Virology 528 (2019) 137–143

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/virology

STAT3 phosphorylation affects p53/p21 axis and KSHV lytic cycle activation

Roberta Santarelli[a], Valentina Carillo[a], Maria Anele Romeo[a], Aurelia Gaeta[b], Cristina Nazzari[b], Roberta Gonnella[a], Marisa Granato[a], Gabriella D'Orazi[c,d], Alberto Faggioni[a,*], Mara Cirone[a,*]

[a] Department of Experimental Medicine, "Sapienza" University of Rome, Italy. Laboratory affiliated to Istituto Pasteur Italia-Fondazione Cenci Bolognetti, Italy
[b] Department of Molecular Medicine, "Sapienza" University of Rome, Italy
[c] Translational Research Area, Regina Elena National Cancer Institute, Rome, Italy
[d] Department of Medical, Oral and Biotechnological Sciences, University "G. d'Annunzio", 66013 Chieti, Italy

ARTICLE INFO

Keywords:
KSHV
STAT3
KAP-1
p53
p21
Lytic cycle
Ser727 STAT3
Tyr705 STAT3

ABSTRACT

The Tyr705 STAT3 constitutive activation, besides promoting PEL cell survival, contributes to the maintenance of viral latency. We found indeed that its de-phosphorylation by AG490 induced KSHV lytic cycle. Moreover, Tyr705 STAT3 de-phosphorylation, mediated by the activation of tyrosine phosphatases, together with the increase of Ser727 STAT3 phosphorylation contributed to KSHV lytic cycle induction by TPA. We then observed that p53-p21 axis, essential for the induction of KSHV replication, was activated by the inhibition of Tyr705 and by the increase of Ser727 STAT3 phosphorylation. As a possible link between STAT3, p53-p21 and KSHV lytic cycle, we found that TPA and AG490 reduced the expression of KAP-1, promoting p53 stability, p21 transcription and KSHV lytic cycle activation in PEL cells.

1. Introduction

Signal Transducer and activator of Transcription 3 (STAT3) is a transcription factor that regulates important cellular processes including cell proliferation, apoptosis and autophagy (Hirano et al., 2000). As for other transcription activators, STAT3 activity is regulated by post-transcriptional modifications such as acetylation at the lysine 685 and phosphorylation that may occurs at least in two different residue: the 705 tyrosine (Tyr705) and 727 serine (Ser727) (Wong LA et al., 2017 Expert opin invest drugs) (Wong et al., 2017). STAT3 is constitutively phosphorylated in one or both residue in several solid and hematological cancers, inducing the transcription of pro-survival molecules such as survivin, c-myc or the anti-apoptotic bcl-2 family proteins (Yu et al., 2008). Interestingly, Tyr705 STAT3 phosphorylation in immune cells negatively regulates their function, as for example it induces a phenotypic and functional impairment of dendritic cells (DCs) (Melillo et al., 2010; Santarelli et al., 2014). Tyr705 phosphorylation is mainly mediated by the Janus protein tyrosine kinase (JAK) 2, activated in response to cytokines such as IL6 (Johnson et al., 2018), growth factors such as VEGF and EGF (Fagard et al., 2013) or by proteins encoded by oncoviruses, including those belonging to human gammaherpesviruses such as KSHV and EBV (Li and Bhaduri-McIntosh, 2016). Indeed it is known that KSHV-encoded proteins together with the release of cytokines, acting in an autocrine fashion, contribute to Tyr705 STAT3 constitutive activation in Primary Effusion Lymphoma (PEL). This is an aggressive KSHV-associated B cell lymphoma that typically arises in immune deficient patients (Aoki et al., 2003; Granato et al., 2015; Santarelli et al., 2015). Previous studies have indicated that the over-expression of STAT3 restrains KSHV replication by activating the transcriptional co-repressor Kruppel-associated box (KRAB)3-associated protein 1 (KAP-1 also named TRIM28 or TIF1β) (King et al., 2015). A similar role of STAT3 in inhibiting viral replication has been also reported for EBV (Hill et al., 2013). However, the impact of Tyr705 and Ser727 STAT3 phosphorylation on KSHV lytic cycle remains to be fully elucidated. It has been shown that Ser727 phosphorylation of STAT3 is promoted by the KSHV latent/lytic protein Kaposin B concomitantly to the phosphorylation and inactivation of KAP-1 in HUVEC cells (King, 2013). Differently from Tyr705, Ser727 STAT3 phosphorylation is mainly mediated by Protein Kinases C (PKCs), such as PKC delta (Gartsbein et al., 2006; Jain et al., 1999), and by Extracellular Signal Regulated Kinase (ERK) 1/2 (Chung et al., 1997). Of note, both PKC delta and ERK1/2 are activated by TPA and play a role in KSHV

Abbreviations: KSHV, Kaposi's Sarcoma-associated Herpesvirus; KAP-1, Kruppel-associated box (KRAB)3-associated protein 1 (also named TRIM28 or TIF1□); OA, okadaic acid; PEL, Primary Effusion Lymphoma; OV, orthovanadate; PKC, Protein Kinases C; STAT3, Signal Transducer and activator of Transcription 3; Stau, Staurosporine; TPA, 12-O-tetradecanoylphorbol 13-acetate

* Corresponding authors.
E-mail addresses: alberto.faggioni@uniroma1.it (A. Faggioni), mara.cirone@uniroma1.it (M. Cirone).

https://doi.org/10.1016/j.virol.2018.12.015
Received 27 July 2018; Received in revised form 19 December 2018; Accepted 20 December 2018
Available online 05 January 2019

lytic cycle activation in tumor cells harboring latent KSHV infection (Deutsch et al., 2004). Moreover, as we have recently shown, the activation of a PKC delta/ERK1/2 axis induced ser15 p53 phosphorylation, promoting the transcription of p21 and activating KSHV lytic cycle in TPA-treated PEL cells (Balistreri et al., 2016; Gonnella et al., 2017). Interestingly, we have also shown that the inhibition of Tyr705 STAT3 phosphorylation may activate 53-p21 axis in PEL cells (Granato et al., 2017). The down-regulation of KAP-1, previously correlated with the inhibition of STAT3 (King et al., 2015), has been reported to prevent p53 degradation and activate p21 transcription (Wang et al., 2005). In addition to kinases, TPA may activate PKC-dependent tyrosine phosphatases mediating Tyr705 STAT3 de-phosphorylation (Oka et al., 2009). However, whether TPA affects STAT3 phosphorylation in PEL cells and how STAT3 phosphorylation influences KSHV lytic cycle remains to be investigated. As STAT3 is frequently targeted to reduce PEL cell survival, investigating how it affects the latency to replication switch is of fundamental importance considering that KSHV replication may contribute to tumorigenesis and tumor persistence (Aneja and Yuan, 2017)

