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IL-32 γ inhibits cancer cell growth through inactivation of NF- κ B and STAT3 signals

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Several studies have shown physiological functions of interleukin (IL)-32, a novel cytokine. However, the role of IL-32 in cancer development has not been reported. In this study, we showed that IL-32 γ inhibited tumor growth in IL-32 γ -overexpressing transgenic mice inoculated with melanoma as well as colon tumor growth in xenograft nude mice inoculated with IL-32 γ -transfected colon cancer cells (SW620). The inhibitory effect of IL-32 γ on tumor growth was associated with the inhibition of constitutive activated nuclear transcription factor-kB (NF-KB) and of signal transducer and activator of transcription 3 (STAT3). The expression of antiapoptotic, cell proliferation and tumor-promoting genes (bcl-2, X-chromosome inhibitor of apoptosis protein (IAP), cellular IAP and cellular FADD-like IL-1_β-converting enzyme-inhibitory protein, cyclin D), cyclin-dependent kinase 4, cycolooxygenase-2 and inducible nitric oxide synthase was decreased, whereas the expression of apoptotic target genes (caspase-3 and -9, bax) increased. In tumor, spleen and blood, the number of cytotoxic CD8⁺ T cells and CD57⁺ natural killer cells and the levels of IL-10 increased, but that of tumor necrosis factor- α (TNF- α), IL-1 β and IL-6 decreased. We also found that forced overexpression of IL-32y inhibited colon cancer cell (SW620 and HCT116) growth accompanied with the inhibition of activated NF-KB and STAT3 in vitro. In addition, when IL-32 γ was knocked down by small interfering RNA (siRNA) or neutralized with an anti-IL-32 γ antibody, IL-32 γ -induced colon cancer cell growth inhibition, the IL-32 γ -induced decrease of TNF- α , IL-1 and IL-6 production, and the increase of IL-10 production were abolished. However, siRNA of NF-κB and STAT3 augmented IL-32γ-induced colon

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cancer cell growth inhibition. These findings indicate significant pathophysiological roles of IL-32 γ in cancer development.

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Introduction

Interleukin-32 (IL-32) was cloned as a gene induced by IL-18 and was formerly known as natural killer (NK) cell transcript 4 (Dahl et al., 1992). IL-32 is produced by mitogen-activated lymphocytes, interferon-y-activated epithelial cells and IL-12-activated NK cells (Kim et al., 2005). IL-32 has six splice variants, IL-32 α , IL-32 β , IL-32 γ , IL-32 δ , IL-32 ϵ and IL-32 ζ , but the functional differences between these isoforms remain unknown (Nold-Petry et al., 2009). IL-32 modulates the generation of tumor necrosis factor- α (TNF- α), IL-1 β , IL-6, IL-10 and two C-X-C chemokine family members involved in inflammatory and/or autoimmune diseases (Dinarello and Kim, 2006; Kang et al., 2009). Although there are several limitations in the studies describing IL-32 pathophysiological functions, significant roles of IL-32 have been reported in the development of several diseases, such as arthritis, psoriasis, ulcerative colitis and Crohn's disease, as well as chronic obstructive pulmonary disease (Dinarello and Kim, 2006; Calabrese et al., 2008). However, the cognate receptor for IL-32 has not yet been identified despite numerous investigations. The lack of an identified receptor as well as the presence of multiple IL-32 isoforms is very uncommon among cytokines (Kim et al., 2005; Nold-Petry et al., 2009). It is also unusual that soluble IL-32 is barely detectable in culture medium and that IL-32 is localized in both the cytosol and the nucleus (Dinarello and Kim, 2006; Kang et al., 2009). Thus, there may be noncanonical and/or cell-associated functions of IL-32 besides its canonical role as a soluble factor.

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Cytokines are involved in inflammatory and/or immune disease-associated cancer development (Lin and Karin, 2007). Cytokines that are released in response to infection can affect tumor development in different ways. IL-8, which induces motility in nonhematopoietic tumor cells, directly modulates endothelial cell proliferation and migration, thus promoting angiogenesis (Fujimoto et al., 2002). Tumor histology revealed the collapse of large blood vessels and disorganized vessel formation in mice that were inoculated with IL-8-transfected cancer cells (Araki et al., 2007). IL-6 increases antiapoptotic activity and, consequently, tumorigenic potency in basal cell carcinoma (Jee et al., 2001). However, Hisada et al. (2004) have reported that IL-27 potently induces tumor-specific antitumor activity. IL-2-stimulated lymphocytes have proven to be effective in destroying tumors (Groscurth et al., 1990). IL-10 inhibits tumor growth and metastasis in an animal model (Huang et al., 1996; Kundu and Fulton, 1997), and it inhibits tumor metastasis through an NK cell-dependent tumor killing mechanism (Zheng et al., 1996). IL-10-deficient transgenic mice are more susceptible to inflammation-associated adenocarcinoma (Westbrook et al., 2009). Moreover, a negative correlation between the expression of IL-10 and human colon cancer development has also been shown (Cacev et al., 2008). A few recent, limited studies showed higher expression or higher protein level of IL-32 in the human lung (Sorrentino and Di Carlo, 2009), pancreas cancer patient tissue (Nishida et al., 2009) and stomach patient tissue (Seo *et al.*, 2008) than in normal tissue or serum. In cancer cell line studies, IL-32 causes apoptotic cell death in a human leukemia cell (Marcondes et al., 2008) and HeLa cell lines (Goda et al., 2006). IL-32 is also involved in the cisplatin-induced apoptotic cell death of HeLa cells (Zhang et al., 2009), but in pancreatic cancer cells, IL-32 stimulates cancer cell growth (Nishida et al., 2009). However, the exact roles and mechanisms of IL-32 in cancer development in vivo have not been reported.

The signal transducer and activator of transcription (STAT) family proteins, especially STAT3 and nuclear transcription factor- κB (NF- κB), have been proposed to be important in inflammatory and/or immune diseaseassociated carcinogenesis (Lin and Karin, 2007). NF-kB and STAT3 are markedly activated in inflammatory and immune cells by cytokines and chemokines, including IL-6, IL-12, IL-17 and IL-23, which could stimulate tumor cell growth (Lu et al., 2004; Lin and Karin, 2007; Yu et al., 2009). Activated NF-kB and STAT3 stimulate pro-survival, proliferative, antiapoptotic and pro-angiogenic genes in cancer development (Bromberg et al., 1999; Lee et al., 2009). Therefore, changes of cytokine levels in immune cells as well as in tumor cells could affect NF-kB and STAT3 activity, thus playing critical roles in tumorigenesis. In this study, we investigated the role and mechanisms of IL-32 γ in melanoma and colon cancer development using IL-32y-transfected colon cancer cells, xenograft nude mice inoculated with IL-32y-transfected colon cancer cells and IL-32y-overexpressing transgenic mice inoculated with melanoma, and we investigated the involvement of STAT3 and NF- κ B pathways in the action of IL-32 γ during cancer development.

