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Macrophage Phenotype in the Ocular Surface of Experimental Murine Dry Eye Disease

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Abstract

To evaluate the phenotype of macrophages in the cornea and conjunctiva of C57BL/6 mice with induced experimental dry eye. C57BL/6 mice exposed to desiccating stress (DS) were evaluated at 1, 5, and 10 days and C57BL/6 mice maintained in non-stressed environment were used as controls. Whole eyes and adnexa were excised for histology or used for gene expression analysis. Location and phenotype of macrophages infiltrating the cornea and conjunctiva was evaluated by immunofluorescence analysis. Quantitative polymerase chain reaction evaluated macrophage

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Conflict of Interest All the authors declare that they have no conflict of interest.

markers and T cell-related and inflammatory cytokine expression in cornea and conjunctiva. Immunofluorescence staining demonstrated that macrophages reside in the conjunctiva of control and dry eye mice and their number did not change with DS. Real-time RT-PCR demonstrated that the level of M1 macrophage marker, iNOS, increased prominently in the conjunctiva at DS 10 days. In contrast, there was a non-significant decrease of the M2 marker Arg1 with DS. The levels of inflammatory cytokine, IL-12a mRNA transcript in the conjunctiva increased significantly at DS1 and decreased at DS5, while levels of IL-18 were significantly increased at DS 10. Macrophages reside in the ocular surface tissues of C57BL/6 mice. Although the number of macrophages in the conjunctiva does not change, evidence of inflammatory M1 activation after desiccating stress was observed. Better understanding of phagocyte diversity and activation in dry eye disease provide a basis for the development of phagocyte-targeted therapeutic strategies.

Keywords

Dry eye disease; Macrophage phenotype; Desiccating stress; Ocular surface

Introduction

Dry eye disease (DED) is one of the most prevalent eye conditions that causes bothersome irritation and can possibly decrease quality of life. DED is not simply a disease of decreased tear production, but has a pathogenesis based on a T cell-mediated autoimmune response. DED has been demonstrated to cause inflammation on the ocular surface, evidenced by increased levels of inflammatory cytokines [interleukin (IL)-1, IL-6, and tumor necrosis factor (TNF)-α] in the tear fluid and corneal and conjunctival epithelia and infiltration of the conjunctiva with $CD4^+$ T cells (De Paiva et al. 2007; Pflugfelder 2003; Pflugfelder et al. 1999; Solomon et al. 2001; Stern et al. 2002). Macrophages interact intimately with $CD4^+$ T cells, serving as both T cell-directed phagocytes and T cell-activating antigen-presenting cells (Zhou et al. 2012). Macrophages are heterogeneous, versatile cells that display remarkable phenotypic plasticity and functional diversity (Galli et al. 2011; Gordon and Taylor 2005; Sica and Mantovani 2012). Hence, activated macrophages often are classified in a spectrum of polarization states. Mirroring T helper type 1–T helper type 2 (T_H1–T_H2) polarization, two distinct states of polarized macrophage activation have been recognized: the classically activated (M1) macrophage phenotype and the alternatively activated (M2) macrophage phenotype (Galli et al. 2011; Gordon and Taylor 2005; Mantovani et al. 2002; Sica and Mantovani 2012). The M1 macrophages are induced by interferon (IFN)-γ or microbial stimuli [lipopolysaccharide (LPS)], and characterized by the expression of high levels of inducible nitric oxide synthase 2 (iNOS) (Mosser and Edwards 2008; Stout et al. 2005). Following activation, M1 macrophages secrete proinflammatory and T cell polarizing cytokines (IL-1β, TNF-α, IL-12 and IL-23) and chemokines (CXCL9 and CXCL10), and induce inflammation and tissue destruction (Mosser and Edwards 2008; Stout et al. 2005). In contrast, the M2 macrophages are driven by T_H2 cytokines IL-4 and IL-13 and are characterized by high expression of mannose receptor (CD206) and production of IL-10, an anti-inflammatory cytokine that resolves inflammation and promotes wound healing (Mosser and Edwards 2008; Stout et al. 2005).

