Published in final edited form as:

Am J Transplant. 2016 May ; 16(5): 1383–1393. doi:10.1111/ajt.13623.

Glutamate receptor interacting protein 1 regulates CD4⁺ CTLA-4 expression and transplant rejection

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Abstract

PDZ domains are common 80–90 amino acid regions named after the first three proteins discovered to share these domains: post-synaptic density 95, disc large, and zonula occludens. PDZ domain-containing proteins typically interact with the C-terminus of membrane receptors. Glutamate receptor interacting protein 1 (GRIP1), a 7 PDZ domain protein scaffold regulates glutamate receptor surface expression and trafficking in neurons. We have found that human and mouse T cells also express GRIP1. T cell-specific GRIP1^{-/-} mice greater than 11 weeks of age had prolonged cardiac allograft survival. Compared to WT T cells, in vitro stimulated GRIP1^{-/-} T cells had decreased expression of activation markers, and increased apoptotic surface marker expression. Surface expression of the strong T cell inhibitory molecule CTLA-4 was increased on GRIP1^{-/-} T cells from mice greater than 11 weeks of age. CTLA-4 increases with T cell stimulation and its surface expression on GRIP1^{-/-} T cells remained high after stimulation was removed indicating a possible internalization defect in GRIP1 deficient T cells. CTLA-4 blocking antibody treatment following heart transplantation led to complete rejection in T cell GRIP1^{-/-} mice indicating that increased CTLA-4 surface expression contributed to the extended graft survival. Our data indicates that GRIP1 regulates T cell activation by regulating CTLA-4 surface expression.

Introduction

T cells express glutamate receptors that have been implicated in regulating T cell adhesion and migration (1–3). Glutamate receptors are trafficked, assembled, and recycled by accessory proteins including glutamate receptor interacting protein 1 (GRIP1) (4). GRIP1 regulates the surface expression of glutamate receptor subunits including the α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) type of glutamate receptor GluR-2 (glutamate receptor 2) (5–6), and the kainic acid receptor subunits, GluK1 (glutamate

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Disclosures: The authors of this manuscript have no conflicts of interest to disclose as described by the *American Journal of Transplantation*.

receptor, ionotropic kainate 1) and GluK2 (glutamate receptor, ionotropic kainate 2) (7–9). GRIP1 is a large protein member of the “PDZ” protein family, named for the first three discovered proteins containing these 80–90 amino acid domains (PDZ is an abbreviation for post-synaptic density 95, discs large, and zonula occludens 1). GRIP1 has 7 PDZ domains and interacts with numerous intracellular and membrane proteins such as kinesin (10–11), ephrins (12–13), and liprins (14–17). Many PDZ domain containing proteins and interacting proteins, including GRIP1 and GluR-2 (5–6), associate via a short consensus sequence in the C-terminus of the target protein. However, identifying PDZ target proteins is difficult due to weak binding interactions with PDZ protein scaffolds (18).

While GRIP1 has been most studied in neurons, it is also expressed and functional in other cells. Complete GRIP1 knockout (GRIP1^{-/-}) mice die in utero due to multi-organ deficits and hypovolemia (19). GRIP1 is highly expressed in the nervous system, muscle, and the epidermis (19). Complete GRIP1^{-/-} mice have defects in epidermal-dermal skin patterning similar to Fraser syndrome, a rare human congenital condition that leads to widespread skin blistering in utero in addition to renal, reproductive, and ocular abnormalities (19–22). GRIP1 interacts with Fras1 (Fraser syndrome 1) and Frem1 (Fras1 related extracellular matrix protein) and absence of any of the three causes Fraser syndrome (19–22). Compared with complete loss of GRIP1, neuron-specific GRIP1^{-/-} mice are viable, but have pro-social behaviors and alterations in long-term potentiation (LTP), indicative of altered synapse formation (23). In humans, a gain-of-function GRIP1 mutation has been associated with autism (23). Other point mutations have been associated with schizophrenia (24) and congenital kidney disease (25). These studies demonstrate that GRIP1 may have important functional roles in many cells and tissues throughout the body.

