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Decay Accelerating Factor Can Control T Cell Differentiation into IFN-γ-Producing Effector Cells via Regulating Local C5a-Induced IL-12 Production

Peter N. Lalli,*† Michael G. Strainic,† Feng Lin,† M. Edward Medof,† and Peter S. Heeger2*‡

A newly recognized link between the complement system and adaptive immunity is that decay accelerating factor (DAF), a cell surface C3/C5 convertase regulator, exerts control over T cell responses. Extending these results, we show that cultures of Marilyn TCR-transgenic T cells stimulated with DAF-deficient (Daf1−/−) APCs produce significantly more IL-12, C5a, and IFN-γ compared with cultures containing wild-type APCs. DAF-regulated IL-12 production and subsequent T cell differentiation into IFN-γ-producing effectors was prevented by the deficiency of either C3 or C5a receptor (C5aR) in the APC, demonstrating a link between DAF, local complement activation, IL-12, and T cell-produced IFN-γ. Bone marrow chimera experiments verified that bone marrow cell-expressed C5aR is required for optimal differentiation into IFN-γ-producing effector T cells. Overall, our results indicate that APC-expressed DAF regulates local production/activation of C5a following cognate T cell/APC interactions. Through binding to its receptor on APCs the C5a up-regulates IL-12 production, this in turn, contributes to directing T cell differentiation toward an IFN-γ-producing phenotype. The findings have implications for design of therapies aimed at altering pathologic T cell immunity.


Following cognate T cell/APC interactions in conjunction with appropriate costimulatory signals, T cells undergo activation, proliferation, and differentiation, yielding type 1 (e.g., IFN-γ) or type 2 (e.g., IL-4, IL-5) cytokine-secreting effectors. APC-secreted IL-12 is a key inducer of effector T cells producing IFN-γ in that in the absence of IL-12 or IL-12R, differentiation into IFN-γ-producing effector T cells is largely prevented (2–5).

The complement system is part of the innate immune response and can be activated through the classical, lectin, and alternative pathways (6). The key amplification step at the convergence of the three pathways is the production of C3/C5 convertases which initiate the formation of the membrane attack complex and subsequent pathogen destruction. Current dogma states that this process must be regulated on host cell surfaces to prevent self injury, and that this intrinsic regulation is mediated in part by decay accelerating factor (DAF); CD55 (7).

Through exploiting mice deficient in the human homolog of the cell surface complement regulator DAF (Daf1) (8), we previously showed that T cell immunity is stronger in the absence of DAF and that this enhanced response is dependent upon alternative pathway complement produced by the APCs. We further showed using wild-type (WT) animals, that alternative pathway complement proteins are up-regulated by T cells and APCs during their cognate interaction (9) and that cell surface expression of DAF is concurrently down-regulated, lifting restraint on local complement activation. Experiments in which added C5a increased the number of Ag-specific effector T cells in vitro and anti-C5 mAb decreased the number in vivo suggested that C5a could influence the strength of the induced T cell response (9). Consistent with this, published work by Sacks (10–14), Carroll (10, 15–17), and by our group (9) have shown that C3 or factor D deficiency limits T cell expansion following exposure to viruses (16, 18, 19), bacteria (20, 21), autoantigens (17), and alloantigens (10–14, 22).

Additional evidence implicating DAF (and complement) in guiding T cell differentiation comes from findings that APC DAF deficiency augments the frequency of IFN-γ-producing T cells (9, 23) while APC C3 deficiency diminishes IFN-γ production (11, 13, 14). It has also previously been found that APC IL-12, a cytokine known to direct T cell differentiation toward an IFN-γ-secreting phenotype, can be influenced by exogenous C5a, although conflicting effects (both up- and down-regulation) have been reported (24–26). Despite these various observations, the specific role of APC DAF in the T cell differentiation process has not been elucidated.

To clarify the interrelationship between DAF, complement, IL-12, and T cell differentiation into IFN-γ-producing effector cells, we exploited double knockout animals deficient in Daf1, in conjunction with C3, C5aR, IL-12, or IL-12R. The results reveal the novel concept that cell surface DAF regulates T cell differentiation in part through controlling local generation of C5a (derived from complement produced by the immune partners). In addition to providing mechanistic insight and explaining several conflicting observations, the findings have implications for therapy aimed at manipulating T cell immunity.
**Materials and Methods**

