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C5aR1 regulates T follicular helper differentiation and chronic graft-versus-host disease bronchiolitis obliterans

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CD4⁺ follicular helper T (Tfh) cells are specialized providers of T cell help to B cells and can function as pathogenic mediators of murine antibody-dependent chronic graft-versus-host disease (GvHD). Using a parent→F1 model of lupus-like chronic GvHD, in which Tfh cell and germinal center (GC) B cell differentiation occurs over 14 days, we demonstrate that absence of CD4⁺ T cell-expressed C5a receptor 1 (*C5ar1*) or pharmacological C5aR1 blockade abrogated generation/expansion of Tfh cells, GC B cells, and autoantibodies. In a Tfh cell-dependent model of chronic GvHD manifested by bronchiolitis obliterans syndrome (BOS), C5aR1 antagonism initiated in mice with established disease ameliorated BOS and abolished the associated differentiation of Tfh and GC B cells. Guided by RNA-sequencing data, mechanistic studies performed using murine and human T cells showed that C5aR1 signaling amplifies IL-6-dependent expression of the transcription factor c-MAF and the cytokine IL-21 via phosphorylating phosphokinase B (AKT) and activating the mammalian target of rapamycin (mTOR). In addition to linking C5aR1-initiated signaling to Tfh cell differentiation, our findings suggest that C5aR1 may be a useful therapeutic target for prevention and/or treatment of individuals with Tfh cell-dependent diseases, including those chronic GvHD patients who have anti-host reactive antibodies.

Introduction

IL-21-producing CD4⁺ follicular helper T cells (Tfh cells) are recognized as specialized providers of T cell help to B cells (1–5). Tfh cells mediate Ig class switching and are required for germinal center (GC) formation and B cell affinity maturation. GC-derived plasma cells and memory T cells require Tfh cells, while pre-Tfh cells, in collaboration with extrafollicular activated B cells, can contribute to short-lived plasmablasts and the early memory B cell pool. Tfh cells can be distinguished from other CD4⁺ T effector cell subsets by their constitutive expression of CXCR5, which causes CXCL13-dependent positioning proximal to B cells in GCs, and by their surface expression of programmed cell death protein 1 (PD1) and inducible costimulator (ICOS) (1–6). Evidence indicates Tfh cell differentiation is a multistep *in vivo* process that principally involves naive T cells sequentially interacting with DCs and B cells. Tfh cell generation requires transduced signals derived from IL-6 and IL-21 as well as ligation of T cell surface-expressed receptor ICOS with its ligand, ICOS ligand, among other interactions. This developmental progression results in upregulation of CXCR5 and induction of a unique lineage-specific gene transcriptional program that includes the transcription factor (TF) STAT3, B cell lymphoma-6 (BCL6), and c-MAF (1, 7–11). Foxp3⁺CD4⁺ T follicular regulatory (Tfr) cells, a subset of Tregs (4, 12–14) that differentiate from naive thymus-derived Tregs, also express CXCR5, which permits them to locally inhibit Tfh cell differentiation and function. Despite the recognition that Tfh cells are essential contributors to physiological antibody affinity maturation and participate in a multitude of antibody-dependent disease processes, the biological signals that initiate and/or modulate *in vivo* Tfh cell differentiation are incompletely characterized.

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Blood and/or marrow transplantation can cure refractory hematological malignancies, but the transferred allogeneic donor lymphocytes commonly cause acute and/or chronic graft-versus-host disease (GvHD) (reviewed in refs. 15, 16). Chronic GvHD, a significant long-term complication that affects approximately 30%–70% of allogeneic blood/marrow recipients, manifests pathologically with inflammation-initiated fibrosis that can involve virtually all organs in the body, including the gastrointestinal tract, joints, mouth, eyes, lungs (bronchiolitis obliterans syndrome [BOS]), liver, and skin (16–19). Current concepts also support pathogenic roles for innate immune cells (20–22) and Th17, Th1, and Th2 cells as well as Tfh cells (regulated by Tfr cells); each have been linked to chronic GvHD (16, 23–25). The latter induce pathogenic IgG, alloantibodies, and/or autoantibodies and together with the T cells, and macrophages initiate a fibrogenic program that results in chronic organ injury in GvHD settings in which potentially pathogenic antibodies have been detected (16, 18, 24, 26).

The complement system, conventionally connected with innate immunity, is known to function as an effector arm of antibody-initiated immune injury (27–29). Complement also regulates B cell activation thresholds (30) and crucially regulates proinflammatory T cell immune responses (31–34), including those to alloantigens (32, 35–39). Despite these established associations, whether the complement system is mechanistically linked to Tfh cell induction and/or function and to the pathogenesis of Tfh cell-dependent diseases, including some models of chronic GvHD, remains incompletely understood. Building upon previous publications implicating signaling transmitted through T cell-expressed C5aR1, a 7-transmembrane-spanning G protein-coupled receptor, as regulating murine and human Th1 immunity (33–35, 40, 41), we tested the hypothesis that T cell-expressed C5aR1 transmits signals that effect Tfh cells. Our studies point to a critical effect of C5aR1 in supporting the differentiation of Tfh cells and indicate that, as a result, C5aR1 signaling drives the GC reaction and downstream GC and antibody-dependent induction of BOS in a model of chronic GvHD.

Results

Recipient C3 mediates Tfh cell-dependent alloimmune responses in vivo. To initially test whether the complement system affects donor Tfh cells formation and function in vivo, we employed a parent→F1 model of alloimmunity in which CD8-depleted B6 spleen cells are injected into nonirradiated semiallogeneic (bx_d)F1 recipients (42). Others showed that in this model system alloreactive donor CD4⁺ T cells expand and differentiate into Tfh cells, with associated induction of GC B cells and production of autoantibodies, including anti-ds-DNA, within 14 days (42). Using this established model, we injected WT CD8-depleted B6 spleen cells into WT (bx_d)F1 recipients or (bx_d)F1 recipients deficient in the central complement component C3, and 14 days later analyzed recipient spleen cells by flow cytometry (Figure 1).

Consistent with previous work in this system, spleens of WT F1 adoptive recipients contained 2-fold more cells than naive F1 controls (160×10^6 vs. 75×10^6 cells, $P < 0.05$, data not shown). The C3^{-/-} F1 spleens contained fewer cells than WT F1 recipients (135×10^6 cells, $P < 0.05$ vs. WT F1, data not shown). We analyzed the effects of recipient C3 on donor Tfh cells by quantifying frequencies and total numbers of TCR⁺CD4⁺PD1⁺CXCR5⁺Foxp3⁻ Tfh cells within the H-2^d (donor cell) gate (Figure 1A). These analyses showed fewer Tfh cells in the C3^{-/-} recipients (Figure 1B). We also observed fewer TCR⁺CD4⁺PD1⁺CXCR5⁺Foxp3⁺ Tfr cells (Figure 1B), although the absolute numbers were at the limit of detection of the assays. Together, the data imply that the absence of C3 inhibits Tfh cell differentiation/expansion.

To test for functional links among C3, Tfh cells, and recipient B cell differentiation, we phenotyped the recipient splenic B cells in the adoptive recipients on day 14 after transfer, quantifying B220⁺Fas⁺GL7⁺ (GC) and B220⁺IgM-IgD⁻ (class-switched) B cells. These analyses (Figure 2, A and B) showed fewer GC B cells and fewer class-switched B cells in the C3^{-/-} F1 mice (percentage and total number). To assess whether the C3-dependent changes in B cells altered autoantibody production, we analyzed day 14 sera for anti-double-stranded DNA (anti-dsDNA) IgG (Figure 2C). The assays showed less anti-dsDNA reactivity in the C3^{-/-} F1 recipients. Together, the data support the conclusion that recipient C3 regulates differentiation and expansion of Tfh cells, GC B cell differentiation, and autoantibody formation in this system.

C5aR1 modulates Tfh cell-dependent alloimmune responses in vivo. C3 is required to form the C5 convertase, which cleaves C5 to biologically active C5a and C5b. Our prior work showed that C5a, the activation product that results from C3- and factor B-dependent complement activation, ligates its receptor, C5aR1, expressed on CD4⁺ T cells, transmitting proliferative and prosurvival signals that augment Th1 immunity (32, 33). Building upon these findings and the above observed effects of C3 deficiency on Tfh cells (Figures 1 and 2), we tested the distinct hypothesis that T cell-expressed C5aR1 controls Tfh cell-differentiation in vivo.

We injected WT or *C5ar1*^{-/-} CD8-depleted spleen cells into WT (bxd)F1 recipients and 14 days later analyzed spleen cell phenotypes (flow cytometry; Figure 3). In confirmation of our previous reports (32, 33), WT CD4⁺ T cells express C5aR1 (Supplemental Figure 1; supplemental material available online with this article; <https://doi.org/10.1172/jci.insight.124646DS1>).

