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TGF β1 ACTIVATED TYPE 2 DENDRITIC CELLS PROMOTE WOUND HEALING AND INDUCE FIBROBLASTS TO EXPRESS TENASCIN C FOLLOWING CORNEAL FULL-THICKNESS HYDROGEL TRANSPLANTATION

Running headline: DENDRITIC CELL ACTIVATION IN OCULAR TISSUE FIBROSIS

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Abstract

We showed previously that 1-ethyl-3-(3-dimethylamino-propyl)-carbodiimide hydrochloride (EDC) crosslinked recombinant human collagen III (RHCIII) hydrogels promoted stable regeneration of the human cornea (continued nerve and stromal cell repopulation) for over four years. However, as EDC crosslinking kinetics were difficult to control we additionally tested a sterically bulky carbodiimide. Here, we compared the effects of two carbodiimide crosslinkers – bulky, aromatic CMC, and non-bulky EDC – in a mouse corneal graft model. Murine corneas undergoing full-thickness implantation with these gels became opaque due to dense retro-corneal membranes (RCM). Corneal epithelial cytokeratin 12 and alpha smooth muscle actin indicative of functional tissue regeneration and wound contraction were observed in RCM surrounding both hydrogel types. However, quantitatively different levels of infiltrating $CD11c^+$ dendritic cells (DC) were found, suggesting a hydrogel-specific innate immune response. More DC infiltrated the stroma surrounding EDC-NHS hydrogels concurrently with higher fibrosis-associated tenascin c expression. The opposite was true for CMC-NHS gels which had previously been shown to be more tolerising to DC. *In vitro* studies showed that DC cultured with transforming growth factor β1 (TGF β1) induced fibroblasts to secrete more tenascin c than those cultured with lipopolysaccharide and this effect was blocked by TGF β1 neutralisation. Furthermore, tenascin c staining was found in 40-50 μm long membrane nano-tubes formed in fibroblast/DC co-cultures. We suggest that TGF β1 alternatively activated (tolerising) DC regulate fibroblast-mediated tenascin c secretion, possibly *via* local production of TGF β1 in early wound contraction, and that this is indirectly modulated by different hydrogel chemistries.

Keywords: biomaterial; corneal transplantation; tissue repair; transforming growth factor β1, membrane nano-tubes, tectonic graft.

1. INTRODUCTION

Strategies employing engineered recombinant human collagen type III (RHCIII) hydrogel templates in corneal regeneration have shown promise in animal models such as mini-pigs (Liu et al., 2009), rabbits (Hackett et al., 2011) and in clinical studies (Per Fagerholm et al., 2010; P. Fagerholm et al., 2014). RHCIII hydrogels crosslinked with 1-ethyl-3-(3-dimethylamino-propyl)-carbodiimide hydrochloride (EDC) and its co-reactant N-hydroxysuccinimide (NHS) grafted as anterior lamellar grafts replacing the majority of the stroma (leaving the most posterior 50-100 μm of stroma and endothelium), promoted regeneration of the human corneal stroma and were seamlessly integrated and stable for over four years. They supported corneal epithelial, stromal and neural regeneration, and importantly retained optical clarity. Notably patients who received EDC-NHS crosslinked hydrogels did not require immuno-suppressants (P. Fagerholm et al., 2014).

Antigen presenting cells such as dendritic cells (DC) function as mediators that link innate and adaptive immunity (Banchereau & Steinman, 1998; Steinman, 2012). They are present in the cornea as immature mononuclear cells and play important roles in immune surveillance by transforming into mature counterparts that upregulate expression of major histocompatibility complex class II (MHC Class II), co-stimulatory molecules, and adaptive immune responses upon encountering pathogens (Janeway & Medzhitov, 2002; Matzinger, 1998; Shortman & Caux, 1997). Dendritic cells are potent discriminatory cells that efficiently regulate either cellular or humoral activation or immuno-inhibitory responses depending on the nature of the stimulus and consequently impact downstream responses (Janeway & Medzhitov, 2002; Matzinger, 1998; Shortman & Caux, 1997). Classical DC activation, for instance through bacterial lipopolysaccharide (LPS), leads to mature, immune-competent DC (mDC), whereas alternative activation through transforming growth factor beta 1 (TGF β1) produces an active but tolerising phenotype (tolDC) that withstands stimulation (Adnan et al., 2016; Naranjo-Gómez et al., 2011). Recent advances in biomaterial immunology have shown that biomaterials can induce both inflammatory, fibrotic responses (Anderson, Rodriguez, & Chang, 2008; Tang & Eaton, 1995) as well as specific immune responses (Babensee, 2008; Badylak & Gilbert, 2008; Sefton, Babensee, & Woodhouse, 2008).

Notably in the grafted RHCIII gels, no mDC were detected at implant sites by *in vivo* confocal microscopy. In control patients who received allografts, however, mDC were present within the grafts (P. Fagerholm et al., 2014).

Despite the immune compatibility and stable regeneration within operated corneas, the EDC crosslinking kinetics were difficult to control during implant fabrication. Hence, a sterically bulky aromatic carbodiimide, N-Cyclohexyl-N0-(2-morpholinoethyl)-carbodiimide (CMC), was tested as an alternative to small aliphatic EDC to slow down the reaction. Both crosslinked hydrogel types were manufactured to give comparable mechanical and optical properties and were functionally equivalent in mouse models as orthotopic grafts (Ahn et al., 2013), with CMC providing more controllable reaction kinetics. However, in companion studies examining the effects of the two differentially crosslinked hydrogels on DC *in vitro*, we found that EDC-crosslinked hydrogels promoted maturation of immune competent DC, while CMC-crosslinked hydrogels promoted a tolerising DC phenotype (Mölzer, Shankar et al, submitted).

