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IL-10 and TGF-β Increase Connexin-43 Expression and Membrane Potential of HL-1 Cardiomyocytes Coupled with RAW 264.7 Macrophages

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ABSTRACT

Cardiac resident macrophages facilitate electrical conduction by interacting with cardiomyocytes via connexin-43 (Cx43) hemichannels. Cx43 is critical for impulse propagation and coordination between muscle contractions. Cardiomyocyte electrophysiology can be altered when coupled with noncardiomyocyte cell types such as M2c tissue-resident macrophages. Using cocultures of murine HL-1 cardiomyocytes and RAW 264.7 macrophages, we examined the hypothesis that cytokine signals, TGF-β1 and IL-10, upregulate Cx43 expression at points of contact between the two cell types. These cytokine signals maintain the macrophages in an M2c anti-inflammatory phenotype, mimicking cardiac resident macrophages. The electrophysiology of cardiomyocytes was examined using di-8-ANEPPS potentiometric dye, which reflects a change in membrane potential. Greater fluorescence intensity of di-8-ANEPPS occurred in areas where macrophages interacted with cardiomyocytes. Suppressor of cytokine signaling 3 (SOCS3) peptide mimetic downregulated fluorescence of this membrane potentiometric stain. Cx43 expression in cocultures was confirmed by fluorescence microscopy and flow cytometry. Confocal images of these interactions demonstrate the Cx43 hemichannel linkages between the cardiomyocytes and macrophages. These results suggest that TGF-β1 and IL-10 upregulate Cx43 hemichannels, thus enhancing macrophage–cardiomyocyte coupling, raising the cellular resting membrane potential and leading to a more excitatory cardiomyocyte. *ImmunoHorizons*, 2022, 6: 334–343.

INTRODUCTION

Cardiac myocytes rely on electrical conduction to facilitate the contractile ability of these important muscle cells. Recent studies have shown that cardiomyocytes and macrophages interact via connexin-43 (Cx43) hemichannels (1). It is this interaction that is critical for raising the resting membrane potential and enabling the contractile ability of the cardiomyocytes. It is important to fully understand what kind of interaction the Cx43 is aiding. Furthermore, understanding how to manipulate this interaction can contribute to future research pertaining to viral or bacterial infections of the heart, including bacterial myocarditis and even COVID-19 infection, as it is known to cause inflammation of the heart tissue (2).

Macrophages are known to play various roles in both innate and adaptive immunity. Despite their good intentions, macrophages can trigger inflammation, promote autoimmune diseases, and even aid in cancer metastasis (3, 4). Hulsmans et al. (5) presented findings showing that murine cardiac resident macrophages interacting with cardiomyocytes facilitate electrical conduction in the heart. The bipolar nature of these cells is due to their two, very different, activation states. The M1 activation state is the macrophages "pro-inflammatory"

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Abbreviations used in this article: AF488, Alexa Fluor 488; AF594, Alexa Fluor 594; CTCF, corrected total cell fluorescence; Cx43, connexin-43; SOCS3, suppressor of cytokine signaling 3.

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phenotype, whereas the M2 activation state primarily aids in tissue repair (3, 4, 6). Macrophages can be activated to an M1 state via introduction of intracellular pathogens (3, 4). Alternatively, an M2 state can be activated via nearby cell death, complement system components, fungal pathogens, and (with vital importance to this study) the presence of IL-10 or TGF-β (7, 8). This activation state will allow the macrophages to aid in the repolarization of the cardiomyocytes (3, 7, 8). The absence of suppressor of cytokine signaling (SOCS3) also pushes macrophages to the inflammatory M1 phenotype (9). This observation suggests that SOCS3 is necessary for the M2 phenotype. Meguro et al. (10) confirmed that SOCS3 is highly expressed in M2 macrophages. Consequently, this study examines the effect of SOCS3 peptide mimetic on the IL-10 and TGF-β pathways in macrophage/cardiomyocyte cocultures in which the RAW 264.7 cells serve the role of the M2 macrophage to mimic the effects of macrophages on cardiomyocyte excitation.

MATERIALS AND METHODS

RAW 264.7 IL-10(p)GFP$^+$ macrophages

PCR oligonucleotide primers were designed to amplify the 1602-bp (−1538/+64) mouse IL-10 promoter in plasmid pGL2B (Addgene, 24942) (11). 5′-ClaI and 3′-BamHI sites were included in the oligonucleotides for subsequent cloning. Following PCR amplification and DNA purification, the promoter was cloned into the ClaI/BamHI sites of the lentiviral construct, pLv-GFP-V5, by excising the CMV promoter to generate pLv- mIL-10(p)-GFP-V5 (12, 13). The promoter construct was transformed, amplified, and confirmed by XhoI restriction digest. To stably express the promoter, RAW 264.7 cells (American Type Culture Collection, TIB-71) were grown in DMEM/10% FBS and maintained at 37°C and 5% CO_2. Cells (2×10^6) in a 35-mm plate were transfected with 4:1 DNA/FuGENE HD and selected for 5 μg/ml blasticidin. The selected, stable pool was then maintained and propagated in petri dishes under blasticidin selection. GFP expression in all cells was confirmed by immunofluorescence. Once stable, this cell line was cultured in DMEM/10%FBS medium containing 5 μg/ml blasticidin. The RAW 264.7 IL-10(p)GFP$^+$ macrophages were used in the flow cytometry experiment (Fig. 1), with the fluorescence experiments resulting in the data in Fig. 3D and 3E and images shown in Figs. 4 and 5A.

RAW 264.7 macrophages

RAW 264.7 macrophages were purchased from American Type Culture Collection (TIB-71). Cells were cultured in an incubator maintained at 37°C with 5% CO_2 and 95% relative humidity as per the manufacturer's recommendations. Culture medium consisted of 10% FBS in DMEM. FBS was purchased from Sigma-Aldrich, and DMEM was purchased from American Type Culture Collection. Cell medium was refreshed every 48–72 h, and cells were split upon reaching at least 70% confluency. Cells were retired after 30 passages to maintain efficacy of the results. These macrophages were used in the fluorescence experiments resulting in the data and images shown in Figs. 2A–C and 3A–C, as well as all confocal imaging (Fig. 5B–H).

HL-1 cardiomyocytes

HL-1 cardiomyocytes were purchased from Sigma-Aldrich (SCC065). Cells were cultured in an incubator maintained at 37°C with 5% CO_2 and 95% relative humidity as per the manufacturer's recommendations. Culture medium consisted of 87% Claycomb medium (from Sigma-Aldrich), 10% FBS, 1% norepinephrine, 1% glutamine, and 1% penicillin/streptomycin. Cell medium was refreshed every 48–72 h and cells were split upon reaching 90% confluency. As cardiomyocytes are only mildly adhesive cells, T75 flasks were prepared with a 0.05% gelatin/fibronectin solution prior to cell culture to facilitate adhesion. Cells were retired after 30 passages to maintain efficacy of the results.

