Pre-incubation of corneal donor tissue with sCD83 improves graft survival via the induction of alternatively activated macrophages and tolerogenic dendritic cells

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Funding information
Deutsche Forschungsgemeinschaft, Grant/Award Number: BO4489/1-2, BO4489/3-1, CRC1181-B03, Cu47/12-1, Cu47/9-1, STE432/15-1 and ZI1225/1-1; Center for Molecular Medicine Cologne, Grant/Award Number: A09

Immune responses reflect a complex interplay of cellular and extracellular components which define the microenvironment of a tissue. Therefore, factors that locally influence the microenvironment and re-establish tolerance might be beneficial to mitigate immune-mediated reactions, including the rejection of a transplant. In this study, we demonstrate that pre-incubation of donor tissue with the immune modulator soluble CD83 (sCD83) significantly improves graft survival using a high-risk corneal transplantation model. The induction of tolerogenic mechanisms in graft recipients was achieved by a significant upregulation of Tgfb, Foxp3, Il27, and Il10 in the transplant and an increase of regulatory dendritic cells (DCs), macrophages (Mφ), and T cells (Tregs) in eye-draining lymph nodes. The presence of sCD83 during in vitro DC and Mφ generation directed these cells toward a tolerogenic phenotype leading to reduced proliferation-stimulating activity in MLRs. Mechanistically, sCD83 induced a tolerogenic Mφ and DC phenotype, which favors Treg induction and significantly increased transplant survival after adoptive cell transfer. Conclusively, pre-incubation of corneal grafts with sCD83 significantly prolongs graft survival by modulating recipient Mφ and DCs toward tolerance and thereby establishing a tolerogenic microenvironment. This functional strategy of donor graft pre-treatment paves the way for new therapeutic options in the field of transplantation.

KEYWORDS
basic (laboratory) research/science, corneal transplantation/ophthalmology, dendritic cell, immunosuppression/immune modulation, macrophage/monocyte biology: differentiation/maturation, soluble CD83, tolerance
1 | INTRODUCTION

Corneal transplantation is performed to restore vision in patients suffering from corneal blindness. With a 5-year median survival of ~80% corneal grafts are commonly well-tolerated, given that the allogeneic tissue is placed into a graft bed which is devoid of blood and lymphatic vessels.1-3 Severe inflammation at the ocular surface can lead to a breakdown of the cornea’s angiogenic and immune privilege.4,5 In this case, the 5-year survival rate of corneal allografts drops to ~40% or lower, even under treatment with non-specific immunosuppressive therapeutics, such as corticosteroids.1,2,6 Standard treatment options for recipients still rely on topical or systemic administration of immunosuppressive agents, which have severe side effects.7-9 There is a great medical need for the development of new therapeutic strategies. One promising approach is the modulation of the local alloimmune response. After transplantation a massive infiltration of inflammatory cells, for example, CD4+ effector T cells and pro-inflammatory macrophages (Mφ), to the graft-host interface lead to graft rejection.10,11

Regarded as orchestrators of the immune response, dendritic cells (DCs) and Mφ represent a promising target in transplant immunology. Resident DCs are characterized by an immature or tolerogenic phenotype during corneal homeostasis.12 After keratoplasty, donor or recipient DCs quickly mature and leave the graft site to migrate to dLNs where they prime transplant specific host T cells.13 DCs treated with pharmacologic inhibitors that prevent maturation have been used to induce tolerance to alloantigens in corneal transplantation.11,14 Mφ can adopt different phenotypes and functions depending on the microenvironment. Mφ populations can be classified into pro-inflammatory, classically activated Mφ (CAM) and anti-inflammatory, alternatively activated Mφ (AAM). In transplantation, the destructive alloimmune response is mainly attributed to CAMs (TNF-α, IL-6, CD86high),15-17 whereas AAMs (MSR-1+, CD86low, CCL-22) have been shown to possess a tolerogenic signature.18,19 Hence, Mφ are crucially involved in wound healing, tissue remodeling, and tolerance induction after transplantation.18,19

Healthy corneas allow a sponge-like adsorption of proteins up to a molecular mass of 56 kDa.11,20-22 Corneal donor tissue can be stored in eye banks for up to 4 weeks before transplantation. These features open up a window for possible therapeutic pre-treatments of corneal donor tissues, thereby using the donor cornea itself as a drug-releasing depot.23 Soluble CD83 (sCD83) is the 16.7 kDa extracellular domain of the membrane-bound CD83 (mCD83) protein, which is expressed by activated DCs, B/T cells and especially Tregs.24-26 Treatment with sCD83 has already been proven to ameliorate symptoms in specific autoimmune disease models.29-32 Systemic administration of sCD83 prevents allograft rejection in animal models.31,33,34 Mechanistically, sCD83 modulates DCs maturation, thereby inducing a tolerogenic DC phenotype,11,28,31,35-37 which impairs DC-mediated T cell proliferation, and induces/expands Tregs.35,36 However, the effect of sCD83 on Mφ has not been investigated in detail so far. In this study, we present a new therapeutic approach to prolong corneal allograft survival after transplantation using sCD83. Pre-incubation of donor corneal tissue with sCD83 increased corneal graft survival in high-risk graft recipients by inducing tolerogenic DCs and AAMs. Moreover, sCD83 directly induces AAMs, which in turn increases the frequency of Tregs.