Results

Generation of IL-32 γ transgenic mice, and expression of IL-32 γ in the mice

To investigate the role of IL-32 γ in tumor growth in vivo, we generated transgenic mice overexpressing human IL-32 γ (hIL-32 γ). Before generating the IL-32 γ transgenic mice, we confirmed that the IL-32y cDNA was properly translated into the IL-32 protein using glutathione S-transferase-fused IL- 32γ protein expression in Escherichia coli. The glutathione S-transferasefused IL-32y protein was detected by western blotting with an anti-IL-32-reactive monoclonal antibody, KU32-52, as described elsewhere (Kim et al., 2008). The IL-32 γ cDNA was subcloned into the mammalian expression vector pCAGGS, and this construct was used for IL-32 γ transgenic mice production (Figure 1a). The nature of the IL-32 γ transgenic mice was confirmed by polymerase chain reaction (PCR) of mouse tail genomic DNA using allele-specific primers (Figure 1b). A 1.4-kb PCR product was amplified from the IL-32y transgenic mice allele. Among the more than 20 resultant pups, three mice were found to be positive in the genotyping analysis. These three founder mice were each backcrossed into the C57BL6/J background for eight generations. The transgene was successfully transmitted to 50% of the pups from each littermate as evaluated by genotyping and western blotting. Reverse transcription (RT)–PCR and western blotting revealed that hIL-32 γ was ubiquitously expressed in various tissues and highly expressed in the intestine, liver, thymus and heart, whereas there was no expression of hIL-32 γ in the tissues of non-transgenic mice (Figure 1c). The male/ female ratio was 50% for the IL-32y transgenic and non-transgenic littermates. IL-32y transgenic mice were viable, fertile and had no tissue or organ abnormalities. IL-32 level in the sera of IL-32 γ transgenic mice (approximately 1.6 ng/ml) detected by enzyme-linked immunosorbent assay method was increased in the wildtype mice (Figure 1d).

Inhibition of tumor growth in IL-32y transgenic mice



Figure 1 Generation of IL-32 γ transgenic mice and detection of IL-32 γ . (a) Scheme for IL-32 γ transgenic generation. (b) PCR analysis was performed to analyze *IL-32\gamma* gene expression, as described in Materials and methods. (c) RT–PCR and western blotting analyses for IL-32 γ in the spleen, thymus, liver, lung, kidney, colon and brain tissues of IL-32 γ transgenic and non-transgenic mice. (d) Detection of IL-32 in the sera of transgenic mice or non-transgenic mice. The results are expressed as mean ± s.d. of three mice. **P*<0.05 compared with non-transgenic mice. A full colour version of this figure is available at the *Oncogene* journal online.

volumes and weights of the transgenic mice were significantly smaller than in those of the non-transgenic mice determined on day 26 (volume: 4056.1 ± 819.5 versus $7217.3 \pm 1243.3 \text{ mm}^3$, P < 0.05; tumor weight: 9.2 ± 2.9 versus 4.8 ± 0.9 g, P < 0.05) (Figure 2a). The histological findings after hematoxylin and eosin staining indicated that the tumor tissues of the transgenic mice, but not those of the non-transgenic mice, contained large areas of necrosis. Immunohistochemically, high levels of cleaved caspase-3 and bax were found more frequently in the transgenic mice than in the non-transgenic mice. The expression levels of cell cycle regulatory proteins, cyclin D and cyclin-dependent kinase 4 (CDK4), proliferation makers, proliferating cell nuclear antigen and Ki-67, as well as inflammatory proteins, cyclooxygenase 2 (COX-2) and inducible nitric oxide synthase (iNOS), were reduced in the transgenic mice (Figure 2b). TdT-mediated dUTP nick-end labeling (assay) (TUNEL) staining revealed a higher frequency of apoptotic cell death in the tumor tissues of the transgenic mice than in those of the nontransgenic mice (Figure 2c). Western blotting showed the expression of IL-32 γ in the transgenic mice tissues, but not in the non-transgenic mice tissues. In the transgenic mice, the expression levels of cell cycle regulatory proteins, cyclin D and CDK 4, and antiapoptotic proteins, bcl-2 and cellular FADD-like IL-1βconverting enzyme-inhibitory protein (c-FLIP₁), as well as inflammatory proteins, COX-2 and iNOS, were decreased, whereas the levels of the pro-apoptotic bax, and cleaved caspase-3 and -9 were increased (Figure 2d).

Inhibition of tumor growth in BALB/c athymic nude mice inoculated with IL-32 γ -transfected colon cancer cells We investigated whether the inhibitory effects of IL-32 γ on colon cancer cell growth could bring about tumor regression in vivo. Vector- or IL-32y-transfected SW620 cells were inoculated subcutaneously into BALB/c athymic nude mice (n = 10), and tumor growth and weight were monitored for 38 days. Tumor growth was gradually and time-dependently retarded in the mice inoculated with colon cancer cells expressing IL- 32γ compared with the mice exposed to the empty vector. On day 38, when the final volumes were recorded, the tumor volume and tumor weight of the mice inoculated with the IL-32 γ -expressing colon cancer cells were 48.7 and 75.3% of the corresponding values for the mice inoculated with the vector-expressing colon cancer cells, respectively (Figure 3a). Immunohistochemical analysis of tumor sections, stained with hematoxylin and eosin, and for proliferation antigens, proliferating cell nuclear antigen and Ki-67, revealed greater inhibitory effects of IL-32 γ on tumor cell growth. We also confirmed that the expression levels of apoptotic cell death regulatory proteins, that is, cleaved caspase-3 and bax, were increased, but inflammatory proteins, iNOS and COX-2, were decreased in the mice tumor tissue that were inoculated with colon cancer cells expressing IL-32 γ (Figure 3b). Apoptotic cell death was significantly induced in the mice tumor tissues that were inoculated with IL-32 γ -expressing colon cancer cells (Figure 3c). Consistent with the immunohistochemical data, western blot analysis showed that IL-32 γ increased the expression levels of cleaved caspase-3 and -9, and bax, whereas it decreased the expression levels of cyclin D, CDK4, bcl-2 and c-FLIP_L, as well as iNOS and COX-2 (Figure 3d).

