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Disabled C3ar1/C5ar1 signaling in Foxp3+ T regulatory cells leads to TSDR demethylation and long-term stability

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Summary

Demethylation of the Treg Specific Demethylation Region (TSDR) of the Foxp3 gene is the hallmark of Foxp3$^+$ T regulatory cell (Treg) stability, but the cellular signaling that programs this epigenetic state remains undefined. Herein, we show that suppressed C3a and C5a receptor (C3ar1/C5ar1) signaling in murine Tregs plays an obligate role. Murine *C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ Foxp3$^+$ cells showed increased SOCS1/2/3 expression, Vitamin C stabilization, and TET1/2/3 expression, all of which are linked to Treg stability. *C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ Foxp3$^+$ cells additionally were devoid of BRD4 signaling that primes Th17 cell lineage commitment. Orally induced ova specific *C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ Foxp3$^+$ OT-II Tregs transferred to ova-immunized WT recipients remained >90% Foxp3$^+$ out to 4 months, whereas identically generated *CD55*$^{-/-}$ (*DAF*$^{-/-}$) Foxp3$^+$ OT-II Tregs (in which C3ar1/C5ar1 signaling is potentiated) lost >75% Foxp3 expression by 14 days. After 4 months *in vivo*, the *C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ Foxp3$^+$ OT-II Tregs fully retained Foxp3 expression even with ova challenge and produced copious TGF-β and IL-10. Their TSDR was de-methylated comparably to that of thymic Tregs (tTregs). They exhibited nuclear translocation of NFAT and NF-κB reported to stabilize tTregs by inducing hairpin looping of the TSDR to the Foxp3 promoter. Thus, disabled CD4$^+$ cell C3ar1/C5ar1 signaling triggers the sequential cellular events that lead to demethylation of the Foxp3 TSDR.

Introduction

Demethylation of the Foxp3 T regulatory cell (Treg) Specific Demethylation Region (TSDR) of the Foxp3 gene is the genomic signature of stable Tregs (1). This insight came from findings that the TSDR is demethylated in thymic Tregs (tTregs) which exhibit durable Foxp3 expression, whereas it is methylated in *ex vivo* TGF-β induced Tregs (iTregs) that rapidly lose Foxp3 expression. Characterizing the processes underlying TSDR demethylation is important immunologically for understanding T cell homeostasis, Treg control of T effector (Teff) responses, avoidance of autoimmunity, and tolerance.

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Author Contributions

MEM: conceived and designed the studies and most experiments were conducted in his lab. SAR: performed TSDR methylation assays: EMS: participated in the configuration and interpretation of the *in vivo* experiments

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Knowledge of the processes that give rise to TSDR demethylation is highly relevant therapeutically as durable Foxp3 expression can enable effective Treg biotherapy. This knowledge also is important for characterizing Treg dysfunction in disease. Because a demethylated TSDR is required for Treg durability, *ex vivo* expanded populations of thymic Tregs (tTregs) that possess this modification and peripherally generated Tregs (pTregs) that acquire it, have been employed. The tTreg and pTreg subsets, however, are restricted to their existing specificities, and can lack full suppressor functionality. De novo induction of polyclonal or antigen-specific iTregs containing a demethylated-TSDR and possessing robust suppressor activity, if achievable, could overcome these limitations in that they could function more potently. An iTreg induction protocol enabling the *ex vivo* generation of potent iTregs with demethylated TSDRs could facilitate the induction of both autologous and allogeneic immunosuppression.

While the signaling cascade in $CD4^+$ cells that leads to TSDR demethylation and durable Tregs remains undefined, a number of processes connected with the acquisition of this state have been implicated. Among these processes are 1) upregulation of Suppressor Of Cytokine Signaling (SOCS1/2/3) expression, 2) suppression of phosphatidylinositol 3,4,5 trisphosphate [PtdIns (3,4,5)P_3] assembly and AKT phosphorylation, 3) remodeling of genomic DNA, and 4) repression of Th17 gene transcription. A final process implicated in the stabilization is 5) hairpin looping of intron 1 Conserved Noncoding Sequence 2 (CNS2) (containing the TSDR) to the Foxp3 promoter (2). While these and other (see Discussion) reported processes seemingly appear unconnected, reversal of any one of them disrupts the induction of stable Tregs. Whether a shared signaling process underlies their collective induction has remained unknown.

In previous studies (3, 4), we found that interacting dendritic cells (DCs) and $CD4^+$ T cells locally produce C3a and C5a from endogenously synthesized complement C3 and C5 proteins and the two anaphylatoxins establish autocrine C3ar1 and C5ar1 signaling loops in both partners. The C3ar1/C5ar1 G protein coupled receptor (GPCR) signaling is controlled by the cell associated regulator CD55 [aka decay accelerating factor (DAF)]. Potentiated C3ar1/C5ar1 signaling occurring as a result of downregulated CD55 promotes Th1 and Th17 Teff responses (4, 5). Conversely, repressed C3ar1/C5ar1 signaling occurring in conjunction with heightened CD55 expression programs T cells to endogenously synthesize TGF-β and IL-10 and become Tregs (6). The Tregs generated in this way show upregulated expression of C5a receptor 2 (C5ar2 aka C5L2), a decoy GPCR devoid of a G protein that scavenges C5a/C3a (7), preventing C3ar1/C5ar1 signaling into the Tregs. The C3ar1/C5ar1 repressed Tregs show disabled AKT phosphorylation (6), absent IL-6 production (6), and inhibited STAT3 activation (6). Functionally they exhibited 4–8-fold > suppressor activity than Tregs conventionally induced by incubating naïve $CD4^+$ cells with exogenous TGF-β (6). They also possessed greater stability in short term (5 day) *in vitro* studies. Methylation assays, however, showed 88% TSDR methylation (6). We hypothesized that demethylation might require longer times and/or *in vivo* conditions.

In this study, we found that disrupted C3ar1/C5ar1 signaling elicits multiple processes that have been implicated in demethylation of the TSDR, including hairpin looping of the CNS2 region to the Foxp3 promoter that has been mechanistically connected with

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stabilizing Foxp3 transcription. We then tested whether disrupted C3ar1/C5ar1 transduction is the signaling condition required for the induction of stable Ag specific Tregs *in vivo*. We transferred sorted ova-specific Foxp3$^-$OT-II CD4$^+$ cells to naïve recipients and orally immunized the recipients by feeding them ova to induce *de novo* ova specific OT-II cell pTregs. We isolated the Foxp3$^+$ cells and transferred the flow purified pTregs to naïve recipients. We then studied their properties over a 4-month period in the absence and presence of ova challenge. We found that pTregs in which CD55 restraint on C3ar1/C5ar1 signaling is lifted rapidly lost Foxp3 expression. In contrast, pTregs disabled in C3ar1/C5ar1 signaling survived > 4 months and retained full immunosuppressive capacity. Phenotypic analyses showed that the 4-month pTregs possessed a demethylated TSDR characteristic of stable tTregs. The analyses showed that the ova specific pTregs expressed high levels of TGF-β and IL-10 characteristic of robust Treg function..

