Signaling through C5a receptor and C3a receptor diminishes function of murine natural regulatory T cells

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Thymus-derived (natural) CD4$^+$ FoxP3$^+$ regulatory T cells (nT reg cells) are required for immune homeostasis and self-tolerance, but must be stringently controlled to permit expansion of protective immunity. Previous findings linking signals transmitted through T cell–expressed C5a receptor (C5aR) and C3a receptor (C3aR) to activation, differentiation, and expansion of conventional CD4$^+$CD25$^-$ T cells (T conv cells), raised the possibility that C3aR/C5aR signaling on nT reg cells could physiologically modulate nT reg cell function and thereby further impact the induced strength of T cell immune responses. In this study, we demonstrate that nT reg cells express C3aR and C5aR, and that signaling through these receptors inhibits nT reg cell function. Genetic and pharmacological blockade of C3aR/C5aR signal transduction in nT reg cells augments in vitro and in vivo suppression, abrogates autoimmune colitis, and prolongs allogeneic skin graft survival. Mechanisms involve C3a/C5a-induced phosphorylation of AKT and, as a consequence, phosphorylation of the transcription factor Foxo1, which results in lowered nT reg cell Foxp3 expression. The documentation that C3a/C3aR and C5a/C5aR modulate nT reg cell function via controlling Foxp3 expression suggests targeting this pathway could be exploited to manipulate pathogenic or protective T cell responses.

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Abbreviations used: DAF, decay-accelerating factor; nT reg cell, natural thymus-derived T reg cell; T conv cell, conventional T cell.

CD4$^+$CD25$^+$ regulatory T cells (T reg cells) expressing the forkhead box transcription factor Foxp3 are required for immune homeostasis and self-tolerance (Fontenot et al., 2003; Hori et al., 2003; Khattri et al., 2003). Mice deficient in Foxp3 exhibit systemic autoimmunity, and CD4$^+$CD25$^+$ T cells obtained from these animals are unable to mediate suppression (Fontenot et al., 2003, 2005; Hori et al., 2003; Khattri et al., 2003). Reconstituting Foxp3 expression rescues suppressive capacity, and adoptive transfer of Foxp3$^+$CD4$^+$ T cells into Foxp3-deficient animals rescues self-tolerance (Fontenot et al., 2003, 2005; Hori et al., 2003; Khattri et al., 2003). CD4$^+$Foxp3$^+$ T reg cells that mature in the thymus, known as thymic or natural T reg cells (nT reg cells), are particularly important for preventing autoimmunity, although a recent publication supports the conclusion that naive T cells induced to express Foxp3 in the periphery (induced T reg cells or iT reg cells) are specifically required for maintaining tolerance at mucosal surfaces, including the gut and the lungs (Josefowicz et al., 2012). CD4$^+$Foxp3$^+$ nT reg cells and iT reg cells have both been shown to regulate pathogenic alloreactive T cells induced to a transplanted organ (Ochando et al., 2006; Nagahama et al., 2007; Joffre et al., 2008; Zhang et al., 2009; Fan et al., 2010; Nadig et al., 2010; Kendal et al., 2011).

Regardless of their origin, the requisite function of T reg cells in preventing autoimmunity must be stringently controlled so as to permit induction, expansion, and function of protective immune responses. Known molecular signals that can inhibit T reg cell function in response to infection include IL-6, IL-1, and multiple

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TLR ligands (Pasare and Medzhitov, 2003; O'Sullivan et al., 2006; Torchinsky et al., 2009; Hu et al., 2011). Signals transmitted by these molecules to T reg cells inhibit or limit Foxp3 expression, preferentially yielding Th1 and/or Th17 effector cells which facilitate expansion of pathogen-reactive T cell responses (Yang et al., 2008). Broad and nonspecific T reg cell inhibitory signals via these mechanisms can potentially overcome self-tolerance, resulting in pathogenic autoimmunity (André et al., 2009; Bettini and Vignali, 2009; O'Sullivan et al., 2006; Radhakrishnan et al., 2008) and prevention of transplant tolerance (Chen et al., 2009; Porrett et al., 2008).

Evidence indicates that Foxp3 expression is regulated more subtly than simply "off/on"; rather, the level of Foxp3 expressed within a given T reg cell affects its suppressive capacity. Genetically induced attenuation (50% reduction), but not absence of Foxp3 in nT reg cells, causes a defect in nT reg cell suppression (Wan and Flavell, 2007; Wang et al., 2010) and lower T reg cell Foxp3 expression has been associated with the development of autoimmunity in humans (Huan et al., 2005; Wan and Flavell, 2007).

The stimuli and signaling pathways that regulate Foxp3 expression in nT reg cells are only partially understood. In $CD4^+CD25^-$ conventional T cells (T conv cells), TCR, and co-stimulatory molecule transmitted signals are associated with PI-3Kγ–mediated conversion of PIP2 to PIP3 leading to the downstream phosphorylation of AKT. In contrast, Foxp3 expression in nT reg cells is associated with suppressed AKT phosphorylation (Crellin et al., 2007; Sauer et al., 2008), a process in part dependent on PTEN, a phosphatase that converts PIP3 back to PIP2 (Carnero et al., 2008), and PHLPP which dephosphorylates p-AKT (Patterson et al., 2011). Studies published in 2010 showed that one mechanism through which p-AKT prevents Foxp3 expression in T reg cells is by phosphorylating the transcription factors Foxo1/3a (Kerdiles et al., 2010; Merkenschlager and von Boehmer, 2010; Ouyang et al., 2010), sequestering them in the cytoplasm through binding to 14–3-3 proteins (Tzivion et al., 2011). The upstream signals that regulate this AKT axis within nT reg cells are incompletely delineated and could represent important mechanisms of self-regulation within the immune system.

In previous works (Lalli et al., 2008; Strainic et al., 2008), we and others showed that co-stimulatory signals transmitted during cognate interactions between T conv cells and APCs unexpectedly induce up-regulation and release of complement components C3, factor B, and factor D, by both partners. We observed simultaneous down-regulation of the cell surface–expressed complement regulator decay-accelerating factor (DAF; CD55), lifting restraint on spontaneous, alternative pathway complement activation and resulting in elevated production of C3a and C5a (Heeger et al., 2005; Lalli et al., 2007; Strainic et al., 2008). The locally produced anaphylatoxins bind to their respective G-protein–coupled receptors, C3aR and C5aR, on the responding T conv cells and on the APC, and independently of TCR signals, activate PI-3Kγ and AKT signaling cascades to promote $CD4^+$ and $CD8^+$ T cell activation, proliferation, differentiation, and survival (Lalli et al., 2008; Peng et al., 2008; Strainic et al., 2008).

Based upon this body of literature, we hypothesized that C3aR and C5aR signaling on nT reg cells would also impact nT reg cell function. Herein, we indeed demonstrate that nT reg cells express C3aR and C5aR and that enhancing signal transmission via these G protein–coupled receptors limits nT reg cell function, whereas blocking signal transduction augments in vitro and in vivo suppressive function in multiple model systems. C3aR/C5aR signaling is biochemically linked to p-AKT–dependent phosphorylation of the transcription factor Foxo1, which alters T reg cell function by modifying the level of Foxp3 expression in the nT reg cell. Together with previous work, our new data delineate a fundamental, immune cell–intrinsic, mechanism that limits nT reg cell function while simultaneously stimulating expansion of effector T cell responses. The findings support the need for testing how targeting C3aR/C5aR signaling could be exploited to therapeutically manipulate T cell immune responses in a variety of disease settings.

