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CD55 Is Essential for CD103^{+} Dendritic Cell Tolerogenic Responses that Protect against Autoimmunity

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Recent studies traced inflammatory bowel disease in some patients to deficiency of CD55 [decay-accelerating factor (DAF)], but the mechanism underlying the linkage remained unclear. Herein, we studied the importance of DAF in enabling processes that program tolerance in the gut and the eye, two immune-privileged sites where immunosuppressive responses are continuously elicited. Unlike oral feeding or ocular injection of ovalbumin in wild-type (WT) mice, which induced dominant immune tolerance, identical treatment of *DAF*$^{-/-}$ mice or *DAF*$^{-/-}$ to WT bone marrow chimeras did not. While 10% to 30% of mesenteric and submandibular lymph node CD4^{+} cells became robust T-regulatory cells (Tregs) in WT forkhead box P3 (Foxp3)—green fluorescent protein mice, few in either site became Tregs with little suppressor activity in *DAF*$^{-/-}$ Foxp3—green fluorescent protein mice. Phenotyping of CD103^{+} dendritic cells (DCs) from the ovalbumin-fed *DAF*$^{-/-}$ mice showed impaired expression of inducer of costimulation (ICOS) ligand, programmed death receptor 1-ligand 1 (PD1-L1), CxxxC chemokine receptor 1 (Cx3CR1), CCR7, and CCR9. Analyses of elicited *DAF*$^{-/-}$ Foxp3^{+} Tregs showed reduced expression of interferon regulatory factor 8 (IRF-8)/aldehyde dehydrogenase 1 family member A2 (Aldh1a2) and glycoprotein A repetitions predominant/latency-associated protein associated with Treg transforming growth factor-β production and presentation, as well as integrin β6/integrin β8 associated with Treg and CD103^{+} DC transforming growth factor-β release. Thus, DAF is required for the properties of CD103^{+} DCs and their naïve CD4^{+} cell partners that together program tolerance. *(Am J Pathol 2019, 189: 1386—1401; https://doi.org/10.1016/j.ajpath.2019.04.008)*

Peripheral tolerogenic responses interdict T-effector (Teff) cell activation against nonpathogenic environmental antigens at mucosal surfaces. In clinical settings, the tolerogenic responses prevent the development of inflammatory bowel disease (IBD), asthma, and other allergic and/or autoimmune conditions. In transplantation, their induction against donor tissue promotes successful organ engraftment. In normal health, tolerogenic responses operate continuously to maintain homeostasis in sites where immune activation must be suppressed. Among these sites are the anterior chamber (a.c.) of the eye and the gastrointestinal (GI) tract, in each setting in coordination with the respective lymphoid organ(s). Determining how acquisition of the tolerogenic properties of antigen-presenting cells (APCs) and CD4^{+} T cells in these sites mechanistically occurs would provide new insights regarding physiological programming of tolerogenic outcomes.

With respect to homeostasis in the eye, early work showed that foreign tissue survived longer when placed in the a.c. as opposed to the peritoneum or the skin,[1,2] suggesting that Teff responses are suppressed in this site. Experimental

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documentation came from studies[1,3–5] showing that in contrast to s.c. injection of ovalbumin (OVA), which evoked a cytotoxic T-lymphocyte response and robust delayed-type hypersensitivity (DTH), a.c. preinjection prevented these and other Teff responses on subsequent s.c. OVA challenge. This phenomenon was designated anterior chamber–associated immune deviation in that it conferred long-lasting dominant tolerance. With respect to the GI tract, early work documented an analogous immune altered state. The immune altered phenomenon there, first termed immunologic unresponsiveness,[6] was later designated oral tolerance.[7] As with anterior chamber–associated immune deviation, antigen uptake by dendritic cells (DCs) or other APCs in the GI tract conferred dominant immune tolerance to the same antigen.[8–10] Although the initial studies attributed the mechanism to anergy or clonal deletion,[11] later studies implicated peripherally induced Foxp3$^+$ T-regulatory cells (pTregs).[8–13] Clinically, ocular tolerance allows corneal transplant engraftment without systemic immunosuppression.[14] Likewise, oral tolerance functions to prevent Teff responses to commensal bacteria and to food antigens.

Decay-accelerating factor (DAF; or CD55) is an intrinsic cellular regulator originally characterized as a control protein that circumvents activation of systemic complement on self-cell surfaces.[15] Recently, DAF has been found to modulate T-cell responses.[16,17] Mechanistic understanding of its T-regulatory function came from the discovery[18] that cognate DC-CD4$^+$ cell partners jointly produce C3a and C5a from endogenously synthesized complement. The two anaphylatoxins establish autocrine signaling loops with C3a and C5a receptors (C3ar1 and C5ar1, respectively) in each partner, which provide costimulatory and survival signals to CD4$^+$ cells.[18] DAF restrains C3a/C5a generation and consequently represses C3ar1/C5ar1 signaling.[18] Autocrine C3ar1/C5ar1 signaling functions comparably in shaping human and mouse Teff responses.[19] Recent studies[20] have highlighted the requisite role of local complement production and its activation in human CD4$^+$ cell responses and provided evidence that the signaling operates intracellularly.

Work building on our finding that autocrine C3ar1/C5ar1 transduction promotes Teff activation led to the insight that the absence of transduced C3ar1/C5ar1 signals into CD4$^+$ cells conversely favors the differentiation of naïve CD4$^+$ cells into pTregs. The extent, however, to which DAF control of immune cell C3ar1/C5ar1 signaling is obligatory or modulatory to the physiological imprinting of Treg commitment has remained unclarified. Moreover, the linkage of DAF's activity with the phenotypic properties of DCs and CD4$^+$ cells that play requisite roles in immune homeostasis also has remained unknown. An important example is the physiological induction of de novo peripheral tolerance, an issue of broad immunologic significance.

Autoimmune uveitis results from broken tolerance in the a.c. Both anterior and posterior disease can occur, and both are connected with CD4$^+$ cell production of tumor necrosis factor-α, interferon (IFN)-γ, and IL-17[21] and decreased numbers of circulating Tregs.[22] This pattern pertains for uveitis occurring in human leukocyte antigen-B27–associated disease[23] and connected with tubulointerstitial nephritis.[24] Although a defect in DAF in human uveitis has not yet been reported, posterior uveitis is closely emulated in mice immunized with retinal antigen interphotoreceptor retinoid-binding protein.[25] Interphotoreceptor retinoid-binding protein–induced disease is markedly heightened in *DAF*$^{-/-}$ mice.[26] Relevant to DAF function, interphotoreceptor retinoid-binding protein–induced disease in mice devoid of C3ar1 and C5ar1 is markedly suppressed.[27]

DAF corresponds to the blood group antigen originally designated Cromer. Three previous reports[28–30] connected individuals having the Cromer subtype INAB (*DAF*$^{-/-}$) with predisposition to IBD. A recent study[31] identified 11 patients with early-onset (aged <2 years) protein losing enteropathy. The patients (six males and five females) belonged to eight families. Their disease was characterized by malnutrition, vomiting, diarrhea, hypoalbuminemia, hypogammaglobulinemia, lymphangiectasia, and inflammation with lymphatic infiltrates. Three patients had thromboses. Family histories included two patients who died. Immunologic analyses showed increased C3 antigen on CD4$^+$ cell blasts (presumed to derive from plasma C3 protein) and increased CD4$^+$ cell production of tumor necrosis factor-α and IFN-γ. Although increased CD4$^+$ cell production of C5a was noted, the pathogenic mechanism underlying the enteropathy was not characterized.

Past work by several investigators on mucosal tolerance has implicated discrete subsets of DCs[32,33] and macrophages[34] in playing critical roles. Important among these subsets is CD103$^+$ DCs. After antigen processing, these DCs migrate to the regional lymph node (LN) and convert CD4$^+$ cells into pTregs. In view of the critical role of this DC subset in the induction of tolerance, we studied whether the loss of DAF function on CD103$^+$ DCs and/or CD4$^+$ cells could underlie the pathogenesis of the above described cases of enteropathy and predispose to IBD and uveitis. The interplay between DAF function, the properties of CD103$^+$ DCs that enable their migration to the LN, and the tailoring of the properties of CD4$^+$ cells that together evoke tolerogenic responses were studied. It was found that DAF control of C3ar1/C5ar1 signaling accounts for many DC and CD4$^+$ cell phenotypic properties reported to be integrally involved in induction of tolerance.

Materials and Methods

Reagents and Antibodies

C5a was from Cell Sciences (Newburyport, MA). C3ar1-A was purchased from Calbiochem (now EMD Millipore, Burlington MA). C5ar1-A synthetic peptide was kindly provided by John Lambris (University of Pennsylvania, Philadelphia, PA). Anti-mouse C3a (I87-1162) and C5a (I52-1486) monoclonal antibodies (mAbs) were from BD Biosciences (San Jose, CA). Mouse IL-2, IL-10, and IFN-γ and

human IL-2, IL-10, IFN-γ, and transforming growth factor (TGF)-β1 were from Prospec Bio (Ness-Ziona, Israel). Anti-murine C5ar1 (20/70) was purchased from Abcam (Cambridge, UK). Abs against mouse CD11c, CD11b, F4/80, CD103, B7-1 (16-10A1), B7-2 (GL1), PD-L1, and ICOS ligand (ICOS-L) were from BD Biosciences. Anti-CD40 ligand mAb was from BioExpress (Kaysville, UT). Anti-murine C3ar1 (sc-20138) was purchased from Santa Cruz Biotechnology (Dallas, TX). Anti-murine C5ar2 (AKA C5L2; HP8015) was purchased from Hycult Biotech (Wayne, PA). Mouse anti-Foxp3 mAbs and Treg staining kits were from eBiosciences (now Thermo Fisher Scientific, Waltham, MA). Anti-CD4 (RM-4-5; fluorescein isothiocyanate), anti-Thy1.1 [HIS51; phosphatidylethanolamine-cyanine 7 (Cy7)], anti-Thy1.2 (53-2.1; APC), anti-CD45.1 (A20; phosphatidylethanolamine), and anti-CD45.2 (104; APC−eFluor 780) were purchased from eBiosciences. Carboxyfluorescein succinimidyl ester (CFSE), CellTracker Red, and CellTracker Violet were from Invitrogen (Thermo Fisher Scientific). Grade VI chicken egg OVA and human IgG were purchased from Sigma (St. Louis, MO). Complete Freund adjuvant (CFA) containing heat-killed *Mycobacterium tuberculosis* strain H37Ra and incomplete Freund adjuvant (IFA) were from Difco Labs (Detroit, MI).