2. Results

2.1. The inhibition of Tyr705 STAT3 constitutive phosphorylation by AG490 leads to the activation of p53-p21 axis and KSHV lytic cycle in PEL cells

The impact of Tyr705 STAT3 phosphorylation on KSHV lytic cycle activation in PEL cells harboring latent KSHV infection was investigated. At this aim, we used AG490, an inhibitor of Jak2, the main kinase that mediates Tyr705 STAT3 phosphorylation. As shown in Fig. 1A, AG490 inhibited Tyr705 STAT3 phosphorylation and activated KSHV lytic cycle, as it induced K-bZIP early lytic antigen expression in both BC3 and BCBL-1 PEL cell lines. Interestingly, this treatment was able to activate also the expression of KSHV late lytic antigens, as evidenced by the expression of the glycoprotein gp64, evaluated by IFA after 48 and 60 h of treatment (Fig. 1B). As we have recently shown, Tyr705 STAT3 phosphorylation negatively influenced the activation of p53-p21 axis (Granato et al., 2017) that is essential for KSHV lytic cycle activation by TPA (Balistreri et al., 2016; Gonnella et al., 2017). Thus, we next investigated whether AG490 could affect p21 expression and found that it increases p21 level in both PEL cell lines (Fig. 1A). Such treatment also resulted in an increase of p53 (Fig. 1C) that could likely be responsible for the increased p21 transcription. To elucidate the role of p53 in p21 and KSHV lytic cycle activation by AG490, we silenced p53 by a specific siRNA in BCBL1 cells. The results shown in Fig. 1D, indicated that p53 knocking-down strongly reduced both p21 and K-bZIP expression induced by AG490 and also down-regulated the basal level of p21 and K-bZIP expression in PEL cells (Fig. 1E). Moreover p53 silencing reduced viral production induced by AG490 (Fig. 1F) highlighting the important role of p53 activation in promoting viral replication. As a possible link between Tyr705 STAT3 inhibition and p53-p21 and KSHV lytic cycle activation, we investigated whether AG490 could influence the expression of KAP-1, a transcriptional target of STAT3 that negatively regulates p53 stability (Wang et al., 2005) and KSHV lytic cycle activation (King, 2015). As shown in Fig. 1A, concomitantly to the increase of p21 and Kb-ZIP, we found that KAP-1 expression was reduced by AG490. Finally, STAT3 was silenced to investigate whether its reduced expression could affect the switch between latency to replication in PEL cells. As shown in Fig. 1G, STAT3 silencing increased the expression of Kb-ZIP lytic antigen, p53 and p21 in comparison to the scramble control (sc), in agreement to previous findings (King et al., 2015). We noticed that STAT3 silencing induced a lower Kb-ZIP expression in comparison to AG490 treatment, and this could suggest that, besides Tyr705 phosphorylation, STAT3 may undergo other post-transcriptional modifications that oppositely influence KSHV replication. However this could also be due to the partial reduction of STAT3 expression obtained by the silencing.

2.2. The inhibition of Tyr705 STAT3 phosphorylation by tyrosine phosphatases contributes to TPA-induced p53-p21 axis and KSHV lytic cycle activation

It has been reported that TPA, one of the most efficient KSHV lytic cycle inducers, is able to activate tyrosine phosphatases (Oka et al., 2009). Therefore, we next investigated whether TPA could influence Tyr705 STAT3 phosphorylation and found that it was reduced by this treatment (Fig. 2A) that, similarly to AG490, reduced also KAP-1 expression (Fig. 2A). Furthermore, the pan-tyrosine phosphatase inhibitor orthovanadate (OV) counteracted STAT3 tyrosine de-phosphorylation, indicating that the activation of tyrosine phosphatases by TPA mediated such effect. Interestingly, OV strongly reduced Kb-ZIP expression (Fig. 2B), suggesting that the de-phosphorylation of Tyr705 STAT3 could contribute to TPA-induced KSHV replication in PEL cells. According to the previous finding that Tyr705 STAT3 phosphorylation may negatively influence the activation of p53-p21 axis (Granato et al., 2017), we found that OV prevented the increase of p21 expression (Fig. 2B) and reduced viral production (Fig. 2C) induced by TPA.Next, to confirm that OV was counteracting TPA-induced KSHV lytic cycle by preventing Tyr705 STAT3 de-phosphorylation, we silenced STAT3 before treating PEL cells with TPA plus OV. As shown in Fig. 2D, STAT3 silencing partially reverted the reduction of K-bZIP and p21 mediated by OV. All together these data suggest that Tyr705 STAT3 de-phosphorylation mediated by tyrosine phosphatases contributed to p21 up-regulation and KSHV lytic cycle induction by TPA.

2.3. Ser727 STAT3 phosphorylation promotes KSHV lytic cycle activation in PEL cells

Besides Tyr705, STAT3 activity can be regulated by the Ser727 phosphorylation. Such phosphorylation can be mediated by PKCs, such as the novel PKC delta (PKCδ) (Jain et al., 1999) or by ERK1/2 (Jain et al., 1998), both known to be activated by TPA and involved in its-induced KSHV replication in PEL cells (Deutsch et al., 2004). In this study, we evaluated whether the activation of PKCs by TPA could induce Ser727 STAT3 phosphorylation in PEL cells. The results shown in Fig. 3A indicate that Ser727 phosphorylation increased upon TPA treatment and that it was reduced by using the PKC pan inhibitor Staurosporine, highlighting the role of PKCs in its activation. As expected, Staurosporine strongly inhibited K-bZIP expression (Fig. 3A) and viral release (Fig. 3B) induced by TPA. Interestingly also p21 expression was reduced by Staurosporin (Fig. 3A) All together these results suggest that PKCs activated by TPA phosphorylated Ser727 STAT3 and induced both p21 expression and KSHV lytic cycle activation. Next, a dose-dependent assay using 10 and 20 nM of Staurosporine was performed to show the dose dependent effect of Staurosporine on the reduction of lytic antigen expression (Fig. 3C) and how this molecule affected PEL cell viability (Fig. 3D). To identify which PKCs could be involved in these effects, we silenced PKC δ and found that it counteracted TPA-mediated Ser727 STAT3 phosphorylation and reduced the expression of p21 and Kb-ZIP (Fig. 3E). To further elucidate the role of pSer727 STAT3 in the regulation of KSHV lytic cycle, we transfected BCBL1 cells with the STAT3 S727A negative dominant before TPA treatment. As shown in Fig. 3F, such treatment that reduced Ser727 phosphorylation down-regulated both K-bZIP and p21 expression and counteracted viral production (Fig. 3G). Finally, the finding that okadaic acid (OA), an inhibitor of serine/threonine phosphatases increased Kb-ZIP and p21 expression (Fig. 3H), indicates that serine/threonine phosphatases are constitutively activated in PEL cells to maintain Ser727 STAT3 de-phosphorylated and to restrain KSHV replication. The role of Ser727 STAT3 phosphorylation in maintaining viral latency was further supported by the use of STAT3 S727A negative dominant that prevented K-bZIP expression induced by OA (Fig. 3I). All together these

