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Purpose. To analyze the effect of a resolvin D1 (RvD1) analogue (RvD1a) on dendritic cell maturation, T-cell sensitization, and allograft rejection in corneal allotransplantation.

Methods. The receptor expression of RvD1 (ALX/FPR2) on bone marrow–derived dendritic cells (BMDC) was measured using quantitative real-time PCR. We determined BMDC maturation after treatment with RvD1a using ELISA to measure interleukin (IL)-12 protein expression and flow cytometry to assess the expression of CD40, major histocompatibility complex (MHC) II, CD80, and CD86. After corneal transplantation in BALB/c mice, we analyzed T-cell infiltration in the cornea and the draining lymph nodes using flow cytometry. The enzyme-linked immunospot (ELISPOT) assay was used to measure T-cell sensitization via the direct and indirect pathway. Angiogenesis and lymphangiogenesis in the cornea after transplantation were measured using immunohistochemistry. Graft opacity and survival were evaluated by slit lamp biomicroscopy.

Results. The receptor for RvD1, lipoxin A4/formyl peptide receptor 2 (ALX/FPR2), was expressed at a significantly lower level on immature than mature dendritic cells (DCs), and RvD1a reduced DC expression of MHC II, CD40, and IL-12 following lipopolysaccharide (LPS) stimulation. Using a murine model of corneal transplantation, RvD1a-treated hosts exhibited significantly reduced allosensitization as demonstrated by decreased frequencies of interferon-gamma–secreting T cells in the draining lymph nodes, and reduced T-cell infiltration into the grafts. Graft survival was significantly enhanced and angiogenesis at the graft site was suppressed in RvD1a-treated hosts compared with vehicle-treated hosts.

Conclusions. These results suggest that RvD1 inhibits DC maturation and reduces alloimmune sensitization following transplantation, thereby establishing a novel connection between resolvin D1 and the regulation of DC-mediated, antigen-specific immunity.

Keywords: transplant rejection, resolvin, dendritic cell

Resolvins are lipid mediators that are biosynthesized from ω-3 polyunsaturated fatty acids.[1] The biosynthesis of resolvins plays an important role in resolution of acute inflammation.[2] Resolvin D1 (RvD1, 7*S*,8*R*,17*S*-trihydroxy-4*Z*,9*E*,11*E*,13*Z*,15*E*,19*Z*-docosahexaenoic acid) is produced via the transcellular biosynthesis of docosahexaenoic acid [DHA] by leukocytes, endothelial and epithelial cells,[3,4] macrophages,[5] and lymphoid tissues.[6] Resolvin D1 mediates potent anti-inflammatory actions and reduces the duration and magnitude of inflammation in complex immune-mediated diseases.[7–12] Earlier, we demonstrated that RvD1 significantly suppresses innate immune responses, including polymorphic neutrophil (PMN) infiltration and interleukin (IL)-1 secretion following acute corneal inflammation.[13] Resolvin D1 has also been shown to limit PMN infiltration in murine peritonitis, block transendothelial migration of human PMNs, and enhance phagocytosis by human macrophages.[14–17] Although a recent report demonstrated that RvD1 promotes clearance of allergens in asthma[18] and resolvin E1 (RvE1) reduces dendritic cell (DC) migration and IL-12 production,[19,20] the function of RvD1 in adaptive immune responses remains largely unexplored.

Resolvin D1 selectively interacts with lipoxin A4/formyl peptide receptor 2 (ALX/FPR2) and G protein-coupled receptor 32 (GPR32) to mediate its proresolving actions.[17,21] The receptor ALX/FPR2 is commonly found on human phagocytic cells such as dendritic cells (DCs), and its murine ortholog ALX/FPR2 is found on macrophages,[22] neutrophils,[23] and DCs.[24] However, the expression of ALX/FPR2 and function of RvD1 on immature versus mature DCs has not been explored.

Dendritic cell maturation is a key factor in host allosensitization and graft rejection, because of the critical function of DCs in mediating alloimmune responses through both the

direct and indirect pathways of sensitization.[25–27] Mature resident and donor DCs express major histocompatibility complex (MHC) class II, costimulatory molecules, and IL-12 after corneal transplantation, and are capable of priming host interferon-gamma (IFN-γ)–secreting Th1 cells in the draining lymph nodes (DLNs),[28,29] which are the main effector cells in corneal allograft rejection.[30–33]

Herein, we aimed to investigate whether an RvD1 analogue (RvD1a, 17R/S methyl RvD1 methyl ester) suppresses the alloimmune response in the host through inhibition of DC maturation. We analyzed the effect of RvD1a on DC maturation using BMDCs in vitro, and investigated the function of RvD1a on T-cell sensitization and allograft rejection using a mouse model of corneal allotransplantation.