Compared with spleens of WT F1 recipients of WT donor cells, the day 14 spleens obtained from WT F1 recipients of *C5ar1*^{-/-} donor cells contained fewer total cells (Figure 3A) and fewer donor-derived Tfh cells, with higher numbers of donor-derived Tfr cells (Figure 3B), resulting in higher Tfr/Tfh cell ratios (Figure 3C). Spleens from the recipients of *C5ar1*^{-/-} donor cells also contained fewer GC B cells (Figure 3D) and fewer class-switched B cells (Figure 3E). Serum ELISAs showed lower anti-dsDNA antibody levels in the recipients of *C5ar1*^{-/-} donor cells [which were not different from background levels in naive (bxd)F1 mice, Figure 3F].

In conversely designed experiments to test the effects of C5aR1 deficiency on host cells, we injected WT CD8-depleted B6 spleen cells into WT and *C5ar1*^{-/-} (bxd)F1 recipients, verifying the absence of C5aR1 on recipient spleen cells (data not shown). Analyses performed 14 days later (Figure 3, G and H) revealed no differences in total numbers of splenic Tfh cells or GC B cells between groups. Together, these findings indicate that Tfh cell differentiation is regulated by host-derived C3 and C5aR1 expression on the responding donor CD4⁺ T cells.

Using a potentially clinically relevant pharmacologic strategy that blocks C5aR1 signaling on all cells, we tested the effects of a specific cyclic peptide C5aR1 antagonist (C5aR1-A), administered at the time of CD8-depleted spleen cell transfer, on the induced immune response (Figure 4). On day 14 after transfer, spleens of C5aR1-A–treated mice were smaller (Figure 4A) and contained fewer donor Tfh cells and Tfr cells (Figure 4B), with a trend toward a statistically insignificant increase in Tfr/Tfh cell ratios (Figure 4C, $P = 0.07$). Spleen cells from the C5aR1-A–treated animals also contained fewer GC B cells (Figure 4D), fewer class-switched B cells (Figure 4E), and lower serum autoantibody levels (Figure 4F) than those from the controls.

C5aR1 blockade reverses multiorgan system chronic GvHD with BOS. While the parent→F1 model is useful for analysis and manipulation of Tfh cells and GC B cell responses in vivo in the absence of irradiation effects, this system does not simulate chronic GvHD induction in conditioned patients nor does the model provide a strong clinical/pathological disease phenotype. To test the hypothesis that specific targeting of C5aR1 limits Tfh cell differentiation and antibody production required for the clinical expression of a Tfh cell–dependent disease process, we employed a multiorgan system chronic GvHD model that we have shown to be Tfh cell dependent and Tfr cell regulated (43, 44). B10.BR mice were given high-dose cyclophosphamide (120 mg/kg/d × 2) followed by total body irradiation (TBI) (8.3 Gy), a conditioning regimen extensively employed in the clinic. Recipients were transplanted with B6 bone marrow supplemented with a low dose of T cells to avoid the intense inflammation associated with acute GvHD while still providing TCR stimulation to induce chronic GvHD. Among other affected organs, by 4 weeks after transplant, WT recipients of fully allogeneic WT donor cells reproducibly develop BOS and pulmonary fibrosis that can be readily measured by pulmonary function tests and tissue analyses.

To test the efficacy of C5aR1 blockade on the outcome of ongoing chronic GvHD with BOS, we initiated continuous therapy with C5aR1-A or vehicle control via an osmotic pump on day 28 (for 30 days). On day 60 after transplant, we tested for clinical evidence of BOS by performing pulmonary function tests. These analyses showed that vehicle-treated recipients of donor bone marrow and T cells developed increased pulmonary resistance and elastance, along with decreased pulmonary compliance, consistent with BOS. Remarkably, C5aR1-A administration beginning on day 28 restored all three of these functional measures to levels observed in control animals that only received donor bone marrow (Figure 5, A–C). Immunophenotyping of spleen cells on day 60 additionally revealed that C5aR1-A treatment reduced the elevated frequencies of GC B cells and Tfh cells and increased the Tfr/Tfh cell ratios compared with those in vehicle control-treated chronic GvHD mice with BOS (Figure 5, D–G). Ig deposition in the lungs trended lower in the C5aR1-A–treated animals but did not reach statistical significance (Figure 5H).

We also tested the therapeutic efficacy of C5aR1-A administration in the B10.D2→BALB/c model of chronic GvHD that recapitulates many clinical characteristics of scleroderma (25, 45). Previous studies have shown that scleroderma pathogenesis in this system involves CD4⁺ Th17 cells as well as STAT3 signaling (46), without clear evidence for involvement of Tfh cell–dependent GCs (25, 45). Therapeutic administration of C5aR1-A beginning on day 14 (after clinical manifestations of the disease have initiated) had

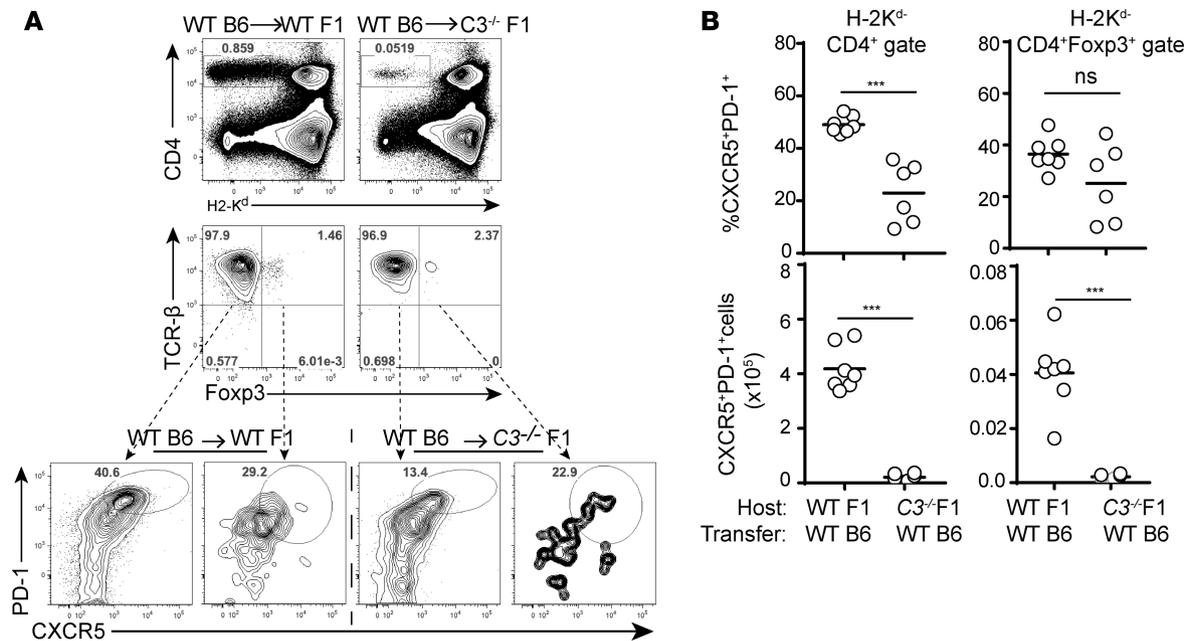


Figure 1. Recipient C3 deficiency prevents Tfh cell induction in a parent→F1 model of alloimmunity. CD8-depleted WT B6 spleen cells were injected i.v. into WT or C3^{-/-} (bx^d)F1 recipients and analyses were performed on day 14. **(A)** Representative flow cytometry histograms and gating strategies for detection of Tfh cells and Tfr cells. **(B)** Quantified percentages (top) and total numbers (bottom) of Tfh cells (left) and Tfr cells (right). Combined data of 2 separate experiments (6–7 mice per group). ****P* < 0.001 by Student's *t* test.

no effect on the progression of the scleroderma phenotype (Figure 5I). Together with the above observed therapeutic effects of C5aR1 antagonism on Tfh cell-dependent BOS (Figure 5, A–C), the data suggest that therapeutic C5aR1 antagonism (initiated after disease was initiated) preferentially modulates Tfh cell development and, as a consequence, Tfh cell function, in these alloimmune settings.

