NEOPLASIA
www.neoplasia.com

Volume 19 Number 7 July 2017 pp. 537–548 537

Activated Natural Killer Cells Mediate the Suppressive Effect of Interleukin-4 on Tumor Development via STAT6 Activation in an Atopic Condition Melanoma Model[1,2]

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Abstract

A protective effect of allergy for cancer has been suggested, but the results are somewhat conflicting, and the mechanism remains elusive. Interleukin-4 (IL-4) signaling has been identified as a potentially important pathway in the development of allergies and the suppression of cancer development. To evaluate the allergy responses in IL-4–mediated tumor development, we compared the growth of B16F10 melanoma cells in 4% phthalic anhydride (PA)-treated *IL-4/Luc/CNS-1* transgenic mice (IL-4 mice) and acetone-olive oil (AOO)–treated IL-4 mice as a control for 3 weeks. Much higher allergic responses and natural killer (NK) and STAT6 activation were found in PA-treated IL-4 mice compared with AOO-treated IL-4 control mice. Tumor volume and weight showed an inverse association with the higher allergic response and were significantly reduced in the PA-treated IL-4 mice when compared with those of AOO-treated IL-4 control mice. Significantly higher activation of STAT6, as well as IL-4 and NK cell activation, was found in the tumor tissues of PA-treated IL-4 mice. Infiltration of immune cells and cytokine levels were also higher in the tumor tissues of PA-treated IL-4 mice. We further found that IL-4–activated NK-92MI cells showed increased anticancer effects in human melanoma cells. Overall, these results showed that allergy responses further accelerated the IL-4–induced inhibition of tumor development through the activation of STAT6 pathways.

Neoplasia (2017) 19, 537–548

Introduction

Malignant melanoma is a malignancy of pigment-producing cells (melanocytes), which are located primarily in the skin [1]. The incidence of melanoma is increasing worldwide, and the prognosis for patients with high-risk or advanced metastatic melanoma remains poor despite advances in the field. With approximately 13,000 annual deaths and a median overall survival of 8 to 18 months, metastatic melanoma is the most aggressive form of skin cancer [2]. The development of melanoma is the result of the interaction between different environmental, genetic, and host factors [3]. Standard treatment for patients is surgery followed by adjuvant therapy or clinical trial enrollment [3]. Currently, the most effective adjuvant therapy is IFN-α. However, because of the limited benefit of

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[1]Financial support: This work was supported by the National Research Foundation of Korea grant of the Korean government (MEST; MRC, 2008-0062275).
[2]Conflict of interest: The authors declare no financial conflicts of interest.
[3]These authors contributed equally to this work.
Received 3 December 2016; Revised 21 February 2017; Accepted 28 February 2017

1476-5586
http://dx.doi.org/10.1016/j.neo.2017.02.014

disease-free survival and the smaller potential improvement of overall survival, the indication for IFN-α treatment remains controversial [4]. Immunotherapeutic approaches such as high-dose interleukin (IL)-2 therapy has been applied to metastatic melanoma patients for a long time, but this is accompanied by severe toxicities that require the patient to be hospitalized for support during treatment [5,6]. In recent years, it was also reported that new molecular target agents (e.g., BRAF inhibitors and MEK inhibitors) and immune checkpoint inhibitors (e.g., against cytotoxic T-lymphocyte–associated protein 4 and programmed cell death protein 1) for immunotherapy of melanoma (PMID = 25993220). Herein, we are also trying to identify the new therapeutic target for melanoma. Atopic dermatitis (AD) is a common, chronic skin disorder that can significantly impact the quality of life of affected individuals as well as their families. Although the pathogenesis of the disorder is not completely understood, it appears to result from the complex interplay between defects in skin barrier function, environmental and infectious agents, and immune abnormalities [7]. The known functions of immunoglobulin E (IgE) antibodies in allergic inflammation suggest that IgE and IgE-mediated mast cell and eosinophil activation contribute to AD [8]. A cohort study conducted in Denmark from 1977 through 2006 suggested a reduced risk of malignant melanoma among AD patients [9]. Recently, AD has also been reported to play a role in tumor formation. Mice lacking the three barrier proteins envoplakin, periplakin, and involucrin ($EPI^{-/-}$ mice) were found to be highly resistant to developing 12-*O*-tetradecanoylphorbol-13-acetate–induced skin carcinogenesis. The 12-*O*-tetradecanoylphorbol-13-acetate response in $EPI^{-/-}$ mice resulted in increased numbers of CD4+ T cells, neutrophils, mast cells, and eosinophils, and many of these leukocyte populations have been shown to be able to exert tumor-protective effects, which could potentially bypass the function of γδ T cells [10]. IgE-coated tumor cells were also found to protect against tumor challenge in a Raucher virus–induced T cell lymphoma–inoculated C57BL/6 tumor model. Moreover, FcεRI IgE receptor activation was found to be essential for IgE antitumor adjuvanticity in a $FcεRIα^{-/-}$ mouse model [11].

IL-4 plays a pivotal role in shaping the nature of immune responses. Upon activation, naive peripheral $CD4^+$ T cells begin to synthesize and secrete cytokines [12]. In T cells, binding of IL-4 to its receptor induces cell proliferation and differentiation into Th2 cells [12]. IL-4 cytotoxin mediates its antitumor activity through the high expression of its receptor IL-4R in lung and gallbladder tumor patients [13,14]. Our previous study also demonstrated that IL-4 has an antitumor effect via high-affinity IL-4Rα to murine melanoma tumors (unpublished data). AD is associated with the elevated production of T helper 2 (Th2) cytokines such as IL-4, IL-5, and IL-13 and low levels of proinflammatory cytokines such as TNF-α, IFN-γ, and IL-1β [15]. Th2 cells are well known to be involved in the allergic response [16]. The development of Th2 cells is controlled by exposure to IL-4. In general, the Th1 immune response is considered to be more effective than the Th2 response for antitumor immunity [17]. However, there is some evidence in mice that the Th2-associated cytokine IL-4 serves to prime Th1-associated, tumor-specific cytotoxic T lymphocytes (CTLs) [18]. IL-4 (–/–) mice showed severe impairment in developing tumor immunity to both a TS/A metastasizing mouse cell line from mammary adenocarcinoma and a CT-26 colon carcinoma line compared with IL-4 (+/+) mice. The lack of tumor immunity in IL-4 (–/–) mice was associated with reduced IFN-γ production and undetectable CTL activity, indicating the role of a defective Th1 response in the lack of the tumor immunity of IL-4 (–/–) mice. Therefore, IL-4 appears to contribute to the generation of Th1-associated and CTL-mediated tumor immunity. It is commonly believed that activation of Th2 cells produces cytokines such as IL-5 and IL-13, which are crucial factors in the induction or prevention of cancer development [19]. IL-1β, IL-6, and TNF-α are well-known prominent agonists that mediate inflammatory and immune-modulatory responses associated with cancer cell growth. IL-1β–deficient mice showed a 50% lower cancer incidence in 3-methylcholanthrene–induced fibroblastic sarcomas compared with wild-type mice [20]. Moreover, BALB/c IL-6 knockout mice are resistant to pristane-induced plasmacytomas development [21]. Another study demonstrated that IFN-γ inhibits pancreatic and gastric cancer development [22,23].

Tumor suppression is associated with enhanced tumor immunosurveillance via the immune system. Several types of immune cells, including CTLs, macrophages, natural killer (NK), and Th cells, play significant roles in developing antitumor immunity [24]. AD skin is characterized by the overexpression of IL-4 and IL-13, Th2 cytokines known to induce atopic responses and downregulate innate immune response genes in the skin [25]. The relationship of AD with hyperreactivity of the immune system can be reinforced by the deviation of the response of T lymphocytes, Th1 cells, for the Th2 response in atopic patients. As a consequence, there are increased immune surveillance and increased cellular destruction, likely including malignant cells [26]. IL-4 is a key factor contributing to the differentiation of precursor T-helper cells into Th2 cells that induce IgE production by plasmocytes. This cytokine is an important regulator in the isotype switching from IgM/IgG to IgE [27].