**Mice**

C57BL/6 (B6), Thy1.1, IL-12p40−/−, IL-12RB1−/−, B6.C3−/− mice (all H-2b), BALB/c (H-2b), and C3H (H-2k) mice were purchased from The Jackson Laboratory. Mice deficient in the dfaf gene (Daf1−/−) were produced as described (8) and backcrossed for >10 generations to B6. B6 mice do not reject Daf1−− skin (data not shown) confirming congenicity. MAR transgenic mice (CD4−, RAG2−/−, anti-HyDβ + I-A*) were a gift from P. Matzinger (National Institutes of Health, Bethesda, MD). C5aR−/− mice (H-2b) were obtained as a gift from C. Gerard (Boston Children’s Hospital, Boston, MA). In some cases, the mouse strains noted above were crossed in our mouse facility to create DAF1/C3−/−, Daf1/IL-12p40−/−, Daf1/C5aR−/−, and IL-12RB1−/− Mar TCR transgenic (all H-2k). All mice were housed in the Cleveland Clinic Biological Resources Unit in accordance with the guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care International.

**Abs and reagents**

Anti-mouse CD4 PE-Cy5, CD11b PerCP-Cy5.5, and IFN-γ PE Abs for flow cytometry were purchased from BD Pharmingen. Anti-IL-12 mAb (clone 17.8.20) was purchased from BioExpress. CFSE was purchased from Molecular Probes (Invitrogen Life Technologies). rIL-12 was purchased from R&D Systems. Molecules Probes (Invitrogen Life Technologies). rIL-12 was purchased from R&D Systems.

**Cell isolation, labeling, and culture**

T cells were isolated from spleen and lymph nodes of naive mice and isolated using a negative T cell selection kit (StemCell Technologies) according to manufacturer’s specification (in the presence of 1.5 μg/ml anti-CD44 (BD Pharmingen) to isolate naive cells) and purified to between 93 and 98% based on CD3 staining. Isolated cells were labeled with CFSE and used for subsequent experiments. In in vitro cultures, T cells were stimulated with peritoneal macrophages obtained by injecting 1 ml of thioglycolate and isolated 5 days later by peritoneal lavage. T cells were activated in 48-well plates in serum-free HL-1 medium (Invitrogen Life Technologies) supplemented with 1% L-glutamine and 1% penicillin/streptomycin in a 2:1 T cell/APC ratio in the presence of 0.1 μM HYDβ for a period of 24–120 h when cells were removed and prepared for flow cytometry. A total of 10 μg/ml anti-IL-12 mAb or control Ab was added at the initiation of selected cultures as indicated.

**In vivo MLR**

CFSE-labeled T cells (10 × 10^6 cells/mouse) were injected i.v. via the tail vein. Spleens from injected mice were harvested at 60 h and stained for intracellular IFN-γ.

**Bone marrow (BM) chimeras**

Male WT Thy1.2− and C5aR−/− mice were irradiated (1200 rad) and injected with 10 × 10^6 WT Thy1.1− or C5aR−/− BM cells and treated with gentamicin for 10 days and used as recipients for cell transfers >21 days after reconstitution. Degree of donor BM chimerism was verified by flow cytometry at the time of sacrifice.

**Flow cytometry**

Cells were stained for surface Ags by incubating with Abs in PBS, 0.1% BSA, and 0.01% sodium azide. For intracellular cytokine staining, cells were initially stained for surface markers then fixed with 4% paraformaldehyde then permeabilized with PBS, 0.1% BSA, 0.1% saponin, and stained with fluorochrome-labeled anti-cytokine Abs. Pilot studies revealed that CFSE staining did not alter IFN-γ production by CD4 TCR-transgenic T cells stimulated with their cognate Ag (data not shown). Samples were collected using either a FACScan or FACSCalibur (BD Biosciences) and analyzed using WinList software (Verity Software).

**ELISAs**

Culture supernatants were collected at the time points indicated and assayed for IFN-γ ELISPOT. IL-2, or IL-4 (BD Pharmingen) using commercially available kits as per the manufacturer’s instructions.

**Real-time PCR**

Cellular RNA was prepared using a Qiagen RNeasy Mini kit and reverse transcribed to cDNA using a High Capacity cdNA Archive kit (Applied Biosystems). Expression levels for mouse IL-12p40 and IL-12p35 was compared with mouse MPRL32 using TaqMan Gene Expression assays (Mm00434165_m1 and Mm00434170_m1 for IL-12p40 and IL-12p35 and Mm00777741_s1 for MPRL32; all obtained from Applied Biosystems) on an Applied Biosystems 7500 Fast system with TaqMan Fast Universal Master Mix.

**IFN-γ ELISPOT**

IFN-γ ELISPOT assays were performed as previously described (9). Briefly, MultiScreen ELISPOT plates (Millipore) were coated overnight with the capture Abs. Following a blocking step, spleen cells (0.2 to 1 × 10^6/well) were plated and incubated with stimulators/Ag at 37°C, 5% CO2 for 24 h. After washing, detection Abs were added overnight. After washing, alkaline phosphatase-conjugated anti-biotin Ab (Vector Laboratories) diluted 1:1000 in PBST was added for 2 h, the plates were developed, and the resulting spots were counted on an ImmunoSpot Series 1 Analyzer (Cellular Technology).