IL-32 γ overexpression inhibits colon cancer cell growth through induction of apoptotic cell death in cultured cells To further investigate whether the introduction of IL-32 γ into cancer cells changes their growth in vitro,





Figure 2 Effect of IL-32 γ on tumor growth in IL-32 γ -overexpressing transgenic mice. (a) Tumor images, volumes and weights. The results are expressed as mean ± s.d. **P*<0.05 compared with the non-transgenic mice. (b) Tumor sections were analyzed by immunohistochemistry. (c) Apoptotic cells were examined by fluorescence microscopy after TUNEL staining. (d) Tumor extracts were analyzed by western blotting. Each image and band is representative of three independent experiments. The values on the right of panels b and d are average percentages of vector control over three independent experiments.

we carried out cell counting and analyses of morphological changes in colon cancer cells transfected with or without the *IL-32* γ gene. IL-32 γ was marginally expressed in colon cancer cells without the introduction of IL-32 γ determined with PCR analysis. The expression of IL-32 γ was found to be increased depending on the amount of introduced IL-32 γ cDNA, and correlated with the inhibition of colon cancer cell growth (Figure 4a). Morphological observations revealed that enforced expression of IL-32 γ caused a gradual reduction in cell size and the rounding of individual colon cancer cells. The introduction of IL-32 γ resulted in the inhibition of colon cancer cell growth in a timedependent manner, as compared with the cells transfected with empty vector (Figure 4b). To confirm further the relationship between the introduction of IL-32 γ and colon cancer cell growth inhibition, the anticancer drug paclitaxel was added to cancer cells, and the levels of growth inhibition were determined. In the presence of paclitaxel (5 nm), the growth inhibition caused by IL-32 γ was augmented (Supplementary Figure 1A). We also found that introduction of IL-32 γ inhibited the prostate, liver and lung cancer cell growth (Supplementary Figure 1B). These results indicate that IL-32 γ inhibits the growth of several cancer cells.

To determine whether apoptotic cell death contributed to the observed inhibitory effect of IL-32 γ on colon cancer cell growth, we evaluated the changes in chromatin morphology of the IL-32y-expressing colon cancer cells using TUNEL staining. The introduction of IL-32 γ increased the number of apoptotic (4',6-diamidino-2-phenylindole-stained TUNEL-positive) colon cancer cells. The frequencies of apoptotic cell death without and with the introduction of IL-32 γ were 10 and 62% in the SW620 cells, and 4 and 49% in the HCT116 cells, respectively (Figure 4c). To elucidate the relationship between the induction of apoptotic cell death by IL- 32γ and the expression of apoptotic cell death-related genes, the expression of apoptotic cell death regulatory proteins was investigated. In the IL-32y-overexpressing colon cancer cells, the expression levels of pro-apoptotic proteins, that is, cleaved caspase-3, -9 and poly-(ADPribose) polymerase, were increased. However, the expression levels of the antiapoptotic proteins, bcl-2, inhibitor of apoptosis protein (IAP), X-chromosome IAP (XIAP), c-FLIP, and of cell proliferation markers,

IL-32 γ inhibits cancer cell growth JH Oh *et al*



Figure 3 Effect of IL-32 γ on tumor growth in the SW620 xenograft *in vivo* model. (a) Tumor images, volumes and weights. The results are expressed as mean \pm s.d. **P* < 0.05 compared with the mice inoculated with vector-transfected colon cancer cells. (b) Tumor sections were analyzed by immunohistochemistry. (c) Apoptotic cells were examined by fluorescence microscopy after TUNEL staining. (d) Tumor extracts were analyzed by western blotting. Each image and band is representative of three independent experiments. The values on the right of panels b and d are average percentages of vector control over three independent experiments.

for example, cyclin D and CDK4, were decreased in the colon cancer cells by IL- 32γ . Furthermore, IL- 32γ reduced the expression of inflammatory marker proteins, COX-2 and iNOS (Figure 4d).

Silencing endogenous expression of IL-32y abolishes

IL-32y-induced cell growth inhibition and apoptotic cell death When the colon cancer cells were co-transfected with the *IL-32* γ gene and IL-32 γ -specific small interfering RNA (siRNA), consistent with the reduced enforced expression of IL-32 γ (Figure 5a), the IL-32 γ transfectioninduced inhibition of cell growth was significantly abolished in the colon cancer cells (Figure 5a). We also found that recombinant IL-32 γ protein inhibited colon cancer cell growth (Figure 5b). However, IL-32 antibody abolished the inhibitory effect of IL-32 γ on colon cancer cell growth in the IL-32y-protein-treated (Figure 5b) or IL-32y-transfected colon cancer cells (Figure 5c). Furthermore, the increase in apoptotic cell number induced by IL-32 γ was diminished by transfection of the IL-32y-specific siRNA (Supplementary Figure 1C) and by treatment of IL-32 antibody in SW620 colon cancer cells (Supplementary Figure 1D).

IL-32 γ decreases NF- κ B activity in tumor tissues and in colon cancer cells

The activation of NF- κ B plays a critical role in cancer cell survival. To determine whether IL-32 γ inhibits the activation of NF-kB in colon cancer and melanoma cells as well as tumors, we determined the DNA-binding activity of NF-kB by electromobility shift assay, and translocation of p50 and p65 into the nucleus and $I\kappa B\alpha$ protein degradation by western blot and immunohistochemical analysis. The DNA-binding activity of NF-KB, and translocation of p50 and p65 into the nucleus and IkB α protein degradation were significantly decreased in the tumor tissues of xenograft nude mice that were inoculated with IL-32 γ -overexpressing colon cancer cells and transgenic mice that expressed IL-32 γ (Figures 6a and b). Immunohistochemical analysis of p50 and p65 confirmed that the intensities of nuclear staining for p50 and p65 were decreased in the tumor tissues of nude and transgenic mice that expressed IL-32 γ (Figure 6c). As NF- κ B is highly activated in colon cancer cells (Ban et al., 2009), the DNA-binding activity of NF-kB was measured after the introduction of the vector or IL-32 γ into the colon cancer cells. The introduction of IL- 32γ inhibited the constitutive DNA-binding activity in the



Figure 4 Expression of IL-32 γ , growth rates and apoptotic cell death in IL-32 γ -transfected colon cancer cells. (**a**) Colon cancer cells (1 × 10⁶) were transfected with various amounts of the IL-32 γ plasmid (1.5–6.0 µg) for 24 h and harvested. IL-32 γ expression was detected by western blotting using monoclonal antibody KU32-52. To determine the effects of different IL-32 γ levels on colon cancer cell growth, the cells were inoculated into 24-well plates (5 × 10⁴ cells per well) and transfected with the IL-32 γ plasmid (0.1–0.4 µg per well) for 72 h. Cell growth was measured by direct counting after Trypan blue staining. (**b**) Colon cancer cells were inoculated into 24-well plates (5 × 10⁴ cells per well) and transfected with the vector or IL-32 γ plasmid (0.4 µg per well). At 24, 48 and 72 h post-transfection, the cells were harvested by trypsinization and counted after Trypan blue staining. Control: untransfected cells. (**c**) Colon cancer cells were transfected with the vector or the IL-32 γ for 72 h. Apoptotic cells were examined under a fluorescence microscope after TUNEL staining. The total number of cells in a given area was determined by 4',6-diamidino-2-phenylindole nuclear staining. The results are expressed as mean ± s.d. of three experiments with each experiment performed in triplicate. **P* < 0.05 compared with the vector or the IL-32 γ for 24 h. Cell extracts were analyzed by western blotting. Each band is representative of three independent experiments.