Materials and Methods

Mice

Six- to 8-week-old male BALB/c and C57BL/6 mice (Taconic Farms, Germantown, NY, USA) were used in all experiments. Animals were housed in a pathogen-free environment at the Schepens Eye Research Institute animal facility and all procedures were approved by the Institutional Animal Care and Use Committee. The animals were treated according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Generation of BMDCs

Bone marrow–derived dendritic cells (BMDCs) were generated as previously described.[34] Briefly, bone marrow cells collected from the femurs of 6- to 8-week-old C57BL/6 mice were cultured in petri dishes at 2×10^6/10 mL RPMI-1640 medium (Lonza Biologics, Inc., Hopkinton, MA, USA) supplemented with 10% fetal calf serum (Atlanta Biologicals, Flower Branch, GA, USA); 2 mM L-glutamine (Lonza Biologics, Inc.); 100 U/mL of penicillin (Lonza Biologics, Inc.); 100 μg/mL of streptomycin (Lonza Biologics, Inc.); 50 mM β-mercaptoethanol (Sigma-Aldrich Corp., Springfield, MO, USA); and 200 U/mL of granulocyte-macrophage colony-stimulating factor (GM-CSF; PeproTech, Inc., Rocky Hill, NJ, USA). Medium was changed every 2 days. On days 5 and 6, nonadherent and loosely adherent immature BMDCs were harvested. To generate mature BMDCs, we subcultured the immature BMDCs in 6-well plates with 100 ng/mL of lipopolysaccharide (LPS; R&D Systems, Inc., Minneapolis, MN, USA) for 24 hours. The purity of the DCs used in the experiments was greater than 97%, as determined by analysis of surface CD11c staining using flow cytometry analysis. To investigate the actions of an RvD1 analogue (17[R/S] methyl- resolvin D1 methyl ester), denoted here as RvD1a (prepared as outlined in Kasuga et al.[35]) on DC function, 6 ng/mL RvD1a were added during subculture (day 6) and LPS stimulation (day 8). Cell surface expression of CD40, MHC II, CD80, and CD86 was analyzed using flow cytometry. The concentration of IL-12 in the supernatants was assayed using a commercial ELISA kit (BioLegend, San Diego, CA, USA). Resolvin D1a was synthesized as outlined in Kasuga et al.[35] and Sun et al.[36]

RNA Isolation and Real-Time PCR

Total RNA was isolated using Trizol (Invitrogen, Carlsbad, CA, USA) and commercial spin columns (RNeasy MinElute Spin Columns; Qiagen, Valencia, CA, USA) as previously described.[37] Reverse transcription of total RNA was conducted using oligo(dT) primer and reverse transcriptase (Superscript III Reverse Transcriptase; Invitrogen). An amount of 1 μL of total cDNA, synthesized from 400 ng of total RNA with random hexamers, was loaded in each well, and assays were performed in triplicates. Real-time qPCR was performed with a commercial master mix (Taqman Universal PCR Mastermix; Applied Biosystems, Foster City, CA, USA) and FAM-MGB dye-labeled predesigned primers (Applied Biosystems) for *ALX/Fpr2* (Mm00484464_s1). Polymerase chain reaction was performed for 2 minutes at 50°C and 10 minutes at 95°C, followed by 35 cycles of 15 seconds at 95°C and 1 minute at 60°C, using a sequence detection system (ABI PRISM 7900 HT; Applied Biosystems). Amplification of PCR of the housekeeping gene encoding *Gapdh* (Mm999999915_gl) was used as internal control. A nontemplate control was included in all experiments to evaluate DNA contamination of isolated RNA and reagents. The results were analyzed by the comparative threshold cycle (C_T) method.

Orthotropic Corneal Transplantation and Assessment of Graft Survival

The procedures for corneal orthotropic transplantation, in which corneal grafts from C57BL/6 (H-2b) mice were transplanted onto BALB/c (H-2d) recipient mice, have been well-established and described previously.[38,39] Briefly, central 2-mm diameter corneal grafts were excised from C57BL/6 mice with a trephine and micro-scissors (Storz Instrument Co., St. Louis, MO, USA). The recipient beds were prepared by excising 1.5 mm pieces of tissue from the central cornea of BALB/c host mice. Prepared donor corneal grafts were then transplanted onto the host beds with eight interrupted 11-0 nylon sutures. The sutures were removed 7 days post transplantation.

A total of 100 μL RvD1a (1 μg/mL) or vehicle (0.01% ethanol with normal saline) were injected intravenously into the recipient mice on days 0 and 7 post transplantation as previously described.[40,41] The RvD1 analogue 17R/s methyl was prepared as previously described,[36] and its structural integrity was assessed prior to experiments using published criteria.[42] All grafts were evaluated using slit-lamp biomicroscopy weekly up to 8 weeks. Grafts were defined as rejected when they became opaque and the iris details could not be recognized (score >2 according to a standardized opacity-grading scheme of 0–5).[38]