2 | MATERIALS AND METHODS

2.1 | Animals

Age-matched BALB/c and C57BL/6N female mice (6–8 weeks, Charles River Laboratories, Germany) were treated in accordance with the institutional and national guide for the care and use of laboratory animals and conformed to the ARVO Statement for the Use of Animals in Ophthalmology and Vision Research. For surgical procedures, mice were anesthetized using a mixture of 120 mg/kg ketamine (Pfizer) and 20 mg/kg xylazine (Bayer AG).

2.2 | Allograft pre-treatment with sCD83

Donor corneal allografts were excised by trephination (2 mm) and incubated ex vivo in Culture Medium I (Biochrom) supplemented with 2% fetal bovine serum and 50 μg/ml human sCD83 for 48 h at 37°C, 5% CO2 or PBS as control. sCD83 was produced in Pichia pastoris as previously described.31,38

2.3 | Corneal high-risk transplantation

For corneal high-risk transplantation, female BALB/c mice (Charles River Laboratories, Germany) served as recipients and age-matched C57BL/6N mice as donors. The procedure has been adapted from Salabarría et al.39 and described in Supplemental Method 1. Animals were euthanized 1 day and 1, 2, or 8 weeks posttransplant.

2.4 | Atto488-conjugated sCD83

Labeling was achieved by incubation of sCD83 with Atto488-NHS-ester in DMSO in a 1:3 ratio for 1 h; reaction was stopped by adding Tris-buffer and excess ester was removed by dialysis against PBS (three times). Protein concentration was determined using the NanoDrop™ 2000/2000c (Thermo Fisher Scientific) and full-thickness corneas and LN is described in Supplemental Method 2.

2.5 | Two-photon microscopy

Confocal imaging of dLNs, was performed by Two-photon microscopy on a Zeiss LSM880 NLO equipped with a 680–1300 nm tunable and fixed 1040 nm 2-photon laser from Newport SpectraPhysics. Two-photon images of dLNs were acquired with a 20x W-Plan Apochromatic objective water dipping lens. The fluorophore Atto-488, AF647 (for CD11c) or AF633 (for F4/80) was excited at 940 nm and specific emission was detected at 500–550 nm and 640–710 nm. The
second harmonic generation (SHG) signal was excited at 940 nm and detected at 300–485 nm to visualize collagen capsules of dLNs.

2.6 | Generation and stimulation of bone-marrow-derived DCs

Bone-marrow derived DCs (BMDCs) were generated from murine bone marrow precursor cells from C57BL/6 mice in R10 medium consisting of RPMI1640 (Lonza), 10% FCS (Merck), 1% Penicillin-Streptomycin-Glutamine-solution (Sigma Aldrich), and 50 µM β-mercaptoethanol supplemented with GM-CSF supernatant, derived from a GM-CSF-expressing cell line, as previously described.

2.7 | Generation and stimulation of bone-marrow-derived Mφ

Bone-marrow derived Mφ (BMDM) were generated from murine BMCs from C57BL/6 mice in D10 medium consisting of DMEM (Lonza), 10% FCS (Merck, Germany), Penicillin-Streptomycin-Glutamine-solution (Sigma Aldrich), and 50 µM β-mercaptoethanol. BMCs were isolated from femur and tibia of mice and seeded in D10 medium supplemented with M-CSF supernatant. M-CSF was generated from a L929 cell line, kindly provided by Prof. Dr. Jochen Mattner (Mikrobiologisches Institut, Universitätsklinikum Erlangen).

2.8 | DC-allogeneic splenocyte mixed lymphocyte reaction (MLR)

BMDCs from C57BL/6 mice were generated in the presence or absence of sCD83 and matured via LPS (100 ng/ml). BMDCs were co-cultured at different ratios with allogeneic splenocytes, derived from BALB/c mice (4 × 10^5 cells/well). T cell proliferation was assessed as described before.

2.9 | Mφ-allogeneic splenocyte MLR

On day 6, BMDMs from C57BL/6 mice were generated in the presence or absence of sCD83, harvested and seeded in 96-well plates. Allogeneic splenocytes derived from BALB/c mice (4 × 10^5 cells/well) were co-cultured with BMDMs in 96-well plates for 72 h in D10 medium (37°C, 5.5% CO₂), at different Mφ:splenocyte ratios (1:2; 1:4). T cell proliferation was assessed as described before.

2.10 | Adoptive transfer of sCD83-treated DCs and Mφ

Bone-marrow-derived DCs and Mφ from C57BL/6N mice were generated ± sCD83 (25 µg/ml). 1 × 10^5 cells were injected via the vein of the tail of BALB/c recipients 7 days before corneal transplantation. Non-vascularized BALB/c mice served as corneal transplant recipients and age-matched C57BL/6N mice served as donors as previously described. Graft survival was determined by opacity grading for 8 weeks (Isolation of dLN is described in Supplemental Method 3).

