HHS Public Access

Author manuscript

J Immunol. Author manuscript; available in PMC 2019 March 15.

Published in final edited form as:

J Immunol. 2018 March 15; 200(6): 2186–2198. doi:10.4049/jimmunol.1701638.

T cell expression of C5a receptor 2 augments murine regulatory T cell generation and regulatory-T-cell-dependent cardiac allograft survival

Divya Verghese[*,†,‡,1], **Markus Demir**[*,†,‡,1], **Nicholas Chun**[*,†], **Miguel Fribourg**[*,§], **Paolo Cravedi**[*,†], **Ines Llaudo**[*,†], **Trent M. Woodruff**[¶], **Pragya Yadav**[*,†], **Sergio A. Lira**[‡], **M. Edward Medof**[‖], and **Peter S. Heeger**[*,†,‡,2]

[*]Nephrology division, Department of Medicine, New York, NY

[†]Translational Transplant Research Center, New York, NY

[‡]Immunology Institute Icahn School of Medicine at Mount Sinai, New York, NY

[§]Department of Neurology Icahn School of Medicine at Mount Sinai

[¶]School of Biomedical Sciences, The University of Queensland, Brisbane St. Lucia, Brisbane, Australia

[‖]Institute of Pathology, Case Western Reserve University, Cleveland OH

Abstract

C5aR2 (C5L2/gp77) is a 7-transmembrane spanning receptor that binds to C5a but lacks motifs essential for G-protein coupling and associated signal transduction. C5aR2 is expressed on immune cells, modulates various inflammatory diseases in mice, and has been shown to facilitate murine and human regulatory T cell (iT_{REG}) generation *in vitro*. Whether and if so how, C5aR2 impacts *in vivo* T_{REG} generation and pathogenic T cell-dependent disease models have not been established. Herein we show that murine T cells express and upregulate C5aR2 during iT_{REG} generation and that the absence of T cell-expressed C5aR2 limits *in vivo* iT_{REG} generation following adoptive transfer of naïve $CD4^+$ T cells into *rag1*$^{-/-}$ recipients. Using newly generated C5aR2 transgenic mice (C5aR2-tg) we show that overexpression of C5aR2 in naïve $CD4^+$ T cells augments *in vivo* iT_{REG} generation. In a model of T_{REG}-dependent cardiac allograft survival, recipient C5aR2 deficiency accelerates graft rejection associated with lower T_{REG}/T_{EFF} ratios

[2]The work was supported by R01 AI071185 awarded to PSH and MEM. NC, MF and PC were supported by T32 AI078892

Corresponding author: Peter S Heeger, MD; Translational Transplant Research Center, Department of Medicine Icahn School of Medicine at Mount Sinai, New York, USA; peter.heeger@mssm.edu, Phone: 212 241 6324; Fax: 212 987 0389.
[1]co-first authors, contributed equally

Contributions

P. Heeger led and funded the project with M Medof, designed the overall direction of the studies, analyzed, reviewed and interpreted data and wrote and edited the manuscript. D Verghese, M Demir and N Chun each performed experiments, analyzed results and edited the manuscript. P Yadav performed experiments and edited the manuscript. S Lira designed the vector for the C5aR2-tg mouse, assisted with the cloning and edited the manuscript. M Fribourg performed all imaging flow experiments and analyses and edited the manuscript. T Woodruff provided the C5aR2 ligand and the C5aR1-A, designed and interpreted the several experiments and edited the manuscript. I Llaudo and P Cravedi assisted with experiments and edited the manuscript.

Disclosure of Conflicts of Interest

None

while overexpression of C5aR2 in immune cells prolongs graft survival associated with an elevation in T_{REG}/T_{EFF} ratios. T cell-expressed C5aR2 modulates T_{REG} induction without altering effector T cell proliferation or cytokine production. Distinct from reported findings in neutrophils and macrophages, T_{REG}-expressed C5aR2 does not interact with β-arrestin or inhibit ERK1/2 signaling. Rather, cumulative evidence supports the conclusion that C5aR2 limits C5aR1-initiated signals known to inhibit T_{REG} induction. Together, the data expand the role of C5aR2 in adaptive immunity by providing *in vivo* evidence that T cell-expressed C5aR2 physiologically modulates iT_{REG} generation and iT_{REG}-dependent allograft survival.

Keywords

complement; C5aR; Treg; transplantation; mouse

Introduction

$CD4^+Foxp3^+$ regulatory T cells (T_{REG}) that crucially mediate peripheral immune tolerance can emerge directly from the thymus or can be induced from naïve $CD4^+$ T cells (induced T_{REG} or iT_{REG}), the latter in response to TCR/costimulatory molecule ligation plus stimuli that include retinoic acid or TGFβ (1–4). Among contexts in which T_{REG} contribute to immune tolerance are the prevention of pathological mucosal immune responses that result in inflammatory bowel disease, autoimmunity, and fetal loss, as well as regulation of injurious alloimmunity that causes transplant rejection (2–9). Despite significant progress, the physiological stimuli that exert control over T_{REG} induction remain incompletely understood.

We have previously demonstrated that complement cleavage products C3a and C5a are produced by immune cells during cognate T cell/APC interactions and these anaphylatoxins ligate their specific 7-transmembrane spanning G-protein coupled receptors (GPCR), C3aR1 and C5aR1, expressed on both partners (10–17). On T cells, these ligations transduce signals via the phosphokinase A (PKA)/cAMP response element-binding protein (CREB) and via phospho-inositol-3-kinase gamma (PI-3Kγ)/pAKT (15, 18). The pAKT prevents intranuclear transport of Foxo1/3 which in turn inhibits Foxp3 production (19, 20), together limiting iT_{REG} generation (18–20). In corollary experiments using several *in vitro* and *in vivo* systems, we also demonstrated that absence or blockade of T cell expressed C3aR1/C5aR1 facilitates human and murine thymic-derived T_{REG} function (19) and iT_{REG} generation, function and stability (18–20).

Human and murine immune cells (21–23), including T cells (18, 24), also express C5aR2 (formerly called C5L2/gp77), a 7-transmembrane spanning receptor that can bind C5a but does not signal as a GPCR (21, 22). Current data indicate that the effects of C5aR2 *in vivo* are context- and cell type-dependent. In support of this conclusion, results of studies using *c5ar2*$^{-/-}$ mice revealed that C5aR2 augments certain inflammatory responses, including models of allergic asthma, sepsis, atherosclerosis (22, 25–30), while others provide evidence that C5aR2 functions to down-modulate T cell-mediated diseases, including murine contact dermatitis (31). Evidence also indicates that the biochemical mechanisms through which

C5aR2 mediates these varied effects include at least two non-mutually exclusive mechanisms. C5aR2 can scavenge and internalize/degrade C5a (and its degradation product C5a-des-arg), a mechanism that indirectly limits C5a/C5aR1 signaling (18, 21, 22, 32). However, in some cells, C5aR2 has also been shown interact with β-arrestin-2 and transduce signals that inhibit ERK1/2 signaling independent of C5aR1 (33–35).

Whether and how C5aR2 impacts *in vivo* murine T cell immune responses has not been reported. In a 2013 publication (18), the Medof laboratory provided evidence that a) C5aR2 is expressed in activated $CD4^+$ T cells including $CD4^+Foxp3^+T_{REG}$, b) T cell-expressed C5aR2 binds to and out-competes C5aR1 for C5a, c) absence of T cell-expressed C5aR2 blunts *in vitro* iT_{REG} induction, and d) C5aR2 scavenges locally produced C5a to limit C5aR1 signaling. Building upon these findings, the goals of the current set of studies were to test the hypothesis that T cell-expressed C5aR2 physiologically modulates iT_{REG} generation *in vivo* and to assess the impact of C5aR2 on T_{REG}-dependent prolongation of cardiac allograft survival.

Materials and Methods

Mice

C57BL/6(B6, *H-2*^{b}), BALB/c (*H-2*^{d}), B6 *rag1*$^{-/-}$, B6 Foxp3-GFP and B6 Foxp3-RFP mice were purchased from The Jackson Laboratory. The B6 *c5ar1*$^{-/-}$ and *c5l2*$^{-/-}$ (*c5ar2*$^{-/-}$) mice (22) were kind gifts from C Gerard (Harvard, Boston MA). The *c5ar2*$^{-/-}$ mice have a GFP reporter knocked in to the *c5ar2* locus (22). We crossed the *c5ar2*$^{-/-}$ mice to the B6 Foxp3-RFP mice to produce *c5ar2*$^{-/-}$ Foxp3-RFP reporter mice.

Production of C5ar2 transgenic (tg) mice: We synthesized a cDNA containing the coding region of the C5aR2 transcript flanked by Kpn1 and Not1 restriction sites and cloned it into an expression vector containing a human CD2 minigene (36, 37). The plasmid was linearized and transgenic mice were produced by the Mouse Genetics Core at the Icahn School of Medicine at Mount Sinai. Four founders expressed the transgene and transmitted the transgene to their offspring. Offspring of the founder with the highest levels of surface and intracellular levels C5aR2 as assessed by flow cytometry on peripheral blood and spleen cells were used for the studies. We also crossed the C5aR2-tg mice with the Foxp3-GFP mice to produce C5aR2-tg Foxp3-GFP reporter animals. Mice were housed in the Icahn School of Medicine at Mount Sinai Center for Comparative Medicine in accordance with guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care International. All experiments were performed using animals that were littermates, or were maintained in the same room and/or were co-housed within the same cages to limit potential effects of microbiome differences.

Antibodies and Reagents

Antibodies against CD3 (145-2C11), CD4 (GK1.5), CD8 (53-6.7), CD11b (M1/70), CD11c (N418), CD44 (IM7), GR1 (RB6-8C5), Foxp3 (FJK-16s), control rat IgG2b (R2B-7C3) and fixable viability dye were purchased from ThermoFisher (Waltham MA). Anti-CD62L (MEL-14), and anti-B220 (RA3-6B2) were purchased from Tonbo (San Diego, CA), anti-

CD88 (C5aR1, 10–92) was purchased from BioRad (Hercules, CA) and anti-C5L2 (C5aR2, 468705) was purchased from R&D systems (Minneapolis MN). Anti-ppS6-kinase (REA454) and pAKT (REA359) were purchased from Miltenyi Biotec, (Bergisch Gladbach, Germany) and anti-β-arrestin 2 was purchased from SantaCruz Biotechnology. MR1 (anti mouse CD40L/CD154) was purchased from BioXCell (West Lebanon, NH). CpG DNA was purchased from invivogen. The selective C5aR2 agonist, P32 (Ac-RHYPYWR-OH), and the selective C5aR1 antagonist C5aR1-A, PMX53 were synthesized as previously described (33).

Cell isolations

Splenic single cell suspensions were passed through a 40μm strainer (BD Falcon) and lysed with red blood cell lysis buffer (Life Technologies, CA). Isolation of naïve and total $CD4^+$ T cells, total T cells/APCs was accomplished using magnetic beads and the AutoMACS Pro machine (Miltenyi Biotec) or manual magnets (EasySep Stemcell; Invitrogen). Flow sorting was done on Sony SH800Z or BD FACS Aria cell sorter.

Mixed lymphocyte responses

Splenic APCs were isolated from naïve mice by magnetic bead separation and were co-cultured with stimulated naïve $CD4^+$ T cells or unfractionated T cells labeled with CFSE or cell trace violet for 4 days in complete medium (RPMI + 10% FCS + L-Glutamine + sodium pyruvate + nonessential amino acids + Pen/Strep + β-mercaptoethanol) at 37°C. CFSE/cell trace dilution, live cell numbers, and surface marker expression were determined by flow cytometry.