STAT6 is one of seven members of the STAT protein family (STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, and STAT6) [28]. The canonical signaling cascade of the JAK-STAT pathway is initiated by the engagement of cytokine receptors. This brings JAK into opposition, resulting in its transphosphorylation and subsequent activation. Once activated, JAK mediates the phosphorylation of specific tyrosine residues. STAT and other molecules that recognize these phosphorylated sites are recruited to the receptor and undergo activation by JAK-driven tyrosine phosphorylation. The activated STAT then dissociates, undergoes dimerization in the cytoplasm, and finally translocates to the nucleus [29]. STAT6 is tightly associated with IL-4 and IL-13 signaling and plays a key role in Th2 polarization of the immune system. In STAT6-deficient mice, expression of CD23 and MHC class II in resting B cells was not enhanced in response to IL-4. Furthermore, production of Th2 cytokines from T cells, as well as IgE and IgGl responses after nematode infection, was profoundly reduced [30]. Thus, STAT6 appears to play a central role in exerting IL-4–mediated biological responses.

Therefore, in the present study, we investigated whether the allergic response could potentiate an inhibitory effect of melanoma tumor growth by IL-4 via activation of STAT6 pathways. We established *IL-4/Luc/CNS-1* transgenic mice (IL-4 mice). To induce the allergy reaction, we used phthalic anhydride (PA) as an allergen. PA is an organic compound and an important industrial chemical, especially for the large-scale production of plasticizers for plastics. In addition, PA is known to provoke dermatitis as a strong irritant associated with skin inflammation [31].

Materials and Methods

Cell Culture

B16F10 mouse melanoma cells, HaCaT human keratinocytes, and NK-92MI human NK cells were obtained from the American Type Culture Collection (Cryosite, Lane Cove, NSW, Australia).

SK-MEL-28 human melanoma cells were obtained from the Korean Cell Line Bank (Seoul, Korea). B16F10 cells and HaCaT cells were grown in Dulbecco's modified Eagle medium (Gibco, Life Technologies, Grand Island, NY) with 10% FBS, 100 U/ml of penicillin, and 100 μg/ml of streptomycin at 37°C in a 5% CO_2 humidified atmosphere. SK-MEL-28 cells were grown in minimal essential medium alpha (Gibco, Life Technologies, Grand Island, NY) with 10% FBS, 100 U/ml of penicillin, and 100 μg/ml of streptomycin at 37°C in a 5% CO_2 humidified atmosphere. NK-92MI cells were grown in minimal essential medium alpha containing 12.5% FBS, 12.5% horse serum, 2 mM L-glutamine, 1.5 g/l of sodium bicarbonate, 0.2 mM inositol, 0.1 mM 2-mercaptoethanol, 0.02 mM folic acid, 100 U/ml of penicillin, and 100 mg/ml of streptomycin. In co-culture, the cells were grown in a mixed medium (1:1) of the SK-MEL-28 and NK-92MI culture media in a Transwell system where the cells were separated by a porous polycarbonate membrane. The SK-MEL-28 cells were first seeded at 5×10^4 cells/well and then cultured overnight; thereafter, the inserts containing NK-92MI cells (5×10^4) or freshly isolated NK cells were added to the plate and cultured with the cancer cells.

NK Cell Culture

NK cells were isolated from mouse spleen cells by negative selection using an NK isolation kit (Miltenyi Biotec, Auburn, CA). Purified NK cells were cultured in RPMI 1640 medium supplemented with 3000 U/ml of recombinant human IL-2 (Bayer HealthCare Pharmaceuticals, Emeryville, CA), 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml of penicillin, 100 μg/ml of streptomycin, and 50 μM 2-mercaptoethanol. Cell purity exceeded 90%. IL-2–activated NK cells were used from day 10 to 12. Activated NK cells were further sorted into $CD226^+$ and $CD226^-$ cells using a FACSAriaII flow cytometer (BD Biosciences, Franklin Lakes, NJ). The purity of $CD226^+$ cells exceeded 90%. B16F10 melanoma cells were purchased from American Type Culture Collection (Manassas, VA).

Human Samples

The human melanoma tissues and human normal skin tissues samples were purchased from the US Biomax Inc. cancer tissue bank collection (US Biomax, Inc., Rockville, MD).

Small Interfering RNA (siRNA) Transfection

Melanoma cells (1×10^4 cells/well) were plated in 96-well plates and transiently transfected with IL-4 siRNA, using a mixture of siRNA and the WellFect-EX PLUS reagent in OPTI-MEN, according to the manufacturer's specifications (WelGENE, Seoul, Korea). The transfected cells were treated with 50 ng/ml of recombinant human IL-4 (rhIL-4; R&D Systems, Minneapolis, MN) for 24 hours and then used for detecting cell viability and protein expression.

Western Blotting

B16F10-bearing melanoma tumor tissues were homogenized with a protein extraction solution (PRO-PREP, Intron Biotechnology) and lysed by 60-minute incubation on ice. The cell lysate was centrifuged at 15,000 rpm for 15 minutes at 4°C. Equal amounts of protein (20 μg) were separated on an SDS/12% polyacrylamide gel and then transferred to a polyvinylidene difluoride membrane (GE Water and Process Technologies, Trevose, PA). Blots were blocked for 1 hour at room temperature with 5% (w/v) skim milk in TBS with Tween-20 (TBST: 10 mM Tris, pH 8.0, and 150 mM NaCl solution containing 0.05% Tween-20). After a brief wash in TBST, the membranes were immunoblotted with primary antibodies targeting the following proteins: caspase-3, caspase-9, caspase-8, BCL-2 (1:1000 dilutions; Cell Signaling Technologies, Beverly, MA), BAX, IL-4Rα, JAK1, p-JAK1, STAT6, p-STAT6 (1:1000 dilutions; Santa Cruz Biotechnology, Santa Cruz, CA), and IL-4 (1:1000 dilution; Thermo Fisher Scientific, Waltham, MA). The blots were performed using specific antibodies followed by secondary antibodies and visualized on an enhanced chemiluminescence detection system.

Electromobility Shift Assay (EMSA)

The DNA binding activity of STAT6 was determined using an EMSA performed according to the manufacturer's recommendations (Promega). Nuclear extracts were prepared and processed for EMSA as previously described. The relative densities of the DNA-protein binding bands were scanned by densitometry using MyImage (SLB) and quantified by Labworks 4.0 software (UVP Inc., Upland, CA).

Immunofluorescence

Human tissues were membrane-permeabilized by exposure to 0.1% Triton X-100 for 2 minutes in PBS and placed in blocking serum (5% bovine serum albumin in PBS) at room temperature for 2 hours. The tissues were then exposed to primary rat polyclonal antibody for CD16 (1:100 dilution, eBioscience Inc., San Diego, CA) and primary mouse polyclonal antibody for p-STAT6 (1:100 dilution, Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C. After washes with ice-cold PBS followed by treatment with an anti-rat secondary antibody labeled with Alexa Fluor 594 (1:100 dilution, Molecular Probes Inc., Eugene, OR) and anti-mouse secondary antibody labeled with Alexa Fluor 488 (1:100 dilution, Molecular Probes Inc., Eugene, OR) for 2 hours at room temperature, immunofluorescence images were acquired using a confocal laser-scanning microscope (TCS SP2, Leica Microsystems AG, Wetzlar, Germany) equipped with a 200× objective.

