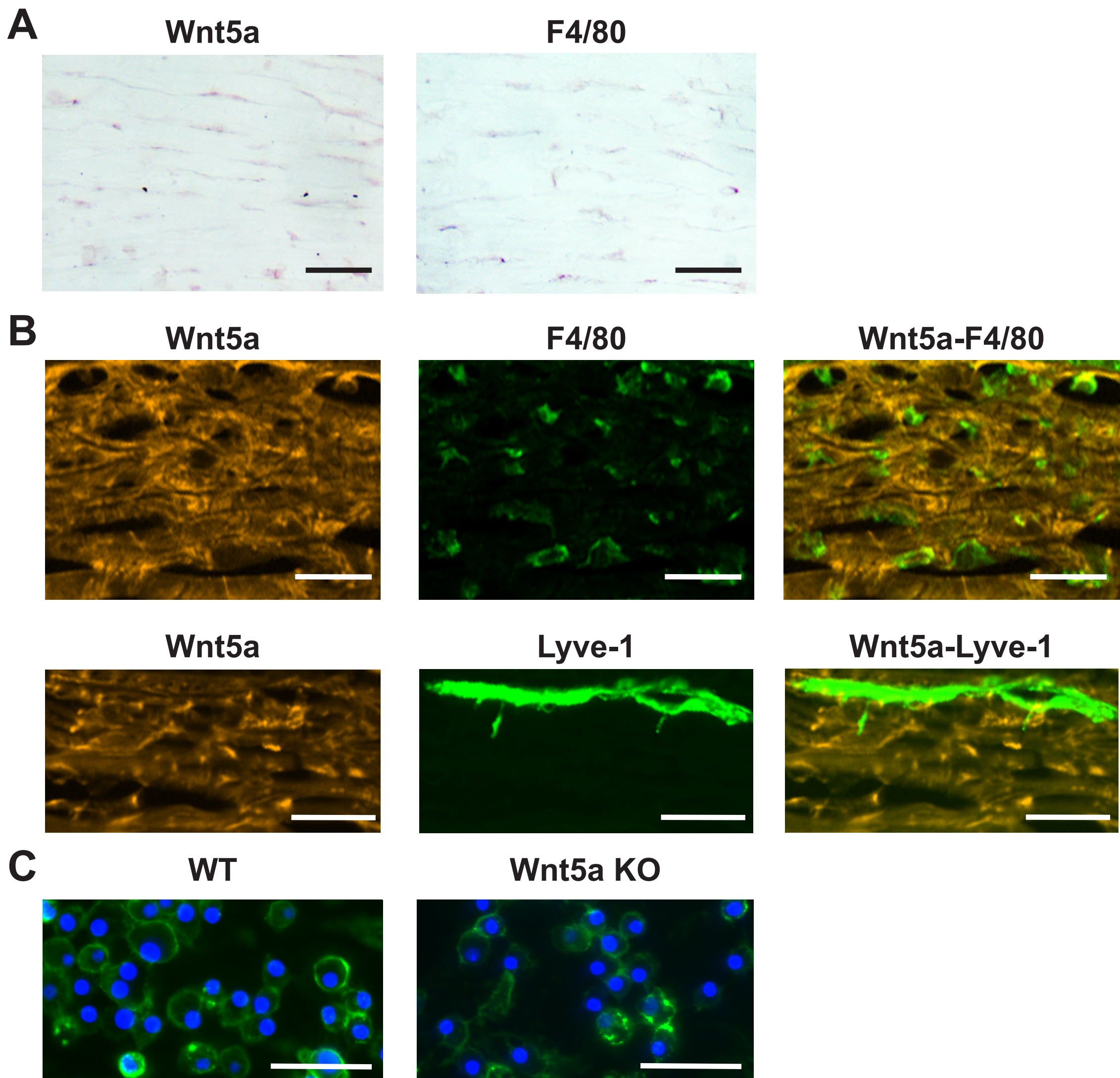

Supplementary Figure 1. Sessa et al.

Supplementary information, Figure S1 (**A**) Representative cross-sectional images of in situ hybridization analysis showing similar expression pattern of Wnt5a and F4/80 transcripts in inflamed corneal stroma. Scale bar: 50 μm. (**B**) Representative cross-sectional images of immunofluorescent microscopic analysis on Wnt5a, F4/80 and LYVE-1 expression in inflamed corneal stroma. Wnt5a distribution was observed on and around F4/80^{+} cells and LYVE-1^{+} lymphatic vessels. Scale bar: 50 μm. (**C**) Representative images of immunocytofluorescent microscopic analysis on CD11b^{+} (green) cells 10 days after bone marrow differentiated macrophage culture with GM-CSF. Fewer cells were observed from *Wnt5a* KO compared to WT. Blue: DAPI nuclear staining. Scale bar: 50 μm.

