Inhibition of Allograft Inflammatory Factor-1 in Dendritic Cells Restrains CD4+ T Cell Effector Responses and Induces CD25+Foxp3+ T Regulatory Subsets

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Allograft inflammatory factor-1 (AIF1) is a cytoplasmic scaffold protein shown to influence immune responses in macrophages and microglial cells. The protein contains Ca2+ binding EF-hand and PDZ interaction domains important for mediating intracellular signaling complexes. This study now reports that AIF1 is expressed in CD11c+ dendritic cells (DC) and silencing of expression restrains induction of antigen-specific CD4+ T cell effector responses. AIF1 knockdown in murine DC resulted in impaired T cell proliferation and skewed polarization away from T helper type 1 and 17 fates. In turn, there was a parallel expansion of IL-10-producing and CD25+Foxp3+ T regulatory subsets. These studies are the first to demonstrate that AIF1 expression in DC serves as a potent governor of cognate T cell responses and presents a novel target for engineering tolerogenic DC-based immunotherapies.

Keywords: dendritic cells, T regulatory cells, tolerogenic, polarization, suppression

INTRODUCTION

Dendritic cells (DC) are professional antigen presenting cells that direct T cell activation, proliferation, and polarization (1, 2). In addition to directing immunity, DC also play prominent roles in modulating peripheral tolerance by inducing anergic states in responder T cells and/or directing fates toward T regulatory cell (Treg) states (3–5). The biological factors and mechanisms that govern direction toward immunity vs. tolerance by DC are not fully understood.

Allograft inflammatory factor-1 (AIF1), also known as ionized calcium-binding adapter molecule 1, is a 17 kD interferon gamma-inducible calcium-binding EF-hand protein (6, 7). The gene, situated in the major histocompatibility class III genomic region (8), has demonstrated diverse roles in both the nervous and immune systems (9–13). For immune cells, it is largely restricted to the monocyte and macrophage lineages. In particular, expression of AIF1 in macrophages has been shown

Abbreviations: AIF1, allograft inflammatory factor-1; DC, dendritic cell; mDC, mature dendritic cell; siRNA, small interfering RNA; siAIF1, AIF1 siRNA knockdown group; siControl, scrambled control siRNA knockdown group; Treg, T regulatory cell; WT, wild type.
to promote pro-inflammatory responses (7, 14–16). Similarly, expression in microglial cells (17), which are derived from the macrophage lineage (18), is important in modulation of synaptic activities and upregulated in response to nerve damage (19). Dysregulation of AIF1 expression has been largely associated with both neuroinflammatory- and autoimmune-related disorders (13, 20, 21). Although reports have shown the importance of AIF1 in macrophage and microglial lineages, no study has determined its role in DC.

In this study, AIF1 function in DC antigen presentation was studied using RNA interference approaches. Results reveal that AIF1 expression in DC supports direction of T cells toward immunity. Silencing of AIF1 in DC induced CD4+ T cells toward Tregs and away from IL-17- and IFN-γ-producing T helper cells. This is the first study to clearly define the presence of AIF1 in DC and its functional role in governing antigen-specific T helper cell responses. Taken together, this investigation provides evidence that AIF1 expression in DC is instrumental in promoting adaptive immune response and that its loss leads to tolerance.

**MATERIALS AND METHODS**

**Mice**

Mice were purchased from Jackson Laboratory and bred in-house. All animal procedures were approved by the Institutional Animal Care and Use Committee. Femurs and tibias were harvested from C57BL/6 [wild-type (WT)] mice between 8 and 14 weeks of age to generate bone marrow-derived DC. WT mice were also used as recipients for in vivo adoptive transfer experiments. Transgenic B6.Cg-Tg(TcraTcrb)425Cbn/J (OT-II) mice were used as a source of naive CD4+ T cells responsive to ovalbumin (OVA323–339).

