Materials and Methods

Evaluation of ccl24 and ccr3 expression in patients with SSc

Measurement of CCL24/Eotaxin 2 Level in Serum

CCL24 serum concentration in healthy volunteers (n=23), diffuse systemic sclerosis (n=27) and limited systemic sclerosis patients (n=10) was measured using a commercial Elisa kit. SSc patients, classified according to ACR/EULAR criteria, from the Internal Medicine Department H, Meir Medical Center, Israel, were consecutively enrolled and blood samples were collected. Whole blood samples were centrifuged at 3000 rpm for 15 minutes. Serum samples were frozen at -80°C. CCL24 levels were determined in the sera of SSc patients and healthy donors using Human CCL24 ELISA Kit (AB100509, Abcam) according to the manufacturer's protocol. All patients were submitted to routine clinical investigations and a complete clinical history, including full biochemical and hematological parameters, was obtained. Study protocol was approved by the Ethics Committee according to guidelines of the 1975 Declaration of Helsinki and all patients gave their written informed consent to the study.

Immunofluorescence on skin sections from early diffuse SSc patients and healthy controls

For evaluation of CCR3 and CCL24 expression in skin tissue, biopsies from the affected skin of the forearm were collected from 5 early diffuse cutaneous SSc patients at the Division of Rheumatology, University of Florence, Italy. Forearm skin biopsies from 5 age- and sex-matched healthy individuals were used as controls. Skin tissue sections (5 µm thick) were deparaffinized, rehydrated and boiled for 10 minutes in sodium citrate buffer (10 mM, pH 6.0). Sections were washed in PBS, incubated in 2 mg/ml glycine for 10 minutes to quench autofluorescence caused by free aldehydes and then blocked for 1 hour at room temperature with 1% bovine serum albumin (BSA) in PBS. The sections were then incubated overnight at 4°C with the following primary antibodies diluted in PBS with 1% BSA: mouse monoclonal anti-human CCL24 (1:100 dilution; Chemomab,

Israel), rabbit polyclonal anti-human CD31/platelet-endothelial cell adhesion molecule-1 (PECAM-1; 1:50 dilution; catalogue number ab28364; Abcam, Cambridge, UK), rat monoclonal anti-human CCR3 (1:100 dilution; Clone 61828; R&D Systems, Minneapolis, MN, USA) and rabbit polyclonal anti-α-smooth muscle actin (a-SMA; 1:100 dilution; catalogue number ab5694; Abcam). Alexa Fluor-488 conjugated goat anti-mouse/rat IgG or Rhodamine Red-X-conjugated goat anti-rabbit IgG (Invitrogen, San Diego, CA, USA) diluted 1:200 in PBS with 1% BSA were used as secondary antibodies. Irrelevant isotype-matched and concentration-matched mouse/rat and rabbit IgG (Sigma-Aldrich, St. Louis, MO, USA) were used as negative controls. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI; Chemicon International, Temecula, CA, USA). Skin sections were then mounted with an antifade aqueous mounting medium (Biomeda Gel Mount; Electron Microscopy Sciences, Foster City, CA, USA) and examined with a Leica DM4000 B microscope equipped with fully automated fluorescence axes (Leica Microsystems, Mannheim, Germany). Fluorescence images were captured with a Leica DFC310 FX 1.4-megapixel digital color camera equipped with the Leica software application suite LAS V3.8 (Leica Microsystems). For quantitative analyses, CCL24-positive cells were counted in 10 randomly chosen microscopic high-power fields (hpf; 40× original magnification) of the dermis (excluding skin adnexal structures) per sample. Only the cells with well-defined nuclei were counted. Counting was performed blindly with regard to the skin biopsy classification. Densitometric analysis of the intensity of CCR3 immunofluorescent staining was performed on digitized images using the ImageJ software (NIH, Bethesda, MD, USA; online at http://rsbweb.nih.gov/ij).

Patient Involvement

This research was done without patient involvement. Patients were not presented with the data acquired or the analysis of the results.

