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ALTERED HOMEOSTASIS OF PERIPHERAL BLOOD B CELLS IN PATIENTS WITH CHRONIC HUMAN HERPESVIRUS-8 INFECTION AND KAPOSI'S SARCOMA: IMPLICATION FOR INFLUENZA VACCINATION

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TESI DI DOTTORATO DI RICERCA

Altered homeostasis of peripheral blood b cells in patients with chronic human herpesvirus-8 infection and kaposi's sarcoma: implication for influenza vaccination

Settore MED/04 - Patologia Generale

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ABSTRACT

Background: Human herpesvirus-8 (HHV-8) is the etiological agent of classic Kaposi's sarcoma (cKS), a lymphoangioproliferative disease found mainly in older men of Eastern European and Mediterranean origin. Due to the dual role of B cells in HHV-8 infection, virus reservoir as well as agents of humoral immune control, we analyzed the subset distribution and the functional state of peripheral blood B cells in HHV-8- infected individuals with and without cKS. Furthermore, in order to investigate whether the chronic HHV-8 infection in B cells could alter the functionality of these cells and particularly may impact on humoral responses to antigenic stimulation, we investigated cKS patients' response to influenza vaccination, in terms of clinical efficacy, antibody production and safety.

Methodology: Circulating B cells and their subsets were analyzed by 6-color flow cytometry in the following groups: 1- patients HHV-8 positive with classic KS (cKS); 2- subjects HHV-8 positive and cKS negative (HSP); 3- healthy controls, HHV-8 negative and cKS negative. Adjuvated trivalent influenza vaccine was administered to cKS patients and age- and sex-matched healthy controls. Influenza symptoms and side effects were recorded by daily diary cards supplied to the patients. Blood analysis and measurement of serum antibodies against vaccine antigens (H1N1, H3N2 and B) were performed before, 1 and 3 months post vaccination.

Principal Finding: The number of B cells belonging to the preimmune/natural effector compartment, including transitional, pre-naı¨ve, naı¨ve and MZ-like subsets, was significantly higher among HHV-8 positive subjects, with or without cKS, while was comparable to healthy controls in the antigen experienced T-cell dependent compartment. The increased number of preimmune/natural effector B cells was associated with increased resistance to spontaneous apoptosis, while did not correlate with HHV-8 viral load. The clinical efficacy of vaccination was similar in cKS patients and controls. Seroconversion and seroprotection occurred equally in both groups. A mild increase in HHV-8 viremia was observed in a proportion of cKS patients after vaccination, without concomitant worsening of cKS lesions. The safety of vaccination did not differ between cKS patients and controls. The frequency of B cell subpopulations was evaluated and did not change after vaccination both in cKS patients and in healthy controls.

Conclusions: For the first time to our knowledge, in this study we report that HHV-8 chronic infection promotes a perturbation of peripheral B cell homeostasis characterized by expansion of B cells of the preimmune/natural effector compartment, in patients with or without cKS. The alterations observed in cKS patients did not lead to an altered response to influenza vaccination that resulted safe and immunogenic in cKS patients as well as in age- and sex-matched controls. This observation may broaden our understanding of the complex interplay between viral and immune factors leading HHV-8-infected individuals to develop HHV-8-associated malignancies. Furthermore, our results have clinical importance because annual influenza vaccination may be particularly recommended for cKS patients considering their advanced age and comorbidity.

SOMMARIO

Introduzione: L' herpesvirus-8 umano (HHV-8) è l'agente eziologico della variante classica del Sarcoma di Kaposi (cKS), una patologia angioproliferativa diffusa principalmente tra gli uomini anziani dell'Est Europa o di origine mediterranea. Nell'infezione caustata da HHV-8 le cellule B hanno un duplice ruolo: reservoir virale e controllo della risposta umorale. Pertanto abbiamo caratterizzato le sottopopolazioni e la funzione delle cellule B di sangue periferico in individui infetti da HHV-8 con o senza cKS. Per determinare se l'infezione cronica di HHV-8 nelle cellule B fosse in grado di alterare la funzionalità di queste cellule e in particolare potesse avere conseguenze nella risposta umorale, abbiamo inoltre analizzato la risposta alla vaccinazione anti-influenzale in pazienti con cKS, in termini di efficacia clinica, risposta anticorpale e sicurezza.

Metodi: Le cellule B circolanti e le loro sottopopolazioni sono state analizzate con metodo citofluorimetrico a 6 colori nei seguenti gruppi: 1- pazienti HHV-8 positivi con cKS; 2- soggetti HHV-8 positivi senza cKS; 3- controlli sani HHV-8 negativi senza cKS. I pazienti con cKS e controlli sani, simili per età e sesso, hanno ricevuto il vaccino anti-influenzale trivalente adiuvato. I sintomi influenzali e gli effetti collaterali sono stati registrati in un diario giornaliero fornito ai pazienti. Le analisi del sangue e la misurazione degli anticorpi sierici contro gli antigeni del vaccino (H1N1, H3N2 e B) sono stati eseguiti prima, uno e tre mesi dopo la vaccinazione.

Risultati: Il numero assoluto delle cellule B appartenente al compartimento preimmune/effettore naturale, che include le sottopopolazioni transitional, pre-naı¨ve, naı¨ve e MZ-like, è significativamente più alto nei soggetti HHV-8 positivi, con o senza cKS, mentre è paragonabile ai soggetti di controllo nel compartimento della memoria. Abbiamo osservato che l'aumento del numero delle cellule B del compartimento preimmune/effettore naturale è associato ad un'aumentata resistenza all'apoptosi spontanea, mentre non è correlato alla carica virale di HHV-8. L'efficacia clinica della vaccinazione, la sieroconversione e la sieroprotezione non differiscono tra pazienti e controlli. E' stato osservato un lieve aumento della viremia di HHV-8 in alcuni pazienti con cKS in seguito alla vaccinazione senza tuttavia un concomitante peggioramento delle lesioni. La sicurezza della vaccinazione è stata dimostrata sia nei pazienti con cKS sia nei soggetti di controllo e infine non sono state osservate variazioni nella frequenza delle sottopopolazioni delle cellule B in seguito alla vaccinazione.

Conclusioni: In questo studio abbiamo dimostrato che l'infezione cronica di HHV-8 promuove alterazioni nell'omeostasi delle cellule B periferiche, caratterizzate da un'espansione delle cellule B del compartimento preimmune/effettore naturale nei soggetti HHV-8 positivi con e senza cKS. Tali alterazioni non influiscono sulla risposta immunitaria alla vaccinazione, che risulta sicura e immunogenica sia nei pazienti con cKS sia nei controlli. I nostri risultati contribuiscono a chiarire il complesso rapporto tra fattori virali e risposta immune che conduce allo sviluppo di patologie associate ad HHV-8. Inoltre è importante sottolineare la rilevanza clinica dei nostri risultati dal momento che la vaccinazione influenzale è raccomandata ai pazienti con cKS a causa della loro età in cui la comorbidità è elevata.

Indice generale

1. Introduction

1.1 KAPOSI'S SARCOMA

Kaposi's sarcoma, first described by Moritz Kaposi in 1872 as "idiopathic multiple pigmented sarcomas of the skin" [1], is an angioproliferative tumour-like lesion usually developing in the skin and eventually disseminating to multiple cutaneous sites, viscera and lymph nodes [2]. Before 1980 KS was primarily known as a disease of older males of Eastern European, Mediterranean, or Jewish background. In the early 1980s, KS was found in approximately 40% of American men with AIDS [3]. Cross-sectional analysis of the population with AIDS KS revealed that the affected population was primarily homosexual men. This phenomenon attracted much publicity to AIDS KS, and hence, the other variants of KS: classic, endemic, and transplant-associated KS. Although phenotypic features are relatively consistent within the 4 subtypes, the clinical course varies [3]. The epidemiology of KS in the AIDS population strongly suggested an infectious agent. In 1994, Chang *et al.*,[4] using representational difference analysis, a novel technique used to determine subtle differences in DNA sequences between two genomes, discovered an uncanny association between a previously unknown virus and KS. This includes all subtypes of KS. This virus was named Kaposi's sarcoma–associated herpesvirus (KSHV), and it was subsequently designated the eighth human herpesvirus, HHV-8. Since then, HHV8 has also been definitively linked with primary effusion lymphoma (PEL) and multicentric Castleman's disease (MCD). Several studies demonstrate that KS is a tumour of multifactorial origin in witch viral oncogenesis by HHV8 and cytokine induced growth together with some state of immunocompromise represent important conditions for developing this rare tumour [5].

1.1.1 Epidemiology and environmental predispositions

Cross-sectional epidemiological studies have determined that HHV8 seropositivity in various populations is strongly correlated with the population's risk of KS,[6,7] and several longitudinal studies have shown that HHV8 infection precedes the onset of KS [8,9]. Ninety to one hundred percent of patients with KS have high antibody titers to HHV8, independent of ethnicity and geographic location [10]. The seroprevalence varies widely in general populations from different geographic areas. The highest seroprevalence of HHV8 is seen in African countries. It is near 100% in the Ivory Coast; 89% in Gambia; 40% in sub-Saharan Africa; 10% in Mediterranean countries; 2% to 4% in Northern Europe, Southeast Asia, and Caribbean countries; and from 5% to 25% (most reports are closer to 5%) in the heterogeneous United States [11,12]. The incidence of KS is highest in those areas with the highest seroprevalence of HHV8 with some exceptions. Although in sub-Saharan Africa the 40% seroprevalence correlates well with the high incidence of endemic KS, this is not true for the Ivory Coast or Gambia [13]. Although the seroprevalence in these countries is extremely high, the incidence of KS is minimal, and endemic KS is not known in these regions. Within the United States, KS is most commonly seen in 5 homosexual men with AIDS [14]. At one time, 40% of this population was reported to have KS. This is a 20-fold increase over non homosexual patients with AIDS,implicating a sexual mode of transmission [14]. In fact, epidemiologic studies have consistently identified the following risk factors for an increased incidence of KS: increased number of sexual partners, HIV-1

seropositivity, history of hepatitis A, a history of other sexually transmitted diseases such as HSV-2, and receptive anal intercourse. Similarly, the risk of seroconversion to HHV8 has been shown to increase with duration of homosexual activity, oral genital contact, insertive and receptive anal intercourse, and insertive and receptive analingus. [15-17]. Among heterosexuals, a predominant mode of HHV8 transmission is less evident. Several studies have shown that women with HIV-1 infection have higher seroprevalences of HHV8 than HIV-1–negative women and that women engaged in prostitution have higher HHV8 seroprevalences [20,21]. However, data from areas where HHV8 is endemic support a nonsexual horizontal mode of transmission.

1.1.2 Clinical manifestation

1.1.2.1 Classic KS

Classic KS (cKS) occurs as a rare and indolent form in elderly Mediterranean men,with particularly high incidence in Italy, Greece, Turkey, and Israel [22, 23].
Historically, KS incidence in the Mediterranean is up to 10-fold higher than in the rest of Europe and the United States [24,25,26]. Classic KS lesions tend to remain confined to the lower extremities and preferentially afflict men rather than women, at approximately a 15:1 ratio. Those affected often live with the disease for 10 years or more and are usually not killed by it [27]. In Italy, seroprevalence and incidence increase with age, particularly in males who have reached their 50th birthdays [28]. Unlike AIDS-associated KS, HIV-1 coinfection is not typical, but the observation that KS develops annually in only 0.03% of HHV8-infected men older than 50 years in the Mediterranean strongly suggests the existence of a cofactor(s) [29]. Extensive epidemiologic studies in Italy demonstrate a strong influence of ethnogeography on HHV8 seropositivity and KS incidence, with a marked gradient increasing from the north to the south. The lowest incidences were in northern Italy (7.3% HHV8 positive, 0.605 case of KS/100,000 population/year), the highest incidences were in the south (24.6% and 1.495/100,000), and a moderate number were in central Italy (9.5% and 0.5/100,000) [12]. Two particular southern Italian hot spots for KS are on the islands of Sardinia and Sicily: for example, a high rate of KS in northern Sardinia is associated with a general seroprevalence of 35%, with a range of 15.3 to 46.3% in five areas [28]. However, hot spots also occur in lowincidence regions: in the Po Valley in northern (mainland) Italy, the incidence is 2.5/100,000 men and 0.7/100,000 women, with one rural zone having double the male rate and four times the female rate [30]. Elsewhere in Mediterranean Europe, a group of blood donors in Spain had a low HHV8 seropositivity similar to that of northern Italians [30].
The indolent nature of classic KS has made the determination of its prognostic 6 factors challenging, but the geographic gradient of seropositivity would appear to make Italy an ideal setting to identify environmental risk factors. In northern Sardinia, the occupation of cereal farming enhances KS risk [31], lending support to Ziegler's hypothesis that chronic exposure to the aluminosilicate-rich volcanic soils specific to southern Italy may contribute to localized immune suppression in the extremities and increased KS risk [32]. However, other studies have failed to confirm the connection between type of soil and KS incidence in northeast Sardinia [33]. In the Po Valley, residence in areas where malaria was formerly endemic corresponded to regions of high HHV8 seroprevalence [34]. Coluzzi *et al*. make a

provocative argument that many of the risk factors identified for classic KS might be attributable to high exposure of those populations to blood-sucking arthropods [35]. The authors hypothesize that local immunosuppression engendered by injection of insect saliva following a bite would prime the tissue microenvironment to enhance (i) transmission of HHV8 (from an infected adult's saliva applied as a “salve” to a bitten child) or (ii) reactivation of HHV8 in an elderly person, whose immune system is already declining. Although this evidence is largely anecdotal, the authors propose prospective studies to test their hypotheses and have published a recent work provided evidence of an association between the density of five mosquito species (Aedes vexans and Aedes caspius, Culex modestus Ficalbi, Culiseta annulata, and Coquillettidia richiardii) and KS [36]. Two other studies explored the risk associated with age and immune function. Brenner *et al*. [37] retrospectively analyzed all of the classic KS patients (a total of 248) treated in two hospitals in northern Israel and Tel Aviv between 1960 and 1995. They showed that increasing age at KS diagnosis was prognostic of increasing disease progression and that immunosuppression (from steroid treatment or renal failure associated with organ transplantation) predicted dissemination. A second study found that KS risk was associated with both mild immunosuppression (lower lymphocyte and CD4+ cell counts) as well as immune activation (increased serum neopterin and β2-microglobulin levels) [38]. Behaviors that influence KS risk have been examined by Goedert *et al*., who demonstrated that increased cigarette smoking (measured both in packs per day and in “pack-years”) reduces the risk of classic KS fourfold [39]. This study used the powerful comparison of patients with both histologically confirmed KS and serologically confirmed HHV8 infection to age- and sex-matched controls who were HHV8 positive without KS. Topical corticosteroid use, infrequent bathing, and asthma independently increased risk for KS in this study [39]. Moreover, recently same Authors have evaluated the virologic, haematologic, and immunologic markers of cKS risk and disease burden, compared with HHV8-seropositive controls without HIV-infection founding thatperipheral blood mononuclear cells (PBMC) HHV8 DNA detection and high HH V8 lytic and latent antibody titers were positively associated with cKS risk. Moreover, independent of PBMC HHV8 DNA, cKS risk was found to be positively associated with reduced hematocrit, hemoglobin, total lymphocytes, including CD4 positive cells and CD8+ cells, and with increased monocytes [40].
Transmission of HHV8 in classic KS probably occurs by both sexual and nonsexual routes. Both the close family members and heterosexual partners of KS patients show increased HHV8 seroprevalence [41,42], while a group of 51 Catholic nuns in Italy had a seroprevalence indistinguishable from that of matched female controls 7 [43]. Similar to AIDS-associated KS patients, the high frequency of detection of HHV8 DNA in tonsillar swabs from HIV-negative KS patients in Italy suggests that transmission in saliva is a predominant route [44].

