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Inhibition of corneal inflammation following keratoplasty by birch leaf extract

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ABSTRACT

The objective of this study was to determine the effect of birch leaf (*Betula pendula*) extract (BPE) on corneal inflammation following keratoplasty in the rat model. T cells were stimulated *in vitro* in the presence of BPE. Proliferation, activation phenotype and the number of apoptotic/necrotic cells in cell culture were analyzed by flow cytometry. Corneal transplantation was performed between Fisher and Lewis rats. Recipient rats were either treated with cyclosporine A at a low dosage (Low-dose CsA = LDCsA) or received LDCsA in combination with BPE (2×1 ml/day). Clinical signs for corneal inflammation and rejection time points were determined. Infiltrating leukocytes were analyzed histologically. BPE specifically inhibited T cell proliferation *in vitro* by inducing apoptosis. The phenotype was not affected. *In vivo*, BPE significantly delayed the onset of corneal opacification ($p < 0.05$). The amount of infiltrating $CD45^+$ leukocytes and $CD4^+$ T cells ($p < 0.001$) was significantly reduced by BPE, whereas infiltration of $CD163^+$ macrophages was not significantly different between the two groups. BPE selectively induces apoptosis of activated T cells. Accordingly, BPE treatment significantly reduces infiltrating T cells and subsequent corneal opacification following keratoplasty. Our findings suggest BPE as a promising anti-inflammatory drug to treat corneal inflammation.

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1. Introduction

Corneal clarity is required for optimal vision and can be severely affected by any form of corneal inflammation. This reduction is mediated in the long-term by infiltrating leukocytes and pathological blood vessel formation. Independently of the causes, any occurrence of corneal inflammation must be treated, especially if the central cornea is involved. Once a corneal scar is established, keratoplasty becomes necessary to restore corneal transparency required for optimal vision.

Although the immune privileged status of the eye facilitates excellent graft survival rates, immune responses against a corneal transplant remain the major cause of irreversible graft failure with subsequent opacification and reduced visual acuity (Niederkorn, 2007; Streilein, 2003). If no inflammation exists prior to transplantation, very good outcomes are achieved regarding clear graft survival (<10% rejection) (Price et al., 1993). However, any form of ocular surface inflammation (e.g. following infection, alkalic burn etc.), systemically occurring immunological disorders (e.g. atopic dermatitis) or a young recipient age present an increased risk of rejection (Reidy, 2001; Schwartzkopff et al., 2010a). Depending on the individual risk factors, long-term rejection occurs in up to 85% of cases (Coster and Williams, 2005). This is mainly due to preexisting corneal blood- and lymph-vessels and an increased frequency of pre-sensitized leukocytes at the ocular surface (Cursiefen et al., 2003; Streilein, 2003). In these patients, focused immunosuppressive therapy is required.

Corticosteroids are the mainstay of any suppression following keratoplasty, in order to reduce subsequent inflammatory corneal reaction. However, even if applied topically, they are associated with complications such as ocular hypertension and cataract development. In cases where corticosteroids are not sufficient, systemic treatment with cyclosporine A (CsA) and/or mycophenolate mofetil (MMF) were shown to significantly improve graft survival (Birnbaum et al., 2005; Reinhard et al., 2001). Even though

Abbreviations: BPE, *Betula pendula* extract; CsA, cyclosporine A; LDCsA, low-dose CsA; MMF, mycophenolate mofetil; CFSE, carboxyfluorescein diacetate succinimidyl ester; MTX, methotrexate; PI, propidium iodide.

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both drug treatments are more specifically directed against the evolving lymphocyte response to the graft, they involve a risk of hepatorenal dysfunction and infections as well as increased incidence of tumor development. Therefore, it is of great clinical interest to develop treatment strategies that have a maximal and specific effect on the respective immune mechanism and which also avoid side effects that could reduce quality of life.

The immune mechanisms of corneal allograft rejection have been intensively investigated in animal models. It is recognized that $CD4^{+}$ T cells are a major factor in graft rejection (Streilein, 2003). Although other leukocyte populations, such as macrophages (Slegers et al., 2000), NK cells (Claerhout et al., 2004; Schwartzkopff et al., 2010b), $CD8^{+}$ T cells (Niederkorn et al., 2006) or antigen-presenting cells (Saban et al., 2010) were also shown to influence the rejection time course, only the depletion of $CD4^{+}$ T cells promoted allograft survival in the long-term (Ayliffe et al., 1992; Yamada et al., 1999). In addition to these cellular components, the intensity of lymph- as well as hem-angiogenesis was also shown to be closely related to graft rejection (Hos et al., 2008). As T lymphocytes have the greatest impact during the inflammatory response following keratoplasty, many immunosuppressive medications aim to act on these cells. Aside from CsA or MMF, alternatives such as FK506 and FK778 also showed positive results (Birnbaum et al., 2007; Sloper et al., 2001). Most of these medications are associated with side effects if given at an effective dosage. Once dosage is reduced below a certain level, both side effects and drug effect cease at the same time. In order to minimize adverse effects but maintain drug efficacy, efforts have been made to combine a reduced dosage of the drug with additional substances that have similar properties (Hackstein et al., 2007).