ELISPOT assays

IFNγ ELISPOT assays were performed using responder B6 spleen cells co-cultured with allogeneic stimulator BALB/c spleen cells on capture plates for 24 h and then analyzed as previously described (38).

Flow cytometry

Data were collected on a FACSCanto II (BD Biosciences) and analyzed using Cytobank (Cytobank Inc., CA).

In vitro T_{REG} induction

Sorted $CD4^+$ $Foxp3GFP^-$ $CD44^{lo}$ $CD62L^{hi}$ T cells or magnetically isolated (EasySep) naïve $CD4^+$ T cells were co-cultured with syngeneic splenic APCs, IL-2 (10ng/ml) and TGF-β (5ng/ml) for 5 days at 37°C in complete medium.

In vivo T_{REG} induction

4×10^5 sorted $CD4^+Foxp3\text{-}GFP/RFP^-$ $CD44^{lo}$ $CD62L^{hi}$ T cells were injected i.v./retro-orbital into sex-matched B6 *rag1*$^{-/-}$ recipients. Fourteen days later spleen cells were isolated, stained for CD4 and Foxp3-GFP/RFP expression was analyzed within the CD4 gate by flow cytometry.

Heart transplantation

Heterotopic heart transplants were performed as previously described (17, 39, 40). Where indicated, recipients were treated with anti-CD40L (anti-CD154) mAb MR1 (0.25 mg on day -1). Heart graft function was monitored every other day by palpation and rejection was defined as the day on which a palpable heartbeat was no longer detectable and was confirmed by histology.

Signaling assays

Spleen cells or purified naïve $CD4^+$ T cells were incubated overnight with anti-CD3 ± anti-CD28 ± fixed numbers of syngeneic APCs in (serum free) HL-1 media (1% L-GLUT, 1% Pen/Strep) at 37°C as indicated. After incubation cells were fixed using PFA (1%), stained for surface antigens, then permeabilized with methanol and stained for intracellular pAKT, p-pS6, and pERK1/2.

Suppression Assays

Naïve CD4 T cells were stimulated with IL-2 (10ng/ml) and TGFβ (10ng/ml) for 3 days at 37°C in complete medium. On day 3, sorted $CD4^+$Foxp3-GFP^+ or -RFP^+ T_{REG} were co-cultured with 2×10^5 CFSE- or cell trace violet-labeled congenic CD45.1 WT B6 T cells and 5×10^4 allogeneic APCs for 4 days at 37°C in complete medium. On day 4, CFSE/cell trace dilution was assessed by flow cytometry and live cell numbers were counted.

Imaging flow cytometry

After staining, cell images were acquired on an ImageStream flow cytometer (Amnis-EMD Millipore Seattle, WA) and analyzed with IDEAS analysis software (Amnis). Single-color controls were used for creation of a compensation matrix that was applied to all files to correct for spectral crosstalk. Debris and dead cells were identified by high pixel intensity correlation across all channels and were excluded from the analysis.

For co-localization the “bright detail similarity” BDS feature of the IDEAS software was used for measurement of the spatial correlation between the fluorescent signal emanating from C5aR1-APC and β2-arrestin-PE. BDS is the log-transformed Pearson’s correlation coefficient of the localized bright spots with a radius of three pixels or less within the masked area in the two input images. The higher the BDS score, the higher the level of co-localization. Positive cutoff values were calculated on the basis of the background “bright detail similarity” (BDS) of C5aR1-APC and β2-arrestin-PE with an irrelevant signal (bright field). BDS scores >1.5 were considered highly-colocalized. A threshold of 1.5 is consistent with previous similar studies (41, 42) and indicative of good performance of the image analysis algorithm.

Statistical analysis

Statistical significance was determined by Student’s t test (unpaired, two-tailed), two-way ANOVA (with Bonferroni post-tests to compare replicate means) or by log-rank (Mantel-Cox) test performed in GraphPad Prism 5 or Prism 6 with a significance threshold values of

$p<0.05$. All experiments were repeated at least twice. Error bars indicate mean ± SEM and ns indicates $p>0.05$, not significant.

Results

C5aR2 on T cells modulates generation of iT_{REG}

To verify that C5aR2 is expressed in effector T cells (T_{EFF}) and in T_{REG}, we took advantage of *c5ar2*$^{-/-}$ mice that have a GFP reporter knocked-in to the C5ar2 gene (22). When we studied GFP expression in T cells from these mice, we did not observe a GFP signal significantly above background in unstimulated T cells (Fig 1A–B). In contrast, following a 5-day stimulation of spleen cells with anti-CD3±TGFβ/IL-2 (the latter to induce T_{REG}) we detected a distinct GFP signal in CD4$^+$ T cells above baseline that was not detected in WT T cells that lack the GFP reporter. The GFP signal was significantly stronger in CD4$^+$ T cells stimulated in the presence of TGFβ/IL-2 (Fig 1C). Notably, the mean fluorescence intensity (MFI) for the GFP signal in CD4$^+$ T cells was ~3-fold lower than in unstimulated neutrophils (Fig 1B–C) known to express high levels of C5aR2 (43).

We next analyzed intracellular and surface C5aR2 expression on CD4$^+$ T cells by imaging flow cytometry and standard flow cytometry after stimulating spleen cells with anti-CD3±TGFβ and IL-2 for 48 h (Fig 1D–F, Supplemental Fig 1A). These assays confirmed that WT (but not *c5ar2*$^{-/-}$) CD4$^+$ T cells expressed intracellular C5aR2 (Fig 1D–E) and confirmed upregulation of surface-expressed C5aR2 under both conditions (Fig 1F). Control experiments showed similar C5aR1 expression on WT and *c5ar2*$^{-/-}$ CD4$^+$ T cells, both at rest and following *in vitro* stimulation with anti-CD3 (Fig 1G, Supplemental Fig 1B). We detected lower C5aR1 expression on WT T cells stimulated with anti-CD3 plus TGFβ and IL-2 compared to parallel cultures containing anti-CD3 alone (Fig 1H).

We next tested whether the absence of C5aR2 alters alloreactive T_{EFF} responses using *in vitro* mixed lymphocyte responses (MLRs). We observed no differences in CD8$^+$ T cell (Fig 2A–B) proliferation or IFNγ production in the presence or absence of T cell-expressed C5aR2. We repeated the experiment with purified naïve CD4$^+$ T cell (Fig 2C–D) and similarly observed no differences.

To confirm the previously reported *in vitro* finding that the absence of C5aR2 from naïve CD4$^+$ T cells limits iT_{REG} generation (18) using an alternative system, we crossed the *c5ar2*$^{-/-}$ mice to a Foxp3-RFP reporter animal, generating Foxp3-RFP *c5ar2*$^{-/-}$ mice. We used Foxp3-RFP rather than Foxp3-GFP because the *c5ar2*$^{-/-}$ mice have a GFP reporter knocked in to the c5ar2 locus (22). Consistent with the previously published work (18), when we stimulated purified, naïve WT and *c5ar2*$^{-/-}$ Foxp3-RFP CD4$^+$ T cells with anti-CD3 plus allogeneic BALB/c APCs, IL-2 and TGFβ for 5 days, we observed significantly fewer Foxp3-RFP$^+$ CD4$^+$ iT_{REG} (Fig 2E–F) with the *c5ar2*$^{-/-}$Foxp3-RFP$^+$ CD4$^+$ T cells. We then flow-sorted the CD45.2$^+$ Foxp3-RFP$^+$ iT_{REG} from the *in vitro* induction assays and tested them for their ability to suppress proliferation of congenic CD45.1 T_{EFF} (Fig 2G). These assays showed similar per-cell suppressive capacities between the WT and *c5ar2*$^{-/-}$ iT_{REG}. Neither did we observe significantly different suppressive capacities of flow-sorted, total Foxp3-RFP$^+$, splenic T_{REG} obtained from naive WT and *c5ar2*$^{-/-}$ mice (Fig 2H).

We next tested the effects of T cell C5aR2 expression on iT_{REG} generation *in vivo* (Fig 3A). We isolated Foxp3-RFP^{neg} CD44^{lo} CD62L^{hi} CD4^{+} T cells from WT or *c5ar2*$^{-/-}$ Foxp3-RFP reporter mice, and transferred equal numbers of them into *rag1*$^{-/-}$ hosts. Two weeks later we quantified the percentage and total numbers of splenic Foxp3-RFP^{+} cells within the transferred CD4^{+} T cells in each recipient (Fig 3B–C). These analyses showed significantly lower percentages and fewer numbers of Foxp3^{+}CD4^{+} T cells in the recipients adoptively transferred with naïve *c5ar2*$^{-/-}$ Foxp3-RFP CD4^{+} T cells, validating that the above *in vitro* findings apply *in vivo*.

Recipient C5aR2 deficiency accelerates cardiac allograft rejection associated with increased anti-donor immunity

To determine the effects of C5ar2 deficiency in a T cell-dependent disease model, we performed heterotopic cardiac allograft transplants in which recipient T cells are requisite mediators of graft rejection. We transplanted sets of B6 WT and *c5ar2*$^{-/-}$ recipients with fully MHC-disparate BALB/c hearts (Fig 4A) and treated them with a single dose of anti-CD154 (MR1), a costimulatory blockade therapy that augments iT_{REG} generation and T_{REG}-induced prolongation of allograft survival (44, 45). Whereas BALB/c hearts transplanted into MR1-treated WT recipients survived with a median survival time (MST) of 58 days, BALB/c hearts transplanted into MR1-treated, congenic B6 *c5ar2*$^{-/-}$ recipients rejected significantly faster, with an MST of 30 d ($p<0.05$ vs. WT treated with MR1). Histological analysis of the graft tissue obtained at the time of rejection from the WT and *c5ar2*$^{-/-}$ recipients showed similar diffuse mononuclear cell infiltrates consistent with cellular rejection (Fig 4B). In control experiments, untreated (no MR1) WT and *c5ar2*$^{-/-}$ recipients rejected the allografts with MST of 8 days (p=ns between groups, Fig 4A).

To determine the impact of recipient C5aR2 deficiency on transplant-induced donor-reactive T cell immunity, we analyzed splenic T cell responses and graft infiltrating lymphocytes (GIL) by ELISPOT three weeks after transplanting additional groups of MR1-treated WT and *c5ar2*$^{-/-}$ recipients with BALB/c allografts (all graft beating at the time of harvest, Fig 4C–D). These assays showed that *c5ar2*$^{-/-}$ recipient spleens contained higher frequencies of donor-reactive IFNγ-producers. Analysis of GILs revealed higher ratios of CD8^{+} effector T cells (T_{EFF}) to T_{REG} in the *c5ar2*$^{-/-}$ recipients of BALB/c hearts. Spleens from naïve WT and *c5ar2*$^{-/-}$ mice were comprised of similar frequencies of T cells, B cells and monocyte/DCs (data not shown) and responded equivalently and weakly (<20/million, not shown) to BALB/c stimulators, together indicating that the above detected donor-reactive immune responses were induced by the transplant.

We assessed mechanisms through which C5aR2 modulates T_{REG} induction, initially by testing the hypothesis that C5aR2 limits signaling transduced by C5a/C5aR1, which we previously have demonstrated inhibits T_{REG} induction (18, 20). Building upon our published observations that C5a ligation of C5aR1 initiates signaling in T cells that results in PI-3Kγ-dependent phosphorylation of AKT (12, 15), we tested whether absence of C5aR2 deficiency augments C5aR1-initiated phosphorylation of AKT (Fig 5A). When we activated WT and *c5ar2*$^{-/-}$ CD4^{+} T cells cultured under T_{REG}-inducing conditions ± a selective C5aR1 antagonist (C5aR1-A) and compared intracellular AKT phosphorylation we observed

a) significantly more pAKT in *c5ar2*$^{-/-}$ CD4^{+} T cells (~10% higher) and b) addition of C5aR1-A reversed this modest but significant increase to levels observed in the stimulated WT control cells.

