*Appendix S1*

## MATERIAL AND METHODS

### *Patients and controls*

Fifty adult patients (18 women and 32 men, mean ± SD age 38 ± 16.7 years) with a diagnosis of non-segmental vitiligo were enrolled in the study after providing informed consent. The patients were divided into 2 groups based on whether new lesions had appeared and/or existing lesions were spreading within the previous 6 months. If a patient provided an affirmative response to one or both of these questions, the patient was included in the progressive vitiligo group. In contrast, patients with no increase in lesion number or size were included in the stable vitiligo group. Thirty-eight patients were classified with progressive vitiligo (14 women and 24 men, age 38 ± 16 years), whereas 12 patients were included in the stable vitiligo group (4 women and 8 men, age 38 ± 20 years). A medication-free period was required for both groups, which consisted of no oral drugs for at least one month and no topical medications or phototherapy for 2 weeks prior to the study procedures. As a control, 20 healthy individuals from Fudan University were enrolled in our study. The institutional review boards of Zhongshan Hospital of Fudan University approved the protocols involving human subjects. Informed consent was obtained in writing from all subjects before study initiation.

Four-millimeter skin biopsies were obtained from 18 vitiligo patients. Six healthy tissue specimens for control and neonatal foreskin for primary human melanocyte cultures were obtained from individuals undergoing orthopedic surgery. The patients and healthy individuals provided informed consent prior to tissue collection. The study protocol was reviewed and approved by the Zhongshan Hospital research ethics committee.

### *Immunohistochemistry*

Tissues from 18 vitiligo patients were processed and embedded in paraffin using routine methods (13). Tissue blocks were serially sectioned to obtain consecutive sections. Immunohistochemistry for IFN-$\gamma^+$ cells and $CD8^+$ T cells was performed using the Novolink Polymer Detection System (Novocastra, Newcastle-upon-Tyne, UK) according to the manufacturer's instructions. Briefly, paraffin-embedded sections were first deparaffinised and then hydrated. After microwave antigen retrieval, endogenous peroxidase activity was blocked by the incubation of slides in 0.3% $H_2O_2$, whereas nonspecific binding sites were blocked with Protein Block (Novocastra). Primary mouse monoclonal antibodies directed against IFN-γ or CD8 (Dako, Copenhagen, Denmark) were used for IFN-γ and CD8 staining. Following serial incubations with primary antibodies, biotinylated goat anti-mouse immunoglobulin and respective anti-mouse HRP-conjugated secondary antibodies (Novolink polymer; Novocastra), the sections were developed in a diaminobenzidine solution and counterstained with haematoxylin. Sections incubated in mouse isotype IgG only served as negative controls.

For IFN-$\gamma^+$ cell and $CD8^+$ cell quantification, serial skin sections were examined microscopically at high power (× 400). Five high-power fields of each sample were selected for digital photographs. Two independent observers determined the number of IFN-$\gamma^+$ cells and CD8+ cells in each field of these same 5 areas by manual count.

### *Flow cytometry detection*

All blood samples were processed on the day of collection. Flow cytometry was performed according to the previously reported method (13). Briefly, peripheral blood mononuclear cells (PBMCs) were purified from Ficoll gradients (GE Healthcare, Uppsala, Sweden). For NK cell detection, PBMCs were stained with PerCP-conjugated anti-CD3 (eBioscience) and PE-conjugated anti-CD56 antibodies (eBioscience, San Diego, CA) for 20 min. Stained cells were then fixed in 1% paraformaldehyde, and 4-colour flow cytometric analyses were performed using FACSCalibur (BD Biosciences, San Jose, CA) and FlowJo software (Tree Star, San Carlos, CA). $CD3^-$ $CD56^+$ NK cells were analysed in the PBMC samples. For IFN-γ detection, PBMCs were incubated for 4–5 h in RPMI 1640 medium (containing 10% foetal bovine serum [FBS]) with 50 ng/ml phorbol myristate acetate (PMA) and 750 ng/ml ionomycin in the presence of 1.7 μg/ml monensin (Enzo Biochem, Farmingdale, New York) in a tissue culture incubator at 37°C. For intracellular staining of IFN-γ, PBMCs were first stained with PerCP-conjugated anti-CD3 and FITC-conjugated anti-CD8 antibodies (eBioscience); the cells were then fixed and permeabilised using FIX & PERM (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Permeabilised cells were then stained with PE-conjugated anti-IFN-γ (eBioscience) for 20 min. Stained cells were then fixed in 1% paraformaldehyde, and 4-colour flow cytometric analyses were performed using FACSCalibur and FlowJo software. For the detection of $CD8^+$ CTLs and Th1 cells, IFN-$\gamma^+$ $CD8^+$ and IFN-$\gamma^+$ $CD8^-$ cells were analysed from the cells first included in the $CD3^+$ gate.