RESULTS

Signaling via C3aR and C5aR alters nT reg cell function in vitro

To address whether and how immune cell–derived C3a and/or C5a impact nT reg cells we performed RT-PCR assays on flow-sorted, peripheral, $CD4^+Foxp3^+GFP^+$ nT reg cells obtained from Foxp3-GFP reporter mice (Fontenot et al., 2005) before and after stimulation with anti-CD3/CD28 (Fig. 1 A). These assays showed mRNA expression of both gene products in resting nT reg cells with increases induced by anti-CD3/CD28 stimulation. Identical findings were observed when we evaluated $CD4^+CD25^{hi}Foxp3^+$ T cells obtained from WT mice (unpublished data). Flow cytometry studies showed surface staining of C5aR on WT nT reg cells that increased with anti-CD3/CD28 stimulation whereas no staining was detected on *C5ar1*$^{-/-}$ nT reg cells, thereby verifying specificity (Fig. 1 B). High surface expression of C5aR on neutrophils (Fig. 1 B) served as a positive control. Surface expression of C3aR was not tested because of lack of specific monoclonal antibody availability.

Because our previous work indicated overlapping but not fully redundant effects of signaling via C3aR and C5aR on T conv cells (Lalli et al., 2008; Strainic et al., 2008; Kwan et al., 2012), we tested how the absence of both receptors affected nT reg cell suppressive function. We compared the ability of WT and *C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ $Foxp3^+CD25^+$ nT reg cells to suppress in vitro proliferation of WT $CD4^+CD25^-$ T conv cells stimulated with anti-CD3 and syngeneic splenic APCs (Fig. 1, C and D). These assays showed significantly enhanced suppressive capacity of the *C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ nT reg cells. nT reg cells isolated from either *C3ar1*$^{-/-}$ or *C5ar1*$^{-/-}$ mice also exhibited enhanced in vitro suppression (Fig. 1 E).

Using an alternative strategy, we blocked C3aR signaling and C5aR signaling on WT nT reg cells using small molecule or peptide antagonists specific for each receptor (C3aR-A

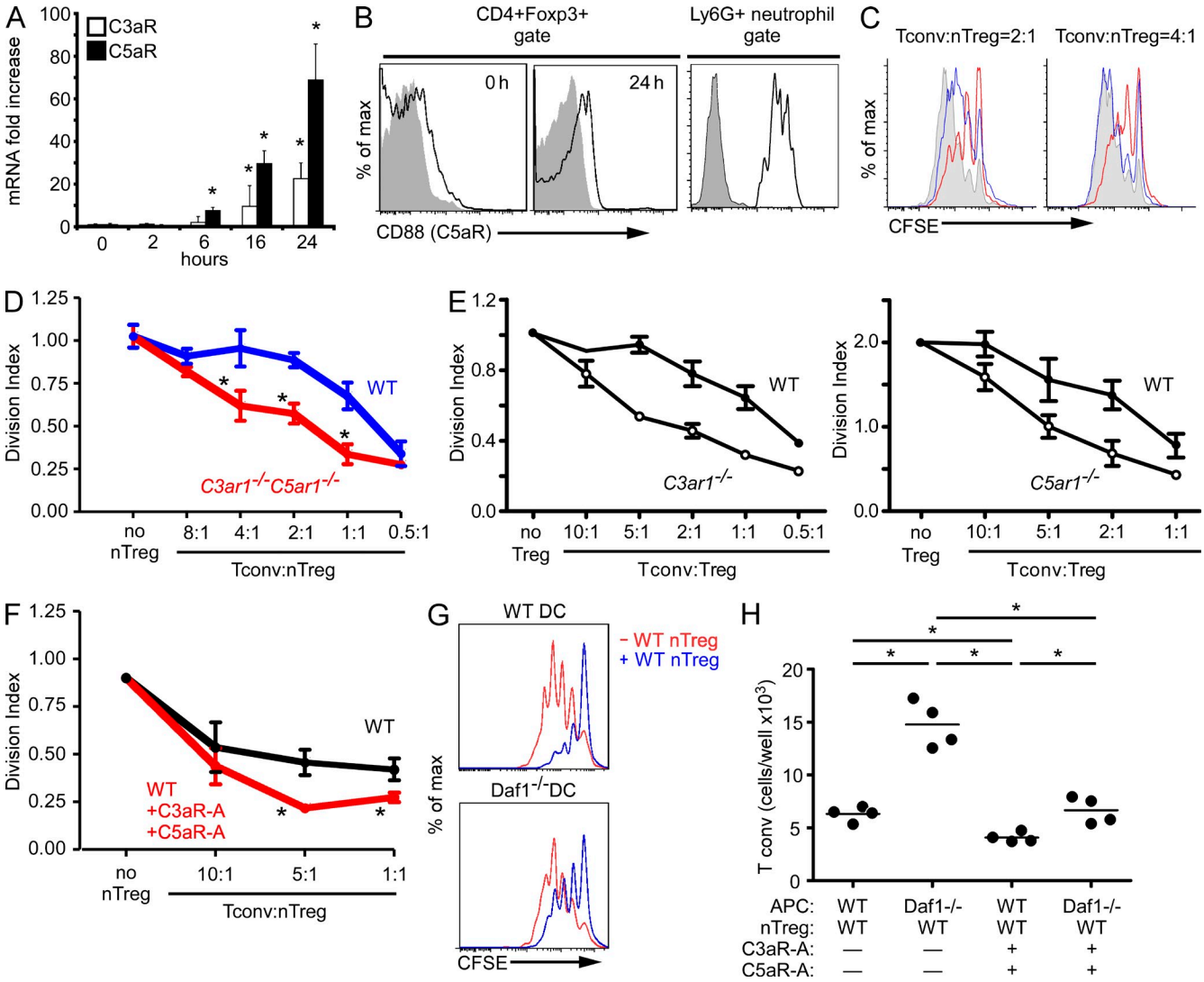

Figure 1. C3aR and C5aR govern in vitro nT reg cell function. (A) Kinetics of C3aR and C5aR gene expression in anti-CD3/anti-CD28–stimulated, flow-sorted CD4$^+$GFP-Foxp3$^+$ nT reg cells. *, $P < 0.05$ versus time 0. (B) C5aR expression (black unfilled) on resting WT nT reg cells (left), 24 h anti-CD3/CD28-stimulated (1 µg/ml) nT reg cells (middle), and Ly6G$^+$ neutrophils (right). Staining of *C5ar1*$^{-/-}$ cells (gray filled) was identical to isotype controls (not depicted). (C) Representative overlays showing in vitro suppression of CFSE-labeled CD45.1$^+$CD4$^+$CD25$^-$ T conv cells mixed with no nT reg cells (gray), CD45.2$^+$ WT nT reg cells (blue) or CD45.2$^+$ *C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ nT reg cells (red) at the ratios indicated. Each histogram is gated on CD45.1$^+$ T conv cells cells. (D) Division indices (mean number of divisions for all of the cells in the original starting population) indicating suppressive capacity of WT (blue) versus *C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ (red) nT reg cells in 72 h suppression assays. (E) Analogous assays performed for WT versus *C3ar1*$^{-/-}$ (left) or *C5ar1*$^{-/-}$ (right) nT reg cells. Assays are representative of at least three individual experiments. (F) Division indices of WT nT reg cells from 72-h suppression assays containing *C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ T conv cells and buffer control (black) or C3aR-A + C5aR-A (red). $P < 0.05$ vs. control. (G) CFSE dilution plots gated on CD45.2$^+$ *C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ T conv cells with (blue) or without (red) WT CD45.1$^+$ nT reg cells at a 1:1 ratio in the presence of either WT (top) or *Daf1*$^{-/-}$ (bottom) DCs. (H) Total number CD45.2$^+$ *C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ T conv cells/well (72-h suppression assays) containing WT nT reg cells, WT, or *Daf1*$^{-/-}$ APCs ± C3aR-A/C5aR-A. *, $P < 0.05$. Each experiment was repeated at least three times with similar results.

and C5aR-A, respectively; Fig. 1 F). In these experiments we used purified T conv cells and splenic CD11c$^+$ DCs, both obtained from *C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ mice, along with CD4$^+$CD25$^+$ nT reg cells from WT mice (that express C3aR and C5aR), so as to isolate the effects of the added antagonists to the nT reg cells. When we added C3aR-A and C5aR-A to the cultures, we observed diminished T conv cell proliferation compared with vehicle controls, verifying that abrogating C3aR/C5aR signaling in nT reg cells enhances nT reg cell function. As a specificity control, we added C3aR-A and C5aR-A to anti-CD3/CD28-stimulated *C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ CD4$^+$CD25$^-$ T conv cells and observed no significant effect (control, 19,957 ± 3,504 cells/well vs. C3aR-A/C5aR-A, 16,953 ± 5,037; $n = 4$; P = ns).