Cell Isolation and Flow Cytometry Analysis

To obtain single-cell suspensions from corneas, corneal tissue samples were digested in 2 mg/mL collagenase D at 37°C, as previously described.[43] Other single-cell suspensions were obtained from ipsilateral DLNs (submandibular and cervical) or from in vitro BMDC cultures. Non-specific staining was blocked with an anti-FcR antibody (R&D Systems), then cells were incubated with the following antibodies: fluorescein isothiocyanate (FITC)-conjugated murine anti-CD3 (BD Pharmingen, San Jose, CA, USA), anti-CD40, anti-CD80, anti-CD86, anti-IAb (MHC II), or phycoerythrin (PE)-conjugated anti-CD11c antibodies (BD Pharmingen). Prior to intracellular IFN-γ staining, cells were stimulated for 4 hours with phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich Corp.) and ionomycin (Sigma-Aldrich Corp.) in the presence of GolgiStop (BD Biosciences, Franklin Lakes, NJ, USA), and subsequently stained with a FITC-conjugated anti-CD3 antibody (BioLegend). After fixation and permeablization (buffers from eBioscience, Inc., San Diego, CA, USA), cells were stained with a PE-conjugated IFN-γ antibody (BD Pharmingen) for 30 minutes in staining buffer at 4°C. The cells were thoroughly washed and analyzed via flow cytometry (EPICS XL; Beckman Coulter, Brea, CA, USA). All experiments were conducted with parallel staining with respective isotype controls (primary antibodies and

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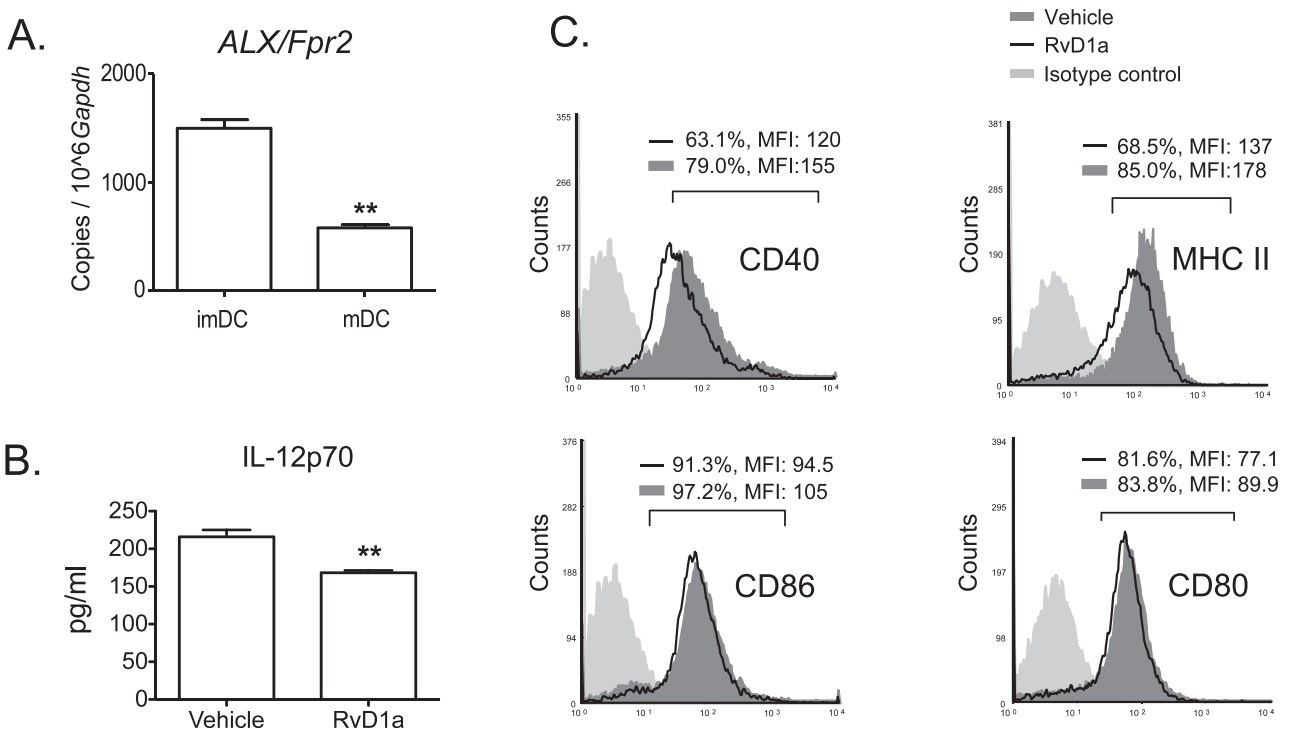