Flow cytometry

RAW 264.7 IL-10(p)-GFP$^+$ macrophages and HL-1 cardiomyocytes were cocultured in T25 flasks at a 1:50 ratio, respectively. Each set of treatments was done in triplicates using identical experimental methods. The treatments varied between no cytokines added, 3 ng/ml TGF-β, 20 ng/ml IL-10, or 3 ng/ml TGF-β and 20 ng/ml IL-10. The cultures were incubated at 37°C with 5% CO_2 for 48 h. At 48 h, the supplemented Claycomb medium was aspirated off, the cells were rinsed with PBS (Ca^{2+}-free and Mg^{2+}-free), and the cells were detached from the flask with 0.05% trypsin. Each set of cells was fixed using 4% paraformaldehyde for 15 min, then rinsed three times with PBS. The fixed cells were resuspended in a blocking buffer consisting of 3% BSA/0.01% saponin in PBS for 39 min. The cells were incubated with either Cx43 conjugated with Alexa Fluor 488 (AF488; Santa Cruz, sc-271837) or an IgG_{2a} isotype conjugated with AF488 (Santa Cruz, sc-3891) for 1 h at room temperature. Both primary Ab and isotype were diluted 1:250 in the 3% BSA/0.01% saponin in PBS blocking buffer. The cells were all washed three times in ice-cold PBS after Ab incubation, and the samples were kept on ice prior to analyzing on an Accuri C6 flow cytometer (BD Biosciences). The raw data were then analyzed in FCS Express 6 flow cytometry software (DeNovo Software).

Di-8-ANEPPS immunofluorescence

Cocultures were prepared in eight-well removable chamber slides. Wells were coated with 0.05% gelatin/fibronectin solution to facilitate adhesion of cardiomyocytes. Cells were cocultured at a ratio of 1:50 RAW 264.7 macrophages to HL-1 cardiomyocytes. This ratio facilitates growth of cardiomyocytes and macrophages without overgrowth of the macrophages, allowing proper observation of the interactions between the two cell lines. After counting cells with a hemocytometer, cell suspensions were diluted (when necessary) to achieve the approximate number of cells per volume. With a suggested seeding density of 40,000–90,000 cells per the manufacturer's recommendation, each well was

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loaded with ~40,000 HL-1 cardiomyocytes and 800 RAW 264.7 macrophages. Wells containing only one cell line (cardiomyocytes or macrophages) were loaded with ~40,000 cells. Cells were cocultured at that ratio in media containing 10% FBS in DMEM.

Preliminary testing provided the optimum dilution for TGF-β1. To maintain physiological relevance, dilutions tested were limited to <5 ng/ml. TGF-β1 was purchased from Fischer Scientific (NC1416454), IL-10 was purchased from Fischer Scientific (50-170-413), and SOCS3 was purchased from GenScript (NM007707.3). Treatments for each well consisted of TGF-β1 at 3.5 ng/ml, IL-10 at 20 ng/ml, SOCS3 at 35 μM, TGF-β1 at 3.5 ng/ml + SOCS3 at 35 μM, IL-10 at 20 ng/ml + SOCS3 at 35 μM, and IL-10 at 20 ng/ml + SOCS3 at 35 μM + TGF-β1 at 3.5 ng/ml, as well as a control well. Cocultures were incubated at 37°C, 5% CO_2, 95% humidity with their respective treatments for 48 h prior to fixing. After a 48-h incubation, all wells were treated with di-8-ANEPPS potentiometric dye at a ratio of 1:250 (di- 8-ANEPPS to DMSO) for 1 h at 4°C (isotypes were loaded with DMSO only). Cells were then fixed with 4% paraformaldehyde in PBS, and blocked with 3% BSA in PBS. After blocking, cells were stained with AF488-F4/80 (or an IgG_{2a} isotype conjugated with AF488) at a 1:200 ratio in 3% BSA in PBS solution. Chambers were removed from the wells and the slide was then mounted using a glycerol mounting medium containing DAPI. Slides were viewed under an FV1000 confocal microscope as well as an Accu-Scope fitted with DAPI (exciter, A350/50x; dichroic, AT410DC; emitter, AT460/50m), GFP/FITC (exciter, AT480/30x; dichroic, AT505DC; emitter, AT535/40m), and Texas Red (exciter, AT575/30x; dichroic, AT600DC; emitter, AT650/60m) LED lasers.

Cx43 immunofluorescence

All wells were prepared and loaded with cells in the same manner as the di-8-ANEPPS experiments. Treatments for each well consisted of TGF-β1 at 3.5 ng/ml, IL-10 at 20 ng/ml, SOCS3 at 35 μM, TGF-β1 at 3.5 ng/ml + SOCS3 at 35 μM, IL-10 at 20 ng/ml + SOCS3 at 35 μM, and IL-10 at 20 ng/ml + SOCS3 at 35 μM + TGF-β1 at 3.5 ng/ml, as well as a control well. Cocultures were incubated at 37°C, 5% CO_2, 95% humidity with their respective treatments for 48 h prior to fixing. After a 48-h incubation, cells were fixed with 4% paraformaldehyde in PBS, permeabilized in 0.5% Triton X-100 in PBS, and blocked with 3% BSA in PBS. After blocking, cells were stained with AF488-F4/80 (or an IgG_{2a} isotype conjugated with AF488) at a 1:200 ratio in 3% BSA in PBS solution as well as Alexa Fluor 594 (AF594)–Cx43 (or an IgG_{2a} isotype conjugated with AF594) at a ratio of 1:250 in 3% BSA in PBS. Chambers were removed from the wells, and the slide was then mounted using a glycerol mounting medium containing DAPI. Slides were viewed under an FV1000 confocal microscope as well as an Accu-Scope fluorescence microscope. Cx43 expression was confirmed by flow cytometry.

ImageJ: measuring fluorescence

ImageJ is a software package provided by the National Institutes of Health to be used for quantifying the number of cells, growth of cells, and amount of fluorescence. For these experiments, ImageJ was used to quantify the amount of fluorescence to indicate presence of macrophages (AF488-F4/80), presence of Cx43 (AF594-Cx43), and change in membrane potential (di-8-ANEPPS dye). To do this, images were taken from each well with an effort made to capture the most fluorescence in each well. Images used for calculating fluorescence were captured on an Accu-Scope fluorescence microscope at ×100 magnification. Using ImageJ, 28 data points were measured for fluorescence from each image for a total of 9408 data points (4704 data points per experiment). Each experiment was repeated three times ($n = 3$). Fluorescence from these data points was analyzed in ImageJ and collected in an Excel spreadsheet. The background subtractor tool was used as well as subtracting isotype numbers and subtracting background measurements to avoid measurement of any background fluorescence. To achieve the corrected total cell fluorescence (CTCF), each raw data point had the average background fluorescence multiplied by the area subtracted from it as indicated in the calculation suggested by the National Institutes of Health: CTCF = integrated density – (area of selected cell × mean fluorescence of background reading).

Statistical analysis

Statistical significance was calculated using one-way ANOVA in SigmaPlot 13.0. All experiments were performed in triplicate. Immunofluorescence experiments were performed with RAW 264.7 IL-10(p)-GFP^+ macrophages in triplicate and then repeated another three times with RAW 264.7 macrophages. Statistically significant differences, determined using p values, are expressed as follows: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$; p values >0.05 (not significant) are not marked.