C5a/C5aR1 modulates Tfh cell differentiation. To provide clues to understanding mechanisms that link C5aR1 signaling on alloreactive T cells to Tfh cell differentiation, we adoptively transferred CD8-depleted WT or *C5ar1*^{-/-} spleen cells into groups of (bx^d)F1 recipients, as in Figure 4. Seven days we later reisolated donor (*H-2^d*) CD4⁺ T cells from the recipient spleens by flow sorting, isolated RNA, and performed RNA sequencing (Supplemental Figure 2), specifically examining effects of absent C5aR1 on genes known to be associated with Tfh cell differentiation. We chose to study the effects on day 7 (rather than day 14, as done for Figures 1–4), because we posited that this earlier time point would permit us to assess differences in gene expression patterns that were occurring during the *in vivo* Tfh cell differentiation process. We also sequenced RNA from naive CD4⁺ WT and *C5ar1*^{-/-} T cells as a baseline comparison. The exploratory analyses indicated that, while we observed increases in signature Tfh cells genes *Ii21*, *Bcl6*, *Cxcr5*, *Irf4*, *Icos*, *Ii4*, *Batf*, and *Maf* (1, 7–10) in the WT CD4⁺ T cells isolated from the F1 recipients compared with naive WT CD4⁺ T cells, none of these genes were upregulated in the *C5ar1*^{-/-} T cells reisolated from the F1 recipients compared with naive *C5ar1*^{-/-} controls (Supplemental Figure 2B). The RNA-sequencing data did not reveal differences between naive and F1-isolated WT or naive and F1-isolated *C5ar1*^{-/-} T cells for transcripts related to T cell immunity in general (*Cd3*, *Cd8*, *Cd4*, *Ii2r*, *Ii2*, *Ii15*), including those related to Th1, Th17, Treg, and T cell survival (Supplemental Figure 2, C and D). Direct comparison in multiple animals through RT-PCR of WT and *C5ar1*^{-/-} F1 cells (sorted, *in vivo*-differentiated WT and *C5ar1*^{-/-} CD4⁺ T cells on day 7 after transfer into F1 recipients) showed no differences in expression of *Bcl6* and *Irf4*, but showed lower expression of *Ii21* and a nonsignificant trend toward less *Maf* in the C5aR1-deficient T cells (Supplemental Figure 2E)

T cell-expressed C5aR1 regulates mTOR-dependent c-MAF and IL-21 production. Guided by the RNA analyses and in an effort to more definitively identify mechanistic links between C5aR1 and Tfh cells, we employed an *in vitro* culture system, noting that Tfh cell differentiation is a multistep process involving IL-6 and IL-21 (in the absence of Th1, Th2, or Th17 cytokines) that is only partially replicated *in vitro* (47, 48). Naive CD4⁺ T cells can be induced to produce IL-21 when stimulated via their TCR with IL-6 while simultaneously blocking TGF-β, IL-4, and IFN-γ (10, 49). When we stimulated WT and

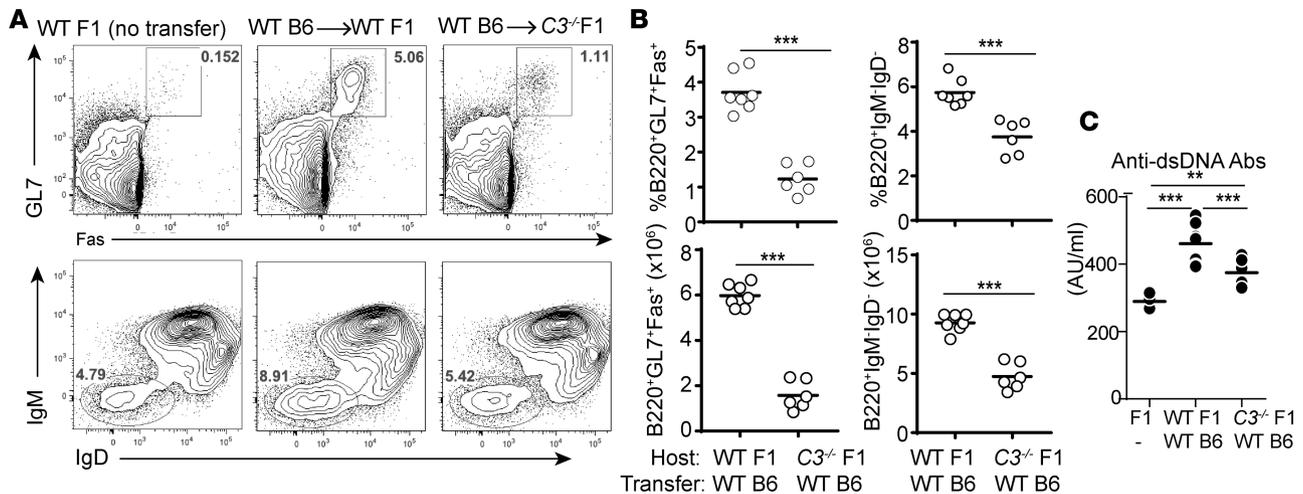


Figure 2. Recipient C3 deficiency prevents GC B cell formation and autoantibody production in a model of parent→F1 alloimmunity. CD8-depleted WT B6 spleen cells were injected i.v. into WT or C3^{-/-} (bx^d)F1 recipients and analyses were performed on day 14. **(A)** Representative histograms depicting percentages of host-derived (H-2K^dB220⁺) Fas⁺GL7⁺ GC B cells (top) and IgM⁺IgD⁻ class-switched B cells (bottom) in naive (bx^d)F1 (left), WT (bx^d)F1 (middle), and C3^{-/-} (bx^d)F1 (right) recipients. **(B)** Total numbers of host-derived B220⁺Fas⁺GL7⁺ cells and IgM⁺IgD⁻ class-switched B cells in day 14 spleens of naive (bx^d)F1 (no cell transfer) and in WT (bx^d) and C3^{-/-} (bx^d)F1 recipients. **(C)** Anti-dsDNA autoantibodies (AU/ml) in sera collected from naive (bx^d)F1 mice or WT (bx^d) and C3^{-/-} (bx^d)F1 recipients on day 14. Combined data of 2 separate experiments (6–7 mice per group). ***P* < 0.01, ****P* < 0.001 by Student's *t* test.

C5ar1^{-/-} CD4⁺ T cells with anti-CD3/anti-CD28 mAbs (Figure 6A) or with allogeneic splenic DCs (Figure 6B) under these Tfh cell-inducing conditions, we observed reduced IL-21 production by C5ar1^{-/-} T cells, verifying that the in vitro-induced Tfh cells behave similarly to those in vivo. Parallel control experiments performed without IL-6 showed essentially no IL-21 production in WT and C5ar1^{-/-} CD4⁺ cells (data not shown). Analogous experiments using WT and C5ar1^{-/-} monoclonal TCR-transgenic TEa CD4⁺ T cells cocultured with specific antigen-expressing (bx^d)F1 stimulators (thereby controlling for any potential development differences in TCR avidity induced by absence of C5aR1) similarly showed less IL-21 production by the C5ar1^{-/-} TEa cells (Figure 6C).

We repeated the in vitro Tfh cell induction experiments using WT and C5ar1^{-/-} CD4⁺ T cells in the presence or absence of the C5aR1-A, this time analyzing c-MAF expression within the CD4⁺ T cells by flow cytometry (Figure 6, D and E). These assays showed that absence or blockade of C5aR1 signaling prevents upregulation of c-MAF protein by approximately 25%–50%, demonstrating that the C5aR1-driven effects suggested by the c-MAF RNA expression data (Supplemental Figure 2) result in alterations in c-MAF protein production. Control experiments verified the C5aR1 specificity of C5aR1-A by showing that the compound had no effect on the lower levels of c-MAF in C5ar1^{-/-} T cells cultured under Tfh cells conditions. The similar effects of C5aR1 deficiency and pharmacological blockade on lowering c-MAF expression indicate that the observed reduction is not attributable to T cell developmental defects that occur in the absence of C5aR1.

To test whether the above observations in murine systems also apply to human T cells, we stimulated naive human CD4⁺CD45RO⁻ T cells with anti-CD3/anti-CD28 with or without C5aR1-A and quantified IL-21 and c-MAF by intracellular flow cytometry 5 days later (Figure 6, F–H). Whereas this Tfh cell-inducing condition augmented IL-21 production compared with stimulation in the absence of IL-6, addition of C5aR1-A prevented IL-6-dependent, IL-21 production and lowered intracellular expression of c-MAF.

To elucidate biochemical links between C5aR1-dependent and IL-6-dependent IL-21 production, we investigated the possibility that C5aR1 expression and/or C5aR1-transduced signals modulate IL-6 receptor (IL-6R) expression or function. When we quantified surface expression of IL-6R and its coreceptor gp130 on murine C5ar1^{-/-} and WT T cells (Figure 7A) we did not observe differences in expression of either molecule between the two cell types. While IL-6 ligation of the IL-6R (CD126)/gp130 heterodimer is known to cause JAK1/STAT3 phosphorylation (50), a kinetic analysis of IL-6-induced pSTAT3 showed no differences in pSTAT3 between WT and C5ar1^{-/-} CD4⁺ T cells (Figure 7B).