colon cancer cells (Figure 6d). Moreover, IL- 32γ prevented the translocation of p50 and p65 into the nucleus through the inhibition of the phosphorylation of $I\kappa B$ (Figure 6e). Confocal microscopy confirmed that the translocation of p50 and p65 into the nucleus was decreased by IL-32 γ (Figure 6f). We also showed that introduction of the other subtype of IL-32 also decreased constitutively activated NF-kB in the SW620 colon cancer cells transfected with IL-32 α , - β and - γ (Supplementary Figure 2A), and IL32- γ transfection also inhibited constitutive or TNF-α-induced NF-κB in other cancer cells such as the prostate (PC3), liver (HepG2) and lung (NICH4610) (Supplementary Figure 2B). In addition, the treatment with recombinant IL- 32γ protein inhibited the constitutive DNA-binding activity in the SW620 cells (Supplementary Figure 2C). The DNA-binding activity of NF-κB was confirmed by the competition and supershift assays (Supplementary Figure 2D). We also found that recombinant IL- 32γ

tional activity (Supplementary Figure 2E). However, treatment with siRNA of IL-32 γ abolished IL-32 γ induced p65 and p50 translocation (Supplementary Figure 3). To further investigate the effect of NF- κ B inactivation on cancer cell growth, we analyzed the growth patterns of colon cancer cells, where p50 or p65 were knocked down by specific siRNAs. The knock down of p50 and p65 augmented the inhibitory effect of IL-32 γ on colon cancer cell growth (Figure 6g). These data indicate that inhibition of NF- κ B is implicated in IL-32 γ -induced cancer cell growth inhibition.

protein decreased TNF-α-induced NF-κB transcrip-

IL-32 γ decreases *STAT3* activity in tumor tissues and in colon cancer cells

STAT3 is also implicated in inflammatory-associated carcinogenesis and in maintaining of the constitutive activation of NF- κ B in cancer cells (Lee *et al.*, 2009;

IL-32 γ inhibits cancer cell growth JH Oh *et al*



Figure 5 Effect of silencing endogenous IL-32 γ expression on colon cancer cell growth. (a) Colon cancer cells were inoculated into 24well plates (5 × 10⁴ cells per well) and co-transfected with the IL-32 γ plasmid (0.4 µg per well) and the IL-32 γ -specific siRNA (siIL-32; 50–100 nM) or anti-IL-32 (5 or 10 µg/ml) for up to 72 h. The expression of IL-32 γ was determined as described above. Thereafter, cell growth was measured by direct counting after Trypan blue staining. siCON: non-targeting control siRNA. (b) Colon cancer cells were treated with either recombinant IL-32 γ alone or with its antibody and cultured for 72 h. (c) Colon cancer cells were inoculated into 24well plates (5 × 10⁴ cells per well) and transfected with the IL-32 γ plasmid (0.4 µg per well) with/without anti-IL-32 (5 or 10 µg/ml) for up to 72 h. All results are expressed as mean ± s.d. of three experiments with triplicate tests in each experiment. #*P*<0.05 compared with the colon cancer cells transfected with vector alone (**a**, **c**) or untreated control (**b**). **P*<0.05 compared with the colon cancer cells transfected with IL-32 γ alone (**a**, **c**) or with recombination IL-32 γ protein (**b**). A full colour version of this figure is available at the *Oncogene* journal online.

Yu et al., 2009). Thus, we investigated whether IL- 32γ inhibits STAT3 activation in colon and melanoma tumors as well as in colon cancer cells. Consistent with the inhibitory effect on NF-kB activation, STAT3 phosphorylation was significantly decreased in the tumor tissues of nude and transgenic mice that expressed IL-32 γ (Figures 7a and b). Co-translocation of p65 and p-STAT3 into the nucleus was also decreased in the tumor tissues of nude and transgenic mice that expressed IL-32 γ (Figures 7c and d). IL-32 γ also inhibited the activated STAT3 activity (phosphorylation of STAT3) in the colon cancer cells (Figure 7e). Confocal microscopy confirmed that the translocation of p-STAT3 into the nucleus, and colocalization of p-STAT3 and p65 were decreased by IL-32 γ in colon cancer cells (Figure 7f). To investigate further the inhibitory effect of IL-32 γ on STAT3 activation on colon cancer cell growth, we analyzed the growth patterns of colon cancer cells, where STAT3 was

knocked down by specific siRNAs. The knock down of STAT3 augmented the inhibitory effect of IL-32 γ on colon cancer cell growth (Figure 7g). These data indicate that inhibition of STAT3 is implicated in IL-32 γ -induced NF- κ B inactivation and cancer cell growth inhibition.

IL-32 γ induces the activation of CD8⁺ cytotoxic T cells and NK cells in the blood, tumor and immune organ tissues, and alters cytokine levels

To investigate whether the inhibition of colon cancer growth by IL-32 γ is related to tumor-specific immune responses, we analyzed the expression patterns of CD8⁺ (cytotoxic T cells) and CD57⁺ (NK cells) in tumor and immune tissues. Immunohistochemically, more CD8⁺ T and CD57⁺ cells were detected in the tumor sections of nude mice inoculated with colon cancer cells expressing IL-32 (Figure 8a). By staining the tumor sections from



Figure 6 Effect of IL-32γ on NF-κB activation in tumor tissues and colon cancer cells. (a) The DNA-binding activity of NF-κB was determined by electromobility shift assay in the nuclear extracts of the xenograft mouse or IL-32γ transgenic mice tumor samples. (b, c) Expression of p50 and p65 in nuclear extracts and IκB phosphorylation in the cytosol of murine tumors, as determined by western blotting (b) and immunohistochemistry (c). Each image and band is representative of three independent experiments. (d) The DNA-binding activity of NF-κB was investigated using electromobility shift assay in nuclear extracts of colon cancer cells that were transfected by IL-32γ. (e, f) Expression of p50 and p65 in nuclear extracts and IκB phosphorylation in the cytosol, as determined by western blotting (e), and fluorescence microscopy (f). (g) Colon cancer cells were co-transfected with the IL-32γ and p50/p65 siRNA for up to 72 h. Cell growth was measured by direct counting of cells stained with Trypan blue. The results are expressed as mean ± s.d. of three experiments with triplicate tests in each experiment. #*P*<0.05 compared with the colon cancer cells transfected with IL-32γ alone. The values on the left of panels **b** and **e** are average percentages of vector control over three independent experiments.