RESULTS

Flow cytometry analysis of Cx43 expression

Samples were gated on the cardiomyocytes by using size exclusion. For this reason, controls were used to gate on larger cells that were greater than the size expected for macrophages. Cardiomyocytes are typically around 100–200 μm, and macrophages are typically around 20 μm (14, 15). Macrophage results were not analyzed because the autofluorescent GFP expressed by the macrophages overlaps with the Cx43 Ab wavelength. TGF-β1 and/or IL-10 show an approximate increase of 20% in Cx43 expression compared with the no treatment coculture (Fig. 1B, 1C). The cocultures with no treatment were used as a baseline for how much Cx43 is to be expected prior to treatments, so the no treatment sample was set to 100%. Each data bar is a percentage rather than a specific value. It was found that both TGF-β1 and/or IL-10 significantly increased Cx43 expression in cardiomyocytes cocultured with macrophages in this preliminary experiment.

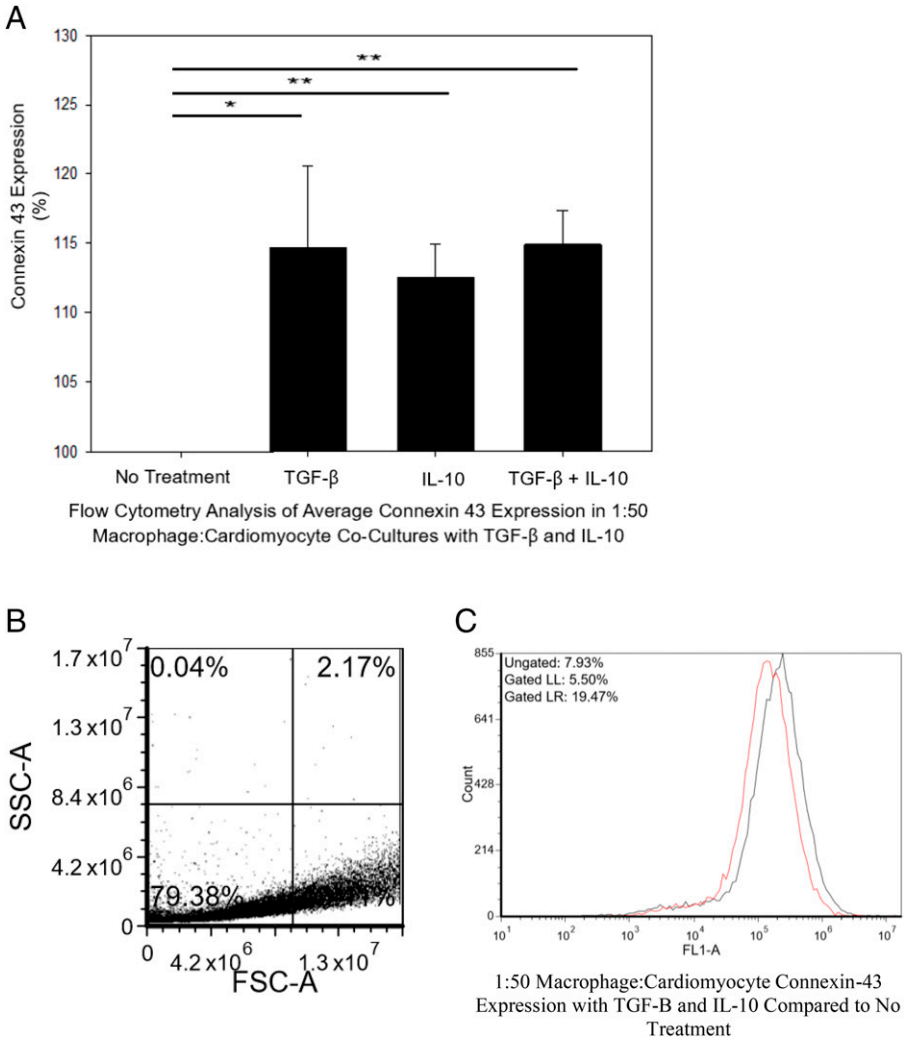

FIGURE 1. Cx43 expression flow cytometry analysis

Flow cytometry analysis of a 1:50 RAW 264.7 IL-10(p)GFP$^+$ macrophage/cardiomyocyte coculture with 3.5 ng/ml TGF-β1 and 20 ng/ml IL-10 stained for Cx43 with a monoclonal Cx43 Ab conjugated with AF488 (Santa Cruz, sc-271837) at a 1:250 dilution. (**A**) Compiled percentages averaged together. The values are expressed as a positive difference percentage compared with the cocultures with no treatment. *$p < 0.05$, **$p < 0.01$. (**B**) Gating to focus primarily on mature cardiomyocytes using size exclusion to remove any macrophages. (**C**) How the histograms were subtracted from one another. The 1:50 coculture without treatment is shown as the red histogram, and the 1:50 coculture with treatments mentioned above is shown as the black histogram.

Cx43 immunofluorescence

Cx43 immunofluorescence was performed to confirm the flow cytometry results. To ensure that the wells for each slide were loaded properly and that there were no macrophages in the cardiomyocyte only wells (e.g., HL-1), AF488-F4/80 fluorescence was quantified in response to the F4/80 protein on the cell membranes of macrophages (Supplemental Fig. 1A). The expression of Cx43 via fluorescence of AF594-Cx43 across the varying treatment groups (Fig. 2) is shown by the slight increase in CTCF or red fluorescence among all of the coculture wells (e.g., "BOTH" wells). Treatment with IL-10 and TGF-β1 significantly increased the expression of Cx43, as shown by the increased CTCF of IL-10– and TGF-β1–treated coculture wells (Fig. 2A). Treatment with SOCS3 inhibits the effect of IL-10 and TGF-β1 while having no statistically significant effect on its own (Fig. 2A). The expression of Cx43 among the cardiomyocyte only wells (i.e., HL-1 wells) is not affected by any of the treatments and shows a fairly steady, but lower, amount of fluorescence on their own (Fig. 2A). Macrophage-only wells (i.e., RAW 264.7 wells) show no fluorescence. The statistical significance, as determined by one-way ANOVA with a Tukey post hoc test in SigmaPlot 13.0, is summarized in Fig. 2B.

This pattern can also be seen in the images of the AF594-Cx43 fluorescence (Fig. 2C). The images in Fig. 2C show cocultures treated with IL-10 and TGF-β separately, as well as a combination of IL-10, TGF-β, and SOCS3 compared with a control coculture with no treatment. For verification, isotype images are provided as well. A clear increase in Cx43 expression can be seen in the cocultures treated with IL-10 and TGF-β, whereas the addition of SOCS3 appears to reduce Cxn43 expression to a level more similar to the control coculture.

di-8-ANEPPS immunofluorescence

Similar to the Cx43 experiment, it is important that the wells for each slide were analyzed to ensure that the materials were loaded properly and that the AF488-F4/80 was not autofluorescing in the cardiomyocyte wells (e.g., HL-1) and macrophages were not present in the HL-1 wells. The F4/80

