Supplement to

MMP-10 Regulates Collagenolytic Activity of Alternatively Activated Resident Macrophages

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Materials and Methods

Macrophage Chemotaxis. BMDM were labeled with Calcein AM (5 μg/ml; Molecular Probes, Eugene, OR) for 1 h at 37°C, washed, and suspended in phenol red-free RPMI with 1% BSA at 10^6 cells/ml. Wells of 96-well ChemoTx plates (Neuro probe, Gaithersburg, MD) were filled with 30 μl of either PBS, 10% FBS, or wound samples homogenates (20 μl/mg tissue in deionized water). An 8-μm pore polycarbonate filter was inserted into each well, and 25 μl (25 x 10^4 cells) Calcein-labeled macrophages were added on top and incubated for 2 h at 37°C. After removing the filter, migration of macrophage through the filter (i.e., bottom) was measured in a fluorescence plate reader (excitation 485 nm, emission 530 nm).

Immunoblotting. Tissue samples were homogenized in cell lytic buffer (Sigma), and protein concentration was assessed using BCA kit (Pierce, Rockford, IL). Homogenates (30 μg protein) were solubilized in 1x NuPage LDS sample and reducing buffer, resolved by SDS-PAGE, and transferred to Immobilon™-P PVDF membranes (Sigma). Blots were blocked in 5% skim-milk and incubated with mouse anti-αSMA IgG (Sigma; 1:10000) or rabbit polyclonal anti-mannose receptor (MRC1; Abcam, Cambridge, MA; 1:1000) overnight at 4°C. Bound antibodies were visualized using horseradish peroxidase-linked secondary antibody (1:4000), followed by detection using SuperSignal West Femto Chemiluminescent substrate (Thermo Scientific, Rockford, IL) according to the manufacturer's instructions.

Fibroblast Culture. Adult mice were euthanized, and skin around underarms was shaved and dissected under aseptic conditions. The skin was cut into 1-mm^2 pieces, incubated in 1 mg/ml bacterial collagenase (Life Technologies, Grand Island, NY) for 30 min at 37°C, washed in PBS, and plated in DMEM with 10% FBS and 1% penicillin/streptavidin. After 7-10 days, by which time the cultures were visibly confluent, the cells were passed. Cells were used at passage 3 or 4.

Macrophage Conditioned Medium. BMDM were activated into M1 or M2 cells with LPS or IL4/IL13, respectively, as described in the main text or were left untreated in Mac medium (M0 macrophages). After 6 h, media were removed, and the cultures were washed and incubated in RPMI with 0.25% BSA and 1% penicillin/streptavidin for 24 h. Media were collected, centrifuged, and stored at -80˚C.

Cell Proliferation Assay. Dermal fibroblasts were plated at 10,000 cells/well in DMEM in 96-well plates. After 6 h, medium was removed, and cells were treated with macrophage-conditioned media at diluted 1:1 with DMEM. Control cells received 1:1 serum-free RPMI/DMEM. After 48 h cell proliferation was assessed by determining the numbers of viable cells using a WST-1 assay (Roche Diagnostics, Indianapolis IN). The plate was read at 450 nm with a reference reading at 630 nm.

Collagen Internalization. Fibroblasts were serum starved overnight, then incubated with DQ collagen (25 μg/ml) for 24 h. Cells were washed and incubated with 0.4% trypan blue for 3 min to quench extracellular fluorescent dye. Then cells were trypsinized, washed in PBS, and suspended in PBS with 10% FBS. Internalized fluorescence was measured by flow cytometry using a GuavaPCA system (Guava Technologies, Hayward, CA), and the data analyzed with FlowJo software (TreeStar, Ashland, OR).

Flow cytometry. Skin samples (2.5 x 2.5 cm) from the shaved backs of wildtype and *Mmp10*$^{-/-}$ mice were minced and digested in RPMI with 0.5 mg/ml Liberase TM (Roche, Indianapolis, IN) at 37°C for 30 min. Samples were triturated by repeated pipetting, filtered through 40-μm filter, and centrifuged gently. The cell pellets were washed twice and suspended in PBS to 1 x 10^6 cells/ml, and the cells were stained with Fixable Viability Dye eFluor 780 (eBioscience), allophycocyanin-conjugated anti-F4/80 (clone BM8, eBioscience), and R-phycoerythrin-indotricarbocyanine-conjugated anti-CD45.2 (clone 104, BioLegend). Cells were analyzed on an LSRII with FACSDiva Software (BD Bioscience, San Jose, CA) and analyzed with FlowJo software.