CTLA-4 surface expression is a potent inhibitor of T cell activation and proliferation by preventing T cell CD28 signaling. CTLA-4 on T cells has a much greater affinity (up to 10X) for CD80 and CD86 on antigen presenting cells (APC) than does CD28. CTLA-4^{-/-} mice die due to immune-mediated organ damage from excessive lymphocyte proliferation (26). CTLA4-Ig has been used in autoimmune conditions and transplantation to blunt APC interactions with conventional T cell CD28 (27). CTLA4-Ig therapy significantly prolongs BALB/c to C57BL6/J murine cardiac allograft survival (28) The CTLA4-Ig drug, belatacept, is currently FDA approved as an immune suppressive agent in kidney transplant recipients, but is not a standard therapy (29). In contrast, CTLA-4 blocking antibodies enhance T cell responses and are used as cancer therapies (30). CTLA-4 is present at low levels on the surface of resting T cells, but with cell activation, CTLA-4 is rapidly trafficked to the surface from an intracellular pool. The increase in CTLA-4 is thought to provide an inhibitory balance to counter CD28 activation (31). However, the mechanisms of CTLA-4 trafficking and internalization remain unclear due to conflicting studies regarding protein interactions and phosphorylation activity (31). CTLA-4 has been shown to be internalized through a YVKM motif near the C-terminus. Endocytosis has been proposed to be regulated by AP-2 binding to dephosphorylated tyrosine 201 in the YVKM motif (32–35). However, recent evidence from a knock-in mouse model suggests that mutant dephosphorylated CTLA-4 does not undergo normal endocytosis, resulting in increased CTLA-4 surface expression (36). In general, CTLA-4 protein interactions during resting and following immune stimulation that lead to dynamic CTLA-4 trafficking are not well described.

We have discovered that GRIP1 is present in T cells and has an important role in regulating CTLA-4 trafficking. An understanding of the role for GRIP1 in regulating CTLA-4 surface expression may lead to novel means to modulate T cell responses.

Materials and Methods

Mice

All protocols were approved by the Institutional Animal Care and Use Committee at the University of Rochester School of Medicine. Lck-Cre⁺ mice on C57BL6/J background were purchased from Jackson Labs. GRIP1 floxed mice on C57BL6/J-129 background were generously provided by Dr. Richard Haganir from The Johns Hopkins University School of Medicine. All mice were C57BL6/J-129 background and WT mice were littermate controls. Hearts from male BALB/c mice from Jackson Labs were grafted into male WT and GRIP1^{-/-} recipient mice. Hearts from BM12 mice from Jackson Labs were grafted into male GRIP1^{-/-} recipient mice. Male littermate WT mouse hearts were grafted into WT and GRIP1^{-/-} female mice. Mice were either 6–7 weeks of age or over 11 weeks of age.

Cardiac Allografts

Cardiac allografts were sutured into the abdominal aorta of the recipient mouse as previously described (38). Abdomens were palpated to determine whether transplanted hearts were beating. Absence of an abdominal beating heart signaled transplant rejection and was confirmed upon necropsy. For CTLA-4 blocking experiments, mice were treated with 200 µg of anti-CTLA-4 (BioXCell) antibody or hamster IgG (BioXCell) beginning on the day of transplantation and continued every 3 days until transplant rejection or end of study. Male-to-female, BM12, and BALB/c cardiac allografts were fixed, sectioned, and hematoxylin and eosin (H&E) stained. Rejection scoring of allografts was performed in a single-blind manner by a veterinary pathologist (0–5 with 5 being most severe).

In Vitro T Cell Experiments

Mouse CD3⁺ T cells were isolated from spleens and placed in 2% fetal bovine serum in phosphate-buffered saline before using T cell enrichment kits (R&D Systems or Stem Cell Technology). Isolated T cells were counted using a VetScan HM5 (Abaxis). T cells were seeded in pre-coated anti-CD3 (1 µg/mL) (ebiosciences or BioXCell) containing wells, and anti-CD28 (1 µg/mL, ebioscience or BioXCell) freshly added. Supernatants from T cells in resting conditions or after 48 hour anti-CD3/28 stimulation were assayed by IL-2 ELISA (R&D).