Animals

CD97$^{-/-}$ were generated as previously described.[35] Foxp3−green fluorescent protein (GFP) mice were a generous gift from Vijay Kuchroo (Harvard University, Boston, MA). Foxp3-GFP and OT-II mice were bred with *Daf1*$^{-/-}$ and *C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ mice in our animal facility. Bone marrow transplants were prepared, as previously described.[17] All studies were approved by the Institutional Animal Care and Use Center (protocol number 2015-0015) in our Assessment and Accreditation of Laboratory Animal Care−accredited facility and conducted according to American Veterinary Medical Association guidelines. Mice were anesthetized with ketamine/xylazine rodent cocktail or isoflurane to reduce suffering. Proparacaine was used as an analgesic to reduce pain caused by intraocular injections.

a.c. Injections and s.c. Challenge

OVA (2 mg/mL) in CFA and OVA (0.5 mg/mL) in IFA were prepared by emulsifying equal volumes of OVA in phosphate-buffered saline (PBS) with each adjuvant. Mice were anesthetized by i.p. injection with 0.6 mL of 1.5 mL ketamine HCl (100 mg/mL), 1.5 mL xylazine HCl (20 mg/mL), and 0.5 mL acepromazine (10 mg/mL). For a.c. injection, the eye was anesthetized topically with proparacaine HCl drops (Alcon Labs, Humacao, Puerto Rico). A paracentesis was performed by inserting a 30-gauge needle at the limbus parallel to the iris. An air bubble then was introduced into the a.c. by injecting 2 μL of air with a Hamilton microsyringe (Hamilton, Reno, NV) fitted with a 33-gauge beveled needle. After this, 50 μg of OVA in 2 μL of PBS or 2 μL of PBS alone was injected. The air bubble sealed the paracentesis, thereby preventing leakage.

Oral Feeding

Wild-type (WT) OT-II, *Daf1*$^{-/-}$ OT-II, and *C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ OT-II mice were fed 20 mg/mL OVA through their drinking water for 5 days, after which the mice were immunized with 100 μg of OVA in IFA, as described by others.[36]

Murine DCs and T-Cell Isolations

CD4^{+} T cells were isolated from spleens and LNs using the CD4^{+} negative selection cocktail from Miltenyi Biotec (Bergisch Gladbach, Germany), per the manufacturer's instructions. CD11c^{+} cells were isolated from spleens, LNs, and eyes (20 mice were used from each genotype for resident eye DC isolation) using the positive selection cocktail from Miltenyi, per the manufacturer's instructions. Both were purified using the Automacs Pro (Miltenyi Biotec). For corneal DCs, 40 mice of each genotype were used.

DTH and Alamar Blue Cytotoxicity Assays

In studies of ocular tolerance, 7 days after a.c. injections, mice were injected subcutaneously with 12.5 μg OVA in IFA in the footpad of the right leg and with PBS in IFA in the footpad of the left leg; and swelling was measured 24 hours later. In studies of oral tolerance, 7 days after oral feeding, mice were injected subcutaneously with 100 μg OVA in CFA at the base of the tail. Seven days later, they were injected subcutaneously with 12.5 μg OVA in IFA in the right leg and with PBS in IFA in the left leg; and swelling was measured 24 hours later.

For cytotoxicity assays, isolated T cells were incubated for 3 days at 37°C with 20 μg/mL OVA. They were then incubated with OVA-transfected EL-4 cells in decreasing ratios and Alamar blue for 24 hours. Cytotoxicity was calculated using the following formula: 100 × ([(AF of targets alone)] − [(AF of mix) − (AF effectors alone)]) × (AF of targets alone), where AF represents the mean of the absolute fluorescence units for the wells minus the average fluorescence units of the media alone.

ELISPOT Assays, Cytokine Enzyme-Linked Immunosorbent Assays, and Ab Isotype Enzyme-Linked Immunosorbent Assays

ELISPOT assays (ImmunoSpot, Shaker Hts, OH) and cytokine assays were performed as previously described.[17] For quantitation of cytokines produced in the eye, explants of eyes were incubated for 48 hours at 4°C in RPMI 1640 medium in the absence of antigen. Supernatants were assayed by the enzyme-linked immunosorbent assay (R&D

Systems, Minneapolis, MN), per the manufacturer's instructions. Plasma samples were collected 10 days after s.c. OVA injection.

Levels of anti-OVA antibodies were measured using the mouse monoclonal Ab ID/SP kit (Zymed, San Francisco, CA).

Foxp3$^+$ Treg Induction and Foxp3$^+$ Treg Suppressor Assays

Foxp3$^+$ or Foxp3$^-$ Cell Isolations and Treg Induction

CD4$^+$ cells were isolated using negative selection beads (Miltenyi Biotec). Foxp3$^+$ or Foxp3$^-$ cells from Foxp3-GFP transgenic mice were sorted using APC-labeled anti-CD4 mAb (Thermo Fisher Scientific) and BD Biosciences Aria Facsorter. Foxp3$^+$ or Foxp3$^-$ cells (1×10^5) were incubated for 24 hours at 37°C with anti-CD3/28 Dynabeads (Invitrogen, Carlsbad, CA), per the manufacturer's instructions, and 2 μg/mL IL-2 (Peprotech, Rocky Hill, NJ) in the presence and absence of WT DCs in RPMI 1640 medium.

Foxp3$^+$ Staining Assays

For both human and murine Treg staining assays, the relevant Foxp3$^+$ T-regulatory staining kits (236A/E7 for human and FJK-16s for mouse) were purchased from Thermo Fisher Scientific and used per the manufacturer's instructions.

Murine Treg Suppressor Assays

Sorted GFP$^-$ Foxp3-GFP OT-II CD4$^+$T cells (1×10^6), labeled with CellTracker Red (Thermo Fisher Scientific), were stimulated with 1×10^5 autologous CD11c$^+$ DCs and OVA$_{323-339}$ alone or with various ratios of sorted GFP$^+$ Foxp3-GFP CD4$^+$ cells. The cells were cultured for 3 days in 96-well flat-bottom plates, and CellTracker Red dilution was analyzed by fluorescence-activated cell sorting.

Phenotyping and Flow Cytometry

Spleens, mesenteric LNs (mLNs), and submandibular LNs (smLNs) were isolated from mice, incubated for 30 minutes in spleen dissociation buffer (Miltenyi Biotec), passed through a 70-μm cell strainer, and incubated for 2 minutes in isotonic red blood cell lysis buffer. After Fc receptor blocking with anti-CD16/32 Ab (BD Biosciences), cells were stained with anti-CD4 and anti-CD11c$^+$ anti-CD103$^+$ or anti-F4/80$^+$ anti-CD11b mAbs. Cells were then further stained for costimulatory molecules, coinhibitory molecules, major histocompatibility complex (MHC)-II, chemokine receptors, or complement fragment receptors, as described in each experiment, and assayed using an Aria, LSR I, or LSR II flow cytometer (BD Biosciences). Flow data were initially analyzed via CellQuest (BD Biosciences) or FACSDiva (BD Biosciences) and then subsequently with FlowJo version 10 (FlowJo LLC, Ashland, OR). Data are presented as mean fluorescent intensity.

Statistical Analysis

All experiments were repeated at least twice. Statistical analyses were performed using a two-tailed *t*-test using Microsoft Excel (Microsoft Corp., Redmond, WA) or GraphPad Prism 6.0 (GraphPad Software, San Diego, CA). All data are presented as means with SD, unless otherwise noted.

Results

Because published studies of ocular and oral tolerance in mice[9,10,36] in large part have used OVA as antigen, OVA was used in the experimental systems.

DAF Is Required for Ocular Tolerance

To study the extent to which DAF function is required for ocular tolerance, the outcome of injecting OVA into the a.c. of *Daf1*$^{-/-}$ (murine equivalent of human DAF) mice versus the a.c. of WT (*Daf1*$^{+/+}$) littermates was compared. After 7 days, the mice were challenged subcutaneously with OVA + IFA in the right leg and with PBS + IFA in the left leg (control); and 24 hours later, leg swelling and lesion histology were measured. WT mice that received OVA in the a.c showed approximately threefold reduced DTH compared with PBS-treated controls (Figure 1A). In contrast, identically treated *Daf1*$^{-/-}$ mice developed lesions (Figure 1A) with even more robust leukocyte infiltration than those of WT recipients that received PBS alone in the a.c. (ie, were susceptible to characteristic DTH) (Figure 1B). The data in WT, but not *Daf1*$^{-/-}$, mice are thus consistent with the anterior chamber−associated immune deviation phenomenon in which antigen that is initially seen in the a.c. elicits a tolerogenic response that inhibits OVA-specific DTH. This is opposed to a.c.-injected PBS in which antigen initially seen systemically elicits a Teff response and DTH consequently is not inhibited. Consistent with the effect being antigen specific, DTH in response to s.c. injection of the nonrelevant peptide was not altered in either WT or *Daf1*$^{-/-}$ mice a.c. preinjected with OVA (data not shown).

The findings that DTH was abrogated in WT mice but heightened in *Daf1*$^{-/-}$ mice argued that a.c. injection in DAF's presence and absence had opposing extraocular effects. In accordance with DAF playing an obligate role in conferring dominate systemic tolerance, splenocytes from OVA a.c. preinjected WT littermates showed <2% of IFN-γ−producing CD4$^+$ cells in response to whole OVA, whereas OVA a.c. preinjected *Daf1*$^{-/-}$ mice conversely showed approximately twofold heightened IFN-γ−producing CD4$^+$ cells (Figure 1C). A comparable difference was observed with the immune dominant peptide of