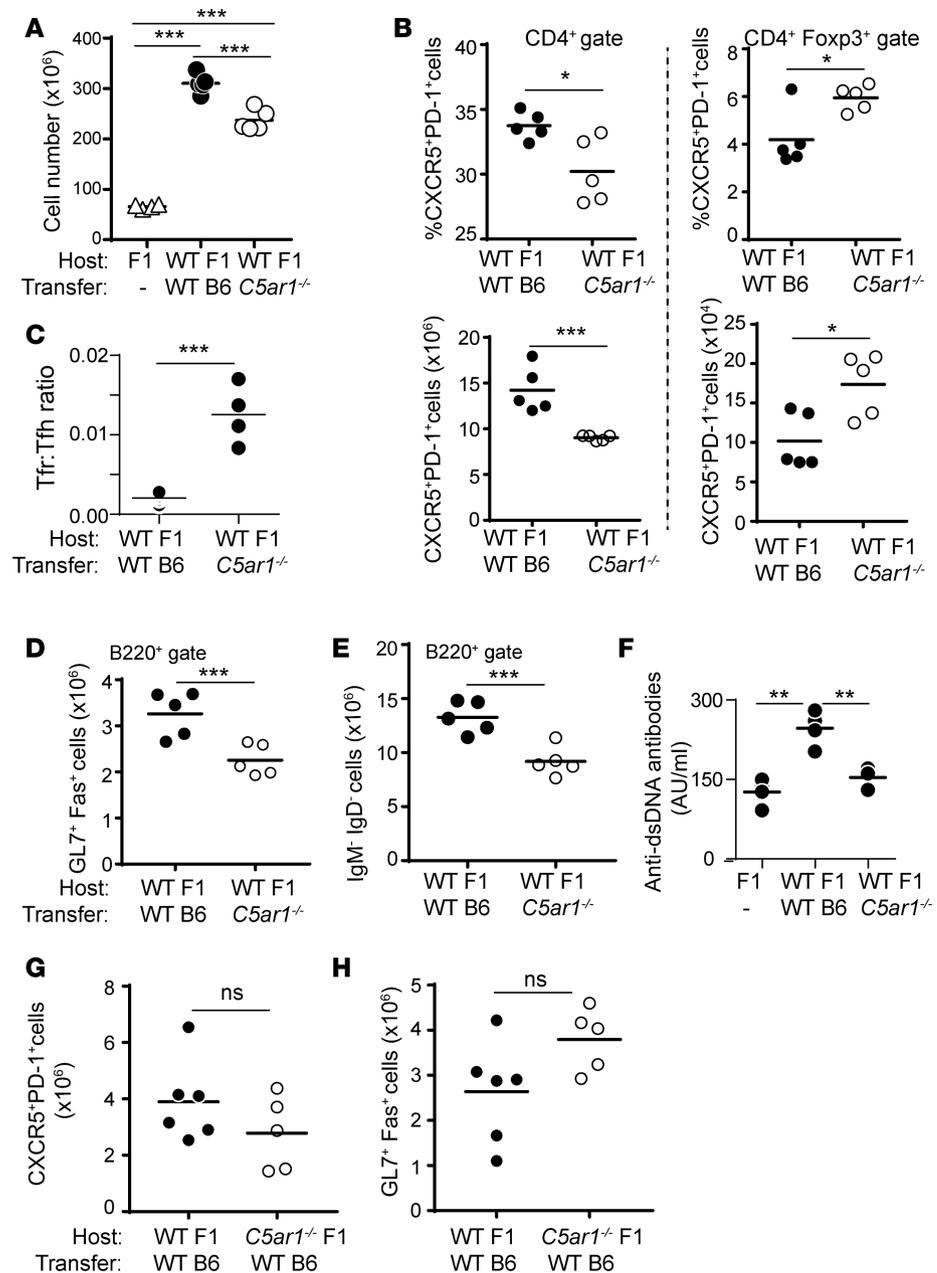


Figure 3. Donor cell deficiency of C5aR1 prevents immunological changes in a model of parent→F1 alloimmunity. CD8-depleted WT or *C5aR1*^{-/-} B6 spleen cells were injected i.v. into WT (bx)F1 recipients and analyses were performed on day 14. Spleen cell numbers (A), percentages and total spleen cell numbers of Tfh cells and Tfr cells (B), Tfr/Tfh cell ratios (C), GC B cells (D), class-switched B cells (E), and serum anti-dsDNA antibodies (F), as determined by analyses shown in Figures 1 and 2. (G and H) CD8-depleted WT B6 spleen cells were injected i.v. into WT or *C5aR1*^{-/-} (bx)F1 recipients, and day 14 analyses (as done in Figures 1 and 2) of donor-derived Tfh cells (G) and recipient GC B cells (H) are shown (spleen cell numbers, percentage and total numbers of Tfr cells, and percentages and total numbers of class-switched B cells did not differ between groups; data not shown). The experiment was repeated twice with similar results. $n = 5$ per group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ by Student's *t* test.

In contrast, we observed that addition of IL-6 augmented AKT phosphorylation (pAKT, S473) in anti-CD3 mAb-stimulated WT CD4⁺ T cells (Figure 7C). In the absence of T cell C5aR1, we observed lower pAKT at baseline and blunted increases in pAKT MFI following stimulation with IL-6. Pharmacological inhibition of AKT or addition of rapamycin (that binds to and inhibits the mammalian target of rapamycin complex [mTORC1] downstream of pAKT) prevented IL-6-induced IL-21 production in both WT and in *C5aR1*^{-/-} CD4⁺ T cells (Figure 7D).

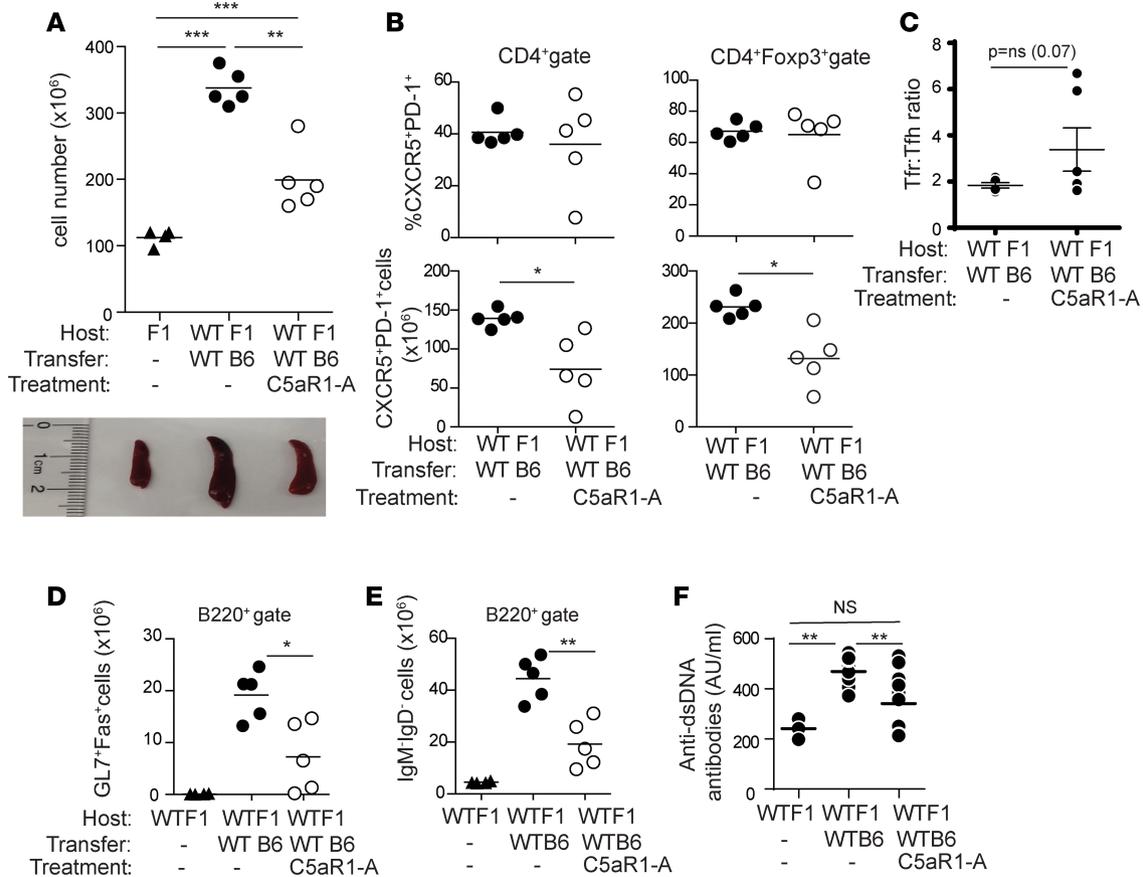


Figure 4. C5aR1 antagonist prevents immunological changes induced during parent→F1 induced alloimmunity. CD8-depleted WT or B6 spleen cells were injected i.v. into WT (bxdf)F1 recipients and given C5aR1-A i.p. daily. Analyses were performed on day 14. Spleen cell numbers with a representative photograph of spleens (A), percentages and total spleen cell numbers of Tfh cells and Tfr cells (B), Tfr/Tfh cell ratios (C), GC B cells (D), class-switched B cells (E), and serum anti-dsDNA antibodies (F), as determined by analyses shown in Figures 1 and 2. $n = 5$ per group. The experiment was repeated twice with similar results. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ by Student's t test.