**C5a Western blot**

Mar T cells stimulated with WT, Daf1−−, C3−−, C5aR−/−, and IL-12p40−/− APCs in serum-free HL-1 medium. Supernatants (equal amounts from each culture well) were collected after 4 days of culture and concentrated 100-fold using Microcon YM-10 centrifugal concentrators (Millipore). Equal volumes of supernatants were loaded and separated by SDS-PAGE using 15% Tris-HCL gels (Bio-Rad) and blotted onto polyvinylidene difluoride membranes. Blots were probed with polyclonal anti-mouse C5a Ab (R&D Systems) and anti-goat HRP (Sigma-Aldrich) and visualized with Immuno-Star HRP substrate kit (Bio-Rad). rC5a (Cell Sciences) was used as a positive control.

**Statistical analysis**

To determine whether groups were statistically different, results were compared using the Student t test. A p value <0.05 was considered significant.

**Results**

**APC-expressed DAF regulates APC production of IL-12 through a complement-dependent mechanism**

To determine whether APC DAF expression influences IL-12 production during T cell/APC interactions, we cultured WT Marilyn-transgenic T cells (Mar, anti-HyDβ plus I-A^d) with Dby peptide-loaded peritoneal macrophages isolated from WT or Daf1−− mice. We used serum-free medium to eliminate exogenous complement. Importantly, extensive previous work by our laboratory and others has shown that this TCR-transgenic T cell behaves in a fashion representative of polyclonal T cells in vitro and in vivo, in multiple models and assay systems (27–33). Specific ELISA performed on 48 and 72 h supernatants revealed significantly more IL-12p70 in cultures containing Daf1−− APCs (Fig. 1A). No IL-12 was detected in control cultures in which we substituted OVA−/−, BM−/− mice (data not shown). Cultures containing Daf1/ C3−/− APCs contained IL-12p70 levels lower than those from WT cultures (Fig. 1A) indicating the augmentation of IL-12 observed with Daf1−− APCs is APC C3 dependent. Mixtures of MAr T cells with Dby-loaded C3−/− APCs produced significantly less IL-12 than WT controls. Similar effects of DAF and C3 on IL-12 production were noted in two different in vitro alloresponses (Fig. 1A). More IL-12p70 was detected in 72-h culture supernatants of BALB/c (H-2^d) or C3H (H-2^k) T cells mixed with B6 (H-2^d) Daf1−− (vs WT) APCs, and the effect was abrogated in the absence of C3. Assays of IL-12 mRNA by quantitative PCR showed that both IL-12 p35 and p40 RNA levels were up-regulated in cell mixtures containing Daf1−− compared with WT APCs (Fig. 1B) and that no up-regulation of either gene product occurred following stimulation with Daf1/C3−/− APCs (Fig. 1B).

**APC-expressed DAF influences T cell IFN-γ production**

Our previous findings that T cell/APC interactions induce up-regulation/release of C3 from both partners (9), that DAF deficiency strengthens T cell immunity (9), and that DAF influences IL-12...
production by APCs (Fig. 1) raised the hypothesis that these observations are mechanistically linked. To test this, we mixed Mar T cells with WT, Daf1/−/−, or C3/−/− APCs (in serum-free medium). We examined IFN-γ in the culture supernatants (72 h) and determined the number of IFN-γ-producing T cells by intracellular cytokine staining on day 3. Extending our previous work (9), more IFN-γ (Fig. 2A, left) and a higher frequency of IFN-γ-producing Mar cells (Fig. 2B) were detected in response to Daf1/−/− APCs. Consistent with reports by others (13), both the IFN-γ in culture supernatants and the percentage of IFN-γ-producing Mar T cells were diminished in response to C3/−/− APCs (Fig. 2A, A and B). Control experiments confirmed that IFN-γ was not generated in cultures containing OVA257−339 (data not shown).

T cells stimulated in the absence of APC DAF produced more IL-2 but less IL-4 (Fig. 2A, left) than those stimulated with WT APCs, consistent with a shift toward a type 1 cytokine phenotype. Conversely, T cells stimulated in the absence of APC C3 produced less IL-2 and more IL-4 (Fig. 2A, left). Polyclonal T cells responding to alloreactive APCs produced the same pattern of cytokines, more IFN-γ and IL-2 with less IL-4 following stimulation with Daf1/−/− APCs (Fig. 2A, right), and less IFN-γ/IL-2 with more IL-4 in the absence of C3.

