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*J Immunol* published online 20 May 2013
http://www.jimmunol.org/content/early/2013/05/19/jimmunol.1300847

Supplementary Material
http://www.jimmunol.org/content/suppl/2013/05/21/jimmunol.1300847.DC1

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Cutting Edge: Receptors for C3a and C5a Modulate Stability of Alloantigen-Reactive Induced Regulatory T Cells

William van der Touw, Paolo Cravedi, Wing-hong Kwan, Estela Paz-Artal, Miriam Merad, and Peter S. Heeger

CD4⁺Foxp3⁺ regulatory T cells (Treg) are critical regulators of immune homeostasis and self-tolerance. Whereas thymic-derived or natural Treg stably express Foxp3, adaptive or induced Treg (iTreg) generated from peripheral CD4 T cells are susceptible to inflammation-induced reversion to pathogenic effector T cells. Building upon our previous observations that T cell–expressed receptors for C3a (C3aR) and C5a (C5aR) drive Th1 maturation, we tested the impact of C3aR/C5aR signaling on induction and stability of alloreactive iTreg. We observed that genetic deficiency or pharmacological blockade of C3aR/C5aR signaling augments murine and human iTreg generation, stabilizes Foxp3 expression, resists iTreg conversion to IFN-γ/TNF-α–producing effector T cells, and, as a consequence, limits the clinical expression of graft-versus-host disease. Taken together, the findings highlight the expansive role of complement as a crucial modulator of T cell alloimmunity and demonstrate proof-of-concept that targeting C3a/C3aR and C5a/C5aR interactions could facilitate iTreg-mediated tolerance to alloantigens in humans.

The Journal of Immunology, 2013, 190: 000–000.

We previously demonstrated that complement activation products C3a and C5a bind their respective G protein–coupled receptors, C3aR and C5aR, expressed on T cells to provide costimulatory signals that enhance Teff activation (9) and limit nTreg function (10). In this study, we investigated whether and how T cell–expressed C3aR/C5aR impact generation and stability of alloreactive iTreg. Our findings indicate that blocking C3aR/C3aR and C5aR/C5aR ligations favors induction and stability of murine and human iTreg. Taken together, the results identify a previously unrecognized set of pharmacological targets that could be exploited to facilitate induction of immune tolerance to alloantigens in the context of human solid organ or hematopoietic cell transplants.

Materials and Methods

Mice

Wild-type (WT), CD45.1, rag1⁻/⁻ (H-2b), BALB/c, CB17-scid (H-2d), and NOD-scid Il2γ⁻/⁻ mice were purchased from The Jackson Laboratory (Bar Harbor, ME). B6 C3ar1⁻/⁻ C5ar1⁻/⁻ mice expressing the Foxp3GFP reporter and B6 Daf1⁻/⁻ mice (10) were developed and maintained through intercrossing at Mount Sinai. Study protocols were approved by the Institutional Animal Care and Use Committee at Mount Sinai School of Medicine (New York, NY).

Reagents

We purchased recombinant human C3a and C5a from R&D Systems (Minneapolis, MN), rat IgG2b and blocking anti-C3a and anti-C5a mAb from BD Biosciences (Franklin Lakes, NJ), C3aR antagonist (C3aR-A) SB2910157 and AKT inhibitor VIII from Calbiochem (San Diego, CA), and C5aR peptide antagonist (Ac-Phe-cyclo(Orn-Pro-dCha-Trp-Arg)) from GenScript (Piscataway, NJ).

Flow cytometry

Cell surface and intracellular staining was performed as described (10). Data were collected on a FACSCanilo II (BD Biosciences) and analyzed using FloJo software (Tree Star, Ashland, OR).

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Received for publication March 29, 2013. Accepted for publication April 18, 2013.

The work was supported by National Institutes of Health Grant R01 AI043578 and American Recovery and Reinvestment Act Supplement to National Institutes of Health Grant R01 AI071185 (to P.S.H.). W.h.-K. was a recipient of a fellowship award from the American Society of Transplantation; E.P.-A. was supported by a grant from the American Heart Association; and W.v.d.T. was supported by National Institutes of Health Grant T32 AI007605.

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The online version of this article contains supplemental material.

Abbreviations used in this article: allo-iTreg, alloantigen-specific induced regulatory T cell; C3α-A, C3a antagonist; C5α-A, C5α antagonist; DMF, decay-accelerating factor; DC, dendritic cell; GVHD, graft-versus-host disease; iTreg, induced regulatory T cell; nTreg, natural regulatory T cell; siRNA, small interfering RNA; Tconv, CD4⁺CD25⁻ conventional T cell; Teff, effector T cell; Treg, regulatory T cell; WT, wild-type.