DAF (CD55) is a cell surface–expressed protein that prevents amplification of the complement cascade by accelerating the decay of C3 convertases. Our previously published work showed that genetically induced DAF deficiency lifts restraint over complement activation, resulting in tonically elevated local production of C3a and C5a by immune cells (Heeger et al., 2005; Strainic et al., 2008). We exploited this effect to test whether enhancing APC-derived C3a/C5a (APCs produce ∼1,000-fold more C3 than T cells; Strainic et al., 2008) would inhibit nT reg cell function. In these assays, we again used *C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ T conv cells so as to avoid any direct proliferative/survival effect of locally produced C3a/C5a on the T conv cells (Lalli et al., 2008; Strainic et al., 2008). Consistent with the hypothesis that enhanced C3a/C5a produced in the context of DAF deficiency inhibits nT reg cell function, we observed increased expansion of the T conv cells in cultures containing *Daf1*$^{-/-}$ DCs compared with WT DCs (Fig. 1, G and H). To verify that these observed effects were mediated through C3aR/C5aR signaling, we repeated the experiments, adding the specific C3aR antagonist and the C5aR antagonist to the culture wells. These assays revealed that C3aR-A plus C5aR-A reduced the DAF deficiency-driven expansion of T conv cells back to baseline, not significantly different from the cultures containing WT DCs (Fig. 1 H). Together, these data support the conclusion that C3aR/C5aR signaling on nT reg cells limits their ability to suppress, whereas blocking C3aR/C5aR signaling enhances their suppressive capacity.

C3aR/C5aR signaling regulates Foxp3 expression levels in nT reg cells

We next examined effects of C3aR/C5aR signaling on Foxp3 expression levels by intracellular Foxp3-staining and/or by measuring the mean fluorescence intensity (MFI) of the GFP signal using Foxp3-GFP reporter mice (pilot studies showed that MFI for GFP directly correlates with MFI of intracellular staining for Foxp3; unpublished data). We consistently observed higher levels of Foxp3, as quantified by GFP MFI in resting nT reg cells from *C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ Foxp3-GFP mice compared with WT Foxp3-GFP controls (Fig. 2, A and B). We assessed Foxp3 expression in nT reg cells 72 h after initiating in vitro suppression and observed an ∼30% higher MFI for Foxp3 (or GFP) when C3aR/C5aR signaling was genetically or pharmacologically blocked (Fig. 2 C). The elevated intracellular expression of Foxp3 detected in the absence of C3aR/C5aR signaling on the nT reg cells was associated with increased surface expression of CTLA4 (Fig. 2, D and E), one molecule through which nT reg cells are known to mediate their suppressive function (Wing et al., 2008).

Conversely, when we added recombinant C3a and C5a to anti-CD3–stimulated nT reg cells from WT B6 mice, we detected a reduction in Foxp3 expression (Fig. 2, F and G). Addition of C3a and C5a had no effect on expression levels of Foxp3 in nT reg cells obtained from C*3ar1*$^{-/-}$*C5ar1*$^{-/-}$ mice (Fig. 2, F and G), confirming that C3a and C5a alter

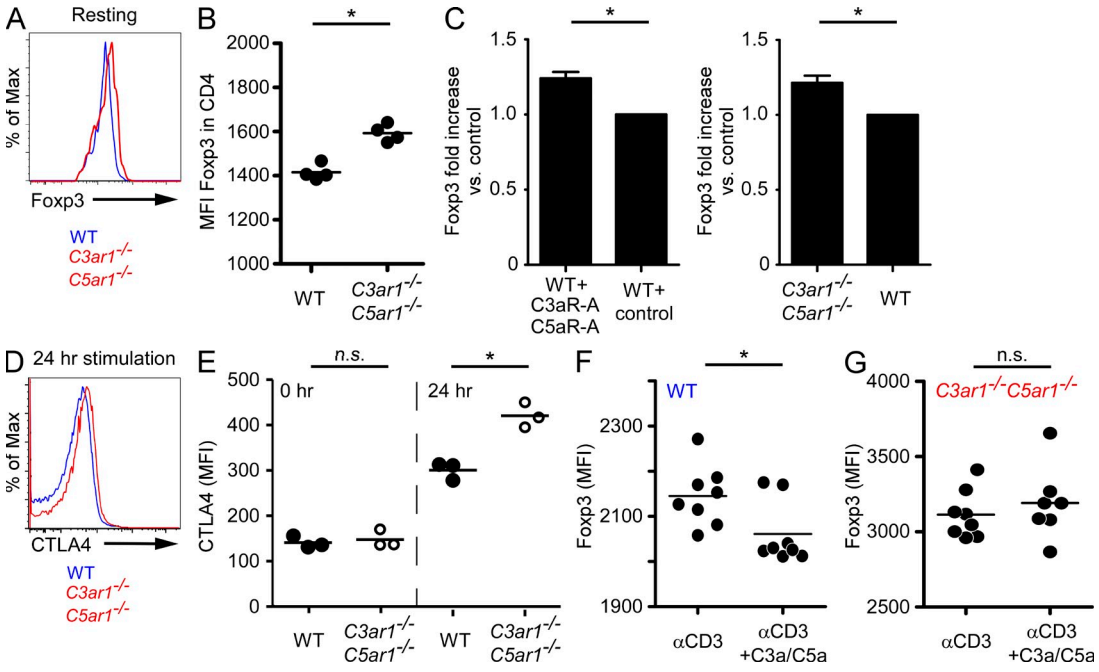

Figure 2. C3aR/C5aR signaling regulates Foxp3 expression. (A) Representative flow plots of intracellular Foxp3 in resting WT (blue) and *C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ (red) Foxp3-GFP^{+} nT reg cells. (B) Quantification of MFI from panel A in each subset. *, $P < 0.05$. (C) Quantified Foxp3 mean fluorescence index in nT reg cells obtained from in vitro suppression assays at 72 h, expressed as a fold increase over simultaneously studied WT controls (considered 100%). Mean C3aR-A/C5aR-A = 131%; mean *C3aR*$^{-/-}$*C5aR*$^{-/-}$ = 126%; $n = 7$ experiments/group; *, $P < 0.05$ vs. untreated WT. Representative histograms (D) and quantified MFI (E) pooled from three experiments for cell surface–expressed CTLA4 on WT and *C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ Foxp3-GFP nT reg cells (gated on GFP^{+} cells) 24 h after stimulation with anti-CD3. *, $P < 0.05$. (F and G) MFI for Foxp3-GFP expression in WT nT reg cells (F) or *C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ nT reg cells (G) 72 h after stimulation with 1 µg/ml anti-CD3 ± C3a/C5a. Each individual experiment was repeated at least once with similar results. *, $P < 0.05$.

Foxp3 expression in nT reg cells directly through C3aR/C5aR signaling on the nT reg cells. Recombinant C3a/C5a also prevented Foxp3 up-regulation in anti-CD3–stimulated, BALB/c CD4^{+}CD25^{hi} nT reg cells (Foxp3-GFP MFI, anti-CD3 + control, 4,748 ± 19.8; anti-CD3 + C3a/C5a, 2,347 ± 24.9; $P < 0.05$; unpublished data), demonstrating that the effects are not mouse strain dependent.

