EDTA, 2 mM Na₃VO₄, 20 mM DTT, 2% SDS, and 20% glycerol). Lysates were then heated to 95 °C for 4 min and sonicated to reduce solution viscosity.

2.2. Cell conjugation assay

Splenocytes from GADS wild-type OT-I or GADS KO OT-I mice and CD8 + T cells were isolated by positive selection using anti-mouse CD8 α Magnetic Particles-DM (BD Biosciences Pharmingen). EL-4 cells were loaded with CFSE as described previously [12]. Briefly, the cell concentration was adjusted to 2 $\times$ 10⁷ cells/mL, and 10 μ M CFSE (Invitrogen, Carlsbad, CA) was added. Cells were incubated for 10 min at 37 °C and the reaction was quenched with cell culture media. Then CFSE-labeled EL-4 cells were pulsed with SIINFEKL at different concentrations for 60 min. Cells were washed to remove peptide and then the EL-4 cells were incubated with purified CD8 + T cells for 90 min. After gentle vortexing (1 s per sample), the cell conjugates were fixed with 1% paraformaldehyde in PBS for 30 min and stained with anti-CD8 before fixing again. Samples were analyzed using a BD LSRII (BD Biosciences, San Jose, CA). The percentage of CD8 + cells bound to CFSE + cells was calculated using BD FACS Diva (BD Biosciences).

2.3. Vectors and shRNA design

Lentiviral pLK4 vectors were donated by Dr. Stephen Bunnell and pLKO.1 lentiviruses were obtained from Addgene (plasmid 8453) [31]. The VSV-G construct was donated by Dr. Bruce Hostager. Packaging plasmids pCL-Eco and Pax2 were a kind gift from Drs. John Colgan and Dawn Quelle, respectively. ShRNA targeting sequences against human GADS (NM_004810.2) were developed utilizing a siRNA/shRNA based algorithm formulated by Dr. Sachidanandam and coworkers (<http://katahdin.cshl.edu>). The following sequences produced the most efficient suppression (GADS sense: 5' GCGAGACAACAAGGGTAATTA 3', GADS anti-sense: 5' TAATTACCCTTGTTGTCTCG 3').

The GADS sense and anti-sense sequences, hairpin loop, and terminal thymidines were inserted into pLKO.1 utilizing AgeI and EcoRI sites. Next, sequences from pLKO.1 vectors containing the U6 promoter driving GADS or control Luciferase (LUC) shRNAs were amplified and cloned into pLK4 lentiviral vectors. To generate a wild-type GADS resistant to shRNA mediated suppression, GADS cDNA was amplified from human CD4 + T cells and mutated using primers containing shRNA-sense bases to produce wild-type GADS with a different nucleotide sequence, but with an identical amino acid sequence. Wild-type GADS cDNA was then amplified and cloned into pLK4 vectors containing U6-GADS shRNA from above.

2.4. Production of lentiviruses

To generate pseudo-typed lentiviral particles, pLK4 vectors were transfected into 293 T cells along with envelope and packaging vectors VSV-G, and Pax2. 18–24 hrs prior to infection, 3 $\times$ 10⁶ 293 T cells were seeded in 10 cm culture dishes at 37 °C and 5% CO₂ in complete DMEM media (DMEM media supplemented with 10% FBS, 50 U/mL penicillin, 50 μ g/mL streptomycin, 1 $\times$ MEM NEAA, and 2 mM L-glutamine) (Gibco). The media was replaced with 5 mL of fresh complete DMEM on the day of transfection. Transfection of 293 T cells was achieved utilizing the calcium phosphate method. Vectors (pLK4 15 μ g, Pax2 10 μ g, VSV-G 7.5 μ g) were added together in 0.5 mL of 1 $\times$ HBS solution (Hepes free acid 21 mM, NaCl 137 mM, D + Glucose monohydrate 5 mM, KCl 50 mM, Na₂HPO₄ 0.35 mM, pH 7.5). Next, 30 μ L of 2.5 M CaCl₂ was added drop-wise to the HBS–DNA solution, mixed through gentle pipetting, and incubated for 20–30 min at room temperature. The HBS–DNA–CaCl₂ mix was then added drop-wise around the plate of 293 T cells and then incubated for 16–18 hrs.

Transfection media was then replaced with fresh media, and viral containing supernatant was collected and filtered through 0.45 µm Durapore Millex (Millipore) filters every 24 hrs for 2 days. Supernatants containing lentiviruses were aliquoted into round-bottom polycarbonate high-speed tubes (Nalgene-Oak ridge). The tubes were centrifuged at 4 °C for 1.5 hrs, 48,000 ×g in Sorvall RC6-Plus centrifuge (SS-34 Rotor). Viral pellets were resuspended and stored in complete RPMI media (RPMI media supplemented with 10% FBS, 50 U/mL penicillin, 50 µg/mL streptomycin, and 2 mM L-glutamine) (Gibco).

2.5. Transduction of HuT78 T cells

3–5 × 10⁵ HuT78 T cells were incubated in 1.5 mL of concentrated viral supernatant in 25 cm² flasks in the presence of 8 µg/mL Hexadimethrine bromide (Polybrene) (Sigma Aldrich). The cells were periodically mixed with the viral supernatant. After 48 hrs, the viral media was removed, and HuT78 cells were resuspended in fresh complete IMDM with 0.5 µg/mL of puromycin and cells were allowed to expand. The concentration of puromycin was then gradually increased each passage to 2 µg/mL.

2.6. Detection of IL-2 and IFN-

HuT78 T cells were washed in complete RPMI 1640, and then resuspended at 5 × 10⁵ cells/mL. To stimulate the cells, 0.5 mL of cell suspension was added 24 well plates coated with various concentrations of anti-CD3 for 24 hrs. Protein concentrations of IL-2 and IFN- in the supernatants were measured by standard TMB ELISA utilizing a spectrophotometric plate reader with a reading absorbance at 450 nm.

2.7. Immunoprecipitations

1–2 × 10⁷ HuT78 T cells were stimulated using soluble anti-CD3 as described above. The cells were then lysed by adding 800–1600 µL of immunoprecipitation buffer to the samples (25 mM Tris pH 8.0, 150 mM NaCl, 1% Brij-97, 0.5% n-Octyl-β-D-glucopyranoside, 5 mM EDTA, 1 mM Na₃VO₄, complete protease inhibitor tablets). Cellular lysates were incubated on ice for 30 min, vortexed every 10 min, and then centrifuged (13,000 rpm, 10 min). To reduce background from the stimulatory antibody, supernatants were pre-cleared by rotating for 30 min with protein A/G plus agarose (Santa Cruz Biotech), centrifuged, and the pre-cleared supernatants were incubated with protein A/G plus agarose with or without immunoprecipitation antibody overnight at 4 °C: PLC-γ1 (clone E-12, Santa Cruz Biotechnology), SLP-76 (clone F-7, Santa Cruz Biotechnology). The beads were washed 3 times by centrifugation (5000 rpm, 2 min) in washing buffer (25 mM Tris pH 8.0, 150 mM NaCl, 5 mM EDTA, complete protease inhibitor tablets). The immunoprecipitated proteins were then eluted by heating at 95 °C for 4 min in 2× lysis buffer.

