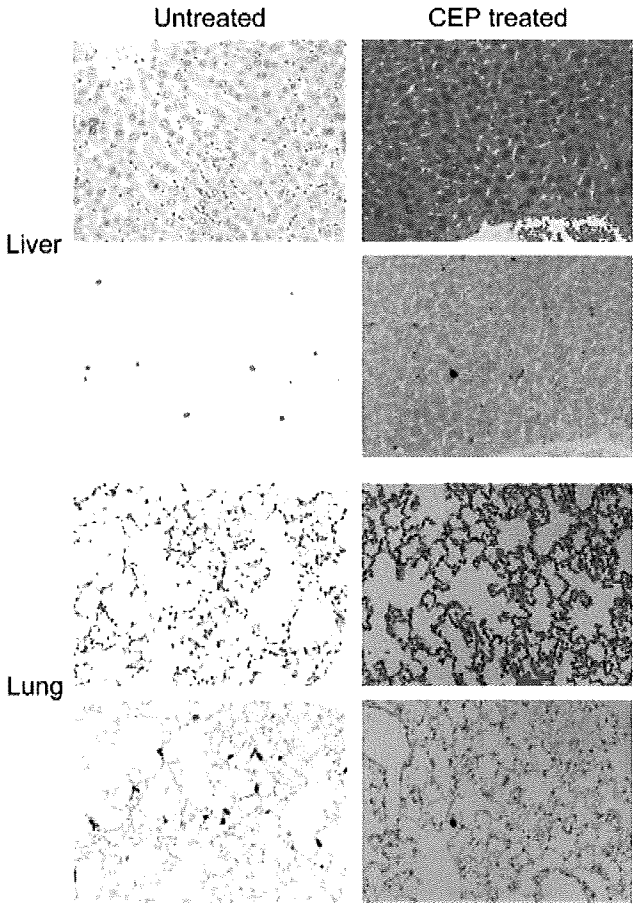


FIGURE 8 – Metastasis of PEL cells into the liver and lungs of the BCBL-1 inoculated mice. Hematoxylin–eosin staining and immunohistochemical staining using anti-LANA (PA1-73N antibody) was performed to detect BCBL-1.

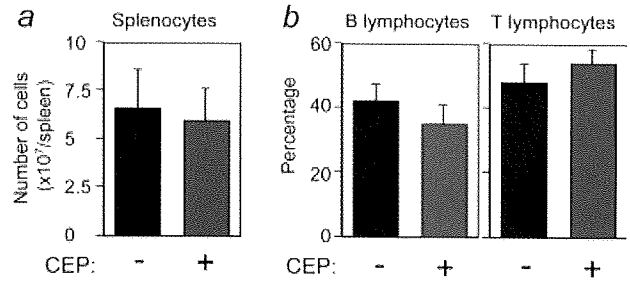


FIGURE 9 – CEP has no adverse effects for murine splenic lymphocytes. (*a*) Number of splenocytes after CEP treatment for 21 days, shown as the mean ± S.D. from 4 mice ($p > 0.05$). (*b*) Percentage of B lymphocytes ($CD19^+$) and T lymphocytes ($CD19^+$) in splenocytes after CEP treatment for 21 days, shown as the mean ± S.D. from 4 mice ($p > 0.05$).

untreated mice. Copy number of KSHV ORF26 revealed that CEP treatment suppressed metastasis (Fig. 7*d*).

Organ infiltration by tumor cells was analyzed and evaluated by Hematoxylin–eosin staining, and LANA immunostaining (Fig. 8). We found that mice inoculated intraperitoneally with BCBL-1 exhibited infiltration into the lung and liver without macroscopic lymphoma formation (Fig. 8). The number of LANA positive cells in CEP treated mice was significantly reduced (0–1cells per field magnification, ×40) compared to the nontreated mice (10–20 cells per field magnification, ×40). These data indicate that CEP significantly inhibits the growth and infiltration of PEL cells *in vivo*.

To investigate the adverse effect of CEP for lymphocytes, a dose of 10 mg/kg CEP in PBS or PBS alone was administrated into Balb/c mice *via* intraperitoneal for 21 days. At the time of sacrifice, the gross anatomical changes were not observed in CEP treated mice including the size of spleen. As shown in Figure 9*a*, the number of splenocytes were not significantly different between CEP treated and untreated mice. Percentages of B lymphocytes and T lymphocytes were also not significantly different (Fig. 9*b*), indicating that there are no significant adverse effects of CEP including for lymphocytes.