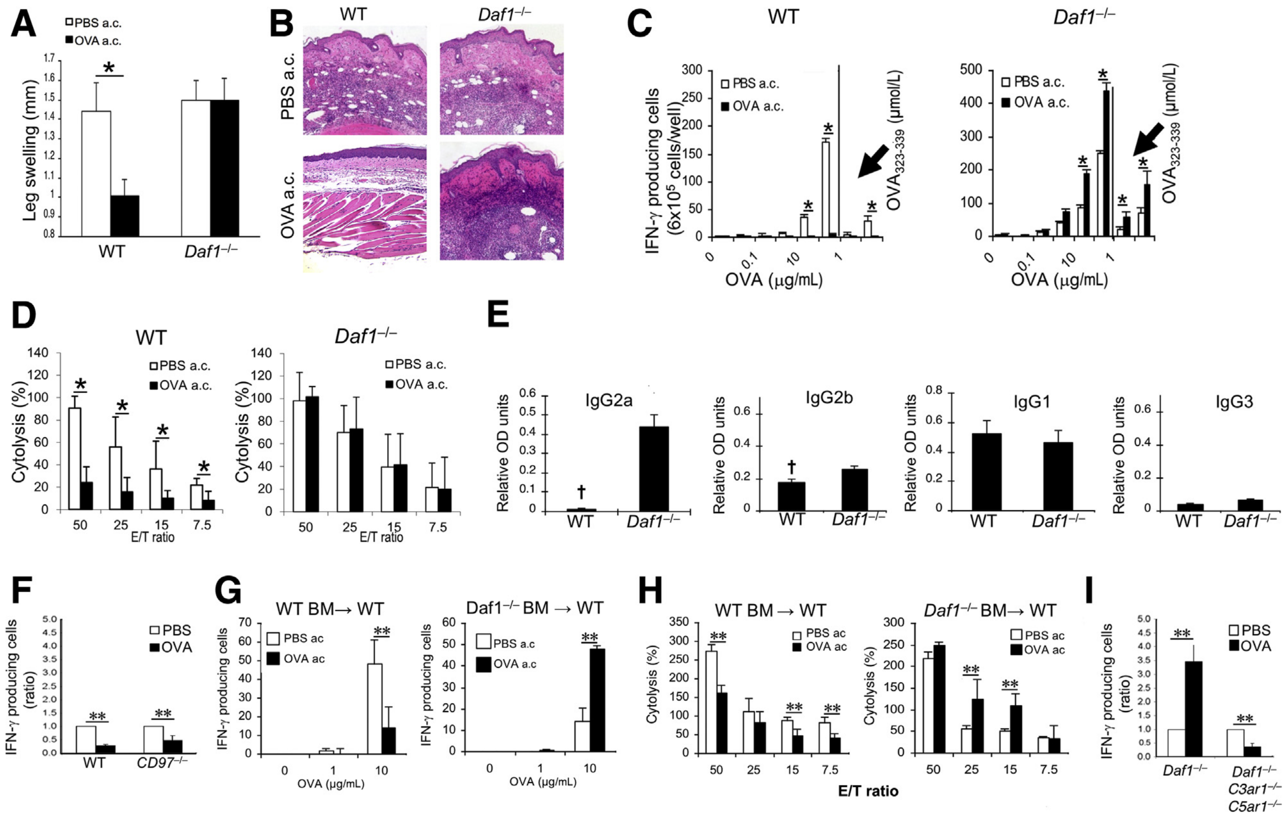

Figure 1 Decay-accelerating factor (DAF) is required for the ocular tolerogenic response to an anterior chamber (a.c.) antigen. **A:** *Daf1*$^{+/+}$ and *Daf1*$^{-/-}$ mice were preinjected in the a.c. with 50 μg ovalbumin (OVA) or phosphate-buffered saline (PBS). Seven days later, the mice were challenged subcutaneously with 12.5 μg OVA in IFA in the right leg and with PBS in IFA in the left leg; and 48 hours later, delayed-type hypersensitivity (DTH) size was measured. **B:** Representative DTH lesions from wild-type (WT) and *Daf1*$^{-/-}$ mice. **C:** Splenocytes (6 × 10^5) from *Daf1*$^{+/+}$ and *Daf1*$^{-/-}$ mice preinjected a.c. with OVA or with PBS (7 days earlier, as in the above protocol) were assayed on interferon (IFN)-γ ELISPOT plates in the presence of a varying amount of whole OVA protein or $OVA_{323\text{-}339}$ peptide. **Arrows** point to the assays with peptide rather than the whole protein. **D:** Lymph node cells [effectors (E)] from mice preinjected a.c. with OVA or PBS were mixed with OVA-transfected EL-4 targets at the designated effector to target (E/T) ratios. Percentage cytolysis was determined using Alamar blue. **E:** *Daf1*$^{+/+}$ and *Daf1*$^{-/-}$ mice were preinjected in the a.c. with OVA or PBS and challenged with OVA in CFA subcutaneously at day 7 (as in **A**–**C**). Sera harvested at day 17 were assayed for anti-OVA antibody isotypes by isotype enzyme-linked immunosorbent assays. **F:** IFN-γ ELISPOT assays were performed on splenocytes from groups of *Daf1*$^{+/+}$ and *CD97*$^{-/-}$ mice 7 days after a.c. preinjection with OVA or PBS. **G** and **H:** IFN-γ ELISPOT (**G**) and cytotoxicity (**H**) assays were performed on splenocytes from WT bone marrow (BM) → WT recipients (**left panels**) or *Daf1*$^{-/-}$ BM → WT recipients (**right panels**) on day 7 after s.c. injection with OVA (day 17 after a.c. injection). **I:** (F12) *Daf1*$^{-/-}$ and (F12) *Daf1*$^{-/-}$ *C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ mice were studied using the protocol described in **F**. Data are shown as ratios of IFN-γ–producing cells by OVA a.c. protected mice normalized to PBS a.c. protected controls. $n = 6$ (**A** and **F**); $n = 3$ per group (**G**–**I**). *$P < 0.05$, **$P < 0.01$; $^{\dagger}P < 0.05$ versus *Daf1*$^{-/-}$. Original magnification, ×40 (**B**).

OVA (ie, $OVA_{323\text{-}333}$) (Figure 1C). Induction of OVA-specific CD8$^+$ T cells similarly was diminished. Although all WT littermates injected in the a.c. with OVA exhibited 75% decreased cytotoxic activity against OVA-transfected (syngeneic H-2^b) EL-4 targets,[4] none of the *Daf1*$^{-/-}$ mice showed a significant decrease ($P < 0.05$) (Figure 1D). Studies with magnetic bead–enriched splenic CD8$^+$ cells (>95% CD8$^+$) showed the same difference (data not shown).

Early work[37] on corneal transplants in the context of ocular tolerance implicated shifts away from complement-fixing Ig isotypes. Anti-OVA antibodies produced in *Daf1*$^{-/-}$ mice that were a.c. preinjected with OVA showed significant increases ($P < 0.05$) in (complement-fixing) anti-OVA IgG2a and IgG2b antibodies as opposed to other isotypes (Figure 1E). No alterations in anti-OVA Abs occurred in response to a nonrelevant antigen (data not shown), consistent with specificity.

Abrogated Ocular Tolerance in *Daf1*$^{-/-}$ Mice Is due to Loss of DAF's Restraint of Immune Cell C3ar1/C5ar1 Signaling

Because DAF function can impact multiple processes, each process was examined in the context of the ocular tolerogenic response. DAF's interaction with CD97, a seven-membrane spanning surface protein expressed on leukocytes and other cell types,[38] promotes leukocyte adhesion at sites of inflammation and so doing contributes to experimental arthritis.[39] The tolerogenic response to OVA was intact in *CD97*$^{-/-}$ mice preinjected with OVA into the a.c. (Figure 1F), excluding DAF-CD97 interactions as being involved.

To test whether increased sensitivity of *DAF*$^{-/-}$ ocular cells to systemic complement attack accounted for the disabled tolerogenic response, lethally irradiated WT recipients were reconstituted with bone marrow cells from

Daf1$^{-/-}$ mice and vice versa. Eight weeks later [after confirming >90% donor bone marrow was comparable in both groups (data not shown)], the ocular OVA challenge experiments were repeated. In WT → *Daf1*$^{-/-}$ chimeras, both OVA-specific CD4$^+$ cell IFN-γ (Figure 1, G and H) and OVA-specific CD8$^+$ cell cytotoxic responses (Figure 1, G and H) were markedly suppressed, as in WTs. In contrast, potent and specific OVA-reactive CD4$^+$ cell and CD8$^+$ cell responses occurred in the *Daf1*$^{-/-}$ → WT chimeras, pointing to effects mediated by immune cells. Because DAF on immune cells suppresses C3ar1/C5ar1 signaling and affects cytokine production as well as cooperates with other regulators in circumventing systemic complement attack,[40,41] the ocular tolerance protocol was repeated in *Daf1*$^{-/-}$*C3ar1*$^{-/-}$ *C5ar1*$^{-/-}$ mice. The tolerogenic response was completely restored in *Daf1*$^{-/-}$*C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ mice (Figure 1I), indicating that the failed tolerogenic response in *Daf1*$^{-/-}$ mice resulted from the loss of DAF restraint of C3ar1/C5ar1 signaling in immune cells. Prior findings[42] that addition of C5a to WT CD4$^+$ cells simulated the effect of DAF deficiency supported the interpretation that DAF's function in the context of tolerance is repression of autocrine C3ar1/C5ar1 signaling in immune cells.

DAF Regulatory Activity Is Required for Induction of Tregs against a.c.-Injected Protein

To decipher mechanisms accounting for the requirement of DAF for the induction of ocular tolerance, the a.c. tolerance protocol was repeated in *Daf1*$^{-/-}$ and WT mice, after which the mice were challenged subcutaneously with OVA in CFA. Seven days later, ocular explants were isolated and cultured with OVA for 48 hours. Supernatants of explants from OVA a.c.-injected WTs showed approximately 1.8-fold more TGF-β1 compared with those from PBS-injected controls (Figure 2A). Explants of identically treated *Daf1*$^{-/-}$ mice conversely showed approximately 1.5-fold less TGF-β than those from WT mice (Figure 2A). Consistent with these findings, splenic cells from OVA a.c.-injected WTs produced increased TGF-β1, IL-10, and little IFN-γ in response to OVA, whereas splenic cells from OVA a.c.-injected *Daf1*$^{-/-}$ mice produced approximately fourfold less TGF-β1, more

