

Manipulation of Panx1 Activity Increases the Engraftment of Transplanted Lacrimal Gland Epithelial Progenitor Cells

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PURPOSE. Sjögren's syndrome is a systemic chronic autoimmune inflammatory disease that primarily targets the salivary and lacrimal glands (LGs). Currently there is no cure; therefore, cell-based regenerative therapy may be a viable option. LG inflammation is facilitated by extracellular ATP and mediated by the Pannexin-1 (Panx1) membrane channel glycoprotein. We propose that suppression of inflammation through manipulation of Panx1 activity can stimulate epithelial cell progenitor (EPCP) engraftment.

METHODS. The expression of pannexins in the mouse and human LG was assayed by qRT-PCR and immunostaining. Acute LG inflammation was induced by interleukin-1 α (IL1 α) injection. Prior to EPCP transplantation, IL1 α -injured or chronically inflamed LGs of thrombospondin-1-null mice (*TSP-1*^{-/-}) were treated with the Panx1-specific blocking peptide (¹⁰panx) or the self-deliverable RNAi (sdRNAi). The efficacy of cell engraftment and the area of inflammation were analyzed by microscopy.

RESULTS. Panx1 and Panx2 were detected in the mouse and human LGs. Panx1 and proinflammatory factors were upregulated during acute inflammation at days 1 to 3 after the IL1 α injection. The analysis of EPCP engraftment demonstrated a significant and reproducible positive correlation between the ¹⁰panx peptide or Panx1 sdRNAi treatment and the number of engrafted cells. Similarly, treatment of the LG of the *TSP-1*^{-/-} mouse (mouse model of chronic LG inflammation) by either Panx1 or Caspase-4 (also known as Casp11) sdRNAi showed a significant decrease in expression of proinflammatory markers and the lymphocyte infiltration.

CONCLUSIONS. Our results suggest that blocking Panx1 and/or Casp4 activities is a beneficial strategy to enhance donor cell engraftment and LG regeneration through the reduction of inflammation.

Keywords: lacrimal gland, Pannexin-1, regeneration, epithelial progenitor cells, injury-induced inflammation

The lacrimal gland (LG) is the primary contributor to the aqueous layer of the tear film in humans. LG dysfunction or degeneration may lead to aqueous tear deficiency or dry eye disease, a condition that currently has no cure. In many ocular tissues, stem/progenitor cell-based therapies have become a highly promising approach to treating diseases previously considered incurable.¹⁻⁵ Similar to other exocrine glands, including the pancreas, salivary, and mammary glands, the "healthy" adult LG has a high regenerative capacity and is able to repair itself even after substantial damage.⁶⁻⁹ Recently, replacement of an adult mouse LG with an embryonic LG-derived epithelio-mesenchymal reaggregate or epithelial cell progenitors has been demonstrated.^{9,10} However, the major cause of therapeutic limitations for cell transplantation in humans results from the poor engraftment of the transplanted cells.¹¹⁻¹³ The use of stem/progenitor cells can be instrumental

in therapeutic LG regeneration¹⁴; however, in contrast to a healthy gland, the chronically inflamed "diseased" LG shows a substantial delay in regeneration.¹⁵ The reason for this is unclear and may be related to disruption of the LG stem cell niche function due to chronic inflammation. We recently reported that engraftment of epithelial cell progenitors (EPCPs) after transplantation into an acutely injured LG varies depending on the state of inflammation: fewer engrafted EPCPs were observed during inflammation than during LG regeneration.¹⁴ We decided to use this acute inflammation model to study whether reduction of inflammation would improve LG progenitor cell engraftment.

An important part of the initiation of the inflammatory process is the formation of the inflammasome.^{16,17} The inflammasome is a cytoplasmic macromolecular complex containing apoptosis-associated speck-like protein (ASC) and



NOD-like receptor protein (NLRP) domains, caspase-1, and/or caspase-4 (also known as caspase-11) convertases in a catalytic core.^{18,19} The main function of this complex is to process precursors and secrete mature IL1 β and IL18.^{20,21} A large body of experimental evidence identifies pannexin-1 (Panx1) and P2X receptors (Panx1/P2XR) as the essential upstream regulators of the inflammasome required for proteolytic activation of caspase 1 and/or Casp4(11).^{18,22-24} Pannexins are a family of ubiquitously expressed gap junction-like proteins that form large membrane channels, permeable to small molecules, including ATP, glutamate, NAD, etc.²⁵⁻²⁷ Panx1 channels were demonstrated to serve as ATP release channels and contribute to purinergic and adenosine signaling.^{28,29} There is ample evidence for a role of Panx1 in various pathologies,^{25,26} ischemic and traumatic brain injuries,^{30,31} postischemic glutamate toxicity,³² and, more recently, in inflammatory damage in the nervous system.^{24,33-35} At the same time, several recent reports implicate Panx1 activity in regeneration, particularly in progenitor cell maintenance, motility, and differentiation.³⁶⁻³⁸ The role of Panx1 in the LG is largely unknown.

In this study, we show that Panx1 is expressed in mouse and human LGs. Panx1 expression has been highly upregulated during LG injury/inflammation. The increase in Panx1 expression in experimentally injured LG was associated with activation of inflammasome complex genes. We recently showed poor EPCP engraftment during this stage of acute LG inflammation.¹⁴ We hypothesized that blocking the Panx1 channel, known to mediate inflammasome activation,^{33,39-41} may improve EPCP engraftment into an injured LG. To test this hypothesis, we deactivated the Panx1 signaling pathway using a Panx1 blocking peptide (¹⁰panx) or self-deliverable Panx1 RNAi (sdRNAi). The analysis of EPCP engraftment showed a significant and reproducible positive correlation between these treatments and the number of engrafted cells per cross section, with an average increase of engrafted cells of approximately 63% after ¹⁰panx treatment and 169% after Panx1 sdRNAi treatment relative to untreated controls. Similarly, treatment of the LGs of thrombospondin-1-null (*TSP-1*^{-/-}) mice, a recently reported model of chronic LG inflammation,^{9,42,43} with sdRNAi to either Panx1 or Casp4(11) showed a significant decrease in mRNA level of proinflammatory genes and LG inflammation.

This study provides new insight into the role of Panx1-mediated inflammation in engraftment of LG progenitor cells and suggests that inhibition of Panx1 and/or Casp4(11) could be a beneficial therapeutic strategy to enhance donor cell engraftment in the LG.

MATERIALS AND METHODS

Animals and Treatments

Because the prevalence for aqueous deficiency dry eye (ADDE) in human population is higher among women than men,⁴⁴ we used female mice in all our experiments. *Pax6-LacZ* female mice (3 to 5 weeks old) on a C57BL/6 background⁴⁵ were used to prepare EPCP cells for transplantation, as described previously.¹⁴ Wild-type C57BL/6 females were used as recipient mice. LG inflammation in recipient mice was induced by intraglandular injection of interleukin-1 α (IL1 α), as previously described.^{6,14} Briefly, C57BL/6 female mice (10 to 12 weeks old) were anesthetized, and the exorbital LGs were injected with either saline (vehicle) or IL1 α (1 μ g; PeproTech, Rocky Hill, NJ, USA) in a total volume of 2 μ L. The LGs from noninjected mice were used as an additional control. The LGs were harvested 1, 2, 3, 4, 5, 7, and 21 days after injection, and total RNA was extracted.

TSP-1^{-/-} mice were originally purchased from the Jackson Laboratory (Sacramento, CA, USA; <https://www.jax.org>) and were bred and maintained on the C57BL/6J background at The Scripps Research Institute (TSRI) vivarium.