2.8. Immunoblotting

Samples containing proteins were loaded onto a 4–15% precast polyacrylamide gels (Biorad). The separated proteins were transferred onto PVDF membranes (Millipore), and then blocked for 1 hr at room temperature in a 1:1 × PBS:SEA Block (Thermo Scientific). The PVDF membranes were incubated with primary antibodies against SLP-76 pY128 (clone J141-668.36.58, BD Pharmingen), SLP-76 (clone F-7, Santa Cruz Biotechnology), AKT pThr 308 (Cell Signaling), GRB2 (clone 23, Santa Cruz Biotechnology), pY783 PLC-γ1 (Cell signaling), PLC-γ1 (Cell Signaling), pY (4G10, Millipore), Actin (clone C4, Millipore), or GAPDH (Meridian Life Sciences). IRDye 800CW or IRDye 680-conjugated secondary antibodies were diluted in SEA Block as above and incubated with the PVDF membranes for 30 min at room temperature. The membranes were then imaged using the Licor Odyssey Infrared detector.

2.9. Immunoblot analysis

Densitometric analysis of protein bands was determined using Odyssey's v3.0 software. Phosphorylated or total forms of proteins were normalized to actin as previously described [32,33]. Normalization of phospho-specific proteins to pan forms may give misleading results due to the inability of pan antibodies to detect phosphorylated forms and steric hindrance that can occur when two antibodies bind to the same protein. However, to account for the reduction of total LAT expression in GADS deficient cells, normalization of phosphorylated LAT in immunoprecipitations was performed by first dividing raw intensities in GADS samples by 0.73145 (compared to 1 in LUC control cells) to account for the reduction of total LAT. The following formula was used for normalization and quantification of the immunoblots and cytokine release data:

1. Normalized data point to actin intensities (NP) = raw band intensity ÷ raw actin intensity.
2. Percent of maximum of LUC stimulation (as indicated) = (NP time point – NP 0 time point of control LUC) ÷ (NP max time point of control LUC – NP 0 time point of control LUC).

2.10. TCR-mediated adhesion assay

HuT78 T cell adhesion assays were performed as previously described utilizing Licor CellVue Burgundy kit [34]. RIA/EIA 96 well plates were coated with various concentrations of anti-CD3. The wells were washed and blocked in 1% BSA–PBS for 2 hrs at 37 °C, and then washed 3 times in PBS. HuT78 T cells were washed and resuspended at 2 × 10⁷ cells/mL in Diluent C and then stained with 4 × 10^{–6} M dye stock for 5 min. Cells were washed 3 times with complete RPMI, suspended at 4.5 × 10⁵ cells/100 µL, and then 100 µL of cell suspension was allowed to adhere to each well for 30 min at 37 °C and 5% CO₂. The plates were then washed twice in PBS, resuspended in 100 µL PBS, and imaged utilizing the Licor Odyssey Infrared detector.

2.11. TCR-induced actin ring formation

1.5 × 10⁵ HuT78 T cells were seeded onto glass chamber slides (LabTek II, Nalgene Nunc International) coated with plate-bound anti-CD3 (10 µg/mL). Cells were activated for 5 min and then fixed with 3% paraformaldehyde–PBS solution for 30 min. The cells were then permeabilized with 0.25% Triton X-100 in PBS for 5 min, washed, and blocked with SEA block for 1 hr at RT. To stain TCR-induced actin polymers, the cells were incubated with 200 nM Phalloidin–Tetramethylrhodamine B isothiocyanate (SIGMA-ALDRICH) for 2 hrs at 37 °C. Cells were washed and the images were acquired using mcherry-epifluorescence channel on the Lonza TIRF microscope located at the Central Microscopy Core Facility at the University of Iowa. All images were taken using the 100× oil objective. Images were processed using Adobe Photoshop software. Three independent experiments (25 cells per group) were used to measure actin intensity and cell area by drawing around each cell and applying “Measure” function in ImageJ.

2.12. Flow cytometry

1 × 10⁶ HuT78 T cells were washed and then resuspended at 1 × 10⁶ cells/mL in FACS buffer (PBS, 10% FBS, and 0.05% sodium azide). The cells were stained with primary conjugated antibodies on ice for 30 min (PE-TCRαβ, BioLegend). The mean fluorescence intensity (MFI) of each sample was obtained using an Accuri C6 flow cytometer.

2.13. Calcium influx

1 × 10⁶ HuT78 T cells were suspended in 1 mL RPMI 1640 without phenol-red (Gibco). 5 µL of 1 mM Fluo-4 AM (Life Technologies), and

10 μ L of 250 mM probenecid (Life Technologies) were added to the cell suspension, and then cells were incubated at 37 °C and 5% CO₂ for 45 min. Cells were washed in RPMI without phenol-red and then resuspended at a concentration of 1×10^6 cells/mL in RPMI-probenecid, and left on ice for 15 min. Prior to stimulation, cells were incubated at 37 °C for 5 min. Basal cytoplasmic calcium levels were obtained for 35 s, and then cells were stimulated with 5 μ g/mL anti-CD3 for 6–7 min. The MFI of each sample was obtained using an Accuri C6 flow cytometer. Statistical analysis per stimulation was calculated based on 10 live cell events per tenth of a second (i.e. 100 cells per seconds). Independent replicates of each group were averaged and graphed based on unstimulated fold increase.

2.14. Statistical analysis

Analysis of Immunoblots and cytokine assays were performed in GraphPad prism software using two-tailed t-test assuming equal variance. Analysis of calcium influx data was performed using Microsoft Excel using a two-tailed t-test. Levels of significance $p < .05$ & $p < .005$ are presented as * & **, respectively.