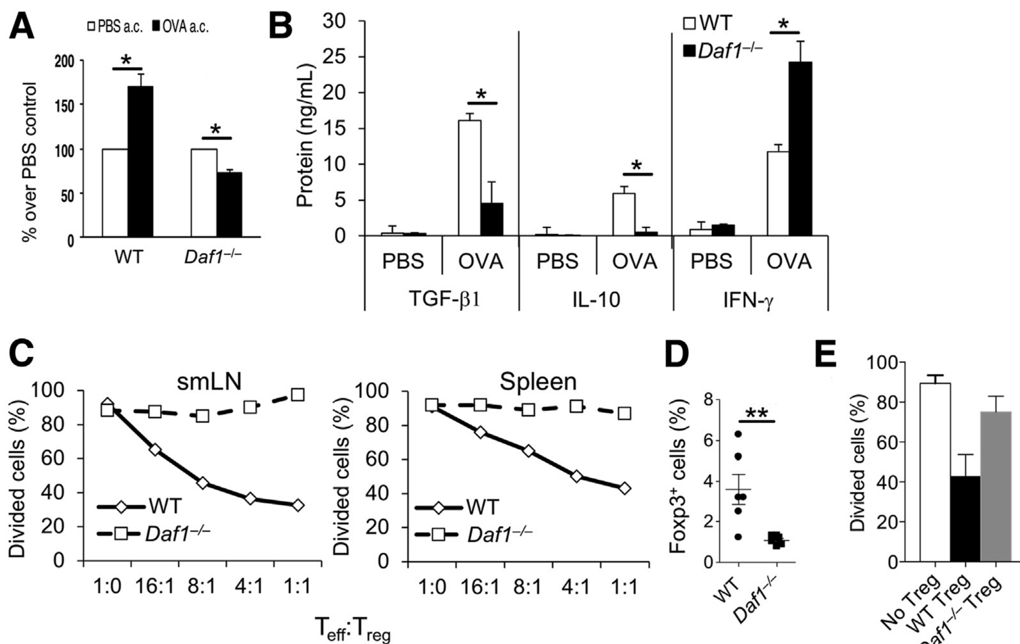

Figure 2 Ocular tolerance requires immunosuppression of the decay-accelerating factor (DAF)—dependent regulatory T cell (Treg). **A:** Ocular explants from anterior chamber (a.c.)—preinjected mice were placed in RPMI 1640 medium for 48 hours. Supernatants were assayed for transforming growth factor (TGF)-β by enzyme-linked immunosorbent assay (ELISA). **B:** Wild-type (WT) or *Daf1*$^{-/-}$ mice were preinjected a.c. with ovalbumin (OVA) or phosphate-buffered saline (PBS), followed by s.c. challenge with OVA in IFA in the right leg 7 days later. Splenocytes were cultured at 37°C for 48 hours with 100 μg/mL of OVA, and culture supernatants were assayed for TGF-β1, IL-10, and interferon (IFN)-γ by ELISAs. **C:** Wild-type (WT) or *Daf1*$^{-/-}$ mice were preinjected a.c. with OVA or PBS, followed 7 days later by s.c. challenge with OVA in IFA in the right leg. After another 7 days, CD4$^+$ cells from draining submandibular lymph node (smLN) and from the spleen were isolated. Sorted Foxp3$^+$ cells from each site were incubated for 5 days in increasing ratios with mixtures of dendritic cells (DCs), $OVA_{323-339}$, and OT-II cells (prelabeled with CellTracker Red), after which suppression of proliferation was assessed by determining percentage dividers. **D:** A total of 4×10^6 sorted green fluorescent protein (GFP)$^-$ CD4$^+$ cells from (Thy1.2) WT or *Daf1*$^{-/-}$ Foxp3-GFP mice were injected intravenously into WT Thy1.1 mice. Two days thereafter, the mice were preinjected a.c. with OVA or PBS and challenged 7 days later with OVA in IFA subcutaneously in the right leg. Seven days after the s.c. challenge, Foxp3$^+$ Thy1.2$^+$ cells in the spleen were quantified by flow cytometry. **E:** Foxp3$^+$ cells from **D** were sorted, and the sorted cells were incubated for 5 days at a 4:1 ratio with mixtures of DCs, $OVA_{323-339}$, and OT-II cells (prelabeled with CellTracker Red). Suppression of proliferation was assessed by determining percentage dividers. $n = 3$ (**C** and **E**); $n = 6$ GFP$^-$ CD4$^+$ cells from (Thy1.2) WT or *Daf1*$^{-/-}$ Foxp3-GFP mice and Foxp3$^+$ Thy1.2$^+$ cells in the spleen (**D**). $^*P < 0.05$, $^{**}P < 0.01$.

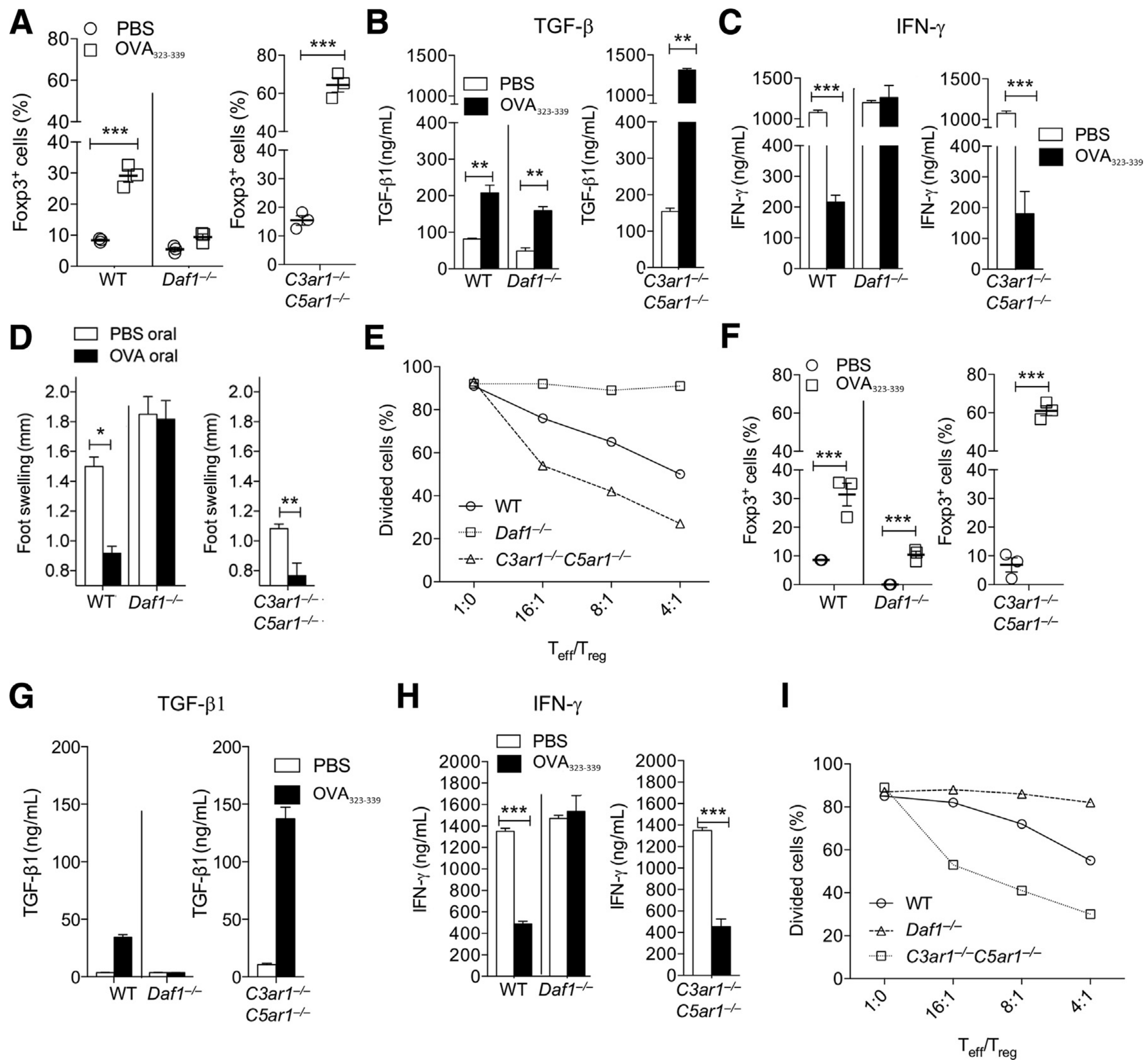

Figure 3 Oral tolerance requires immunosuppression of decay-accelerating factor (DAF)–dependent regulatory T cell (Treg). **A–E:** Data are organized showing wild-type (WT) versus *Daf1*$^{-/-}$ mice on the left, and for side-by-side comparison, *C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ mice on the right. WT, *Daf1*$^{-/-}$, and *C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ OT II mice were fed 20 mg/mL ovalbumin (OVA) or phosphate-buffered saline (PBS) in their drinking water for 5 days, after which mesenteric lymph nodes (mLNs) were isolated and CD4^{+} and CD11c^{+} cells were separated via sorting. **A:** Percentage of CD4^{+} cells that were CD25^{+} Foxp3^{+} in the initial isolate were quantified by flow cytometry. **B** and **C:** After overnight culture of the remainder of the cells with OVA (100 μg/mL), amounts of transforming growth factor (TGF)-β1 (**B**) and interferon (IFN)-γ (**C**) released into culture supernatants were assayed by enzyme-linked immunosorbent assays (ELISAs). **D:** In other animals, delayed-type hypersensitivity responses were measured 48 hours after OVA injection into the footpad. **E:** Sorted CD25^{+} cells that were Foxp3^{+} in other aliquots from **A** were incubated for 5 days at increasing T-effector (Teff)/Treg ratios with mixtures of dendritic cells (DCs), $OVA_{323-339}$, and WT OT-II cells prelabeled with CellTracker Red. Suppression of CellTracker Red–labeled OT-II cell proliferation was assessed by quantifying percentage dividers. **F–I:** Sorted Foxp3^{-} OT-II cells from WT, *Daf1*$^{-/-}$, and *C3ar1*$^{-/-}$ *C5ar1*$^{-/-}$ OT-II Foxp3–green fluorescent protein mice were adoptively transferred into Thy1.1 recipients. The Thy1.1 recipients were fed 20 mg/mL OVA or an equal volume of PBS in their drinking water for 5 days and immunized subcutaneously with OVA in IFA in the hind leg. Seven days later, mLNs were harvested and the cells were isolated. **F:** Percentage of CD4^{+} cells that were Foxp3^{+} CD25^{+} in the initial mLN isolate were quantified by flow cytometry. **G** and **H:** After 48 hours of culture of the remainder of the cells with OVA, the amounts of TGF-β1 and IFN-γ released into the supernatants were quantified by ELISAs. **I:** Sorted Foxp3^{+} cells in the initial isolate (**F**) were incubated for 5 days with mixtures of DCs, $OVA_{323-339}$, and sorted WT OT-II cells (prelabeled with CellTracker Red) at varying ratios. Suppression of proliferation was assessed by quantifying percentage dividers. $n = 6$ mice for each genotype, OVA group, and PBS group (**F–I**); $n = 12$ for each recipient group (**F–I**). *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.

than ninefold less IL-10, and approximately twofold more IFN-γ (Figure 2B) than WTs.

Because TGF-β1 and IL-10 are connected with Treg induction and function, WT and *Daf1*$^{-/-}$ Foxp3-GFP reporter mice were prepared and pretreated with OVA in the a.c., after which Treg induction was assessed. The gating strategy is shown in Supplemental Figure S1A. Basal percentages of Foxp3^{+} cells among CD4^{+} cells in the draining smLN and spleen of unimmunized WT mice were 5.7% ± 0.7% and 8.0% ± 0.5%, respectively. This compared with only 1.4% ± 0.4% and 3.5% ± 0.4%, respectively, in unimmunized *Daf1*$^{-/-}$ mice ($P < 0.05$ for both sites). After a.c. preinjection of OVA or PBS in WT Foxp3-GFP mice and subsequent tail immunization (Figure 1), Foxp3^{+} cell

percentages in the spleen and smLN increased from 5.7% to 6.7% ± 0.8% and from 8.0% to 10.2% ± 0.6%, respectively ($P < 0.05$ in each site). In contrast, after identical treatment of *Daf1*$^{-/-}$ Foxp3-GFP mice, no increases from the lower basal Foxp3^{+} cell levels occurred (ie, from 1.4% ± 0.4% to 1.3% ± 0.5% in the spleen and from 3.5% ± 0.4% to 3.0% ± 0.3% in the smLN; $P < 0.0002$ and $P < 0.0001$, respectively, compared with WTs). Flow-sorted Foxp3^{+} cells from the smLN or spleen (Figure 2C) of the WTs efficiently suppressed OT-II cell proliferation, whereas the *Daf1*$^{-/-}$ Foxp3^{+} cells showed minimal, if any, suppressor activity. Thus, in the context of this *in vivo* a.c. antigen initial encounter system, DAF function is required for Treg induction and the ocular tolerance response.