To test whether the effects of DAF (and C3) on T cell IFN-γ production apply in vivo, we injected CFSE-labeled Mar T cells (10 × 10^6 cells/mouse i.v.) into WT, Daf1/−/−, or C3/−/− male hosts, isolated the spleens on day 3, and analyzed the percentage of Mar cells that were IFN-γ+ by flow cytometry (Fig. 2, C and D), and a lower percentage of Mar T cells produced IFN-γ after injection into C3−/− mice, when compared with injections into WT controls. DAF and C3 expression in the recipient influenced the total number of Mar cells in the spleen (2-fold more in the absence of Daf1 and ~30% less in the absence of C3, Fig. 2D), a result that in part reflects our previously documented effect of Daf1 and C3 on T cell proliferation/expansion (9).

Blockade/absence of IL-12 or IL-12R prevents T cell IFN-γ production regardless of APC DAF expression

Our above results suggested that the increased T cell differentiation into IFN-γ producers by Daf1/−/− APCs (see Fig. 2) might be dependent APC IL-12 production. To test this, we mixed Mar T cells with Daf1/−/− APCs ±25 μg/ml blocking anti-IL-12 Ab (clone 17.8.20 or control IgG) and on day 3 evaluated T cell IFN-γ production. The IL-12 neutralization lowered the total amount of IFN-γ in the culture supernatants (Fig. 3A) and diminished the percentage of proliferating Mar cells that produced IFN-γ (Fig. 3B, top). A corresponding effect (less IFN-γ production) was observed when Mar T cells were stimulated with APCs deficient in both DAF and IL-12 (Daf1/IL-12p40−/−, Fig. 3, A and B), thereby demonstrating through two independent approaches that the augmented IFN-γ detected in the absence of APC DAF required local production of IL-12. Controls revealed low-level baseline production of IFN-γ despite IL-12 deficiency, as reported previously (34). In additional control experiments, adding back 10 ng/ml rIL-12 to syngeneic T cells plus APCs (data not shown). *, p < 0.05 vs WT; †, p < 0.01 vs Daf1/−/−. B, Relative quantification of mRNA for IL-12p40 (top) and p35 (bottom) in whole culture lysates of Mar T cells stimulated with Dby peptide-loaded WT, Daf1/−/−, C3/−/−, and Daf1/C3/−/− peritoneal macrophage at 72 h. Data are normalized to WT APCs before stimulation. Results represent mean SE of at least two experiments for each group.

*, p < 0.05 vs WT; †, p < 0.01 vs Daf1/−/−.
cultures containing Daf1/IL-12p40−/− APCs increased secreted IFN-γ (Fig. 3A) and the percentage of IFN-γ+ T cells (Fig. 3B) to that detected in response to APCs deficient in Daf1 alone (Fig. 3, A and B), confirming the link between APC DAF/IL-12 and T cell differentiation into IFN-γ-producing effector cells. This control also shows that the diminished IFN-γ produced in the absence of APC Daf1 and IL-12 is due to the absence of IL-12 not to an unanticipated genetic abnormality related to the intercross.

As we published previously (9), Mar T cells underwent approximately one additional cell division over 3 days in response to Daf1−/− compared with WT APCs (2.5 vs 1.8 divisions; Fig. 3B, bottom panels, p < 0.05). However, the presence or absence of IL-12 had a minimal effect on this augmented proliferation (Fig. 3B, bottom). Together, the data suggest that the increased APC IL-12 produced in the absence of DAF regulates IFN-γ production independent of any effect on proliferation.

As another approach for evaluating the link between APC DAF, IL-12, and T cell IFN-γ production, we intercrossed IL-12RB1−/− mice with Mar mice to produce Mar tg/IL-12RB1−/− animals. CD4 T cells were purified from these mice (and from WT Mar controls) and tested for IFN-γ production following in vitro stimulation with WT or Daf1−/− APCs plus Dby peptide. A significantly lower percentage of IL-12R−/− Mar T cells produced IFN-γ compared with WT Mar cells in response to WT or Daf1−/− APCs (Fig. 3C), further demonstrating that Daf1−/− APCs augmented T cell IFN-γ production through IL-12. In accordance with the above IL-12 experiments, the absence of IL-12R had no detectable effect on proliferation following stimulation with Daf1−/− APCs (average number of cell divisions: WT Mar, 3.8 vs IL-12R−/− Mar, 3.9, not significant).

