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Modulation of immune responses is one of the main research aims in transplant immunology. In this study, we investigate the local immunomodulatory properties of soluble CD83 (sCD83) at the graft-host interface using the high-risk corneal transplantation model. In this model, which mimics the inflammatory status and the preexisting vascularization of high-risk patients undergoing corneal transplantation, allogeneic donor corneas are transplanted onto sCD83-treated recipient animals. This model allows the direct and precise application of the immune modulator at the transplantation side. Interestingly, sCD83 was able to prolong graft survival after systemic application as well as after topical application, which is therapeutically more relevant. The therapeutic effect was accompanied by an increase in the frequency of regulatory T cells and was mediated by the immune-regulatory enzyme IDO and TGF-β. In vitro, sCD83 induced long-term IDO expression in both conventional and plasmacytoid dendritic cells via autocrine or paracrine production of TGF-β, a cytokine previously shown to be an essential mediator of IDO-dependent, long-term tolerance. These findings open new treatment avenues for local immune modulation after organ and tissue transplantation. The Journal of Immunology, 2013, 191: 1965–1975.

Immune-mediated allograft rejections remain the most common cause for graft failure after organ and tissue transplantation. There is an unmet medical need for pharmacologic strategies to promote graft survival without unduly compromising the health of the recipient (reviewed in Ref. 1). This also holds true for cornea transplants. More than 40,000 cornea transplants are performed per year in North America. Although the early outcomes of corneal allograft transplants are typically very good (90% survival at 1 y after surgery), the survival of penetrating corneal allografts at 15 y is only 55% (2). The situation is even more dramatic if the recipient cornea is inflamed because of chemical or thermal burns, herpes virus infections, or transplant rejection. In this case, the 1-y survival rate decreases below 50% (2, 3). The majority of clinical studies have shown that irreversible rejection is the most important cause of corneal graft failure (4, 5).

The gold standard to inhibit the immune reaction driving corneal graft failure is the use of corticosteroids. However, using these immunosuppressive agents topically or systemically leads to severe side effects, including cataract, glaucoma, conjunctival necrosis, and impaired wound healing (6). Others agents, such as cyclosporine A, have not shown a significant improvement of graft survival and lack good corneal penetration (7–9). Corneal allograft rejection is histologically characterized by massive infiltration of CD4+ T cells and macrophages. Significant numbers of neutrophils and NK cells (NK cells) were also found in the cellular infiltrates (10–14). Furthermore, CD11c+ CD11b+ myeloid dendritic cells (DCs) are present throughout the anterior stroma. Inflammatory stimuli determine the maturation stage of DCs and their immunogenic properties. It is known that an increasing number of MHC class II–bearing, Ag-specific DCs in cervical draining lymph nodes arises only a few hours after transplantation (14). This finding implies that DCs at different maturation stages might be the main initiators of this devastating tissue destruction (14–18).

The expression of costimulatory membrane-bound ligands, such as CD80 and CD86, regulating T cell activation determines the immune-regulatory capacity of DCs, among other factors (19, 20). The membrane-bound glycoprotein CD83 (mCD83) is a well-known surface marker for mature DCs, in humans and mice (21, 22). On mature DCs, mCD83 acts as a costimulatory molecule, thereby increasing T cell stimulation (23, 24).

In addition, Hock et al. (25) reported the existence of a soluble form of CD83 (sCD83) released by activated DCs and B cells; it was also detectable in normal human sera and, in increased concentrations, in sera of patients with hematologic malignancies (26). A direct correlation between increased sCD83 levels and treatment-free survival has been observed in patients with chronic...
lymphocytic leukemia, indicating that CD83 might modulate antitumoral immune responses (27). In subsequent studies using recombinant CD83 molecules, it could be shown that CD83 has prominent immunosuppressive properties. We and others reported that recombinant CD83 inhibits DC maturation (28, 29) and blocks T cell stimulation (28, 30). It has also been reported that CD83 is a regulator of B cell function (31, 32).

Using the experimental autoimmune encephalomyelitis (EAE) model in vivo, we showed that prophylactic and therapeutic CD83-treatment prevents paralysis associated with EAE (33). Because animals were also protected from a second EAE relapse without an additional CD83 administration, CD83-specific and long-term tolerogenic mechanisms should have been induced (33). In addition, it has been reported that CD83 could modulate transplant-related immune responses in murine models of allograft transplantation (29, 34, 35).

Thus, considering the failure of current therapeutics in preventing corneal transplant rejection and, on the other hand, the long-lasting tolerogenic effects of CD83, we investigated CD83-specific effects in the cornea transplantation model (36). To our knowledge, we show for the first time that CD83 suppresses corneal graft rejection by systemic and, therapeutically more importantly, by topical application in the form of eye drops. In both cases, the CD83 tolerogenic effect was clearly associated with an increase of regulatory T cells in the eye-draining lymph nodes. Furthermore, by means of in vivo and in vitro approaches, we demonstrate thatIDO and TGF-β are major mediators of immune regulation by topical CD83 treatment. These findings open interesting new therapeutic options in the field of cornea transplantation and for other solid organ and tissue transplantation studies that require long-term immunosuppression.

