HHS Public Access
Author manuscript
J Immunol. Author manuscript; available in PMC 2024 September 15.

Published in final edited form as:
J Immunol. 2023 September 15; 211(6): 917–922. doi:10.4049/jimmunol.2300138.

Induced loss of Rasgrp1 in peripheral CD4+ T cells of conditional Rasgrp1-deficient mice reveals an essential role for Rasgrp1 in TCR/CD28-induced Ras-MAPK signaling

Yating Chang[1], **Praveen Manivannan**[1], **Abbas Doosti**[1], **Philip E. Lapinski**[1], **Di Chen**[1], **Jeroen Roose**[2], **Philip D. King**[1,2]

[1]Department of Microbiology and Immunology, University of Michigan Medical School, Ann Arbor, Michigan, USA

[2]Department of Anatomy, University of California, San Francisco, USA

Abstract

Rasgrp1 is a Ras guanine nucleotide exchange factor that participates in the activation of the Ras-ERK signaling pathway in developing T cells and is required for efficient thymic T cell positive selection. However, the role of Rasgrp1 in mature peripheral T cells has not been definitively addressed, in part because peripheral T cells from constitutive Rasgrp1-deficient mice show an abnormal activated phenotype. Here, we generated an inducible Rasgrp1-deficient mouse model to allow acute disruption of Rasgrp1 in peripheral CD4+ T cells in the context of normal T cell development. TCR/CD28-mediated activation of Ras-ERK signaling was blocked in Rasgrp1-deficient peripheral CD4+ T cells. Furthermore, Rasgrp1-deficient CD4+ T cells were unable to synthesize IL-2 and the high affinity IL-2R and were unable to proliferate in response to TCR/CD28 stimulation. These findings highlight an essential function for Rasgrp1 for TCR/CD28-induced Ras-ERK activation in peripheral CD4+ T cells.

Introduction

Ras is a small GTP-binding protein tethered to the inner leaflet of plasma membrane that cycles between inactive GDP-bound and active GTP-bound states (1). In its active state, Ras triggers the MAPK signaling cascade that culminates in the activation of ERK protein kinases (2). Active ERKs translocate to the cell nucleus where they phosphorylate and activate transcription factors that drive different cell responses such as proliferation and differentiation. Activation of Ras is mediated by Ras guanine nucleotide exchange factors (RasGEFs) that upon localization to the cell membrane displace GDP from the Ras guanine nucleotide binding pocket, thereby permitting Ras to bind free GTP (3). In T cells, two important RasGEFs are mammalian son of sevenless (Sos1)[3] and Ras guanine nucleotide releasing protein 1 (Rasgrp1) that participate in the activation of Ras downstream of the

Corresponding author: Philip D. King, Ph.D. Department of Microbiology and Immunology, University of Michigan Medical School, 6606 Med Sci II, 1150 West Medical Center Drive, Ann Arbor, MI 48109-5620, Tel: 734-929-9713, Fax: 734-764-3562, kingp@umich.edu.

Disclosure
The authors have no financial conflicts of interest.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

TCR (4, 5). Both RasGEFs are recruited to the T cell plasma membrane as a result of TCR-mediated phosphorylation of the transmembrane adapter protein, linker of activated T cells (LAT). Sos1 complexes with the Grb2 adapter protein that binds phosphorylated LAT leading to indirect membrane recruitment (6). Rasgrp1 is recruited to the plasma membrane through recognition of diacyl glycerol (DAG) that is generated as a result of phospholipase C-gamma binding to phosphorylated LAT (4, 5, 7). Studies performed in the Jurkat T leukemia cell line together with computational modeling indicate that Sos1 is able to deliver strong digital Ras-ERK signals in response to TCR stimulation, whereas Rasgrp1 delivers more modest analog Ras-ERK signals in response to the same stimulus (8, 9). However, Rasgrp1-generated RasGTP efficiently induces a RasGTP-SOS positive feedback loop that ensures high-level digital Ras-ERK signaling in Jurkat cells (8).

Analyses of T cell-specific Sos1 and constitutive Rasgrp1-deficient mice have confirmed important roles for Sos1 and Rasgrp1 in T cell development (4, 5). Loss of Sos1 in the T cell lineage results in impaired pre-TCR signaling at the TCR-beta selection checkpoint whereas later thymocyte positive selection is unaffected (10). In contrast, loss of Rasgrp1 has a major impact upon T cell positive selection associated with reduced TCR-induced Ras-ERK activation in CD4+CD8+ double positive (DP) thymocytes (11, 12). In Sos1 and Rasgrp1 double-deficient mice, transition through the TCR beta selection checkpoint is completely blocked showing that Rasgrp1 contributes at this stage of T cell development also (13). In addition, Sos1 and Rasgrp1 appear to cooperate to mediate T cell negative selection in the thymus. Thus, whereas loss of either RasGEF alone does not affect negative selection, loss of both Sos1 and Rasgrp1 impairs negative selection (13).

The role of Sos1 and Rasgrp1 in TCR plus CD28 costimulatory receptor-induced Ras-ERK activation in primary peripheral T cells is less clear. Peripheral T cells from Rasgrp1-deficient mice showed normal TCR/CD28-induced expression of the high affinity receptor for the IL-2 T cell growth factor and only partially reduced proliferation that was evident at later but not early time points after stimulation (12). Likewise, peripheral T cells from Rasgrp1-deficient humans show only partial impairment of TCR/CD28-induced proliferation (particularly CD4+ T cells) that is associated with only partially reduced Ras-ERK signaling (14). However, results obtained with peripheral T cells from constitutive Rasgrp1-deficient mice and humans should be interpreted with caution since they exhibit an abnormal activated and exhausted phenotype that in the case of mice at least is a consequence of peripheral homeostatic proliferation in a chronically immunocompromised host (14, 15).

Some studies have examined the effect of knockdown of Sos1 or Rasgrp1 in human peripheral T cells. Knockdown of Sos1 has a modest or no effect upon TCR/CD28-induced Ras-ERK activation, IL-2 synthesis, and proliferation (9, 16, 17). Knockdown of Rasgrp1 more consistently reduces TCR/CD28-induced Ras-ERK activation, IL-2 synthesis, and proliferation but only partially (9, 16, 17). In one study, reduced ERK activation upon Rasgrp1 knockdown was completely restored upon additional knockdown of Sos1 (17). Complicating interpretation of these knockdown studies was that the targeted RasGEFs were often reduced in abundance rather than ablated.

