METHODS

Cytokines and reagents

SCF, thrombopoietin, TNF-α, GM-CSF, FLT3L, IL-6, IFN-γ, IL-4, and macrophage colony-stimulating factor were purchased from PeproTech (Rocky Hill, NJ). TGF-β1 was from R&D Systems (Minneapolis, Minn). PanCSK4 was purchased from InvivoGen (San Diego, Calif). The recombinant extracellular domain of Notch ligand Delta-1 (Delta-1ext–IgG) was kindly provided by I. Bernstein (Seattle, Wash). Coating of Delta-1ext–IgG was performed, as previously described.

Sources of cells and skin tissue

CD34+ cells were obtained from cord blood samples from healthy donors. Blood samples were collected during healthy full-term deliveries and prepared, as previously described. CD34+ hematopoietic progenitors were isolated by means of immunomagnetic positive selection (EasySep; STEMCELL Technologies, Vancouver, British Columbia, Canada). For the microarray screen, CD34+CD19+ cells were subsorted into CD45RAlow/−, CD45RA+ and CD45RA− subsets by means of FACS and analyzed separately. For all differentiation experiments, the total CD34+ cell fraction was used.

Isolation of immune cells for microarray studies

Epidermal LCs and keratinocytes, as well as dermal cell populations, were isolated from healthy human skin. Briefly, after separation of dermal and epidermal sheets by means of incubation with Dispase I (3 U/mL; Roche), single cells were released from epidermal sheets by using 0.25% trypsin/EDTA (Invitrogen) for 30 minutes at 37°C. CD11b+CD1a+ LCs (n = 4) and CD11b+CD1a− keratinocytes (n = 1) were sorted from epidermal cell suspensions on a FACSAria (BD Biosciences). For preparation of dermal cell suspensions, dermal sheets were dissociated with 0.5 U/mL collagenase IV (Worthington Biochemical, Lakewood NJ) for 90 minutes at 37°C. Dermal cells were sorted into CD1a−dDCs (CD1a+CD1b+CD14+, n = 3), and CD14+ dDCs (CD14+CD1b+CD1a−, n = 3). Monocytes were isolated from buffy coats obtained from the Austrian Red Cross. Briefly, after Ficoll Hypaque (Pharmacia, Uppsala, Sweden) density gradient centrifugation, PBMCs were depleted of Lin− cells (CD3, CD16, CD34, CD56, and glycoporphin A), and CD14+CD11b+CD19−CD1c+ monocytes were sorted by using a FACSAria. The purity of all cell populations used was at least 98%. Sorted cells were pelleted and lysed in TRI Reagent (Sigma-Aldrich), and RNA was isolated, according to the manufacturer’s recommendations.

mRNA microarray and data analysis

Cells were collected at indicated time points (0, 6, and 24 hours after addition of TGF-β1). Total RNA from 6 independent donors was isolated by using the RNeasy Micro Kit (Qiagen). RNA samples were then combined into 2 separate pools (each containing RNA from 3 independent donors), labeled, and hybridized onto U133 Plus 2.0 Affymetrix GeneChips (Affymetrix, Santa Clara, Calif). Top hundred nanograms of total RNA per sample was processed by using Ambion’s MessageAmp II Biotin Enhanced Labeling Kit (Ambion, Thermofisher, Waltham, Mass). Gene chips were cleaned, washed, and scanned according to Affymetrix standard procedures. The probe level data (CEL files) were processed for local normalization, and expression values were generated by using the robust multiarray average algorithm of the “affy package” in the R software environment (http://www.R-project.org). The microarray data have been deposited in the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo) and can be accessed as GSE31318. For mRNA profiling of immune cells isolated from skin, total RNA was subjected to 2 rounds of linear amplification, as previously described. Biotin-labeled ribonucleotides were incorporated by using the ENZO Bio-Array High-Yield RNA Transcript Labeling Kit (Affymetrix) during the second round of in vitro transcription. Fragmented cDNA (10 μg) was hybridized to Human Genome U133 Plus 2.0 Array (Affymetrix). Microarray data were normalized by using the robust multiarray analysis, as implemented in Bioconductor.