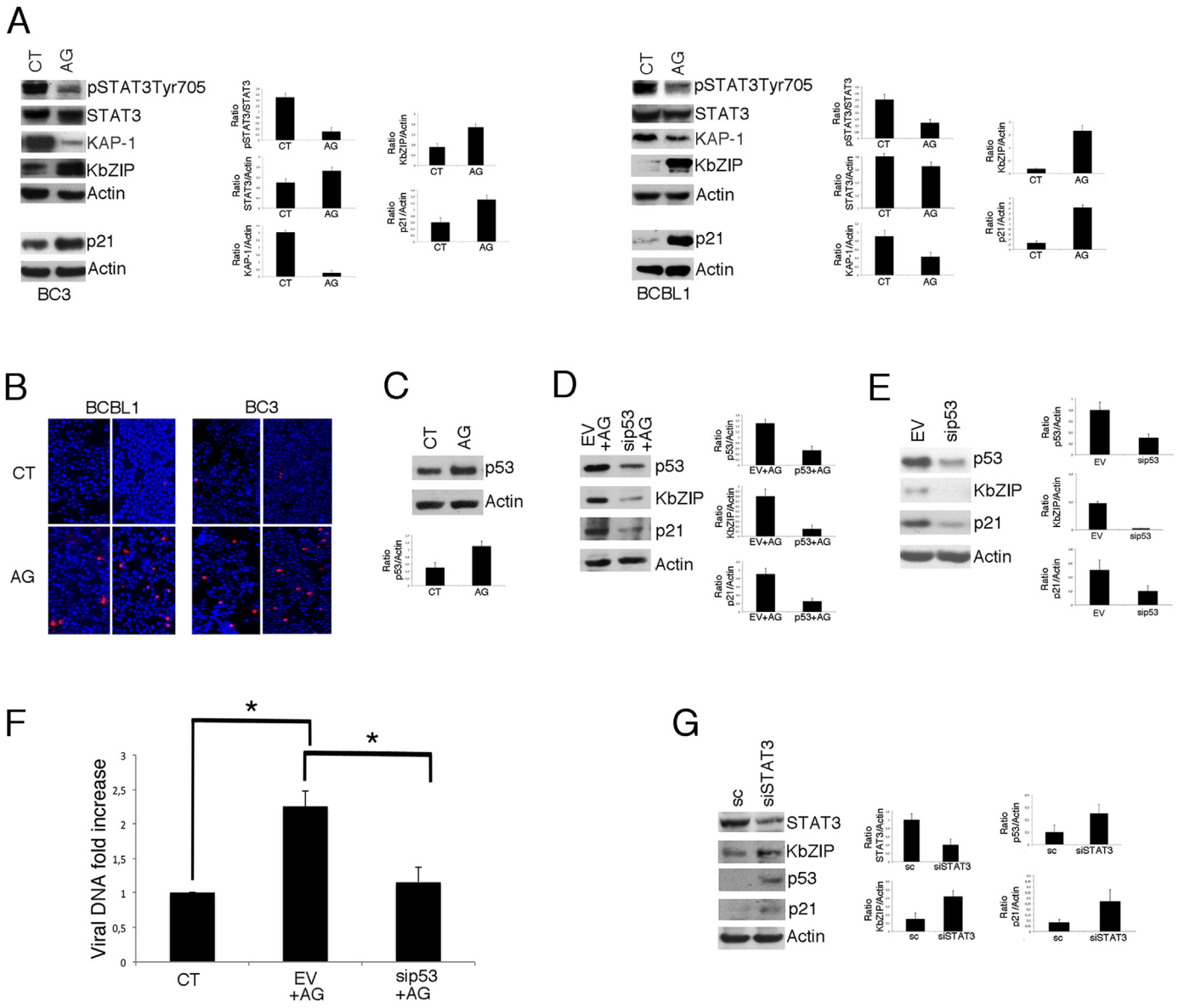


Fig. 1. The inhibition of STAT3 705 tyrosine constitutive phosphorylation by AG490 activates the p53-p21 axis and KSHV lytic cycle in PEL cells. (A) BC3 and BCBL1 cells were cultured in presence of AG490 50 μM (AG) or vehicle (CT) and pSTAT3Tyr705, total STAT3 (STAT3), KAP-1, KbZIP and p21 expression was assessed by western blotting analysis. (B) The expression of gp64 KSHV lytic protein (red staining) was evaluated by IFA performed on BCBL1 cells cultured in presence of AG490 50 μM for 48 and 60 h. Nuclei were stained with DAPI (blue). (C) BCBL1 cells were cultured in presence of AG490 (AG) or with vehicle (CT) and p53 expression was analysed by western blotting.) (D) p53, KbZIP and p21 were assessed by western blotting analysis in BCBL1 cells transfected with a specific si-p53 plasmid (sip53) or with empty vector (EV). (E) BCBL1 cells were transfected with sip53 or with EV and then p53, KbZIP and p21 expression level was evaluated by western blotting analysis. (F) qPCR of viral DNA extracted from KSHV particles released in the medium by BCBL1 cells transfected with EV or sip53 and cultured in the presence of AG490 50 μM, for 96 h. Hystograms represent viral DNA fold increase of treated versus untreated samples. (G) Western blot analysis of STAT3, KbZIP, p53 and p21 level of BCBL1 cells silenced with a specific STAT3 siRNA or a scrambled siRNA (sc). In the figure, actin was used as loading control and one representative experiment out of three is shown. Furthermore, the histograms represent the mean plus S.D. of the densitometric analysis of the ratio of pSTAT3 Tyr705/STAT3, STAT3/Actin, KAP-1/Actin, KbZIP/Actin, p53/Actin and p21/Actin of three different experiments. * $p < 0.05$.

results indicate that STAT3 may undergo Ser727 phosphorylation and that such phosphorylation plays an opposite role in comparison to Tyr705 STAT3 phosphorylation in the reactivation of KSHV from latency in PEL cells.