Whilst partial thickness corneal transplants (lamellar keratoplasties) are beneficial in cases of stromal opacity, full-thickness corneal transplants or penetrating keratoplasties may be the only therapeutic options available for patients with stromal opacity extending to endothelial involvement. Such cases comprise the major cause of corneal blindness in developing nations. Our laboratory previously published that EDC-NHS or N-Cyclohexyl-N0-(2-morpholinoethyl) carbodiimide metho-*p*-toluenesulfonate (CMC)-NHS crosslinked RHCIII hydrogels employed in full-thickness murine corneal transplants resulted in reduced corneal clarity by day 22 post transplantation (p.t.) due to the formation of dense retro-corneal membranes (RCM) (Ahn et al., 2013). Retro-corneal membranes develop as complications of penetrating keratoplasty, especially in high risk patients, and commonly after corneal prosthesis (Buznyk et al., 2015). They present as dense collagenous membranes, effectively forming a partial capsule surrounding the implant. Previous studies have shown that inflammatory macrophages (MΦ 1) are implicated in RCM formation, a process akin to exaggerated scar tissue formation. However, T cells have also been implicated in scar tissue formation (Ghiasi, Cai, Perng, Nesburn, & Wechsler,

2000) and are usually activated by DC. The significance of DC in RCM formation has not been previously explored.

The impact of biomaterials on modulating DC maturation and allo-stimulation are well-documented (Park & Babensee, 2012; Shankar, Petrie, García, & Babensee, 2010). We previously examined the effects of EDC *vs.* CMC on the activation or maturation status of corneal DC, and suggested a possible role in the wound healing responses, in maintaining tissue homeostasis, and their possible involvement in directing tissue repair (Ahn et al., 2013). In corneal epithelial wound repair, bi-directional interactions between intraepithelial DC and epithelial cells lead to enhanced wound closure mediated by the chemokine CXCL10, thymic stromal lymphopoeitin or interleukin-1β, while depleting corneal DC delayed this outcome (Gao, Yin, Yoon, Mi, & Yu, 2011), demonstrating key roles for DC in wound contraction. Specifically, epithelial cells promoted DC recruitment into corneas and DC activation, while DC impacted epithelial apoptosis and limited the deleterious effects of polymorphonuclear leukocyte penetration into healing corneas (Gao et al., 2011). Others have shown that beta-glucan-driven activation of human monocyte-derived DC *via* engagement of the C-type lectin receptor dectin-1 expressed on DC triggered the migration and multiplication of human skin-derived keratinocytes resulting in wound closure *in vitro* and *ex vivo* (van den Berg, Zijlstra-Willems, Richters, Ulrich, & Geijtenbeek, 2014). These studies reveal the multi-faceted nature of wound healing and highlight potentially central roles for DC in coordinating cohesive responses in tissue repair.

In the context of corneal hydrogel implants, we propose in an accompanying report that differences in hydrogel properties may result in the differential recruitment of and/or recognition by immune cells including DC (Mölzer, Shankar et al, submitted). This may in turn differentially impact DC-directed responses of fibroblasts, epithelial cells, keratinocytes and smooth muscle cells involved in tissue repair. It is important to consider these interactions when designing regenerative extracellular matrix-inspired biomimetics in a site-specific manner (Shankar, Griffith, Forrester, & Kuffová, 2015). To this end, we designed a range of *in vitro* experimental panels, including three connective tissue cell lines (*i.e.* fibrosarcoma WEHI-164, subcutaneous L929, and embryonic NIH-3T3; alone or in co-culture with DC), to cover different stages and conditions of physiological and pathological development which

we investigated. To explore corneal wound healing responses *in vivo*, EDC-NHS and CMC-NHS cross-linked hydrogels were grafted into BALB/c mice. The methods utilised included immunohistochemistry, confocal fluorescence microscopy, and flow cytometry.

Here we show that the immune-tolerizing hydrogel CMC-NHS markedly reduced levels of DC infiltration *in vivo*, compared to the more pro-inflammatory EDC-NHS gel. We also report that the CMC-NHS gel was associated with RCM formation and wound healing, with markedly lower levels of tenascin c expression in the epithelial and sub-epithelial RCM stroma. We have shown *in vitro* CMC-NHS gels promote "tolerant" DC (tolDC) which express lower levels of co-stimulating molecules along with increased levels of apoptosis (Mölzer, Shankar et al, submitted), and demonstrate here that CMC-NHS induced tolDC only in low numbers. Dendritic cells can directly impact fibroblast-mediated production of tenascin c. As this protein is secreted by keratinocytes and fibroblasts involved in tissue regeneration (Kim S. Midwood & Orend, 2009; Pearson, Pearson, Shibahara, Hofsteenge, & Chiquet-Ehrismann, 1988), we are therefore proposing an association between DC and tissue fibrosis. Bone marrow DC are recruited within hours to transplanted RHCIII hydrogel corneas (Shankar et al., 2015) and may be uniquely positioned to modify wound healing and thereby determine hydrogel acceptance and functional integration or conversely, fibrosis and hydrogel opacity.