Figure 1. Resolvin D1 analogue inhibits LPS-induced BMDC maturation. (**A**) Quantitative RT-PCR analysis showing ALX/Fpr2 mRNA expression in immature DCs (imDC) and mature DCs (mDC; 1495.6 ± 110.0 vs. 588.8 ± 11.5, $n = 3$, $P = 0.008$); P values were calculated using the Student's t-test and *error bars* represent mean ± SEM. Data shown are representative of three independent experiments. (**B**) The concentration of IL-12p70 in culture supernatant was measured 24 hours after LPS stimulation in RvD1a and vehicle-treated BMDCs using ELISA (216.0 ± 9.1 vs. 168.2 ± 3.2, $n = 4$, $P = 0.0025$, t-test). (**C**) Gating on CD11c-positive cells, the expression of CD40, MHC II, CD80, and CD86 in RvD1a- and vehicle-treated BMDCs were measured by flow cytometry. We cultured BMDCs from days 0 to 6 and treated with 6 ng/mL RvD1a on days 6 and 8. Lypolysaccharide was added to stimulate maturation on day 8 for 24 hours. Data shown are representative of three independent experiments.

isotype controls were purchased from eBioscience, Inc., unless noted otherwise).

ELISPOT Assay

The enzyme-linked immunospot assay was performed to measure the frequencies of directly and indirectly primed T cells, as described previously.[43] Briefly, 96-well ELISPOT plates (Whatman Polyfiltronics, Newton, MA, USA) were coated with 4 μg/mL primary anti–IFN-γ mAb (BD Pharmingen) in sterile PBS overnight. Then, the plates were washed and blocked for 1.5 hours with PBS containing 1% bovine serum albumin and cells were added to these plates. Purified T cells (5×10^5 CD90.2-positive magnetic activated cells [MACS]–sorted) from the DLNs of the grafted BALB/c mice (eight of each group, 3 weeks post transplantation of C57BL/6 donor corneas) were pooled and then incubated in triplicates with C57BL/6 antigen-presenting cells (APCs; 5×10^5 CD90.2-negative, MACS-sorted splenocytes) for 48 hours to quantify frequencies of directly allosensitized T cells. To quantify frequencies of indirectly allosensitized T cells, syngeneic (BALB/c) APCs (1×10^6) pulsed with sonicated donor antigen (2×10^7 C57BL/6 APCs/mL) were incubated with BALB/c recipients' T cells; T cells harvested from lymph nodes of naive BALB/c animals served as controls. After washing, biotinylated anti–IFN-γ detection mAb was added at 2 μg/mL (BD Pharmingen) and incubated overnight at 4°C. The plates were then washed, incubated for 1 hour with avidin-HRP and developed using amino-ethylcarbazole staining solution (Sigma-Aldrich Corp.). The resulting spots were analyzed using the computer-assisted ELISPOT image analyzer (Cellular Technology Ltd., Cleveland, OH, USA).

Corneal Wholemount and Immunofluorescent Staining

Freshly excised corneas were washed in phosphate-buffered saline, fixed in acetone for 15 minutes, and then double stained with CD31 and lymphatic vessel endothelial hyaluronan receptor (LYVE)-1 as described previously[44] using goat anti–mouse CD31 FITC as a panendothelial marker (Santa Cruz Biotechnology, Dallas, TX, USA), diluted 1:100; and purified rabbit anti–mouse LYVE-1 as a lymphatic endothelial marker (Abcam, Inc., Cambridge, MA, USA), diluted 1:400. Rhodamine-conjugated goat anti-rabbit (BD Pharmingen, 1:100) was used as a secondary antibody.

Statistical Analysis

Dunnett's multiple comparison tests and ANOVA or Student's t-test were applied for the data analysis. Kaplan-Meier survival curves were analyzed in a log-rank (Mantel-Cox) test. Results are presented as the mean ±95% confidence interval (CI), and error bars represent the 95% CI. Values of $P \leq 0.05$ were considered statistically significant.

Results

RvD1a Inhibits LPS-Induced BMDC Maturation

We first investigated the gene expression level of the RvD1 receptor ALX/*Fpr2* on both DCs and T cells. We cultured BMDCs for up to 6 days in the presence of GM-CSF to obtain immature DCs, then LPS was added for 24 hours to generate