3. Discussion

It has been previously demonstrated that the constitutive Tyr705 STAT3 phosphorylation plays a pro-survival role in PEL (Chung et al., 1997; King et al., 2015) as well as in other solid and hematological tumor cells (Gunning et al., 2008). STAT3 may be also phosphorylated at the Ser727 residue (Jain et al., 1998; Sengupta et al., 1998) and this phosphorylation may inhibit STAT3 activity, as it may negatively influence 705 tyrosine activation (Chung et al., 1997; Sengupta et al., 1998). However, other studies have indicated that the Ser727 phosphorylation may result in a complete activation of STAT3 (Wen et al., 1995), suggesting that its role in regulating STAT3 activity is still controversial. Of note, STAT3 cross-talks with other cellular pathways to control fundamental cellular processes such as survival, apoptosis and autophagy (Carpenter and Lo, 2014). In this study, we investigated the role of STAT3 phosphorylation in reactivating KSHV from latency in naturally infected lymphoma cells. We found that Tyr705 and Ser727 STAT3 phosphorylation play an opposite role as both the inhibition of Tyr705 and the increase of Ser727 phosphorylation of STAT3 promote

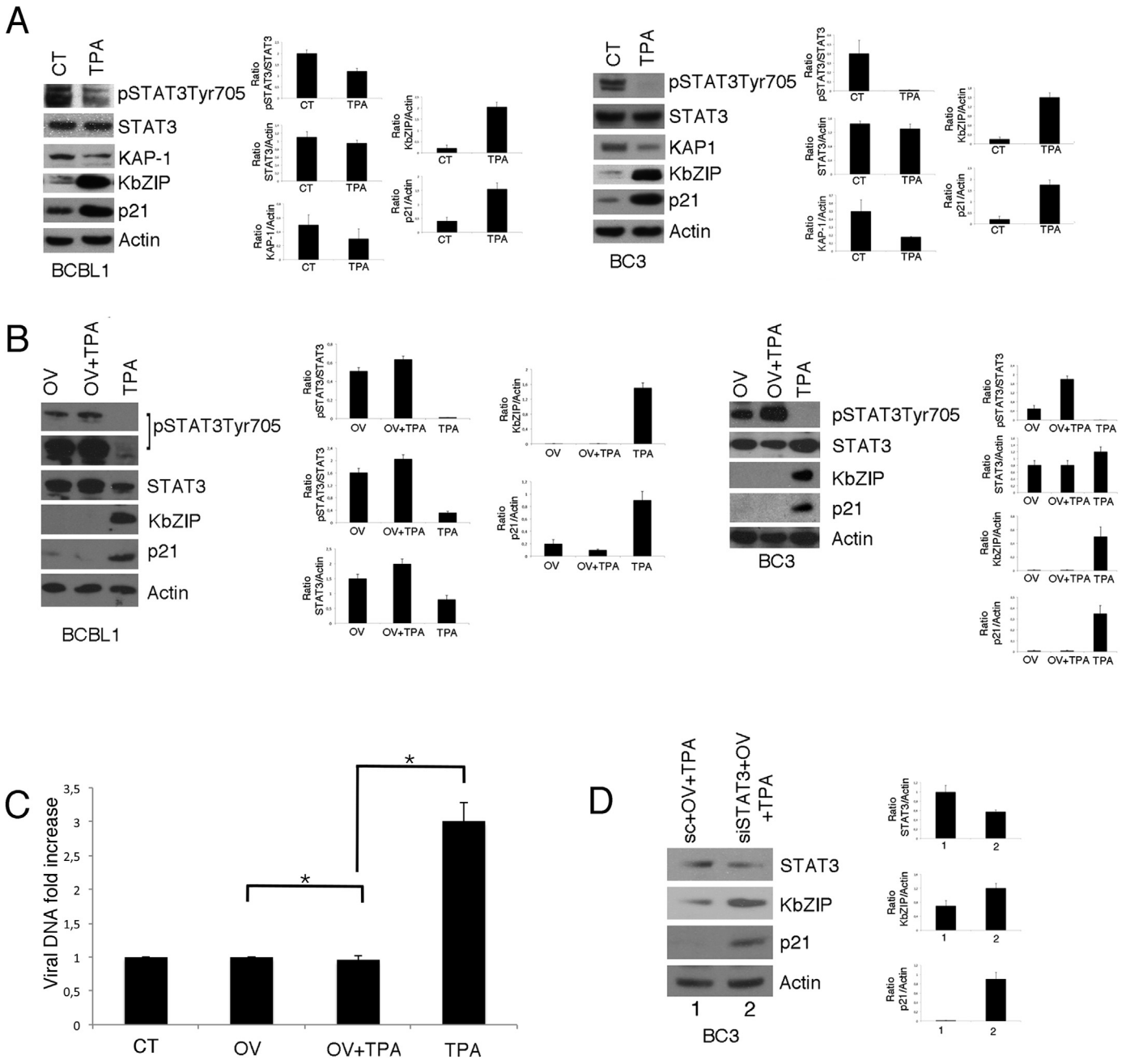
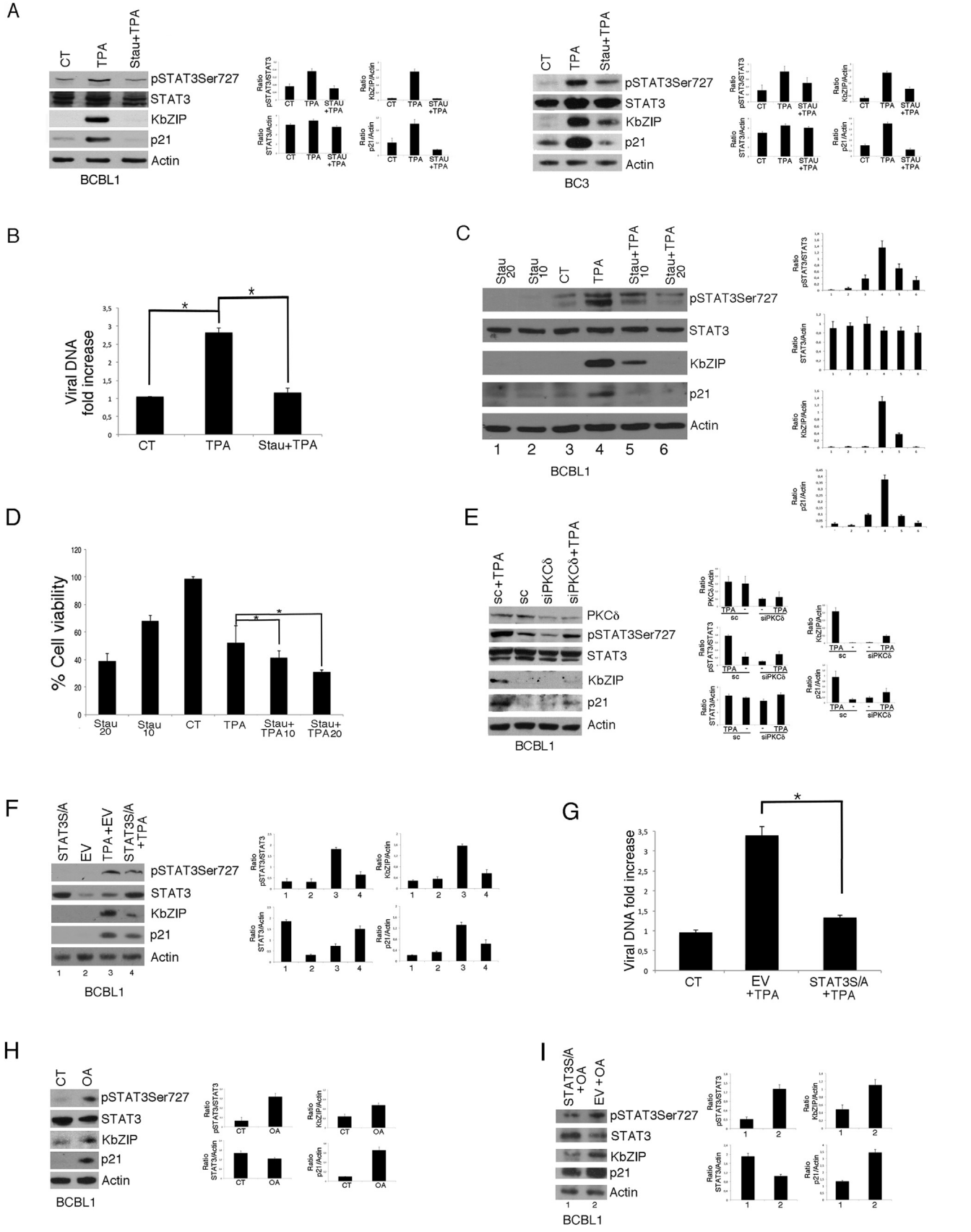