2. MATERIALS AND METHODS

2.1. Hydrogels

Recombinant human collagen type III (RHCIII) hydrogels were fabricated as previously described (Ahn et al., 2013). Type III collagen was chosen over other types, as it is comparably more flexible, facilitating surgical handling during grafting. Very briefly, 600 mg (w/w) of an 18 % (w/w) aqueous RHCIII solution (Fibrogen, San Francisco, CA, USA) was mixed with 150 µl of a 0.625 M solution of morpholinoethanesulfonic acid (MES; EMD Chemicals Inc., USA). A predetermined quantity of either EDC or CMC crosslinker was added based on a molar equivalent ratio of crosslinker:collagen-NH2. Carbodiimide:NHS was used at a 1:1 M equivalent (ME), where the molar

equivalent is defined as the amount of carbodiimide to the amino content in collagen. After reaction, the mixture was cast into 150 µm thick sheets between glass plates separated by spacers and cured. The resulting hydrogels were demoulded, washed thoroughly and stored in phosphate buffered saline containing 1 % chloroform to maintain sterility until use.

2.2. Implantation of hydrogel into mouse corneas

All animal experiments complied with the ARRIVE guidelines and were carried out by well-trained, experienced staff in accordance with the UK Animals (Scientific Procedures) Act, 1986 and associated guidelines. Naïve, 6 – 8 weeks old BALB/c mice (obtained from the Medical Research Facility, University of Aberdeen, Scotland, UK) underwent full-thickness right-sided corneal implantation with either EDC-NHS- or with CMC-NHS- (Sigma Aldrich, St. Louis, MO) crosslinked RHCIII hydrogels. Hydrogel implants had been trephined from whole sheets into 2 mm diameter discs and sterilised with gentamicin in phosphate buffered saline (PBS; Oxoid Ltd., Hampshire, UK) as described in detail elsewhere (Ahn et al., 2013). Whole eyes were removed at day 2 (baseline) or day 22 p. t., respectively (Ahn et al., 2013), embedded in OCT on dry ice and stored frozen at -20 °C until use.

2.3. Immunohistochemistry and confocal microscopy

Eyes were cut into 6 µm thick sections and fixed in 4 % (v/v) methanol-free formalin (Thermo Fisher Scientific Inc., Waltham, MA) in PBS (15 min, 4 °C) and in 100 % methanol [(Sigma Aldrich) 20 min, room temperature (RT)] and washed in PBS three times for 2 min each. Sections were permeabilised with 0.2 % (v/v) Triton X-100 (Sigma Aldrich) (overnight, 4 °C) in a humid chamber, washed as before and blocked with PBS-BGEN for 30 mins at RT [10 % (v/v) normal mouse serum diluted in 3 % (v/v) bovine serum albumin (Fisher Scientific Waltham, MA), 0.25 % gelatin (Sigma Aldrich), 5 mM ethylene diamine tetraacetic acid (EDTA) (Sigma Aldrich) and 0.025 % octylphenoxypolyethoxyethanol (IGEPAL CA-630, Sigma Aldrich)]. Sections were incubated with 5 - 10 µg/mL of polyclonal anti-α-smooth muscle actin (α-SMA) (Novusbio, UK), polyclonal anti-

cytokeratin 12 (Santa Cruz, Heidelberg, Germany), anti-tenascin c (578) (Novusbio) or fluorescently-labelled anti-CD11c (HL3) (BD Biosciences, Franklin Lakes, NJ) in PBS-BGEN, (overnight, 4°C). After washing, sections were stained with species-specific fluorescently labelled secondary antibodies corresponding to α-SMA (eBiosciences, San Diego, CA), cytokeratin 12 or tenascin c (both from Invitrogen, Carlsbad, CA) [2 hours (hrs), RT) in PBS-BGEN], and protected against photo-bleaching. After washing, sections were stained against 4',6-diamidino-2-phenylindol (DAPI; Thermo Fisher; 20 min, RT) nuclear counterstain. After washing, sections were mounted in anti-fade Vectashield (Vector Laboratories Inc., Burlingame, CA), protected by coverslips (VWR, Radnor, PA), covered in foil and stored at 4 °C. To confirm signal specificity, positive controls of healthy mouse small intestine for α-SMA, healthy mouse eyes for corneal cytokeratin 12, WEHI-164 (ATCC, LGC Standards, Teddington, Middlesex, UK) mouse fibrosarcoma cell line treated with 5 and 50 ng/mL activated transforming growth factor β1 (TGF β1; R&D Systems, Minneapolis, MN; 48 hrs, 37 °C, 5 % CO_2) for tenascin c or healthy mouse spleens for CD11c were included. Appropriate controls of secondary antibodies alone or relevant isotype controls to assure specific staining or unstained controls to measure intrinsic cellular auto-fluorescence were included. Images were captured using an LSM700 confocal microscope (Carl Zeiss, Oberkochen, Germany) and were analysed using Zen Black software (Carl Zeiss, Oberkochen, Germany). Fluorescence intensity of antibody markers was quantified using the Volocity programme (Perkin Elmer, Inc., Waltham, MA, USA) by measuring fluorescence associated with designated regions of interest or entire pictures, respectively, as per the manufacturer's instructions.