The interaction between APC DAF, APC IL-12, and T cell IL-12R was then tested in vivo by injecting CFSE-labeled WT or IL-12RB1−/− Mar T cells into male WT, Daf1−/−, IL-12−/−, and Daf1/IL-12−/− recipients (Fig. 3D). Injection of WT Mar cells into Daf1−/− recipients resulted in a significantly higher percentage of IFN-γ+ effectors compared with injections performed in WT controls. This augmentation was not detected following injection into Daf1/IL-12−/− recipients. A significantly lower percentage of Mar tg/IL-12RB1−/− T cells (compared with WT Mar T cells) produced IFN-γ in both WT and Daf1−/− hosts (Fig. 3D), consistent with our in vitro findings (Fig. 3C).

Exogenous IL-12 can overcome the decrease in IFN-γ production in response to C3−/− APCs

The data in Fig. 2 show that C3−/− APCs induce less T cell proliferation and, in addition, cause a lower percentage of the responding cells to produce IFN-γ. If the inability to produce IFN-γ in response to C3−/− APCs is due to decreased APC IL-12 (as

**FIGURE 2.** APC-expressed DAF regulates C3-dependent T cell cytokine production. *A. Left,* Cytokines detected in 72 h (IFN-γ) or 48 h (IL-2/IL-4) culture supernatants of Mar T cells stimulated with WT, Daf1−/−, or C3−/− peritoneal macrophages. *Right,* Cytokines detected in 72 h culture supernatants of BALB/c or C3H T cells stimulated with B6 WT, Daf1−/−, or C3−/− spleen cell APCs. *B,* IFN-γ production by CFSE-labeled Mar T cells stimulated with WT, Daf1−/−, or C3−/− peritoneal macrophage. Representative flow plots from 3-day cultures (*B*) are depicted. Numbers in upper left quadrant represent percent IFN-γ+ T cells undergoing more than one division. *C and D,* In vivo effects of DAF on T cell IFN-γ production. Percentages of IFN-γ+ Mar T cells in the spleens (*C*) and the total numbers of Mar T cells in the spleens (*D*) 60 h after injection into WT, Daf1−/−, or C3−/− male mice (10 × 10⁶ cells/mouse). Horizontal bars represent mean values for each group; n = 4/group. *p < 0.05 vs WT.
opposed to some alternative unanticipated effect), then addition of exogenous IL-12 to the culture should induce IFN-γ production. To formally test this, we cultured Mar T cells with WT or C3−/− APCs in the presence or absence of 10 ng/ml rIL-12 and assayed T cell intracellular IFN-γ production on day 3. As shown in Fig. 4, the IL-12 added to cultures with C3−/− APCs restored the percentage of IFN-γ+ T cells (Fig. 4A) and the amount of IFN-γ in the culture supernatants (Fig. 4B) to that found following stimulation with WT APCs. Despite equal percentages of IFN-γ+ T cells in cultures in C3−/− APCs plus IL-12 was significantly lower than found in WT cultures (data not shown, see Fig. 3D) because the IL-12 did not alter T cell proliferation (average number of cell divisions; WT APCs 2.0 divisions, C3−/− APCs 0.9 divisions, C3−/− APCs plus IL-12, 1.0 division). Overall, the data reveal that the C3-dependent effects on T cell differentiation (which we have shown are controlled in part by APC-DAF) function through APC IL-12 release, distinct from our previously published results (9).

**FIGURE 3.** DAF regulation of T cell IFN-γ production is dependent on APC-expressed IL-12 and T cell expressed IL-12R. A, IFN-γ detected in 72-h culture supernatants of Mar T cells mixed with WT, Daf1−/−, or Daf1/IL-12p40−/− APCs plus anti-IL-12 mAb (25 μg/ml blocking anti-IL-12 mAb) or rIL-12. *, p < 0.05 vs WT; †, p < 0.05 vs Daf1−/−. B, Representative flow plots of CFSE-labeled Mar T cells stimulated in vitro for 3 days with HYDhy-loaded WT, Daf1−/−, or Daf1/IL-12−/− peritoneal macrophages ±25 μg/ml blocking anti-IL-12 mAb or control Ab and stained for intracellular IFN-γ. Upper plots, Single-color histograms for IFN-γ (IFN-γ+ in each panel); lower panels, CFSE dilution of responding T cells (the average number of cell divisions is shown in each panel, n = 2–3/group). Representative of more than three individual experiments per condition are shown. C, Flow cytometry plots of CFSE-labeled WT or IL-12Rβ1−/− Mar T cells stimulated with WT or Daf1−/− APCs in vitro on day 3. Upper plots, Single-color histograms for IFN-γ; lower panels, CFSE dilution of responding T cells. Percentages of IFN-γ+ cells or average number of cell divisions for each plot are shown. Representative of more than three individual experiments are shown. D, Percentages of IFN-γ+ WT or IL-12Rβ1−/− Mar T cells in recipient spleens at 60 h after injection into male WT, Daf1−/−, IL-12p40−/−, or Daf1/IL-12p40−/− recipients. Data represent mean values for groups plus SD n = 4/group. *, p < 0.05 vs WT T cells; †, p < 0.05 vs WT APCs; ‡, p < 0.05 vs Daf1−/− APCs.

