OX40 and 4-1BB downregulate Kaposi’s sarcoma-associated herpesvirus replication in lymphatic endothelial cells, but 4-1BB and not OX40 inhibits viral replication in B-cells

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Kaposi’s sarcoma-associated herpesvirus (KSHV) belongs to the human gammaherpesvirus subfamily and is associated with malignancies of endothelial origin (Kaposi’s sarcoma, KS) and B-cell origin [primary effusion lymphoma (PEL) and multicentric Castleman’s disease (MCD)]. Viral lytic replication is known to be required for KS and MCD. As KSHV-related tumours mostly develop in human subjects when the immune system is compromised by immunosuppressive regimen, human immunodeficiency virus infection or some genetic deficiencies, KSHV-specific immune responses are believed to be important in the control of KSHV replication. However, analysis of the roles of immune cells in viral pathogenesis has been difficult due to the lack of an adequate animal model. Recently, congenital OX40 deficiency, as determined by genome-wide exome sequencing, was shown to be associated with aggressive childhood KS in a patient, suggesting that disrupted OX40–OX40L interactions might be implicated in disease development. Here, we report that interaction of recombinant OX40 protein with OX40L expressed on endothelial cells severely impaired KSHV lytic replication. Furthermore, 4-1BB–4-1BBL interactions were also capable of efficiently inhibiting viral replication in B-cells and endothelial cells. To the best of our knowledge, this is the first direct evidence that ligation of tumour necrosis factor superfamily members and their cognate receptors is important for the control of viral lytic replication. These data are likely to pave the way for the development of KSHV-specific therapies for KS and MCD, in which viral lytic replication is a disease-determining factor.

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INTRODUCTION

Kaposi’s sarcoma-associated herpesvirus (KSHV) is a lymphotropic gammaherpesvirus that is associated with several human malignancies, including Kaposi’s sarcoma (KS) and at least two types of B-cell lymphomas – primary effusion lymphoma (PEL) and multicentric Castleman’s disease (MCD) (Cesarman et al., 1995; Chang et al., 1994; Dupin et al., 1995; Moore & Chang, 1995; Soulier et al., 1995; reviewed by Ganem, 2007a, b, 2010). Among these, KSHV lytic replication is known to be important for the tumorigenesis of MCD (Casper et al., 2004; Polizzotto et al., 2013; Uldrick et al., 2011) and KS (Martin et al., 1999). When viral replication is inhibited, KS and MCD regress, suggesting that KSHV replication is intricately involved in the pathogenesis of these KSHV-induced malignancies. Although AIDS-related KS displays aggressive disease progression, classical KS is a mostly indolent tumour (Davidovici et al., 2001; Iscovich et al., 2000). It has been estimated that only 1 in 10 000 KSHV carriers develop KS and typically not until old age; KS development is extremely rare in paediatric patients (Byun et al., 2013 and references therein) unless the immune system is compromised (Serraino et al., 2010; Shiels et al., 2011). AIDS and immunosuppression after organ transplantation profoundly increase the risk for KSHV-induced tumours (epidemic and iatrogenic KS) at least 3000-fold (Shiels et al., 2011) and 100-fold (Serraino et al., 2010), respectively. In fact, a number of studies point to the critical importance of an intact immune system for the control of KSHV replication (reviewed by Ganem, 2007a). Over the past decades,
an accumulating body of evidence has suggested that interactions of tumour necrosis factor superfamily members (TNFSF) and their cognate receptors (TNFRSF) provide costimulatory signals for T-cell activation for the control of viral replication (reviewed by Vinay & Kwon, 2009). However, the role of TNFSF–TNFRSF interactions in the control of KSHV replication remains largely unknown. Only recently, Byun et al. (2013) elegantly showed that an inherited defect in OX40 impairs T-cell-mediated control of viral infections, thus possibly predisposing the paediatric patient with OX40 deficiency to the development of aggressive childhood KS.