All analyses were performed with log2-transformed data. The Ingenuity Pathway Analysis (http://www.ingenuity.com) tool was used to assign microarray data sets to common biological pathways and to define gene sets attributed to Notch signaling. Analysis was performed for genes showing regulated expression under TGF-β1–supplemented versus nonsupplemented conditions after 6 and 24 hours (cutoff: fold change, 1.3). Differential expression of Notch was determined by using a heat map with Spotfire software (http://www.spotfire.com/).

Transfection of packaging cell lines and gene transduction

Gene transduction was performed, as previously described. Briefly, the packaging cell line Phoenix-Gag-Pol (Ph-GP) was used for generating GALV envelope-containing retroviral particles. Target cells were plated on RetroNectin (Takara Bio, Shiga, Japan)-coated non-tissue culture plates coated with virus (3-5 hours) in specific growth medium. Infections were repeated 2 to 3 times at intervals of 12 to 24 hours. CD34+ cells were infected in expansion mix (SCF, FLT3L, and thrombopoietin; each 50 ng/mL). The retroviral tetracycline-inducible system (tet-on system) was described previously. Briefly, the first vector encoded the Tet activator TA-mCD8a. The second vector encoded human KLF4-ires-GFP under the control of a Tet-responsive element. CD34+ cells were first infected with TA-mCD8a, followed by infection with the Tet-response vector. Expression of the KLF4 transgene was induced by addition of 1 μg/mL doxycycline. Fresh doxycycline was added every 2 to 3 days of LC differentiation culture to sustain KLF4 expression (GM-CSF, TNF-α, and TGF-β1; secondary cultures).

Retroviral vectors

RV-GFP and RV-KLF4-GFP vectors were kindly provided by M. W. Feinberg. KLF4-coding DNA was inserted into the BglII/XhoI sites of the MCV-IRES-GFP vector. Cutting RV-KLF4 with BglII and XhoI and inserting it into MCV-IRES-NGFR vector generated MIN-KLF4. MIC-RUNX3 was kindly provided by S. Sakaguchi (Vienna, Austria). HR-KLF4-IGFP was generated by cutting RV-KLF4-GFP with BglII and XhoI and inserting it into the BamHI/XhoI site of the pHR-IGFP vector (kindly provided by F. Rossi, Vancouver, British Columbia, Canada).

Immunohistochemistry staining

Double-labeled immunohistochemical staining was performed on paraffin-embedded sections or cytospin preparations by using the LabVision MultiVision Polymer Detection System (anti-mouse AP; anti-rabbit horseradish peroxidase), according to the commercial protocol (Thermo Fisher Scientific). The following primary antibodies were used: monoclonal mouse anti-CD1a (Novus Biologicals, Littleton, Colo), polyclonal rabbit anti-activated Notch-1 (Abcam, Cambridge, Unite Kingdom), and polyclonal rabbit anti-KLF4 (Sigma-Aldrich).

Confocal microscopy

Multicolor immunofluorescent staining procedures were performed on frozen sections, as previously described. Negative controls were obtained in all staining experiments by substituting primary antibody with the isotype-matched IgG. Slides were mounted in Permafluor (Thermo Fisher Scientific) or VECTASHIELD (Vector Laboratories, Burlingame, Calif). Immunofluorescently labeled sections were analyzed with a Zeiss LSM 510 confocal microscope (×40/1.3 NA; Zeiss, Oberkochen, Germany), and
images were captured with Zen 2008 Software (Zeiss). The following primary antibodies were used: polyclonal rabbit anti-KLF4 (Sigma-Aldrich); mouse anti-CD207 (Immunotec, Vaudreuil-Dorion, Quebec, Canada), fluorescein isothiocyanate (FITC)–conjugated mouse anti-CD11b (Immunotec), FITC-labeled mouse anti–HLA-DR (BD Biosciences), polyclonal rabbit anti-activated Notch-1 (Abcam), monoclonal mouse anti-CD1a (Novus Biologicals), polyclonal goat anti-CD14 (Novus Biologicals), and FITC-labeled mouse anti-CD14 (BioLegend, San Diego, Calif). Alexa Fluor 488–conjugated anti–Laminin-5 (Millipore, Temecula, Calif) was used to visualize the dermal-epidermal junction. Anti-FITC polyclonal goat IgG (Invitrogen) and anti-rabbit polyclonal goat F(ab)2 fragment (Jackson ImmunoResearch, West Grove, Pa) or goat anti-rabbit Alexa Fluor 647 (Jackson ImmunoResearch) antibodies served as secondary reagents. All secondary antibodies have been cross-absorbed to avoid cross-reactivity with IgG of other species.