To further investigate this mechanistic link, we stimulated WT and *C5ar1*^{-/-} spleen cells with or without IL-6 and quantified intracellular levels of the phosphorylated form of the mTOR substrate p70S6 kinase (p-pS6K) within CD4⁺ T cells by flow cytometry (Figure 7, E and F). These assays showed significant upregulation of intracellular p-pS6K in IL-6/anti-CD3 treated WT T cells, but the p-pS6K levels in identically stimulated *C5ar1*^{-/-} CD4⁺ T cells were reduced by >50%. Pharmacological AKT inhibition prevented IL-6-induced p-pS6K expression in WT and *C5ar1*^{-/-} T cells (Figure 7F). When we stimulated human CD4⁺ T cells under Tfh cell-generating conditions in the presence or absence of C5aR1-A, we observed that, analogous to the murine findings, C5aR1 blockade limited phosphorylation of AKT and pS6 in the human T cells (Figure 7, G–J).

Discussion

Through the use of genetic knockout mice, a pharmacological antagonist, in vitro studies of human and murine T cells, and established models of chronic GvHD in mice, our findings delineate a previously unrecognized and potentially clinically relevant mechanism that connects C5aR1, a receptor for the complement activation product C5a, to Tfh cell differentiation and function. While complement is traditionally considered a component of innate immunity, we show herein that C5aR1-initiated signals on murine and human CD4⁺ T cells responding to anti-CD3/CD28 and to alloantigens induce pAKT- and mTOR-dependent upregulation of multiple gene products (including IL-21 and c-MAF) that have been previously shown by others to be required for Tfh cell differentiation. In the absence/blockade of C5aR1, CD4⁺ T cells responding in allogeneic recipients were restrained from differentiating into Tfh cells, and C5aR1 antagonism ameliorated the clinical manifestations of Tfh cell-dependent BOS in a model of chronic GvHD.

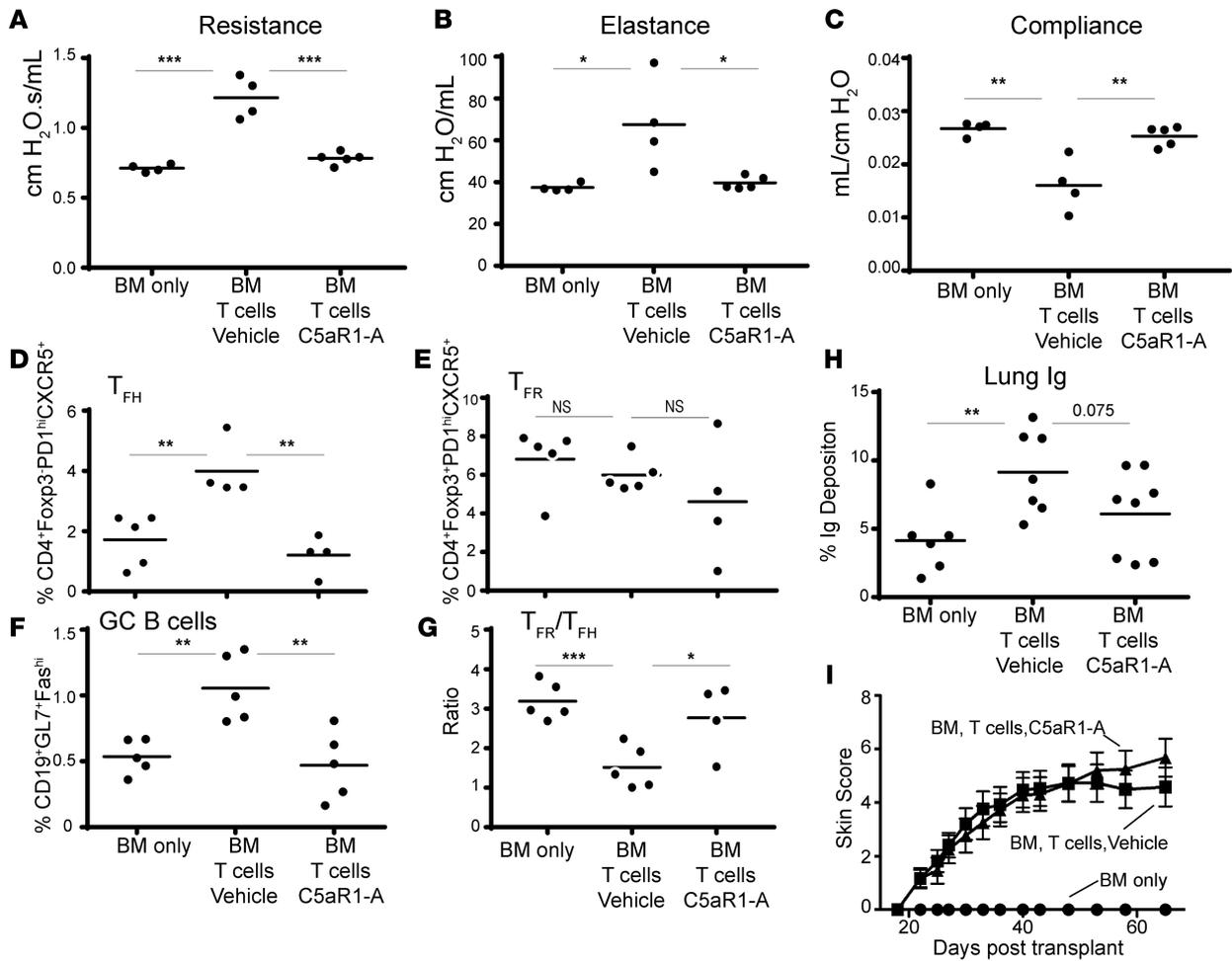


Figure 5. C5aR1 antagonism abrogates chronic GvHD that results in BOS. (A–C) Day 60 analysis of pulmonary function tests (A, resistance; B, elastance; and C, compliance) of mice that received donor BM or donor BM plus donor T cells and either DMSO vehicle or 0.6 mg C5aR1-A via osmotic pump beginning on day 28. Ten animals total per group were assessed. Pulmonary function was analyzed in 5 animals per group for the experiment shown (1 of 3 individual experiments); all experiments showed the same statistical differences. (D–G) Frequencies of Tfh cells (D), Tfr cells (E), and GC B cells (F) and ratios of Tfr/Tfh cells (G) in spleens of transplanted mice assessed on day 60. (H) Lung deposition of Ig in animals shown in A–C, assessed on day 60. (I) Skin scores in BALB/c recipients that received donor BM alone (circles) or donor BM plus donor spleen and either DMSO (squares) or 0.6 mg C5aR1-A (triangles) via osmotic pump beginning on day 14 (no difference between the latter 2 groups). *n* = 15–20 animals per group. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 by Student's *t* test.

In each of these *in vivo* systems, the lower frequencies/absolute numbers of Tfh cells observed in the absence of C5aR1 signaling on the donor CD4⁺ T cells were associated with decreased numbers of GC B cells and less autoantibody production compared with controls, together demonstrating that the reduced frequencies of Tfh cells result in defective T cell help to B cells. While C5aR1-initiated signals can activate APCs, including DCs and macrophages (33, 37, 51), our finding that donor CD4⁺ T cells differentiate equivalently into Tfh cells regardless of C5aR1 expression in the recipients supports the conclusion that the dominant effects of C5aR1 signaling on Tfh cell differentiation in this system are mediated through C5aR1 expressed on the responding CD4⁺ T cells.

We showed that C5aR1 antagonism was efficacious in the BOS model but was not effective in the B10.BR→BALB/c model of scleroderma, a disease that is principally attributed to Th17 alloimmunity and not autoantibody (52). These data are consistent with previous work in which we showed that a small-molecule BCL6 peptidomimetic functioned as a potent inhibitor in the BOS model but had no effect in the same scleroderma system, as reported in this manuscript (25). Coupled with the observations that pharmacological syk inhibition potently affects Tfh cell-dependent chronic GvHD in mice and is efficacious in human chronic GvHD, yet has little clinical benefit in the scleroderma model (53), our findings suggest that C5aR1 inhibition preferentially restrains Tfh cell-dependent GC formation when administered after disease initiation. These observations build upon prior murine studies in which chronic GvHD could be reversed by