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Cell isolation
T cell depletion from bone marrow suspensions and isolation of murine splenic and lymph node CD4⁺ T cells and CD11c⁺ dendritic cells (DCs) was accomplished using magnetic beads (Miltenyi Biotec, Auburn, CA). CD4⁺Foxp3⁺GFP⁺ cells were isolated using a MoFlo Legacy cell sorter from Beckman Coulter (Indianapolis, IN). Human CD4⁺CD25⁺ T cells and CD14⁺ monocytes were obtained from PBMCs of healthy controls (under Mount Sinai Institutional Review Board consent) using appropriate kits (Miltenyi Biotec).

Cell stimulation
B6 anti-BAFc4/8 alloantigen-specific iTreg (allo-iTreg) were generated by culturing 2 × 10⁶ CD4⁺CD25⁻ conventional T cells (Tconv) with 5 × 10⁶ CD11c⁺ DCs (BALB/c) plus IL-2 and TGFB for 5–7 d. Stability was assessed by restimulating 5 × 10⁶ allo-iTreg with 5 × 10⁶ CD11c⁺ DCs plus IL-2 for 5 d. Human iTreg were generated by culturing 2 × 10⁶ Tconv with 5 × 10⁶ monoctye-derived DCs with anti-CD3, IL-2, and TGFB for 5 d. To generate DCs, CD14⁺ monocytes were matured for 5 d with GM-CSF and IL-4 followed by LPS stimulation for 48 h. Human iTreg stability was assessed by restimulating 5 × 10⁶ CD25⁺ iTreg with 5 × 10⁶ DCs plus anti-CD3 and IL-2 for 3 d. C3ar⁻/⁻C5ar⁻/⁻, or vehicle control was added to cultures as indicated.

In vivo models
Homeostatic proliferation and in vivo iTreg conversion were tested by transfer of 2 × 10⁶ naïve Foxp3⁺GFP⁺CD62L⁺CD44⁻ Tconv in rag⁻¹/⁻ mice. Short-term acute graft-versus-host disease (GVHD) experiments were done in lethally irradiated (850 rad) BALB/c hosts receiving 2.5 × 10⁶ CD11c⁺ DCs plus IL-2 and TGFB for 5 d. Stability was assessed by restimulating 5 × 10⁶ allo-iTreg with 5 × 10⁶ CD11c⁺ DCs plus IL-2 for 5 d. Human iTreg were generated by culturing 2 × 10⁶ Tconv with 5 × 10⁶ monocyte-derived DCs with anti-CD3, IL-2, and TGFB for 5 d. To generate DCs, CD14⁺ monocytes were matured for 5 d with GM-CSF and IL-4 followed by LPS stimulation for 48 h. Human iTreg stability was assessed by restimulating 5 × 10⁶ CD25⁺ iTreg with 5 × 10⁶ DCs plus anti-CD3 and IL-2 for 3 d. C3ar⁻/⁻, C5ar⁻/⁻, or vehicle control was added to cultures as indicated.

In vivo suppression assays
Murine suppression assays were performed as described (10): 2 × 10⁶ CFSE-labeled CD45.1⁺ Tconv (B6) were mixed with 5 × 10⁶ APcs (BALB/c) in 96-well plates with and without titrated numbers of CD45.2⁺ Foxp3⁺GFP⁺ iTreg and CSFE dilution was assessed on day 5. Human suppression assays were performed similarly with 2 × 10⁶ CFSE-labeled PBMC anti-CD3 with and without CD25⁻-isolated iTreg.

Immunoblots
Immunoblots for Foxo1 and phospho-Foxo1 were detected using Abs from Cell Signaling Technologies (Beverly, MA) as described (10). We obtained decay-accelerating factor (DAF) or control scrambled small interfering RNA (siRNA) from Thermo Scientific (Waltham, MA). DCs were transfected using the NEON electroporation system (Life Technologies) by guest on April 16, 2017 http://www.jimmunol.org/ Downloaded from
Absence or blockade of C3ar/C5ar enhances allo-iTreg stability

iTreg are susceptible to Foxp3 loss and can acquire effector phenotypes (8), prompting us to test the effects of absent C3ar/C5ar on iTreg stability. Flow-sorted Foxp3GFP+ allo-iTreg (>98% purity) were restimulated in secondary MLRs in the absence of TGF-β and examined for Foxp3GFP expression, revealing higher percentages and total numbers of Foxp3GFP+ cells in cultures containing the C3ar1−/−C5ar1−/− cells (Fig. 2A). Absent C3ar/C5ar signaling also prevented conversion of former (ex-)iTreg to IFN-γ/TNF-α-secreting effectors (Fig. 2B). No IL-17A was detected (not shown). Blocking C3ar/C5ar signaling on WT cells using receptor antagonists had similar effects (Fig. 2C).