Mice were housed under standard conditions of temperature and humidity, with a 12-hour light/dark cycle and free access to food and water. All experiments were performed in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the Guidelines for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and were preapproved by TSRI Animal Care and Use Committee.

Immunostaining and Confocal Microscopy

Dissected LGs were fixed with 2% paraformaldehyde in PBS (pH 7.4) for 20 minutes and frozen in 2-methylbutane (isopentane; Sigma-Aldrich, St. Louis, MO, USA) cooled by liquid nitrogen, and 15- μ m frozen sections were cut with a Microm HM500 cryostat (MICROM International GmbH, Dreieich, Germany). Sections were blocked with 5% goat serum in Tris-buffered saline containing 0.1% Tween 20 (TBST). The following primary antibodies were used for immunostaining: rabbit polyclonal antibody to Panx1 (Sigma-Aldrich; HPA016930), affinity-purified rabbit polyclonal antibody against the carboxyl terminus of human PANX1,⁴⁶ affinity-purified rabbit Panx1 antibody CT-395 (Px-34),⁴⁷ kindly provided by Dale W. Laird (University of Western Ontario, Ontario, Canada), rabbit polyclonal antibody to Panx2 (Aviva Systems Biology Corp., San Diego, CA, USA; Cat# ARP42778_T100), mouse monoclonal α -smooth muscle actin antibody (clone 1A4; cat.# A2547; Sigma-Aldrich). Appropriate secondary antibodies were obtained from Invitrogen (Waltham, MA, USA). Images were taken using a Zeiss LSM 780 laser (San Diego, CA, USA) scanning confocal microscope (LSCM). The isotype-specific immunoglobulins (normal rabbit or mouse IgGs; Sigma-Aldrich) or preimmune serum, as a substitute for the primary antibody, were used for negative controls.

Immunohistochemistry on Human LG Paraffin Sections

Human LGs from three donors were obtained from Advanced Tissue Services (Phoenix, AZ, USA). The LG were removed \leq 24 hours after death. Tissues were preserved immediately in RNAlater and shipped at 4°C overnight. All donors were females, and their ages at the time of death were 62, 84, and 90 years. The LGs were embedded in paraffin, and 5- μ m sections were prepared. Endogenous peroxidase activity on rehydrated sections was blocked by treating slides with 3% hydrogen peroxide in absolute methanol for 30 minutes. Antigen retrieval was performed for 40 minutes using 0.01 M citrate (pH 6.39) in a humidified heated chamber. Sections were blocked with 5 g/L casein (Sigma Aldrich) in PBS containing 0.5 g/L thimerosal (Sigma-Aldrich; cat# T5125-25G) for 30 minutes, incubated with primary antibodies, and diluted in casein buffer 1:50 overnight at 4°C. Biotinylated goat anti-rabbit IgG antibodies (Vector Labs, Burlingame, CA, USA) were used at a 1:300 dilution. Visualization was achieved using biotin/avidin-peroxidase (Vector Labs) and Nova Red (Vector Labs). Counterstaining was made with Gill's hematoxylin (Fisher Scientific, San Diego, CA, USA; CS400).

LG Cell Dissociation and Fluorescence Activated Cell Sorting

To obtain sufficient cells for flow cytometric analysis and fluorescence activated cell sorting (FACS), we pooled LGs from

6 to 12 mice. The mice were euthanized, and the skin was sterilized with 70% ethanol before surgically exposing the LG. The LG capsule was removed with tweezers, and a cell suspension was prepared as described by Gromova et al.¹⁴ To remove red blood cells, 25 mL cold red blood cell lysis buffer (RBCLB: 0.2% wt/vol Tris, pH 7.5, 0.74% wt/vol NH₄Cl) was added to each tube of LG cells suspended in growth media. Purified LG cells were collected by centrifugation at 1000g, resuspended in 100 μg/mL DNase and 5 mM MgCl₂ in Hank's salt (HBSS) (DNase Sigma D-4513; HBSS, Sigma H-6648), and incubated for 15 to 30 minutes at room temperature. The cells were pelleted at 1000g, washed in HBSS, resuspended in staining buffer PBE (1× PBS, 0.5% BSA, and 1 mM EDTA), and counted. For FACS analysis, approximately 0.5 × 10⁶ cells were pelleted at 1200g for 10 minutes at 4°C and resuspended in 100 μL staining buffer containing the appropriate conjugated antibody. A panel of surface markers for isolation of LG progenitor cells was based on existing publications⁴⁸⁻⁵⁴ and tested in our recent study.⁹

The following antibodies were used: R-phycoerythrin (PE) rat anti-mouse Ly-6A/E (Sca1, #553336; BD Pharmingen, San Jose, CA, USA); FITC rat anti-mouse CD34 (#553733; BD Pharmingen); anti-mouse CD117 (c-Kit) Adenomatous polyposis coli (APC)-eFluor 780 (#47-1171-80; eBiosciences, Waltham, MA, USA); and anti-mouse CD326 (EpCAM) APC (#17-5791-80; eBiosciences). The cells were stained on ice for 1 hour with gentle vortexing every 15 to 20 minutes, pelleted as above, and resuspended in 1 mL cold PBE in FACS tubes. Flow cytometric analysis and FACS were performed at the TSRI Flow Cytometry Core Facility using Digital LSRII and FACS Vantage DiVa instruments. Data analyses were performed using FlowJo software. Cells were sorted as described previously.¹⁴ Control samples labeled with isotype control antibodies and with a single primary antibody were used to determine the background noise due to nonspecific antibody binding and to establish proper compensation for optimum separation between signals.

Self-Deliverable RNAi Injections

Modified RNAi compounds (sdRNAi) (Supplementary Fig. S1) silencing the expression of Pannexin 1 (Px23, cat. 40423) and Caspase 4 (Cs12, cat. 30412) were developed in collaboration with Advirna, LLC (Cambridge, MA, USA). Px23 and Cs12 duplexes with the highest efficacy were selected based on in vitro screening using the luciferase reporter assay (Supplementary Methods, Supplementary Fig. S2, and Supplementary Table S1). Prior to the experiments on LG, the Px23 and Cs12 compounds were tested in cell base and in vivo assays (Supplementary Fig. S3). In in vivo experiments female mice were anesthetized, and 2 to 3 μL solution of sdRNAi (diluted with saline to 250 nM) was injected into several lobes of the LG on the right side of the mouse. The left LG was injected with control sdRNAi or vehicle. As additional controls, we used LGs from noninjected mice or mice injected with only control sdRNAi. In addition to intraglandular injection, each sdRNA was premixed with 30 μL pluronic gel 30% Pluronic F-127 gel (Sigma-Aldrich; cat. P2443-250G) to a final concentration of 250 nM and injected under the LG capsule. The pluronic gel solution in PBS was prepared in advance and kept at 4°C. The left LG was injected with vehicle (pluronic gel) and used as a control. On contact with tissues, the pluronic gel solidifies and stays in contact with tissue for 8 to 12 hours.⁵⁵ Injections were performed once a week for 4 to 5 weeks, and the mice were euthanized 2 weeks after the last injection.