Author Manuscript

In the current study, we sought to clarify the role of Rasgrp1 in Ras-ERK activation in mature peripheral T cells. For this purpose, we generated a Rasgrp1 conditional deficient mouse strain to permit inducible acute disruption of Rasgrp1 in peripheral CD4+ T cells. Analyses of CD4+ T cells from these mice revealed an essential function for Rasgrp1 in TCR/CD28-induced Ras-ERK activation and progression through the cell cycle.

Materials and Methods

Rasgrp1 conditional mice

A conditional floxed *Rasgrp1* allele was generated by CRISPR/Cas9 targeting in Agouti C57BL/6N JM8.F6 ES cells (18). Briefly, a Cas9 cut was introduced into exon 2 (guide RNA 5'-GATCACCCAGTTCCGAATGA-3') and homology directed repair was achieved with a DNA donor containing a floxed exon 2 cassette with 1.1 kb 5' and 0.7 kb 3' homology arms plus a FRT-flanked Neo^R cassette inserted 3' of the 3' *loxP* site. After G418 selection, correctly targeted clones were injected into blastocysts to generate chimeras that were subsequently bred with $ACTB^{Flpe}$ mice (The Jackson Laboratory, strain 005703) to delete the Neo^R cassette. Neo^R-deleted progeny were then crossed with $Cd4^{ert2cre}$ mice (The Jackson Laboratory, strain 022356) to generate $Rasgrp1^{fl/fl}$ $Cd4^{ert2cre}$ and littermate $Rasgrp1^{fl/fl}$ controls (19). Mice of both sexes were used in experiments. All mice were on a predominantly C57BL/6J background. To disrupt *Rasgrp1* in CD4+ T cells, 7-week-old mice were given 3 i.p. injections of tamoxifen (TM; Sigma; 0.05 mg/g body weight per injection, dissolved in corn oil) on consecutive days. Mice were euthanized 7 days later and T cell function was analyzed. Cre-mediated removal of exon 2 is expected to result in a *Rasgrp1* mRNA that contains 12 codons from exon 1 followed by 8 nonsense codons and a premature stop codon leading to nonsense mediated RNA decay (20). Exon 2 was targeted since it is the most 5' exon that following its excision would generate a stop codon. All experiments were performed in compliance with University of Michigan guidelines and were approved by the University Committee on the Use and Care of Animals.

Flow cytometry

Single cell suspensions from thymus and spleen, were stained with fluorochrome-labeled CD4 (GK1.5), CD8α (53–6.7), CD69 (H1.2F3), TCRβ (H57–597), CD44 (IM7), CD62L (MEL-14), and CD25 (PC61) mAb (all BD Pharmingen). Cell staining was analyzed by flow cytometry on a FACSCanto (BD Biosciences).

Rasgrp1 expression

CD4+ T cells were isolated from spleens by negative selection (Miltenyi Biotech). *Rasgrp1* gene copy number was determined by qPCR of genomic DNA in a 7500 Fast PCR machine (Applied Biosystems) using Taqman primer/probe sets that detect intact *Rasgrp1* exon 2. Forward Primer 5'ATCAACCAACAGCCCTCTCC3'; Reverse Primer 5'AAGCGCTACAGACTGTCTCC3'; Internal probe 5'GAGCCAGCCTGGATGATCTT3'. The transferrin receptor gene (*Tfrc*) was used as the standard reference for copy number analysis (Applied Biosystems). Total amounts of Rasgrp1(M-199, Santa Cruz) and actin (4967, Cell Signaling Technology) protein were determined by Western blotting.

Author Manuscript

T cell activation

Purified CD4+ T cells were activated by incubation with PMA (30 ng/ml, Sigma) or with immobilized CD3 mAb (1 μg/ml, 145–2C11, Invitrogen) plus soluble CD28 mAb (1 μg/ml, clone 37.51, Invitrogen) in complete RPMI medium (RPMI 1640 medium with 10% FBS). ERK and mTORC1 activation was determined by Western blotting using p-ERK and p-S6 antibodies respectively (9101 and 2211, Cell Signaling Technology). Total amounts of ERK and S6 were determined by probing for the respective proteins using specific antibodies (4695 and 2317, Cell Signaling Technology). Expression of activation markers was determined by flow cytometry. IL-2 synthesis was determined by ELISA of culture supernatants (Invitrogen). For proliferation experiments, CD4+ T cells were labelled with CellTrace™ Violet (Invitrogen) before CD3/CD28 mAb stimulation. To assess apoptosis, cells from proliferation cultures were stained with Annexin V (Biolegend). In some experiments, IL-2 (3000 pg/ml; R&D Systems) was added to wells. Cell proliferation and apoptosis was determined by flow cytometry. To assess ERK activation and mTORC1 activation by flow cytometry, whole splenocytes were stimulated with PMA (30 ng/ml; Sigma) or soluble CD3 and CD28 mAb (both 1 μg/ml) in complete RPMI medium. Flow cytometry was performed as described using phospho-specific antibodies (197G2 and D57 2.2E, Cell Signaling) (21).

Cell cycle analysis

To determine entry of CD4+ T cells into and progression through the G1 stage of the cell cycle, whole splenocytes were stimulated CD3 and CD28 mAb (both 1 μg/ml) in complete RPMI. RNA content of CD4+ T cells was determined by incubation with Pyronin Y (Sigma) and flow cytometry.

Macropinocytosis

For macropinocytosis assessment, whole splenocytes were stimulated with CD3 and CD28 mAb in complete medium for 4 or 12 hours. BSA-Alexa488 (Thermo Fisher, 0.4 mg/ml) was added to wells for the last 4 hours of culture before assessment of probe uptake by flow cytometry.

