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Title: Physical and functional interactions between STAT3 and Kaposi's Sarcoma-associated Herpesvirus-encoded LANA

Running title: Physical and functional interactions between STAT3 and LANA

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Key words: STAT3; KSHV; LANA; transcription

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Abbreviations: STAT, signal transducer and activator of transcription; KSHV, Kaposi sarcoma-associated herpesvirus; LANA, latency-associated nuclear antigen, PEL, primary effusion lymphoma

Abstract

The Kaposi's sarcoma-associated herpesvirus (KSHV)-encoded latency-associated nuclear antigen (LANA) is known to modulate viral and cellular gene expression. We show that LANA directly associates with an IL-6 signal transducer, STAT3 and that LANA enhances the transcriptional activity of STAT3. Coimmunoprecipitation studies documented a physical interaction between LANA and STAT3 in transiently transfected 293T cells as well as the KSHV-infected primary effusion lymphoma (PEL) cell line. Furthermore, small-interfering RNA-mediated reduction of LANA expression decreased the STAT3-dependent transcription in KSHV-positive PEL cells, whereas overexpression of LANA enhanced STAT3 activity in KSHV-negative B lymphoma cells. These data demonstrate that LANA is a transcriptional co-activator of STAT3, and may have implications for the pathogenesis of KSHV-associated diseases.

1. Introduction

STAT3 was originally cloned as an acute-phase response factor activated by interleukin-6 (IL-6) in mouse liver, and also by homology to STAT1 [1,2]. Growth factors, such as epidermal growth factor, platelet-derived growth factor and colony-stimulating factor-1, can also stimulate STAT3 activity [1,2]. STAT3 is also known to play crucial roles in early embryonic development as well as in other biological responses including cell growth and apoptosis [1,2,3]. Furthermore, STAT3 is constitutively activated in oncogene-transformed cells and various primary tumors and cell lines [4]. Several tumor viruses are also known to be associated with STAT3 activation. STAT3 is constitutively activated in human T cell lymphotropic virus I -transformed T cells and Epstein-Barr virus (EBV)-related lymphoma cell lines [5,6,7]. The herpesvirus saimiri tyrosine kinase-interacting protein Tip-484 also activates STAT3 [8].

The Kaposi sarcoma-associated herpesvirus (KSHV), also known as human herpesvirus 8, has been associated with HIV-related and -unrelated cases of Kaposi sarcoma, primary effusion lymphoma (PEL), and multicentric Castleman disease [9,10,11]. The majority of KSHV-infected cells harbor the virus in a latent form. During viral latency, latency-associated nuclear antigen (LANA), is critical to the persistence of KSHV episomes and functions in this capacity by tethering viral episomes to chromosomes during mitosis [12,13,14]. Viral IL-6, the KSHV homolog of human IL-6, is known to serve as an autocrine growth factor for KSHV-infected PEL cells [15]. Furthermore,

STAT3 plays a critical role in promoting survival of KSHV-infected PEL cells [16]. LANA physically interacts with cellular proteins, such as p53, pRB and GSK3 β , resulting in inhibition of p53-mediated apoptosis, dysregulation of β -catenin and the Wnt signaling pathway [17,18,19].

In present study, we examined the physical and functional interactions between STAT3 and LANA in a KSHV-infected PEL cell line. Furthermore, RNA interference and overexpression experiments confirmed that LANA modulates STAT3 activity in the KSHV-infected or -uninfected human B lymphoma cells. Thus, these data show that LANA is a transcriptional co-activator of STAT3.