Cell Transplantation Experiments

Donor EPCPs were obtained by cell sorting from uninjured LGs (see above) of 3- to 4-week-old *Pax6-LacZ* mice, as described

previously.¹⁴ Cells were pelleted and suspended in minimum essential medium α (α -MEM) medium (Corning, Manassas, VA, USA). Control LGs of 2- to 3-month-old wild-type (WT) recipient mice were injured by a single injection of IL1 α ,^{14,56} and EPCP transplantation was performed 1 day later (during the inflammation stage). Experimental LGs were injected with IL1 α + specific mimetic peptide inhibitor of Panx1¹⁰ panx (100 μM, sequence WRQAAFVDSY)⁵⁷⁻⁵⁹ or Panx1 sdRNAi (diluted with saline to 250 nM), and sorted EPCP cells were transplanted 1 day after the IL1 α and inhibitor treatment. As an additional control, we used LGs injected with vehicle (saline or with control sdRNAi). Transplantation was repeated for a total of three independent experiments. Analysis of EPCP engraftment was carried out 40 days after transplantation as we described previously.⁹ Briefly, engraftment was assessed as the percent of LacZ⁺ donor-derived cells in each injected LG lobe. Cells were counted in 200-μm² fields, with approximately 5 to 6 fields per section and 10 to 15 sections per LG. Four LGs were assessed in each of three independent experiments. LacZ⁺ cells per field were enumerated blindly.

X-Gal Staining

Fixed tissue was rinsed three times in 0.1 M PBS, pH 7.4, for 20 to 30 minutes and stained overnight at room temperature in the dark in a X-gal staining solution. Solution was comprised of 5 mM K₃[Fe(CN)₆], 5 mM K₄[Fe(CN)₆], 0.1 mM MgCl₂, 0.02% NP40, 0.02% deoxycholate, and 1 mg/mL of X-gal in 0.1 M PBS, pH 7.4. A 50 mM stock solution of X-gal (Sigma #B4252) in N,N-dimethylformamide (DMF) or dimethylsulfoxide (DMSO) was prepared in advance and stored at -80°C.

RNA Extraction and Real-Time RT PCR

RNA was isolated using a RNeasy Mini kit (cat. 74104; SABiosciences, Qiagen, Valencia, CA, USA); cDNA was made using a RT2 First Strand Kit (cat. 330401; SABiosciences, Qiagen). To compare expression of Panx1, 2, and 3 in the LGs at mouse postnatal day 1 (P1) with their expression in the adult LG at P60, we used sets of primers reported previously⁶⁰ and housekeeping genes *β-actin* (forward: 5'-GATCATTGC TCCTCTGAGC-3', reverse: 5'-CCGGACTCATCGTACTCCTG-3') and *GAPDH* (forward: 5'-GAACGGATTTGGCCGTATT-3', reverse: 5'-ITGCCGTGAGTGGAGTCATA-3'). The qRT-PCR amplification products were examined by melting curve analysis. Expression of the housekeeping genes was used to normalize the level of target gene expression.

In all other experiments, primers to Panx1 (NM_019482, cat. PPM31514B), Panx2 (NM_001002005, cat. PPM59262A), IL1 β (Il1b: NM_008361, cat. PPM03109F), Il6 (NM_031168, cat. PPM03015A), Il18 (NM_008360, cat. PPM03112B), Nlrp3 (NM_145827, cat. PPM29506F), CCL2 (NM_011333, cat. PPM03151G), Ccl7 (NM_013654, cat. PPM02955B), Ccl12 (NM_011331, PPM02977E), P2RX7 (NM_011027, cat. PPM04252A), P2RY2 (NM_008773, cat. PPM04914A), Col1a1 (NM_007742, cat. PPM36469A), Casp8 (NM_009812, cat. PPM02923F), Casp1 (NM_009807, cat. PPM02921E), Casp3 (NM_009810, cat. PPM02922F) Casp4 (NM_007609, cat. PPM03075C), and housekeeping genes PrS13 (forward: 5'-TCCCTCCCAGATAGGTGTAATCC-3' and reverse: 5'-TCCTTTCTGTTCCCTCTCAAGGT-3') and PrL27 (forward: 5'-AAAGCCGTCATCGTGAAGAAC-3' and reverse: 5'-GCTGTCACCTTCCGGGGATAG-3'), as well as RT² SYBR Green qPCR Mastermix were purchased from Qiagen. Quantitative RT-PCR was performed on an ABI 7300 system (Life Technologies, Grand Island, NY, USA), and the data were analyzed using online normalization and analysis tools (provided in the public domain, <http://sabiosciences.com/pcrarrayda>

taanalysis.php). Three independent experiments were performed.

Statistical Analysis and Data Presentation

Statistical analyses were performed using Prism Software (GraphPad, San Diego, CA, USA). In bar graphs, data are presented as means \pm SD of replicates from a representative experiment or of the normalized data from several experiments. In the latter case, mean fold changes were calculated by first determining the ratio of the test conditions over the appropriate control conditions for each individual measurement and then averaging these ratios. The Anderson-Darling normality test was performed prior to further data analyses. The unpaired two-tailed Student's *t*-test was used to determine significance ($P < 0.05$) in the differences between data sets.

RESULTS

Panx1 and 2 Are Expressed in the Mouse and Human LG

The pannexin family of channel-forming glycoproteins includes three members: Panx1, 2, and 3. The presence and distribution of Panx1, 2, and 3 proteins in the mouse LG were studied by qRT-PCR and immunohistochemistry. Analysis of Panx1, 2, and 3 mRNA expressions in the LGs of newborn pups at P1, when the gland is still undergoing substantial growth/remodeling, was compared with their expression in adult LGs (at P60), when LG morphogenesis is largely completed. Panx1 mRNA expression was found in the LGs at P1 and P60. The mean cycle threshold (Ct) values were 25.7 ± 0.5 (at P1) and 28.4 ± 0.2 (at P60). Expression levels of Panx2 were generally lower than of Panx1, with mean cycle threshold (Ct) values of 30.1 ± 0.8 (at P1) and 27.5 ± 0.8 (at P60). Expression of Panx3 mRNA in the LGs was not detectable at any stage. Moreover, Panx1 expression was 2.5 times higher in the LGs at P1 compared with adult LGs at P60 (Fig. 1A). In contrast, Panx2 expression increased 9.8-fold in mature LGs compared with its expression level at P1 (Fig. 1B). These findings suggest that Panx1 and 2 may have different roles in LG morphogenesis and homeostasis. Panx1 protein was highly expressed in the epithelial component of the LGs during embryonic and early postnatal development (E19 to P0; Fig. 1C). Later in postnatal development (P20), a punctate expression of Panx1 was found in the epithelial ducts and acini (Fig. 1D). Moreover, the highest level of Panx1 protein at P20 was detected in the LG ducts (Fig. 1D, duct, yellow arrows), whereas acinar cells expressed a lower level of the protein (Fig. 1D; acini are surrounded by myoepithelial cells [MECs], red). In adult LGs at P60, Panx1 punctate labeling was found in both ducts and acini at similar levels (Fig. 1E). Sections through the acinar compartment of the LGs showed a higher level of labeling in more apical parts of acinar cells (Fig. 1E, apical parts of acinar cells labeled with white arrowheads). The expression of Panx1 in the MECs of the LGs was lower and less conspicuous (Figs. 1F–1H, Supplementary Fig. S4, and Supplementary Movie S1). In contrast to Panx1, expression of the Panx2 protein was almost absent at early postnatal development at P1 (Fig. 1I), but Panx2 was expressed in mature LGs (Figs. 1J, 1K). The pattern of Panx2 expression was similar to that of Panx1; it was highly expressed in the apical parts of the acinar cells (Figs. 1J, 1K). It was also found in the LG ducts (Fig. 1J, yellow arrow) and blood vessels (Fig. 1J, white arrows).