OX40 belongs to the TNFRSF, which consists of a growing number of structurally similar receptors (~30 receptors identified to date) (Croft, 2009; Croft et al., 2012; Li et al., 2013; Sedy et al., 2014; Summers deLuca & Gommerman, 2012). TNFRSF members bind to one or more ligands (TNFSF; ~19 ligands known). Examples include OX40 (CD134)–OX40L (CD252), 4-1BB (CD137)–4-1BBL (CD137L), CD27–CD70 and TL1A–DR3. The receptors (OX40, 4-1BB, CD27 and TL1A) are expressed mainly on T-cells upon activation and their ligands are constitutively or inducibly expressed on antigen-presenting cells (APCs; macrophages, B-cells and dendritic cells) as well as endothelial cells (ECs). Upon receptor–ligand ligation, a signalling cascade is generated either unidirectionally or bidirectionally between the two interacting partners. TNFSF–TNFRSF interactions have been shown to provide co-stimulatory signals to T-cells, inducing cytokine expression and cell proliferation. The importance of such interactions in the control of viral infection has been reviewed elsewhere (Sedy et al., 2014; Vinay & Kwon, 2009) and with special emphasis on OX40–OX40L (Croft, 2010) or 4-1BB–4-1BBL (Vinay & Kwon, 2011).

The role of TNFSF–TNFRSF interactions in the development of KS is beginning to be revealed. Byun et al. (2013) reported a paediatric patient with childhood KS who had a mutation in the OX40 gene. This patient with OX40 deficiency developed leishmaniasis and KS early in life, most likely due to impaired T-cell function because the multitude and magnitude of T-cell responses from the blood of the patient were significantly reduced compared with the control group. Moreover, the peripheral T-cells could not mount memory responses to vaccine strains such as BCG and measles, suggesting that T-cell activation and the development of memory T-cells was diminished in the absence of OX40–OX40L interaction between T-cells and APCs. These data underscore the importance of OX40–OX40L interactions in immune control of the development of KS. However, direct evidence for its involvement in the regulation of KSHV replication has been elusive. We hypothesized that TNFSF–TNFRSF interactions, including OX40–OX40L and 4-1BB–4-1BBL, play a role in the control of KSHV replication in endothelial and B-cells, and thus in the development and pathogenesis of KS and MCD, respectively.

Which cells would be best for testing this hypothesis? Selecting the most relevant cell type for in vitro infection studies is particularly important because there is no animal model available to study KSHV pathogenesis in vivo. Spindle cells, so-called because of their elongated morphological features, are proliferating tumour cells of endothelial origin in KS lesions. Infection of most transformed cell lines with KSHV in vitro does not induce dramatic changes in morphology, including actin rearrangement and cell elongation. However, human primary dermal microvascular endothelial cells are a prominent exception (Ciufolo et al., 2001; Gao et al., 2003). Dermal microvascular endothelial cells consist of lymphatic and blood endothelial cells (LECs and BECs, respectively). Of these, only LECs undergo spindle formation upon KSHV infection while BECs do not (Chang & Ganem, 2013 and unpublished observations). As spindle formation is the most characteristic feature of KS lesions, LECs are considered to be the most relevant cell type for in vitro studies of KS. Moreover, it should be noted that LECs produce a large quantity of viral progeny upon de novo infection whereas KSHV infection in BECs is relatively latent. Virus production in LECs subsides after initially efficient viral replication, eventually reaching a plateau. However, aberrant lytic replication continues even after 2 weeks of culture in vitro, producing detectable levels of viruses in the culture supernatant (Chang & Ganem, 2013, and unpublished observations). Therefore, LECs seem to serve a suitable in vitro model for investigating the roles of T-cells and the consequences of their interactions with ECs in the control of productive KSHV replication. In the present study, we set out to test our hypothesis using primary human dermal endothelial cells (LECs and BECs), vascular endothelial cells (human umbilical vein endothelial cells, HUVECs) and tonsillar T-cells as the interacting partners. We report that activation of both OX40–OX40L and 4-1BB–4-1BBL interactions in infected LECs, but not BECs, significantly inhibits infectious viral progeny production, suggesting that intracellular signals emitted from activated OX40L and 4-1BBL in LECs inhibit KSHV replication. On the other hand, neutralization of 4-1BB–4-1BBL, but not OX40–OX40L, interactions between T-cells and infected tonsillar B-cells prevents T-cell-mediated inhibition of KSHV replication in B-cells, implying that activation of 4-1BBL on B-cells has inhibitory effects on viral replication. These data suggest the presence of cell type-specific mechanism(s) by which TNFRSF interactions are involved in the regulation of KSHV replication through the crosstalk between the communicating cells: T-cell–EC or T-cell–B-cell. To the best of our knowledge, this is the first report that TNFRSF interactions may play an important role in T-cell-mediated control of KSHV replication. Furthermore, these data may at least in part explain why OX40 deficiency on T-cells predisposes the patient to the development of KS. It is possible that KSHV replication proceeds uncontrolled in KS lesions in the absence of functional OX40, suggesting the importance of interactions between infiltrating T-cells and ECs in the control of viral replication.
RESULTS