2. Materials and Methods

2. 1. Reagents and antibodies

Recombinant human IL-6, interferon (IFN)- α and erythropoietin (EPO) were kindly provided from Ajinomoto (Tokyo, Japan), Sumitomo Pharmaceuticals (Osaka, Japan) and Kirin Company. (Tokyo, Japan), respectively. Recombinant human LIF was purchased from INTERGEN (Purchase, NY). Expression vectors, epitope-tagged STAT3, STAT3YF, STAT3-LUC, STAT3-C, EPO receptor, ISRE-LUC, STAT5-LUC were provided by Dr. T. Hirano (Osaka University, Osaka, Japan), Dr. J. F. Bromberg (Rockefeller Univ., New York, NY), Dr. J. N. Ihle (St. Jude CRH, Memphis, TN) and Dr. D. Wang (The Blood Res. Inst., Milwaukee, WI), respectively [20,21]. FLAG-LANA (pDY52) and LANA-SiRNAs (siN-LANA and siC-LANA) cloned into pSuper plasmid were previously described [18]. FLAG-LANA deletion mutants were generated by PCR and sequenced (primer sequences are available upon request). Anti-LANA mAb was purchased from Advanced Biotechnologies Inc. (Maryland USA). Anti-Myc, anti-GST, anti-STAT3, anti-cyclin D1 and anti-cdk4 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phosphoSTAT3 (Tyr705) was purchased from Cell signaling Technologies (Beverly, MA). Anti-FLAG M2 mAb, rabbit polyclonal anti-FLAG antibody were purchased from Sigma (St Louis, MO). Fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG, rhodamine-conjugated anti-mouse IgG, anti-Actin were purchased from Chemicon (Temecula, CA).

2. 2. Cell culture, transfections, and luciferase assays

Human embryonic kidney carcinoma cell line, 293T was maintained in DMEM containing 10% fetal bovine serum (FBS) and transfected by the standard calcium precipitation protocol. Luciferase assay was performed as described [22]. Human hepatoma cell line Hep3B was maintained in DMEM containing 10 % FCS and transfected with using jetPEI (PolyPlus-transfection, Strasbourg, France) according to the manufacturer's instructions [23]. Before stimulation, the cells were cultured for 12 hrs in DMEM containing 1% FCS followed by treatment with IL-6.

2. 3. Nucreofection and Reporter Assay of B cells

HBL6 cells (KSHV⁺, EBV⁺) and DG75 cells (KSHV⁻, EBV⁻) were grown in RPMI 1640 medium containing 10% FBS. 5×10^6 of HBL6 (or DG75) cells were nucreofected with 2.5 μ g of STAT3-LUC, 0.3 μ g of pRL-TK (for internal control) and 2.5 μ g of LANA siRNA plasmid (or FLAG-LANA) [19] by Human B Cell Nucleofector Kit (Amaxa biosystems, Cologne, Germany). Cells were resuspended in 0.2 ml of lysis solution, Passive Lysis Buffer (Promega, Madison, WI) for luciferase assay.

2. 4. Immunoprecipitation, immunoblotting and indirect immunofluorescence

Immunoprecipitation, western blotting and indirect immunofluorescence were performed as described previously [24].

3. Results and Discussion

3. 1. STAT3 and LANA physically interact in vivo

IL-6/STAT3 signaling has been documented to play crucial roles in the development of the KSHV-associated diseases [15,16]. These facts led us to examine the molecular interactions between an IL-6 signaling molecule, STAT3 and a most abundant latent protein, LANA in KSHV-infected cells. We first examined whether LANA physically interacts with STAT3. To demonstrate in vivo binding of LANA to STAT3, we performed coimmunoprecipitation studies in 293T cells that were transiently transfected with FLAG-LANA and Myc-STAT3 expression constructs. The transfected 293T cells were lysed and subjected to immunoprecipitation with anti-FLAG antibody. Immunoprecipitates were then used in western blot analysis with anti-Myc antibody. As shown in Figure. 1A, STAT3 interacted with LANA in 293T cells. To further establish the relevance of the interaction between endogenous LANA and STAT3, we performed co-immunoprecipitation studies on the KSHV-positive BC3 PEL cell line. In KSHV-positive PEL cells but not negative B lymphoma cells, STAT3 was constitutively tyrosine-phosphorylated (data not shown). Immunoprecipitates with anti-STAT3, but not control IgG, successfully pulled down LANA as demonstrated by western blotting with an anti-LANA antibody (Figure 1B). Similar immunoprecipitation studies on KSHV-negative B lymphoma DG75 cells as well as immunoprecipitation with an IgG control antibody, served as negative controls (Figure 1B).

To next delineate the domains in the LANA that mediate the protein–protein interactions

between LANA and STAT3, co-immunoprecipitation experiments were performed with a series of mutant LANA proteins (Figure. 1C). As shown in Figure 1D (left panels), Deletion of LANA central repeat domain (LANA-NC, Δ 330-927) and the C-terminal domain of LANA (LANA-C, 928-1162) interacted with STAT3, whereas the N-terminal domain of LANA (LANA-N, 1–329) failed to interact with STAT3. These data suggest that the C-terminal domain is required for LANA to interact with STAT3.