Methods

Animals

Foxp3-GFP OT-II mice *CD55*$^{-/-}$, WT and *C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ backgrounds were prepared in the Medof lab. Foxp3-GFP mice were kindly provided by Dr. V Kuckroo (Harvard Medical School). They were maintained in the Case Western Reserve Animal Facilities. All experiments were approved by CWRU Institutional Animal Care and Use Committee (IACUC).

Reagents and Antibodies

Ovalbumin (ova) was obtained from Sigma-Aldrich (St Louis, Mo). qPCR primers were designed by obtained Integrated DNA Technologies (IDT) (Coralville, IA). Vitamin C was obtained from Fisher Scientific. C3ar1-A 559410-10mg and C5ar1-A-234415-5mg were from Millipore 31908021. Fluorescein isothiocyanate–anti-CD4 (RM-4-5), phycoerythrin–indotricarbocyanine–anti-Thy-1.1 (53–2.1), allophycocyanin–anti-Thy-1.2 (53–2.1), phycoerythrin–anti-CD45.1 (A20) and allophycocyanin–eFluor 780–anti-CD45.2 (104) were from eBioscience.

Foxp3$^+$ T regulatory cell Induction

CD4$^+$ T cells were isolated from spleens and LNs via CD4$^+$ Negative Selection Cocktail Beads per the manufacturer's instructions (Miltenyi, Biotec) and purified with an Automacs Pro. Foxp3$^-$ and Foxp3$^+$ CD4$^+$ T cells were purified by cell sorting gating on GFP$^+$ and GFP$^-$ populations as described in (6) As described (6), iTregs were prepared by incubating Foxp3$^-$ (GFP$^-$) CD4$^+$ cells with anti-CD3/28 Dynabeads plus IL-2 (5 ng/ml) for 3–5 days after which Foxp3$^+$ (GFP$^+$) Tregs were purified by cell sorting. pTregs were prepared by adoptive transfer of Thy-1.2$^+$ (CD45.1$^+$) CD4$^+$ OT-II cells (4×10^6) on each genotype via tail vein into ova-fed WT Thy-1.1$^+$ mice followed by immunizing the recipients with ova (323–339) (100 ug) in IFA.

Flow Cytometry and Cell Sorting

All FACS analyses and studies involving cell sorting were performed on an LSR2 Flow Cytometer in the Flow Cytometry Core of the Comprehensive Cancer Center at Case

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Western Reserve University. Fluorescent labeled anti-Thy-1.2 and ova mAbs were obtained from Bioscience. Florescent labeled anti-human TGF-β and IL-10 mAbs were obtained from Bioscience. Staining and gating were performed as described in (4, 6).

Amnis Cytometry

Assays for nuclear NFAT and NF-kB were carried out on an Amnis Cytometer (Beckman-Coulter San Diego, CA) (Dept of Pathology, Case Western Reserve University) using fluorescent labeled anti-NFAT and NF-kB mAbs (Cell Signaling Inc. Danvers MA). A gate was placed on the nuclear stain and NFAT and NF-kB signals in and outside of the gate were assessed.

TSDR Methylation Analysis

Measurements of TSDR methylation was performed by bisulfite sequencing as described in (8, 9) in the Shevach lab (NIAID, NIH, Bethesda, MD).

Statistics.

Statistical significance was determined by Student's t-test (unpaired, two-tailed) with Microsoft Excel or GraphPad Prism 5.

Animal Studies

All mouse breeding and adoptive transfers, long-term follow-up and harvesting of spleen and lymph node cells were approved by the Case Western Reserve Institutional Animal Care and Use Committee (IACUC).

Results

Disrupted C3ar1/C5ar1 signaling in Tregs induces expression of SOCS1, 2, and 3

Prior work by others on Treg stability found that STAT3 activation in Tregs leads to their conversion to "exTregs" or Th17 cells (10, 11). Signaling studies showed that SOCS1 and SOCS3 which inactivate STAT3 participate in inhibiting this conversion and thereby promote Treg stability (12). Our previous studies of Treg induction (6) linked disabled $CD4^+$ cell C3ar1/C5ar1 signaling with inhibited STAT3 activation but did not provide a mechanism for the inhibition.

To determine if absent C3ar1/C5ar1 signaling induces the expression of the STAT3 phosphatases SOCS1 and/or 3 in $CD4^+$ cells, we sorted $Foxp3^+$ $CD25^+$ cells deriving from Treg induction cultures of $Foxp3^-$*CD55*$^{-/-}$, WT, and *C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ $Foxp3^-$ $CD4^+$ cells and quantitated SOCS 1,2 and 3 mRNA levels in induced $Foxp3^+$ $CD25^+$ cells Levels of SOCS1 and 3 were 200–300% higher in $Foxp3^+$ *C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ $CD4^+$ cells than in $Foxp3^+$ WT $CD4^+$ cells, whereas they were >500% lower in $Foxp3^+$ *CD55*$^{-/-}$ $CD4^+$ cells (Fig 1A). Similar results were observed for SOCS2 which inhibits IL-4 induction of STAT6 activation (Fig 1A).

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Disrupted C3ar1/C5ar1 signaling in Tregs augments upregulation of the ten-eleven translocation enzymes (TET1/TET3) and enables Vitamin C enhancement of their upregulatory effect on Foxp3 stability

TET enzymes [which oxidize 5-methylcytosine (5mC) to 5-hydroxymethylcytosine and other oxidized methylcytosines] participate in demethylation of genomic DNA (13, 14). There are three TET genes. A previous Treg study (14) connected upregulation of TET2 and TET3 with demethylation of the TSDR. Knock-out of these genes led to loss of Foxp3 expression comparably to knockout of the CNS2 region itself (14). Another Treg study (13) found that Vitamin C participates in demethylation of the CNS2 region via promoting TET2 upregulation.