We formally tested the hypothesis that C3a/C5a-induced down-regulation of Foxp3 diminishes nT reg cell suppressive capacity. We flow-sorted Foxp3-GFP^{+} nT reg cells from WT and *C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ mice (Fig. 3 A) and activated them in vitro with anti-CD3, IL-2, and syngeneic *Daf1*$^{-/-}$ DCs, the latter used as a source of locally produced C3a/C5a (Lalli et al., 2008; Strainic et al., 2008). On day 5, we examined Foxp3-GFP expression levels (Fig. 3 B); quantified IL-10 (Fig. 3 C) and TGFβ (Fig. 3 D) in culture supernatants; and tested the suppressive capacity of the activated WT and *C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ nT reg cells in secondary suppression assays (Fig. 3 E). These experiments showed that the activated WT nT reg cells expressed lower levels of Foxp3; produced less IL-10 and TGFβ; and exhibited diminished suppressive capacity compared with the activated *C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ nT reg cells. In parallel control experiments, we re-sorted Foxp3-GFP^{hi} cells from WT and *C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ cultures (documenting similarly high expression of Foxp3; Fig. 3 F), and tested them in suppression assays (Fig. 3 G). These experiments showed that activated and sorted Foxp3^{hi}, WT, and *C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ nT reg cells exhibited indistinguishable suppressive capacities. Together, the data support the conclusion that C3a/C3aR and C5a/C5aR ligations on nT reg cells cause Foxp3 down-regulation and the reduced Foxp3 expression results in lessened suppressive capacity.

Although controversial, evidence indicates that pro-inflammatory signals can limit the function of circulating, predominantly nT reg cells by blocking or down-regulating Foxp3 expression (Degauque et al., 2008; Rubtsov et al., 2010; Zhou et al., 2009a,b), particularly within a CD25^{lo} subset of the Foxp3^{+} T reg cells (Miyao et al., 2012). We tested whether C3aR/C5aR signaling contributes to this phenomenon. When we compared CD25 expression levels on Foxp3-GFP^{+}CD4^{+} cells obtained from naive WT and *C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ Foxp3-GFP reporter mice (Fig. 4, A and B) we observed similar percentages of CD25^{hi} nT reg cells. We flow-sorted the CD25^{hi} and CD25^{lo} nT reg cells from WT and *C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ mice and stimulated each sorted population with IL-2 alone (control) or with anti-CD3/CD28,

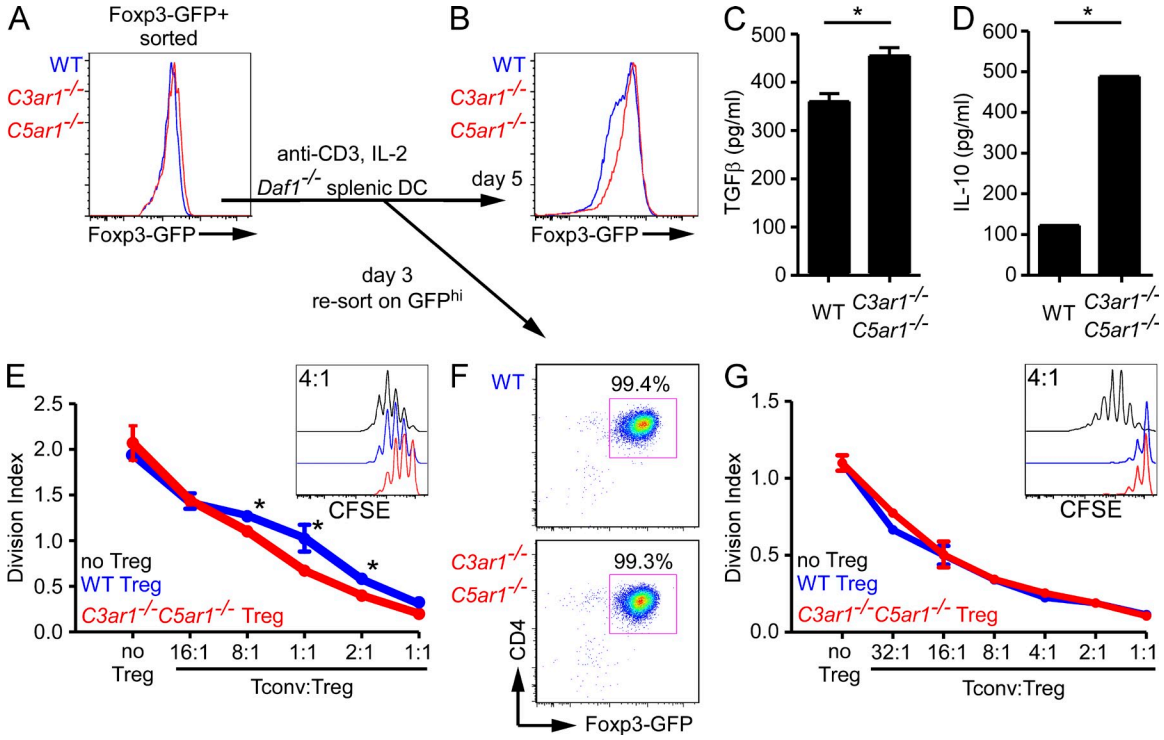

Figure 3. C3aR/C5aR-induced Foxp3 down-regulation results in diminished suppressive capacity. Representative overlay histograms for flow-sorted Foxp3-GFP^{+} cells obtained from WT (blue) or *C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ (red) mice before stimulation (A) or after a 5-d in vitro stimulation with anti-CD3, IL-2 and *Daf1*$^{-/-}$ DCs (B). (C-D) Results of ELISAs (triplicate wells) performed on 5 d culture supernatants for TGFβ (C) and IL-10 (D). (E) Representative quantification (left) and 4:1 T conv cells/nT reg cells histogram (right) of suppression assays using nT reg cells obtained from B. (F and G) Re-sorted Foxp3-GFP^{hi} WT and *C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ nT reg cells after 3 d in culture with anti-CD3, IL-2, and *Daf1*$^{-/-}$ DCs were used in suppression assays. Foxp3-GFP expression on re-sorted cells (F) and suppressive capacity (G) did not differ between groups. Histogram inset shows representative CSFE dilution without nT reg cells (black), with WT nT reg cells (blue), or *C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ nT reg cells (red) at 4:1 T conv cells/nT reg cells. The data are representative of 3–4 independent experiments for each panel. *, $P < 0.05$.

IL-2/IL-6, an activating and proinflammatory stimulus shown by others to limit T reg cell Foxp3 expression (Miyao et al., 2012). 5 d later, we observed that anti-CD3/CD28, IL-2/IL-6 induced down-regulation of Foxp3$^+$ in WT nT reg cells, such that only ∼75% of the CD25^{hi} and ∼40% of the CD25^{lo} subsets remained Foxp3$^+$ (Fig. 4, C and D). In contrast, the same stimulus did not down-regulate Foxp3 expression in the *C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ nT reg cells; >92% were Foxp3$^+$ on day 5 of the culture, regardless of their original CD25^{hi}/CD25^{lo} status (Fig. 4 C). We also observed two-fold higher Foxp3 levels (MFI) in the remaining Foxp3$^+$ *C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ nT reg cells compared with the WT (Fig. 4 E). Control experiments showed that Foxp3 expression was maintained in WT and *C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ nT reg cells cultured with IL-2 alone (Fig. 4, C and D). Together, the data support the conclusion that Foxp3 down-regulation induced by this proinflammatory stimulus administered during nT reg cell activation is dependent on C3aR/C5aR signaling in the nT reg cells.

C3aR/C5aR signaling are linked to Foxp3 expression via AKT and Foxo1

In previous work, we and others showed that C3aR and C5aR transmit PI-3Kγ/AKT-dependent signals that stimulate proliferation and prevent cell death in T conv cells (Lalli et al., 2008; Strainic et al., 2008). PI-3Kγ signaling and downstream AKT phosphorylation have also been documented to prevent Foxp3 expression in nT reg cells (Haxhinasto et al., 2008; Sauer et al., 2008; Hedrick, 2009), together raising the possibility that C3aR/C5aR signaling and Foxp3 expression are linked in nT reg cells through AKT. To test this hypothesis, we stimulated flow-sorted Foxp3-GFP WT nT reg cells with C3a, C5a, or both and performed immunoblots for p-AKT on cell lysates 15 min later (Fig. 5 A). We consistently observed that both the anaphylatoxins, alone and together, up-regulated p-AKT without altering total AKT.

