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INVESTIGATIVE REPORT

Interferon-gamma Inhibits Melanogenesis and Induces Apoptosis in Melanocytes: A Pivotal Role of CD8$^+$ Cytotoxic T Lymphocytes in Vitiligo

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Increased expression of the cytokine interferon (IFN)-γ plays a pivotal role in vitiligo-induced depigmentation. However, the major source of IFN-γ in vitiligo patients and the mechanisms underlying melanocyte destruction are unknown. In this study, a large number of skin infiltrating IFN-γ$^+$ cells and CD8$^+$ T cells were detected in progressive vitiligo. Among the peripheral blood mononuclear cells (PBMCs) of vitiligo patients, CD8$^+$ cytotoxic T lymphocytes (CTLs) that express IFN-γ exhibited significant expansion, which suggests that activated CTLs are the main source of increased IFN-γ in progressive vitiligo. An *in vitro* analysis demonstrated that IFN-γ inhibits melanogenesis in primary cultured human melanocytes by altering melanogenic enzyme mRNA expression and, more importantly, that IFN-γ directly induces melanocyte apoptosis. Our data indicate that vitiligo pathophysiology may be linked to globally activated CD8$^+$ CTL subpopulations, which produce increased IFN-γ and induce melanocyte dysfunction and apoptosis. *Key words: interferon-gamma; vitiligo; cytotoxic T lymphocytes; melanocytes; apoptosis.*

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Vitiligo is characterised by a loss of melanocytes from the epidermis, which results in white macula on the skin (1). Numerous histological and laboratory data support the hypothesis that apoptosis, rather than necrosis, is the mechanism for melanocyte depletion (1). Apoptosis can be induced by a variety of factors, including immune cytokines, various environmental chemicals or other molecular mechanisms. The exact mechanism for melanocyte apoptosis remains unknown, but autoimmune factors have been strongly implicated in the development of the disease, especially non-segmental vitiligo.

Cytokines are small immune-regulatory molecules that can generate an inappropriate immune response when imbalanced (2). Previous studies (3) have demonstrated cytokine imbalance in the skin of vitiligo patients, suggesting a role for cytokines in autoimmunity. Cytokines, such as interferon (IFN)-γ, soluble interleukin-2 receptor (sIL-2R), IL-10, IL-13 and IL-17A (4–6), have been reported to be increased in vitiligo skin; however, no direct evidence for the role of cytokines in vitiligo melanocyte loss has been observed (7).

IFN-γ is a pleiotropic cytokine that is a key regulator of the immune system (8). In addition to host defence, IFN-γ also contributes to autoimmune pathology by inducing autoantibodies, activating autologous cytotoxic T cells and inducing target cell apoptosis (9, 10). It has been proposed that melanocyte death in vitiligo is mediated by apoptosis in the context of autoimmunity and that increased IFN-γ may play a role in the pathogenesis of this disease (1). However, solid evidence for this hypothesis is lacking. The effect of IFN-γ on melanogenesis and melanocyte cell fate remains unknown. Moreover, the major IFN-γ-secreting cells in human are T helper 1 (Th1) cells, natural killer (NK) cells and CD8$^+$ cytotoxic T lymphocytes (CTLs) (11, 12). To date, the primary source of increased IFN-γ in vitiligo patients has not been fully elucidated.

To address these issues, 50 vitiligo patients were enrolled in this study. The IFN-γ-producing cell frequency was analysed in the skin samples or peripheral blood of vitiligo patients with progressive or stable disease, respectively. The ability of recombinant IFN-γ to affect melanogenesis in primary culture melanocytes and to induce melanocyte apoptosis was also assessed *in vitro*.

MATERIAL AND METHODS (see Appendix S1[1])

RESULTS

Increased frequency of IFN-γ$^+$ cells and CD8$^+$ T cells in vitiligo skin tissue samples

To visualise IFN-γ$^+$ cells and CD8$^+$ T cells in vitiligo skin, we performed IFN-γ and CD8 immunohistochemical staining. The number of cells staining positive for these markers was identified from non-lesional, perilesional and lesional skin samples from vitiligo

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patients and normal controls. Perilesional skin samples from patients with progressive vitiligo exhibited pathologic changes, including a large number of infiltrating IFN-γ^+ cells and $CD8^+$ cells predominantly adjacent to the epidermal basal layer (Fig. 1a).