3. Results

3.1. Knockdown of GADS protein expression through shRNA-mediated suppression

The current model is that GADS recruits SLP-76 to the LAT signalosome, resulting in increased phosphorylation of SLP-76. Activated SLP-76 then binds to ITK, which phosphorylates and activates PLC- γ 1, and the VAV1-NCK-WASp complex, which alters actin cytoskeleton rearrangement. Thus, the GADS/SLP-76 complex is critical for calcium influx, transcriptional regulation and T cell adhesion. However, these studies are based on inhibition of the ability of SLP-76 to bind to GADS and potentially other partners in GADS deficient murine thymocytes and T cells. The role of GADS in mediating downstream functions in mature human CD4+ T cell has not been fully examined.

To address this question, we have utilized an online shRNA generating algorithm as described in the [Materials and methods](#) to target GADS expression in human T cells. The identified sequences were cloned into mammalian expression lentiviral packaging plK4 vectors. Potential shRNA sequences against GADS were then experimentally tested by stably transducing human CD4+ HuT78 T cells that have similar TCR signaling kinetics to primary human CD4+ T cells [35]. The most efficacious sequences produced >90% GADS suppression (Fig. 1A).

To confirm that GADS suppression does not alter other functions of HuT78 T cells, expression of signaling molecules associated with TCR and LAT complexes were examined. Overall, there was no statistically significant difference in total protein expression of GRB2, PLC- γ 1, LCK, AKT, and PI3 kinase (Fig. 1B and 1C). Additionally, the surface expression of TCR $\alpha\beta$ was similar between GADS deficient and LUC control cells (Fig. 1D). The expression level of LAT was significantly reduced in the absence of GADS, while SLP-76 expression was variable but was overall less in quantity when compared to LUC (Fig. 1D). The reduction of LAT and SLP-76 indicates a role for GADS in the homeostatic expression of these two proteins (Fig. 1B and 1C). These studies show that shRNAs can be used to produce a human CD4+ T cell with greatly reduced GADS expression without markedly altering the expression of other signaling proteins.

3.2. GADS is required for the LAT recruitment, but not phosphorylation of SLP-76

To understand the role of GADS in mediating TCR-induced signaling, we first examined the main function of GADS, specifically the phosphorylation of SLP-76 and its recruitment to LAT. HuT78 T cells carrying

shRNAs against GADS or LUC were stimulated with soluble anti-CD3 for various times, and then SLP-76 was immunoprecipitated from cellular lysates. As expected, upon TCR stimulation SLP-76 interacted with phosphorylated LAT in LUC control cells, but the interaction was significantly diminished in GADS deficient HuT78 T cells (Fig. 2A). When the immunoprecipitated phospho-LAT was normalized to total LAT expression, we observed that phosphorylated LAT had a substantial reduction in the interaction with SLP-76 in the absence of GADS (Fig. 2A). Next, to determine if the diminished recruitment of SLP-76 to LAT resulted in impaired activation, we probed the phosphorylation of Y128 on SLP-76 in the absence of GADS. Surprisingly, given the inability of SLP-76 to interact with LAT and the reduction of total SLP-76 proteins in GADS deficient cells (Fig. 1B and 1C), there was a trend towards an increase in total tyrosine and site specific Y128 phosphorylation of SLP-76 in the absence of GADS (Fig. 2A and 2B). Our results are in agreement with GADS KO T cells where SLP-76 fails to associate with LAT, but still undergoes complete phosphorylation [10]. The activation of AKT, shown previously to be dependent on SLP-76, was also not altered between GADS and LUC control cells (Fig. 2B) [4]. These results indicate that there is redundancy in the activation of SLP-76 and downstream effectors in the absence of the ability of GADS to recruit SLP-76 to the LAT signaling complex.

3.3. TCR-mediated adhesion is not impaired in the absence of GADS

To examine if the recruitment of SLP-76 to LAT by GADS is essential for TCR-mediated actin rearrangement and cellular adhesion, we first examined the ability of GADS deficient T cells to polymerize actin. HuT78 T cells were allowed to spread on anti-CD3 coated glass chambers, and then polymerized actin was stained and imaged. Surprisingly, given the current paradigm of SLP-76/LAT-mediated cytoskeletal rearrangement, we observed similar actin ring formation in GADS deficient compared to LUC control cells (Fig. 3A). The mean of maximum actin intensity per cell was slightly increased in the absence of GADS, but the mean cell area between GADS deficient and control cells was similar (Fig. 3B). Moreover, we utilized a near-infrared imaging technique that allows live cells to adhere onto anti-CD3 coated plates in an actin polymerization dependent process [34]. We observed increased TCR-mediated adhesive capacity in GADS deficient HuT78 T cells when compared to LUC controls (Fig. 3C). To confirm these findings in a more physiological setting, we utilized primary murine GADS KO CD8+ T cells. GADS KO and wild-type CD8+ T cells from OT-I mice were allowed to adhere to EL-4 cells pulsed with various concentrations of SIINFEKL for 60 min. The percentage of CD8+ cells bound to CFSE+ cells were calculated using flow cytometry. In agreement with GADS deficient HuT78 cellular adhesion, GADS KO primary CD8+ T cells had significantly increased EL-4 cell-conjugate formation when compared to wild-type CD8+ T cells (Fig. 3D). Our results indicate a non-essential requirement for GADS in mediating TCR-induced actin polymerization and cytoskeletal arrangement from the LAT complex.

3.4. GADS deficient HuT78 T cells exhibit impaired PLC- γ 1 recruitment to LAT and calcium mobilization

We next inquired if the defects observed in GADS deficient cells resulted in impaired PLC- γ 1-induced signaling. Upon TCR activation PLC- γ 1 is recruited to LAT and subsequently phosphorylated, resulting in IP3 induction and calcium mobilization. To determine if GADS controls PLC- γ 1-calcium signaling in human CD4+ T cells, HuT78 T cells were incubated with Fluo-4 calcium indicator, and then stimulated with soluble anti-CD3. HuT78 T cells expressing GADS shRNA had marked reduction in the peak levels and timing of calcium influx (Fig. 4A). To confirm the requirement of GADS in mediating TCR-induced calcium influx, we cloned a shRNA-resistant wild-type GADS protein in the same construct expressing the GADS shRNA (Fig. 4B). Add-back of shRNA-resistant wild-type GADS into GADS deficient