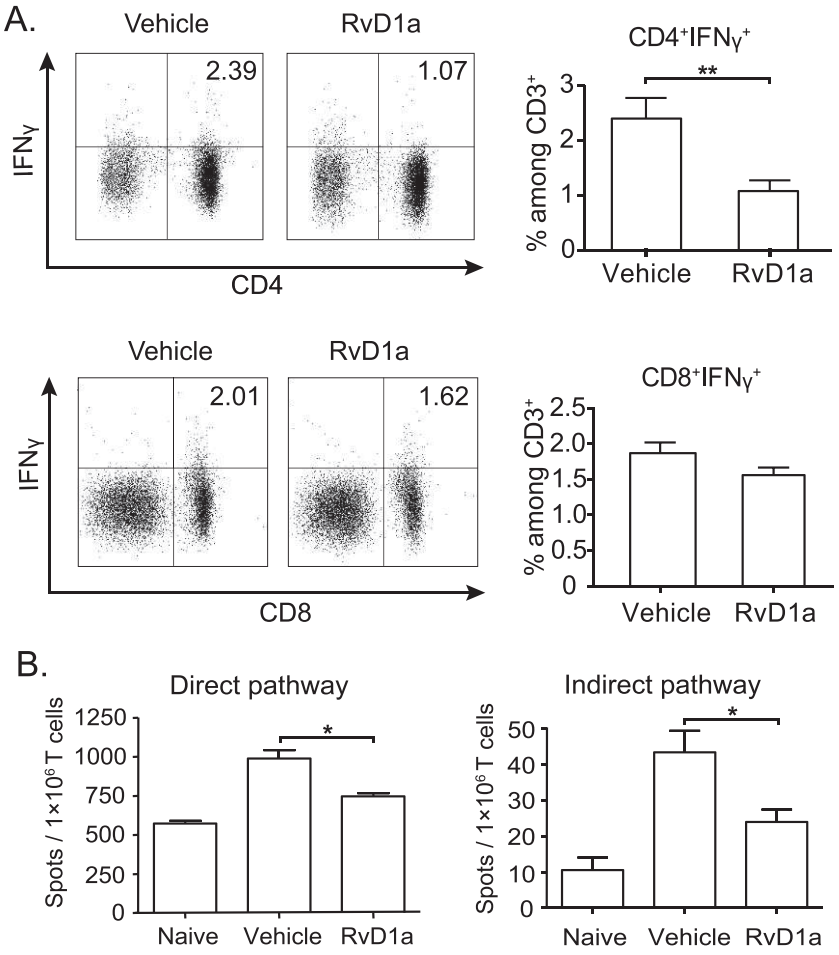

Figure 2. Resolvin D1 analogue treatment suppresses host T-cell allosensitization. Allografted BALB/c mice were treated intravenously with 100 ng RvD1a or vehicle on days 0 and 7 post transplantation. Mice were analyzed 21 days post transplantation. (**A**) Flow cytometry analysis of IFN-γ expressing $CD4^+$ and $CD8^+$ T cells among $CD3^+$ T cells in the draining lymph nodes from RvD1a- and vehicle-treated host DLNs 3 weeks post transplantation is shown (representative plot, 500,000 events/sample). $CD4^+IFN\text{-}\gamma^+$: 2.4% ± 0.4% vehicle versus 1.1% ± 0.5% RvD1a treated, $n = 8$, $^{**}P = 0.009$); $CD8^+IFN\text{-}\gamma^+$: 1.9 ± 0.4% vehicle versus 1.6% ± 0.2% RvD1a treated, $n = 8$, ns). (**B**) ELISPOT assay shows $IFN\text{-}\gamma^+$ T cells primed by either donor (direct) or host (indirect) APCs. Direct pathway (T cells stimulated by C57BL/6 splenic APCs), vehicle 988 ± 54 versus RvD1a 746 ± 21 spots/10^6 T cells, $n = 8$, $^*P = 0.014$; indirect pathway (stimulated by BALB/c splenic APCs and sonicated with C57BL/6 antigen), vehicle 43 ± 6 versus RvD1a 24 ± 4 spots/10^6 T cells, $n = 8$, $^*P = 0.048$; *P* values were calculated using the Student's *t*-test and *error bars* represent mean ± SEM.

mature DCs. Real-time PCR was performed showing that ALX/*Fpr2* is expressed 2.5-fold higher on immature DCs than mature DCs (Fig. 1A). However, no ALX/Fpr2 expression was detectable in T cells isolated from DLNs of either naïve or grafted mice 3 weeks after allogeneic corneal transplantation (data not shown). Since anti-murine ALX/FPR2 antibody is not commercially available, we were unable to assess its protein expression. To investigate the functional effect of RvD1a on DC maturation, BMDCs were stimulated with LPS on day 8 and treated with 6 ng/mL RvD1a or vehicle on days 6 and 8. Twenty-four hours after LPS addition, we analyzed the supernatant using ELISA and observed a significant lower IL-12 concentration in RvD1a-treated BMDCs compared with the vehicle-treated cells. Gating on CD11c positive cells, flow cytometry analysis revealed that RvD1a treatment reduces the frequencies of $CD40^+$ (63.1% vs. 79.0% vehicle) and MHC II^+ (68.5% vs. 85.0% vehicle) by approximately 20% compared with the vehicle-treated BMDCs. However, RvD1a treatment did not significantly alter the expression of $CD80^+$ and $CD86^+$ on BMDCs (Fig. 1C).

RvD1a Inhibits Host T-Cell Allosensitization Through Both Direct and Indirect Pathways

Interferon gamma–producing T cells are the principal mediators of acute corneal allograft rejection. Following corneal transplantation, both $CD4^+$ and $CD8^+$ T cells are activated and have the ability to reject allograft.[45] Therefore, we determined the frequencies of total IFN-γ producing $CD4^+$ as well as $CD8^+$ T cells in the DLNs 3 weeks after transplantation using $CD3^+$, $CD4^+$, $CD8^+$, and IFN-γ costaining. We observed significantly decreased frequencies of $IFN\text{-}\gamma^+$ CD4 T cells among total $CD3^+$ T cells in the RvD1a treated group compared the vehicle-treated group. Frequencies of $IFN\text{-}\gamma^+$ CD8 T cells were lower in RvD1a treated mice compared with vehicle-treated mice, however not significantly (Fig. 2A).