To distinguish whether the Treg differences reflected the generation of de novo pTregs versus the expansion of preexisting (thymic Tregs and/or pTregs), sorted Foxp3^{-} (GFP^{-}) CD4^{+} cells were adoptively transferred from WT or *Daf1*$^{-/-}$ Foxp3-GFP mice (both Thy-1.2) into WT Thy-1.1 recipients. Repeat studies using the above protocol and gating on Thy-1.2 CD4^{+} cells showed an increase of 3.8% ± 0.04% Thy-1.2 GFP^{+} cells in recipients of WT (Thy-1.2) Foxp3^{-} cells, compared with 0.8% ± 0.02% in recipients of *Daf1*$^{-/-}$ (Thy-1.2) Foxp3^{-} cells ($P < 0.008$) (Figure 2D). This argued that the Tregs induced by the a.c. OVA injection derived from uncommitted CD4^{+} cells. Although only small numbers of Thy-1.2 *Daf1*$^{-/-}$ Foxp3^{+} cells could be retrieved from the recipients of GFP^{-} *Daf1*$^{-/-}$ CD4^{+} cells, their inclusion at a 4:1 Teff/Treg ratio in Treg suppression assays confirmed that they possessed little suppressive activity (Figure 2E). This contrasted with Thy-1.2 Foxp3^{+} WT cells from the recipients of Foxp3^{-} WT cells (Figure 2E). These data argued that failed ocular tolerance in *Daf1*$^{-/-}$ mice is connected with impaired induction as well as impaired function of pTregs. The data, thus, argued that DAF control of DC and CD4^{+} cell C3ar1/C5ar1 signaling is obligatory for the *in vivo* induction of tolerance in this site rather than constituting a redundant or modulatory process.

DAF Is Required for Oral Tolerance

Unlike the a.c., the GI tract is in constant contact with commensal bacteria and is exposed to numerous dietary antigens, which past studies[43] have shown associate with lamina propria DCs within 30 minutes. Much work[7] has documented that initial antigen encounter with DCs and CD11b^{+} F4/80^{+} macrophages in gastrointestinal tract lymphoid tissue elicits dominant immune tolerance.[44] This and other work have implicated CD103^{+} DC induction of pTregs[45] as being critical for the oral tolerogenic response.

To determine the importance of DAF in the induction of oral tolerance, OVA or PBS was added to the drinking water of naïve WT and *Daf1*$^{-/-}$ (OVA-specific) OT-II mice for 5 days, after which both groups were immunized subcutaneously with OVA in IFA. Seven days later, CD4^{+} cells were harvested from the mLNs. Intracellular staining for Foxp3 showed that in WT mice fed PBS, the percentage of OT-II cells that were Foxp3^{+} was 9.0% ± 0.5%; and in those fed OVA, the percentage increased to 30% ± 2% (Figure 3A). In contrast, in *Daf1*$^{-/-}$ OT-II mice fed PBS, <5.4% ± 4% of the OT-II cells were Foxp3^{+}; and OVA feeding did not significantly increase the percentage (Figure 3A). Culture supernatants of mLN CD4^{+} cells and OVA-primed DCs from WT OT-II mice fed OVA produced approximately 2.5-fold more TGF-β1 and approximately 5-fold less IFN-γ compared with those of PBS-fed controls (Figure 3B). In contrast, culture supernatants from *Daf1*$^{-/-}$ OT-II mice fed OVA showed a slightly smaller increase in TGF-β1 production compared with those of PBS-fed controls, but no decrease in IFN-γ production (Figure 3C). As occurred in the a.c., DTH in OVA-fed WTs decreased from 1.5 to 0.9 mm, whereas DTH did not decrease in OVA-fed *Daf1*$^{-/-}$ mice (Figure 3D). Likewise, sorted CD25^{+} cells (>95% Foxp3^{+} in other aliquots) harvested from mLNs of WT OT-II mice showed robust suppressor activity, whereas those harvested from mLNs of *Daf1*$^{-/-}$ OT-II mice exerted little suppressor activity (Figure 3E).

To determine whether the differences in Treg induction and function reflected differences in the induction of de novo pTregs, Thy-1.1 WT recipients of sorted Thy-1.2 Foxp3^{-} WT and *Daf1*$^{-/-}$ Foxp3^{-} cells were studied, as in ocular tolerance. In view of the finding that ocular tolerance in *Daf1*$^{-/-}$ → WT chimeras was restored in *Daf1*$^{-/-}$*C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ mice, linking DAF's repression of C3ar1/C5ar1 signaling with tolerance (Figure 1I), *C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ mice were included in the comparisons of oral tolerance. OT-II Foxp3-GFP mice were prepared on each genetic background, and sorted WT, *Daf1*$^{-/-}$, or *C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ Foxp3^{-} OT-II cells (all Thy-1.2) were transferred to Thy-1.1 recipients, and the protocol used with the manipulated mice on each background was followed. Thy-1.2 Foxp3^{+} cells increased from 10% to 30% in OVA-fed WTs, from 9% to 64% Foxp3^{+} cells in OVA-fed *C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ mice, and from <0.5% to only 12% in OVA-fed *Daf1*$^{-/-}$ mice (Figure 3F). Cultures of CD4^{+} and CD11c^{+} cells plus OVA from the mLN of *Daf1*$^{-/-}$ mice generated approximately eightfold less TGF-β and threefold more IFN-γ than those of recipients of WT cells (Figure 3, G and H). Conversely, cultures from *C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ mice generated 140-fold more TGF-β1 and approximately threefold less IFN-γ than WTs (Figure 3, G and H). As observed in ocular tolerance, Thy-1.2 Foxp3^{+} *Daf1*$^{-/-}$ OT-II cells, recovered from the mLN, showed little, if any, suppressor activity, whereas Foxp3^{+} *C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ OT-II cells showed more than fourfold more suppressor activity than those recovered Foxp3^{+} *C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ OT-II cells (Figure 3I). Returning to the comparisons of DTH in *Daf1*$^{-/-}$ versus WT mice, now including *C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ mice (Figure 3D), *C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ OT-II mice fed OVA showed a more profound reduction in DTH than in WTs (Figure 3D). Consistent with the failed immunosuppressive activity of *Daf1*$^{-/-}$ Tregs, intracellular staining of CD4^{+} cell precursors on each genetic background showed that *Daf1*$^{-/-}$ CD4^{+} cells produced markedly lower amounts of TGF-β and IL-10,

whereas *C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ cells produced markedly more of both immunosuppressive cytokines (Supplemental Figure S2, A and B). These findings indicated that repression of C3ar1/C5ar1 signaling by DAF is required for oral tolerance. They argued that this control is an obligate process.

MHC Class II Is Up-Regulated and PD-L1/ICOS-L Are Constitutively Down-Regulated on APCs in the smLN and mLN of Naïve *Daf1*$^{-/-}$ Mice

To decipher mechanism(s) accounting for the abrogated versus enhanced tolerogenic responses in *Daf1*$^{-/-}$ versus *C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ mice, DCs and CD4$^+$ cells were first compared for their C3a/C5a production and C3ar1/C5ar1 levels in both cases, assaying levels both on the cell surface and intracellularly. These data were then correlated with immune cell processes connected in the literature with tolerance. Tolerogenic responses occur as a result of the following: i) uptake of antigen by resident APCs, which are immature; ii) transit of the primed APCs to the draining LN; and iii) stimulation of (naïve) cognate CD4$^+$ cells by the immature entrant APCs.[46]

To determine how disabled DAF affects APC immaturity in the eye and gastrointestinal tract, MHC class II and costimulatory molecule expression levels were compared on unstimulated resident DCs in the cornea, smLN, and mLN of naïve WT and *Daf1*$^{-/-}$ mice. The gating strategy is shown in Supplemental Figure S1B. While unstimulated DCs in the cornea of naïve WT mice showed constitutive PD-L1 (B7-H1)[47] and ICOS-L (B7-H2) expression in conjunction