**Generation of Bone Marrow-Derived DC and Small Interfering RNA (siRNA) Knockdown**

Bone marrow-derived DC were generated as described by a modified protocol of Inaba et al. (22). Briefly, bone marrow cells were cultured in IMDM (Thermo Fisher Scientific, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific), 2 mM L-glutamine (Thermo Fisher Scientific), 100 U/ml penicillin/streptomycin (Thermo Fisher Scientific), and 20 ng/ml GM-CSF for 8 days in culture. On day 6 (of the 8-day culture), cells were purified for a homogenous DC population using CD11c microbeads (Miltenyi Biotec, Auburn, CA, USA) for positive selection. AIF1 was knocked down using an ECM 830 (BTX, Holliston, MA, USA) square wave electroporator with 1 nmol (6.65 µg) of siRNA oligos in 4 mm gap cuvettes with the following settings: 310 V, 10 ms, 1 pulse. AIF1 siRNA (siAIF1) sequence used: 5’-GGCAAGAGAACUCCCAUCUU-3’ (Thermo Fisher Scientific, Grand Island, NY, USA). Scrambled siRNA (siControl) was used as controls (siControl): 5’-GGGCTCTACGAGCAGATTAA-3’. Additionally, studies used silencer pre-designed siRNA 73668 targeting AIF1 purchased from Thermo Fisher Scientific: 3’-GGUGAAAGUCACUGGAGUUG-5’. After electroporation of siRNA on day 6 in CD11c+sorted DC, cells were placed back into culture. On day 7, 24 h after siRNA transfection, DC were matured with 250 ng/ml of LPS (or other TLR agonists) for an additional 24 h. On day 8, these siRNA transfected mature DC were used to assess immunophenotype and prime naïve CD4+ OT-II T cells. For all in vivo studies, DC were adoptively transferred into mice 24 h after siRNA transfection to compensate for the trafficking time required to enter the draining lymph nodes and prime T cell responses.

**Isolation of CD4+ T Cells for In Vitro Stimulation and CFSE Proliferation Assays**

For isolation of naïve CD4+ T cells from OT-II mice, CD8+ cytotoxic T cells and MHC class II+ antigen presenting cells were depleted by negative selection from spleen and lymph nodes using primary antibodies to CD8 and MHC class II (BioLegend, San Diego, CA, USA) followed by secondary labeling with anti-rat IgG magnetic microbeads (Qiagen, Hilden, Germany). Cells were then depleted by passing through a magnetic column. The approach yielded 96 ± 2.1% purity of CD4+ T cells. These naïve CD4+ T cells were cultured with 1.0, 0.3, or 0.1 µg/ml of OVA peptide (ISQQVHAAHAEINEAGR)-323–339-pulsed siAIF1 or siControl LPS-matured DC at a ratio of 10:1, respectively. Peptides were purchased from AnaSpec (Fremont, CA, USA). Scrambled non-specific peptides served as controls for some experiments, with the following sequences: VAAGIAQAHESIREHAN and IENHQIAGAAERSAAVH.

OVA323–339-pulsed siAIF1 or siControl mature DC stimulated OT-II CD4+ T cells were harvested at the 24 h time point to evaluate early activation markers CD69, CD62L, and CD25; antibodies purchased from BioLegend. For proliferation assays, CD4+ T cells pre-labeled with 2.5 µM CFSE (Thermo Fisher Scientific) were cultured with OVA323–339-pulsed siAIF1 or siControl DC for 96 h. Cells were co-stained with antibodies to IL-2 (BioLegend) for in vitro suppression assays. For isolation of naïve CD4+ T cells responsive to OVA323–339, cells were expanded for 12–14 days by siAIF1 or siControl DC for CD25, Foxp3, and of primary antibodies to CD8 and MHC class II (BioLegend, San Diego, CA, USA) followed by secondary labeling with anti-rat IgG magnetic microbeads (Qiagen, Hilden, Germany). Cells were then depleted by passing through a magnetic column. The approach yielded 96 ± 2.1% purity of CD4+ T cells. These naïve CD4+ T cells were cultured with 1.0, 0.3, or 0.1 µg/ml of OVA peptide (ISQQVHAAHAEINEAGR)-323–339-pulsed siAIF1 or siControl LPS-matured DC at a ratio of 10:1, respectively. Peptides were purchased from AnaSpec (Fremont, CA, USA). Scrambled non-specific peptides served as controls for some experiments, with the following sequences: VAAGIAQAHESIREHAN and IENHQIAGAAERSAAVH.