2.11 | Flow cytometric analyses

Surface staining of dLN cells, BMDCs, BMDMs or cells from MLRs was performed in PBS containing a LIVE/DEAD marker and the appropriate antibodies for 30 min. For intracellular staining, cells were permeabilized and fixed in Permeabilization Reagent (Thermo Fisher Scientific). The following specific antibodies were used (from BioLegend or otherwise stated): B220 (RA3-6B2), CD3 (17A2), CD4 (RM4-5), CD25 (M1/70), CD40 (HM40-3), CD80 (16-10A1), CD86 (GL-1), CD11c (N418), CD40 (HM40-3), IFN-γ (XMG1.2), RORγT (Q31-378), GATA3 (L50-823; BDBiosciences), T-BET (O4-46,BD Pharmingen), FOXP3 (FJK-16s; Thermo Fisher Scientific).

2.12 | RNA isolation

Total RNA was isolated from dLNs of recipient mice 1, 2 as well as 8 weeks posttransplant as well as from BMDMs, BMDCs or Mφ was derived from MLRs, using the RNeasy Plus Mini Kit (Qiagen), according to the manufacturer’s instructions. To yield sufficient RNA, a pool of three corneas was transferred to innuSPEED lysis Tube W (Analytik Jena), dissociated using a homogenizer and subsequently centrifuged (13,000 rpm, 3 min, 4°C). Finally, RNA was isolated by RNeasy Plus Micro Kit (Qiagen), according to the manufacturer’s instructions.

2.13 | qPCR

Total RNA was reverse transcribed using the First strand cDNA synthesis Kit (Thermo Fisher Scientific) as described by the manufacturer. qPCR analyses were performed using the Sybr Green Super mix (Biozym), a CFX96 Real time system (BioRad), and normalized to the reference gene transcripts Rpl4 (for dLNs and DCs) or Hprt (for cornea and Mφ). For primer sequences see Table 1.

2.14 | Western blot analyses

To assess the sCD83 protein content within corneas which have been pre-incubated with 50 µg/ml sCD83 or PBS as a control for 48h, Western blot analyses were performed. A pool of 15 corneas were lysed as described above. Proteins were purified from the
flow-through of RNAeasy Spin columns by precipitation in ice-cold acetone for 1h at −20°C. Subsequently, proteins were separated via SDS-polyacrylamide gel electrophoresis and blotted onto a nitrocellulose membrane (GE Healthcare). After blocking (1x Rotiblock; Carl Roth, Germany) membranes were incubated with a mouse anti-human-CD83 mAB (Clone F-5, Santa Cruz) overnight (4°C). Specific signals were detected using the appropriate HRP-labeled secondary antibody and ECL Prime Western Blotting Detection Reagent (GE Healthcare).

2.15 | Cytometric bead array (CBA)

Supernatants of BMDM cultures or MLRs were analyzed using the LEGENDplex™ Mouse Macrophage/Microglia or LEGENDplex™ MuTh Cytokine panel (BioLegend), respectively, according to the manufacturer’s instructions.

2.16 | Statistics

All statistics were calculated with GraphPad Prism 8.0 using the two-tailed unpaired student's t-test or Mann–Whitney U test for nonparametric data, 1 or 2-way ANOVA. Data are presented as mean values including the Standard Error Mean (SEM) or Standard Deviation (SD). *p < .05; **p < .01; ***p < .001; and ****p < .0001.

2.17 | Study approval

Animal care and all experimental procedures of the present study were performed in accordance with the institutional and national guide for the care and use of laboratory animals and conformed to the ARVO Statement for the Use of Animals in Ophthalmology and Vision Research.

3 | RESULTS

3.1 | Pre-incubation of corneal allografts with sCD83 establishes a protein reservoir that induces Tgf-β mRNA expression and leads to infiltration of the recipient

To investigate whether pre-incubation of donor cornea with sCD83 would establish a protein reservoir within the graft, we analyzed mock- and sCD83-incubated corneal grafts for the presence of human sCD83 by Western blotting (Figure 1A). Recombinant human sCD83 protein was absent in mock-treated corneas, whereas it was clearly present in sCD83-pre-incubated grafts (Figure 1A). Noteworthy, sCD83 pre-treatment significantly upregulated Tgf-β as well as Ido1, by trend, within the corneal donor tissue, compared to mock-treated controls (Figure 1B). When incubating the corneal graft with labeled sCD83-Atto488 and transplanting it in a high-risk graft bed, sCD83 was not only present within the graft, but also