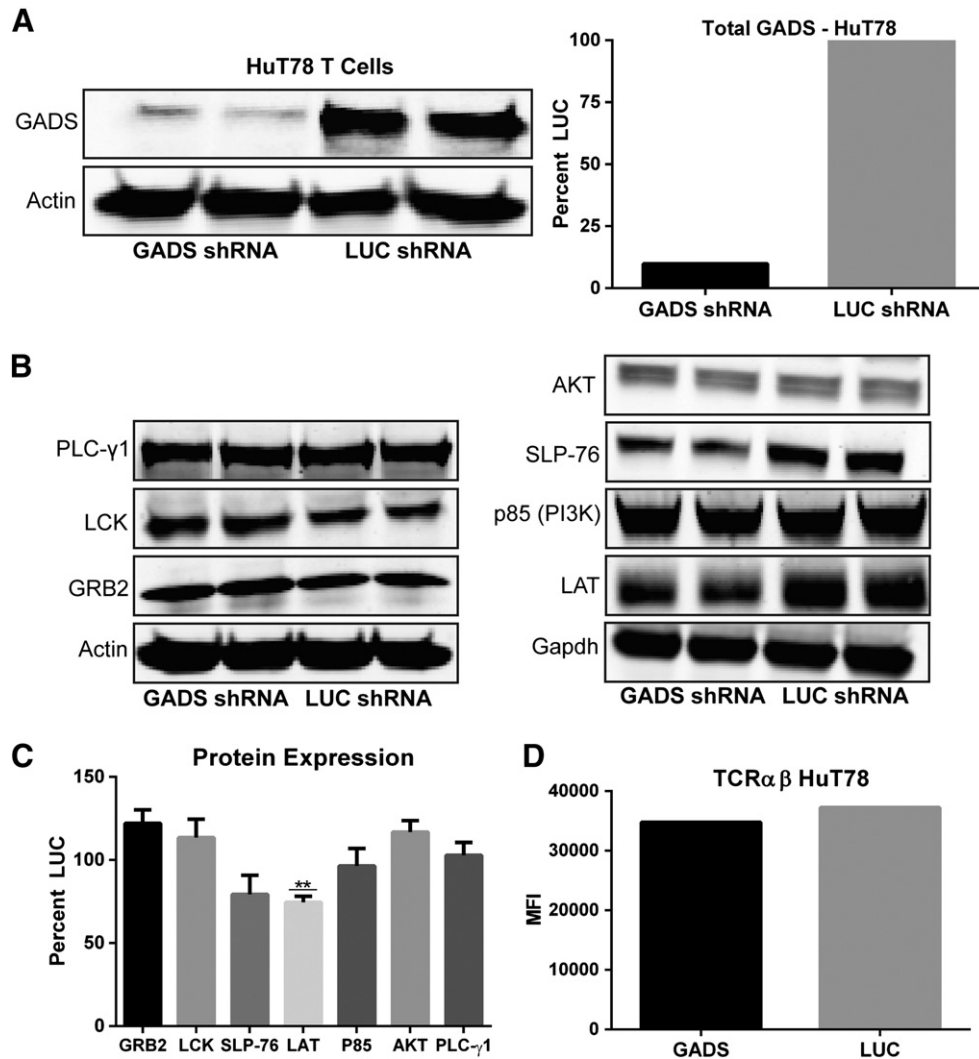


Fig. 1. Suppression of GADS protein expression in HuT78 CD4+ T cells. (A) Left: HuT78 T cells were transduced with lentiviruses carrying GADS or control Luciferase (LUC) shRNA, and protein levels of GADS and actin were detected by immunoblotting. Right: quantification of GADS knockdown in HuT78 T cells. (B) GADS and LUC HuT78 T cells were probed for the total GRB2, LAT, PLC-γ1, LCK, SLP-76, AKT, and PI3K-p85 protein expression via immunoblotting. Three separate quantifications were pooled and graphed as percent LUC $\pm$ SEM. (C) Quantification of total protein expression in GADS deficient HuT78 T cells. (D) GADS deficient or LUC HuT78 T cells were probed for TCRαβ surface expression using flow cytometry. Analysis is displayed as mean fluorescence intensity (MFI).

HuT78 T cells significantly rescued calcium mobilization to a higher extent than LUC control cells (Fig. 4B and 4C). These results demonstrate that the defects observed are specific to the absence of GADS.

Based on these observations, we next examined if GADS deficient HuT78 T cells had impaired phosphorylation and recruitment of PLC-γ1 to LAT. As expected, after TCR ligation there was a substantial reduction in the interaction between PLC-γ1 and LAT (Fig. 4D). Surprisingly, the amount of phosphorylated PLC-γ1 was similar between GADS and LUC control HuT78 T cells (Fig. 4D and 4E). Overall, our results demonstrate that GADS is not required for the phosphorylation of PLC-γ1, but is essential for the stable recruitment of PLC-γ1 to the LAT signalosome and its subsequent ability to induce cytoplasmic calcium influx.

3.5. GADS is required for TCR-mediated IL-2 and IFN-γ release

To determine if GADS is required for TCR-mediated downstream function, such as cytokine release, in mature human CD4+ T cells, HuT78 T cells carrying GADS or control LUC shRNA were stimulated with plate-bound anti-CD3 for 24 hrs and then the supernatants were examined for the release of IL-2 and IFN-γ. GADS deficient HuT78 T cells had significant reductions in both IL-2 and IFN-γ protein levels

as determined by ELISA (Fig. 5A). The reduced production of IL-2 in GADS deficient T cells could be rescued by the addition of wild-type shRNA-resistant GADS, showing that the defect in cytokine release was due to the lack of GADS expression (Fig. 5B). These results demonstrate a critical role for GADS in mediating TCR-induced cytokine release in CD4+ T cells.

4. Discussion

In the current study, we have utilized lentiviruses carrying shRNAs against GADS to effectively suppress the expression of GADS in human CD4+ T cells. Our results demonstrate that GADS is dispensable for TCR-mediated cytoskeletal rearrangement and adhesion but is required for TCR-induced release of immunomodulatory cytokines IL-2 and IFN-γ. The defect in cytokine production is not due to decreased phosphorylation of SLP-76 or PLC-γ1, but instead attributed to impaired recruitment of SLP-76 and PLC-γ1 to LAT complex, which results in diminished calcium influx. Overall, our studies reveal that the current model for how GADS regulates TCR-mediated signaling in human T cells is incomplete and requires further characterization.

