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α-1 Antitrypsin Promotes Semimature, IL-10–Producing and Readily Migrating Tolerogenic Dendritic Cells

Eyal Ozeri, Mark Mizrahi, Galit Shahaf, and Eli C. Lewis

Tolerogenic IL-10–positive CCR7-positive dendritic cells (DC) promote T regulatory (Treg) cell differentiation upon CCR7-dependent migration to draining lymph nodes (DLN). Indeed, in human DC deficiencies, Treg levels are low. α-1 antitrypsin (AAT) has been shown to reduce inflammatory markers, promote a semimature LPS-induced DC phenotype, facilitate Treg expansion, and protect pancreatic islets from alloimmune and autoimmune responses in mice. However, the mechanism behind these activities of AAT is poorly understood. In this study, we examine interactions among DC, CD4⁺ T cells, and AAT in vitro and in vivo. IL-1β/IFN-γ–mediated DC maturation and effect on Treg development were examined using OT-II cells and human AAT (0.5 mg/ml). CCL19/21-dependent migration of isolated DC and resident islet DC was assessed, and CCR7 surface levels were examined. Migration toward DLN was evaluated by FITC skin painting, transgenic GFP skin tissue grafting, and footpad DC injection. AAT-treated stimulated DC displayed reduced MHC class II, CD40, CD86, and IL-6, but produced more IL-10 and maintained inducible CCR7. Upon exposure of CD4⁺ T cells to OVA-loaded AAT-treated DC, 2.7-fold more Foxp3⁺ Treg cells were obtained. AAT-treated cells displayed enhanced chemokine-dependent migration and low surface CD40. Under AAT treatment (60 mg/kg), DLN contained twice more fluorescence after FITC skin painting and twice more donor DC after footpad injection, whereas migrating DC expressed less CD40, MHC class II, and CD86. Intracellular DC IL-10 was 2-fold higher in the AAT group. Taken together, these results suggest that inducible functional CCR7 is maintained during AAT-mediated anti-inflammatory conditions. Further studies are required to elucidate the mechanism behind the favorable tolerogenic activities of AAT. The Journal of Immunology, 2012, 189: 146–153.
injected clinical-grade plasma-derived human AAT (30). In addition, AAT has recently been shown to promote IL-10–producing DC in three animal models for graft-versus-host disease (31) and to promote Treg cells in experimental autoimmune encephalomyelitis (32).

Most circulating AAT is synthesized during steady-state conditions by the liver, and its levels rise up to 6-fold during acute-phase responses. In addition to exerting anti-inflammatory effects, AAT is a potent inhibitor of multiple inflammatory and immune-activating serine proteases and exhibits tissue-protective properties (33). Nevertheless, the tolerogenic activities of AAT are still under investigation.

In the current study, we focus on the DC as one of the cellular targets of AAT that holds a tolerogenic potential. Specifically, we examine the ability of AAT to facilitate Treg-promoting, IL-10–producing, and readily migrating smDC.

Materials and Methods

Mice

Wild-type (WT) C57BL/6, BALB/c, and chicken β-actin promoter GFP transgenic mice (C57BL/6 background) were obtained from Harlan Laboratories. Foxp3-GFP knock-in mice that express GFP under the promoter for Foxp3 were provided by T. Strom (Harvard Institutes of Medicine, Boston, MA; transgenic animals described in Ref. 34), both the Institute of Science; mice originally generated by Barnden et al. (35), both Boston, MA; transgenic animals described in Ref. 34), and OVA-specific OT-II mice were provided by T. Strom (Weizmann Institute of Science; mice originally generated by Barnden et al. (35)), both C57BL/6 background. Male F1, from Foxp3-GFP/OT-II mice were used for OVA-responsive CD4+ T cell experiments (34). All animals were males between the ages of 7 and 10 wk. Experiments were approved by the Ben-Gurion University of the Negev Animal Care and Use Committee, and animals were bred and maintained in accordance with the guidelines of the Care and Use of Laboratory Animals published by the Israeli Ministry of Health.