To determine whether pannexins are expressed in human LGs, we performed immunostaining of human LG paraffin sections. All human LGs were obtained from female donors of

age 62, 84, and 90. To confirm a specific pattern of Panx1 expression in humans, we used three different antibodies to Panx1 (Materials and Methods). Analysis of pannexin expression in human LGs showed that all three antibodies detected Panx1 in an identical pattern to that seen in the adult mouse LGs (Fig. 1, Supplementary Fig. S5). In human LG sections, Panx1 protein was expressed within the epithelial lobes (Figs. 2A, 2B and Supplementary Figs. S5A, S5B, and S5E–S5G, LG lobes labeled with white arrowheads), whereas the surrounding tissue had low or no Panx1 expression. Similar to the mouse LGs, Panx1 was highly expressed in the epithelial compartments (ducts and acini) of the human LGs (Figs. 2B, 2C and Supplementary Figs. S5B, S5E, S5G, duct, ac). It was also found in the blood vessels (Fig. 2A; Supplementary Figs. S5A, S5E, bv). In the LGs obtained from 84- and 92-year-old donors, we found some periductal mononucleated cell infiltrates (Figs. 2A, 2C, 2E, yellow arrows), suggesting the presence of LG inflammation. In these glands, Panx1 was also detected within the infiltrating mononucleated cells (Figs. 2A, 2C). No staining was found in controls in which the tissue was incubated with preimmune serum or isotype-specific immunoglobulins (Fig. 2E and Supplementary Figs. S5C, S5D, S4H).

Panx2 protein expression was detected in the human LGs in a similar pattern to the one described for the mouse LGs (Fig. 2D). Negative control did not have any specific staining (Fig. 2F).

Panx1 and 2 Expression During Injury-Induced LG Inflammation and Regeneration

To investigate the role of Panx1 and 2 during LG regeneration, we used an acute LG injury model.⁶ LG injury was induced by a single injection of IL-1 α , which induces extensive inflammation and LG damage, followed by a regenerative phase that restores LG morphology and function between 7 and 21 days after injury.⁶ Recent studies suggest that pannexins and pannexin-mediated purinergic signaling play important roles in activation of both postinjury inflammatory response and the subsequent process of tissue regeneration.^{33,61} In particular, Panx1 has been associated with elevated levels of proinflammatory cytokines.⁶² We reasoned that Panx1 and 2, which are expressed in the epithelial component of the gland, play a role in the processes of inflammation or active cellular remodeling during regeneration. LGs of 2-month-old WT mice were injected with IL1 α or saline (vehicle control) and collected at days 1, 2, 3, 7, and 21 after injection for RNA extraction. Levels of the Panx1 and Panx2 and PrL27 (noncoding RNA, poly[A]-bearing) and PrS13 (16S ribosomal RNA gene) housekeeping mRNAs were determined by qRT-PCR (Table; Fig. 3A). We found that Panx1 mRNA expression was increased six- to eightfold on days 1 to 3 after LG injury when inflammation is at its highest peak, whereas Panx2 mRNA expression was increased later between days 3 and 21 after LG injury, reaching a peak on day 7 during the LG differentiation phase (Fig. 3A; Table). Thus, Panx1 may be involved in regulation of inflammation and Panx2 may have a role in LG differentiation and maturation.

Expression of Inflammatory Factors After Acute LG Injury

ATP is a major indicator of stress in many tissues, released from both dying and viable cells when challenged by cytokines⁶³ or mechanical stress.⁶⁴ Extracellular ATP is a very potent proinflammatory factor, which induces inflammasome activation via the Panx1-P2RX7 complex.^{18,22–24} To determine whether acute LG injury affects inflammasome-related gene expression, levels of their mRNAs were

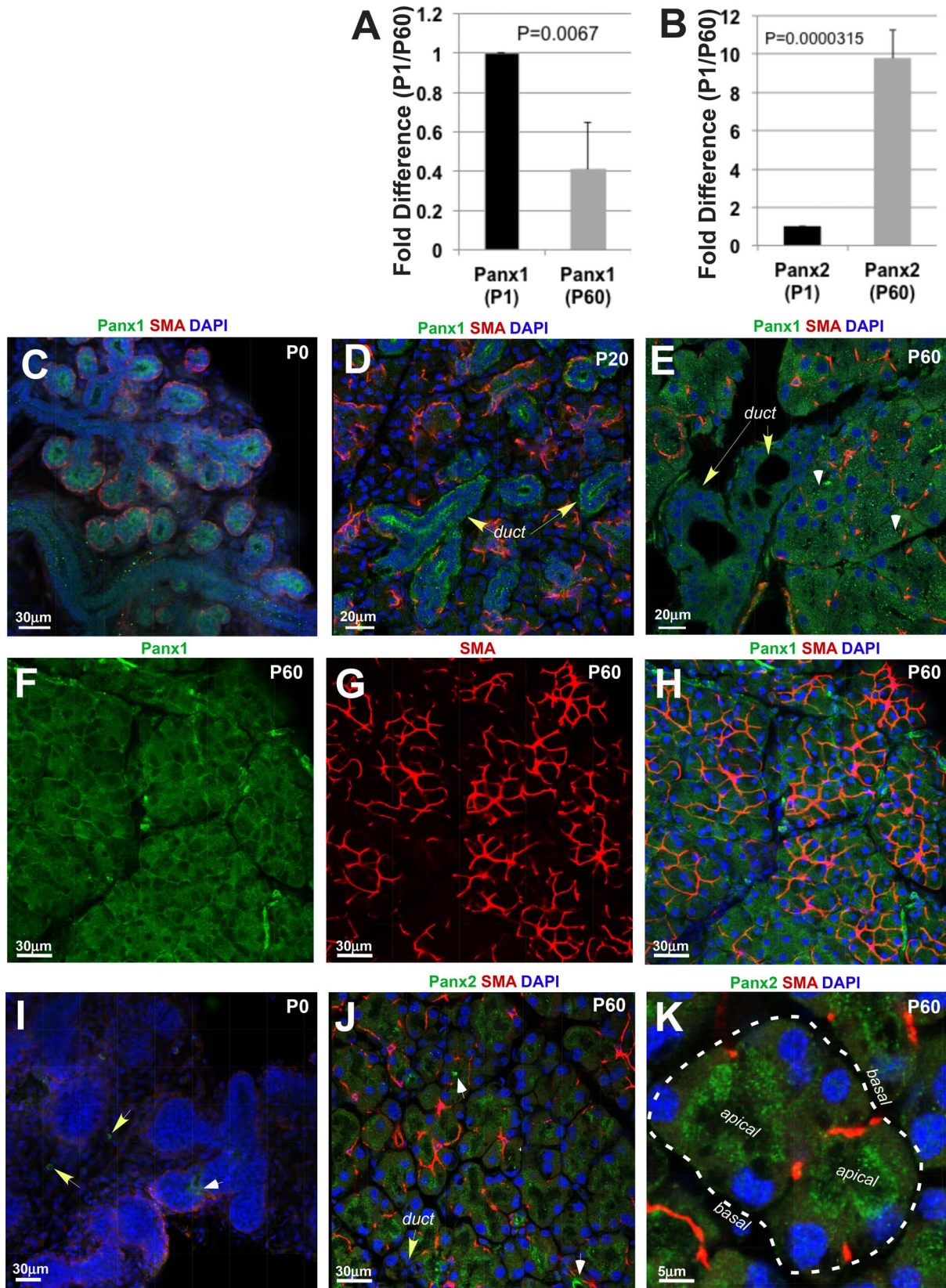


FIGURE 1. Panx1 and 2 expression in mouse LG. (A) Panx1 expression slightly decreases in the LG of adult (postnatal day 60 [P60]) mice compared with the LG obtained from mice at postnatal day 1 (P1). (B) Expression of Panx2 increases in adult LGs compared with LGs at P1. In A and B, histograms represent the normalized expression of Panx1 and 2 to GAPDH assessed by qRT-PCR in three independent experiments. (C–H) Panx1 expression in mouse LGs at P0 (C) during postnatal (D: P20) development and adulthood (E–H). At P and P20, Panx1 (green) is highly expressed in the developing LG ducts (C, D). Panx1 expression in the developing acini is low (D). Myoepithelial cells (red) are localized around acini, nuclei labeled with 4',6-Diamidino-2-phenylindole (DAPI) (blue). (E, F, H) Panx1 expression in adult LG (P60). (F–H) MECs have low level of Panx1

expression. Low level of Panx1 expression in MEC is confirmed in nonmerged images (F, Panx1 expression; *green*; and G, SMA [marker of MECs] expression, *red*). (I) Low level of Panx2 expression in the epithelial components of the LG at P. Panx2 was only detected in blood vessels (*white arrow*) and some mesenchymal cells (*yellow arrows*) At P60, Panx2 (J, K) is expressed in the acinar cells. High levels of Panx2 protein is found in blood vessels (J, *white arrows*) and apical parts of acinar cells (K, apical; basal parts of acinar cells are labeled with puncture line). Myoepithelial cells (*red*). Nuclei are labeled with DAPI (*blue*).