C5aR1-initiated signaling in T cells also leads to pAKT-dependent mTOR activation (18). We observed increased phosphorylation of p-pS6 kinase, an mTOR-substrate in anti-CD3/CD28-stimulated *c5ar2*$^{-/-}$ CD4^{+} T cells (Fig 5B). To test for a functional link, we performed *in vitro* iT$_{REG}$ generation cultures with naïve *c5ar2*$^{-/-}$ CD4^{+} T cells ± the C5aR1-A (Fig 5C). These assays showed that C5aR1 inhibition partially rescued (increased) the lower frequency of Foxp3-expressing T cells in the cultures containing *c5ar2*$^{-/-}$ T cells.

Previous studies have indicated that in some cell types C5aR2 facilitates association of β2-arrestin with C5aR1, and β2-arrestin is known to dissociate G-proteins and internalize GPCRs (46). Using imaging flow cytometry of WT and *c5ar2*$^{-/-}$ CD4^{+} T cells stimulated under iT$_{REG}$-generating conditions for 24 and 48 h (Fig 5D–E), we observed similar amounts of co-localized C5aR1 and β2-arrestin in WT and *c5ar2*$^{-/-}$ CD4^{+} T cells, providing no evidence for a link between C5aR2 and β-arrestin-dependent regulation of C5aR1 in T cells. C5aR2 ligation has also been shown to inhibit ERK1/2 signaling in myeloid cells (31, 33, 47). To test whether this mechanism is operative in T cells, we stimulated WT and *c5ar2*$^{-/-}$ CD4^{+} T cells under iT$_{REG}$-generating conditions ± a specific C5aR2 peptide ligand agonist P32 (33) or vehicle control and measured pERK1/2 by phosphoflow cytometry. These assays showed no differences in ERK activation between WT and *c5ar2*$^{-/-}$ CD4^{+} T cells (Fig 5F) and no effect of the C5aR2 agonist (data not shown). Together with our previously published findings (18), these data support the conclusion that C5aR2 limits C5a/C5aR1 signaling on iT$_{REG}$ induction potentially by scavenging C5a.

Transgenic overexpression of C5aR2 in T cells prolongs graft survival and augments iT$_{REG}$ generation

As a complementary strategy to test the function of immune cell expressed C5aR2 on *in vivo* T cell immunity we generated a congenic B6 C5aR2 transgenic mouse (C5aR2-tg) in which we overexpressed C5aR2 under the CD2 promoter. Characterization of immune cells from offspring of the founder with the highest expression of C5aR2 showed constitutively elevated and surface expression of C5aR2 on CD4^{+} and CD8^{+} T cells, as well as on B cells, CD11c^{+} and CD11b^{+} myeloid cells (Fig 6A–C). The spleens of the C5aR2-tg contained similar percentages of T cells, T cell subsets, B cells, monocytes, DCs and granulocytes (Fig 6D). *In vitro* MLRs showed similar alloreactive proliferative capacities of WT and C5aR2-tg CD4^{+} and CD8^{+} T cells (Fig 6E).

When we transplanted BALB/c cardiac allografts into MR1-treated C5aR2-tg recipients we observed that survival of the donor hearts was significantly prolonged in the C5aR2-tg recipients compared to allografts transplanted into WT controls (Fig 7A). We halted the experiment after 100 days and examined the grafts histologically. The grafts that were beating at the end of the experiment showed mononuclear cell infiltrates and vasculopathy consistent with chronic rejection (and not tolerance, Fig 7B). Untreated (no MR1) C5aR2-tg recipients rejected the BALB/c heart grafts with a MST of 8 day (Fig 7A), not different from untreated WT recipients, indicating that overexpression of C5aR2 is insufficient to prolong

graft survival in the absence of peri-transplant CD154 blockade. Immune cell analyses performed in separate sets of MR1-treated recipients 4 weeks posttransplant showed that the spleens of C5aR2-tg mice contained fewer donor-reactive IFNγ-producers (Fig 7C–D) a higher frequency of $CD4^+Foxp3^+$ T_{REG} (Fig 7E), and higher T_{REG}/T_{EFF} ratios (Fig 7F).

To directly test effects of transgenic C5aR2 overexpression on *in vitro* iT_{REG} generation, we crossed the C5aR2-tg mouse with a Foxp3-GFP reporter to produce C5aR2-tg Foxp3-GFP animals. We isolated naïve splenic GFP^{neg} $CD4^+$ from these mice and from WT GFP^{neg} Foxp3-GFP controls and cultured them under iT_{REG}-generating conditions (syngeneic APCs, anti-CD3, IL-2+TGFβ, Fig 8A–B). These assays showed significantly higher frequencies of $Foxp3^+$ iT_{REG} in the cultures containing C5aR2-tg $CD4^+$ T cells. The augmentation of iT_{REG} generation in the cultures containing C5aR2-tg T cells was partially abrogated when assays were performed using APCs deficient in C3 and C5 (Fig 8C), confirming that the C5aR2-dependent augmentation of iT_{REG} is mediated by regulating effects of locally produced complement. When we sorted Foxp3-GFP^+ WT and C5aR2-tg cells and tested them in *in vitro* suppression assays, we observed similar per cell suppressive capacities of the WT and C5aR2-tg iT_{REG} (Fig 8D). Finally, to directly test whether transgenic C5aR2 overexpression alters *in vivo* iT_{REG} generation, we adoptively transferred GFP^{neg} C5aR2-tg Foxp3-GFP or GFP^{neg} WT Foxp3-GFP $CD4^+$ T cells into *rag1*$^{-/-}$ recipients and quantified frequencies of $CD4^+GFP^+$ cells within the recipient spleens 2 weeks later (Fig 8E–F). These assays showed significantly higher frequencies of $CD4^+GFP^+$ cells in the adoptive recipients of the naïve C5aR2-tg $CD4^+$ T cells.

Discussion

Our findings add to the emerging understanding that C5aR2 functions as a modulator of immune responses by showing for the first time that T cell-expressed C5aR2 has clinically relevant effects on iT_{REG} generation and T_{REG}-dependent transplant survival *in vivo*. Using, a GFP reporter system to quantify C5aR2 gene expression as well as standard flow and imaging flow methodologies to verify protein expression, we unequivocally demonstrate that murine T cells express C5aR2 and that *in vitro* stimulation under T_{REG}-inducing conditions (in the presence of TGFβ) upregulates C5aR2 expression (Fig 1). In a model of T_{REG}-dependent allograft survival, absence of recipient C5aR2 accelerated rejection, a clinical phenotype associated with higher frequencies of donor-reactive IFNγ-producing T cells and with lower ratios of splenic and intragraft iT_{REG} to T_{EFF} cells, compared to WT controls (Fig 4). Conversely, allogeneic hearts transplanted into anti-CD154-treated, C5aR2-tg recipients survived longer than in WT controls and the prolonged survival was associated with weaker anti-donor T cell responses and higher iT_{REG}/T_{EFF} ratios (Fig 7). *In vitro* assays showed that while the absence of C5aR2 did not alter allo-induced T_{EFF} proliferation or IFNγ production (Fig 2), absence of T cell C5aR2 inhibited iT_{REG} induction [verifying our previous report (18)]. Conversely, transgenic overexpression of C5aR2 in T cells augmented *in vitro* iT_{REG} induction (Fig 8). This T cell-intrinsic role for C5aR2 as a modulator of iT_{REG} induction was verified *in vivo* using adoptive transfer of naïve T cells into *rag1*$^{-/-}$ hosts (that express all complement components including C5aR2). Absence of C5aR2 only on the transferred T cells limited (Fig 3), while overexpression of C5aR2 only on the transferred T cells augmented (Fig 8), *in vivo* iT_{REG} generation. Despite our

documentation that C5aR2 expression (deficiency or transgenic overexpression) alters transplant outcomes and modulates iT_{REG} induction *in vivo*, we acknowledge that the design of our transplant experiments does not permit us to definitively conclude that the observed changes in allograft survival are solely dependent upon C5aR2-driven alterations in T_{REG}. Mechanistically, our *in vitro* signaling and iT_{REG} induction assays (Fig 5), in aggregate with our previous findings (18), are consistent with the conclusion that T cell-expressed C5aR2 facilitates iT_{REG} generation by scavenging locally produced C5a and limiting C5aR1-transduced signals within the responding T cells. We acknowledge that an alternative possible mechanism is that C5aR2 could form heterodimers with C5aR1 in T_{REG} and that such heterodimers could limit/alter C5aR1 signal transduction, analogous to what has been shown for other GPCRs (48); as a consequence, absence of C5aR2 would prevent heterodimer formation and lift restraint on C5aR1 signaling. Regardless, the data provide strong supportive evidence that C5aR2 serves as a break on C5aR1 signaling during T_{REG} induction.

The mechanism through which C5aR1 limits T_{REG} induction has been documented previously by our group (12, 18); C5aR1 (and C3aR1) blockade limits PKA-induced cAMP/CREB and prevents PI3Kγ-dependent phosphorylation AKT and pAKT-dependent phosphorylation of Foxo1, facilitating transport into the nucleus (19, 20), together upregulating Foxp3 expression and iT_{REG} suppressive function. While others have shown that C5aR2 can transmit a signal in part by interaction with β-arrestin and/or that stimulating C5aR2 using a specific peptide ligand inhibits ERK1/2 signaling in some cell types (e.g. macrophages) (33, 43, 49), our data do not support that either of these two mechanisms are operative in T cells.

While we observed that T cell-expressed C5aR2 augments iT_{REG} generation and prolongs transplant survival, absence or overexpression of C5aR2 did not alter *in vitro* alloreactive T_{EFF} cell proliferation or IFNγ-production (in the absence of iT_{REG}). We speculate that higher amounts of T cell/APC derived and locally activated complement during effector responses (in the absence of TGFβ) and the relatively low levels of C5aR2 expressed on T_{EFF} compared to T_{REG} accounts for these differences. While one report implicates surface-expressed C5aR2 on human T cells as a modulator of intracellular complement activation that drives inflammasome activity (24), this mechanism does not apply to murine systems.

Published work by others, predominantly using C57BL/6 mice, show that the effects of C5aR2 deficiency on inflammation and on *in vivo* disease manifestations depend on the model system employed, as well as the cellular molecular mediators that result in the injury (50). C5aR2 deficiency was shown to exacerbate murine contact dermatitis, a T cell-dependent, Th1-like, type IV hypersensitivity response that is known to be modulated by T_{REG} (similar to our system) (31). While these investigators showed that the worsened dermatitis observed in *c5ar2*$^{-/-}$ mice was associated with augmented LN and effector site expression of proinflammatory chemokines (e.g. IFNγ) and chemokines, they did not directly test whether C5aR2 deficiency altered T_{EFF} or T_{REG}. Our data suggest that the worsened disease in the absence of C5aR2 is at least in part related to suboptimal T_{REG} generation and consequently uncontrolled T_{EFF} responses.