Because DAF accelerates decay of C5 convertases (7) that generate C5a and because it has been reported that C5a added to APCs in vitro can influence IL-12 production (25), we postulated that the DAF-dependent effects on T cell differentiation functioned in part through local C5a. Confirming and extending our previous work...
duced IL-12, consistent with the published literature (35–39). The tants. Exogenous C5a down-regulated the production of LPS-in-
of nanogram quantities of this cytokine into the culture superna-
duce any detectable IL-12, the addition of LPS resulted in release
of IL-12 noted by one group (25) but is contrary to the dose-depen-
treatment is also dependent on cognate T cell/APC interactions. We mixed
marrow-derived macrophages in the presence of increasing concentrations of
C5a (data not shown) confirming that the up-regulated C5a pro-
duction is also dependent on cognate T cell/APC interactions.
Based on these results, we next tested the hypothesis that APC
regulates IL-12 production during cognate T cell/APC interactions through a C5aR-dependent mechanism. We mixed
Mar T cells with male WT, Daf1/C5aR deficient APCs. The stimula-
tion of APC IL-12 is dependent on APC C5aR expression. We mixed
Mar T cells with C5aR deficient APCs and WT APCs and stimulated the su-
interpretation of our data is that APC DAF regulates IFN-
production induced by APCs at 48 and 72 h. Results represent mean plus SD of duplicate wells of two individual experi-
ments. *, p < 0.05 vs WT; †, p < 0.01 vs Daf1 alone. This result is consistent with C5a-induced up-regulation of IL-12 noted by one group (25) but is contrary to the dose-depen-
down-regulatory effects of C5a on LPS- or anti-CD40-induced IL-12 production as described by others (35–39). To resolve this discrepancy, we compared IL-12 production between LPS-pretreated and control untreated peritoneal macrophages (Fig. 5C). Although untreated macrophages did not produce any detectable IL-12, the addition of LPS resulted in release of nanogram quantities of this cytokine into the culture superna-
tants. Exogenous C5a down-regulated the production of LPS-in-
duced IL-12, consistent with the published literature (35–39). The

FIGURE 4. Diminished production of IFN-γ by T cells stimulated with C3−/− APCs is overcome by exogenous IL-12. A, Flow cytometry plots of CFSE-labeled WT Mar T cells stimulated with WT or C3−/− APCs for 3 days in the presence or absence of 10 ng/ml rIL-12 and stained for intracellular IFN-γ. Upper plots, Single-color histograms for IFN-γ; lower panels, CFSE dilution of responding T cells. Percentages of IFN-γ− cells or average number of cell divisions for each plot are shown. Representative of more than three individual experiments are shown. B, IFN-γ in 72-h culture supernatants of Mar T cells responding to WT or C3−/− APCs with or without 10 ng/ml rIL-12. Results are representative of two individual experiments. *, p < 0.05 vs WT.

FIGURE 5. C5a is produced by cognate interactions and promotes production of APC IL-12. A, Western blot of culture supernatants for C5a from cultures of Mar T cells with male WT, Daf1−/−, and C3−/− APCs. rC5a is shown as a positive control. B, IL-12p70 ELISA of culture supernatants taken from cultures of Mar T cells stimulated with WT, Daf1−/−, C5aR−/−, or Daf1/C5aR−/− APCs at 48 and 72 h. Results represent mean plus SD for each group. *, p < 0.05 vs WT; †, p < 0.01 vs Daf1 alone. C, IL-12p70 protein in supernatants of unstimulated (top) or LPS stimulated (bottom, 10 ng/ml LPS) WT APCs in the presence of increasing concentrations of rC5a as determined by ELISA. No IL-12p70 was detected after stimulation of C5aR−/− macrophages with the same concentrations of C5a (data not shown). Data represents mean plus SD for each group. *, p < 0.05 vs no C5a control.
FIGURE 6. APC-expressed C5aR is required for optimal effector T cell production of IFN-γ. A, IFN-γ detected in 72-h culture supernatants of WT Mar T cells (■) or IL-12Rβ2−/− Mar T cells (□) 3 days after stimulation with WT, Daf1−/−, C5aR−/−, or Daf1/C5aR−/− HYDby-loaded APCs in the presence or absence of 10 ng/ml rIL-12. Data represent mean plus SD for each group. *, p < 0.05 vs WT T cells; †, p < 0.01 vs Daf1−/− APCs. B, Percentages of IFN-γ-producing WT (■) or IL-12Rβ2−/− (□) Mar T cells on day 3 of cultures with each APC type as depicted in the panel. C, IFN-γ ELISPOTs produced by WT polyclonal C3H (H-2k) T cells stimulated in vitro with WT, Daf1−/−, C5aR−/−, or Daf1/C5aR−/− (H-2k) splenocytes. Data represent mean plus SD for each group. *, p < 0.05 vs WT; †, p < 0.01 vs Daf1−/−. D, Percentages of IFN-γ-producing WT (■) or IL-12Rβ2−/− (□) Mar T cells 60 h after injection into WT, Daf1−/−, C5aR−/−, or Daf1/C5aR−/− male recipients. Data represent mean values for groups plus SD; n = 4/group. *, p < 0.05 vs WT T cells.