To determine if $CD4^+$ cell expression of CD55 and its suppression of autocrine C3ar1/C5ar1 signaling are involved in TET gene upregulation and Vitamin C augmentation of Foxp3 associated TET activity, we performed two experiments. In the first, we assayed TET mRNA expression in sorted $Foxp3^+$ cells derived from Treg induction cultures of flow sorted WT, *CD55*$^{-/-}$, or *C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ $Foxp3^-$ $CD4^+$ cells. Sorted WT $Foxp3^+$ $CD4^+$ cells showed ~4-fold increased mRNA levels of all three TET enzymes, whereas sorted $Foxp3^+$ *CD55*$^{-/-}$ $CD4^+$ cells showed no changes in any of the TETs. In contrast, sorted $Foxp3^+$ *C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ cells showed ~ 9-fold increases in TET1 and TET3 mRNAs (Fig 1B). Previous studies showed that TET mRNA levels closely correlate with TET protein levels and TET function (14). In the second experiment, we repeated the above iTreg induction protocol in the absence and presence of Vitamin C. Kinetic analyses of the sorted $Foxp3^+$ cells in cultures showed that the inclusion of Vitamin C augmented Treg stability most profoundly in *C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ cells and least profoundly in *CD55*$^{-/-}$ cells (Fig 1C). While Vitamin C reversed the loss of $Foxp3^+$ in the *CD55*$^{-/-}$ cells from 70% to 60% (a ~ 14% difference), it decreased the loss from ~40% to 10% in the *C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ cells (a 75% increase). Thus, TET gene upregulation is essential for Treg induction as well as stability while Vitamin C is Foxp3 modulatory as it enhances TET effects.

Disrupted C3ar1/C5ar1 signaling in Tregs represses expression of BRD4 needed for Th17 gene expression.

Bromodomain and Extra-Terminal (BET) family proteins enable the productive transcription of genes that drive cell growth and enable proinflammatory processes (15, 16). They do so by binding to acetylated lysines on histones and TFs and in so doing de-repress transcriptional elongation. There are four BET protein family members BRD2, BRD3, BRD4 and BRDT (testes specific). Recent studies (17) showed that BRD4 plays an obligatory role in Th17 lineage commitment. In line with this, the BET protein inhibitor JQ1 totally abolishes Th17 cell generation (17). Motivated by findings that Treg instability, i.e. the production of “exTregs”, involves the conversion of Tregs into Th17 cells, we examined whether BRD4 expression and C3ar1/C5ar1 signaling are interconnected. Sorted WT $Foxp3^-$ CD4 cells activated with anti-CD3/28 under Teff conditions upregulated BRD4. The activated $CD4^+$ cells concurrently upregulated the endogenous production of C3 and the aurora kinases aura1 and aura2 which participate in mitosis. The inclusion of C3ar1/C5ar1 antagonists inhibited all of the upregulations. The pharmaceutical C3ar1/C5ar1 blockade inhibited BRD4 mRNA expression similarly to JQ1 (Fig 1D). Collectively, the findings

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argue that absent C3ar1/C5ar1 signaling in CD4+ cells not only functions endogenously to confer the signature phenotypic markers of Tregs, but at the same time endogenously represses a process that would promote the conversion of Tregs to Th17+ "ex Tregs".

Repressed C3ar1/C5ar1 signaling in developing Tregs evokes nuclear translocation of NFAT and NF-kB.

While the above studies showed that disabled C3ar1/C5ar1 signaling in Tregs induces several of the reported processes that lead to a demethylated TSDR and long term pTreg stability, they did not provide a molecular mechanism.

A previous study (2) linked nuclear translocation of NFAT to the TSDR-containing CNS2 region to hairpin looping of the CNS2 intronic region to the Foxp3 promoter. Other studies linked concomitant nuclear translocation of NF-kB to the Foxp3 promoter (18, 19). To determine if these translocations are interconnected with CD55 restraint of C3ar1/C5ar1 signaling in CD4+ cells, we activated sorted Foxp3− WT, *CD55*−/−, and *C3ar1*−/−*C5ar1*−/− CD4+ cells with anti-CD3/28 Dynabeads and IL-2 plus TGF-β (Treg conditions). After 3 d, we assayed sorted Foxp3+ cells induced from sorted Foxp3− cells of each genotype for nuclear NFAT and NF-kB by Amnis Cytometry. Both nuclear NFAT and NF-kB were upregulated in Foxp3+ cells generated from sorted Foxp3−*C3ar1*−/−*C5ar1*−/− cells, but not in Foxp3+ cells induced from sorted Foxp3− WT or *CD55*−/− CD4+ cells (Fig 2). These data link upregulated TET expression with their targeting to the CNS2 region in Foxp3+ cells that develop on a background disabled in C3ar1 and C5ar1 signaling.

The *in vivo* stability of Tregs depends on CD55 restraint of autocrine C3ar1/C5ar1 signaling in pTregs.

To determine the extent to which restraint of C3ar1/C5ar1 signaling in pTregs impacts the stability of Foxp3 expression and consequent durability of Tregs *in vivo*, we performed *in vivo* studies with ovalbumin (ova) specific Tregs generated from ova specific OT-II CD4+ cells. We adoptively transferred 4 × 10^6 flow sorted Foxp3− (GFP−) WT, *CD55*−/−, or *C3ar1*−/−*C5ar1*−/− OT-II cells (all Thy-1.2) to Thy-1.1 recipients. We added 20 mg/ml of ova to the drinking water for 5 d, after which we immunized the mice with 100 ug of ova in IFA and followed the mice thereafter without further manipulation. After 8 months without any other intervention, we harvested Thy-1.2 cells on each genetic background. Following flow sorting of the Thy-1.2+ Foxp3+ (GFP+) CD4+ cells, we examined the TSDR in the sorted cells. While the recovered Thy-1.2+ Foxp3+ *CD55*−/− and WT OT-II cells contained methylated TSDRs, the recovered Thy-1.2+ Foxp3+ *C3ar1*−/−*C5ar1*−/− OT-II cells contained a de-methylated TSDR (Fig 3A).

The above data prompted two subsequent antigen challenge experiments directed at Treg durability. In the first, we added 20 mg/ml of ova to the drinking water of WT, *CD55*−/−, and *C3ar1*−/−*C5ar1*−/− mice all on the OT-II-Foxp3-GFP background. After 5 d, we immunized each mouse group with 100 ug of ova in IFA as above. Seven d later, we harvested CD4+ cells from the mLNs. We then transferred 4 × 10^6 sorted Foxp3+ (GFP+) OT-II cells (Thy-1.2) from each genotype to 3 sets (18 mice each) of naïve Thy-1.1 recipients for analyses at 1, 2, and 4 m. At each time point thereafter, we immunized half of the recipient

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mice with ova in IFA and half with IFA alone as control. Five d later, we assayed (Thy-1.2) $CD4^+$ cells in the inguinal LNs and in the spleen for $Foxp3^+$ cells (diagramed in Fig 3B). This monitoring of Foxp3+ OT-II cells over time showed that the number of transferred (Thy-1.2) OT-II cells remained stable for all groups over the full 4 m period (Fig 3C left and right). In recipients that received IFA alone, Foxp3 expression on $CD55^{-/-}$ (Thy-1.2) $Foxp3^+$ OT-II cells progressively declined, and that on WT (Thy-1.2) $Foxp3^+$ OT-II cells began to decline at 4 m. In contrast, Foxp3 expression on $C3ar1^{-/-}C5ar1^{-/-}$ (Thy-1.2) $Foxp3^+$ OT-II cells remained uniformly stable out to 4 m (Fig 3D left). Moreover, in recipients that were immunized with ova, only $C3ar1^{-/-}C5ar1^{-/-}$ OT-II $Foxp3^+$ cells retained $Foxp3^+$ expression (Fig 2D right) supporting the proposition that disabled C3ar1/C5ar1 signaling sustains Foxp3 expression even following activation with specific antigen.