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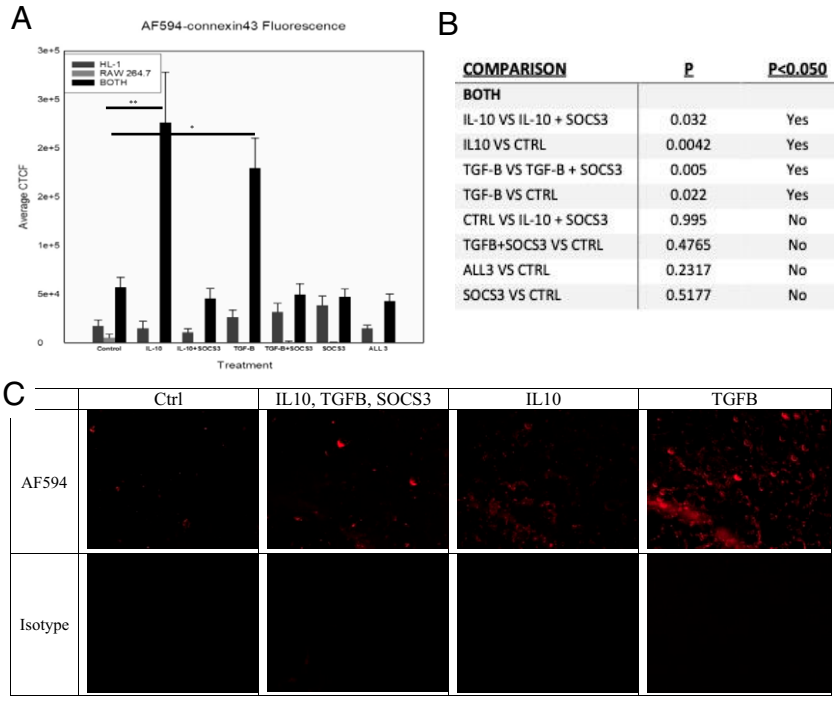


COMPARISON	P	P<0.050
BOTH		
IL-10 VS IL-10 + SOCS3	0.032	Yes
IL10 VS CTRL	0.0042	Yes
TGF-B VS TGF-B + SOCS3	0.005	Yes
TGF-B VS CTRL	0.022	Yes
CTRL VS IL-10 + SOCS3	0.995	No
TGFB+SOCS3 VS CTRL	0.4765	No
ALL3 VS CTRL	0.2317	No
SOCS3 VS CTRL	0.5177	No

FIGURE 2. Cx43 expression immunofluorescence and data analysis

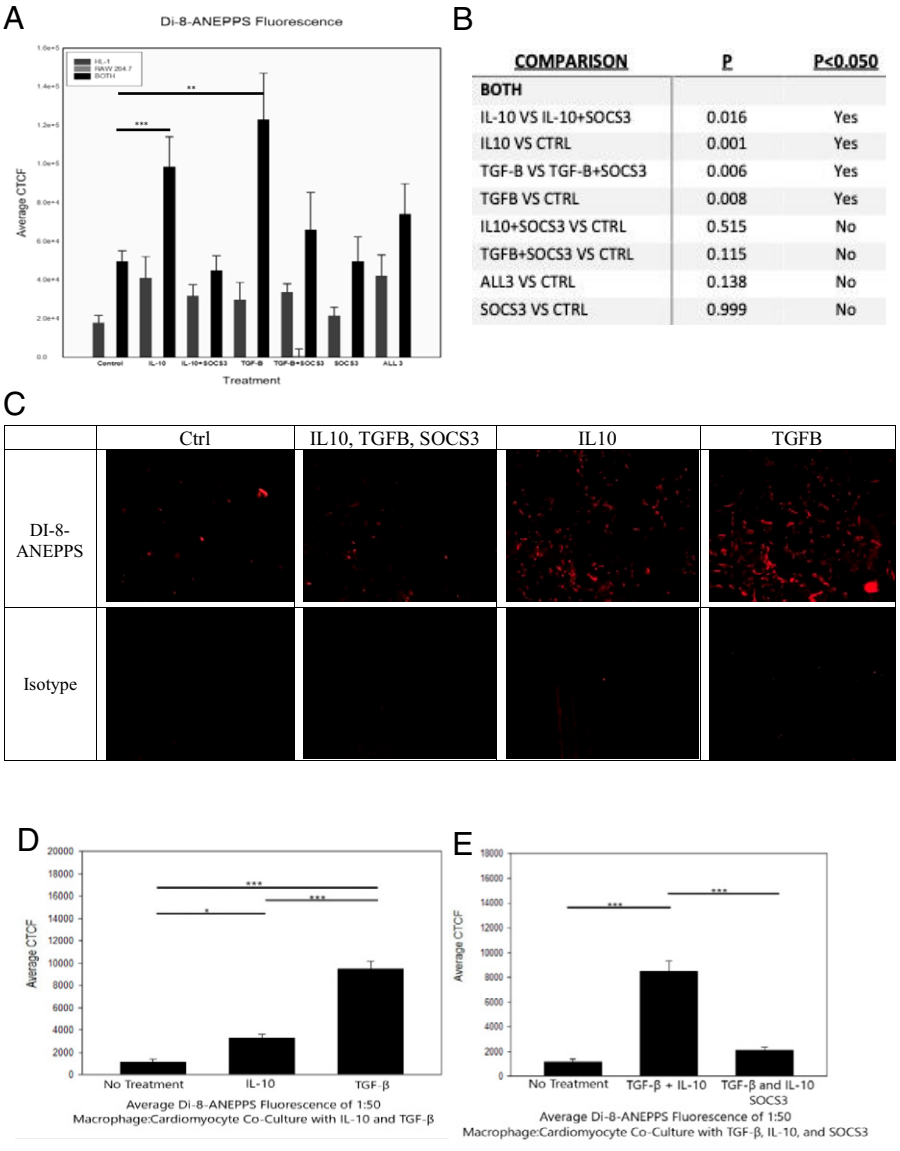
Macrophages cocultured with cardiomyocytes (BOTH) increase fluorescence of AF594-Cx43. Fluorescence is further increased by the addition of IL-10 and TGF-β1. SOCS3 appears to inhibit the effects of IL-10 and TGF-β1 while having no effect on its own. (**A**) Average CTCF of Cx43 via AF594 fluorescence compared between all treatment groups as well as macrophage-only wells, cardiomyocyte-only wells, and coculture wells. Bars represent mean CTCF and error bars represent the SE for each group. *$p < 0.05$, **$p < 0.01$. CTCF, corrected total cell fluorescence. (**B**) Determination of significance and corresponding p values for the coculture wells of the histogram from (A). (**C**) Images showing the increase in AF594-Cx43 fluorescence in coculture wells treated with IL-10, TGF-β1, all three (IL-10, TGF-β1, and SOCS3), as well as a control well with no treatment. Isotype images were added for verification. Presence of Cx43 is indicated by the red fluorescence. Images were taken at an original magnification of ×100 with a standard fluorescence microscope fitted with GFP, Texas Red, and DAPI lasers.

protein fluorescence on the macrophage cell membranes is quantified in Supplemental Fig. 1B. The change in membrane potential via fluorescence of the bright red di-8-ANEPPS dye was compared among the varying treatment groups (Fig. 3A). A similar pattern emerges that nearly mirrors the results seen in the Cx43 experiments (Fig. 3A). A slight increase in calculated CTCF or fluorescence can be seen among all of the coculture wells (e.g., BOTH wells). Treatment with IL-10 and TGF-β1 clearly created a statistically significant increase in CTCF of the coculture wells (Fig. 3A). Treatment with SOCS3 inhibited the effects of IL-10 and TGF-β1 while having no statistically significant effect on its own (Fig. 3A). The change in membrane potential, as indicated by the CTCF among the cardiomyocyte-only wells (e.g., HL-1 wells), is not affected by any of the treatments and shows a steady, but lower, amount of CTCF on its own. Macrophage-only wells (e.g., RAW 264.7 wells) show no statistically significant change in membrane potential (Fig. 3A). The di-8-ANEPPS experiments were first performed using the RAW 264.7 IL-10(p) GFP^+ macrophages (Fig. 3D, 3E) and were then repeated with the nontransfected RAW 264.7 macrophages (Fig. 3A–C).