**Treg In Vitro Suppression Assays**

OT-II T cells were expanded for 12–14 days by siAIF1 or siControl DC pulsed with OVA peptide. After expansion, these T cells were then labeled with Cell Tracker Violet dye (Thermo Fisher Scientific). These labeled cells were referred to as suppressors. Next, naïve CD4+ or CD8+ T cells were isolated from WT mice by negative depletion and confirmed CD25+ by flow cytometric cell analysis.
analyses. Briefly, the CD4⁺CD25⁻ or CD8⁺CD25⁻ T cells were labeled with 2.5 μM CFSE. These CFSE-labeled naïve T cells are the responders. The purpose of labeling with different dyes was to distinguish suppressor from the responder populations. The suppressor and responder T cells were then cultured together at a 3:1, 1:1, 1:3, and 1:10 ratio, respectively, prior to stimulation with anti-CD3/CD28-coated microbeads (Dynabeads; Thermo Fisher Scientific). Cells were incubated for 72–96 h prior to collection, staining, and analysis of responder T cell proliferation using a modified approach by Collison and Vignali (23).

**In Vivo Adoptive Transfer of DC and Assessment of T Cell Responses**

CD4⁺ T cells were isolated from OT-II mice and labeled with CFSE. Next, 5 x 10⁶ of these CFSE-labeled OT-II CD4⁺ T cells were intravenously injected into WT mice, as described by Moon et al. (24). Next, 2 x 10⁶ control (siControl) or AIF1 knockdown (siAIF1) DC pulsed with OVA 323–339 were subcutaneously (s.q.) injected once at 6 h or twice at 6 and 12 h after the transfer of the CFSE-labeled OT-II cells. For these studies, DC were treated with siRNA 24 h prior to adoptive transfer, with LPS stimulation at the 12 h time point. Finally, the DC were incubated with 5 μg/ml of OVA 323–339 peptide in the last 4 h prior to adoptive transfer. Subcutaneous injection sites were the scruff of the neck and/or the upper thigh-just above the hind leg. The draining lymph nodes nearest to the s.q. injection were harvested 3.5 or 7 days post-injection to assess CD4⁺ T cell proliferation and effector responses. In each experiment, the same lymph nodes were collected: axillary, brachial, inguinal, lumbar, and superficial cervical. The tissues were then dissociated into single suspension, washed, fixed, and stained for intracellular assessment of cytokines.

**Cytometric Bead Array Multiplex Analysis and ELISA**

After various timepoints of DC or DC-T cell co-culture incubation periods, cells were spun down and supernatants collected. Mouse Inflammation Kit Cytometric Bead Array (CBA; BD Biosciences) and Legendplex Mouse Th Cytokine Panel (BioLegend) were used to detect cytokine levels in the supernatant; CBA kit analyzed IL-12p70, IL-10, IL-6, TNFα, MCP-1, and Legendplex was used to analyze IL-17A, IL-4, and IFNγ. Median fluorescence intensity values were normalized to cytokine concentration based on internal standard controls following manufacturer recommended protocols.

**Cryosection, Staining, and Microscopy**

Lymph nodes and spleens from WT mice were harvested and immediately fixed in 3% paraformaldehyde in PBS overnight at 4°C. After fixation, tissues were placed in 10% sucrose for 1 h prior to cryosectioning at 25 and 50 μm. Next, sections were permeabilized and stained with CD11c-Alexa488 (BioLegend), rabbit monoclonal AIF1 (EPR16588 or EPR178847; Abcam, Cambridge, MA, USA), and DAPI. Secondary anti-rabbit Alexa-594 was used to detect AIF1. Sections were then imaged using a three-channel wide-field fluorescence microscope and analyzed by ImageJ.

**Western Blotting**

Cell lysates were prepared by incubating Nonidet P-40 cell lysis buffer (Amresco, Solon, OH, USA) with cells for 30 min before high-speed centrifugation. Lysates were collected and run in 15% gels using vertical gel electrophoresis. Protein content was transferred from gels to nitrocellulose blots using the Pierce Power Blotter (Thermo Fisher Scientific). GAPDH served as a loading control. After primary antibody staining, secondary antibodies conjugated to fluorochromes were used for visualizing protein bands on the Odyssey imaging system (LI-COR, Lincoln, NE, USA). Image Studio 5.2 software (LI-COR) was used to calculate relative fluorescence intensities.

