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Avian leukosis virus p27 inhibits tumor necrosis factor α expression in RAW264.7 macrophages after stimulation with lipopolysaccharide

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Summary. – Avian leukosis is a widely distributed disease caused by Avian leukosis virus (ALV). ALV p27 is a capsid protein and group specific antigen, whose role in host immune response is poorly understood. To explore ALV p27, we developed a RAW264.7 macrophage cell line stably expressing p27-GFP fusion protein. The cells of this line and control cell line expressing GFP protein only were compared in production of tumor necrosis factor α (TNF-α), interleukins IL-1β, IL-6, IL-12, and in proliferative activity stimulated by the lipopolysaccharide (LPS). It was found that the ALV p27 expression markedly reduced the production of TNF-α, but did not affect the production of IL-1β, IL-6, and 12, and cell proliferative activity. These results demonstrate that ALV p27 specifically inhibits TNF-α expression in the macrophages stimulated by LPS, suggesting a possible contribution of ALV p27 to immunosuppression detected in ALV-infected hosts.

Keywords: Avian leukosis virus; p27; tumor necrosis factor α; interleukins; macrophages; lipopolysaccharide

Introduction

Avian leukosis virus (the family *Retroviridae*, the genus *Alpharetrovirus*) is a common occurring avian pathogen transmitted vertically from hen to the progeny and horizontally from bird to the bird. ALV induces neoplastic diseases and other production problems in chickens (Stedman and Brown, 1999). The genome of ALV is about 7.2 kb in size and encodes structural and enzymatic proteins, such as gag, pol, and env. The capsid protein ALV p27 is encoded by the gag-gene and acts as a major group-specific antigen (Weiss, 2006). Localization of ALV p27 in the tissues of ALV-infected chickens indicated that ALV primarily replicates in the lymphocytes of bursa of Fabricius and in the spleen, suggesting that the lymphoid tissue is a target for ALV infection in chickens (Hortling *et al*., 1975; Spencer and Gilka, 1982). In addition, ALV is also present in the heterophils of ALV-infected chickens and decreased their bactericidal ability (Stedman *et al*., 2001). ALV p27 could also be detected in the serum of ALV-infected chickens and was determined to be the indicator of ALV infections (Barbour *et al*., 1999). These findings suggest that ALV infection may induce immunosuppression in chickens and ALV p27 may play a role in this process.

The virus-induced immune suppression has long been recognized and antiretroviral therapy has been thoroughly studied in some hosts (Wainberg and Mills, 1985; Dorrell, 2005; Feola *et al*., 2006; Weiss, 2000). However, very few data regarding a cytokine response against ALV infection in the host were available except for the finding that transforming growth factor-β_2 (TGF-β_2) might be related to the ALV infection (Barbour *et al*., 1999). Therefore, the exact role of individual ALV components in eliciting the immune response is not very clear.

Since macrophages are important contributors to innate immunity and also participate in the adaptive responses via presentation of antigen and secretion of cytokines and

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Abbreviations: ALV = Avian leukosis virus; GFP = green fluorescence protein; LPS = lipopolysaccharide; p.i. = post infection; TGF-β_2 = transforming growth factor β_2; TNF-α = tumor necrosis factor α

chemokines (Zheng *et al.*, 2004; Burleson and Burleson, 2007), the aim of this study was to explore the effect of ALV p27 expression on host immune response, namely on the production of TNF-α and some interleukins and on cell proliferative activity following stimulation by LPS.