Materials and Methods

Animals

Six- to 8-week-old female BALB/c mice (Charles River Germany, Sulzfeld, Germany) were used for vessel-inducing suturing of the corneal stroma and further served as recipients for corneal transplantation. Donor buttons were generated by trephination of corneas from 6–8-week-old female C57BL/6 mice. No animal showed any symptoms suggesting of systemic disease because of the experiment. For surgical procedures, mice were anesthetized using a mixture of ketamine and xylazine (120 mg/kg and 20 mg/kg body weight, respectively).

Corneal transplantation

For the high-risk setting, corneal stromas of 6–8-week-old BALB/c mice were sutured with three interrupted 11-0 nylon sutures (Serag Wiessner, Nailsa, Germany). The sutures were left in place for 2 wk. Penetrating corneal transplantation was performed after suture removal. Keratoplasty was performed as described previously (37). Donor corneas were excised by trephination using a 2-mm bore, and cut with curved Vannas scissors. Until grafting, corneal tissue was placed in chilled PBS. Recipients were anesthetized using a mixture of ketamine and xylazine (120 mg/kg and 20 mg/kg body weight, respectively).

Recipient sCD83 (16.7 kDa) was generated as described previously (38, 39). Recipients receiving the systemic application were injected i.p. with 100 μg sCD83 1 d before corneal transplantation and subsequently every second day for 14 d. The pellets containing 1-methyl-tryptophan (1-MT; the gold standard inhibitor of IDO catalytic activity; Innovative Research of America) were implanted immediately after cornea transplantation. The pellets released the agent for 2 wk and were left in place until the end of the experiment to minimize traumatic effects.Mock-treated animals received the same volume of PBS. Recipients from the topical application group received one drop of sCD83 (corresponding to 7.5 μg/3 μl) three times a day for 15 d, starting 1 d before cornea transplantation. Mock-treated animals received the same volume of PBS for the same time period. For local treatment with 1-MT, recipient mice received sCD83 (6 μg/3 μl) in the form of eye drops, three times a day (corresponding to 18 μg/d) starting 1 d before transplantation and every day afterward (for a total of 15 d). For the systemic treatment with the anti-TGF-β–Ab (a-TGF–β, clone 1D11, reactive to mouse TGF-β1, TGF-β2, and TGF-β3; Bioceres, Utrecht, the Netherlands) mice received 150 μg a-TGF–β in 200 μl PBS i.p. the day before transplantation and four times every other day afterward. Mock-treated animals received 100 μl PBS. For local treatment, mice received the a-TGF–β (15 μg per 6 μl per mouse, three times per day) the day before transplantation and four times every other day afterward. Treatment with recombinant TGF-β was performed as described previously (40), applying 200 ng/ml of recombinant TGF-β (three times per day, 3 μl per drop; PeproTech) on the day before transplantation and 14 d postoperatively.

RT-PCR

Total RNA was prepared from cornea and draining neck lymph nodes using the RNeasy Plus Mini Kit (Qiagen). Traces of genomic DNA were removed by DNase digestion with the RNase-free DNase Set (Qiagen). Subsequently, 1 μg RNA was reverse transcribed into c cDNA using the First Strand cDNA Synthesis Kit (Fermentas) as specified by the manufacturer. Real-time RT-PCR was performed using the LightCycler 2.0 (Roche Diagnostics, Penzberg, Germany) and specific primers for Hprt (5′-gtgatgacgagcaagcctgt-3′ and 5′-gattaaccttgctctctctag-3′), Ido1 (5′-cggaagaggacacagactgt-3′ and 5′-acataacctggtatgtatagct-3′), and Foxp3 (5′-ccggaaagacagacacattctt-3′ and 5′-tggcttccttcctcaggg-3′). The levels of gene expression normalized to Hprt were calculated using relative quantification (∆∆Ct) study software (LightCycler Software 4.05).

Restimulation assay

Lymphocytes (2.5 × 10^6 cells) from spleen, draining, and contralateral nondonor draining neck lymph nodes were removed from sCD83 and mock-treated responder BALB/c mice. These experiments were performed with 2 or 8 wk after corneal transplantation. Cells were seeded into 96-well flat-bottom plates (Falcon) in R10 Medium (see bone marrow–derived DC [BMDC] culture methods) either alone or cocultured with irradiated C57/BL6 stimulator spleen cells (2.5 × 10^5) for 72 h at 37°C. Cells were additionally stimulated with exogenous IL-2 (200 U/ml; PeproTech). To analyze their proliferation capacity, cells were subsequently pulsed with [3H]-thymidine (1 μCi/well; PerkinElmer, Rodgau, Germany) for 8–16 h. Culture supernatants were harvested onto Glass Fiber FilterMates using an ICH-110 harvester (Inotech, Dottikon, Switzerland), and filters were counted in a 1450-microplate (Wallac, Turku, Finland). Supernatants of cultures were used to determine cytokine secretion.