IL-32 γ transgenic mice, we found that there were also a significant higher number of CD8⁺ T cells in the tumor, as well as an increase in the number of NK cells (Figure 8b). To investigate further the differences in the numbers of CD8⁺ T cells and NK cells between the control and IL-32y-expressing cells in immunity-related organs, we analyzed the levels of CD8⁺ (cytotoxic T cells) and CD49⁺ reactive cell (NK cells) numbers in spleen and thymus tissues. With respect to the spleen, the numbers of CD8⁺ T cells were much higher in the IL-32 γ transgenic mice than those in the non-transgenic mice, whereas there was a slight higher number in the xenograft nude mice inoculated with IL-32 γ -expressing colon cancer cells (Figure 8c). Thymus sections of the IL-32 γ transgenic mice also showed increased levels of CD8⁺ T cells and NK cells as compared with those of the non-transgenic mice (Figure 8d). Moreover, fluorescence-activated cell sorting (FACS) analysis also showed that the CD8 $^{\scriptscriptstyle +}$ T- and NK cell number as well as total B and T cells in the tumor tissues was higher in the blood of IL-32 γ transgenic mice compared with those of the non-transgenic mice (Figure 8e). Similar increase of CD8⁺ T- and NK cell number was found in the blood and spleen even though the total number of T and B cells was not much different (Supplementary Table 1). These data suggest that IL-32 γ induces antitumor immunity by promoting the activation of CD8⁺ T cells and NK cells in both tumor and immune organ tissues, which are potent cytotoxic effectors for tumors.

To investigate whether IL-32 γ alters other cytokine levels in tumor tissues and immune organ (spleen), and thus effect on the cancer cell growth, NF- κ B and STAT3 signal and activation of immune cells in the tumor and/ or immune organ, we analyzed cytokine levels in tumor tissues by quantitative RT–PCR. The levels of the tumor growth-prompting cytokines, TNF- α , IL-6 and IL-1 β , were significantly decreased, whereas the level of IL-10, a tumor growth-inhibiting cytokine, was significantly elevated in the tumor tissues of IL-32 γ transgenic mice

IL-32γ inhibits cancer cell growth JH Oh et al



Figure 7 Effect of IL-32 γ on STAT3 activation in tumor tissues and colon cancer cells. (**a**, **b**) Phosphorylation of STAT3 in whole extracts of murine tumors, as determined by western blotting (**a**) and immunohistochemistry (**b**). (**c**, **d**) Cellular localization p-STAT3 (green) and p65 (red) in tumor tissues of xenograft mice (**c**) and IL-32 γ transgenic mice (**d**). Each image and band is representative of three independent experiments. (**e**) Colon cancer cells were transfected with the vector or the IL-32 γ for 24 h. Whole-cell extracts were prepared and analyzed for phosphorylated STAT3 by western blotting. Each band is representative of three independent experiments. (**f**) Cellular localization of p-STAT3 (green) and p65 (red) was observed by confocal microscopy after immunofluorescence staining of colon cancer cells transfected with IL-32 γ . (**g**) Colon cancer cells were co-transfected with the IL-32 γ and STAT3 siRNA for up to 72 h. Cell growth was measured by direct counting of cells stained with Trypan blue. The results are expressed as mean ± s.d. of three experiments with triplicate tests in each experiment. *P < 0.05 compared with the colon cancer cells transfected with IL-32 γ alone.

than those in the non-transgenic mice (Figure 8c). Similar alteration of cytokine levels was found in the xenograft nude mice tumor tissues (Supplementary Figure 4A) as well as SW620 colon cancer cells expressing IL-32 γ (Supplementary Figure 4B). However, treatment with siRNA of IL-32 γ abolished IL-32 γ transfection-induced alteration of cytokine levels (Supplementary Figure 4C).

Discussion

In this study, using IL-32 γ , the most biological active form of the IL-32s (Choi *et al.*, 2009), we found that tumor growth was inhibited in the xenograft BALB/C

athymic nude mice inoculated with IL-32 γ -overexpressing colon cancer cells and in the IL-32 γ -overexpressing transgenic mice inoculated with B16 melanoma cells. The inhibitory effect of IL-32 γ on tumor growth was associated with the inhibition of constitutively activated NF- κ B and STAT3. This antitumor activity was also associated with decreased expression of antiapoptotic, cell proliferation and tumor-promoting genes (*cleaved caspase-3* and *-9*, *bax*, *cyclin D* and *CDK4*, *COX-2* and *iNOS*), but with increased expression of their target apoptotic genes (*bcl-2*, *xIAP*, *cIAP* and *c-FLIP_L*). In tumor, blood and immune tissues, the number of cytotoxic CD8⁺ T cells and CD57⁺ NK cells and the levels of IL-10 increased. When IL-32 γ was knocked down



Figure 8 Effect of IL-32 γ on the infiltration of CD8⁺ T cells and NK cells into tumor tissues and immune organ tissues and on cytokine levels in tumor tissues. (**a**–**d**) Immunohistochemistry was used to determine the level of CD8⁺ - and CD57-reactive cell number in tumor sections and immunity-related organs, as described in Materials and methods. Shown are the expression patterns of CD8⁺ and CD57 in the tumor sections of nude mouse xenografts (**a**) and IL-32 γ transgenic mice (**b**), as well as in the spleens (**c**) and thymuses (**d**) of IL-32 γ transgenic mice. The images shown are representative of three sections from each mouse (*n* = 3). Each image and band is representative of three independent experiments. (**e**) Subpopulation of immune cells determined after FACS analysis, as described in detail in Materials and methods. The values in each area are the average subpopulation of immune cells (NK cells and CD8⁺ cells). Each image is representative of three independent experiments. (**f**) The levels of IL-10, TNF- α , IL-1 β and IL-6 in the tumor tissues of transgenic mice (*n* = 3) were measured using quantitative real-time PCR, as described in Materials and methods. **P*<0.05 compared with non-transgenic mice tumor tissues.

by siRNA IL-32, NF- κ B or STAT3, or neutralized with an anti-IL-32 antibody, the IL-32 γ -induced colon cancer cell growth inhibition; IL-32 γ -induced decrease of TNF- α , IL-1 and IL-6 production; and increase of IL-10 production were abolished. These findings suggest that IL-32 γ has an inhibitory influence on tumor development.