Materials and Methods

Plasmid constructs. ALV p27 gene was subcloned from plasmid pET28α-p27 (kindly provided by Dr. Jing Chen) into expression plasmid vectors pEGFP-N1 vector (Clontech). ALV p27 gene was amplified for 30 cycles with a specific set of primers (5'-AAG CTT GCC ACC ATG CCT GTA GTG ATT AAG ACA GAGG-3', 5'-GG ATC CGA GGG CTG GAT AGC AGA CGA CAT AGC-3'). The PCR reaction was performed on Techne TC512 Gene Amp PCR System (Techne Corporation). The program consisted of an initial denaturation step at 94°C for 4 mins and 30 amplification cycles, each with 1 min at 94°C, 30 secs at 55°C and 40 secs at 72°C, followed by an additional extension step at 72°C for 10 mins. The sequence of ALV p27 gene plasmid was confirmed by sequence analysis (Shanghai Shenergy Biocolor Bioscience & Technology Company). pEGFP-N1-p27 construct was confirmed by restriction analysis after digestion with *Hind*III and *Bam*HI at 37°C for 1 hr. The plasmid DNA was prepared with Maxiprep Kits (Vigorous Biotech Co.).

Agarose gel electrophoresis. PCR products or restriction endonuclease digestion products were resolved on 1% agarose gels. Results were photographed using Alpha Imager TM 2200.

Development of RAW264.7 cell lines expressing GFP or p27-GFP. RAW264.7 cells were seeded into 96-well plates at a density of 5×10^4 cells/well and cultured for 24 hrs before transfection with pEGFP-N1-p27 or pEGFP-N1 plasmids using the FuGENE® HD Transfection Reagent (Roche) with reference to the protocol provided by the manufacturer. The transfected cells were selected with 0.5 mg/ml G-418 for 2 weeks before the survival cells were trypsinized, serially diluted, and cultured with 96-well plates. p27-GFP/GFP-stable RAW264.7 cells were maintained in DMEM culture medium in the presence of 0.25 mg/ml G-418. The expression of GFP or p27-GFP fusion proteins by the selected RAW 264.7 clones were visualized under a fluorescence microscope and ALV p27 was examined by Western blot analysis with specific ALV antiserum.

Stimulation of macrophages with lipopolysaccharide (LPS). RAW264.7 cells stably expressing p27-GFP fusion or GFP proteins were seeded into 48-well plates and cultivated using DMEM with 10% FCS in the presence of 100 ng/ml LPS for 12, 24, and 48 hrs before supernatants were examined for the presence of the cytokines.

Western blot analysis. 293T cells (transfected with pEGFP-N1-p27 or pEGFP-N1 plasmid as a negative control) or stable RAW264.7 p27-GFP/GFP- cells were washed twice with cold PBS and lysed in 100 µl of lysis buffer containing 0.05 mol/l Tris, 0.15 mol/l NaCl, 0.01 mol/l EDTA, 1% NP40, and 1:100 diluted protease inhibitor cocktail (Shanghai Shenergy Biocolor Bioscience & Technology Company) for 20 mins on ice. The cell lysates were centrifuged at 12,000 rpm for 10 mins at 4°C and the supernatant was subjected to PAGE with 12% gel. Separated proteins were transferred to nitrocellulose membrane at 100V for 1 hr. The blots were blocked with 5% nonfat milk, incubated with either ALV rabbit antiserum diluted at 1:400 or β-actin monoclonal antibodies diluted at 1:600 (Clone sc-1616, Santa Cruz Biotechnology), and subsequently with corresponding HRP-labeled secondary antibodies (goat anti-rabbit or goat anti-mouse IgG antibodies) (DingGuo Biotech). The blots were developed using chemiluminiscence detection reagents (Vigorous Biotech Co.).

Immunofluorescence assay. The transfected 293T cells or stable RAW264.7 p27-GFP/GFP cells were observed under the fluorescence microscope (Nikon-Eclipse80i). Original magnification is 100x.

ELISA of TNF-α, IL-1β, IL-6, and IL-12. The supernatants from RAW264.7 cell cultures after LPS stimulation were examined for the presence TNF-α, IL-1β, IL-6 or IL-12 using ELISA sets (BD Pharmingen) according to the manufacturer's instruction.