Orally induced pTregs in the absence of C3ar1/C5ar1 signaling show durable Foxp3 expression, de-methylated TSDRs and robust TGF-β and IL-10 expression.

Because $Foxp3^+$ OT-II cells recovered from the recipients in the above study could have contained tTregs, in a second study we adoptively transferred sorted (Thy-1.2) $Foxp3^-$ OT-II cells on each genetic background to Thy1.1 recipients. We fed the recipients 20 mg/ml of ova and transferred sorted Thy-1.2 $Foxp3^+$ OT-II that were orally induced to a second set of naïve recipients (diagrammed in Fig 4A). We then studied the stability of Foxp3 expression on the WT, $CD55^{-/-}$ and $C3ar1^{-/-}C5ar1^{-/-}$ OT-II Tregs at 14 days, 1 month, and 2 months as described in the prior experiment. Analyses of the recovered Thy1.2 $CD4^+$ cells at each time point yielded similar results (Fig 4B). We isolated $Foxp3^+$ WT, $CD55^{-/-}$, and $C3ar1^{-/-}C5ar1^{-/-}$ (Thy-1.2) OT-II cells from the LNs and spleen and expanded the cells in cultures with anti-CD3/28 Dynabeads and IL-2. Analyses of the TSDR of the Foxp3 gene of each genotype at 2 months showed that it was ~80% methylated in $CD55^{-/-}$ or WT OT-II $Foxp3^+$ cells similar to conventional T cells. In contrast, the level of demethylation in $C3ar1^{-/-}C5ar1^{-/-}$ OT-II $Foxp3^+$ cells was similar to that of tTregs (Fig 4C). A replicate analysis of the TSDR gave the same result. This finding of sustained TSDR de-methylation only in $C3ar1^{-/-}C5ar1^{-/-}$ OT-II $Foxp3^+$ cells despite TCR stimulation with ova further validates the interpretation that disabled C3ar1/C5ar1 signaling into Tregs is a requisite condition that sustains antigen specific Treg stability. In line with this interpretation, an earlier experiment without the use the Foxp3-GFP transgene in which we used adoptively transferred $CD25^+$ cells from the parental WT, $CD55^{-/-}$, or $C3ar1^{-/-}C5ar1^{-/-}$ OT-II mice and analyzed $Foxp3^+$ expression on recovered Thy-1.2 cells by intracellular staining showed stability of Foxp3 expression only in $Foxp3^+$ $C3ar1^{-/-}C5ar1^{-/-}$ OT-II cells (not shown).

In a subsequent experiment, we examined the properties of the recovered WT, $CD55^{-/-}$, or $C3ar1^{-/-}C5ar1^{-/-}$ OT-II Tregs that were orally induced *in vivo*. To do this, we used an identical protocol of feeding ovalbumin to Thy-1.1 recipients of sorted Thy-1.2 $Foxp3^-$ OT-II cells on the three genotypes. We then harvested the Thy-1.2 $Foxp3^+$ OT II cells in the mLNs after 2 months. Intracellular staining of $C3ar1^{-/-}C5ar1^{-/-}$ $Foxp3^+$ OT-II cells showed more TGF-β1 and IL-10 than in WT $Foxp3^+$ OT-II cells and little of either immunosuppressive cytokine in $CD55^{-/-}$ $Foxp3^+$ OT-II cells (Fig 4D).

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Summarizing the in vivo data, the experiment in Fig 3A analyzed ova specific *CD55*$^{-/-}$, WT, and *C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ Tregs generated *in vivo* out to 8 months at which time the TSDR was demethylated only in the *C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ Tregs. The experiment in Fig 3B examined *CD55*$^{-/-}$, WT, and *C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ Tregs at 1, 2 and 4 months and found that *CD55*$^{-/-}$ and WT Tregs markedly lost Foxp3 expression with immunization (activation of their TCR), whereas *C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ mice fully retained Foxp3 expression. The experiment in Fig 4 used the same protocol, except the mice received adoptively transferred Tregs orally generated in a prior set of mice. At 2 months, only the *C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ cells again were stable and uniformly demethylated, A fourth experiment employing the same protocol with non Foxp3-GFP cells staining intracellularly for Foxp3 showed the same full stability uniquely in the *C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ Tregs. A fifth experiment used the same protocol and isolated the Tregs at 2 months. Only the *C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ cells retained robust production of the prototypical Treg cytokines TGF-β and IL-10. The five experiments taken together combined with the *in vitro* data thus argue that absent C3ar1/C5ar1 signaling in Tregs enables TSDR demethylation, Treg stability, and promotes Treg immunosuppressive function.

Discussion

The experiments in this study show that disrupted C3ar1/C5ar1 signaling in naïve $CD4^+$ cells triggers multiple processes connected in the literature with TSDR demethylation in antigen specific $CD4^+$ cells. The processes ultimately give rise to Tregs that exhibit long-term stability and robust Foxp3 expression when activated *in vivo* with specific antigen. Specifically, they show that disabled C3ar1/C5ar1 signaling is connected with upregulation of the TET demethylases which together with Smad2 activation documented in our previous study (6) empower activation of the TET proteins shown by others (20–22) to target the Foxp3 locus. In conjunction with the TET upregulation, repressed C3ar1/C5ar1 signaling is connected with Vitamin C augmentation of Foxp3 transcriptional stabilization mediated via enhanced TET demethylation (13, 14, 23). Disabled Treg C3ar1/C5ar1 signaling inactivates BRD4 that otherwise would favor Th17 cell differentiation (17, 24). The experiments additionally show that SOCS1/2/3 mRNA expression levels are upregulated 5-fold sustaining stable Foxp3 expression, since one process linked to decay of Foxp3 expression is STAT3/4 signaling (25–27).