## Discussion

In the present study, we investigated the direct effects of the biscoclaurine alkaloid CEP on PEL cells *in vitro* and *in vivo*. Our results show that CEP exhibits potent proapoptotic effects on PEL cells and provides evidence that such apoptosis occurs *via* inhibition of NF-κB activity. The rapid and efficient engraftment of PEL cells in NOD/Scid/Jak-3 deficient (NOJ) mice displays characteristics of the human disease and this small animal system is an important step towards developing an *in vivo* model for studying PEL and HHV-8 pathogenesis. Immunodeficient NOD/Scid mice have been previously used as the recipient of human tumor xenograft especially for hematological malignancies,[39,40] as they exhibit complete deficiencies in mature T and B lymphocytes and complement protein, and partial deficiencies in NK cells, macrophages and dendritic cell function.[41,42] Recent advances suggest that complete deficiency of NK cells allows more efficient engraftment of human tumor cells and human hematopoietic stem cells.[43–45] The NOJ mice display a complete deficiency of NK cells like the NOD/Scid/common γ deficient (NOG) mice and provide the ideal microenvironment for the propagation and expansion of PEL cells. The formation of malignant ascites without solid lymphoma formation displayed in PEL xenografted NOJ mice resembles the diffuse nature of human PEL.[1,2]

PEL is an incurable, aggressive B-cell malignancy and most of the patients that suffer from it respond poorly to traditional chemotherapy and develop chemoresistance.[1,2] Several agents have been tested in the search for a more effective treatment for PEL. It is now postulated that the mechanisms of lymphomagenesis involve deregulation of several signaling pathways that may act either independently or crosstalk with each other. These include NF-κB, JAK/STAT and PI3 kinase[4–6] in the case of PEL. PEL is associated with KSHV/HHV-8 infection and KSHV/HHV-8 contains a homologue of the cellular FLIP protein vFLIP, which has the ability to activate the NK-κB pathway.[7,38,46] Moreover, inhibition of NK-κB activity leads to apoptosis of KSHV-infected PEL cells.[5,11] These results suggest that inhibition of NK-κB is an effective target for the treatment of PEL. Activation of NK-κB is involved in various kinds of cancer development and progression[47–49] as well as in virus associated lymphomas,[9] indicating that NF-κB is the good molecular target for cancer treatment.[50]

CEP is one of the biscoclaurine alkaloids that is widely used for the treatment of many acute and chronic diseases in Japan. Antitumor effects and induction of apoptosis by CEP have been reported in the last 10 years.[17–21] Recently, CEP has been shown to inhibit the activation of NF-κB;[12,22,23,51] however, the mechanisms are largely unknown. In our study, we demonstrated that CEP is able to suppress the growth of PEL cells and induce apoptosis *via* the inhibition of NF-κB activity especially by blocking the phosphorylation of p65 NF-κB (Fig. 5*a*). KSHV vFLIP binds to the IKK complex to induce constitutive kinase activation.[10] Consequently, PEL cells have high levels of nuclear NF-κB activity, which is necessary for the survival of PEL cells.[9] Treatment of PEL cells with CEP abrogates the NF-κB activity by blocking phosphorylation of NF-κB p65 (Fig. 5*a*), inhibiting NF-κB nuclear translocation (Fig. 5*b*) and DNA binding activity (Fig. 6*b*). However, as

CEP may not affect KSHV vFLIP, vFLIP continuously activates IKK and results in the retention and accumulation of nonactivated NF-κB p65 (Fig. 5*a*).

CEP has been shown to protect human lymphocytes against radiation-induced apoptosis[52] and oxidative damage.[53] In addition, CEP has been used in Japan for a long time[12] and has shown few side effects,[54–56] and a pharmacokinetic study did not show any adverse effects in volunteer subjects.[57] In this study, we observed administration of CEP showed no significant adverse effects for Balb/C mice (Fig. 8). Moreover, Kikukawa *et al.* recently showed that CEP treatment was effective for the patient with chemotherapy resistant multiple myeloma without obvious side effects.[58] Taken together, CEP is the very useful for the treatment of patients with PEL without side effects. However, the effects of CEP on PEL cells other than NF-κB pathway are still unclear because CEP exerts diverse pharmacological effects.[12] There may be other pathways for inducing the apoptosis of PEL cells other than *via* inhibition of NF-κB. Indeed, CEP has been shown to have a multidrug resistant-reversing effect,[12] to induce cell cycle regulators such as p27Kip1 and p21WAF1,[59] and to activate JNK1/2.[21] Further investigations aimed at determining the efficacy of CEP are warranted and may lead to the development of new effective therapies for this intractable lymphoma.

In conclusion, we have shown the ability of CEP to induce cell death through blocking the NF-κB pathway in PEL cells. Our study provides the rationale for a clinical trial of CEP in patients with PEL and other NF-κB activated tumors.

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