Given that host T cells are allosensitized either directly by donor APCs or indirectly by host APCs, we further investigated the frequencies of directly or indirectly primed of IFN-γ producing T cells using the ELISPOT assay. In the RvD1a-treated group, the frequency of allosensitized T cells in the host DLNs were significantly lower in both direct (746 ± 21 vs.

988 ± 54 spots/10^6 T cells, $P = 0.014$) and indirect (24 ± 4 vs. 43 ± 6 spots/10^6 T cells, $P = 0.048$) pathways, compared with the respective vehicle groups (Fig. 2B).

RvD1a Inhibits Graft T-Cell Infiltration, Graft Angiogenesis, and Promotes Corneal Allograft Survival

Systemic RvD1a administration led to a 22% reduction of host T-cell recruitment into the grafts compared with the control group 3 weeks post transplantation (Fig. 3A). Graft bed vascularity is the leading risk factor for allorejection in corneal transplantation.[46] Thus, we examined the angiogenic and lymphangiogenic response after transplantation using biomicroscopy weekly for up to 8 weeks. Over the 8-week observation period, the graft angiogenesis score was consistently lower in the RvD1a-treated than in the vehicle-treated group (Fig. 3B). In addition, we examined the blood ($CD31^+$) and lymphatic vessels ($LYVE\text{-}1^+$) of the grafted corneas using immunofluorescence staining 21 days after transplantation (Fig. 3C). Quantification of the vascularized areas in corneal wholemounts showed that RvD1a treatment significantly reduced the de novo generation of $CD31^{high}$ $LYVE\text{-}1^-$ blood vessels as well as $CD31^{low}$ $LYVE\text{-}1^{high}$ lymphatic vessels (CD31: 7.6% ± 0.8% vs. 12.9% ± 1.6%, $n = 6$, $P < 0.00001$; LYVE-1: 6.5% ± 1.0% vs. 11.1% ± 1.4%, $n = 6$, $P < 0.00001$; Figs. 3D, 3E) compared with vehicle-treated mice. Finally, graft opacity scores were significantly lower (Fig. 3F) and graft survival (%) was significantly higher in RvD1a-treated mice compared with the vehicle-treated control mice (graft survival: 76.9% in the RvD1a-treated group, 37.5% in the controls; log-rank analysis, $n = 16$, $P = 0.02$; Fig. 3G).

Discussion

In the current study, using a stable analogue of the proresolving mediator RvD1, we investigated its actions on in vitro generated BMDCs as well as its effect on alloantigen-specific T-cell responses in an in vivo model of corneal transplantation.

Previous studies have demonstrated that RvD1 plays a prominent role in resolving acute inflammation through reduced neutrophil recruitment, enhancing phagocytosis, and reducing cytokine secretion in corneal angiogenesis, vascular inflammation, and pulmonary inflammation.[13,17,47,48] The importance of ALX/Fpr2 in mediating the anti-inflammatory functions of RvD1 has previously been demonstrated.[17,49] Recently, the receptor ALX/FPR2 was found to mediate RvD1 actions in human phagocytes,[16] and evoke homeostatic tissue functions of RvD1 in resolution through specific proresolving microRNAs.[21] In human monocytes, ALX/FPR2 (also known as FPRL1) is downregulated as they differentiate into DCs.[50] We demonstrate here that there is a 60% reduction in the expression of *ALX/Fpr2* in LPS-stimulated mature murine BMDCs (Fig. 1A), suggesting that RvD1 may primarily affect immature BMDCs and that furthermore, the expression level of ALX/FPR2 on DCs may be functionally relevant. In contrast, the mRNA expression of *ALX/Fpr2* in both naïve and primed T cells was undetectable (data not shown), indicating that RvD1 may not have a direct effect on T cells. We additionally found that RvD1a-treated BMDCs displayed reduced expression of MHC II, CD40, and IL-12p70 (Figs. 1B, 1C). Our results are in line with previous studies using DHA, arachidonic acid, and eicosapentaenoic acid to inhibit maturation of LPS-stimulated BMDC cultures.[51,52] Docosahexaenoic acid has been shown to effectively suppress IL-12 production by DCs.[51] However, the expression of CD80/CD86 costimulatory molecules was not significantly changed, suggesting that RvD1 may modulate DC–T-cell interactions through the CD40-CD154 pathway, but not through the CD80/86-CD28 pathway. Both MHC II and CD40 are critical for T-cell sensitization after corneal transplantation,[53] which suggests that RvD1 may indirectly affect the graft survival through maintaining the low maturation state of DCs. Similar results were reported for another member of the resolvin family, RvE1. Dendritic cells treated in vitro with RvE1 maintained an immature phenotype because RvE1 prevents upregulation of maturation markers as well as costimulatory molecules and reduces IL12p70 production.[19,20] The original RvE1 structural assignment of stereochemistry and total organic synthesis showed that this action of RvE1 is similar to LXA_4 and its receptor ALX on PMNs.[19] These data support our findings that RvD1 regulates inflammation and adaptive immune response through ALX on DCs.