determined by qRT-PCR. The LGs of female BALB/c mice (three mice per treatment group) were injected with either saline (control) or with IL1 α , and the LGs were harvested 1, 2, 3, 4, 5, 7, and 21 days after injection (Table; Fig. 3). Our analysis showed that mRNA expression of P2RX7 and P2RY2 was significantly upregulated during LG inflammation (first 2 to 3 days after the injury), whereas expression of collagen I (Col1a1, fibril-forming collagen), which is implicated in tissue remodeling/regeneration and maintenance of the integrity of extracellular matrix,⁶⁵⁻⁶⁷ was increased during the phase of LG regeneration, days 5 to 7 (Fig. 3A). Expressions of several inflammasome related (NLRP3, Casp1, Casp4[11], and Casp8) factors were significantly upregulated at days 1 to 3 after injury (Table; Figs. 3B, 3C). Expressions of major proinflammatory cytokines such as IL1 β , IL6, and IL18 were upregulated substantially during LG inflammation stage. We also found increases in the expression of the interleukin-1 family member 9, which is known to act as a proinflammatory stimulator of chemokine production,⁶⁸ and the upregulation of specific proinflammatory chemokines Ccl2, Ccl6, Ccl7, and Ccl12 (Table; Figs. 3C, 3D).

Blocking the Panx1 Function Improves Engraftment of EPCPs Into Injured LG

We recently demonstrated that transplantation of EPCPs isolated from WT LGs could mediate the structural and functional recovery of injured LGs and LGs of *TSPI*^{-/-} mice (a new mouse model of human Sjögren's syndrome).¹⁴ Moreover, we reported that efficacy of EPCP transplantation into injured LGs varied depended on how much time passed after the injury by IL1 α injection. We demonstrated that cells injected into the gland during the regeneration phase (3 days after injury) engrafted more efficiently than cells injected during the acute inflammation phase (1 day after injury).¹⁴ Here we show that the increase in Panx1 expression correlates with the activation of acute inflammatory responses induced by the IL1 α injection. Panx1 is also expressed in immune cells such as monocyte and macrophages and mediates IL1 β processing and release from macrophages in response to P2X₇-R activation⁵⁸ and promotion of inflammation. Thus, blocking Panx1 during LG inflammation may improve cell engraftment efficacy. To test this hypothesis, we used an interfering peptide that targets Panx1 function, ¹⁰panx (a

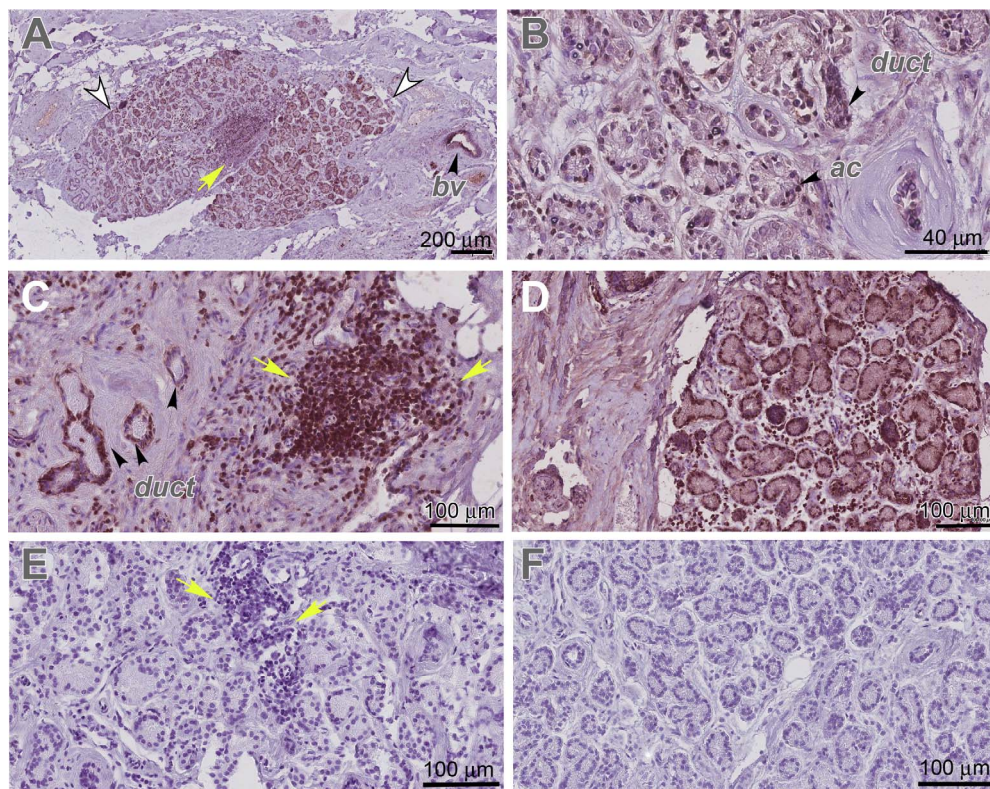


FIGURE 2. Panx1 and 2 expression in human LG. (A–D) Panx1 (*brown*) is specifically expressed in the LG (in LG lobes labeled with *white arrowheads*) ducts (*duct*), acini (*ac*), blood vessels (*bv*), and infiltrating lymphocytes (*I*; *yellow arrows*) of the human LG. Panx1 expression was visualized by different antibodies to Panx1: (A, B) rabbit polyclonal antibody to Panx1 (Sigma-Aldrich; HPA016930) and (C) affinity purified rabbit Panx1 antibody CT-395 (Px-34, also see Supplementary Fig. S1). (D) Panx2 (Aviva Systems Biology, Cat# ARP42778_T100) is expressed in the LG. (E, F) Negative controls to Panx1 and 2 antibodies in which primary antibodies were substituted with the isotype-specific immunoglobulins (normal rabbit IgG, Sigma-Aldrich). (A–F) LG was obtained from a 90-year-old female donor.