| TABLE 1 | Primer sequences used in qPCR experiments |
|-----------|------------------------------------------|
| Gene      | Forward primer                          | Reverse primer                      |
| Hprt      | GCTGGATACAGCACAGCTTTTGTG               | GATCCAACTTGTCATCTTTAGG               |
| Rpl4      | GCTGACCCCTTCGCAGAAGA                   | TCTCGATTTGTTGAGGGCAGT                |
| Il27      | TTCCAAGTGGTCTGGACTTTT                | AAGGTTGGTAGAGGGAGAGCA                |
| Il10      | CCAAGGTTCTGAGAATGGACGTCTTC           | TTTTCCAGGAGGAAGATCGC                 |
| Foxp3     | CCCAGGAAAAGCACAGCAACCTT               | CTTGGCCTTTCTCATCCAGGA                |
| Tgfb      | TGGAGCACAATGGAGAAGACCTA              | AGACAGCAGTACAGGGCTAC                |
| Ido1      | ATTTGTTGAAATGGACGTCTTC               | ACAACTGCACGATCCCTCTTTAAA             |
| Rorgt     | TTTCTGAGGAGTGGAGATTGC                | TTGTGCGATGAGTCATTGCAGA               |
| Tbx21     | AGCAAGGAGGAGGGAATGTT            | GGGGTTGAGCATATAAGGCGGTC              |
| Gata3     | CCAAGGGAAGGCTTGGCAGCA               | TCCCTGAGGCGGTCATGACAC                |
| Ilng      | GCTTTGCAGCTCTTCCAT                  | GTCAACATCCCTTTGAGCAGT                |

FIGURE 1 | Pre-incubation of corneal grafts with sCD83 establishes a protein reservoir within the graft that infiltrates the recipient post transplantation and is DC- and Mφ-associated. Corneal allografts were treated ± sCD83 (50 µg/ml) for 48 h. For (C) and (D), sCD83 was conjugated to Atto488. (A) Assessment of the sCD83 protein reservoir in corneal allografts prior to transplantation via Western blotting. Proteins were isolated from mock versus sCD83-treated corneas (pool of 15 corneas per lysate; see also uncut gels in Supplemental Figure 1A) (B) qPCR of Tgf-β and Ido1 in mock versus sCD83 pre-incubated corneas, n = 15, in pools of 3. Data are represented as mean ± SEM. Statistical analysis was performed using unpaired t-test. *p < .05. (C) Epi-fluorescence image of a whole, transplanted cornea 24 h post high-risk transplantation with sCD83-Atto488 (green) and DAPI (blue); the dotted line indicates the graft-host interface; inset shows the graft-host interface in higher magnification with cell associated sCD83 signals (arrow heads). Scale bar (whole mount): 500 µm; scale bar (magnification): 100 µm. (D) Ex vivo confocal microscopy of a whole, transplanted cornea 24 h post high-risk transplantation with Atto488 signal (green), CD11c (red), F4/80 (magenta), and DAPI (blue). Upper row: 25x magnification; scale bar = 50 µm; dotted line: graft-host interface; arrow heads: CD11c+ dendritic cells; asterisks: F4/80+ macrophages. (E) Ex vivo two-photon microscopy of dLNs recovered 24h post high-risk corneal transplantation. sCD83-Atto488 (green), F4/80 (upper panel; red); CD11c (lower panel; red) and the collagen structure of the LN capsule (white), which was detected by second harmonic generation (SHG) signals. Scale bar = 50 µm [Color figure can be viewed at wileyonlinelibrary.com]
(A) cornea
mock sCD83 2 µg sCD83
26 kDa
17 kDa
pre-incubated corneas

(B) cornea
relative mRNA expression x-fold induction
mock sCD83
Tgfb
Ido1

(C) cornea

(D) cornea
CD11c F4/80 sCD83 DAPI merge
24 h

(E) draining lymph node
F4/80 SHG sCD83 merge
24 h

CD11c SHG sCD83 merge
24 h
crossed the graft border and infiltrated the recipients bed 1 day posttransplant (Figure 1C). We co-localized sCD83 with CD11c⁺ DCs as well as F4/80⁺ Mφ in the recipient bed 24 h posttransplant (Figure 1D). Specific Atto488 signals were detected in the dLNs 24 h posttransplant which were co-localized with either F4/80⁺ or CD11c⁺ cells (Figure 1E).

3.2 | Pre-incubation of corneal allografts with sCD83 induces immune regulatory mechanisms in the recipient

We next analyzed the effect of sCD83 graft pre-incubation on allogeneic immune response after corneal high-risk transplantation at different time points. Donor grafts were pre-incubated for 48 h ± sCD83, grafted into pre-vascularized recipient beds, and subsequently corneas as well as dLNs were isolated 1 or 2 weeks post-transplant (Figures 2A and 3A). One week post keratoplasty, flow cytometric analyses of dLNs-derived cells demonstrated significantly decreased frequency of CD11c⁺MHCI⁺CD80⁺ DCs in the sCD83 group (Figure 2B).

Two weeks posttransplant, qPCR analyses of corneal allografts, pre-incubated with sCD83, revealed significantly increased levels of Foxp3, Tgf-β, Il-27p28, Il-10 and a trend regarding Ido1 (Figure 3B). Expression levels of Il-10 and Ido1 (Figure 3D), as well as the frequencies of CD4⁺Foxp³ Tregs (Figure 3C), were significantly increased in dLNs. Animals with sCD83 pre-incubated grafts showed significantly increased numbers of CD11c⁺MHCI⁺CD200R⁺ DCs (Figure 3E). Furthermore, the frequencies of F4/80⁺CD86⁺ Mφ, were significantly decreased, while the percentage of F4/80⁺Msr-1⁺ Mφ was significantly increased in dLNs of recipients with sCD83 pre-incubated corneas (Figure 3F).