Cytokine expression patterns or the ratio of iNOS to arginase 1 (Arg1) gene expression is commonly used as readouts of macrophage phenotype and functional status (Chiang et al. 2008; Mosser 2003; Stout et al. 2005). However, these functional phenotypes are reversible.

Although mechanisms and molecules associated with macrophage plasticity and polarized activation have been studied in tumors, diseases of the central nervous system, or tissue transplantation (Biswas and Mantovani 2010; Oh et al. 2013; Sica 2010; Shechter and Schwartz 2013), there have been few studies on differentiation and distribution of macrophages in dry eye associated inflammation. Thus, we investigated the phenotype of macrophages in ocular surface tissues of experimental dry eye in mice. Greater understanding of macrophage plasticity and polarization in the ocular surface response to desiccating stress may provide a basis for macrophage-centered diagnostic and therapeutic strategies.

Materials and Methods

Animals

This research protocol was approved by the Baylor College of Medicine Center for Comparative Medicine, and it conformed to the standards of the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research.

Six- to eight-week old of female C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME) for establishment of mouse colonies in our vivarium. They were housed in our vivarium in a pathogen-free environment that is managed by Baylor Center for Comparative Medicine. Mice were exposed to a 12-h light cycle while in the vivarium with timers controlling the light/dark cycle.

Induction of Desiccating Stress

C57BL/6 mice were exposed to desiccating stress (DS). DS was induced by subcutaneous injection of scopolamine hydrobromide (0.5 mg/0.2 ml; Sigma–Aldrich, St. Louis) four times a day (0800, 1100, 1400, 1700 hours), alternating flanks, for 1, 5 or 10 consecutive days (DS1, DS5, DS10), as previously described (Coursey et al. 2013; Dursun et al. 2002; Luo et al. 2004). Mice were placed in a cage with a perforated plastic screen on one side to allow airflow from a fan placed six inches in front of it for 16 h/day. Room humidity was maintained at 20–30 %. Control mice were maintained in a non-stressed environment at 50–75 % relative humidity without exposure to a forced air draft.

Histology and Immunofluorescent Staining

Immunofluorescence staining was performed to detect F4/80 (general macrophage marker) expression by infiltrating macrophages in corneal and conjunctival tissue sections. Eyes and adnexa from three mice of each group were surgically excised, embedded in optimal cutting temperature compound (VWR, Swannee, GA), and flash frozen in liquid nitrogen. Briefly, cryosections were fixed in methanol for 10 min, and permeabilized with 0.1 % Triton X-100 in phosphate buffer solution (PBS). After blocking with 20 % normal goat serum in PBS for

30 min, monoclonal rat antibody against F4/80 (Novus Biologicals, Littleton, CO) was applied, and the sections were incubated for 1 h at room temperature. After rinsing with PBS, Alexa-Fluor 488 conjugated goat anti-rat IgG (1:100 dilution) secondary antibodies were then applied, and the sections were incubated in a dark chamber for 1 h, and followed by nuclear counterstaining with Hoechst (Sigma-Aldrich, St. Louis, MO). Tissues without primary antibody were used as negative controls. The images were photographed using an epifluorescence microscope (Eclipse 400; Nikon, Garden City, NY). Positively stained cells were counted in the goblet cell rich area of the palpebral conjunctiva, over a length of at least 500 μm in the epithelium and to a depth of 75 μm below the epithelial basement membrane in the stroma for a distance of 500 μm using image-analysis software (NIS Elements Software, version 3.0; Nikon). Results were expressed as the number of positive cells per 100 μm.

