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uniroma1.it)STAT3 activation by KSHV correlates
with IL-10, IL-6 and IL-23 release and an
autophagic block in dendritic cellsRoberta Santarelli¹, Roberta Gonnella¹, Giulia Di Giovenale¹, Laura Cuomo², Angela Capobianchi³,
Marisa Granato¹, Giuseppe Gentile³, Alberto Faggioni¹ & Mara Cirone¹¹Istituto Pasteur-Fondazione Cenci Bolognetti, Department of Experimental Medicine, Sapienza University, Viale Regina Elena 324, 00161 Rome, Italy, ²Department of Clinical Pathology, A.C.O., S. Filippo Neri, Via Giovanni Martinotti, 20, 00135 Rome, Italy, ³Department of Cellular Biotechnologies and Hematology, Sapienza University, Via Benevento 6, 00161 Rome, Italy.

Kaposi's sarcoma associated herpesvirus (KSHV) has been reported to infect, among others, monocytes and dendritic cells DCs impairing their function. However, the underlying mechanisms remain not completely elucidated yet. Here we show that DC exposure to active or UV-inactivated KSHV resulted in STAT3 phosphorylation. This effect, partially dependent on KSHV-engagement of DC-SIGN, induced a high release of IL-10, IL-6 and IL-23, cytokines that in turn might maintain STAT3 in a phosphorylated state. STAT3 activation also correlated with a block of autophagy in DCs, as indicated by LC3II reduction and p62 accumulation. The IL-10, IL-6 and IL-23 release and the autophagic block could be overcome by inhibiting STAT3 activation, highlighting the role of STAT3 in mediating such effects. In conclusion, here we show that STAT3 activation can be one of the molecular mechanisms leading to KSHV-mediated DC dysfunction, that might allow viral persistence and the onset of KSHV-associated malignancies.

Dendritic cells (DCs) are at crossroad between innate and adaptive immunity, being able to prime naive T lymphocytes to novel antigens and to initiate a specific immune response^{1,2}. Kaposi's sarcoma-associated herpesvirus (KSHV) is a human gammaherpesvirus found in all forms of Kaposi's sarcoma (KS) and is also highly associated with lymphoproliferative disorders, such as primary effusion lymphomas (PEL) and multicentric Castleman's disease (MCD)^{3–5}. As other members of Herpesvirus family^{6–8}, KSHV is able to infect plasmacytoid DCs, myeloid DCs or their monocyte precursors^{9–11}, leading to reduction of costimulatory molecules, altered cytokine release and impaired allostimulatory capacity^{11,12}. Monocytes exposure to KSHV results also in down-regulation of chemokine receptors and consequently in reduced migration capacity in response to chemokines¹³. DC functional impairment has also been observed *in vivo*, in patients with KSHV-associated disease, such as Kaposi's Sarcoma^{14,15}. To date, the molecular mechanisms underlying the KSHV-mediated immunosuppressive effects are not completely known. KSHV entry into myeloid DCs is mediated by dendritic cell-specific ICAM-3-grabbing nonintegrin (DC-SIGN; CD209), although other receptor molecules seem to be involved¹⁰. Furthermore, KSHV binds heparan sulfates (HS) on the surface of monocytes and THP-1 monocytic cell line, activating molecules such as PI3K and NF- κ B¹⁶. These signaling pathways are also involved in the modulation of DC function^{17–20} in addition to the signal transducer and activator of transcription 3 (STAT3), whose activation by immunosuppressive factors, released by tumor cells, leads to DC dysfunction^{21–23}. Recently STAT3 activation by IL-10, in bystander macrophages/monocytes, in the course of HIV infection, has been reported to interfere also with the autophagic process in these cells²⁴. STAT3 inhibitors, on the other hand, are reported to be potent autophagy inducers²⁵.

Autophagy is characterized by the formation of double-membrane vesicles that, surrounding aggregated proteins or damaged organelles, fuse with lysosomes, leading to the degradation of their content²⁶. It may allow cells to survive in stressful conditions, such as nutrient shortage and microbial infections²⁷. Moreover, autophagy plays an essential role in pathogen clearance and MHC-presentation by antigen presenting cells (APC)²⁸ and has been reported to be required also for monocyte differentiation into macrophages and DCs^{29,30}. Given its important role in the immune response, it is not surprising that pathogens have developed strategies to interfere with the autophagy to avoid the immune control³¹.