2.4. Culture of primary dendritic cells

Dendritic cells were cultured as described elsewhere (Inaba et al., 1992) and as modified in (Martin-Granados et al., 2015). In brief, primary mouse DC were generated by obtaining hind-limbs of naïve C57BL/6 mice aged 5-8 weeks (mice were bred and maintained in the Medical Research Facility, University of Aberdeen, Scotland, UK), flushing bone marrow, lysing red blood cells and depleting cultures of differentiated cells [CD4 (GK 1.5), CD8a (53-6.7)], B cells [CD45R/B220 (RA3-6B2)] and MHCII cells (P7/7; Serotec) or granulocytes (Gr-1, RB6-8C5) (all antibodies from BD Biosciences, Franklin Lakes, NJ) by antibody-based magnetic bead isolation (Dynal Biotech). Dendritic cells were

cultured in media containing RPMI (Sigma) with 5 % low IgG foetal calf serum (FCS) (Gibco) supplemented with 1 % (v/v) sodium pyruvate (Gibco), 0.1 mM non-essential amino acids (NEAA; Gibco) and 10^{-5} M 2-mercaptoethanol (2-ME; Gibco), 1 % (v/v) penicillin/streptomycin (P/S; Biowhitaker) and 10 ng/mL granulocyte-macrophage colony-stimulating factor (GM-CSF; R&D Systems) in 10 cm bacteriology culture plates (BD Biosciences). Following seeding (day 0), DC cultures were maintained for another 6 days, with media changes on days 2 and 4.

2.5. Culture of fibroblast cell lines

The WEHI-164 mouse fibrosarcoma or subcutaneous L929 (ATCC, LGC Standards, Teddington, Middlesex, UK) mouse connective tissue cell lines were sub-cultured in cDMEM [DMEM (Sigma), 1 % (v/v) L-glutamine (Gibco), 10 % (v/v) FCS and 1 % (v/v) penicillin/streptomycin (Biowhitaker)] in T25 flasks (Sigma) and were passaged every 3 days using 0.025 mM trypsin-EDTA solution (Sigma). The NIH-3T3 embryonic fibroblast cell line (ATCC, LGC Standards, Teddington, Middlesex, UK) was cultured in cRPMI [RPMI (Sigma Aldrich), 1 % (v/v) L-glutamine (Gibco), 10 % (v/v) FCS, 1 % P/S (Biowhitaker)] in T25 flasks and passaged every 3 days using 1 mL of Accutase solution per flask (Sigma Aldrich; 3min, 37 °C). The seeding concentration was 1 X 10^6 cells per 10 mL of cDMEM/cRPMI and the cells were cultured in 10 cm bacteriology dishes at 37 °C with 5 % CO_2 and used at 6-10 passages.

2.6. Co-culture of DC and fibroblasts

$CD11c^+CD11b^+$ DC harvested on day 6 of culture (~ 60-70 % confluent, with day 0 being the day of seeding) were plated in 24-well plates (Greiner) for 24 hrs (37 °C, 5 % CO_2) at 5 X 10^5 cells/well, and either left untreated or treated with different concentrations of activated TGF β1 (1 – 50 ng/mL), 1 μg/mL bacterial lipopolysaccharide (LPS) from *E. coli* (Invivogen, San Diego, CA) or 2.5 ng/mL anti-TGF β1 neutralisation antibody (Thermo, clone 1D11.16.8). In parallel, WEHI-164, L929 or NIH-3T3 cells were plated separately in 24-well plates at a density of 2 X 10^5 cells/well and left untreated for 24 hrs (37 °C, 5 % CO_2) to facilitate fibroblast adherence.

After 24 hours, the loosely adherent DC clusters (Inaba et al., 1992) were collected, washed with sterile PBS (Oxoid, Hampshire, UK) by centrifugation at 300 g, at 18 °C and re-suspended in cDMEM (for L929 and WEHI-164) or cRPMI (for NIH-3T3). Then 500 µL of medium from each WEHI-164/L929/NIH-3T3 well was replaced with the respective pre-treated DC suspension (500 µL containing approx. 5 X 10^4 DC). Also, controls of DC alone or WEHI-164/L929/NIH-3T3 alone were left untreated or cultured in the presence of TGF β1 or LPS at similar concentrations and time points as for co-cultured counterparts. Every other day 500 µL of the DC-fibroblast co-culture media were replenished. Depending on the respective experiment, cells from co-cultures were collected at various time points (24 hrs, 48 hrs, 72 hrs, 5 days, and 6 days). For confocal microscopy, WEHI-164 and L929 were dissociated with trypsin-EDTA and fixed in 4 % formalin in PBS. For flow cytometry NIH-3T3 cells were detached using Accutase solution (Sigma Aldrich), and fixed using 1 % formalin in PBS. Fixations were performed over 15 min at 4 °C.

Alternately, DC and L929 cells were co-cultured at a DC to L929 ratio of 7.5 X 10^5:1.5 X 10^5 cells/well in 16-well chamber slides (Thermo Scientific) for 24 hrs (37 °C, 5 % CO_2) in a total of 200 µL/well after DC had been separately pre-treated with TGF β1 or LPS for 24 hrs, respectively. 100 µL of the medium was changed every other day. As before, adherent cells on chamber slide wells were fixed in 4 % formalin in PBS for 15 min at 4 °C at 48 hrs, 72 hrs, 5 days or 6 days for subsequent analysis by confocal microscopy (LSM700 confocal microscope; Carl Zeiss, Oberkochen, Germany). Fluorescence intensity was quantified using the Volocity programme (Perkin Elmer, Inc., Waltham, MA, USA) by measuring fluorescence associated with entire images, as per the manufacturer's instructions.