Fig. 2. TPA-induced p53-p21 axis and KSHV lytic cycle activation involves the inhibition of Tyr705 STAT3 phosphorylation mediated by tyrosine phosphatases. (A) BCBL1 and BC3 cells were cultured in presence of TPA (7,5 ng/ml) or vehicle (CT) for 20 h and pSTAT3Tyr705, STAT3, KAP-1, KbZIP and p21 expression was evaluated by western blotting analysis. (B) BCBL1 and BC3 cells were pre-treated with orthovanadate (OV, 100 μM) for 45 min and then cultured for additional 20 h in presence of TPA (7,5 ng/ml). pSTAT3Tyr705, STAT3, KbZIP and p21 level was assessed by western blotting. For BCBL1, pSTAT3Tyr705 short and long exposures are shown. (C) qPCR of viral DNA extracted from KSHV particles released in the medium by BCBL1 cells cultured in presence of OV, OV + TPA or TPA for 96 h. Hystograms represent viral DNA fold increase of treated versus untreated samples. (D) Western blot analysis of STAT3, KbZIP and p21 expression of BCBL1 cells silenced with a specific STAT3 siRNA or a scrambled siRNA (sc) for 24 h and subsequently incubated with OV (100 μM) for 45 min and finally cultured in presence of TPA or vehicle (CT) for additional 20 h. In the figure, actin was used as loading control and one representative experiment out of three is shown. Furthermore, the histograms represent the mean plus S.D. of the densitometric analysis of the ratio of pSTAT3Tyr705/STAT3, STAT3/Actin, KAP-1/Actin, KbZIP/ Actin, p21/Actin and p53/Actin of three different experiments. * $p < 0.05$.

KSHV lytic cycle in PEL cells treated with TPA. We found that PKC delta, a kinase known to play a fundamental role in the activation of KSHV replication by TPA, was responsible for Ser727 STAT3 phosphorylation in PEL cells. Indeed, the pan PKC inhibitor Staurosporin as well as PKC delta silencing reduced Ser727 phosphorylation and KSHV lytic antigen expression in TPA-treated PEL cells. Interestingly, we have shown that the two different STAT3 phosphorylation, besides KSHV lytic cycle, regulated in opposite fashion the activation of p53-p21 axis, that has been reported to be essential for the activation of viral replication (Balistreri et al., 2016; Gonnella et al., 2017). Previous studies have reported a cross-talk between STAT3 and p53 (Niu et al., 2005), molecules often dysregulated in cancers, that play an opposite role in cell survival. Interestingly, Ser727 STAT3 phosphorylation has been reported to occur concomitantly with the inactivation of KAP-1, a protein that represses KSHV lytic cycle (King, 2015). On the other hand, the inhibition of the Tyr705 STAT3 phosphorylation, has been reported



(caption on next page)