Despite the connection of TSDR demethylation with Treg stability 14 years ago (28), how this epigenetic state confers Treg stability has remained incompletely clarified. The studies by Li et.al. in 2014 (2) implicating hairpin looping of the TSDR-containing CNS2 region to the Foxp3 promoter triggered by nuclear recruitment of NFAT to the former and NF-kB to the latter provided a critical insight into the mechanics of how the transcriptional stabilizing effect occurs. The Treg signaling that enables this Foxp3 promoter alteration, however, remained unclarified. The experiments herein are in line with the Li et al findings. They document by Amnis cytometry that nuclear translocation of both NF-kB and NFAT occurs when C3ar1/C5ar1 signals are absent but not when they are present. Taken together with the above findings, they thus for the first time provide a coherent signaling connection that is directly tied to TSDR demethylation and stabilized Foxp3 transcription.

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The finding that CNS2 looping to the Foxp3 promoter is associated with nuclear translocation of NF-kB as well as NFAT seemingly differs from recent findings by Mikami et.al (29) that NF-kB is suppressed in connection with TSDR demethylation occurring when Tregs are generated under conditions of CD28 deficiency. We are unable to explain the difference. However, our previous findings (4, 6) that C3a/C5a production is induced by CD28 costimulation argue that *CD28*$^{-/-}$ CD4^{+} cells are likely disabled in C3ar1/C5ar1 signaling. As referenced in that communication, many past studies (18, 19, 30–34) provided evidence that strong CD28 signaling and consequent NF-kB activation is needed for tTreg and pTreg TSDR demethylation and stability. Another point is that while the MikamI et al study focused on TSDR demethylation, it did not investigate long-term Treg stability. Further studies will be needed to precisely determine the role of CD28 costimulation and NF-kB in programming i-, p-, and tTreg induction, function, and stability.

Other studies have connected additional Foxp3 Treg processes with repression of C3ar1/C5ar1 signaling. Relevant to tolerance and to checkpoint therapy in cancer, studies of oral and ocular tolerance (35) showed that suppression of C3ar1/C5ar1 signaling upregulates GARP that delivers latent TGF-β to TGFβR1/2. The consequent autocrine TFG-β signaling upregulates ICOS, PD1 and CTLA-4 (35) which transmit coinhibitory signals into CD4^{+} cells and reciprocally downregulates CD28, CD40 ligand expression needed for Teff responses. Repression of C3ar1/C5ar1 signaling concomitantly upregulates CD37, CD73 and adenosine that inhibits Teff. Repressed C3ar1/C5ar1 signaling additionally upregulates interferon regulatory factor-8 (IRF-8) that enables the expansion of Tregs (36) and aldehyde dehydrogenase 1 family member A2 (Aldh1a2) that generates retinoic acid in the GI tract. It additionally upregulates CX3CR1, CCR7, and CCR9 which enable CD4^{+} cell transit to LNs and the lamina propria (35). Studies of vascular endothelial cell (EC) homeostasis (35) (*Feng-Qi A, et.al. under review*) showed that absent C3ar1/C5ar1 signaling enables the recruitment of CREB binding protein (CBP) or p300, a co-activator tied to p-CREB co-transcriptional activity in the Foxp3 promoter (37). Studies of growth factor signaling (38) showed that disruption of C3ar1/C5ar1 signaling derepresses PTEN activity which dephosphorylates PtdIns (3,4,5)P_3 and thereby suppresses AKT activation, a hallmark of Tregs. The repressed PtdIns (3,4,5)P_3 and p-AKT prevent Th1/Th17 differentiation but amplify autocrine TGF-β production. The growth factor studies additionally showed that repressed PI-3Kγ resulting from disabled C3ar1/C5ar1 signaling evokes PHLLP phosphatase activity that inactivates any preexisting p-AKT and prevents activation of CK2 and Fyn (38) that otherwise would introduce phosphorylations in PTEN that inactivate its phosphatase activity. They additionally showed that the absence of C3ar1/C5ar1 signaling disables autocrine IL-6 production and IL-6R-gp130 signaling (38, 39) and consequently impairs the transition of Tregs to Th17 “ex-Tregs”. The absence of C3ar1/C5ar1 signaling is thus connected with many cellular processes tied to Treg induction and TSDR demethylation. Because of the collective immunoinhibitory effects of these multiple signaling alterations, *C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ Tregs possess more robust suppressor activity than Tregs conventionally induced by TGF-β (6).

Although the full signaling cascade(s) underlying the linkage of absent C3ar1/C5ar1 signaling in Tregs with TSDR de-methylation remains to be characterized, the findings herein that circumvention of C3ar1/C5ar1 signaling into CD4^{+} cells activates TET1/2/3,

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shown by others to target the Foxp3 gene (20–22) provides a mechanism for demethylating the TSDR and thereby enabling its NFAT uptake. Our prior findings (6) that C3a/C5a activate STAT3 dependent Th1/Th17 responses and repress SOCS1/3/4 activation (this study) provide one mechanistic insight into the unwanted outcome of the collapse of Tregs into "ex Tregs" in Treg biotherapy (28, 40).

In support of the physiological connection of repression of C3ar1/C5ar1 signaling with TSDR demethylation and durable Foxp3 transcription, the *in vivo* experiments in this study showed that disabled $CD4^+$ cell C3ar1/C5ar1 signaling in Tregs is an essential condition for long term Foxp3 expression and Treg stability. The experiments with (ova-specific) orally induced pTregs showed that suppressed C3ar1/C5ar1 signaling prevented the loss of Foxp3 expression for 8 months. The experiments in which we adoptively transferred Thy-1.2 WT, $CD55^{-/-}$, and $C3ar1^{-/-}C5ar1^{-/-}$ $Foxp3^-$ OT-II pTregs prepared *in vivo* in Thy-1.1 recipients to a second set of naïve Thy-1.1 recipients revealed that Foxp3 expression of $CD55^{-/-}$ $Foxp3^+$ OT-II cells was unstable at 14 days even in the absence of ova immunization. They conversely showed that Foxp3 expression in transferred $C3ar1^{-/-}C5ar1^{-/-}$ $Foxp3^+$ OT-II cells was stable at 4 months even with TCR stimulation (i.e. ova immunization). Consistent with Foxp3 stability occurring on the $C3ar1^{-/-}C5ar1^{-/-}$ $Foxp3^+$ background, the TSDR in the 4-month $C3ar1^{-/-}C5ar1^{-/-}$ $Foxp3^+$ OT-II cells was de-methylated. In contrast, the TSDR in $CD55^{-/-}$ and WT $Foxp3^+$ OT-II cells was methylated directly tying repressed C3ar1/C5ar1 signaling to TSDR de-methylation. In further support of this relationship, the analysis of the $C3ar1^{-/-}C5ar1^{-/-}$ $Foxp3^+$ OT-II cells at 8 months without adoptive transfer showed that Foxp3 stability in $C3ar1^{-/-}C5ar1^{-/-}$ Tregs likewise was associated with TSDR demethylation. Our findings are similar to those of Van der Touw et al (38) who found that alloantigen-induced $C3ar1^{-/-}C5ar1^{-/-}$ iTreg were more stable than WT iTreg upon transfer to $Rag^{-/-}$ recipients after 5 days (41). Our findings of TSDR demethylation and-long term stability of ova specific Tregs generated *in vivo* however differ. One possible explanation for the difference is that the Van der Touw studies examined iTregs induced *in vitro* by allo-stimulation.