IFN-γ^+ cell counts in each high-power field (×400) were significantly increased in 16 perilesional skin samples from progressive vitiligo patients (mean ± SD 9.87 ± 3.43) compared with skin from 6 healthy individuals (3.50 ± 1.85, $p<0.01$), 2 lesional skin samples from patients with stable disease (4.59 ± 1.32, $p<0.05$) or 8 non-lesional vitiligo skin samples (4.64 ± 1.43, $p<0.05$) (Fig. 1b). Consistent with the results of IFN-γ^+ cell counts, we observed that $CD8^+$ T lymphocyte infiltration was also significantly increased in the 16 perilesional skin samples from patients with progressive vitiligo (22.96 ± 2.29) compared to any of the other sample types: skin samples from 6 healthy individuals (12.79 ± 6.89, $p<0.05$), lesional skin from patients with stable disease ($n=2$; 10.83 ± 6.53, $p<0.05$) or non-lesional vitiligo skin ($n=8$; 11.19 ± 4.4, $p<0.05$) (Fig. 1c). Importantly, there was a positive correlation between the number of IFN-γ^+ cells and $CD8^+$ T cells in 16 perilesional skin samples from progressive vitiligo patients (Spearman correlation coefficient, $r=0.839$, $p<0.001$) (Fig. 1d). No significant difference in IFN-γ^+ or $CD8^+$ cell counts was observed between lesional skin and non-lesional skin from vitiligo patients or healthy skin from controls ($p>0.05$). These results provide evidence that IFN-γ-producing cells infiltrate in close proximity to $CD8^+$ T cells in the perilesional skin of vitiligo patients, and both cells may be involved in the vitiligo disease process.

Highly enriched circulating IFN-γ^+ $CD8^+$ cytotoxic T lymphocytes in vitiligo patients

NK cells, $CD4^+$ Th1 lymphocytes and $CD8^+$ CTLs are reported to be the major producers of IFN-γ (11, 12). In our study, PBMCs obtained from 38 progressive vitiligo patients, 12 stable vitiligo patients, and 20 healthy controls were analysed for the frequency of $CD3^-$ $CD56^+$ NK cells, $CD3^+$ $CD8^-$ IFN-γ^+ Th1 cells and $CD3^+$ $CD8^+$ IFN-γ^+ CTLs using flow cytometry (Fig. 2). A significantly increased number of circulating IFN-γ^+ $CD8^+$ CTL cells among $CD3^+$ T cells was observed in progressive vitiligo (14.80 ± 3.56%; $p<0.01$) and stable vitiligo (13.46 ± 3.69%; $p<0.01$) patients compared with healthy controls (8.66 ± 2.59%) (Fig. 2a, b). No significant difference in the numbers of circulating IFN-γ^+ $CD8^-$ Th1 cells in $CD3^+$ T cells was observed between patients with progres-