3.3 | sCD83 pre-incubated corneal allografts have a prolonged allograft survival in a high-risk transplantation model by long-term induction of Tregs

We evaluated whether pre-incubation of donor corneas with sCD83 could promote long-term allograft survival. Therefore, graft survival was assessed by weekly examination of corneas for up to 8 weeks (Figure 4A). Graft survival was significantly improved when grafts were pre-incubated with sCD83 (Figure 4B), and flow cytometric
(A) Suture Placement  | Corneal Transplantation  | Suture Removal
Day 0  | Day 12  | Day 14  | Day 21/1 week post transplantation  | Day 28/2 weeks post transplantation

Incubation of donor corneal xenograft with 50 μg/ml sCD83, 48h prior to transplantation

(B) mRNA

- Foxp3
- Tgfb
- IL-27p28
- IL-10
- Ido1

Draining lymph node

(C) T cells

- Foxp3

% of Foxp3 + T cells

(D) mRNA

- Tgfb
- IL-27p28
- Ido1
- IL-10

(E) DCs

- CD200R

(F) Macrophages

- CD86
- Msr-1

mock  | sCD83
Incubation of donor corneal allograft with 50 μg/ml sCD83, 48h prior to transplantation.

Corneal Graft Survival

Opacity Scores

draining lymph node

T cells

CD4+Foxp3+ Cells [%]
analyses of dLNs revealed significant increase in CD4⁺Foxp3⁺ regulatory T cells (Figure 4C). No differences were detected for DC and Mϕ phenotypes in dLNs between the two groups (data not shown).

In conclusion, our in vivo experiments reveal a new therapeutic option to manage corneal graft rejection. Pre-incubation of donor corneal tissue with sCD83 modulates the alloimmune response,

![Diagram](image-url)
FIGURE 6  sCD83 induces a regulatory AAM phenotype when present during Mφ differentiation promoting the induction of CD4+Foxp3+ T cells. (A) Experimental set-up of in vitro Mφ assays. (B) Assessment of supernatants of Mφ derived cultures by CBA, revealed increased levels of the AAM-associated cytokines CCL22 and CCL17. (C) After generation, Mφ were seeded into 24-well plates, containing fresh medium without the addition of sCD83. After resting, the protein expression of F4/80 (n = 32), CD11b (n = 32), Msr-1 (n = 17), CD83 (n = 10), CD86 (n = 32), Ox40L (n = 7), on F4/80+CD11b+ Mφ, was assessed via flow cytometry. (D) Analyses of the proliferative capacity of allogeneic splenocytes in coculture with mock- or sCD83-treated Mφ (left graph), and assessment of the corresponding cytokine secretion via CBA (right graphs, n = 7). (E) Analyses of different T cell subsets via flow cytometry (upper left graph, n = 4), and representative dot plots for CD4+Foxp3+ T cells (upper right graph). Experiments were performed at least three times. All graphs demonstrate mean ± SEM; statistical analysis was performed using an unpaired t-test (B-C, D) or two-way ANOVA (E). *p < .05; **p < .01*** p < .001. ****p < .0001 [Color figure can be viewed at wileyonlinelibrary.com]
**Figure 7** sCD83 modulates CAM as well as AAM and enables them to increase the frequency of CD4+Foxp3+ T cells in vitro. (A) Experimental set-up for the in vitro assays. Mφ were generated and sCD83 (25 µg/ml) was added or left out, on days 1 and 3 during Mφ generation. Mφ were either differentiated into CAM using IFNγ (300 U/ml), or into AAM using IL-4 (40 ng/ml), without further sCD83 administration overnight. Afterwards, surface molecule expression was analyzed by flow cytometry or cells were used for subsequent MLR assays. (B) Analyses of Mφ phenotype via flow cytometry. After stimulation of Mφ, the surface expression of F4/80 (n = 32), CD11b (n = 32), Msr-1 (n = 28), CD86 (n = 32), and OX40L (n = 9), was assessed on F4/80+CD11b+ CAMs or AAMs, via flow cytometry. (C) Bar graphs show the frequencies of different T cell subsets, derived from MLRs using either CAMs (upper bar graph) or AAM (lower bar graph), as APCs. Cells were gated on the CD4+ T cell population. Representative FACS dot plots (right site) show the frequencies of CD4+Foxp3+ T cells in co-cultures with CAMs (upper panel) or AAMs (lower panel). All experiments were performed at least three times. All graphs demonstrate mean ± SEM; Statistical analyses were performed using two-way ANOVA. *p < .05; **p < .01**p < .001. ****p < .0001 [Color figure can be viewed at wileyonlinelibrary.com]
(B) **Adoptive transfer - DCs**

**Corneal Graft Survival**

- iDCs mock
- iDCs sCD83

![Graph showing survival over weeks posttransplant for iDCs mock and iDCs sCD83.]