Conventional and plasmacytoid DC cultures

DCs were isolated from the spleen or a pool of lymph nodes (cervical, axillary, inguinal, and mesenteric) by means of MACS using CD11c MicroBeads and MidiMacs (Miltenyi Biotech) in the presence of EDTA to disrupt DC–T cell complexes, as described previously (41). For the purification of conventional (cDCs) and plasmacytoid DCs (pDCs), CD11c+ cells were further fractionated using CD11b and mPDCA-1 MicroBeads (Miltenyi Biotech), respectively.

BMDC cultures

BMDCs were generated from murine bone marrow precursors in RPMI 1640 medium containing 10% FCS (R10) as described previously (42). Bone marrow precursor cells were cultured for 8 d in R10 medium at a starting density of 2 × 10^6 cells per 10-cm dish (Falcon) in a humidified 5% CO_2 incubator at 37°C. BMDCs were generated from murine bone marrow precursors in RPMI 1640 medium containing 10% FCS (R10) as described previously (42). Bone marrow precursor cells were cultured for 8 d in R10 medium at a starting density of 2 × 10^6 cells per 10-cm dish (Falcon) in a humidified 5% CO_2 incubator at 37°C. BMDCs were generated from murine bone marrow precursors in RPMI 1640 medium containing 10% FCS (R10) as described previously (42). Bone marrow precursor cells were cultured for 8 d in R10 medium at a starting density of 2 × 10^6 cells per 10-cm dish (Falcon) in a humidified 5% CO_2 incubator at 37°C.

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6, and cells were fed again with fresh 10 ml of 1:10 diluted R10-medium containing GM-CSF supernatant. At day 9, cells were used for the different experiments.

**DC–T cell cocultures**

On day 9, BMDC cultures were harvested and matured at a density of $3 \times 10^6$ cells/ml R10 culture medium in a 96-well plate for 24 h with TNF (500 U/ml; PeproTech). Simultaneously with the maturation stimulus, BMDCs were also inoculated with increasing concentrations of sCD83 or were left untreated (mock). These cells were then cocultured with T cells ($4 \times 10^5$/ml) derived from BALB/c mice for additional 120 h. Cells were then analyzed with FACS, and supernatants were used to determine cytokine secretion.

**FACS analyses**

Following maturation, cells were analyzed using flow cytometry. Directly fluorochrome-conjugated mAbs specific for surface staining of CD4 (clone RM-4-5 PerCP) or CD25 (clone 7D4 FITC) were purchased from BD Pharmingen. For surface staining of splenic and lymph node cells, CD3 (clone 500-A2 Pacific Blue; BD Pharmingen) and anti-mouse CD4 (clone RM-4-5 APC; eBioscience) were used. Cell surface staining was performed for 30 min on ice using a standard method described previously (42). Samples were analyzed by a FACSscan flow cytometer using the FlowJo analysis software (Tree Star). Nonviable cells were gated out on the basis of their light scatter properties. Cells were gated on lymphocytes and CD4" cells. Intracellular Foxp3 staining was performed according to the manufacturer’s instructions (Foxp3 anti-mouse/rat [Clone FJK-16S PE], Foxp3 staining set; eBioscience). Samples were analyzed with a FACSscan flow cytometer (BD Pharmingen) using FlowJo analysis software.

**Cytotoxic analyses of intracellular IDO and membrane TGF-β1**

Lymph node DCs were first treated with rat anti-CD16/32 (2.4G2) for 30 min at 4°C for the blockade of Fc receptors before assaying on an EPICS flow cytometer using EXPO 32 ADC software (Beckman Coulter). For detection of intracellular IDO, DCs were stained with PE-conjugated anti-CD11c (clone HL3; BD Pharmingen), fixed, permeabilized, and sequentially incubated with a rabbit anti-mouse monoclonal IDO (cv152) or isotype control rabbit IgG, and Alexa Fluor 488 F(ab)₂ fragment of goat anti-rabbit IgG (H+L; Invitrogen, Molecular Probes) as described previously (41). Purified CD4" T cells were costained with anti-mouse CD4 (clone GK1.5 PE; BD Pharmingen) and anti-human TGF–β1 (clone TWA-2F8 FITC, cross-reacting with mouse TGF–β1; Biolegend).

**Cytometric bead array**

Determination of inflammatory cytokines secreted by DC–T cell cocultures and restimulation assays were assessed by a mouse inflammatory cytometric bead array (BD Biosciences) according to the manufacturer’s protocol.

