Different Innate Immune Responses in BALB/c and C57BL/6 Strains following Corneal Transplantation

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Keywords

Keratoplasty · Corneal graft rejection · Macrophage · C57BL/6 · BALB/c

Abstract

Purpose: To investigate immunological differences and the role of CD38+/F4/80 + M1 macrophages in C57BL/6J- and BALB/c-recipient mouse corneal transplantation models.

Methods: Allogeneic transplantation was performed cross-wise in BALB/c mice and C57BL/6J mice; syngeneic transplantation was performed in both strains. Anterior chamber depth (ACD) was measured before and central corneal thickness (CCT) was measured both before and after transplantation. In vivo graft rejection was monitored using anterior eye segment optical coherence tomography (ASOCT) evaluating the CCT and grading of corneal oedema using a well-established clinical score (CS). Histology of corneal grafts was performed 18 or 21 days after surgery. Immunohistochemistry with anti-F4/80 antibody and anti-CD38 antibody was used for detecting M1 macrophages within the grafts. **Results:** High CS and CCT values after allogeneic transplantation persisted in both BALB/c ($n = 18$) and C57BL/6J recipients ($n =$

20). After syngeneic transplantation, CS and CCT values increased in both models in the early phase after surgery due to the surgical trauma. Surprisingly, in the syngeneic C57BL/6J model, high CCT values persisted. Furthermore, anterior synechiae developed in C57BL/6 recipients after both syngeneic and allogeneic transplantation, whereas BALB/c recipients showed almost no synechiae – even though C57BL/6J animals tended to have a deeper anterior chamber (281 ± 11 pixels [mean $\pm$ SD]) compared with BALB/c animals of the same age (270 ± 9 pixels [mean $\pm$ SD]). Immunohistochemistry revealed numerous CD38+/F4/80 + M1 macrophages in grafts of C57BL/6J recipients following both syngeneic and allogeneic transplantation. However, in BALB/c-recipient mice only sparse M1 macrophages were detectable (CD38 + M1 macrophages relative to all F4/80 + cells: 75 vs. 17% [after allogeneic transplantation] and 66 vs. 17% [after syngeneic transplantation]; $p < 0.05$). **Conclusions:** Allogeneic corneal transplants are rejected in BALB/c as well as C57BL/6J mice, but tissue alterations with anterior synechiae are more pronounced in C57BL/6J recipients. Fol-

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lowing syngeneic transplantation, C57BL/6J-recipient animals show a persistent graft swelling with increased numbers of CD38+/F4/80 + M1 macrophages in grafted tissue, in contrast to the common model using BALB/c-recipient mice. Our data strongly suggest that strain-dependent differences convey different innate immune responses in BALB/c and C57BL/6J strains. Accordingly, in murine keratoplasty experiments, the mouse line of both donor and recipient animals must be carefully considered. C57BL/6J-recipient mice might be particularly suited to study corneal graft rejection in a clinical setting considered "high risk."

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Introduction

Corneal transplantation is one of the most commonly performed forms of tissue transplantation worldwide [1] and enjoys a 10-year graft survival rate of ~90% in *low-risk* situations. However, the rejection rate is over 50% in *high-risk* hosts such as those with corneal ulcers, corneal vascularization, or a history of graft rejections. This occurs despite maximal topical and systemic immune suppression [2]. Corticosteroids are the commonly used treatment for acute rejection [3]. Applied systemically or locally, they also cause adverse effects, such as cataract, steroid response with an increased intraocular pressure, and secondary infections [4, 5]. Thus, it is imperative to develop new strategies for preventing or treating corneal graft rejection.