Generation of DC

Splenic DC were enriched using magnetic bead enrichment kit (Miltenyi Biotec). Bone marrow-derived DC (BMDC) were generated, as described (36). Briefly, cells that were released by flushing of mouse femur and tibia with PBS were cultured (2 × 10^6 per well) in 10 ml RPMI 1640 containing 50 U/ml penicillin, 50 μg/ml streptomycin, 10% FBS (all Biological Industries), and 2-ME 50 μM (Sigma-Aldrich), and supplemented with rGM-CSF (10 ng/ml; ProSpec). On day 3, 10 ml medium containing 10 ng/ml GM-CSF was added, and half of the medium was replaced by medium containing 10 ng/ml GM-CSF. On day 9, >95% of the cells were CD11c+ according to FACS staining.

In vitro and in vivo DC maturation and migration experiments

According to each experimental setting, unless otherwise specified, BMDC and splenic DC were pretreated overnight with AAT (0.5 mg/ml; Aralast, Baxter, Westlake Village, CA) and then stimulated with murine rIFN-γ (5 U/ml; Sigma-Aldrich) and then stimulated with murine rIL-1β (1 ng/ml; Sigma-Aldrich). DC were then washed and stained with anti–IL-10 PE. Fluorescent intensity was measured using a LSR II or Canto II (each experiment was repeated using at least two different FACS instruments). Data were analyzed by FlowJo (version 7.6.3; Tree Star, Ashland, OR).

Footpad-injected DC. BMDC were injected into the footpad of recipient mice (2 × 10^6 cells per animal). Twenty-four hours prior to cell injection, footpads were preconditioned by IL-1β and IFN-γ (200 U/ml each), and AAT was administered systemically. Ingual lymph nodes were harvested 24 h later and analyzed by FACS. In related experiments, spleen was harvested on day 7 for evaluation of Treg population. In addition, AAT was loaded on BMDC as in the in vitro settings, and cells were introduced into footpads of OT II mice; spleen were harvested on day 7 for evaluation of Treg population.

Footpad-injected DC migration. Skin was removed from ventral sides of GFP transgenic donor mouse ears, as described (37), and then placed s.c. at the hypogastric abdominal area of recipient mice. Ingual lymph nodes were collected in separate experiments for RT-PCR and FACS analysis. In related experiments, skin grafting was performed into DTR CD11c+ mice with or without preconditioning using subthelial doses of diphtheria toxin (Sigma-Aldrich; 100 ng/injection), as described (38); spleens were harvested on day 7 for evaluation of Treg population.

Transwell migration assay. BMDC (5 × 10^5 cells per 5-μm Transwell inserts; Costar) were submerged in culture medium. After overnight incubation, cell migration to the bottom chamber was determined by an automatic cell counter (Countess; Invitrogen).

RT-PCR and cytokine analysis

Total RNA was extracted using PerfectPure RNA Tissue Kit (5 PRIME), and reverse transcription was followed was using Verso cDNA kit (Thermo Scientific). Mouse β-actin forward, 5′-GGTCTCAAACATGATCTGGG-3′ and reverse, 5′-GGTGTCAGAGGATGTCTTATG-3′ and reverse, 5′-TCCA-GAACATCAGGAACACCCAGC-3′ and reverse, 5′-CAGGTTACTGATGGTGCGATAC-3′, CCR7: forward, 5′-GAGAAGAGATGTCGGTCCAGC-3′ and reverse, 5′-GCCC-TCTTTCTGGATTTTCCAG-3′ and CCL19 forward, 5′-CCTTCCGCTACCTTTAATG-3′ and reverse, 5′-CTTCTTGCTTGGTTCCTTTG-3′. Equal amounts of total RNA were used to generate cDNA for all the samples. Prior to gene expression analysis, β-actin levels were compared between the samples and had displayed no significant differences. For semi-quantitative PCR, each sample was amplified by at least two different numbers of cycles to ensure that amplification was in the exponential phase of PCR. Gene expression profile was analyzed by densitometry using NIH ImageJ (http://rsbweb.nih.gov/ij/) and normalized to β-actin. Quantitative real-time PCR was performed to confirm results obtained in the latter technique (data not shown). Cell culture supernatants were analyzed for IL-1β, IL-2, IL-6, and IL-10 using Q-Plex mouse cytokine chemiluminescence-based ELISA on a Q-View imager (Quansys Biosciences, Logan, UT) and quantified using Quansys Q-View software (Quansys Biosciences).

