

Supplemental Materials and Methods

DNA Microarray Hybridization and Analysis. For biotin-labeled target synthesis starting from 5 μ g of total RNA, which was isolated using the RNeasy Mini Kit (QIAGEN), reactions were performed using standard protocols supplied by the manufacturer (Affymetrix). In brief, 5 μ g of total RNA was converted to dsDNA using 100 pmol of a T7T23V primer (Eurogentec) containing a T7 promotor. The cDNA was used directly in an in vitro transcription reaction in the presence of biotinylated nucleotides. 12.5 μ g of the cleaned and biotinylated cRNA preparation was fragmented and placed in a hybridization cocktail. Samples were hybridized to an identical lot of Affymetrix MG-U74Av2 for 16 h. After hybridization, the GeneChips were washed, stained with PE-labeled streptavidin, and read using an Affymetrix GeneChip fluidic station and scanner. Analysis was done with gene expression software (GeneChip, MicroDB, and Data Mining Tool; all Affymetrix) with a filter for regulated genes using fold changes >2 or <-2 , change p -values <0.001 or >0.999 , respectively, and signal differences of ≥ 40 .

Real-Time Quantitative RT-PCR Assay for Foxp3. Reverse transcription (TaqMan Reverse Transcription Reagent; Applied Biosystems) was performed in a conventional thermocycler (10 min at 25°C, 40 min at 48°C, and 5 min at 95°C) with 1 μ g of total RNA and a 1:1 mixture of oligo(dT) and random hexamer primers in a final volume of 20 μ l. Real-time PCR was performed with the LightCycler instrument and the FastStart DNA Master SYBR Green I kit (Roche Diagnostics). The primer sequences and the cycling program for murine forkhead box p3 (Foxp3) and the housekeeping gene hypoxanthine guanine phosphoribosyl transferase (HPRT), which was used for normalization, were as follows: Foxp3, 5'-CTGCTCCTCTATTCCTCGTAAC-3' and 5'-AGCTAGAGGCTTTGCCTTCG-3', HPRT, 5'-GCTGGTGAAGAGGACCTCT-3' and 5'-CACAGGACTA-GAACACCTGC-3', cycling program (10 min at 95°C followed by 40 cycles of 15 s at 95°C, 15 s at 60°C for Foxp3 or 65°C for HPRT, and 15 s at 72°C). Data were evaluated using the Lightcycler software version 3.5.28 (Roche Diagnostics) and the second derivative maximum algorithm. Specificity of the PCR product was confirmed by agarose gel electrophoresis and melting curve analysis. Serial dilutions of murine CD4⁺ cells were used to generate the standard curves.

Real-Time Quantitative RT-PCR Assay for Chemokine Receptors. Total RNA was reverse transcribed with 200 U M-MLV reverse transcriptase (GIBCO BRL) for 45 min at 42°C in 20- μ l assays containing 1 μ l oligo(dT) primers (GIBCO BRL) and 10 mM dNTP. The reaction was stopped by adding 40 μ l Tris-EDTA buffer (pH 8.0) and heat inactivation for 10 min at 90°C. Quantitative real-time PCR was performed with a GeneAmp 5700 Sequence Detection System (Applied Biosystems) to quantify mRNA levels of selected genes. Amplifications were run in 50- μ l assays with the TaqMan Universal Master Mix (Applied Biosystems) containing SYBR green and PCR core reagents (corresponding primers were synthesized by MWG-Biotech; see Table SI). The cycling program was as follows: initial annealing for 2 min at 50°C followed by activation of Taq polymerase for 10 min at 95°C. 40 amplification cycles of a 15-s denaturing interval at 95°C and a 1-min annealing step at 54/58°C, respectively (depending on primer T_m) were run. Standard curves were generated using serially diluted cDNA probes. The RPS9 housekeeping gene (Schrader et al. 2002. *Br. J. Cancer*. 86:1250) was used for normalization.

Quantification of TRECs by Real-Time PCR. In this PCR, binding of a specific probe containing a quencher (TAMRA) and a reporter (6FAM) dye to the amplification products results in Taq DNA polymerase-mediated cleavage of the probe. This leads to separation of the quencher from the reporter, thereby inducing fluorescence of the reporter dye. The amount of target in analyzed samples is established by measuring the threshold cycle (C_T), defined as the fractional cycle number at which the fluorescence generated by cleavage of the probe passes a fixed threshold above baseline, and uses the standard curve to determine starting copy number. To precisely determine the percentage of cells carrying the mouse TREC, a duplex vector was constructed containing a fragment of the δ Rec- ϕ J α signal joint (TREC) and a fragment of the mouse RAG2 gene, used as a reference. The RAG2 was cloned first in the T-A acceptor site and subsequently the TREC was cloned into the EcoRV restriction site of the pCR2.1 TOPO TA Vector (Invitrogen). The vector was linearized, measured by spectrophotometry and confirmed by a quantitative gel electrophoresis. Standard dilutions of the vector from 10⁷ to 10¹ copies were prepared. In brief, PCR of 50 μ l total volume was performed with \sim 100 ng of genomic DNA, 25 pmol of each primers, 5 pmol of 6FAM-TAMRA probe, and the TaqMan PCR Core Reagent Kit (Applied Biosystems). The kit includes 1.5 U AmpliTaq gold polymerase and 4 mM MgCl₂ PCR buffer with 10 nmol of ATP, CTP, GTP, and UTP, and 0.5 U AmpErase Uracil N-glycosylase, to prevent a carry-over contamination. After the initial incubation at 50°C for 2 min, to activate the Uracil N-glycosylase, followed by denaturation at 95°C for 10 min and activating the AmpliTaq Gold polymerase, 45 two-step cycles consisting of 95°C for 30 s and 62°C for 1 min for RAG2 or 66°C for 1 min for TREC were performed. For TREC analysis, the 5' primer ϕ J α (-115), 5'-AAGATGGGCCTCTCT-GAGGAACAC-3'; and the 3' primer δ Rec(+108), 5'-GCTGAACTTATTGCAGCTCCTGAG-3', amplifying the signal joint sequence generated by the δ Rec- ϕ J α rearrangement (TREC), were used together with the TREC-specific probe as follows: 6FAM-CACAgCTgTgTgCCCTACCCTgCC-TAMRA. For RAG2 analysis, the 5' primer RAG2

(1115), 5'-GCAACATGGGAAATGGAAGT-3'; the 3' primer, RAG2 (1358), 5'-GGTTCAAATTCATCGTCAC-CATC-3'; and the RAG2-specific probe, 6FAM-CCCCAGGATCTTCTGTTGATGTCTGACTGTTGGAGA-TAMRA, were used.

DTH-type Skin Inflammation Model. Th1 cells were generated in vitro as described previously (Austrup, F., D. Vestweber, E. Borges, M. Löhning, R. Bräuer, U. Herz, H. Renz, R. Hallmann, A. Scheffold, A. Radbruch, and A. Hamann. 1997. *Nature*. 385:81–83.) from DO11.10 mice. 10⁶ cultured Th1 cells were injected i.v. into naive BALB/c mice, and 24 h later the DTH response was induced by s.c. injection of 250 ng OVA^{323–339} peptide (synthesized at the Dept. of Biochemistry, Humboldt-University, Berlin, Germany) together with incomplete Freund's Adjuvant (Sigma-Aldrich) into the left footpad. As control, the right footpad was injected with PBS and incomplete Freund's Adjuvant. 24 h after antigen injection, a strong acute inflammatory reaction was measurable using an Oditest micrometer gauge (Kröplin Längenmesstechnik).