Although the actions of RvD1 on DCs and T cells are not yet studied,[54] the RvD1 precursor DHA leads to reduction of costimulatory molecules and cytokine production in human monocyte-derived DCs and murine BMDCs.[51,55–57] Dendritic cells treated with DHA show reduced ability to activate naive T cells to differentiate into Th1/Th17 effectors in an experimental encephalitis model.[58] Previous data have shown that IFN-γ secreting T cells,[31] especially the Th1 cells, play a key role in corneal allograft rejection.[45,59] In corneal transplantation, the allograft rejection is mediated by adaptive immunity, whereas innate immune responses conducted by PMNs and macrophages may contribute to the nonspecific inflammation. However, these innate cells are not responsible for graft rejection upon alloantigen recognition. Nevertheless, previous data showed that RvD1 acts on human PMNs and macrophages in vitro and regulates their trafficking and functions in many in vivo mouse and rabbit models.[2,8,15,16] These effects may reduce nonspecific inflammation in the host microenvironment; some innate cells also play a role as APCs and thus indirectly contribute to the suppression of adaptive alloimmune response to the graft, although the principal APCs in corneal allograft transplantation are DCs.[60] Accordingly, we show herein that RvD1a treatment reduces not only the $IFN\text{-}\gamma^+$ CD4 and CD8 T-cell differentiation in the DLNs (Fig. 2A), but also suppressed $CD3^+$ T-cell infiltration into the graft (Fig. 3A), demonstrating that RvD1a effectively inhibits allosensitization. As a result, we observed improved opacity scores and higher graft survival rates in the RvD1a-treated group compared with the vehicle-treated group (Figs. 3F, 3G).

Both donor- and host-derived DCs are able to sensitize T cells, via the direct and indirect pathways, respectively.[31] Thus, we used the ELISPOT assay to differentiate between both pathways, and found both pathways to be impaired after RvD1a treatment (Fig. 2B), suggesting that RvD1a actions are not dependent on the origins (donor versus host) of DCs, and are not strain-specific.

The normal cornea is both alymphatic and avascular.[61] However, corneal lymphangiogenesis significantly enhances the trafficking of graft antigens and alloantigen-bearing APCs to the DLNs,[33] while corneal angiogenesis significantly promotes effector T-cell trafficking to the graft.[62,63] In addition to its effects on DC function and sensitization, we demonstrate here that RvD1a treatment significantly reduced the extent of angiogenesis and lymphangiogenesis after corneal grafting (Figs. 3B–E). Our current data are in accord with the results of a previous study applying RvD1 locally in a suture-induced neovascularization model.[13] Thus, we speculate that in addition to its suppressive effect on allosensitization through downregulation of DC maturation, RvD1 and its stable analogue may also suppress alloimmunity via inhibition of local angiogenic responses.

In summary, we demonstrate here, for the first time, that RvD1a inhibits DC maturation and that systemic application of

