Supplemental Data – CXCR3 letter to JID

Mice. C3H/HeJ +/+ (JR# 659) inbred mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Mice were maintained in a humidity-, temperature-, and light cycle (12:12) controlled vivarium under specific pathogen-free conditions (http://jaxmice.jax.org/html/health/quality_control.shtml#Animalhealth). Mice were housed in double-pen polycarbonate cages (330 cm^2 floor area) at a maximum capacity of four mice per pen. Mice were allowed free access to autoclaved food (NIH 31, 6% fat; LabDiet 5K52, Purina Mills, St. Louis, MO) and acidified water (pH 2.8-3.2).
All studies were done using Institutional Animal Care and Use Committee approved protocols.

Histology. Mice were euthanized by CO_2 asphyxiation. The dorsal skin was surgically removed at the time of necropsy, laid flat on aluminum foil in a cranio-caudal orientation, and fixed overnight by immersion in Fekete's acid-alcohol-formalin solution and then transferred to 70% ethanol (Seymour *et al.*, 2004). The skin was trimmed, processed routinely, embedded in paraffin, sectioned at 6 um, and stained with hematoxylin and eosin (H&E).

Affymetrix® gene arrays. To follow the progression of alopecia areata we performed full thickness skin grafts (from mice with AA or normal mice) onto young normal C3H/HeJ mice as described in detail elsewhere (McElwee *et al.*, 1998) and collected skin distant from the engraftment site for RNA extraction and histopathology at 5, 10, and 20 weeks after surgery as well as age and gender matched mice with spontaneous AA or that were unaffected, both clinically and histologically normal. RNA samples from 3 AA graft and 3 normal graft-recipient mice were used at each time point. RNA was extracted by our Institutional Gene Expression Shared Service. Samples were tested using the Affymetrix *GeneChip Mouse Genome 430 2.0 Array*® (Affymetrix, Santa Clara, CA).

Briefly, skin and spleen samples were stored in RNAlater (Ambion, Austin, TX) per the manufacturer's instructions and homogenized in TRIzol (Invitrogen, Carlsbad, CA). Total RNA was isolated by standard TRIzol methods, and quality assessed using a 2100 Bioanalyzer instrument and RNA 6000 Nano LabChip assay (Agilent Technologies, Palo Alto, CA). Following reverse transcription with an oligo(dT)-T7 primer (Affymetrix, Santa Clara, CA), double-stranded cDNA was synthesized with the GeneChip Expression 3' –Amplification One-cycle kit (Affymetrix).

In an *in vitro* transcription (IVT) reaction with T7 RNA polymerase, the cDNA was amplified and labeled with biotinylated nucleotides (Affymetrix). Ten micrograms of biotin-labeled and fragmented cRNA were hybridized onto MOE430v2.0 GeneChip™ arrays (Affymetrix) for 16 hours at 45°C. Post-hybridization staining and washing was done according to the manufacturer's protocols using a Fluidics Station 450 instrument (Affymetrix). Finally, the arrays were scanned with a GeneChip™ Scanner 3000 laser confocal slide scanner. The images were quantified using GCOS 1.0 software (GeneChip™ Operating Software, Affymetrix). Data were imported into the R software environment and analyzed using the R/MAANOVA package comparing AA engrafted mice versus sham controls at the various time points. After graphical diagnostics and appropriate transformations, an analysis of variance (ANOVA) model was applied to the

data, and F1, F2, F3 and Fs test statistics constructed along with their permutation p-values.

Large scale quantitative real time RT-PCR arrays.

Generation of a T cell and NK activation specific QPCR array. We first used an updated, 384-immune response gene set version of the original 96-gene set ImmunoQuant Array (IQA) developed at The Jackson Laboratory (Akilesh *et al.*, 2003). Non-informative gene sets were replaced with all the genes in the canonical pathway for cytotoxic T cell, helper T cell, and NK cell activation (Ingenuity Pathways Analysis®, Redwood City, CA) as well as genes predicted to be important in human alopecia areata. QPCR primer pairs for modifying the array were designed using a proprietary primer selection algorithm optimized for SYBR Green® QPCR technology (Bar Harbor BioTechnolgy, Inc.). Pipmaker analysis (http://bio.cse.psu.edu/pipmaker/) was used to update definitions of conserved sequence elements between the mouse and human genomes for relevant genes.