Statistics

Statistical significance was determined using Student's 1-sample and 2-sample t-tests. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$; ****; $P<0.0001$

Results and Discussion

To assess the role of Rasgrp1 in peripheral CD4+ T cells, we administered TM to *Rasgrp1*$^{fl/fl}$ *Cd4*^{ert2cre} and littermate *Rasgrp1*$^{fl/fl}$ control mice at 7 wk of age and analyzed mice 1 week later. Flow cytometric analyses of thymocytes did not reveal any statistically significant differences in the total numbers of $CD4^{-}CD8^{-}$ double-negative, $CD4^{+}CD8^{+}$ double-positive (DP), or $CD4^{+}$ or $CD8^{+}$ single-positive (SP) thymocytes at this time point (Supplemental Fig. 1A,B). However, the ratio of post-positive selection DP thymocytes ($CD69^{+}TCR\beta^{int}$) to pre-positive selection DP thymocytes ($CD69^{-}TCR\beta^{lo}$) was reduced in *Rasgrp1*$^{fl/fl}$ *Cd4*^{ert2cre} mice as was the total number of $CD69^{+}$ $CD4^{+}$ and $CD69^{+}CD8^{+}$ SP

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

thymocytes that are the immediate descendants of post-positive selection DP thymocytes (Supplemental Fig. 1B). These findings are consistent with activity of this Cre driver at the DP stage of T cell development and a role for Rasgrp1 in positive selection as previously reported (11, 12, 19). Nonetheless, no statistically significant differences in the total numbers of peripheral $CD4^+$ or $CD8^+$ naïve ($CD44^{lo}CD62L^{hi}$), central memory ($CD44^{hi}CD62L^{hi}$), or effector memory ($CD44^{hi}CD62L^{lo}$) T cells were apparent between *Rasgrp1$^{fl/fl}$ Cd4^{ert2cre}* and *Rasgrp1$^{fl/fl}$* mice 1 wk after TM administration (Supplemental Fig. 1C,D). In addition, peripheral CD4+ T cells from *Rasgrp1$^{fl/fl}$ Cd4^{ert2cre}* mice did not express the CD69 activation marker and expression of the CD25 IL-2R alpha chain on a subset of CD4+ T cells was comparable with that observed in *Rasgrp1$^{fl/fl}$* mice, and consistent with their identity as T regulatory cells (Supplemental Fig. 1D). In contrast, peripheral T cells from constitutive Rasgrp1-deficient mice display an activated $CD44^{hi}CD62L^{lo}$CD69+ phenotype that develops as a result of peripheral T cell lymphopenia and subsequent homeostatic proliferation (15). These findings, therefore, indicate that the vast majority of peripheral T cells in *Rasgrp1$^{fl/fl}$ Cd4^{ert2cre}* mice treated with TM 1 wk previously are phenotypically normal and are likely derived from Rasgrp1-expressing thymocytes that have undergone normal T cell development, i.e. prior to the time of TM administration.

To determine the efficiency of *Rasgrp1* gene disruption in TM-treated *Rasgrp1$^{fl/fl}$ Cd4^{ert2cre}* peripheral CD4+ T cells, splenic CD4+ T cells were purified from mice and TM-treated *Rasgrp1$^{fl/fl}$* controls and relative intact *Rasgrp1* gene copy number was determined by qPCR. These analyses showed that gene disruption in *Rasgrp1$^{fl/fl}$ Cd4^{ert2cre}* peripheral CD4+ T cells was efficient and consistent (mean 16.3, SEM 3.7 percent of *Rasgrp1* intact gene copy number compared to *Rasgrp1$^{fl/fl}$* controls) (Fig. 1A). Consistent with this, CD4+T cells from TM-treated *Rasgrp1$^{fl/fl}$ Cd4^{ert2cre}* mice showed much reduced amounts of Rasgrp1 protein by Western blotting (mean 11.6, SEM 4.7 percent of Rasgrp1 protein amount of *Rasgrp1$^{fl/fl}$* controls) (Fig. 1B, 1C).

To confirm functional deletion of Rasgrp1 in peripheral CD4+ T cells, we examined ERK activation in response to PMA stimulation. PMA is a DAG analog that activates the Ras-ERK pathway in a Rasgrp1-dependent manner. As shown by Western blotting and flow cytometry using anti-active phospho-specific ERK antibodies, PMA induced minimal activation of ERK in CD4+ T cells from TM-treated *Rasgrp1$^{fl/fl}$ Cd4^{ert2cre}* mice compared to TM-treated *Rasgrp1$^{fl/fl}$* controls (Fig. 1D–F). In contrast, PMA-induced activation of ERK was normal in CD8+ T cells of these mice (Fig. 1E, 1F).

We next examined ERK activation in response to stimulation with anti-CD3 (against the TCR complex) and anti-CD28 mAb. As shown by both Western blotting and by flow cytometry, CD3/CD28 mAb-induced ERK activation was much reduced in CD4+ T cells, but not CD8+ T cells, from TM-treated *Rasgrp1$^{fl/fl}$ Cd4^{ert2cre}* mice Fig. (2A–C). In contrast, activation of the mechanistic target of rapamycin (mTORC1) signaling pathway was intact in CD4+ T cells from TM-treated *Rasgrp1$^{fl/fl}$ Cd4^{ert2cre}* mice as detected using anti-phospho-S6 antibodies (Fig. 2D–F) (22).

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

We next examined the ability of *Rasgrp1$^{fl/fl}$ Cd4^{ert2cre}* peripheral CD4+ T cells to enter into the cell cycle and proliferate in response to CD3/CD28 stimulation. Murine peripheral CD4+ T cells simulated with CD3/CD28 mAb *in vitro* synchronously enter the G1 phase of the cell cycle 12 h after stimulation (21, 23). Thereafter, T cells asynchronously progress through G1 with the first cells entering S phase at 20 h (21, 23). Entry into G1 can be detected by staining with Pyronin Y that binds RNA that increases in content as cells enter G1, whereas progression through G1 can be determined by an increase in cell size (21). Pyronin Y staining performed 12 h after CD3/CD28 stimulation showed that peripheral CD4+ T cells from TM-treated *Rasgrp1$^{fl/fl}$ Cd4^{ert2cre}* mice were able to enter G1 (Fig. 3A). However, in comparison to control CD4+ T cells, Rasgrp1-deleted T cells showed less of an increase in cell size between 12 and 18 h post-stimulation, as determined by flow cytometry (Fig. 3B). These findings indicate that Rasgrp1-deficient T cells are impaired in their ability to progress through G1.