Results and Discussion

Expression of p27-GFP fusion protein in 293T cells

ALV p27 from pET28α-p27 plasmid was subcloned into the expression plasmid pEGFP-N1 (Clonetech) using specific primers as described in Materials and Methods. The insertion of ALV p27 gene into pEGFP-N1 was confirmed by PCR assay and restricted enzymatic analysis (Figs. 1, 2). To determine the expression of ALV p27 gene in eukaryotic cells, 293T cells were transiently transfected with the pEGFP-N1-p27 or control plasmids (Fig. 3a,b) and examined for the presence of ALV p27 protein by Western blot analysis. ALV p27 protein could be detected in 293T cells 48 hrs post transfection with pEGFP-N1-p27 plasmids as detected by ALV antiserum using Western blot analysis (Fig. 3c). This result clearly indicates the antigenic activity of ALV p27 protein expressed in eukaryotic cells after transfection with pEGFP-N1-p27.

Establishment of macrophage cell lines stably expressing p27-GFP fusion protein or GFP protein

Since RAW264.7 macrophages are generally used as model cells to elucidate the function of macrophages *in vitro* (Gao *et al.*, 1999; Weber *et al.*, 2006), we examined the effect of ALV p27 on cytokine response in mononuclear cells after LPS treatment using RAW264.7 macrophages as the model cells. As the efficiency of transient transfection in RAW264.7 cells is usually poor and not sufficient for analysis of the transfected cells phenotype, a stable transfection of RAW264.7 macrophages with expression plasmids is usually performed to get over-expression of target genes for determination of their effects on macrophages (Dahlberg *et al.*, 1996; Weber *et al.*, 2006). We developed RAW264.7 cell line stably expressing p27-GFP fusion protein via transfection of

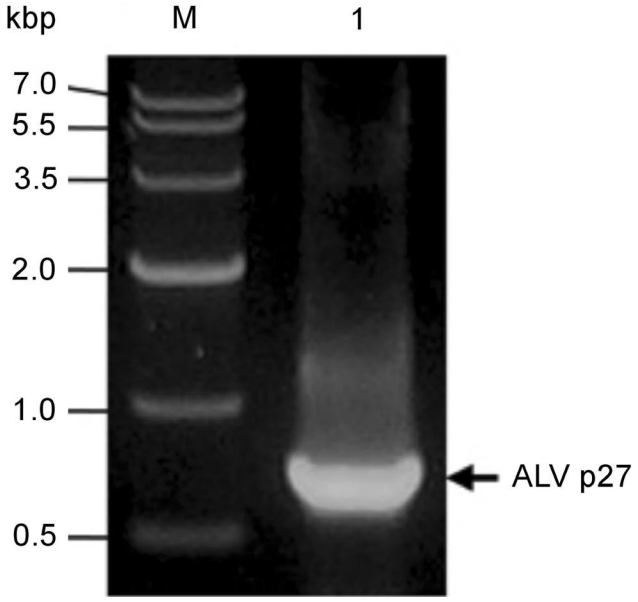

Fig. 1

Amplification of ALV p27 gene from pET28α-p27 plasmid

Agarose gel electrophoresis of PCR product (lane 1). DNA ladder (lane M).

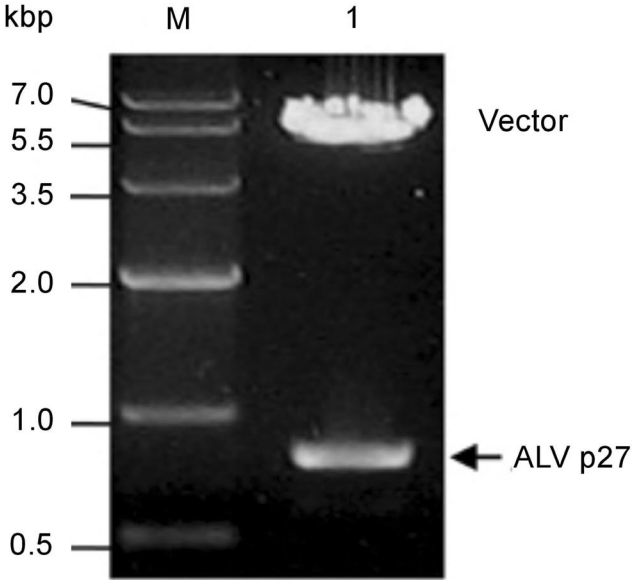

Fig. 2

Restriction analysis of pEGFP-N1-p27 plasmid

Agarose gel electrophoresis of pEGFP-N1-p27 digested with *Hind*III and *Bam*HI (lane 1). DNA ladder (lane M).