The aim of our study was to investigate which molecular pathways could be activated by KSHV in human DCs. We showed that both active and UV-inactivated KSHV induced STAT3 phosphorylation in DCs. STAT3



activation was partially dependent on DC-SIGN engagement and correlated with a high release of IL-10, IL-6 and IL-23 cytokines. Moreover, a reduced IL-12 production upon LPS stimulation was observed. Finally, we found that, by activating STAT3, KSHV induced a block of autophagy, essential for the immune function of these cells.

Results

KSHV activates STAT3 in DCs. DCs, overnight serum-starved, were mock-treated or exposed to KSHV ($\sim 9 \times 10^6$ viral DNA copies/ 1×10^6 DCs) for 15 and 30 minutes at 37°C . STAT3 phosphorylation was then analyzed by western blot. We observed that STAT3 phosphorylation increased after 15 min of KSHV-exposure, in comparison to mock-treated DCs, and was sustained for 30 min (fig. 1a). We next investigated whether KSHV would also influence the activation of other molecules, such as NF- κ B and AKT, involved in the regulation of DC function and activated by KSHV in other cell types^{16,32}. Differently from STAT3, KSHV slightly influenced the phosphorylation of NF- κ B p65 subunit and AKT after 15 or 30 minutes of viral exposure (fig. 1a). Next, to establish whether viral gene expression was required for STAT3 activation, we performed the same experiment with UV-inactivated KSHV. As shown in fig. 1b, UV-inactivated KSHV was still able to induce STAT3 phosphorylation in DCs, suggesting that viral binding/entry is sufficient for STAT3 activation and it occurs independently of viral gene expression. As for active KSHV, the UV-inactivated virus slightly modified the phosphorylation of NF- κ B and AKT (fig. 1b).

STAT3 activation by KSHV is partially dependent on viral engagement of DC-SIGN. We then focused our study on STAT3 since it was highly phosphorylated in DCs exposed to active or UV-inactivated KSHV. We first evaluated if STAT3 activation could be induced by the engagement of KSHV with DC-SIGN, receptor molecule known to mediate KSHV binding and/or internalization into DCs¹⁰. For this purpose, we inhibited KSHV interaction with DC-SIGN by pre-incubating DCs with anti-DC-SIGN antibody (20 $\mu\text{g}/\text{ml}$ for 1 hour at 4°C), according to a previous study¹⁰. The

results obtained showed that anti-DC-SIGN pre-treatment led to $\sim 60\%$ reduction of KSHV-mediated STAT3 phosphorylation, based on densitometric analysis of phosphorylated vs total STAT3 protein ratio (fig. 2a), suggesting that STAT3 phosphorylation is partially mediated by viral interaction with DC-SIGN, although other molecules seem to be involved. Similar results were obtained with UV-inactivated virus (fig. 2b). Of note, the exposure to anti-DC-SIGN, in the absence of KSHV, did not affect STAT3 phosphorylation (fig. 2c). Finally, by inhibiting viral interaction with DC-SIGN, we strongly reduced KSHV entry into these cells, as measured by real-time DNA PCR (fig. 2d), confirming the importance of DC-SIGN as KSHV receptor on DC surface¹⁰.

The KSHV-mediated STAT3 activation influences cytokine production in DCs. STAT3 phosphorylation has been reported to induce a release of cytokines^{33–36} that are able in turn to phosphorylate STAT3, by engaging their specific receptors³⁷. Hence, we investigated if STAT3 activation by KSHV in DCs could influence the production of IL-10, IL-6 and IL-23. To this aim, serum-starved DCs were exposed to active or UV-inactivated KSHV and cultured overnight at 37°C in serum-free medium. Active and, to lesser extent, UV-inactivated KSHV induced a high release of IL-10, IL-6 and IL-23, in comparison to mock-treated DCs, as detected by ELISA assay (fig. 3). Moreover, DCs exposed to KSHV and then treated with LPS showed a reduced production of IL-12p70, the main cytokine responsible for TH1 polarization. Next, to demonstrate that the altered cytokines release was dependent on KSHV-mediated STAT3 activation, we pre-treated DCs with STAT3 inhibitor AG490, before exposure to KSHV¹⁹. As shown in fig. 3, AG490 pre-treatment strongly reduced the production of IL-10, IL-6 and IL-23. Conversely, the IL-12 production was restored by AG490 pretreatment. These results suggest that STAT3 activation is the main signaling that leads to an altered cytokine release by DCs exposed to KSHV.