OX40 and 4-1BB are expressed in a subset of tonsillar CD4⁺ T-cells

OX40 and 4-1BB are known to be expressed on peripheral blood T-cells. To examine whether those molecules were expressed on tonsillar CD4⁺ T-cells as well, human lymphoid aggregate cultures (HLAC) were employed. The focus of this study was CD4⁺ T-cells because CD4⁺, but not CD8⁺ T-cells, are responsible for the T-cell-mediated inhibition of KSHV lytic replication in tonsillar B-cells (Myoung & Ganem, 2011a). Approximately 10% of tonsillar CD4⁺ T-cells of the unfractionated HLAC expressed OX40 in the absence of PHA stimulation, while PHA stimulation increased OX40 expression to ~16% (Fig. 1). Notably, a lower proportion of CD4⁺ T-cells among purified T-cells expressed OX40 without (3.1%)

Figure 1. Expression of OX40 and 4-1BB on tonsillar lymphocytes. OX40 and 4-1BB expression on tonsillar CD4⁺ T-cells (a) or CD19⁺ B-cells (b) was analysed by flow cytometry. Unfractionated or fractionated cells were left untreated or stimulated with phytohaemagglutinin (PHA) for 48 h before subjected to cytometric analysis. For the scheme of cell purification, see Methods.

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or with (5.8 %) PHA stimulation, suggesting a positive role of other cell types, possibly B-cells, in the expression of OX40 in the HLC. On the other hand, approximately twofold more CD4+ T-cells in the uninfected LAC expressed 4-1BB even in the absence of PHA stimulation (20.5 %), and PHA stimulation further upregulated expression to 27.2 %. When T-cells were cultured in the absence of B-cells, a much lower proportion of CD4+ T-cells expressed 4-1BB without (5.8 %) or with (7.9 %) PHA stimulation, further supporting the notion that B-cells in the HLC enhance the expression of these two TNFRSF members on CD4+ T-cells. In fact, addition of increasing numbers of purified B-cells to T-cells augmented OX40 and 4-1BB expression on T-cells (Fig. S3, available in the online Supplementary Material). On the contrary, as expected, CD19+ B-cells, whether purified or not, did not express either OX40 or 4-1BB.

**Blockade of 4-1BB–4-1BBL interactions between T- and B-cells prevents KSHV lytic replication in B-cells**

To investigate the effect of OX40–OX40L and 4-1BB–4-1BBL interactions between lymphocytes on viral lytic replication, KSHV-infected B-cells were co-cultured in the presence of neutralizing antibodies (10 μg ml−1) specific to OX40, OX40L or 4-1BB (Fig. 2). When infected B-cells were co-cultured with PHA-stimulated T-cells alone, KSHV titres in the culture supernatant were significantly (~70 %) reduced compared with those of the infected B–uninfected B-cell co-culture, which is in line with our previous finding that activated T-cells inhibit viral replication in B-cells (Myoung & Ganem, 2011a). Surprisingly, blockade of the 4-1BB–4-1BBL interaction partially relieved the T-cell-mediated inhibition of KSHV lytic replication in B-cells to ~72 % of the level in the control (Fig. 2a) in a dose-dependent manner (Fig. 2b), suggesting that activation of 4-1BB signalling is important for T-cells to inhibit viral replication in B-cells. However, blockade of OX40–OX40L interactions between T- and B-cells had little effect on KSHV replication.

**Expression of OX40L and 4-1BBL on primary human lymphatic endothelial cells is induced upon KSHV infection**

To examine the expression levels of TNFSF members on primary human endothelial cells, including two dermal microvascular endothelial types (LECs and BECs) and HUVECs, the cells were stained with specific antibodies to OX40L, 4-1BBL, CD70, TL1A and CD30L (Fig. 3, top panels). MHC class I and II antibodies were included as controls (Fig. 3, bottom panels). To activate endothelial cells, a separate group of cells were stimulated with IFN-γ for 48 h (histograms in red). Expression of OX40L was detected on all of the primary human endothelial cells; however, IFN-γ treatment did not further enhance OX40L expression. Notably, KSHV-infected cells displayed much higher levels of OX40L expression, suggesting KSHV infection might upregulate the surface expression of OX40L. The same was true for expression of 4-1BBL. Although uninfected LECs displayed low, if any, expression of 4-1BBL, KSHV infection significantly enhanced its expression. The expression of CD70, TL1A and CD30L was neither detected nor enhanced by KSHV infection in any of the cells examined. As expected, IFN-γ greatly augmented MHC class I and II expression whereas KSHV infection diminished the surface expression of MHC class I, as reported previously (Brulois et al., 2014; Goto et al., 2010; Ishido et al., 2000).