FACS analysis

Analysis was performed using Cytomics FC 500 Beckman Coulter, BD Canto II, and BD FACScalibur (each experiment was repeated using at least two different FACS instruments). Data were analyzed by FlowJo (version 7.6.3; Tree Star, Ashland, OR). In vitro-derived DC migration required detection of rare populations and was analyzed using MACSQuant FACS analyzer (Miltenyi Biotec). After washing with FACS buffer (PBS containing 0.1% BSA, 0.1% sodium azide, and 2 μM EDTA) (10^6 per sample) were incubated with FcRII/III blocker (BioLegend) and then stained with the following Abs: anti-CD69 FITC, anti-MHCII PE, anti-CD39 PE, anti-CD11c allophycocyanin, and anti-Foxp3 allophycocyanin (from eBioscience, according to manufacturer’s guidelines); anti-CD4 Pacific Blue, anti-GITR PE, anti-CD3 FITC, anti-CD40 PE, and anti-CD25 allophycocyanin–Cy7 (from BioLegend, according to manufacturer’s guidelines); and anti-CD69 PE, anti-CD40 PE, and anti-CD25 allophycocyanin–Cy7 (from BioLegend, according to manufacturer’s guidelines). Intracellular IL-10 staining was performed according to BD CytoFLEX/ Cytopep Plus Fixation/Permeabilization Kit (BD GolgiStop). Briefly, stimulated cells were incubated with GolgiStop for 5 h, then harvested and stained for surface molecules, and washed twice with FACS staining buffer and resuspended in fixation/permeabilization solution. Cells were then washed and stained with anti-IL-10 PE. Fluorescent intensity <10 was defined negative, as obtained from unstained samples and isotype controls (CT) (data not shown). Fluorescent intensity in the range of 10^2–10^3 was defined high for CD40 and CD86 (CD86[high] and CD86[high]), and in the range of 10^3–10^4 was defined high for MHCII (MHCII[high]).
**Statistical analysis**

Results are expressed as mean ± SEM. Two-way ANOVA, followed by Student *t* test, was used to assess differences among groups. A *p* value <0.05 was considered significant.

**Results**

**AAT promotes tolerogenic smDC**

Although the appearance of LPS-induced smDC in the presence of AAT has been established (28, 31), we sought to examine the effect of AAT on DC stimulated by rIL-1β/IFN-γ (Fig. 1A–C). Splenic DC were incubated overnight in the presence of AAT (0.5 mg/ml), and then stimulated with IL-1β/IFN-γ. For FACS analysis, 24-h cultures were examined, and CD11c+ cells were identified to determine the surface levels of MHCII, CD40, and CD86. Fig. 1A depicts representative FACS images from each condition and analyte. As shown, stimulated cells displayed elevated levels of all three surface markers, whereas stimulated cells that were added AAT displayed reduced surface levels of all three markers to different extents; by setting the stimulated amplitude for each marker at 100%, we calculate that stimulation in the presence of AAT allowed MHCII to reach 72.9 ± 8.8% of stimulation levels (*p* = 0.048), CD40 16.6 ± 0.55% of stimulated levels (*p* = 0.052), and CD86 35.4 ± 2.05% of stimulated levels (*p* = 0.039). The kinetics of CD86 expression was examined by RT-PCR. As shown in Fig. 1B, CD86 transcript levels increased in stimulated cells by up to 1.14 ± 0.03-fold from CT in the absence or presence of AAT in the first 5 h after stimulation. However, CD86 transcript levels decreased to nonstimulated values at 9 and 24 h after stimulation in the presence of AAT.