To examine proliferation, purified CD4+ T cells were labelled with CellTrace™ Violet (CTV) and were stimulated with CD3/CD28 antibodies for 3 d before determination of cell proliferation by dilution of CTV fluorescence (Fig. 3C,D). These experiments revealed that peripheral CD4+ T cells from TM treated *Rasgrp1$^{fl/fl}$ Cd4^{ert2cre}* mice were severely impaired in their ability to proliferate with few T cells having entered into division at this time point. Co-staining of CD4$^+$ T cells with the Annexin V marker showed that of the small number of *Rasgrp1$^{fl/fl}$ Cd4-ert2cre* CD4$^+$ T cells that were able to divide that they were more susceptible to apoptosis (Fig. 3C,D).

TCR/CD28-induced proliferation of CD4+ T cells is driven by T cell-synthesized IL-2 that binds the high affinity IL-2R present on the T cell surface. The high affinity IL-2R comprises of the IL-2R beta and gamma chains together with the CD25 alpha chain, the last of which is not expressed on the surface of resting non-T regulatory cells but is induced upon T cell activation. Therefore, to understand the basis of an inability of *Rasgrp1$^{fl/fl}$ Cd4^{ert2cre}* CD4$^+$ T cells to proliferate, we examined their ability to synthesize and secrete IL-2 and to express CD25 in response to CD3/CD28 stimulation. *Rasgrp1$^{fl/fl}$ Cd4^{ert2cre}* CD4$^+$ T cells secreted much reduced amounts of IL-2 compared to control *Rasgrp1$^{fl/fl.}$* T cells and essentially failed to express increased amounts of CD25 on their cell surface (Fig. 4A–C). Therefore, deficient production of both IL-2 and the high affinity IL-2R accounts for the blocked proliferative response. Consistent with this, exogenous IL-2 could not rescue the proliferation of *Rasgrp1$^{fl/fl}$ Cd4^{ert2cre}* CD4$^+$ T cells when added to wells at the peak concentration present in supernatants of control CD4+ T cells, i.e. 3 ng/ml at 48 h (Fig. 4A,D,E).

We also examined expression of the CD69 activation marker in *Rasgrp1$^{fl/fl}$ Cd4^{ert2cre}* CD4$^+$ T cells in response to CD3/CD28 antibody stimulation (Fig. 4B, 4C). Surprisingly, a similar percentage of *Rasgrp1$^{fl/fl}$ Cd4^{ert2cre}* and *Rasgrp1$^{fl/fl}$* CD4$^+$ T cells were induced to express CD69, although the level of expression of CD69 among CD69$^+$ *Rasgrp1$^{fl/fl}$ Cd4^{ert2cre}* CD4$^+$ T cells was significantly lower than that in CD69$^+$ *Rasgrp1$^{fl/fl}$* CD4$^+$ T cells. These latter findings indicate that a component of CD69 expression is independent of Rasgrp1 and ERK activation.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Last, we examined an ability of TM-treated *Rasgrp1$^{fl/fl}$ Cd4^{ert2cre}* CD4$^+$ T cells to engage in a large-scale form of endocytosis known as macropinocytosis. For this purpose, we tested the ability of CD3/CD28-stimulated CD4+ T cells to take up fluorochrome-labeled BSA that is internalized into T cells by macropinocytosis (21). T cell macropinocytosis during the G0 and G1 stages of the cell cycle does not require Ras activation (21). Therefore, we reasoned that CD4+ T cell macropinocytosis during G0 should not be affected by loss of Rasgrp1. Indeed, CD4+ T cells from *Rasgrp1$^{fl/fl}$ Cd4^{ert2cre}* mice acquired similar amounts of BSA as CD4$^+$ T cells from *Rasgrp1$^{fl/fl}$* mice when exposed to probe for the first 4 h and between 8 and 12 h post CD3/CD28 stimulation (Fig. 4F).

In summary, in the current study we use an inducible CD4+ T cell-specific Rasgrp-1 deficient mouse model to demonstrate a critical function for Rasgrp1 in the activation of the Ras-ERK pathway in peripheral CD4+ T cells. In the absence of Rasgrp1, CD4+ T cells show severely impaired progression through the cell cycle and proliferation in response to CD3/CD28 stimulation that can be accounted for by much reduced IL-2 secretion and cell surface expression of the high afinity IL-2R. These findings clarify long-standing unresolved questions concerning the mechanism by which Ras is activated in peripheral CD4+ T cells downstream of the TCR and CD28.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We acknowledge the Transgenic Animal Model Core of the University of Michigan's Biomedical Research Core Facilities for assistance with the design and production of the Rasgrp1 floxed mice.

This work was supported by National Institutes of Health Grant RO1 R01 AI165381 (P.D.K.) and P01 AI091580 (J.P.R.)

Abbreviations used in this article:

DP CD4+CD8+ double positive thymocytes

LAT linker of activated T cells

Sos1 mammalian son of sevenless

Rasgrp1 Ras guanine nucleotide releasing protein 1

SP CD4$^+$ or CD8$^+$ single-positive thymocytes

TM tamoxifen

References

1. Buday L, and Downward J. 2008. Many faces of Ras activation. Biochim Biophys Acta 1786: 178–187. [PubMed: 18541156]
2. Chang L, and Karin M. 2001. Mammalian MAP kinase signalling cascades. Nature 410: 37–40. [PubMed: 11242034]