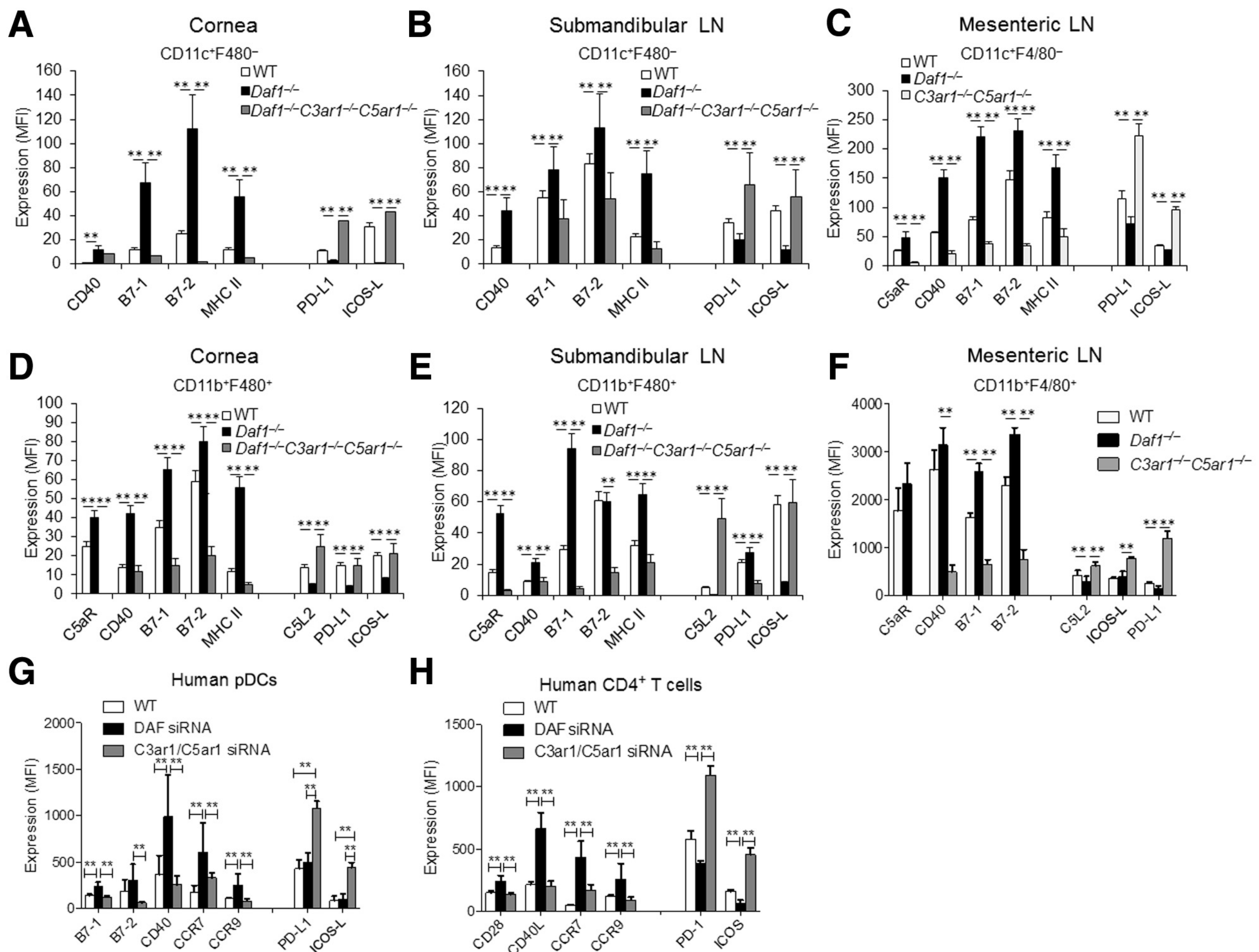

Figure 4 Decay-accelerating factor (DAF) is required for the tolerogenic properties of CD103$^+$ dendritic cells (DCs) and regulatory T cells. **A–C:** CD11c$^+$ DCs harvested from the eyes (**A**), draining submandibular lymph nodes (smLNs; **B**), and mesenteric lymph nodes (mLNs; **C**) of wild-type (WT), *Daf1*$^{-/-}$, and *Daf1*$^{-/-}$*C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ mice were assayed for CD40, B7.1, B7.2, major histocompatibility complex (MHC) II, PD-L1, and ICOS-L by flow cytometry (*Daf1*$^{-/-}$ versus WT and *Daf1*$^{-/-}$*C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ versus WT). **D–F:** CD11b$^+$F4/80$^+$ macrophages harvested from the eyes (**D**), draining smLNs (**E**), and mLNs (**F**) of WT, *Daf1*$^{-/-}$, and *Daf1*$^{-/-}$*C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ mice were assayed for the same markers. Cells were also assayed for C5ar1 and C5L2. The fact that differences in ICOS-L levels between *Daf1*$^{-/-}$ and WT cells reach statistical significance reflects mouse numbers as other studies in the laboratory (M.G.S., J.L., F.A., and M.E.M., unpublished data) reproducibly have documented statistical significance. **G** and **H:** Human plasmacytoid DCs (pDCs; **G**) and CD4 T cells (**H**) were treated with siRNA targeting DAF or C3ar1 and C5ar1 for 72 hours, after which expression of CD40, B7.1, B7.2, CCR7, CCR9, PD-L1, and ICOS-L on pDCs (**G**) and expression of CD28, CD40 ligand, CCR7, CCR9, PD-1, and ICOS on CD4$^+$ T cells (**H**) was assessed by flow cytometry. $n = 10$ per group (**A–F**); $n = 3$ (**G** and **H**). $^{**}P < 0.01$. MFI, mean fluorescent intensity.

with low MHC class II expression and essentially no B7-1,2 or CD40 expression (Figure 4A), those in naive *Daf1*$^{-/-}$ mice showed virtually no PD-L1 and ICOS-L expression, but 450% to 1200% ($P < 0.01$) increased constitutive expression levels of MHC class II, 560% ($P < 0.01$) higher levels of B7-1, 450% ($P < 0.01$) higher levels of B7-2, and 1200% ($P < 0.01$) higher levels of CD40. Resident corneal CD11b^{+} F4/80^{+} macrophages (Figure 4D), smLN DCs and macrophages (Figure 4, B and E), as well as mLN DCs and macrophages (Figure 4, C and F), in WT and *Daf1*$^{-/-}$ mice exhibited the same differences. A similar pattern was observed for inguinal LN (iLN) DCs (Supplemental Figure 1C). The increases in MHC class II and costimulatory molecule expression levels were completely reversed in *Daf1*$^{-/-}$*C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ mice or *C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ mice (Figure 4A). As in Figure 1I, *Daf1*$^{-/-}$*C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ cells were included to show that DAF's effect in the context of tolerance was solely related to its repression of autocrine C3ar1/C5ar1 signaling. Consequently, the effects of its deficiency were completely overcome by disabled C3ar1/C5ar1 signaling in immune cells. More important, selective studies showed the increases in PD-L1 and ICOS-L in WT and *C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ DCs were accompanied by increases in C5L2 (ie, C5ar2), a C5ar G protein−coupled receptor (GPCR) devoid of a G protein, which is implicated in scavenging C5a so as to prevent C5ar1 signaling. Also more important, studies with human plasmacytoid DCs and CD4^{+} cells silenced in either DAF or C3ar1/C5ar1 with siRNA(s) showed comparable changes (Figure 4, G and H), indicating that these findings apply in human immune cells.

Although the previous studies examined immune cell C3ar1/C5ar1 signaling in the context of proinflammatory experimental autoimmune encephalitis, they did not study it in the context of peripheral tolerance.[42] They also did not study the properties of immune cells that program the de novo

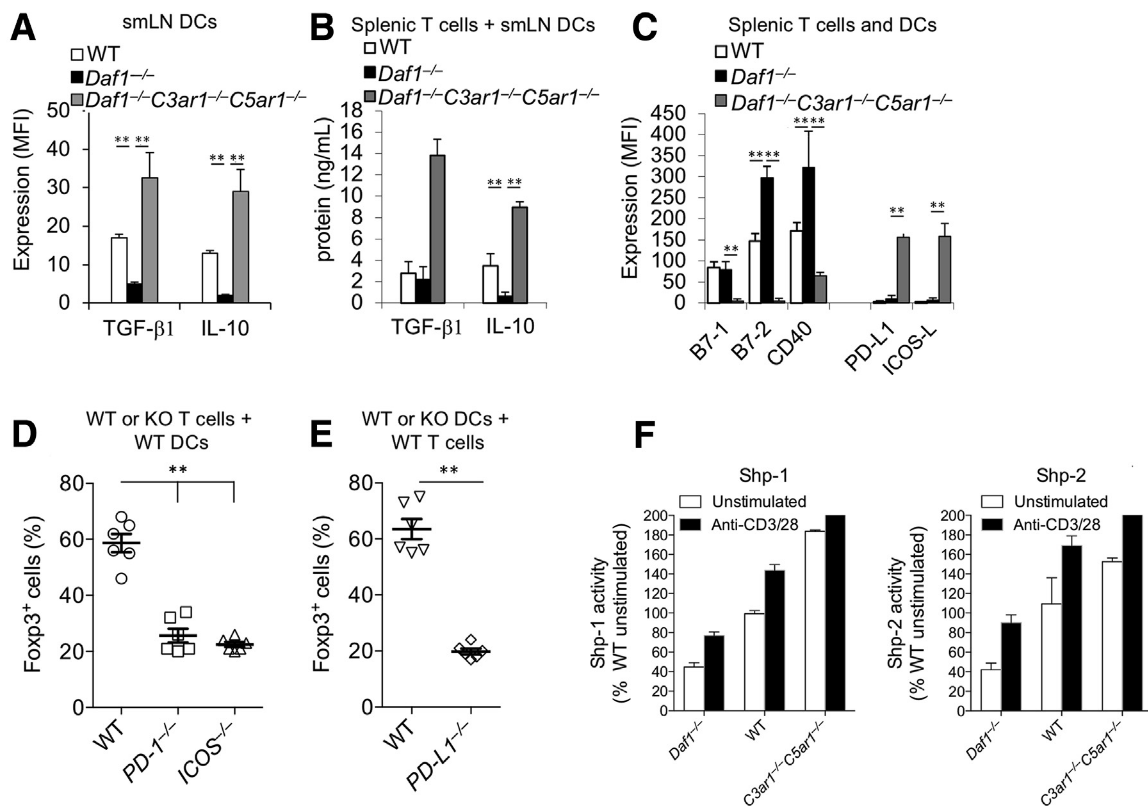

Figure 5 The expression of T-cell and dendritic cell (DC) decay-accelerating factor (DAF) regulates cytokine and surface protein expression. **A:** A total of 1×10^6 CD11c^{+} cells from the submandibular lymph node (smLN) of wild-type (WT), *Daf1*$^{-/-}$, and *Daf1*$^{-/-}$*C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ mice were incubated in RPMI 1640 media for 24 hours, after which IL-10 and transforming growth factor (TGF)-β1 were assayed by internal staining. **B:** A total of 2.5×10^5 WT dendritic cells (DCs) were incubated for 24 hours with 1×10^6 CD4^{+} T cells from WT, *Daf1*$^{-/-}$, and *Daf1*$^{-/-}$*C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ mice and anti-CD3/28 (1 μg/mL each), after which IL-10 and TGF-β1 were assayed by enzyme-linked immunosorbent assays. **C:** A total of 2.5×10^5 WT DCs were incubated for 24 hours with 1×10^6 WT, *Daf1*$^{-/-}$, and *Daf1*$^{-/-}$*C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ CD4^{+} T cells and anti-CD3/28, after which B7-1, B7-2, CD40, PD-L1, and ICOS-L expression levels on the DCs were assayed by flow cytometry (Supplemental Figure S2B). **D:** A total of 1×10^6 CD62L^{hi}CD25^{-} CD4^{+} cells from WT, *ICOS*$^{-/-}$, or *PD-1*$^{-/-}$ mice were incubated for 3 days with 1×10^5 CD11c^{+} WT DCs + anti-CD3/28 (1 μg/mL each) or IL-2 and C3aR-A/C5aR-A (10 μg/mL each), after which percentage of Foxp3^{+} cells was assayed by intracellular staining. **E:** A total of 1×10^5 CD11c^{+} WT or *PD-L1*$^{-/-}$ DCs were incubated for 3 days with 1×10^6 CD62L^{hi}CD25^{-} CD4^{+} cells from WT mice + anti-CD3/28 (1 μg/mL each) or IL-2 and C3aR-A/C5aR-A (10 μg/mL each), after which percentage Foxp3^{+} cells was assayed by intracellular staining. **F:** A total of 1×10^6 sorted *Daf1*$^{-/-}$, WT, and *C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ Foxp3-GFP CD4^{+} cells were incubated for 2 hours without or with anti-CD3/CD28 Dynabeads in the presence of IL-2 and Shp-1, and Shp-2 mRNA level was quantitated by real-time quantitative PCR. The results are shown in comparison to WT cells set as 100%. As in the above studies, each group contained three mice and experiments were repeated three times. $n = 6$ (**A** and **C**); $n = 5$ (**B**). $^{**}P < 0.01$. KO, knockout; MFI, mean fluorescent intensity.

induction of tolerance. DCs in the smLN of *Daf1*$^{-/-}$ mice contained less TGF-β1 and IL-10 than DCs in the smLN of WT mice (Figure 5A), whereas those of *Daf1*$^{-/-}$*C3ar1*$^{-/-}$ *C5ar1*$^{-/-}$ mice contained more of both immunosuppressive cytokines ($P < 0.01$ versus WTs). There was greater up-regulation of TGF-β and IL-10 production in *Daf1*$^{-/-}$ *C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ T cells compared with WT T cells. This increase is because of the fact that disabled C3ar1/C5ar1 signaling overcomes the effect of DAF deficiency because augmented C3a/C5a production, resulting from DAF's absence, is unable to signal through C3ar1/C5ar1. Moreover, the C3ar1/C5ar1 deficiency induces immunosuppressive TGF-β and IL-10 that would overcome any effect of DAF. Comparable differences were obtained for DCs in the mLN (data not shown). Co-cultures of WT DCs and *Daf1*$^{-/-}$ CD4^{+} cells produced less active TGF-β1 and IL-10 than co-cultures of WT DCs and WT CD4^{+} cells. In contrast, co-cultures of WT DCs and *Daf1*$^{-/-}$*C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ CD4^{+} cells produced more active TGF-β1 and IL-10 (Figure 5B), indicative of CD4^{+} cells modifying APC partners to become tolerogenic. Accordingly, WT DCs in co-cultures containing stimulated *Daf1*$^{-/-}$ CD4^{+} cells expressed markedly up-regulated B7-1,2 and CD40 levels compared with DCs in co-cultures with stimulated WT CD4^{+} cells, whereas WT DCs in co-cultures with stimulated *Daf1*$^{-/-}$ *C3ar1*$^{-/-}$*C5ar1*$^{-/-}$CD4^{+} cells expressed more coinhibitory PD-L1 and ICOS-L (Figure 5C). Adding C3a/C5a or C3ar1/C5ar1 antagonists to WT DCs induced differences corresponding to those in the different genotypes and anti-C3a/anti-C5a treatment could not overcome the maturity of WT *Daf1*$^{-/-}$ cells (Supplemental Figure S2, C and D)