Establishment of Transgenic IL-4 Mice

The plasmid pIL/Luc/CNS-1 was constructed by cloning the human *IL-4* promoter with the CNS-1 enhancer into pTransLucent (Panomics, Fremont, CA) harboring the firefly luciferase gene *Luc*. The pIL/Luc/CNS-1 plasmid was microinjected into the male pronuclei of fertilized embryos obtained by crossing C57BL/6 (female) mice with DBA/2 (male) mice. The transgene was identified via DNA-PCR analysis of the genomic DNA isolated from the tails of the 3-week-old founder mice as described elsewhere [32]. The *IL-4/Luc/CNS-1* transgenic mice used in this study were kindly provided by the National Institute of Food and Drug Safety Evaluation of the Korea FDA (Cheongju, Chungbuk, Korea). The mice were housed and bred under specific pathogen-free conditions at the Laboratory Animal Research Center of Chungbuk National University, Korea. All protocols involving mice in this study were reviewed and approved by the Chungbuk National University Institutional Animal Care and Use Committee and complied with the Korean National Institute of Health Guide for the Care and Use of Laboratory Animals (CBNU-278-11-10).

Experimental Design

Given that a high level of IL-4 can amplify the development of Th2 cells and allergic inflammation [33], we used the transgenic IL-4 mice for this study. Eight-week-old IL-4 mice ($n = 14$) were injected subcutaneously with B16F10 melanoma cells (1×10^6 tumor cells in 0.1 ml of PBS

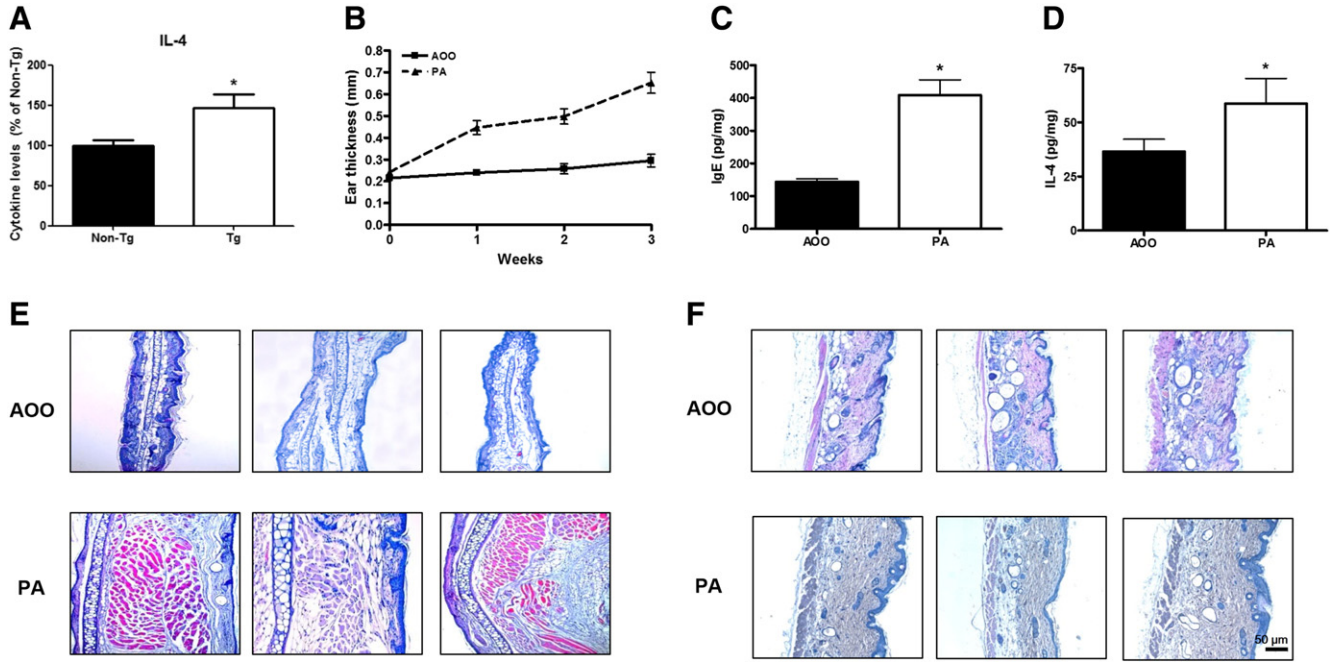

Figure 1. PA-induced allergy reaction. Induction of the allergenic response to PA in IL-4 mice and AOO-treated IL-4 control mice. (A) IL-4 levels in the serum of *Luc/IL-4/CNS-1* transgenic mice and nontransgenic (Non-Tg) mice. The concentration of IL-4 in the Non-Tg mice was 20.34 pg/mg. Changes in various immune-related factors such as ear thickness (B), IgE concentration (C), IL-4 concentration (D), and increases in epidermal thickness in the ear tissue (E) and skin tissue of the dorsal region (F) were detected. * ($P < .05$) indicates a significant difference from the AOO group ($n = 8$).

per animal). The mice were divided randomly into two subgroups. For the first subgroup, 100 μl of 4% PA solution (Sigma-Aldrich, St. Louis, MO) in vehicle (acetone-olive oil [AOO], 4:1 v/v) was sprayed repeatedly on each ear and on the shaved dorsal region of each mouse three times a week for 3 weeks to induce an allergy response. For the second subgroup, 100 μl of vehicle (AOO, 4:1 v/v) was sprayed repeatedly on each ear and on the shaved dorsal region of each mouse three times a week for 3 weeks. To induce the allergy reaction, we used PA as an allergen. Ear thickness was measured using a thickness gauge to determine the degree of allergic skin inflammation induced by the PA treatment. Tumor volumes were estimated by the following formula: length (mm) × width (mm) × height (mm)/2, at the end of the experiment.

Measurement of IgE, IL-4, IFN-γ, IL-10, IL-1β, IL-6, and TNF-α

Serum of the blood and the lysate of the tumor tissue were used to measure the levels of IgE, IL-4, IFN-γ, IL-10, IL-1β, IL-6, and TNF-α using each specific ELISA Kit (R&D System and Koma Biotech). The lysates of the tumor tissue were obtained through protein extraction buffer containing a protease inhibitor. In brief, 100 μl of sample was added to a precoated plate and incubated overnight at 4°C. After washing each well of the precoated plate with a washing buffer, 100 μl of labeled antibody solution was added, and the mixture was incubated for 1 hour at 4°C in the dark. After washing, chromogen was added, and the mixture was incubated for 30 minutes at room temperature in the dark. Finally, the resulting color was assayed at 450 nm using a microplate absorbance reader (Tecan, Switzerland) after adding stop solution.

Immunohistochemistry

All tissues were fixed in 4% paraformaldehyde and cut into 5-μm sections using a freezing microtome (Thermo Scientific, Schwerte, Germany). The sections were stained with hematoxylin and eosin for pathological examination. For immunohistological staining, tumor sections were incubated with the primary antibody. After rinsing in PBS, the sections were incubated with a biotinylated secondary antibody. The tissues were incubated for 1 hour in an avidin-peroxidase complex (ABC, Vector Laboratories Inc., Burlingame, CA). After washing in PBS, the immunocomplex was visualized using 3,3-diaminobenzidine solution (2 mg/10 ml) containing 0.08% hydrogen peroxide in PBS. The sections were dehydrated in a series of graded alcohols, cleared in xylene, and coverslipped using Permount (Fisher Scientific, Suwanee, GA).

Statistical Analysis

The data were analyzed using the GraphPad Prism 4 version 4.03 software (Graph-Pad Software, La Jolla, CA). Data are presented as mean ± SD. The differences in all data were assessed by one-way analysis of variance. When the P value in the analysis of variance test indicated statistical significance, the differences were assessed by the Turkey's test. A value of $P \leq .05$ was considered to be statistically significant.