RNA Isolation and Real-Time PCR

Total RNA from conjunctiva and corneal epithelium (individually collected and individually pooled) from C57BL/6 mice was extracted using a Qiagen MicroPlus RNA Isolation Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions, measured by a NanoDrop ND-1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA), and stored at −80 °C. One sample consisted of pooled samples of right and left eyes of the same animal. Samples were treated with DNase to prevent genomic DNA contamination according to the manufacturer's instructions (Qiagen, Valencia, CA, USA).

First-strand complementary DNA was synthesized from 1 μg of total RNA using random hexamers and M-MuLV reverse transcriptase (Ready-To-Go You-Prime First-Strand Beads; GE Healthcare, Arlington Heights, NJ, USA), as previously described (Luo et al. 2004).

Quantitative real-time PCR was performed with specific minor groove binder (MGB) probes (Taqman; Applied Biosystems) and PCR master mix (Taqman Gene Expression Master Mix), in a commercial thermocycling system (StepOnePlus Real-Time PCR System; Applied Biosystems). Murine MGB probes were NOS2 (iNOS, Mm00440485_m1), Arg1 (Mm00475988_m1), IL-9 (Mm00434304_m1), IL-4 (Mm00445259_m1), IL-13 (Mm00434204_m1), IL-18 (Mm 00434225_m1), and IL-12a (Mm00434165_m1). The hypoxanthine phosphoribosyltransferase 1 (HPRT-1) (Mm00446968_m1) gene was used as an endogenous reference for each reaction. The results of quantitative PCR were analyzed by the comparative C_t method in which the target of change $=2^{-\Delta\Delta C_t}$ and were normalized by the C_t value of HPRT-1 and the mean C_t of relative mRNA level in the normal control group (non-stressed) was used as the calibrator.

Statistical Analysis

One-way analysis of variance (ANOVA) with Tukey's post hoc testing was used for statistical comparisons of multiple groups. $P \leq 0.05$ was considered statistically significant. These tests were performed using GraphPad Prism 6.0 software (GraphPad Software Incorporation, San Diego, CA).

Results

Macrophage Infiltration in the Ocular Surface of Unstressed and Desiccating Stressed C57BL/6 Mice

F4/80$^+$ macrophages were detected in the conjunctival stroma and epithelium by immunofluorescence staining (Fig. 1a). These F4/80$^+$ cells also infiltrated the subepithelial and stromal compartment of cornea (Fig. 1a), the number of F4/80$^+$ cells in the cornea did not change with DS. The number of F4/80$^+$ cells in the conjunctiva did not change with DS (Fig. 1b).

Effects of Desiccating Stress on Expression of Macrophage Phenotype Markers in the Cornea and Conjunctiva of C57BL/6 Mice

To investigate the macrophage phenotype, we compared expression of macrophage phenotype markers in the conjunctiva and corneal epithelium of C57BL/6 mice in non-stressed conditions and after at 1, 5 and 10 days after desiccating stress. Real-time RT-PCR demonstrated that the level of M1 macrophage marker, iNOS, increased significantly in the conjunctiva at DS10. In contrast, Arg1, a marker of M2 macrophages, showed a gradual non-significant decrease with DS (Fig. 2a). Expression of Th1 and Th2 associated cytokine transcripts in the conjunctiva was also evaluated (Fig. 2b). A significant increase in IL-12a was noted at DS1 and decreased to baseline levels at DS5 (Fig. 2b), while levels of IL-18 were significantly increased at DS10. No significant change in levels of IL-9, IL-4, and IL-13 transcripts in the conjunctiva was noted.

In the cornea of C57BL/6 mice, there was no change in the levels of iNOS, Arg1, IL-4, IL-9, and IL-13 transcripts with DS (Fig. 3). Increased expression of IL-12a in the cornea was noted with DS, but this did not reach significance (Fig. 3b). iNOS and Arg1 are known to utilize L-arginine as a common substrate, and they are considered signature enzymes of M1 and M2 macrophages, respectively. We calculated the ratio of iNOS to Arg1 to determine the predominant macrophage phenotype in non-stressed mice at each time point after induction of DS. Our results presented in Fig. 2c show greater iNOS to Arg1 ratio in the conjunctiva at DS10, whereas this ratio decreased in the cornea with DS (Fig. 3c).