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(C) **Adoptive transfer - Mφ**

**Corneal Graft Survival**

- Mφ mock
- Mφ sCD83

![Graph showing survival over weeks posttransplant for Mφ mock and Mφ sCD83.]

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(D) **Restimulation assay**

**DCs**

- IFNγ
- Foxp3

**Mφ**

- IFNγ
- Foxp3

![Graphs showing relative mRNA expression for IFNγ and Foxp3 in DCs and Mφ for mock and sCD83 conditions.]

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induces long-term tolerance mechanisms and improves graft survival.

3.4 | Soluble CD83 modulates DCs toward a tolerogenic phenotype in vitro

Since sCD83 treatment of donor corneal tissue led to the modulation of DCs as well as MΦ in the recipient in vivo, we next investigated the mechanistic effect of sCD83 on these cells in vitro. Previous in vitro analyses revealed an inhibiting effect of sCD83 on DC maturation. In our study, we investigated the specific effect of sCD83 on BMDC differentiation (experimental set-up is depicted in Figure 5A).

We analyzed the phenotype of BMDCs via flow cytometry, directly after differentiation and frequencies of CD11c\(^+\)MHC\(_{\text{high}}\) cells were significantly reduced after sCD83-treatment (Figure 5B). Within the CD11c\(^+\)MHC\(_{\text{high}}\) population we detected a strong reduction of the co-stimulatory molecules CD86 and CD80, while the inhibitory receptors CD200R and Msr-1 were significantly upregulated (Figure 5C). Upon maturation, sCD83-treated BMDCs showed significantly decreased expression levels of CD25 and costimulatory molecules (Supplemental Figure 4). In MLRs with sCD83-treated mDCs, a decreased proliferative response of allogeneic T cells was observed (Figure 5D).

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FIGURE 8  Adoptive transfer of iDCs and MΦ differentiated in the presence of sCD83 improves corneal graft survival. (A) Experimental set-up of the adoptive transfer experiment. C57BL/6N-derived iDCs and MΦ were generated in the presence or absence of sCD83 (25 µg/ml). 1x10\(^6\) cells were injected into the tail vein of the recipient mice 7 days prior low-risk keratoplasty. Graft survival was determined by weekly opacity grading using a slit lamp. Corneal graft survival with corresponding opacity scores of mice receiving (B) iDCs (\(n = 15\) for sCD83-treated iDCs, \(n = 13\) for mock-treated iDCs) and (C) MΦ (\(n = 10\) each group). Data are represented as mean ± SEM. (D) mRNA expression of IFN\(_{\gamma}\) and Foxp3 in dLN cell suspensions after restimulation with ionomycin and PFA 8 weeks posttransplant. Data are represented as mean ± SEM. Statistical analyses were performed using Log-rank test (B+C), two-way ANOVA (B+C) or unpaired t-tests (D). * \(p < .05\); ** \(p < .01\) [Color figure can be viewed at wileyonlinelibrary.com]

FIGURE 9  Schematic overview of the sCD83-induced mode of action in donor induced corneal transplant tolerance. Sponge-like absorption of sCD83 by donor corneal allografts establishes a sCD83 protein reservoir, which can directly act on tissue-resident, as well as infiltrating responder cells, including MΦ and DCs. sCD83 pre-incubation of corneal tissues induces tolerogenic APCs (i.e., increased frequencies of CD11c\(^+\)CD200\(^R\) and F4/80\(^-\)CD11b\(^+\)Msr-1\(^+\) cells), within dLNs posttransplant. In this context, sCD83 induces graft tolerance by downregulating allogeneic T cell proliferation, TNF-α and IL-6 secretion, expansion of CD4\(^+\)Foxp3\(^+\) T cells and upregulation of immunoregulatory mediators, such as IL-10, Ido1, Tgf-β, IL-27p28, which are important to induce long-term tolerance mechanisms. Thus, we hypothesize that sCD83-pre-incubation of donor corneal allografts induces regulatory mechanisms, associated with resolution of inflammation and promotion of allograft acceptance. This represents a completely novel treatment approach in cornea transplantation [Color figure can be viewed at wileyonlinelibrary.com]
3.5 | sCD83 induces a regulatory Mφ phenotype and increases the frequency of Foxp3+ Tregs in MLRs in vitro

Our in vivo data revealed that sCD83 alters the phenotype of Mφ in dLNs two weeks posttransplant. Since no data were available regarding the effects of sCD83 on Mφ, we performed in vitro experiments (Figure 6A). When sCD83 was present during Mφ differentiation, a highly significant increased secretion of the AAM-associated chemokines CCL22 and CCL17 was detected in supernatants (Figure 6B). Flow cytometric analyses revealed that F4/80, CD11b, and Msr-1 were significantly upregulated on sCD83-treated Mφ, while CD83 and CD86 were significantly downregulated (Figure 6C). Functionally, we observed a strong reduction in allogeneic splenocyte proliferation in MLRs with sCD83-treated Mφ (Figure 6D). Analyses of cytokines present in MLR supernatants revealed significantly lower levels of TNF-α and IL-6 (Figure 6D). Next, we analyzed the presence of different T cell subsets within MLRs by flow cytometry and qPCR. We observed a significant increase in the frequency of CD4+FoxP3+ T cells in the sCD83 group, while other T cell subsets, including CD4+GATA3+, CD4+ RORɣT+, and CD4+T-BET+ cells, were not influenced (Figure 6E, left bar graph). In line with that, Foxp3 mRNA expression was significantly increased in MLRs with sCD83-treated Mφ. Expression of other T cell subset transcription factors, such as T-bet, RoRɣT, or Gata3, was not modulated (Figure 6E, right bar graph).