Both PD-L1−PD-1[48,49] and ICOS-L−ICOS[50] interactions are centrally implicated in Treg lineage commitment. To determine whether these linkages are mechanistically interconnected with absent C3ar1/C5ar1 signaling in DC-CD4^{+} cell partners, sorted CD25^{-} WT, *PD-1*$^{-/-}$, or *ICOS*$^{-/-}$ CD4^{+} cells were incubated with WT DCs, or WT CD4^{+} cells were incubated with *PD-L1*$^{-/-}$ DCs, in all cases with IL-2 in the presence of C3ar1 and C5ar1 pharmaceutical antagonists (C3ar1-A and C5ar1-A). After 5 days, the CD4^{+} cells were assayed intracellularly for Foxp3. While >60% of the CD4^{+} cells became Foxp3^{+} in the presence of C3ar1-A/C5ar1-A when both the WT CD4^{+} cells and DCs possessed PD-1-PD-L1 and ICOS-ICOS-L couplings in PD-1−PD-L1 and ICOS−ICOS-L couplings, <20% became Foxp3^{+} when PD-1 or ICOS was deficient in the CD4^{+} cells (Figure 5D) or PD-L1 was deficient in the DCs (Figure 5E). These data showed that the participation of PD-1−PD-L1 and ICOS−ICOS-L couplings in Treg induction is dependent on C3ar1/C5a1 antagonism. To gain mechanistic insight, WT and *C3ar1*$^{-/-}$ *C5ar1*$^{-/-}$ CD4^{+} cells were examined for Src homology region 2 domain-containing phosphatase-2 (Shp-2), a phosphatase tied to PD1 expression.[51−54] Anti-CD3/28 activation of flow-sorted *Daf1*$^{-/-}$, WT, or *C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ Foxp3^{-} CD4^{+} cells with IL-2 showed that basal and stimulated Shp-2 as well as Shp-1 mRNA levels in *Daf1*$^{-/-}$ cells were lower than in WT cells and in *C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ cells. Both Shp-1 and Shp-2 were downregulated in unactivated and activated *Daf1*$^{-/-}$ CD4^{+} cells (Figure 5F). These data indicated that the linkages of Shp-2−dependent PD-1−PD-L1 and ICOS−L-ICOS couplings with Treg commitment are tied to absent C3ar1/C5ar1, which our past studies have shown represses phosphoinositide-3 kinase-γ (PI-3Kγ) activation.[42]

DAF Expression on CD103^{+} DCs Is Essential for Processes Enabling Their Tolerogenic Function

CD11c^{+}CD11b^{-}CD103^{+} migratory DCs are centrally connected with oral tolerance.[45,55] In lymphoid organs potentially relevant to ocular tolerance, they have been identified in the salivary LN[56] but have not been studied in the smLN draining the a.c. CD103^{+} DCs were analyzed in the mLN. The gating strategy used for examining CD103^{+} DCs is shown in Supplemental Figure S3. Compared with CD11c^{+}CD11b^{-}CD103^{+} DCs from WT mice, CD103^{+} DCs from *Daf1*$^{-/-}$ mice showed reduced ICOS-L and PD-L1 (Figure 6A), paralleling reduced levels in whole DCs (Figure 4, A and B). They also showed reduced expression of Cx3CR1 involved in priming CD8^{+} cells,[55,57] CCR7 involved in DC transit to the mLN,[44] and CCR9 involved in DC transit to the lamina propria (Figure 6B). CD11c^{+}CD103^{+} DCs were identified in the smLN in proportions corresponding to those in the mLN (Figure 6, C and D). These cells as well as macrophages in both sites (Supplemental Figure S1D) showed the same alterations as those measured earlier in *Daf1*$^{-/-}$ mice.

A recent study[58] provided evidence that CD103^{+} DCs are indispensable for oral tolerance. Phenotyping distinguished CD8a^{+} CD11b^{-} DCs, which are IRF-8 positive, as the relevant CD103^{+} DC subset. High transcription of the *Aldh1a2* and *TGFB2* genes (which encode retinaldehyde dehydrogenase 2 and TGF-β2, respectively) in both this CD103^{+} DC subset and CD4^{+} cells was linked with high-efficiency Treg induction. To determine how DAF deficiency impacts the expression of these markers, their expression was compared in WT, *Daf1*$^{-/-}$, and *C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ CD4^{+} cells in Treg induction cultures of WT, *Daf1*$^{-/-}$, and *C3ar1*$^{-/-}$ *C5ar1*$^{-/-}$ CD4^{+} cells supplemented with C3ar1-A/C5ar1-A. Unlike WT CD4^{+} cells activated under Treg conditions, *Daf1*$^{-/-}$ CD4^{+} cells showed reduced IRF-8, Aldh1a2, and TGF-β2 mRNAs, whereas *C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ CD4^{+} cells showed increases in each (Figure 6E). Cx3CR1, CCR7, and CCR9 as well as IRF-8, Aldh1a2, and TGF-β2 were not measured in other APC sets.

DAF Expression on Tregs Is Essential for Processes Implicated in Their Tolerogenic Function

Tregs possess inactive (latent) TGF-β1 on their surface in association with latency-associated protein (LAP), which is bound to glycoprotein A repetitions predominant.[59] The interaction of these complexes with integrin β6 (Itg-β6; pulmonary epithelial cells) and Itg-β8 on either the Tregs

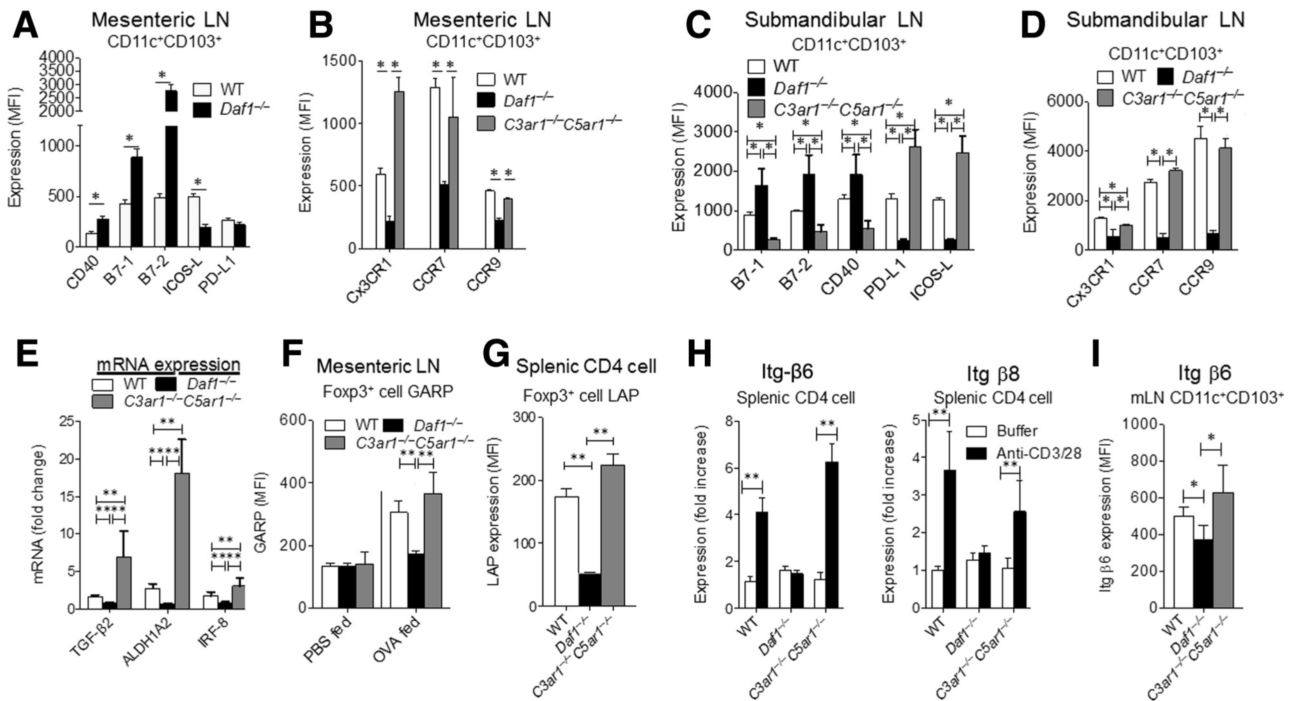

Figure 6 $CD103^+$ dendritic cell (DC) expression of decay-accelerating factor (DAF) is required for tolerance. **A:** $CD11c^+$ $CD103^+$ DCs were harvested from mesenteric lymph nodes (mLNs) of wild-type (WT) and *Daf1*$^{-/-}$ Foxp3—green fluorescent protein mice and assayed for expression of CD40, B7.1, B7.2, ICOS-L, and PD-L1 by flow cytometry. **B:** $CD11c^+CD103^+$ DCs harvested from mesenteric LNs of WT, *Daf1*$^{-/-}$, and *C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ mice were assayed for expression of CxCR3, CCR7, and CCR9 by flow cytometry. **C:** $CD11c^+CD103^+$ DCs were harvested from submandibular lymph nodes (LNs) of WT, *Daf1*$^{-/-}$, and *C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ mice and assayed for expression of CD40, B7.1, B7.2, ICOS-L, and PD-L1 by flow cytometry. **D:** $CD11c^+CD103^+$ DCs harvested from submandibular LNs of WT, *Daf1*$^{-/-}$, and *C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ mice were assayed for expression of CxCR3, CCR7, and CCR9 by flow cytometry. **E:** A total of 2.5×10^5 WT DCs were incubated for 24 hours with 1×10^6 $CD4^+$ T cells from WT and *Daf1*$^{-/-}$*C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ mice and anti-CD3/28 (1 μg/mL each), after which cells were assayed for *TGFB2*, *ALDH1A2*, and *IRF8* mRNA expression by real-time quantitative PCR (qPCR). **F:** $Foxp3^+$ $CD4^+$ T cells from WT, *Daf1*$^{-/-}$, and *C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ mice fed ovalbumin or phosphate-buffered saline were assayed for glycoprotein A repetitions predominant (GARP) expression by flow cytometry. **G:** Sorted $Foxp3^-$ $CD4^+$ T cells from WT, *Daf1*$^{-/-}$, and *C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ mice were activated for 3 days with anti-CD3/28 Dynabeads + C3aR-A/C5aR-A (10 μg/mL each), after which sorted $Foxp3^+$ cells were assayed for latency-associated protein (LAP) expression by flow cytometry. **H:** Sorted $Foxp3^-$ $CD4^+$ T cells from WT, *Daf1*$^{-/-}$, and *C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ mice were activated for 3 days with anti-CD3/28 Dynabeads + C3aR-A/C5aR-A (10 μg/mL each), after which sorted $Foxp3^+$ cells were assayed for integrin β6 (Itg-β6) and Itg-β8 mRNA expression by qPCR. **I:** $CD103^+CD11c^+$ cells from the mLN of WT, *Daf1*$^{-/-}$, and *C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ mice were assayed for Itg-β6 expression by flow cytometry. $n = 10$ (**A**–**D**). $^*P < 0.05$, $^{**}P < 0.01$. MFI, mean fluorescent intensity.