**Flow Cytometry**

Cell surface staining was performed in PBS supplemented with EDTA and 2.5% FBS (FACS buffer). Single cell suspensions were washed with FACS buffer two to three times prior to staining with fluorochrome-tagged-antibodies. Cells were stained for 15 min at 4°C with 10 μl of a 10 μg/ml working concentration per 2 x 10⁶ cells. Cells were then washed and fixed for 20 min in 3% paraformaldehyde at 4°C. For intracellular staining, fixed cells were permeabilized with 0.2% saponin in PBS for 1 h. Next, primary antibodies were added and cells incubated for 1 h. For primary unconjugated antibodies, secondary-tagged fluorochrome-labeled antibodies were prepared. These secondary antibodies were diluted to 1:1,000–1:3,000 working concentrations and 10 μl were added per 2 x 10⁶ cells. Cells were allowed to incubate for 1 h or overnight followed by extensive washing. Samples were acquired using a BD FACSVersa flow cytometric analyzer. Datasets were analyzed using FlowJo v10 (TreeStar, Ashland, OR, USA). Respective isotype controls and/or fluorochrome-labeled isotype controls were used in all assays. Gating strategies were established based on respective isotype controls.

**Statistical Analysis**

GraphPad Prism v6.0 (GraphPad Software, La Jolla, CA, USA) was used to determine statistical significance. Student unpaired two-tailed t-test was used to evaluate the significance of two groups. One-way or two-way analysis of variance was used to evaluate the significance between the means of three or more independent groups. A p-value ≤0.05 was considered statistically significant; *<0.05, **<0.01, and ns, non-significant. Error bars for all figures indicate SEs.

**RESULTS**

**AIF1 Is Expressed in DC**

To assess expression of AIF1 in DC, both lymph nodes and spleens were harvested from WT mice and evaluated for co-expression with CD11c⁺ subsets. WT mice were injected with either PBS or an LPS and IFNγ cocktail for 24 h. Spleen and lymph node tissues
were then isolated, cryosectioned, and stained. Fluorescence microscopy revealed co-localization of AIF1 with CD11c+ cells in both lymph nodes and the spleen (Figures 1A, B). Next, total cells isolated from the lymph nodes or the spleen were analyzed by flow cytometry for expression of AIF1. Live cells were gated on MHC class II and evaluated for co-expression of CD11c and AIF1. Results revealed high expression of AIF1 in MHC class II+CD11c+ DC from both lymph nodes and the spleen (Figures 1C, D). Finally, LPS and IFNγ stimulation resulted in significant increase in AIF1 expression in these MHC class II+CD11c+ subsets.

Bone marrow-derived DC were generated and sorted to yield a pure population of CD11c+ subsets (Figure S1A in Supplementary Material). Transfection of siRNA oligonucleotides targeting AIF1 (siAIF1) was used to knockdown the gene expression in CD11c+ DC. Scrambled non-targeting siControl. Results yielded a consistent 70–75% knockdown of AIF1 from endogenous levels as measured by western blot analyses (Figure 2A). Flow cytometric analyses confirmed that siRNA targeted knockdown of AIF1 in the CD11c+ DC reduced expression from 60.6 down to 16.0% 48 h post-transfection (Figure 2B). Results also revealed that optimal siRNA knockdown of AIF1 occurred during the 48–72 h time point post-transfection and that sustained depression of AIF1 lasted up to 5 days in culture (Figure S1B in Supplementary Material). Knockdown using siRNA (both in-house and pre-design oligonucleotides) did not yield any major differences in viability in comparison with controls (Figure 2C). Phenotypic characterization of LPS-matured CD11c+ DC transfected with siAIF1 showed no significant differences in expression of CD80, CD86, CD83, CD40, 33D1, CD11b, MHC class I, MHC class II, and F4/80 compared with siControl treated (Figure 2D).

Furthermore, no significant differences were found in secretion of the pro-inflammatory cytokines TNFα, IL-6, IL-12p70, IFNγ, IL-10, and MCP-1 (Figure 2E). These observations were recapitulated using other TLR agonists (i.e., TLR3-Poly I:C, TLR5-Flagellin, TLR9-CpG; data not shown).