**Luciferase assay**

The plasmid mIDOprom900-luc, which contains the mouse Ido1 promoter (900 bp) and 70 nucleotides of noncoding sequence in Ido1 exon 1 upstream of the firefly luciferase coding sequence, was transferred by electroporation into DCs as described previously (44). The Renilla reporter plasmid pRL-TK (Promega) was transferred by electroporation as an internal control of the transfection process. Luciferase activity was assayed with the Dual Luciferase Reporter Assay Kit (Promega). For the neutralization of TGF–β1 in vitro, an affinity-purified mAb (1D11) to TGF–β1 was used at a final concentration of 40 μg/ml.

**Statistics**

Statistical calculation was performed with GraphPad Prism. Kaplan–Meier analysis was used for survival of the investigated groups. Restimulation capacity and cytokine production was assessed with ANOVA. Relative expression of RT-PCR analyses and frequency of IDO expression in cDCs was assessed using the Student t test; p < 0.05 was considered significant.

**Results**

**Systemic sCD83 treatment inhibits corneal allograft rejection and induces IDO and Foxp3 expression during systemic application**

To investigate whether sCD83-treatment could have a beneficial effect on the survival of transplanted corneas in the fully mismatched high-risk transplantation model, we first administered sCD83 i.p. to BALB/c mice as recipients ($n = 10$) of allogeneic graft transplants (C57/BL6) after suture-induced inflammation (Fig. 1A). This is a good defined approach to overwrite the immune privilege of the cornea and analyze rejection processes (36). Compared with mock-treated animals, the systemic injection of sCD83 resulted in a significantly increased graft acceptance (Fig. 1B).

IDO is recognized as an authentic regulator of immunity in several physiopathologic conditions, including autoimmunity, allergy, chronic inflammation, viral infection, transplantation, and neoplasia (45, 46). IDO immunoregulatory effects are mainly mediated by DCs and involve both tryptophan deprivation and production of kynurenine. As a result, IDO-expressing DCs mediate multiple effects on T lymphocytes, including inhibition of proliferation, apoptosis, and differentiation toward a regulatory Foxp3" T cell phenotype (47). To elucidate the mechanistic effects of systemic sCD83 treatment of high-risk corneal graft transplantation, we investigated the expression of IDO1 and Foxp3 as...
markers for immune regulatory processes. Thus, the mRNA levels in the transplanted corneal grafts and in the draining submandibular, superficial cervical, and internal jugular lymph nodes (neck lymph nodes) were analyzed. Corneas and lymph nodes were harvested at the end of the experiment (i.e., 8 wk after corneal transplantation; \(n = 10\)). By means of RT-PCR, we found a 30-fold increased IDO1-expression in corneas of mice treated with sCD83 compared with the mock-treated animals (Fig. 1C, left panel). IDO1 expression was also detectable in draining lymph nodes (Fig. 1C, right panel). Furthermore, the expression of Foxp3 was significantly upregulated in corneas and in lymph nodes of the sCD83-treated group compared with mock-treated mice (Fig. 1C, right panel). From these findings, we concluded that sCD83-treatment increases IDO1 and Foxp3 expression in mice subjected to allogeneic cornea transplantation. Expression of IDO2 was not detectable in any of the analyzed animals (data not shown).

**IDO plays a key role in sCD83-mediated immunosuppression**

To ascertain whether IDO has a major role in the immunoregulatory effects exerted by sCD83 in the cornea transplantation model, we administered 1-MT to mice in the form of slow-release pellets (Fig. 2A). Analyzing the survival rate, we found that the sCD83-treated group has a highly significant, better outcome compared with both, the sCD83 plus 1-MT treated group (\(p < 0.0006\)) and the mock treated group (\(p < 0.0003\)) (Fig. 2B). Thus, our data indicate that sCD83 induces long-term graft survival and that IDO activity is a critical requirement for the immunosuppressive effects exerted in vivo.

**Topical sCD83-treatment leads to increased corneal graft tolerance**

Data reported above demonstrate the proof of principle that sCD83 improves graft survival when administered systemically similar as in solid organ transplant models (29, 34, 35, 48). However, regarding a possible therapeutic application of scCD83 also for humans, topical application would be highly advantageous in comparison with systemic administration. Therefore, we applied sCD83 directly at the graft-host interface in the form of eye drops. After high-risk corneal transplantation with allogeneic C57/BL6 donor cornea onto BALB/c recipient mice, sCD83 was dripped three times a day for two weeks (Fig. 3A). The mock-treated animal group received the same volume of PBS. Mice were scored for a period of 8 wk in terms of graft rejection. As shown in Fig. 3B, administration of sCD83 reduced the rejection of allografts significantly even at 8 wk after transplantation. In contrast, in the mock-treated group almost 70% of the animals rejected their graft already at 2 wk after transplantation (\(p < 0.025\)). Thus, our data suggest that sCD83 has also a potent immunosuppressive effect when applied topically, directly at the graft-host interface.