However, the fundamental mechanisms underlying these phenomena are not clear. Because of their abilities to induce the expression of a large array of inflammatory mediators and their roles as core transcription factors in diverse immune responses, NF- κ B and STAT family signals have been recognized as major pathways responsible for cytokine-associated cancer development or antitumor immunity (Mantovani *et al.*, 2008; Baud and Karin, 2009). When the colon cancer cells or the *in vivo* tumor tissues were overexpressed with IL-32 γ , the expression of *NF*- κ B target genes, especially inflammatory and tumor development-associated genes, such as *iNOS* and *COX-2*, and antiapoptosis genes, Bcl-2, cIAP, xIAP and c-FLIP_L, and the DNA-binding activity of NF- κ B, were significantly inhibited, but the expression of cell death NF- κ B target genes, such as *Bax* and *caspase-3* and -9, was enhanced. In addition, p50 and p65 translocation into the nucleus and phosphorvlation of IkBa was also inhibited. The inhibitory effect on NF-kB activity was also found in conjunction with other subtypes of IL-32, such as α and β , and in other cancer cell lines, such as the prostate, liver and lung (data not shown). The colon cancer cell lines and human tumor samples, as well as the nuclei of stromal macrophages in sporadic adenomatous polyps, had increased levels of NF-κB (Hardwick et al., 2001; Lind et al., 2001), which is likely acting as a survival factor for colon and melanoma cancer cell growth. Constitutively activated NF-kB has been associated with several aspects of tumorigenesis, including tumor cell growth, antiapoptosis and tumor promotion in colon cancer (Karin et al., 2002). Thus, the inhibitory effects on NF-κB activity and on the expression of target proteins are critical in the IL-32y-induced inhibition of tumor growth. Although it remains to be investigated, it is worth noting that NF- κ B is activated or inactivated by many cytokines. It has been reported that IL-6 induces the activation of NF- κ B in the intestinal epithelia, resulting in the induction of intercellular adhesion molecule 1 expression (Wang et al., 2003), increase of cell cycle progression and suppression of apoptotic cell death-leading, tumor promotion (Lin and Karin, 2007). In contrast, IL-10 blocks NF-kB activity, causing apoptotic cell death of cancer cells (Schottelius et al., 1999), and it inhibits the production of proinflammatory cytokines, such as TNF- α , IL-6, IL-1 β and IL-12, which are closely associated with tumor development (Lin and Karin, 2007). We found that the levels of TNF- α , IL-1 β and IL-6 were significantly reduced, but IL-10 expression increased in the tumor tissues of BALB/C athymic nude mice inoculated with colon cancers expressing IL-32y and of IL-32y-overexpressing transgenic mice inoculated with B16 melanoma cells. In addition, siRNA of IL-32 γ or a neutralization with anti-IL-32 antibody abolished the constitutive activity of NF-KB (p50 and p65 translocation into the nucleus) accompanied with the abolishment of cell growth inhibition; decreased TNF- α , IL-1 β and IL-6 production; increased IL-10 production; and the induction of apoptotic cell death in cultured colon cancer cells. Moreover, siRNA of p50 and p65 augmented IL-32y-induced colon cancer cell growth inhibition. Very similar to this finding, it was also found that associated with attenuated tissue damage, IL-6 and TNF- α was lower, but IL-10 level was higher in IL-32y-overexpressed transgenic mice compared with those levels of wild-type mice having dextran sodium sulfate-inflamed colitis (Choi et al., 2010). Therefore, it is possible that the inhibition of the NF- κ B signal by IL-32 γ could play important roles in the inhibitory effect of IL-32 γ on colon cancer development and reduced production of pro-inflammatory cytokines (TNF- α , IL-1 β and IL-6), but an increase in the production of anti-inflammatory cytokine (IL-10) could be critically involved in this tumor killing effect of IL-32 γ .

We also found that STAT3 activation was significantly reduced in the tumor tissues of xenograft BALB/ C athymic nude mice inoculated with IL-32y-overexpressing colon cancer cells and in those of the IL-32 γ -overexpressing transgenic mice inoculated with B16 melanoma cells. Recently, the role of STAT3 and NF-kB and their relationship in inflammatory and cytokine-associated cancer development have been studied frequently (Lee et al., 2009; Yu et al., 2009). Activated nuclear STAT3 has been detected in many forms of cancer, including breast, colon, gastric, lung, head and neck, skin, prostate and others (Lee et al., 2009). Activation of STAT3 in the tumor contributes to the expression of pro-cancer inflammatory mediators and growth factors, leading to increased tumor growth. STAT3 also induces the expression of antiapoptotic genes in tumors (Lee et al., 2009). Conversely, conditional STAT3 ablation inhibited tumor development and progression (Chan et al., 2004; Jenkins et al., 2005). In addition to the inhibitory effect of IL-32 γ on cancer

cell growth and STAT3 activation, we found that knock down of STAT3 with siRNA further augmented the IL-32y-induced inhibition of colon cancer cell growth. Therefore, it is also possible that inhibition of the STAT3 signal by IL-32 γ could play important roles in the inhibitory effect of IL-32 γ on cancer development too. Activated STAT3 can increase NF-kB activity in cancer, and persistent activation of NF-kB in tumors is dependent on STAT3 activation (Lee et al., 2009). In agreement with these findings, we also found that STAT3 and NF- κ B were co-activated in colon cancer cells and tumor tissues, and the introduction of IL- 32γ reduced the co-activation of STAT3 and NF-kB. Functional interaction between NF-KB and STAT3 can occur as well. Many genes whose products play important roles in tumor development are controlled by NF- κ B and STAT3, either synergistically or individually (Bromberg et al., 1999; Bollrath and Greten 2009; Lee et al., 2009; Grivennikov and Karin, 2010). The expression of antiapoptotic genes, such Bcl-2, xIAP2 and cIAP2, which are prominent targets for NF-kB and STAT3, was increased by IL-32 γ ; however, the expression of NF-kB-dependent tumor-promoting genes, such as *c-FLIP*, *COX-2* and *iNOS*, was inhibited. The expression of STAT3 and NF- κB target genes controlling cell cycle and proliferation, such as cyclins D and B (Levy and Darnell, 2002; Jenkins et al., 2005; Naugler and Karin, 2008), also was decreased by treatment with IL-32 γ , and it was accompanied with G_0/G_1 -phase cell cycle arrest. Thus, the blocking ability of IL-32 γ on STAT3 alone and/or coordination of the inhibitory effect on NF- κ B could be significantly associated with the inhibition of tumor growth by IL- 32γ . It remains to be determined how IL- 32γ inhibited activated STAT3 and NF-kB, but it is also worth noting that several cytokines can modulate STAT3 and NF-KB activities to increase gene transcription. IL-6 activates STAT3, whereas IL-10 inhibits the activation of STAT3 during tumor growth inhibition (Yu et al., 2009). The acetylation and/or recruitment of p300 protein are critical in cytokine-induced activation of STAT3 and NF- κ B, and this cytokine (IL-6) prolongs their retention in the nucleus (Choi et al., 2002; Hou et al., 2008; Ray et al., 2008; Ma et al., 2010). We found that acetylation of p65 and expression of p300 in the nucleus of colon cancer cells were inhibited by the introduction of IL-32 γ and in the nucleus of tumor tissues overexpressing IL-32 γ (data not shown). Thus, direct inhibition of acetylation and/or recruitment of p300 protein may partially contribute to the inhibitory effects of IL-32 γ on the inactivation of STAT3 and NF-κB. Indirect inactivation via modulation of other cytokines in the tumor tissues (decreases of TNF- α , IL-6, and IL-1 β and/ or an increase of IL-10) may also play a role in the inhibitory effects of IL-32 γ on the inactivation of STAT3 and NF-κB.