Based on the current paradigm of the role of the GADS/SLP-76 complex in TCR-mediated actin polymerization, we were surprised to

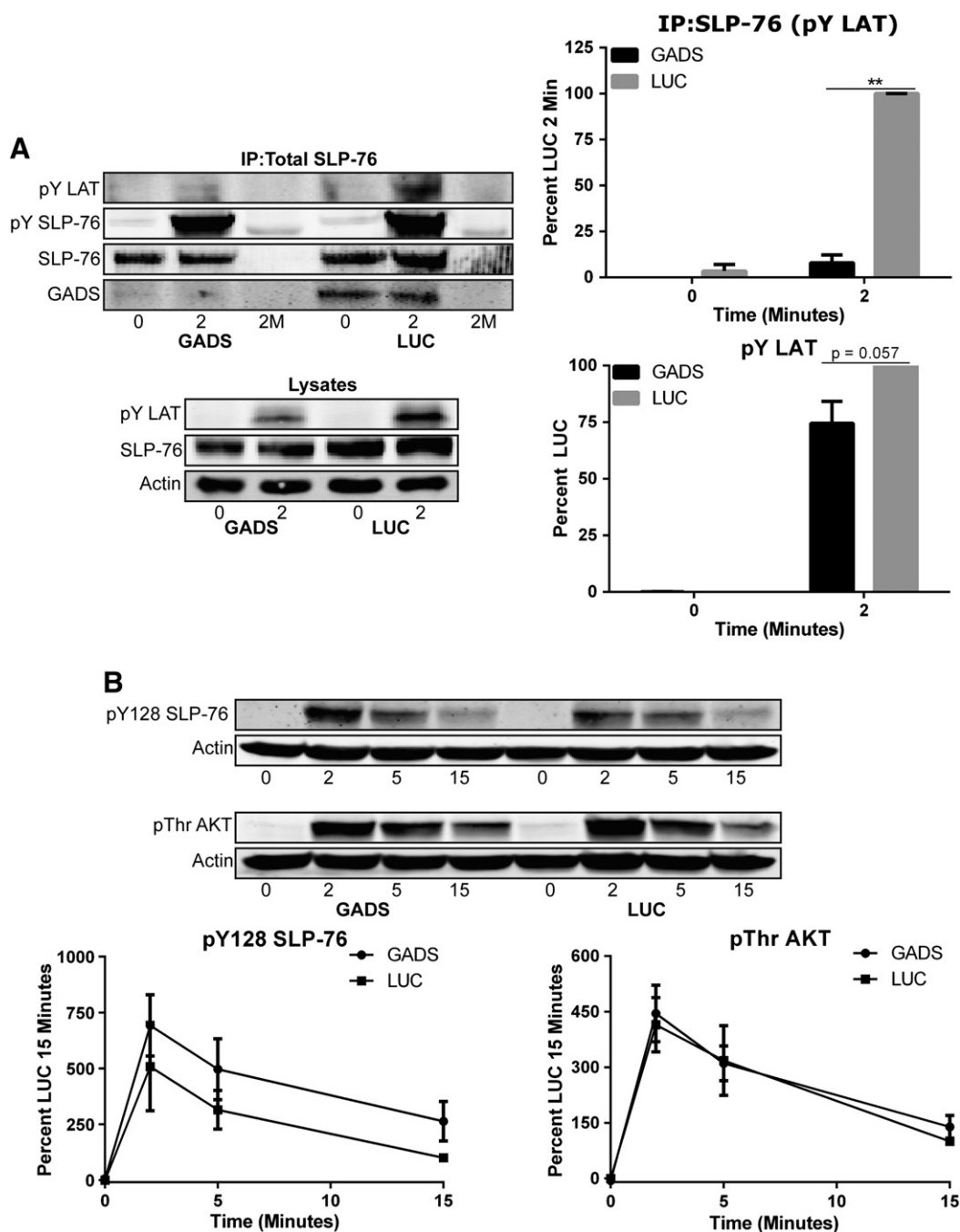


Fig. 2. Impaired SLP-76 and LAT complex formation but normal SLP-76 phosphorylation in GADS deficient HuT78 T cells. (A) Left: HuT78 T cells were stimulated with 2 μ g/mL soluble anti-CD3 for 2 min, and the lysates were immunoprecipitated with total SLP-76 antibody or mock “no antibody” control (2M). The protein levels were detected by immuno-blotting using phospho-tyrosine, SLP-76, GADS, and actin antibodies. Right: “Top” quantification of the interaction between SLP-76 and phospho-LAT in immunoprecipitates after normalization to the total LAT expression; “Bottom” quantification of the total phospho-LAT in lysates. Three independent experiments were graphed as percent LUC $\pm$ SEM. (B) HuT78 T cells were stimulated with 2 μ g/mL soluble anti-CD3 for the indicated time points (minutes), and the lysates were probed with antibodies against pY128 SLP-76, pThr 308 AKT, and actin. Immunoblots of four independent replicates were normalized to actin and illustrated as mean percentage phosphorylation of LUC $\pm$ SEM as described in materials and methods.

observe increased binding of GADS deficient HuT78 and primary murine CD8⁺ T cells to anti-CD3 coated plates or antigen presenting cells, respectively. The role of LAT and SLP-76 in inducing TCR-mediated cytoskeletal arrangement by recruiting cytoskeleton associated proteins has been well documented [3,11,22,24]. The role of GADS in these processes was largely extrapolated from experiments targeting the ability of SLP-76 to interact with GADS. However, these methods could also potentially inhibit the binding of SLP-76 to other proteins. In fact, SLP-76 deficient Jurkat T cells were still able to polymerize actin post-TCR activation, indicating a non-essential requirement for TCR-induced actin polymerization [11]. Additionally, transfection of

murine T cells with a peptide corresponding to the GADS binding site on SLP-76 has no effect on T cell-APC conjugate formation [28]. Our data demonstrate that the recruitment of SLP-76 to LAT is dispensable for TCR-mediated adhesion. One possibility for these results is that SLP-76 can mediate adhesion utilizing different receptors such as CD6 [36,37]. Upon engagement of TCR, CD6 associates with SLP-76 and VAV1, indicating a potential in mediating cytoskeletal arrangement [37]. Additionally, SLP-76 independent cytoskeletal rearrangement may still occur through other pathways. Specifically, adaptor proteins such as GRB2 can induce actin polymerization in multiple cells through the binding and mediating the activation of WASp [38–40].