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

3. Bos JL, Rehmann H, and Wittinghofer A. 2007. GEFs and GAPs: critical elements in the control of small G proteins. Cell 129: 865–877. [PubMed: 17540168]
4. Kortum RL, Rouquette-Jazdanian AK, and Samelson LE 2013. Ras and extracellular signal-regulated kinase signaling in thymocytes and T cells. Trends Immunol 34: 259–268. [PubMed: 23506953]
5. Lapinski PE, and King PD 2012. Regulation of Ras signal transduction during T cell development and activation. Am J Clin Exp Immunol 1: 147–153. [PubMed: 23205323]
6. Jun JE, Rubio I, and Roose JP 2013. Regulation of ras exchange factors and cellular localization of ras activation by lipid messengers in T cells. Front Immunol 4: 239. [PubMed: 24027568]
7. Roose JP, Mollenauer M, Gupta VA, Stone J, and Weiss A. 2005. A diacylglycerol-protein kinase C-RasGRP1 pathway directs Ras activation upon antigen receptor stimulation of T cells. Mol Cell Biol 25: 4426–4441. [PubMed: 15899849]
8. Das J, Ho M, Zikherman J, Govern C, Yang M, Weiss A, Chakraborty AK, and Roose JP 2009. Digital signaling and hysteresis characterize ras activation in lymphoid cells. Cell 136: 337–351. [PubMed: 19167334]
9. Roose JP, Mollenauer M, Ho M, Kurosaki T, and Weiss A. 2007. Unusual interplay of two types of Ras activators, RasGRP and SOS, establishes sensitive and robust Ras activation in lymphocytes. Mol Cell Biol 27: 2732–2745. [PubMed: 17283063]
10. Kortum RL, Sommers CL, Alexander CP, Pinski JM, Li W, Grinberg A, Lee J, Love PE, and Samelson LE 2011. Targeted Sos1 deletion reveals its critical role in early T-cell development. Proc Natl Acad Sci U S A 108: 12407–12412. [PubMed: 21746917]
11. Dower NA, Stang SL, Bottorff DA, Ebinu JO, Dickie P, Ostergaard HL, and Stone JC 2000. RasGRP is essential for mouse thymocyte differentiation and TCR signaling. Nat Immunol 1: 317–321. [PubMed: 11017103]
12. Priatel JJ, Teh SJ, Dower NA, Stone JC, and Teh HS 2002. RasGRP1 transduces low-grade TCR signals which are critical for T cell development, homeostasis, and differentiation. Immunity 17: 617–627. [PubMed: 12433368]
13. Kortum RL, Sommers CL, Pinski JM, Alexander CP, Merrill RK, Li W, Love PE, and Samelson LE 2012. Deconstructing Ras signaling in the thymus. Mol Cell Biol 32: 2748–2759. [PubMed: 22586275]
14. Salzer E, Cagdas D, Hons M, Mace EM, Garncarz W, Petronczki OY, Platzer R, Pfajfer L, Bilic I, Ban SA, Willmann KL, Mukherjee M, Supper V, Hsu HT, Banerjee PP, Sinha P, McClanahan F, Zlabinger GJ, Pickl WF, Gribben JG, Stockinger H, Bennett KL, Huppa JB, Dupre L, Sanal O, Jager U, Sixt M, Tezcan I, Orange JS, and Boztug K. 2016. RASGRP1 deficiency causes immunodeficiency with impaired cytoskeletal dynamics. Nat Immunol 17: 1352–1360. [PubMed: 27776107]
15. Priatel JJ, Chen X, Zenewicz LA, Shen H, Harder KW, Horwitz MS, and Teh HS 2007. Chronic immunodeficiency in mice lacking RasGRP1 results in CD4 T cell immune activation and exhaustion. J Immunol 179: 2143–2152. [PubMed: 17675473]
16. Poltorak M, Meinert I, Stone JC, Schraven B, and Simeoni L. 2014. Sos1 regulates sustained TCR-mediated Erk activation. Eur J Immunol 44: 1535–1540. [PubMed: 24497027]
17. Warnecke N, Poltorak M, Kowtharapu BS, Arndt B, Stone JC, Schraven B, and Simeoni L. 2012. TCR-mediated Erk activation does not depend on Sos and Grb2 in peripheral human T cells. EMBO Rep 13: 386–391. [PubMed: 22344067]
18. Pettitt SJ, Liang Q, Rairdan XY, Moran JL, Prosser HM, Beier DR, Lloyd KC, Bradley A, and Skarnes WC 2009. Agouti C57BL/6N embryonic stem cells for mouse genetic resources. Nat Methods 6: 493–495. [PubMed: 19525957]
19. Aghajani K, Keerthivasan S, Yu Y, and Gounari F. 2012. Generation of CD4CreER(T(2)) transgenic mice to study development of peripheral CD4-T-cells. Genesis 50: 908–913. [PubMed: 22887772]
20. Conti E, and Izaurralde E. 2005. Nonsense-mediated mRNA decay: molecular insights and mechanistic variations across species. Curr Opin Cell Biol 17: 316–325. [PubMed: 15901503]

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

21. Charpentier JC, Chen D, Lapinski PE, Turner J, Grigorova I, Swanson JA, and King PD 2020. Macropinocytosis drives T cell growth by sustaining the activation of mTORC1. Nat Commun 11: 180. [PubMed: 31924779]

22. Huang H, Long L, Zhou P, Chapman NM, and Chi H. 2020. mTOR signaling at the crossroads of environmental signals and T-cell fate decisions. Immunol Rev 295: 15–38. [PubMed: 32212344]

23. Mishima T, Toda S, Ando Y, Matsunaga T, and Inobe M. 2014. Rapid proliferation of activated lymph node CD4(+) T cells is achieved by greatly curtailing the duration of gap phases in cell cycle progression. Cell Mol Biol Lett 19: 638–648. [PubMed: 25424911]

Author Manuscript
Author Manuscript
Author Manuscript
Author Manuscript

Key points

- Rasgrp1 is necessary for TCR/CD28-induced Ras-Erk in murine peripheral CD4+ T cells
- TCR/CD28-induced CD4+ T cell IL-2 and IL-2R expression is dependent on Rasgrp1
- TCR/CD28-induced CD4+ T cell proliferation is severely impacted by Rasgrp1 deficiency