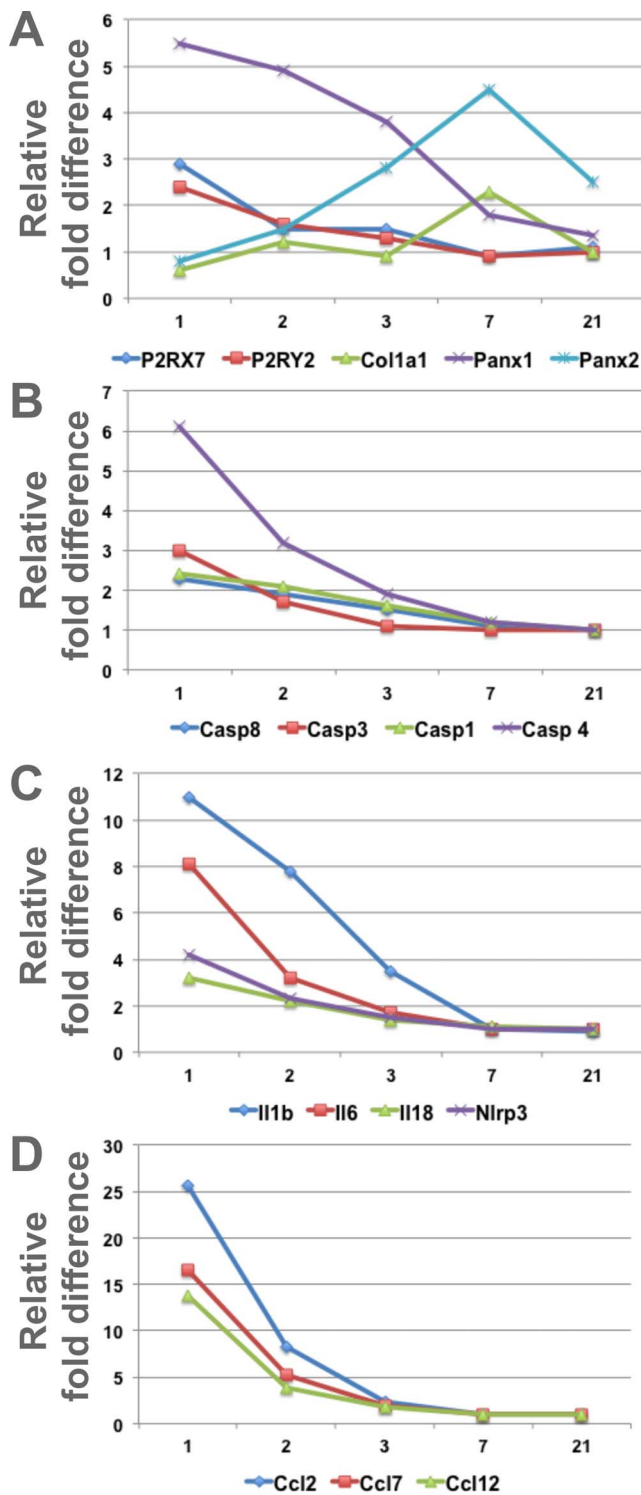


FIGURE 3. Expression of Panx1, Panx2, and proinflammatory and differentiation factor mRNAs after LG injury and during LG regeneration. Graphic representation of the Table. LGs were injected with IL-1 or saline (vehicle control) and collected at days 1, 2, 3, 7, and 21 after injection for RNA extraction. Levels of mRNAs were determined by qRT-PCR. (A) Expressions of Panx1 and purinergic receptors P2RX7 and P2RY2 were upregulated during first 1 to 3 days after the LG injury (inflammation stage), whereas expressions of Panx2 and collagens I and XVI (A: Col16a1 and Col1a1) were increased during the regeneration phase, on days 5 to 7 after IL1 β injection. Expressions of several inflammasome related factors (B, C: NLRP3, Casp1, Casp4 [11] and Casp8); proinflammatory cytokines (C: IL1b, IL6, IL18), and

TABLE. Changes in Gene Expression of Proinflammatory and Differentiation Markers After IL-1 α LG Injection

Gene Name	1	2	3	7	21	Control
<i>P2rx7</i>	2.9	1.5	1.5	0.9	1.1	1.1
<i>P2ry2</i>	2.4	1.6	1.3	0.9	1.0	1.0
<i>Panx1</i>	5.5	4.9	3.8	1.8	1.4	1.1
<i>Panx2</i>	0.8	1.5	2.8	4.5	2.5	1.1
<i>Col1a1</i>	0.6	1.2	0.9	2.3	1.0	0.8
<i>Casp8</i>	2.3	1.9	1.5	1.1	1.0	1.0
<i>Casp3</i>	3.0	1.7	1.1	1.0	1.0	0.9
<i>Casp1</i>	2.4	2.1	1.6	1.2	1.0	0.9
<i>Casp4(11)</i>	6.1	3.2	1.9	1.2	1.0	0.9
<i>Il1b</i>	11.0	7.8	3.5	1.0	0.9	0.9
<i>Il6</i>	8.1	3.2	1.7	1.0	1.0	1.0
<i>Il18</i>	3.2	2.2	1.4	1.1	1.0	0.8
<i>Nlrp3</i>	4.2	2.3	1.5	1.0	1.0	0.9
<i>Ccl2</i>	25.7	8.3	2.4	1.0	1.0	0.9
<i>Ccl7</i>	16.5	5.2	1.9	1.0	1.0	1.1
<i>Ccl12</i>	13.8	3.9	1.8	1.0	1.0	0.9

The qRT PCR experiments show a substantial increase in expression of Panx1 and several proinflammatory factors within first 1 to 3 days after IL-1 α LG injury (acute inflammation phase), whereas expression of Panx2 and LG differentiation markers (collagen I [Col1a1]) increases on days 4 to 7 (regeneration/differentiation phase). Statistically significant changes corresponding to $P < 0.01$ values are shown in bold. Casp1, Caspase 1; Casp3, Caspase 3; Casp4(11), Caspase 4, also known as 11; Casp8, Caspase-8; Ccl2, C-C motif chemokine ligand 2; Ccl7, C-C motif chemokine ligand 7; Ccl12, C-C motif chemokine ligand 12; Il1b, interleukin 1 β ; Il6, interleukin 6; Il18, interleukin 18; Nlrp3, NOD-like receptor protein-3; P2rx7, P2X purinoceptor 7; P2ry2, P2Y purinoceptor 2.

selective Panx1 blocker)⁵⁷⁻⁵⁹ or sRNAi, a novel class of nontoxic stable RNAi compounds capable of efficient cellular uptake in vitro and in vivo without the use of transfection reagents for cell penetration.^{69,70} The compounds were designed in silico and initially tested in cell culture or in vivo by passive transfection: adding sRNA directly to the cell culture medium or by injecting sRNA into tissue (Supplementary Methods, Supplementary Table S1, Supplementary Figs. S3, S4). The LG of a recipient mouse was injected with IL1 α alone (control), whereas the gland on the other side was injected with IL1 α plus ¹⁰panx inhibitor peptide or Panx1 IL1a plus sRNAi (experimental) (Fig. 4A). One day after injury, EPCPs isolated from *Pax6-LacZ* mice^{45,71} were injected into the injured LGs, as we described previously¹⁴ (Fig. 4A). Analysis of cell engraftment was carried out on the 40th day after cell transplantation (Fig. 4A). The percentage of engrafted LacZ⁺ cells was quantified in serial LG sections stained with X-gal and Fast red to visualize transplanted cells and nuclei, respectively. In both cases, EPCPs injected into the LG were found in the ductal and acinar epithelial cell compartments; however, the efficacy of cell engraftment was significantly higher in ¹⁰panx peptide or Panx1 sRNAi treated glands. Approximately 63% more engrafted cells were found after ¹⁰panx peptide and 169% more engrafted cells were found after sRNAi administration (Figs. 4B-4D). Control LGs injected with the vehicle did not show any LacZ⁺ cells (data not shown).

IL1 family member 9 (C: Il1f9, proinflammatory stimulator of chemokine); and specific proinflammatory chemokines (D: Ccl2, Ccl6, Ccl7, and Ccl12) were significantly increased at days 1 to 3 after LG injury.