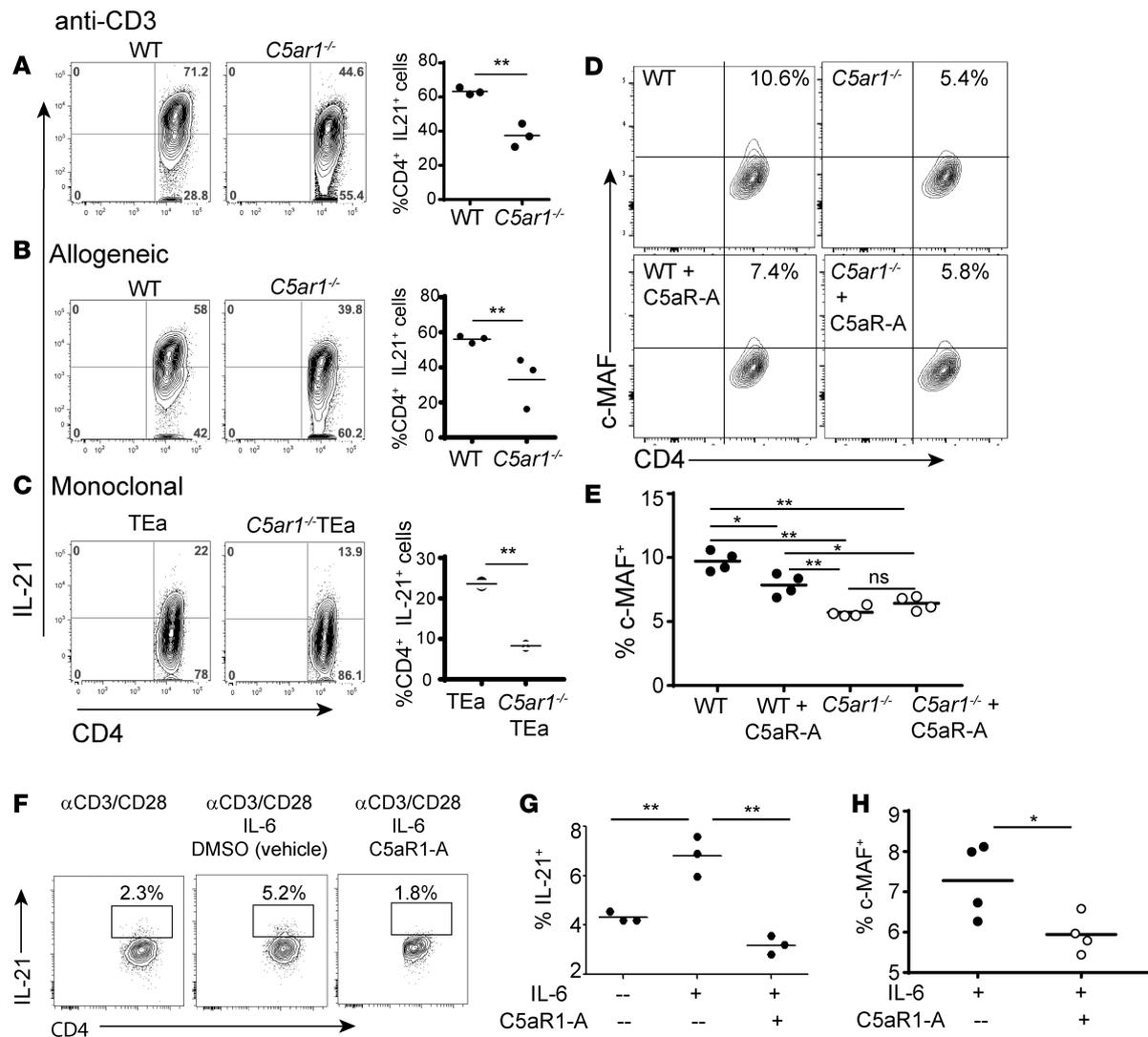


Figure 6. T cell-expressed C5aR1 regulates in vitro induction of IL-21-producing CD4⁺ T cells. Representative flow plots for IL-21-producing CD4⁺ T cells and summarized data of naive B6 CD62L^{hi}CD44^{lo}CD4⁺ T cells from WT and *C5aR1*^{-/-} mice cultured with αCD3/CD28 (A) or allogeneic BALB/c splenic APCs (B) under Tfh cell-inducing conditions for 5 days ($n = 3$ per group, repeated twice with similar results). (C) Representative flow plots for IL-21-producing CD4⁺ T cells and summarized data of naive B6 CD62L^{hi}CD44^{lo}CD4⁺ T cells from WT TEa or *C5aR1*^{-/-} TEa TCR-transgenic mice cultured with (bx)F1 APCs under Tfh cell-inducing conditions for 5 days ($n = 3$ /group, repeated once with similar results). (D and E) Representative flow plots (D) and quantitation (E) for c-MAF expression from WT and *C5aR1*^{-/-} mice cultured with αCD3/CD28 with or without C5aR1-A for 5 days ($n = 4$ /group, repeated twice with similar results). (F–H) Representative flow cytometry plots (F) and quantification (G) of IL-21 producers and quantitation of intracellular c-MAF (H) within human CD4⁺ T cells stimulated for 5 days under the conditions shown, with or without human C5aR1-A. $n = 3$ –4/group. Each experiment shown is representative of at least 3 independent experiments for each set of studies. * $P < 0.05$, ** $P < 0.01$ by Student's t test.

targeted GC disruption using a lymphotoxin- β fusion protein (54) or administering T cells or bone marrow as a source of B cells defective in supporting GC formation (25, 43, 53–55). Relevant to human chronic GvHD patients, serum CXCL13 levels are elevated (44) and are associated with low Tfh cell numbers in peripheral blood (44, 53), suggesting that circulating Tfh cells have been recruited into GCs where high-affinity antibody-secreting cells can be generated. Whether targeting C5aR1 will be effective in treating other Tfh cell-dependent disease processes, including other models of chronic GvHD in which extrafollicular CD4 T cell–B cell interactions are sufficient to generate autoimmune-like chronic GvHD (26), remains to be determined. Nonetheless, as we have previously provided evidence for increased Tfh cell function in humans with chronic GvHD (56), our data raise the possibility that C5aR1 blockade could have efficacy in some forms of steroid-refractory chronic GvHD, a heterogeneous and highly morbid condition.

Tfh cells have also been shown to be crucially involved in autoantibody formation (57–59), including in models of systemic lupus erythematosus. Indeed the parent→F1 model used here to interrogate the role of