The KSHV-mediated STAT3 activation interferes with the autophagic flux in DCs. It has been reported that IL-10 activates STAT3 in bystander monocytes, in the course of HIV infection, and that STAT3 activation interferes with the autophagic process in these cells²⁴. These observations prompted us to investigate whether KSHV-mediated STAT3 activation could interfere with the autophagic process in DCs. For this purpose, DCs were exposed to UV-inactivated KSHV, to rule out the known influence of viral encoded proteins on this process^{38,39}. Mock or UV-inactivated KSHV exposed DCs were overnight serum-starved to induce autophagy, in the presence or in the absence of STAT3 inhibitor AG490. The autophagic flux was then evaluated by analyzing the expression levels of LC3I/II and p62, the two main autophagic markers, by western blot. As shown in fig. 4a, DCs, exposed to UV-inactivated KSHV, displayed a reduction of LC3II, concomitantly to the persistent STAT3 activation. The KSHV/STAT3-mediated increase of p62 expression level further indicates that an autophagic block was occurring in virus exposed DCs. The inhibition of STAT3 phosphorylation by AG490 pre-treatment (fig. 4a) was able to overcome the autophagic block, suggesting that it was mainly dependent on STAT3 activation by KSHV. Although STAT3 was activated after 15 minutes of viral exposure, the expression level of LC3II and p62 was slightly affected (fig. 4b), indicating that the interference with the autophagic process required longer viral exposure. More recently, STAT3 activation has been shown to inhibit autophagy by up-regulating Mcl-1, protein that, like BCL-2 and BCL-xL, is able to bind and sequester Beclin1^{40,41}. Hence, we evaluated if UV-KSHV autophagic block could be due to an up-regulation of Mcl-1 in DCs, dependent on STAT3 activation. We found that UV-KSHV induced an up-regulation of Mcl-1 in DCs, that was prevented by AG490 pre-treatment (fig. 4c). Furthermore, we analysed the expression level of BCL-2 and BCL-xL,

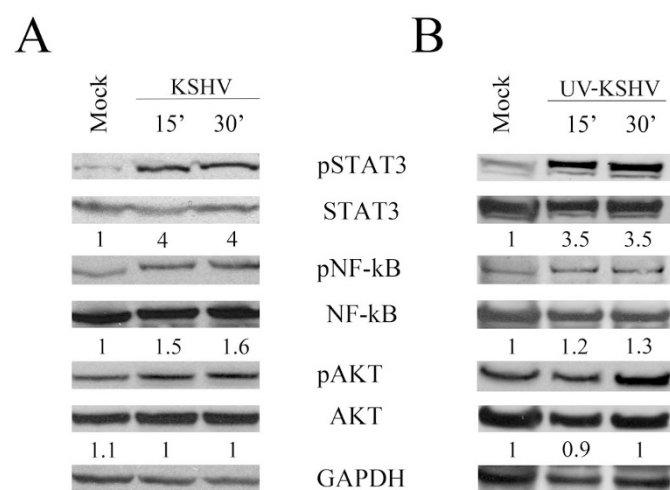


Figure 1 | Short exposure to KSHV induces STAT3 activation in human DCs. DCs were mock treated or exposed to (A) active or (B) UV-inactivated virus (UV-KSHV) for 15 and 30 min and STAT3, NF- κ B p65 and AKT phosphorylation were analysed by western blot. Total STAT3, NF- κ B p65, AKT are also shown. STAT3, NF- κ B p65 and AKT phosphorylation and their fold of induction, based on densitometric analysis of the ratio of phospho/total molecules, are shown. GAPDH was included as protein loading control. 1×10^6 overnight serum-starved DCs/point were used in all experiments. One representative experiment out of three is shown.