We further investigated the effect of sCD83 on Mφ polarization and analyzed the influence on CAM and/or AAM differentiation, as illustrated in Figure 7A. We confirmed the successful switch of Mφ into CAMs or AAMs, using IFNγ or IL-4, respectively, by flow cytometry (Supplemental Figure 5). Treatment of Mφ with sCD83 revealed a significant increase of F4/80 and CD11b on the surface, while CD83 and CD86 were significantly decreased compared to controls on both CAMs (Figure 7B, upper panel) and AAMs (Figure 7B, lower panel). Ox40L was significantly downregulated on sCD83-treated CAMs (Figure 7B upper panel), whilst MSR-1 was significantly increased on sCD83-treated AAMs (Figure 7B, lower panel). We next assessed sCD83-treated CAMs and AAMs to induce Tregs using MLR assays. Both CAMs as well as AAMs differentiated in presence of sCD83 significantly increased the frequency of CD4+FoxP3+ T cells compared to mock (Figure 7C, bar graphs and representative FACS plots). Flow cytometric analysis of Mφ phenotypes, after MLR assays, revealed significantly enhanced levels of F4/80 by both, CAMs and AAMs treated with sCD83, while CD40 and CD86 levels were reduced compared to controls (Supplemental Figure 6).

Conclusively, administration of sCD83 leads to a shift toward a regulatory phenotype in unstimulated Mφ, AAMs and even more surprising also in CAMs. This shift is characterized by reduced expression levels of T cell-activating molecules and higher frequencies of Tregs.

3.6 | Adoptive transfer of sCD83-treated DCs and Mφ suppress corneal allograft rejection

To further decipher the role of sCD83-treated DCs and Mφ in corneal transplantation, adoptive transfer experiments were performed (Figure 8A). DCs and Mφ were generated ± sCD83 from C57BL/6N mice and the sCD83-induced regulatory cell phenotype was confirmed by flow cytometry (Supplemental Figure 7). Adoptive transfer of both sCD83-treated DCs and Mφ significantly prolonged corneal graft survival compared to the mock-treated controls (Figure 8B,C). At the eight week end point of experiment we performed re-stimulation experiments with dLN cells and we detected less Ifnγ expression when animals were adoptively transferred with sCD83-treated Mφ. Concomitantly, significantly more Foxp3 expression was observed in restimulated dLN cells when mice were adoptively transfected with sCD83-treated DCs (Figure 8D).

4 | DISCUSSION

To improve the outcome of high-risk corneal transplants, alternatives, and supplements to treatment approaches primarily targeting the recipient and to non-specific immunosuppressive agents targeting host and recipient are urgently needed.

We and others have shown that the immunomodulatory properties of the sCD83 protein can prolong allograft survival in several transplantation models. Here, we report that the pre-incubation of donor corneal tissue with sCD83, represents a new therapeutic approach to prevent graft rejection without further treatment of the graft recipient. We demonstrated that pre-treatment of the corneal graft establishes a drug-depot that allows the diffusion or transport of the sCD83 molecule not only into the recipient graft bed but also to the dLNs (Figure 1), thereby inducing immune regulatory processes in the recipient.

A previous study showed that subconjunctival injection of TGF-β and IL-10 in the living donor prior to graft isolation induces tolerogenic DCs in the donor cornea and subsequently improves corneal graft survival after transplantation. Within our study, we demonstrate that ex vivo pre-incubation of donor corneal tissue with sCD83 induces the tolerogenic mediator Tgf-β in the transplant itself. This further supports the establishment of an immunoregulatory microenvironment prior to transplantation. Noteworthy and in line with these results, previous studies reported that the protective effects of sCD83 rely on the induction of Tgf-β expression.

A recent study showed that soluble antigen is transported from the corneal tissue via lymphatic vessels to the lymph node already after 30 min and is cleared within 6–8 h, whilst cell-associated proteins persist for longer and are detectable for up to 24 h. Myeloid cells in the LNs play a central role in sequestering particulate material as it moves from the afferent lymph into the subcapsular lymph node sinus. Consequently, using whole LN imaging, we show for the first time the co-localization of fluorescence-labeled sCD83 on F4/80+ Mφ, mostly located at the subcapsular sinus, the place of antigen capture. Furthermore CD11c+ DCs were localized with sCD83 in the diffuse cortex of the dLN, where migrating DCs drive T cell activation and initiate an alloimmune response. Insights regarding the dynamic bioavailability and locomotion of sCD83 within the graft and recipient’s graft bed have to be addressed in future studies.
In our in vivo experiments, we observed reduced frequencies of the costimulatory molecules on DCs and \( M_\Phi \) in dLNs, when corneas were pre-treated with sCD83. Low expression of costimulatory molecules results in diminished antigen-specific stimulation of T cells, induction of T cell tolerance and immune-regulatory cytokine expression,\(^{51,52} \) which is consistent with our results (Figures 2–4). Interestingly, we showed for the first time that DCs and \( M_\Phi \) in dLNs of mice receiving sCD83-pre-incubated grafts, upregulate the tolerogenic molecules CD200R and Msr-1. Different studies already confirmed a regulatory role of Msr-1 on DCs, since Msr-1-deficient DCs were more potent in stimulating T cells, via TLR4 activation.\(^{53,54} \)