Most of the research in corneal transplantation and in conducting research on pharmacological interventions is performed using mouse models, with C57BL/6 and BALB/c mice most frequently used as recipients [6–9]. In murine keratoplasty, a 2.0–2.5-mm graft is obtained from the donor animal and sutured into the recipient animal by interrupted sutures. Usually, allogeneic mouse strains are transplanted [10], while syngeneic animals serve as controls. In case of rejection, the transplant is subsequently clouded due to an immune reaction. This clouding is the result of cellular infiltration of the graft as well as immune-cell-induced damage to the endothelium of the graft, which leads to oedema [11]. Until now, the readout parameter "opacity" has only been assessed semi-quantitatively by means of a score; more recent work has also established anterior eye segment optical coherence tomography (ASOCT) for measuring thickness of the rejected graft in order to generate a parameter that can be documented and standardized [12]. Regarding the use of individual mouse strains, there is neither standardization

nor are there any studies comparing immune reactions between mouse strains.

In the 1980s, Scott and Farrell [13] and Heinzel et al. [14] observed that C57BL/6 mice are resistant to *leishmanial major* infections, whereas BALB/c mice die after infection with the same pathogen. Compared with BALB/c mice, C57BL/6 mice also showed stronger protection against melanoma metastases and higher resistance to various types of infection, including tuberculosis, *Pasteurella pneumotropica*, and Chlamydia [15–18]. Other studies revealed that in contrast to C57BL/6 mice, which show a Th1- and M1-dominant immune response, BALB/c mice show a more Th2- and M2-dominant immune response [17, 19]. The differences in susceptibility strongly indicate significant differences in the innate immune system function, which were eventually attributed to M1/M2 differences.

As of 2019, 4 studies comprising a murine keratoplasty model were published (Table 1). Half of them grafted C57BL/6 donor tissue into BALB/c recipients. Both mouse strains are the de facto standard to investigate the innate immune system and immunological processes during graft rejection. However, it remains unclear to what extent these models are mimicking immunological mechanisms during corneal graft rejection in humans and if the differences in the innate immune system of these 2 mouse strains affect the outcome after corneal transplantation. In the present study, we therefore compared the outcome of corneal transplantation after syngeneic and allogeneic transplantation into BALB/c or C57BL/6J mice, respectively. In addition, we investigated the macrophage subtypes in rejected corneal grafts.

Materials and Methods

Ethics

All animal experiments were approved by the Faculty of Medicine, University of Freiburg, and the federal state of Baden-Württemberg represented by the regional council Freiburg. This animal study was conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Animals were also treated in compliance with German, European, and Federation of Laboratory Animal Science Associations (LAS) regulations for the care of experimental animals.

Animals

Inbred adult female C57BL/6J mice (Charles River, Sulzfeld, Germany) and female BALB/c mice (Charles River, Sulzfeld, Germany) were used for syngeneic or allogeneic transplantation. All animals were 6–12 weeks old; the exact age and weight of the animals used can be found in online suppl. Fig. 1; for all online suppl. material, see www.karger.com/doi/10.1159/000509716.

Table 1. Mouse models used to investigate corneal transplantation [7, 12, 20–28]

Donor	Recipient	Outcome	Exemplary publications	Articles published in 2017	Articles published in 2018	Articles published in 2019
BALB/c	BALB/c	Nearly no rejection	Lapp, 2016 [7] Pastak, 2018 [12] Wang, 2019 [20]	0	2	1
C57BL/6	C57BL/6	Rejection in all cases	Yamada, 1997 [24] Tan, 2013 [25]	0	0	0
C57BL/6	BALB/c	Rejection in all cases	Tahvildari, 2017 [26] Pastak, 2018 [12] Inomata, 2018 [27] Li, 2019 [21] Jiang, 2019 [22]	11	4	2
C3H/He	BALB/c	Rejection in all cases	Lapp, 2016 [7]	0	0	0
B10D2	BALB/c	Rejection in 90% of cases	Sano, 1996 [28]	0	0	0
C57BL/6	B6D2F1	Rejection in all cases	Shamloo, 2019 [23]	0	0	1