To address whether the enhanced stability was associated with reduced epigenetic methylation of the CNS2 region within the Foxp3 promoter (15, 16), we performed bisulfite sequencing in the WT and C3ar1−/−C5ar1−/− allo-iTreg. These assays showed similar methylation of the CNS2 regions within the Foxp3 promoter in the WT and C3ar1−/−C5ar1−/− allo-iTreg (Supplemental Fig. 1). We assessed the impact of C3ar/C5ar signaling on iTreg stability in vivo by injecting B6 CD45.2 WT or C3ar1−/−C5ar1−/− allo-iTreg into lethally irradiated BALB/c hosts with B6 bone marrow and congenic CD45.1 Tconv and examined Foxp3GFP expression 5d later (design/gating in Supplemental Fig. 2). These assays showed more Foxp3GFP+ iTreg (Fig. 2D) and fewer IFN-γ/TNF-α-producing ex-Treg (Fig. 2E, 2F) in recipients of C3ar1−/−C5ar1−/− allo-iTreg, C3ar1−/−C5ar1−/− allo-iTreg better suppress GVHD onset.

To test the functional significance of the observed enhanced iTreg stability resulting from C3ar/C5ar deficiency, we used a model of acute GVHD in which Tconv with and without iTreg are adoptively transferred into 4-wk-old allogeneic SCID recipients (17). Adoptive transfer of Tconv alone induced GVHD manifested in part as preventing the weight gain that normally occurs as the animals age to maturity (Fig. 3). Although WT allo-iTreg delayed disease onset, we observed better weight gain and lower clinical scores in animals transferred with C3ar1−/−C5ar1−/− allo-iTreg (Fig. 3A, 3B). Spleens and livers from recipients of C3ar1−/−C5ar1−/− allo-iTreg 4 wk after transfer contained up to 6-fold more Foxp3GFP+ cells (Fig. 3C), indicating that absent C3ar/C5ar signaling augments long-term iTreg stability in vivo during GVHD.

C3ar/C5ar signaling modulates generation and stability of human iTreg

Verifying the murine findings with human cells, we observed that addition of recombinant C3a/C5a reduced Foxp3 expression during human iTreg generation (Supplemental Fig. 3) whereas antagonizing C3ar/C5ar signaling (small molecule inhibitors) augmented the frequency of Foxp3+ effectors (Fig. 4A). The induced CD4+CD25+Foxp3+ cells generated with and without C3ar-A/C5ar-A exhibited equal suppressive capacities (Fig. 4B). When we knocked down human DAF on monocyte-derived DCs with siRNA (Fig. 4C) and used these APCs for in vitro iTreg generation, we observed 2- to 4-fold

**FIGURE 2.** Absent C3ar/C5ar signaling enhances allo-iTreg stability. (A) Percentage (left middle) and number (right) of Foxp3GFP+ cells from sorted allo-iTreg 5 d after restimulation with allogeneic DCs plus IL-2. (B) Representative intracellular IFN-γ/TNF-α cytokine staining of cultures gated on ex-Treg. (C) Percentage (left middle) and number (right) of Foxp3GFP+ cells stimulated as in (A) with and without C3ar-A/C5ar-A (Antag). (D-F) Sorted CD45.2 allo-iTreg were injected into lethally irradiated BALB/c mice with B6CD45.1 bone marrow transplant plus Tconv. Foxp3 expression in splenic CD45.2 (D) and intracellular IFN-γ (E) and TNF-α (F) in CD45.2 Foxp3+ (ex-Treg), and Foxp3GFP+ (iTreg) populations are shown. Results are each representative of at least three independent experiments. Error bars indicate mean ± SD. *p < 0.05.

**FIGURE 3.** Allo-iTreg deficient in C3ar/C5ar suppress acute GVHD and exhibit enhanced in vivo stability. (A) Weight changes and (B) clinical scores in CB17-scid mice that received CD45.1+ Tconv alone (black, n = 4), Tconv plus WT (red, n = 7), or C3ar1−/−C5ar1−/− (blue, n = 8) allo-iTreg. Normal growth curve in PBS controls (green, n = 5) is shown. (C) Quantified percentages of Foxp3GFP+ iTreg among CD45.2+ T cells in the spleen and liver of recipients at 4 wk. Results are cumulative data of three independent experiments. Error bars indicate mean ± SEM. *p < 0.05.
higher quantities of C3a/C5a from knocked down DAF cultures \((p < 0.05)\) by ELISA, not shown, verifying lifted restraint on local complement activation), which were associated with generation of fewer Foxp3+ iTreg (Fig. 4D). The effects were reversed by adding a C3aR-A. Following restimulation in MLRs, we observed higher percentages (Fig. 4E) and total numbers (Fig. 4F) of Foxp3+ cells in the presence of C3aR-A/C5aR-A, indicating that blocking C3aR/C5aR signaling augments phenotypic stability.