Results

PA-Induced Allergy Reaction

To identify the expression of IL-4 in *Luc/IL-4/CNS-1* transgenic mice, we measured the cytokine level of IL-4 in the serum of *Luc/IL-4/CNS-1* mice and nontransgenic mice. The data showed that the level of IL-4 was significantly higher in the serum of *Luc/IL-4/CNS-1* mice than in that of the control, nontransgenic mice (Figure 1*A*). We next conducted an *in vivo* analysis to determine tumor development in the PA-induced atopic condition. To determine the allergenic response in IL-4 mice, we detected various immune-related factors such as ear thickness, IgE concentration, and IL-4 concentration in the

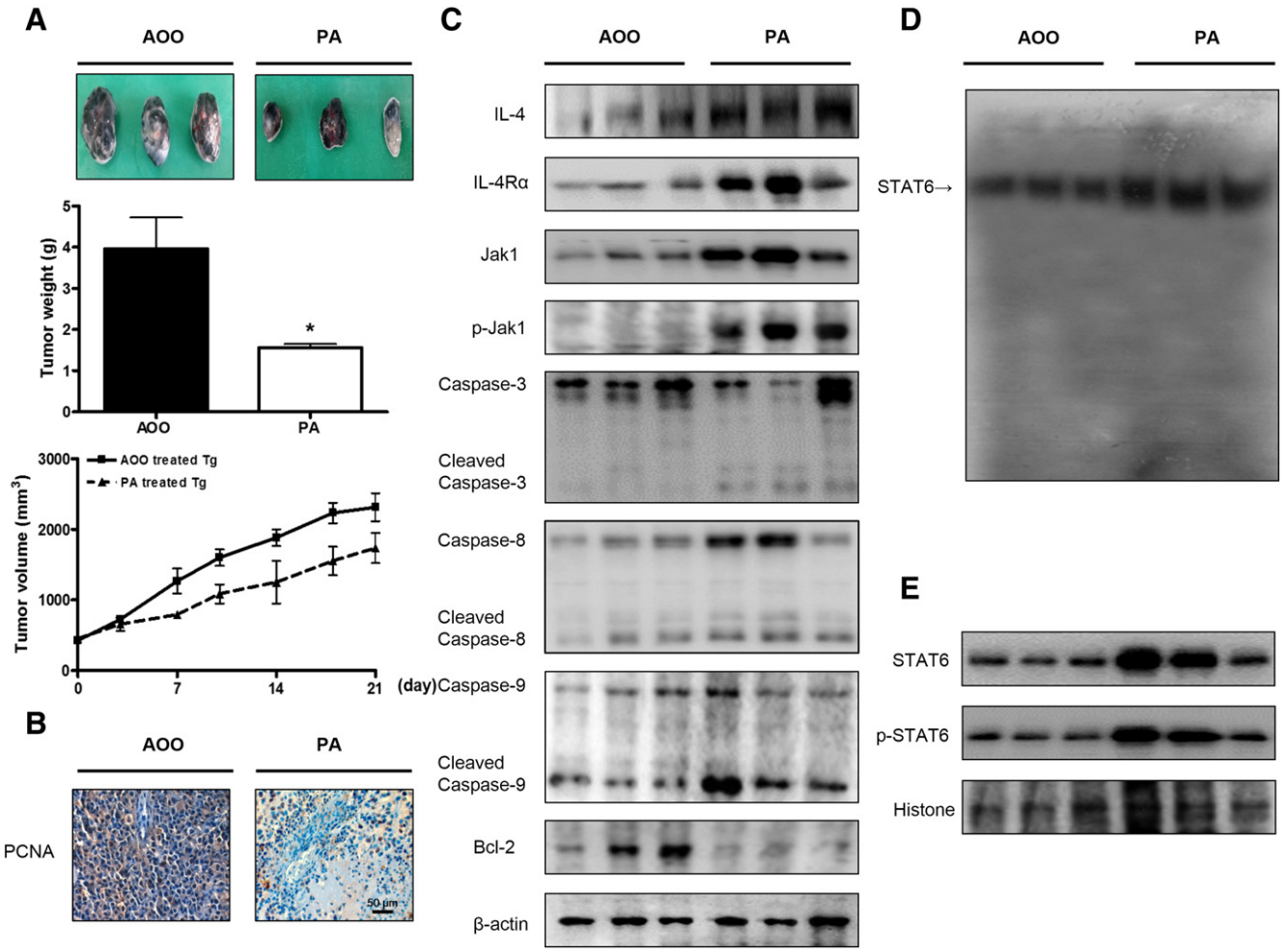

Figure 2. Allergy responses inhibited IL-4–mediated tumor growth in a melanoma model. (A) Tumor growth inhibition (as assessed by tumor weight and volume) in B16F10-bearing PA-treated IL-4 mice and AOO-treated IL-4 control mice. The tumor burden was measured twice per week using a caliper, and the volume was calculated according to the following formula: volume length (mm) × width (mm) × height (mm)/2. Tumor weight and volume are presented as means ± SD from eight mice. (B) Immunohistochemistry was used to determine the expression levels of proliferating cell nuclear antigen in the melanoma tissues of PA-treated IL-4 mice and AOO-treated IL-4 mice. (C) The expression of IL-4 and apoptotic proteins was detected by Western blotting using specific antibodies: IL-4, IL-4Rα, JAK1, p-JAK1, cleaved caspase-3, -8, -9, BCL-2, and β-actin; β-actin was used as an internal control. (D) STAT6 activity in tumor tissues was detected by EMSA, and (E) the nuclear location of STAT6 was determined by Western blotting; histone protein was used as an internal control. All values represent the mean ± SD from eight animal tumor sections. * ($P < .05$) indicates a significant difference from the AOO group.

PA-treated IL-4 mice and AOO-treated IL-4 control mice. The results of ear thickness analysis revealed that the PA treatment induced an increase in ear thickness, whereas AOO treatment induced no such changes (Figure 1*B*). The concentration of IgE, as an indicator of atopic reaction, revealed that the PA treatment increased the serum of IgE level, whereas AOO treatment induced no such changes (Figure 1*C*). As an indicator of the allergy response, the level of IL-4 was also increased in the serum of PA-treated IL-4 mice compared with that in AOO-treated IL-4 control mice (Figure 1*D*). Furthermore, the greater epidermal thickness was detected in the ear tissues of the PA-treated IL-4 mice (Figure 1*E*) and in the skin tissues of the dorsal area (Figure 1*F*).

Allergy Responses Inhibited IL-4–Mediated Tumor Growth in the Melanoma Model

To identify the effect of the allergic response on tumor growth, we measured the tumor growth in a B16F10-inoculated melanoma model. We previously found that IL-4 mice have a protective effect against melanoma growth (unpublished data). Similarly, the present results revealed that tumor volume and weight in the PA-treated IL-4 mice were much lower than those of the AOO-treated IL-4 control mice (~4 g vs 1-2 g, respectively; Figure 2*A*). Expression of proliferating cell nuclear antigen was also decreased in PA-treated IL-4 mice (Figure 2*B*). Furthermore, the expression levels of IL-4, IL-4Rα, phosphorylated JAK1, and proapoptotic proteins, including cleaved caspase-3, -8, and -9, were concomitantly increased in the PA-treated IL-4 mice, but the expression level of BCL-2 was decreased (Figure 2*C*). Moreover, we found a higher binding activity of STAT6 in B16F10-bearing IL-4 mice compared with AOO-treated IL-4 control mice (Figure 2*D*). In line with the increment of STAT6 activity, the nuclear phosphorylation of STAT6 was increased in PA-treated B16F10-bearing IL-4 mice (Figure 2*E*).