The statistical significance for the data collected in Fig. 3A is summarized in Fig. 3B. Treatment with IL-10 to the coculture wells demonstrates a statistically significant difference from both the control and the combined treatment with IL-10 and SOCS3. Treatment with TGF-β1 shows a significant difference from both control and the combined treatment of TGF-β1 and SOCS3 (Fig. 3B). Finally, the control (no treatment) group showed no difference from treatment with SOCS3 alone, TGF-β1 and SOCS3, IL-10 and SOCS3, or all three treatments combined.

This pattern can also be seen in the images of the di-8-ANEPPS fluorescence (Fig. 3C). Within these images, an increase in di-8-ANEPPS fluorescence shows an increase in membrane potential among the coculture wells treated with IL-10 and TGF-β1 when compared with the control well (Fig. 3C). Images of the coinciding isotype wells were provided for verification purposes. Coculture wells treated with IL-10, TGF-β, and SOCS3 do not show a difference in fluorescence compared with the control well (Fig. 3C).

Fig. 4 shows side-by-side images of macrophages coupled with cardiomyocytes, and the di-8-ANEPPS fluorescence drastically increases at points of contact between the two cell lines. These points of contact are depicted by the addition of colored arrows to the images.



COMPARISON	P	P<0.050
BOTH		
IL-10 VS IL-10+SOCS3	0.016	Yes
IL10 VS CTRL	0.001	Yes
TGF-B VS TGF-B+SOCS3	0.006	Yes
TGFB VS CTRL	0.008	Yes
IL10+SOCS3 VS CTRL	0.515	No
TGFB+SOCS3 VS CTRL	0.115	No
ALL3 VS CTRL	0.138	No
SOCS3 VS CTRL	0.999	No

FIGURE 3. Di-8-ANEPPS fluorescence and data analysis

Macrophages cocultured with cardiomyocytes (BOTH) increase fluorescence of di-8-ANEPPS dye. Fluorescence is further increased by the addition of IL-10 and TGF-β1. SOCS3 appears to inhibit the effects of IL-10 and TGF-β1 while having no effect on its own. (**A**) Average CTCF of Cx43 via AF594 fluorescence compared between all treatment groups as well as macrophage-only wells, cardiomyocyte-only wells, and coculture wells. (**B**) Determination of significance and corresponding *p* values for the coculture wells of the histogram from (A). (**C**) Images showing the increase in AF594-Cx43 fluorescence in coculture wells treated with IL-10, TGF-β1, all three (IL-10, TGF-β1, and SOCS3), as well as a control well with no treatment. Isotype images were added for verification. Presence of Cx43 is indicated by red fluorescence. Images were taken at an original magnification of ×100 with a fluorescence microscope fitted with GFP, Texas Red, and DAPI lasers. (**D**) Histogram compares average CTCF of RAW 264.7 IL-10(p)GFP$^+$ macrophages coupled with HL-1 cardiomyocytes at a 1:50 ratio treated with either IL-10, TGF-β1, or no treatment. (**E**) Histogram compares average CTCF of RAW 264.7 IL-10(p)GFP$^+$ macrophages coupled with HL-1 cardiomyocytes at a 1:50 ratio treated with either TGF-β1 and IL-10, TGF-β1, IL-10 and SOCS3, or no treatment. Error bars indicate SE. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. CTCF, corrected total cell fluorescence.

Confocal microscopy

To visualize the Cx43 connection between macrophages and cardiomyocytes, confocal images were taken. Using confocal microscopy to view the interactions involving Cx43 between cardiomyocytes and macrophages allows for not only enhanced images, but the ability to view these interactions in a three-dimensional manner. Confocal images present a much cleaner background, enhanced coloring, and increased definition. Fig. 5B and 5C show a comparison between two images taken from the same IL-10–treated

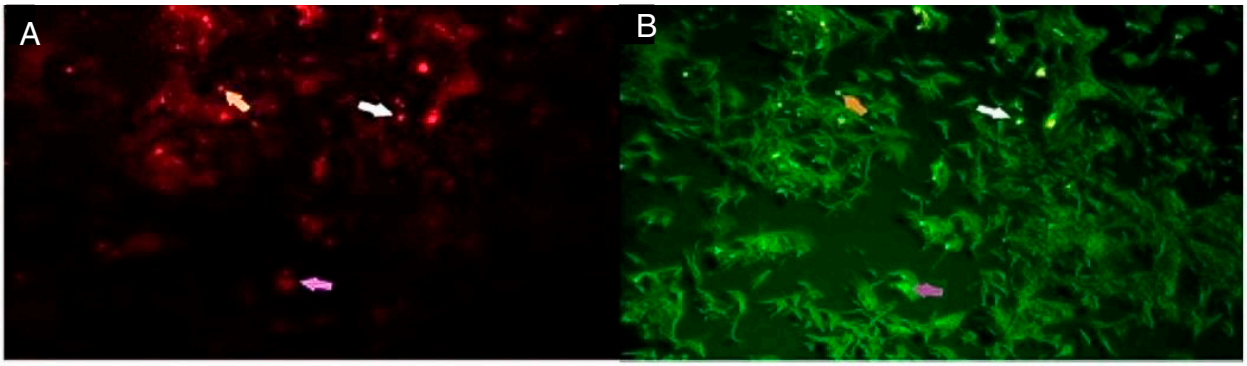


FIGURE 4. Coculture coupling effects of Cx43 expression

(**A**) RAW 264.7 IL-10(p)GFP$^+$ macrophages coupled with HL-1 cardiomyocytes at a 1:50 ratio stained with AF594-Cx43. Red fluorescence shows Cx43, expression and the arrows point to locations of peak fluorescence. (**B**) Same frame as in (A), but using the GFP laser instead of Texas Red shows the GFP-labeled macrophages fluorescing at peak GFP fluorescence locations. Arrows from (A) were layered, indicating areas of peak Cx43 expression to show that these areas coincide with areas where macrophages are coupled with cardiomyocytes. (A and B) Original magnification ×100.

coculture well in the Cx43 experiments. Fig. 5A is an image captured on the AccuScope (fluorescence microscope) at ×500 magnification to compare with confocal images such as Fig. 5B and 5C. Fig. 5B and 5C were captured on the FV1000 confocal microscope at ×600 magnification. Although the interaction (and Cx43) can be seen in the regular fluorescence image, the muted colors make the Cx43 less apparent.

Clear confocal images, as in Fig. 5B and 5C, show the interactions between cardiomyocytes and macrophages as well as the Cx43 that links them. Green arrows indicate macrophages (fluorescing green), and blue arrows identify cardiomyocytes (depicted by DAPI and the lack of green fluorescence). Red arrows indicate Cx43 (fluorescing red) that can be seen stretching between and around the cells. Fig. 5E–H show how Cx43 can stretch quite far to connect cardiomyocytes to macrophages.