Flow Cytometry

T cells or total spleen cells were incubated with antibody for 15 minutes, fixed, and analyzed on an Accuri C6 (BD) flow cytometer. Intracellular stained samples were permeabilized using buffers and protocols from R&D Systems. Proliferation was detected by total positive CD3⁺ cells after 48 hours or with carboxyfluorescein diacetate succinimidyl ester (CFSE) labeling and decay. Antibodies used included anti-CD69 (ebiosciences; Biolegend), CD3 (ebiosciences), CD25 (Biolegend; ebiosciences), Annexin V (Biolegend), propidium iodide

(Biolegend), CTLA-4 (BD), CD4 (ebiosciences), CD8 (ebiosciences; BD), IL-17 (ebiosciences), IFN γ (R&D), IL-4 (ebiosciences), and FoxP3 (Biolegend).

Immunofluorescence

Jurkat T cells were seeded on 10 mm poly-L-lysine coated coverslips in 3.5 cm wells (MatTek) and permeabilized with methanol, incubated with anti-GRIP1 (BD) and goat anti-mouse Alexa Fluor 488 (Thermo Fisher Scientific), stained with 4',6-diamidino-2-phenylindole (DAPI), fixed with formalin, and coated with Mowiol. Images were taken with an Olympus Confocal microscope at 60X using Fluoview 1000 software (Olympus).

Western Blot

T cell lysates were created with 1X NP-40 lysis buffer. T cells and brain (control) lysates were run on sodium dodecylsulfate polyacrylamide gel electrophoresis, transferred to nitrocellulose, immunoblotted with anti-GRIP1 (BD) and goat anti-mouse Alexa Fluor 750 (Thermo Fisher Scientific), and visualized with a Licor infrared scanner (Licor).

Statistics

All graphs (using standard error of the mean (SEM) for bar and scatter graphs) were created and statistics were analyzed using Graphpad Prism 5.0 (Graphpad). Statistical tests were primarily students T-test as indicated. For survival experiments, Kaplan-Meier curves were used and significance was assessed using log-rank statistical tests. Mann-Whitney test was used to determine significance of graft rejection scoring. $P < 0.05$ or lower was considered statistically significant. Lowercase n indicates numbers per group.

Results

GRIP1 is a multi-PDZ domain scaffolding protein that interacts with a variety of proteins associated with protein trafficking and internalization, and stabilizes surface receptor expression (5–17, 23, 39–42). We have found that GRIP1 is expressed in T cells. Cell lysates were prepared from mouse brain (positive control), mouse T cells, or the Jurkat T cell line and immunoblotted for GRIP1 (Figure 1A). Immunofluorescence staining of Jurkat T cells for GRIP1 further confirmed its expression (GRIP1 green, DAPI blue) (Figure 1B). Complete GRIP1^{-/-} mice are embryonic lethal. By crossing Lck (lymphocyte-specific protein tyrosine kinase)-Cre recombinase mice and GRIP1^{fl/fl} mice, T cell-specific GRIP1^{-/-} mice were generated. An acute cardiac allograft model was used to determine whether immune responses were altered in T cell GRIP1^{-/-} mice. Hearts from BALB/c mice were transplanted into WT and T cell GRIP1^{-/-} recipient mice at either 7 weeks (wks) of age or 11–12 wks of age and rejection monitored by palpating the transplanted heart graft daily. GRIP1^{-/-} mice greater than 11 (11+) wks had greatly prolonged transplant survival compared to the other transplanted groups (Figure 1C). All WT recipients rejected their hearts by about 10 days as anticipated, similar to GRIP1^{-/-} T cell mice at 7 wks of age. However, about 60% of the heart allografts from 11+ wk old T cell GRIP1^{-/-} recipient mice were still beating 24 days after transplantation when the study concluded (Figure 1C).

To begin to assess how a lack of T cell GRIP1 leads to prolonged allograft survival in the 11+ wk old GRIP1^{-/-} T cell recipients, we determined T cell numbers in WT and T cell GRIP1^{-/-} mice. GRIP1^{-/-} mice had significantly fewer circulating CD4⁺ and CD8⁺ T cells as a percentage of circulating leukocytes (Figure 2A). Total splenic T cell numbers after CD3⁺ enrichment and isolation were also significantly less in GRIP1^{-/-} mice (Figure 2B) with an equivalent decrease in CD4⁺ and CD8⁺ T cell populations compared to WT (Figure 2C). While fewer T cells may impact GRIP1^{-/-} transplant rejection, T cell numbers were not likely reduced enough to account for the greatly prolonged rejection responses in 11+ wk old mice. 6–7 wk old GRIP1^{-/-} mice also had a significant reduction in CD4⁺ and CD8⁺ T cell numbers (Figure 2D), but similar transplant rejection times as WT mice.