2.7. Quantification of fluorescence intensity

To quantify expression of CD11c and tenascin c, respectively, fluorescence intensity was measured using Volocity Image Analysis Software (PerkinElmer, Inc., Waltham, MA, USA), with brightness set

to maximum and resolution (12 bit) at 1024x1024 pixels/inch. Laser and microscope settings were kept constant between analyses (laser gain, objective, pinhole).

2.8. Flow cytometry

At 24 hrs and 48 hrs, supernatants over NIH-3T3/DC co-cultures were collected for further analysis. The remaining cells were detached from 24 wells using 150 µL of Accutase solution per well (5 min, 37 °C), washed using PBS, stained using fixable eFluor 450 viability dye (Thermo) for 30 min at 4 °C in PBS, washed again, fixed using 1 % formalin in PBS for 15 min at 4 °C, permeabilised (1.5 % Triton X-100 in PBS, 25 min, 4 °C) washed twice, stained using PE-CF594 conjugated pSmad 2/3 (phosphorylated small worm phenotype mothers against decapentaplegic protein 2 and 3; BD Biosciences; clone O72-670) and anti-tenascin c APC (Biotechne: clone 4C8MS) antibodies (30 min, 4 °C) in FACS staining buffer (1 % BSA, 0.09 % Na-azide, and 2 mM EDTA in PBS). Fluorescence minus one (FMO) controls were included in each experiment to enable accurate gating. All analyses were completed on a BD LSR Fortessa device. Correct compensation was confirmed prior to each experiment. The gating strategy for data analysis included live cells only.

2.9. Statistical analysis

Statistical analyses of normally distributed data were performed using a one-way analysis of variance (ANOVA) with Tukey *post-hoc* test (based on homogeneous variances) or Student's *t*-test. Based on a 95 % level of confidence, $p \leq 0.05$ was considered significant. For these procedures, Graph Pad Prism V.5 (Graph Pad Software, La Jolla, CA) was used. As for skewed FACS data, the Mann-Whitney U-test was used. These procedures were performed, and boxplots generated using IBM SPSS 24.

3. RESULTS

3.1. Hydrogel corneal transplants are re-epithelialised and induce activation of fibroblasts

Both EDC-NHS and CMC-NHS gels induced RCM. In addition, both gels became re-epithelialised with cytokeratin 12^+ host corneal epithelium by day 22 p.t. (Figure 1A). Continuous layers of epithelium from host cornea onto the hydrogel surface were observed. αSMA^+ fibroblasts were found in the RCM particularly at the host-gel-interface and were associated with cytokeratin 12^+ epithelial downgrowth (Figure 1B), with occasional infiltration into the hydrogel matrix.

3.2. EDC-NHS-induced RCM are markedly infiltrated by $CD11c^+$ DC

$CD11C^+$ DC were detected extensively in RCM associated with EDC-NHS gels in contrast to CMC-NHS RCM (Figure 1C). Staining for $CD11c^+$ DC extended beyond the RCM onto the anterior chamber surfaces in the presence of EDC-NHS gels, and appeared to be associated with marked tissue swelling as a sign of inflammation. In contrast to EDC-NHS gels, DC infiltration of CMC-NHS gels was qualitatively lower (Figure 2), a trend quantitatively confirmed by fluorescence evaluation on the Volocity programme, and RCM formation appeared less oedematous (Figure 1).

Figure 1 here (in b/w in print)

Figure 2 here (in b/w in print)

3.3. Expression of tenascin c is particularly abundant in RCM surrounding EDC-NHS gels

Interestingly, significant differences in expression of tenascin c were noted for EDC-NHS vs. CMC-NHS hydrogels (p 0.0079) in the RCM at day 22 p.t.. Notably, tenascin c was produced within the re-epithelialised layers, at hydrogel edges in proximity to peripheral host corneal tissue and in the stroma of the RCM surrounding hydrogels. However, strongest tenascin c staining was detected at the borders of RCM with re-epithelialised corneas surrounding EDC-NHS hydrogels (Figure 3A), while little staining was observed with CMC-NHS gels (Figure 3B).

Figure 3 here (in b/w in print)

3.4. TGF-β1 pre-treated DC induce expression of tenascin c in L929 and NIH-3T3 fibroblasts

Since tenascin c expression appeared to correlate with the degree of RCM swelling and infiltration of DC, we next investigated whether there was a direct relationship between conditioned, tolDC and tenascin c production. TGF β1 is a major cytokine known to induce tolDC and also involved in fibrosis and scar tissue formation. We therefore asked whether TGF β1-treated DC had a role in the production of tenascin c, directly or indirectly.