Moreover, engagement of CD200R during DC generation gives rise to tolerogenic DCs.\(^{55} \) Upon injection of these DCs into mice, allogeneic tolerance for skin transplants was established accompanied by a significant increase in regulatory T cells, constituting \(-60\%\) of all infiltrating cells in rejected corneal allografts.\(^{10} \) To further substantiate our in vivo findings, we administered sCD83 during \( M_\Phi \) in vitro generation and detected significantly upregulated AAM-like cytokines such as CCL22 and CCL17, both very important recruiters for Tregs and thus for the resolution of inflammation.\(^{57,58} \) Furthermore, we showed that the on one hand sCD83 significantly enhances the expression of CD11b, F4/80, and Msr-1 and on the other hand, downregulates CD68 and OX40L. F4/80 is important for the establishment of peripheral tolerance and Treg induction by \( M_\Phi \), since F4/80\(^{+}\) mice are unable to induce Tregs in an anterior chamber associated immune deviation model.\(^{59} \)

Similar effects have been reported for the knock-out of CD11b.\(^{60} \) Consequently, we show that sCD83 induces regulatory \( M_\Phi \) which are able to induce Tregs in vitro. These data raised the question whether both or one of these in vitro generated regulatory cell types is able to improve corneal graft survival. The adoptive transfer of donor-derived sCD83 treated regulatory DCs and \( M_\Phi \) revealed no superior role of either cell type since the graft survival was improved to the same extent. Interestingly, in preclinical kidney transplantation studies it has been shown, that adoptively transferred AAM-like regulatory \( M_\Phi \) are able to expand CD4\(^{+}\)Foxp3\(^{+}\) T cells and control effector T cell immunity.\(^{15,17,61} \) Future studies are necessary to characterize the observed kinetics of Treg induction and donor-induced tolerance in more detail.

Conclusively, pre-incubation of donor grafts with sCD83 induces tolerogenic mechanisms in the recipient. This includes the induction of regulatory DC, \( M_\Phi \), and Tregs, which lead to improved corneal graft acceptance (Figure 9). Within this study, we report for the first time a new therapeutic concept of donor-mediated tolerance induction in the field of cornea transplantation, which possibly could also be transferred to other tissue/solid organ transplantation settings.

**ACKNOWLEDGMENTS**

Special thanks go to Manuel Koch who provided the labeled sCD83-Atto488 protein. Furthermore, we thank Andrea Deinzer for help with qPCR analyses. Moreover, we thank Prof. Dr. Jochen Mattner for providing the murine M-CSF producing cell line. This work was supported by the German Research Foundation (DFG), funding the research unit FOR2240 (www.FOR2240.de), projects BO4489/1-2, BO4489/3-1 (F.B.), Cu47/9-1 and Cu47/12-1 (C.C.), project ZI1225/1-1 (E.Z.), project STE432/15-1 (A.S.), and the CRC1181 project B03 (A.S.) and the Center for Molecular Medicine Cologne (CMMC) (F.B.; C.C.: A09). Multiphoton microscopy was performed on a Zeiss LSM880 NLO Intravital Microscope, funded by Deutsche Forschungsgemeinschaft (DFG, German Research Foundation) – project 261193037.

**DISCLOSURE**

The authors of this manuscript have no conflicts of interest to disclose as described by the *American Journal of Transplantation*.

**AUTHOR CONTRIBUTIONS**

K. Peckert-Maier and A. Schönberg designed, conducted and analyzed the majority of experiments and prepared the manuscript. A. B. Wild, L. Stich performed the experiments, analyzed the data and edited the manuscript. G. Braun and K. Hadrian performed the experiments. P. Tripal supported two-photon microscopy analyses. D. Royzman provided and generated essential reagents and edited the manuscript. C. Cursiefen and A. Steinkasserer provided scientific input and helped in experiments. B. Wild, L. Stich performed the experiments, analyzed the data and prepared the manuscript. All authors critically revised the manuscript and approved the final version.

**DATA AVAILABILITY STATEMENT**

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION
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How to cite this article: Peckert-Maier K, Schönberg A, Wild AB, et al. Pre-incubation of corneal donor tissue with sCD83 improves graft survival via the induction of alternatively activated macrophages and tolerogenic dendritic cells. Am J Transplant. 2022;22:438–454. https://doi.org/10.1111/ajt.16824