Chromatin immunoprecipitation assay

CD14+ peripheral blood monocytes were induced to differentiate with GM-CSF (100 ng/mL) and IL-4 (25 ng/mL) into moDCs. KLF4 chromatin immunoprecipitation was performed with the KLF4 ExactaChIP Kit (R&D systems), according to the manufacturer’s protocol. Briefly, moDCs (6 × 10^6) were treated for 15 minutes at 37°C with 1% (vol/vol) formaldehyde, followed by addition of glycine (final concentration, 125 mmol/L). Cells were pelleted, resuspended in lysis buffer, lysed on ice, and sonicated to obtain chromatin fragments of 0.5 to 1 kb in length. Equal amounts (5 μg) of goat anti-human KLF4 antibody or of normal goat IgG were used per reaction. The following primers were used for detection of the RUNX3 promoter region: 5′-GCAGCCCCAGAACAAATC-3′ and 5′-GGTGCTACGACCGAGGAGG-3′. The abundance of distinct DNA fragments was quantified by means of semiquantitative PCR; PCR products were resolved by using 2% agarose gel electrophoresis.

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FIG E1. Immunohistochemical analysis of healthy human skin and *in vitro*-generated LCs. **A**, Paraffin sections of healthy human skin were stained for CD1a (red) and aN1 (blue). *Bars* = 10 μm. **B**, p-LCs and moLCs were sorted by using anti-CD207 mAb with magnetic beads. Cells were spun on slides, fixed, permeabilized and stained for CD1a and aN1. *Bars* = 10 μm.
FIG E2. Analysis of the inhibitory role of KLF4 on LC-like cell differentiation. A and B, KLF4-IRES-GFP–, RUNX3-IRES-GFP–, or control-transduced day 5 precursors were cultured under LC-promoting conditions (+TGF-β1). GFP⁺CD1a⁺-gated cells were analyzed for expression of CD207, DC-specific intercellular adhesion molecule–grabbing nonintegrin (DC-SIGN; CD209), CD11b, and CD14. C, KLF4-IRES-GFP– or control-transduced cells were cultured under LC-promoting cytokine conditions (+TGF-β1). After 24 hours, GFP⁺ cells were sorted, and quantitative PCR analysis for ID2 mRNA expression was performed. Bar diagrams represent mean ± SEM values of 3 independent experiments.
| Primer name | Orientation | Sequence                  |
|------------|-------------|---------------------------|
| RFX2       | Forward     | ATA GAT GTC TCC CAC TGC TTC |
|            | Reverse     | TCT CGA TGT AGT GGA ACT GGA G |
| TIEG       | Forward     | CCA GGA TGT GGC AAG ACA TAC |
|            | Reverse     | TTC ACA ACC TTT CCA GCT ACA G |
| BLIMP-1    | Forward     | CGG CAA GAT CAA GTA CGA ATG |
|            | Reverse     | GAG CTG AGT AAA GCC CTT GTT G |
| ETS-2      | Forward     | TTG TGG GTG ACA TTC TCT GG |
|            | Reverse     | ATG AGG AAC GGA GGT GAG G |
| DEC2       | Forward     | CCT ACC GTC CCA CAG ATT G |
|            | Reverse     | CCT TGG TGT CGT CTC GTT TC |
| DEC1       | Forward     | TGA CCG GAT TAA CGA GTG C |
|            | Reverse     | GAG CAG AAC ATC TCT TGA CCT G |
| KLF4       | Forward     | GCC GCT CCA TTA CCA AGA G |
|            | Reverse     | GTG CCT TGA GAT GGG AAC TC |
| PPARδ      | Forward     | TCA CAC AGT GGC TTC TGC TC |
|            | Reverse     | TCT ACA GGG TGG TTC CCA TC |
| RXRα       | Forward     | CGA CCC TGT CAC CAA CAT TTG C |
|            | Reverse     | GAG CAG CTC ATT CCA GCC TGC C |
| VDR        | Forward     | AGA TGA CCC TTC TGT GAC CC |
|            | Reverse     | AGC TTG TTC AGT CCC ACC TG |
| HPRT       | Forward     | GAC CAG TCA ACA GGG GAC AT |
|            | Reverse     | AAC ACT TCG TGG GGT CCT TTT C |
| Antigen   | Conjugate | Distributor                  |
|-----------|-----------|------------------------------|
| CD34      | FITC      | BD Biosciences               |
| CD14      | FITC      | BD Biosciences               |
| HLA-DR    | FITC      | BD Biosciences               |
| CD1a      | FITC      | BD Biosciences               |
| CD1c      | FITC      | Miltenyi Biotec GmbH        |
| CD11b     | FITC      | Immunotech                   |
| CD207     | PE        | Miltenyi Biotec GmbH        |
| CD203     | PE        | Immunotech                   |
| CD1a      | PE        | BD Biosciences               |
| CD14      | PE        | BD Biosciences               |
| HLA-DR    | PE        | BD Biosciences               |
| CD45RA    | PE        | BD Biosciences               |
| CD11c     | PE        | BD Biosciences               |
| Lactoferrin| PE        | Caltag/An der Grub           |
| CD14      | PE        | ImmunoTools                  |
| Jagged-2  | PE        | BioLegend                    |
| CD19      | PerCP     | BD Biosciences               |
| NGFP      | PerCP-Cy5.5| BD Biosciences               |
| CD45      | ECD       | Immunotech                   |
| CD117     | CyChrome  | BD Biosciences               |
| CD1a      | APC       | BD Biosciences               |
| CD14      | APC       | BD Biosciences               |
| E-Cadherin| APC       | BioLegend                    |
| Notch-1   | APC       | BioLegend                    |
| E-Cadherin| AF647     | BD Biosciences               |
| CD11b     | PE-Cy7    | BioLegend                    |
| CD11b     | APC-Cy7   | BD Biosciences               |
| CD80      | Biotinylated| BD Biosciences               |
| CD86      | Biotinylated| BD Biosciences               |
| CD209     | Biotinylated| BD Biosciences               |
| CD11b     | Biotinylated| BD Biosciences               |
| CD1a      | BV412     | BD Biosciences               |
| CD1a      | Pacific Blue| BioLegend                   |