Extract of birch leaves was reported to have an anti-inflammatory function (Klinger et al., 1989). We observed a cytostatic effect of a commercially available birch leaf extract (*Betula pendula* extract: BPE) on human lymphocytes *in vitro* (Gründemann et al., 2011). Therefore, we hypothesized that a combination of BPE with sub-therapeutic CsA dosage would effectively suppress corneal inflammation following keratoplasty. In this series of experiments, the immunosuppressive capacity of BPE was analyzed on rat T cells *in vitro* and in combination with a low dosage of CsA during the inflammatory response following corneal transplantation *in vivo*.

2. Material and methods

2.1. Cells and animals

Inbred female Fisher ($Rt1^{lv}$) and Lewis ($Rt1^{l}$) rats (Charles River, Sulzfeld, Germany) were used as donors and recipients of corneal transplants. All animals were handled according to EU Directive 2010/63/EU. For *in vitro* studies, T cells were isolated from spleens; monocytic cells were obtained from cell line U937.

2.2. Groups

Lewis rats aged 8 weeks were divided into two groups: Group 1 ($n = 13$) received daily cyclosporine A (Novartis AG, Basel, Switzerland) at a low, sub-therapeutic dosage (LDCsA) (1 mg/kg body weight). Group 2 ($n = 8$) received LDCsA together with BPE (2×1 ml/day), respectively. Therapy was administered intraperitoneally for 14 days. The investigated aqueous *B. pendula* extract (BPE) is an injectable plant extract from the leaves of the birch *B. pendula* Roth, which is marketed by ABNOBA GmbH (Pforzheim, Germany) as an officially registered preparation according to § 38/39 of the German Drug Law (Betula Folium D3 ABNOBA, batch-no. 706 A41 and 910 A06 was used in the present study). For legal reasons, the extract is manufactured according to method no. 32 of the German Homeopathic Pharmacopoeia (GHP). Harvested birch leaves undergo maceration in a patented pressing machine under protective atmosphere to avoid oxidation. The resulting BPE contains 1.88 mg of fresh plant material, corresponding to an anhydrous mass of 0.63 mg/ml. The finished product is sterile filtered, filled in glass ampoules and released for sale if it meets all current European Pharmacopoeia (EP) specifications for solutions intended for injection. All specifications for parental medications were fulfilled according to the EP. Good Manufacturing Practice (GMP) and quality control, defined by the EP, are monitored by the German authorities (Federal Institute for Drugs and Medical Devices; BfArM); this includes proof of plant source identity and absence of contamination by heavy metals, pesticides, aflatoxins and microorganisms. Cell biological experiments were performed at our laboratory in Freiburg, Germany using ampoules from the sales stock. For each experiment, a fresh ampoule of *B. pendula* extract was used and concentrations were tested as indicated.

2.3. Corneal transplantation and anesthesia

Anesthesia was performed with a short inhalation of isoflurane (ABBOTT GmbH&Co.KG, Wiesbaden, Germany), deepened by a combination of ketamine (Essex, München, Germany), xylazine (Bayer, Leverkusen, Germany) and atropine (Braun, Melsungen, Germany) intraperitoneally. Orthotopic penetrating keratoplasties were performed as described previously (Birnbaum et al., 2007). In brief, Fisher donor buttons (2.5 mm) were obtained and the animals were sacrificed afterward. Recipients were anesthetized as described above. The central cornea was removed using a 2.0 mm trephine. The donor cornea was fixed with 8 interrupted sutures (11.0 Ethilon, Ethicon, Norderstedt, Germany). Finally, a blepharorrhaphy was applied for three days.

2.4. Clinical graft assessment

After removal of the blepharorrhaphy, the grafts were examined by two independent investigators for signs of opacity, vascularization, and edema according to an internationally accepted scoring method (Birnbaum et al., 2007; Schwartzkopff et al., 2010c) explained in Table 1. Rejection was defined as complete opacification (grade 4). The animals were continuously monitored during the assessment for signs of toxic side effects such as weight loss.

2.5. Histological analyses

Four rats per group were sacrificed for immunohistological evaluation on day 9. $CD45^{+}$ leukocytes, $CD4^{+}$ T cells and $CD163^{+}$

Table 1
Clinical graft assessment. Evaluation of opacification, edema and vascularization (Birnbaum et al., 2007; Schwartzkopff et al., 2010c).

Opacification	
0	No opacity
1	Slight opacity, details of iris clearly visible
2	Moderate opacity, some details of iris no longer visible
3	Strong opacity, pupil still recognizable
4	Total opacity, pupil no longer visible
Vascularization	
0	No vessels
1	Vessels on host not in the transplant
2	Vessels in the periphery of the transplant
3	Vessels reaching the center of the transplant
Edema	
0	No edema
1	Slight edema
2	Strong edema, margin of the transplant slightly elevated
3	Severe edema, margin of the transplant elevated

macrophages were stained on cryosections, as described previously (Birnbaum et al., 2007; Schwartzkopff et al., 2010c). In brief, obtained bulbi were snap-frozen, and 6 μm-thick cryosections were prepared and fixed in acetone at −20 °C. Unspecific binding was blocked by incubation in Tris buffer containing 10% calf serum. Primary mouse-anti-rat antibodies (anti-CD4: clone W3/25, anti-CD45: clone IBL-3/16; anti-CD163: clone ED2, obtained from AbD Serotec, Düsseldorf, Germany) were applied to the sections. A biotinylated rabbit-anti-mouse secondary mAB was used, followed by incubation with streptavidin-conjugated alkaline phosphatase (both Dako, Hamburg, Germany) and the corresponding substrate (Vector, Burlingame, VT, USA). After incubation, sections were counterstained with Mayer's hematoxylin. Positively stained cells were counted within three squares on the graft by two independent investigators and calculated as mean cellular infiltrate per mm^2. Positive and negative controls were accomplished with parallel stained slices of rat spleen.