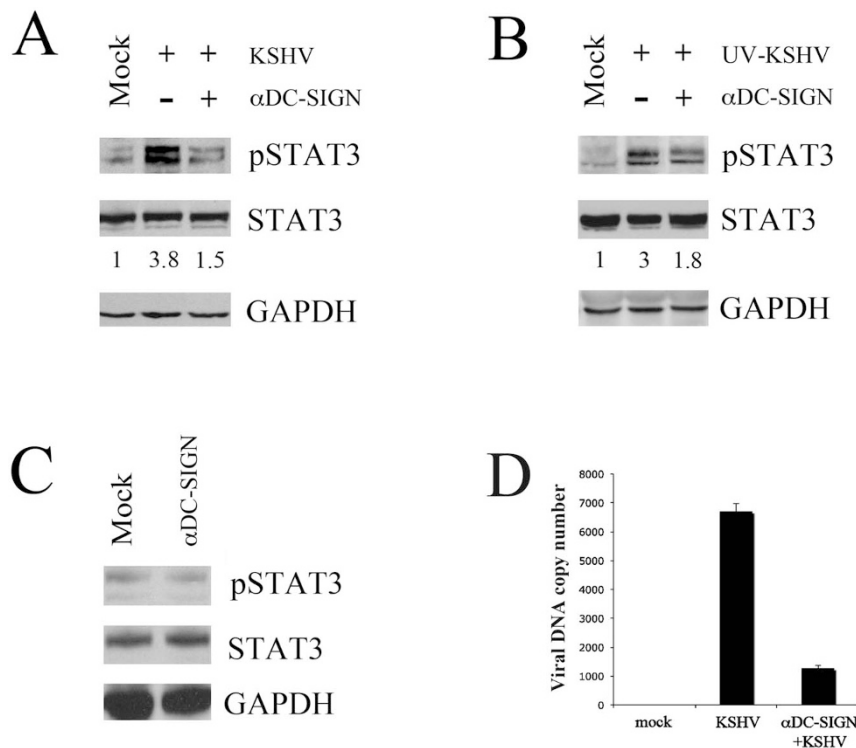


Figure 2 | STAT3 activation by KSHV is partially dependent on viral engagement of DC-SIGN. DCs were mock-treated or pre-incubated with anti-DC-SIGN (20 μg/ml) for 1 hr at 4°C, before exposure for 15 min at 37°C to (A) active KSHV or (B) UV-KSHV. STAT3 phosphorylation and its fold of induction, based on densitometric analysis of the ratio of phospho/total STAT3, are shown. (C) DCs were incubated with anti-DC-SIGN antibody alone for 15 min at 37°C and STAT3 phosphorylation was analyzed by western blotting. Total STAT3 and GAPDH were included as control. 1×10^6 /point overnight serum-starved DCs were used in all experiments. One representative experiment out of three is shown. (D) Real-time PCR showing KSHV DNA copy number/10⁵ mock and KSHV-exposed DCs, pre-treated or not pre-treated with anti-DC-SIGN (20 μg/ml). Viral exposure was performed for 15 min at 37°C. Mean \pm SD of three experiments is reported.

two proteins belonging to the same family of Mcl-1 and also able to interact with Beclin1. The results shown in fig. 4c indicate that UV-KSHV slightly affected BCL-xL and BCL-2 and that Beclin1 itself was also not influenced, suggesting that KSHV mediated a specific Mcl-1 up-regulation through STAT3 that could be responsible for the interference with the autophagic process. To confirm the role of KSHV-mediated STAT3 activation in the autophagy inhibition, we knocked-down the STAT3 gene expression in DCs with specific siRNA (fig. 5a). According to the results obtained with AG490, the STAT3 silencing was able to prevent the increase of p62 and Mcl1 (fig. 5a) and the LC3II decrease (fig. 5b) induced by UV-KSHV in DCs. To confirm that the reduction of LC3II was due to an autophagic block, 3-Methyladenine (3-MA) was used as control to inhibit autophagy (fig. 5c).

Discussion

Based on our results, we suggest that STAT3 activation could be one of the molecular mechanisms underlying KSHV-mediated immunosuppression in human DCs. STAT3 has been previously reported to be activated by KSHV in endothelial cells⁴². To the best of our knowledge, this is the first report showing that STAT3 is also activated by KSHV in human DCs. The importance of this observation relies on the fact that STAT3 activation correlates with an immunosuppressive phenotype and function of DCs that is observed in the immune cells in tumor microenvironment and also in the peripheral blood of tumor bearing patients^{22,35,36}, such as KS patients^{14,15}. Here we show STAT3 activation in DCs occurs following KSHV-engagement of its DC receptor, DC-SIGN, and that it occurs independently of viral replication. We correlated STAT3 activation with a reduced production of IL12p70 in response to LPS stimulation and with an higher