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

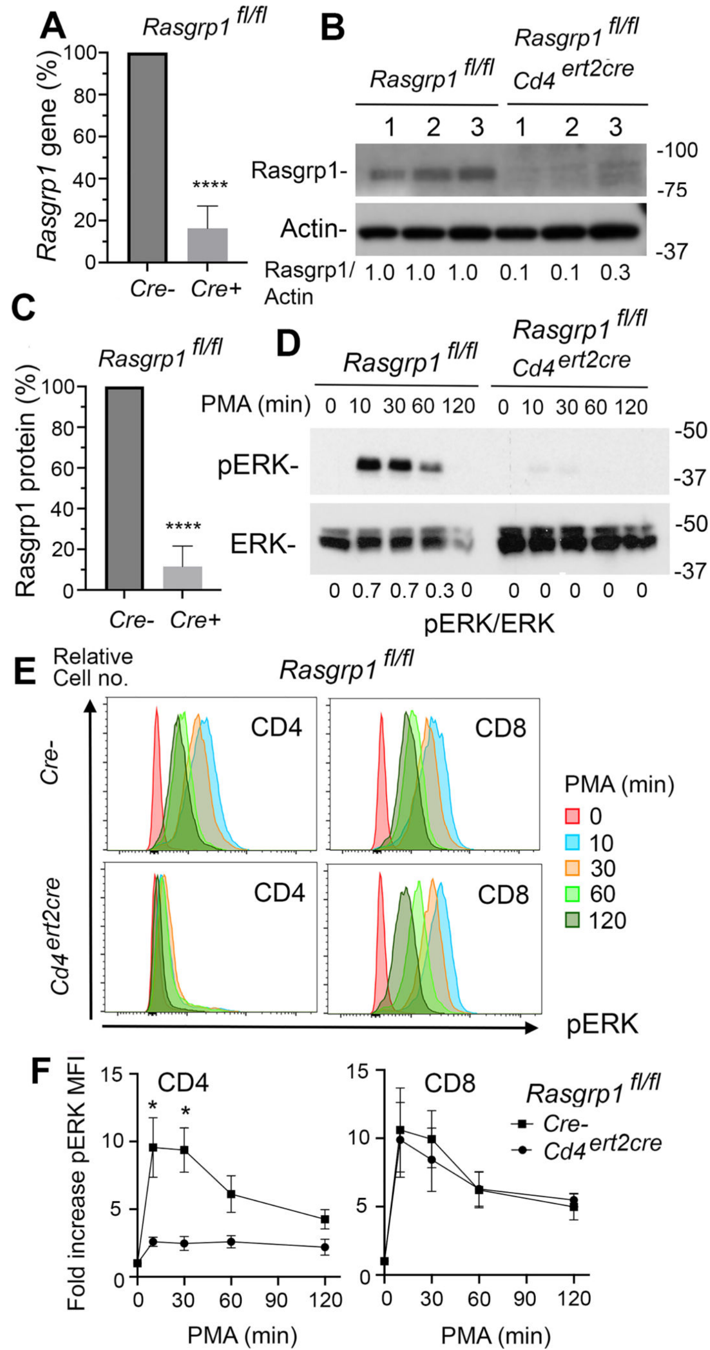

FIGURE 1.

PMA-induced ERK activation in Rasgrp1-deficient peripheral CD4+ T cells. (**A-D**) Splenic CD4+ T cells were isolated from TM-treated littermate mice of the indicated genotypes. (**A**) Mean + 1 SEM of percent *Rasgrp1* gene copy number determined by qPCR (*Rasgrp1$^{fl/fl}$*, n=4; *Rasgrp1$^{fl/fl}$ Cd4^{ert2cre}*, n=8). (**B**) Rasgrp1 protein expression determined by Western blotting. (**C**) Mean + 1 SEM of percent Rasgrp1 protein abundance (n=5 each genotype). (**D**) Western blot analysis of ERK phosphorylation (pERK) following stimulation with PMA for the indicated times. Representative of 2 independent experiments using pooled CD4+

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

T cells from 3 different mice. (**E** and **F**) Whole splenocytes were stimulated with PMA for the indicated times. (**E**) Representative flow cytometry plots of pERK staining in CD4 +and CD8+ T cells. (**F**) Mean +/− 1 SEM of fold increase in mean fluorescence intensity (MFI) compared to unstimulated T cells in flow cytometry experiments (*Rasgrp1*$^{fl/fl}$, n=4; *Rasgrp1*$^{fl/fl}$ *Cd4*^{ert2cre}, n=3).