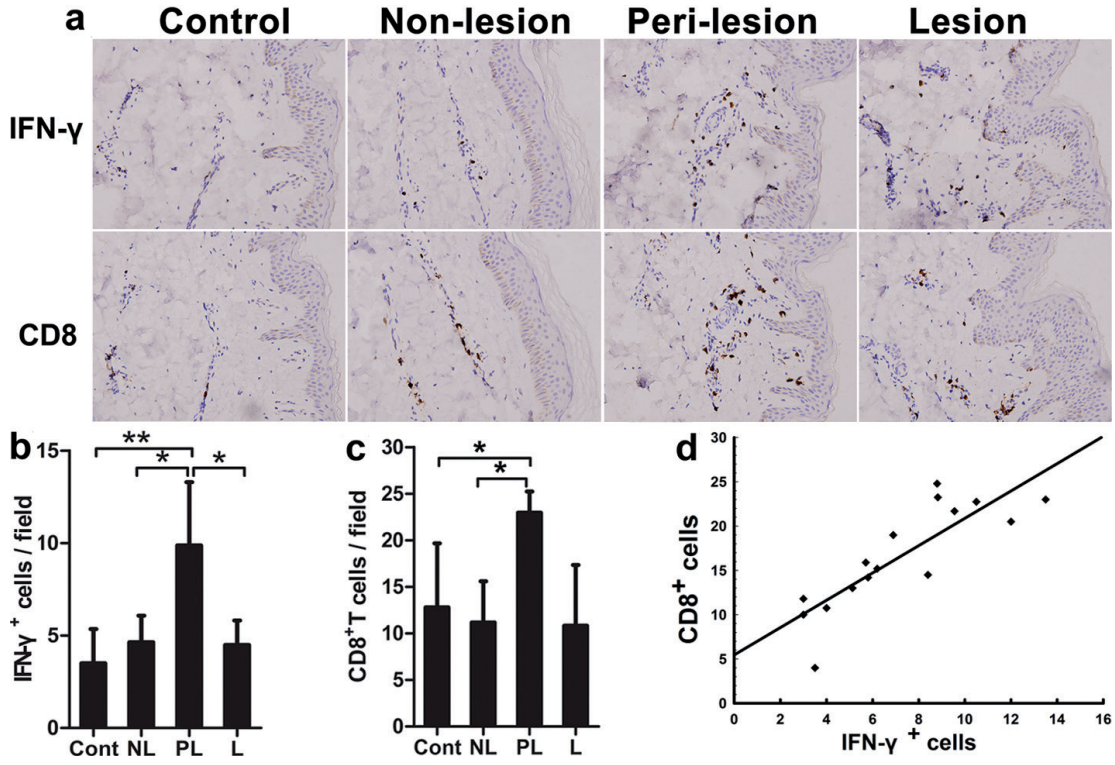

Fig. 1. Skin-infiltrating interferon (IFN)-γ^+ cells and $CD8^+$ cells in vitiligo patients. (a) Consecutive sections were used for immunohistochemical detection of T cells expressing IFN-γ or CD8 in skin samples from healthy controls or vitiligo patients (representative fields, ×200). Positive cells appear brown. Both IFN-γ^+ (b) and $CD8^+$ (c) T-cell numbers were increased significantly in the perilesional (PL) tissues of progressive vitiligo patients ($n=16$). In contrast, no significant differences were observed between lesional (L) ($n=2$) and non-lesional vitiligo skin (NL) ($n=8$), as compared to normal skin from unaffected adults (Cont) ($n=6$). *$p<0.05$, **$p<0.01$. (d) The number of IFN-γ^+ and $CD8^+$ T cells in perilesional vitiligo skin samples exhibited a positive correlation ($r=0.839$, $p<0.001$; $n=16$). Values in B–C are the mean and SD.