2.6. Cell preparation and culture media

Lymphocytes of rat spleen were centrifuged and purified on Ficoll (PAN BIOTECH GmbH, Aidenbach, Germany). Cells were then washed twice with medium and cell viability and concentration were determined by trypan blue staining.

Splenic lymphocytes and U937 cells were cultured in IMDM containing 1% glutamine (Invitrogen, Carlsbad, USA), 1% penicillin–streptomycin mixture (Biochrom AG, Berlin, Germany) and 1.1% supplement comprising 45.5% insulin–transferrin (Roche, Mannheim, Germany), 45.5% HEPES-buffer and 9% non-essential amino acids (both Biochrome AG, Berlin, Germany).

2.7. Stimulation and proliferation

Peripheral blood lymphocytes from four individual Lewis rats and U937 cells were washed twice in cold PBS and resuspended in PBS at a concentration of 5×10^6 cells/ml. Carboxyfluorescein diacetate succinimidyl ester (CFSE; 5 mM; Sigma Aldrich, Taufkirchen, Germany) was diluted 1/1000 and incubated for 10 min at 37 °C. Labeling was stopped by washing twice with medium containing 10% FCS. Lymphocytes were stimulated with anti-rat CD3 and anti-CD28 mAB (anti-CD3, clone: G4.18; anti-CD28, clone: JJ319, both by eBioscience Inc., San Diego, USA). Monocytes were stimulated with lipopolysaccharide (Sigma Aldrich, Taufkirchen, Germany). Cell division was analyzed by flow cytometry.

2.8. Activation phenotype assessment

T cells were stimulated in the absence or presence of increasing concentrations of BPE and changes in the activation phenotype were analyzed after 24 h of culture. Briefly, $CD4^+$ T cells were stained for CD25- and CD62L-expression (CD4: clone: W3/25: CD25: clone: OX-39 and CD62L: clone: OX-85) and analyzed by flow cytometry. All antibodies were purchased from Serotec (Düsseldorf, Germany). Cells were obtained from four individual Lewis rats.

2.9. Apoptosis assay

The amounts of apoptotic and necrotic cells in T cell cultures were assessed by staining with annexin-V and propidium iodide (eBioscience Inc., San Diego, USA) following 72 h of culture. Staining was performed with annexin-V, that was diluted in specific binding buffer according to the manufacturer's instructions. After 15 min of incubation with 50 μl per well, 2 μl propidium iodide was added and incubated for a further 10 min before analysis by flow cytometry. Cells were obtained from four individual Lewis rats.

2.10. Statistics

The time interval to the onset of rejection was analyzed with the Kaplan–Meier method and compared with the log-rank test between the groups. The densities of infiltrating immune cells in corneal allografts were compared statistically using the paired Student's t-test. *In vitro* findings were also analyzed by means of the *t*-test. Results were considered statistically significant at $p < 0.05$.

3. Results

3.1. BPE specifically inhibits T cell proliferation

BPE was reported to have anti-inflammatory action in various immune mechanisms (Gründemann et al., 2011). In order to analyze its immunosuppressive capacity, increasing concentrations of BPE were added to T cell cultures ($n = 4$) in the presence of T cell receptor (TCR)-driven stimulation and to monocytic cells ($n = 3$) *in vitro*. Cells were labeled with CFSE beforehand and loss of CFSE-intensity was calculated by flow cytometry. It was found that already 40 μg/ml of BPE led to significant inhibition of T cell proliferation ($p < 0.01$) (Fig. 1A and B), whereas no inhibition of dividing monocytic cells was observed (Fig. 1C). CD25-upregulation and CD62L-downregulation on activated T cells were analyzed in addition to proliferation ($n = 4$). The activation phenotype of $CD4^+$ T cells was not altered by the presence of BPE in the culture medium (Fig. 2A and B).