RAW264.7 cells with pEGFP-N1-p27 plasmid. The stable clones of RAW264.7 cells were selected with G-418 (Hou and Hsu, 2005). They expressed p27-GFP fusion protein or GFP protein as control and were visualized under fluorescence microscope (Fig. 4a,b). ALV p27 protein could be detected in pEGFP-N1-p27 transfected RAW264.7 cells, but not in control cells using Western blot analysis (Fig. 4c). These results demonstrated that the stable clones of RAW264.7 cell lines expressing GFP or p27-GFP fusion proteins were successfully established and allowed us to explore the effect of ALV p27 on cytokine response of macrophages after LPS treatment.

Effect of ALV p27 expression on production of TNF-α and interleukins in RAW264.7 cells and their proliferative activity

To analyze the effect of ALV p27 on cytokine response in macrophages, the stable RAW264.7 cell lines expressing GFP or p27-GFP fusion proteins were treated with LPS, a potent activator of macrophage proliferation that binds to toll-like receptor (TLR)-4 (Hoshino *et al.*, 1999; Beutler, 2002). Because macrophages activated by LPS produce primarily proinflammatory cytokines such as TNF-α, IL-1, IL-6, and IL-12 (Zheng *et al.*, 2004, 2005), we examined the concentrations of these cytokines in the supernatant of stimulated cells. Interestingly, among all these cytokines, the production of TNF-α in RAW264.7 p27-GFP cells dramatically decreased as compared to that of controls ($p < 0.01$) (Fig. 5). Although IL-1, IL-6, and IL-12 were detectable in the supernatants of RAW264.7 p27-GFP cells and controls, no significant difference was observed between RAW264.7 p27-GFP and control cells in IL-1β (15.5 ± 1.1 pg/ml vs. 17.3 ± 1.2 pg/ml, $p = 0.28$), IL-6 (20.1 ± 4.8 pg/ml vs. 29 ± 12.5 pg/ml, $p = 0.33$), and IL-12 (13.2 ± 6 ng/ml vs. 7.6 ± 3.3 ng/ml, $p = 0.29$). In addition, the proliferative activities of stable RAW264.7 cells did not change with or without the presence ALV p27 protein (Fig. 6), which rules out the possibility that ALV p27 affected cytokine expression via alteration of the cell cycle. These results clearly demonstrate that ALV p27 specifically inhibits the expression of TNF-α in RAW264.7 macrophages after LPS treatment.

One of the mechanisms retroviruses are able to induce tumors in the host is the inactivation of tumor suppressor genes. It has long been recognized that TNF cytokines are toxic to the tumor cells and reduce a tumor regression in mice (Aggarwal, 2003). Therefore, TNF family members have been extensively studied for their anti-tumorigenic properties leading to promising results (Aggarwal, 2003; Gaur and Aggarwal, 2003). In this study, ALV p27 was found to inhibit the production of TNF-α by RAW264.7 cells after LPS treatment, suggesting an important role of ALV p27 in facilitating tumor progression through down-regulation of TNF-α expression. In addition to its anti-tumorigenic activity, TNF