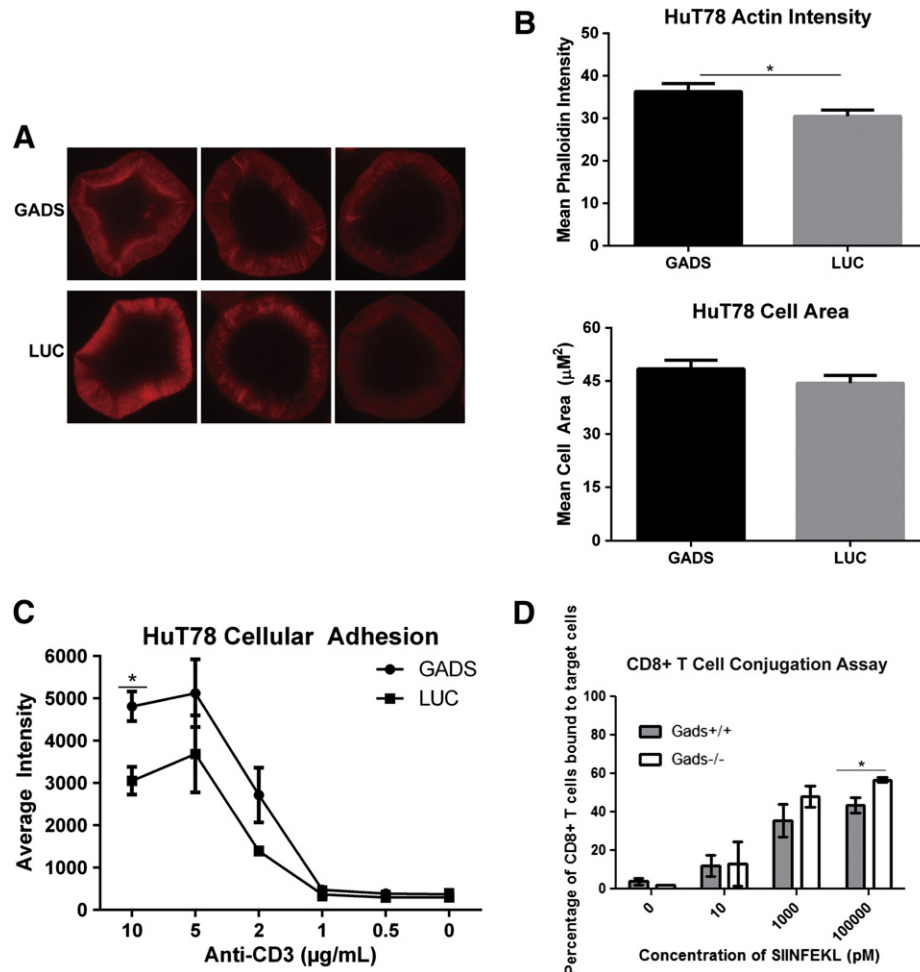


Fig. 3. TCR-induced actin polymerization and cellular adhesion are not impaired in the absence of GADS. (A) HuT78 T cells were stimulated for 5 min on anti-CD3 coated glass coverslips. Cells were stained with Phalloidin–Tetramethylrhodamine and visualized using epifluorescence. (B) Quantification of Phalloidin intensity and cell area from “A” utilizing ImageJ “Measure” functions. Data was collected from three independent replicates (25 cells per group) and graphed as $\pm$ SEM. (C) GADS and LUC HuT78 T cells were allowed to adhere to 96 well plates coated with various concentrations of anti-CD3, and intensity of adhered cells was measured using an Odyssey infrared detector. The average intensity of triplicate wells in each of three independent replicate is illustrated as $\pm$ SEM. (D) GADS KO or wild-type murine OT-I CD8 + T cells were allowed to form conjugates with CFSE-labeled EL-4 cells pulsed with different concentrations of SIINFEKL. The percentage of CD8 + cells bound to CFSE + cells were calculated using flow cytometry, and graphed as $\pm$ SD of 2 independent replicates.

Interestingly, although in our studies the GADS/SLP-76 complex was dispensable for LAT-mediated adhesion in T cells, it has been shown to control the actin cytoskeleton in non-T cells through alternate signaling pathways. Recent studies demonstrated that the transforming factor for chronic myelogenous leukemia, the BCR–Abl kinase, mediates actin rearrangement through the GADS/SLP-76 complex [41].

The impaired T cell function in GADS deficient HuT78 T cells was not due to reduced SLP-76 activation, as we observed increased SLP-76 phosphorylation in the absence of GADS (Fig. 2A and 2B). The latter observation is in agreement with GADS KO murine T cells where SLP-76 phosphorylation was normal in the absence of LAT interaction [10]. However, LAT deficient Jurkat T cells had reduced SLP-76 phosphorylation implicating that maximal activation of SLP-76 by ZAP-70 occurs at LAT presumably through GADS-mediated recruitment [42,43]. The results from previous work and our studies suggest that one or a combination of the following may occur. First, LAT enhances but is not absolutely required for SLP-76 phosphorylation, as LAT independent signaling may still occur in T cells [44]. In this case, CD6 or other receptors may also be involved in the regulation of SLP-76 phosphorylation; this is likely as the interaction between CD6 and SLP-76 was ZAP-70 dependent [37]. Second, the GADS homologs, GRB2 or GRAP may compensate for the deficiency of GADS by facilitating the interaction of SLP-76 with LAT and/or other adaptors and receptors that could mediate its phosphorylation by ZAP-70 [45]. It is possible that the ability of GRB2 or GRAP to

facilitate the interaction of SLP-76 with LAT is weak and transient, thus unstable in immunoprecipitation studies, but sufficient for SLP-76 phosphorylation. Finally, similar to the differences seen between GADS KO CD4 + and CD8 + murine cells, differential requirement of GADS for SLP-76 phosphorylation could be due to altered signaling in T cell subsets (i.e. CD4 vs. CD8, immature vs. mature or naïve vs. effector vs. memory) [12,30,45]. In fact, SLP-76 expression and phosphorylation is altered between naïve, effector, and memory CD4 + T cells [45,46]. Likewise, Jurkat T cells express higher GADS protein levels when compared to HuT78 T cells (data not shown). Thus, alteration of protein levels between cell types may modulate the requirement of GADS for the induction of SLP-76 phosphorylation [45]. Overall, our data demonstrate a non-essential role for GADS in mediating adhesion and SLP-76 phosphorylation downstream of LAT complexes.