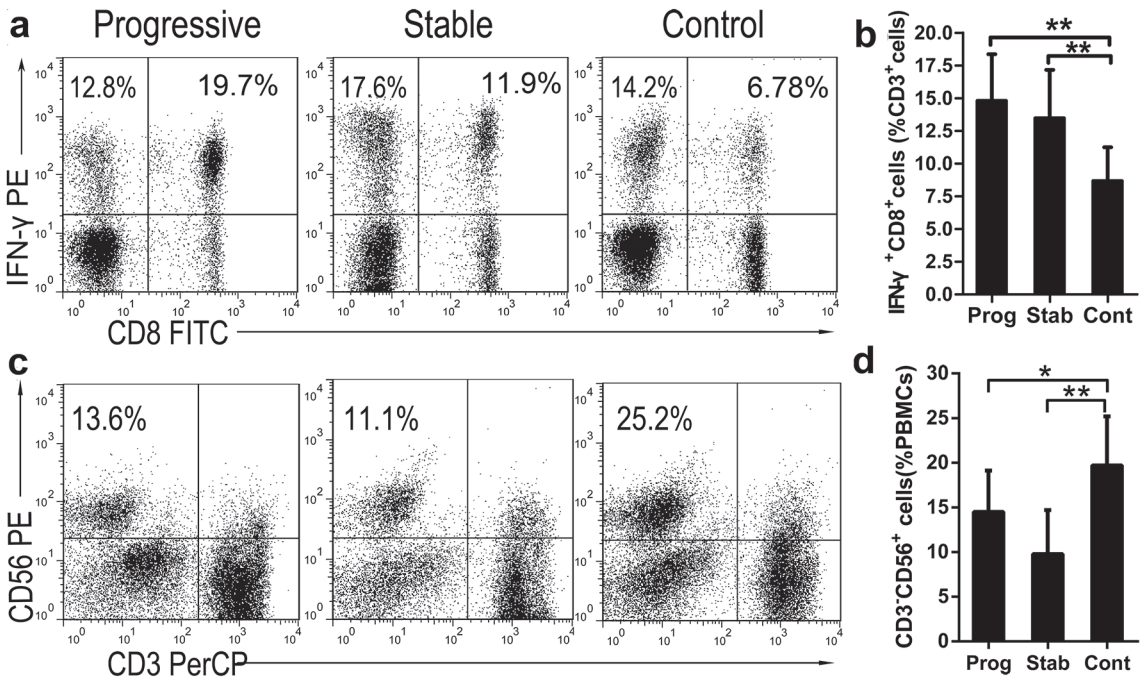

Fig. 2. Increased $CD8^+$ cytotoxic T lymphocytes (CTLs) and reduced natural killer (NK) cells in peripheral blood mononuclear cells (PBMCs) from vitiligo patients. (a) Interferon (IFN)-γ was detected by intracellular staining. The values are the percentage of IFN-γ^+ $CD8^-$ and IFN-γ^+ $CD8^+$ cells among $CD3^+$ T cells (Representative cases). (b) Flow cytometric analysis of IFN-γ^+ $CD8^+$ cells within the $CD3^+$ T-cell populations from patients with progressive vitiligo ($n=38$), stable vitiligo ($n=12$) and control subjects ($n=20$). (c) Human PBMCs were labelled with antibodies specific for NK cells (CD3 and CD56). The values are the percentage of $CD3^-$ $CD56^+$ NK cells among PBMCs (representative cases). (d) The results of flow cytometric analysis of NK cells in patients with progressive vitiligo ($n=38$), stable vitiligo ($n=12$) and control subjects ($n=20$). $^*p<0.05$, $^{**}p<0.01$.

sive vitiligo (13.53 ± 7.54%; $p>0.05$) and stable vitiligo (15.40 ± 6.62%; $p>0.05$) compared with healthy controls (15.06 ± 6.55%). Interestingly, our data demonstrate significantly reduced percentages of $CD3^-$ $CD56^+$ NK cells among PBMCs in active vitiligo (14.50 ± 4.62%; $p<0.05$) and inactive vitiligo (9.78 ± 4.93%; $p<0.01$) patients compared with healthy controls (19.70 ± 5.50%) (Fig. 2 c, d). Our results suggest that activated $CD8^+$ CTLs are the major cellular source of IFN-γ in progressive vitiligo patients.

IFN-γ inhibits melanocyte melanogenesis by altering melanogenic enzyme mRNA expression

To determine the effect of IFN-γ on melanocyte melanogenesis *in vitro*, we treated melanocytes with 100–400 ng/ml recombinant human IFN-γ for 48 h and determined the intracellular melanin concentration. Our data indicate that IFN-γ inhibits melanin synthesis in a dose-dependent manner compared with control melanocytes without IFN-γ treatment (Fig. 3a). To investigate the mechanism underlying the IFN-γ-mediated inhibition of melanin synthesis, we analysed the transcription levels of melanogenic enzymes, including tyrosinase, tyrosinase-related protein 1 (TRP1) and microphthalmia-associated transcription factor (MITF), using real-time PCR. As shown in Fig. 3b, 3 concentrations of IFN-γ significantly inhibited tyrosinase mRNA expression. In addition, increased IFN-γ altered MITF mRNA expression, whereas TRP-1 expression was slightly increased in melanocytes treated with 3 doses of IFN-γ. These data suggest that

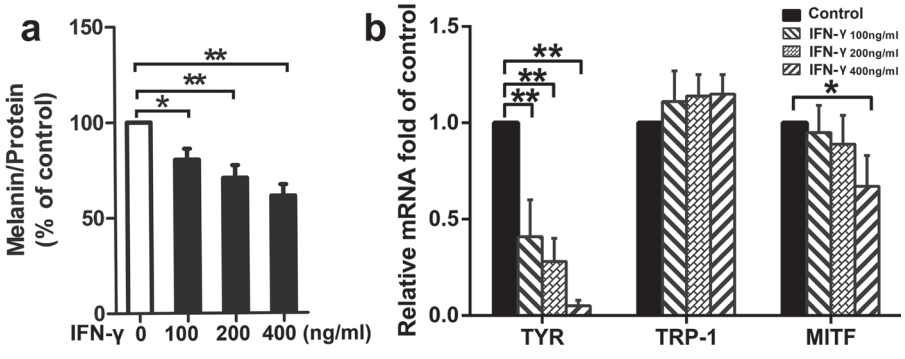

Fig. 3. Inhibitory effects of interferon (IFN)-γ on melanogenesis in melanocyte. Melanocytes were incubated with or without 100–400 ng/ml IFN-γ for 48 h. (a) Melanin synthesis in melanocytes were determined. (b) Real-time PCR were performed to analyse transcription levels of melanogenic enzymes, including tyrosinase (TYR), tyrosinase-related protein 1 (TRP-1) and microphthalmia-associated transcription factor (MITF). The values represent the mean of 3 independent experiments performed in triplicate ± SD. $^*p<0.05$, $^{**}p<0.01$.