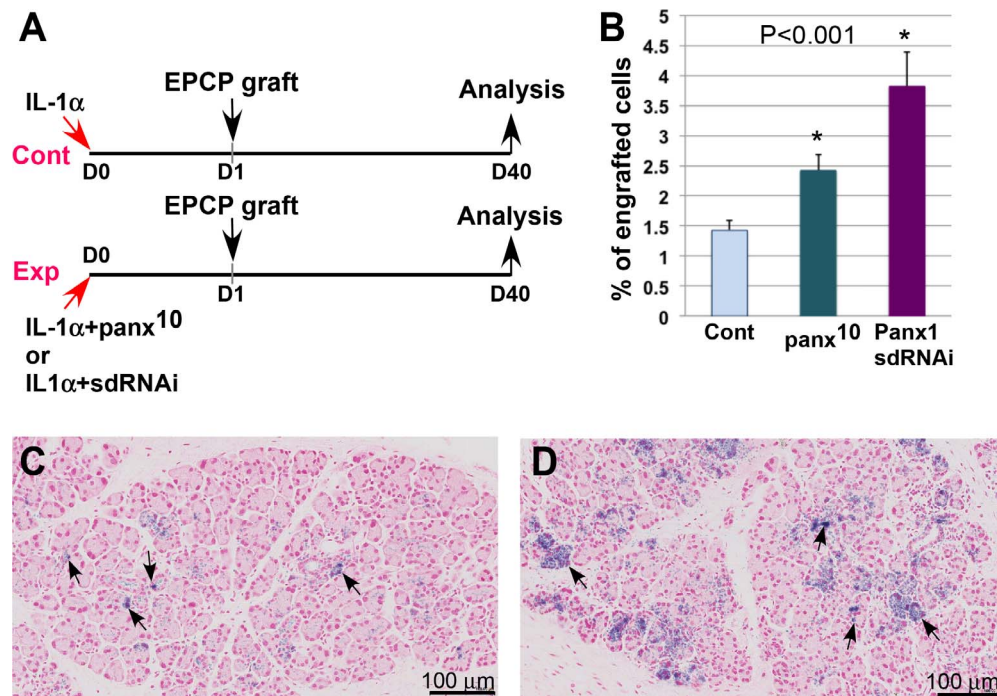


FIGURE 4. Pharmacologic blockade of Panx1 increases engraftment of transplanted EPCPs into the injured host LG. (A) Experimental paradigm. Recipient LGs were injected with IL1 α alone or combined with a mimetic peptide inhibitor of Panx1 10 panx (100 mM) or Panx1 sdrRNAi and donor cells, sorted from R26-LacZ mouse, and were transplanted 1 day after injury and analyzed 40 days after transplantation. (B) Blocking Panx1 channels by 10 panx or Panx1 sdrRNAi increased the engraftment of EPCP cells relative to control LGs. Engraftment of transplanted EPCPs into control (C) or sdrRNAi-treated LGs (D) (black arrows, LacZ $^{+}$ engrafted cells. In (B) * $P < 0.01$.

Blocking Panx1 or Casp4(11) in the LGs of *TSP-1* $^{-/-}$ Mice Decreases Inflammation

Next, we tested whether silencing of Panx1 or Casp4(11) genes in the LG of the thrombospondin-1-null (*TSP-1* $^{-/-}$) mouse, a recently reported mouse model of ADDE, would decrease LG inflammation. Although born with no apparent abnormalities of the LG, *TSP-1* $^{-/-}$ mice develop a severe inflammation of the LG with a significant loss of LG secretory function around 24 weeks of age. Thus, the *TSP-1* $^{-/-}$ mouse fully mimics the development of chronic autoimmune dry eye disease in humans.^{42,43}

Modified RNAi compounds (sdrRNA) for silencing the expression of Panx1 and Casp4(11) were developed. Duplexes with the highest efficacy were selected (Supplementary Fig. S2) and tested in cell base and in in vivo assays (Supplementary Fig. S3) prior to LG application. We used the most efficient Panx1 (Px23) or Casp4(11) (Cs12) sdrRNAi compounds for intraglandular injections. As demonstrated previously, inflammation in *TSP-1* $^{-/-}$ LGs starts at approximately 10 to 12 weeks as periductal lymphocytic infiltrates^{14,43} and could be found in periacinar regions at 3 to 4 months of age.^{42,43} Thus, injections were started when *TSP-1* $^{-/-}$ mice were around 4 months old and performed once a week for at least 5 weeks. We injected the right LGs of female *TSP-1* $^{-/-}$ mice with a Panx1 or Casp4(11) sdrRNAi, whereas the LGs on the other side were injected with a control sdrRNAi or vehicle. Each sdrRNAi was also mixed with a pluronic gel and injected under the LG capsule (a thin film surrounding the gland). Pluronic gel is liquid at low temperatures, 0–4°C, but sets at physiologic temperature, remaining in place for 8 to 12 hours.^{55,72} The gel has the additional advantage of being a mild surfactant and provides local controlled sdrRNAi release for an extended time. As we and others showed previously these features of the

pluronic gel appeared to markedly expedite penetration of oligonucleotides into the cells.^{72,73}

Mice were euthanized at the end of the experiment (2 weeks after the last injection), and the LGs were processed for RNA isolation and histology. Both Panx1 and Casp4(11) sdrRNAi treatments resulted in significant reduction of Panx1 and Casp4(11) mRNA expression (Fig. 5A). Analysis of IL1 β and Nlrp3 (inflammation markers) mRNA expression by qRT-PCR showed that downregulation of Panx1 mRNA levels resulted in a significant decrease of expression of both IL1 β and Nlrp3, whereas blocking Casp4(11) reduced only IL1 β but not Nlrp3 mRNA level (Fig. 5B).

To further assess the level of inflammation in the LGs after sdrRNAi treatment, we measured the cumulative area of inflammation (summed area of infiltration foci per field, measured in μm^2) in Panx1-treated, Casp4(11)-treated, and control LGs, as described previously.¹⁴ We found that both Panx1 and Casp4(11) sdrRNAi-treated LGs had a slight but a statistically significant reduction of cumulative area of inflammation (Fig. 5C).

Combined these data indicates that manipulation of Panx1 signaling may reduce LG inflammation.

DISCUSSION

Endogenous epithelial progenitor cell transplantation is a promising tool for regeneration of the damaged epithelial components of the LG in cases of severe dry eye disease. In our previous work, we showed that EPCP transplantation could improve the structure and function of chronically inflamed LGs of *TSP-1* $^{-/-}$ mice.¹⁴ Our experiments also suggested that endogenous stem cell function and engraftment of transplanted cells depends on LG inflammation level.^{14,74} These data imply that using anti-inflammatory approaches may have