**Loss of AIF1 in DC Impairs Antigen-Specific T Cell Responses**

Antigen presentation capacity of DC was evaluated upon AIF1 knockdown. In these studies, siControl vs. siAIF1 LPS-matured DC pulsed with OVA_{323–339} peptide was cultured with naïve OT-II CD4+ T cells. These isolated T cells were confirmed to be naïve CD4+CD25− subsets (Figure S1C in Supplementary Material). After 24 h, impaired early activation responses were observed in cognate T cells primed by siAIF1 DC compared with siControl, as measured by reduced CD25 (63.4–36.3%) and CD69 (63.7–34.1%) expression and restrained down regulation of CD62L (32.4–52.3%) (Figure 3A). Supernatant harvested at 72 h of culture was also assessed. These studies found decreased levels of IL-2, TNFα, and IFNγ in the CD4+ T cells primed by AIF1 knockdown DC compared with siControl (Figure 3B); the no stimulation group contained T cells without the presence of OVA peptide-pulsed DC. Lastly, CFSE-labeled CD4+ T cell responders were primed by siControl or siAIF1 DC at varying concentrations of peptide (1.0, 0.3, and 0.1 μg/ml) and allowed to incubate for 96 h prior to measurement. Cells were then harvested and co-stained with antibodies targeting IL-2. The results revealed a significant reduction in proliferative capacity of the siAIF1 DC primed T cells compared with the siControl group (Figure 3C); non-stimulated CFSE-labeled T cells served as internal controls. There was a significant impairment in T cell proliferative capacity.
in the siAIF1 cohort, reducing proliferation by as much as 50% at 0.1 µg/ml of OVA peptide vs. siControl DC groups. T cell responses were found to be antigen dose dependent, where higher levels of peptide stimuli could override the phenotype observed in the siAIF1 DC stimulated groups. To confirm antigen-specificity, scrambled OVA peptide was employed as an additional internal control. No proliferation occurred in the scrambled OVA control from either siAIF1 or siControl DC stimulated groups (Figure S1D in Supplementary Material).

For evaluating polarization responses, T helper cell-associated cytokine expression was measured 12 days after initial stimulation by OVA-pulsed siAIF1 vs. siControl DC. These investigations revealed restrained IFNγ from 23.7 to 2.6% expression in siControl vs. siAIF1, respectively, and IL-17A from 6.6 to 2.2% (upon PMA/ionomycin re-stimulation in the presence of brefeldin A; Figures 4A,B). Additionally, in DC primed CD4+ T cells, there was a concomitant threefold increase in IL-10 production from 17.5 to 48.8% in siControl vs. siAIF1 DC primed CD4+ T cells, respectively (Figure 4B). The datasets are representative of three independent experiments. Intracellular cytokine flow analysis results were further validated using multiplex cytometric bead array assays. Cytokines were measured from supernatant collected 4 h after the PMA/ionomycin re-stimulation. IFNγ, IL-4, and IL-17A cytokine expressions were decreased in siAIF1 group compared with siControl, whereas IL-10 expression was markedly increased (Figure 4C). These findings suggest that AIF1 expression in DC plays an immunomodulatory role in promoting pro-inflammatory responses in T helper cells.
CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs Expended by AIIF1
Knockdown DC Are Functionally Suppressive

Due to increased IL-10 and decreased levels of IFNγ and IL-17A, the studies next measured Treg generation. siAIIF1 DC expanded OT-II CD4<sup>+</sup> T cells resulted in a threefold increase in CD25<sup>+</sup>Foxp3<sup>+</sup> subsets (7.35–22.2%) compared with siControl DC by day 14 (Figure 5A). Further evaluation found a consistent, albeit moderate, increase in CD27 and CTLA-4, as well as a decrease in CD44 in the siAIIF1 group compared with siControl (Figure 5B). This corroborated literature reports that Foxp3<sup>+</sup> cells are largely present within the CD27<sup>+</sup> population and that CTLA-4 is associated with attenuation of antigen presenting cells via B7 co-stimulatory engagement (25–27).