**Activation of OX40L and 4-1BBL signalling by recombinant ligands inhibits KSHV replication in lymphatic endothelial cells**

To investigate the potential role of augmented OX40L and 4-1BBL upon KSHV infection in endothelial cells, recombinant cognate ligands (10 μg ml−1) were added into infected LEC or BEC cultures. Recombinant CD27, a ligand for CD70, was included as a control. Interestingly, both OX40 and 4-1BB suppressed infectious virion production by roughly 70 % in LECs (Fig. 4a) but not in BECs (Fig. 4b). In addition, treatment of recombinant TNFRSF members did not affect cell viability (Fig. S1) and the expression of viral lytic genes was inhibited by the treatment of recombinant OX40 and 4-1BB (Fig. S2). These data demonstrate that OX40–OX40L and 4-1BB–4-1BBL interactions inhibit KSHV lytic replication in LECs, the physiologically relevant cell culture model for KS (Chang & Ganem, 2013; Weninger et al., 1999). Furthermore, both recombinant OX40 (Fig. 4c) and 4-1BB (Fig. 4d) inhibited KSHV replication in infected LECs in a dose-dependent manner.

**DISCUSSION**

KS is the most common AIDS-related malignancy. It is a complex disease and is considered to be a proinflammatory, angioproliferative state, especially at the early stages of tumorigenesis (Ensoli et al., 2001; Ganem, 2007a, 2010). One of the prominent features of KS lesions is massive immune cell infiltration, including B-cells, T-cells and monocytes. Immune cells in KS lesions are rarely infected and produce a wide variety of proinflammatory cytokines, whose roles in the control of KSHV infection remain poorly defined (Parravicini et al., 2000 and reviewed by Ensoli & Stürzl, 1998). KSHV infection in KS lesions is detected mainly in endothelial cells (Boshoff et al., 1995; Staskus et al., 1997), so-called ‘spindle cells’ with characteristic elongated cell morphology. The majority of spindle cells are latently infected with fewer than 10 % of cells lyrically activated. As in vitro infection models for KS display a highly lytic nature (Boshoff et al., 1995; Chang & Ganem, 2013), it has been postulated that infiltrating immune cells in KS lesions may modulate KSHV replication in
OX40 and 4-1BB inhibit KSHV replication

(a)

(b)
Fig. 2. Blockade of 4-1BB signalling de-suppresses KSHV lytic replication in B-cells. Tonsillar B-cells were purified by negative selection and infected with recombinant KSHV.219 viruses (rKSHV.219) at an m.o.i. of 3. After extensive washing, 10^6 purified B- or T-cells were mixed with the same number of infected B-cells. (a) Isotype or neutralizing antibodies to human OX40, OX40L and 4-1BB (10 mcg/ml) were added into the co-culture at d0 and d2. Two different neutralizing antibodies to OX40L, raised in mouse or goat, were used: the goat antibodies are indicated. (b) In a separate experiment, serial dilutions of anti-4-1BB antibodies were made before addition to the co-culture to examine dose dependence. Cells were cultured in the presence of neutralizing antibodies for 5 days before infectious titres in the culture supernatant were determined. Infected cells in the co-culture are indicated in bold. The mean ± SEM of three independent experiments is plotted. *P<0.05; **P<0.01; ***P<0.001 by Student’s t-test.