Orthotopic Corneal Transplantation

Syngeneic and allogeneic corneal transplantation was performed as described previously [12]. Corneal transplantation was performed in only one eye per animal in accordance with Animal Welfare Regulations. In brief, anaesthesia was performed by injecting 10 mg/kg body weight xylazine (Bayer, Leverkusen, Germany) and 100 mg/kg body weight ketamine (Essex, München, Germany) intraperitoneally. A 2.0-mm diameter central corneal graft from the donor was placed into a 1.5-mm recipient corneal bed and sutured with 8 interrupted 11–0 nylon sutures (ETHILON® Nylon Suture, Ethicon Inc., Somerville, NJ, USA). All corneas were completely clear before grafting. Following surgery, ofloxacin ointment (Floxal, Dr. Gerhard Mann, Chem.-pharm. GmbH, Germany) was applied once to the cornea. The eyelids were closed with a 7.0 SERALON® suture (Serag-Wiessner GmbH & Co. KG, Naila, Germany) to protect the surgical area for 72 h. After suture removal, the eyes were examined using a surgical microscope from the third post-operative day and every 3 days afterwards. Animals exhibiting surgical complications, such as intraocular haemorrhage, signs of infection, collapse of the anterior chamber, or cataract, were excluded. In total, we included 18 BALB/c mice (10 syngeneic and 8 allogeneic) and 20 C57BL/6J mice (10 per group).

Clinical Score, Central Corneal Thickness, and Anterior Chamber Depth Measurement

Evaluation of the corneal opacity was performed as previously described [12]. In brief, the opacity of corneas was assessed according to the following scoring system using a slit-lamp biomicroscope (Table 2). Onset of graft rejection was defined when the corneal opacity exceeded grade 2 (no iris vessels discernible).

Table 2. Definition of the clinical score

Grade	Clinical description
0	Completely transparent cornea
1	Minimal corneal opacity with iris vessels easily detectable
2	Visible moderate corneal opacity with iris vessels still visible
3	Moderate corneal opacity with only the pupillary margin visible
4	Complete corneal opacity, pupil not visible

For ASOCT measurements, animals were anesthetized as described above and analyzed using a Micron IV Retinal Imaging System (Phoenix Research Laboratories, Pleasanton, CA, USA) and an Image-Guided OCT for mice (Cat. No. 9002; Phoenix Research Laboratories). To ensure that only the central areas of the graft were measured when determining the corneal thickness, the cutting axis was additionally monitored by means of a visual image during the measurement. Tomographic images were used to measure transplant thickness in pixels, defined in a central vertical section using Cell Finder software [29]. Since ASOCT tends to distort anatomic structures, we excluded systemic errors by measuring the vertical and the horizontal central CCT at the same time and used both measurements to calculate the average central thickness. ASOCT was performed every 3 days starting with day 6 after transplantation. The ACD was measured using the same technique.

Fig. 1. CCT, CS, and corresponding Kaplan-Meier estimates. CS (a) and CCT (c) are displayed from the 6th to the 18th day after surgery. Each point represents a post-operative examination; points are jittered to prevent overplotting ($n = 18$ BALB/c mice and 20 C57BL/6 mice). The grey ribbons indicate the standard error of the mean (a, c). The statistics for the corresponding Kaplan-

Meier estimates (defined by the CS in b and by the transplant oedema in d) were calculated using a log-rank test. With regard to transplant swelling, the first 10 days were excluded in the Kaplan-Meier estimate to exclude errors related to corneal oedema caused by surgery (d). CCT, central corneal thickness; CS, clinical score.

Histology and Immunohistochemistry

The animals were sacrificed by cervical dislocation, and eye-balls were harvested 18 and 21 days after transplantation. Staining for histology was performed in 10 BALB/c and 10 C57BL/6 mice (5 syngeneic and 5 allogeneic animals per group, respectively). All specimens collected were frozen in Tissue-Tek®