Author Manuscript

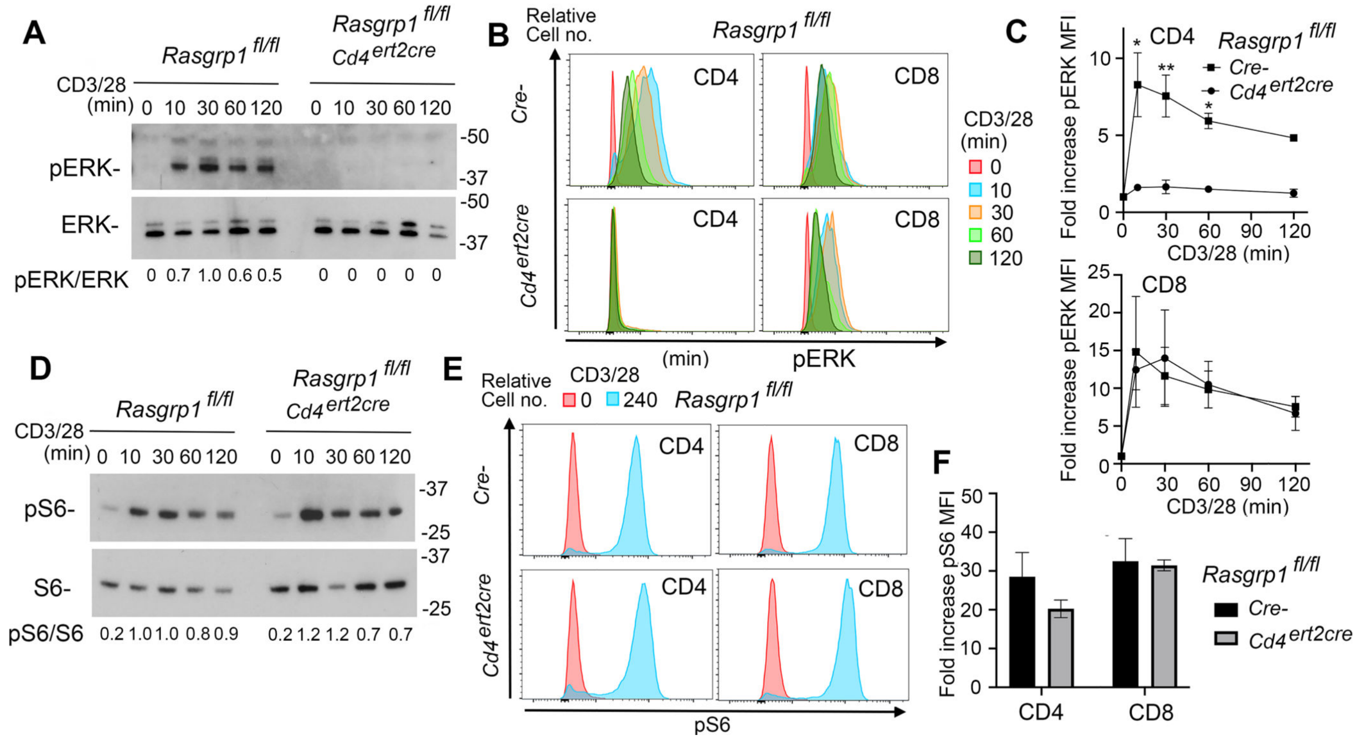

FIGURE 2.

CD3/CD28-induced ERK activation in Rasgrp1-deficient peripheral CD4+ T cells. Purified splenic CD4+ T cells (**A** and **D**) and whole splenocytes (**B,C,E,** and **F**) from littermate TM-treated mice were stimulated with CD3 and CD28 mAb for the indicated times before assessment of ERK and S6 phosphorylation by Western blotting (**A** and **D**) and flow cytometry (**B,C,E,** and **F**). (**A** and **D**) Representative of 2 independent experiments using pooled CD4+ T cells from 3 different mice. (**B** and **E**) Representative flow cytometry plots. (**C** and **F**) Mean +/− 1 SEM of fold increase in MFI compared to unstimulated T cells (*Rasgrp1$^{fl/fl}$*, n=3; *Rasgrp1$^{fl/fl}$ Cd4^{ert2cre}*, n=3).

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

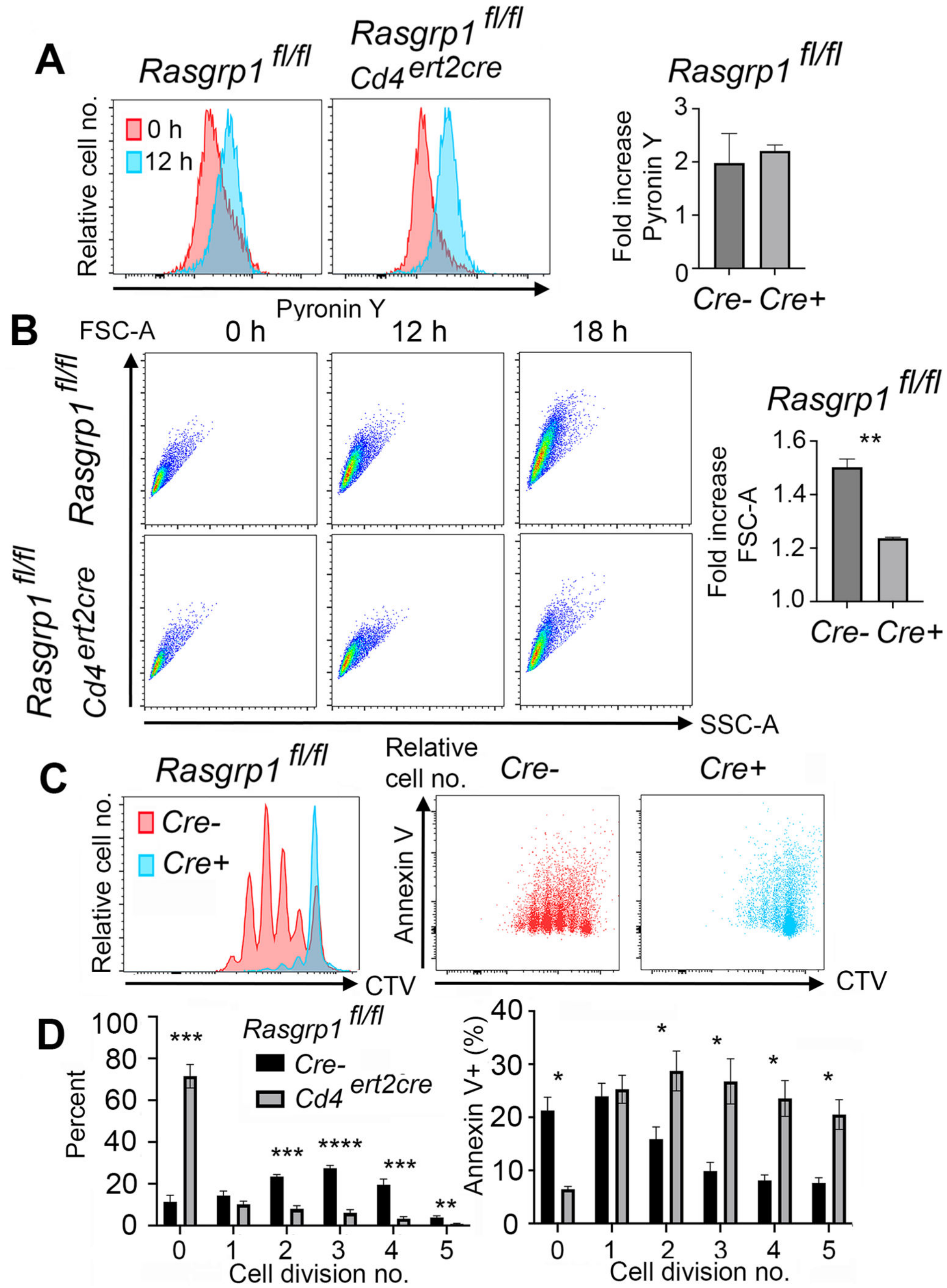

FIGURE 3.