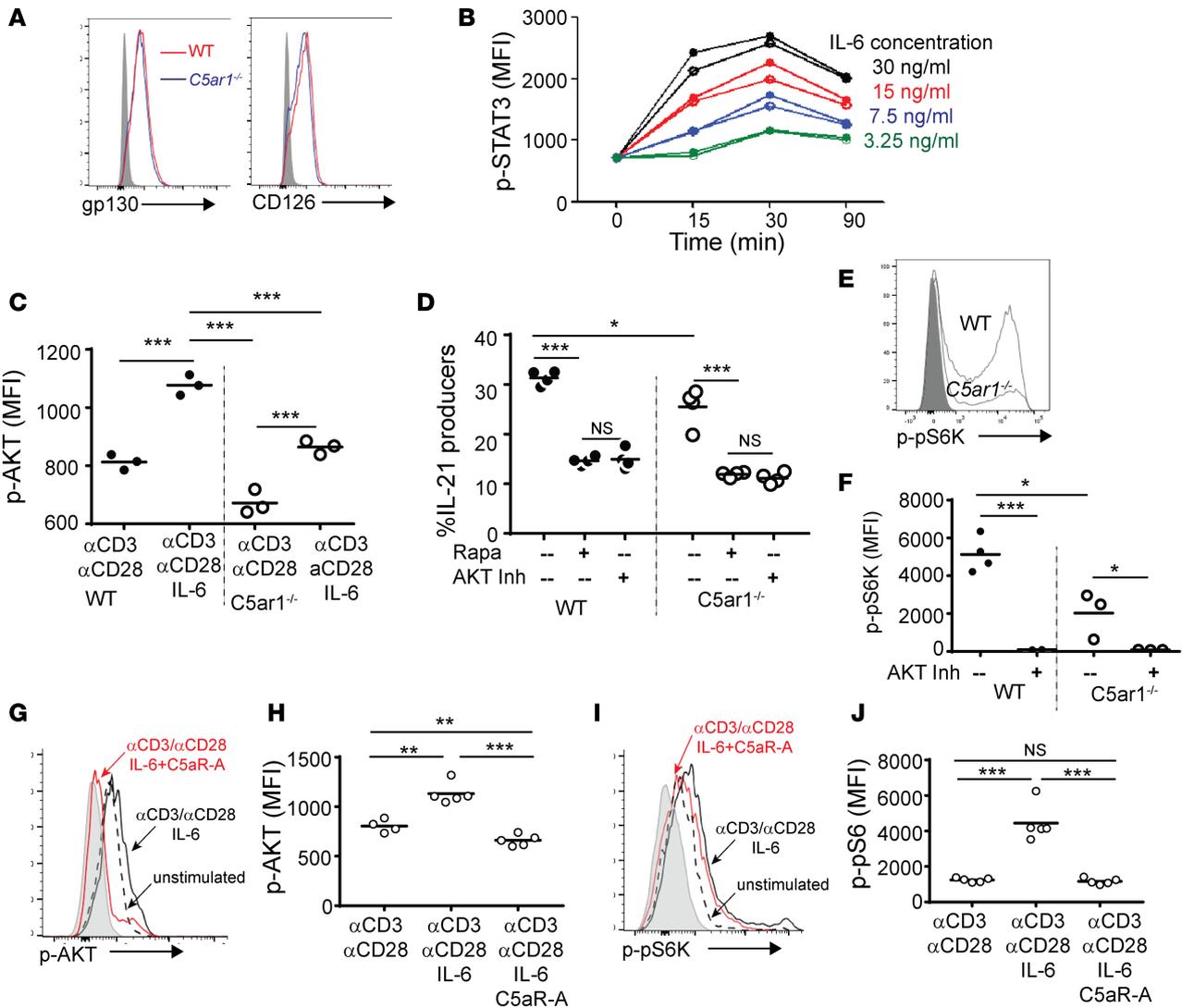


Figure 7. C5aR signaling augments IL-6-induced pAKT-dependent mTOR activation and IL-21 production. (A) Representative flow cytometry histograms of WT and *C5ar1*^{-/-} B6 spleen cells 7 days after injection into (bxid)F1 recipients to induce activation (gated on CD4⁺H-2K^d cells). MFI for IL-6R and for gp130 were not different between groups (*n* = 3–4 animals/group). (B) Kinetics of pSTAT3 expression (intracellular flow cytometry) of WT (closed circles) and *C5ar1*^{-/-} (open circles) spleen cells stimulated with aCD3/aCD28 with or without IL-6 at concentrations indicated. There were no significant differences between WT and *C5ar1*^{-/-} cells at any of the times or IL-6 concentrations tested (*n* = 3–6/group, repeated at least 3 times with similar results). (C) pAKT expression within WT or *C5ar1*^{-/-} CD4⁺ T cells after 18 hours of cultures with anti-CD3/CD28 with or without IL-6 as indicated (*n* = 3/group repeated twice with similar results). (D) Percentage of IL-21⁺ WT and *C5ar1*^{-/-} CD4⁺ T cells (intracellular flow cytometry) on day 5 of IL-21-inducing culture conditions with or without rapamycin (50 nM) with or without AKT inhibitor (1 μM) for 5 days (*n* = 4/group, repeated twice with similar results). (E and F) Representative flow cytometry histogram (E) and quantified results (F) of p-pS6K expression (intracellular flow cytometry) in WT and *C5ar1*^{-/-} CD4⁺ T cells cultured under IL-21-inducing conditions for 18 hours with or without AKT inhibitor (1 μM) (*n* = 3–4/group, repeated twice with similar results). (G–J) Representative flow cytometry histograms (G and I) and quantified results (H and J) for pAKT (G and H) or p-pS6K (I and J) in human CD4⁺ T cells stimulated under the conditions listed (*n* = 4–5/group, repeated twice with similar results). **P* < 0.05, ***P* < 0.01, ****P* < 0.001 by Student's *t* test.

C5aR1 signaling in a lupus-like chronic GvHD model (42) demonstrated the key requirement of Tfh cells in autoantibody and chronic GvHD generation. By deciphering a mechanism through which C5aR1 modulates Tfh cells, our data provide an alternative explanation to account for the efficacy of C5aR1 inhibition in abrogating kidney and central nervous system disease in MRL *lpr/lpr* mice (60, 61), which was attributed to inhibitory effects on neutrophils/phagocytes and vascular endothelial integrity.

The documented effects of absent C5aR1 signaling on Tfh cells described herein are distinct from our published work in which we previously tied immune cell-derived and locally activated alternative pathway complement to proinflammatory Th1 and Th17 responses (31–33, 51, 62). These previous studies showed that, during cognate T cell/APC interactions, the locally produced C3a and C5a bind to C3aR1 and C5aR1, respectively,

through autocrine and paracrine loops (32, 33, 37). The resultant PI3-K γ - and pAKT-dependent costimulatory signals drive T cell proliferation, amplify Th1 differentiation, and promote effector T cell survival. Absence of C3aR1/C5aR1 signaling limits T cell expansion and differentiation and enhances apoptosis, despite the presence of traditional costimulatory molecule interactions (i.e., CD80/86:CD28 and CD40:CD154). The effects apply to murine and human T cell immunity induced by alloantigens, autoantigens, model antigens, infectious antigens, and polyclonal stimuli (32, 33, 35, 37, 38, 62–64). While C5aR1 antagonism did not alter disease progression in the Th17-dependent scleroderma progression in the B10.D2→BALB/c model (Figure 5I), we speculate that by the time the treatment was started (day 14) the Th17-dependent fibrogenetic effector program was already irreversibly engaged. Whether C5aR antagonism can prevent induction of CD4⁺ Th17 immunity in the B10.BR→BALB/c model remains to be tested. Regardless, our published findings and the data herein, together with studies by the Kemper group showing that intracellular C5a promotes NLRP3 inflammasome formation and Th1 differentiation (34) and a publication from the Botto laboratory indicating that C1q inhibits self-reactive CD8⁺ T cells by modulating mitochondrial metabolism (65), support an expansive role for complement as a modulator of various T cell immune responses via multiple distinct mechanisms.

Current concepts are that Tfh cell differentiation is a multistep process involving IL-6-, IL-21-, and ICOS-dependent signals that induce the TFs BCL6 and c-MAF, among several others (7, 11, 48, 66). ICOS-initiated signaling, which is required for Tfh cell differentiation (8), is transmitted in part through PI3-K γ p110 δ (67), an intermediary that we previously showed is activated by C5aR1, a 7-transmembrane-spanning G protein-coupled receptor (33). PI3-K γ phosphorylates AKT and subsequently activates mTOR complex 1, which was mechanistically linked by other reports to Tfh cell differentiation (10). As the magnitude of PI3-K γ signaling has been shown to correlate directly with GC size and Tfh cell numbers (68), it is likely that PI3-K γ serves as a nexus between ICOS and C5aR1 (among other) signaling pathways, with signaling through the two pathways converging to amplify Tfh cell differentiation. *Bcl6* expression is driven by IL-6-dependent STAT1/3 signaling (among other signals) that is not known to intersect with C5aR1-initiated signaling intermediaries (1, 69), providing an explanation for our observation that absence of C5aR1 limits c-MAF expression without affecting BCL6.

Our current findings additionally build upon previous studies by our group in which we showed that absence/blockade of C5aR1 (and C3aR1) signaling on murine and human CD4⁺ T cells stabilizes and in fact augments thymic-derived (natural) Treg function (70). Herein, we demonstrate that Tfr cells, which differentiate from Foxp3⁺, thymic-derived Tregs (rather than differentiating from naive CD4⁺ T cells) and specifically regulate Tfh cell function (4, 13), are largely unaffected by the absence/blockade of C5aR1 signaling, an effect manifested by significantly higher Tfr/Tfh cell ratios in the GvHD models. Constitutively functional intrinsic phosphatase activities limit AKT activation in thymic-derived Tregs (71) (and by inference in Tfr cells), providing a molecular explanation for their stable suppressive capacity, despite C5aR1 expression. Tfr cells have also been shown to express constitutively upregulated mTORC1, which likely facilitates c-MAF expression required for their function (72, 73), regardless of C5aR1 signaling.

In sum, our results link complement and C5a/C5aR1 ligations on CD4⁺ T cells to IL-6-dependent Tfh cells and GC B cell formation in vivo, in part through modulating mTOR activation and upregulating c-MAF, thereby guiding Tfh cell differentiation programs. Together with recent evidence that Tfh cells likely participate in the pathophysiology of certain human antibody-dependent disease processes, including chronic GvHD (44, 56), and the pharmaceutical industry's recent interest in targeting complement (28, 74, 75), our findings raise the possibility that targeting C5aR1 signaling (76) could be a useful therapeutic approach for prevention and/or treatment of Tfh cell-dependent disease processes, including some forms of human chronic GvHD.

Methods

Additional details, including an accession number for sequencing data, are provided in the Supplemental Methods. Briefly, flow-sorted H-K^d- CD4⁺ T cells were isolated from recipient spleens 7 days after transfer, RNA was isolated (Qiagen RNeasy kit), single-end sequencing at 75-bp read length was performed on a HiSeq 4000 (Illumina), and differential analysis by LIMMA test was performed to identify significantly dysregulated genes at $P < 0.05$, which were then subjected to Gene Ontology function and Canonical pathway enrichment analysis by Fisher exact test.