IFN-γ may inhibit melanogenesis by, at least in part, reducing mRNA expression of the primary melanogenic enzymes.

IFN-γ induces melanocyte apoptosis in vitro

To determine the effect of IFN-γ on melanocyte apoptosis *in vitro*, we treated melanocytes with 100, 200 and 400 ng/ml recombinant human IFN-γ for 48 h. Then, cellular apoptosis was evaluated by microscopy, Hoechst 33258 fluorescence staining and an Annexin V-PI double staining assay. Using polarised light microscopy, the melanocytes appear aggregated and varied in shape after treatment with low levels of IFN-γ. Medium and high levels of IFN-γ caused more marked changes in melanocytic morphology, including cell flattening and partial loss of bipolar dendrites, whereas the untreated control cells presented with a spindle-shaped morphology (Fig. 4a). These results indicate that melanocyte survival is drastically affected by IFN-γ.

Using Hoechst 33258 staining, we demonstrate that IFN-γ treatment results in concentration-dependent increases in chromatin condensation and nuclear fragmentation, which is the most significant change in an apoptotic cell (Fig. 4b). Apoptotic melanocyte counts in each high-power field (×400) were significantly increased in melanocytes treated with 400 ng/ml recombinant human IFN-γ (mean ± SD 22.2 ± 7.16) compared with those with 200 ng/ml IFN-γ (11 ± 2.65, $p<0.05$), 100 ng/ml IFN-γ (4 ± 1.58, $p<0.01$) or negative control (0.6 ± 0.89, $p<0.01$). Significant difference was also observed between melanocytes treated with 200 ng/ml IFN-γ and those with 100 ng/ml IFN-γ ($p<0.05$) or negative control ($p<0.01$). Even the number of apoptotic melanocytes in melanocytes treated with 100 ng/ml IFN-γ was significantly increased compared to those