In contrast, in disease models driven by T cell-independent mechanisms, including dextran sulfate sodium-induced colitis, thioglycollate-induced peritonitis, acute lung injury, sepsis, and kidney ischemia perfusion injury, C5aR2 deficiency led to improved outcomes (27, 49, 51, 52). Together with the current work, these previous findings suggest that signaling via C5aR2 on T cells is an important modulator of T_{REG} which indirectly exert control over Th1 immune responses, while signaling via C5aR2 predominantly on innate immune cells can modulate their pro-inflammatory functions. The complexity of C5aR2's *in vivo* effects is highlighted by observations made in murine models of allergic asthma in BALB/c mice prone to Th2 immunity (26) in which *c5ar2*$^{-/-}$ mice are protected. Importantly, these studies showed that C5aR2 expressed on DCs amplifies Th2 T cell differentiation required for development of asthma, while absence of C5aR2 shifted the cytokine profile towards Th1/Th17 which is protective in this strain. The authors did not report any effects of C5aR2 deficiency on T_{REG} in their system. Thus in this Th2-driven disease in BALB/c mice, the evidence indicates that the dominant effect of C5aR2 is related to DC-dependent T cell differentiation processes.

We note that in a 2017 publication (34) one research group concluded that murine T cells do not express C5aR2 because the investigators could not identify a knocked-in tomato reporter signal in peripheral T cells. Our data refute this claim by directly demonstrating C5aR2 gene expression (using a different reporter) and protein expression in murine T cells, including T_{REG}. Additionally, we provide direct functional evidence that levels of C5aR2 expressed on T cells alters *in vivo* T_{REG} induction following adoptive transfer into *rag1*$^{-/-}$ recipients.

In sum, our data expand the known functions of C5aR2 in adaptive immunity by providing new *in vivo* evidence that T cell-expressed C5aR2 physiologically modulates iT_{REG} generation and iT_{REG}-dependent allograft survival. Our findings add to the increasingly recognized function of the complement system as modulating adaptive T cell immune responses and provide the foundation for designing therapeutics that upregulate T cell C5aR2 to increase T_{REG} generation and thereby suppress pathogenic T cell immunity, including those induced by transplantation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors thank Peter Boros, Jinhua Liu, and Yansui Li (Mount Sinai) for their microsurgery support, the Flow Cytometry Core at the Icahn School of Medicine at Mount Sinai for flow sorting support, and Kevin Kelley and the Mouse Genetics and Gene Targeting Core at the Icahn School of Medicine at Mount Sinai for mouse re-derivation and production.

References

1. Maynard CL, Hatton RD, Helms WS, Oliver JR, Stephensen CB, Weaver CT. Contrasting roles for all-trans retinoic acid in TGF-beta-mediated induction of Foxp3 and Il10 genes in developing regulatory T cells. J Exp Med. 2009; 206:343–357. [PubMed: 19204112]
2. Fontenot JD, Gavin MA, Rudensky AY. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. Nat Immunol. 2003; 4:330–336. [PubMed: 12612578]

3. Kronenberg M, Rudensky A. Regulation of immunity by self-reactive T cells. Nature. 2005; 435:598–604. [PubMed: 15931212]
4. Samstein RM, Josefowicz SZ, Arvey A, Treuting PM, Rudensky AY. Extrathymic generation of regulatory T cells in placental mammals mitigates maternal-fetal conflict. Cell. 2012; 150:29–38. [PubMed: 22770213]
5. Kim JM, Rasmussen JP, Rudensky AY. Regulatory T cells prevent catastrophic autoimmunity throughout the lifespan of mice. Nat Immunol. 2007; 8:191–197. [PubMed: 17136045]
6. Issa F, Wood KJ. CD4+ regulatory T cells in solid organ transplantation. Current opinion in organ transplantation. 2010; 15:757–764. [PubMed: 20881492]
7. Regateiro FS, Howie D, Cobbold SP, Waldmann H. TGF-beta in transplantation tolerance. Curr Opin Immunol. 2011; 23:660–669. [PubMed: 21839624]
8. Hilbrands R, Howie D, Cobbold S, Waldmann H. Regulatory T cells and transplantation tolerance. Immunotherapy. 2013; 5:717–731. [PubMed: 23829623]
9. Kendal AR, Chen Y, Regateiro FS, Ma J, Adams E, Cobbold SP, Hori S, Waldmann H. Sustained suppression by Foxp3+ regulatory T cells is vital for infectious transplantation tolerance. J Exp Med. 2011; 208:2043–2053. [PubMed: 21875958]
10. Heeger PS, Lalli PN, Lin F, Valujskikh A, Liu J, Muqim N, Xu Y, Medof ME. Decay-accelerating factor modulates induction of T cell immunity. J Exp Med. 2005; 201:1523–1530. [PubMed: 15883171]
11. Lalli PN, Strainic MG, Lin F, Medof ME, Heeger PS. Decay accelerating factor can control T cell differentiation into IFN-gamma-producing effector cells via regulating local C5a-induced IL-12 production. J Immunol. 2007; 179:5793–5802. [PubMed: 17947652]
12. Lalli PN, Strainic MG, Yang M, Lin F, Medof ME, Heeger PS. Locally produced C5a binds to T cell-expressed C5aR to enhance effector T-cell expansion by limiting antigen-induced apoptosis. Blood. 2008; 112:1759–1766. [PubMed: 18567839]
13. Lin M, Yin N, Murphy B, Medof ME, Segerer S, Heeger PS, Schroppel B. Immune cell-derived c3 is required for autoimmune diabetes induced by multiple low doses of streptozotocin. Diabetes. 2010; 59:2247–2252. [PubMed: 20584999]
14. Liu J, Lin F, Strainic MG, An F, Miller RH, Altuntas CZ, Heeger PS, Tuohy VK, Medof ME. IFN-gamma and IL-17 production in experimental autoimmune encephalomyelitis depends on local APC-T cell complement production. J Immunol. 2008; 180:5882–5889. [PubMed: 18424707]
15. Strainic MG, Liu J, Huang D, An F, Lalli PN, Muqim N, Shapiro VS, Dubyak GR, Heeger PS, Medof ME. Locally produced complement fragments C5a and C3a provide both costimulatory and survival signals to naive CD4+ T cells. Immunity. 2008; 28:425–435. [PubMed: 18328742]
16. Vieyra M, Leisman S, Raedler H, Kwan WH, Yang M, Strainic MG, Medof ME, Heeger PS. Complement regulates CD4 T-cell help to CD8 T cells required for murine allograft rejection. The American journal of pathology. 2011; 179:766–774. [PubMed: 21704012]
17. Sheen JH, Strainic MG, Liu J, Zhang W, Yi Z, Medof ME, Heeger PS. TLR-Induced Murine Dendritic Cell (DC) Activation Requires DC-Intrinsic Complement. J Immunol. 2017; 199:278–291. [PubMed: 28539427]
18. Strainic MG, Shevach EM, An F, Lin F, Medof ME. Absence of signaling into CD4(+) cells via C3aR and C5aR enables autoinductive TGF-beta1 signaling and induction of Foxp3(+) regulatory T cells. Nat Immunol. 2013; 14:162–171. [PubMed: 23263555]
19. Kwan WH, van der Touw W, Paz-Artal E, Li MO, Heeger PS. Signaling through C5a receptor and C3a receptor diminishes function of murine natural regulatory T cells. J Exp Med. 2013; 210:257–268. [PubMed: 23382542]
20. van der Touw W, Cravedi P, Kwan WH, Paz-Artal E, Merad M, Heeger PS. Cutting Edge: Receptors for C3a and C5a Modulate Stability of Alloantigen-Reactive Induced Regulatory T Cells. J Immunol. 2013
21. Okinaga S, Slattery D, Humbles A, Zsengeller Z, Morteau O, Kinrade MB, Brodbeck RM, Krause JE, Choe HR, Gerard NP, Gerard C. C5L2, a nonsignaling C5A binding protein. Biochemistry. 2003; 42:9406–9415. [PubMed: 12899627]

22. Gerard NP, Lu B, Liu P, Craig S, Fujiwara Y, Okinaga S, Gerard C. An anti-inflammatory function for the complement anaphylatoxin C5a-binding protein, C5L2. J Biol Chem. 2005; 280:39677–39680. [PubMed: 16204243]

23. Kim H, Erdman LK, Lu Z, Serghides L, Zhong K, Dhabangi A, Musoke C, Gerard C, Cserti-Gazdewich C, Liles WC, Kain KC. Functional roles for C5a and C5aR but not C5L2 in the pathogenesis of human and experimental cerebral malaria. Infect Immun. 2014; 82:371–379. [PubMed: 24191300]

24. Arbore G, West EE, Spolski R, Robertson AAB, Klos A, Rheinheimer C, Dutow P, Woodruff TM, Yu ZX, O'Neill LA, Coll RC, Sher A, Leonard WJ, Kohl J, Monk P, Cooper MA, Arno M, Afzali B, Lachmann HJ, Cope AP, Mayer-Barber KD, Kemper C. T helper 1 immunity requires complement-driven NLRP3 inflammasome activity in CD4(+) T cells. Science. 2016; 352:aad1210. [PubMed: 27313051]

25. Selle J, Asare Y, Kohncke J, Alampour-Rajabi S, Shagdarsuren G, Klos A, Weber C, Jankowski J, Shagdarsuren E. Atheroprotective role of C5ar2 deficiency in apolipoprotein E-deficient mice. Thromb Haemost. 2015; 114:848–858. [PubMed: 26084965]

26. Zhang X, Schmudde I, Laumonnier Y, Pandey MK, Clark JR, Konig P, Gerard NP, Gerard C, Wills-Karp M, Kohl J. A critical role for C5L2 in the pathogenesis of experimental allergic asthma. J Immunol. 2010; 185:6741–6752. [PubMed: 20974988]

27. Rittirsch D, Flierl MA, Nadeau BA, Day DE, Huber-Lang M, Mackay CR, Zetoune FS, Gerard NP, Cianflone K, Kohl J, Gerard C, Sarma JV, Ward PA. Functional roles for C5a receptors in sepsis. Nat Med. 2008; 14:551–557. [PubMed: 18454156]

28. Ward PA. The harmful role of c5a on innate immunity in sepsis. J Innate Immun. 2010; 2:439–445. [PubMed: 20588003]

29. Bachmaier K, Guzman E, Kawamura T, Gao X, Malik AB. Sphingosine kinase 1 mediation of expression of the anaphylatoxin receptor C5L2 dampens the inflammatory response to endotoxin. PLoS One. 2012; 7:e30742. [PubMed: 22355325]

30. Bosmann M, Haggadone MD, Zetoune FS, Sarma JV, Ward PA. The interaction between C5a and both C5aR and C5L2 receptors is required for production of G-CSF during acute inflammation. Eur J Immunol. 2013; 43:1907–1913. [PubMed: 23575697]

31. Wang R, Lu B, Gerard C, Gerard NP. Disruption of the complement anaphylatoxin receptor C5L2 exacerbates inflammation in allergic contact dermatitis. J Immunol. 2013; 191:4001–4009. [PubMed: 24043888]

32. Honczarenko M, Lu B, Nicholson-Weller A, Gerard NP, Silberstein LE, Gerard C. C5L2 receptor is not involved in C3a / C3a-desArg-mediated enhancement of bone marrow hematopoietic cell migration to CXCL12. Leukemia. 2005; 19:1682–1683. author reply 1684–1685. [PubMed: 15990859]

33. Croker DE, Monk PN, Halai R, Kaeslin G, Schofield Z, Wu MC, Clark RJ, Blaskovich MA, Morikis D, Floudas CA, Cooper MA, Woodruff TM. Discovery of functionally selective C5aR2 ligands: novel modulators of C5a signalling. Immunol Cell Biol. 2016; 94:787–795. [PubMed: 27108698]