1.1.2.2 AIDS-Associated KS

KS is the most common neoplasm in homosexual and bisexual men with AIDS [45]; the disease is extremely aggressive in this population and displays a more frequent mucosal progression than in the other epidemiologic forms [23]. It commonly presents multifocally and frequently on the upper body, head, and neck [27], and it evolves quickly, both in local progression of lesions to tumors and in visceral dissemination leading to organ dysfunction and high mortality. A direct

correlation between HIV-1 infection, AIDS pathogenesis, and KS progression has been demonstrated in numerous studies [46-48]. Many conflicting studies have demonstrated, however, that the relative contributions of HIV-1 burden and immunosuppression to KS development are not always directly proportional [49]. Likewise, specific T-cell responses to purified HHV8 virions were reduced in HIV-1 positive men but were proportional to KS risk in HIV-1-negative men; however, both were independent of CD4+ counts [50]. Finally, Veugelers, *et al*. found that age at the time of HHV8 seroconversion may carry more risk than absolute CD4+ numbers: older HIV-1-positive men have higher CD4+ numbers at the time of seroconversion than do younger men [51]. Furthermore, in the Amsterdam Cohort, decreasing CD4+ numbers had no influence on the rate of HHV8 seroconversion but, instead, correlated with increased HHV8 replication; this was suggested by an increase of antibody titers to a viral structural protein but no change in antibody titers to a viral latent protein as CD4+ levels decreased [52]. This study also demonstrated that HHV8 seroconversion following HIV-1 seroconversion carries a higher risk for KS development than does HHV8 seroconversion prior to HIV-1 seroconversion [52].

Sexual transmission of HHV8 in the HIV-infected population is supported by numerous data, and sexual behavior is the most significant epidemiologic variable in AIDS-associated KS [46]. The risk of developing KS is >10,000 times higher in HIV-infected homosexuals than in the general population [45]. Conversely, hemophiliacs and injection drug users with AIDS have historically had a markedly reduced HHV8 prevalence and KS risk [53]. Furthermore, the disease is 300 times more frequent in homosexual and bisexual men than in other immunosuppressed individuals [53] and women with AIDS have a fourfold-increased risk of KS if their partners are bisexual rather than addicted intravenous drug users [53].

Recent studies have suggested that orogenital rather than anogenital sex may be the most significant behavioral risk factor for HHV8 infection. Although receptive anal intercourse was a HHV8 risk factor among homosexual men in the Amsterdam Cohort Study [49], participation in orogenital sex was a predictor for HHV8 seroconversion [15]. In agreement, many studies have found HHV8 in the saliva of seropositive patients [55-57], and HHV8 has been detected in prostate tissue and the male urogenital tract [57-60]; detection of HHV8 in the ejaculate has been reported but remains controversial [56,59, 61-63]. Taken together, these studies affirm a route of sexual transmission for HHV8 that differs significantly from that for 8 HIV-1. The increasing and successful use of highly active antiretroviral therapy (HAART) has afforded important clinico-epidemiological insights into the reversal of AIDS and the consequent fate of HHV8 pathogenesis. Widespread HAART has resulted in a yearly declining trend in AIDS-related deaths [64]. There is clear evidence that immune restoration due to HAART is one key to improving the prognosis of AIDS-KS patients [65]. HAART may reverse KS progression not only by inhibiting HIV-1 replication but also by exerting direct effects on KS tumors. Considering only current therapies, the fate of AIDS-associated KS in dually infected patients will arguably depend on the continued success of HAART treatment.

1.1.2.3 Endemic (African) KS

Prior to the emergence of HIV, African KS was an endemic disease that affected mainly two age groups: young men with an average age of 35 years and children

with an average age of 3 years [66]. In Cameroon from 1986 to 1993, the latter form accounted for about 4% of childhood cancers [67]. The highest prevalence of African KS prior to AIDS was found in a broad band crossing equatorial Africa, with particularly high rates in northeastern Zaire and western Uganda and Tanzania. This geographic pattern conformed to areas of frequent podoconiosis, a lymphatic disease of the legs presenting as lymphedema etiologically associated with chronic, barefoot exposure to volcanic soils [68]. In podoconiosis, microparticles of silica dust in these soils penetrate the skin of the foot during barefoot walking and are then taken up by the lymphatics and cause localized inflammation and lymphedema. Aluminosilicates are cytotoxic to macrophages in animal models [69] and may contribute to localized immune suppression; this had been proposed as an environmental contributor to African KS [68]. However, KS on that continent has evolved to an epidemic magnitude as the spread of the AIDS pandemic has exacerbated the already elevated prevalence of endemic KS. Current clinicoepidemiologic studies of African KS as an independent disease entity are thus virtually impossible in most contemporary African populations. Clinically, KS in Africa is more frequent in children [32, 67, 70] and females than anywhere else worldwide and occurs in four forms [27]. One form is similar to classic KS in its course but strikes young adults. The other three forms are more aggressive andare similar to AIDS-associated KS in their progression: one of these, however, remains cutaneous with local tissue invasion, while another occurs most often in young children with a mean age of 3 years, is aggressive with visceral progression, but often lacks the cutaneous involvement [27]. A comparison of the prevalence of KS prior to AIDS with its prevalence during the AIDS pandemic in Africa clearly demonstrates the brutal potentiation of KS by HIV-1 coinfection [66]. KS is also the leading cancer in Zimbabwe, comprising almost 33% of cancers there [71], andaccounts for 15% of male cancers in Zaire [72]. Since the emergence of AIDS, the incidence of KS in children has increased 40-fold in Uganda [32], while in South African women it has increased at a higher rate than in men: the male-to-female ratio has decreased from 7:1 in 1988 to 2:1 in 1996 [73]. Overall, while the prevalence of KS within the HIV-positive community has skyrocketed, even the prevalence of KS in the HIV-negative, “contemporary”-endemic population has 9 risen. Despite clinical heterogeneity, all forms of KS in Africa are associated with HHV8 infection [11,74-77]. HHV8 seropositivity in Africa is quite heterogeneous geographically, ranging from 36 to 100% in different countries and populations in sub-Saharan Africa [10]. Similar to the situation for U.S. populations, numerous studies support the inference that HHV8 was highly prevalent preceding the emergence of HIV-1; nonetheless, these studies affirm the critical role of HIV-1 as a cofactor in African KS progression.

The endemic pattern of KS in Africa, especially the high prevalence in childhood, reflects the occurrence of primary HHV8 infection before puberty (including during infancy). In fact, in many African populations, seroprevalence reaches adult levels during adolescence. This early appearance of HHV8 in children in Africa suggests that transmission is primarily nonsexual, possibly occurring from mother to fetus [78], with clear evidence of intrafamilial and horizontal spread. Indeed, a significant risk factor for seroconversion of a child is having a HHV8-infected mother: 42% of children born to seropositive mothers were also seropositive when tested between 0 and 14 years of age, while only 1% of those born to seronegative mothers were seropositive [79]. In this study, the potential for vertical transmission of HHV8 was

suggested, since one of four infants younger than 18 months born to an infected mother was also seropositive. However, many studies argue that a majority of seropositive infants acquire maternal immunoglobulins passively from their mothers, with vertical transmission of virus remaining possible but rare. For example, 83% of Zambian infants were HHV8 seropositive if born to a mother who was also seropositive; however, only 3% of the infants had detectable HHV8 DNA in their PBMCs [80]. Frequent studies have demonstrated a loss of HHV8 seropositivity in children after infancy [81-83]. Childhood transmission in Africans therefore seems to follow mostly horizontal patterns, with a few vertical instances.

1.1.2.4 Iatrogenic KS

Iatrogenic KS represents an additional clinicoepidemiologic peculiarity of HHV8 infection that presents either chronically or with rapid progression [27]. The induction of iatrogenic KS by immunosuppressive therapy and its subsequent regression on removal of immunosuppression provided some of the earliest clinical recognition of the reversibility of KS [27]. Iatrogenic KS shows extreme ethnogeographic associations, occurring in only about 0.4% of transplant patients in the United States and Western Europe [84,85], but in about 4.0 to 5.3% of renal transplant patients in Saudi Arabia [86,87]. Strikingly, KS represents 87.5% of posttransplantation neoplasia in Saudi Arabia [88], and a recent study found KS in 80% of posttransplantation cancers in Turkey. In the latter study, KS developed within 1 year in 46% of those cases. The high frequency of iatrogenic KS in Saudi Arabia reflects the 7% endemic seroprevalence of HHV8 in healthy Saudi donorsor patients with non-KS malignancies [88]. Interestingly, transplant-associated KS is seen predominantly in kidney allograft recipients and not other solid-organ or bone marrow transplant recipients [22]; kidney recipients have a greater than two fold-higher seroprevalence than those at low risk of KS in France [89]. Although Andreoni *et al*. found that 21.4% of liver recipients, but only 8.6% of kidney recipients, seroconverted for HHV8, the risk of KS was higher in the kidney 10 transplant recipients. In fact, 75% of those who progressed to KS were HHV8 seropositive prior to transplantation [90].

Differences in immunosuppressive therapy might favor HHV8 reactivation in the kidney recipients. KS has historically been seen more often in patients whose treatment includes cyclosporine [91], a drug that can reactivate HHV8 from latencyto lytic replication in tissue culture [61]. Likewise, remission of iatrogenic KS on cessation of immunosuppression is the norm. A recent study in Turkey demonstrated that 8 of 11 iatrogenic KS patients showed complete regression of visceral and cutaneous KS within 6 months of cessation of cyclosporine [92]. A recent case study showed regression of cutaneous and mucosal KS after cessation of immunosuppressive therapy in a kidney allograft recipient; however, HHV8 DNA persisted in the regressed lesions [93]. In recent years, sirolimus has been demonstrated as possessing antineoplastic and immunosuppressive properties. These effects of sirolimus are due to a common mechanism. Sirolimus inhibits mTOR, which links mitogen-induced stimulation of protein synthesis and cell-cycle progression [94]. Since 2004, more than 25 recipients with post-transplantation KS were treated successfully with sirolimus associated with calcineurin inhibitors (Cyclosporine A) (CNI) withdrawal [94,95]. The CNI/sirolimus switch,performed immediately after KS diagnosis, is now considered as the first-line treatment of KS for transplant recipients [94,95]. Many data support the inference that most

iatrogenic KS patients are HHV8 positive prior to transplantation, suggesting that reactivation of latent viral infection leads to disease [96]. Although less frequent, seroconversion following transplantation suggests that seronegative recipients can be infected by HHV8 from the donated organ [96]. Barrozzi *et al*. studied whether HHV8 and the elusive KS progenitor cells could be transmitted from the donor through the grafts and showed that the HHV8-infected neoplastic cells in posttransplant
KS harbored either genetic or antigenic markers of their matched donors. These data suggest the use of donor-derived HHV8-specific T cells for the control of post-transplant KS [97].

1.1.2.5 Mucocutaneous involvement

Initial clinical presentations at mucocutaneous surfaces begin typically as macular and papular lesions that progress to plaque-like or nodular tumours (Fig. 1). Lesions vary in size and shape and are generally non-pruritic and painless. The colours of the lesions range from pink to deep purple and appear brownish in classic KS. While lesions can be found on any body surface they seem to have a predilection for the upper body, head, and neck areas in AIDS-KS. Classic and iatrogenic KS most often have a preference for the lower extremities. Macular lesions are virtually always symptomless and the correct diagnosis is frequently missed. Papular and nodular lesions are mostly 1–2 cm in diameter and follow the skin tension lines. Lymphatic involvement can give rise to cutaneous and subcutaneous complications such as pain, oedema, maceration, and ulceration. KS is often accompanied or preceded by local lymphoedema. The coexistence of the two events (lymph stasis and vascular neoformation) is probably meaningful in these patients [32,98,99]. The observed acral hyperkeratosis may result from proliferation of keratinocytes by keratinocyte growth factor (KGF) secreted from 11
activated fibroblasts [98]. Other variants of KS include: telangiectatic KS (translucent nodules with prominent telangiectasia), ecchymotic KS that appears as periorbital ecchymoses with a large number of extravasated red blood cells, keloidal KS (brown-violaceous nodules with a keloidal component), cavernous KS as a rare type of locally aggressive KS that histologically resembles cavernous haemangiomas, and lymphangioma-like KS that is a rare variant in which dilated vascular spaces produce a bullous-appearing eruption, typically on the lower legs. The lesions are easily compressible and appear clinically to be fluid-filled. Visible lesions carry a social stigmatisation. Dermal and lymphatic infiltration with KS may result in debilitating and cosmetically unacceptable oedema in the periorbital areas, genitals, or extremities causing difficulty in walking. Oral lesions are usually symptomless, but can occasionally produce obstructive symptoms, pain and problems with swallowing [27].

1.1.2.6 Visceral involvement

Visceral involvement is common in AIDS-KS but is often symptomless. Post-mortem studies suggest that more than 25% of patients with AIDS-KS also have visceral lesions. They most commonly involve the stomach, bowel, liver, spleen, and lungs. Gastric outlet obstruction, enteropathy with small bowel involvement and occasionally bleeding of ulcerated KS lesions have been reported. Gastrointestinal KS has a typical red, raised appearance and is difficult to diagnose by biopsy because of the submucosal location of many lesions. Pulmonary KS is

severely debilitating and rapidly fatal if left untreated [47]. Presenting symptoms include dyspnoea without fever and occasionally haemoptysis. Chest radiograms typically demonstrate diffuse, reticular- nodular infiltrates, mediastinal enlargement, and sometimes pleural effusions [100]. Once the diagnosis of KS is clinically suspected, it is confirmed by biopsy and histological examination. Although most KS lesions are readily recognised, early lesions may be difficult to diagnose and other diseases (eg, bacillary angiomatosis or cat-scratch fever) may mimic KS.