Supplementary information, Data S1 Materials and Methods

Animals

Mice were treated according to ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and the protocols were approved by the Animal Care and Use Committee of the institutes. Mice were anesthetized using a mixture of ketamine, xylazine, and acepromazine (50 mg, 10 mg, and 1 mg/kg body weight, respectively) for each surgical procedure. *Wntless* (*Wls*) or *Wnt5a* gene was targeted for conditional deletion in murine monocytes[1,2]. The transgenic mice were generated by cross-breeding *Csf1R-cre* and *Wls-flox* or *Wnt5a-flox* mice[3-5].

Corneal Inflammatory Lymphangiogenesis

The standard suture placement model was used to induce corneal inflammatory LG, as previously described[6,7]. Briefly, three interrupted sutures (11-0 nylon, Arosurgical, Newport Beach, CA) were placed into corneal stroma without penetrating into the anterior chamber.

Immunofluorescent Microscopic Assay and Quantification

The experiments were performed as previously described[6,7]. For whole-mount corneal staining, full thickness tissues one or two weeks after suture placement were harvested, fixed in acetone, and stained with rabbit-anti-mouse F4/80 or LYVE-1 antibodies (Abcam, Cambridge, UK) for macrophages and lymphatic vessels, respectively. Samples were processed with Vectashield mounting medium (Vector Laboratories, Burlingame, CA) and examined by an AxioImager M1 epifluorescence deconvolution microscope (Carl Zeiss AG, Germany) or an upright Fluoview FV1000 confocal microscope (Olympus, Japan). Digital images were evaluated by NIH Image J software for lymphatic invasion area and macrophage infiltration. The percentage scores of lymphatic coverage areas (n = 6 and n = 7 for two experiments on *Wls* KO and *wnt5a* KO, respectively) and macrophage numbers (n = 3 for two experiments on *Wnt5a* KO) were obtained by normalizing to control groups where the values were defined as being 100%. Mann-Whitney test was used for the determination of significance levels in LG between different groups. For cross-section staining, sutured eyeballs (n = 3) two weeks after suture placement were harvested, fixed in 4% paraformaldehyde, and embedded in Optimal Cutting Temperature (OCT) compound. Sections of 14 μm thickness were

stained with the specific antibodies against F4/80 (Abcam), LYVE-1 (Abcam), or Wnt5a (R&D Systems, Minneapolis, MN), respectively. Samples were processed with Vectashield mounting medium and examined by the AxioImager M1 epifluorescence deconvolution microscope (Carl Zeiss AG).

In Situ Hybridization

The experiment was performed as previously described[8]. Briefly, the hybridization was performed using digoxigenin-labeled probes and detected with an alkaline phosphatase-conjugated anti-digoxigenin antibody and BCIP/NBT substrates (n=3). Two probes were used for both Wnt5a and F4/80 on 14 µm frozen sections of the corneas two weeks after suture placement. The sets of oligos used to PCR amplify the templates for *Wnt5a* were the following: Wnt5a primer set 1: 5' – TGCAGGTGCTCTGGTACAAA – 3' and 5' – CTGAACATGGCTCCTCGTGT – 3'; Wnt5a primer set 2: 5' – AAAGGGAACGAATCCACGCT – 3', and 5' – TCTCCGTGCACTTCTTGCAT – 3'. The sets of oligos used to PCR amplify the templates for the F4/80 probes were the following: F4/80 primer set 1: 5' – AGCTGTAACCGGATGGCAAA – 3' and 5' – GGGGCCAATCTGGAAAATGC – 3'. F4/80 primer set 2: 5' – GCCTGGACGAATCCTGTGAA – 3' and 5' – CTGGGGCCCCTGTAGATACT – 3'. A T7 promoter sequence was added to the 5' end of the reverse oligo for each probe. For both Wnt5a and F4/80, the two probes targeted non-overlapping regions and were combined.