While these results differ from our previous 5 day *in vitro* studies (6), they argue that either more time, an *in vivo* process, or both are required for stability of the TSDR demethylated state. Consistent with the interpretation that Foxp3 stability did not derive from the outgrowth of another contaminating cell type, a short term (14 day) study in which Treg "fate mapping" mice (42) crossed with $C3ar1^{-/-}C5ar1^{-/-}$ or WT mice were treated with CpG showed Foxp3 stability on pTregs in the $C3ar1^{-/-}C5ar1^{-/-}$ mice but not the WT mice. Thus, absent $CD4^+$ cell C3ar1/C5ar1 signaling gives rise to processes that both enable pTreg generation and prevent pTreg instability. As examples, the inductive processes include upregulation of CD55 which downregulates C3ar1/C5ar1 signaling (3), interference with C3ar1/C5ar1 signaling by upregulation of non-G-protein coupled C5ar2 (6) which competes for C5a, upregulation of CTLA-4, PD1 and PD-L1 which transmit coinhibitory signals into $CD4^+$ cells (35), expression of SOCS1/4/3 which inactivate STAT1/2/3 [this paper] and repression of MyD88 signaling which inactivates TLR signaling (43). While the inherent Foxp3 instability of *ex vivo* TGF-β prepared Tregs has limited the effectiveness of biotherapy connected with their use, it remains to be determined how the stability of Tregs prepared by C3ar1/C5ar1 antagonism will be affected by inflammation. This will

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require more experiments as studies by others of antigen (MOG_{35-55}) specific pTregs in experimental autoimmune encephalitis (EAE) have shown loss of Foxp3 expression and function (44). However, our past studies of MOG_{35-55} specific Tregs generated in *C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ CD4^{+} cells or prepared by C3ar1/C5ar1 antagonism in EAE have shown retained Foxp3 (GFP) expression, robust immune suppression, and resolution of disease (6).

The findings herein that suppression of C3ar1/C5ar1 signaling in pTregs induces stabilized Foxp3 transcription and immunosuppressive TGF-β and IL-10 expression by iTregs argue that it may be possible to achieve durable immune suppression to an exogenous antigen in a recipient if Tregs can be generated in a way that corresponds to the *C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ background in *in vivo* conditions, i.e. disabled C3ar1/C5ar1, but retained C5ar2 function. This formulation could have important clinical relevance for immune-mediated conditions, e.g. autoimmunity, transplantation, allergic airway disease, or other disorders in which suppression of T effector responses would be beneficial.

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Key Points

1. Foxp3 TSDR demethylation is governed by autocrine C3ar1/C5ar1 signaling in Tregs.
2. Treg generation under *in vivo* conditions is required for TSDR demethylation

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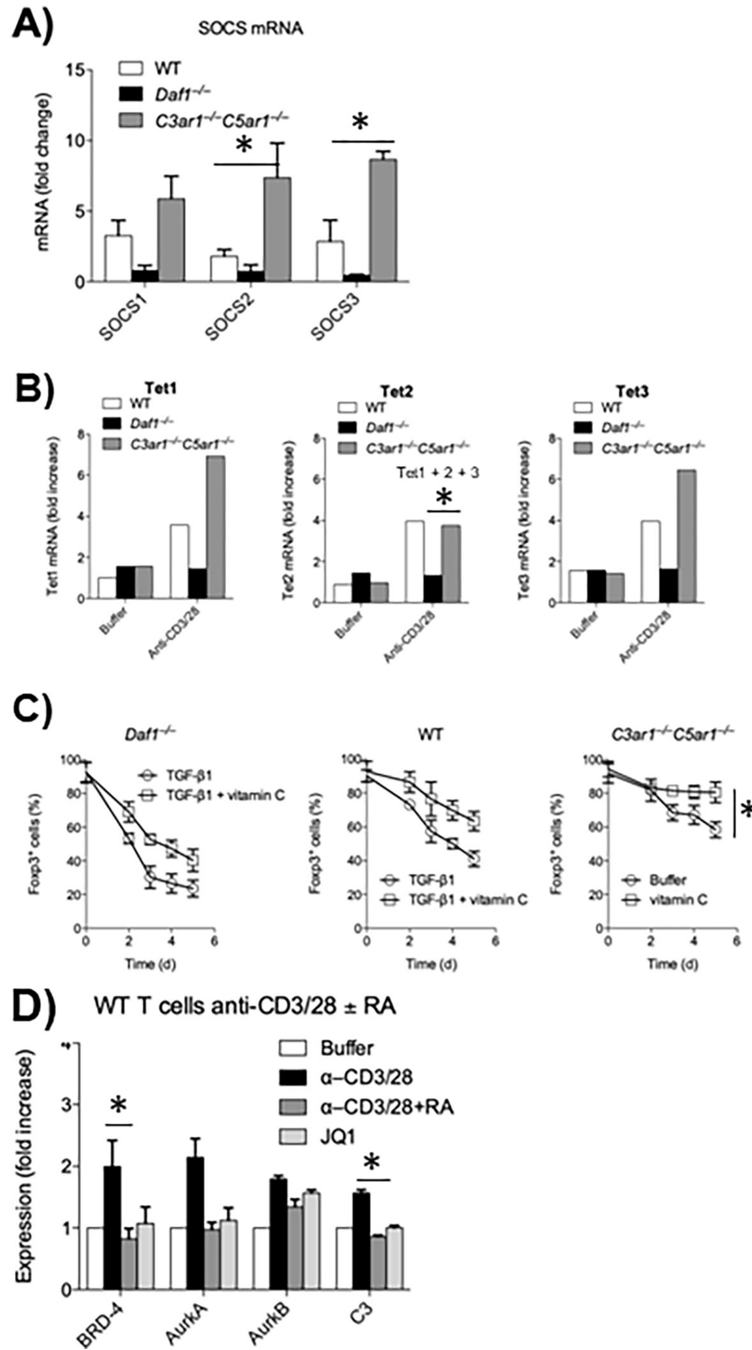

Figure 1: Metabolic pathways connected with absent C3ar1/C5ar1 signaling in activated CD4+ cells.