### *Primary human melanocyte cultures*

Primary human melanocyte cultures were obtained from neonatal foreskin. The epidermis was separated from the dermis after an overnight incubation of skin samples in a 0.25% Dispase solution (Sigma-Aldrich, St. Louis, USA) in PBS at 4–8°C. To separate cellular elements, epidermal sheets were incubated at 37°C in a solution of 0.25% trypsin and 0.02% ethylenediamine-tetraacetic acid (EDTA) in PBS for 10 min. The cellular suspension was then filtered through a 70-mm cell strainer (BD Falcon) and centrifuged at 1,000 rpm for 5 min to harvest the cells. Then, the melanocytes were selectively grown in defined medium254 with human melanocytes growth supplements (Cascade Biologics, Portland, USA). The cells were maintained in a humidified incubator with 5% $CO_2$ at 37°C. The cells were fed every 3 days and further passaged at 1:2 when they became 80% confluent. All experiments were performed with cells grown for 3 to 4 passages.

### *Measurement of cellular melanin contents*

Melanin production was determined as described previously (14). Briefly, one day after plating $5\times10^4$ melanocytes in a 6-well culture dish, the cells were treated with 100–400 ng/ml of recombinant IFN-γ for 48 h. To determine the melanin content, the treated cell pellets were washed with PBS and dissolved in 500 μl of 1 N NaOH for 2 h in a 70°C water bath. Each lysate (100 μl) was placed in a well on a 96-well microplate, and the absorbance at 562 nm was measured with a microplate spectrophotometer (BD Bioscience, San Jose, USA). The protein concentration of each sample was determined by the Bio-Rad Protein Assay (Bio-Rad, Richmond, USA). The intracellular melanin concentration is displayed as percentage values. Each percentage value in the IFN-γ-treated cells was calculated with respect to the medium254-treated control cells. This assay was performed in triplicate over 3 experiments.

### *Analysis of transcription factor mRNA expression*

Total RNA was purified with the TRIzol regent (Invitrogen). Complementary DNA (cDNA) samples were synthesised using a PrimeScript RT reagent kit (TAKARA Bio INC, Dalin, China), and mRNA expression was examined with a Bio-Rad iCycler optical system (Bio-Rad, USA) using SYBR Premix Ex Taq (TAKARA Bio INC). GAPDH was used as a positive control. The 2−△△Ct method was used to normalise gene transcription

to GAPDH cDNA and to calculate the fold induction relative to the control. The PCR primer sequences were designed as follows:
TYR fwd. (5'-GAGGAGTACAACAGCCATCAGTC-3'); TYR rev. (5'-AGGCATCCGCTATCCCAGT-3'); TYP-1 fwd. (5'-TTCCACTCTAATAAGCCCAAACT-3'); TYP-1 rev. (5'-AAGCACTGAGCGACATCCTG-3'); MITF fwd. (5'-AGACATGCGCTGGAACAAG-3'); MITF rev. (5'-ACCCGTGGATGGAATAAGG-3'); GAPDH fwd. (5'-AGAAGGCTGGGGCTCATTTG-3'); and GAPDH rev. (5'-AGGGGCCATCCACAGTCTTC-3'). *TYR=tyrosinase; TYP-1=tyrosinase related protein-1; MITF=microphthalmia associated transcription factor; GAPDH=Glyceraldehyde-3-phosphate dehydrogenase.

### *Cell apoptosis analysis*

The analysis of changes in apoptotic melanocyte morphology was evaluated by microscopy and Hoechst fluorescence staining (15). Briefly, after a 48-h incubation with recombinant human IFN-γ, melanocytes were observed microscopically under polarised light, and then were fixed, washed twice with PBS, and stained with Hoechst 33258 staining solution according to the manufacturer's instructions (Invitrogen). Changes in the melanocyte nuclei after Hoechst 33258 staining were observed under a confocal laser scanning microscope (LSM 510 META DuoScan; Carl Zeiss, Jena, Germany), and apoptotic melanocytes with chromatin condensation and nuclear fragmentation were evaluated quantitatively by analysing at least 5 different fields (× 400) by 2 independent observers.

The quantitative evaluation of apoptotic cells was performed via an Annexin V-PI double staining assay using a FACScan flow cytometer (Becton-Dickinson, USA). After 48 h of treatment with 100, 200 and 400 ng/ml recombinant human IFN-γ in a 6-well plate at 37°C, the cells were harvested, washed with ice-cold PBS, resuspended in 100 µl of binding buffer and incubated with 5 µl Annexin V-FITC (Invitrogen) and 5 µg/ml propidium iodide (PI) (Invitrogen) for 15 min at room temperature in the dark. The samples were analysed by flow cytometry to identify apoptotic cells. Cells without treatment were used as a negative control. The results were analysed using Flowjo 7.6 software. Annexin $V^+/PI^-$ cells were characterised as early or primary apoptotic cells, and Annexin $V^+/PI^+$ cells were characterised as late apoptotic or secondary apoptotic. Annexin $V^-/PI^+$ cells were characterised as necrotic (16). This assay was performed in triplicate over 3 experiments.

### *Statistical analysis*

Data analysis was performed with SPSS version 12.0 for Windows software (SPSS Inc., Chicago, IL). Quantitative data were expressed as the mean ± SD. Statistical significance was determined by an analysis of variance followed by the Bonferroni *post hoc* test for comparisons of multiple means or Student's *t*-test. Correlations were determined by Spearman's ranking. A $p$-value of $< 0.05$ was considered significant.