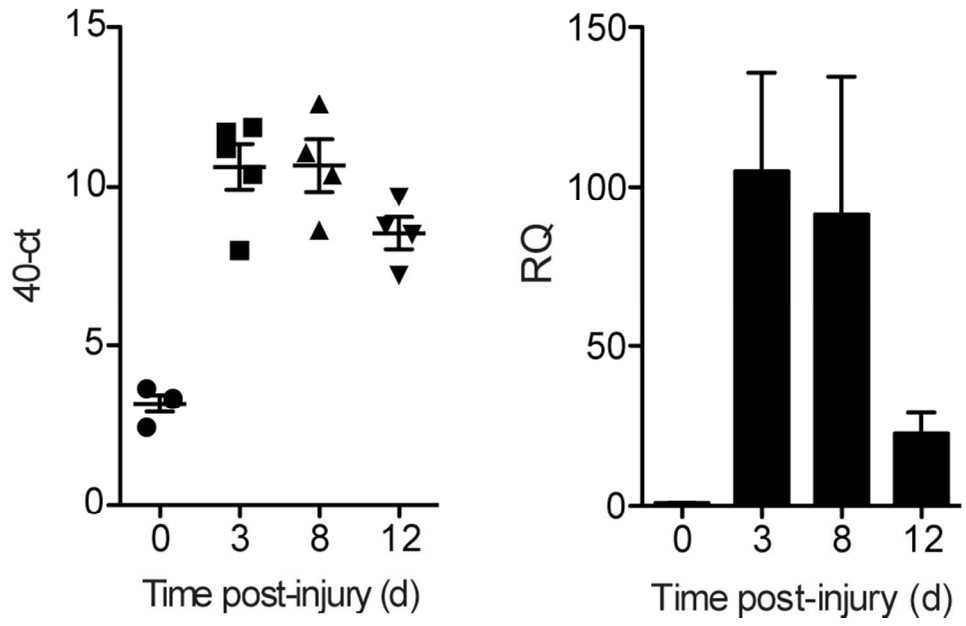

Figure S1. MMP-10 is Induced in Response to Skin Injury.

Total RNA was isolated from uninjured skin or wounds on days 3, 8 and 12 post-injury from wildtype mice and reverse transcribed to cDNA. *Mmp10* mRNA was quantified by qRT-PCR. Data are shown both as 40-Ct and relative quantification (RQ) normalized to *Hprt* mRNA (n = 4 mice/time point).

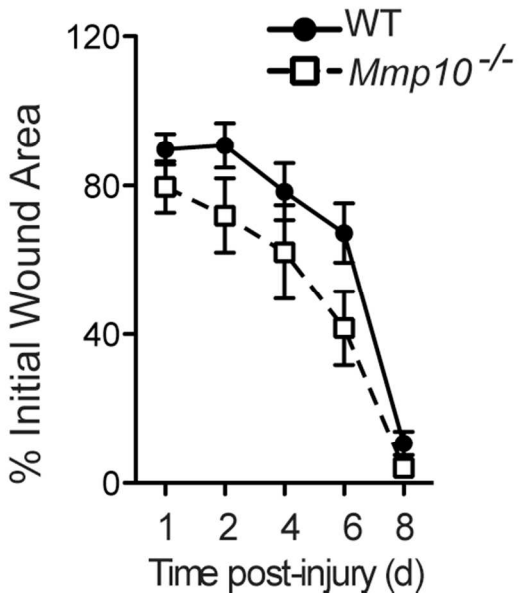

Figure S2. MMP-10 does not Significantly Impact Wound Closure.

Wound sites were photographed at indicated days. The wound size was measured using ImageJ (http://imagej.nih.gov/ij/), and the average of 4 wounds from each mouse was quantified. Wound closure was measured compared to the original wound area (n = 4 mice/genotype/time point).