Release of IL-6 and IL-10 was determined 72 h after stimulation (Fig. 1C). As shown, stimulated cells displayed an increase in IL-6 and IL-10, albeit without significant difference from CT. However, IL-10 was elevated in the presence of AAT. (Fig. 1D). Cytokine release at 24 h. Cultured DC (5 × 10^5 well in triplicates) were either untreated (CT) or cytokine stimulated, in the absence or presence of AAT. Representative dot plots; shown are percentage events out of CD11c+ in separate gates for high (upper gate) and low (bottom gate) expression levels of surface MHCII, CD40, and CD86. Representative experiment of five independent experiments. (B) RT-PCR. Gene expression kinetics for CD86, as analyzed in 5 × 10^5 cells/well in multiples of seven at 0, 5, 8, and 24 h after exposure to cytokine stimulation. Results are shown as fold from untreated (CT) cells. Mean ± SEM, *p* < 0.05 between conditions in each time point. (C) Cytokine release at 24 h. Supernatants cytokines release at 72 h after stimulation for IL-6 and IL-10. Mean ± SEM, *p* < 0.05, **p** < 0.01. (D) Supernatant cytokines 10^6/well in triplicates) were either untreated (CT) or cytokine stimulated, in the absence or presence of AAT. Representative dot plots; shown are percentage events out of CD11c+ in separate gates for high (upper gate) and low (bottom gate) expression levels of surface MHCII, CD40, and CD86. Representative experiment of five independent experiments. (E) Cytokine release at 24 h. Splenic bead-enriched CD11c+ cells (1 × 10^7 cells per round-bottom 96-plate wells in multiples of 12) were either untreated or preincubated with AAT, and then OVA Ag (10 μg/ml) was added overnight and washed. Splenic bead-enriched CD4+ T cells from OT II mice were added to the culture (4 × 10^5 cells/well) and allowed to interact with DC for 3 and 6 d. (D) Supernatant cytokines. IL-1β, IL-6, IL-2, and IL-10 were measured; day of supernatant collection is depicted. Mean ± SEM, *p* < 0.05, **p** < 0.01. (E) FACS for surface CD40. Left, Representative overlay of CD11c+ cells out of the coculture of DC and CD4+ T cells under the various treatment protocols; right, representative bar graph from three independent experiments. Mean ± SEM, **p** < 0.01. (F) FACS for surface CD4 and CD25, and depiction of Foxp3+ cells. Representative FACS contour images as obtained by collecting gated data from non-CD11c+ cells. Center, Cells positive for Foxp3 (using the transgenic Foxp3-driven GFP signal) out of CD4+CD25+ cells. Representative histogram and bar graph from pooled results (right). Representative experiment of five independent experiments. Mean ± SEM, **p** < 0.01.
in the presence of AAT, IL-6 release was decreased 2.5-fold from stimulated levels, and IL-10 release was increased 2.7-fold from stimulated levels.

To assess the ability of AAT-treated DC to promote Treg differentiation, a mixed culture of DC and T lymphocytes was studied (Fig. 1D–F). BMDC obtained from WT mice were cultured overnight in the presence of AAT. Cells were then incubated with synthetic OVA Ag for 24 h. Splenic Foxp3-GFP×OT-II OVA-reactive CD4+ T cells were washed distracted with DC. Supernatant content was examined on days 3 and 6 (Fig. 1D). As shown, the presence of OVA resulted in an increase in the release of IL-1β and IL-2, and, to a lesser extent, IL-6 (day 3), in accordance with reports on cytokine release by exposure to OVA (39). However, in the presence of AAT, IL-1β and IL-6 levels were significantly reduced from stimulated levels. Unexpectedly, IL-2 release was further increased by added AAT. Whereas IL-10 levels were below detection on day 3, day 6 supernatants contained greater levels of IL-10 in cocultures that contained AAT-pretreated (OVA-treated) DC and (OT-II OVA-reactive) CD4+ T cells.

According to FACS analysis (Fig. 1E), a significantly lower population size of CD40+ cells was observed in the presence of AAT, compared with OVA-treated cells. Also, as shown in Fig. 1F, in two representative FACS images (left), T cells that were cultured in the presence of OVA/AAT-pretreated DC exhibited reduced population proportions of CD4+CD25+ T cells (from 16.93 ± 1.12% to 8.8 ± 1.0% out of CD11c+ cells). The CD4+CD25+ cells, we determined the percentage of CD4+CD25+Foxp3+ cells (center, representative overlay; right, pooled results). The Foxp3 signal in these experiments is derived from transgenic GFP under Foxp3 promoter, which was confirmed in a separate transplant experiment to overlap with direct intracellular Foxp3 staining (data not shown). As illustrated in Fig. 1F, the proportion of Foxp3-expressing cells out of cells gated for CD4+CD25+ displayed a significant increase in the AAT-treated group (from 2.88 ± 0.53% to 7.9 ± 0.83%, respectively). In related in vivo experiments, we examined the changes in Treg population size using cultured BMDC that were preincubated with AAT and then added IL-1β/IFN-γ (Supplemental Fig. 1). As shown, once injected to animals, the population of CD3+CD4+CD8−Foxp3+CD39− cells in the spleen increased 1.72 ± 0.26-fold in the presence of AAT. Similarly, when OVA-loaded BDMC were introduced to OT II mice (Supplemental Fig. 2), CD3+CD4+CD25+GITR+ cell population size increased 2.71 ± 0.69-fold in the AAT-treated group. Furthermore, the requirement of DC for the process of AAT-induced Treg elaboration gained support by the use of DTR-CD11c mice; as shown in Supplemental Fig. 3, skin allograft recipient animals exhibited a larger Treg population size when treated systemically with AAT, whereas animals that were pretreated with DC-depleting doses of diphtheria toxin exhibited a lack of AAT-mediated increment in Treg population size.