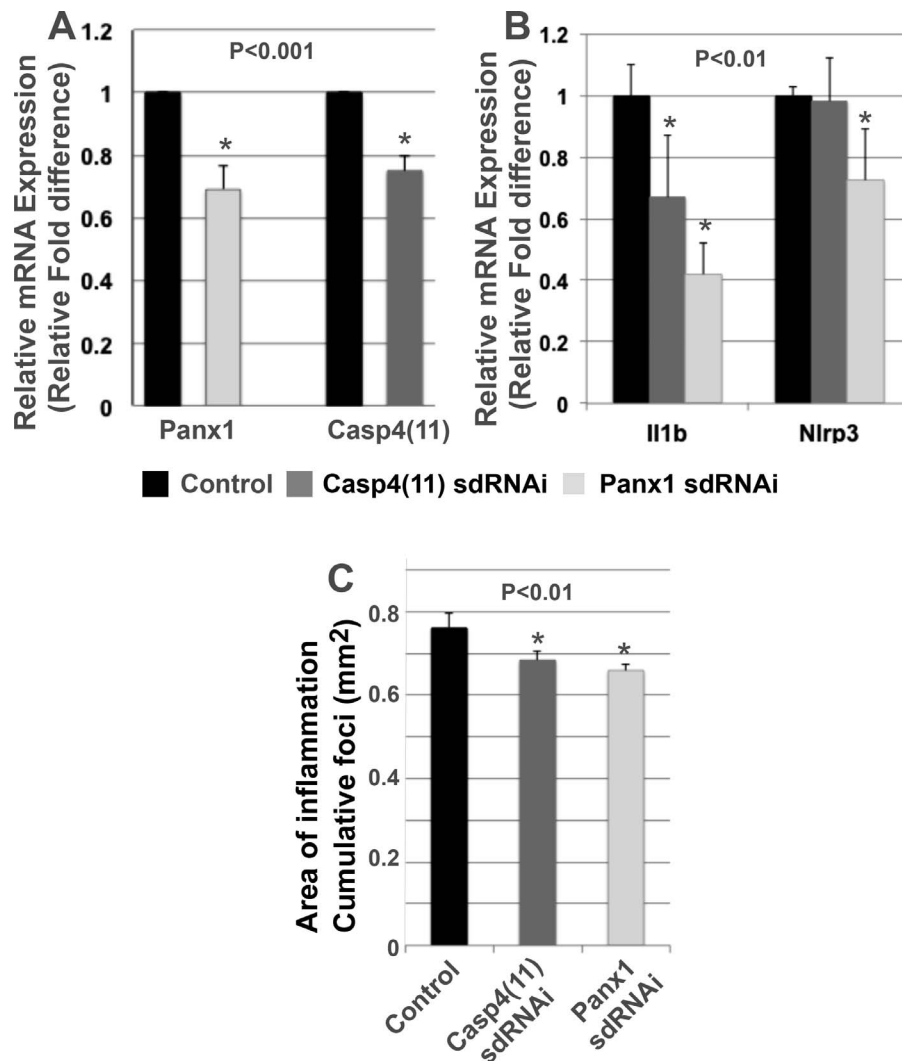


FIGURE 5. Blocking Panx1 or Casp4(11) with sdRNAi in *TSP1*^{-/-} LGs decreases LG inflammation. Two to 3 μ L solution of Panx1 or Casp4(11) sdRNAi (at a final concentration of 250 nM) was injected into the LGs on the right side of 4-month-old *TSP1*^{-/-} mice. The left LG was injected with a control sdRNAi or vehicle (saline). Mice were injected once a week for the following 5 weeks and euthanized 2 weeks after the last injection. LGs were processed for qRT-PCR or histology. (A) LG treated with specific sdRNAi (to Panx1 or Casp4[11]) showed significant reduction in Panx1 or Casp4(11) mRNA expression compared with LG treated with control sdRNAi. (B) Decrease of Panx1 mRNA level resulted in a significant reduction of expression of both IL1 β and Nlrp3, whereas blocking Casp4(11) significantly reduced only IL1 β but not Nlrp3 expression. (C) Measurement of the cumulative area of inflammation (summed area of infiltration foci per field, measured in mm²) showed that Panx1 or Casp4(11) sdRNAi treatments of the LG of *TSP1*^{-/-} mice significantly decreased the cumulative area of inflammation compared with controls. To quantify statistical difference, a two-tailed Student's *t*-test was used. Panx1 sdRNAi ($P = 1.6 \times 10^{-6}$) and Casp4 sdRNAi ($P = 4.0 \times 10^{-5}$).

important therapeutic implications in ongoing dry eye disease or as a therapy combined with stem/progenitor cell transplantation. The acute LG injury model reported previously¹⁵ was useful to study cell engraftment. Although this model has numerous clinical limitations compared with chronic models of LG inflammation for long-term studies, such as concise period of inflammation and fast and robust recovery of LG after acute injury, it also had certain benefits for our study. The inflammation process in this model is very local (observed only within injured LG), its timing is well defined and the mechanism of this acute inflammation is very well studied.^{6,15,75-77} In addition the role of Panx1 in acute inflammation has been reported.^{78,79} In current study, we demonstrated that Panx1 is highly expressed in the epithelial components of the mouse and human LGs and that progenitor cell engraftment into injured LGs was significantly improved by the blocking of Panx1 function. These findings suggest that stem/

progenitor cells function more efficiently when inflammation is downregulated and the regeneration process is induced.¹⁴ Moreover, these findings support a consensus view that Panx1 is an important mediator of inflammation.^{78,79} Our model suggests that transplantation of progenitor cells at high level of inflammation results in their poor survival (Fig. 6A), whereas blocking Panx1 in the LG decreases the level of inflammation and promotes progenitor cell survival/function within the LG (Fig. 6B). This finding also indicates that persistent LG inflammation sustains dysfunction of the endogenous LG stem cell niche and challenges the potential efficacy of stem cell therapies aimed at mobilizing endogenous and/or transplanted progenitor cells. Although the process of acute inflammation is an important step toward its resolution,³³ a delay in resolution may lead to chronic inflammation and progressive cell death. Although our previous study showed that progenitor cell transplantations could temporarily improve recovery and

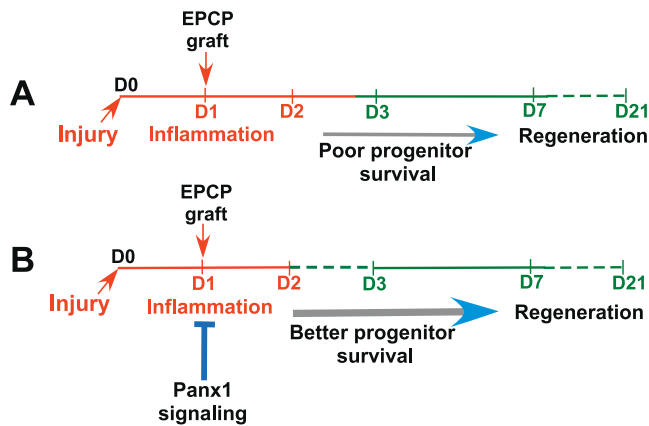


FIGURE 6. Blocking Panx1 improves donor LG progenitor cell engraftment by decreasing the inflammation and promoting transplanted cell survival. (A) Cell transplantation during a high level of inflammation results in poor transplanted cell survival, whereas (B) blocking Panx1 in the LG decreases the level of inflammation and promotes progenitor cell survival/function within the LG. The diagram represents our understanding of how Panx1 inhibition may control inflammation and therefore progenitor cell engraftment.

function of the chronically inflamed LG, we observed some variations in stem cell survival/engraftment depending on severity of inflammation; thus, the longer-term studies are necessary to analyze function of transplanted cells and to determine the mechanisms/treatments that would increase long-term disease remission. Our study also shows that “local” blockage of Panx1 or Casp4(11) could decrease expression of proinflammatory factors and to some extent improve LG structure reducing size of infiltration foci during chronic inflammation. Because we still did not observe any statistically significant changes in tear production in treated $TSPI^{-/-}$ mice, future studies need to use general anti-inflammatory treatments or a complete “reset” of the immune system⁸⁰ to stop it attacking the body.

Our previous^{14,74} and current studies support the idea that persistent chronic LG inflammation mediates changes in the stem/progenitor cell survival/function that recovers when inflammation abates.

These findings have implications for therapeutic strategies targeting Panx1 signaling pathways in the LG prior to or at the time of progenitor cell transplantation. The effectiveness of this strategy will depend on successful suppression of inflammation, as well as on the long-term plasticity of the transplanted progenitor cells. Studies of Panx1 function in human cells are needed to develop new therapeutic strategies for human dry eye disease.

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