34. Karsten CM, Wiese AV, Mey F, Figge J, Woodruff TM, Reuter T, Scurtu O, Kordowski A, Almeida LN, Briukhovetska D, Quell KM, Sun J, Ender F, Schmudde I, Vollbrandt T, Laumonnier Y, Kohl J. Monitoring C5aR2 Expression Using a Floxed tdTomato-C5aR2 Knock-In Mouse. J Immunol. 2017; 199:3234–3248. [PubMed: 28864475]

35. Pundir P, MacDonald CA, Kulka M. The Novel Receptor C5aR2 Is Required for C5a-Mediated Human Mast Cell Adhesion, Migration, and Proinflammatory Mediator Production. J Immunol. 2015; 195:2774–2787. [PubMed: 26283482]

36. Zhumabekov T, Corbella P, Tolaini M, Kioussis D. Improved version of a human CD2 minigene based vector for T cell-specific expression in transgenic mice. J Immunol Methods. 1995; 185:133–140. [PubMed: 7665895]

37. Yang TY, Chen SC, Leach MW, Manfra D, Homey B, Wiekowski M, Sullivan L, Jenh CH, Narula SK, Chensue SW, Lira SA. Transgenic expression of the chemokine receptor encoded by human herpesvirus 8 induces an angioproliferative disease resembling Kaposi's sarcoma. J Exp Med. 2000; 191:445–454. [PubMed: 10662790]

38. Benichou G, Valujskikh A, Heeger PS. Contributions of direct and indirect T cell alloreactivity during allograft rejection in mice. J Immunol. 1999; 162:352–358. [PubMed: 9886406]
39. Valujskikh A, Pantenburg B, Heeger PS. Primed allospecific T cells prevent the effects of costimulatory blockade on prolonged cardiac allograft survival in mice. Am J Transplant. 2002; 2:501–509. [PubMed: 12118893]
40. Purroy C, Fairchild RL, Tanaka T, Baldwin WM 3rd, Manrique J, Madsen JC, Colvin RB, Alessandrini A, Blazar BR, Fribourg M, Donadei C, Maggiore U, Heeger PS, Cravedi P. Erythropoietin Receptor-Mediated Molecular Crosstalk Promotes T Cell Immunoregulation and Transplant Survival. J Am Soc Nephrol. 2017; 28:2377–2392. [PubMed: 28302753]
41. Khalil AM, Cambier JC, Shlomchik MJ. B cell receptor signal transduction in the GC is short-circuited by high phosphatase activity. Science. 2012; 336:1178–1181. [PubMed: 22555432]
42. Rao RR, Li Q, Gubbels Bupp MR, Shrikant PA. Transcription factor Foxo1 represses T-bet-mediated effector functions and promotes memory CD8(+) T cell differentiation. Immunity. 2012; 36:374–387. [PubMed: 22425248]
43. Bamberg CE, Mackay CR, Lee H, Zahra D, Jackson J, Lim YS, Whitfeld PL, Craig S, Corsini E, Lu B, Gerard C, Gerard NP. The C5a receptor (C5aR) C5L2 is a modulator of C5aR-mediated signal transduction. J Biol Chem. 2010; 285:7633–7644. [PubMed: 20044484]
44. Ochando JC, Homma C, Yang Y, Hidalgo A, Garin A, Tacke F, Angeli V, Li Y, Boros P, Ding Y, Jessberger R, Trinchieri G, Lira SA, Randolph GJ, Bromberg JS. Alloantigen-presenting plasmacytoid dendritic cells mediate tolerance to vascularized grafts. Nat Immunol. 2006; 7:652–662. [PubMed: 16633346]
45. Burrell BE, Bromberg JS. Fates of CD4+ T cells in a tolerant environment depend on timing and place of antigen exposure. Am J Transplant. 2012; 12:576–589. [PubMed: 22176785]
46. DeWire SM, Ahn S, Lefkowitz RJ, Shenoy SK. Beta-arrestins and cell signaling. Annu Rev Physiol. 2007; 69:483–510. [PubMed: 17305471]
47. Croker DE, Halai R, Kaeslin G, Wende E, Fehlhaber B, Klos A, Monk PN, Cooper MA. C5a2 can modulate ERK1/2 signaling in macrophages via heteromer formation with C5a1 and beta-arrestin recruitment. Immunol Cell Biol. 2014; 92:631–639. [PubMed: 24777312]
48. Fribourg M, Moreno JL, Holloway T, Provasi D, Baki L, Mahajan R, Park G, Adney SK, Hatcher C, Eltit JM, Ruta JD, Albizu L, Li Z, Umali A, Shim J, Fabiato A, MacKerell AD Jr, Brezina V, Sealfon SC, Filizola M, Gonzalez-Maeso J, Logothetis DE. Decoding the signaling of a GPCR heteromeric complex reveals a unifying mechanism of action of antipsychotic drugs. Cell. 2011; 147:1011–1023. [PubMed: 22118459]
49. Hsu WC, Yang FC, Lin CH, Hsieh SL, Chen NJ. C5L2 is required for C5a-triggered receptor internalization and ERK signaling. Cell Signal. 2014; 26:1409–1419. [PubMed: 24631530]
50. Li R, Coulthard LG, Wu MC, Taylor SM, Woodruff TM. C5L2: a controversial receptor of complement anaphylatoxin, C5a. FASEB J. 2013; 27:855–864. [PubMed: 23239822]
51. Gao H, Neff TA, Guo RF, Speyer CL, Sarma JV, Tomlins S, Man Y, Riedemann NC, Hoesel LM, Younkin E, Zetoune FS, Ward PA. Evidence for a functional role of the second C5a receptor C5L2. FASEB J. 2005; 19:1003–1005. [PubMed: 15784721]
52. Poppelaars F, van Werkhoven MB, Kotimaa J, Veldhuis ZJ, Ausema A, Broeren SGM, Damman J, Hempel JC, Leuvenink HGD, Daha MR, van Son WJ, van Kooten C, van Os RP, Hillebrands JL, Seelen MA. Critical role for complement receptor C5aR2 in the pathogenesis of renal ischemia-reperfusion injury. FASEB J. 2017; 31:3193–3204. [PubMed: 28396344]

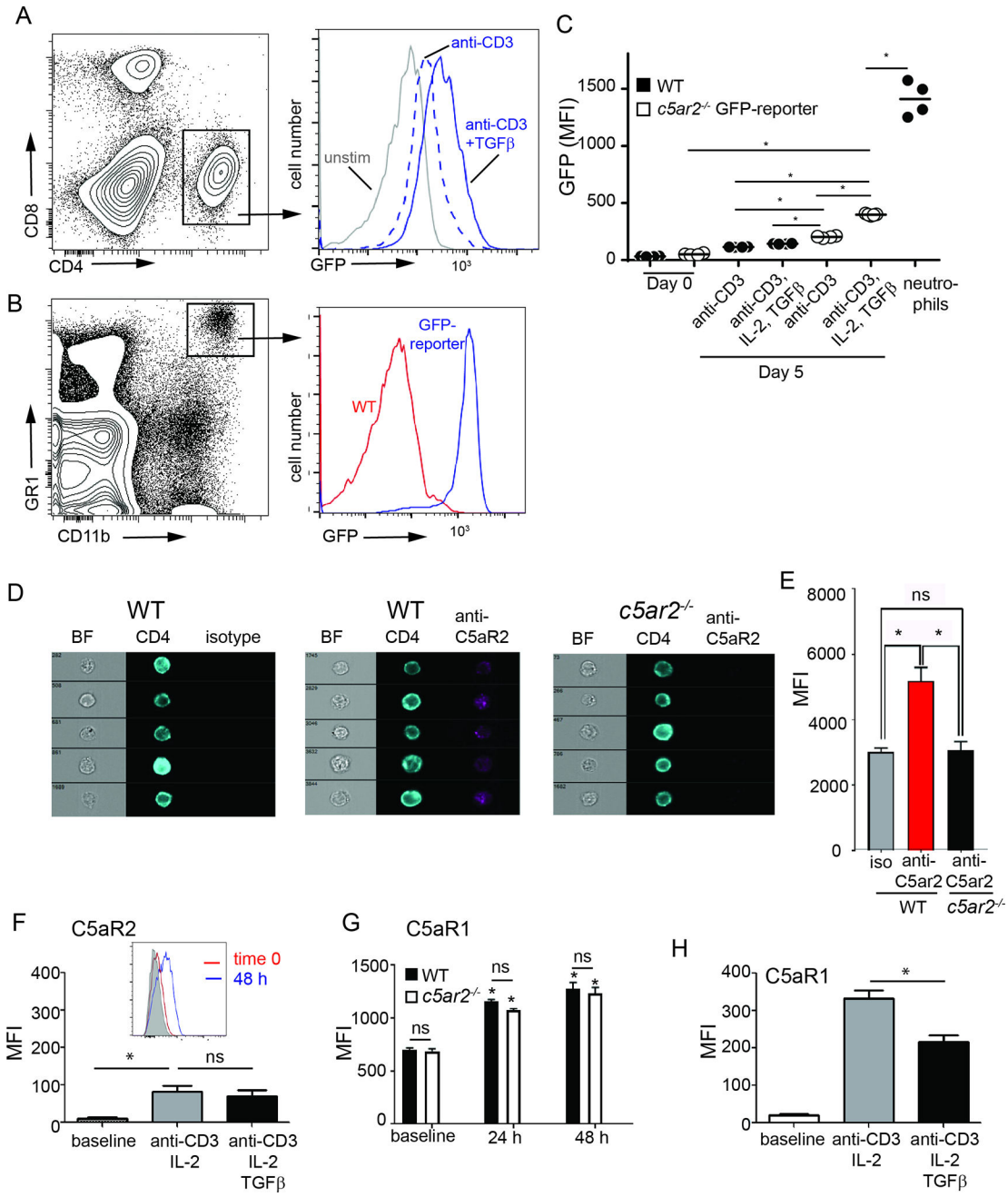

Figure 1.

C5aR2 is expressed in CD4$^+$ T_{REG}. A. Representative flow cytometry plot gated on CD4$^+$ cells (left panel) showing GFP expression (right panel) within splenic *c5ar2*$^{-/-}$ C5aR2-GFP reporter mice at baseline (grey line) and on day 5 following stimulation with anti-CD3 (blue dashed lines) or anti-CD3 + TGFβ/IL2 (blue solid lines). B. Representative flow cytometry plot showing GFP expression (right panel) from WT (no reporter, red) and *c5ar2*$^{-/-}$ GFP-reporter (blue) mice gated on unstimulated GR1$^+$CD11b$^+$ neutrophils (left panel). C. Quantified results of GFP expression in WT and *c5ar2*$^{-/-}$ GFP reporter CD4$^+$ T cells under the conditions listed. n=4/group. D–E. Five representative images (D) and quantified results

(E) from imaging flow studies of spleen cells from WT or *c5ar2*$^{-/-}$ mice stimulated *in vitro* for 48 h with anti-CD3, TGFβ and IL-2. Bright field (BR) images and images stained with anti-CD4, anti-C5aR2 or isotype control as indicated are shown (a duplicate enlarged version of 1D is provided as Supplemental Figure 1A). F. Insert: representative standard flow cytometry plot of C5aR2 expression on unstimulated (red) or anti-CD3 stimulated (48 h, blue) WT spleen cells gated on CD4^{+} T cells. Gray filled histogram: isotype control. Median fluorescence intensity (MFI) of surface expressed C5aR2 on spleen cells gated on the CD4^{+} subset, at (baseline) and 48 h after stimulation with anti-CD3+IL-2 or anti-CD3+IL-2+TGFβ as indicated. n=4/group. G. Surface expression of C5aR1 on WT and *c5ar2*$^{-/-}$ spleen cells within the CD4+ gate at baseline and 24 and 48 h after *in vitro* stimulation with anti-CD3. Representative flow cytometry plot is shown in Supplemental Figure 1. H. Median fluorescence intensity (MFI) of surface expressed C5aR1 on spleen cells gated on the CD4^{+} subset, at (baseline) and 48 h after stimulation with anti-CD3+IL-2 or anti-CD3+IL-2+TGFβ as indicated. n=4/group. All experiments were repeated at least once. ns=not significant. *p<0.05 throughout; in panel G, * refers to comparison with expression level at baseline.