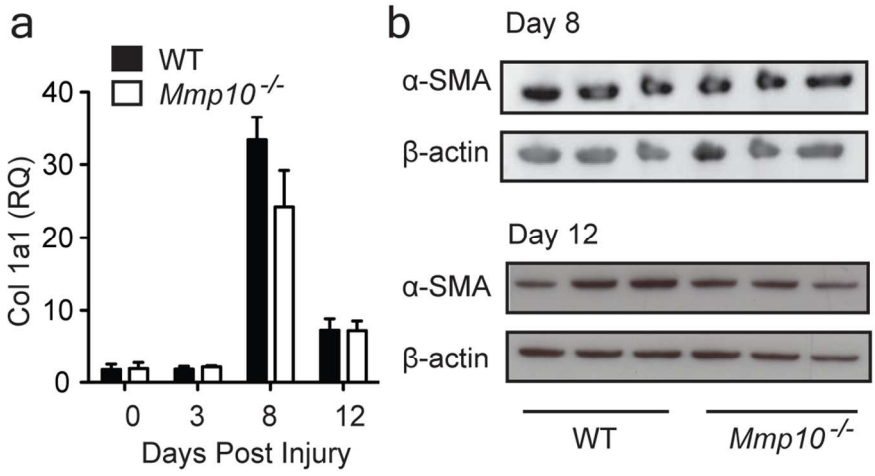

Figure S3. MMP-10 does not Influence Levels of Type I Collagen mRNA or α-SMA Protein in Skin Wounds.

(a) *Col1a1* mRNA levels were quantified by qRT-PCR using total RNA from wounds collected at the indicated days post injury (n = 4-6/genotype).

(b) Wound samples were collected at days 8 and 12 post-injury and homogenized. α-SMA levels were assessed by immunoblotting. β-actin was used as the loading control (n = 3/genotype).

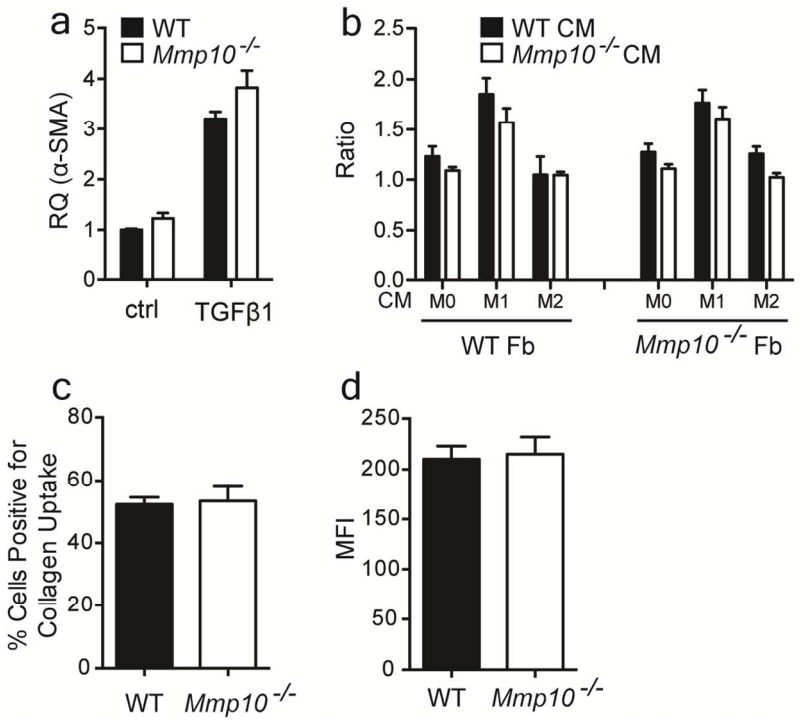

Figure S4. Similar Behavior of Dermal Fibroblasts from Wildtype and *Mmp10*$^{-/-}$ Mice.

(a) Primary dermal fibroblasts from wildtype and *Mmp10*$^{-/-}$ mice were treated with 10 ng/ml TGFβ1 (Cell Signaling Technology, Danvers, MA) for 6 h, then total RNA was extracted for determination of αSMA (*Acta2*) mRNA by qRT-PCR (n = 3/genotype).

(b) Fibroblasts (Fb) from wildtype and *Mmp10*$^{-/-}$ mice were treated for 48 h with medium conditioned (CM) by M0 or M1- or M2-differentiated macrophages, and numbers of viable cells were measured using a colorimetric assay (WST-1). Data were normalized to wildtype fibroblasts treated with serum free RPMI. The assay was done in triplicate for each line of fibroblasts; n = 9 wildtype and 6 *Mmp10*$^{-/-}$ fibroblast lines. Media conditioned by M0 and M2 macrophages did not affect fibroblast proliferation. Media from M1 macrophages modestly stimulated fibroblast proliferation but this response did not differ between genotypes.

(c,d) The ability to internalize type I collagen did not differ between fibroblasts from wildtype and *Mmp10*$^{-/-}$ mice. Data shown are (c) percent of the population that was positive for internalized collagen and (d) the mean fluorescence intensity per positive cell (n=3/ genotype).