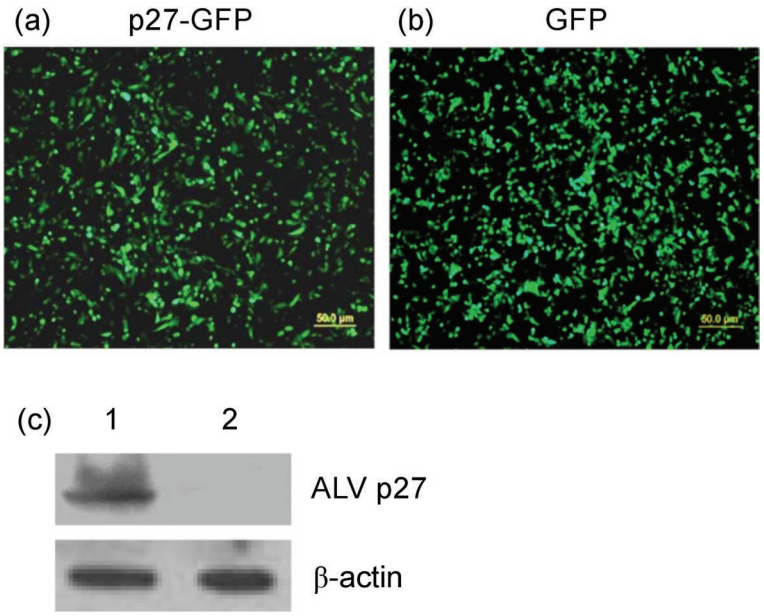

Fig. 3

Transient expression of p27-GFP fusion protein in 293T cells transfected with pEGFP-N1-p27 plasmid

Immunofluorescence assay of cells transfected with pEGFP-N1-p27 plasmid (a) and pEGFP-N1 plasmid (b). Western blot analysis of ALV p27 (c).

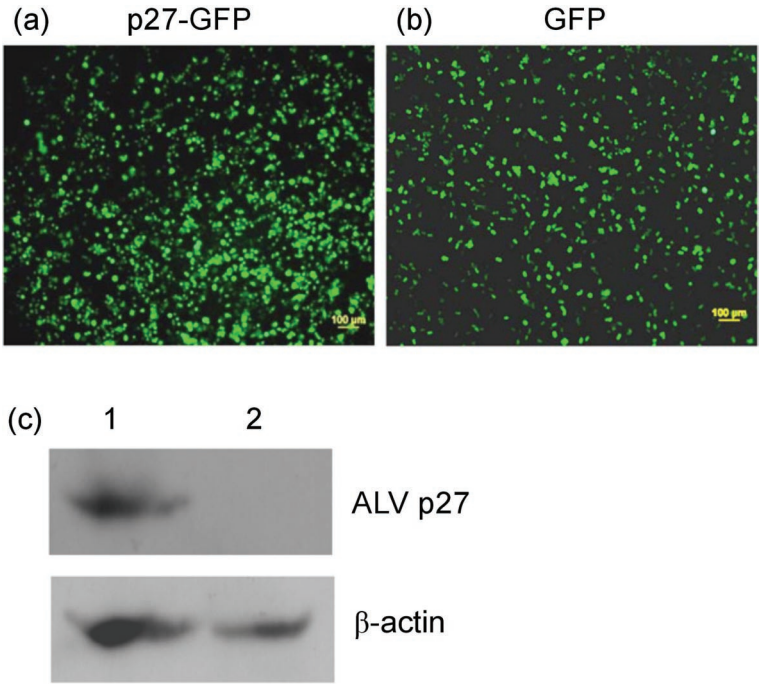

Fig. 4

Stable expression of p27-GFP fusion protein in RAW264.7 cells

Imunofluorescence assay of RAW264.7 cells expressing p27-GFP fusion protein (a) and GFP protein (b). Western blot analysis of ALV p27 (c).

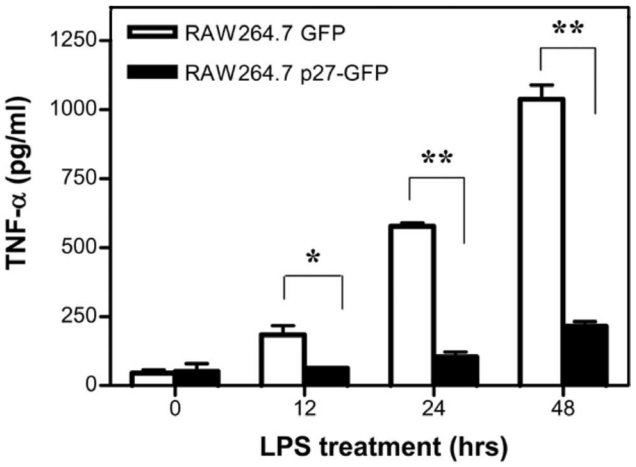

Fig. 5

Inhibition of TNF-α production by ALV p27 expression in RAW264.7 cells stimulated with LPS

Results are representative of two independent experiments and presented as means ± SEM. *Stands for significant difference between two groups ($p < 0.05$) and ** for significant difference between two groups ($p < 0.01$).