To characterize further the nature of the interaction between STAT3 and LANA, we attempted to determine where this interaction occurs in cells. LANA localized in the nucleus in the presence or absence of IL-6-stimulation, while IL-6 stimulation induced translocation of STAT3 into nucleus in Hep3B cells (Figure 1E). The cytoplasmic STAT3 did not co-localize with LANA. However, after IL-6 stimulation, STAT3 translocated into nucleus and co-localized with LANA (Figure 1D). Consistent with the *in vivo* interaction data presented above, these results suggest that the activated STAT3 interacts with LANA in the nucleus.

3. 2. LANA augments transcriptional activation of STAT3 in 293T cells

To assess the functional relevance between LANA and STAT3, we tested whether LANA affects STAT3-mediated transcriptional activation using transient transfection experiments in 293 cells. The STAT3-mediated transcriptional responses were measured by using STAT3-LUC, in which the α 2-macroglobulin promoter drives expression of the luciferase (LUC) reporter gene [20]. 293T cells were transfected with STAT3-LUC together with or without LANA, and treated with LIF and LUC activities were

determined. When cells were co-transfected with LANA, the transcriptional activation of STAT3-LUC augmented in a dose-dependent manner (Figure. 2A). Co-expression of a dominant negative STAT3, STAT3YF inhibited LANA-mediated augmentation of STAT3 activation, suggesting that LANA affected the STAT3-mediated transcriptional activity. To next assess the direct interaction between STAT3 and LANA, we used a constitutively active form of STAT3, STAT3-C [21]. 293T cells were transfected with STAT3-LUC and expression vectors for LANA and/or STAT3-C. As shown in Figure. 2B, STAT3-LUC activation by STAT3-C was augmented by LANA in a dose dependent manner. We also tested whether LANA induces IL-6 mRNA in 293T cells by PCR. However, any IL-6 mRNA expression was observed after LANA expression in 293T cells (data not shown). We next examined the effects of LANA on the IFN- β -induced STAT1 and erythropoietin (EPO)-induced STAT5 activation using similar transient transfection experiments. However, LANA affected neither STAT1 nor STAT5 activation (Figure 2C and D). These results indicate that LANA activates STAT3 transcriptional activity but not STAT1 and STAT5, suggesting LANA acts on STAT3 in a specific manner. To further understand the molecular mechanisms responsible for LANA-mediated enhanced STAT3 activation, we examined whether LANA expression affects phosphorylation, dimerization, nuclear translocation/retention or DNA binding activity of STAT3, however, we could not observe any significant effect on these issues by LANA expression in 293T cells (data not shown). We also examined whether LANA physically interacts with unphosphorylated STAT3, such as STAT3YF. In 293T cells, we could detect a direct interaction between LANA and STAT3YF (data not shown),

suggesting that phosphorylation of STAT3 may be not necessary for their physical interactions. However, LANA-mediated enhanced STAT3 activation was observed only after LIF stimulation. These results indicate that STAT3 can interact with LANA in the nucleus after its phosphorylation and translocation. Further detailed work will be required to clarify the molecular mechanisms of LANA-mediated enhancement of STAT3 activation.

3. 3. LANA acts as a transcriptional activator for STAT3 in B cells

To understand the physiology of molecular interactions between STAT3 and LANA, we further examined the effects of LANA gene silencing on STAT3 activity in KSHV-infected PEL cells. We co-transfected STAT3-LUC construct with the LANA or control siRNA into HBL6 cells and assessed reporter gene expression. LANA siRNA reduced expression of cellular LANA expression by about 50% compared to control siRNA (Figure 3A). Importantly, the LANA siRNA markedly reduced the STAT3 target gene expression, such as cyclin D1 and cdk4 [21] as well as the reporter gene expression from the STAT3-LUC construct compared to the control siRNA, although the phosphorylation level of STAT3 did not alter by treatment of LANA siRNA (Figure 3A). To further confirm the enhanced effect of LANA on STAT3 activation, we overexpressed LANA together with the STAT3-LUC construct in a KSHV-negative DG75 cells. As shown in Figure 3B, ectopically expressed LANA markedly augmented STAT3-LUC in DG75 cells. These data indicate that LANA plays a critical role in the enhanced STAT3 activation in KSHV-infected PEL cells.