AAT-treated DC readily migrate

Because DC typically express CCR7 and migrate under inflammatory conditions, the concern was raised that AAT-treated DC might fail to migrate. We therefore sought to examine whether the addition of AAT to DC allows for chemokine-directed migration in vitro and in vivo. Using an agarose-based migration assay (Fig. 2A), AAT-pretreated cytokine-stimulated BMDC were allowed to migrate overnight through an agarose gel prepared in RPMI 1640, on the surface of poly-L-lysine–coated plates, toward neighbor wells that contained rCCL19/CCL21. As shown, CT untreated cells demonstrated steady-state migration to an average distance of 187.3 μm. Cytokine-stimulated cells, however, migrated to a distance 1.18-fold greater than CT. Unexpectedly, in the presence of AAT, cells migrated to a distance 1.977-fold greater than CT.

In light of the ability of AAT to promote tolerance in islet transplantation models and in the NOD mouse, islet-resident DC were next examined (Fig. 2B). The phenomenon of APC emigration from cultured islets has been described (40). As shown in representative images, a migrating mass of cells can be observed extending away from cultured primary pancreatic islets (representative micrographs and pooled automatic cell-counter results), with a clear increase in cell migration upon added cytokine stimulation. However, in the presence of AAT, a 2.8-fold further increase in the number of emigrating cells was observed. Cell count was consistent with FACS analysis results, which depict

**FIGURE 2.** DC migration in vitro. (A) Agarose migration assay. BMDC (1 × 106 per well) were treated with AAT (0.5 mg/ml) and then stimulated overnight with IL-1β/IFN-γ (5 ng/ml each). CCL19/CCL21 (30 ng/ml each) added in the well next to BMDC. Migration distance was determined after 24 h. Data pooled from five independent experiments. Mean ± SEM, **p < 0.01. (B) Primary islet resident cell migration assay. Freshly isolated pancreatic islets (50 per well in multiples of six) were treated with AAT (0.5 mg/ml) and then stimulated overnight with IL-1β/IFN-γ (5 ng/ml each). Wells were added to rCCL19/CCL21 (30 ng/ml each), and islet resident cells were allowed to migrate on the surface of the wells for 24 h. Photomicrographs of representative fields containing 1–3 islets (original magnification ×40, unstained). Arrows, adherent and migrating cells. Right, Automatic cell counter results obtained from scraped adherent cells. The cells were then examined by FACS analysis for the surface markers. (C) CD11c (representative overlay and pooled results) and (D) CD40 (representative dot plot). Insets, Percentage of high and low CD40 expression. Representative experiment of four independent experiments. Mean ± SEM, *p < 0.05. CT, Untreated cells.

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changes in CD11c+ cell population size (Fig. 2C, representative overlay on the left and a graph of pooled results on the right).

Being that the migrating CD11c+ cells are considered a rare population in islets, FACS analysis required MACSQuant FACS analyzer (Miltenyi Biotec), which further allowed the identification of CD40 on the surface of the minute population of migrating CD11c+ cells (Fig. 2D). As shown, in stimulated cells the population of CD40+CD11c+ migrating cells was elevated 7.76 ± 3.4-fold from CT, but only 1.43 ± 0.62-fold in the presence of AAT (representative dot plots). Of note, the FACS analysis incorporates all adherent cells in the plate and thus includes also nonmigrating adherent cells.