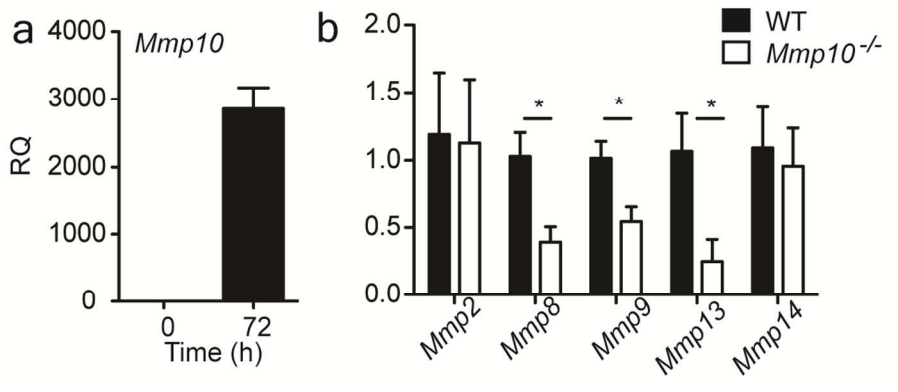

Figure S5. Expression of *Mmp* mRNAs in Cultured Skin Explants.

(a) Wildtype skin explants were harvested and cultured for 72 h. Total RNA was extracted from homogenized samples, and *Mmp10* mRNA levels were quantified by qRT-PCR normalized to *Hprt* mRNA levels. Data are presented as ratio compared to WT samples (n = 6).

(b) Wildtype (WT) and *Mmp10*$^{-/-}$ explants were harvested and cultured for 72 h, and the expression levels of the indicated *Mmp* mRNAs was measured. Data are presented as ratio compared to wildtype samples and normalized to *Hprt* expression (n = 3/genotype); **p* <0.05.

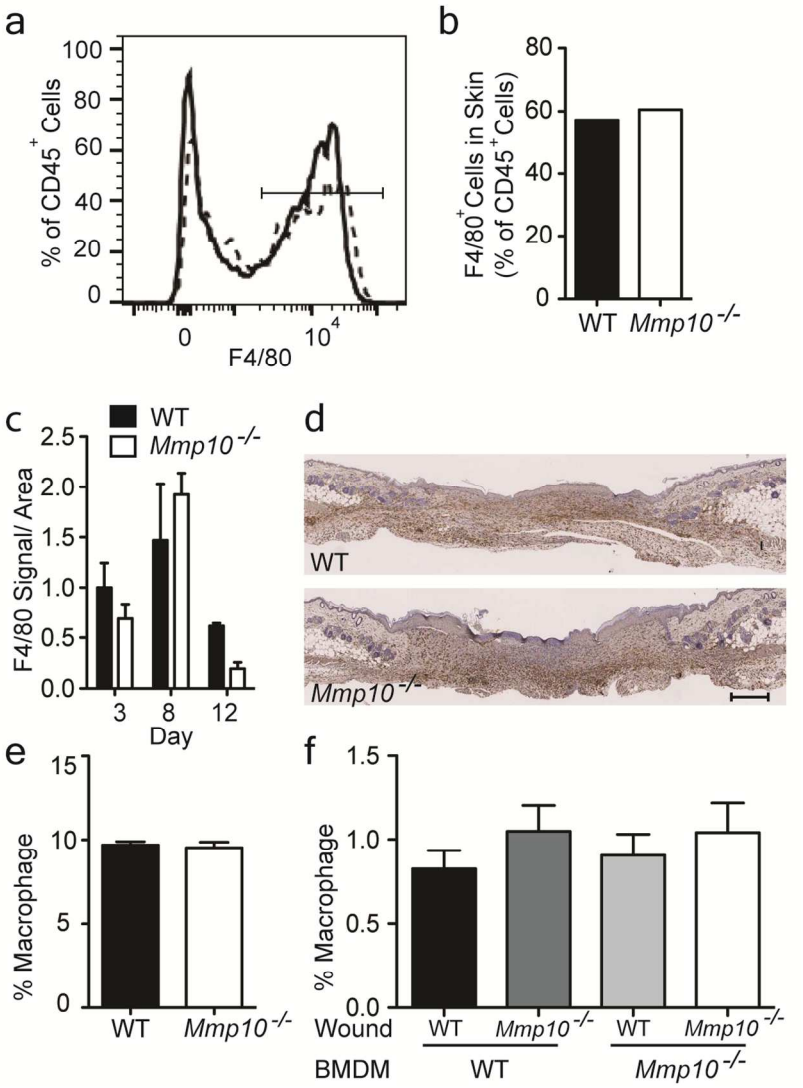

Figure S6. Equivalent Numbers of F4/80$^+$ Macrophages in Normal (Unwounded) Wildtype and *Mmp10*$^{-/-}$ Skin; MMP-10 does not Influence Macrophage Influx into Wounds.