3. 4. Concluding remarks

STAT3 is constitutively phosphorylated and activated in various primary human tumors and in transformed cell lines and is implicated in tumorigenesis [2,3,4]. However, molecular mechanisms of the persistent activation of STAT3 in human tumor cells are largely unknown. Recently, the hepatitis C virus (HCV) core protein has been shown to directly interact with and activate STAT3 through phosphorylation of the critical tyrosine residue [25]. Chronic infection by HCV is known to associate with development of liver cirrhosis and hepatocellular carcinoma. HCV core protein is also proposed to be involved in the virus-induced transformation, indicating that the HCV core protein cooperates with STAT3, which leads to cellular transformation.

In this study, we propose a novel interaction between STAT3 and the KSHV-derived LANA in PEL cells. LANA augmented transcriptional activity of STAT3 in 293T cells. Furthermore, small-interfering RNA-mediated reduction of LANA expression decreased the STAT3-dependent transcription in KSHV-positive PEL cells, whereas overexpression of LANA enhanced STAT3 activity in KSHV-negative B cells. Thus, further understanding of the detailed molecular interactions between STAT3 and LANA may provide a novel therapeutic strategy for the KSHV-associated tumors.

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Figure Legends

Fig. 1. STAT3 and LANA physically interact in vivo

(A) 293T cells (1×10^7 cells) were transfected with FLAG-LANA (10 μg) together with or without Myc-STAT3 (7.5 μg). Forty-eight hrs after transfection, the cells were lysed, and immunoprecipitated with anti-FLAG antibody and immunoblotted with anti-Myc (upper panel) or anti-LANA antibody (middle panel). Total cell lysates (1%) were blotted with anti-Myc or anti-LANA antibody (lower panels).

(B) Human Burkitt's lymphoma DG75 (KSHV-negative) or primary effusion lymphoma (PEL) (KSHV-positive) cells (2×10^7) were lysed, and immunoprecipitated with control IgG or anti-STAT3 antibody and immunoblotted with anti-LANA antibody (upper panels) or anti-STAT3 antibody (lower panels).

(C) Domain structure of LANA and mutant fragments are schematically shown.

(D) 293T cells (1×10^7 cells) were transfected with Myc-STAT3 (5 μg) together with or without LANA-N (1–329), LANA-C (928–1162) and LANA-NC (Δ 330–927)(10 μg). Forty-eight hrs after transfection, the cells were lysed, immunoprecipitated with anti-Myc antibody and immunoblotted with anti-FLAG or anti-Myc antibody (left panels). Total cell lysates (1%) were blotted with anti-FLAG or anti-Myc antibody (right panels) to monitor the expression of LANA or STAT3 proteins. The asterisks indicate the migration position of LANA mutants.

(E) Hep3B cells were transfected with Myc-STAT3 (1 μg) and FLAG-LANA (1 μg). Thirty hrs after transfection, cells were treated with or without IL-6 (50 ng/ml) for 30

min, and then fixed and reacted with rabbit anti-LANA polyclonal and mouse anti-Myc monoclonal antibody and visualized with FITC-conjugated anti-rabbit antibody or rhodamine conjugated anti-mouse antibody.

Fig. 2. LANA augments transcriptional activation of STAT3 in 293T cells

(A) 293T cells in a 12-well plate were transfected with STAT3-LUC (0.4 μ g) and/or indicated amounts of empty vector, expression vector for LANA or STAT3YF. Thirty-six hrs after transfection, the cells were stimulated with LIF (100 ng/ml) for additional 8 hrs. The stimulated cells were harvested, and luciferase activities were measured.

(B) 293T cells in a 12-well plate were transfected with STAT3-LUC (0.4 μ g) together with/without STAT3-C (500 ng) and/or indicated amounts (100-400 ng) of expression vector for LANA. Forty eight hrs after transfection, the cells were harvested, and luciferase activities were measured.