Among numerous substrates, p-AKT phosphorylates the forkhead box o transcription factor Foxo1 resulting in Foxo1 sequestration in the cytoplasm (Brunet et al., 2002; Sauer et al.,

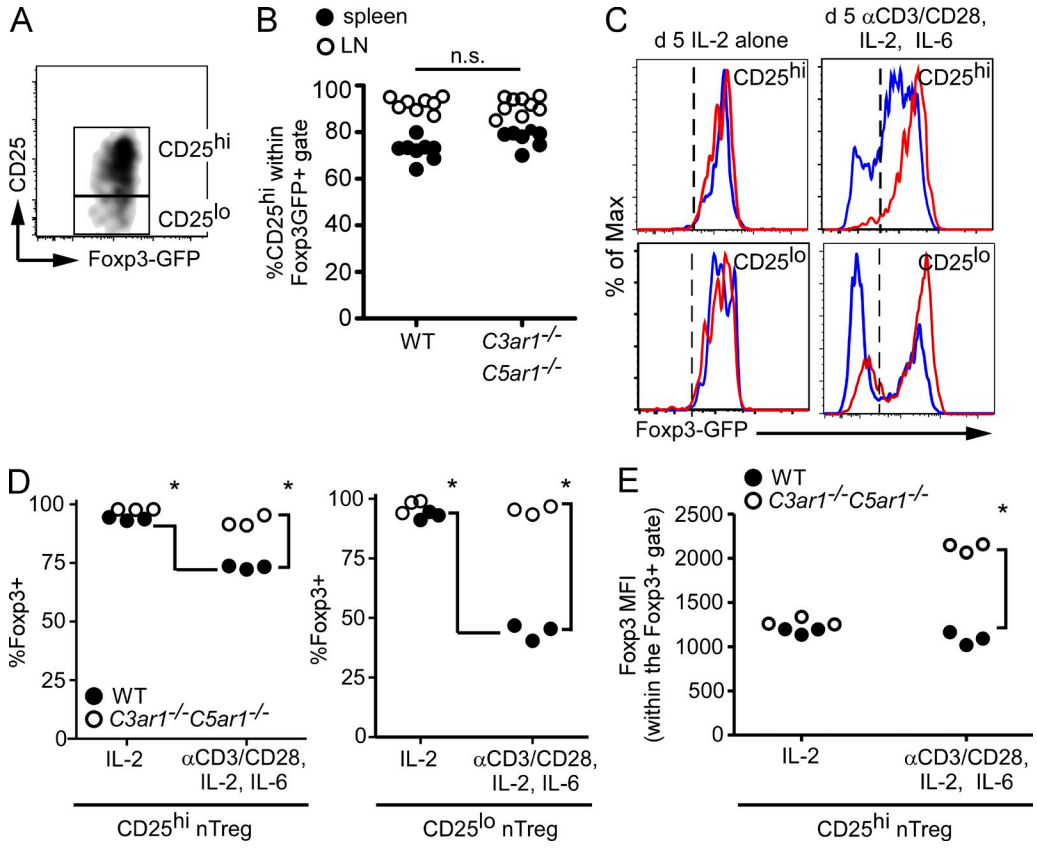

Figure 4. C3aR/C5aR signaling regulates stability of Foxp3 expression in response to a proinflammatory stimulus in vitro. (A) Representative flow plot of Foxp3 and CD25 expression levels and gating strategy to define CD25^{hi} versus CD25^{lo} subsets. (B) Percentages of CD4$^+$Foxp3-GFP$^+$CD25^{hi} cells in spleen (closed circles) and peripheral lymph nodes (open circles) of naive WT and *C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ mice. (C) Representative histograms of Foxp3 expression in CD25^{hi} (top) and CD25^{lo} (bottom) WT (blue) and *C3ar1*$^{-/-}$*C5ar1*$^{-/-}$(red) nT reg cells 5 d after stimulation with IL-2 alone (left) or anti-CD3/CD28, IL-2/IL-6 (right). (D) Quantified results from CD25^{hi} (left) and CD25^{lo} (right) T reg cells ($n = 3$). WT (closed circles) and *C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ (open circles) are shown. *, $P < 0.05$. (E) MFI for Foxp3-GFP within the remaining Foxp3$^+$ cells of the CD25 ^{hi} WT and *C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ after 5 d in IL-2 or anti-CD3/CD28, IL-2/IL-6. WT (closed circles) and *C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ (open circles) are shown. *, $P < 0.05$. All experiments were performed at least three times with similar results.

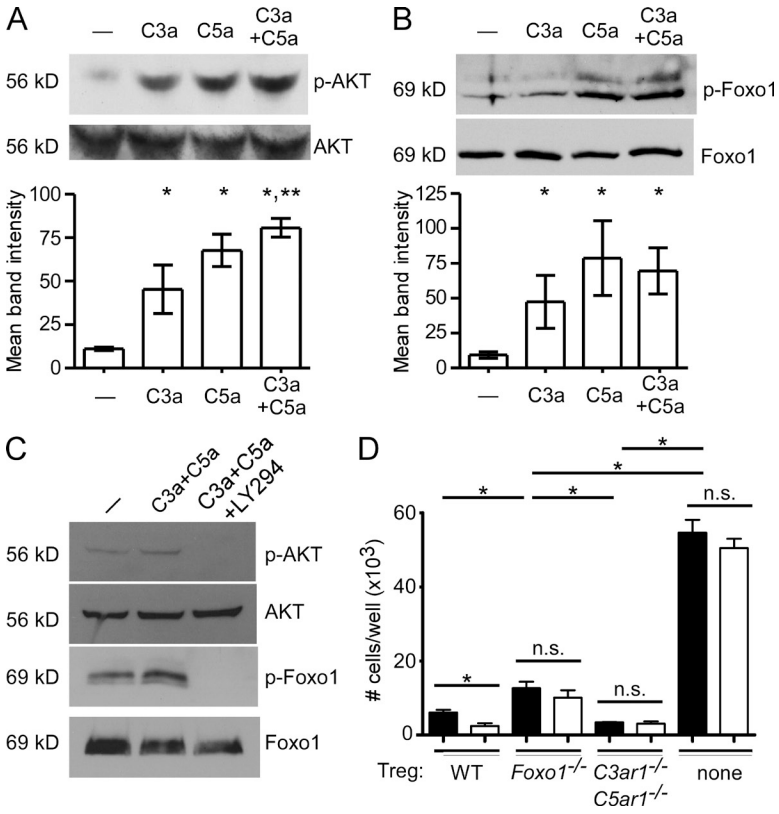

Figure 5. AKT and Foxo1 link C3aR/C5aR signaling to Foxp3 expression. (A and B) Representative immunoblots of flow-sorted Foxp3-GFP$^+$ CD4$^+$ nT reg cells lysates 15 min after stimulation with C3a, C5a, both, or control (buffer alone). p-AKT/total AKT (A) and p-Foxo1/total Foxo1 (B) shown with quantification normalized to nonphosphorylated bands (bottom of each panel). (C) Representative immunoblot of flow-sorted Foxp3-GFP$^+$CD4$^+$ nT reg cells lysates 15 min after stimulation with C3a$^+$C5a ± PI-3K inhibitor LY294 for p-AKT, total AKT, p-Foxo1, and total Foxo1. No signal above background was detected for p-AKT or p-Foxo1 in lysates from the LY294-treated cells. Blots are representative of at least independent three experiments. (D) Total number of T conv cells from suppression cultures using WT, *Foxo1*$^{-/-}$, or *C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ nT reg cells + C3aR-A/C5aR-A (white) or buffer control (black). *, $P < 0.05$. The experiment was repeated twice with similar results.