O.C.T.™ Compound (Sakura Finetek Europe, Alphen aan den Rijn, The Netherlands) and stored at -20°C . For histology, globes were cut in half and sections of 6- μm thickness were cut in a Microtome 3050S (Leica, Wetzlar, Germany) beginning from the central cornea towards the periphery. The sections were stained with haematoxylin and eosin (H&E) or periodic acid-Schiff and

examined with a light microscope. Corneal thickness evaluation was performed only in central sections of the graft; for anterior chamber and synechia assessment, histological sections from the center to the periphery were scanned to avoid missing peripheral synechiae. For immunohistochemistry, sections were blocked using 1% BSA and 0.3% Tween in PBS for 60 min. Thirteen sections (3 BALB/c syngeneic and 3 allogeneic, as well as 3 C57BL/6J syngeneic and 4 allogeneic) were incubated with anti-mouse F4/80 (1:200, rabbit monoclonal to mouse, Abcam: ab111101), anti-mouse CD68 (1:200, CD68 polyclonal antibody, rabbit/IgG, Invitrogen: PA532330), or anti-mouse CD38 (1:100, rat monoclonal to mouse, Lifespan Biosciences: LS-C43670) at +4°C overnight. After washing 3 times in PBS and 0.1% Tween, the corneas were incubated with Anti-Rabbit Alexa 488 (1:500, Donkey Anti-Rabbit IgG (H + L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 488, Invitrogen: A21206) and Anti-Rat Cy3 (1:500, Donkey Anti-Rat IgG (H + L) Cy3 Affinity Purified F(ab')₂ Fragment, Jackson ImmunoResearch: 712-166-150) for 1 h at room temperature. The samples were mounted in Mowiol supplemented with 0.1% DAPI.

Statistical Analysis and Data Plotting

Data from the clinical score (CS) and the central corneal thickness (CCT) were analyzed descriptively using the R system [30], package *ggplot2* [31] and *Hmisc* [32]. We used the Kruskal-Wallis test to compare numerical data between groups and Kaplan-Meier curves to visualize the respective incidence rates. These curves were statistically tested using the log-rank test. Data were calculated and plotted using the R platform as mentioned above.

Results

Dynamics of Corneal Graft Rejection in Both Recipient Strains

To investigate whether mouse strains show a different level of inflammation after syngeneic or allogeneic transplantation, we examined the CS and CCT of grafts using ASOCT every 3 days from day 6 to day 18 after surgery. We observed no relevant differences between allogeneic models with regard to the CS (Fig. 1a, upper 2 curves) and the CCT (Fig. 1c, upper 2 curves). Kaplan-Meier estimations confirmed this course for CS (Fig. 1b), allowing differentiation between syngeneic and allogeneic transplantation ($p < 0.04$, log-rank test) and showing fewer events in both BALB/c groups in comparison with the C57BL/6 groups. In syngeneic models, the CS between the 2 mouse strains showed no difference (Fig. 1a, lower 2 curves). However, CCT, which is more objective and repeatable [12], was higher in C57BL/6J mice (Fig. 1c, lower 2 curves) and remained higher from day 15 onward. This finding was confirmed in the Kaplan-Meier estimation ($p < 0.01$, log-rank test).

Histopathology

H&E staining was performed on corneal grafts 18 or 21 days after transplantation. Examples of H&E staining are shown in Figure 2. Two schematic drawings (1 normal mouse eye and 1 transplanted eye) and a histological overview illustrate the relevant structures. The border between the transplant and the recipient cornea can be determined in the histological overview.

In the course of transplant rejection, inflammatory infiltration and swelling of the transplant occur. This swelling and cell infiltration are more pronounced after allogeneic (Fig. 2a1, b1) than after syngeneic (Fig. 2c1, d1) transplantation. For C57BL/6J mice, anterior synechiae were much more prominent after allogeneic transplantation than after syngeneic transplantation (see Fig. 2b1, b4, d4 and asterisks in Fig. 2d1). In the syngeneic group, 5 out of 10 C57BL/6-recipient mice and 1 out of 10 BALB/c-recipient mice showed synechiae, whereas in the allogeneic group, the ratio was 8 out of 10 in the C57BL/6 group and 1 out of 10 in the BALB/c group.

Anatomical Influences as Possible Confounders

To exclude any possible anatomical confounders, control CCT and anterior chamber depth (ACD) were measured before transplantation (see Fig. 3). CCTs of these 2 mouse strains ($n = 10$ animals per group) were almost the same with an average value at about 60 pixels (see right boxes in Fig. 3; $p = 0.72$, Kruskal-Wallis test). The ACD ($n = 12$ animals per group) showed no statistically relevant difference either, but C57BL/6J animals tended to have a deeper anterior chamber, making the formation of anterior synechiae even less likely (see left boxes in Fig. 3; $p = 0.06$, Kruskal-Wallis test).