(C) 293T cells in a 12-well plate were transfected with IRSE-LUC (0.4 μ g) and/or indicated amounts (100–400 ng) of expression vector for LANA. Thirty-six hrs after transfection, the cells were stimulated with IFN- β (100 U/ml) for additional 8 hrs. The stimulated cells were harvested, and luciferase activities were measured.

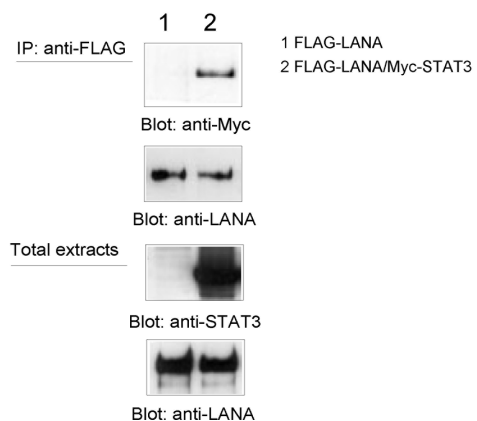
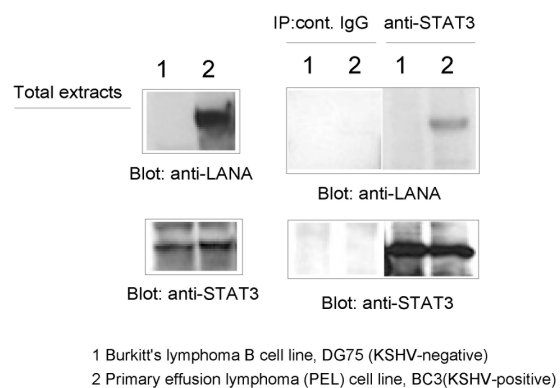
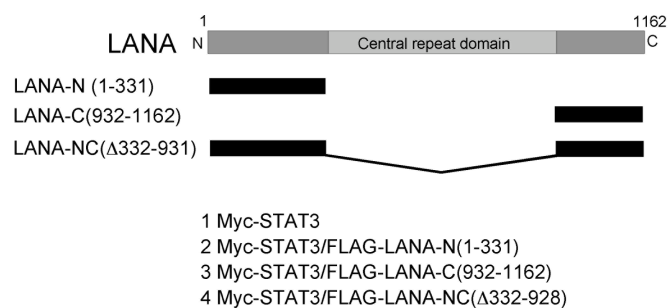
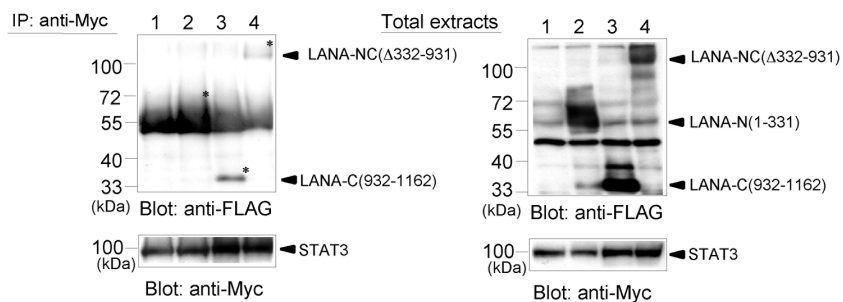
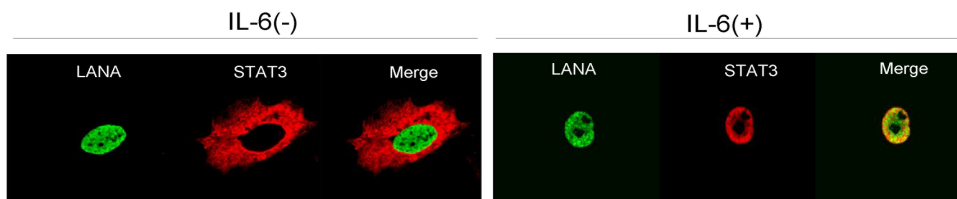
(D) 293T cells in a 12-well plate were transfected with STAT5-LUC (0.4 μ g) together with EPO receptor (1 μ g) and/or indicated amounts (100–400 ng) of expression vector for LANA. Thirty-six hrs after transfection, the cells were stimulated with EPO (1 U/ml) for additional 8 hrs. The stimulated cells were harvested, and luciferase activities