A) Sorted *CD55*$^{-/-}$, WT, and *C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ CD4^{+} cells were incubated with anti-CD3/28 Dynabeads plus IL-2 for 5 days after which Foxp3^{+} cells were sorted. **A)** SOCS1, 3, and 4 mRNA levels. Induction in *C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ vs WT cells and **B)** TET1, TET2, and TET3 mRNA levels were quantitated by qPCR. Representative of 2 assays. Induction in *C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ vs *CD55*$^{-/-}$ cells. **C)** Sorted Foxp3^{-} *CD55*$^{-/-}$, WT, and *C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ CD4^{+} cells were incubated with anti-CD3/28 Dynabeads and IL-2 plus TGF-β in the

absence and presence of Vitamin C and percent Foxp3^{+} cells quantitated daily. Induction in *C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ vs WT^{-} cells. **D**) WT CD4^{+} cells were incubated for 1 h with anti-CD3/28 Dynabeads without or with C3ar1/C5ar1 receptor antagonists (RA) or the BRD inhibitor JQ1 after which BRD4, AuraA, AuraB and C3 mRNA levels were assayed by qPCR. Induction in *C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ vs WT^{-} cells. All n=2. * = p<.05.

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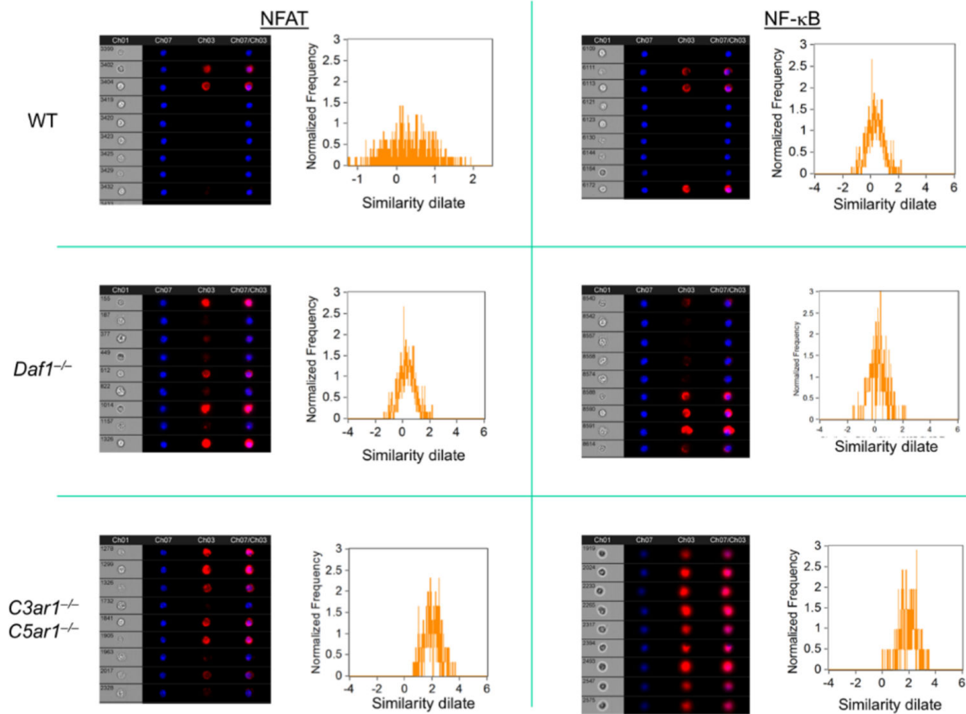

Figure 2: Nuclear translocation of NFAT and NF-kB in Tregs devoid of C3ar1/C5ar1 signaling.
Sorted induced Foxp3^{+} cells on each genotype were assayed for nuclear NFAT and NF-kB by Amnis Cytometry. The flow plots show colocalization of NFAT and NF-kB with the nuclear stain. Representative of 2 assays. The red lines are the anti-TGF-β and anti-IL-10 stains. The black lines are nonrelevant controls.

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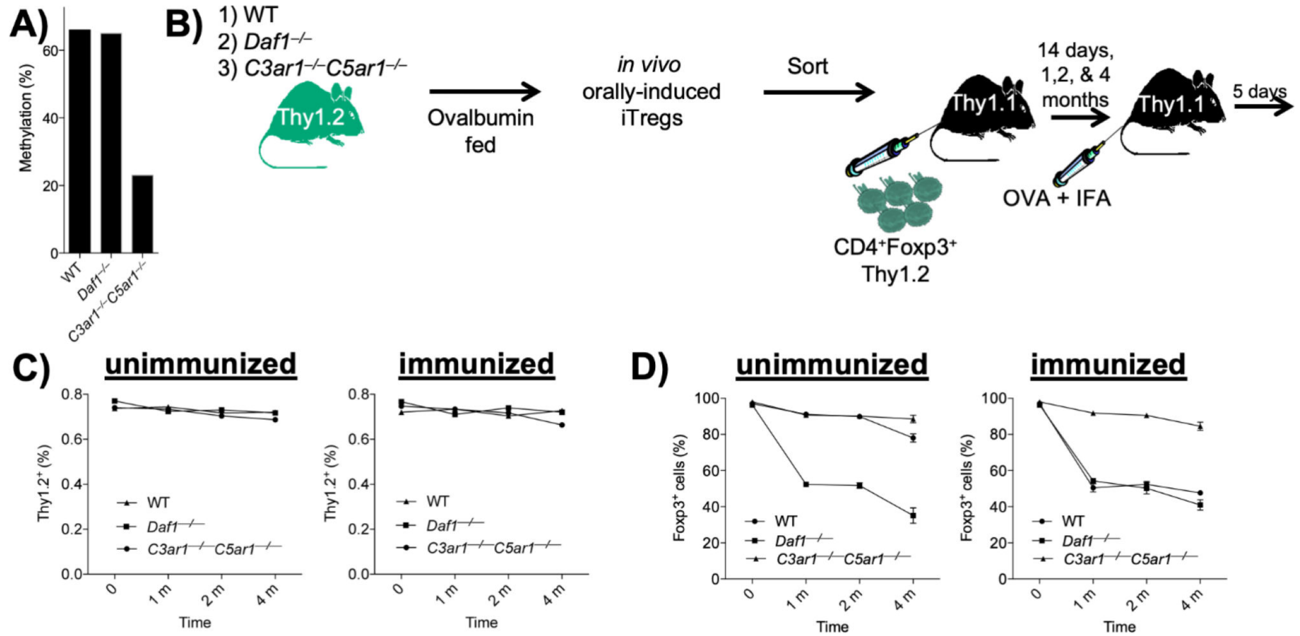

Figure 3: Long-term *in vivo* stability of Tregs induced in the absence of C3ar1/c5ar1 signaling.