vivo (Parravicini et al., 2000). Consistent with this notion, several recent studies have highlighted the importance of activated functional T-cells in the regulation of viral replication: (1) Myoung & Ganem (2011a, d) demonstrated that infected tonsillar B-cells display spontaneous lytic replication, which is inhibited by activated CD4^+ T-cells in a contact-dependent and killing-independent manner and (2) Byun et al. (2013) reported that congenital deficiency of OX40 is responsible for the inability of T-cells to control viral replication, possibly leading to the development of an aggressive childhood KS. These groundbreaking pieces of work open up an intriguing possibility that OX40–OX40L interaction, and possibly other TNFSF–TNFRSF interactions, may be involved in the regulation of KSHV replication between T-cells and the two main virus targets in vivo: endothelial and B-cells. Therefore, it is important to determine whether TNFSF or TNFRSF molecules are expressed on the surface of endothelial cells and lymphocytes in order to investigate if those cells communicate with one another through TNFSF–TNFRSF interactions to modulate the outcomes of KSHV infection. Previously, it was shown that the expression of OX40 and 4-1BB is not ubiquitous and is highly induced, especially on CD4^+ T-cells upon immune activation. In line with these data, significant proportions of tonsillar CD4^+ T-cells expressed both OX40 and 4-1BB (Fig. 1), and blocking 4-1BB de-suppressed KSHV lytic replication in infected B-cells (Fig. 2a) in a dose-dependent manner (Fig. 2b). Even without PHA stimulation, a considerable proportion of tonsillar CD4^+ T-cells expressed OX40 and 4-1BB molecules on their surface, indicating that those cells had undergone a recent inflammatory event: indeed, the tonsils utilized in the present study were taken from paediatric patients with tonsillitis. It is important to note that tonsillar T- and B-cells interact through 4-1BB–4-1BBL and inhibition of this interaction partially prevents T-cell-mediated inhibition of KSHV replication in B-cells (Fig. 2). It would be very interesting to see whether recombinant 4-1BB alone can recapitulate T-cell-mediated inhibition. LECs, on the other hand, expressed OX40L and 4-1BB at high levels especially upon KSHV infection (Fig. 3), and activation of them with recombinant cognate ligands, OX40 and 4-1BB, respectively, inhibited the production of infectious progeny (Fig. 4a). These data suggest that activation of signalling pathways downstream of OX40–OX40L and 4-1BB–4-1BBL is capable of limiting viral replication. Delineation of the underlying molecular mechanisms is now currently underway.

Of note, 4-1BB–4-1BBL interactions on both B- and endothelial cells resulted in inhibition of KSHV replication while OX40–OX40L interactions induced the similar effect only on endothelial cells. These data imply that there may be cell type-specific mechanism(s). It is not clear what the basis of this interesting phenomenon is. Plausible mechanisms include the following: (1) Differential levels of OX40L expression on B-cells compared with those of endothelial cells may provide a possible explanation. While infected LECs upregulated surface OX40L expression, that of B-cells was not determined in this study. Once determined, it would provide an insight on the basis of cell type-specific mechanisms. (2) It has been reported that LECs undergo an aberrant lytic programme (unpublished observations; Chang & Ganem, 2013) while B-cells support a full lytic replication (Myoung & Ganem, 2011a, d). Differences in viral lytic programmes in B- and endothelial cells may account for the cell type-specific responses; how the differences affect cellular gene expression programmes, and thus host responses to infection, remains to be investigated. (3) Many other factors might be responsible as well, such as differences in intracellular signalling cascades, inherent differential cellular transcription programmes and/or disparate regulatory mechanisms of gene expression in B- and endothelial cells. Attempts are currently being made to investigate the basis of the differential responses to OX40–OX40L interactions on those two in vivo targets of KSHV.

The differential levels of OX40L and 4-1BBL on ECs warrant a comment. The levels of OX40L on the surface of various primary endothelial cells (Fig. 3) are comparable to those reported by Byun et al. (2013). In addition, activation of endothelial cells by IFN-γ had little effect on the OX40L levels of LECs, BECs and HUVECs, suggesting that OX40L is constitutively expressed. On the other hand, expression of 4-1BBL was almost negligible on the primary endothelial cells examined and remained unchanged after treatment with IFN-γ, even though these primary endothelial cells were capable of upregulating MHC class I and II upon IFN-γ treatment (Fig. 3, bottom panels), indicating EC activation. Furthermore, KSHV infection downregulated MHC class I expression on the cell surface,
as reported previously (Brulois et al., 2014; Goto et al., 2010; Ishido et al., 2000). In line with our data, Imura et al. (1996) also detected similar levels of OX40L on HUVECs, human aorta endothelial cells (HAECs) and human dermal microvascular endothelial cells (HDMECs), and showed that interaction of OX40L with OX40 on CD4^+ T-cells mediated T-cell adhesion. A few years later, the same group of researchers (Kunitomi et al., 2000) demonstrated that the interaction of OX40L on ECs with OX40 on CD4^+ T-cells stimulated T-cell proliferation.