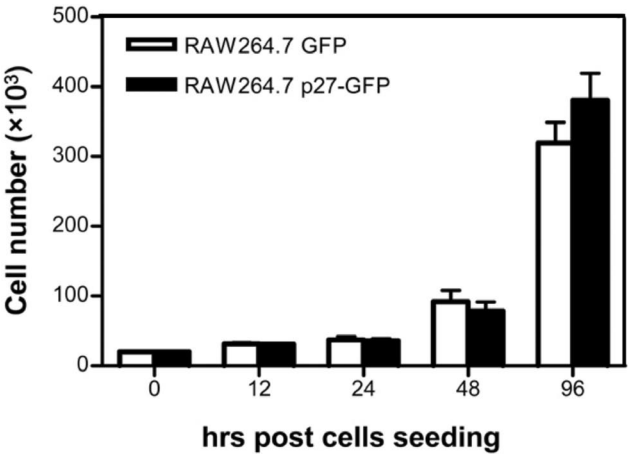

Fig. 6

ALV p27 expression showed no effect on the proliferative activity of RAW264.7 cells

Results are representative of two independent experiments and presented as means ± SEM.

acts as an important immune mediator involved in multiple processes to regulate immune responses. The macrophages are the major source of this cytokine (Aggarwal, 2003). ALV p27 may also directly compromise the immune response to pathogens through down-regulation of TNF-α expression in macrophages, which leads to a reduced bacterial uptake and killing. Monocyte-macrophages are considered as the first line of the immunological defense and may initiate and modulate both specific and nonspecific immune responses via processing and presentation of antigen, phagocytosis, killing of pathogens, and secretion of immune mediators including TNF (Aggarwal, 2003; Burleson and Burleson, 2007). ALV infection may increase flock morbidity and/or mortality due to its immunosuppressive effects that allows the chance of secondary infections by opportunistic bacterial agents. in the epidemic surveillance ALV p27 protein has been targeted as an indicator of ALV infection. High levels of ALV p27, as high as 2899 pg/ml in the serum, could be detected in ALV-infected chickens (Barbour *et al.* 1999). In addition, the presence of another tumor suppressor TGF-β_2 was also found to correlate with chronic ALV infections (Barbour *et al.*, 1999). Since TGF-β_2 is primarily produced by T cells and macrophages and plays an important role in the inhibition of lymphocyte and tumor cell proliferation, it will be interesting to investigate the role of ALV p27 in the induction of TGF-β_2 expressions in the host. Although our study indicated that ALV p27 inhibited TNF-α production by RAW264.7 macrophages, more efforts will be required to elucidate the exact mechanisms underlying the immunosuppressive effects of ALV infection.

In this study we stably transfected RAW264.7 cells with pEGFP-N1-p27 or pEGFP-N1 with the purpose to directly visualize cells expressing the target gene. However, GFP protein, as a popular gene expression marker, may have toxic effects on cells since it induces the secretion of IL-6 in muscle cells (Mak *et al.*, 2007). In the present study, both RAW264.7 p27-GFP/GFP cells expressed GFP, thus reduces as much noise as possible from GFP expression. In addition, there was no significant difference between p27-GFP RAW264.7 cells and controls in terms of producing IL-1β, IL-6, and IL-12. This may suggest a direct contribution of ALV p27 to the immunosuppressive effects of ALV on host via reducing TNF-α expression in macrophages.

In conclusion, ALV p27 inhibits TNF-α expression in RAW264.7 cells after LPS treatment, suggesting the immunosuppressive effects of this protein on the host defense.

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