2008; Hedrick, 2009; Merkenschlager and von Boehmer, 2010). Preventing Foxo1 translocation to the nucleus prevents its ability to bind the Foxp3 locus, where it has been shown to regulate Foxp3 promoter activity. Genetically induced Foxo1 deficiency in CD4 T cells limits induction and function of T reg cells, resulting in systemic autoimmunity (Harada et al., 2010; Kerdiles et al., 2010; Ouyang et al., 2010). We tested whether C3a/C5a signaling in nT reg cells is linked to p-Foxo1, and if so, through AKT, by stimulating WT nT reg cells with C3a and/or C5a and performing immunoblots on cell lysates (Fig. 5 B). These experiments showed that addition of C3a and C5a to WT nT reg cells induced phosphorylation of Foxo1. When we next pharmacologically inhibited AKT we prevented the C3a/C5a-induced increase in p-Foxo1 (Fig. 5 C), thereby linking C3aR/C5aR signaling in nT reg cells to p-Foxo1 via an AKT-dependent mechanism.

To assess the functional significance of the biochemical link between C3aR/C5aR signaling and Foxo1, we added C3aR-A and C5aR-A to suppression assays containing WT or *foxo1*$^{-/-}$ nT reg cells and compared the expansion of T conv cells in the culture wells (Fig. 5 D). Whereas addition of C3aR-A and C5aR-A enhanced the in vitro suppressive capacity of WT CD4$^+$CD25^{hi} nT reg cells, limiting T conv cells cell expansion (Fig. 5 D), the same antagonists did not alter the already reduced suppressive capacity (reflected by more T conv cells) of *foxo1*$^{-/-}$ CD4$^+$CD25^{hi} nT reg cells. Parallel, control experiments confirmed enhanced suppressive ability of *C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ CD4$^+$CD25^{hi} nT reg cells (culture wells contained fewer T conv cells than in wells containing WT nT reg cells) and confirmed that addition of C3aR/C5aR antagonists did not alter the suppressive function of these nT reg cells, thereby verifying specificity of the effects (Fig. 5 D). Together, these data support the conclusion that C3aR/C5aR signaling on nT reg cells modulates nT reg cell suppressive function in part, via controlling AKT-dependent Foxo1 phosphorylation and downstream Foxp3 expression.

Absence of C3aR/C5aR signaling enhances in vivo nT reg cell suppressive function

To test the impact of C3aR/C5aR signaling in T reg cells on their in vivo function, we quantified expansion of splenic CD45.1$^+$CD4$^+$CD25$^-$ T conv cells 6 d after co-transfer with WT or *C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ CD45.2$^+$CD4$^+$Foxp3$^+$ nT reg cells into syngeneic *rag1*$^{-/-}$ recipients (Fig. 6, A and B). These experiments showed that *C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ nT reg cells reduced T conv cell expansion in immunodeficient hosts by ~40% more than WT nT reg cells. We enumerated splenic nT reg cells in each animal on day 6 (Fig. 6, C and D) and found fewer *C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ nT reg cells than WT nT reg cells. These results argue against inadvertent transfer of more *C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ nT reg cells and against the possibility that C3aR/C5aR deficiency confers a survival advantage.

To assess the effect of C3aR/C5aR signaling on nT reg cells in disease models, we compared the ability of WT and *C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ nT reg cells to suppress autoimmune

colitis when co-transferred with WT T conv cells into *rag1*$^{-/-}$ hosts (Fig. 7 A). 6 wk after the adoptive transfers, recipients of T conv cells alone lost 15% body weight (which is consistent with reports by others; Powrie et al., 1993; Read et al., 2000; Wan and Flavell, 2007), whereas recipients co-transferred with WT nT reg cells maintained their baseline weights. The recipients of T conv cells plus *C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ nT reg cells gained 10% body weight ($P < 0.05$ vs. WT nT reg cells). When we examined the histology of the colon tissue (Fig. 7 B), we observed lower pathology scores (Murthy et al., 1993) in those given WT nT reg cells (vs. no nT reg cells), whereas the colons from mice given *C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ nT reg cells were essentially normal. Spleens of animals transferred with WT or *C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ nT reg cells contained similar numbers of nT reg cells (Fig. 7 C). We also observed higher Foxp3 levels in recovered splenic *C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ nT reg cells (Fig. 7 D), validating in vivo the in vitro finding that C3aR/C5aR signaling impacts Foxp3 expression. An independent experiment in which we transferred fewer cells but followed animals for 14 wk showed similar results (percentage of baseline body weight: no nT reg cells, 91%; WT nT reg cells, 101%; *C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ nT reg cells, 111%; $P < 0.05$ among groups; $n = 4$ per group; unpublished data).

We also compared the ability of WT and *C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ nT reg cells to prevent allogeneic skin graft rejection. We transplanted BALB/c tail skin onto B6 *rag1*$^{-/-}$ recipients, allowed the grafts to fully heal (>3 wk), and then performed adoptive transfers of T conv cells without or with flow-sorted WT or *C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ CD4$^+$ Foxp3-GFP$^+$ nT reg cells based on a published protocol (Nagahama et al., 2007). We observed prolonged graft survival in the recipients given *C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ nT reg cells compared with WT nT reg cells (Fig. 7, E and F), confirming enhanced in vivo suppressive function in another model system. As published (Nagahama et al., 2007), control animals transferred with T conv cells alone rejected their grafts faster than those given nT reg cells from either source.

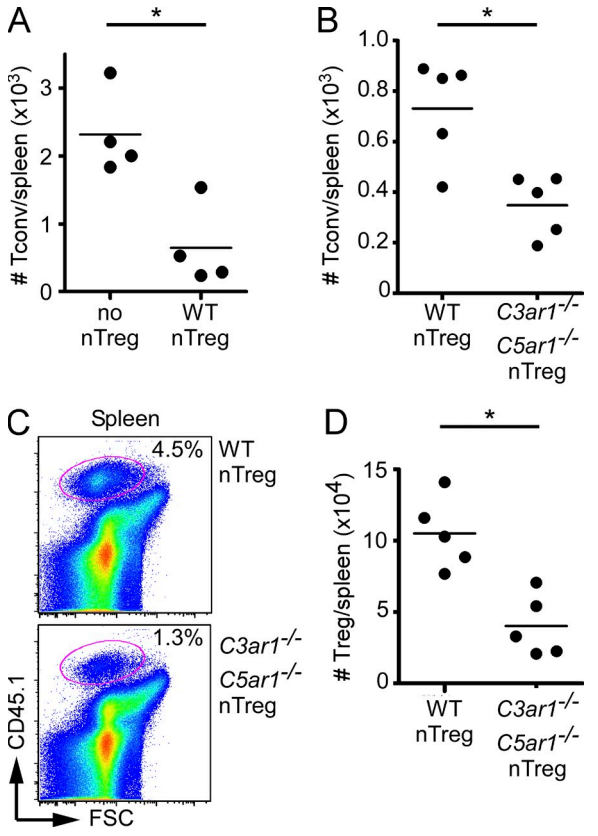

Figure 6. ***C3aR*$^{-/-}$*C5aR*$^{-/-}$ nT reg cells exhibit enhanced ability to inhibit homeostatic proliferation.** CD45.1 T conv cells were injected alone or 1:1 with CD45.2$^+$ WT or with *C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ nT reg cells into syngeneic *rag1*$^{-/-}$ recipients. Homeostatic proliferation was assessed on day 6. (A) Total number of splenic T conv cells per mouse after co-transfer with no nT reg cells or WT nT reg cells. (B) In a separate experiment, WT and *C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ nT reg cells suppress homeostatic proliferation. Representative flow plots from two individual experiments depicting percentages of nT reg cells per spleen(C) and the total number of nT reg cells per spleen (D). *, $P < 0.05$.

DISCUSSION

Our data establish a key role for C3a/C3aR and C5a/C5aR signaling on nT reg cells as molecular modulators of nT reg cell function. C3aR- and C5aR-transduced signals inhibit the nT reg cell's ability to suppress in vitro (Fig. 1), whereas blockade of C3aR/C5aR signaling on nT reg cells enhances their in vitro (Fig. 1) and in vivo (Figs. 6 and 7) suppressive capacity. The in vitro cultures performed in serum free-medium (Fig. 1) show that C3a and C5a produced during cognate T cell–APC interactions are sufficient to mediate the effects. C3a and C5a have extremely short in vivo half-lives, as both are degraded by ubiquitously expressed carboxypeptidase activities (Mueller-Ortiz et al., 2009), providing a physiological mechanism through which the immune cell–produced complement activation products could locally impact T conv and T reg cells without systemic effects. The previously observed role for immune cell–derived complement as a T cell co-stimulatory intermediary that promotes T cell expansion (Heeger et al., 2005; Lalli et al., 2007, 2008; Peng et al., 2006, 2008; Strainic et al., 2008; Zhou et al., 2006), in conjunction with the new data indicating that C3aR/C5aR mediate inhibition of nT reg cell function, provides a mechanism to explain how the immune system can optimize inflammatory T cell responses to an inciting antigen without compromising systemic regulatory mechanisms that could result in pathological autoimmunity.