Fig. 3. 727 serine STAT3 phosphorylation contributes to KSHV lytic cycle activation in PEL cells. (A) BCBL1 and BC3 cells were pre-treated with Staurosporin (Stau) 20 nM, or with vehicle (CT) and then cultured in presence of TPA (7,5 ng/ml). pSTAT3Ser727, STAT3, KbZIP and p21 expression was assessed by western blotting. (B) qPCR of viral DNA extracted from KSHV particles released in the medium by BCBL1 cells cultured in presence of vehicle (CT), TPA or Stau + TPA for 96 h. Hystograms represent viral DNA fold increase of treated versus untreated samples. (C) BCBL1 cells were pre-treated with Stau 10 and 20 nM, or with vehicle (CT) and then cultured in presence of TPA. Then, pSTAT3Ser727, STAT3, KbZIP and p21 expression was assessed by western blotting. (D) Cell viability of BCBL1, pre-treated with Stau 10 and 20 nM, and then cultured in presence of TPA (Stau + TPA) or with vehicle (CT), was evaluated by Trypan blue exclusion assay. (E) BCBL1 cells were transfected with a specific PKCδ siRNA or a scrambled siRNA (sc) and then cultured in the presence of TPA. The expression level of PKCδ, pSTAT3Ser727, STAT3, KbZIP and p21 was assessed by western blotting. (F) BCBL1 cells were transfected with STAT3 S727A (STAT3S/A) or with empty vector (EV) and then treated with TPA (5 ng/ml) for 20 h (TPA + EV and STAT3S/A + TPA, respectively). (G) qPCR performed on viral DNA extracted from KSHV particles released in the medium by BCBL1 cells after EV or STAT3S/A transfection and subsequent TPA treatment for 96 h (EV + TPA and STAT3S/A + TPA, respectively). Hystograms represent viral DNA fold increase of treated versus untreated samples. (H) BCBL1 cells were cultured in the presence of Okadaic acid (OA, 20 nM) or vehicle (CT) for 20 h and pSTAT3Ser727, STAT3, KbZIP and p21 expression was analysed by western blotting. (I) BCBL1 cells were transfected with STAT3 S727A or with empty vector and then cultured for additional 20 h in the presence of Okadaic acid (EV + OA and STAT3S/A + OA, respectively). The level of pSTAT3Ser727, STAT3, KbZIP and p21 was assessed by western blotting. In the figure, actin was used as loading control and one representative experiment out of three is shown. Furthermore, the histograms represent the mean plus S.D. of the densitometric analysis of the ratio of pSTAT3Ser727/STAT3, STAT3/Actin, KbZIP/Actin, p21/Actin and PKCδ/Actin of three different experiments. * $p < 0.05$.

to reduce KAP-1 expression (King, 2015). The data obtained in this study suggest that the down-regulation of KAP-1 could be the link between STAT3 and p53-p21 axis, as it has been reported that KAP-1 promotes p53 degradation and reduces p21 transcription (Wang et al., 2005). These evidences suggest that drugs targeting Tyr705 or Ser727 STAT3 phosphorylation may oppositely affect p53-p21 axis and KSHV lytic cycle activation in PEL cells. The understanding of the mechanisms that regulate viral replication is of fundamental importance as this process, although leading to tumor cell lysis, promotes viral persistence and spread that are essential for the maintenance of KSHV-driven malignancies.

4. Materials and methods

4.1. Cell culture, reagents and treatments

BC3 (ATCC, CRL-2277) and BCBL1 (kindly provided by Prof. P. Monini, National AIDS Center, Istituto Superiore di Sanità, Rome, Italy) are human B-cell lines derived from Primary Effusion Lymphoma (PEL) carrying latent KSHV. Both cell lines were cultured in RPMI medium 1640, containing L-glutamine (2 mM), streptomycin (100 µg/ml) (Corning, NY, USA; 30–002), penicillin (100 U/ml) (Corning, NY, USA; 25–005) and supplemented with 10% Fetal Bovine Serum (FBS) (Corning, NY, USA; 35–079). Cells were grown at 37 °C in a 5% CO_2 incubator. KSHV lytic cycle was induced by treating BC3 and BCBL1 cells with 12-O-tetradecanoylphorbol 13-acetate (TPA, 7.5 ng/ml; Sigma Aldrich, P8139) for 20 h. Depending on the experiments, cells were incubated with AG490 (AG, 50 µM), a Jak2 inhibitor, or with okadaic acid (OA, 20 nM), a serine/threonine phosphatases inhibitor, for 20hrs. Also, in some experiments, BC3 and BCBL-1 cells were pre-incubated with Staurosporine (Stau, 10 and 20 nM), a PKC pan inhibitor, or with orthovanadate (OV, 100 µM) a pan-tyrosine phosphatase inhibitor, for 45 min before adding TPA. Then the cells were cultured for additional 20hrs. To quantitate the extracellular virus, the treatments were extended up to 96 h.

4.2. Western blot analysis

Cells were washed twice with phosphate-buffered saline (PBS; Gibco, 18912–014) and lysed in a RIPA buffer containing 150 mM NaCl, 1% NP-40 (Calbiochem, 492015), 50 mM Tris-HCl, pH 8, 0.5% deoxycholic acid (SIGMA, D-6750), 0.1% SDS (SERVA, 39575.02) 1% Triton X-100, protease and phosphatase inhibitors (SIGMA, S8830, S6508 and 450022). 10 µg of each lysate were subjected to electrophoresis on 4–12% NuPage Bis-Tris gels (Life Technologies/Novex, NP0323) and transferred to nitrocellulose membranes (GE Healthcare/Amersham, 10600002 Protran). The membranes were then blocked in PBS-0.1% Tween-20 (SIGMA, P1379) containing 3% BSA (SIGMA, A4503) and probed with specific primary antibodies. After several washes in PBS-0.1% Tween 20 the membranes were incubated with appropriate secondary antibodies conjugated to horseradish peroxidase. Finally, the membranes were washed in PBS-0.1% Tween-20 and immunoreactivity was detected using an enhanced chemiluminescence kit (Thermo Scientific, 32209). All the primary and secondary antibodies were diluted in PBS-0.1% Tween20 solution containing 3% of BSA (SERVA, Reno, NV, USA; 11,943.03). Densitometric analysis was performed using ImageJ software (http://imagej.nih.gov).

4.3. Antibodies

In western blotting analysis, we used the following primary antibodies: rabbit polyclonal anti p21 (1:100) (Santa Cruz Biotech, Heidelberg, Germany; sc-397), mouse monoclonal anti-p53 (1:500) (Santa Cruz Biotech, Heidelberg, Germany; sc-126), mouse monoclonal anti-STAT3 (1:1000) (BD Transduction Laboratories, New Jersey, USA; #610189), mouse monoclonal anti-phospho-STAT3 (pTyr705) (1:100) (Santa Cruz Biotec, Heidelberg, Germany; sc-8059), mouse anti-phospho-STAT3 (pSer727) (1:500) (BD Biosciences #612542); KbZIP (1:300) (Santa Cruz, sc-69797), PKC delta (1:50, Santa Cruz Biotech, Heidelberg, Germany; sc-2013), KAP-1(Santa Cruz, sc-515790). Mouse monoclonal anti-β-Actin (1:10,000) (Sigma Aldrich, St Louis. MO, USA; A5441) was used as loading control. The goat polyclonal anti-mouse IgG-Horseradish Peroxidase (HRP) (Santa Cruz Biotechnology Inc., Heidelberg, Germany; sc-2005) and anti-rabbit IgG-HRP (Santa Cruz Biotechnology Inc., Heidelberg, Germany; sc-2004) were used as secondary antibodies.