To determine whether GRIP1^{-/-} T cell activation is decreased in 11+ wk old GRIP1^{-/-} mice compared to WT and 6 wk old GRIP1^{-/-} mice, equivalent numbers of WT and GRIP1^{-/-} T cells were isolated and stimulated *in vitro* using anti-CD3 and anti-CD28 antibody stimulation. 48 hours later activation was assessed by expression of the markers of T cell activation, CD69 and CD25. T cell activation was similar between WT and 6 wk old GRIP1^{-/-} T cells (Figure 3A–3B). However, 11+ wk old GRIP1^{-/-} T cells had significantly decreased percentages of CD69 and CD25 positive cells after activation (Figure 3C–3D). T cell proliferation was also significantly decreased in 11+ wk old GRIP1^{-/-} T cells as shown by reduced decay in CFSE labeling (Figure 3E) and in a separate experiment, by counting fewer cell T cell numbers 48 hours post-stimulation (Figure 3F). GRIP1^{-/-} T cells also had decreased post-stimulation survival as evidenced by more propidium iodide/Annexin V positive T cells compared to WT (Figure 3G). Post-activation, GRIP1^{-/-} T cells produced similar amounts of IL-2 as WT cells (Figure 3H) indicating that a lack of IL-2 secretion does not cause decreased proliferation in GRIP1^{-/-} T cells.

The composition of the basal effector CD4⁺ cell population in the spleens of WT and T cell GRIP1^{-/-} mice was assessed by determining the numbers of Th1, Th2, Th17, and T regulatory (T reg) cells. There was no significant difference in the percentage of Th1 and Th17 populations, but Th2 and T reg cell populations were increased in T cell GRIP1^{-/-} mice (Figure 4A). The T reg population is largely inhibitory for T cell activation and proliferation, but we found that the T regulatory cell population in 6 wk old GRIP1^{-/-} T cells was similarly elevated (data not shown), indicating that increased T reg cells are unlikely to cause the prolonged allograft survival noted in the older mice. The post-transplant effector cell populations in WT and GRIP1^{-/-} mice given BALB/c cardiac grafts were also determined. At 5 days post-transplant, the percentages of Th1 and Th2 cells were similar, while the per cent of Th17 cells was less in T cell GRIP1^{-/-} mice (Figure 4B). Representative images of WT and GRIP1^{-/-} mouse allografts from these mice on day 5 post-transplant demonstrated increased inflammation and necrosis in hearts from WT compared to GRIP1^{-/-} recipients (Figure 4C).

To investigate why *in vivo* and *in vitro* T cell responses were decreased in GRIP1^{-/-} T cells we examined CTLA-4 expression. CTLA-4 is similar to the glutamate receptor subunit GluR-2. Each is found in an internal pool and in the cell membrane. Upon stimulation both GluR-2 and CTLA-4 dynamically traffic into and out of the membrane. GluR-2 trafficking is partly dependent on GRIP1, particularly GluR-2 removal from the membrane and recycling

to the internal pool (39–42). Small changes in CTLA-4 surface expression have potent inhibitory effects on T cell function. CTLA-4 expression increases with age and cell stimulation (43, 44). Because CD4⁺ T cells are required for cardiac transplant rejection (38), CTLA-4 trafficking is most understood in CD4⁺ T cells, and the GRIP1^{-/-} T cell phenotype is consistent with altered CTLA-4 trafficking, we investigated CTLA-4 surface expression on CD4⁺ T cells. CTLA-4 surface expression on 8 wk and 24 wk old WT and GRIP1^{-/-} CD4⁺ cells was simultaneously assessed by flow cytometry. CTLA-4 was greatly increased on 24 wk old GRIP1^{-/-} CD4⁺ cells compared to WT CD4⁺ cells and 8 wk old GRIP1^{-/-} CD4⁺ cells (Figure 5A). To further investigate early aging changes in CTLA-4 expression on GRIP1^{-/-} T cells, CTLA-4 was separately assessed on 6 wk old and 11 wk old WT and GRIP1^{-/-} CD4⁺ cells. 6 wk old mice had similar CTLA-4 expression, but by 11 wks of age CTLA-4 was increased on GRIP1^{-/-} cells (Figure 5B).