Immunohistochemistry

To characterize infiltrating immune cells, anti-F4/80 and anti-CD38 antibodies were used for immunohistochemistry (see Fig. 4). F4/80 antigens have been widely used to detect macrophages in tissues, while CD38 is a novel marker for M1 macrophages [46–50]. We found almost no double-positive cells in corneas from syngeneic- and allogeneic-transplanted BALB/c mice (see Fig. 4a3, c3, e) but a significant number of double-positive immune cells in both C57BL/6J groups (see Fig. 4b3, d3, e; $p = 0.03$ in the allogeneic group and $p = 0.07$ in the syngeneic group; Kruskal-Wallis test). In untreated, non-transplanted animals, only sparsely F4/80 + antigen-presenting cells (APC) are present in both BALB/c and C57BL/6 animals; additional staining for CD68 did not indicate the presence of APC either (see Fig. 4f1–4).

Fig. 2. Histology of eyes 18 to 21 days after corneal transplantation. The schematic drawing and the histological overview provide an insight into the anatomical structures. After allogeneic transplantation (**a, b**), there is a distinct swelling of the graft in both mouse lines; this swelling is less pronounced after syngeneic transplantation (**c, d**). The dark pigmentation of the iris allows the recognition of the C57BL/6 animals; in the BALB/c animals, the iris has almost

no pigmentation. After transplantation, anterior synechiae (**b1** and asterisk in **d1**) occur mostly in C57BL/6 animals. Figures 2–4 show further details; the epithelium is located in 2, the stroma in 3, and the endothelium – if still present – in 4. Note the pigment adhesions to the endothelium, which are a result of the synechiae (**b4** and **d4**). Representative images of a total of $n = 10$ BALB/c and $n = 10$ C57BL/6J mice per group are given.

Discussion

Our study revealed significant strain-dependent differences in the innate immune response following mu-

rine corneal transplantation. While allogeneic corneal transplants were rejected to a similar extent in BALB/c and C57BL/6J mice, inflammation, transplant infiltration, and anterior synechia formation were less pro-

Fig. 3. ACD (a) and CCT (b) of BALB/c and C57BL/6J mice prior to surgery. Box and whiskers plots of ACD and CCT before transplantation of both murine models revealed no statistically significant differences. The ACD even turned out to be higher in C57BL/6 mice in comparison with BALB/c mice. CCT, central corneal thickness; ACD, anterior chamber depth.

nounced in syngeneic corneal transplants in BALB/c-recipient mice compared with C57BL/6J-recipient mice. This is of particular interest since various recipient and donor mouse strains have been used in previously published studies neglecting possible confounders related to the innate immune response following corneal transplantation in mice.

When evaluating corneal graft rejection in mice, using a subjective CS system has been the gold standard. This CS system describes the corneal opacity through integration of the underlying parameter oedema due to an endothelial dysfunction as well as a cellular infiltration. In contrast, the ASOCT objectively measures the CCT as read-out for corneal swelling and has a significantly higher resolution with regard to the degree of tissue changes than the CS [12]. In our study, both the CS as well as the CCT values of allogeneic models increased until day 18, suggesting an aggravated and ongoing inflammation within the graft after allogeneic transplantation. In corresponding OCT images, allogeneic grafts appeared thick with the Descemet membrane being unrecognizable.

CS and CCT values of the syngeneic models were always lower than the corresponding allogeneic models. The peak of CCT after syngeneic transplantation appeared right after transplantation, being most likely related due to the surgical manipulation. The CS and CCT of BALB/c syngeneic grafts decreased afterwards. However, although the CS of C57BL/6 syngeneic model declined after the early phase, the CCT did not. It is important to point out that possible anatomical confounders

leading to increased inflammation and anterior synechia formation such as pre-operative CCT or ACD were ruled out in this study. There was no statistical difference in pre-operative CCT or ACD between the 2 mouse strains indicating that the differences in corneal oedema and anterior synechiae between strains were most likely due to different levels of inflammation following corneal transplantation.