Fig. 3. Expression levels of TNF receptor superfamily members on the surface of human primary endothelial cells. Lymphatic endothelial cells (LECs, left panels), blood endothelial cells (BECs) and human umbilical vein endothelial cells (HUVECs) were left uninfected or infected with rKSHV.219 viruses at an m.o.i. of 3 before flow cytometric analysis. As a control, uninfected cells in a separate well were stimulated with IFN-γ to induce the expression of TNFRSF and MHC molecules. Shaded histogram, isotype antibody; cyan, IFN-γ untreated; red, IFN-γ treated; black, GFP negative; green, GFP positive. One representative data from three independent experiments is shown.
suggesting that OX40–OX40L interactions can function to provide a co-stimulatory signal to T-cell activation. As the cytoplasmic tail of OX40 on T-cells is too short to generate signal cascades in T-cells, it is not clear how T-cells receive co-stimulatory signals upon ligation of OX40 on T-cells with OX40L expressed on endothelial cells. On the other hand, 4-1BB–4-1BBL interaction is known to generate bidirectional signals in both interacting partners (Vinay & Kwon, 1998): in this case, T-cells and endothelial cells. The expression of 4-1BB on LECs, BECs and HUVECs was low or undetectable, and IFN-γ had little effect on its expression (Fig. 3). Of note, KSHV infection induced the expression of 4-1BBL as well as OX40L on the surface of LECs, but not on BECs and HUVECs, and activation of signalling cascades with recombinant 4-1BB and OX40 inhibited productive KSHV lytic replication (Fig. 4a, b) in a dose-dependent manner (Fig. 4c, d). Virus-induced expression of TNFSF co-stimulatory molecules is not unprecedented: coxsackievirus B3 (CB3) infection induces the expression of CD30L, CD70, OX40L and 4-1BBL (Seko et al., 2001), thus playing a role in the pathogenesis of viral myocarditis. However, unlike CB3 infection, the expression of CD70 and CD30L remained unchanged upon KSHV infection.

**Fig. 4.** OX40 and 4-1BB inhibit KSHV lytic replication in LECs but not in BECs. (a, b) LECs (a) or BECs (b) were infected with rKSHV.219 (m.o.i. = 3). After extensive washing, recombinant OX40, 4-1BB or CD27 protein (10 μg ml⁻¹) was added to the culture at d0 and d2. Cells were cultured for 3 days before infectious titres in the culture supernatant were determined. (c, d) LECs cells were prepared as in (a), and serially diluted recombinant OX40 (c) or 4-1BB (d) proteins were added to the culture as indicated to examine dose-dependent responses. The infectious titre of each sample was normalized to that of the medium alone control, denoted as ‘None’. The mean ± SEM of three independent experiments is plotted. *P<0.05; **P<0.01 by Student’s t-test.

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infection in LECs, suggesting the existence of virus-specific mechanisms.

It is tempting to speculate why infected endothelial cells are mostly latent in vivo while in vitro infection of LECs, the most physiologically relevant cell type for KS, is highly lytic. It is possible that in vivo KSHV infection of endothelial cells induces the expression of OX40L and 4-1BB and that their subsequent ligation with OX40 and 4-1BB molecules, respectively, on the surface of infiltrating T-cells may render infected endothelial and B-cells latent. This hypothesis is at least partly supported by a recent study showing that spindle cells in KS lesions, the tumour element of KS, express high levels of OX40L on their surface (Byun et al., 2013). If OX40 or 4-1BB-mediated inhibition of viral replication actually takes place in KS lesions, it may serve as an immune evasion mechanism as fully lytic replication produces an array of viral antigens that activate immune cells, eventually leading to immune clearance of viruses. Therefore, although it may at first seem counterintuitive for KSHV to induce expression of OX40L and 4-1BB in infected endothelial cells that inhibits KSHV lytic replication, TNFSF-mediated inhibition of productive viral replication may in fact be advantageous for viral persistence through evasion of full-blown immune attacks against the myriad of antigens that are expressed during productive replication.

Taken together, the results of the present study demonstrate that selected TNFSF–TNFRSF interactions (e.g. OX40–OX40L and/or 4-1BB–4-1BBL) between activated T-cells and the two main in vivo KSHV targets (B-cells and endothelial cells) have a profound inhibitory effect on KSHV lytic replication in vitro. These data may provide an intriguing rationale for activating OX40–OX40L and/or 4-1BB–4-1BBL signalling pathways in KSHV-induced tumours when designing antiviral agents for diseases such as KS and MCD where lytic replication plays a critical role in pathogenesis.