In resting T cells CTLA-4 normally has low surface expression with an intracellular pool that is trafficked to the cell membrane within 48 hours of cell stimulation to limit T cell activation responses (31) and CTLA-4 may be internalized and recycled or degraded following T cell stimulation (45). WT and GRIP1^{-/-} T cells (11+ wk old) were stimulated *in vitro* and CTLA-4 expression was measured after activation to determine the activation-induced change in CTLA-4 expression. At 48 hours post-T cell activation CTLA-4 expression was increased on GRIP1^{-/-} T cells compared to WT T cells (Figure 5C). Cells were then removed from the stimulus for an additional 72 hours and CTLA-4 surface expression was again determined. After the additional 72 hours in resting conditions, previously stimulated WT T cell CTLA-4 expression was similar to the resting control, indicating CTLA-4 internalization after stimuli removal. However, GRIP1^{-/-} T cell CTLA-4 surface expression remained significantly elevated (Figure 5D) indicating an internalization defect, an overall steady-state increase in CTLA-4, or continual CTLA-4 exocytosis to the surface. To address whether GRIP1^{-/-} T cells have increased intracellular CTLA-4 (this pool makes up about 90% of the total CTLA-4 (31)), intracellular CTLA-4 was determined at rest (Figure 5E). Internal CTLA-4 expression did not differ between WT and GRIP1^{-/-} T cells. While the role of CTLA-4 on CD8⁺ T cells is less clear than what is known for CD4⁺ T cells, CTLA-4 surface expression was also significantly elevated on the surface of 11+ wk old GRIP1^{-/-} T cells (Figure 5F). These data indicate that GRIP1 has a role in CTLA-4 surface receptor trafficking.

These experiments suggested that increased CTLA-4 expression may lead to prolonged GRIP1^{-/-} transplant survival. To determine whether increased CTLA-4 on GRIP1^{-/-} T cells contributes to decreased rejection, we transplanted BALB/c donor hearts into T cell GRIP1^{-/-} mice. T cell GRIP1^{-/-} transplant recipients were treated with either CTLA blocking antibody (anti-CTLA-4 antibody) or IgG starting the day of transplant to limit CTLA-4 signaling. Treatment was continued every 3 days until loss of transplant heart beat or day 21. GRIP1^{-/-} mice given anti-CTLA-4 antibody had a significantly shortened time to transplant rejection compared to IgG-treated GRIP1^{-/-} mice (Figure 5G). WT mice treated with anti-CTLA-4 antibody or WT mice with IgG rejected their heart transplants at a similar time point as anti-CTLA-4 treated GRIP1^{-/-} mice. These data indicate that increased CTLA-4 expression may significantly contribute to the prolonged allograft rejection in GRIP1^{-/-} mice.

To determine the effect of a lack of GRIP1 on a more chronic heart transplant rejection model female WT and T cell GRIP1^{-/-} mice were given a heart transplant from male donors (46). After 18 days heart grafts were collected to assess rejection responses. Eighty per cent of the WT mice had pathologic evidence of an advanced rejection response, whereas GRIP1^{-/-} recipients had more moderate rejection responses (Figure 6A–6B). CTLA-4 expression was also increased on T cells isolated from the spleen of transplant recipient GRIP1^{-/-} mice (Figure 6C). As further evidence, we also used the BM12 donor model of allograft rejection and harvested allografts 10 days post-transplant. T cell GRIP1^{-/-} recipients had decreased severity of rejection compared to WT recipients (Figure 6D–6E). CTLA-4 expression was also significantly increased on T cells from the spleens of GRIP1^{-/-} transplant recipients (Figure 6F). It is interesting to note that if left to complete rejection (no palpable heart beat) WT and T cell GRIP1^{-/-} mice that received BM12 hearts had comparable allograft survival (data not shown). This may indicate that the effects of GRIP1 regulation of CTLA-4 expression in the BM12 model are most significant in the early T cell responses to the allograft. These data also indicate that GRIP1 has an important role in T cell function in part by regulating CTLA-4 surface expression and T cell responses to transplantation.