Our results support the conclusion that C3aR/C5aR signaling on circulating nT reg cells modulates their function by controlling the level of Foxp3 expression in the T reg cells (Figs. 2–4). We observed more *C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ Foxp3$^+$ nT reg cells (fewer became Foxp3$^-$) after in vitro stimulation (Figs. 3 and 4) and observed higher Foxp3 expression levels in Foxp3$^+$ T reg cells from *C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ mice compared with WT controls, at rest, after TCR stimulation (Fig. 2–4) and in vivo after adoptive transfer into *rag1*$^{-/-}$ hosts (Fig. 7). Together with the detected increased surface expression of

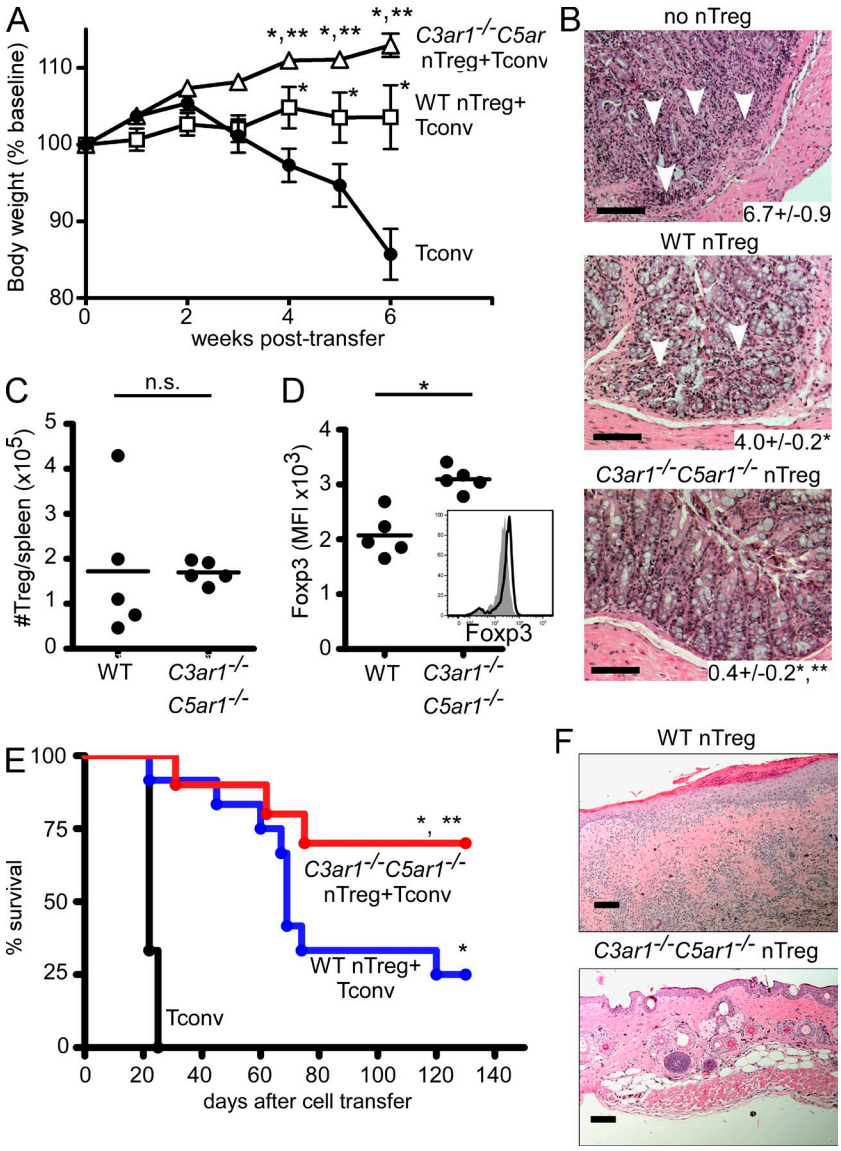

Figure 7. *C3aR*$^{-/-}$*C5aR*$^{-/-}$ nT reg cells more efficiently prevent autoimmune colitis and skin allograft rejection in vivo. Autoimmune colitis was assessed over 6 wk. (A) Weights of animals given CD45.1 T conv cells alone (closed circles, *n* = 3), T conv cells plus 2:1 CD45.2$^+$ WT nT reg cells (open squares, *n* = 10), or T conv cells plus 2:1 *C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ nT reg cells (open triangles, *n* = 10). Data are pooled from two individual experiments. (B) Representative H&E-stained colon sections with quantitative scores (*n* = 5–6 per group) shown in bottom right. Bar, 100 µm. *, P < 0.05 versus no nT reg cells; **, P < 0.05 versus WT nT reg cells. (C) Number of CD45.2$^+$ nT reg cells per spleen of each mouse from A at 6 wk. (D) Representative Foxp3 staining gated on splenic CD45.2 nT reg cells at 6 wk from each mouse in A. Representative Foxp3 expression histogram shown in bottom inset. WT, filled; *C3ar1*$^{-/-}$*C5ar1*$^{-/-}$, open. *, P < 0.05. (E) BALB/c tail skin graft survival in B6 *rag1*$^{-/-}$ recipients receiving T conv cells alone (black, *n* = 3), T conv cells + WT nT reg cells (blue, *n* = 12), or T conv cells + *C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ nT reg cells (red, *n* = 12). *, P < 0.05 versus no T reg cells; **, P = 0.05 versus WT nT reg cells. (F) Representative skin grafts in recipients receiving WT nT reg cells sacrificed at day 65 rejection (left) or *C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ nT reg cells sacrificed at day 120 were H&E stained. Bar, 100 µM.

CTLA4 on *C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ nT reg cells (Fig. 2), a molecule that mediates suppression and is regulated by Foxp3 (Wing et al., 2008), and the functional data using activated nT reg cells from WT and *C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ mice (Fig. 3), our data indicate that C3aR/C5aR signaling physiologically regulates these circulating T reg cells in part by modulating Foxp3 expression. Our results also suggest that IL-6–mediated inhibition of T reg cells (manifested by Foxp3 down-regulation) is dependent on C3aR/C5aR signaling, as *C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ nT reg cells are resistant to the effects (Fig. 4). Several groups showed that TLR-stimulated APCs up-regulate IL-6, IL-1, and complement protein production, coincident with inhibiting T reg cell function (Pasare and Medzhitov, 2003; Hu et al., 2011; Peng et al., 2006; Zhou et al., 2006), and that the IL-6–mediated effects are linked to C5aR in other cell types (Riedemann et al., 2003, 2004), thereby providing additional associative evidence in support of this concept.

The mechanism through which C3aR/C5aR signaling on the T reg cell lowers Foxp3 expression is at least partially mediated via a canonical signaling pathway which enhances AKT phosphorylation, leading to p-AKT–dependent phosphorylation of the transcription factor Foxo1 (Fig. 4). Although the prevention of AKT and Foxo1 phosphorylation have been previously described as required for inducing/maintaining Foxp3 and T reg cell function (Crellin et al., 2007; Sauer et al., 2008), the proximal stimuli initiating these signaling pathways were not described, thereby highlighting the novelty of our results. C3aR and C5aR are seven transmembrane spanning, G protein–coupled receptors that have been demonstrated to transmit signals via both their Gβγ subunit that induces PI-3Kγ–dependent AKT phosphorylation, and through their Gαi subunit, which leads to cAMP-dependent PKA activation and subsequent phosphorylation of the transcription factor CREB. Although our results linking

C3aR/C5aR signaling to p-AKT and p-Foxo1 in nT reg cells are clear (Fig. 4), they do not rule out additional contributing effects, including those involving the cAMP–PKA–CREB pathway, and or cross-talk with NF-κB, JAK/STAT5, and ERK pathways, the latter of which have also been implicated in modulating Foxp3 (Ohkura et al., 2011). The specific differences and overlapping signaling pathways induced by stimulation of C3aR and C5aR, which each signal via PI-3Kγ to induce p-AKT and p-Foxo1 (Fig. 5), and each of which impact T reg cell function (Fig. 1), remain to be elucidated.