Discussion

Macrophages are very flexible in adapting their transcription profile to changes in their microenvironment (Stout et al. 2005). Our results demonstrated that after subjecting C57BL/6 mice to desiccating stress, there was no change in number of macrophages, but increased production of M1 macrophage markers in the conjunctiva. Also, the expression of inflammatory cytokines IL-18 and IL-12 that are produced by activated M1 macrophages was found to increase in the conjunctiva.

Desiccating stress, factors that disrupt tear film stability and increase tear osmolarity, can induce ocular surface damage and initiate an inflammatory cascade that generates innate and adaptive immune responses (Stevenson et al. 2012). In our study, transiently increased IL-12 expression was noted after exposure to desiccating stress. IL-12 expression peaked one day after exposure to desiccating stress. The transient appearance of IL-12 would suggest that

although the number of macrophages did not change in the early period of DS, there is a shift toward M1 phenotype that may be induction of the Th1 response and increased IFN-γ production that we have previously reported (De Paiva et al. 2007).

Macrophages can also be polarized into an “M2-like” state, which shares some but not all the signature features of M2 cells (Biswas and Mantovani 2010). For example, various stimuli, such as antibody immune complexes together with LPS or IL-1, transforming growth factor-β and IL-10, give rise to M2-like functional phenotypes that share properties with IL-4 or IL-13-activated macrophages (such as high expression of mannose receptor, IL-10 and angiogenic factors) (Mantovani et al. 2004). Adipose tissue macrophages from obese mice have a mixed profile, with upregulation of several M1 and M2 gene transcripts (Shaul et al. 2010). In our study, there was an increase in levels of M1 macrophage markers in the conjunctiva in response to DS. This study suggests there is plasticity of macrophage functional state on the ocular surface with a shift towards M1 shortly after exposure to desiccating environmental conditions (Biswas and Mantovani 2010; Mosser and Edwards 2008).

Macrophages might exert direct cytotoxicity and act as effector immune cells by producing reactive nitrogen species (Biswas and Mantovani 2010; Sica and Mantovani 2012). M1 macrophages express high levels of iNOS that compete with Arg1 for L-arginine, the common substrate of both enzymes (Cho et al. 2014). iNOS converts L-arginine to nitric oxide (NO), competing with Arg1, which converts NO into urea and ornithine (Cho et al. 2014). By inhibiting iNOS, Arg1 may promote the M2 phenotype and contributes to the suppression of the M1 phenotype (Cho et al. 2014). Our data show the level of iNOS and the ratio of iNOS to Arg1 gradually increased in the conjunctiva with desiccating stress, peaking at DS10.

Previous report stated that local depletion of peripheral conjunctival antigen-presenting cells using subconjunctival injection of clodronate blocked the ability of dry eye specific $CD4^+$ T cells to accumulate within ocular surface tissues and did not induce robust production of proinflammatory cytokines (Schaumburg et al. 2011). Depleting dendritic cell and macrophages will turn off the immune response of dry eye. Complete understanding of macrophage biology and mechanisms underlying macrophage differentiation and phenotypic maturation might provide novel therapeutic targets for preventing ocular surface inflammation in response to DS.

In conclusion, our results show that C57BL/6 mice have macrophages residing in the conjunctiva and cornea. Although there is no change of number of macrophages after DS, inflammatory M1 activation was observed. Better understanding of phagocyte diversity and activation in dry eye disease may show efficacy for phagocyte-targeted therapeutic strategies.