Figure 4 here (in b/w in print)

In initial experiments, using semi-quantitative analysis of expression of tenascin c in co-cultures of DC with L929 fibroblasts, DC-fibroblast co-cultures alone induced moderate but significant expression of tenascin c (Figure 4A; Figure 5A). Addition of TGF β1 (50 ng/mL) markedly increased tenascin c expression in the co-cultures, while lower levels of additional TGF β1 made little difference (Figure 4A, B; Figure 5B). L929 fibroblasts alone produced little tenascin c while LPS induced a late, and hence shorter-lived surge in tenascin c levels (Figure 4A, B; Figure 5A). These data suggested that DC have the ability to induce tenascin c in tissue fibroblasts in response to TGF β1. To follow up on this and assess if embryonic cells would respond differently to TGF β1, a series of NIH-3T3 fibroblast DC co-culture experiments were performed (Figure 6). This was also done to confirm activation of the TGF β signalling pathway in fibroblasts and its progression through the Smad protein family, eventually resulting in tenascin c expression. To this end, phosphorylation of Smad 2/3 was measured in NIH-3T3 cells, together with intracellular tenascin c production after 24 hrs and 48 hrs, using flow cytometry. This was done after complete removal of DC from the co-cultures. Absence of DC was confirmed by a lack of $CD11b^+$ and $CD11c^+$ staining signals (data not shown). For the co-cultures, DC pre-treated with 1, 2.5, 5, 10, 25, and 50 ng/mL TGF β1, anti-TGF β1 neutralisation antibody or both (2.5 ng/mL antibody and/or 2.5 ng/mL TGF β1) were used. Both direct TGF β1 administration to NIH-3T3 and TGF β1 pre-treatment of DC, induced significant phosphorylation of Smad 2/3 and tenascin c protein expression in NIH-3T3 fibroblasts for the majority of test conditions, compared to untreated controls, even at TGF β1 concentrations that were lower by orders of magnitude (1 vs. 10, and 5 vs. 50 ng/mL)

as compared to L929 cells (Figures 5 and 6). This effect was blocked by anti-TGF β1 neutralising antibody (Figure 6). The observed increased responsiveness of NIH-3T3 cells is likely rooted in their embryonic nature that is characterised by a higher, more reactive tenascin c expression and accelerated, more complete wound healing.

Figure 5 here (in b/w in print)

Figure 6 here (in b/w in print)

3.5. Co-cultured L929 cells form distinctive tenascin c based nano-tubes

L929 fibroblasts cultured alone presented punctate, extracellular tenascin c secretion patterns surrounding adherent cells in culture at 48 hrs and hazy peri-cellular accumulations peaking at 72 hrs, which were less visible by day 5 or 6 (Figure 4A).

Interestingly, when co-cultured with untreated DC, L929 fibroblasts expressed a distinct tenascin c staining pattern, and when TGF β1-treated DC were used, cells formed tenascin c based nano-tubes of about 40-50 μm long between non-adjacent cells by the 72 hrs time point (Supplementary Figure S1A). Comparable nano-tubes also became evident at 72 hrs between WEHI-164 fibrosarcoma cells in co-culture with TGF β1-treated DC (Supplementary Figure S2) and were first detected as membrane protrusions as early as 48 hrs in culture (Supplementary Figure S2A). Strongest staining corresponding to tenascin c presence was found at edges (*vs.* centres) of adherent cell clusters for L929 cells co-cultured with DC that had been pre-treated with 50 ng/mL TGF β1 after 5 days (Supplementary Figure S1B) or at 6 days (Supplementary Figure S1C).

Taken together, these results suggest that tenascin c expression patterns in co-cultures were distinctive. Specifically, membrane nano-tubes comprising tenascin c were detected in DC-fibroblast co-cultures and tenascin c persisted for the longest durations at outer edges of co-culture clusters whilst tenascin c staining remained equally low over time in L929 only experiments (Figure 4).

4. DISCUSSION

We previously showed that murine corneas of naïve BALB/c mice after full-thickness transplantation with engineered optically clear EDC-NHS or CMC-NHS crosslinked RHCIII hydrogels developed a non-transparent dense RCM at day 22 p.t., causing loss of corneal transparency (Ahn et al., 2013) while collagen hydrogels incorporating a network of a synthetic phosphorylcholine polymer were transparent in the corneas of guinea pigs as full-thickness grafts at the end of a six-month observation period (McLaughlin et al., 2010). Here, we examined whether RHCIII hydrogel implants fabricated using different chemistries trigger different wound healing responses.

In agreement with the above *in vivo* evidence (Ahn et al., 2013), in the present study the mouse corneal graft model while remaining optically clear at baseline by clinical evaluation (day 2 p.t.; data not shown), also resulted in the formation of RCM after implantation by day 22 p.t.. Interestingly, the implanted gels showed that although EDC and CMC gels share structural and functional similarities and are not incorporated into the collagen hydrogels they crosslink, the resulting stable hydrogels triggered different innate immune responses. In an earlier accompanying *in vitro* study to the present one (Mölzer, Shankar et al, submitted), EDC-crosslinked hydrogels were found to exert pro-inflammatory effects on DC, while CMC-crosslinked hydrogels were more immune-tolerising *in vitro* (Mölzer, Shankar et al, submitted). Along the lines of these results, in the present *in vivo* study, CMC-NHS implants showed less $CD11c^+$ DC infiltration, compared to pro-inflammatory EDC-NHS hydrogels. Furthermore, the extensive oedematous swelling encountered in corneas grafted with EDC-crosslinked hydrogels was absent in CMC-crosslinked hydrogel implants, further emphasising pro-inflammatory activation of infiltrating DC and aggravated inflammation in and around EDC hydrogels. In RCM surrounding both hydrogels, we found functional *de novo* re-epithelialisation (Tanifuji-Terai, Terai, Hayashi, Chikama, & Kao, 2006) by day 22 p.t. and activated fibroblasts in and around corneal hydrogel transplants, as determined by αSMA and cytokeratin 12 expression (Figure 1A, B). This

demonstrates that both types of hydrogel implants guided regenerating tissue (Sadtler et al., 2016), were stably integrated with native tissue, and allowed for extracellular matrix (ECM) remodelling.