To obtain further insight regarding the cellular mechanisms leading to the observed increased graft survival, we first analyzed the Ag-specific T cell response in the recipient mice 8 wk after transplantation. sCD83-treated and mock-treated BALB/c animals were sacrificed after allogeneic transplantation with C57/BL6 corneas, and single-cell suspensions of spleens and neck lymph nodes were prepared (\(n = 7\)). These cells were used as responders and were cocultured with irradiated C57/BL6 spleen cells, which act as allogeneic stimulators. As shown in Fig. 3C, the proliferation capacity of lymph node cells, derived from sCD83-treated mice, is significantly reduced when compared with the mock saline-treated group, in response to donor Ags in the presence of exogenous IL-2. Interestingly, proliferation in the presence of exogenous IL-2 alone was still visible in both treatment groups, indicating that sCD83 treatment does not lead to general T cell deletion or anergy. The reduced proliferation capacity could be observed only in lymph nodes but not in cultures of restimulated splenic cells derived from different treatment groups (Fig. 3D). These data indicate that specificity to donor Ag is restricted to the site of action; therefore, sCD83-treated animals are not generally immune suppressed.

Because sCD83-mediated immunosuppression in lymph nodes was detectable even 8 wk after allogeneic transplantation, we hypothesized that tolerance induction is long lasting and speculated that sCD83 influences the frequency of Foxp3-expressing T cells. Therefore, we analyzed the expression of CD4^+Foxp3^ T cells prepared from spleen and neck lymph nodes of sCD83- and mock-treated mice. Using flow cytometric analyses, we demonstrated that topical sCD83-treatment leads to an increased frequency of CD4^+Foxp3^ regulatory T cells in the draining lymph nodes compared with mock-treated mice (Fig. 3E, upper panel). Again, this effect was not detectable in splenic cells (Fig. 3E, lower panel).

**Topical sCD83 administration leads to IDO expression and reduction of Ag-specific proliferation**

In the experiments described in this study, the immune status was assessed 8 wk after transplantation. Next, we asked whether immunomodulatory or suppressing effects could already be induced at an earlier time point. First, we examined whether sCD83 treatment could affect the percentage of IDO^+ cells in cDCs (CD11c^{high}) and pDCs (CD11c^{dim}) purified from the spleen and lymph nodes of transplanted mice 2 wk after transplantation, a time point that corresponds to the end of sCD83 treatment. No significant increase could be observed in the number of IDO^+ cells of cDCs or pDCs purified from spleens or of pDCs purified from lymph nodes of sCD83 treated mice compared with mock-treated animals (data not
shown). However, we found a significant increase in the percentages of cDCs expressing IDO in the draining lymph nodes of transplanted mice on sCD83 treatment compared with controls (Fig. 4A; \( n = 6 \)). Interestingly, at the same time after transplantation, the sCD83 treatment also significantly increased the percentage of TGF-\( \beta \)1+ cells in the CD4+ T cell population purified from eye-draining lymph nodes (Fig. 4B). As a result, our data indicate that sCD83 induces early immunoregulatory effectors in the DC (i.e., IDO) and in the CD4+ cell (i.e., TGF-\( \beta \)1) compartment that could have a major role in the control of transplant rejection.

To investigate this finding further, we assessed T cell responses to donor Ags at this earlier time point. Spleen and neck lymph nodes from the recipient BALB/c mice were removed postmortem, and single-cell suspensions were prepared. These responder cells from sCD83- and mock-treated animals (\( n = 7 \)) were cultured with irradiated C57/BL6 splenic stimulator cells. As shown in Fig. 4C (left panel), immune responses to C57/BL6 alloantigen were suppressed in lymph node cultures derived from animals topically treated with sCD83. Although exogenous IL-2 partially restored the reduced proliferative capacity to donor Ags, the proliferation response was nevertheless significantly suppressed. The right panel in Fig. 4C shows the percent suppression of three independent experiments normalized to mock controls.

We next assessed the cytokine production in these restimulated cell cultures. As shown in Fig. 4D, IFN-\( \gamma \) production was strongly reduced in sCD83-treated animals, and this reduced secretion could not be restored by exogenous IL-2. In the case of TNF-\( \alpha \) and IL-6, a significant reduction could be observed in the presence of IL-2. Production of IL-17A, IL-10, and TGF-\( \beta \) was low or could not be detected (data not shown). Interestingly, the observed reduction in Ag-specific responses was unique to draining lymph node cells, because restimulation of splenic cells did not reveal a significantly reduced proliferation capacity (Fig. 4E). Interestingly, local sCD83 treatment did not induce these tolerogenic effects in the contralateral lymph nodes (Fig. 4F). From contralateral eyes, which were left untreated, lymph nodes were removed and restimulated with donor Ag; however, these cocultures showed almost no proliferation. In addition, cytokine production was not detectable in these cocultures (Fig. 4G).