release of IL-10, IL-6 and IL-23. This cytokine pattern could skew the TH1/TH2 profile towards TH2 and/or TH17, promoting immunosuppression and inflammation. These cytokines might also, in turn, maintain STAT3 persistently phosphorylated, by binding on their specific receptors on DC surface, as previously reported in bystander monocytes in the course of HIV infection²⁴. Finally, KSHV-induced STAT3 activation correlated with an interference with the autophagic process that could be overcome by the pre-treatment with AG490 or by STAT3 specific siRNA. We found that STAT3 activation correlated an up-regulation of Mcl-1 expression that was reversed by inhibiting STAT3 activation. Mcl-1 is one of the proteins able to bind and sequester Beclin1, hampering its essential role in autophagosome formation. Indeed, it has been recently shown that the reduction of STAT3 phosphorylation by Sorafenib treatment, in a hepatocarcinoma cell line, resulted in the down-regulation of Mcl-1 and disruption of Beclin1-Mcl-1 complex⁴¹. The Mcl-1 increased expression, induced by STAT3 activation by KSHV in DCs, could be a possible mechanism leading to the block of autophagy observed in this study.

The interference with the autophagic process is of pivotal importance for pathogens to impair antigen processing and presentation by DCs and therefore to avoid their own clearance. Of note, we observed that the autophagic block occurred after UV-inactivation of KSHV, excluding the involvement in such effect of vBCL2 and vFLIP viral proteins, that are reported to mediate such effect by binding Beclin1 and Atg3, respectively^{38,39}. Moreover, these data suggest that KSHV is able to mediate an immunosuppressive effect also by simply interacting with its receptor/s on the surface of DCs. In conclusion, by unveiling a new molecular pathway underlying KSHV-induced DC dysfunction, this study may help to find new therapeutic