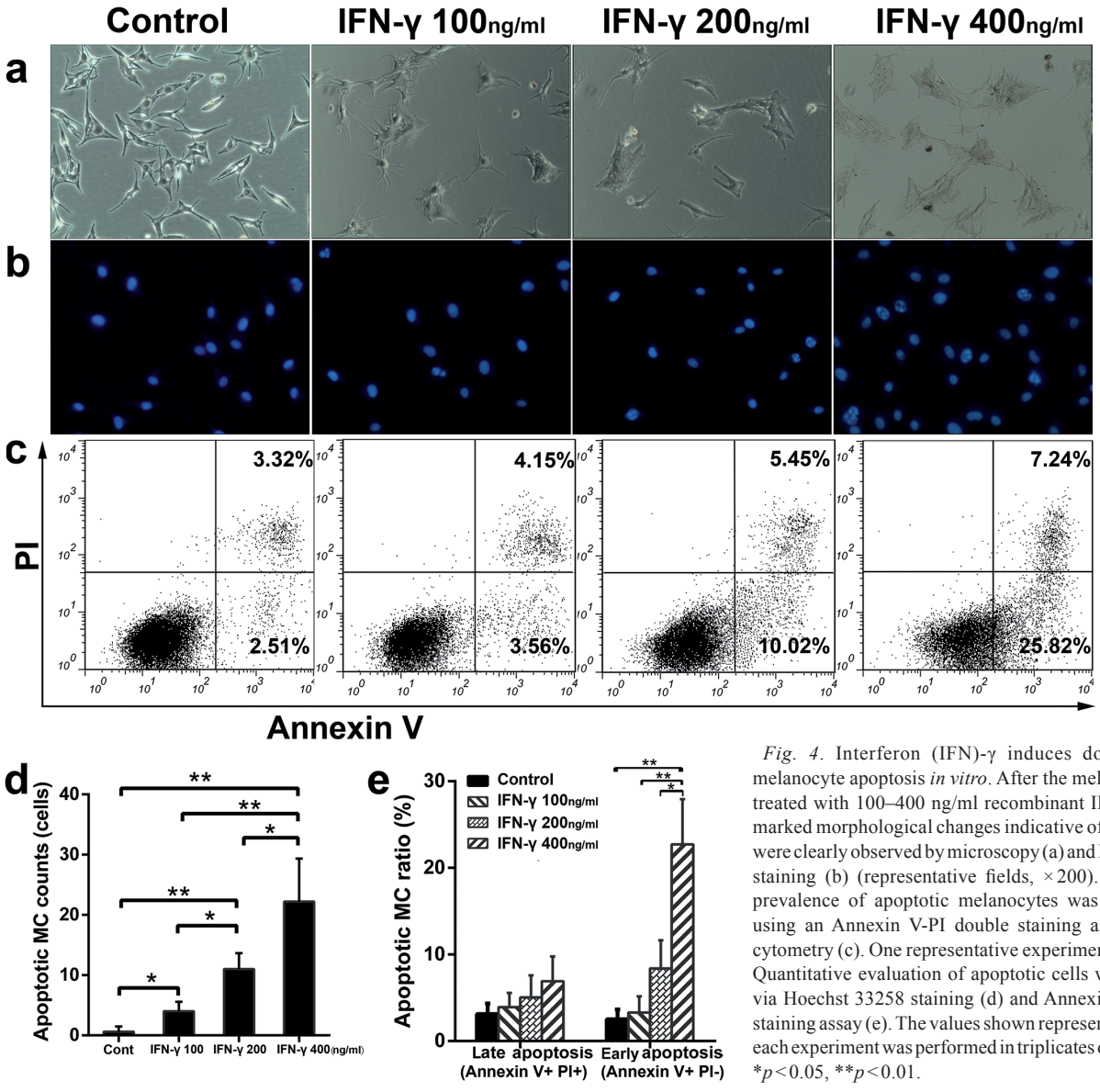

Fig. 4. Interferon (IFN)-γ induces dose-dependent melanocyte apoptosis *in vitro*. After the melanocytes were treated with 100–400 ng/ml recombinant IFN-γ for 48 h, marked morphological changes indicative of cell apoptosis were clearly observed by microscopy (a) and Hoechst 33258 staining (b) (representative fields, ×200). An increased prevalence of apoptotic melanocytes was demonstrated using an Annexin V-PI double staining assay and flow cytometry (c). One representative experiment is presented. Quantitative evaluation of apoptotic cells was performed via Hoechst 33258 staining (d) and Annexin V-PI double staining assay (e). The values shown represent means ± SD; each experiment was performed in triplicates over 3 samples. *$p<0.05$, **$p<0.01$.

without IFN-γ treatment ($p < 0.05$) (Fig. 4d). These results indicate that IFN-γ appears to directly induce melanocyte apoptosis in a concentration-dependent manner.

We next performed an Annexin V-PI double staining assay to quantify melanocyte apoptosis using a FACScan flow cytometer. Fig. 4c shows the frequency of apoptotic melanocytes treated with various IFN-γ concentrations. The majority of untreated melanocytes were viable and non-apoptotic (Annexin V^- PI^-), and few melanocytes were necrotic (Annexin V^-/PI^+). However, increasing IFN-γ doses resulted in an increase in early or primary apoptotic populations (Annexin V^+ PI^-), accompanied by an increase in late apoptotic or secondary apoptotic cells (Annexin V^+ PI^+). Significant difference in the percentage of early apoptotic melanocyte was observed between melanocytes treated with 400 ng/ml IFN-γ (22.70 ± 5.24) and those with 200 ng/ml IFN-γ ((8.38 ± 3.26, $p < 0.05$), 100 ng/ml IFN-γ (3.30 ± 1.87, $p < 0.01$) or negative control (2.50 ± 1.16, $p < 0.01$) (Fig. 4e). No significant difference in the proportion of late apoptotic cells was observed between melanocytes treated with IFN-γ and negative control ($p > 0.05$), which might be caused by relatively short IFN-γ-incubation period. These data indicate that IFN-γ induces dose-dependent melanocyte apoptosis *in vitro*.

DISCUSSION

Since the first description of type II IFN activity more than 4 decades ago, a considerable amount has been learned about the biological effects of IFN-γ. As one of the most important endogenous mediators of immunity and inflammation, IFN-γ plays a key role in macrophage activation, inflammation, host defence against intracellular pathogens, Th1 cell responses, tumour surveillance and immunoediting (17). In humans, IFN-γ is implicated in the pathology of several autoimmune diseases, including Systemic Lupus Erythematosus (18, 19), multiple sclerosis (20), allergic encephalomyelitis (21), rheumatoid arthritis (22, 23), type-1 diabetes (24) and vitiligo (25).