CD3/CD28-induced cell cycle analysis of Rasgrp1-deficient peripheral CD4+ T cells. (**A**) left, flow cytometry plots showing Pyronin Y staining of CD4+ T cells from TM-treated mice of the indicated genotypes activated with CD3/CD28 mAb for 12 h; right, mean + 1 SEM of fold increase in Pyronin Y staining at 12 h compared to unstimulated T cells (n=2 each genotype). (**B**) left, FSC-A vs SSC-A profiles of CD4+ T cells from TM-treated mice of the indicated genotypes activated with CD3/CD28 mAb for the indicated times; right, fold increase in FSC-A at 18 h compared to unstimulated T cells (n=3 each genotype). (**C** and

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

D) Splenic CD4+ T cells from TM-treated littermate mice of the indicated genotypes and stimulated with CD3/CD28 mAb for 72 h. (**C**) Representative flow cytometry plots showing dilution of CTV fluorescence and Annexin V staining at 72 h. (**D**) Mean +/− 1 SEM of percent of live cells at each division (left) and percent of Annexin V+ cells at each division (right; n=3 each genotype).

Author Manuscript

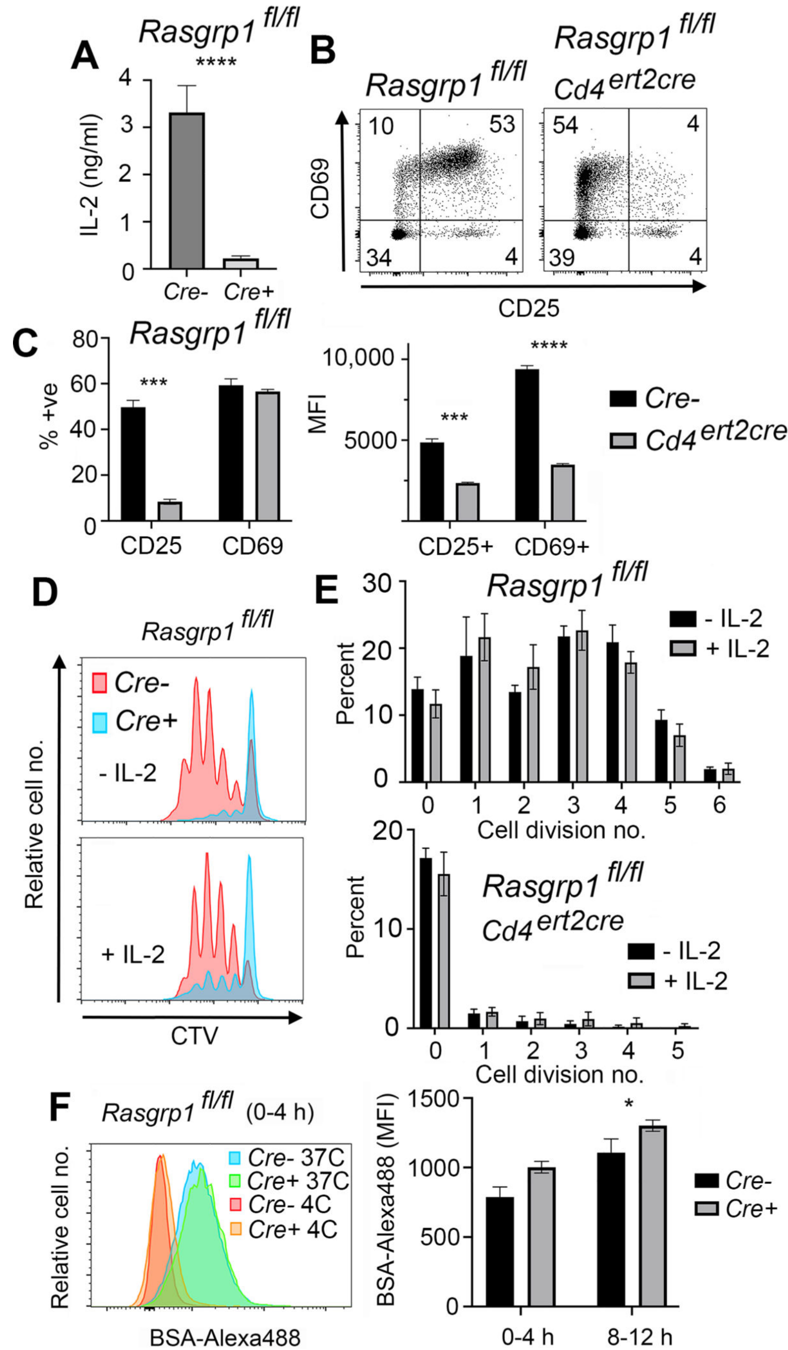

FIGURE 4.

CD3/CD28-induced IL-2/IL2R expression and macropinocytosis in Rasgrp1-deficient peripheral CD4+ T cells. Splenic CD4+ T cells from TM-treated littermate mice of the indicated genotypes were stimulated with CD3/CD28 mAb. (**A**) Mean + 1 SEM of concentration of IL-2 in culture supernatants at 48 h (n=3 each genotype). (**B**) Representative flow cytometry dot plots of CD25 and CD69 expression at 12 h. (**C**) Mean + 1 SEM of percent CD25+ or CD69+ cells (left) and MFI of CD25+ and CD69+ cells (right) (n=3 each genotype). (**D**) Representative flow cytometry plots of CTV fluorescence at 72 h

in the presence and absence of IL-2. (**E**) Mean +/− 1 SEM of percent of live cells at each division in the presence and absence of IL-2 (n=3 each genotype). (**F**) Splenocytes from tamoxifen-treated mice of the indicated genotypes were incubated with CD3/CD28 mAb for 4 h in the presence of BSA-Alexa488 at 4C or 37C. Left, representative histograms of Alexa488 fluorescence. Right, mean + 1 SEM of MFI of Alexa488 fluorescence in repeat experiments performed for 4 h or 12 h (with probe addition at 8 h) at 37C (n=2 each genotype).

Author Manuscript Author Manuscript Author Manuscript Author Manuscript