In vitro sCD83-treatment leads to an increased frequency of Foxp3+ regulatory T cells

The experiments described above clearly showed that sCD83 acts directly at the graft-host interface and induces an immunosuppressive environment leading to prolonged allograft survival. To mimic this situation in vitro and to gain further mechanistic insights, we next assessed the cytokine production in these restimulated cell cultures. As shown in Fig. 4D, IFN-\( \gamma \) production was strongly reduced in sCD83-treated animals, and this reduced secretion could not be restored by exogenous IL-2. In the case of TNF-\( \alpha \) and IL-6, a significant reduction could be observed in the presence of IL-2. Production of IL-17A, IL-10, and TGF-\( \beta \) was low or could not be detected (data not shown). Interestingly, the observed reduction in Ag-specific responses was unique to draining lymph node cells, because restimulation of splenic cells did not reveal a significantly reduced proliferation capacity (Fig. 4E). Interestingly, local sCD83 treatment did not induce these tolerogenic effects in the contralateral lymph nodes (Fig. 4F). From contralateral eyes, which were left untreated, lymph nodes were removed and restimulated with donor Ag; however, these cocultures showed almost no proliferation. In addition, cytokine production was not detectable in these cocultures (Fig. 4G).
FIGURE 4. Topical application of sCD83 induces IDO expression and a recipient-specific T cell hyporesponsiveness to donor alloantigen in the draining lymph node, but not in the spleen. (A) Frequency of IDO expression in cDCs purified from lymph nodes of mice 2 wk after transplantation (left side, means ± SEM of three experiments, each performed in triplicates; right side, dot plots of one representative experiment). Numbers in the top right quadrants indicate the percentage of IDO+ cells, which are also CD11c+. (B) Frequency of membrane TGF-β1 expression in CD4+ T cells purified from lymph nodes as in (A) (left side, means ± SEM of three experiments, each performed in triplicates; right side, dot plots of one representative experiment). Numbers in top right quadrants indicate the percentage of CD4+TGF-β1+ cells. (C) Left panel, Restimulation assays were performed 2 wk (Figure legend continues)
we performed experiments with allogeneic DC–T cell cocultures. C57/BL6-derived bone marrow DCs were cultured in the presence of increasing concentrations of sCD83, matured, and cocultivated afterward with allogeneic T cells from BALB/c mice for 120 h. These cocultures were then analyzed by FACS for the presence or expansion of CD4+CD25+Foxp3+ cells. DC–T cell cocultures, which were incubated with sCD83, exhibited a clear increase of CD4+CD25+Foxp3+ cells (Fig. 5A, upper panel). In contrast, T cells cultured alone, regardless of sCD83 presence, did not show an increase in CD4+CD25+Foxp3+ cells (Fig. 5A, lower panel). 

As depicted in Fig. 5B, the relative mean of Foxp3 expression (of three independent experiments) is elevated in the sCD83-treated groups. These data indicate that the presence of sCD83 during DC maturation leads to the induction or expansion of Foxp3-expressing T cells, possibly reducing the functionality of effector T cells.

We next examined whether this was indeed the case by means of assessing the production of key inflammatory cytokines. Secretion of the proinflammatory cytokines IFN-γ, IL-17A, and IL-6 was strongly reduced in cultures in which sCD83 was present (Fig. 5C). In contrast, production of immunosuppressive TGF-β was increased in the presence of sCD83 (Fig. 5C, right lower panel).

sCD83 induces significant Ido1 promoter activity

Recently, Ido1 has also been shown to possess a signal-transducing activity in pDCs that leads to long-term expression of Ido1 and immune tolerance in vivo (44). This effect is triggered by TGF-β and requires both autocrine and paracrine production of TGF-β by pDCs. We therefore investigated whether the previously observed sCD83-mediated, endogenous TGF-β production could induce Ido1 expression in DCs in vitro. For this purpose, cDCs and pDCs were purified from the spleen of naive mice and transfected with a plasmid construct containing the mouse Ido1 promoter upstream of the luciferase gene (44), with prior incubation with different concentrations of sCD83 for short (3 h) and long (24–48 h) periods in the presence or absence of a neutralizing a-TGF–β. TGF-β and medium alone were used as positive and negative control stimuli, respectively. An isotype mouse Ig was used as a control for TGF-β neutralization. Fig. 6A shows that both recombinant TGF-β and sCD83 significantly induced Ido1 promoter activity at late (24–48 h) but not at early (3 h) time periods in pDCs and cDCs, although to a lesser extent. The addition of the TGF-β neutralizing Ab, but not of the control isotype, significantly inhibited sCD83-induced Ido1 promoter activity (Fig. 6B).

Our data indicate that sCD83 induces TGF-β in cDCs and pDCs, and this effect in turn drives long-term Ido1 expression, which is necessary for the long-lasting effects of immunoregulatory agents in transplantation.