Analyzing the immune response following allogeneic transplantation, rejection occurs as published, but inflammation as well as formation of anterior synechiae is more pronounced in C57BL/6J-recipient mice, suggesting a different aggressiveness of rejection. This may also be a reason for the predominant use of BALB/c-recipient mice reported in the literature. Using histology and immunohistochemistry, our observations suggest that rejection intensity correlates with the presence of M1 macrophages. This finding is in line with earlier reports of differing innate immune system activation after infection with various pathogens and the reported M1/M2 differences in BALB/c and C57BL/6 mice [10–16]. The origin of these M1 macrophages in C57BL/6 animals cannot be answered definitively. However, in line with the previous observation that M1 macrophages dominate the response in C57BL/6 animals to bacterial challenge, our data suggest that graft rejection is also dominated by macrophages of the M1 phenotype in this strain.

Macrophages have been found not only in the limbus, iris, or trabecular meshwork but are also known to be present within the corneal stroma [33, 34]. Macrophages

Fig. 4. Examples of immunohistochemistry of corneal transplants 18 to 21 days after transplantation. **a–d** The epithelium is located at the top of the image and the endothelium at the bottom. Anti-mouse F4/80 (red, first row) and anti-mouse CD38 (green, second row) antibodies were used to identify M1 macrophages. DAPI (blue) was used for nuclear staining. The box plots (**e**) represent the percentages of CD38 + cells among the F4/80 + macrophages

as shown in **a–d**. The percentages of CD38 + cells among the F4/80 + cells were counted from the immunohistochemical slides as above ($n = 3$ animals were analyzed for the calculation, except $n = 4$ in the allogeneic BALB/c group). Statistics were calculated using the Kruskal-Wallis test; the results are presented as mean ± SEM. In untreated, non-transplanted mice, only sparse CD68 + or F4/80 + cells can be detected, that do not stain for CD38 (**f**).

were recognized in 1985 as one of the major cell types infiltrating rejected human corneal grafts [35]. Depletion of macrophages by subconjunctival injection of clodronate liposomes, a macrophagicidal drug, significantly prolonged corneal graft survival [36] thereby strongly suggesting a significant role of macrophages in the initiation of corneal graft rejection. When Liu and colleagues transplanted corneas from GFP + mice to WT mice, GFP + APC appeared in the lymph nodes of WT recipients [37], confirming the evasion of donor APC from the transplant. Beige nude mice failed to reject corneal grafts from BALB/c mice, indicating that macrophages alone are insufficient to induce rejection. However, rejection occurred in the presence of CD4+ T cells sensitized against BALB/c mice [38]. Taken together, these data indicate that macrophages play an important role as APC in the afferent arm of corneal graft rejection.

When stimulated with human allogeneic corneal tissue, human monocyte-derived macrophages (MDM) induced the recruitment of further immunocompetent cells, which is seen as an initial step of corneal rejection [39]. In clinical samples from human patients, there is a significant influx of CD45+ monocyte-derived macrophages into the anterior chamber in the early phase of graft rejection [40]. Mounting evidence suggests that macrophages differentiate into a wide spectrum of intermediate phenotypes with different immunoregulatory capabilities. The categorization of macrophages in M1 and M2 subtypes and further differentiation into M2a–M2d is controversial and anything but simple, but most accepted [41].

