

Supplementary Information for

Topographical modulation of macrophage phenotype by shrink-film multi-scale wrinkles

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Materials and Methods

Material fabrication

The flat, 1D and 2D wrinkled substrates were fabricated from polyethylene (PE) films (Cryovac D-film, LD935, Sealed Air Corporation). Both 1D and 2D wrinkled substrates were treated with oxygen plasma (Plasma Prep II, SPI Supplies) for 5 minutes. The treated PE films were constrained on two opposite sides, and were thermally-shrunk at 150°C for 3 minutes to creating 1D wrinkles. To create the 2D wrinkles, the treated films were shrunk on all sides without any constrain.

Characterization of wrinkles

Both SEM and AFM were performed to characterize the wavelengths and depths, respectively, of the wrinkles. To characterize the wavelengths, the wrinkled substrates were coated with 4 nm gold/palladium (Polaron SC7620), and SEM images were obtained at various magnifications (1,000x to 20,000x) (FEI Quanta 3D FEG). The wavelengths of the wrinkles were calculated by using an in-house developed MATLAB (MathWorks Inc., Natick, MA, USA) code based on fast Fourier transformation. To characterize the depths, AFM was conducted on the wrinkled substrates in tapping mode using silicon tip with a resonant frequency of about 75 kHz and force constant of 3 N/m on a MFP-3D inverted optical microscope (Asylum Research, Santa Barbara, CA). The software used for data acquisition and analysis was IGOR Pro 6.0 (Wavemetrics, Portland, OR).

Cell isolation and culture

All protocols involving animals were approved by University of California Irvine's Institutional Animal Care and Use Committee, which is accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International (AAALACi). Bone marrow cells were isolated from femurs of C57BL/6 mice (Jackson Laboratories) as previously described. Cells were treated with ACK lysis buffer (Invitrogen) to remove red blood cells, centrifuged, and then resuspended and cultured in D10 media composed of high glucose DMEM supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 100 units/mL, 100 µg/mL streptomycin (all from Invitrogen), and 10% conditioned media containing macrophage colony stimulating

factor (M-CSF) produced by CMG 12-14 cells to induce differentiation to bone marrow derived macrophages (BMDMs). BMDMs were maintained at 37°C in a humidified 5% CO₂ incubator. After 7 days of culture, cells were dissociated using Cell Dissociation Buffer (Invitrogen) and seeded on wrinkled substrates. After 4 h of culture to allow adhesion of cells, substrates were transferred to a new culture well with fresh media and then cultured for an additional 24 h.

Cell shape and morphological analysis

Cells were incubated with 10 µM CellTracker Red CMTPX (Invitrogen) in serum-free DMEM at 37 °C in a humidified 5% CO₂ incubator for 45 min, and then transferred to fresh D10 media. After 30 min, the samples were rinsed with sterile PBS before imaging. For analysis of cell morphology, cells were visualized using an Olympus inverted microscope with a 20x or 40x objective. The long axis, or longest length of the cell, and short axis, or the length across the nucleus, of each cell were manually traced and measured using ImageJ software (National Institutes of Health). Elongation factor was calculated as the ratio of the long axis to the short axis.

Immunofluorescence staining and Western blotting

Cells were fixed with 100% cold methanol on ice for 15 min, washed thoroughly with PBS, and then blocked with 5% normal donkey serum (Jackson ImmunoResearch) in PBS overnight at 4°C. Cells were then incubated with goat anti-arginase-I (Santa Cruz

Biotechnology Inc.), washed thoroughly with 1% BSA, incubated with Alexa Fluor-594 donkey anti-goat fluorescent secondary antibodies (Jackson ImmunoResearch) for 1 h, and then counterstained with Hoechst 3342 dye (Invitrogen). Samples were imaged using an Olympus inverted microscope. For Western blot, cells were rinsed in PBS and lysed in RIPA buffer supplemented with 1% protease inhibitor cocktail (both from Sigma Aldrich). Cells were scraped and agitated at 4°C for 20 minutes and centrifuged to pellet cellular components. The supernatant was collected and total protein content was measured by BCA protein assay (Thermo Fisher). Samples were then denatured in a Laemmli sample buffer (Biorad) at 95°C, and separated by standard SDS-PAGE using 7.5% polyacrylamide gels. Proteins were transferred to nitrocellulose membranes and then detected using goat anti-arginase-1, rabbit anti-iNOS (both from Santa Cruz) or mouse anti-tubulin (Sigma) antibodies, followed by HRP-conjugated secondary antibodies (Santa Cruz). HRP substrate solution (Millipore) was applied to the membrane and imaged using a ChemiDoc XRS System (Biorad).

Cytokine secretion

BMDMs were cultured on substrates as described above for 24 h, after which supernatants were collected and analyzed for TNF- α and IL-10 by enzyme-linked immunosorbent assay (ELISA) following the manufacturer's instructions (BioLegend, San Diego, CA).