Subsequent experiments assessed whether these T cells expanded by siAIIF1 DC were functionally suppressive. Briefly, total OT-II CD4<sup>+</sup> T cells expanded from siControl or siAIIF1 DC were harvested, washed, and labeled with a violet marker; this group is termed the suppressors. These suppressors were then counted and cultured with newly isolated naïve WT CD4<sup>+</sup> or CD8<sup>+</sup> T cells labeled with CFSE at approximately a 3:1, 1:1, and 1:3 ratio; these CFSE-labeled naïve WT T cells are termed the responders (Figure 5C). This approach allowed distinguishing between the two populations to distinctly assess proliferation of each respective group. Upon anti-CD3/CD28 stimulation, both CFSE-labeled CD4<sup>+</sup> and CD8<sup>+</sup> T cell responders cultured with siAIIF1 expanded suppressors were restrained in proliferation after 72 h in culture (Figures 5D,E). In three independent experiments, results identified markedly reduced proliferation of responder T cells in the presence of siAIIF1 expanded suppressor T cells compared with that of the siControl group. This confirmed that the siAIIF1 expanded CD4<sup>+</sup> Tregs were functionally suppressive in their ability to restrain neighbor T cell expansion.

Adoptive Transfer of AIF1 Knockdown DC Restains T Effector Responses In Vivo

In vitro adoptive cell transfer studies were performed to corroborate in vitro results. Briefly, CFSE-labeled OT-II CD4<sup>+</sup>...
T cells were adoptively transferred into WT recipient mice. Approximately 10% of CFSE+ OT-II cells were identified in the lymph nodes 36 h after adoptive transfer into the WT recipients (Figure 6A). siAIF1 or siControl OVA<sub>323–339</sub>-pulsed LPS-matured DC were then injected subcutaneously either once (1×) at 6 h or twice (2×) at the 6 and 12 h time points after initial transfer of the CFSE-labeled OT-II CD4+ T cells into the WT recipients. After 5.5 days, the draining lymph nodes of recipient mice were then analyzed for proliferation and co-expression of IL-2 and IFNγ from the adoptively transferred CFSE-labeled OT-II T cells. For the mice cohort receiving adoptively transferred DC twice (at 6 and 12 h time points), results showed restrained T cell proliferation, as assessed by CFSE dilution assays, from 37.0% in the siControl to 12.6% in the siAIF1 transfected groups (Figure 6C). For the mice cohort receiving adoptively transferred DC once (at 6 h) after initial transfer of the CFSE-labeled OT-II cells, proliferation decreased from 28.0% for siControl to 10.4% for the siAIF1 cohort (Figure 6D). Adipotively transferred siAIF1 or siControl DC into the WT recipients without OVA peptide pulsing served as control (Figure 6C). These results describe a novel role of AIF1 in governing DC antigen presentation capacity for directing T cell proliferation and effector responses.

**FIGURE 4** | Restrained T cell cytokine production of IL-17A and IFNγ upon stimulation by allograft inflammatory factor-1 (AIF1) knockdown dendritic cells (DC). AIF1 knockdown or control LPS-matured CD11c+ DC were used to prime naive CD4+ T cells from OT-II mice for 12 days in culture. T cells were then stimulated with PMA/ionomycin in the presence of brefeldin A for 4 h prior to intracellular staining of CD4, IFNγ, IL-17A, and IL-10. (A,B) Results show dot plots of CD4 vs. IFNγ and IL-17A vs. IL-10 in siControl vs. siAIF1 DC primed OT-II CD4+ T cells. (C) Additionally, 4 h after PMA/ionomycin stimulation, supernatant was collected and assessed for IFNγ, IL-4, IL-17A, and IL-10 by cytometric bead array. Bar graph presents siControl as red (*) and siAIF1 as blue □. Data are representative of four independent experiments in three replicate wells. A p-value ≤0.05 was considered statistically significant; *<0.05, **<0.01, and ns, non-significant.