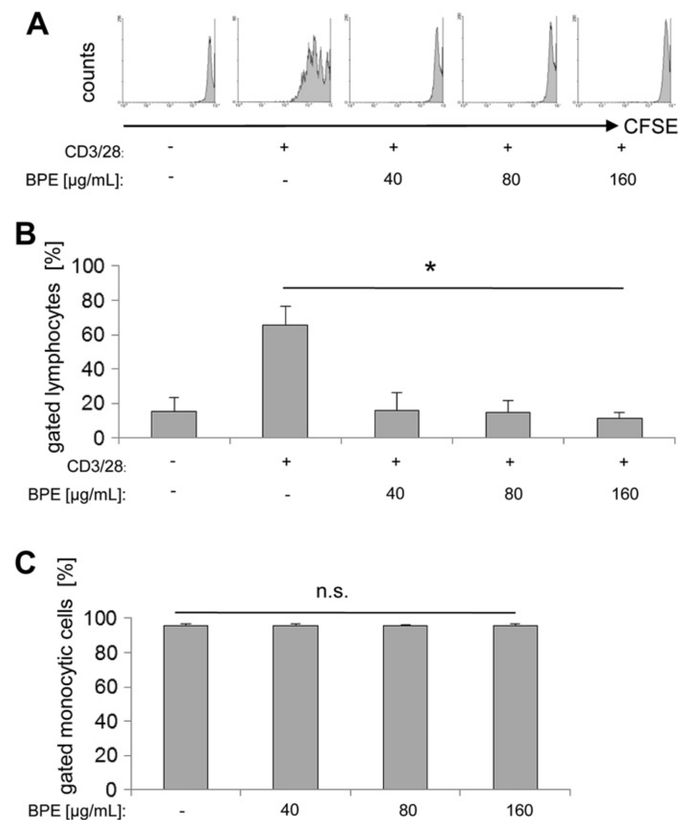

Fig. 1. BPE selectively inhibits T cell proliferation. $CFSE^+$ lymphocytes were stimulated with anti-CD3/anti-CD28 and incubated with increasing concentrations of BPE. (A + B) It was found that already 40 μg/ml BPE significantly inhibited T cell proliferation (*$p < 0.01$). (C) Monocytic cells (cell line U937) stimulated with lipopolysaccharide were similarly cultured in the presence of BPE. However, no inhibitory effect on cell division was detected. Data shown are representative of three different, independent experiments. The results are depicted as mean percentage of gated lymphocytes or monocytic cells by flow cytometry with standard deviation (SD).

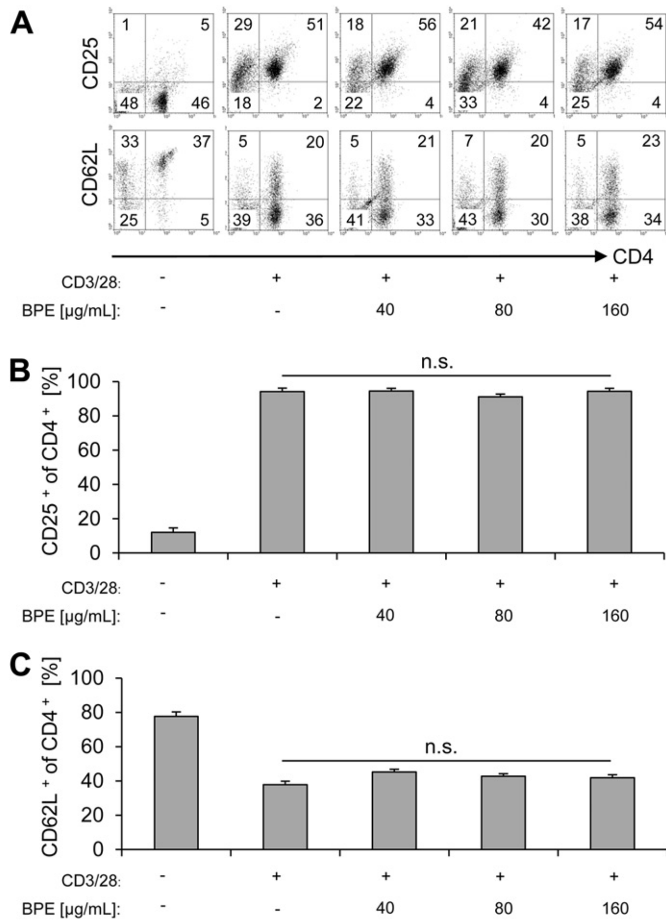

Fig. 2. BPE does not affect activation phenotype of T cells. Stimulated CD4$^+$ T cells were analyzed for CD25- and CD62L-expression by flow cytometry. (A) As representative flow cytometric data plots indicate, (B) neither CD25- nor CD62L-expression were affected by incubation with BPE. Bars represent mean percentage of PE positive cells (CD25 or CD62L) of total CD4$^+$ cells with SD for five different, independent experiments.