A) Thy-1.2 flow sorted Foxp3− (GFP−) WT, *CD55*−/−, or *C3ar1*−/−*C5ar1*−/− OT-II cells were adoptively transferred to Thy-1.1 recipients. Ova (20 mg/ml) was added to the drinking water of the recipients for 5 d after which the recipients were immunized with 100 ug/ml of ova in IFA. Eighty d later Thy-1.2 Foxp3+ cells in the inguinal LNs and spleen of the recipients were harvested and methylation status of the TSDR assayed. Values representative of 2 assays. **B)** Orally induced Thy-1.2 Foxp3+ OT-II cells on the *CD55*−/−, WT, and *C3ar1*−/−*C5ar1*−/− backgrounds were prepared as in A) and sorted Foxp3+ cells on each background were transferred to a second set of naïve Thy-1.1 recipients. The recipients were immunized with ova in IFA or IFA alone, and Thy-1.2 CD4+ cells in the inguinal LNs and spleens of the recipients were assayed at 1, 2, and 4 months for retention of Foxp3 expression. The cartoon diagrams the experimental protocol. **C)** The number of Thy-1.2 CD4+ on each background recovered at each time point. **D)** The percentage of Thy-1.2 CD4+ cells on each genetic background retaining Foxp3 (GFP+) expression at each time point.

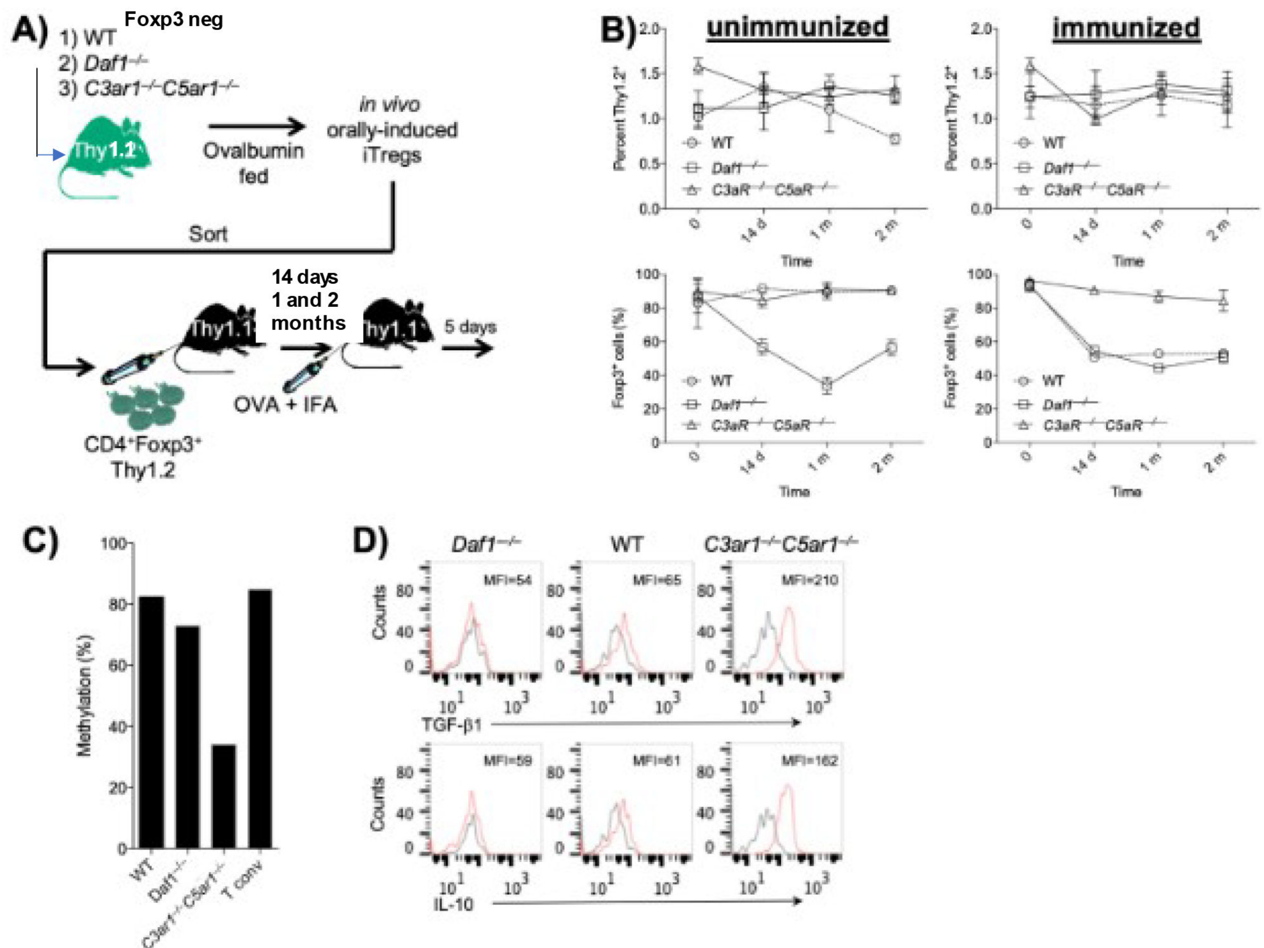

Figure 4: Demethylation of the TSDR in Tregs devoid of C3ar1/C5ar1 signaling.

A) Sorted Foxp3$^-$ *CD55*$^{-/-}$, WT, and *C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ OT-II cells (all Thy-1.2) were adoptively transferred to naïve Thy-1.1 recipients. After feeding the recipients 20 mg/ml of ova and immunizing them with 100 ug of ova in IFA as above, sorted Thy-1.2 Foxp3$^+$ OT-II cells on each genotype in the spleens and inguinal LNs of the recipients were transferred to a second set of naïve Thy-1.1 recipients. Thy-1.2 OT-II cells on each genotype were recovered from the spleens and inguinal LNs of the second set of recipients at 1, 2 or 4 months and analyzed as in Fig 2. **A)** Cartoon of the experimental protocol. **B)** numbers (Upper) and Foxp3 (GFP+) expression (Lower) of Thy1.2 OT-II cells on each genotype recovered at each timepoint. **C)** The methylation status of TSDR in the sorted Foxp3$^+$ OT-II cells on each genotype was assayed. N=2. **D)** Intracellular TGF-β and IL-10 in the sorted Foxp3$^+$ OT-II cells on each genotype were assayed by intracellular FACS.

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