The biology and functions of the different macrophage subtypes have been studied extensively in mouse and man. However, very little is known about the function of different macrophage subtypes in corneal graft rejection. In mice, M1 macrophages display pro-inflammatory properties, representing an important source of reactive oxygen and nitrogen intermediates and of pro-inflammatory cytokines. On the other hand, M2 macrophages mediate resistance to parasites, wound healing, tissue remodelling, and resolution of inflammation [19]. Immunohistochemistry revealed a striking predominance of CD38 + F4/80 + cells in rejected grafts of C57BL/6 mice in our study. The presence of these M1 macrophages thus could be linked to a more aggressive graft rejection, altering the macrophage phenotype into a M2-dominated pattern, therefore might be a promising therapeutic approach. Tahvildari et al. [26] injected IL-10 subconjunctivally to induce the generation of “tolerant APC” in mice, which significantly prolonged corneal graft survival. In-

terestingly, IL-10 is the major cytokine for M2 induction [41, 42]. Thus, the effect of IL-10 may be M2 mediated. This suggests IL-10 as an interesting compound to suppress corneal graft rejection by modulation of the macrophage phenotype within the graft.

We acknowledge that this study is limited by the fact that we evaluated the M1 phenotype in F4/80 macrophages only using CD38 as a marker. Immunophenotyping of both – human and murine – corneas remains incomplete compared with other tissues. Work in this area shows that various APCs are present in the cornea; these cells include macrophages, dendritic cells, and Langerhans cells [43]. Further work is needed on both: the phenotyping of immune cells in the cornea as well as on the function of different APC in the cornea. Moreover, the role of CD38 in the eye is still unclear. Under basal conditions, corneas in our study did not show a positive staining for CD38; only after transplantation, a large number of CD38-positive cells could be detected. These cells were mainly co-stained with F4/80 in C57BL/6 animals but not in BALB/c animals.

In summary, our study provides evidence on why BALB/c and C57BL/6J mice respond differently after syngeneic corneal transplantation. M1 macrophages appear to play a crucial role in this process. Our study is the first to compare multiple aspects of corneal rejection between M1- and M2-dominated immune response models, namely C57BL/6J and BALB/c mice. These mouse strains are two of the most widely used models for rejection studies [7–9, 12, 24, 25, 27, 44, 45]. The results of our study indicate that these studies deserve cautious interpretation. On the other hand, the clear differences between these 2 models suggest a wide range of clinical and scientific applications. The BALB/c-recipient model could be used as a surgical control for corneal transplantation experiments, whereas models using C57BL/6 as recipients with the severe inflammation and the high rejection rate may serve as “high-risk” models for corneal transplantation.

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Statement of Ethics

All animal experiments were approved by the Faculty of Medicine, University of Freiburg, and the federal state of Baden-Württemberg represented by the regional council Freiburg. This animal study was conducted in accordance with the ARVO Statement for

the Use of Animals in Ophthalmic and Vision Research. Animals were also treated in compliance with German, European, and Federation of Laboratory Animal Science Associations (LAS) regulations for the care of experimental animals.

Conflict of Interest Statement

We confirm that all authors concur with the submission. All funding for the studies in the manuscript, together with the names of the principal funding recipients, are listed in the text. This work has not been published elsewhere, neither completely, nor in part, nor in another form. The manuscript has not been submitted to another journal and will not be published elsewhere. The manuscript does contain data derived from animal experiments. This study was approved by the Faculty of Medicine of the University of Freiburg and by the federal state of Baden-Württemberg represented by a regional council. Animals were treated in accordance with the European Union regulations for the care of experimental animals. This animal study was conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Animals were also treated in

compliance with German, European, and Federation of Laboratory Animal Science Associations (LAS) regulations for the care of experimental animals. No financial/commercial conflicts of interest have been disclosed.

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Author Contributions

T.L., T.B., C.L., and G.S.: conceptualization. T.B., X.Z., A.H., and D.B.: data curation. T.B., X.Z., A.H., T.L., and D.B.: formal analysis. T.B. and T.L.: funding acquisition. T.B., X.Z., A.H., and D.B.: investigation. T.B., X.Z., A.H., C.L., D.B., G.S., T.R., and T.L.: methodology. T.L. and G.S.: project administration and supervision. T.B., X.Z., C.L., G.S., T.L., and D.B.: validation. T.B., X.Z., T.L., and D.B.: visualization. T.B. and X.Z.: writing – original draft. A.H., C.L., D.B., G.S., T.R., and T.L.: writing – review and editing.

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