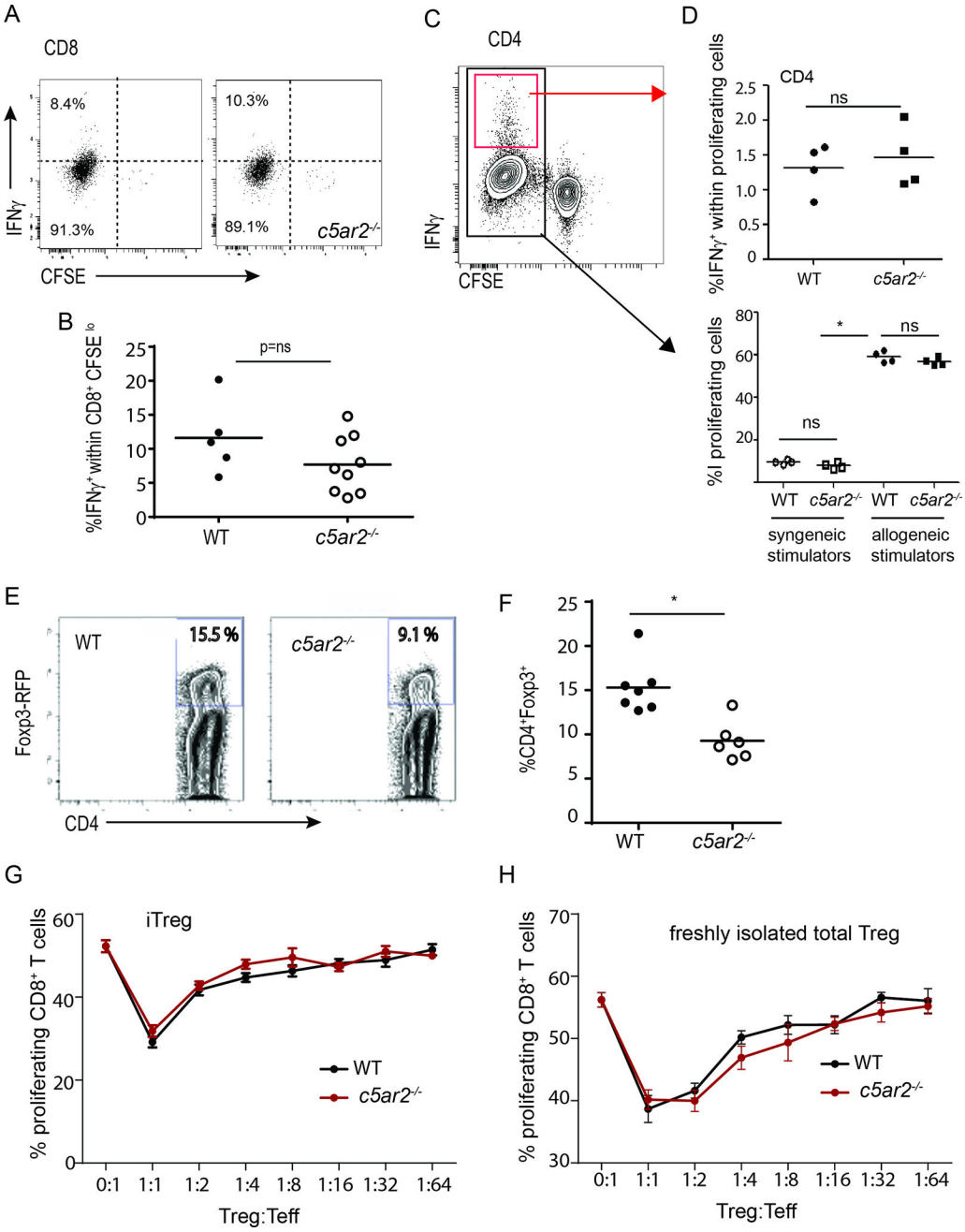

Figure 2.

Absence of C5aR2 limits iT_{REG} generation. A–B. Representative flow plots (A) and quantification for IFNγ expression (B) in an MLR gated on $CD8^+$ T cells from spleens of WT B6 mice stimulated with BALB/c stimulators for 4 days. C. Representative flow plot for CFSE and IFNγ expression in an MLR using naive $CD62L^{hi}CD44^{lo}CD4^+$ T cells from WT B6 mice stimulated with BALB/c stimulators for 4 days. D. Quantification of proliferating $CSFE^{lo}$ naive WT vs *c5ar2*$^{-/-}$ $CD4^+$ T cells stimulated with syngeneic (control) or allogeneic stimulators (bottom),and percent $IFN\gamma^+$ within the $CFSE^{lo}$ population (top), gated on $CD4^+$ T cells. n=4/group, one of 2 representative experiments. E–F. Representative

flow plots (E) and quantified percentages of Foxp3-RFP$^+$ iT^{REG} (F) on day 4 after stimulating naïve WT or *c5ar2*$^{-/-}$ Foxp3-RFP T cells with allogeneic APCs, IL-2 and TGFβ. G–H. *In vitro* suppression assays using flow sorted WT or *c5ar2*$^{-/-}$ Foxp3-RFP$^+$ iT$_{REG}$ obtained from cultures in C–D (G) or flow sorted splenic Foxp3-RFP$^+$CD4$^+$ T cells from WT or *c5ar2*$^{-/-}$ Foxp3-RFP$^+$ mice (H). no significant differences were noted at any of the T$_{REG}$:T$_{EFF}$ ratios in either experiment. ns=not significant, *p<0.05.

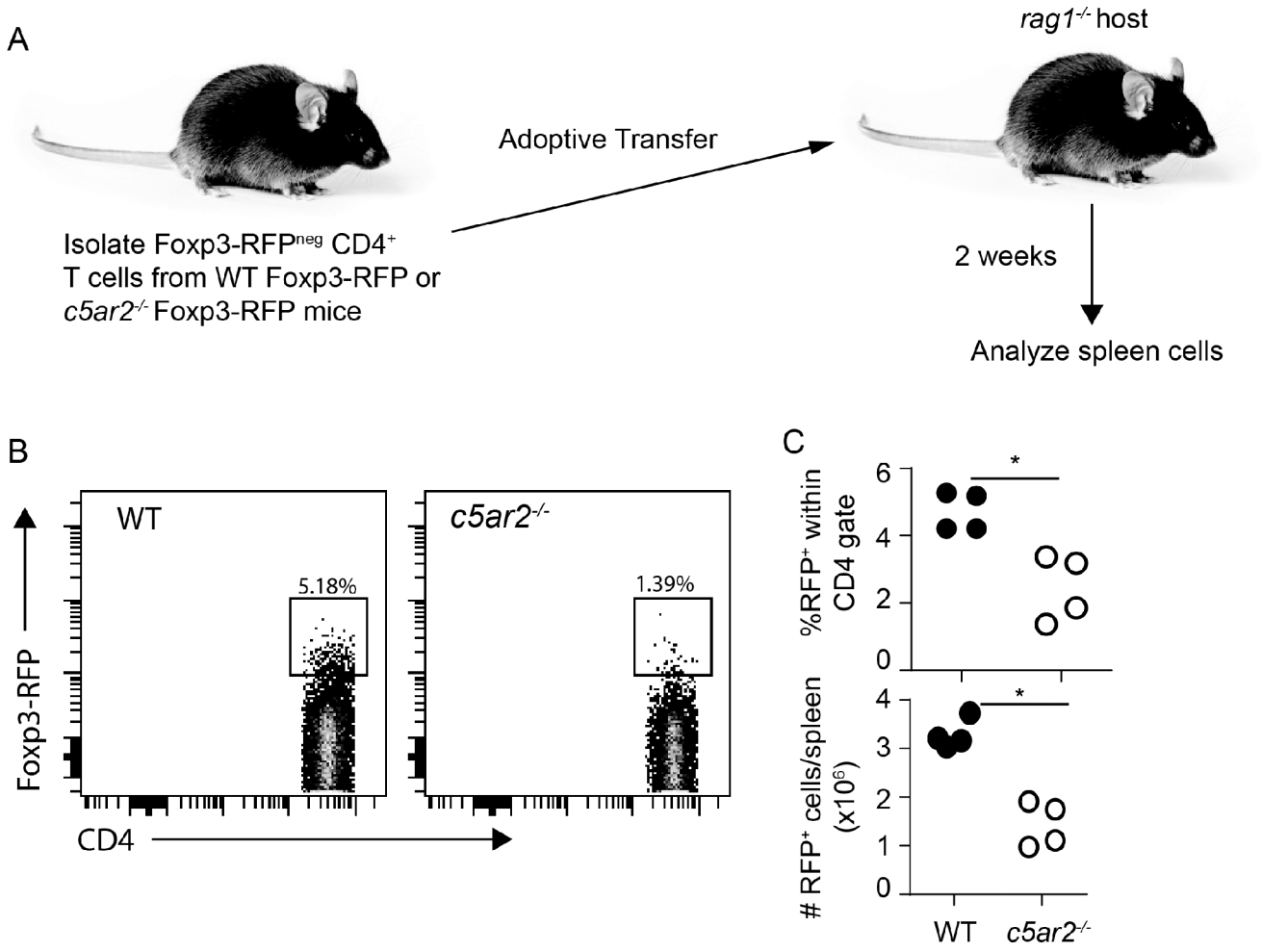

Figure 3.

C5aR2 deficiency limits iT_{REG} generation *in vivo*. A. Schematic of experimental design. B. Representative flow cytometry plots depicting splenic Foxp3-RFP^{+} cells within the CD4 gate 2 weeks after adoptive transfer of Foxp3-RFP^{neg}, naïve WT or *c5ar2*$^{-/-}$ Foxp3-RFP CD4^{+} T cells into *rag1*$^{-/-}$ recipients. C. Quantification of results showing percentages (top) and total number (bottom) of iT_{REG} in each animal. *p<0.05.