FIGURE 7. BM-derived cell expression of C5aR is required for optimal T cell IFN-γ production in vivo. A, Representative flow cytometry plots of CFSE-labeled CD4+ Mar T cells of Mar T cells injected into WT (Thy1.1)→C5aR−/− (Thy1.2) or C5aR−/− (Thy1.2)→WT (Thy1.1) BM chimeric mice. Chimerism was determined to be >90% donor BM based on Thy1.1/Thy1.2 polymorphisms (inset). B, Percentages of injected IFN-γ+ Mar T cells 72 h after injection into WT→WT, C5aR−/−→C5aR−/−, WT→C5aR−/−, or C5aR−/−→WT BM chimeric mice. Data represent mean values for groups plus SD; n = 3/group. *, p < 0.05 vs WT→WT.

with Daf1/C5aR−/− APCs resulted in less IFN-γ in the culture supernatants (Fig. 6A, ■) and in a lower percentage of IFN-γ-producing T cells (Fig. 6B, ■) than stimulations with Daf1−/− or WT APCs. Control cultures with C5aR−/− macrophages behaved analogously the Daf1/C5aR−/−. The diminished IFN-γ induced by C5aR−/− APCs could be overcome by exogenous IL-12 (Fig. 6, A and B), but only if the responding T cells expressed IL-12R (compare with responses in IL-12R−/− T cells, Fig. 6, A and B, □). The requirement for APC C5aR was not restricted to monoclonal Mar-transgenic T cells as IFN-γ production by polyclonal allogeneic C3H T cells was also regulated by APC-expressed C5aR (Fig. 6C).

In vivo experiments confirmed that the increase in IFN-γ production by Mar T cells was dependent on APC-expressed C5aR as a lower percentage of cells injected into Daf1/C5aR−/− recipients developed into IFN-γ producers compared with those injected into Daf1−/− controls (Fig. 6D, ■). Again,
this augmentation was dependent upon T cell expressed IL-12Rβ1 as there were no differences in the percentage of IFN-γ producers among recipient groups given adoptive transfers of T cells deficient in the IL-12R (Fig. 6D, □).

In a final set of experiments, we formally examined whether the C5aR expression on BM-derived cells mediates T cell differentiation into IFN-γ-producing effectors in vivo by producing and testing responses in BM-chimeric mice. As shown in Fig. 7, the percentage of Mar T cells that differentiated into IFN-γ-producing effectors in male mice with C5aR expressed on BM-derived, but not parenchymal cells (WT BM → C5aR<sup>−/−</sup> chimeras) was similar to the percentage induce in WT mice and in WT → WT chimeric controls. In contrast, a significantly lower percentage of Mar T cells injected into male mice with C5aR expressed on parenchymal but not BM-derived cells (C5aR<sup>−/−</sup> BM → WT chimeras) differentiated into IFN-γ producers.

**Discussion**

These results expand upon our previous report that cognate T cell/APC interactions stimulate complement release by both partners and that the absence or physiological down-regulation of APC-expressed DAF permits complement activation (9). The current data delineate a novel role for DAF as a regulator of T cell differentiation into IFN-γ-producing effector T cells. Cognate interactions between Mar CD4<sup>+</sup> T cells and Daf1<sup>−/−</sup> APCs augment IL-12 (Fig. 1) and the absence of Daf1 and IL-12 or IL-12R (Fig. 3) prevents the increase in total IFN-γ production and in the number of IFN-γ<sup>+</sup> Mar cells. Together, these findings suggest that DAF can regulate T cell differentiation in this system through controlling APC IL-12 secretion which in turn stimulates more responding T cells to become IFN-γ-producing effectors.