Importantly, not only were EDC-crosslinked gels associated with the generation of a more pro-inflammatory environment, but also with an increased fibroblastic ("ECM") tenascin c expression *in vitro* (Figure 3 and Supplementary Figure S3). Both of which was true for CMC hydrogels, albeit to a markedly lesser extent. As pro-adhesive tenascin c is secreted by keratinocytes and fibroblasts and involved in tissue regeneration (Kim S. Midwood & Orend, 2009; Pearson et al., 1988), we propose an association between (differentially activated) DC with tissue regeneration or conversely with inflammation and tissue disruption. This suggests itself, as bone marrow DC are recruited within hours to transplanted RHCIII hydrogel corneas (Shankar et al., 2015) and may be uniquely positioned to determine graft outcome. The relationship between DC and tenascin c in connection with corneal tissue injury and recovery is unknown, especially in the context of inflammation (K.S. Midwood, Chiquet, Tucker, & Orend, 2016). However, with reference to implant functionality, tenascin c has been connected with opacification of the posterior lens capsule. Tenascin c is a known key-player in embryonic development around migrating cells during embryonic to mesenchymal transition (Tucker & Chiquet-Ehrismann, 2009), in tumour stroma, and in connective tissue repair and remodelling (Jones & Jones, 2000; Kim S. Midwood & Orend, 2009). It is not typically expressed in the human cornea, but found in the limbal region of the adult eye (Maseruka, Ridgway, Tullo, & Bonshek, 2000). Importantly, tenascin c is secreted by epithelial cells, keratinocytes and fibroblasts *in vitro* upon treatment with TGF β1 (Jinnin et al., 2004), mimicking an injury-type situation in which tolerising TGF β1 is liberated from epithelial tissue. In line with this report, we found that TGF β1 was a strong inducer of tenascin c expression on co-culture of L929 cells/DC. We also found that LPS induced tenascin c expression on similar co-culture of L929 cells/DC, but the timing of expression was delayed by 24 hours. However, this confirmed that tenascin c is elaborated in an inflammatory milieu as well as in response to TGF β1 (Figure 4). Interestingly, co-culture of different fibroblast cell lines (L929 and WEHI-164) with DC in the presence of TGF β1 was furthermore observed to be associated with the formation of tenascin c-rich nano-tube like structures (Supplementary Figures S1 and S2).

In an attempt to replicate the anti-inflammatory wound-healing response we had encountered in our previous *in vivo* work (Ahn et al., 2013) and following our earlier observation of a hydrogel-specific activation of cultured DC, they were pre-treated *in vitro* using TGF β1 at different concentrations, and/or kept in co-culture with different fibroblast lines, mimicking RCM, in the absence of hydrogels. Transforming growth factor β1 is an important mediator of wound healing, alternatively activating DC to make them tolerogenic and refractory against maturation signals (Adnan et al., 2016; Naranjo-Gómez et al., 2011), thereby creating tolDC. In these experiments, we found that TGF β1 alternatively activated tolDC induced expression of tenascin c in fibroblasts (Figures 4 and 5), by signalling through the TGF β1 pathway (Figure 6). As expected, the effects were lost in the presence of TGF β1 neutralising antibody blocking downstream TGF β1 effects (Figure 6), or pro-inflammatory LPS, provoking classical DC activation thereby mimicking the "pro-inflammatory EDC-NHS hydrogel effect" discovered earlier (Figures 4 and 5). In agreement with those *in vitro* data presented in our related report (Mölzer, Shankar et al, submitted), the above results emphasize the importance of hydrogel composition in graft outcome. Drawing from the evidence presented in this report, we suggest that CMC-NHS gels provide a more tolerising environment to DC, causing less inflammation, hence supporting wound healing and graft integration. This lays the foundation for functional re-epithelialisation and stabilisation and may allow for some degree of graft coverage with host endothelium (Plsková, Kuffová, Holán, Filipec, & Forrester, 2002) of corneal tectonic grafts with less tissue disruption than in a pro-inflammatory environment. Retro-corneal membrane formation in the absence of inflammation and overt levels of tenascin c expression would allow for tissue regeneration and eventual remodelling or extrusion of the tectonic graft.

Our results imply that the apparent pro-/anti-inflammatory properties of the respective hydrogel antigen encountered may (indirectly) differentially impact the extent and rate of tenascin c secretion by fibroblasts that populate fibrous capsules and therefore importantly, the rate, timing and quality at which early wound healing progresses into later stages, and ultimately determine graft outcome. We propose that in a pro-inflammatory graft environment, TGF β1-mediated alternative DC activation is futile,

redirecting TGF β1 to the surrounding ECM, where it directly triggers overt tenascin c expression (Supplementary Figure S3).