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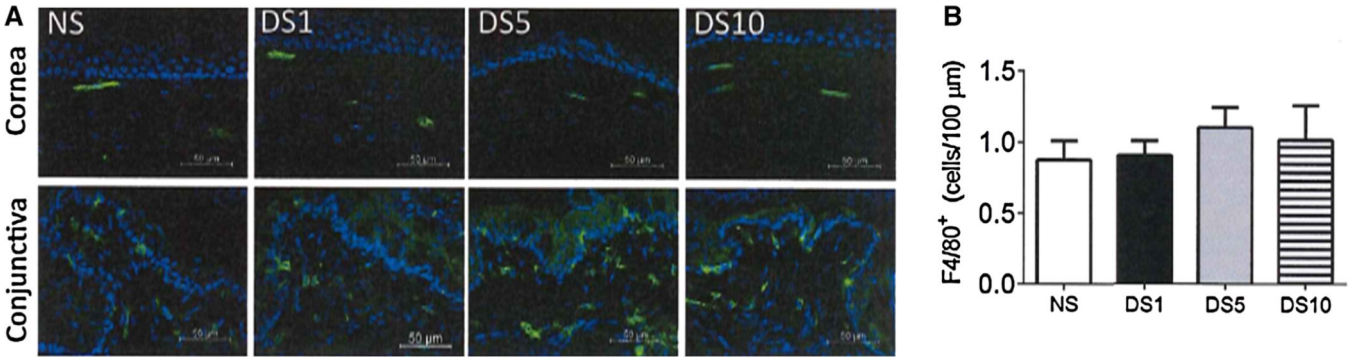

Fig. 1.

Histologic analysis of macrophage infiltration in the ocular surface of C57BL/6 mice after desiccation stress. **a** Representative images showed macrophage localization [F4/80 (*green*)] in the cornea and conjunctiva by immunofluorescence staining with Hoechst (*blue*) as nuclear counterstaining. **b** Macrophage density in the conjunctiva. *NS* non-stressed, *DS1* desiccating stress (DS) day 1, *DS*5 DS day 5, *DS*10 DS day 10

