Online Supplement

Material and methods

Mice and therapeutics

DBA/2, FVB, C57/Bl6 and BALB/c mice were purchased from Janvier (Le Genest Saint Isle, France), B10.D2 and Tight-skin-1 (Tsk-1) mice from Jackson laboratories (Bar Harbor, Maine, USA). T0901317 was dissolved in DMSO and further diluted in PBS (1:10) for per os-application (Sigma-Aldrich, Taufkirchen, Germany). T0901317 was applied once daily during the bleomycin challenge. The anti-interleukin-6 antibody 20F3 was provided by Prof. S. Rose-John and applied once weekly in a dose of 250 µg/mouse [21]. All animal experiments were performed with the approval of the local ethics authorities.

Generation of LXR knockout mice

We derived the conditional targeting vectors using the Lambda knockout shuttle (KOS) system [1]. The Lambda KOS phage library, arrayed into 96 superpools, was screened by PCR. Two and four pKOS genomic clones were isolated from the library screen for the LXRα and LXRβ genes, and confirmed by sequence and restriction analysis. For the LXRα vector, gene-specific arms (5′-AATTCCAGTTACCCTGGAGGCTGAGACAAGATCAC-3′) and (5′-CTACCCAGATAAGGAAGTTTTAATCCACACTCAG-3′) were appended by PCR to a yeast selection cassette containing the URA3 marker. The yeast selection cassette and pKOS-30 were cotransformed into yeast, and clones that had undergone homologous recombination to replace a 512–basepair (bp) region containing exon 4 with the yeast selection cassette were isolated. This 512-bp fragment was independently amplified by PCR and cloned into the intermediate vector pLF-Neo, introducing flanking LoxP sites and a Neo selection cassette (LXRα–pLFNeo). The yeast cassette was subsequently replaced with the LXRα–pLFNeo

selection cassette to complete the conditional LXR α targeting vector that has exon 4 flanked by LoxP sites. For the LXRβ vector, gene-specific arms (5′-GTTCAGCCACATAGTTCGGGTAGCCTGAGCTGTAT-3′) and (5′-CTGGCTGGCTCTCTGGAGGCTCCAGATACCTTGTT-3′) were appended by PCR to a yeast selection cassette containing the URA3 marker. The yeast selection cassette and pKOS-68 were cotransformed into yeast, and clones that had undergone homologous recombination to replace a 1619-bp region containing exons 3 to 7 with the yeast selection cassette were isolated. This 1619-bp fragment was independently amplified by PCR and cloned into the intermediate vector pLF-Neo, introducing flanking LoxP sites and a Neo selection cassette (LXRβ–pLFNeo). The yeast cassette was subsequently replaced with the LXRβ–pLFNeo selection cassette to complete the conditional LXRβ targeting vector that has exons 3 to 7 flanked by LoxP sites. The Not I linearized targeting vectors were electroporated into 129/SvEvBrd (Lex-1) embryonic stem (ES) cells. G418/FIAU-resistant ES cell clones were isolated, and correctly targeted clones for the different genes were identified and confirmed by Southern blot analysis. For the LXRα recombination analysis, a 609-bp 5′ Neo internal probe (N2/N5) was generated by PCR using primers Neo-2 (5′-CCTCAGAAGAACTCGTCAAG-3′) and Neo-5 (5′-GGCAGCGCGGCTATCGTG-3′), and a 310-bp 3′ external probe (6/7) was amplified by PCR using primers LXRa-6 (5′-AACCTCTCGACTCACAGC-3′) and LXRa-7 (5′-CCAAGCTGGATATCTGG-3′). Southern analysis using probe N2/N5 detected a 13.0-kb mutant band in Kpn I–digested genomic DNA whereas probe 6/7 detected a 1.5-kb wild-type band and 3.4-kb mutant band in Bgl II–digested genomic DNA. For the LXRβ recombination analysis, a 328-bp 5′ external probe (68/69) was generated by PCR using primers LXRb-68 (5′-GCAAGAATTTGTGCTTAATAAAG-3′) and LXRb-69 (5′-GCATTCAGCTTCATGATAG-3′), and a 317-bp 3′ external probe (87/96) was amplified by PCR using primers LXRb-87 (5′-GTGATCAAGCGGGAGTTGCTC-3′) and LXRb-96 (5′-GTATCATGTGCCTTTACCTGC-

3′). Southern analysis using probe 68/69 detected a 12.8-kb wild-type band and 6.5-kb mutant band in Xba I–digested genomic DNA, whereas probe 87/96 detected a 10.0-kb wild-type band and 9.6-kb mutant band in Stu I–digested genomic DNA. The targeted clones from each project were microinjected into C57BL/6 (albino) blastocysts. The resulting chimeras were mated to C57BL/6 (albino) females to generate mice that were heterozygous for the LXRα and LXRβ conditional mutations. These conditional heterozygous animals were further bred to a protamine-Cre–expressing line (O'Gorman and colleagues [2]) to create compound heterozygous mice that were further bred to obtain mice with the universal excision (mutation) of the LXR genes. The LXRα and LXRβ mutant mice were generated by Lexicon Pharmaceuticals, Inc. (The Woodlands, TX, USA).