PCR amplicon development. Primer sets (MWG Biotech, High Point, NC) were searched against GenBank via the NCBI Blast algorithm to ensure specificity to the desired gene target. Each PCR product was subjected to bidirectional sequencing using each end-specific primer on the ABI Prism 3700 Sequencer. SYBR Green® dissociation curves were generated via the ABI 7900HT Real-Time PCR System to further ensure the generation of a single PCR product under experimental reaction conditions.

RNA preparation. RNA was obtained from mice in the longitudinal skin graft study along with spontaneously affected and clinically normal controls as described above.

Real-time quantitative PCR. AA QuantArray 384-well plates were prepared robotically by adding primers to each well, and then drying down the mixture to maintain an extended shelf-life. SYBR Green® PCR Master Mix (ABI), and sample cDNA were then added to the plates. The plate was sealed using an Optical Adhesive Cover (ABI) and the fluid spun down in a swinging bucket centrifuge. Real-time PCR data were collected on an ABI 7900HT Real-Time PCR System using the default reaction conditions (1 cycle 50°C for 2 minutes, 1 cycle 95°C for 10 minutes, 40 cycles 95°C for 15 seconds and 60°C for 1 minute). The baseline and threshold were set to experimentally determined values and the Experimental Report data (a table of Ct values for each of the 384 reactions) exported for data analysis.

Global Pattern Recognition (GPR). The GPR is a novel, robust algorithm used to compute significant changes in gene expression profiles from complex QPCR data set (Livak and Schmittgen, 2001). It overcomes the ambiguities in comparing QPCR data sets using conventional single gene normalization procedures by utilizing all QPCR dataset for normalization purposes. Results comparing AA engrafted mice versus sham controls at the various time points were reported as a GPR score which is the fraction of normalizer genes in the entire dataset. This score was validated using a novel geometric-mean statistics (Akilesh *et al.*, 2003).

ELISA. Recipient mice were purchased (The Jackson Laboratory) and donor mice were shipped from Dr. Sundberg to The Ohio State University three weeks before mice were grafted as describe above. Starting two weeks before surgery, recipient mice were fed a control unpurified diet (chow, Harlen Teklad 7912, Indianapolis, IN). Body weight, food

intake, and hair loss were measured (Tang *et al.*, 2004) and photographs taken weekly. At 5, 10, 15, and 20 weeks post grafting, dorsal and ventral skin was collected and flash frozen in liquid nitrogen. All procedures were done with approval of The Ohio State University IACUC. Frozen skin sections were thawed in protein isolation solution containing protease and phosphatase inhibitors (ThermoFisher, Waltham, MA). The skin was homogenized using a tissue homogenizer and then underwent three cycles of sonication, freezing, and thawing. The resulting solutions were then spun to remove nucleic acids and lipids. The supernatants were assayed for total protein concentration uisng a *DC* Protein Assay Kit (Bio-Rad, Hercules, CA). The assayed supernatants were used to determine CXCL9 levels via Enzyme-Linked Immunosorbant Assays (ELISAs; R&D Systems, Minneapolis, MN). Briefly, 96-well plates with high protein binding affinity were incubated overnight with a solution containing the appropriate capture antibody. The next day, the plates were blocked (1% BSA, Fraction V, Fisher Scientific, Pittsburgh, PA) prior to being incubated with the skin supernatants and protein standards. Then the plates were washed and incubated with the appropriate biotin-labelled detection antibody. The plates were washed and incubated with streptavidin-HRP, then washed and incubated with substrate (TMB Ultra 1 Step, Fisher Scientific, Pittsburgh, PA). The reaction was stopped with 2N sulfuric acid. The resulting changes in color were determined using a plate reader (Spectra Max 190; Molecular Devices, Sunnyvale, Ca) and concentrations calculated in Excel (Microsoft, Redman, WA). Data were analyzed using SPSS, v19 (IBM; Armonk, New York) after consultation with the OSU statistical consulting services. CXCL9 was analyzed by univariate analysis of variance using a full factorial model of graft type and week post surgery after log transformation and outlier removal. This was followed by Tukey post hoc tests when appropriate.

References:

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