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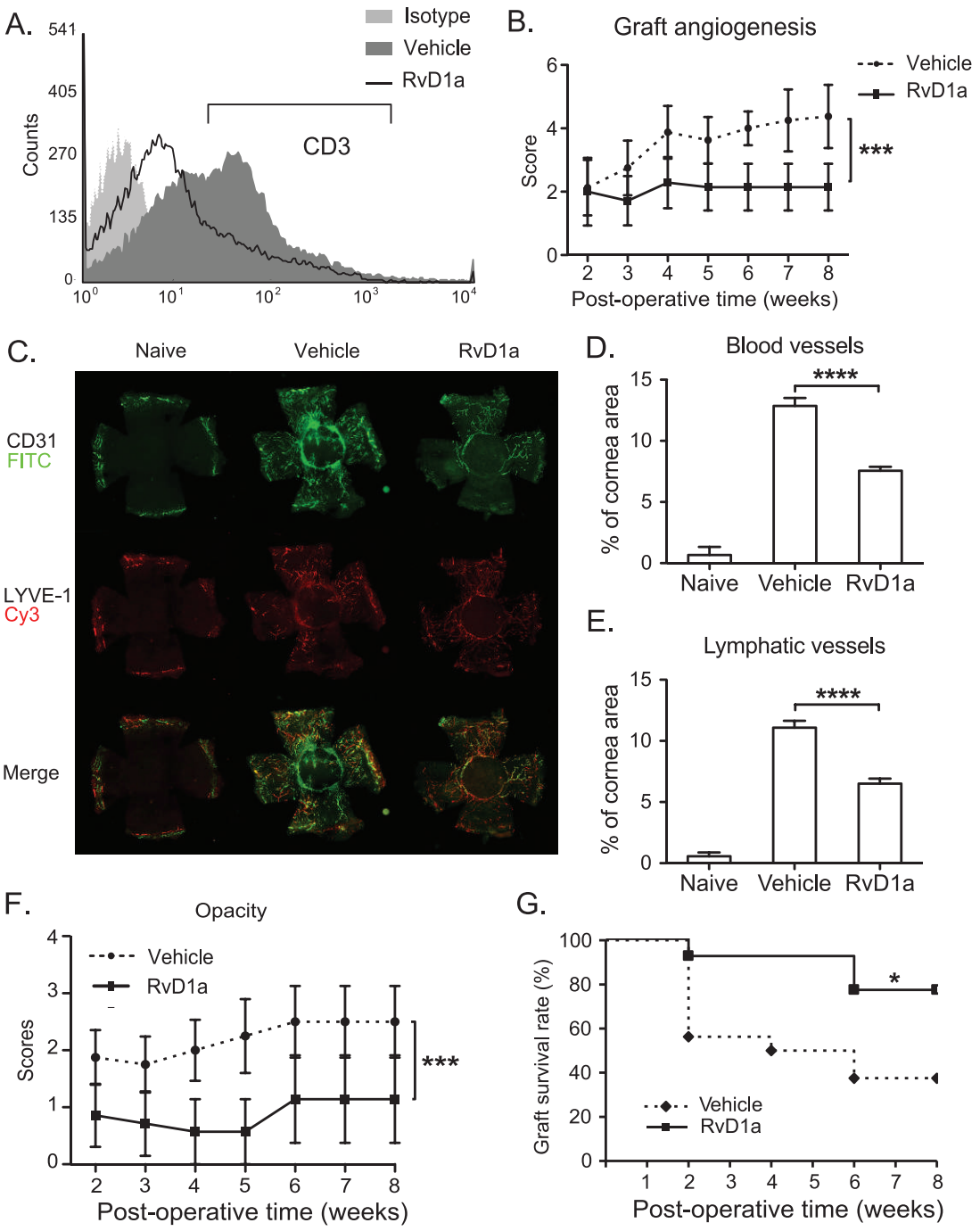

Figure 3. Resolvin D1 analogue promotes corneal allograft survival by suppressing graft T-cell infiltration and angiogenesis. Allografted BALB/c mice were treated systemically with 100 ng RvD1a or vehicle on days 0 and 7 post transplantation. (**A**) Flow cytometry analysis shows the frequencies of $CD3^+$ T cells among the total digested corneal cells from the RvD1a- and vehicle-treated hosts 21 days post transplantation. Corneas were harvested and digested in collagenase D at 37°C to obtain a single cell suspension. The frequencies of infiltrated $CD3^+$ T cells in the cornea were reduced in RvD1a-treated mice compared to vehicle-treated mice (pooled sample $n = 8$/group, total events 50,000/sample). Data shown are representative for three independent experiments. (**B**) Corneal graft angiogenesis was scored biomicroscopically in a masked fashion with a slit lamp according to a standardized scoring grid. Scores in the RvD1a-treated group were significantly lower compared with the vehicle group (Two-way ANOVA with Bonferroni posttest, $n = 8$, $P < 0.0001$). (**C**) Representative epifluorescence micrographs display whole corneas from naïve and RvD1a-treated as well as vehicle-treated hosts ($n = 6$/group). Tissues were harvested 21 days post transplantation and stained with anti-CD31 Ab (green) and anti-LYVE-1 Ab (red; ×4 magnification). (**D**, **E**) The percentage of the corneal area covered with blood vessels ($CD31^{high}$/LYVE-1^-) 7.6% ± 0.8% RvD1-treated versus 12.9% ± 1.6% vehicle-treated, $n = 6$, $P < 0.00001$ one-way ANOVA with Tukey's posttest (**D**) and lymphatic vessels ($CD31^{low}$/LYVE-1^{high}) 6.5% ± 1.0% vs. 11.1% ± 1.4%, $n = 6$, $P < 0.00001$ (**E**) of the total cornea. Representative data from three independent experiments are shown. (**F**) Graft opacity scores of RvD1a-treated and vehicle-treated mice (two-way ANOVA with Bonferroni posttest, $n = 8$, $^{***}P < 0.0001$). (**G**) Kaplan-Meier graft survival curve of allografted BALB/c hosts treated with 100 ng RvD1a intravenously once a week for up to 8 weeks after corneal transplantation (log-rank analysis $n = 8$, $^*P = 0.02$).

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RvD1a significantly increased corneal allograft survival. Increased graft survival is associated with reduced host T-cell allosensitization, graft T-cell infiltration, and corneal angiogenesis. The proresolving mediator RvD1a may thus serve as a novel therapeutic for controlling alloimmunity after corneal transplantation.

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