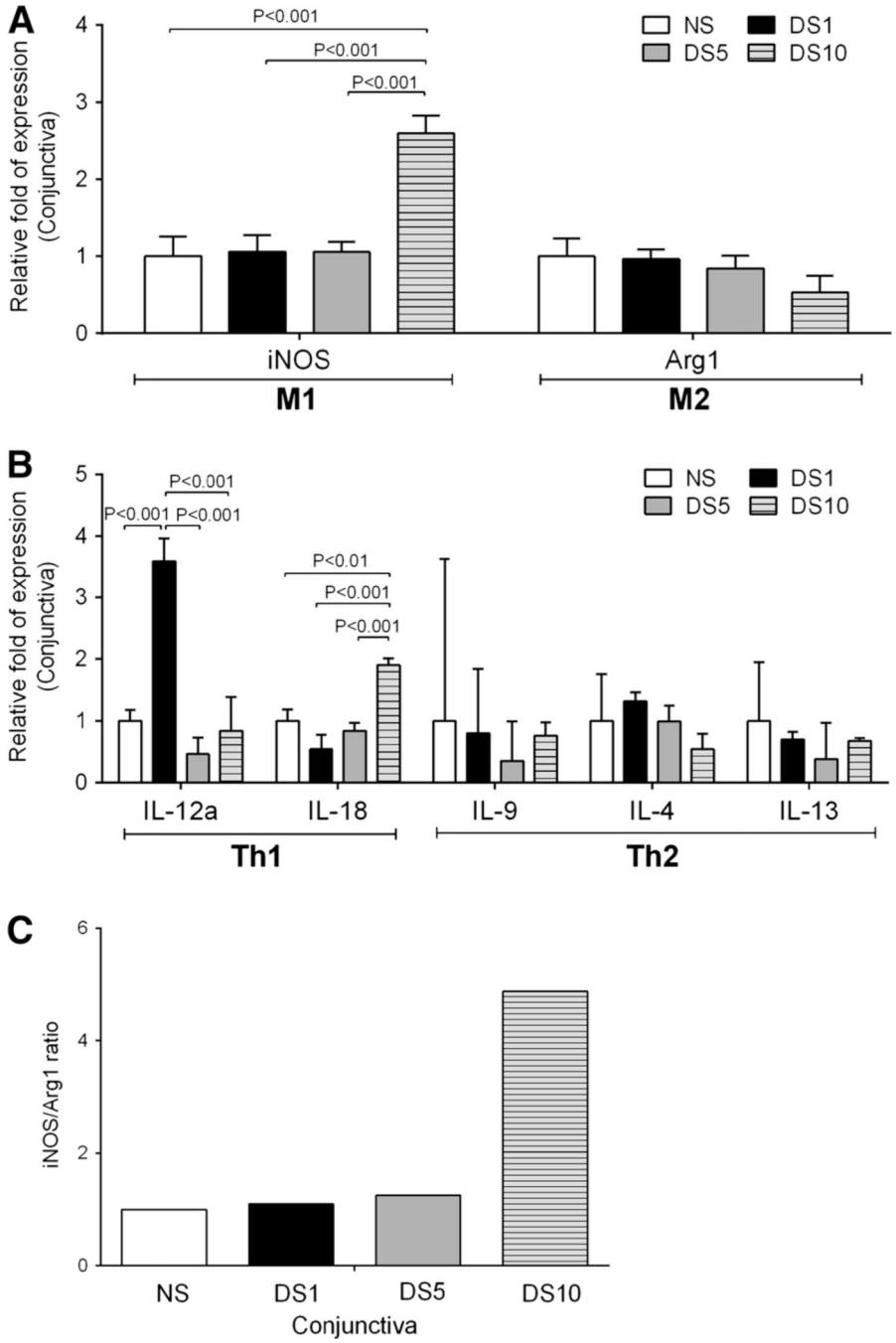

Fig. 2.

Quantitative gene analysis of mRNA transcripts in the conjunctiva of C57BL/6 mice after desiccation stress. **a** Relative fold of expression of M1 and M2 macrophage markers in conjunctiva of C57BL/6 mice at desiccating stress 1, 5, and 10 days. *Bar graphs* show mean ° SEM of one representative experiment with 7–10 samples/time point (experiment was repeated twice with similar results). One sample consisted of pooled right and left conjunctivas of the same animal. **b** Relative fold of expression of T cell-related cytokines and inflammatory cytokines. **c** iNOS to Arg1 ratio in the conjunctiva of C57BL/6 mice after

desiccation stress. *NS* non-stressed, *DS1* desiccating stress (DS) day 1, *DS5* DS day 5, *DS*10 DS day 10, *iNOS* inducible nitric oxide synthase (NOS2), *Arg1* arginase 1, *IL*-9 interleukin-9