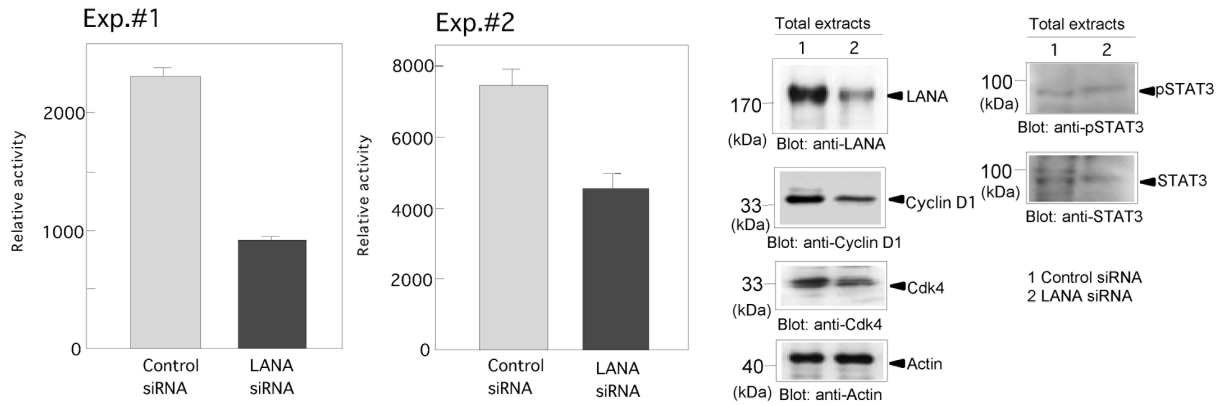
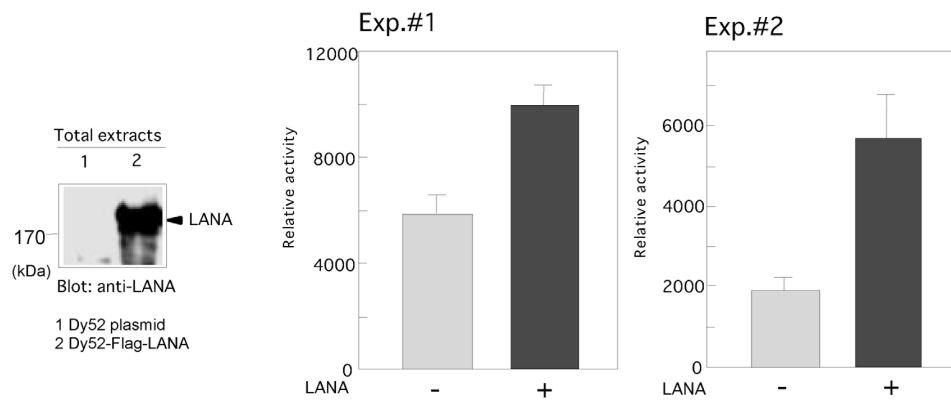
were measured. The above results are indicated as fold induction of luciferase activity from triplicate experiments, and the error bars represent the S.D.