1.1.3 Histopathology

Spindle cells represent the main cell type of the advanced final nodular stage of KS lesions. Despite some clinical and epidemiological differences, the four KS display very similar histopathological features, with the proliferation of spindle cells (considered as the KS tumor cells) associated with inflammation and neo-angiogenesis [101].
Multicentric neoformation of atypical lymphatics represents the primary process in histogenesis of KS. Important cell interactions are presumed to occur between fibroblasts, monocytes and endothelial cells. The remarkable symmetry of lesions and the concept of multicentric origin of KS fit well with this hypothesis. Patch-stage KS, the earliest pattern, typically arises in the reticular dermis as a clinically macular lesion. A proliferation of small, irregular and jagged endothelium lined spaces surrounding normal dermal vessels and adnexal structures accompanied by a variable, inflammatory lymphocytic infiltrate (with or without plasma cells) is characteristic [102]. Plaque-stage KS, the histological correlate of small palpable lesions, represents the expansion of a spindle-cell vascular process throughout the entire dermis (Fig. 2), at times extending to the subcutaneous fat. Spindle cells are dispersed throughout dermal collagen bundles forming irregular, cleft-like, angulated vascular channels containing varying numbers of erythrocytes. Haemosiderin deposits and eosinophilic hyaline globules are typically present. A perivascular inflammatory infiltrate is usually found [103]. Histiocytes with the phenotype of either factor XIIIa or S-100 dermal dendritic cells as well as T-cells and B-cells including plasma cells are also present [103,104]. Nodular-stage KS lesions are composed of sheets and fascicles of spindle cells with mild to moderate cytological atypia, single cell necrosis, and trapped erythrocytes within an extensive network of slit-like vascular spaces. Vessels lack a prominent investment of pericytes and have a fragmented basal lamina and frequent discontinuities in the endothelial lining. Erythrophagocytosis and necrosis of individual endothelial cells may also occur [105].

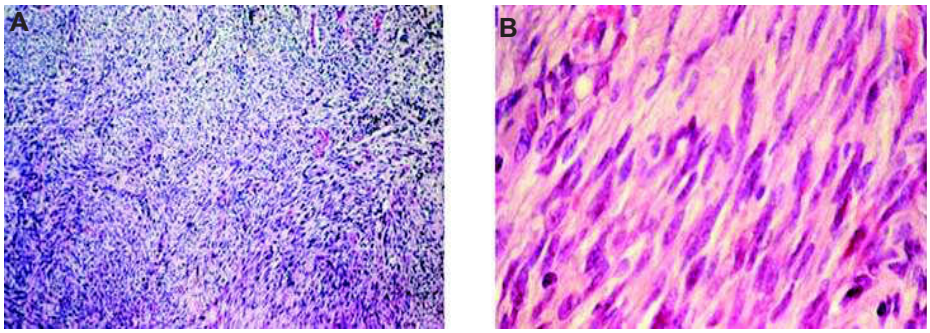


Figure 1: Hystology of cKS lasion. A) Hematoxilin and eosin stained section of dermal KS showing cord of spindle cells and slit-like vascular spaces. B) High magnification view of KS spincle cells showing their drammaticaly elongated morphology [101].

1.1.4 Staging

Staging this multicentric disorder has been a challenge. The value of each scheme is limited. Moreover, KS may sometimes have an acute onset and rapidly progressive course, or undergo sudden worsening that complicates a previously chronic and generally undemanding evolution. Taylor's first classification [106] referred to the classical and endemic forms considered clinical, behaviour and localization criteria common to all variants. The recognition of AIDS-related KS made it necessary to broaden the staging criteria: Kriegel suggested a 4-stage subdivision that included the parameters of visceral sites and systemic involvement [107], whereas the classifications of Mitsuyasu [108] and Chachowa and Krown [109,110] introduced the concept of prognosis but only referred to the AIDS-related variant. There are other staging ideas, including thoughtful ones by Cottoni and Montesu [111] and Schwartz *et al* [112]. However, the staging systems elaborated after the rise of AIDS-related KS are not suitable for cKS, as the former worsens rapidly with early spreading to visceral organs in the context of severe immunodeficiency mostly lacking in cKS. Moreover, the heterogeneity of clinical presentation and the variability of evolution of cKS make difficult the staging of the disease. Brambilla *et al*. [113] have identified additional clinical parameters that have been included in a new staging system which is applicable to the classic forms of KS and may represent a useful tool for therapeutic choices. Table 1 summarises staging system proposed by Authors which includes four clinical stages:

- Stage I (maculo-nodular): small isolated angiomatous maculae and/or nodules, prevalently localised in lower limbs;
- Stage II (infiltrative): prevalent violet-grey plaque lesions involving wide areas of lower limbs, sometimes associated with a few nodules;
- Stage III (florid): exuberant angiomatous plaques and nodules, often ulcerated, involving one or more limbs;
- Stage IV (disseminated): presence of a significant number of angiomatous

plaques and nodules involving other skin districts in addition to the limbs.
Moreover, in Table 1, the "*Evolution*" column refers to the speed of disease progression (A: slow and B: rapid), with rapid being defined as an increase in the total number of nodules/plaques or in the total area of plaques in the three months following an examination. Furthermore, complications which can be present in all the stages are considered: objective ones, such as ulcerations, bleeding, lymphedema, lymphorrhea, and subjective ones, such as pain, functional grip and/or ambulatory impotence. The presence of lesions in visceral sites (the gastro-enteric tract, including the oral cavity, the lymph nodes and the lungs) is also taken into account, although internal localization is a rare event, prevalently confined to the oral cavity and gastric area in stages III and IV. Considering the speed of evolution and the presence of complications may be helpful for prognostic and therapeutic evaluations in the context of a modified disease staging.

Table 1. cKS staging and therapeutic choices

Stage	**Skin lesions**	**Localization**	**Behaviour**	**Evolution**	**Complications***	**Therapy**
I Maculo-nodular (±v)	Nodules and/or macules	Lower limbs	Non aggressive	Slow (A) Rapid (B)	•Lymphedema •Lymphorrea	Elastic stocking; intralesional chemotherapy only for nodule
II Infiltrative (±v)	Plaques	Lower limbs	Locally aggressive	Slow (A) Rapid (B)	•Hemorrhage •Pain	Elastic stocking; intralesional chemotherapy only for nodule
III Florid (±v)	Angiomatous nodules and plaques	Limbs, lower prevalent	Locally aggressive	Slow (A) Rapid (B)	•Functional impairment	Elastic stocking; intralesional and systemic chemotherapy-
IV Disseminated (±v)	Angiomatous nodules and plaque	Limbs, trunk, head	Disseminated aggressive	Rapid (B)	•Ulceration	Systemic and intralesional chemotherapy, elastic stocking

v: visceral involvement (pharyngo-oral cavity, gastroenteric tract, lymph nodes, bone marrow, lungs).
Rapid: increase in total number of nodules/plaques or in total area of plaques in the three months following an examination.
*All of them prevalent in stage III and IV, lymphedema and lymphorrea often observed in stage II, lymphedema and hemorrhage sometimes present in stage I.

Moreover, Authors have found that most of the patients with maculo-nodular (stage I) or infiltrative (stage II) disease have a slow evolution without complications, thus allowing to avoid systemic chemotherapy administration. These patients, who can be adequately monitored by means of examinations every 2-3 months, could be treated with intralesional chemotherapy and/or elastic stockings. However, systemic chemotherapy is used in the case of stage II patients with slowly evolving complicated disease or rapidly evolving disease with or without complications, and in all variants of stage III and IV disease. Brambilla and co-authors employ classic chemotherapy and not interferon immunotherapy because the latter causes clinically significant systemic side-effects in elderly patients. Proposed therapeutic choices based on this classification of cKS patients, are summarised in Table 1.

1.1.5 Pathogenesis of KS

Although necessary for KS development, HHV8 infection is not sufficient to engender KS. From 2%–7% of the U.S. population has serologic evidence of infection [6,74], yet most of these individuals have no discernable KS risk, which clearly indicates that cofactors are required for tumorigenesis. In AIDS-related KS, the cofactor is obviously HIV infection. HIV's cofactor role in KS is strongly supported by (a) the dramatic reduction in KS incidence that has accompanied reduction of HIV viremia via antiretroviral therapy [114,115] and (b) the remission of clinical KS in patients treated with HAART alone [116,117]. Exactly how HIV infection promotes KS development is still a matter of controversy. There is laboratory evidence that in certain settings HIV infection can augment HHV8 replication in both cell-autonomous and paracrine fashions [118-120], but the in vivo relevance of these observations is unclear. (For instance, because dually infected cells are rare in vivo [60], cell-autonomous interactions between these viruses are unlikely to be critical). Ensoli and colleagues have argued for a role for secreted HIV tat protein as a growth factor for KS spindle cells, again on the basis of experiments in cultured cells (reviewed in Reference 121). However, the interpretation of these data is complicated by the fact that cultured spindle cells tend to lose the HHV8 genome (see below) and so cannot be relied upon to model faithfully all aspects of KS biology. Certainly, in the context of the whole organism, HIV infection is associated with cytokine upregulation, and such an environment should be conducive to sustaining spindle-cell survival and growth. Moreover, the T cell depletion and immunodeficiency induced by unchecked HIV replication is certain to be a factor in the progression of KS as it manifestly is in KS linked to iatrogenic immunosuppression. The controversy about HIV's role in KS development is unlikely to be resolved in the near future. However, it is worth pointing out that none of the models just summarized are mutually exclusive.

Instead, the identity of the cofactor(s) in classical KS remains unknown. Accumulating evidences suggests that cKS is a multistep process involving both viral and cellular factors, which include infection of spindle cells by HHV8 and production of several inflammatory cytokines, chemokines and angiogenic factors. From an initial reactive and inflammatory process, some selective pressure and or/genetic alterations may give rise to nodular tumoral KS lesions, consisting of a neoplastic oligoclonal proliferation of spindle cells. There are three extraordinary

and overlapping developments with regards to the pathogenesis of KS: the HHV8 infection, the deregulation of immune system and the role played by the inflammatory microenvironment.

1.1.5.1 HHV8

Concordant with its homology to lymphotropic viruses, the discovery of HHV8-DNA in KS specimens was soon followed by detection of the virus in cultured cells from patients with primary effusion lymphoma (PEL), a rare AIDS-associated B-cell non-Hodgkin's lymphoma. The ability of PEL cells to support continuous HHV8 infection and conditional, productive replication was crucial for the subsequent cloning and nucleotide sequencing of the entire viral genome and for direct visualization of mature herpesvirus-like HHV8 virions by electron microscopy [122]. The complete nucleotide sequence of HHV8 confirmed its classification as a rhadinovirus, or gamma-2-herpesvirus, joining the gamma-1-herpesvirus EBV as the only human gammaherpesviruses. Similar to the HVS and EBV genomes, the HHV8 genome is maintained during latency in PEL cell lines as a circular, multicopy episome and contains multiple GC-rich, 801-bp terminal repeats flanking approximately 145 kb of “unique” sequence [123] (Fig. 3).

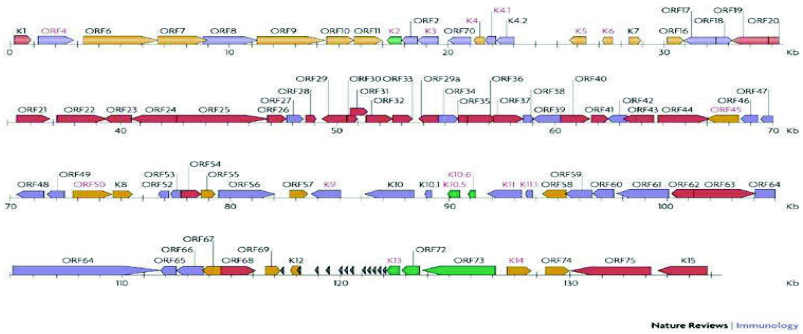


Figure 2: HHV8 genome map. Upon infection, HHV8 genome is delivered to the nucleus where it circularizes. During the latent programme, a small number of proteins (indicated in green) are expressed to maintain the viral episome, to avoid host antiviral immune responses and to provide a growth advantage to infected cells. Latently infected cells can be induced to enter the lytic cycle under specific physiological conditions. Lytic replication leads to extensive viral gene expression. Lytic gene expression is tightly regulated (viral genes transcribed early upon reactivation are indicated in yellow, those transcribed during intermediate kinetics in orange, and those transcribed during late kinetics in red). HHV8-encoded microRNAs are indicated by grey arrowheads [149].

During productive replication, viral progeny DNA is ultimately synthesized as linear, single-unit genomes destined for incorporation into independent virions. Cryoelectron microscopy and digital reconstruction demonstrate that the capsid is icosahedral with a diameter of approximately 1,140 to 1,300 Å [124]. The characteristic herpesvirus tegument and envelope presumably are added to the

capsids during egress. HHV8 contains at least 87 open reading frames (ORFs) [123] (Fig. 3). Comparison of its genome with that of HVS (the prototype gammaherpesvirus) reveals a remarkably similar genetic organization. The two family members share 68 conserved genes that are arranged colinearly, with short, interspersed regions of genes unique to each virus. Each conserved gene has been given the prefix “ORF” and numbered consecutively from left to right along the genome, while the unique genes have been designated K1 to K15 [125]. The 87 HHV8 genes iclude both homologues of cellular proteins (named like the cellular protein with a v- before the name to indicate the viral origin) and unique HHV8 proteins (like Latency-associated nuclear antigen LANA and the Kaposins).

As noted above, HHV8 DNA is present in all KS tumors, irrespective of clinical type. Viral infection selectively targets spindle cells [60,126,127], with little or no infection of other cell types (though monocytes in the lesions may be infected) [128]. Like all herpesviruses, HHV8 can adopt either of two replicative programs, which are known as latency and lytic replication. In latency, viral gene expression is heavily restricted, with only a handful of viral genes being expressed; viral DNA is retained as a circular episome in the nucleus, and no progeny virions are produced. In contrast, lytic replication involves the temporally regulated expression of virtually the entire viral genome, with viral DNA replication and production of infectious progeny, in the course of which the host cell dies. Lytic replication can be induced from the latent state by the induction of theexpression of a single viral gene, which encodes a transcription factor known as RTA (replication and transcription activator) [129,130]. In vitro, this can be achieved by the treatment of latently infected cells with phorbol esters or sodium butyrate or by transfection with constitutively active RTA alleles. The stimuli to HHV8 lytic reactivation in vivo are unknown, but there is evidence that some cytokines can trigger such reactivation in culture [131,132]. The latency program is assigned the primary role in oncogenesis, by both promoting cell growth and impairing apoptosis. The lytic cycle, by contrast, has traditionally been thought not to contribute directly to oncogenesis because cells that enter this program invariably die. Of course, it has long been acknowledged that lytic replication plays an important early role in viral oncogenesis by allowing the virus to spread throughout the host. This exposes many tissues to the virus and allows the establishment of many latent foci, which can then begin their long mutational march to cancer. Most KS spindle cells are latently infected with only a small subpopulation of spindle cells (1%–3%) display lytic replicative markers [60, 126]. This finding accords with conventional notions of the role of latency in tumors. Examination of early (patch) lesions of KS shows that only 10%–30% of spindle-like cells express latent genes [126], whereas in later lesions virtually all such cells are latently infected [60,126]. This clearly implies that latently infected cells have a growth or survival advantage in vivo.