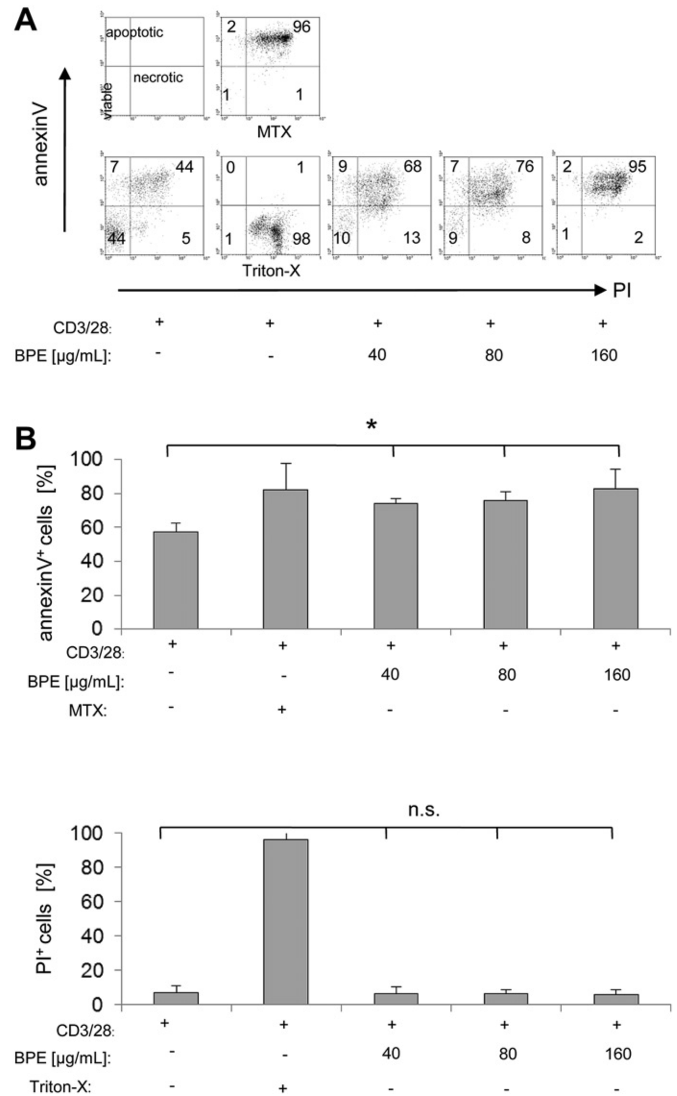

Fig. 3. BPE induces apoptosis of activated T cells. The numbers of apoptotic and necrotic cells within T cell-stimulation cultures were analyzed by annexin-V and propidium iodide (PI) staining. (A) shows representative data plots of flow cytometric analyses. (B) Similar to MTX, BPE demonstrated a cytotoxic potential on activated T cells ($p < 0.01$). (C) Necrosis was not induced *in vitro* by BPE. Data are presented as mean and standard deviation of the percentage of apoptotic (annexin-V$^+$) and necrotic (PI$^+$) cells for four different, independent experiments.

3.2. Induction of apoptosis but not necrosis in activated T cells

As the inhibition of T cell activation could be excluded, the induction of cell death was analyzed as another potential mechanism to reduce the number of proliferated T cells. In order to differentiate between apoptosis and necrosis, annexin-V and propidium iodide staining was performed and analyzed by flow cytometry ($n = 4$). The addition of BPE to T cell cultures under stimulation conditions induced a significant increase of apoptotic cells ($p < 0.01$) similar to that observed in the presence of methotrexate (MTX) (Fig. 3A). The amount of necrotic T cells was not increased in the presence of BPE (Fig. 3B).

3.3. BPE delays onset of corneal allograft opacification

As allograft rejection depends on T cells and BPE selectively induces apoptosis in T cells with a cytostatic potency similar to MTX, BPE was analyzed following corneal transplantation *in vivo*. We hypothesized that BPE may have an additive anti-inflammatory effect to a low cyclosporine A (CsA) dosage. Therefore, recipient rats were either treated once daily with a subtherapeutic dosage of CsA after keratoplasty (1 mg/kg body weight) (LDCsA) or with LDCsA in combination with two injections of 1 ml BPE ($n = 4$, respectively). Opacity, edema and vascularization were assessed as clinical signs of emerging allograft rejection according to an internationally accepted scoring method (Table 1). After an initial iatrogenic opacity grade of 1–2 in both treatment groups (that also occurs in syngeneic corneal grafts; not shown), the subsequent rate of opacification was significantly slowed by BPE (Fig. 4A) ($p < 0.05$). No clinically relevant differences were observed in strength of corneal edema or vascularization between the two groups (Fig. 4B and C). Corneal allograft rejection is defined as complete opacification (grade 4). In the rat model used, this is achieved after a median of 15 days postoperatively (Schwartzkopff et al., 2010a). After the significant initial discrepancy observed in corneal opacification, this parameter equalized and allograft rejection occurred in both treatment groups within statistically similar timescales. However, a tendency toward better graft survival was observed in BPE-treated recipients (Fig. 5A). No obvious toxic side effects were observed (Fig. 5B).

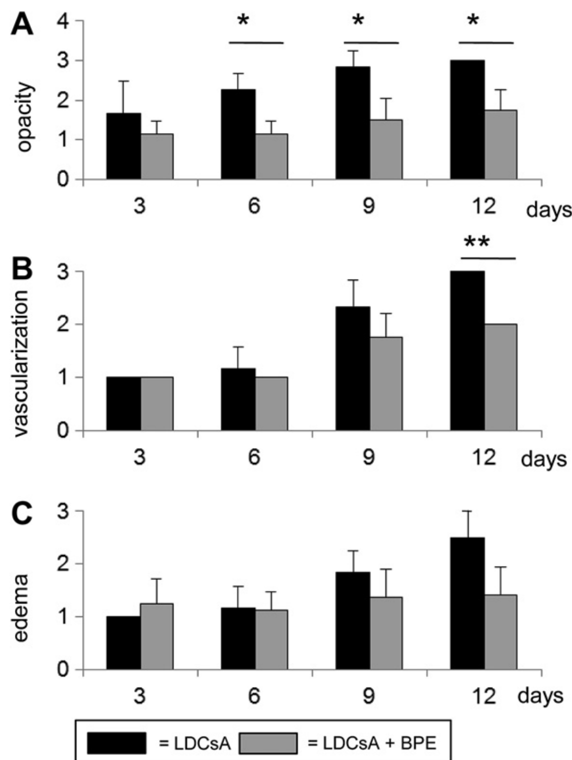

Fig. 4. BPE reduces corneal opacification following keratoplasty. Orthotopic penetrating keratoplasties were performed between Lewis and Fisher rats and clinically evaluated for signs of opacification (A), vascularization (B), and edema (C). Bars show LDCsA animals (control, black) in comparison to BPE + LDCsA (grey) with SD.