Discussion

CD4⁺ T cells are central regulators of acquired immune responses, including transplant rejection. Inhibition or elimination of CD4⁺ T cells in cardiac allograft recipient mice can completely abrogate transplant rejection (38). While the complete absence or a severe decline in CD4⁺ T cell numbers can prevent transplant rejection, mild but significant decreases in the number of CD4⁺ cells, such as seen in the GRIP1^{-/-} mice, do not appear to have an effect on transplant rejection. In our studies younger and older T cell GRIP1^{-/-} mice had similar T cell numbers, but only the older mutant mice demonstrated prolonged allograft survival and increased CTLA-4 surface expression. Even small changes in CTLA-4 expression can result in significant inhibition of CD4⁺ cell responses. Others have shown that murine cardiac allograft rejection is reduced by CTLA4-Ig due to decreased interaction of CD80 and CD86 with the T cell-activating receptor CD28 (27). Increased CTLA-4 also inhibits intracellular inhibitory signaling in addition to its CD80 and CD86 dependent T cell inhibition functions. Increased CTLA-4 surface expression also limits cell activation in response to T cell receptor stimulation by mechanisms that are not well understood (31). This is evidenced by our data showing GRIP1^{-/-} T cells have decreased activation in response to anti-CD3/CD28 stimulation. Because CTLA-4 therapeutics currently in clinical use can have significant side effects, increased knowledge of the molecular mechanisms of CTLA-4 internalization and trafficking may lead to novel strategies to target CTLA-4 to either increase or inhibit its surface expression in a variety of applications.

Our data indicates that T-cell GRIP1^{-/-} mice have significantly fewer post-transplant Th17 cells, and a trend towards fewer Th2 cells compared to WT mice. However, Th1 cells were unchanged between WT and T cell GRIP1^{-/-} mutants. The relative post-transplant decrease in Th17 and Th2 cells, but not Th1 cells, may be further indicative of an important role for GRIP1 in regulating CTLA-4 expression and CD4⁺ responses. Increased CTLA-4 expression has less impact on limiting Th1 responses than it does other T-helper cells.

CTLA-4 engagement inhibits the polarization of naïve CD4⁺ cells towards Th2 cells to a greater extent than it does Th1 differentiation (47,48). Blocking CTLA-4 also potentiates Th17 cell differentiation and increases the susceptibility of mice to experimental autoimmune myocarditis (49). Th17 memory cells express higher levels of CTLA-4 compared to Th1, and *ex vivo* stimulation of these Th17 cells in the presence of inhibitors of CTLA-4 interactions augmented Th17 responses more than it did Th1 responses (50,51). These studies are consistent with our data that increased CTLA-4 in GRIP1^{-/-} CD4⁺ cells resulted in fewer post-transplant Th17 and Th2 cells, with no change in Th1 cells.

The mechanisms of CTLA-4 exocytosis, internalization, recycling and degradation during rest and after T cell receptor-induced activation are not well understood (31). While the YVKM motif in the C-terminal end of CTLA-4 appears to be needed for internalization, it remains unclear what other binding partners besides AP-2 and clathrin machinery participate in CTLA-4 endocytosis (31). One mouse model has reported increased surface CTLA-4 expression in a knock in dephosphorylated mutant of the YVKM motif. This mutation was originally hypothesized to induce internalization and less CTLA-4 surface expression as tyrosine phosphorylation was thought to stabilize CTLA-4 surface expression (31, 36). This indicates that additional unknown mechanisms contribute to the regulation of CTLA-4 trafficking. Our results indicate that CTLA-4 interacts either directly or indirectly with GRIP1. This may be via a direct PDZ domain interaction or through GRIP1-mediated interactions between CTLA-4 and other regulatory proteins. GRIP1 regulates GluR-2 trafficking, in part by recruiting and localizing phosphatase and adenosine triphosphatase proteins (39). GRIP1 may also provide a scaffold to recruit and localize other proteins to allow for CTLA-4 internalization or recycling in an indirect manner. GRIP1 is required to internalize and recycle GluR-2 in endosomes after N-methyl-D-aspartate (NMDA)-induced activation in neurons (39–42). CTLA-4 is also reported to be recycled through an endosome-dependent pathway and degraded by an alternate pathway (44). GRIP1 may mediate endosomal trafficking of CTLA-4 through similar mechanisms. More work must be done to determine the nature of GRIP1 interactions with CTLA-4 and other trafficking-related proteins.