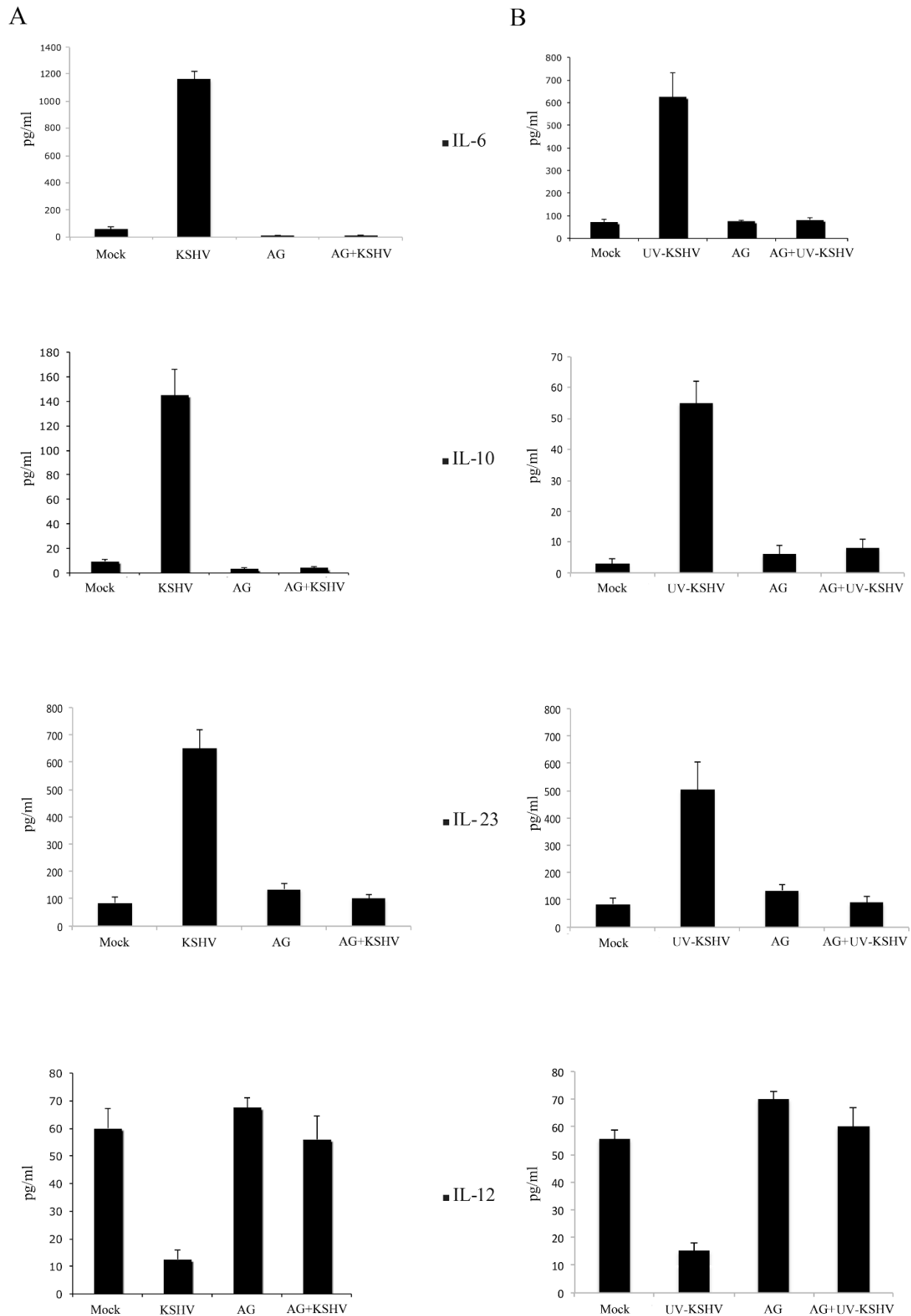


Figure 3 | STAT3 activation by KSHV alters DC cytokine release. ELISA assay to measure the IL-10, IL-6 and IL-23 release by DCs or IL-12p70 release by LPS-treated DCs, exposed to (A) active or (B) UV-KSHV. Histograms (mean \pm SD of three independent experiments) represent the amount of cytokines produced by mock and KSHV-exposed DCs, in the presence or in the absence of STAT3 inhibitor AG490 (50 μ g/ml).

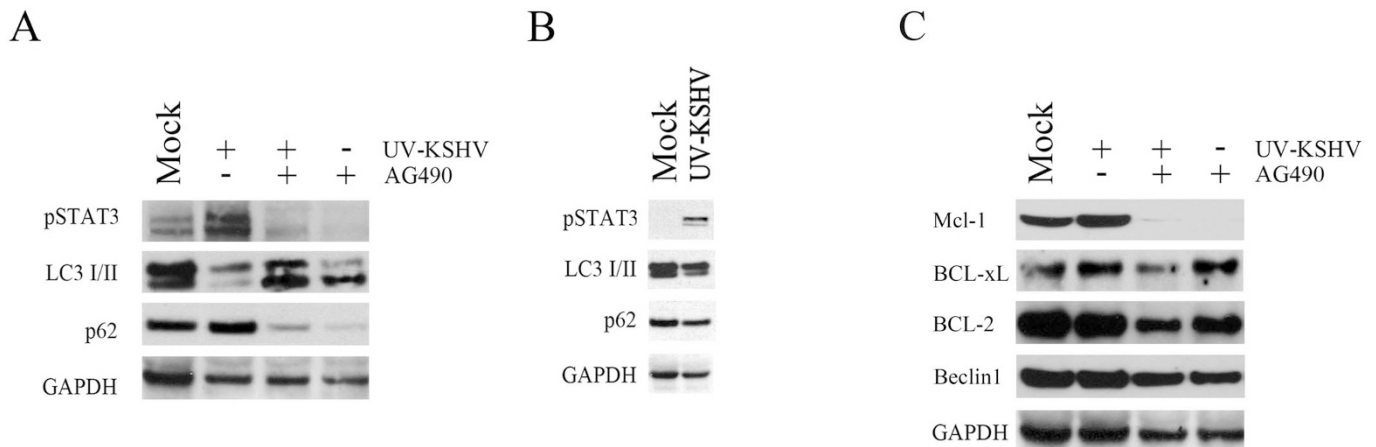


Figure 4 | STAT3 activation by KSHV correlates with an autophagy block in DCs. (A) Western blot analysis showing pSTAT3, LC3I/II and p62 expression level in DC mock or exposed to UV-KSHV for 15 min and cultured 24 hours in serum free conditions, in the presence or in the absence of STAT3 inhibitor AG490 (50 μ g/ml). (B) Western blot analysis of pSTAT3, LC3I/II and p62 after 15 min of DC exposure to UV-KSHV (C) Western blot analysis of Mcl-1, BCL-xL, BCL-2 and Beclin1 expression in DCs mock or UV-KSHV-exposed, in the presence or in the absence of STAT3 inhibitor AG490 (50 μ g/ml). GAPDH was included as protein loading control. 1×10^6 overnight serum-starved DCs/point were used in all experiments. Data, representative of three independent experiments, are reported.

strategies to prevent and/or improve the outcome of KSHV-associated malignancies.