However, the discovery that the HHV8 latency program does not appear to be strongly immortalizing has raised questions about whether latency really suffices to drive KS pathogenesis unassisted. In fact, independent evidence from clinical studies suggests that lytic replication also plays a pivotal role in KS development. In patients with advanced AIDS, treatment with ganciclovir (GCV), a drug that blocks lytic but not latent HHV8 infection, results in a prompt and dramatic decline in the incidence of new KS tumors [133]. How might lytic replication contribute to KS tumor development? There are three nonexclusive possibilities: (*i*) spindle cells are not immortalized in vivo, then in order for a tumor mass to expand, HHV8-

positive cells that die must be replaced by new latently infected cells. The most obvious source of such cells would be from de novo infection of endothelia with viruses produced by lytic replication; (*ii*) If, as noted above, HHV8 latency is unstable, then as spindle cells proliferate, they would be expected to rapidly lose the viral genome. For KS to progress, cells that lose the HHV8 genome must be replaced by cells newly recruited to latency by de novo infection with viruses produced by lytic replication. (*iii*) The third manner by which lytic HHV8 replication may contribute to KS pathogenesis harkens back to the view that KS is a composite of three processes: proliferation, inflammation, and angiogensis. Because lytically infected cells die, they cannot contribute significantly to the proliferative component of the disease. However, if lytically infected cells produce paracrine signaling molecules, they could promote both the inflammatory and angiogenic components of the lesion. In fact, examination of the HHV8 genome [134] reveals numerous viral genes whose products are secreted signaling molecules—many of which are homologs of cellular cytokines or chemokines. The majority of these are lytic-cycle genes, including the three viral CC chemokines and v-IL6.

1.1.5.2 Deregulation of immune system

To efficiently establish a persistent infection, HHV8 dedicates a large amount of its coding potential to produce proteins that antagonize the immune system of its host. These viral immunomodulators interfere with both the innate and adaptive immune responses and most of them are homologous to cellular proteins, suggesting that they have been pirated from the host during viral evolution. The HHV8 genome contains 87 genes, of which at least 22 are potentially immunomodulatory (Table 2) on both adaptive and innate immune responses, including T- and B-cell functions, complement activation, the innate antiviral interferon response and natural killer cell activity. Reports of CD4+ T-cell responses to HHV8 antigens are very limited, but such antigens have been described [135]. By contrast, many reports have described responses to HHV8 by CD8+ cytotoxic T lymphocytes (CTL), and multiple epitopes have been identified in latent and lytic proteins [136-137]. Nevertheless, CTL responses to HHV8 in KS patients are considerably less frequent than in asymptomatic HHV8-seropositive subjects, regardless of CD4+ T-cell count [135]. Based on current evidence, the explanation of this observation could be related to a loss or sequestration in KS lesions of HHV8-specific CTLs from the periphery and/or to a suppression of their anti-HHV8 function. Down-regulation of the levels of MHC-I molecules on the surface of antigen presenting cells (APC) could be an effective method of suppressing CTL responses to virus-infected cells. HHV8 infects myeloid dendritic cells, macrophages and B cells through a receptor called DC-SIGN [138]. This infection reduces MHC-I expression in B cells [139] in addition to antigen processing and presentation in dendritic cells and macrophages. Several HHV8 proteins have the capacity to effect MHC-I down-regulation. They might function in concert, at different times during the viral lifecycle or in different host cells. The MIR1 and MIR2 protein trigger endocytosis and proteasomal degradation of MHC-I by ubiquitinating its cytoplasmic tail [14]. MIR1 and MIR2 have different requirements for the proximity to the transmembrane domain of their target residues for ubiquitination, which could, therefore, determine the portfolio of cellular proteins down-regulated by each of

these viral proteins: MIR2 preferentially ubiquitinates residues closer to the membrane than MIR1 [140].

Table 2. KSHV immunomodulatory proteins

KSHV protein	KSHV gene	Cellular homologue	Immunomodulatory function
1) Modulation of cytokines and cytokine receptors			
vIRF-1, vIRF-2, vIRF-3, vIRF-4	*K9, K11.1/K11, K10.6/K10.5, K10.1/K10*	Interferon-regulatory factors	Anti-interferon
ORF45	*ORF45*	None	Inhibition of IRF-7
RTA	*ORF50*	None	Inhibition of IRF-7
vIL-6	*K2*	IL-6	Cell growth
vCCL-1, vCCL-2, vCCL-3	*K6, K4, K4.1*	Macrophage inflammatory proteins	Chemokines
vGPCR	*ORF74*	IL-8 receptor	Cell growth
Kaposin B	*K12*	None	Increased stability of mRNAs containing AU-rich elements
2) Modulation of apoptosis			
vFLIP	*K13 (ORF71)*	FLIP	Inhibition of apoptosis
vBcl-2	*16*	Bcl-2	Inhibition of apoptosis
vIAP	*K7*	IAP (survivin)	Inhibition of apoptosis
Regulation of complement			
KCP	*ORF4*	DAF	Complement regulation
Deregulation of cell–cell contact			
MIR1, MIR2	*K3 (ORF12), K5*	None	MHC class I downregulation (E3 ligase)
K1	*K1*	ITAM	Downmodulation of B cell-receptor expression
K15	*K15*	None	B cell-receptor signalling
Other immunomodulatory activities			
vOX2	*K14*	CD200	Myeloid-cell regulator
SOX	*ORF37*	None	Host shutoff

Classically, the corollary to MHC down-regulation on the cell's surface is increased recognition of that cell by natural killer (NK) cells. Likewise, NK cells express a battery of activating receptors, including NKG2D, 2B4 and NKp80. NKG2D recognizes MHC-I-related chains (MIC) A and B, whereas NKp80 recognizes the activation-induced C-type lectin (AICL). In HHV8 infected cells, MIR2 reduced surface expression of ICAM-1 and B7-2 [141] and MICA, MICB and epitope-tagged AICL [142], protecting against NK-mediated lysis. However, the contribution of NK cells to HHV8 infection biology and the relative importance of modulating their activity remain to be determined unequivocally. Given that HHV8 can infect APCs, including B cells and dendritic cells, its down-regulation of the MHC-II antigen presentation pathway might be anticipated. However, we are not aware of any evidence to support this possibility.
As weel as T cells, the virus seems to influence several aspects of B-cell biology, with modulation of the immune response (Fig. 4). HHV8 also encodes miRNAs: twelve have been identified within the latency-associated region [143,144]. One of them, miR-K12–11, is an orthologue of miR-155. This miRNA is a contributor to B-cell differentiation and phenotype implicated in germinal centre development and, by regulating the activity of the enzyme activation-induced-deaminase (AID), antibody diversity. HHV8-miRNAs seem to regulate either the same cellular genes or genes involved in similar cellular processes [145]. Thus, there is every expectation that HHV8 miRNAs will be found to modulate immune function, and perhaps other aspects of pathogenesis, including oncogenesis [38]. Moreover, The HHV8 type I membrane glycoprotein K1 e the protein K15 might also influence B-cell biology. K1 retains BCR complexes in the endoplasmic reticulum, hence reducing the presence of BCR on the cell surface [146], instead K15 interacts with components of the clathrin-mediated endocytic pathway and might accelerate BCR internalisation (Fig. 4) [147].

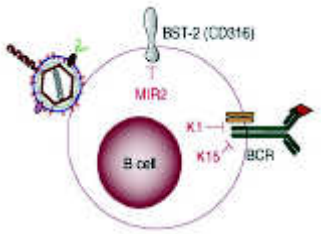


Figure 3: Summary of the mechanisms of HHV8 modulation of B cell function. The B cell is one likely reservoir of latent KSHV infection. However the three immunoregulatory proteins depicted are most abundantly expressed during lytic replication. K1 reduces the presence of BCR on the cell surface. Phosphorylation of the ITAM motif from K1 results in recruitment of various signalling proteins containing SH2-domains that participate in the production of cytokines, inhibition of apoptosis and cell transformation. K15 can also repress signalling from the BCR. MIR2 protein down-regulates BST-2 (also called tetherin), which is implicated in normal and malignant B-cell differentiation [123].

Little is known about the phagocytosis of opsonised HHV8 virions, although the HHV8 v-OX2 protein can inhibit neutrophil function [123]. HHV8 could also interact with the Complement system. In particular HHV8 ORF4 encodes a protein, known as HHV8 complement regulatory protein (KCP) [148], possessing complement regulatory activity. The protein is a lytic cycle protein that is incorporated on the surface of the virion [148]. Here it can participate in cell infection via the known HHV8 cell-attachment factor heparan sulphate, in addition to inhibiting complement-mediated immune responses. Molecular studies of recombinant KCP have revealed that it accelerates the decay of the classical pathway C3 convertase, and acts as a cofactor for factor I-mediated inactivation of C3b and C4b (components of the C3 and C5 convertases) facilitating binding of the virus to the cell and evasion of complement.
Arguably IFN-α and IFN-β are the most important and certainly the most understood of the IFNs associated with innate immunity to be activated in response to virus infection. Remarkably, HHV8 encodes four proteins (called v-IRFs) with limited homology to the cellular IRFs. In addition to the effect on MHC-I (see earlier), v-IRFs negatively regulate cellular IFN signalling. v-IRF1 inhibits the transcriptional programs induced by exogenous IFNs specifically by blocking transcriptional activation by the cellular proteins IRF1 and IRF3. The other lytic viral IRF, v-IRF3, is most closely related to the cellular IRF4 and also blocks IFN signaling by functioning as a dominant negative inhibitor of IRF3 and IRF7. Other HHV8 proteins also participate in regulating the IFN-mediated antiviral response. The K8 -encoded K-bZIP protein, the virion-associated ORF45 protein and RTA. The plethora of strategies adopted by HHV8 to inhibit the antiviral IFN response indicates that such modulation is important to HHV8 survival. However, complete abrogation of the IFN response is rarely achieved [149].
Chemokines activate leukocytes and regulate their trafficking. HHV8 encodes three chemokine homologues expressed during lytic replication and called viral CC-chemokine ligand 1 (v-CCL1), v-CCL2 and v-CCL3. Together they have numerous immune regulatory activities, but overall they probably polarize the adaptive immune response toward a Th2-like, or humoral, response [123]. Aside from their roles in immune modulation, these viral chemokines are angiogenic and anti-apoptotic for endothelial cells [150]; these activities might contribute to HHV8 pathogenesis. Moreover, HHV8 produce v-IL6 that retains sequence and functional homology to cellular IL-6. It stimulates multiple cellular pathways to induce cell proliferation and extrahepatic acute-phase responses through engagement of the gp130 coreceptor independently of the IL-6 (gp80) receptor [149]. v-IL-6, but not human IL-6, protects PELs and heterologous cells from the antiviral, cytostatic effects of IFN-α, which down-regulates the surface expression of gp80 but not gp130. In fact, v-IL6 transcription is induced indirectly by IFN-α and v-IL6 induces human IL6 secretion, supports the growth of IL6 dependent cell lines. Cells stably expressing v-IL6 secrete increased VEGF and induce hematopoiesis, tumorigenesis, and angiogenesis when injected into nude mice [149].

1.1.5.3 Kaposi's sarcoma: a model of chronic inflammation

Many aspects of KS suggest that chronic inflammation plays a role in its pathogenesis. At first glance, it seems counter-productive for a virus to encode

both proteins that evade the immune system and proteins that activate the immune system. However, in the context of chronic inflammation it makes perfect sense. It appears that inflammation is primarily mediated by hyperactivation of the humoral arm of the immune system (Th2-mediated responses) and is often accompanied by a decrease in the cellular immunity (Th1-mediated responses), which is the more effective antiviral response. We have already discussed many of the mechanisms used by HHV8 to downregulate the Th1-mediated responses such as suppression of IFNs and MHC-1 downregulation, so we will now turn to the mechanisms that the virus uses to activate the inflammatory responses, which lead to continued inflammation and KS tumor progression.

1.1.5.3.1 Pro-inflammatory cytokines

Several studies indicated that a network of soluble inflammatory cytokines promotes KS lesion development and progression by acting both in an autocrine and paracrine fashion. In particular, all patients with KS or at risk for KS show signs of immune activation such as increased levels in blood of intercellular adhesion molecule 1, neopterin, soluble CD8, and inflammatory cytokines, including IL1, IL6, TNF, IFN-γ (reviewed in Reference 132). In addition, high levels of inflammatory cytokines are detected in early KS lesions, which are infiltrated by numerous CD8+ T cells and monocytes that precede KS spindle cells appearance. Altogether, these data suggest that inflammatory cytokines may behave as KS "initiating factors" since they trigger the production of angiogenic factors which, in turn, promote KS development and progression. Clinical evidence confirms that immune activation rather than immunodeficiency may play a role in lesion development. In particular, both patients with classic KS and those with African KS show evidence of CD8+ T-cell activation with production of Th-1-type cytokines [151]. In addition, AIDS-KS not only can arise in the absence of immune deficiency [152], but it also develops in individuals showing signs of immune activation and Th-1-type cytokine production [153]. Furthermore, treatment of AIDS patients with recombinant inflammatory cytokines has resulted in KS onset or progression [154]. Finally, immune activation is likely to occur also in post-transplant KS in which, in spite of immune suppressive therapy, allogeneic stimulation may induce local foci of activated immune cells. Thus, immune activation and inflammatory cytokine production initiate KS, whereas immune defects and HHV8 escape mechanisms may be key for KS progression. Paradoxically, the immune response against HHV8 may even exacerbate KS progression via production of inflammatory cytokines [155,156]. In fact, the increased inflammatory cytokines in individuals with (or at risk for) KS induce both endothelial cells and leukocytes to express adhesion molecules which mediate the adhesion of HIV-infected, HHV8-infected, or uninfected leukocytes to the endothelium and their migration into tissues [155]. The recruitment of leukocytes in the lesion is enhanced by several chemokines (e.g., MIP1α and -β, RANTES, monocyte chemotactic protein-1, IL-8, Mig, and IFN-γ-inducible protein 1) that are expressed by KS spindle cells, activated leukocytes, or endothelial cells [157]. It is noteworthy that inflammatory cytokines reactivate latent HHV8, increasing the virus load and inducing virus spread to all blood cells [118,156].

Moreover, HHV8 encodes for a protein that stimulates the proinflammatory immune response: vGPCR. The constitutive activity of the v-GPCR induces myriad

signaling molecules. These include NF-κB, which has become increasingly linked to chronic inflammation and cancer progression. The activation of these various pathways by v-GPCR in monocytes and T cells leads to the production of proinflammatory cytokines including IL-1β, TNFα, IL-6, IL-2, IL-4 [158] v-GPCR induced activation of NF-κB in endothelial cells results in expression of RANTES, IL8 and GM-CSF as well as the adhesion molecules VCAM-1, ICAM-1 and E-selectin [159]. Due to the lytic expression of vGPCR, any effects this signaling has on tumor formation or progression probably occurs via a paracrine mechanism. HHV8 also induces NF-κB via the expression of v-FLIP. Because v-FLIP is latent and, therefore, widely expressed in KS and PEL tissues, the activation of NF-κB is constitutive, not transient. This constant signaling from the tumor tissue could be a major stimulus for both proliferation and chronic infiltration by leukocytes. Very important is the role played by IL6, a cytockine that has long been associated with KS and MCD tumor development. It has been shown to enhance proliferation of KS cells in culture and was found in high levels in blood from MCD patients, which correlated with disease progression [149]. The viral homologue v-IL6 induces all of the same signals as its cellular counterpart, but because it does not require IL-Rα for signaling, more cells are susceptible to activation [149].