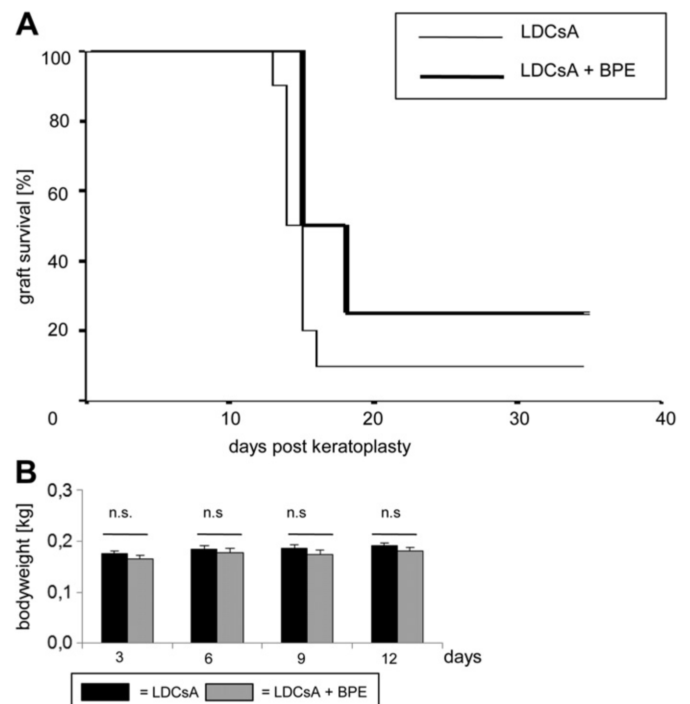

Fig. 5. Graft survival and toxicity. Total graft survival post-keratoplasty was analyzed and displayed by Kaplan–Meier survival curve. Treatment with LDCsA ($n = 11$, thin line) as control compared with BPE + LDCsA ($n = 4$, thick black line) did not promote total graft survival ($p = 0.17$) (A). While treating the animals with BPE, health care checks were routinely conducted and body weight quantified. Bars with SD in Figure (B) illustrate body weight development of LDCsA (control, black) compared to BPE + LDCsA (grey) treated animals. No significant variations were found between the two groups.

3.4. Reduced T cell infiltration in corneal grafts of BPE-treated rats

Clinically apparent corneal opacification is known to correlate with the extent of mononuclear cell infiltration in the graft (Slegers et al., 2000), which is a sign of corneal inflammation occurring during allograft rejection. In order to analyze whether the observed lower opacity-score also correlated with cellular corneal inflammation, corneas of both treatment groups were analyzed histologically ($n = 4$, respectively). The number of graft-infiltrating $CD45^{+}$ leukocytes was significantly lower in recipients that had received BPE ($p < 0.001$) (Fig. 6A). Moreover, the difference in the number of $CD4^{+}$ T cells in corneal grafts was even more pronounced in BPE-treated recipient rats ($p < 0.0001$) (Fig. 6B). No effect of BPE was seen regarding the strength of infiltrating $CD163^{+}$ macrophages (Fig. 6C).

4. Discussion

Corneal transparency is affected by corneal inflammation that may result from infection, trauma or occur postoperatively. The long-term risk of scar-establishment and subsequent reduced vision is considerable if left untreated. Following keratoplasty, a primary traumatic inflammatory response occurring soon after transplantation is a risk factor in graft rejection and irreversible graft failure. Topical corticosteroids are the mainstay treatment throughout the world to control any emerging immune reaction following keratoplasty (Hill, 1995). Where the risk of corneal inflammation and rejection are increased, this may be supplemented by systemic immunosuppressive therapy. Independently of the application route (topically or systemically), these additional treatments are all accompanied by possible side effects, which may appear locally or even systemically and thereby become life threatening. A focused concept for treatment of corneal inflammatory responses following keratoplasty is therefore desirable. This should be mediated by pharmaceuticals that specifically target T cells and involve minimal side effects.