**DISCUSSION**

These studies demonstrate that AIF1 is well expressed in dendritic cell and involved in directing cognate T cell responses in an antigen-specific manner. AIF1 is expressed in the lymph nodes and the spleen, but not all cells expressing AIF1 co-localized with CD11c. This was not surprising, as others have shown AIF1 expression in macrophages, microglial cells, and monocytes (16, 28, 29). AIF1 expression in DC was further confirmed by flow cytometric analysis, whereby the approach assessed expression in MHC class II<sup>+</sup>CD11c<sup>+</sup> subsets. This, coupled with LPS and IFNγ stimulation, helped to identify that both steady state and inflammatory DC express AIF1. IFNγ increased expression of AIF1 in DC corroborated literature reports of similar induction in macrophages (15, 28). Finally, in vitro bone marrow-derived DC also expressed AIF1. Although an increase in AIF1 was observed upon DC maturation, this was directly proportional to other associated maturation markers (i.e., CD80, CD86, and CD40).

RNAi-mediated silencing of AIF1 in DC resulted in greater than 70% knockdown. Potential artifacts introduced into our system caused by offsite target effects were limited by using two different siRNA oligos. There were no significant changes in associated DC co-stimulatory markers or pro-inflammatory cytokine production between AIF1 knockdown and control
promoted increased IL-10 expression in responder T cells and expanded functionally suppressive CD25+Foxp3+ Tregs. During these investigations, evaluating CD25 and Foxp3 12–14 days after primary stimulation with siAIF1 or control DC ensured that expression was not a transient byproduct of initial T cell activation (31). Finally, the suppressive function of siAIF1 DC expanded CD25+Foxp3+ T cells were evaluated for ability to restrain neighboring T cells proliferative responses. Even with robust stimulation using anti-CD3/CD28 microbeads, the expanded T cells from the siAIF1 DC cohort retracted proliferation of naïve CD4+ or CD8+ T cells. These results corroborate literature reports in that AIF1 has a dominant role in pro-inflammatory-associated diseases (7, 32, 33).

For the in vivo experiments, subcutaneous injection of control and AIF1 knockdown DC each resulted in accumulation in the draining lymph nodes at comparatively equal numbers. This would suggest that AIF1 knockdown does not directly impair the migratory abilities upon adoptive transfer. However, within the lymph node compartment, adoptive transfer of antigen bearing AIF1 knockdown DC into WT recipient mice led to impaired cognate T cell proliferation, as well as restrained IL-2 and IFNγ co-expression, when compared with control DC. This corroborates in vitro data of impaired effector T cell responses. Taken together, these studies show that AIF1 expression in DC plays an important role in antigen presentation and T cell function.
Adoptive transfer of allograft inflammatory factor-1 (AIF1) knockdown dendritic cells (DC) restrains T cell proliferation and IFNγ production in vivo. OT-II CD4+ T cells were harvested from spleen and lymph nodes prior to labeling with CFSE. These OT-II CFSE-labeled CD4+ T cells were then intravenously injected into wild-type (WT) recipient mice. Next, OVA323–339-pulsed LPS-matured control (siControl) or AIF1 knockdown (siAIF1) DC were then subcutaneously injected once (1×) at 6 h or twice (2×) at 6 and 12 h after initial injection of the OT-II CFSE-labeled CD4+ T cells into the respective WT recipient mice. (A) Thirty-six hours after intravenous injection of OT-II labeled CD4+ T cells into WT recipients, tissues were harvested to assess frequency of CFSE+ cells. x-axis is FSC and y-axis is CFSE.

(B,D) After 5.5 days post OT-II CFSE-labeled CD4+ T cell followed by the siAIF1 or siControl DC adoptive transfers, mice were sacrificed and draining lymph nodes harvested. Cells were washed, fixed, permeabilized, and co-stained with antibodies to IL-2 and IFNγ prior to flow cytometric analysis. Histogram plots (top) show CFSE expression on x-axis as a measure of proliferation in the siControl vs. siAIF1 DC adoptive transfer groups. All gates defining the CFSE+ T cells were established using isotype and unstained controls. Dot plots show IFNγ (middle) and IL-2 (bottom) co-expression with CFSE. Bar graphs below show percent of proliferating subsets and statistical relevance. (C,E) No peptide-pulsed LPS-matured siControl or siAIF1 DC adoptively transferred with OT-II CFSE-labeled CD4+ T cells into the WT recipient mice served as internal controls. Data are representative of three independent experiments with three to four mice per group. A p-value ≤0.05 was considered statistically significant; *<0.05, **<0.01, and ns, non-significant.