1.1.5.3.2 Angiogenesis

The formation of new blood vessels to increase blood supply to a site of injury is another normal inflammatory response of the immune system that, when aberrantly stimulated, enhances tumor pathogenesis. Among the angiogenic factors expressed by KS spindle cells in vitro and in vivo, basic fibroblast growth factor (bFGF) is a key mediator of KS lesion formation since it promotes both KS spindle cells and endothelial cells locomotion and proliferation in an autocrine and paracrine manner, respectively [131,132]. Injection of bFGF in mice results in the formation of KS-like lesions, while bFGF antagonists that include specific antisense oligodeoxynucleotides or neutralizing antibodies block the development of angioproliferative lesions induced by primary KSC upon injection in nude mice [131.132]. Another angiogenic molecule expressed in vitro and in vivo by KS spindle cells is the vascular endothelial cell growth factor type A (VEGF-A) [160,161], a homodimer belonging to the cysteine knot family of growth factors. In KS lesions, VEGF-A is responsible for edema formation and synergizes with bFGF in inducing endothelial cell growth and angiogenesis [160,161]. Moreover, VEGF-C, another member of the VEGF family, stimulates the growth of some KS spindle cells isolates which, similarly to lymphatic endothelial cells, express the receptor for this angiogenic factor. HHV8 encodes several other proteins that induce VEGF. For example, v-IL6 and all three HHV8-encoded chemokines (vCCLs) have been shown to induce angiogenesis in experimental models, and the mechanism for this activity is likely the induction of VEGF [162]. Similarly, it has also been shown that in HHV8-infected endothelial cells, vGPCR upregulates both VEGF and its receptor VEGF-R2.44 It has been postulated that this may create a paracrine feedback loop for continued cellular proliferation and angiogenesis [163].

In conclusion, most of the proinflammatory cytokines and angiogenic factors produced or induced by HHV8 likely evolved to create a highly proliferative environment for viral genome maintenance and reactivation from latency. Both lytic

and latent genes are responsible suggesting that both autocrine and paracrine mechanisms are involved. In addition to the virally induced factors, the persistent infection induces a constant infiltration of leukocytes, which also secrete a large variety of cytokines, chemokines, MMPs and IFNs that favor the growth of the infected cells and adds to the progression of KS. Theese evidence suggest that, from a pathogenetic point of view, it is useful to think of KS as being composed of three parallel processes: a proliferative component (involving chiefly spindle cells), an inflammatory component, and an angiogenic component. Although it is likely that these processes are interdependent and perhaps mutually reinforcing, thinking of them as individual entities allows the virologist to entertain different models for how viral gene expression may influence each component and thus affect the lesion as a whole.

1.1.6 Spindle cells and their role in Kaposi's sarcoma

The term spindle cell was first used by the German pathologist Kobner in 1883 and then by Philippson in 1902. Spindle cells are defined as the characteristic cells, exhibiting a typical spindle-shaped morphology, which are mostly present in the advanced stage of the KS lesions.

Indeed, these peculiar cells, which are considered to be the KS tumor cells, represent the most prominent histo-pathological feature of the final nodular stage of KS. These cells clearly represent the main proliferative element in KS, but analysis of their clonality has shown that many KS lesions are oligo- or polyclonal—again reinforcing how different KS is from traditional cancer [164,165]. Although this conclusion has been disputed by some research [166], even these dissenting studies have shown evidence of non-clonality in numerous tumors, and the recent work by Duprez *et al*. [167] strongly suggests the oligoglonal origin of KS lesion. Although spindle cells are considered the driving force behind KS histogenesis, they are by no means the sole cell type in the tumor. As described, all KS lesions also contain significant numbers of inflammatory cells (B and T cells, plasma cells, and monocytes) and a profusion of aberrant, slit-like neovascular spaces [126]. Moreover, one criterion for malignant transformation in cell culture is the reduced dependence upon exogenous growth factors. However, cultured KS spindle cells, unlike classical tumor cells, remain highly dependent upon exogenous growth signals when cultured in vitro. Ensoli and colleagues were the first to grow KS spindle cells in culture and found them to be strongly dependent upon a cytokine-rich medium derived from supernatants of activated T cell cultures [75,132]. To date, no less-complex medium that regularly allows spindle-cell outgrowth has been found, and certainly such lines do not grow in low-serum medium, nor do they display genetic instability, another hallmark of traditional cancers. KS spindle cells are generally diploid, and no characteristic chromosomal rearrangements are shared by multiple tumors. Classical KS lesions do not display microsatellite instability, although some advanced AIDS-KS lesions do [168]. Cultured KS spindle cells also lack other markers of transformation. They do not form foci, nor grow in soft agar, nor form tumors in nude mice [101]. However, an interesting phenotype is observed when the cells are injected subcutaneously in nude mice. Although they do not generate a tumor, they do survive for a brief interval, during which slit-like new vessels of murine origin appear in the

surrounding tissue. When the human implant involutes, these vessels likewise disappear [167]. This suggests a KS model in which spindle cells require growth factors from their microenvironment (perhaps from infiltrating inflammatory cells) for their survival and proliferation, but also produce angiogenic and proinflammatory substances to recruit the other components of the lesion. In this view, none of the partners in this process are fully autonomous; each depends upon the other (Fig. 5).
All in all, most clinical and experimental observations about KS suggest that the differences between this disorder and classical cancers are at least as numerous as their similarities. It should then come as no surprise that the viral contributions to KS pathogenesis may be more variegated than those of other tumor viruses to more traditional cancers.

1.1.6.1 Cell origin

The question of the spindle cell origin has been a matter of animated debate and several cell types have been considered during the last decades. Researchers have long thought that spindle cells are of endothelial lineage, and indeed they bear many endothelial markers, including CD31, CD34, CD36, factor XIII, En-4, and PAL-E [103,131,132,170]. However, most spindle cells lack staining for factor VIII, a classical marker of differentiated vascular endothelium, and Weibel-Palade bodies are absent ultrastructurally [101]. Confusion has been compounded by the finding that KS spindle cells display significant heterogeneity in marker expression. For example, a small number of cells in some biopsies do stain for factor VIII, whereas other cells stain for smooth muscle α-actin [103,131,170], leading some researchers to speculate that spindle cells may arise from bipotential mesenchymal precursors of vascular cells. Another view is that the cells arise from lymphatic endothelium, a notion that has been encouraged further by the observation that clinical KS virtually never arises in tissues that lack lymphatics (e.g., the central nervous system) [3], and that molecular markers of lymphatic endothelium (VEGF-C, VEGF-R3, podoplanin, and LYVE-1) are also expressed regularly by spindle cells in vivo [171,172]. Two recent studies have examined the effects of KS-associated herpesvirus (HHV8) infection on the gene-expression programs of cultured vascular and lymphatic endothelium using DNA microarrays to profile host-gene transcription [173,174]. Although these studies are conducted in vitro perforce, they may help explain the heterogeneity observed in marker expression in biopsies, and they certainly complicate attempts to discern the lineage of infected cells on the basis of patterns of marker expression alone. They reveal that viral infection reprograms endothelial marker expression, which causes vascular endothelium to express certain lymphatic markers, whereas lymphatic endothelium shifts to a more vascular-like marker profile. Theese findings indicates that KS spindle cells do not faithfully represent either cell lineage, which could be a consequence of HHV8 infection [173]. Based on these evidences, the more likely scenario is that HHV8 infected endothelial precursor cells circulate in blood driving these precursors towards lymphatic phenotype and that after infections, they may go to permissive sites to proliferate as KS lesion or grow following transmission of HHV8 to dermal microvascular endothelial cells [175]. Moreover, others two considerations suggest that KS lesions may originate from the seeding of previously infected endothelial precursors. The first consideration derives from the

observation that KS lesions often progress during or following states of systemic inflammation, and that KS tumors sometimes arise precisely at sites of previous local inflammation, such as surgical wounds (a property known as the Koebner phenomenon) [176], thus suggesting that an inflammatory environment can elicit spindle cell proliferation from circulating HHV8-infected precursors. The second and more stringent consideration derives from the demonstration that recipients of kidney allografts HHV8-negative prior to transplantation may develop KS lesions containing HHV8-infected neoplastic cells of donor origin. This observation clearly indicates that infected progenitor cells from HHV8-infected donors without KS can be seeded and undergo neoplastic transformation and progression in the recipient immunosuppressed hosts [97]. Although it is possible that donor-derived KS precursors might be mature endothelial cells of the kidney, it appears far more likely that they may originate from circulating endothelial progenitors entrapped within the graft.

1.1.6.2 In vitro culture of spindle cells

Several laboratories have succeeded in establishing cell lines derived from KS cells using conditioned media and/or diverse inflammatory cytokines, in vitro. These cells, originating from classic/epidemic KS lymph node, from skin biopsies or from pleural effusion of KS patients, exhibit a spindle-shaped morphology. However, they are quite heterogeneous, as some cell lines express mainly endothelial cell markers (with or without blood vessel or lymphatic markers, depending on the cell-line), while others present a smooth muscle phenotype or even mesenchymal markers [170]. Theese cell lines have been extensively characterized and used in several animal models (such as nude mice), in order to gain new insights into KS pathogenesis. However, one of the most puzzling features of spindle cell biology has been the fact that every spindle cell line that has been derived has been shown to lack the HHV8 genome [177,178], despite the fact that in advanced primary KS tumors nearly all spindle cells are latently infected. The reasons for this have been uncertain, but it is noteworthy that numerous cell lines have been derived from HHV8-associated primary effusion lymphomas (PELs) in which HHV8 genomic persistence is readily demonstrable. This indicates that there are circumstances under which stable latency is achieved in vivo and that standard culture conditions are not incompatible with this state, at least in B cells. Moreover, this marked propensity to segregate latent viral genomes is shered also by proliferating cells infected with HHV8 in vitro. In fact, they display only a variable but small subpopulation being capable of stable episome maintenance [179]. To explain this paradox many studies suggest that stable maintenance of viral genome is not due to the enhanced production of viral or host *trans*-acting factors, but is associated with *cis*-acting, epigenetic changes in the viral chromosome [179]. These results indicate that acquisition of stable HHV8 latency is a multistep process that proceeds with varying degrees of efficiency in different cell types.

1.1.7 B cells and HHV8 infection

Soon after the discovery of HHV8 in the spindle cells of KS tumors, HHV8 was found associated with two B-cell lymphoproliferative disorders: multicentric Castleman disease (MCD) and primary effusion lymphoma (PEL). There are further

reports of HHV8-positive lymphomas with clinical, histological and immunophenotypic features distinct from the above HHV8-associated lymphoproliferative disorders in patients both with and without immunodeficiency [5]. Together, these studies show that HHV8 is associated with a wide spectrum of lymphoproliferative disorders with varied clinicopathological features. Moreover, several studies demonstrated that in KS patients, HHV8 establishes persistent infection in peripheral blood B lymphocytes witch represent the major virus reservoir. The virus is able to interact with B lymphocyte in several ways: (1) generating a immune evasion strategy for elicit B cell response; (2) skipping the B cell developmental pathways; (3) inducing B cell lymphoproliferation by the switch of both latent and lytic genes and by the affection of specific pathways of B cell proliferation, including cell cycle check points and the Notch and Wnt signalling pathways. The first point was already discussed (see Figure 4), in this section we will examine the others points to clearly understand the mechanisms of interaction between HHV8 and B cells in KS and in the lymphoproliferative diseases associated with HHV8 infection.

1.1.7.1 HHV8 interferes with the B cell developmental pathways

In the HHV8 positive plasmablastic variant of MCD of plasma cells type and in the frank plasmablastic lymphoma, the HHV8-positive plasmablasts phenotypically resemble mature B-cells. For example, they express abundant cytoplasmic immunoglobulin and many express CD27 [180,181], a surface marker for memory B-cells. However, HHV8-positive plasmablasts in MCD consistently show a lack of somatic mutations in their rearranged immunoglobulin heavy and light chain genes [181], indicating that they originate from pre-germinal centre B-cells. This, together with the predilection of HHV8-positive plasmablasts to localise in the mantle zone of B cell follicles, suggests that HHV8 may preferentially target IgM expressing naïve B-cells in patients with MCD and drive the infected cells to differentiate into plasmablasts without going through the germinal centre reaction (GCR), a critical process for normal B-cell maturation [180,182]. Therefore, HHV8 may infect IgM-positive naïve B cells and drive these cells to differentiate into plasmablasts and form lymphoproliferative lesions without undergoing the GCR. Among all the HHV8 genes, the first responsible for these effects could be v-IL6 that is a potent factor of B cell survival. Indeed, it has been suggested that HHV8 encoded v-IL6 may play a direct role in driving HHV8 infected naïve B cells to differentiate to plasmablasts and contribute to the development of lymphoproliferative lesions, without the need for the transition of the lymphoma precursor cell through the GCR [180,182]. Also the latency protein LANA could participate to this process, as suggests by the recent study by Fakhari et al. [183]. The Authors, using transgenic mice that expressed LANA protein in mature B cells, found that LANA activated mature B cells in the absence of antigen stimulation, which predisposed the animals to lymphoma development. In particular, all of the transgenic mice developed splenic follicular hyperplasia due to an expansion of IgM+IgD+ B cells [183].
Moreover, HHV8 encodes for two proteins, K1 and K15, able to subvert BCR signalling during lytic replication and perhaps also during latency in B cell. The viral protein K1 protein interacts with the heavy chains of the BCR, retaining this complex in the endoplasmatic reticulum and preventing it from reaching the cell surface [146]. The viral protein K15, instead, can inhibit the BCR signal

transduction by reducing tyrosine phosphorylation and intracellular calcium influx [141]. This strategy may then usurp control of infected-cell signalling altering the normal developmental pathway and extending the life of the infected cell to maximize progeny virion production.
Another possible mechanism of interaction between HHV8 and the developmental pathway of B cells may come from the study of viral micro-RNAs. Computer analysis of potential mRNA targets for these viral micro-RNAs, has identified a number of interesting candidate cellular genes, involved in a range of cellular activities, including apoptosis and signalling, one of which is, for example, BCL-6, targeted by HHV8 micro-RNA-K4 [143]. The theoretical possibility is raised that targeting BCL-6 by viral micro-RNA-K4 may lead to its down-regulation, followed by the up-regulation of BLIMP-1 and by the shift of a B cell towards a plasma cells stage of differentiation. This and other studies on the possible role of HHV8 micro-RNAs in B cell proliferation and transformation may contribute to shed lights into the various and reciprocal interactions between host and herpesviral genes, helping to dissect the fine tuning of B cell fate.