METHODS

Cells and reagents. Primary human LECs, BECs and HUVECs were purchased from Lonza. LECs and BECs were cultured in EBM-2 medium supplemented with the EGM-2MV BulletKit while HUVECs were cultured in medium supplemented with EGM BulletKit. HLACs were prepared and used in this study as described previously (Eckstein et al., 2001; Myoung & Ganem, 2011a, d). Neutralizing or activating antibodies to the following TNFSF or TNFRSF members were purchased from various vendors: human OX40 ligand (goat IgG), human OX40 ligand (mouse IgG1), human CD27 (mouse IgG1), human CD30 ligand (mouse IgG2B) from R&D Systems; LEAF human CD137 (4-1BB, mouse IgG1), LEAF human TL1A (mouse IgG1), LEAF human CD134 (mouse IgG1), LEAF mouse isotype IgG1 and IgG2b and normal goat IgG control from BioLegend; preservative-free CD70 (mouse IgG1) and CD30 (IgG2b) from Ancell. FACS antibodies to TNFRSF members or TNFRSF proteins were purchased from various manufacturers: recombinant human 4-1BB, recombinant human 4-1BBL and recombinant human OX40 Ligand from PeproTech; human CD134 (OX40)–muIg fusion protein, human CD137 (4-1BB)–huIg fusion protein, human CD27–muIg fusion protein, human CD137L–muCD8/biotin fusion protein and human CD252 (CD134L)–muCD8/biotin from Ancell; CD134 (OX40) Fc chimera protein from Abcam.

Purification of T- or B-cells using magnetic beads. Untouched T- or B-cells were purified by an indirect magnetic labelling system using the Pan T Cell Isolation Kit II (human) or B-cell isolation Kit II (human), both of which were purchased from Miltenyi Biotec. Non-T or non-B-cells were indirectly magnetically labelled with a cocktail of biotin-conjugated monoclonal antibodies with subsequent addition of anti-biotin microbeads. Labelled non-T- or non-B-cells were depleted by retention on a MACS column whereas the unlabelled T- or B-cells passed through the column. The purity of B-cells was greater than 99 % while that of T-cells was over 95 %, as revealed by staining cells with anti-CD3 and anti-CD19 antibodies. Fractionated or unfractionated tonsillar cells were stimulated with phytohaemagglutinin (PHA) at 1 µg ml⁻¹ for 12 h before being subjected to flow cytometric analysis of OX40 and 4-1BB expression.

Virus preparation, infection and titration. Recombinant KSHV.219 (rKSHV.219) viruses (Vieira et al., 1997) were prepared from induced iSLK.219 with doxycycline and titrated on QBI293A cells (Q-Biogene) as described previously (Myoung & Ganem, 2011b, c). Briefly, induced culture medium was collected and cleared by low-speed centrifugation before pelleting. Virus pellets were resuspended in an appropriate cell culture medium. Infectious units (IU) of virus stocks and experimental samples were determined on QBI293A cells as described previously (Myoung & Ganem, 2011b, c). Virus stocks were diluted and mixed with tonsillar B-cells for infection at an m.o.i. of 3 for 6 h before extensive washes. Infected tonsillar B-cells were co-cultured with uninfected T- or B-cells in the presence/absence of neutralizing antibodies. Primary human endothelial cells were infected with diluted rKSHV.219 stocks by spinoculation (Yoo et al., 2008) at an m.o.i. of 3 for 90 min with subsequent washes. Infected primary human endothelial cells were cultured for 3 days and IU in the culture supernatant were determined using QBI293A cells.

Flow cytometry. Cells were subjected to flow cytometric analysis with LSRII (BD Biosciences) and the data were analysed by FlowJo software. Cells were stained with specific antibodies for 60 min at 4 °C. Primary tonsillar cells were washed and stained with various antibodies. Primary endothelial cells were trypsinized before staining with specific antibodies to TNFSF or TNFRSF members and stained cells were extensively washed before being subjected to flow cytometry.

Statistics. Data are shown as the mean ± SEM of triplicated samples. The significance of differences in the mean values was evaluated by two-tailed Student’s t-test. P < 0.05 was considered statistically significant.

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