Recent studies have demonstrated increased IFN-γ mRNA in vitiligo skin (26, 27) and PBMCs (28) compared with controls. The use of IFN-γ inhibitors as vitiligo treatment has demonstrated positive therapeutic responses (29). Moreover, increased IFN-γ expression was observed during the early and active stages of Smyth line chickens, a classical animal model for autoimmune vitiligo (30). And TCR transgenic mouse model studies for vitiligo have also confirmed the role of IFN-γ in vitiligo pathogenesis (31, 32). These reports suggest a crucial role for IFN-γ in vitiligo pathogenesis. However, the major source of IFN-γ in vitiligo patients remains unclear. Moreover, few papers have investigated the effect of IFN-γ on melanogenesis and viability in melanocyte *in vitro*. Direct evidence for IFN-γ-mediated apoptotic induction is still lacking.

In our study, we detected the frequency of IFN-γ-producing cells in skin samples and in the peripheral blood of vitiligo patients with progressive or stable disease, respectively. Herein, we demonstrated that numerous infiltrating IFN-γ^+ cells were predominantly localised adjacent to the perilesional skin in progressive vitiligo, which may be involved in disease development. To explore the major source of increased IFN-γ in vitiligo, we assessed the prevalence of circulating Th1, $CD8^+$ CTLs and NK cells in PBMCs of vitiligo patients, as these cells are the primary IFN-γ-secreting cells in healthy controls (11, 12). Our data indicate that $CD8^+$ CTLs that express IFN-γ were significantly increased and circulating NK cells were significantly reduced in the PBMCs of progressive vitiligo patients. No significant difference was observed for the number of circulating Th1 cells. We further detected the frequency of $CD8^+$ cells in the same skin sample of vitiligo patients, and the results showed that an increased proportion of $CD8^+$ T cells also accumulated in the perilesional skin of progressive vitiligo patients, especially in areas adjacent to disappearing melanocytes. More importantly, the number of IFN-γ^+ cells and $CD8^+$ T cells in perilesional vitiligo skin samples exhibited a positive correlation ($r = 0.839$, $p < 0.001$). These data suggest that activated $CD8^+$ CTLs may be the major cellular source of IFN-γ in progressive vitiligo, and circulating $CD8^+$ CTLs could migrate to sites of inflammation and participate in the destruction of melanocyte by producing IFN-γ, thereby promoting vitiligo-associated depigmentation. Interestingly, our data indicate NK cell population deficiency in vitiligo patients, suggesting that NK cells may not be critical to vitiligo pathogenesis. This result is consistent with a report from Zhou et al. (33) confirming that percentages of peripheral invariant natural killer T cells were significantly decreased in vitiligo patients compared to that in healthy controls.

In the study, we observed an obvious expansion of $CD8^+$ CTLs in the circulating and in perilesional skin of vitiligo patients. This finding is consistent with substantial evidence that suggests that an increased number of melanocyte-specific cytotoxic $CD8^+$ T cells contribute to the pathogenesis of vitiligo (34–36). These $CD8^+$ T cells are primarily found to be skin-homing, polarised toward type-1 effector function, and evidently cytotoxic while clustering near disappearing melanocytes (37, 38). In the late 20th century, the characterisation of circulating $CD8^+$ T cells with an Elispot assay and a developed technique of HLA-peptide tetrameric complexes favoured the idea of a melanocyte-specific cytotoxic attack in vitiligo. Melan-A is one of the melanocyte-specific differentiation antigens often recognised by CTLs in melanoma (39). High frequencies of Melan-A specific $CD8^+$ T cells in the peripheral blood and skin of vitiligo patients were detected and were found to correlate with disease severity, and these melanocyte-specific T cells were able to destroy melanocytes *in vitro* (34–36). Based on the

number of cases observed in patients with melanoma, immunotherapies against melanocytes differentiation antigens such as gp100 and tyrosinase may lead to $CD8^+$ CTLs infiltration both in the specific melanoma area as in vitiligo lesions (40–42). In addition, an *in vitro* study showed that $CD8^+$ CTLs infiltrated in common vitiligo perilesional area destroyed neighbouring melanocytes in unaffected skin, while they did not induce apoptosis in lesional skin, which is devoid of melanocytes, thereby indicating the melanocyte-specific cytotoxic activity of these $CD8^+$ CTLs in human vitiligo (43). Taken together with our findings, these data indicate that vitiligo pathophysiology may be linked to an excessive activation of $CD8^+$ CTL subpopulation, most of which may be melanocyte-specific, producing increased IFN-γ and promoting an active immune response against melanocyte.