Mice. B10.BR (*H-2^b*), BALB/c (*H-2^d*), C57BL/6 (B6) *C3^{-/-}* (*H-2^b*), *C5ar1^{-/-}* BALB/c, and WT (B6 × BALB/c)F1 [(bx)F1] mice were purchased from The Jackson Laboratories. WT B6 (*H-2^b*) mice were

purchased at The Jackson Laboratories or National Cancer Institute. BALB/c $C3^{-/-}$ were backcrossed >12 generations from B6 $C3^{-/-}$ mice and were crossed with B6 $C3^{-/-}$ mice to produce $C3^{-/-}$ (bx)F1 animals. B6 $C5ar1^{-/-}$ mice were a gift from C. Gerard (Harvard, Cambridge, Massachusetts, USA) and were backcrossed >15 generations. B6 $C5ar1^{-/-}$ mice were crossed with BALB/c $C5ar1^{-/-}$ mice (The Jackson Laboratories) to produce (bx)F1 $C5ar1^{-/-}$ animals. T cell receptor–transgenic (TCR-transgenic) TEa mice ($H-2^b$, $CD4^+$ TCR reactive to I-A^b+ I-E^d α_{52-68}) were obtained from A. Rudensky (Memorial Sloan Kettering, New York, New York, USA) and were intercrossed with the B6 $C5ar1^{-/-}$ mice. Colonies of WT B6 and B6 $C5ar1^{-/-}$ animals (The Jackson Laboratory) were bred, maintained, and housed in a specific pathogen–free facility at the Icahn School of Medicine at Mount Sinai. To limit genetic drift, animals from these WT and $C5ar1^{-/-}$ mouse colonies were backcrossed with B6 mice (The Jackson Laboratory) every 2 years. Experiments were performed using littermates or with mice bred within the same room and cohoused within the same cages to limit potential effects of microbiome differences. B10.BR, BALB/c, and B6 mice were also obtained from The Jackson Laboratory for use in the chronic GvHD models at the University of Minnesota.

Reagents. The murine C5aR1-A, AcF-[OPdChaWR], PMX53, was synthesized as previously described (77). This compound potently inhibits C5aR1, but not C5aR2 (76–79). C5aR1-A was dissolved in a sterile solution of 5% glucose/water and administered by daily i.p. injection (1 mg/kg/d) or via subcutaneously implanted osmotic pumps (0.6 mg C5aR1-A or vehicle in model 1004 sufficient for 30-day delivery, Azlet). Mouse anti-dsDNA Ig was quantified using an ELISA kit (Alpha Diagnostic International).

Parent→F1 model of *in vivo* alloimmunity. Spleen cells were CD8 depleted (Dynabeads Flow Comp Mouse CD8 kit, Invitrogen) and resultant spleen cells containing 10×10^6 to 15×10^6 $CD4^+$ cells were injected i.v. into nonirradiated (bx)F1 recipients. Flow cytometric analysis verified <1% $CD8^+$ T cells with equal numbers of $CD4^+$ T cell transfers between groups within an experiment.

Chronic GvHD models. For the bronchiolitis obliterans system, B10.BR recipients were conditioned with cyclophosphamide (120 mg/Kg) on day –3 and –2 and TBI of 8.3 Gy on day –1 (23, 43, 44, 54, 80). Recipients received 10×10^6 T cell–depleted bone marrow cells from WT B6 mice with or without splenic B6 T cells (0.07×10^6 to 0.1×10^6) on day 0. Where indicated, on day 28 mice were anesthetized and filled osmotic pumps were inserted subcutaneously. To quantify BOS, anesthetized mice were weighed, intubated, and lung function was assessed by whole body plethysmography using the Flexivent system (SCIREQ) and analyzed using the Flexivent software version 7.3.

In the scleroderma system, BALB/c recipients were conditioned with TBI of 700 cGy on day –1 (25). Recipients received 10×10^6 T cell–depleted bone marrow cells from WT B10.BR mice with or without splenic 10^7 B6 spleen cells on day 0. Where indicated, on day 28 mice were anesthetized and filled osmotic pumps were inserted subcutaneously. Skin scores were quantified by a blinded investigator as described previously (25).

Reagents for flow cytometry and sorting. The following mAb clones were purchased from BD Biosciences: CD44 (IM-7, catalog 553133), biotinylated CXCR-5 (2G-8, catalog 551960), Fas (Jo2, catalog 554258), and GL-7 (GL-7, catalog 562080), with appropriate isotype controls. From eBioscience/Invitrogen (part of Thermo Fischer Scientific), we purchased H-2K^d (SF1-1.1.1, catalog 48-5957-82), CD45 (30-F11, catalog 47-0451-82), CD8 (5.3–6.7, catalog 25-0081-82), CD62L (MEL-14, catalog 17-0621-82), CD25 (PC61.5, catalog 53-0251-82), Foxp3 (FKJ-16s, catalog 11-5773-82), c-MAF (sym0F1, catalog 46-9855-42), TCR β (H57-597, catalog 45-5961-82), streptavidin (catalog 48-4317-82), B220 (RA3-6B2, catalog 45-0452-82), IgM (eB121-15F9, catalog 25-5890-82), and IgD (11-26, catalog 47-5993-82) as well as appropriate isotype controls. Anti-gp130 (4H1B35, catalog 149401), IL-6R (D7715A7, catalog 115803), CD4 (GK1.5, catalog 100449), PD-1 (RMP1-30, catalog 109110), and secondary goat anti-rat FITC (poly4054, catalog 405404) were purchased from Biolegend. C5aR1/CD88 (10/92, catalog MCA2456A647) was purchased from Bio-Rad (formerly AbD Serotec).

For intracellular cytokine staining, cells were stimulated with PMA (50 ng/ml; MilliporeSigma) and Ionomycin (500 ng/ml; MilliporeSigma) for 4 hours and Golgiplug (BD Biosciences). After surface staining, cells were fixed, permeabilized, and stained for intracellular cytokines. For IL-21, IL-21R subunit/Fc chimera (R&D Systems, catalog 596-MR) was primary, with AffiniPure F(ab')₂ Fragment Goat Anti-Human IgG, Fc γ (Jackson ImmunoResearch, catalog 109-136-098) as secondary. Analysis was performed on a FACSCanto II flow cytometer (BD Biosciences). Data were analyzed using FlowJo software (Tree Star Inc.). Flow sorting was performed on a SH800S cell Sorter (Sony Biotechnology).

In vitro Tfh cell differentiation. Murine splenic CD4⁺ T cells (EasySep, Stemcell Technologies) were stimulated in RPMI, 10% fetal calf serum, 2 mM Glutamine (Gibco), 100 U/ml penicillin/streptomycin (Gibco/Thermo Fisher), 50 μM β-mercaptoethanol with BALB/c or (bx)F1 APCs or with anti-CD3/anti-CD28 mAbs (8 μg/ml each, clones 145-2c11/37.51, catalog 16-0031-85/16-0281-85, respectively, from Invitrogen) at 37°C for 5 days with 30 ng/ml murine IL-6 (PeproTech), 20 μg/ml anti-TGF-β (clone 11D.11.15.8, catalog BE0057, BioXCell), 10 μg/ml anti-IFN-γ, (clone XMG1.2, catalog BE0055, BioXCell), and 10 μg/ml anti-IL-4 (clone 11B11, catalog BE0045, BioXCell). For human cell assays, we isolated naive CD4⁺ T cells from buffy coats (EasySep, Stemcell) and activated them overnight in cRPMI with anti-huCD3/CD28 dynabeads (10 μl/10⁶ cells, Thermo Fisher) and then washed and replated them in cRPMI with plate-coated anti-huCD3/CD28 mAbs (5 μg/ml each, clone OKT3, catalog 16-0037-81, Invitrogen, and clone CD28.2, catalog 555725, BD Bioscience, respectively) and recombinant human IL-6 (50 ng/ml, PeproTech) with or without C5aR antagonist (10 μg/ml W-54011, Calbiochem, a division of MilliporeSigma) for 5 days. Intracellular staining for IL-21 was performed using clone A3N2 (eBioscience, catalog 50-7219-42).

Signaling assays. Murine spleen cells or purified CD4⁺ T cells were rested in HL-1 medium (Lonza) for 1 hour at 37°C and then stimulated with anti-CD3/CD28 mAbs (1 μg/ml) with or without murine IL-6 with or without C5aR1-A for 30 minutes to 18 hours as indicated. Cells were fixed and stained for surface markers and intracellular pAKT (REA359, catalog 130-105-250, Miltenyi Biotec), p-pS6K (REA454, catalog 130-107-470, Miltenyi), or pSTAT3 (4/P-STAT3, catalog 562072, BD Bioscience).

Statistics. Statistical significance was determined by Student's *t* test (unpaired, 2-tailed), 2-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 significance threshold values of $P \leq 0.05$. All experiments were repeated at least twice. Data are presented as mean values with SD. Error bars indicate mean \pm SEM and "ns" indicates $P > 0.05$ (not significant).

Study approval. All animals were used with the approval of the institutional animal care and usage committees of the Icahn School of Medicine at Mount Sinai and the University of Minnesota.

Author contributions

DAV and NC performed and analyzed experiments at Mount Sinai using the parent→F1 model system, performed all in vitro murine and human Tfh cell induction and signaling assays and wrote/edited the manuscript. KP, RF, and JD performed and analyzed chronic GvHD experiments at the University of Minnesota and edited the manuscript. MF assisted with signaling assays, performed imaging flow analyses, analyzed the RNA-sequencing data, and edited the manuscript. TMW provided C5aR antagonists, designed experiments, and edited the manuscript. HX and YH performed in vitro experiments and edited the manuscript. WZ and ZY performed the RNA-sequencing analyses. BRB and PSH obtained funding for the project, designed and oversaw the project, interpreted the work, wrote and edited the manuscript.

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