In this context, a commercially available extract from birch leaves (BPE) was shown to inhibit human T cells (Gründemann et al., 2011). Members of the *Betulaceae* (birch) family are known to contain appreciable amounts of polyphenolic compounds (Keinänen and Julkunen-Tiitto, 1998) that were shown to be effective anti-inflammatories in animal models (Guardia et al., 2001; Mamani-Matsuda et al., 2006). Birch leaf extracts are known to contain mainly flavonoid-glycosides, such as quercetin-3-galactoside, quercetin-3-glucoside, quercetin-3-rhamnoside and quercetin-3-glucuronide (Keinänen and Julkunen-Tiitto, 1998). In the aqueous birch leaf extract used for this study, the presence of typical polyphenolic compounds was previously confirmed using LC-MS analysis (Gründemann et al., 2011). Birch leaf extracts were traditionally used to treat rheumatism and arthritic diseases. Aside from its anti-inflammatory properties, no severe side effects were reported. As the immune rejection of a corneal graft is mainly mediated by $CD4^{+}$ T cells, we hypothesized whether BPE would affect corneal inflammation following keratoplasty in the rat model. In this experimental setting, it was found that a severe inflammatory response occurs, which mediates rejection and graft failure of the transplanted cornea.

In order to test whether BPE has an effect on rat T cells, BPE was added to stimulated $CD4^{+}$ T cell cultures. As shown in Fig. 1, BPE specifically and significantly inhibited proliferation of $CD4^{+}$ T cells *in vitro*. This was mediated by apoptosis of dividing T cells (Fig. 3), whereas resting T cells were not affected (not shown). Surprisingly,

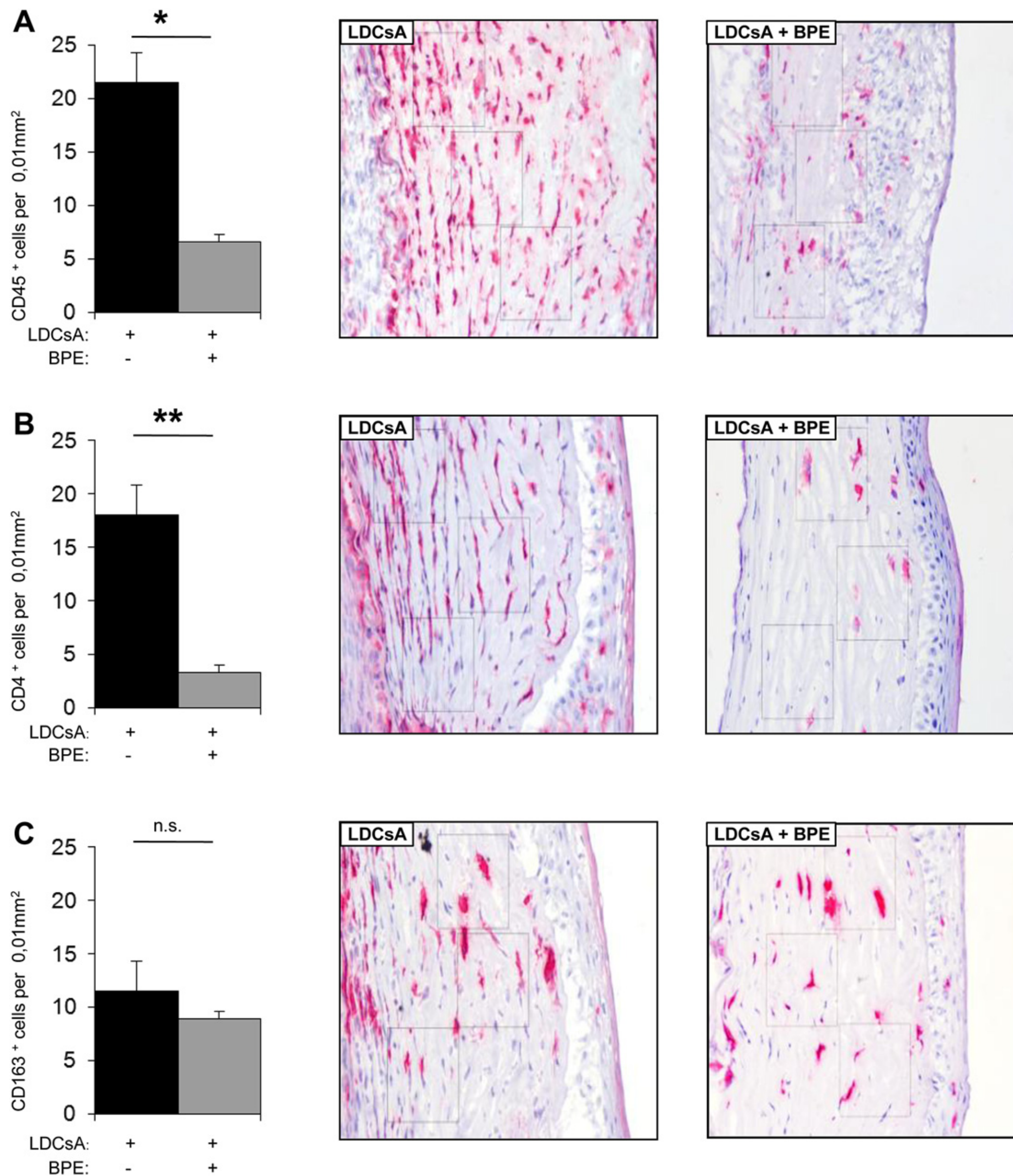

Fig. 6. BPE reduces leukocytic cell infiltration. Immunostaining of cryosections of LDCsA (control, black) and LDCsA + BPE (grey) treated animals ($n = 4$, respectively) were performed at day nine. $CD45^+$ leukocytic infiltrate (A), $CD4^+$ T cell infiltrate (B), and $CD163^+$ macrophage infiltrate (C) in the central corneal stroma were counted in three different squares per animal. Significant reductions of infiltrating leukocytes ($p < 0.001$) and of infiltrating $CD4^+$ T cells ($p < 0.0001$) were identified in the BPE group by two independent investigators. Macrophage-infiltrate was not affect by BPE treatment. Bars represent cell infiltration per 0.01 mm^2 with SD.