Similar to SLP-76, phosphorylation of PLC- γ 1 was not reduced in the absence of GADS. However, the recruitment of PLC- γ 1 to the LAT complex was substantially lower in GADS deficient HuT78 T cells. The instability of PLC- γ 1 at LAT resulted in a reduction of TCR-induced calcium mobilization. Our results are similar to murine GADS KO T cells where TCR-mediated calcium influx was diminished compared to control wild-type T cells [10,12]. However, these cells had reduced tyrosine phosphorylation of PLC- γ 1 which impaired calcium entry into the cells [10]. In our hands, recruitment of PLC- γ 1 to the LAT complex rather than its phosphorylation was the primary factor in the reduction of

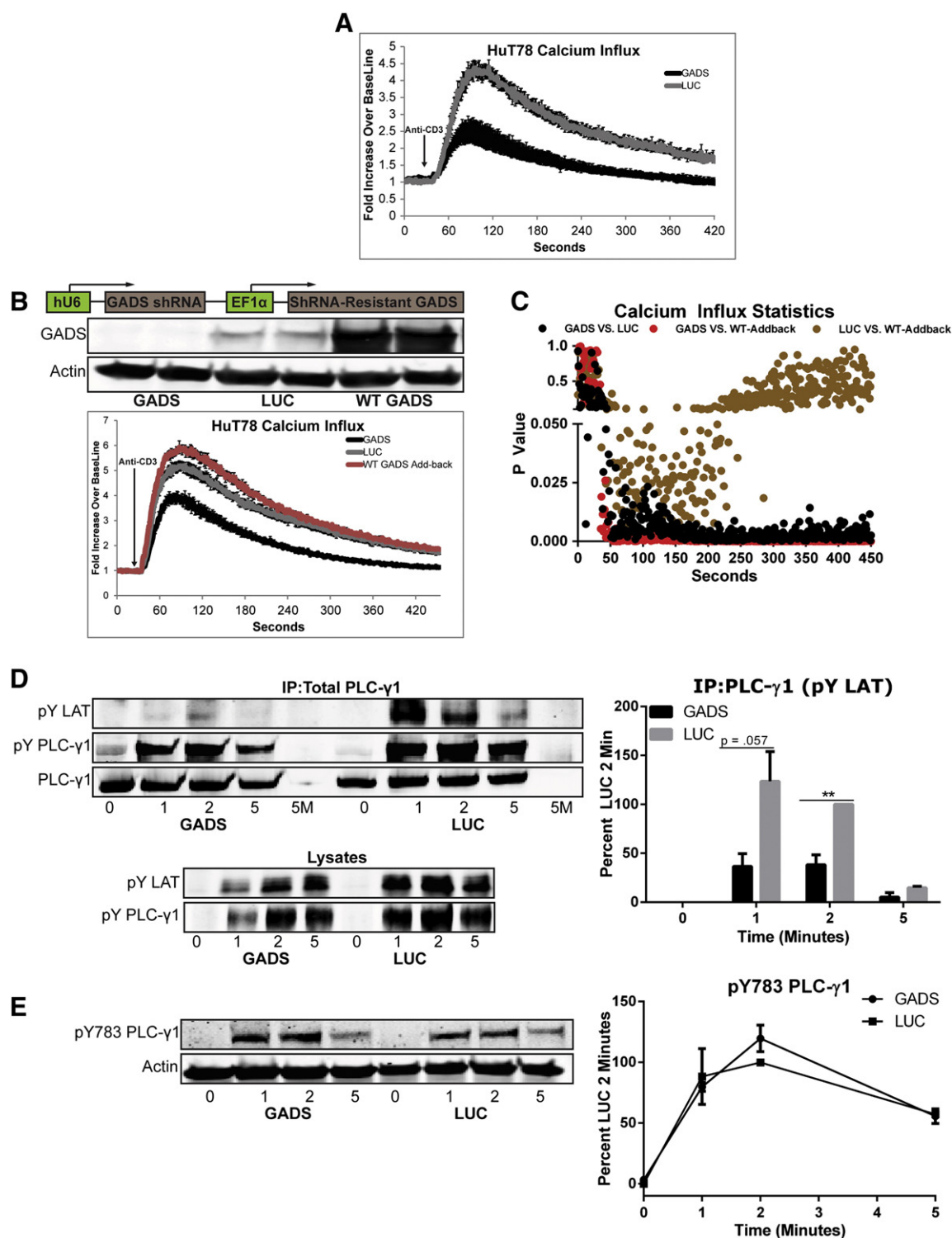


Fig. 4. Reduced calcium mobilization and PLC- γ 1 recruitment to LAT in GADS deficient HuT78 T cells. (A) GADS deficient or LUC HuT78 T cells were incubated with Fluo-4 AM, and then stimulated with 5 μ M soluble anti-CD3. Independent experiments were quantified as fold-increase MFI over unstimulated baseline $\pm$ SEM of four replicates. (B) Top: schematic diagram and immunoblot of HuT78 T cells transduced with lentiviral constructs carrying LUC, and GADS shRNA with or without reconstitution of shRNA-resistant GADS. Bottom: HuT78 T cells expressing LUC shRNA or GADS shRNA with or without reconstituted shRNA-resistant wild-type GADS were incubated with Fluo-4 AM, and then stimulated with 5 μ M soluble anti-CD3. Independent experiments were quantified as fold-increase MFI over unstimulated baseline $\pm$ SEM of three replicates. (C) Scatterplot illustrating statistical analysis performed on data from "B". (D) Left: HuT78 T cells were stimulated with 2 μ M soluble anti-CD3 for various times (minutes), and the lysates were immunoprecipitated with total PLC- γ 1 antibody or mock "no antibody" control (5M). The protein levels were detected by immuno-blotting using phospho-tyrosine, and PLC- γ 1 antibodies. Illustrated immunoprecipitation is a representative of three experiments. Right: quantification of the interaction between PLC- γ 1 and phospho-LAT in immunoprecipitates after normalization to total LAT expression. (E) HuT78 T cells were stimulated with 2 μ M soluble anti-CD3 for the indicated time points (minutes), and the lysates were probed with antibodies against pY783 PLC- γ 1, and actin. Immunoblots of four independent replicates were normalized to actin and illustrated as mean percentage phosphorylation of LUC $\pm$ SEM as described in [Materials and methods](#).