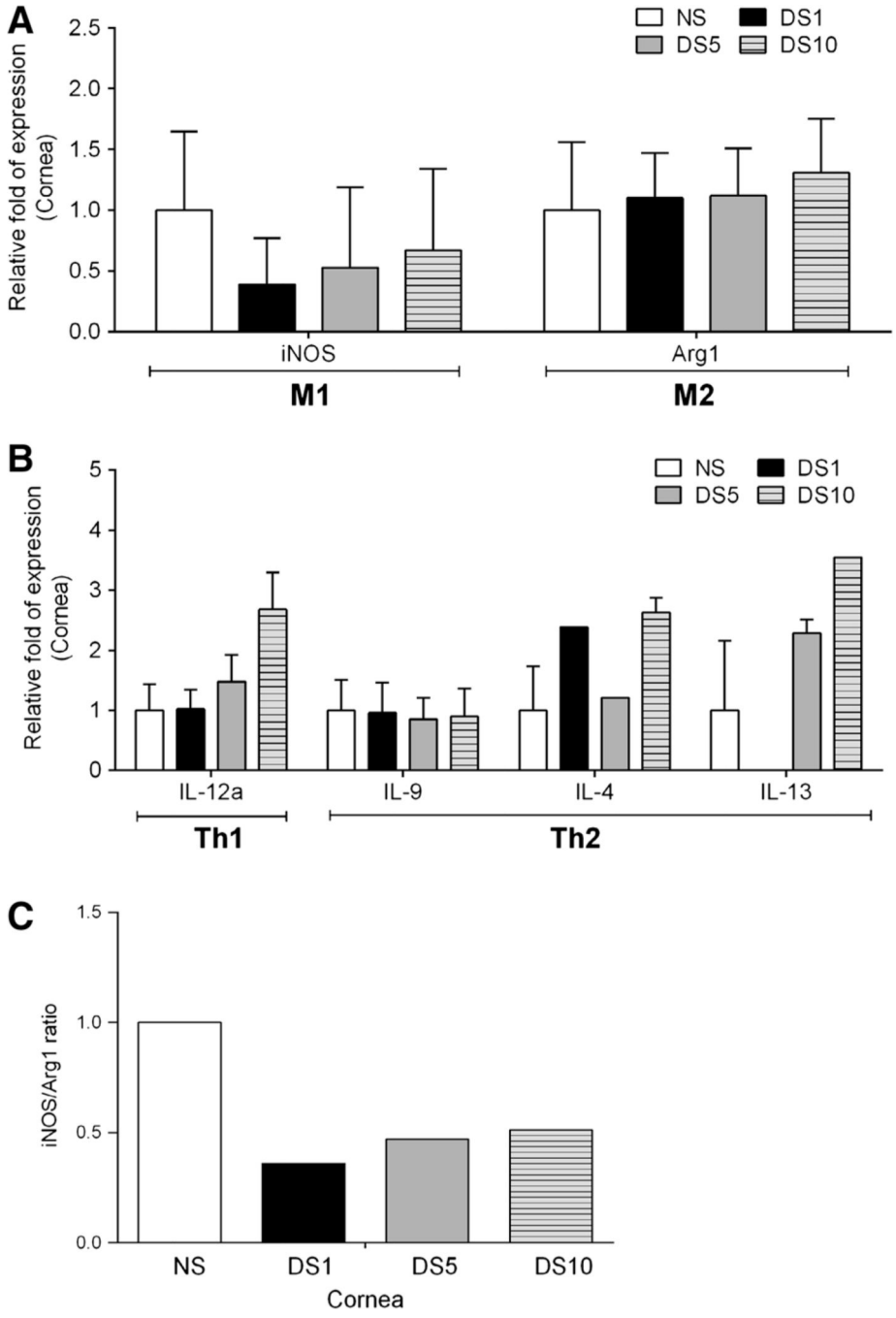

Fig. 3.

Quantitative gene analysis of mRNA transcripts in the cornea of C57BL/6 mice after desiccation stress. **a** Relative fold of expression of M1 and M2 macrophage markers in cornea of C57BL/6 mice at desiccating stress 1, 5, and 10 days. *Bar graphs* show mean ± SEM of one representative experiment with 7–10 samples/time point (experiment was repeated twice with similar results). One sample consisted of pooled right and left cornea of the same animal. **b** Relative fold of expression of T cell-related cytokines and inflammatory cytokines. In results of IL-4 and IL-13, there was 1/5 less only sample to amplify at DS1,

DS5, and DS10. **c** iNOS to Arg1 ratio in the cornea of C57BL/6 mice after desiccation stress. *NS* non-stressed, *DS1* desiccating stress (DS) day 1, *DS5* DS day 5, *DS10* DS day 10, *iNOS* inducible nitric oxide synthase (NOS2), *Arg1* arginase 1, *IL*-9 interleukin-9