The second-step reagent for biotinylated antibodies was streptavidin-PerCP (BD Biosciences).

APC, Allophycocyanin; ECD, Phycoerythrin-Texas Red; PE, phycoerythrin; PerCP, peridinin-chlorophyll-protein complex.
**TABLE E3. Microarray data: mRNAs induced in TGF-β1–stimulated versus nonstimulated cultures**

| Probe set     | Gene symbol | 0 h  | 6 h  | 24 h | 0 h  | 6 h  | 24 h | Sum of calls | P value |
|---------------|-------------|------|------|------|------|------|------|--------------|---------|
| 201131_s_at   | CDH1        | 13.3*| 14.9 | 26.1 | 69.8 | 470.0| 8   | <.0001       |
| 222549_at     | CLDN1       | 8.8  | 11.2 | 12.8 | 34.6 | 253.8| 6   | <.0001       |
| 220428_at     | CD207       | 7.2  | 6.8  | 8.2  | 7.7  | 197.9| 2   | <.0001       |
| 206337_at     | CCR7        | 19.0 | 16.3 | 25.4 | 51.9 | 77.9 | 6   | .0015        |

*Microarray expression values generated by using the robust multiarray average algorithm and processed for global normalization.
**TABLE E4.** Microarray data: mRNAs repressed in TGF-β1–stimulated versus nonstimulated cultures

| Probe set  | Gene symbol | 0 h | 6 h | 24 h | 6 h | 24 h | Sum of calls | P value |
|------------|-------------|-----|-----|------|-----|-----|--------------|---------|
| 209555_s_at | CD36        | 312.3* | 389.9 | 730.6 | 165.1 | 343.5 | 10 | 0.00007 |
| 206682_at  | CLEC10A     | 217.2 | 295.3 | 517.1 | 214.7 | 350.1 | 10 | 0.00310 |
| 204438_at  | MRC1        | 661.0 | 775.3 | 1771.1 | 372.7 | 93.6 | 10 | 0.00001 |
| 203305_at  | FXIII X     | 75.7 | 73.8 | 73.8 | 64.4 | 42.1 | 7 | 0.00435 |