(a,b) Flow cytometric analysis of F4/80$^+$ cells in intact skin. (a) Histograms of the percent of F4/80$^+$ cells (a pan macrophage marker; gate indicated by horizontal line) within total CD45$^+$ live cells (doublet cells excluded) isolated from intact skin. Solid line: *Mmp10*$^{-/-}$; dashed line: wildtype. (b) Quantification of F4/80$^+$ cells as a percent of total CD45$^+$ cells.

(c,d) Sections of day-3, -8 and -12 wounds were stained with anti-F4/80. Total area of F4/80-signal was quantified and normalized to total tissue area. Background values for control sections (i.e., no primary antibody) for each sample were subtracted. (d) Representative images of F4/80-stained day-8 wounds. Scale bar = 500 μm.

(e,f) Chemotactic ability of wildtype and *Mmp10*$^{-/-}$ BMDM to (e) 10% non-heat inactivated FBS or (f) homogenates of day-8 wound samples. Data are the percent of macrophages that migrated to the lower well (n = 3/genotype).

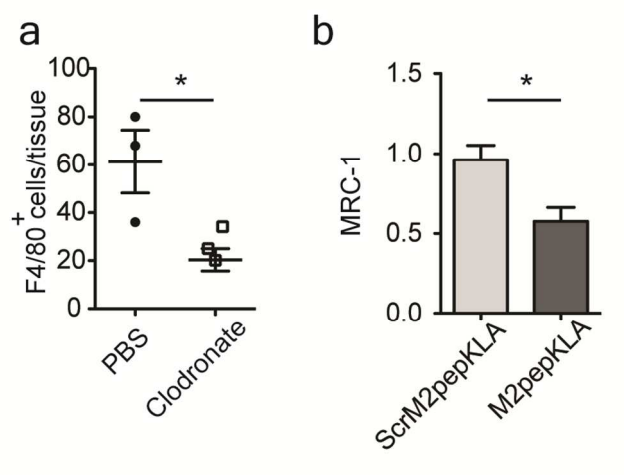

Figure S7. Depletion of Total Macrophages with Clodronate-liposomes and Depletion of M2-polarized Macrophages with a Targeted Pro-apoptotic Peptide.

(a) Skin explants treated with PBS- or clodronate-liposomes were harvested after 72 h in culture, fixed in 4% paraformaldehyde, and embedded in paraffin. Skin sections were immunostained with anti-F4/80 antibody. Number of positive cells per section was quantified (n = 3/genotype).

(b) Skin samples were treated with 20 μg/ml control peptide Src-M2-pep-KLA or pro-apoptotic peptide M2-pep-KLA and harvested 72 h later. Tissues were homogenized in lysis buffer, and equal amounts of protein lysate (30 μg) were resolved by SDS-PAGE and transferred to PVDF membranes. Immnuoblotting of M2-biased macrophages was done using anti-mannose receptor (MRC1) antibody. β-actin was used as the loading control. Immunoblot signal intensities were quantified using ImageJ. Data are presented as signal for MRC-1 normalized to β-actin (n = 3/genotype, * $p<0.05$).