Effect of Allergy Responses on the Infiltration of Immune Cells in Tumor Tissues

To investigate whether the inhibition of melanoma growth by PA (Figure 3*A*) is related to tumor-specific immune responses, we analyzed the expression patterns of CD4+ (helper T cells), CD16

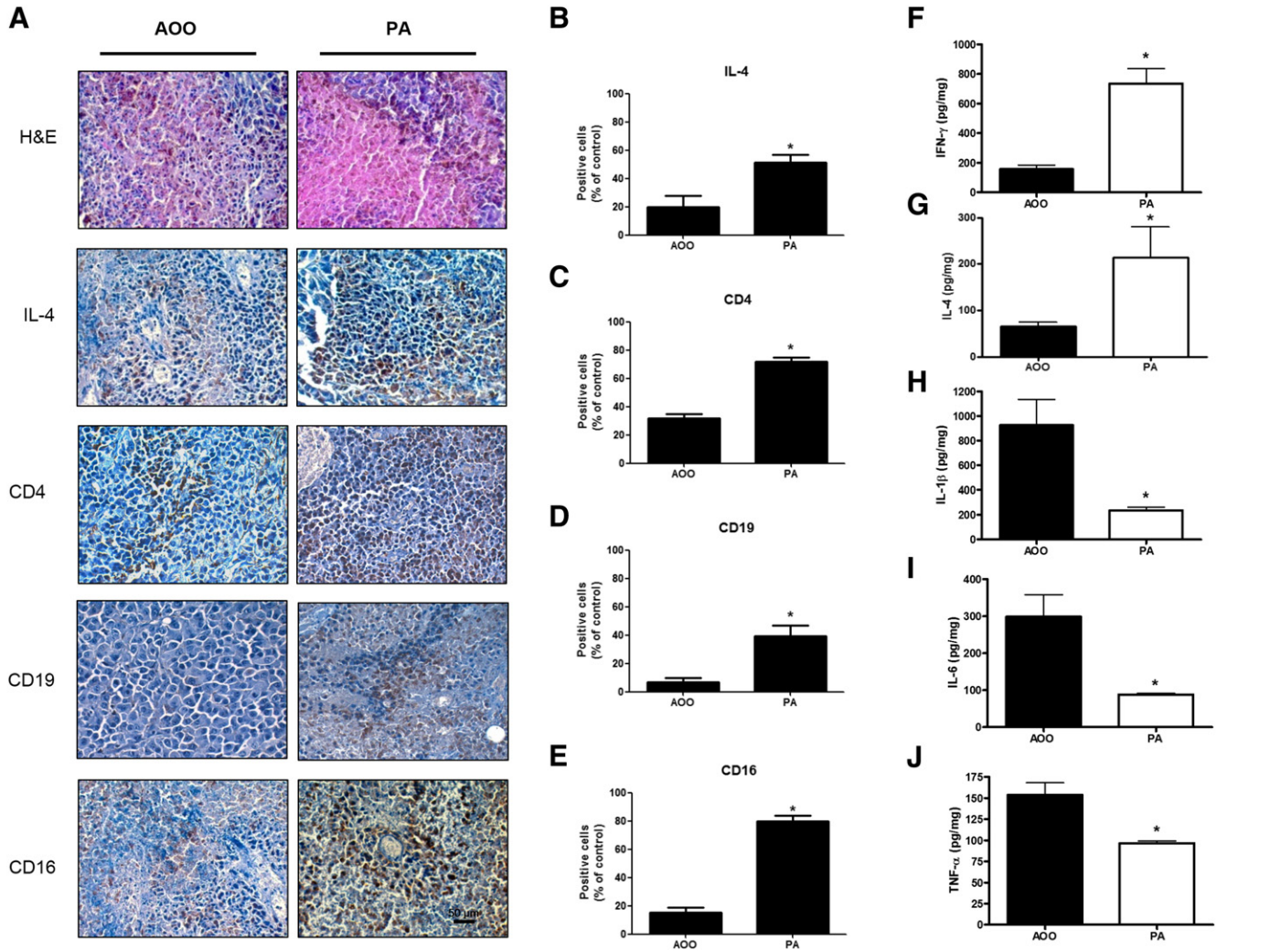

Figure 3. Effect of allergy responses on the infiltration of immune cells in tumor tissues. (A) Immunohistochemistry was used to determine the expression levels of IL-4, CD4, CD19, and CD16 in the tissues of PA-treated IL-4 mice and AOO-treated IL-4 mice. (B–F) Percentages of IL-4–, CD4-, CD19-, and CD16-positive cells of the total number of cells counted in the AOO group. (F–J) Changes in various cytokine levels, such as IFN-γ (F), IL-4 (G), IL-1β (H), IL-6 (I), and TNF-α (J). The results are presented as the mean ± SEM from eight mice. * ($P < .05$) indicates a significant difference from the AOO group.

(NK cells), and CD19 (B cells) in the tumor tissues. Immunohistochemically, more IL-4, CD4, CD19, and CD16 were detected in the PA-treated IL-4 mice (Figure 3, *B–E*). The percentage of CD16-positive cells in PA-treated samples was significantly higher compared with that of controls (Figure 3*E*). These data suggest that promotion of the infiltration of CD16-expressing cells, potent cytotoxic effectors of tumors, could play a role in the inhibition of tumor growth in PA-treated IL-4 mice. We also analyzed the levels of cytokines in the tumor tissue. The levels of IFN-γ and IL-4 were much higher in PA-treated IL-4 mice compared with the AOO-treated IL-4 control mice (Figure 3, *F–G*). However, the levels of tumor growth-promoting cytokines IL-1β, IL-6, and TNF-α were significantly reduced in the PA-treated IL-4 mice (Figure 3, *H–J*).

Effects of IL-4 on LPS- or TNF-α–Induced Human Keratinocyte Cell Growth

To evaluate the apoptotic cell death of the atopic condition *in vitro*, we performed Western blot analysis to investigate the apoptotic effect of IL-4 on LPS- or TNF-α–induced skin inflammation in human keratinocytes. LPS (Sigma-Aldrich, St. Louis, MO) and TNF-α were used as inducers of the atopic condition in human keratinocytes, and both treatments inhibited the growth of human keratinocytes. To assess the inhibitory effect of IL-4 in the presence of LPS or TNF-α on the cell growth of human keratinocyte cells (HaCaT cells), we analyzed cell viability with the MTT assay. The cells were treated with high and low concentrations of IL-4 (10 ng/ml and 50 ng/ml) for 24 hours in the presence of LPS or TNF-α. IL-4 augmented the LPS- and TNF-α–induced growth inhibition of HaCaT cells (Figure 4, *A* and *B*). Expression levels of IL-4 and proapoptotic proteins, including p53, p21, and BCL-2, were significantly enhanced by LPS and TNF-α treatment (Figure 4, *C* and *D*). Moreover, IL-4 siRNA abolished the cell growth inhibitory effect of LPS and TNF-α as well as expression of p53 (Supplementary Figure 1).

Effects of IL-4 on LPS- or TNF-α–Induced STAT6 Activation in Human Keratinocytes

To investigate whether IL-4 activates LPS- or TNF-α–induced STAT6 activation, we conducted an EMSA for detecting the DNA binding activity of STAT6. We found that IL-4–untreated