The role of the inflammatory cytokines IFN-γ in autoimmune diseases is not yet fully defined. In vitiligo patients, IFN-γ is expressed in lesional skin and can be produced by autoreactive $CD8^+$ CTLs (43). Gene expression revealed an IFN-γ–specific signature; chemokine CXCL10 was elevated in both vitiligo patient skin and serum, and CXCR3, its receptor, was expressed on pathogenic T cells (7). Previous studies have demonstrated that IFN-γ released by lymphocytes can inhibit cell growth, function and initiate apoptosis in multiple cell lines from various histologies (44, 45). This finding led us to evaluate the ability of recombinant cytokine IFN-γ to affect the melanogenesis of primary culture melanocyte and to induce melanocyte apoptosis *in vitro*. Melanin production in melanocytes treated with IFN-γ was significantly suppressed, and mRNA expression levels of tyrosinase and MITF in those cells were reduced, demonstrating that IFN-γ could inhibit melanocyte melanogenesis *in vitro* by suppressing the expression levels of melanogenic enzymes. These findings are consistent with reports from Natarajan et al. (46) and Son et al. (47). Natarajan et al. confirmed that IFN-γ signalling mediated its hypopigmenting effect through the transcription factor IFN regulatory factor-1, which in turn, impeded maturation of the key organelle melanosome (46). And Son et al. demonstrated that IFN-γ inhibited basal and α-MSH-induced melanogenesis by inhibiting MITF expression (47).

Apoptosis is one of the cell death pathways thought to be involved in melanocyte destruction in vitiligo. However, the complex interactions during vitiligo pathogenesis are difficult to mimic *in vitro*. Few studies focus primarily on the cytotoxic/apoptotic effects of IFN-γ in melanocytes *in vitro*, and therefore, the question remains whether IFN-γ and/or perilesional T cells are a cause or a consequence of melanocyte destruction. Our studies indicate that IFN-γ can directly induce melanocyte apoptosis *in vitro*, suggesting that IFN-γ plays a crucial role in the depigmentation of vitiligo (Fig. 4). Our findings are consistent with a recent report from Gregg et al. (31) and Harris et al. (32) confirming that vitiligo is strongly dependent on IFN-γ produced by $CD8^+$ T cells in a TCR transgenic mouse model. Genetic studies also demonstrated that IFN-γ allele polymorphisms are associated with vitiligo susceptibility and that IFN-γ expression affects disease onset and progression (48). These data suggest an important role for IFN-γ in vitiligo progression. Notably, the amount of IFN-γ we used in inducing melanocytes apoptosis is much higher (100–400 ng/ml) than that found in the serum of vitiligo patient (mean ± SD 2.5 ± 1.05 pg/ml, unpublished data). In the perilesional epidermis, however, the effective concentration of IFN-γ secreted by infiltrating CD8+ CTLs could be much higher, leading to toxicity similar to the experiments. It is clear that $CD8^+$ CTLs control target cell destruction rapidly and precisely. Upon encountering cognate Ag and appropriate co-stimulatory molecules on professional APCs, naive $CD8^+$ T cells become activated and produce a rapid pulse of IFN-γ, accompanied by accumulating granzyme B and perforin (49). IFN-γ synthesis by $CD8^+$ T cells is explosive, but transient, peaking at 12 h after secondary Ag-recognition and terminating hours thereafter (50). It is therefore hypothesised that the transient pulse of IFN-γ production may provide a microenvironment or concentration at which IFN-γ can exert its cytotoxic effects on target melanocytes efficaciously and precisely. The latter may explain why keratinocyte bystanders destroy are rarely detected in the vicinity of apoptotic melanocytes in vitiligo, and no significant difference in the serum level of IFN-γ is observed between vitiligo patients and healthy individuals. Interestingly, an *in vitro* study demonstrated that keratinocyte apoptosis could be induced by IFN-γ at concentrations of 100 U/ml to 1000 U/ml (5–50 ng/ml) in a dose-dependent manner (51), which are much lower than those in melanocyte apoptosis (100–400 ng/ml). The difference may be attributed to the cell specificity, and keratinocyte may be more sensitive upon IFN-γ cytotoxin than melanocyte. Taken together, these data indicate that the pathophysiology of vitiligo might be linked to an expansion of activated $CD8^+$ CTL subpopulations, most of which might be melanocyte-specific $CD8^+$ CTL that produce increased levels of IFN-γ, inhibiting melanogenesis and inducing melanocyte apoptosis. The identification of a medication that specifically protects melanocyte against IFN-γ and/or $CD8^+$ CTLs might serve as the key to halting vitiligo spreading.

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