Methods

Cell lines, viral production, quantitation and UV-inactivation. BC3, a human B-cell line derived from PEL, carrying latent KSHV, was cultured in RPMI 1640 (SIGMA, R0883) supplemented with 10% fetal bovine serum (Invitrogen) in the presence of 5% CO₂.

KSHV was obtained by inducing viral lytic cycle in BC3 cells treated with 0.3 mM Na-butyrate and TPA (10 ng/ml) for 96 hours, as described⁴³. BC3 were then analyzed for the expression of K8.1A lytic antigen by IFA and the virus was collected when the percent of K8.1A positive cells was higher than 30%. The virus was then concentrated 200 \times in serum-free medium, in aliquots containing $\sim 9 \times 10^6$ viral DNA copies, as measured by real-time DNA PCR (Nanogen Advanced Diagnostics, Milan, Italy), using primers amplifying the KSHV capsid protein gene, as previously described⁴⁴. KSHV UV-inactivation was carried out at 1500 mJ in a UV cross-linker for 8 min.

Reagents and antibodies. TPA and Na-butyrate were purchased by SIGMA (cat.no P1585 and B5887, respectively). AG490 was purchased by Calbiochem (cat.no 658411). The following antibodies were used: mouse-anti-STAT3 and mouse anti-phosphoSTAT3 (pY705) (BD Transduction Laboratories, cat.no 610189 and 612356, respectively), rabbit anti-phospho-NF-kB p65 (Cell Signaling, cat.no S536), rabbit anti-NF-kB p65 (Santa Cruz, cat.no sc-109), mouse anti-GAPDH (Santa Cruz, cat.no sc-137179), mouse anti-DC-SIGN (Abcam, clone 120507), rabbit polyclonal anti-

LC3 (Novus Biologicals, cat.no NB 100-222055), mouse monoclonal anti-p62 (BD Transduction Laboratories, cat.no 610833), rabbit polyclonal anti-BCL-xL (Cell Signalling, cat.no 5446), rabbit polyclonal anti-BCL-2 (Cell Signalling, cat. No 50E3) and rabbit polyclonal anti-Mcl-1 (Cell Signalling, cat.no D35A5).

Generation of dendritic cells and treatments. To generate monocyte-derived DCs, human peripheral blood mononuclear cells (PBMC), obtained from healthy donors (under informed consent), were isolated by Fycoll-Paque gradient centrifugation (Pharmacia, Uppsala, Sweden) from buffy coats. CD14⁺ monocytes were positively selected using anti-CD14 MAb-conjugated magnetic microbeads (Miltenyi Biotec, cod. no.130-050-301). Purified monocytes were cultured at a density of 10^6 cells/3 ml in 12-well plates for 6 days in RPMI 1640 containing 10% fetal calf serum (FCS), glutamine (300 μ g/ml), 100 U/ml penicillin G, 100 μ g/ml streptomycin and recombinant human granulocyte-macrophage colony stimulating factor (GM-CSF, 50 ng/ml) and interleukin 4 (IL-4, 20 ng/ml) (Miltenyi Biotec, cod. no 130-095-372 and 130-093-917, respectively) to generate DC (DC). Cytokines were replenished every other day with 10% fresh medium⁴⁴. Depending on the experiments, over-night serum-starved DC were exposed to KSHV for different times at 37°C, or DCs were pre-incubated with anti-DC-SIGN monoclonal antibody (20 μ g/ml) for 1 hour at 4°C and then exposed to KSHV or incubated with anti-DC-SIGN alone for 15 min at 37°C. To study the effect of KSHV-mediated STAT3 activation on autophagy, DCs were pretreated with STAT3 inhibitor, AG490 (50 μ g/ml), or with DMSO as control, for 2 hours at 37°C and then exposed to KSHV for 15 min at 37°C. In some experiments STAT3 knock-down with specific siRNA (Santa Cruz) was performed before viral exposure, using INTERFERin (Polyplus) to transfect DCs. Cells were subsequently cultured overnight in serum-free medium. In some experiments, as control, 3-Methyladenine (3-MA) (5 mM) was used to inhibit autophagy.