These findings suggest that GRIP1 has similar functions in T cells and neurons; GRIP1 has a central role in surface receptor internalization and trafficking. Loss of GRIP1 *in vitro* led to decreased T cell activation and proliferation. Using murine cardiac allograft models GRIP1^{-/-} mice had greatly prolonged transplant survival in an acute rejection model. We have discovered that GRIP1 regulates CTLA-4 surface expression in T cells and that GRIP1 may be a crucial interacting protein that modulates inhibition of T cell function.

Acknowledgments

Funding via American Heart Association (13EIA14250023) and National Institutes of Health (R01HL124018) to CNM.

Non-Standard Abbreviations

GRIP1 glutamate receptor interacting protein

PDZ	Post-synaptic density 95, discs large, and zonula occludens 1
CTLA-4	cytotoxic T-lymphocyte antigen-4
AMPA	α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
GluR-2	glutamate receptor 2
Fras1	Fraser syndrome 1
Frem1	Fras1 related extracellular matrix protein
GluK1	glutamate receptor, ionotropic kainate 1
GluK2	glutamate receptor, ionotropic kainate 2
LTP	long-term potentiation
CFSE	carboxyfluorescein diacetate succinimidyl ester
DAPI	4',6-diamidino-2-phenylindole
SEM	standard error of the mean
<i>n</i>	number in group
NS	not significant
H&E	hematoxylin and eosin
kDa	kiloDaltons
RBC	red blood cells
MFI	geometric mean fluorescence intensity
FC	flow cytometry
CBC	Coulter blood count
NMDA	N-methyl-D-aspartate
PI	propidium iodide
Lck	lymphocyte-specific protein tyrosine kinase
IL-17	interleukin-17
IL-4	interleukin-4
FoxP3	forkhead box P3
IFNγ	interferon gamma
IL-2	interleukin-2

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A. **B.**

Figure 1.

T cells express GRIP1. A) Western blot of GRIP1 with CD3⁺ mouse T cells and Jurkat T cells. Brain as positive control. All samples run on same blot, unrelated samples removed as marked by black line. Size is marked by kilo Daltons (kDa). B) Immunofluorescence of GRIP1 (green) in Jurkat T cells (DAPI in blue). Images at 60X (scale bar = 10 μ M). C) WT and T cell GRIP1^{-/-} mice either 7 wk or 11 wk of age (B6/129 background) were given heart transplants from MHC mismatched BALB/c mice. Transplants were palpated for rejection up to 24 days post transplantation (n=3–6 per group, *p=0.0148 by log rank test between 11 week old WT and GRIP1^{-/-} mice).

Figure 2. GRIP1^{-/-} mice have fewer circulating and spleen derived T cells. A) Peripheral blood was collected, red blood cells (RBC) lysed, and cells analyzed by flow cytometry (FC) for percentages of CD4⁺ and CD8⁺ cells (n=3–4 per group, ***p<0.001, *p=0.026 by students T-test). B) Total T cells from 11+ wk old mice measured by complete blood count (CBC) after CD3⁺ T cell isolation (n=11 per group, *p=0.011). C-D) Total T cells isolated from spleens of C) 11 wk old and D) 6 wk old mice analyzed for percentages of CD4⁺ and CD8⁺ T cells by flow cytometry C) n=4–5 per group, **p=0.004, ***p<0.001 by students T-test; D) n=3–4 per group, ***p<0.001, *p=0.015 by students T-test).

E. **F.**

Figure 3. GRIP1^{-/-} T cells have reduced activation and proliferation. A–B) Percentage of A) CD69 and B) CD25 positive WT and GRIP1^{-/-} T cells from mice at 6 wk of age in control or anti-CD3/28 activating conditions (A) n=3 per group, p=0.974, *p=0.038 by students T-test; B)