4.4. Indirect immunofluorescence assay (IFA)

BC3 and BCBL1 cells were incubated with AG490 (50 µM) for 48 and 60 h, washed with PBS, applied onto multispot microscope slides (Thermo Scientific, ER-301B-CE24) and air dried. Cells were fixed in 2% paraformaldehyde for 30 min, washed 3 times in PBS, permeabilized with 0.5% Triton X-100 for 5 min, blocked with a solution containing 3% BSA 1% glycine for 30 min and incubated with a monoclonal antibody against gp64 (1:50, Santa Cruz Biotech, Heidelberg, Germany; sc-65444), a glycoprotein located on the surface of KSHV virion envelope, for 30 min at room temperature. Slides were then washed 3 times with PBS and the cells were further incubated with a Cy™3-conjugated sheep-anti-mouse secondary antibody (1:1000; Jackson Imm. Res. 515–165-062) for 30 min at room temperature. After 3 washes in PBS, cells were stained with DAPI (4,6-diamidino-2-phenylindole) (1:5.000, SIGMA, D9542) for 1 min at room temperature. Finally, slides were further washed in PBS, mounted with glycerol: PBS (1:1) and observed under a fluorescence microscope (Olympus BX53, USA).

4.5. Quantitative PCR (qPCR)

To quantify KSHV in the cell supernatant, DNA extraction was performed by Nuclisens EasyMag instruments (BioMerieux spa). Extracted samples were analysed by quantitative TaqMan real-time PCR using the HHV-8 Elite MGB kit.

4.6. p53, STAT3 and PKC delta silencing

BCBL1 cells were plated at a density of 8×10^5 cells/well, in 1.25 ml of complete medium without antibiotics in 6 well plates. For p53 interference, cells were transfected with 12 μg of empty vector (EV) or with sip53 plasmid (Cecchinelli et al., 2006) using Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA, USA; 1880,845) according to the manufacturer's instructions. Cells were cultured overnight and then in the presence of AG490 (50 μM) or vehicle (DMSO) for additional 20hrs. STAT3 and PKC delta (δ) silencing was also performed using Lipofectamine 2000 by transfecting 30pmol of specific or scrambled (sc) siRNAs, according to the manufacturer's instructions. Depending on the experiment, after 24hrs of culture, STAT3-silenced BCBL1 cells were incubated for 45 min with OV (100 μM) and then further cultured with TPA (7.5 ng/ml) for 20hrs. Finally, PKCδ silencing was performed and the day after, the cells were cultured in the presence of TPA (7.5 ng/ml) for additional 20hrs.

4.7. STAT3 S727 transfection and treatments

To investigate the role of STAT3 Ser727 phosphorylation, BCBL-1 cells were transfected with a plasmid for the expression of a dominant-negative STAT3 Ser727 mutant (STAT3 Ser727A, kindly provided by Prof. Larner A.C.) (Meier et al., 2017). To this purpose, cells were plated at a density of 1×10^6 cells/well, in 1 ml of medium without antibiotics in 6 well plates, and transfected using Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA, USA; 1880,845), according to the manufacturer's instructions. Cells were then cultured overnight and, depending on experiments, TPA or OA were added for further 20 or 96 h.

4.8. Statistical analysis

Results are represented by the mean ± standard deviation (SD) of at least three independent experiments. A two-tailed Student's *t*-test was used to demonstrate statistical significance and differences were considered statistically significant when p-value was at least < 0.05.

Funding and acknowledgment

This work was supported by grants from ASI (Agenzia Spaziale Italiana) (2014-033-R.O.), from AIRC - Associazione Italiana per la Ricerca sul Cancro (IG 15858), Italy and from Istituto Pasteur Italia-Fondazione Cenci Bolognetti. We thank Prof. Larner A.C. for providing STAT3 S727A plasmid and Dr. Antonella Battista for technical assistance.