Confocal imaging allows for layered scanning of a sample. This feature permits visual enhancements, such as three-dimensional plots, video clips, and models. The image collected in Fig. 5D is depicted three-dimensionally to show that differences in cell shape and size can be used as another way to differentiate cell lines in coculture. Extracellular structures such as Cx43 can be given dimension, and any cells laying above or below another cell can be revealed (Fig. 5D). Fig. 5E–H show layered images of a cardiomyocyte–macrophage interaction. The images in Fig. 5E–H were taken at 88, 92, 95, and 98 μm from the slide, respectively. In Fig. 5E, closest to the slide, we begin to see the nuclei of the cells (DAPI-blue) as well as Cx43 at the base of a cardiomyocyte. In Fig. 5F and 5G, the Cx43 can be seen stretching between the cardiomyocyte and the macrophage at the top. In Fig. 5H, farthest from the slide, AF488-F4/80 green fluorescence appears, marking the macrophages, and Cx43 disappears.

DISCUSSION

Cocultures of murine HL-1 cardiomyocytes and RAW 264.7 macrophages were used to explore the hypothesis that cytokine signals, IL-10 and TGF-β1, induce the M2 activation state of macrophages (mimicking cardiac resident M2c macrophages), which enhances cardiomyocyte–macrophage interactions and increases the expression of Cx43. Significant increases were seen in Cx43 expression and in di-8-ANEPP fluorescence in coculture wells treated with IL-10 or TGF-β1, providing evidence that these cytokine signals stimulate an increase in Cx43 expression in cardiomyocyte–macrophage couples at points of hemichannel formation. This provides evidence that IL-10 and TGF-β1 would also maintain the macrophages in a resident macrophage (M2c) state in the heart.

SOCS3 inhibited the effects of IL-10 and TGF-β1, as CTCF or fluorescence of coculture wells treated with combinations of IL-10 and SOCS3 or TGF-β1 and SOCS3 showed no significant difference from control wells. Treatment with SOCS3 alone appeared to have no effect on coculture wells, as its CTCF also showed no significant differences from the control wells. These results were expected considering that the balancing role that SOCS3 plays in both the TGF-β signaling pathway and the IL-10 signaling pathway is well known, as shown in Supplemental Fig. 2, and SOCS3 treatment alone would have no effect, as it would not have a cytokine to regulate.

di-8-ANEPPS fluorescence was used to detect depolarization of cardiomyocytes. Controls in each experiment, cardiomyocytes and macrophages, were cultured separately and received the same treatments as the cocultures. Di-8-ANEPPS probes did not fluoresce in macrophage-only cultures. This is expected because macrophages do not depolarize and repolarize when they are not coupled to an excitable cell (5). Cardiomyocytes exhibited a faint fluorescence with di-8-ANEPPS when they were treated and cultured without macrophages. This is expected because HL-1 cardiomyocytes are able to spontaneously depolarize (16). Although they are capable of spontaneous depolarization on their own, it was not surprising that cardiomyocytes showed increased di-8-ANEPPS fluorescence while coupled to macrophages. Fig. 6A shows a Cx43 protein and its subunits. Cx43 is made up of six

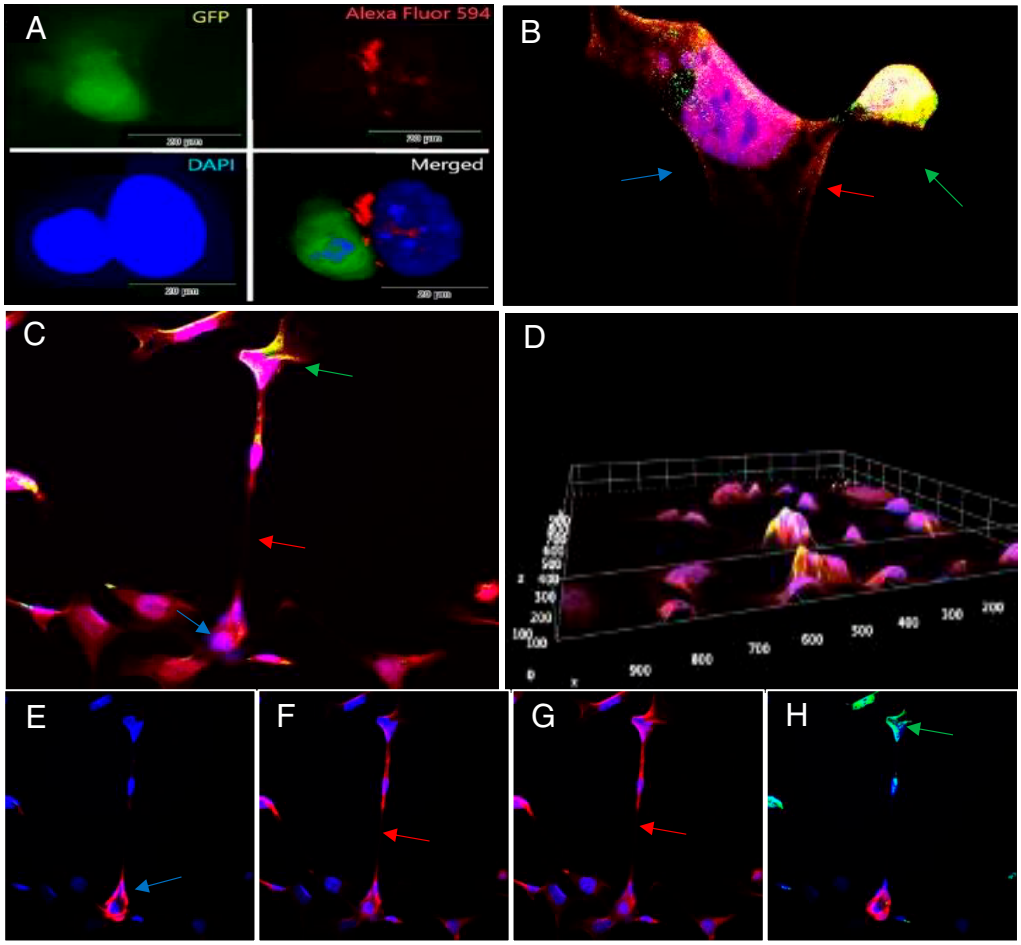


FIGURE 5. Confocal imaging and layering

Macrophages (green arrows) can be seen interacting with cardiomyocytes (blue arrows) via Cx43 (red arrows). (**A**) Image taken at ×500 original magnification on a fluorescence microscope (AccuScope). Macrophage (fluorescing green, AF488-F4/80) and a cardiomyocyte (DAPI-blue with no green fluorescence) can be seen coupled with Cx43 connecting the cells in the center (red, AF594-Cx43). (**B**) Image taken at ×600 original magnification on confocal microscope (FV1000). To be compared with image in (A). Macrophage (fluorescing green, depicted by a green arrow) and a cardiomyocyte (DAPI-blue with no green fluorescence, depicted by a blue arrow) with Cxn43 connecting the cells (red, depicted by a red arrow) can be seen. (**C**) Macrophage (green arrow) fluorescing green/yellow interacting with a cardiomyocyte (blue arrow) via Cx43 (red arrow) fluorescing in red in between the cells. Original magnification ×600. (**D**) Three-dimensional depiction of coculture as it is laying on the slide. Height differences can be seen between cardiomyocytes (flat) and macrophages (tall). Original magnification ×600. (**E**–**H**) Series of images captured at varying depths (88, 92, 95, and 98 μm, respectively) via an FV1000 confocal microscope. Original magnification ×600. This shows the layering capabilities of the confocal microscope and how it can be useful in determining where cells lie in a three-dimensional field. Again, blue arrows point to cardiomyocytes, red arrows point to Cx43, and green arrows point to macrophages.

connexins, each one composed of four transmembrane domains, and the full hexamer is known as a connexon. When two connexons from two different cells are lined up and adhere to each other properly, a hemichannel is formed, as seen in Fig. 6B. The zoomed-in portion of Fig. 6B shows a frontal section of an open Cx43 hemichannel between a coupled macrophage and cardiomyocyte. Fig. 5C shows an autocrine and paracrine system between macrophages and cardiomyocytes where the two cells are coupled via Cx43. The autocrine and paracrine systems shown are thought to keep anti-inflammatory conditions prevalent while also inducing noninflammatory phagocytosis if needed (1, 17).