themselves or $CD103^+$ DCs (see *Discussion*) is implicated in the release of active TGF-β1 and consequent maintenance of gut homeostasis.[60] Phenotyping of $Foxp3^+$ cells, isolated from the mLN of OVA-fed WT, *Daf1*$^{-/-}$, and *C3ar1*$^{-/-}$ *C5ar1*$^{-/-}$ OT-II Foxp3-GFP mice, showed that although glycoprotein A repetitions predominant and LAP expression increased on WT and *C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ pTregs, neither protein did so on *Daf1*$^{-/-}$ Tregs (Figure 6, F and G). Unlike WT or *C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ pTregs, which showed up-regulated Itg-B6 and Itg-B8 mRNA (Figure 6H), *Daf1*$^{-/-}$ pTregs showed little mRNA up-regulation of either integrin. Although Itg-β8 on $CD103^+$ DCs could not be measured because of insufficient cell recovery for real-time quantitative PCR and the lack of a suitable anti-mouse Itg-β8 antibody, as reported by others,[60] the same difference between the three genotypes was found for Itg-β6 protein on $CD103^+$ DCs (Figure 6I). Thus, the normal expression of multiple factors on both $CD103^+$ DCs and pTregs, which play requisite roles in ocular and oral tolerance, depends on DAF.

Discussion

The maturation status of APCs that process antigens and migrate to draining LNs, in large part, determines whether priming of cognate naïve $CD4^+$ cells in the LN produces effector or tolerogenic responses.[12,13,46] In this study, two independent sites where tolerogenic responses are continually and physiologically mounted were examined: the a.c. of the eye and the GI tract. Our prior studies showed absence of C3ar1/C5ar1 signaling in $CD4^+$ cells enables autocrine TGF-β production by DC and $CD4^+$ cell partners and programs pTreg induction[42]; but it was unclear whether this is a requisite, redundant, or modulatory process in T-lineage commitment. In addition, they did not address physiological processes that underlie T-cell homeostasis versus activation, such as tolerance, or interrogate the immune cell properties of APCs and Tregs traditionally linked with tolerance. The experiments herein, together with the recent clinical findings that IBD-like enteropathy develops in DAF's absence, show that DAF expression on immune cells is obligatory for many

of these properties and is needed for CD103$^+$ DC induction of peripheral gut and ocular tolerance. They show that physiological DAF expression does the following: i) represses DC MHC class II expression, ii) induces coinhibitory PD-L1/ICOS-L expression and PD1/ICOS expression on the interactive partners while repressing costimulatory molecule expression, iii) enables CD103$^+$ DC IRF-8$^+$Aldh1a2$^+$TGF-β2$^+$ expression connected with CD103$^+$ DC migration to the regional LN, iv) increases Shp-2 expression needed for PD1 expression, v) increases glycoprotein A repetitions predominant and increases surface LAP and Itg-β6/Itg-β8 connected with Treg TGF-β1 secretion, and vi) enables CCR7 and CCR9 expression needed for transit of CD4$^+$ cells to and from tolerogenic LNs, where Tregs are generated. More important, the studies show that DAF expression on CD4$^+$ cells as well as on APCs is required for tolerogenic APC processes in the GI tract and in the a.c.

The experiments on ocular and oral tolerance yielded essentially identical results. They showed that resident DCs and CD4$^+$ cells in the smLN and mLN of unmanipulated *Daf1*$^{-/-}$ mice tonically are more mature than in WTs, reflecting their acquisition of an M1 rather than a tolerogenic M2 phenotype. Consequently, lineage commitment to pTregs in *Daf1*$^{-/-}$ mice, needed for a tolerogenic response, was virtually abolished. In accordance with the effect being due to restraint of immune cell C3ar1/C5ar1 signaling, this contrasted with *Daf1*$^{-/-}$*C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ or *C3ar1*$^{-/-}$ *C5ar1*$^{-/-}$ mice in which the M1 maturation status of DCs possessing *Daf1*$^{-/-}$ deficiency alone was reversed and pTreg commitment was augmented above WTs. The superphysiological tolerogenic properties when C3ar1/C5ar1 are deficient are due to the fact that DAF in WT cells does not fully block C3ar1/C5ar1 signaling, allowing constitutive signaling through these GPCRs, which supports the viability of conventional Teff cells.[18] The data herein, thus, argue that the default state of APCs is connected, at least in part, with low-level APC C3a/C5a production, which, when increased, would serve as an APC maturation signal and render the APCs able to convert naïve CD4$^+$ cells to a Teff lineage. In support of this characteristic being DAF specific rather site specific and alterations of DAF control of C3ar1/C5ar1 signaling being site specific via elaboration of cytokines, the same connections of DAF with immature DCs and its absence with mature DCs were found in the inguinal LN absent a toll-like receptor or maturation signal. In the presence of restraint of autocrine C3ar1/C5ar1 signaling in *C3ar1*$^{-/-}$*C5ar1*$^{-/-}$ DCs, TGF-β1/IL-10 production by smLN and mLN CD4$^+$ cells was increased above WTs and IFN-γ production reciprocally decreased, whereas the opposite pattern was observed in the presence of potentiated C3ar1/C5ar1 signaling in *Daf1*$^{-/-}$ DCs.

Collectively, the data herein on DAF control of autocrine C3ar1/C5ar1 signaling tie together many individual APC and CD4$^+$ cell processes associated in the literature with tolerance.[61,62] These findings may explain earlier observations[63] interpreted in the context of serum complement that dextran sodium sulfate−induced colitis is markedly more severe in *Daf1*$^{-/-}$ mice. They likewise provide a mechanism for reports that i) the absence of C5ar1 signaling is important for tolerance in the dextran sodium sulfate model,[64] ii) dextran sodium sulfate colitis is attenuated in *C5ar1*$^{-/-}$ mice,[65] and iii) blockade of C5a suppresses intestinal damage in trinitrobenzene sulfonic acid−induced colitis.[66] As discussed, the same presence or absence of DAF control of autocrine C3ar1/C5ar1 signaling in macrophages, which biases between PD1-L1/ICOS-L expression and TGF-β1/IL-10 production, versus B7/CD40 expression and IFN-γ production is likely relevant to M1 versus M2 macrophage polarization. This macrophage polarization is linked with type 2 diabetes, atherosclerosis, and cirrhosis,[67] among other disease states.

Several different immune processes have been proposed as playing a principal role in ocular tolerance. A common denominator has not been identified. Among the processes proposed as being central have been the following: i) local production of TGF-β1 in the a.c.[68]; ii) decreased expression of B7-1,2 and CD40 by DCs in the cornea and in the a.c.[69,70]; iii) altered transit of DCs from the a.c. to the spleen and diminished IL-10 production there[71]; iv) defective generation of pTregs in the a.c.[72,73]; v) local production of neuropeptides, in particular alpha-melanocyte stimulating hormone (α-MSH), in the a.c.[74]; vi) transmission of apoptotic signals to Teff by FasL expressed on corneal endothelium[75]; and vii) increased natural killer T-cell activity.[76] The data herein provide a common mechanism for processes i) through iv), in part reconciling this previous literature. Further work will be needed to determine whether there are mechanistic linkages with processes (v) α-MSH, (vi) corneal and endothelial cell FasL expression, and (vii) natural killer T cells.

Although tolerance in the gastrointestinal tract and airways is more complex than that in the a.c. in that it involves IL-10−producing Tr1 cells,[77] TGF-β−producing type 3 helper T cells, and myeloid-derived suppressor cells,[78] numerous studies have shown that pTreg induction resulting from the migration of immature DCs to regional LNs plays an essential role in tolerance at both sites.[64−66] A recent study[79] implicated initial antigen interaction with macrophages, followed by transfer to DCs, both of which, according to our data, would depend on DAF expression to maintain their immaturity. More important, a more recent study distinguished that the critical APC is the CD103$^+$ CD8a$^+$ CD11b$^-$ IRF-8$^+$Aldh1a2$^+$TGF-β2$^+$ DC.[80] In addition to linking expression of coinhibitory PD-L1, ICOS-L, IRF-8$^+$Aldh1a2$^+$TGF-β2$^+$ CCR7, and CCR9 on CD103$^+$ DCs with DAF, the data herein show that surface expression of glycoprotein A repetitions predominant, which anchors LAP−TFG-β1 complexes to the surface of Tregs,[59,60] depends on DAF. Most important, they show

that expression levels of CD103, the integrin mechanistically linked with conversion of surface-associated LAP−TGF-β1 complexes into active TGF-β1 on $CD103^+$ DCs,[45,59,60] are decreased. They likewise show that up-regulation of Itg-β6 and Itg-β8 on pTregs is dependent on DAF. $CD103^+$ DCs in the smLN having the same properties essential to ocular tolerance are identified for the first time.

DAF's modulatory effect on Teff responses is mechanistically due to the fact that Gβγ signaling of the G proteins of the C3ar1/C5ar1 GPCRs evokes PI-3Kγ activation.[18] The activated PI-3Kγ is needed for optimal assembly of inner leaflet phosphatidylinositol 3,4,5 trisphosphate, on which AKT is phosphorylated.[81] One molecular mechanism connecting the loss of DAF with loss of tolerance is that lifted DAF restraint on C3ar1/C5ar1 GPCR signaling would increase PI-3Kγ activation.[18] Activated PI-3Kγ promotes AKT phosphorylation,[18] which augments phosphorylated AKT−dependent activation of mammalian target of rapamycin complex 1 and thereby restrains TGF-β1 production.[82] Because C3ar1 and C5ar1 are Gi-coupled GPCRs, the Gα subunits of their G proteins repress adenylyl cyclase and consequently suppress protein kinase A activation. Increased AKT phosphorylation and decreased protein kinase A activation prevent the nuclear translocation of Foxo1[83] and the generation of phosphorylated cAMP response element binding protein (CREB),[18] two transcription factors needed for Foxp3 expression.[18]

The physiological and pathogenic relevance of C3ar1/C5ar1 signaling in DCs and $CD4^+$ cells has remained unclear, and whether this pathway operates redundantly as one of several pTreg lineage commitment mechanisms in the context of tolerance has remained unstudied. The data herein argue that, although other processes operate to provide immunosuppression, DAF is indispensable in the context of physiological tolerance−associated pTreg induction that protects against IBD. They show that immune cell DAF is essential for expression of $CD103^+$ cell and $CD4^+$ cell markers that play requisite roles in the induction of tolerance.

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Supplemental Data

Supplemental material for this article can be found at *http://doi.org/10.1016/j.ajpath.2019.04.008*.

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