1.1.7.2 HHV8 induced B cell proliferation

HHV8 genome contains viral genes which are homologous to cellular genes involved in the control of proliferation and apoptosis. Such viral genes that are pathogenetically relevant for HHV8 driven lymphoproliferation are expressed during either the latent or the lytic phase of the viral cycle and control cellular functions, directly [184]. Several viral protein are able to to induce B cell proliferation in different ways.
Normally, during viral infection, host cells induce the antiviral factor IFN-a to up-regulate p21, initiate cell cycle arrest and inhibit viral replication. Moreover, IFN-a down-regulates the human IL6 receptor gp80. However, a viral transcription programme exists in which IFN-α also activates the HHV8 v-IL6 promoter to induce the secretion of v-IL6, which, in turn, by-passes the gp80 negative regulatory check point, by binding directly to its other receptor, the gp130 receptor, which is not down-regulated by IFN-a and thus is free to induce B cell survival and proliferation [185,186]. Additionally, HHV8 encode proteins that inactivate p53 or Rb [182]: LANA and v-cyclin, both expressed in HHV8 positive lymphoproliferative lesions in vivo, inactivate p53 and Rb, respectively. LANA inhibits p53-mediated apoptosis by inactivating Rb, v-cyclin induce the release of E2F, which is normally inhibited by Rb, and initiate the transcription of genes involved in DNA synthesis (such as DNA polymerase, thymidine kinase, etc.) [182].
Moreover, HHV8 could affect specific pathways of B cell proliferation, including the Notch and Wnt signalling pathways. The Notch receptor and the Notch ligands are transmembrane proteins that influence cell fate decisions, proliferation and differentiation. HHV8 has developed a mechanism to alter this pathway through the protein RTA that binds the nuclear mediator of Notch and activates either cellular (CD21, CD23) or viral (v-IL-6) genes [187]. The Wnt signalling pathway regulates development and proliferation. In particular, the Wnt signalling regulates the effector molecule Beta-catenin through a complex containing glycogen synthase kinase 3beta (GSK-3beta), adenomatosis polyposis coli, and axin. This complex efficiently phosphorylates Beta-catenin, promoting its degradation by the proteosome. In PEL, HHV8 LANA binds and traps GSK-3beta in the nucleus,

resulting in inefficient phosphorylation and degradation of Beta-catenin, which, thus, may accumulate and is free to enter the nucleus and to activate genes involved in cell proliferation [187].

1.1.7.3 HHV8 infects B cells in KS patients

Several reports have demonstrated that B lymphocytes are preferentially infected by HHV8 [188-190]. However, there are contrasting results on the percentage KS patients that harbour HHV8-infected B cells, ranging from low percentage [188] to 81% [189]. Although the percentage of infected B cells in these patients is a very small fraction of B lymphocytes, B cells are now defined as one of the principal circulating virus reservoir. This hypothesis is supported by the finding that B cells isolated from patients with KS or at risk of KS sustain the reactivation of HHV8 infection after exposition to inflammatory cytokines [156]. Therefore, several Authors suggest that circulating B cells may recruit the virus into tissues and may undergo lytic infection and transmit the virus to neighbour cells.
Peripheral B cells are HHV8-infected in KS patients, and HHV8 might also be involved in the transformation of B cells. Therefore, several studies have investigated a possible association between cKS and lymphorpoliferative disorder (LD). The first report of an association between KS and a LD dates back approximately 90 years [191]; later, a large number of reports of followed but the results collected in various studies of large series of homogeneous patient populations with nonepidemic KS have not always been concordant [192-195]. A recent work by Hiatt *et al*. [196] demonstrates an increased incidence of multiple myeloma and non-Hodgkin's lymphoma in a cKS population in USA. These non-Hodgkin lymphomas include angioimmunoblastic lymphadenopathy, B-cell immunoblastic lymphoma, autoimmune hemolytic anemia, and hairy cell leukemia. These contrasting results of association could be explained by considering the temporal relationship of occurrence of KS and LD. It has been postulated that the development of secondary malignancies, including LD, after the diagnosis of KS in HIV negative and positive patients is not increased [192,193]. Conversely, the risk of developing KS after LD is high due to immunosuppression secondary to chemotherapeutics, infectious agents, and radiotherapy, and even in those patients with benign lesions treated with corticosteroids and/or azathioprine [196]. Therefore, further studies are needed to definitively asses the relation between KS and LD, and to verify the hypothesis that infected B cells could be driven towards a transformed phenotype.

Thus, B cells in KS patients are viral reservoir able to sustain the lytic reactivation and dissemination of the virus. In addiction, B cells are one of the target of viral proteins able to promote a modulation of B cell functions. It is conceivable that evolutionary pressure has selected B lymphocytes as the site of HHV8 latency also for making these cells relative incapable to stimulate a strong immune response against the virus.

1.2 B cell development: from precursor to antybody producing plasma cell

The discovery that lymphocyte subpopulations participate in distinct components of the immune response focused attention onto the origins and function of lymphocytes more than 40 years ago. Studies in the 1960s and 1970s demonstrated that B and T lymphocytes were responsible primarily for the basic functions of antibody production and cell-mediated immune responses, respectively. The decades that followed have witnessed a continuum of unfolding complexities in B-cell development, subsets, and function that could not have been predicted. The phenotypic and functional diversity of B lymphocytes, their regulatory roles independent of antibody production, and the molecular events that make this lineage unique are also poorly defined [197]. Recently many studies demonstrated that B cells outside the marrow are morphologically homogenous, but their cell surface phenotypes, anatomic localization, and functional properties reveal still-unfolding complexities [197]. B lymphocytes are generated via several sequential steps of differentiation (Fig. 8), from precursor to antibody producing cells.

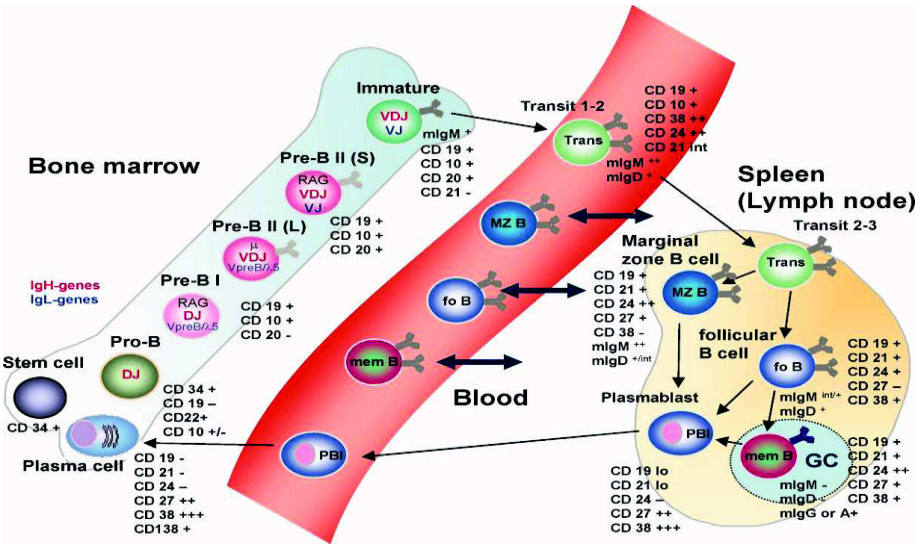


Figure 4: Scheme of B cell development. The central and peripheral B cell developments are outlined. Blood represents circulating B cell populations. Relevant surface markers for each differentiational step are listed. fo B cell, follicular B cell (naïve B cell); GC, germinal center; mem B, memory B cells; MZ B cell, marginal zone B cell; Pbl, plasmablast; Trans or Transit, transitional B cell [261].

1.2.1 Central B cell development

The early development of human pro and pre B cells from pluripotent stem cells is antigen independent and takes place in the bone marrow.
Pro B cells (CD19-CD10+/-CD20-CD22+CD24- vpreB-Ig□-/+) are characterized by the expression of the first B cell specific surface marker CD22. Late pro and early pre B cells commence assembling the immunoglobulin surface receptor (BCR) and its signaling components. The completed rearrangement of the heavy chain and pairing with the surrogate light chain on the surface as the Pre B cell receptor (preBCR) marks the pre B cell stage [199].
Pre B cells (CD19+ CD10+CD20-CD24^{high}vpreB+Ig□+ intracellular □+) expressing correctly assembled preBCR on the surface are positively selected for proliferation and further differentiation. Deletion of B cell specific transcription factors BSAP, E2A, EBF, responsible for the transcription of the BCR, the recombinase-activating genes RAG1 and -2, responsible for the rearrangement of the □-heavy (and later light) chains, pre BCR components (□-chain, Ig□, Ig□, □5, vpreB) and downstream signaling components, like Syk, Slp65, and btk, cause severe blocks at the pro-/pre B cell transition [200]. Defects of some of these signaling molecules have been identified in immunodeficient humans [201].

After the successful assembly of the preBCR and several rounds of expansion, kappa or lambda light chains replace the surrogate light chain. The pairing of the heavy chain and a light chain finally leads to the surface expression of IgM, the hallmark of immature B cells. Immature B cells (CD19+CD10+CD20+CD24++IgM+) are most likely positively selected for the successful recombination of heavy and light chain. Simultaneously, the expression of the BCR on the surface allows for the first time antigen-specific negative selection. At this stage the central development of B cells is accomplished and so-called transitional B cells leave the bone marrow for full maturation in secondary lymphoid organs, especially the spleen. Neither pro-, pre-B cells are found in the circulation of healthy humans [202].

1.2.2 Peripheral B cell development

B cells generated in the bone marrow migrate to the periphery at the transitional B-cell stage, when they are still short-lived and functionally immature [203,204]. Transitional B cells are transported by the bloodstream to the spleen. Here they develop into long-lived mature B cells. Similar to the early stages in the bone marrow [205], this phase of development in the spleen also requires the presence of a functional B-cell receptor (BCR) and of a supporting microenvironment.
In mice, peripheral B cells pass through several consecutive developmental stages termed transitional B cell T1-2 or 3 [206,207]). Transitional type 1 (T1) B cells (IgM^{high}IgD-CD21-CD23-) resemble the immature B cells of the bone marrow. In the splenic environment T1 B cells differentiate into T2 B cells (IgM^{high}IgD+CD21^{int}CD23+). Beside BCR-derived signals, BAFF (synonym: Blys) and its receptor BAFF-R (synonym: BR3) [208] have been shown to be key regulators for the survival at this stage. BAFF-R belongs to the TNF receptor family

and is almost exclusively expressed on B cells. While early precursors have no detectable expression, transitional T2 B cells are the first to express BAFF-R on the surface [209,210]. The development of human transitional B cell seems to resemble the described murine differentiation at least in part [211].
It is unclear how T1, T2 and T3 subsets relate to stages of human B cell maturation. However, it is clear in both mice and humans, B cell development during the transition from immature to mature naïve B cells is a multistep process, and at each step B cells are influenced by selection pressure to ensure tolerance before full maturation [211]. Intermediate cell subsets during human peripheral B cell development have not been fully characterized.

Recently, circulating human transitional B cells were identified in the peripheral blood oh adult by studing CD38, CD10, and CD24 expression, previously identified as immature B-cell markers in bone marrow [212]. Phenotype analysis revealed that approximately 14% of cord blood B cells and 2-4% of peripheral B cells expressed these immature B-cell markers and a phenotype similar to that described for murine transitional type I (T1) B cells: these cells are CD19+, $CD24^{high}$,$CD38^{high}$, $CD21^{low}$, $CD23^{neg/low}$, IgM^{high}, IgD^{int}, and $CD62L^{low}$. The increased expression of CD20 and reduced expression of CD40 and CD22 was found on these human B cells have also been reported on murine T1 B cells [206,213]. The lack or diminished expression of activation markers, such as CD69, CD11b, CD80, CD86, and CD95, compared with memory or recently activated B cells indicates that these cells are unlikely to have responded to antigen recently [212]. The absence of both CD27 expression and Ig somatic hypermutation demonstrates that these cells are not memory B cells [214]. There were differences between murine T1 B cells and their putative human counterparts. The most notable difference was the expression of CD5 on the latter [211,214]. CD5 expression is associated with a subset of murine B cells known as B1a cells that are predominately found in the peritoneum and express low affinity BCRs with autoreactive specificity [215]. The immature markers on T1 B cells, particularly the increased expression of CD24, low expression of CD11b, and their poor survival, distinguishes them from murine B1a B cells [216]. Moreover, Ig gene usage and substantial nontemplated nucleotide addition are not characteristic of either murine peritoneal B1a cells or human fetal B-cell repertoires [217]. Notably, the initial B cells emerging from bone marrow following human stem cell transplantation are CD5+ [218]. Therefore, CD5-expressing immature B cells represent T1 B cells rather than B1a cells, and could be an useful marker for identification of human transitional B cells [211,212].
Since T1 B cells express low levels of the adhesion molecules CD62L and CD44 compared with mature B cells their capacity to migrate is limited [219]. Nevertheless, the expression profile of circulating T1 B cells and the relative stability of this population following immunization distinguish these cells from pre-GC and plasma cell precursors [212]. Moreover, human T1 B cells died rapidly in culture without any stimulation, a feature common to mouse splenic T1 B cells [207,218]. Furthermore, $CD24^{high}CD38^{high}$ B cells displayed other immature characteristics, including: unmutated Ig V genes, elevated levels of surface IgM, lacked expression of Bcl-2, greatly reduced capacity to proliferate, secrete Ig, and migrate in vitro compared with mature B cell populations [220].Thus, $CD24^{high}CD38^{high}$ B cells appear to represent a unique population of human B cells,

with morphological, phenotypic, and functional characteristics that distinguish them from mature B cells and would be consistent with their designation as transitional B cells.

Importantly, intermediates in human B cell development can be found in the peripheral blood [212], raising questions about the site(s) of maturation of these cells in humans. Recently, Lee *et al*. [220] identified a distinct pre-naïve B cell population circulating in human peripheral blood that exhibits an intermediate phenotype between transitional and naïve B cells. Like human transitional B cells, these cells express CD5 but have intermediate densities of CD38, CD10, CD9, and the ABCB1 transporter compared with transitional and naïve B cells. These pre-naïve B cells account for a majority of circulating human CD5 B cells. Importantly, CD5 pre-naïve B cells could be induced to differentiate into cells with a naïve phenotype in vitro. CD5 pre-naïve B cells show only partial responses to BCR stimulation and CD40 ligation and undergo more spontaneous apoptosis and cell death than do naïve B cells, whereas BAFF/BLyS (B cell-activating factor belonging to the TNF family) did not enhance their survival compared with naïve B cells. In contrast, CD5 pre-naïve B cells carry out certain functions comparable to naïve B cells, including the capacity to differentiate into plasma cells and the ability to function as APCs [220].

After passing the transitional stage, the human mature B cell compartment consists of naïve B cells and marginal zone B cells [201]. It is not known whether a human equivalent of the murine B1 B cell compartment exists. The murine marginal zone of the spleen contains a sessile $IgM^{high}CD21^{high}CD23^{low}CD9+$ B cell population (MZ B cells) which is a crucial player of the early antibody response, especially to blood-borne, T independent but also dependent antigens [221].
In humans, MZ B cells have been identified as $CD27+IgM^{high}IgD+$ B cells and represent about 15-25% of the circulating B cell pool [222]. BCR specificities for polysaccharides like pneumococcal polysaccharides (PnPS) are enriched among the MZ B cells and a decrease of circulating MZ B cells is associated with a poor PnPS vaccination response [221].
Naïve B cells express CD23, CD21 and are IgM+, and IgD+, but do not express CD27. They represent the main B cell population in the blood and secondary lymphoid tissues of mice and men. Most of their BCRs recognize proteins and therefore naïve B cells are typically recruited in T cell dependent responses. This allows for the participation in germinal center reaction and differentiation into long-lived memory B cells or plasma cells [200].