The interaction between tumor and immune cells is primarily responsible for overall tumor progression (or regression) and the spreading or induction of antitumor immune responses and tumor rejection (Shurin *et al.*, 2006). Cytokines can act as inducers, causing the

activation of T cells (cytotoxic CD8+ T cells) and NK cells in the tumor tissues, which results in the apoptotic cell death of tumor cells (Moretta et al., 2000). The expression of IL-2 and IL-10 at the site of tumors enhances tumor killing ability by inducing the generation of tumor-reactive cytotoxic T lymphocytes and by allowing the increased infiltration of activated T cells into the tumors (Lee *et al.*, 1998). We found that IL-32 γ overexpressing transgenic mice and xenograft nude mice have higher levels of CD8 + T cells and CD57-positive NK cells in the tumor area, blood, spleen and thymus. Thus, activation of immune cells, such as cytotoxic T cells and NK cells, could be important in the inhibition of colon cancer development by IL-32 γ . Several recent studies have reported that activation of STAT3 and NF-κB signals in tumors by cytokines, such as IL-6 and IL-12, impedes dendrite maturation and inhibits the generation of T cells, giving rise to immune tolerance against cancers (Lee et al., 1998; Moretta et al., 2000; Chan et al., 2004; Jenkins et al., 2005; Shurin et al., 2006). Thus, it is possible that inactivation of STAT3 and NF- κ B by the overexpression of IL-32 γ may also enhance immune responses to the tumor via an increase of traffic into the tumor area or maturation of immune cells, especially cytotoxic T cells and NK cells, thereby causing indirect inhibition of tumor growth. We are currently studying the roles and mechanisms of IL-32s on the immune responses against cancer development. The present data conclusively show that IL- 32γ can act as a cytokine-inhibiting cancer cell growth and that the inhibition of NF- κ B and STAT3 signaling and the reduced production of pro-inflammatory cytokines (but increased production of anti-inflammatory cytokines) play an important role in the inhibitory effect of IL-32y on colon cancer and melanoma cell growth.

Materials and methods

Generation of IL-32y transgenic mice

To generate transgenic mice that express hIL- 32γ , concentrated hIL-32 γ cDNA was prepared. The pCAGGS/hIL-32 γ plasmid was prepared using the Qiagen MIDI-Prep Kit. To generate IL-32 γ transgenic mice, a 705-base pair fragment of the hIL-32 γ gene was subcloned into the EcoRI sites of the pCAGGs expression vector. The sequence of the IL-32 γ insert was confirmed by automated sequencing. The IL-32y genecontaining genomic fragment was released from the vector using Sall/HindIII digestion, and then separated from the pCAGGs vector using low-melting-point agarose and transverse alternating field-gel electrophoresis. The fragment was purified and microinjected at a concentration of 4 ng/µl into the embryos of BDF1 mice, as described previously (Wang et al., 2005; Kim et al., 2008). The experimental treatments were carried out according to the guidelines for animal experiments of the Faculty of Disease Animal Model Research Center, Korea Research Institute of Bioscience and Biotechnology (Daejeon, Korea). IL-32 γ insertion was confirmed by amplification of genomic DNA isolated from the transgenic mice tails using Super Taq PLUS Pre-mix (RexGeneBioTech, Seoul, Korea) and the following specific primer set: sense, 5'-GAAGGTCCTCTCTGATGA

CA-3'; and antisense, 5'-GAAGAGGGACAGCTATGAC TG-3' (nt 2245–2225). pCAGGS/IL-32 γ was used as a positive control, and pCAGGS (4.8 kb) was used as a negative control. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control. Genomic DNA samples were extracted from transgenic mice tails and PCR analysis was performed for $IL-32\gamma$ gene expression. The following conditions were used for the TaKaRa PCR Thermal Cycler: 94 °C for 10 min, followed by 35 cycles of 94 °C for 1 min, 63 °C for 1 min and 72 °C for 1.5 min, with a final step of 72 °C for 10 min. To analyze IL-32 gene expression in various tissues of transgenic mice, total RNAs were extracted and RT-PCR analysis was performed using primer sets as follows: sense, 5'-GAAGGTCCTCTCTGATGACA-3'; antisense, 5'-GGGG TTCAGAGCACTTCT-3' (371 bp). GAPDH was used as an internal control as follows: sense, 5'-ACCACAGTCCATGC CATCAC-3'; antisense, 5'-TCCACCACCCTGTTGCTGTA-3' (450 bp). IL-32 γ protein expressed in the tissues of transgenic mice was detected by western blotting with an anti-IL-32 monoclonal antibody KU32-52 (Kim et al., 2008).

Antitumor activity of IL-32y in IL-32y transgenic mice

Male, 6- to 8-week-old IL-32 γ transgenic and non-transgenic mice were maintained in accordance with the guidelines proscribed by the Chungbuk National University Animal Care Committee (Chungbuk, Korea). B16 melanoma cells were injected subcutaneously (5 × 10⁵ tumor cells in 0.1 ml phosphate-buffered saline per animal) into transgenic mice and non-transgenic mice. The weights and tumor volumes of the animals were monitored twice weekly. The tumor volumes were measured with Vernier calipers and calculated using the following formula: ($A \times B^2$)/2, where A is the larger and B is the smaller of the two dimensions. At the end of the experiment, the animals were killed and the tumors were separated from the surrounding muscles.

Detection of IL-32 in the sera of transgenic mouse

The level of IL-32 in the sera was detected by enzyme-linked immunosorbent assay methods as described elsewhere (Lee *et al.*, 2010).

In vivo antitumor activity of IL-32 γ in a xenograft animal model Six-week-old male BALB/c athymic nude mice were purchased from Japan SLC (Hamamatsu, Japan). All experiments were approved and carried out according to the Guide for the Care and Use of Animals (Chungbuk National University Animal Care Committee). Human colon cancer SW620 cells that had been transfected with the vector or the IL-32 γ plasmid (6 µg/ 1×10^6 cells) were injected subcutaneously (1×10^7 tumor cells in 0.1 ml PBS per animal) into the right-lower flanks of the carrier mice. The body weights and tumor volumes of the animals were monitored twice weekly. The formula described above was used to calculate tumor volume. At the end of the experiment, the animals were killed by cervical dislocation. The tumors were separated from the surrounding muscles and dermis, excised and weighed.

Immunohistochemistry

All specimens were fixed in formalin and embedded in paraffin for examination. Sections (4-µm thickness) were stained with hematoxylin and eosin and analyzed by immunohistochemistry as described elsewhere (Son *et al.*, 2007) using primary mouse anti-human proliferating cell nuclear antigen, Ki-67, bax (1:200 dilution), CD3 (1:10) and CD57 (1:50) monoclonal antibodies or primary rabbit anti-human cleaved caspase-3

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polyclonal antibody (1:100) and secondary biotinylated antimouse and anti-rabbit antibodies.

Detection of apoptosis

TUNEL assays were performed using the *In situ* Cell Death Detection Kit (Roche Diagnostics GmbH, Mannheim, Germany), as described elsewhere (Son *et al.*, 2007). The total cell number in a given area was determined based on 4',6-diamidino-2-phenylindole nuclear staining. The apoptotic index was calculated as the number of 4',6-diamidino-2-phenylindole-stained, TUNEL-positive cells divided by the total number of cells counted \times 100.