References

Aneja, K.K., Yuan, Y., 2017. Reactivation and lytic replication of kaposi's sarcoma-associated herpesvirus: an update. Front. Microbiol. 8, 613.
Aoki, Y., Feldman, G.M., Tosato, G., 2003. Inhibition of STAT3 signaling induces apoptosis and decreases survivin expression in primary effusion lymphoma. Blood 101, 1535–1542.
Balistreri, G., Viiliainen, J., Turunen, M., Diaz, R., Lyly, L., Pekkonen, P., Rantala, J., Ojala, K., Sarek, G., et al., 2016. Oncogenic herpesvirus utilizes stress-induced cell cycle checkpoints for efficient lytic replication. PLoS Pathog. 12, e1005424.
Carpenter, R.L., Lo, H.W., 2014. STAT3 target genes relevant to human cancers. Cancers 6, 897–925.
Cecchinelli, B., Lavra, L., Rinaldo, C., Iacovelli, S., Gurtner, A., Gasbarri, A., Ulivieri, A., Del Prete, F., Trovato, M., et al., 2006. Repression of the antiapoptotic molecule galectin-3 by homeodomain-interacting protein kinase 2-activated p53 is required for p53-induced apoptosis. Mol. Cell. Biol. 26, 4746–4757.
Chung, J., Uchida, E., Grammer, T.C., Blenis, J., 1997. . STAT3 serine phosphorylation by ERK-dependent and -independent pathways negatively modulates its tyrosine phosphorylation. Mol. Cell. Biol. 17, 6508–6516.
Deutsch, E., Cohen, A., Kazimirsky, G., Dovrat, S., Rubinfeld, H., Brodie, C., Sarid, R., 2004. Role of protein kinase C delta in reactivation of Kaposi's sarcoma-associated herpesvirus. J. Virol. 78, 10187–10192.
Fagard, R., Metelev, V., Souissi, I., Baran-Marszak, F., 2013. STAT3 inhibitors for cancer therapy: have all roads been explored? Jak.-Stat. 2, e22882.
Gartsbein, M., Alt, A., Hashimoto, K., Nakajima, K., Kuroki, T., Tennenbaum, T., 2006. The role of protein kinase C delta activation and STAT3 Ser727 phosphorylation in insulin-induced keratinocyte proliferation. J. Cell Sci. 119, 470–481.
Gonnella, R., Yadav, S., Gilardini Montani, M.S., Granato, M., Santarelli, R., Garufi, A., D'Orazi, G., Faggioni, A., Cirone, M., 2017. Oxidant species are involved in T/B-mediated ERK1/2 phosphorylation that activates p53-p21 axis to promote KSHV lytic cycle in PEL cells. Free Radic. Biol. Med. 112, 327–335.
Granato, M., Chiozzi, B., Filardi, M.R., Lotti, L.V., Di Renzo, L., Faggioni, A., Cirone, M., 2015. Tyrosine kinase inhibitor tyrphostin AG490 triggers both apoptosis and autophagy by reducing HSF1 and Mcl-1 in PEL cells. Cancer Lett. 366, 191–197.
Granato, M., Gilardini Montani, M.S., Santarelli, R., D'Orazi, G., Faggioni, A., Cirone, M., 2017. Apigenin, by activating p53 and inhibiting STAT3, modulates the balance between pro-apoptotic and pro-survival pathways to induce PEL cell death. J. Exp. Clin. Cancer Res.: CR 36, 167.
Gunning, P.T., Glenn, M.P., Siddiquee, K.A., Katt, W.P., Masson, E., Sebti, S.M., Turkson, J., Hamilton, A.D., 2008. Targeting protein-protein interactions: suppression of Stat3 dimerization with rationally designed small-molecule, nonpeptidic SH2 domain binders. Chembiochem: a Eur. J. Chem. Biol. 9, 2800–2803.
Hill, E.R., Koganti, S., Zhi, J., Megyola, C., Freeman, A.F., Palendira, U., Tangye, S.G., Farrell, P.J., Bhaduri-McIntosh, S., 2013. Signal transducer and activator of transcription 3 limits Epstein-Barr virus lytic activation in B lymphocytes. J. Virol. 87, 11438–11446.
Hirano, T., Ishihara, K., Hibi, M., 2000. Roles of STAT3 in mediating the cell growth, differentiation and survival signals relayed through the IL-6 family of cytokine receptors. Oncogene 19, 2548–2556.
Jain, N., Zhang, T., Fong, S.L., Lim, C.P., Cao, X., 1998. Repression of Stat3 activity by activation of mitogen-activated protein kinase (MAPK). Oncogene 17, 3157–3167.
Jain, N., Zhang, T., Kee, W.H., Li, W., Cao, X., 1999. Protein kinase C delta associates with and phosphorylates Stat3 in an interleukin-6-dependent manner. J. Biol. Chem. 274, 24392–24400.
Johnson, D.E., O'Keefe, R.A., Grandis, J.R., 2018. Targeting the IL-6/JAK/STAT3 signalling axis in cancer. Nat. Rev. Clin. Oncol. 15, 234–248.
King, C.A., 2013. Kaposi's sarcoma-associated herpesvirus kaposin B induces unique monophosphorylation of STAT3 at serine 727 and MK2-mediated inactivation of the STAT3 transcriptional repressor TRIM28. J. Virol. 87, 8779–8791.
King, C.A., Li, X., Barbachano-Guerrero, A., Bhaduri-McIntosh, S., 2015. STAT3 Regulates Lytic Activation of Kaposi's Sarcoma-Associated Herpesvirus. J. Virol. 89, 11347–11355.
Li, X., Bhaduri-McIntosh, S., 2016. A Central Role for STAT3 in Gammaherpesvirus-Life Cycle and -Diseases. Front. Microbiol. 7, 1052.
Meier, J.A., Hyun, M., Cantwell, M., Raza, A., Mertens, C., Raje, V., Sisler, J., Tracy, E., Torres-Odio, S., et al., 2017. Stress-induced dynamic regulation of mitochondrial STAT3 and its association with cyclophilin D reduce mitochondrial ROS production. Sci. Signal. 10.
Melillo, J.A., Song, L., Bhagat, G., Blazquez, A.B., Plumlee, C.R., Lee, C., Berin, C., Reizis, B., Schindler, C., 2010. Dendritic cell (DC)-specific targeting reveals Stat3 as a negative regulator of DC function. J. Immunol. 184, 2638–2645.
Niu, G., Wright, K.L., Ma, Y., Wright, G.M., Huang, M., Irby, R., Briggs, J., Karras, J., Cress, W.D., et al., 2005. Role of Stat3 in regulating p53 expression and function. Mol. Cell. Biol. 25, 7432–7440.
Oka, M., Sumita, N., Sakaguchi, M., Iwasaki, T., Bito, T., Kageshita, T., Sato, K., Fukami, Y., Nishigori, C., 2009. 12-O-tetradecanoylphorbol-13-acetate inhibits melanoma growth by inactivation of STAT3 through protein kinase C-activated tyrosine phosphatase(s). J. Biol. Chem. 284, 30416–30423.
Santarelli, R., Gonnella, R., Di Giovenale, G., Cuomo, L., Capobianchi, A., Granato, M., Gentile, G., Faggioni, A., Cirone, M., 2014. STAT3 activation by KSHV correlates with IL-10, IL-6 and IL-23 release and an autophagic block in dendritic cells. Sci. Rep. 4, 4241.
Santarelli, R., Granato, M., Faggioni, A., Cirone, M., 2015. Interference with the Autophagic Process as a Viral Strategy to Escape from the Immune Control: lesson from Gamma Herpesviruses. J. Immunol. Res. 2015, 546063.
Sengupta, T.K., Talbot, E.S., Scherle, P.A., Ivashkiv, L.B., 1998. Rapid inhibition of interleukin-6 signaling and Stat3 activation mediated by mitogen-activated protein kinases. Proc. Natl. Acad. Sci. USA 95, 11107–11112.
Wang, C., Ivanov, A., Chen, L., Fredericks, W.J., Seto, E., Rauscher 3rd, F.J., Chen, J., 2005. MDM2 interaction with nuclear corepressor KAP1 contributes to p53 inactivation. EMBO J. 24, 3279–3290.
Wen, Z., Zhong, Z., Darnell Jr., J.E., 1995. Maximal activation of transcription by Stat1 and Stat3 requires both tyrosine and serine phosphorylation. Cell 82, 241–250.
Wong, A.L.A., Hirpara, J.L., Pervaiz, S., Eu, J.Q., Sethi, G., Goh, B.C., 2017. Do STAT3 inhibitors have potential in the future for cancer therapy? Expert Opin. Investig. Drugs 26, 883–887.
Yu, L.J., Wu, M.L., Li, H., Chen, X.Y., Wang, Q., Sun, Y., Kong, Q.Y., Liu, J., 2008. Inhibition of STAT3 expression and signaling in resveratrol-differentiated medulloblastoma cells. Neoplasia 10, 736–744.