Role of TGF-β in high-risk cornea transplantation

To confirm the role of TGF-β in sCD83-mediated immune regulation in vivo, we performed additional high-risk corneal transplantations. This time, however, the topical sCD83-treatment was combined with the topical application of a neutralizing a-TGF–β.

In a comparison with the sCD83-treated group, TGF-β-blockade in sCD83-treated animals resulted in a clearly reduced graft survival rate (Fig. 7A). In addition, topical coadministration of 1-MT together with sCD83 strongly reduced sCD83-mediated cornea graft survival comparable to the systemic effect of 1-MT (Fig. 2).

To elucidate and confirm the in vitro findings regarding the role of TGF-β, we performed transplantation experiments using recombinant human TGF-β as a single topical treatment agent. As shown in Fig. 7B, TGF-β-treatment also leads to an increased graft survival. As already observed in the case of sCD83, the protective effect could be reversed effectively by the coadministration of 1-MT (Fig. 7B). Taken together, these data demonstrate that TGF-β also has an important role in the sCD83-mediated immune deviation in vivo.

Discussion

The challenge of graft rejection in solid organ and tissue transplantation is a demanding topic for surgeons and patients. Current treatments use a general immunosuppressive regimen, which makes the patient highly vulnerable to common and easily defendable pathogens and usually has to be taken lifelong with severe side effects. Thus, there is medical need for more specific, locally acting compounds with fewer side effects. In this study, we used the high-risk model of corneal transplantation because of the easy accessibility of the host interface and the insufficient treatment options for high-risk patients undergoing cornea transplantation. In initial experiments, we tested the therapeutic efficacy of a systemic treatment with sCD83 to improve graft survival in this model. We observed a significant increase in graft survival, providing the proof of principle for a possible therapeutic use of sCD83. In addition, we found that the therapeutic effect of sCD83 was accompanied by the significantly increased expression of the immune-modulatory enzyme Ido1 in the cornea as well as in draining lymph nodes.

These data correlate well with a previous study reporting that systemic application of sCD83 prevents kidney allograft rejection and inhibition of Ido1 by 1-MT, which is a gold standard inhibitor of the catalytic function of Ido1, reverts graft acceptance (48). However, sCD83-induced Ido1 expression in the transplanted organ and expansion of Foxp3+ cells in draining lymph nodes of sCD83-treated recipients has not been shown previously. As in the abovementioned work by Lan et al. (48), we could also demonstrate the critical involvement of Ido1 in sCD83-mediated effects using 1-MT, which completely ablated the in vivo immunoregulatory effects of sCD83 in this cornea transplantation model. Thus, sCD83 treatment can induce a cascade of Ido1-dependent tolerogenic mechanisms, which eventually led to the induction and expansion of regulatory T cells, needed for long-term surveillance of adaptive immunity.

Regarding the functional importance of Ido1 in cornea transplantation, Beutelspacher et al. (49) have shown that overexpression of Ido1 in murine corneas prolongs allograft survival. Furthermore, the topical and systemic application of the tryptophan catabolite, 3-hydroxykynurenine, and the tryptophan metabolite analog N-(3,4-dimethoxyxycinnamonyl)anthranilic acid prevents allograft rejection (50, 51), emphasizing the important role of Ido1 in the cornea transplantation.

Study of the effects of sCD83 have far focused on the systemic application form, but the site of action has never been investigated in detail. Therefore, we wanted to determine whether sCD83...
application at the graft-host interface (i.e., topically) also induces IDO-mediated immune regulatory effects. Because of the molecule size of 16.7 kDa, sCD83 is able to penetrate the corneal stroma and is therefore suitable for topical treatment, because it has been shown that molecules up to 56 kDa can penetrate a healthy cornea and that larger molecules (up to 149 kDa) can be used to penetrate the inflamed cornea (52, 53). The great advantage of topical drug administration to the cornea is 1) easy deposition of the drug by the patient himself, 2) the reduction of drug concentration, and 3) reduction of possible side effects, which can occur via systemic application.

The protein was applied topically on the grafted cornea. Eight weeks after transplantation, a significantly increased graft survival was observed in the group treated topically with sCD83. Interestingly, T cells isolated from the draining lymph nodes of sCD83-treated mice had a significantly lower response capacity, whereas cells derived from the spleen did not show such an increase (lower panel). The data shown here represent one representative experiment of three. (B) For better comparison, values of Foxp3 expressing cells were normalized to DC–T cell cultures in the absence of sCD83 (corresponding to 100%). Relative mean of Foxp3 (%) of three independent experiments is shown. Values are ± SEM. (C) Supernatants of these cocultures were also examined for their content of IFN-γ, IL-6, IL-17A, and TGF-β. A reduced secretion of IFN-γ, IL-17A, and IL-6 was observed in the sCD83-treated cocultures. TGF-β expression, however, was clearly enhanced in cultures where sCD83 was present. Data represent the average of three independent experiments (±SEM).