*Microarray expression values generated by using the robust multiaarray average algorithm and processed for global normalization.
**TABLE E5. Microarray data: KLF family member mRNA regulation in TGF-β1–stimulated versus nonstimulated cultures**

| Probe set   | Gene symbol | 0 h     | 6 h | 24 h | 6 h     | 24 h | Sum of calls | P value |
|-------------|-------------|---------|-----|------|---------|------|--------------|---------|
| 210504_at   | KLF1        | 35.9*   | 34.7| 38.9 | 55.6    | 41.8 | 10           | .827    |
| 219371_s_at | KLF2        | 18.6    | 16.8| 19.2 | 15.6    | 17.4 | 1            | .531    |
| 222913_at   | KLF3        | 65.8    | 63.0| 67.0 | 63.1    | 67.2 | 10           | .953    |
| 221841_s_at | KLF4        | 111.4   | 137.6|205.7 |52.2    | 65.0 | 10           | .057    |
| 209212_s_at | KLF5        | 31.5    | 38.4| 31.7 | 26.6    | 32.9 | 7            | .444    |
| 1555832_s_at| KLF6        | 442.8   | 411.8|605.6 |352.4   | 555.3|10           | .010    |
| 1555420_a_at| KLF7        | 28.8    | 32.0| 29.9 | 30.3    | 29.5 | 10           | .976    |
| 219930_at   | KLF8        | 5.0     | 4.6 | 5.4  | 4.5     | 5.2  | 0            | .295    |
| 203543_s_at | KLF9        | 4.8     | 5.0 | 6.2  | 4.7     | 7.5  | 5            | .056    |
| 202393_s_at | KLF10       | 116.9   | 94.9|110.3 |221.3   | 181.6|10           | .014    |
| 218486_at   | KLF11       | 52.0    | 47.1| 55.7 | 47.2    | 63.4 | 10           | .211    |
| 227261_at   | KLF12       | 89.1    | 77.4| 76.8 | 88.2    | 104.2|10           | .062    |
| 225390_s_at | KLF13       | 490.0   | 458.2|578.2 |586.3   | 635.8|10           | .129    |
| 1552814_a_at| KLF14       | 9.9     | 8.9 | 9.7  | 8.4     | 9.4  | 0            | .747    |
| 221302_at   | KLF15       | 25.7    | 23.4| 27.6 | 28.2    | 26.0 | 0            | .668    |
| 226328_at   | KLF16       | 51.8    | 50.6| 60.8 | 46.5    | 58.4 | 2            | .976    |
| 1553891_at  | KLF17       | 6.7     | 7.4 | 7.3  | 5.9     | 5.9  | 0            | .628    |

*Microarray expression values generated by using the robust multiarray average algorithm and processed for global normalization.
**TABLE E6.** Ingenuity Pathway Analysis: Notch pathway genes regulation in TGF-β1–stimulated versus nonstimulated cultures

| Gene symbol | −TGF-β1 0 h vs 6 h | −TGF-β1 0 h vs 24 h | +TGF-β1 0 h vs 6 h | +TGF-β1 0 h vs 24 h |
|-------------|-------------------|-------------------|------------------|------------------|
| HES1        | 1                 | 1                 | 1.974            | 1.926            |
| JAG1        | 1                 | 1.365             | −1.975           | 1.857            |
| JAG2        | 1                 | 1                 | 1.764            | 1.814            |
| MAML2       | 1                 | 1                 | 1.419            | 1.707            |
| PSEN1       | 1                 | 1.354             | 1.522            | 1.573            |
| MAML3       | 1                 | 1                 | 1               | 1.319            |
| NOTCH1      | 1                 | −1.341            | 1.446            | 1.316            |
| ADAM17      | 1                 | 1                 | 1               | 1.3              |
| CNTN1       | 1                 | 1.304             | 1               | 1               |
| PSEN2*      | 1                 | 1                 | −1.356           | 1               |
| MAG         | −1.305            | 1                 | 1               | 1               |
| MFNG        | 1.342             | 1                 | 1               | 1               |
| PSEN2*      | 1.375             | 1                 | 1               | 1               |
| NOTCH2      | 1                 | −1.367            | 1               | −1.545           |
| DTX4        | 1                 | 1.374             | −2.293           | −1.994           |

*PSEN2 expression was detected by using 2 Affymetrix probe sets: 204261_s_at for “0 h vs 6 h −” and 211373_s_at for “0 h vs 6 h +.”*