Anti-CCL24 Antibody (CM-101)

CM-101 is a proprietary antibody that binds human CCL24 with high affinity. It is a humanized IgG1, kappa light chain monoclonal antibody, manufactured in Chinese Hamster Ovary cells. The antibody was generated from a parental hybridoma (CM-101-D8) that contains a murine backbone and cross-reacts with murine CCL24 with high affinity.

Chemotaxis assay

Chemotactic migration of Normal human dermal fibroblasts (NHDF) (Promocell) was studied in transwells with 8-µm pores (Costar). Serum starved NHDF cells (5 $x10^4$ cells/insert) were placed in the upper chamber and migration toward CCL24 (Peprotech) was assessed. CCL24 (100 ng/ml) alone or CCL24 (100 ng/ml) preincubated with CM-101 (100, 50 or 10nM) or IgG (100 nM) for 30 minutes at 37°C were placed in lower chamber. Cells were incubated for 4h at 37 °C. Transmigrated cells were fixed with 4% paraformaldehyde for 10 minutes at room temperature. The transwells were washed, stained with Comassie blue and washed again. Cell migration is documented by 4-6 random snap shot of each transwell. Analysis of cell counts was performed using image pro-5 software.

Calcium flux assay

Fluo-4 AM was used as the fluorescent Ca2+-sensitive indicator for these experiments. $2x10^5$ NHDF cells were loaded with 4 µM Fluo-4 AM (Invitrogen) in fibroblasts medium for 30 min at 37°C. The cells were then washed with phosphate buffered saline (PBS) twice to remove extra Fluo-4 AM. agonist induced changes in free cytosolic Ca2+ in NHDF cells was measured during 180 seconds from stimulation using FACSCalibur flow cytometer (Becton Dickinson). 0.1% serum (from SSc patients) was used as agonist. For the assessment of CM-101inhibitory effect, CM-101 was preincubated with human serum for 30 minutes at 37°C prior to its addition to the cells.

Endothelial cells activation

Human Umbilical Vein Endothelial Cells (HUVEC) (ATCC) were cultured for 48 hours with or without 0.1 % serum from SSc patients. Expression of vascular cell adhesion molecule-1 (VCAM-1) was tested using anti-VCAM-1 FITC-conjugated antibody (R&D systems) and detected by flow cytometry. For the assessment of CM-101 inhibitory effect, CM-101 was preincubated with human serum for 30 minutes at 37°C prior to its addition to the cells.

Mouse bleomycin-induced dermal fibrosis model

Female C3H mice at age 6 weeks were acclimatized for 7 days at the animal house. All animal work was performed following approval of the National Board of Animal Studies in the Ministry of Health by the Kaplan medical center.

Bleomycin (BLM) (Sigma) was diluted to 200 µg/ml with PBS. Bleomycin or PBS (100 µl) were injected subcutaneously into a single location on the shaved back of female mice once daily for 3 weeks. For the prevention model, CM-101 at doses of 5µg, 10µg and 20µg or immunoglobulin G1 (IgG1) at a dose of 50µg or PBS was injected every other day (starting on Day 1) in parallel with the initiation of bleomycin injections (n=8).

For a treatment protocol, SSc was induced similarly as in the prevention model. Mice were treated subsequently by i.p. injections of CM-101, at 2.5 mg/kg, 3 times a week from day 8 until day 21. Control mice were treated by IP injections of PBS. A baseline group was sacrificed on day 8 prior to mAb administration to verify disease onset (n=10).

CCL24 knockout mice

CCL24 -/- mice were generated using CRISPR/Cas9-mediated genome engineering by injecting Mouse Ccl24-gRNA2 (VB150827-10036) targeted to exon 2 of the CCL24 gene into fertilized eggs (Cyagen Biosciences Inc). The positive founders (F0) created different chimera mice that were bred to the next generations (with WT C57BL/6) to generate F1 founders. All F1 founder mice were genotyped by polymerase chain reaction and DNA sequencing analysis. Six F1 mice were obtained, two with an 8bp deletion and four with an 11bp deletion, both types of deletions creating nonsense mutations in exon 2.

F1 mice were then bred to each other to create CCL24 -/- mice. Off-target was assessed and found negative.