the activation status of remaining T cells did not change compared to untreated activated cells and CD25 was upregulated, whereas CD62L-expression reduced (Fig. 2) unless proliferation did not occur. This suggests that BPE not only induced cell death but also influenced T cell differentiation *in vitro*. It remains to be determined whether the remaining $CD25^+$ T cells are early activated, anergic or regulatory T cells. Further *in vitro* analyses would require the isolation of BPE-treated cells from cultures.

As the pro-apoptotic ability of BPE *in vitro* was similarly effective to methotrexate (MTX), we questioned whether its immunosuppressive capacity would be transferable to an *in vivo* system that is mediated by $CD4^+$ T cells. Keratoplasty possibly resembles one of the most severe and complex corneal inflammatory responses. Although graft rejection is a very complex immunological scenario, complete abrogation of the $CD4^+$ T cell response at the time of surgery can promote long-term survival (Ayliffe et al., 1992). Pharmacologically, this effect is used during cyclosporine-A therapy, where T cells are inhibited and graft survival is achieved if cyclosporine serum levels do not fall below a certain value. If the initial value is too low or subsequently drops below the threshold, the therapy becomes ineffective and rejection is possible. In our pilot experiments, no positive effect of BPE monotherapy following keratoplasty was observed (not shown). Following previous studies, in which the substance of interest proved to be effective only if combined with a low, subtherapeutic CsA dosage (LDCsA) (Hackstein et al., 2007) we suggested an additive effect of BPE with LDCsA. Therefore, LDCsA was injected daily, either alone or in combination with BPE, over a period of 14 days, beginning on the day of keratoplasty. Compared to LDCsA monotherapy, the combination with BPE

significantly inhibited the clinical progress of corneal opacification ($p < 0.01$), whereas the effect on edema formation and vascularization was clinically marginal (Fig. 4). We recently demonstrated that strength of graft infiltration and opacification is directly correlated (Schwartzkopff et al., 2010b). We therefore conclude that BPE exhibited an inhibitory effect on corneal inflammation if combined with LDCsA. However, although intracorneal inflammation following keratoplasty was clinically reduced, no clinical survival-promoting effect was observed (Fig. 5). It is well known that corneal allograft rejection involves many immunological mechanisms and that these can compensate for each other. Aside from the fundamental role of $CD4^+$ T cells, other cellular (e.g. antigen-presenting cells or NK cells) or angiogenic factors are also involved. In the model presented, compensatory or altered rejection mechanisms could be present and may potentially explain why rejection occurred despite reduced initial inflammation.

Moreover, it is important to note that different treatment strategies may exhibit different effects even if a single cell type (e.g. $CD4^+$ T cells) is targeted: Whereas $CD4^+$ T cell-depletion abrogates graft rejection in the long-term, the outcome of T cell-specific treatment with CsA correlates with the time of therapy.

In order to better understand the initial cellular mechanisms that subsequently mediate graft rejection, staining of leukocytes, T cell populations and macrophages was performed in both treatment groups (Fig. 6). Corresponding with our clinical findings, the number of graft-infiltrating $CD45^+$ leukocytes was significantly reduced ($p < 0.001$). More importantly, and correlating to our *in vitro*-results, intragraft $CD4^+$ T cells showed an even greater reduction ($p < 0.0001$), whereas no changes of $CD163^+$ macrophages were observed. Similarly to the experiments shown in Fig. 1, BPE influenced T cells but not macrophages. Therefore, we conclude that the cytostatic effect of BPE appears to be transferred to the *in vivo* situation. However, it remains to be determined whether BPE also induces apoptosis of T cells in this experimental animal model. Therefore, we would suggest analyzing T cells, obtained by draining lymph nodes, for the presence of apoptotic T cells.

Our results demonstrate that BPE has a strong cytostatic capacity and leads to significant and clinically relevant reduction of corneal inflammation following corneal transplantation. Importantly the anti-inflammatory effect is achieved without obvious negative side effects such as weight loss or behavioral changes in the rat model. Further consideration should be given to analyzing the individual components of BPE in order to isolate single fractions that may be responsible for the cytostatic effect. BPE contains many plant-derived components (flavonoids etc.) that may cause allergic reactions, if high BPE-concentration would be necessary for an effective treatment. Therefore, isolation and pharmacological application of the “molecule of interest” would be desirable to reduce the risk of allergy development. Taken together, BPE or individual constituents may be used to reduce T cell-mediated inflammatory reactions in the cornea.

Disclosure statement

KW, CG, YK, LB, RH, TR and JS have no financial interest in this work.

CG received financial support from ABNOBA GmbH for lab consumables.

RH previously acted as a consultant to ABNOBA GmbH on issues unrelated to the present work.

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