n=4 per group, $p=0.658$, $**p=0.004$ by students T-test). C-D) Percentage of C) CD69 and D) CD25 positive WT and GRIP1^{-/-} T cells from mice 11+ wk of age in control or anti-CD3/28 activating conditions (C) n=4 per group, $p=0.658$, $**p=0.004$ by students T-test); n=3 per group, $p=0.974$, $*p=0.038$ by students T-test. E) T cell CFSE dilution 72 hours after anti-CD3/28 activation (11+ wk old mice). Histograms and bar graph show percentage proliferation (n=2–4 per group, $p=0.125$, $***p<0.001$ by students T-test). F) Number of WT or GRIP1^{-/-} T cells after 48 hours with or without anti-CD3/28 stimulation (n=7–9 per group, $p=0.890$, $*p=0.029$ by students T-test). G) Percentage of WT and GRIP1^{-/-} T cells negative for propidium iodide/Annexin V after 48 hours with or without anti-CD3/28 activation (n=4 per group, $*p=0.032$, $***p<0.001$ by students T-test). H) IL-2 release from resting and 48 hour activated CD3/28 T cells from 11+ wk old WT and GRIP1^{-/-} mice (n=7–9 per group, $p=0.0973$, $p=0.436$) by students T-test).

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C.**WT****GRIP1^{-/-}** **4X** **20X****Figure 4.**

Immunophenotype of GRIP1^{-/-} and WT T cells. A) Percentage of T helper cells from freshly isolated spleen cells of WT and T cell GRIP1^{-/-} mice (IFN γ p=0.744, IL-4 *p=0.038, IL-17 p=0.077, Foxp3 *p=0.026, by students T-test). B) Percentage of T helper cells from 11+ wk old WT and GRIP1^{-/-} mice 5 days after BALB/c cardiac allograft (students T-test for IFN γ p=0.230, IL-4 p=0.155, and IL-17 *p=0.022). C) Representative images of BALB/c hearts 5 days post-transplant (at 4X and 20X).

B. **C.**

Figure 5.

GRIP1 regulates CTLA-4 surface expression. A) CTLA-4 surface expression is greatly increased on CD4⁺ cells from 24 wk old GRIP1^{-/-} mice compared to WT and 8 wk old GRIP1^{-/-} mice (mean fluorescence intensity (MFI), n=3–4 per group, *p<0.01 by students T-test). B) CTLA-4 on CD4⁺ T cells from younger and older WT and GRIP1^{-/-} mice (MFI, n=7–9 per group, ***p<0.001 by students T-test). C) CTLA-4 expression on CD4⁺ T cells

from WT and GRIP1^{-/-} mice after 48 hours with or without anti-CD3/28 T cell stimulation (n=7–9 per group, p=0.084, ***p<0.001 by students T-test). D) CTLA-4 expression on CD4⁺ T cells from WT and GRIP1^{-/-} mice an additional 72 hours after removal from 48 hrs of anti-CD3/28 T cell stimulation (n=7–9 per group, p=0.814, *p=0.015 by students T-test). E) Internal CTLA-4 expression of CD4⁺ T cells from WT and GRIP1^{-/-} mice (n=7–9 per group, p=0.187 by students T-test). F) CTLA-4 expression on CD8⁺ T cells from WT and T cell GRIP1^{-/-} mice (n=7–9 per group, **p=0.004 by students T-test). G) 11+ wk old WT and GRIP1^{-/-} mice given BALB/c heart grafts and administered either anti-CTLA4 antibody or isotype control IgG and heart rejection monitored (n=3–4 per group, *p=0.025 by log-rank test between GRIP1^{-/-} IgG and GRIP1^{-/-} anti-CTLA-4).

B.

Score = 1

Score = 5

E.**4X****20X****WT** **GRIP1^{-/-}**

F.

Figure 6. GRIP1^{-/-} graft recipients in chronic rejection models have reduced graft inflammation. A) Rejection scores from female 11+ wk old WT and T cell GRIP1^{-/-} male heart transplant recipients on day 18 post-transplant (n=5 per group, p=0.105 by Mann-Whitney test). B) Representative images of low and high rejection scores. (1 = no rejection, 5 = complete rejection). Scale bar = 1 mm. C) CTLA-4 expression on CD4⁺ T cells from WT and GRIP1^{-/-} transplant recipients 18 days post-transplant (n=4–5 per group, p=0.078 by students T-test). D) Rejection scores from WT and T cell GRIP1^{-/-} BM12 heart transplant recipients 10 days post-transplant (n=4–5 per group, p=0.098 by Mann-Whitney test). E) Representative images of allografts from 11+ wk old BM12 donors 10 days post-transplant (4X and 20X). F) GRIP1^{-/-} CD4⁺ cells have increased CTLA-4 expression 10 days after BM12 heart transplant compared to WT CD4⁺ cells (n=3–4, **p<0.001 by students T-test).