These in vitro findings prompted us to examine whether the observed trend in enhanced smDC migration by AAT is reproducible in vivo. FITC skin painting model allows evaluation of DC migration from skin to DLN by analysis of carryover fluorescence. The population of cells in this model found in the DLN has been shown to be CD11c+ (3, 41). As shown in Fig. 3A, fluorescence in DLN increased 2.1 ± 0.3-fold in AAT-treated mice compared with untreated mice (CT, p = 0.03). DC footpad injection and skin tissue grafting were next performed (Fig. 3B, C–E, respectively). BMDC from GFP transgenic mice were added overnight and then stimulated by IL-1β/IFN-γ (Fig. 3B). Twenty-four hours after cytokine stimulation, 2 × 10^6 cells were injected into the rear footpads of WT mice. As shown, after 24 h from cell injection, DLN contained 10.59 ± 3.4% donor-derived CD11c+GFP+ cells out of total CD11c+ cells in mice that received untreated cells (CT), and 26.88 ± 1.22% in mice that received cytokine-stimulated cells. DLN from mice that were injected with AAT-treated stimulated cells contained 54.11 ± 1.3% CD11c+GFP+ cells out of total CD11c+ cells, representing a 2 ± 0.12-fold increase in the proportion of donor-derived DC.

In the tissue-grafting model (Fig. 3C), recipient mice were divided into untreated and systemically pretreated AAT mice. Skin tissue from GFP transgenic mice was grafted to recipient animals, and 16 h later inguinal lymph nodes were collected and analyzed by FACS for CD11c+GFP+ donor-derived cells. The proportion of graft-derived CD11c+GFP+ cells that migrated to DLN was 2.28-fold greater on average in the AAT-treated recipient mice (n = 9) compared with untreated graft recipients (n = 7) (p = 0.0003). Moreover, as shown in Fig. 3D, surface levels of MHCII, CD40, and CD86 on donor-derived GFP+CD11c+ cells were consistently lower in the AAT-treated group (2.64 ± 0.11-, 2.12 ± 0.07-, and 4.16 ± 0.16-fold reduction in markers, respectively).

Unlike the downregulation of maturation markers observed in migrating donor cells in the AAT group, intracellular DC IL-10 exhibited a positive response to AAT. As shown in Fig. 3E, in the AAT-treated group IL-10 levels increased 2 ± 0.07-fold in the total population of CD11c+ cells (left), compared with untreated graft recipients (center, pooled results), and were similarly elevated in migrating donor-derived GFP+ cells (Fig. 3E, right).

Because inducible CCR7 expression appears to require inflammation to promote DC migration, we sought to examine whether the anti-inflammatory environment exerted by AAT allows for CCR7 expression and for CCR7-dependent migration. BMDC were cultured in the presence of AAT 24 h before stimulation with IL-1β/...
IFN-γ. Cells were then analyzed for CCR7 expression by RT-PCR after 4 h and by FACS after 12 and 24 h (Fig. 4A, 4B). As shown, relative CCR7 mRNA transcript levels displayed a positive response upon inflammatory stimulation, and instead of being reduced by AAT, appeared to have maintained their inducible levels. Greater changes were observed in CCR7 surface levels (Fig. 4B); AAT-treated cells exhibited a 3.7-fold increase in CCR7 levels at 12 h compared with CT. At 24 h, cytokine stimulation resulted in a 5.3-fold rise in CCR7 surface levels, and a consistent rise in AAT-treated cells (5.5-fold greater than CT). Thus, surface CCR7 is upregulated earlier in DC that are inflamed and exposed to AAT.

Surface CCR7 was also evaluated in CD11c⁺ cells that emigrated from cultured islets (Fig. 4C, extended readouts from the experiment described in Fig. 2B). As shown, CCR7 surface levels were consistently greater in resident CD11c⁺ cells that emigrated from islets in the presence of AAT. Of note, unlike in Fig. 2C, the FACS analysis in this study incorporates only cells that migrated through the pores of the Transwell and are thus predominantly CCR7 positive.

To assess whether inflammation-triggered lymph node-derived ligands for CCR7 are consistent with an active CCR7 pathway in the presence of AAT in vivo, the expression of CCL19 was examined in DLN from tissue-grafted mice (Fig. 4D). As shown, CCL19 exhibited an increase in DLN transcript levels after tissue transplantation, and maintained values in the AAT-treated group that are comparable to the stimulated group. CCL21 did not respond to transplantation at this time point and was unchanged in the presence of AAT (data not shown); these constitutive expression levels are consistent with reports regarding posttranscriptional proteolytic regulation of CCL21 (6).

The functional role of CCR7 in the migratory effects of AAT on DC was examined using neutralizing anti-CCR7 Ab in a Transwell migration assay (Fig. 4E). As shown, cells readily migrated to the bottom compartment in stimulated conditions, and also in stimulated wells that were added AAT. However, when CCR7 was blocked by neutralizing Ab, there was a significant reduction in DC migration in stimulated AAT-treated wells, indicating that AAT drives DC migration in a CCR7-dependent manner.