Bleomycin-induced dermal fibrosis

Skin fibrosis was induced in 6-week-old DBA/2 or 6-week-old LXR knockout mice by subcutaneous injections of bleomycin as described previously [3-9]. Controls were injected with 0.9 % NaCl. After a 4-week bleomycin challenge, mice were sacrificed and the injected skin processed for further analysis.

Sclerodermatous, chronic Graft-versus-Host Disease

The B10.D2→Balb/c [H-2(d)] minor histocompatibility antigen-mismatched modelwas used as an additional model for early inflammatory stages of SSc [10-15]. PBS was used to flush bone marrow cells from bone marrow cavities of tibias and femurs followed by erythrocyte hemolysis. No further purification or in vitro expansion of a particular subset of cells was performed. Recipient mice [BALB/c (H-2d)] underwent total body irradiation with 700 cGy. Sixteen hours after the second irradiation, each recipient mouse received 2×10^6 splenocytes and 1×10^6 bone marrow cells, dissolved in 100 μL PBS, from either BALB/c (H-2d) in a

syngeneic or B10.D2 (H-2d) in an allogeneic, multiple minor mismatched transplantation via tail vein injection. Mice were kept under low-germ conditions and received antibiotic prophylaxis via the drinking water from -2 to day +21 after transplantation. The treatment was started at the onset of first clinical signs of disease 21 days after BMT. At day 42, mice were sacrificed and the skin was processed for further analysis.

TSK-1 mice

Treatment of Tsk-1 as well as pa/pa control mice started at the age of 5 weeks [3, 4, 6, 9]. After 5 weeks of treatment, mice were sacrificed and the skin processed for further analysis.

Analysis of murine skin

Skin thickness, α-smooth muscle actin counts, hydroxyproline content and inflammatory infiltrates were analyzed as described previously [3-9]. In brief, specimens were fixed in 4% paraformaldehyde, embedded in paraffin, and sliced into 5 µm-sections to determine skin thickness and stain for α-smooth muscle actin. For determining skin thickness, slices were stained with Masson's trichrome reagents. For the bleomycin-model, dermal thickness at the injection sites was analyzed at 100-fold magnification by measuring the distance between the epidermal-dermal junction and the dermal-subcutaneous fat junction. For analysis of Tsk-1 mice, a 40-fold magnification was used and hypodermal thickness was defined as the thickness of the subcutaneous, loose connective tissue layer (i.e., the hypodermis or superficial fascia) beneath the panniculus carnosus. To analyze the collagen content in skin samples, hydroxyproline assay was performed as described by Woessner [16]. To detect myofibroblasts, skin sections were stained with a monoclonal antibody against α-smooth muscle actin (clone 1A4; Sigma-Aldrich, Taufkirchen, Germany). Slices were further

processed with peroxidase reaction (horseradish-peroxidase-labed secondary antibody and a substrate kit, Vector, Burlingame, CA). Irrelevant isotype-matched antibodies were used as controls. In each section, α-smooth muscle actin-positive cells were counted in three randomly chosen high-power fields at a magnification of 200-fold. Dermal infiltration by leukocytes was assessed by a semi-quantitative scoring with 0 = no / 1 = slight / 2 = moderate / 3 = severe inflammatory infiltrate / 4 = very severe inflammatory infiltrate on hematoxylin- and eosin-stained sections at 100-fold magnification.

Immunofluorescence for F4/80 and IL-6

After deparaffinization and blocking with 5% horse serum, murine skin sections were incubated with a polyclonal rat anti-F4/80 antibody (AbD Serotec, Düsseldorf, Germany) and a polyclonal rabbit anti-IL-6 antibody (Abcam, Cambridge, UK). Irrelevant isotype-matched antibodies were used as controls. Donkey anti-rat Alexa Fluor 594 and goat anti-rabbit Alexa Fluor 488 were used as secondary antibodies. The cell nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI; 1:800; Santa Cruz Biotechnology, Heidelberg, Germany). Images were captured at 200-fold magnification using a Nikon Eclipse 80 microscope (Nikon, Badhoevedorp, The Netherlands). F4/80 positive macrophages were counted and IL-6 staining was assessed by semi-quantitative scoring (with 0 = no / 1 = slight / 2 = moderate / 3 = strong inflammatory infiltrate / 4 = very strong staining) in 8 different sections of lesional skin for each mouse at 200-fold magnification.

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