FIGURE 5. In vitro treatment with sCD83 of DC–T cell cocultures leads to an increased frequency of Foxp3-expressing T cells and a modulation of inflammatory cytokines. (A) In DC–T cell cocultures, where DCs were pretreated with sCD83, CD4+CD25+ Foxp3+ T cells were clearly increased (upper panel). In contrast, T cells alone did not show such an increase (lower panel). The data shown here represent one representative experiment of three. (B) For better comparison, values of Foxp3 expressing cells were normalized to DC–T cell cultures in the absence of sCD83 (corresponding to 100%). Relative mean of Foxp3 (%) of three independent experiments is shown. Values are ± SEM. (C) Supernatants of these cocultures were also examined for their content of IFN-γ, IL-6, IL-17A, and TGF-β. A reduced secretion of IFN-γ, IL-17A, and IL-6 was observed in the sCD83-treated cocultures. TGF-β expression, however, was clearly enhanced in cultures where sCD83 was present. Data represent the average of three independent experiments (±SEM).
expression by the endogenous production of TGF-β-luciferase gene, we investigated whether sCD83 can induce IDO. sCD83 induced significant IDO activity in DCs transfected with a firefly luciferase construct containing the *Ido1* promoter. Cells were incubated for 3–48 h with TGF-β (20 ng/ml) or different concentrations of sCD83 as indicated. (B) Neutralizing a-TGF–β (40 μg/ml) or an isotype control (None) was added in combination with sCD83 (50 μg/ml) to 24 h cultures of cDCs and pDCs. Results are normalized to the activity of a cotransfected constitutive reporter and are presented relative to those in untreated cells (dashed line, 1-fold). Data are mean ± SD of three experiments, each performed in triplicate. *p < 0.01, **p < 0.001.

It has been shown recently that, in addition to its enzymatic function, IDO has a crucial role in the sCD83-mediated immunosuppressive effect, by downmodulating harmful inflammatory responses (56). TGF-β, however, has been shown to induce long-lasting regulatory properties mediated by the induction or expansion of regulatory T cells (57). The long-term prevention of graft rejection observed in sCD83-treated animals further suggests a TGF-β-mediated regulatory effect.

To address this question further, in vitro studies were performed showing that sCD83 increases the number of CD4<sup>+</CD25<sup>5<sup>Foxp3<sup> T cells in DC–T cell cocultures. Interestingly, this effect could not be observed when T cells were cultured in the absence of DCs, underlining the fact that IDO-specific effects are mainly mediated by DCs. In line with the immunosuppressive, IFN-γ, IL-17A, and IL-6 expression were reduced in DC–T cell cocultures. In contrast, TGF-β expression was increased when DCs and T cells were present at the same time.

It has been reported that under inflammatory conditions, such as in the context of microbial infections, IDO is rapidly induced by IFN-γ to downmodulate harmful inflammatory responses (56). TGF-β, however, has been shown to induce long-lasting regulatory properties mediated by the induction or expansion of regulatory T cells (57). The long-term prevention of graft rejection observed in sCD83-treated animals further suggests a TGF-β-mediated regulatory effect.

An important finding was that sCD83 causes IDO-mediated immunosuppressive effects in vivo (44). Interestingly, this effect is triggered by TGF-β and requires both autocrine and paracrine production of TGF-β by pDCs. Using an in vitro transfection system, including a plasmid construct containing the mouse *Ido1* promoter upstream of the luciferase gene, we investigated whether sCD83 can induce IDO expression by the endogenous production of TGF-β. Interestingly, sCD83 induced significant *Ido1* promoter activity at late (24–48 h) but not early (3 h) time points in DCs. This effect was comparable to that exerted by recombinant TGF-β and could be blocked by the addition of neutralizing a-TGF–β.

To verify the in vivo relevance of these TGF-β-related in vitro findings, additional corneal transplantation experiments were performed, again using sCD83 in topical application form. Interestingly, when a TGF–β–neutralizing Ab was coadministered together with sCD83, graft survival was clearly reduced, indicating that TGF–β has a crucial role in the sCD83-mediated immunomodulatory effects. On the other hand, topical administration of recombinant TGF–β to transplanted recipient animals resulted in a similar graft survival rate as observed by sCD83. Using a combined heart and skin transplantation model, Ag-specific tolerance induction has been reported for sCD83 and thus this molecule might have additional advantages (35).

Our data indicate that sCD83 induces long-term IDO expression via TGF–β in DCs both in vitro and in vivo and that IDO activity is a critical requirement for the potent immunoregulatory effects exerted by sCD83. The fact that sCD83 induces long-lasting allograft tolerance in combination with a locally restricted immunosuppressive environment, mediated by the induction of regulatory
T cells, makes sCD83 a promising candidate for further clinical development in organ and tissue transplantation.

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Disclosures

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References


CORAINE TRANSPANTATION AND SOLUBLE CD83