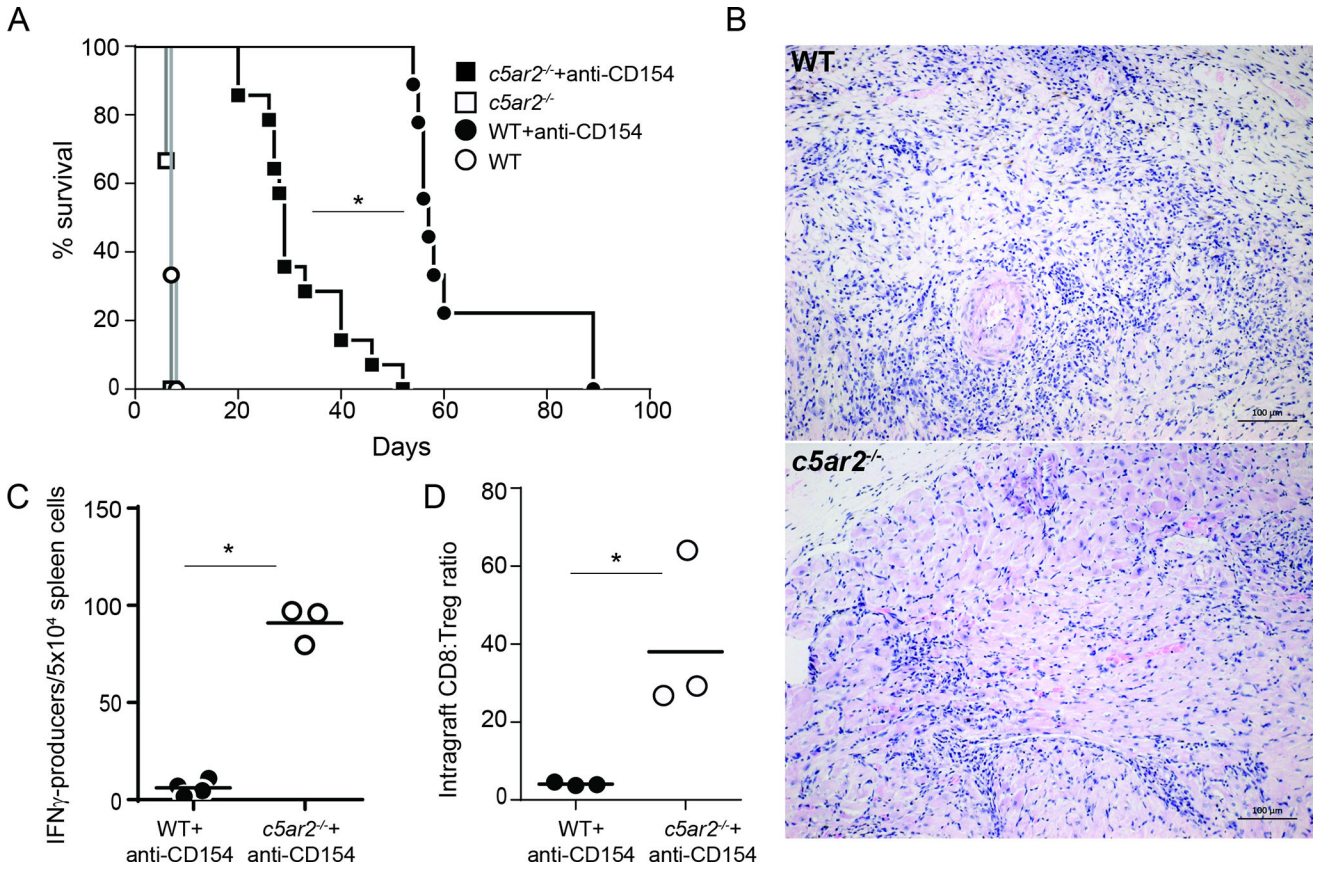

Figure 4.

Recipient C5aR2 deficiency accelerates T cell mediated cardiac allograft rejection. A. Survival of WT and congenic *c5ar2*$^{-/-}$ B6 recipients transplanted with allogeneic BALB/c hearts ± peri-transplant anti-CD154 mAb (MR1, 250 μg). B. Representative H&E stained sections of transplanted BALB/c heart tissue (7–9 animals per group) obtained at the time of rejection showing diffuse mononuclear cell infiltration consistent with acute cellular rejection in the WT and congenic *c5ar2*$^{-/-}$ B6 recipients. C–D. Groups of MR1-treated WT and *c5ar2*$^{-/-}$ allograft recipients were sacrificed on day 21 posttransplant. C. Splenic donor-reactive IFNγ producers were quantified by ELISPOT (one of three independent experiments with similar results). D. Intragraft ratios of $CD8^+$ T cells to $CD4^+Foxp3^+$ T cells as determined by flow cytometric analysis of graft infiltrating lymphocytes. *$p<0.05$

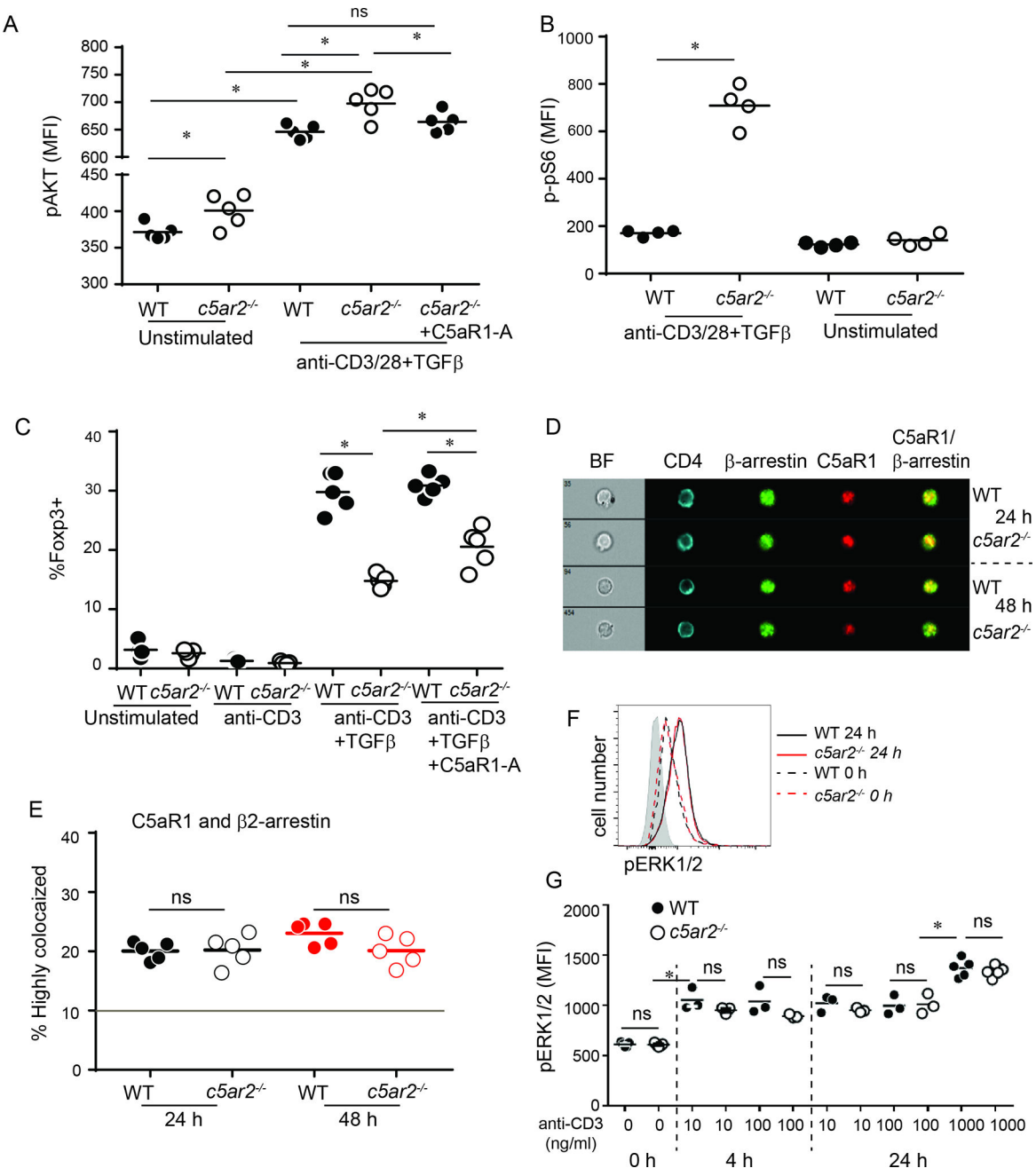

Figure 5.

C5aR2 expression augments iT_{REG} generation by limiting C5a/C5aR1 signaling on $CD4^+$ T cells. A–B. Quantification of intracellular phosphoflow cytometry analysis of pAKT (A) and p-pS6 (B) in naïve WT and *c5ar2*$^{-/-}$ $CD4^+$ T cells cultured with syngeneic APCs for 24 h under the conditions listed (anti-CD3, 100 μg/ml). Results are representative of ≥ 2 independent experiments. C. Foxp3 expression at the end of 5 day iT_{REG} generation assays using magnetically sorted naïve $CD4^+$ WT and *c5ar2*$^{-/-}$ cells under the conditions listed (anti-CD3 added at 1000 μg/ml). D-E. Representative imaging flow images (D) and quantified co-localization of C5aR1 and β2-arrestin (E) in WT and *c5ar2*$^{-/-}$ $CD4^+$ T cells

cultured for 24 or 48 h under iT_{REG} generating conditions (anti-CD3 1000 μg/ml). F. Representative flow cytometry plot (top) and quantification of intracellular phosphoflow cytometry analysis of pERK1/2 in WT and *c5ar2*$^{-/-}$ CD4^{+} T cells cultured for 4-24 h with anti-CD3 at the concentrations listed + IL-2 and TGFβ. ns=not significant. *$p<0.05$

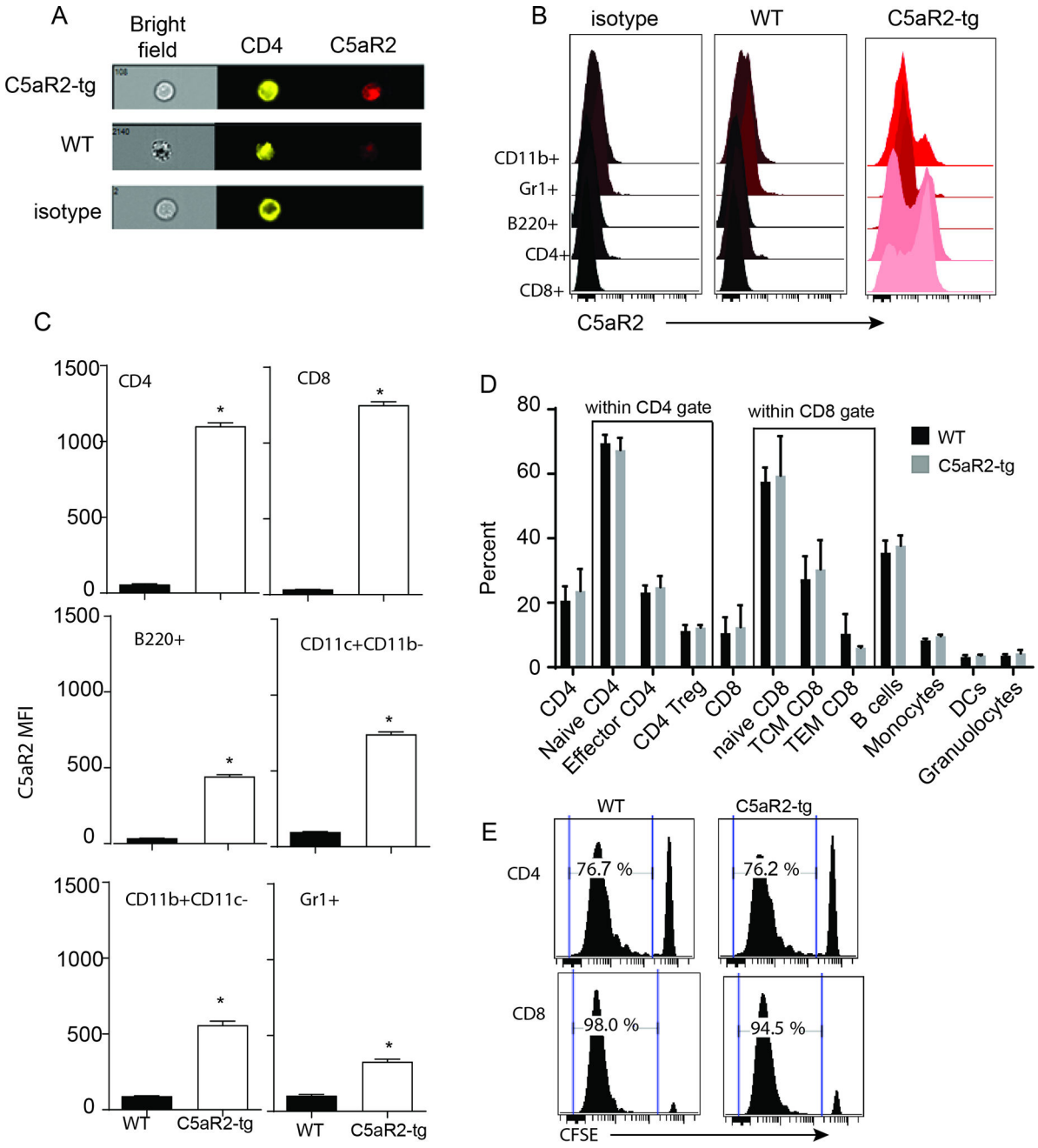

Figure 6.