5. CONCLUSION

This study may have important consequences for corneal or other transplant-related wound healing sites and may suggest new biomarkers or targets for therapeutic intervention. Independent of their origin and developmental stage, all three fibroblast cell lines tested behaved in a comparable manner upon direct contact with TGF β1 or pre-treated tolDC, respectively. Differentially crosslinked hydrogels not only function as adjuvants and affect DC maturation *in vitro* (Mölzer, Shankar et al, submitted), but may also differentially regulate downstream wound healing responses and secretion of tenascin c from fibroblasts *in vivo,* thereby impacting corneal regeneration and ultimately graft success.

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7. DISCLOSURES

The authors have no competing interest to declare.

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FIGURES

Figure 1: Both alpha smooth muscle actin, αSMA (A), and cytokeratin 12 (B) could be detected in EDC-NHS and CMC-NHS singly-crosslinked full-thickness hydrogel transplants of murine corneas at day 22 post-transplantation (p.t.). Staining corresponding to αSMA presence observed in anterior re-epithelialised membranes or posterior retro-corneal membranes for both EDC-NHS and CMC-NHS hydrogels, or cytokeratin 12 presence observed in anterior re-epithelialised membranes or in native peripheral non-transplanted corneal epithelia for both EDC-NHS or CMC-NHS hydrogels. Representative images from murine hydrogel corneal transplants; N=2 with EDC-NHS hydrogels, N=2 with CMC-NHS hydrogels.

Higher abundance of $CD11c^+$ DC (C) surrounding EDC-NHS crosslinked hydrogel transplants. Staining corresponding to increased presence of $CD11c^+$ DC observed in posterior retro-corneal membranes for EDC-NHS hydrogels versus CMC-NHS counterparts. Representative images from murine hydrogel corneal transplants; N=2 with EDC-NHS hydrogels, N=2 with CMC-NHS hydrogels. Pictures were taken using confocal microscopy.

Figure 2: Higher signal for CD11c+ dendritic cells in and around transplanted EDC-NHS vs. CMC-NHS crosslinked hydrogels (non-significant result). Quantification was completed using fluorescence intensity. N=2 with EDC-NHS hydrogels, N=2 with CMC-NHS hydrogels.

Figure 3: Increased abundance of tenascin c surrounding EDC-NHS crosslinked hydrogel transplants. Red staining corresponding to tenascin c accumulation observed in peripheral layers of anterior re-epithelialized membranes or at invasive edges of posterior retro-corneal membranes for EDC-NHS hydrogels (A), versus CMC-NHS counterparts (B). Representative images from murine hydrogel corneal transplants; N=2 with EDC-NHS hydrogels, N=2 with CMC-NHS hydrogels. Quantification of tenascin c expression (p 0.0079; *$p < 0.05$) was established using fluorescence intensity (C), following confocal microscopy.

Figure 4: Tenascin c secretion by L929 fibroblasts under different culture conditions (untreated, TGF β1, or LPS) assessed by confocal microscopy (A), and quantification of tenascin c expression using fluorescence intensity for each of the cultures (B). Representative confocal microscopy images at 40x.

Figure 5: Tenascin c is secreted by L929 cells when cultured alone in the presence of TGF β1 (A), or when co-cultured with DC pre-treated with TGF β1 (B). Mean ± SEM; N=3; ****$p<0.0001$, ***$p<0.001$, **$p < 0.01$, *$p < 0.05$.

Figure 6: Phosphorylation of Smad 2/3 (A) and downstream tenascin c protein expression (B) in NIH-3T3 fibroblast DC co-cultures, assessed using flow cytometry. Where applicable, DC had been pre-treated with different concentrations of biologically active TGF β1, ranging from 1 – 50 ng/mL. NIH-3T3 alone as well as in co-culture with untreated DC served as controls. To confirm that the effects

seen are TGF β1 specific, neutralisation antibody (2.5 ng/mL) was used in additional controls together with or without TGF β1 (2.5 ng/mL). Values are expressed as mean fluorescence intensity (MFI) within the singlet cell population over that of the negative control. MFI were measured using flow cytometry at 24 and 48 hrs, respectively. Significances are indicated using asterisks (*) with p-values provided above the respective boxplot. Significant results are based on a 95 %, trends ("T") on a 90 % level of confidence.

Additional control experiments (C and D) show the effect of biologically active TGF β1 when directly administered to NIH-3T3 fibroblasts. Fibroblasts were treated with different concentrations of biologically active TGF β1, ranging from 1 – 10 ng/mL. Untreated NIH-3T3 served as controls. To confirm that the effects seen are TGF β1 specific, neutralisation antibody (2.5 ng/mL) was used. Phosphorylation of Smad 2/3 (C) and downstream tenascin c protein expression (D) are expressed as MFI within the singlet cell population over that of the negative control at 24 and 48 hrs, respectively. Significant results (*) are based on a 95 %, trends ("T") on a 90 % level of confidence; p-values are given above the respective boxplot.

Graphical abstract: Upon corneal tectonic grafting of biomaterials, biologically active Transforming Growth Factor β1 (TGF β1) is liberated from its latency associated peptide. In a pro-inflammatory environment when DC have been activated (*e.g.* by a specific hydrogel; mDC), TGF β by-passes mDC and instead signals towards fibroblasts to produce and release overt levels of tenascin c. In the absence of inflammation, immature DC (iDC) are tolerised by TGF β1 which ultimately controls tenascin c production.