To assess effects of C5aR blockade in vivo, we adoptively transferred human PBMC into NOD-scid \(i^{IL2rg}\) null recipients, an immunodeficient mouse that permits the expansion of human lymphocytes resulting in GVHD during 4–6 wk (18), and notably lacks murine C5 (19). We tested the effects of administering a specific human C5aR-A (20) with and without CTLA4Ig (21) and to facilitate iTreg generation (22, 23) on disease outcome. CTLA4Ig attenuated disease scores and weight loss compared with IgG-treated controls, and cotreatment with C3aR-A further improved outcome (Fig. 4G). Treatment with C5aR-A alone partially delayed disease onset.

At day 45 after transfer, we quantified human CD4+ Foxp3+ cells in the spleens of each animal. Treatment with C5aR-A plus CTLA4Ig decreased the total number of human CD4+ cells yet increased the numbers of human CD4+ Foxp3+ cells, resulting in an overall increase the percentage of CD4+ Foxp3+ cells compared with untreated controls (or C5aR-A–treated controls, Fig 4H). In contrast, CTLA4Ig alone reduced total CD4+ T cell numbers without altering the number of CD4+Foxp3+ cells, consistent with previous reports (24, 25).

That C3aR and C5aR signaling on T cells modulates induction and stability of murine and human iTreg supports an expansive role for these receptors beyond their traditional contribution to innate immunity. Consistent with previous studies evaluating C3aR/C5aR modulation of Treg cells (9), our data indicate that the effects on iTreg are mediated through locally produced, spontaneously activated, immune cell–derived complement. C3aR and C5aR signal in part through AKT-dependent Foxo1/3a phosphorylation, although others have demonstrated links to cAMP/CREB (26) that may contribute as well. Whether and how additional stimuli known to inhibit iTreg generation and stability (including IL-6) interact with C3aR/C5aR-initiated signals, including pFoxo1, will require additional studies. Our findings support the need to test C3aR/C5aR antagonism as a strategy to improve the success of adoptive iTreg immunotherapy aimed at preventing and/or treating GVHD, transplant rejection, and, potentially, autoimmune diseases in humans.

Acknowledgments
We thank Parth Lakhani for technical assistance and the personnel of the Mount Sinai Flow Cytometry Shared Resource Facility for reliable cell sorting service.

Disclosures
The authors have no financial conflicts of interest.

References


SUPPLEMENTAL FIGURE 1: Improved stability of $C3aR^{-/-}C5aR^{-/-}$ iTreg is independent of epigenetic regulation (A) Genomic locus of the Foxp3 promoter showing promoter, CNS1, CNS2, and CNS3 regions in light blue, non-coding exons in white, and open-reading frame exons in black. Individual CpG residues in the CNS2 region are depicted below with site-directed primers indicated by arrows. (B) Percent methylation of individual CpG residues from the Foxp3 CNS2 region determined by bisulfate sequencing. Sorted splenic nTreg, Tconv, Foxp3$^{GFP^+}$ allo-iTreg from 5-day stimulation. Data represents combined analysis of 3 independent experiments, each containing methylation state of 9 CpG residues. ***p<0.005, n.s.= not significant
SUPPLEMENTAL FIGURE 2: Experimental schematic of acute short-term GVHD (A) WT or C3ar1⁻/⁻C5ar1⁻/⁻ B6 anti-BALB/c allo-iTreg are generated by coculture of naïve B6 WT or C3ar1⁻/⁻C5ar1⁻/⁻ Foxp3<sup>GFP</sup>neg CD45.2<sup>+</sup> Tconv with BALB/c splenic DCs + IL-2 and TGFβ for 5 days. The Foxp3<sup>GFP</sup>⁺ allo-iTreg are flow sorted and intravenously injected with B6 CD45.1 Tconv and B6 T cell depleted bone marrow cells into lethally irradiated BALB/c hosts. Cell fates are analyzed five days later. (B) Gating strategy for flow cytometric analysis, CD4 positive cells were separated from apoptotic cells stained with Viability dye eFluor450. Gating on H-2 K<sup>d</sup>neg cells excludes contaminating BALB/c and FSC+W gates out doublets. Gating on pathogenic Teff are CD45.1⁺ and the transferred allo-iTreg are CD45.2⁺CD45.1⁻neg. Within the later population Foxp3<sup>GFP</sup>⁺ cells are considered stable allo-iTreg, and Foxp3<sup>GFP</sup>neg cells are unstable former allo-iTreg (ex-Treg).