Our results further demonstrate that more C5a is produced in the absence of APC DAF (Fig. 5A), the enhanced IL-12 production detected in absence of APC Daf1 is abrogated in the absence of APC Daf1 and C5aR (Fig. 5), and the enhanced induction of IFN-γ-producing Mar T cells by Daf1<sup>−/−</sup> APCs is prevented in the absence of APC C5aR (Fig. 6). Other work from our joint group using WT mice revealed that B7 and CD40 signaling up-regulates complement production by APCs and that the produced anaphylatoxins are required for APC production of IL-12 (40). Together, the findings suggest that one mechanism through which DAF regulates IL-12-induced T cell differentiation in this system is through controlling local production of C5a. Although we do not yet know in detail how C5a regulates IL-12, it is intriguing to note that C5aR signal transduction includes phosphorylation of p38 MAPK. p38 MAPK is a known intermediary in the CD40-induced up-regulation of IL-12 transcription (via NF-κB) (41–43), providing one potential mechanism.

The above-documented effect of DAF on locally produced C5a on IL-12 up-regulation does not preclude the contribution of other/additional physiological stimuli. DAF regulates the formation of C3a (via controlling C3 convertase activity) and APCs express C3aRs, raising the hypothesis that local production of C3a could also influence IL-12 production, although this has yet to be formally tested. Although it is now established that DAF interacts with the seven transmembrane spanning protein CD97 independent of its complement regulatory effects and this interaction can influence T cell responses (44, 45), blocking anti-CD97 had no effect on augmented T cell immunity induced by Daf1<sup>−/−</sup> APCs (9), limiting the likelihood that DAF/CD97 interactions account for our results. Overall, in conjunction with our previous publication (9), the current data are consistent with the interpretation that APC-expressed DAF regulates activation of locally produced complement (C5a), which in turn shifts the innate cytokine balance toward IL-12 production, thereby guiding T cell differentiation toward IFN-γ production.

Results from this monoclonal TCR-transgenic system may not be fully translatable to all polyclonal T cell immune responses, but the findings are largely consistent with observations reported by others. Polyclonal T cell responses induced in a different Daf1<sup>−/−</sup> mouse following immunizations in CFA also exhibited augmented IFN-γ and IL-2 production, although effects on IL-4 were background strain and potentially model dependent (23). An association between DAF down-regulation, autoimmune disease, and augmented IL-4 production has been reported, but mechanistic links were not explored (46). Mechanistic studies elucidating how DAF and complement regulate production of other cytokines, including type 2 cytokines (IL-4, IL-13) requires further study.

Our data provide a potential explanation for the reported link between C5a and clinical expression of immune-mediated diseases (24, 25, 47–49). The absence of C5a or its receptor exacerbates murine tuberculosis, a disease that requires IL-12-induced T cell IFN-γ for protective immunity (47). Analogously, murine allergic asthma is exacerbated in C5-deficient animals, a phenotype associated with diminished IL-12/IFN-γ (24, 25, 48). These observations, in the context of the data provided in this study, raise the intriguing possibility that complement produced during cognate T cell/APC interactions (C5a in particular) could regulate T cell cytokine profiles during effector differentiation. Whether previously reported factors known to influence T cell cytokine phenotype, including the specific costimulatory molecules expressed by the cognate interacting pair (50) and the avidity of the TCR:peptide/MHC interaction (51, 52), function through regulating local complement production is not known. This newly documented link between DAF, complement, and IL-12 also raises the intriguing speculation that the preferential development of type 1 immunity by C57BL/6 (among other strains) (53–55) and the type 2 immunity by BALB/c (among other strains) could be related to differences in DAF and/or complement polymorphisms (56, 57), although there are currently no published data to support this contention.

Our data help to clear up an apparent paradox in the literature that C5a mediates both up-regulation and down-regulation of IL-12 by APCs (25, 35–39). In side-by-side experiments, we showed that the different results could be attributed to significant differences in experimental design. C5a-induced down-regulation of IL-12 occurred if APCs were prestimulated in vitro with LPS (Fig. 5). This stimulus induced nanogram quantities of IL-12 which was partially abrogated by exogenous C5a. In contrast, log-fold lower levels of IL-12 were produced during more physiological cognate T cell/APC interactions and under these conditions C5a augmented IL-12 production. Understanding the molecular basis of the different responses to C5a under each condition, including the possibilities that C5aR expression and/or intracellular signaling pathways are differentially affected by pretreatment with LPS, will require additional study.

In summary, our results show that DAF, by controlling local complement activation which in turn functions to regulate APC IL-12 production, can function as a regulator of T cell differentiation. This newly described link between DAF, complement, and IL-12 provides a mechanism to account for previously reported findings that allogeneic T cells to produce less IFN-γ in response to C3<sup>−/−</sup> APCs (11, 13, 14) and provides
new insights into the factors that influence T cell immune responses in vivo. The results have important implications for the design of further therapies aimed at altering pathological T cell immune responses in a wide array of conditions.

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Disclosures

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