1.2.3 Antigen Driven B Cell Differentiation of Mature B Cell Compartments

Depending on BCR signal strength, activation of costimulatory receptors, and other factors, antigen activation of mature B cell leads either to death or further differentiation into the memory or plasma cell pool [200].
When successfully activated, B cells enter the FDC network inside the B cell follicles and give rise to germinal centers around day 3-5 of T dependent antibody responses [223]. Germinal centers are a highly specialized niche, crucial for class switch recombination, affinity maturation, selection, and expansion of antigen-specific B cell clones [224]. Additional T cell derived signals, foremost by CD40L,

allow these cells to survive and acquire characteristics of memory B cells. Other stimuli including IL10, IL21 [225] induce a part of germinal center cells to enter an alternative pathway of differentiation generating plasma cells [226]. During the germinal center phase B cells go through several stages (BM1-5) [227], of which only the pre (naïve, BM1-2) and post germinal center (memory, BM5) stages can be found in the circulation.

1.2.4 B Cell Memory and Plasma cells

It is well established that following an immune response two types of differentiated B cells persist in the memory pool: plasma cells, which confer immediate protection by the secretion of specific antibodies; and memory B cells, which confer rapid and enhanced response to secondary challenge [228]. Longevity remains the key aspect of immunological memory and applies to both plasma cells and memory B cells. In humans all memory B cells recirculate through the blood, but their main reservoir is represented by lymphoid tissues, such as the bone marrow and the spleen [229]. Their survival requirements remain to be defined. Mouse studies indicate that BlyS is required for the maintenance of naïve B cells and APRIL for the maintenance of plasma cells, while both ligands appear to be dispensable for the survival of memory B cells [230,231]. However, there is evidence that under steady-state conditions human memory B cells are slowly dividing [232] suggesting that the memory B cell pool may be maintained through homeostatic proliferation as it is the case for memory T cells. This view is supported by the observation that memory B cells are highly responsive to polyclonal stimuli [232]. At present a B 'memory stem cell' has not been identified and remains a matter of speculation.

While the identification of murine B cell memory suffered from the lack of specific surface markers, human memory B cells can be identified by the expression of CD27 [229]. About 16-55% (5-95% confidence interval) of circulating B cells are CD27+ B cells of which about 50% express IgM and IgD while the rest has undergone class switch recombination with only a very low percentage of post germinal center IgM-only (IgM+IgD-CD27+) B cells in the peripheral blood [248]. Several lines of evidence point out that the IgM+IgD+CD27+ B cells represent marginal zone B cells (see above) [228,229]. Therefore only IgD- are true post germinal center memory B cells, and could be further divided in class-switched IgM- IgD- CD27+ B cells and IgM memory B cells IgM+ IgD- CD27+, a small popolation representig 1% of total B cells [230].

Plasma cells emerge from extrafollicular as well as germinal center responses. During primary antigen encounter plasmablasts form antibody forming foci (AFC) in the medullary cords of lymph nodes and in the red pulp of the spleen [232]. These plasma cells can switch to IgG1, carry only few SHM and are short lived [296]. In contrast, postgerminal center responses can create a long lasting humoral immunity. Plasma cells are not circulating and only few plasmablasts are detectable in the peripheral blood. The number depends on recent activation and increases around day 6 after vaccination to return soon back to below 2% [295].

1.3 Influenza vaccine responses in older adults

1.3.1 Influenza—a significant cause of morbidity in older adults

Vaccination is of crucial importance in preventing infection and protecting the vulnerable elderly population from disease. Because the efficacy of a vaccine depends on the quality of the immune response, immunocompromised persons, such as very young infants and elderly people, are likely to be insufficiently protected. Thus, over the past decade, a large number of studies have shown that a variety of vaccines are less efficient in elderly persons. Annual vaccination against influenza, for example, is recommended in most developed countries for persons with underlying chronic diseases and for everybody aged >60 or >65 years, depending on individual national recommendations.

The full impact of influenza is increasingly recognized as an illness that goes well beyond pneumonia and influenza statistics. Peak months of mortality due to respiratory illness, ischemic heart disease, cerebrovascular events and diabetes in adults 70 years and older coincide with annual influenza epidemics, suggesting that influenza illness is the major cause of excess mortality in this population during the winter months [233]. Hospitalization and death rates from influenza are rising in spite of widespread influenza vaccination programs implemented in the 1990s. Influenza on average resulted in 36,000 deaths annually in the U.S. from 1990 to 1999 [234], almost double that of the period from 1976 to 1990. A similar rise in hospitalization rates for acute respiratory illnesses and cardiovascular diseases during the influenza season was also observed over these two time periods [235], [236].

1.3.2 Link to immune senescence

A decline in immune function is a hallmark of aging and affects the ability to resist influenza infection and respond to vaccination. It is recognized that multiple components of immune function, particularly cell-mediated immunity, are affected during the aging process. As a consequence, there has been a paradigm shift in understanding the limitations of antibody titers as a sole measure of influenza vaccine efficacy in older people [237], [238], [239], [240], [241] and [242]. In this population, adequate antibody titers may not provide sterilizing immunity, where antibody fails to bind the influenza virus to prevent infection of the cell [243],[244]. Further, statistically significant increases in antibody titers that correlate with protection in response to vaccination, may not translate to clinically important improvements in influenza outcomes in older adults [245]. Thus, the goal of vaccination may be clinical protection against illness when infection occurs and is mediated by both humoral and cellular immune mechanisms. The challenge to new vaccine development is that antibody titers as a sole predictor of vaccine efficacy may fail to detect important changes in cellular immunity that enhance vaccine-mediated protection in older people.

1.3.3 Antibody and cell-mediated immune responses to influenza vaccine

Influenza virus stimulates an antiviral response in both B and T lymphocytes resulting in humoral and cell-mediated immunity, respectively. Virus-activated T-cells, through cytokine mediators, stimulate B-cells to differentiate and produce antibodies specific for a particular vaccine strain. These specific antibodies bind to the surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA), to neutralize the virus particle. Influenza vaccines must be updated annually to include the predicted circulating strains of influenza A/H3N2 and A/H1N1 and influenza B. Antibody responses to influenza vaccination have largely been evaluated by the hemagglutination inhibition (HI) assay. While the HI test is convenient and considered a gold standard, virus neutralization assays are gaining more acceptance as they are considered more functional.

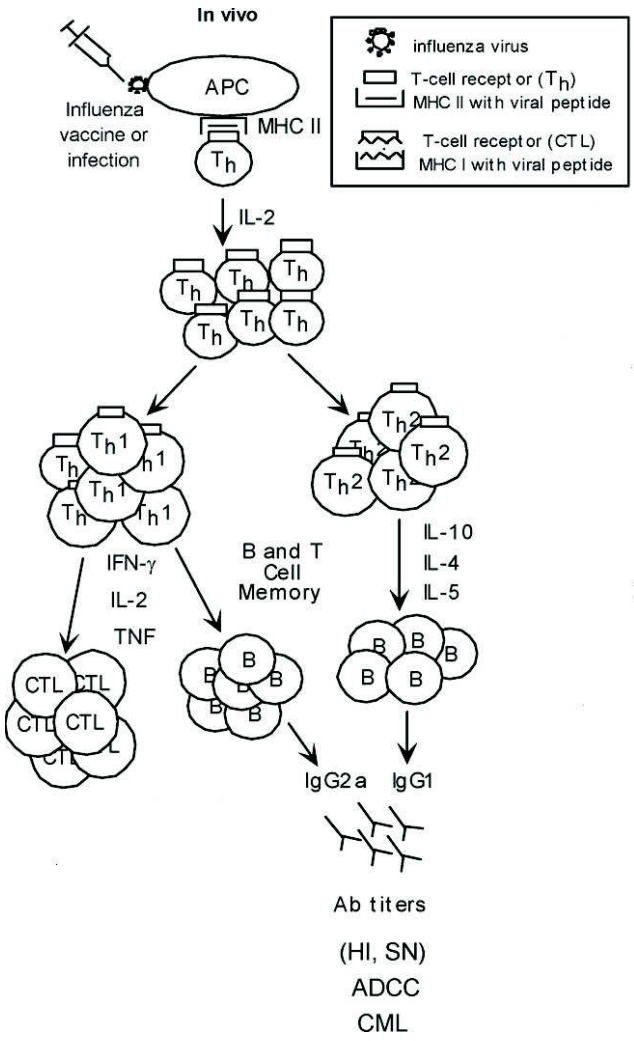


Figure 5: Scheme of the response to vaccination; *APC, antigen presenting cell; Th, helper T-cell; type 1, Th1; type 2, Th2; CTL, cytotoxic T lymphocyte; B, B cell; IL, interleukin; IFN, interferon; TNF, tumor necrosis factor; Grz B, granzyme B; Ab, antibody; HI, hemaglutination inhibition; SN, serum neutralization; ADCC, antibody dependent cell-mediated cytotoxicity, CML, complement-mediated lysis.*

2 . Aim of the study

Human herpesvirus-8 (HHV-8) is the etiological agent of Kaposi's sarcoma (KS), a lympho-angioproliferative disease present in four different variants among the population: 1) Classic KS, 2) AIDS-Associated KS, 3) Endemic (African) KS and 4) Iatrogenic KS. To avoid the interference of factors related to the other variants of KS, we studied patients affected by the classic Mediterranean variant of the disease, that is mainly found in aged men of Eastern European and Mediterranean origin. HHV-8 has a deep impact on B cells through different mechanisms: 1) affecting their development; 2) allowing infected B cells to escape from the control of the immune system; 3) triggering clonal B cell proliferation by affecting their cell cycle check-points and 4) mimicking cell signals that control cell proliferation.
Moreover, B cells of peripheral lymphoid organs and blood of cKS patients represent a major virus reservoir endowed with the ability to sustain the lytic reactivation and dissemination of the virus. Importantly, lymphoproliferative disorders of B cell origin, including primary effusion lymphoma (PEL) and multicentric Castleman's disease (MCD), can occur concomitantly with cKS, confirming the existence of a deep pathogenetic linkage between these malignancies. The persistence of HHV-8 infection is the result of a delicate balance between immune control, viral latency, viral reactivation and persistent replication. However, only a minor proportion of infected hosts eventually develop malignancies due to immune breakdown. Thus, despite the high prevalence of HHV-8 infection in the European population (24.1% in Italian population), the majority of HHV-8-positive subjects do not develop clinically evident malignancies.
To better understand the role of B cells in chronic HHV-8 infection and associated malignancies, we first characterized peripheral B cells in HHV-8 positive patients with or without cKS compared to healthy controls by flow cytometry. Peripheral B cells were counted, their different subsets were characterized in depth and functional assays were performed to test their state of activation, spontaneous apoptosis and proliferation rate.
In the second part of this study we investigated whether the chronic HHV-8 infection in B cells could alter the functionality of these cells and particularly may impact on humoral responses to antigenic stimulation. To this purpose, we assessed the protective efficacy and safety of licensed influenza vaccination in the same patients. The advantage of this T-dipendent stimulation relies on the fact that influenza vaccination is a common practice of cKS patients, as cKS mainly occurs in the elderly, when influenza vaccination is recommended because people aged 65 and older are at a higher risk of debilitating complications, hospitalizations and deaths from influenza. To assess the protective efficacy, we measured the antibody response using standard parameters: seroconversion, seroprotection and GMT. Also, we evaluated anti-nuclear antibodies (ANA) levels in the cKS patients sera to determine if autoreactive B cells, occuring frequently in transitional and MZ-like B cell developmental stages, could be activated by the vaccination.
Moreover, we monitored HHV-8 plasma viral load to evaluate whether any progression of the disease occured after influenza vaccination; side effects were monitored through a daily diary card and finally B cell subpopulations were evaluated after vaccination.
To our knowledge, this is the first study that describes altered B cell homehostasis in HHV-8 infected subjects and evaluates the benefit of influenza vaccination in KS patients; this observation may broaden our understanding of the complex interplay between viral and immune factors leading HHV-8-infected individuals to

develop HHV-8-associated malignancies. Moreover, the second part of this study demonstrates that influenza vaccination is effective and safe in cKS patients despite their B cell alterations. This is relevant because annual influenza vaccination may be particularly recommended for cKS patients considering their advanced age and comorbidity.

3. Materials and Methods

3.1 Patients and Controls

All cKS patients enrolled in this study were followed in the Institute of Dermatological Sciences of the Fondazione IRCCS Ospedale Maggiore Policlinico, Mangiagalli e Regina Elena. The mean age at the time of the first observation was 70.6 years. Approximately 40 % of the patients already had a diagnosis of KS when they were referred to Department for staging and, if required, treatment. Once the clinical and histological diagnosis of KS had been made, the patients underwent the following protocol: clinical photography; physical evaluation, including a careful evaluation of the main lymph nodal sites (in the case of an increase in nodal volume, the patients were asked to undergo lymph node ultrasonography and, if necessary, biopsy); routine blood chemistry examinations and HIV test; HHV8 test (serology by immunoperoxidase); chest X-ray; ENT examination to evaluate pharyngo-oral sites; esophagogastroduodenoscopy and, if necessary, biopsy; rectosygmoidoscopy, except in the case of patients with angina pectoris, arrhythmias (even if treated), severe respiratory insufficiency, untreated severe arterial hypertension or previous vascular accidents (all of these patients are given an opaque clyster with a double contrast medium despite the diagnostic limitations of the procedure); bronchoscopy and bone marrow biopsy, performed only in patients whose overall clinical data suggested possible pulmonary or bone marrow involvement. All patients had histologically confirmed diagnosis of KS, were positive for anti-HHV-8 antibody and negative for HIV. The selection of patients affected by the classic variant of the disease was aimed to avoid the confounding effects of HIV-coinfection or immunosuppressive therapy that are present in the other clinical variants of KS. Patients in systemic chemotherapy were excluded. Staging was performed in accordance with our classification that takes into account the prevalent type of lesions, localization, clinical behavior, evolutive pattern, and presence of complications [113]. Age- and sex-matched healthy subjects were included as controls. Ethics approval was obtained from the local Institutional Review Committee, and a signed informed consent was obtained from all participants. An accurate description of all clinical characteristics of cKS patients and control enrolled for each parts of this study will be reported in result section.