**Discussion**

Tolerogenic DC and CD4⁺CD25⁺Foxp3⁺ Treg cells hold the capacity to influence alloageneic and autoimmune immune responses (17). Typically, DC that are tolerogenic exhibit reduced surface levels of inducible CD40, CD86, and MHCII; release less inflammatory cytokines; and secrete more IL-10 (11), thus termed semimature. In a previous report of ours, we demonstrated that the anti-inflammatory acute-phase circulating protein, AAT, promotes islet allograft tolerance, as well as smDC (28). Consistent outcomes were generated in the NOD mouse (29), in experimental autoimmune encephalomyelitis (32), and in three animal models for graft-versus-host disease (31). Uniformly, treatment with human AAT results in an elevated population size of CD4⁺CD25⁺Foxp3⁺ Treg cells in the lymph nodes and also at local antigenic sites. Importantly, other groups as well as ours have established that AAT does not interfere with IL-2–mediated T cell responses, thus presumably allowing Treg expansion (28, 29).

In the current study, we revisited the capacity of AAT to generate smDC by extending the stimuli to include IL-1β and IFN-γ. Consistent with our previous report, we observed favorable changes in cell surface markers, less proinflammatory cytokine release, and more IL-10 production. When cocultured with T cells, smDC increased the proportion of Treg cells. It was interesting to observe that IL-2 was increased in mixed cultures of AAT-pretreated DC and T cells. At this point in the study, at least two major questions remained to be addressed, as follows: 1) can these smDC migrate toward lymph nodes, and 2) do they convert T cells into Treg cells?

As shown, AAT allowed in vivo migration of smDC, resulting in the accumulation of smDC in DLN in multiple experimental setups. AAT promoted DC migration in an agarose plate toward a chemokine gradient, and also away from stimulated cultured islets. AAT facilitated DC migration in skin FITC-painting model and in

**FIGURE 4.** smDC CCR7 expression and CCR7-dependent migration. (A and B) In vitro CCR7 expression. BMDC (2 × 10⁶ in multiples of six) were pretreated with AAT (0.5 mg/ml) and then stimulated with IL-1β/IFN-γ for 4, 12, and 24 h (5 ng/ml each). (A) RT-PCR for CCR7 at 4 h, normalized to β-actin, shown as fold-change from CT. Mean ± SEM, representative experiment of two independent repeats, *p < 0.05. (B) FACS analysis for CCR7 surface levels at 12 and 24 h. Representative experiment of 10 independent repeats. Mean ± SEM, *p < 0.05. (C) Islet-derived DC. Islets were incubated as in Fig. 2 and then analyzed by FACS for CD11c⁺CCR7⁺ migrating cells (representative overlay and pooled results). (D) In vivo expression of CCL19. Mice were treated with AAT and then grafted with allogeneic skin (n = 6 per group). Four hours later, inguinal DLN was analyzed by RT-PCR for CCL19 and normalized to β-actin. **p < 0.01. (E) CCR7-dependent AAT-facilitated Transwell migration. BMDC (0.5 × 10⁶) were cultured on a Transwell insert in wells containing CCL19/CCL21 (30 ng/ml each) with or without neutralizing anti-CCR7 Ab. Cells were pretreated with AAT (0.5 mg/ml) and then stimulated with IL-1β/IFN-γ (5 ng/ml each). Migrating cells were determined in the bottom compartment after 24 h by automatic cell counter. Mean ± SEM. CT, Untreated cells; Tx, grafted mice.
the injected AAT-treated stimulated BMDC model. We further demonstrated that the migrating smDC produce more IL-10 by intracellular staining. In related experiments that are not shown in this work, smDC generated by AAT were repeatedly introduced into allogeneic mice (2 \times 10^6 cells/mouse/day i.v., days 0, 3, 5, 9, and 12, n = 3 per condition) in the absence of systemic AAT therapy, causing a 1.2 ± 0.91-fold rise in splenic CD4^+CD25^+ Foxp3^+ cells on day 15. Similarly, a rise in CD39^+ Treg cells was observed on day 7 postinjection of AAT-pretreated BMDC, and a consistent rise in GITR^+ Treg cells was achieved by injection of AAT-pretreated OVA-loaded DC into OT II mice (data not shown). These findings are consistent with a study reported by Fu et al. (17), regarding the effects of footpad-injected smDC on Treg cells. Importantly, being a pretreatment ex vivo protocol and not a systemic AAT therapy protocol, this setup focuses on the DC as a cellular target of AAT.