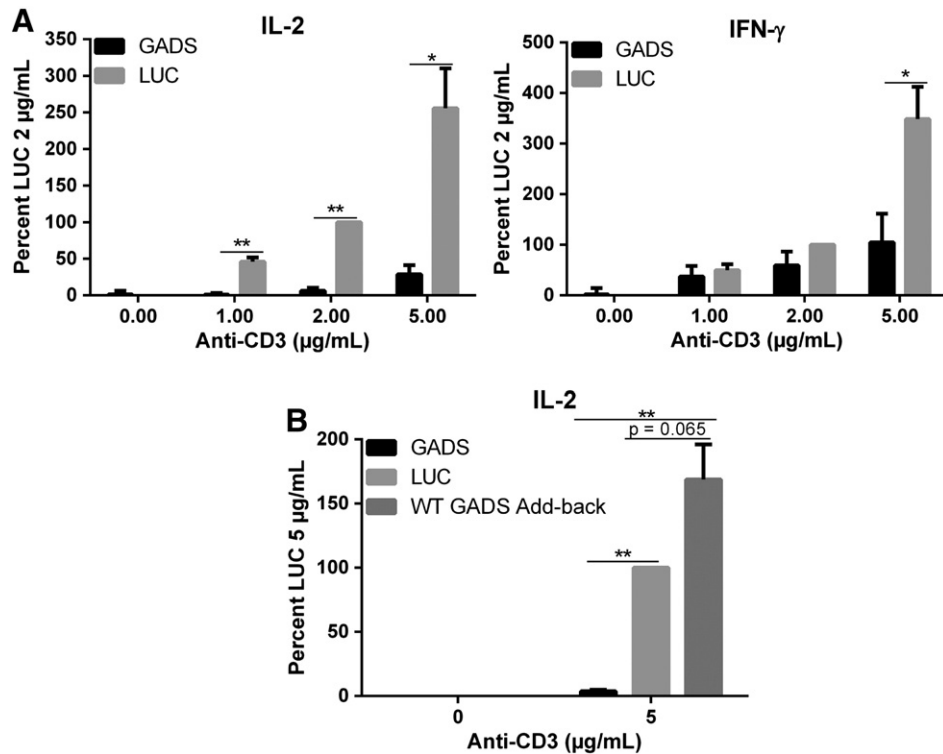


Fig. 5. Substantial reduction of TCR-induced cytokine release in the absence of GADS. (A) GADS deficient and LUC HuT78 T cells were stimulated on anti-CD3 coated plates with various concentrations for 24 hrs. The supernatants were probed for IL-2 and IFN- γ production via ELISA. The mean normalized value $\pm$ SEM of five (IL-2) and four (IFN- γ) independent experiments is shown. (B) HuT78 T cells were stimulated as in “A” and the supernatants were probed for IL-2 production via ELISA. The mean normalized value $\pm$ SEM of three independent experiments is shown.

calcium mobilization. It is unclear why the phosphorylation of PLC- γ 1 is reduced in murine GADS KO T cells and similar in mature HuT78 GADS deficient T cells. Similar to SLP-76, differences of signaling and expression of PLC- γ 1 or other proteins between different T cell types may produce alternative signaling [47]. Interestingly, in contrast to T cells, GADS negatively controls calcium influx in B cells upon BCR ligation [48]. Together, these results confirm the requirement of GADS in mediating TCR-induced calcium influx due to its ability to stabilize the LAT-PLC- γ 1 complex, but not by mediating the phosphorylation of PLC- γ 1.

Silencing of GADS substantially reduced the ability of HuT78 T cells to secrete both IL-2 and IFN- γ . Our results are in contrast to GADS KO

murine CD8 + T cells, where IFN- γ secretion was not affected by the absence of GADS. The differences between these data could stem from the signaling differences between CD8 + and CD4 + T cells [29]. For example, murine CD4 + T cells were more dependent on GADS for survival and homeostasis when compared to CD8 + counterparts [12]. The homeostatic deficiencies in GADS KO murine CD4 + T cells correlate with our cytokine release data. Our data also coincide with GADS over-expression studies where the synergistic co-expression of GADS and SLP-76 in Jurkat T cells, and not separate over-expression, enhanced NFAT and IL-2 promoter activity [8]. Thus, GADS drives TCR-mediated calcium influx and subsequent NFAT nuclear translocation, thereby inducing the transcription of cytokine mRNA.

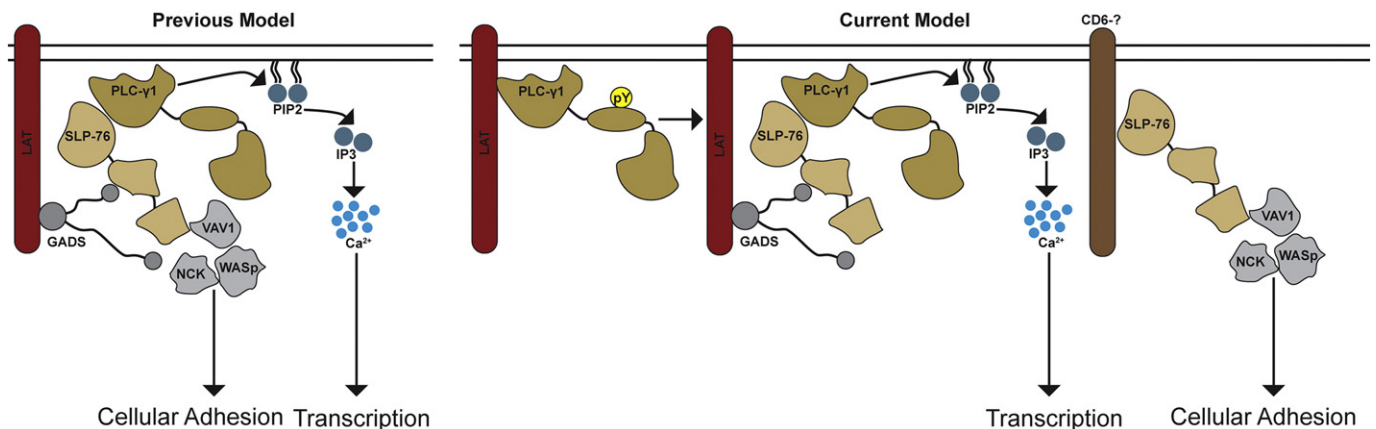


Fig. 6. GADS drives TCR-mediated calcium influx and cytokine production but not cellular adhesion. Upon TCR activation, SLP-76 is recruited to receptors or adaptor proteins other than LAT, such as CD6, which drive actin polymerization and cellular adhesion by recruiting SLP-76-bound proteins (NCK, VAV1, WASp) to the cellular membrane, thereby inducing cytoskeletal rearrangement. Simultaneously, phosphorylated SLP-76 is recruited to the LAT signaling complex via GADS. SLP-76 stabilizes phosphorylated PLC- γ 1 at the LAT-cellular membrane junction thereby driving calcium mobilization. These processes subsequently induce cellular motility and adhesion, transcription, and cytokine production, all of which are critical for T cell function.

Collectively, our data demonstrate that the current model for how GADS regulates TCR-mediated signaling is incomplete. Specifically, we demonstrate that the recruitment of SLP-76 to LAT by GADS is not required for TCR-mediated cytoskeletal rearrangement in CD4⁺ and CD8⁺ T cells. Instead, actin polymerization is induced through other receptors or a compensatory mechanism at LAT (Fig. 6). We also highlight GADS as an essential player in TCR-induced calcium mobilization and cytokine release. Collectively, our data identify GADS as a critical player in the biology of T cells by transmitting TCR signal transduction. It also suggests the potential of therapeutic targeting of GADS in T-cell mediated autoimmune disorders, GADS-mediated leukemias and other hematopoietic malignancies [49].

Acknowledgments

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