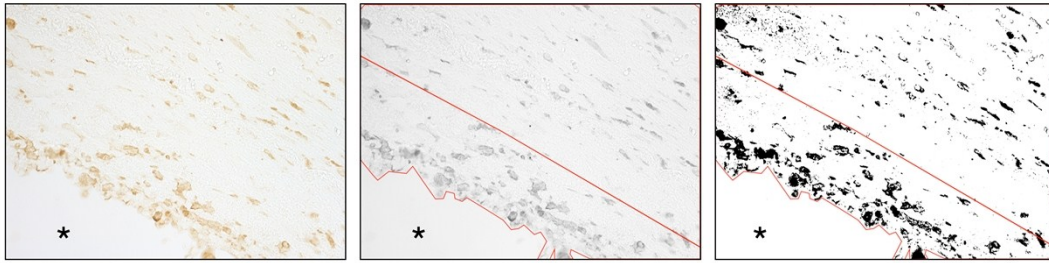
Material implantation and Immunohistochemistry

All protocols involving animals were approved by the Institutional Animal Care and Use Committee at the University of California Irvine prior to initiation of the study. Sterilized wrinkle and flat control materials were subcutaneously implanted in 6-week-old female C57BL/6J mice (Jackson Laboratories) anesthetized by 2% isoflurane inhalation. At 7 days after implantation, mice were euthanized, then skin tissue containing materials were collected and fixed in 10% formalin solution for 24 h at 4°C. Fixed tissues were embedded in paraffin blocks, sectioned and stained with hematoxylin and eosin (H&E) and Masson's trichrome by AML laboratories (Baltimore, MD). For immunohistochemistry, sections were heated to 60°C for 50 min, then deparaffinized and rehydrated using Histo-Clear II (National Diagnostics) and an ethanol gradient before staining. After incubating in target retrieval solution (Dako), dual endogenous enzyme-blocking reagent (Dako), and washing with TBS-A (Tris-buffered saline with 0.1% Triton X-100), the sections were blocked with TBS-B (TBS-A with 2% BSA and 1.5% donkey serum), then incubated with goat anti-arginase-I and goat anti-F4/80 antibodies (both from Santa Cruz Biotechnology Inc.) for 16 h at 4°C, followed by HRP conjugated donkey anti-goat IgG antibodies (Santa Cruz Biotechnology Inc.) for 1 hour. After the avidin-peroxidase reaction was developed using DAB (diaminobenzidine) and DAB plus Chromogen solution (Dako), the sections were counterstained with hematoxylin (Vector Laboratories). Slides were imaged using Nikon Eclipse E800 microscope with a 20x objective and an Olympus camera. Implants were placed in at least three separate animals and representative images are shown.

Statistical analyses

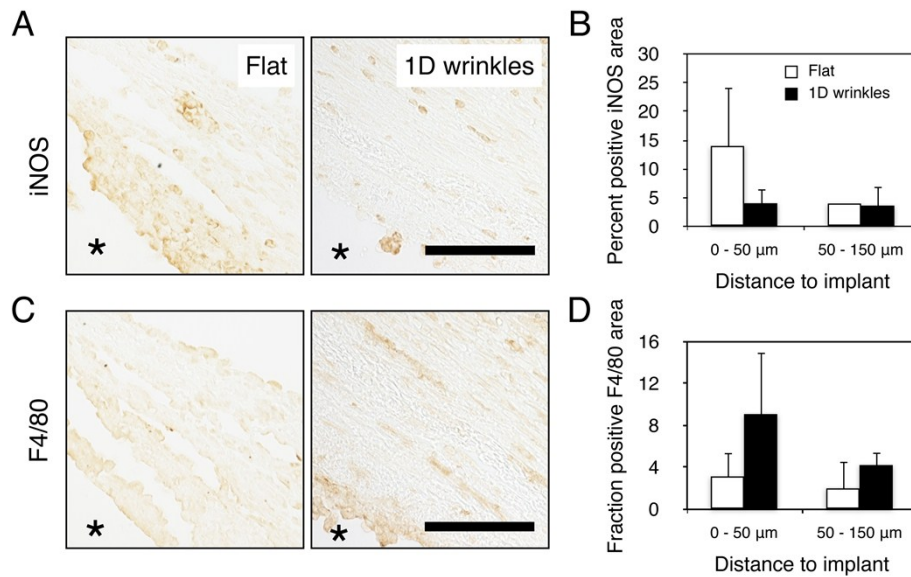
Statistical analysis was performed using Student's t-test or one-way ANOVA with Sidak's post hoc test (multiple comparisons test). $p < 0.05$ was considered statistically significant.

Supplemental Figures



Supplemental Figure 1. Method for quantification of DAB-stained sections.

Original image (left), grayscale image (middle) and thresholded image (right) with 0 – 50 μm and 50 – 150 μm distance from implant regions indicated with red outline. Percent positive area was calculated by the number of positive pixels in the thresholded image divided by the total area within the indicated region, multiplied by 100.



Supplemental Figure 2. Surface wrinkles modulates iNOS and F4/80 expression.

Representative iNOS (A) and F4/80 (C) stained images of flat and 1D wrinkle surface implants at 7 days after subcutaneous implantation in C57BL/6J mice. Asterisks denotes

location of material. Quantification of percent positive iNOS (B) and F4/80 (D) area at indicated distances from the implant. Mean $\pm$ SD of n = 2, scale bar = 50 μ m.