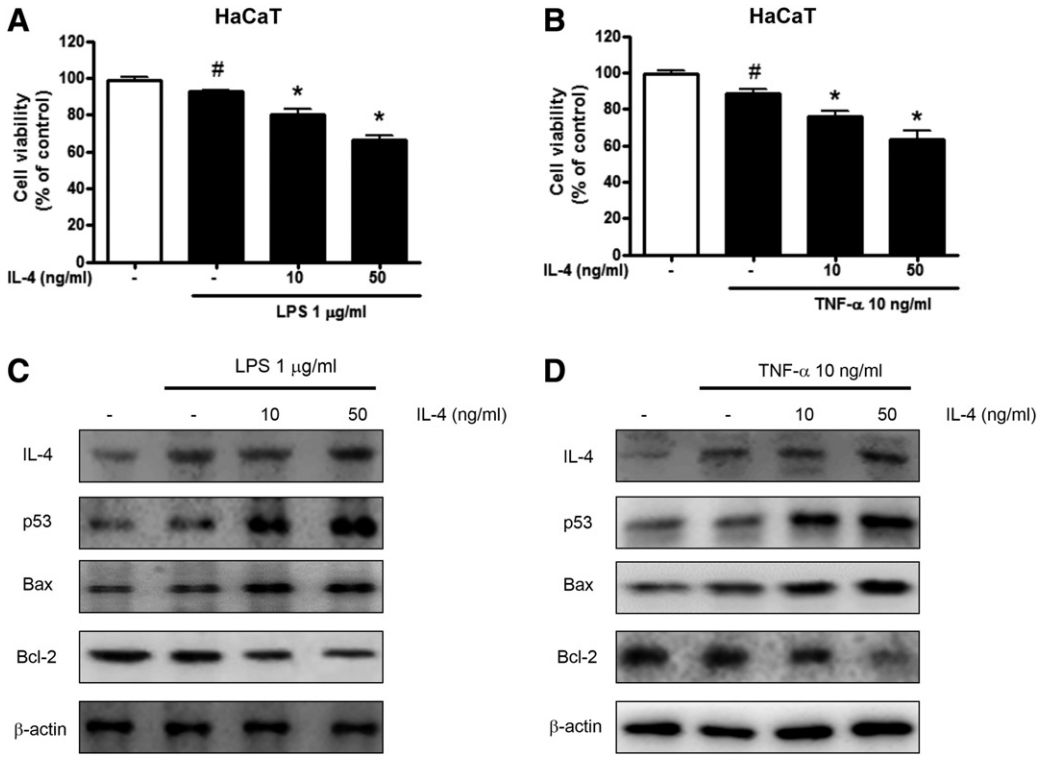

Figure 4. Apoptotic effect of IL-4 on LPS- or TNF-α–induced cell viability and apoptotic protein expression in human keratinocytes. IL-4–treated human keratinocytes were treated with LPS (1 μg/ml) or TNF-α (10 ng/ml). After treatment, cell viability was measured by the MTT assay (A and B), and the expression of apoptosis regulatory proteins (C and D) was determined. β-Actin was used as an internal control. Each blot is representative of three experiments. # ($P < .05$) indicates statistically significant differences from the control group. * ($P < .05$) indicates statically significant differences from the LPS- or TNF-α–treated group.

keratinocytes and LPS- or TNF-α–induced keratinocytes showed lower constituted activation of STAT6 in HaCaT cells; however, the IL-4 treatment enhanced the LPS- or TNF-α–induced DNA binding activity of STAT6 in a dose-dependent manner (Figure 5, *A* and *B*). In line with the increment of DNA binding activity of STAT6, the phosphorylation of STAT6 in the nuclei was enhanced by IL-4 treatment in LPS- or TNF-α–induced HaCaT cells (Figure 5, *C* and *D*).

Effect of IL-4–Activated NK-92MI Cells on the Expression of Apoptosis Regulatory Proteins in Human Melanoma Cells and Colocalization with CD16 and p-STAT6 in Human Tissues

To investigate the anticancer effect of NK cells, SK-MEL-28 human melanoma cells were co-cultured with NK-92MI cells. There was a significant decrease in the cell viability of SK-MEL-28 melanoma cells in the presence of IL-4 (25 ng/ml)–activated NK-92MI cells (Figure 6*A*). In addition, the morphological features of the SK-MEL-28 cells showed a reduction in cell size and the rounding up of cells in the presence of IL-4 (25 ng/ml)–activated NK-92MI cells when compared with treatment with either IL-4 (25 ng/ml) or NK-92MI cells alone (Figure 6*B*); however, NK cell and IL-4 inhibitory was abolished in the presence of TNF-α may be significant in the NK cell mediating IL-4 cancer cell growth inhibitory effect. We also showed that freshly isolated murine NK cells from mice spleen effectively decreased the cell viability of melanoma cells similar with NK-92MI cells (Supplementary Figure 2). We further analyzed the expression levels of apoptotic signaling proteins. The results showed an increase in IL-4Rα, IL-4, and apoptotic regulatory proteins such as caspase-3, caspase-9, and BAX but a decrease in BCL-2 levels in melanoma cells co-cultured with IL-4–activated NK-92MI cells (Figure 6*C*). We also found significant activation of STAT6 in SK-MEL-28 melanoma cells co-cultured with IL-4–activated NK-92MI cells (Figure 6*D*). To demonstrate the significance of NK cells for STAT6 activation, co-expression of CD16 and p-STAT6 in the normal skin and human melanoma tissue was investigated. We found much higher activation of NK cells and STAT6 and colocalization of p-STAT6 with CD16 in melanoma tissues compared with normal skin (Figure 6*E*).

IL-4 siRNA Reversed the IL-4–Induced Cell Growth Inhibition and STAT6 Activation

To determine the specific relationship of IL-4 expression for the inhibitory effect of the growth of B16F10 melanoma cells, we transfected B16F10 melanoma cells with 100 nM of IL-4 siRNA for 24 hours, which were then treated with IL-4 (50 ng/ml) for 24 hours. Knockdown of IL-4 almost completely reversed the cell growth inhibitory effect of IL-4 in B16F10 cells (Figure 7*A*). The expression levels of IL-4, IL-4Rα, caspase-3, caspase-8, BCL-2, and p53 were also reversed (Figure 7*B*). Moreover, the increased activity of STAT6 by IL-4 was abolished by transfection with IL-4 siRNA in B16F10 melanoma cells (Figure 7*C*).

Discussion

In the present study, we found that the PA-induced allergy response further inhibited melanoma tumor growth through activation of STAT6 and NK cells in PA-treated IL-4 mice compared with AOO-treated IL-4 control mice. An association between cancer and allergy has been previously recognized [34]. The incidences of glioma, colorectal, larynx, esophageal, oral, pancreatic, stomach, and uterine cancers, as well as melanoma and non-Hodgkin's lymphoma, were

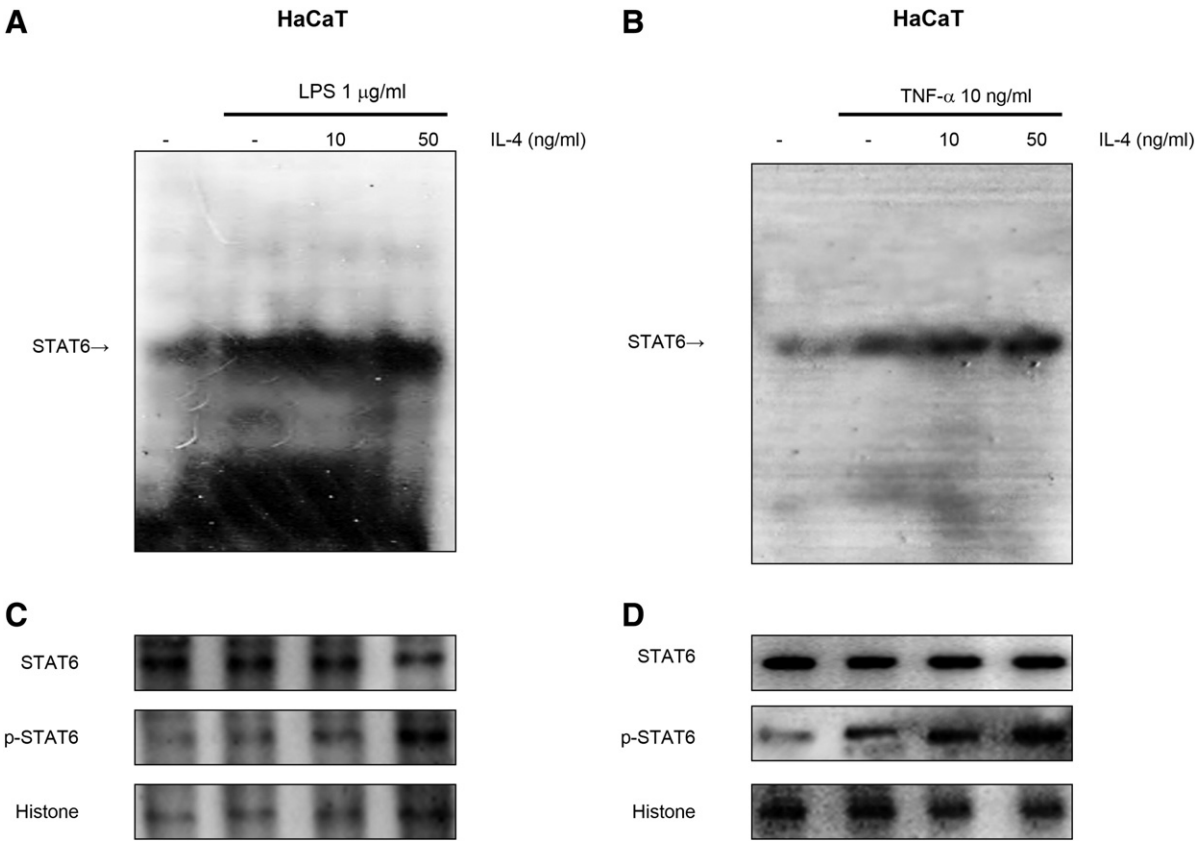

Figure 5. Effects of IL-4 on LPS- or TNF-α–induced STAT6 activation in human keratinocytes. (A and B) STAT6 activity was detected by EMSA. (C and D) The expression of STAT6 and p-STAT6 was detected by Western blotting using specific antibodies. Histone protein was used as an internal control. Each blot is representative of three experiments.