Confocal imaging was used to further analyze the Cx43 connections between cardiomyocytes and macrophages (Fig. 5). Although the two-dimensional confocal images provided a better view of cell structures and specifically the elucidation of Cx43 by showing a cleaner background with vibrant coloring compared with regular fluorescence microscopy (Fig. 5B, 5C), the three-dimensional images were able to provide new information

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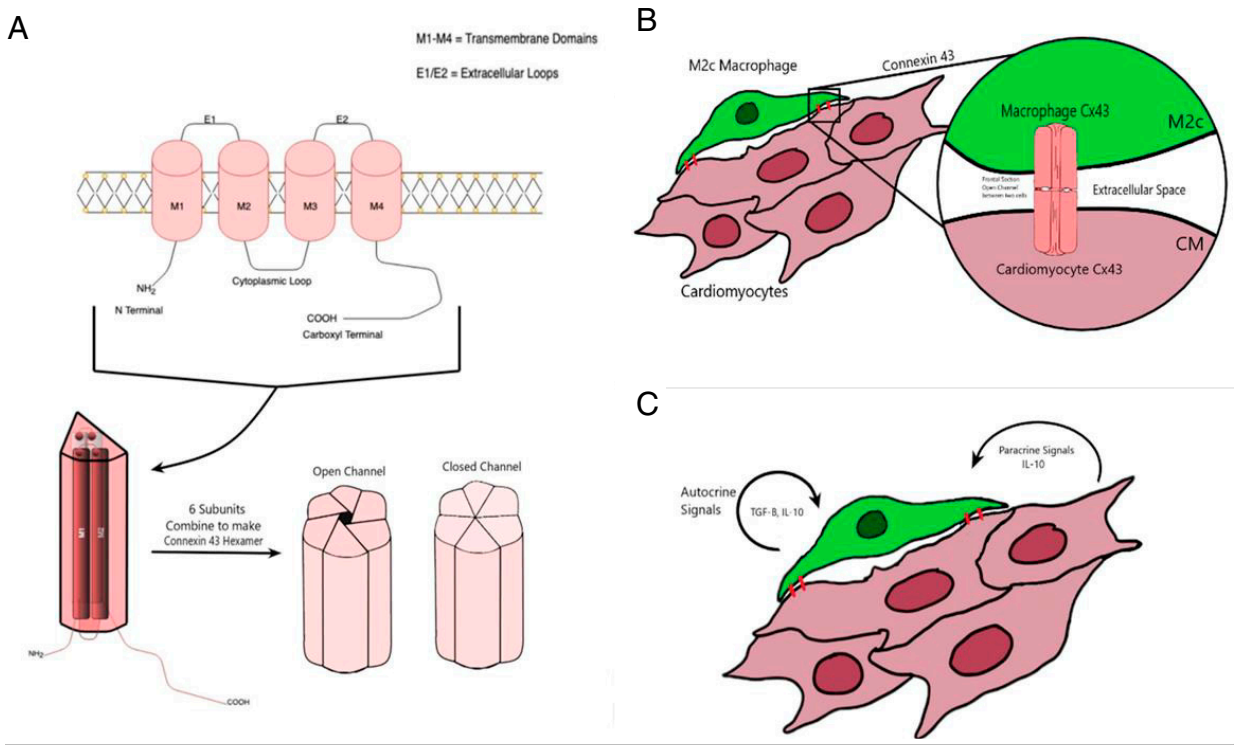

FIGURE 6. Graphic image of Cx43 structure and interactions between cardiomyocytes and macrophages

(**A**) Cx43 protein and its subunits. Cx43 is made up of six connexins, each one composed of four transmembrane domains, and the full hexamer is known as a connexin. (**B**) When two connexons from two different cells are lined up and adhered to each other properly, a hemichannel is formed. The zoomed-in portion shows a frontal section between an open Cx43 hemichannel between a coupled macrophage and cardiomyocyte. (**C**) An autocrine and paracrine system between macrophages and cardiomyocytes where the two cells are coupled via Cx43.

that was unable to be seen in two-dimensional images (Fig. 5D–H). Cells were identified based on their morphology in addition to the inherent fluorescence of the macrophages. It could be seen that macrophages retained their shape in cocultures and exhibited more height than the cardiomyocytes, whereas cardiomyocytes tend to flatten out in coculture (Fig. 5D). The shape of the Cx43 hemichannels was also visible and showed that Cx43 lies flat on the slide with very little height as it stretches from cardiomyocytes and connects to the base of the macrophage cells (Fig. 5D). Finally, with two-dimensional nonconfocal images such as in Fig. 5A, it could be argued that a cardiomyocyte could be hidden below a macrophage, making it appear that Cx43 is interacting between a cardiomyocyte and a macrophage when it is actually just interacting with another cardiomyocyte. The confocal images and three-dimensional images, however, provide clear evidence of cardiomyocytes and macrophages interacting via Cx43 as shown (Fig. 4D–H).

This macrophage and cardiomyocyte coculture provided a system to examine the effects of an M2c macrophage in stimulating fluorescent potentiometric signals on both the macrophage and the cardiomyocyte at points of contact. TGF-β1 and IL-10 appear to be the major stimulating signals for increases in fluorescence of di-8-ANEPPS. Individual treatment or a combination of the two treatments are also shown to significantly increase Cx43 expression. Because both cytokines can be produced by cardiomyocytes, and both signals induce the M2 state of macrophages, it is possible that both cells contribute to enhanced cellular interactions as measured by our experiments showing that TGF-β1 and IL-10 upregulate Cx43 expression and membrane potential in our cocultures. SOCS3 strongly attenuated the effects of TGF-β1 and IL-10 on the di-8-ANEPPS fluorescence as well as the Cx43 fluorescence. In accordance with the role of SOCS3 in the TGF- β pathway and the IL-10 pathway, SOCS3 appears to be modulating TGF-β1 and IL-10 signaling.

Although treatments with IL-10 and TGF-β1 stimulated similar levels of Cx43 upregulation, it is likely that TGF-β1 is better at creating "proper" coupling between the two cell types as described by Hulsmans et al. (5). TGF-β is an important factor in cardiomyocyte coupling with nonmyocytes, as it is highly expressed at all stages of cardiogenesis, where proper cell interactions need to take place prior to heart formation (18, 19). TGF-β is essential for spontaneous Ca^{2+} oscillations in cultured rat cardiomyocytes (20). TGF-β–treated cocultures of rat fibroblasts and rat cardiomyocytes exhibited spontaneous depolarizations compared with cocultures without treatment. Once the coupled fibroblast was killed, the cardiomyocyte did not spontaneously depolarize during the observed period (21). In the current study, TGF-β1 treatments enhanced fluorescence in our cocultures at

points of contact between macrophages and cardiomyocytes, which is dependent on ion exchanges to excite the di-8-ANEPPS probe. The data from our present study using murine cardiomyocytes are similar to rat cardiomyocyte studies that show TGF-β increases spontaneous depolarization events, especially when a nonmyocyte is coupled to the cardiomyocyte (21).