The rise in Treg cells by preconditioned DC also suggests that donor DC may communicate a tolerogenic phenotype to host cells, as proposed by others (42). Indeed, we show that DLN display both greater proportions of IL-10-containing donor-derived CD11c^+ cells, as well as greater proportions of IL-10-containing total lymph CD11c^+ cells.

CCR7-dependent DC migration is the primary means for inflamed, Ag-loaded mDC to relocate from the periphery to DLN (6). Indeed, the expression of CCR7 in DC is upregulated during inflammation, coinciding with concomitant DC maturation. Thus, one would suspect that during diminished inflammatory conditions, such that result in smDC, the levels of CCR7 expressed by DC might diminish accordingly. In this study, we present results from multiple migration models that establish that AAT-induced smDC maintain surface CCR7 levels comparable to those of mDC. In fact, in the presence of AAT, CCR7 appeared to rise on the surface of smDC earlier than in mDC and persists at least for 24 h. The levels of surface CCR7 are therefore potentially independent of the effect of AAT on the anti-inflammatory phenotype of DC.

Because CCR7 is stored in vesicles prior to its cell surface display (43), it was not unexpected to find that the changes in CCR7 mRNA transcript levels were minor in comparison with changes in surface CCR7 levels. The fact that AAT is an anti-protease is tempting to associate with extended half-life of CCR7, and changes in maturation markers on activated DC shown in this study, is a novel finding. The possibility that DC viability is enhanced by AAT, particularly in culture studies, was raised in light of its antiapoptotic attributes (47, 48). However, we found no evidence for increased DC viability in the presence of AAT according to live-dead cell staining and trypan blue exclusion (data not shown). In addition, because neutralizing anti-CCR7 Ab blocked AAT-related DC migration, it appears that the large cell numbers at the end of migration assays are indeed CCR7 dependent and not affected by enhanced viability. It would be of interest to investigate whether CCR7^−/− mice that have been shown to fail to evolve Foxp3^+ Treg cells (9) can achieve Foxp3^+ Treg expansion under systemic AAT treatment.

Our findings support the notion that AAT affects both host and donor CD11c^+ cells in a manner that achieves semimature migrating tolerogenic cells. However, further studies are required to elucidate the potential use of AAT for ex vivo graft preconditioning, or, conversely, for manipulating host DC toward immune adaptation.

A diverse variety of agents has been shown to promote smDC, most of which inhibit nuclear translocation of members of the NF-κB family of transcription factors (reviewed in Ref. 49). With this regard, the reports by Churg et al. (50) and Tawara et al. (31), in which AAT reduces NF-κB translocation to the nucleus, provide a possible mechanism for the elaboration of smDC by AAT. In addition, IL-1β has been shown to break Treg-mediated tolerance (51); because AAT induces the endogenous antagonist for IL-1β, IL-1R antagonist (52), and also reduces IL-1β and IL-6 levels (53, 54), it appears that AAT promotes a tolerogenic profile of cytokines. Whether the mechanism behind these changes is restricted to the ability of AAT to inhibit proteases or whether it is serine protease independent, is a topic of recent interest (33, 48, 55, 56).

In conclusion, the current study describes a particular aspect of AAT-mediated immune tolerance, namely, the DC as a cellular target of immune modulation. In addition, we describe the unexpected observation that inducible surface CCR7 is maintained during AAT-mediated anti-inflammatory conditions, and that modulated DC migrate despite the lack of an inflammatory cytokine profile. Consistent with the availability of AAT as augmentation therapy for individuals with genetic AAT deficiency, its favorable safety profile (57), and its islet-protective attributes in the context of unwarranted immune responses (28, 29), the rapid testing of weekly administration of i.v. human AAT for youngsters with recently diagnosed autoimmune type 1 diabetes is currently investigated in three clinical trials (National Institutes of Health clinical trial registry codes NCT01304537, NCT01319331, and NCT01183468). Nevertheless, further studies are required to fully elucidate the mechanism behind the favorable tolerogenic activities of AAT.

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**Disclosures**

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