found to be decreased in the allergic condition, whereas the incidences of bladder cancer, lymphoma, and myeloma, and prostate cancer were increased in the allergic condition [35,36]. Case-control studies, multicenter case-control studies, and rigorous meta-analyses have provided stronger support for the relationship between allergy and cancer development [37–40]. However, epidemiological data also indicate that the apparent relationship between tumor incidence and the allergic condition could vary depending on the type of tumor and allergy [41]. Many factors could play a role in mediating the influence of the allergic condition on cancer development. It was also reported that the interaction of tumor cells bound to IgE results in the massive release of mediators such as cytokines, which in turn promote the recruitment of effector cells such as mast cells and basophils and ultimately kill the tumor [42,43].

Typically, human melanoma tumors can constitutively express cytokines and growth factors. Expression of the cytokines IL-1β, IL-6, leukemia inhibitory factor, IL-7, Gro-alpha, IL-8, and the p35 chain of IL-12 was detected in more than 60% of melanomas. Receptors for IL-6 and IL-7 were also detected, but IL-1α, IL-5, IL-10, IFN-β, and TNF-α were all expressed at lower levels in human melanoma tumors [44]. In line with these results, we demonstrated that IL-4 has a suppressive effect on B16F10 melanoma cell growth. Moreover, the pattern of IL-4 siRNA-transfected melanoma cell growth was reversed compared with that of the nontreated melanoma cells. We found that the concentrations of IL-1β, IL-6, and TNF-α were decreased, but those of IL-4 and IFN-γ were increased, in PA-treated IL-4 mice. Our previous study demonstrated that IL-4 suppresses tumor growth (unpublished data). Thus, our data suggest that IL-4 mainly plays an anticancer role in melanoma growth by the downregulation of IL-1β, IL-6, and TNF-α and the upregulation of IFN-γ.

In general, there are a variety of defects in the innate immune system that collectively affect the development and severity of AD. Infiltration of polymorphonuclear leukocytes, plasmacytoid dendritic cells, and NK cells to the skin; epithelial barrier disruption; and Toll-like receptor 2 defects are the most credible explanations for AD patients' susceptibility to pathogens [45]. Skin lesions in AD are induced by allergen-specific T cells that infiltrate the skin at the site of allergen exposure. It has been reported that infiltration of CD4+, CD19+, and CD16+ cells into the skin tissue was also increased in the allergic condition. CD19+ cells play a critical role in antigen-specific $CD4^+$ T-cell proliferation and Th2 responses in a murine model of AD [46]. Increased expression of CD16 was also demonstrated in AD skin compared with healthy and nonlesional AD skin [47]. NK cells are best known for their ability to recognize and kill tumor cells and virally infected cells [48]. Recent research has substantially expanded the function of NK cells in cancer and autoimmunity, and the contributions of NK cells to allergies and various skin diseases have been demonstrated [49,50]. In fact, activated NK cells have been noted in AD patients [50]. It was also reported that NK cells arise after stimulation with the type 2 cytokine IL-4 [51]. Recent studies also indicate that the cytokines produced by these cells of the innate defense system play an essential role in influencing the immune response towards protective antitumor immunity. Indeed, in the present study, infiltration of immune cells such as CD4+, CD16+, and CD19+ cells into tumor tissues was increased in PA-treated IL-4 mice compared with that in AOO-treated IL-4 control mice. Among