In summary, these experiments have provided evidence that cardiomyocytes and macrophages interact via Cx43 hemichannels and that this interaction results in an increase in membrane potential for the cardiomyocytes. The introduction of IL-10 or TGF-β1 appears to induce the M2c activation state of macrophages, resulting in a more reparative role of the macrophage, further increasing the amount of Cx43 hemichannels and, in turn, increasing change in membrane potential of the cardiomyocytes. In the future, a tri-cell culture system consisting of murine macrophages, cardiomyocytes, and fibroblasts would be useful in studying the formation of cardiac myopathies. Lucas et al. (22) suggest that communication between fibroblasts and other cardiac cells via exosomes may influence myocardial fibrosis and remodeling.

DISCLOSURES

The authors have no financial conflicts of interest.

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REFERENCES

1. Ribeiro-Rodrigues, T. M., T. Martins-Marques, S. Morel, B. R. Kwak, and H. Girão. 2017. Role of connexin 43 in different forms of intercellular communication—gap junctions, extracellular vesicles and tunnelling nanotubes. *J. Cell Sci.* 130: 3619–3630.
2. Weckbach, L. T., A. Curta, S. Bieber, A. Kraechan, J. Brado, J. C. Hellmuth, M. Muenchhoff, C. Scherer, I. Schroeder, M. Irlbeck, et al. 2021. Myocardial inflammation and dysfunction in COVID-19-associated myocardial injury. *Circ. Cardiovasc. Imaging* 14: e012220.
3. Edholm, E.S., R.H. Rhoo, and J. Robert. 2017. Evolutionary aspects of macrophages polarization. *Results Probl. Cell Differ.* 62: 3–22.
4. Martinez, F. O., and S. Gordon. 2014. The M1 and M2 paradigm of macrophage activation: time for reassessment. *F1000Prime Rep.* 6: 13.
5. Hulsmans, M., S. Clauss, L. Xiao, A. D. Aguirre, K. R. King, A. Hanley, W. J. Hucker, E. M. Wülfers, G. Seemann, G. Courties, et al. 2017. Macrophages facilitate electrical conduction in the heart. *Cell* 169: 510–522.e20.
6. Mills, C. D. 2001. Macrophage arginine metabolism to ornithine/urea or nitric oxide/citrulline: a life or death issue. *Crit. Rev. Immunol.* 21: 399–425.
7. Murray, P. J., J. E. Allen, S. K. Biswas, E. A. Fisher, D. W. Gilroy, S. Goerdt, S. Gordon, J. A. Hamilton, L. B. Ivashkiv, T. Lawrence, et al. 2014. Macrophage activation and polarization: nomenclature and experimental guidelines. *Immunity* 41: 14–20.
8. Stein, M., S. Keshav, N. Harris, and S. Gordon. 1992. Interleukin 4 potently enhances murine macrophage mannose receptor activity: a marker of alternative immunologic macrophage activation. *J. Exp. Med.* 176: 287–292.
9. Qin, H., A. T. Holdbrooks, Y. Liu, S. L. Reynolds, L. L. Yanagisawa, and E. N. Benveniste. 2012. SOCS3 deficiency promotes M1 macrophage polarization and inflammation. *J. Immunol.* 189: 3439–3448.
10. Meguro, K., D. Nakagomi, K. Suzuki, J. Hosokawa, T. Fukuta, M. Yokota, Y. Maezawa, A. Suto, and H. Nakajima. 2016. SOCS3 expressed in M2 macrophages attenuates contact hypersensitivity by suppressing MMP-12 production. *J. Invest. Dermatol.* 136: 649–657.
11. Brightbill, H. D., S. E. Plevy, R. L. Modlin, and S. T. Smale. 2000. A prominent role for Sp1 during lipopolysaccharide-mediated induction of the IL-10 promoter in macrophages. *J. Immunol.* 164: 1940–1951.
12. Kaufman, M. R., R. E. Albers, C. Keoni, K. Kulkarni-Datar, D. R. Natale, and T. L. Brown. 2014. Important aspects of placental-specific gene transfer. *Theriogenology* 82: 1043–1048.
13. Albers, R. E., M. R. Kaufman, B. V. Natale, C. Keoni, K. Kulkarni-Datar, S. Min, C. R. Williams, D. R. C. Natale, and T. L. Brown. 2019. Trophoblast-specific expression of Hif-1α results in preeclampsia-like symptoms and fetal growth restriction. *Sci. Rep.* 9: 2742.
14. Judd, J., J. Lovas, and G. N. Huang. 2016. Isolation, culture and transduction of adult mouse cardiomyocytes. *J. Vis. Exp.* (114): 54012.
15. Krombach, F., S. Münzing, A. M. Allmeling, J. T. Gerlach, J. Behr, and M. Dörger. 1997. Cell size of alveolar macrophages: an interspecies comparison. *Environ. Health Perspect.* 105(Suppl 5): 1261–1263.
16. White, S. M., P. E. Constantin, and W. C. Claycomb. 2004. Cardiac physiology at the cellular level: use of cultured HL-1 cardiomyocytes for studies of cardiac muscle cell structure and function. *Am. J. Physiol. Heart Circ. Physiol.* 286: H823–H829.
17. Pinto, A. R., J. W. Godwin, and N. A. Rosenthal. 2014. Macrophages in cardiac homeostasis, injury responses and progenitor cell mobilisation. *Stem Cell Res. (Amst.)* 13(3 Pt B): 705–714.
18. Engelmann, G. L., K. D. Boehm, M. C. Birchenall-Roberts, and F. W. Ruscetti. 1992. Transforming growth factor-beta$_1$ in heart development. *Mech. Dev.* 38: 85–97.
19. Kaur, K., and J. Jalife. 2017. Is TGF-β_1 (transforming growth factor-β_1) an enabler of myofibroblast-cardiomyocyte cross talk? *Circ. Arrhythm. Electrophysiol.* 10: e005289.
20. Neylon, C. B., S. M. Bryant, P. J. Little, and A. Bobik. 1994. Transforming growth factor-β 1 regulates the expression of ryanodine-sensitive Ca^{2+} oscillations in cardiac myocytes. *Biochem. Biophys. Res. Commun.* 204: 678–684.
21. Salvarani, N., A. Maguy, S. A. De Simone, M. Miragoli, F. Jousset, and S. Rohr. 2017. TGF-β_1 (transforming growth factor-β_1) plays a pivotal role in cardiac myofibroblast arrhythmogenicity. *Circ. Arrhythm. Electrophysiol.* 10: e004567.
22. Lucas, T., A. Waisman, R. Ranjan, J. Roes, T. Krieg, W. Müller, A. Roers, and S. A. Eming. 2010. Differential roles of macrophages in diverse phases of skin repair. *J. Immunol.* 184: 3964–3977.