Characterization of C5aR2-tg mice. A. Representative imaging flow images demonstrating elevated expression of C5aR2 within freshly isolated unstimulated $CD4^+$ T cells from C5aR2-tg animals. B–C. Representative standard flow cytometry overlay histogram plots (B) and quantified MFI values (C) depicted surface C5aR2 expression levels gated on the indicated cell types from WT and C5aR2-tg mice. D. Flow cytometric analysis of spleen cells from WT and C5aR2-tg mice showing no significant differences in the percentages of the major immune cell populations between groups. n=3–5 per group per experiment. E. Representative *in vitro* MLRs using CFSE labeled spleen cells from WT and C5aR2-tg mice

stimulated for 3 days in culture with BALB/c APCs demonstrate no significant differences between groups (p=ns for 4 replicates per condition, repeated with same results). *p<0.05.

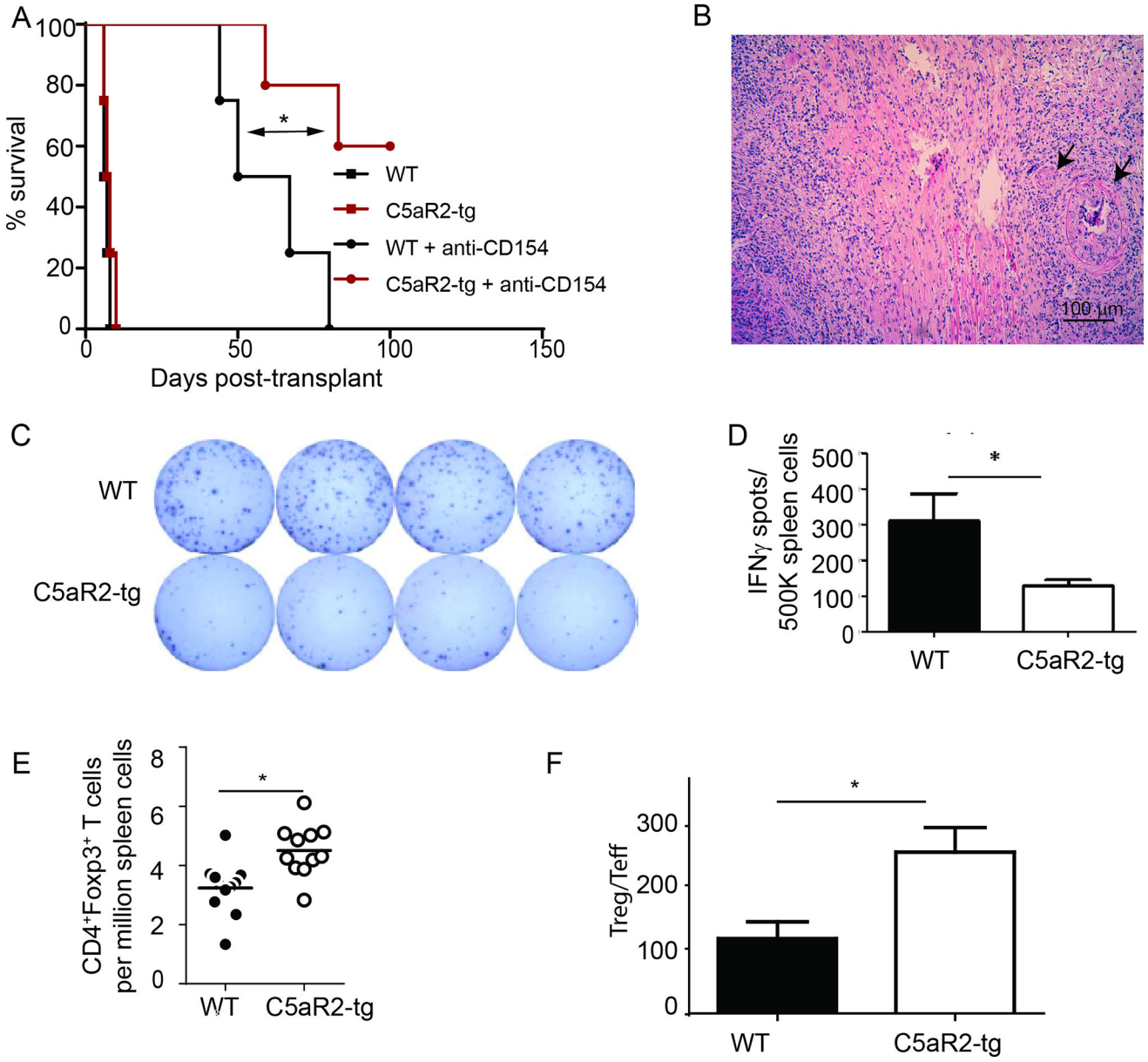

Figure 7.

Transgenic overexpression of C5aR2 under CD2 promoter promotes graft survival associated with fewer T_{EFF} and more T_{REG}. A. Survival of BALB/c hearts transplanted into WT or C5aR2-tg recipients ± peri-transplant MR1 (250 μg). The experiment was halted at 100 days posttransplant. Survival of the WT and the C5aR2-tg recipients treated with MR1 were significantly longer than untreated WT and C5aR2-tg recipients. Survival of grafts transplanted into anti-CD154-treated C5aR2-tg was statistically longer than those transplanted into anti-CD154-treated WT controls (*$p<0.05$). B. Representative H&E stained section from a BALB/c heart transplanted into an anti-CD154-treated C5aR2-tg recipient that was beating on day 100 posttransplant, demonstrating diffuse mononuclear cell infiltrates and vasculopathy (arrowheads). C–D. Representative IFNγ ELISPOT wells (C) and quantification of splenic donor-reactive IFNγ-producers (D) on day 28 posttransplant in

MR1-treated WT and C5aR2-tg recipients of BALB/c hearts. E–F. Total numbers of $CD4^+Foxp3^+$ splenic T cells (E) and ratios of T_{REG} to splenic donor-reactive IFNγ producers (T_{EFF}, F) in same animals depicted in C. *$p<0.05$.

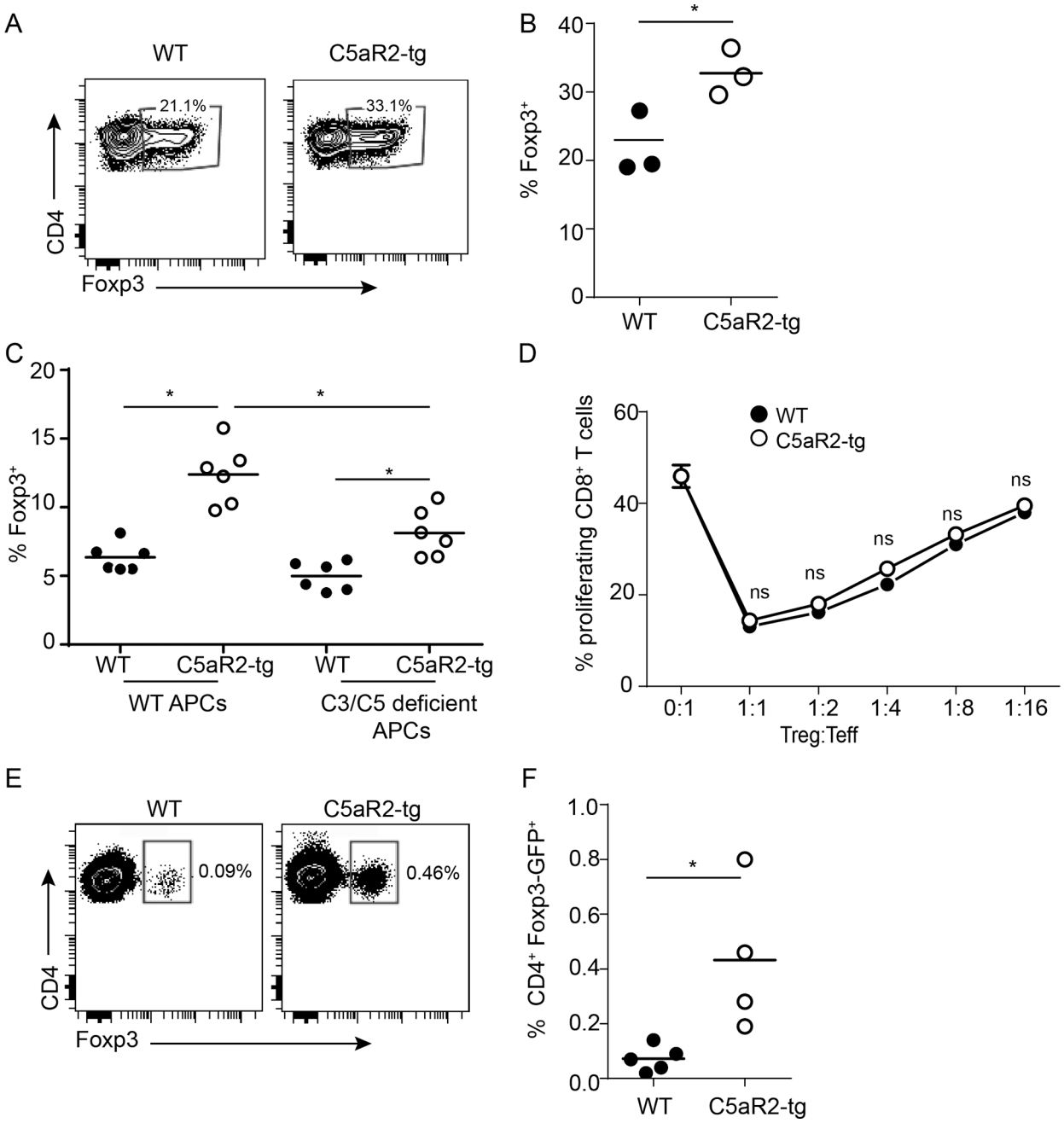

Figure 8.

Transgenic overexpression of C5aR2 promotes iT_{REG} generation *in vitro* and *in vivo*. A–C. Representative flow cytometry plots (A) and quantified Foxp3-GFP^{+} percentages on day 3 of iT_{REG} generation cultures (B–C) using flow sorted Foxp3-GFP^{neg} naïve WT and C5aR2-tg Foxp3-GFP CD4^{+} T cells and WT (B–C) or C3/C5 deficient allogeneic APCs (C). D. *In vitro* suppression assays using flow sorted Foxp3-GFP+ iT_{REG} induced in B. E–F. In vivo iT_{REG} generation assays. Representative flow cytometry plots (E) and quantification of splenic Foxp3-GFP^{+} CD4^{+} in spleens (F) of *rag1*$^{-/-}$ recipients 2 weeks after adoptive

transfer of equal numbers of flow sorted Foxp3-GFP^{neg} WT or C5aR2-tg Foxp3-GFP CD4^{+} T cells. *$p<0.05$, ns indicates $p>0.05$, not significant.