Measurement of KSHV internalization by real-time DNA PCR. DCs untreated or exposed to KSHV for 15' at 37°C, with or without pretreatment with anti-DC-SIGN, were washed twice in PBS and incubated with 0.25% trypsin-EDTA for 30' at 37°C, to remove the non-internalized virus. The internalized KSHV was then quantified by real-time DNA PCR (Nanogen Advanced Diagnostics, Milan, Italy), using primers amplifying the KSHV capsid protein gene, as previously described⁴⁴. Briefly, to standardize the assay, we used a plasmid containing part of ORF26. Serial dilutions of this plasmid ranging from 5 to 5 000 000 copies were used to characterize the linearity, precision, specificity, and sensitivity of the real-time DNA PCR. Amplification of human genomic β -globin DNA was also used to assess the absence of PCR-inhibitory substances^{45,46}.

Western blotting. Cells (1×10^6) were washed twice with PBS and lysed in a modified RIPA buffer containing 150 mM NaCl, 1% NP-40, 50 mM Tris-HCl (pH 8), 0.5% deoxycholic acid, 0.1% SDS, 1% Triton X-100, protease and phosphatase inhibitors. The lysates were subjected to electrophoresis on 4–12% NuPage Bis-Tris gels (Life Technologies) and transferred to nitrocellulose membranes (Protran BA 85, Whatman). The membranes were blocked with 3% BSA (SIGMA, cod.no A4503) and probed with specific primary antibodies, overnight at 4°C. The membranes were washed and incubated with appropriated secondary antibody conjugated to horseradish peroxidase (Santa Cruz), used at 1 : 10.000. The membranes were washed and immunoreactivity was detected by enhanced chemiluminescence kit (Thermo Scientific, cod.no 32209).

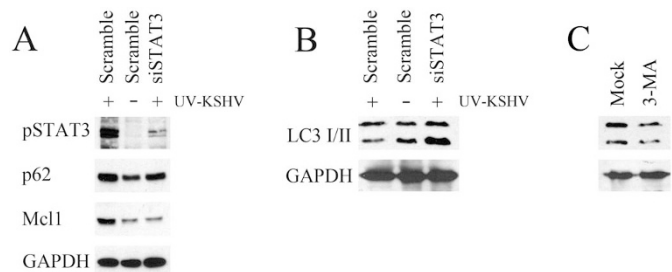


Figure 5 | STAT3 silencing prevents the KSHV-mediated autophagic block in DCs. (A) Western blot analysis showing pSTAT3, p62 and Mcl1 expression and (B) LC3I/II in DC treated with control (scramble) or STAT3 specific siRNA exposed to UV-KSHV for 15 min and cultured 24 hours in serum free conditions. (C) LC3I/II was also evaluated in the presence of 3-MA autophagic blocker. GAPDH was included as protein loading control. 1×10^6 overnight serum-starved DCs/point were used in all experiments. Data, representative of three independent experiments, are reported.



ELISA assay. Serum-starved DCs mock were pretreated with STAT3 inhibitor, AG490 (50 µg/ml), or with DMSO as control, for 2 hours at 37°C and exposed to KSHV for 15 min at 37°C. Cells were then cultured for an additional 24 hours for IL-6, IL-10 and IL-23 production. For IL12p70 release DCs, during the 24 hours LPS (1 µg/ml, Salmonella abortus equi, SIGMA) was added to the cell culture. ELISA assays were performed using commercially available reagents and standards (for IL-6, IL-10 and IL-12 RayBiotech, Inc and for IL-23 Abcam ELISA kits were used). The supernatants were added in duplicate to appropriate pre-coated plates. After the plates were washed, horseradish peroxidase-conjugated detection antibody was added. The substrate used for color development was tetramethylbenzidine (TMB). The optical density was measured at 450 nm with a microplate reader (Multiskan Ex, Thermo Labsystem). The minimum detection dose is typically less than 1 pg/ml.

Statistical analyses. All data are represented by the mean \pm standard error of at least three independent experiments.

Ethics statement. The study was approved by the ethical Committee of Policlinico Umberto I, Sapienza University, Rome, Italy.

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Author contributions

M.C. and R.S. conceived the experiments, R.S., G.D.G., R.G. and M.G. performed Western blot analysis, G.G. and A.C. performed Real Time PCR, L.C. performed ELISA assay, M.C.,



R.S. and A.F. interpreted results and wrote the paper. All authors reviewed and approved the manuscript.

Additional information

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