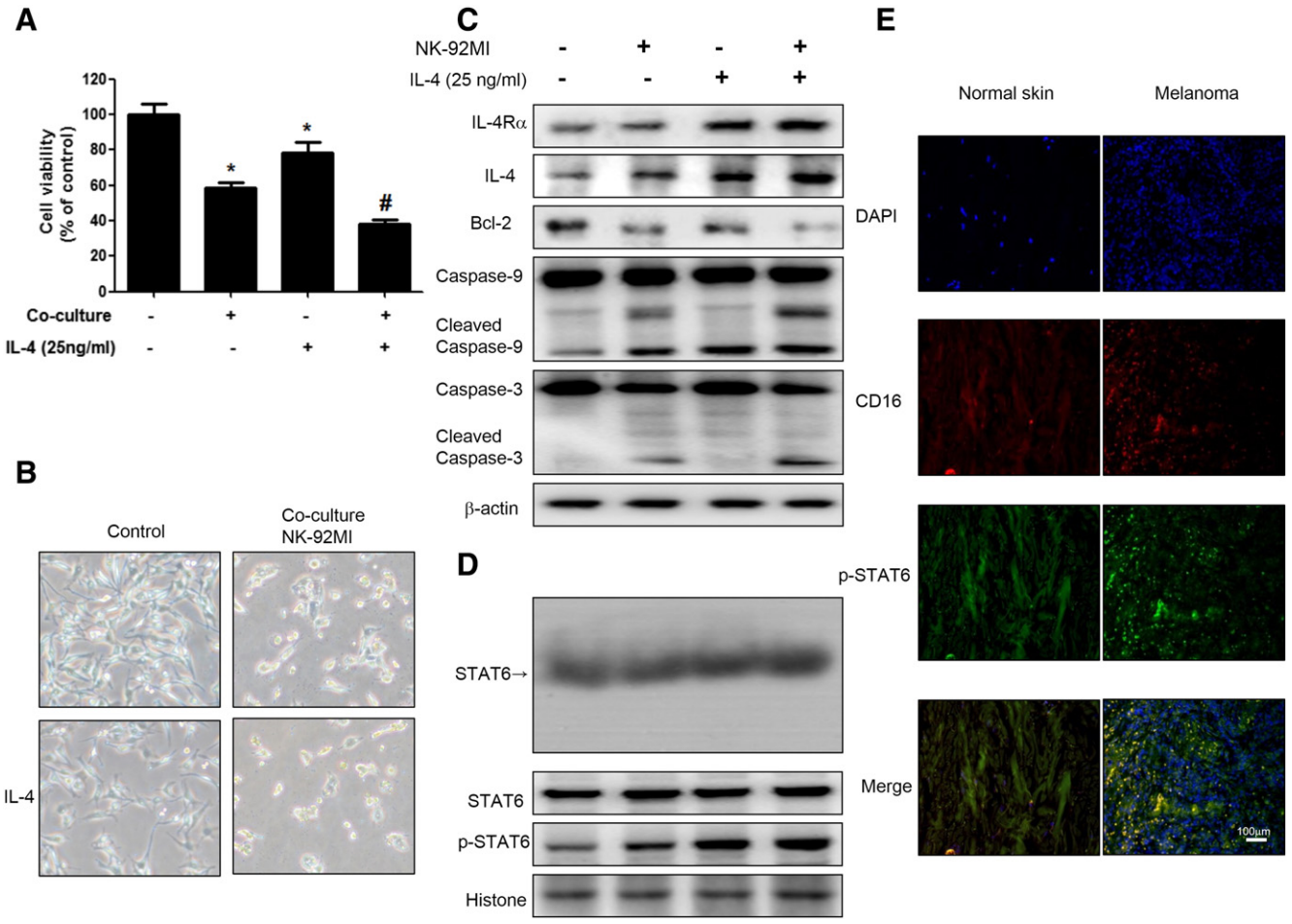

Figure 6. Effect of IL-4–activated NK-92MI cells on the expression of apoptosis regulatory proteins in human melanoma cells and colocalization with CD16 and p-STAT6 in human tissues. The SK-MEL-28 human melanoma cells were co-cultured with NK-92MI cells treated with IL-4 (25 ng/ml). After treatment, (A) cell viability was measured by the MTT assay, and (B) morphological changes were observed. Values are the mean ± SD of three experiments with replicates. *($P < .05$) indicates statistically significant differences from the control group. # ($P < .05$) indicates statistically significant differences from co-culture or IL-4–treated group. (C) The expression of IL-4Rα, IL-4, and apoptotic proteins was detected by Western blotting using specific antibodies. Equal amounts of total proteins (50 g/lane) were subjected to 10% SDS-PAGE. Expression of IL-4Rα, IL-4, BCL-2, caspase-3, -9, and β-actin was detected by Western blotting using specific antibodies. β-Actin was used as an internal control. Each band is representative of three experiments. (D) STAT6 activity in tumor tissues was detected by EMSA, and the nuclear location of STAT6 was determined by Western blotting; histone protein was used as an internal control. Each blot is representative of three experiments. (E) The colocalization with CD16 and p-STAT6 was determined by immunofluorescence using anti-CD16 and anti-p-STAT6 antibody. Pictures were taken with a confocal scanning microscope (magnification, 20×).

them, infiltration of CD16+ cells was highest. We also found much higher levels of the colocalization of p-STAT6 with CD16 in melanoma tissues compared with normal skin. CD16+ NK cells hold great promise for adoptive cancer immunotherapy, and IL-4 is one of the most prominent immunosuppressive factors [52]. NK cell cytotoxic activity is also enhanced by the activation of Th2 cells. Memory Th2 cells were found to induce antitumor effects via IL-4–mediated NK cell activation in an OVA-specific B cell–bearing tumor model [53]. It was also reported that IL-4 induces STAT6-dependent IFN-γ secretion by NK and NK T cells [54]. These findings demonstrate the mechanism by which IL-4 can contribute to Th1 cytokine-associated immune effector functions. We also found that the anticancer effect of IL-4–activated NK-92MI cells in SK-MEL-28 human melanoma cells was increased; activation of STAT6 in SK-MEL-28 cells was increased following co-culture with IL-4–activated NK-92MI cells. Activated NK cells are thus in a position to directly or indirectly exert their antitumor activity to control tumor growth and prevent the rapid dissemination of metastatic tumors [55]. Therefore, it is possible that enhanced IL-4 in an allergic condition could induce more antitumor immunity by promoting the activation of CD16+ NK cells for tumor growth inhibition.

Among the STAT proteins, STAT6 is activated by IL-4 and IL-13. The critical roles of STAT6 on the biological response of IL-4 have been demonstrated. STAT6 (−/−) mice show no response to IL-4 and IL-4α and fail to produce IgE following OVA sensitization [30,56]. IgE in response to IL-4 was also not detected in STAT6-deficient B lymphocytes [57]. Studies on the antitumor effects of STAT6 and NK cells via IL-4 have been widely reported. For example, NK T cell–mediated IL-13 was shown to be related to the repression of tumor immune surveillance by the IL-4R-STAT6 pathway in IL-4R (−/−) and STAT6 (−/−) mice [58]. The Th2 cytokine production of NK cells was found to be dependent on STAT6 and to play certain roles in specific immune responses, including the regulation of allergic diseases [59]. It is known that STAT6 promotes the pathogenesis of

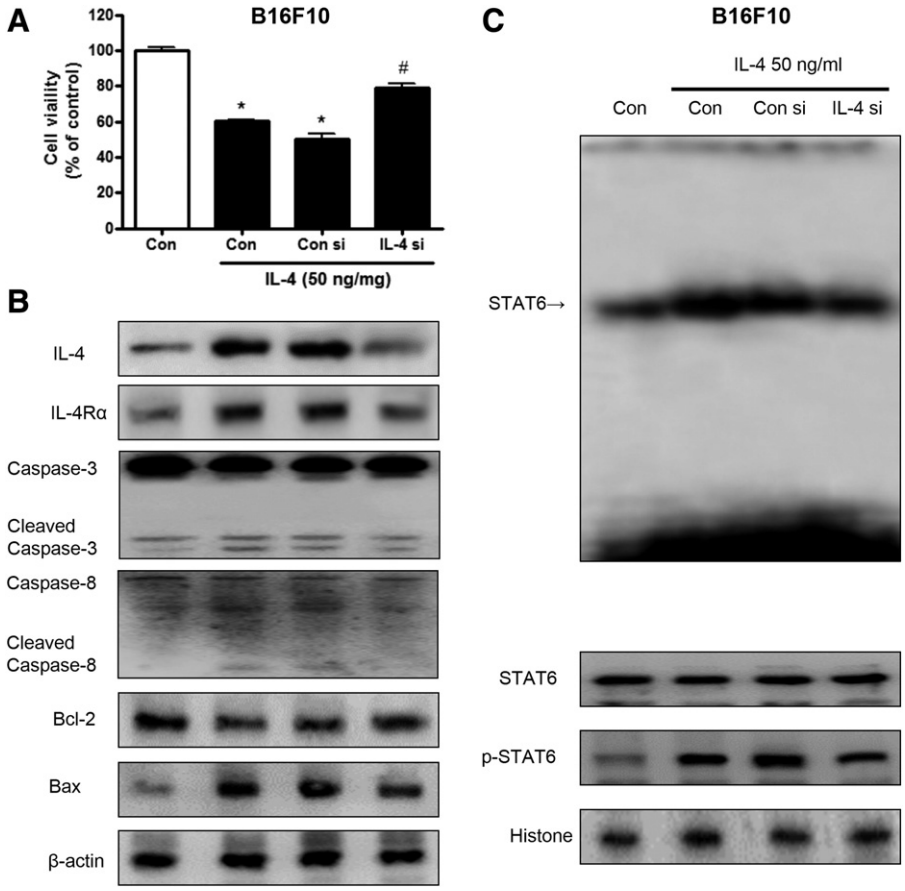

Figure 7. Reversed effect of IL-4 siRNA on IL-4–induced cell growth inhibition and STAT6 activation. The melanoma cells were transfected with IL-4 siRNA (100 nM) for 24 hours, and the cells were then treated with IL-4 (50 ng/ml) for 24 hours. After treatment, (A) cell viability was measured by the MTT assay, and (B) the expression of apoptosis regulatory proteins and (C) STAT6 activity were determined as described in the legend to Figure 6; histone protein was used as an internal control. Cell growth is presented as the means ± SD of three experiments. *($P < .05$) indicates statistically significant differences from the control group. # ($P < .05$) indicates statically significant differences from the rhIL-4–treated group.

allergic disorders like AD [60]. It was also reported that NK cells from healthy people produce more type 1 cytokines compared with those of atopic patients whose NK cells secrete more type 2 cytokines. The ratio of IL-4 + CD56+ NK2 cells in the blood of patients with an allergic disorder in response to Th2 immunity is higher than that in healthy people, and the level of INFγ+CD56+ NK1 cells is lower in these patients [61]. In addition, STAT6 is known as a key regulator for type 2 cytokines [62]. In particular, STAT6 signaling plays an essential role in the induction of hypersensitivity. For example, 2,4,6-trinitrochlorobenzene– or 2,4-dinitrofluorobenzene–induced ear swelling was significantly reduced in STAT (−/−) mice compared with wild-type mice. The expression level of IL-4 in 2,4,6-trinitrochlorobenzene–challenged skin tissues was also profoundly reduced in STAT6 (−/−) mice compared with wild-type mice [63]. Moreover, OVA exposure in STAT6 (−/−) mice reduced the expression levels of IL-4 and IL-5 and the production of OVA-specific IgE compared with wild-type mice [64]. Thus, our data suggest that the allergy response accelerates the tumor-suppressive effect of IL-4 via the activated NK cell–mediated activation of STAT6 in IL-4–overexpressed transgenic mice.

Appendix A. Supplementary Data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.neo.2017.02.014.

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