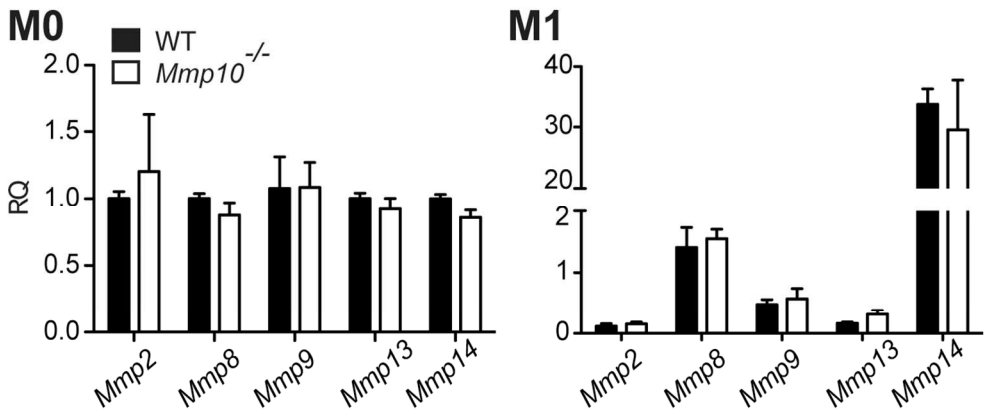

Figure S8. Expression Patterns of Collagenolytic MMPs in Naïve (M0) and M1-differentiated Macrophages do not Differ between Cells from Wildtype and *Mmp10*$^{-/-}$ Mice.

Bone marrow cells from wildtype and *Mmp10*$^{-/-}$ mice were cultured for 7 days in Mac medium to differentiate them into macrophages (M0). For M1 polarization cells were cultured with 100 ng/ml *E. coli* LPS for 6 h, washed, and cultured for another 24 h. RNA was isolated, and the expression levels of the indicated *Mmp* mRNAs was measured by qRT-PCR. Data are presented as ratio compared to wildtype samples and normalized to *Hprt* expression (n = 3/genotype).

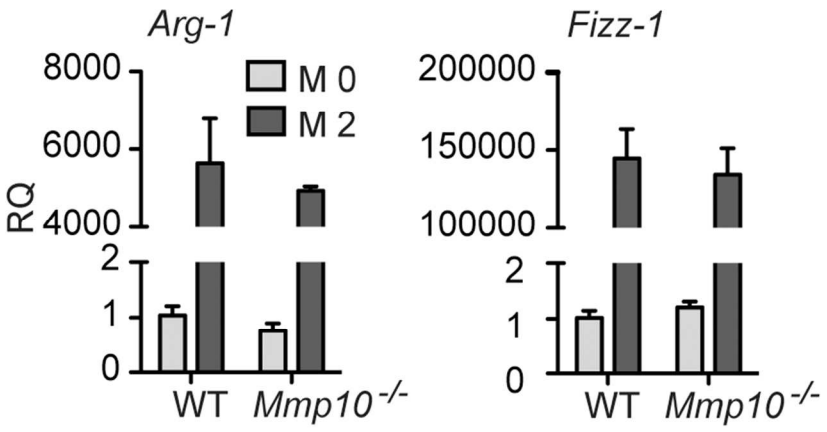

Figure S9. Equivalent Activation of M2 Markers in Wildtype and *Mmp10*$^{-/-}$ Macrophages.

BMDM from wildtype and *Mmp10*$^{-/-}$ mice were differentiated with IL4 and IL13 (10 ng/ml each) to differentiate them into M2 macrophages. After 48 h, cells were harvested, and levels of mRNA for *Arg-1* and *Fizz-1* (markers of M2 macrophages) were analyzed by qRT-PCR. Data are normalized to naïve cells from wildtype mice (n = 4 wildtype, 5 *Mmp10*$^{-/-}$).

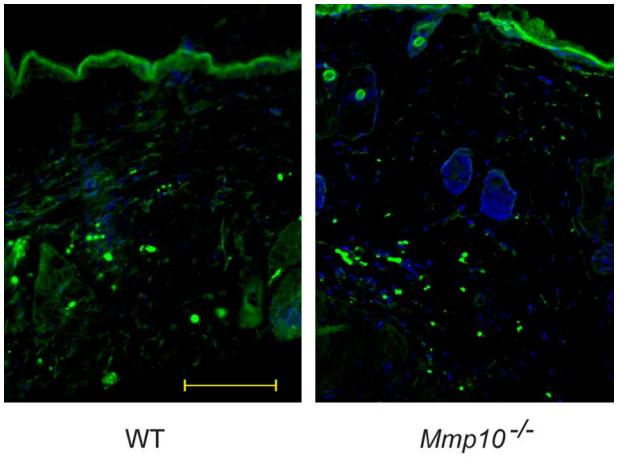

Figure S10. Influx of EGFP^{+} Macrophages into Wildtype and *Mmp10*$^{-/-}$ Wounds.

EGFP^{+} BMDM were isolated from wildtype MacGreen mice and were injected retro-orbitally into wildtype and *Mmp10*$^{-/-}$ mice on day 10 post-wounding. Wounds were excised on day 12. Presence of EGFP^{+} cells in wounds of both genotypes was confirmed by fluorescence microscopy of frozen sections. Scale bar= 100 μm