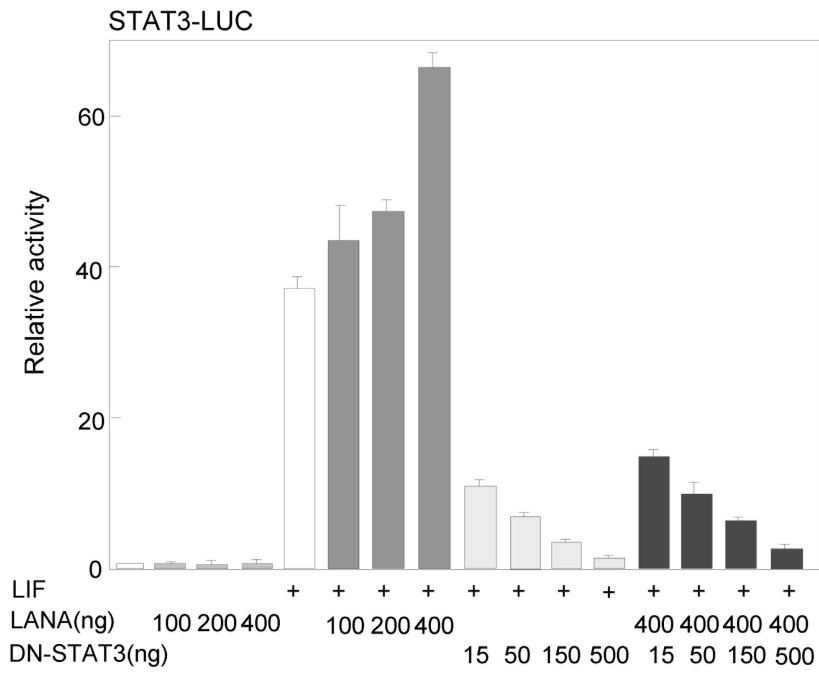
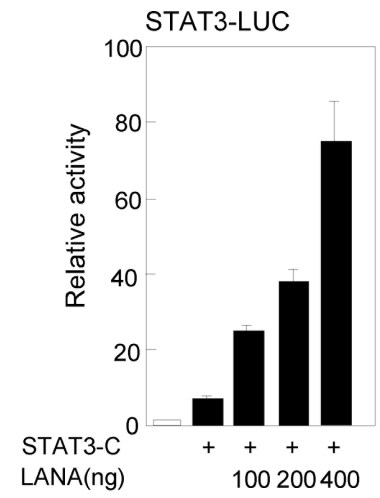
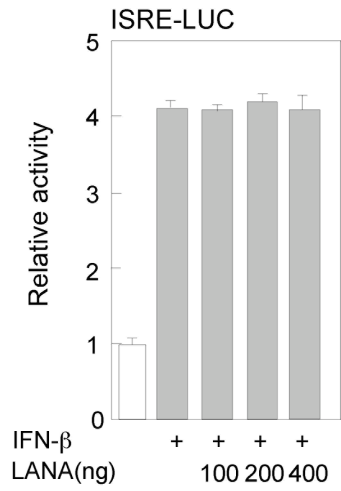
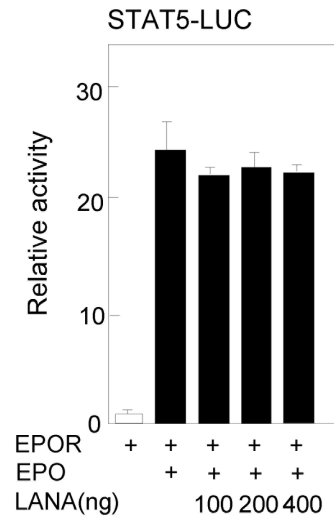
Fig. 3. LANA acts as a transcriptional activator for STAT3 in B cells

(A) To abolish expression of endogenous LANA and measure luciferase activities from STAT3 signaling, HBL6 cells were nucleofected with 2.5 μ g of luciferase reporter (STAT3-LUC), 0.3 μ g of pRL-TK and 2.5 μ g of LANA siRNA expressing plasmid (1.25 μ g of siN-LANA and 1.25 μ g of siC-LANA) or 2.5 μ g of control siRNA plasmid by nucleofection with Human B Cell Nucleofector Kit according to their optimized protocol. Cells were harvested after 2 (Exp.#2) and 3 days (Exp.#1) and resuspended in 0.2 ml of lysis solution for luciferase assay. One part of lysate was subjected to western blotting analysis using anti-LANA rat mAb to confirm the knock down of LANA expression. The same samples were also subjected to western blotting analysis using anti-phosphoSTAT3 (Tyr705), anti-STAT3, anti-cyclin D1 or anti-cdk4 antibodies.

(B) DG75 cells were nucleofected with 2.5 μ g of STAT3-LUC, 0.3 μ g of pRL-TK and 2.5 μ g of FLAG-LANA by Nucleofector as well as HBL6 and cells were harvested after 5 (Exp.#1) and 6 days (Exp.#2). Nucleofection was done with a total of 5 μ g of DNA (adjusted with vector plasmid as a carrier) per each sample. Cells lysate were subjected to luciferase assay and western blotting with anti-LANA rat mAb.

A**B****C****D****E****Fig. 1**

A**B****Fig. 3**

A**B****C****D****Fig. 2**