Mouse bleomycin induced lung fibrosis model

8 weeks old C57Bl/6 male mice (n=11 per group) were administered with bleomycin (BLM) (3mg/kg in 100 µl) or PBS via intratracheal injection under isoflurane (2%) anesthesia after intubation. BLM was dissolved in PBS. After injections, the mice were placed in a vertical position and rotated for 1 min to allow even distribution of BLM in lung alveoli. PBS, 2.5mg/kg murine CM-101 or 2.5mg/kg murine IgG were administered intraperitoneal (IP) three times a week from day 10 until day 20 (treatment protocol). Pirfenidone (400mg/kg) or Nintedanib (50mg/kg) (Cellagen Technology LLC) were administered orally once a day from day 10 until day 20. Control group was administered with PBS via intratracheal injection at day 0. Baseline group was administered with BLM and scarified at day 10 to estimate fibrosis prior to treatments administration.

Preparation of BAL fluid

The BAL assay was performed as previously described (Komai et al, 2010). After the bleeding procedure, the trachea was cannulated and the left bronchi were tied for histological examination. The right air lumen was then washed 4 times with 0.5 ml calcium- and magnesium-free PBS containing 0.1% BSA and 0.05 mM EDTA-2Na. This procedure was repeated three times (total volume: 1.3 ml, recovery >85%). BAL fluid from each animal was pooled in a plastic tube, cooled on ice, and centrifuged (150 × g) at 4°C for 10 min. Cell pellets were resuspended in the same buffer (0.5 ml). Total white blood cells and mononuclear cells were assessed using flow cytometry. Alternatively, WBC count was conducted using COBAS 6000 instrument.

Histological evaluations

Skin and lung tissues were formalin-fixed and embedded in paraffin. Sections were then stained with H&E and Masson's trichrome for microscopic evaluation. Dermal thickness,

defined as the thickness of dermis was assessed in 5 different fields per section, in 2 different sections from each animal.

Measurement of collagen in skin and lung samples

Soluble collagen was quantified using the Sircol soluble collagen assay (Biocolor, Belfast, UK). Skin or lung samples were obtained from scleroderma/IPF induced mice. The samples were extracted into acid–pepsin solution. The samples were analyzed for collagen content according to the manufacturer's protocol. Briefly, 100µl of sample was added to 1 ml of the colorimetric reagent and agitated for 30 min followed by centrifugation at 10,000g for 10 min. The SR dye was released from the pellet with alkali reagent (1 N NaOH) and spectrophotometric readings were taken at 555 nm using a microplate reader.

Detection of *α*-SMA expression by Western blot, FACS and Real-Time PCR

Skin Protein lysate (75 g) were resolved by SDS-polyacrylamide gel electrophoresis (PAGE) and electrophoretically transferred to nitrocellulose membrane. Membranes were blotted with anti-mouse α-SMA antibody (1:1000, ab5694 Abcam) or anti-tubulin (1:1000 Sigma). Immunoreactive bands were quantified by densitometry with Image Master VDS-CL using TINA 2.0 software (Ray Tests).

For detection of intracellular α-SMA by FACS, NHDF cells were incubated for 48 hours with systemic sclerosis patients' serum (0.1%) with or without CM-101 (5 or 10 µg/ml). Intracellular α-SMA expression was detected using human α-smooth muscle actin PE-conjugated antibody (R&D systems) and Flow Cytometry Fixation & Permeabilization Kits (#IC1420P R&D systems).

Real-Time PCR was done using Taqman® assay Mm00725412_s1 for Acta2 levels and Mm99999915_g1 Gapdh as normalizing control (Applied Biosystems, ThermoFisher Scientific). Reaction were run in the C1000™ Thermal cycler, CFX96 Real-Time (BioRad). Acta2 levels were normalized to sample Gapdh and fold change in mRNA levels were calculated between control and treatments (ddCT).

Statistical Tests

To evaluate the differences of CCL24 serum levels the non-parametric Mann-Whitney U-test was employed. All other statistical analyses were done by Student's t-test. $P < 0.05$ was considered statistically significant. Results were expressed as mean ± SEM.