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Supplementary Material

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autoimmune diseases. *Autoimmun Rev* (2016) 15:1071–80. doi:10.1016/j.autrev.2016.07.032

6. Zhao YY, Yan DJ, Chen ZW. Role of AIF-1 in the regulation of inflammatory activation and diverse disease processes. *Cell Immunol* (2013) 284:75–83. doi:10.1016/j.cellimm.2013.07.008

7. Utans U, Arcici RJ, Yamashita Y, Russell ME. Cloning and characterization of allograft inflammatory factor-1: a novel macrophage factor identified in rat cardiac allografts with chronic rejection. *J Clin Invest* (1995) 95:2954–62. doi:10.1172/JCI118003

8. Harney SM, Vilarino-Guell C, Adamopoulos IE, Sims AM, Lawrence RW, Cardon LR, et al. Fine mapping of the MHC class III region demonstrates association of AIF1 and rheumatoid arthritis. *Rheumatology* (Oxford) (2008) 47:1761–7. doi:10.1093/rheumatology/ken376

9. Broglio L, Erne B, Tolnay M, Schaeren-Wiemers N, Fuhr P, Steck AJ, et al. Allograft inflammatory factor-1: a pathogenetic factor for vasculitic neuropathy. *Muscle Nerve* (2008) 38:1272–9. doi:10.1002/mus.21033

10. Schluesener HJ, Seid K, Kretzschmar J, Meyermann R. Allograft inflammatory factor-1 in rat experimental autoimmune encephalomyelitis, neuritis, and uveitis: expression by activated macrophages and microglial cells. *Glia* (1998) 24:244–51. doi:10.1002/(SICI)1098-1136(199810)24:22<244::AID-GLIA9>3.0.CO;2-3

11. Chen ZW, Ahren B, Ostenson CG, Cintra A, Bergman T, Moller C, et al. Identification, isolation, and characterization of daintain (allograft inflammatory factor-1), a macrophage polypeptide with effects on insulin secretion and abundantly present in the pancreas of prediabetic BB rats. *Proc Natl Acad Sci USA* (1997) 94:13879–84. doi:10.1073/pnas.94.25.13879

12. Pashenkov M, Efendic S, Zhu J, Zou LP, Ostenson CG, Mustafa M. Augmented inflammatory factor-1 in rat experimental autoimmune encephalomyelitis, neuritis. *Immunity* (1994) 1:405–13. doi:10.1016/0897-6136(94)90071-X

13. Alkassab F, Gourh P, Tan FK, McNearney T, Fischbach M, Ahn C, et al. Imaging, isolation, and characterization of daintain (allograft inflammatory factor-1) expression in spleen, peripheral nerves and sera during experimental autoimmune neuritis. *J Biol Chem* (2002) 277:16202–10. doi:10.1074/jbc.M205001200

14. Sibinga NE, Feinberg MW, Yang H, Werner F, Jain MK. Macrophage-restricted and interferon gamma-inducible expression of the allograft inflammatory factor-1 gene requires Pu.1. *J Biol Chem* (2002) 277:16202–10. doi:10.1074/jbc.M205001200

15. Orsmark C, Skoog T, Jeskanen L, Kere J, Saarialho-Kere U. Expression of allograft inflammatory factor-1 in inflammatory skin disorders. *Acta Derm Venereol* (2007) 87:223–7. doi:10.2340/00015555-0225

16. Pawlik A, Kurzawski M, Drzdziejko V, Safranow K, Paczkowska E, Maśliński W, et al. Allograft inflammatory factor-1 gene polymorphisms in patients with rheumatoid arthritis. *Genet Test Mol Biomarkers* (2012) 16:341–5. doi:10.1089/gtmb.2011.0201

17. Inaba K, Inaba M, Romani N, Aya H, Deguchi M, Ikehara S, et al. Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. *J Exp Med* (1992) 176:1693–702. doi:10.1083/jem.176.6.1693

18. Collins LW, Vignali DA. In vitro Treg suppression assays. *Methods Mol Biol* (2011) 707:21–37. doi:10.1007/978-1-61779-974-6_2

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