In conclusion, in this report we have shown that signaling through C3aR and C5aR physiologically down-regulates the function of $CD4^+Foxp3^+$ circulating T reg cells. The delineation of this previously unrecognized mechanism raises the possibility that blocking C3aR/C5aR signaling on nT reg cells could be exploited to treat transplant rejection or autoimmunity and that augmenting C3aR/C5aR signaling on nT reg cells could be used to limit T reg cell function, thereby enhancing effector T cell responses to pathogens and tumors.

MATERIALS AND METHODS

Mice. C57BL/6, CD45.1 C56BL/6, BALB/c, and B6 *rag1*$^{-/-}$ were purchased from The Jackson Laboratory. Mice deficient in the *Daf1* gene (*Daf1*$^{-/-}$; Heeger et al., 2005) were backcrossed for >13 generations to B6. *C5ar1*$^{-/-}$ mice (*H-2*^{b}) were obtained from C. Gerard (Boston Children's Hospital, Boston, MA). *H-2*^{d} *C3ar1*$^{-/-}$ mice (The Jackson Laboratory) were backcrossed >9 generations to B6 and then intercrossed with the *C5ar1*$^{-/-}$ mice to produce *C3ar1*$^{-/-}$*C5ar1*$^{-/-}$. *foxo1*$^{-/-}$ mice were produced as described (Ouyang et al., 2010). Foxp3-GFP reporter mice obtained from A. Rudensky (Sloan-Kettering Institute, New York, NY; Fontenot et al., 2005) were crossed with the *C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ mice to produce *C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ Foxp3-GFP. All mice were housed in the Mount Sinai School of Medicine Center for Comparative Medicine and Surgery or Sloan Kettering Animal Facility (*foxo1*$^{-/-}$) in accordance with guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care International.

Abs and reagents. Antibodies against CD4, Foxp3, GR1, CTLA4 (eBioscience), p-AKT (Ser473; BD), CD88, CD45.1 (BioLegend) were used for flow cytometry. Antibodies against p-AKT (Ser473), AKT, p-Foxo1 (Thr24), Foxo1, and secondary HRP-conjugated antibodies (Cell Signaling Technology) were used for immunoblotting. C3aR antagonist (559410; EMD Millipore) was used at 200 nM. C5aR peptide antagonist (Ac-Phe-cyclo [Orn-Pro-dCha-Trp-Arg]; GenScript) was used at 10 μM. CFSE was obtained from Molecular Probes/Invitrogen. IL-6 was purchased from BD. IL-10 (Cell Sciences) and TGFβ ELISAs (BD) were performed as per the manufacturer's recommendations.

Suppression assay. Single-cell suspensions were isolated from pooled spleen and lymph nodes from age- and sex-matched mice. APCs were isolated using CD90.2 depleting microbeads (Miltenyi Biotec) and irradiated (800 rad). Responding $CD4^+$ T cells were isolated using $CD4^+CD62L^+$ T cell isolation kit (Miltenyi Biotec) and labeled with CFSE (Invitrogen). nT reg cells were isolated using $CD4^+CD25^+$ regulatory T cell isolation kit (Miltenyi Biotec) to >94% purity and enriched by flow sorting ($CD4^+GFP^+$ or $CD4^+CD25^{hi}$) yielding >98% purity. 5×10^4 responding T cells were cultured with 5×10^4 APCs in complete medium (RPMI + 10% FCS + L-Glutamine + sodium pyruvate + NEAA + Pen/Strep + β-mercaptoethanol) and 1 μg/ml anti-CD3ε (2C11; eBioscience) for 72 h at 37°C ± nT reg cells.

Flow cytometry. Dead cells were excluded using viability dye eFluor 450 (eBioscience). Intracellular Foxp3 staining was performed using Foxp3 staining kit (eBioscience). Samples were collected using a FACSCanto II (BD) flow cytometer and analyzed using Flow Jo software (Tree Star).

Western blot. nT reg cells were rested in complete RPMI medium (30 min), followed by stimulation at 37°C with anti-CD3 (clone 2C11; eBioscience), anti-CD28 (clone 37.51; eBioscience). huC3a and/or mC5a (R&D Systems) were each added at 100 ng/ml. Cell lysates were prepared in 2X Laemmli Sample Buffer + β-Me (Bio-Rad Systems) and boiled. Lysates were analyzed by SDS-PAGE/immunoblot. Blots were blocked and blotted according to antibody manufacturer's recommendations. Results were quantified by densitometry.

Real-time PCR. RNA isolation was performed using RNeasy Mini kit (QIAGEN) and cDNA was reverse-transcribed using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems) as per the manufacturer's instructions. RT-PCR (TaqMan probes; Applied Biosystems) was performed using the CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories). PCR products were normalized to the control gene (MRP123) and expressed as fold increase compared with unstimulated cells using the ΔΔCt method.

Homeostatic proliferation. *Rag1*$^{-/-}$ mice were injected i.v. with 3×10^5 $CD45.1^+CD4^+CD25^{neg}$ T conv cells ± 1.5×10^5 purified $CD45.2^+$ WT or *C3aR*$^{-/-}$*C5aR*$^{-/-}$$CD4^+CD25^+$ nT reg cells and analyzed on day 6.

Colitis. *Rag1*$^{-/-}$ mice were injected i.v. with 5×10^5 $CD45.1^+CD4^+CD25^{neg}$ T conv cells ± 2.5×10^5 purified $CD45.2^+$ WT or *C3ar1*$^{-/-}$*C5ar1*$^{-/-}$$CD4^+CD25^+$ nT reg cells. Animals were weighed weekly and sacrificed after 6 wk (in a second experiment the animals were followed for 14 wk) or until the animal lost >20% body weight. Spleen cells were analyzed by flow cytometry, and gut histology was processed, stained with H&E, and analyzed by blinded investigator using sum of an 8-point scale (Murthy et al., 1993) involving inflammation (0–4) and epithelial injury (0–4).

Inflammation. 0, no inflammation. 1, low level of inflammation with mildly increase inflammatory cells in the lamina propria. 2, moderately increased inflammation in the lamina propria (multiple foci). 3, high level of inflammation with evidence of wall thickening by inflammation. 4, maximal severity of inflammation with transmural leukocyte infiltration and or architectural distortion.

Epithelial injury. 0, normal or no neutrophils. 1, occasional epithelial lesion (focal and superficial or rare cryptitis). 2, foci of cryptitis, including rare crypt abscess. 3, multiple crypt abscesses and or focal ulceration. 4, grade 3 plus extensive ulceration.

Skin grafting. BALB/c tail skin was transplanted onto B6 *rag1*$^{-/-}$ mice (Heeger et al., 2005) and permitted to fully heal (≥2 wk). 2×10^5, flow-sorted B6 $CD45.1^+CD4^+CD25^{neg}$ naive T conv cells with or without 2×10^5 flow-sorted WT or *C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ $CD45.2^+CD4^+Foxp3\text{-}GFP^+$ nT reg cells were injected intravenously into the recipients and the skin grafts were monitored weekly. Rejection was defined as >80% necrosis as assessed by visual inspection. Histology was assessed by examining H&E-stained sections.

Statistical analysis. To determine whether groups were statistically different, results were compared using the Mann-Whitney test or the Student's *t* test. We used the Log-Rank (Mantel-Cox) test to compare skin graft survival. A p-value ≤0.05 was considered significant.

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The authors report no conflicts of interest.

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