Real-Time PCR

The experiments were performed as previously decribed[6,7]. Total RNA was extracted and purified from mouse corneal stroma harvested two weeks after suture placement or macrophages in culture with TRIzol Reagent (Life Technology, Thermo Fisher Scientific, Waltham, MA). Reverse transcription was performed using the iScript Reverse Transcription Supermix for RT-PCR (Bio-Rad Laboratories, Inc., Hercules, CA). Real-time PCR was performed using Fast SYBR Green Master Mix (Applied Biosystem, Thermo Fisher Scientific, Waltham, MA) with the CFX96 sequence detection system (Bio-Rad Laboratories, Inc., Hercules, CA). Gene relative expression was calculated from the Δ-Ct (threshold cycle) of the targeted gene normalized to the Δ-Ct of GAPDH. Primers sequence were as follow: GAPDH: 5' – AACAGCAACTCCCACTCTTC – 3' and 5' – CTGTTGCTGTAGCCGTATTC – 3'; Wnt5a: 5' – CGCTAGAGAAAGGGAACGAATC – 3' and 5' – CTCCATGACACTTACAGGCTAC – 3'; Arg 1: 5' – GGAATCTGCATGGGCAACCTGTGT – 3' and 5' – AGGGTCTACGTCTCGCAAGCCA – 3'; VEGF-C 5' – GGGAAGAAGTTCCACCATCA – 3' and 5' – ATGTGGCCTTTTCCAATACG – 3'; TNF*α* 5' – CATCTTCTCAAAATTCGAGTGACAA– 3' and 5' – TGGGAGTAGACAAGGTACAACCC– 3'. Experiments were repeated twice (n = 4 for Figure panels C and F and n = 3 for Figure panel M).

Flow Cytometry

Peritoneal cell extract was obtained using the standard protocol[9]. Briefly, peritoneal cavities were washed with ice-cold RPMI-1640 supplemented with 10% FCS, and cells

extracted were treated with red blood cell lysis solution, blocked for Fc receptors (CD16/32, BD Bioscience, Franklin Lakes, NJ), and incubated with antibodies against F4/80-Cy5.5 PerCP (Life Technologies, Thermo Fisher Scientific, Waltham, MA). Data were acquired using a Guava easyCyte HT cytometer (Millipore, Billerica, MA) and the InCyte 2.6 software (Millipore). Experiments (n = 3) were repeated three times.

Isolation and Culture of Bone Marrow Derived Macrophages

Bone marrow derived macrophages were isolated and differentiated using the standard protocol[9]. Briefly, macrophages were derived from total bone marrow isolation, and cultured in RPMI-1640 supplemented with 10% FCS and GM-CSF (10 ng/ml, PeptroTech, Rocky Hill, NJ) for 10 days. Activation of *in vitro* differentiated macrophages was performed with INF-γ (30 ng/ml, R&D System) as pre-treatment for 6 hours, followed by LPS (100 ng/ml, Invitrogen, Thermo Fisher Scientific) overnight for M1 activation and IL-4 (20 ng/ml, R&D System) overnight for M2 activation, as previously described[10].

Immunocytofluorescent Microscopic Assay

In vitro differentiated macrophages were immunostained as previously reported[6,7]. Briefly, cells were seeded on culture slide chamber (BD Bioscience, San Jose, CA) and stained with specific antibodies against F4/80 (Abcam) or CD11b (BD Bioscience), respectively. Samples were mounted with Vectashield mounting medium with DAPI (Vector Laboratories), and digital images were taken with an epifluorescence microscope (Zeiss Axioplan 2; Carl Zeiss). Macrophage coverage was analyzed by NIH Image J

software by normalizing to control condition where the values were defined as being 100%. Experiments (n = 3) were repeated three times.

Cytokine Antibody Array and Analysis

Cultured macrophages were analyzed with a mouse cytokine antibody array (L-Series, RayBiotech, Inc., Norcross, GA) according to the manufacturer's instructions. Briefly, the cytokine array was blocked with blocking buffer, and incubated with the pre-biotin labelled proteins from macrophage supernatant. Cy3-conjugated streptavidin antibody mix was applied to the arrays and reading of the fluorescence signal was performed using the GenePix 4000B (Molecular Devices, Sunnyvale, CA). Data were processed for background subtraction and normalization by positive spots on the reference sub-array. Linear mixed model was used to analyze VEGF-C expression (n = 3, experiments repeated twice), and the intensity ratios of KO compared to WT were calculated to evaluate the reduced ratios.

Statistical Analysis

The results are reported as mean ±S.D. and Student *t*-test was used for the determination of significance levels between different groups using Prism software (GraphPad, La Jolla, CA) unless otherwise indicated. The differences were considered statistically significant when $p < 0.05$.

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