Reagents and cell culture

The HCT116 and SW620 colon cancer cell lines and B16 melanoma cells were obtained from the American Type Culture Collection (Manassas, VA, USA). Colon cancer cells were grown at 37 °C in 5% CO₂ humidified air in RPMI 1640 medium that contained 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μ g/ml streptomycin. B16 melanoma cells were grown at same conditions in DMEM medium. RPMI 1640, DMEM, penicillin, streptomycin and FBS were purchased from Gibco Life Technologies (Grand Island, NY, USA). siRNA species for IL-32, p50 and p65, and a non-targeting control siRNA were purchased from Bioneer (Daejeon, Korea) and siRNA for STAT3 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Transfection

Colon cancer cells $(5 \times 10^4$ cells per well) were plated in 24-well plates and transiently transfected with $0.4 \,\mu g$ of the empty vector or the constitutively activated full-length IL-32 γ plasmid per well, using a mixture of plasmid and the WelFect-EX PLUS reagent in OPTI-MEN, according to the manufacturer's specification (WelGENE, Seoul, Korea).

Cell viability

To determine viable cell numbers, the colon cancer cells were seeded onto 24-well plates (5×10^4 cells per well). The cells were trypsinized, pelleted by centrifugation for 5 min at 1500 r.p.m., resuspended in 10 ml of PBS and 0.1 ml of 0.2% Trypan blue was added to the tumor cell suspension in each solution (0.9 ml each). Subsequently, a drop of suspension was placed in a Neubauer chamber, and the living cancer cells were counted. Cells that showed signs of Trypan blue uptake were considered to be dead, whereas those that excluded Trypan blue were considered to be viable. Each assay was carried out in triplicate.

Western blotting

Western blot analysis was performed as described previously (Son *et al.*, 2007). The membranes were immunoblotted with the following primary antibodies: mouse monoclonal antibodies directed against p65, p50 and c-FLIP (1:500 dilutions; Santa Cruz Biotechnology, Santa Cruz, CA), rabbit polyclonal antibodies directed against bax, poly-(ADP-ribose) polymerase, COX-2, iNOS and CDK4 (1:500 dilutions; Santa Cruz Biotechnology) and against caspase-3, cleaved caspase-3, caspase-9, Bcl-2, XIAP, c-IAP-1 and Akt (1:1000 dilutions; Cell Signaling Technology, Beverly, MA, USA). The monoclonal anti-hIL-32 antibody KU32-52 was used as reported previously (Kim *et al.*, 2008).

Gel electrophoretic mobility shift assay

A gel electromobility shift assay was performed according to the manufacturer's recommendations (Promega, Madison, WI, USA), as described elsewhere (Son *et al.*, 2007).

Fluorescence microscopy

The fixed cells and tissues were exposed to following primary antibodies: p50, p65 and p-STAT3 (1:100 dilutions in blocking serum; Santa Cruz Biotechnology) at room temperature for 1 h. After incubation, the cells were washed twice with ice-cold PBS and incubated with an anti-rabbit or -mouse secondary antibody conjugated to Alexa Fluor 488 or 568 (Invitrogen– Molecular Probes, Carlsbad, CA, USA) at room temperature for 1 h. Immunofluorescence images were acquired using an inverted fluorescent microscope Zeiss Axiovert 200 M (Carl Zeiss, Thornwood, NY, USA).

Quantitative real-time PCR

For mRNA quantification, total RNA was extracted using the RNAqueous kit and the cDNA was synthesized 1 µg of total RNA using High Capacity RNA-to-cDNA kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol. Quantitative real-time PCR was performed using specific primers for GAPDH (Mm99999915 g1), IL-6 (Mm00446190 m1), TNF-α (Mm00443258 m1), IL-10 (Mm00439616_m1) and IL-1ß (Mm00434228_m1) in a 7500 Real-Time PCR System (Applied Biosystems). Thermocycling conditions consisted of an initial denaturation of 20 s at 95 °C, followed by 60 cycles of 95 °C for 30 s and 60 °C for 30 s. The values obtained for the target gene expression were normalized to GAPDH and quantified relative to the expression in control samples. For the calculation of relative quantification, the 2^{- $\Delta\Delta CT$} formula was used, where $-\Delta\Delta CT = (C_{T,target})^{-1}$ $C_{T,GAPDH}$) experimental sample- $(C_{T,target} - C_{T,GAPDH})$ control sample.

FACS analysis for immune cell populations

Immune cell populations in the whole blood, spleen and tumor were analyzed by FACS analysis. One hundred microliters of whole blood was collected using a hematocrit capillary tube and blocked with Fc block (eBioscience, San Diego, CA, USA) to reduce nonspecific antibody binding for 3 min at room temperature. Cells were then incubated in the dark with 10 µl of the appropriate fluorochrome-conjugated antibodies from eBioscience-T cells (anti-CD3-FITC, 1:25), B cells (anti-CD19-PE, 1:25) and NK cells (anti-CD49-APC, 1:50) for 20 min at 4 °C. Cells were washed with 500 µl of FACS buffer containing 0.02% sodium azide and 2% FBS in PBS. The red blood cells were lysed for 5 min with FACS lysis buffer (BD Bioscience, Franklin Lakes, NJ, USA) at room temperature, and then re-washed with FACS buffer. Finally, each sample was fixed with 1% paraformaldehyde until further analysis. Flow cytometry analysis was performed on the FACSCalibur system (BD Biosciences, Franklin Lakes, NJ, USA). Control samples were matched for each fluorochrome. Data were analyzed using the CellQuest software (Becton Dickinson, Franklin Lakes, NJ, USA). For immune cell population analysis in the spleens and tumor, tissues were disrupted by forcing them through 70-um cell strainer into 10 ml of cold PBS using a rubber-tipped syringe plunger. The cell suspensions were then centrifuged at 1500 r.p.m. for 10 min and the supernatants were discarded. The cells were resuspended in 3 ml ACK lysing buffer (Lonza, Walkersville, MD, USA) for 3 min and the debris was sedimented by centrifuging at 1500 r.p.m. for 10 min. Cell concentrations were determined by hemocytometer counting using Trypan blue dye exclusion,

3358

and were adjusted to 1×10^6 cells per ml. Single-cell suspensions were stained with the following fluorochrome-conjugated antibodies from BD Biosciences: B cells (anti-B220-APC, 1:100), T cells (anti-CD3-FITC, 1:400), NK cells (anti-CD49-APC, 1:50) and cytotoxic T cells (anti-CD8-FITC, 1:100). Flow cytometry and data analysis was carried out as mentioned above in the blood.

Statistical analysis

The data were analyzed using the GraphPad Prism 4 ver. 4.03 software (La Jolla, CA, USA). Data are presented as means \pm s.d. The homogeneity of variances was assessed using the Bartlett test in tumor incidence test. 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 Dunnett's test. A value of *P*<0.05 was considered to be statistically significant.

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Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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