3.2 Sample preparation, staining and flow cytometry for B cell characterization

Sodium-citrate peripheral blood (PB) sample obtained from all individual enrolled were processed within 6 hours after blood withdrawal. 100 µl of PB were washed two times in PBS plus 1% bovine serum albumin (BSA), and the incubated with fetal bovine serum (FBS) for 30 min at 37°C. Then PB were incubated for 30 min with with the following antibodies: FITC-conjugated anti-human IgD (Serotec), kappa light chain (Miltenyi-Biotec) or CD86 (BD); PE-conjugated anti-human IgM (Serotec), lambda light chain (Miltenyi), CD80 (BD), CD38 (Dako) or HLA-DR (Caltag); PerCP-Cy5.5-conjugated anti-human CD19 (BD); PE-Cy7-conjugated anti-human CD20 (eBiociences, San Diego, CA), APC- conjugated anti-human 54 CD5 (BD) and APC-Cy7- conjugated anti-human CD27 (eBiosciences). After incubation, erythrocytes were lysed using by incubation with ammonium chloride for 10 min. All operations were done at 4°C in the dark. Data acquisition and

analysis was performed on a FACSCanto flow cytometer (BD) using a FACSDiva software (v6.1) immediately after staining. As isotype controls were not used, those cells that did not express a certain marker were considered as negative control for positive cells. Lymphocytes were gated on forward (FSC) vs side scatter (SSC) plot and B lymphocytes were identified as cells CD19+ and/or CD20+. 150,000 events were routinely collected to visualize and gate on this population.

3.2.1 Identification of B cell subsets in the peripheral blood

B cells were identified as $CD19^+$ cells and further gated based on their CD27 surface expression. Gated on $CD27^-$ B cells, transitional and pre-naı¨ve B lymphocytes were identified as $CD5^+CD38^{hi}$ and $CD5^+CD38^{int}$ cells, respectively. Gated on $CD27^-CD5^-$ B cells, naı¨ve and $CD27^+$ memory B lymphocytes were identified as IgD^+IgM^+ and IgD^-IgM^- cells, respectively. Gated on $CD27^+$ B cells, switched memory, IgM-only memory and MZ-like B lymphocytes were identified as IgD^-IgM^-, IgD^-IgM^+ and $IgD^{lo}IgM^+$ cells, respectively.

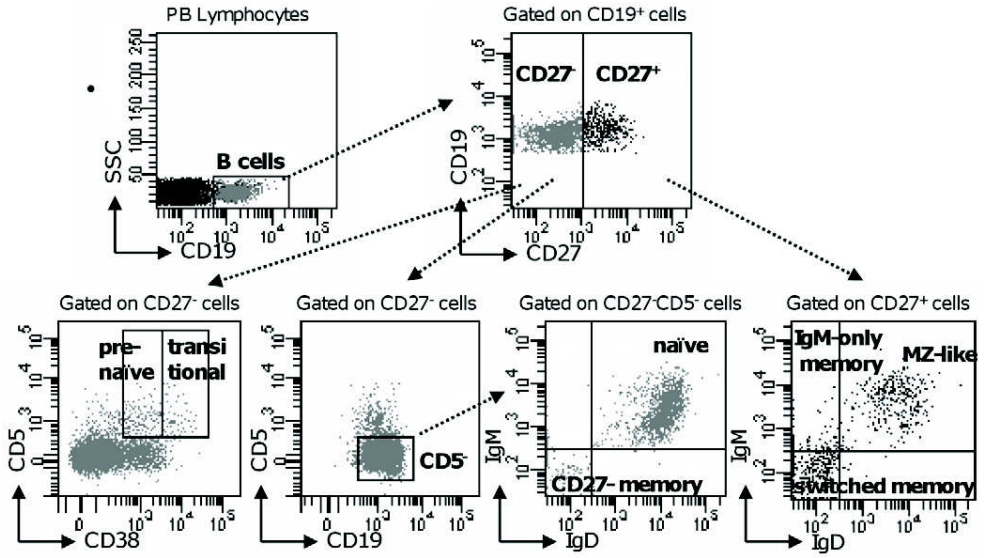


Figure 6. Gating strategy for the identification of B cell subsets in the peripheral blood.

3.2.2 Assessment of spontaneous apoptosis of B cell subsets

The sensitivity of B cell subsets to spontaneous apoptosis was evaluated in cultured cells. Isolated PBMCs from 6 cKS patients and 6 HHV-8-seronegative healthy controls were cultured in 24-well plated for 24 h in RPMI 1640 (Euroclone, Wetherby, UK) containing 10% fetal calf serum (Gibco, Invitrogen Co., Carlsbad, CA, USA) and incubated in a 5% CO_2 incubator at 37°C for 24 hours. Cells were harvested and stained for flow cytometric analysis using an annexin-V APC apoptosis detection kit (BD Pharmingen, San Jose, CA, USA) in combination with anti-CD19-PerCP-Cy5.5, anti-CD27-PE (BD Biosciences) and anti-IgD-FITC (Serotec). Apoptotic cells were identified as annexin-V + cells gated on different B cell subsets.

3.2.3 Ex vivo Ki67 staining

PBMCs from 6 cKS patients and 6 HHV-8-seronegative healthy controls were stained for flow cytometric analysis with anti-CD19-PerCP-Cy5.5 anti-CD27-PE (BD Biosciences). Samples were then fixed, permeabilised and stained for the cell cycleassociated antigen Ki67 (FITC-labeled B56; BD Biosciences), using the Leucoperm Reagent (AbD Serotec), according to the manufacturer's instructions. Cells that had recently divided were identified as $Ki67^+$ cells and provided an estimate of the in vivo cell turnover.

3.2.4 Isolation of human B cells

B lymphocytes were isolated from 7 cKS patients and 6 HSP controls, chosen among subjects with B cell count either over the 75th percentile or under the 25th percentile calculated for each group. Peripheral blood mononuclear cells (PBMCs) were obtained by Ficoll density gradient centrifugation (Ficoll-Hypaque Pharmacia Biotech AB, Sweden). $CD19^+$ cells were purified using immunomagnetic selection with mini-MACS cell isolation Kit (Milteny-Biotec) according to the manufacturer's instructions. Total PBMCs, B cells and non-B cells were further processed for virological analyses.

3.2.5 Assessment of HHV-8 infection in isolated cells

Isolated PBMCs, B cells and non-B cells from 7 cKS patients and 6 HSP controls were tested for the presence and load of HHV-8 DNA sequences. Briefly, DNA was extracted from at least $2x10^5$ cells, and HHV-8 viral load was measured by quantitative real-time polymerase chain reaction (PCR), and results were reported as gEq/105 cells.

3.2.6 IL-6, IL-7 and BAFF measurement in plasma samples

Plasma samples were obtained under sterile conditions from single donors. The amounts of plasmatic IL-6, IL-7 and BAFF were determined by ELISA kits from R&D Systems (Minneapolis, MN). All individual steps were performed according to the manufacturer's instructions.

3.2.7 Immunogenicity assessment

Serum samples were assessed for HAI antibodies to each hemagglutinin of the H1N1, H3N2 and B strains contained in the vaccine, using a standard microtiter assay, as previously described (Grilli *et al.*, 1997; Pregliasco *et al.*, 2001). HAI antibody titers that were below the detection limit (i.e., <1:10) were assigned a value of 1:5. The co-primary immunogenicity outcomes were: a) the proportion of subjects with antibody titer ≥1:40 (seroprotection rate); b) the proportion of subjects with either a prevaccination titer ≤1:10 and a postvaccination titer ≥1:40 or a prevaccination titer ≥1:40 and a minimum 4-fold increase in postvaccination titer (seroconversion rate); c) the geometric mean titer (GMT); and d) the geometric mean of the within-subject ratios of pre- and postvaccination reciprocal antibody titers (GMT increase).

3.2.8 Safety assessment

At first visit, enrolled subjects were given a diary card containing a list of solicited adverse events and their grades. On the immunization day, subjects were observed at the study site for a period of 30 min after vaccination to detect any immediate adverse reactions. For the next 7 days, subjects were educated to record the severity of solicited local and systemic adverse events, axillary temperature and concomitant medications in the diary card. Subjects used a standard scale to grade adverse events .

3.2.9 Determination of plasmatic HHV-8 DNA and anti-HHV-8 antibodies

cKS patients underwent measurement of plasmatic HHV-8 DNA before, and 4 and 12 weeks after vaccination. HHV-8 DNA was quantified by HHV-8 Q-PCR Alert Kit (Nanogen Advanced Diagnostics, Milan, Italy), according to the manufacturer's instructions. Results were expressed in genome Equivalents (gEq). Anti-HHV-8 specific IgG antibodies were tested in serum samples using a commercial immunofluorescence assay (HHV-8 IgG IFA, Biotrin, Ireland).

3.2.10 ANA testing

ANA were monitored in the sera before and after immunization by a standard indirect immunofluorescence technique on Hep-2 cells (Virgo ANA-Hep-2 IgG Kit, Hemagen Diagnostics Inc, Columbia, MD). ANA titer of 1:80 or higher was considered positive.

3.2.11 Statistical Analysis

For comparison between B cell subpopulations, data are presented as mean ± SE. Comparisons of samples to establish the statistical significance of difference were determined by the Student t test for independent samples. The Mann-Whitney U test was also used when indicated. All statistical analyses assumed a 2-sided significance level of 0.05. Data analyses were performed with Openstat3 software.
To assess vaccine immunogenicity, pre- and post-vaccination HAI antibody titers were compared within each group using Wilcoxon signed-rank tests. For comparison between patients and controls, differences of HAI antibody titers were tested by use of the Mann-Whitney *U* test; proportions were compared by use of χ^2 tests and Fisher's exact tests, as appropriate. All analyses were performed in

GraphPad Prism 4.03 (GraphPad Software, San Diego, CA), using a cutoff of $P < .05$ for 2-tailed tests.

4. Results

4.1 Characterization of b lymphocytes and their subpopulations in cks patients

4.1.1 Clinical characteristic of cKS patients

Forty-seven cKS patients were included in the study, 38 males and 9 females, mean age 72 years (range 49-95). All patients had histologically confirmed diagnosis of KS, were positive for anti-HHV-8 antibody and negative for HIV. Staging was performed in accordance with our classification that takes into account the prevalent type of lesions, localization, clinical behavior, evolutive pattern, and presence of complications. Patients in systemic chemotherapy were excluded. B cells characterization was performed at a single time point on fresh peripheral blood samples; staging at this time is reported in Table 3. Forty-three age- and sex-matched HHV-8-seronegative healthy subjects were included as controls. Ten age- and sex-matched HHV-8-seropositive controls without KS were also enrolled. None of the subjects had suffered from chronic inflammatory, autoimmune and cancer disease other than KS, nor they had clinically evident infections. Peripheral blood samples were obtained by venipuncture and collected into sodium citrate Vacutainer tubes (Becton Dickinson, San Jose, CA, US). Ethics approval was obtained from the local Institutional Review Committee, and a signed informed consent was obtained from all participants.

Table 3. Clinical characteristics of patients with cKS

Characteristics	Healthy controls	cKS patients
No. of patients	43	47
Age, yr[a]	74.4 ± 5	72.2 ± 1.4
Sex, no.		
Male		39
Female		8
KS stage[b], no.		
I: maculo-nodular		
A		15
B		14
II: infiltrative		
A		4
B		7
III: florid		
A		1
B		3
IV: disseminated		
A		0
B		3

[a]Mean ± standard error.
[b]cKS patients were classified according to our classification that takes into account the prevalent type of lesions, localization, clinical behaviour, evolutive pattern and presence of complications. A = slow evolution; B = rapid evolution; rapid denotes an increase in the total number of nodules /plaques or in the total area of plaques in the three months following the last examination.

4.1.2 Absolute number of total B cells and their subpopulations in cKS patients

4.1.2.1 Absolute number of total B cells

We examined the number and the frequency of B cells and their subpopulations in the peripheral blood of 47 cKS patients compared with 43 healthy controls, by using 6-color flow cytometry. B lymphocytes were identified as cells positive for staining with anti-CD19. As show in Figure 7, B cells were increased in cKS patients (234 ± 35 vs 129 ± 13 cells/µl, cKS patients vs healthy controls; $P < 0.05$). The frequency of B cells within lymphocytes in healthy aged controls was 6.7% ± 0.5, as expected and it was significantly higher in cKS patients (10.9% ± 1.1; $P < 0.001$).

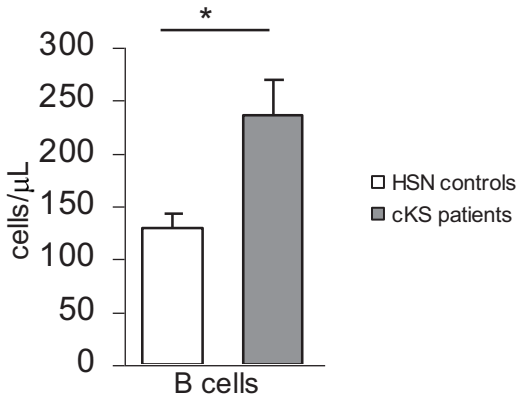


Figure 7: Absolute number of circulating B cells in control subjects (white) and in cKS patients (grey). Data presented as mean ± SE. The statistical significance was determined by the two-tailed Mann–Whitney test.

4.1.2.2 Absolute number of preimmune/natural effector and antigen-experienced B cell compartment

To more precisely define the phenotype of the expanded populations we examined the distribution of B cells along the various steps of peripheral B cell maturation and differentiation. Gated on B cells, the expression of CD27 was used to roughly discriminate between naïve ($CD27^-$) and memory ($CD27^+$) B cells. As shown in Figure 8, the analysis of $CD27^-$ and $CD27^+$ compartments showed that the absolute number of $CD27^-$ B lymphocytes was significantly higher in cKS that controls (172

± 24 vs 86 ± 9 cells/µl; $P < 0.002$), while there was no difference between the absolute number of $CD27^+$ B cells (59 ± 12 vs 43 ± 6 cells/µl, *P*=ns). This resulted reflected the significant increase of the frequency of $CD27^-$ (73.6% ± 2.1 vs 67.1% ± 2.6; $P < 0.05$).

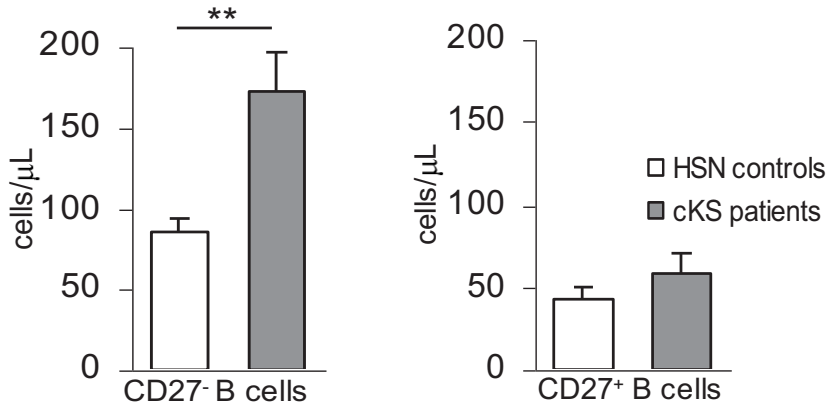


Figure 8: Absolute number of circulating B cells in control subjects (white) and in cKS patients (grey). Data presented as mean ± SE. The statistical significance was determined by the two-tailed Mann–Whitney test.

Within $CD27^-$ cells, we defined transitional B cells, pre-naïve B cells, unswitched naïve B cells and $CD27^-$ memory B cells. The absolute number of transitional (15 ± 2 vs 5 ± 2 cells/µl) , pre-naïve (27 ± 4 vs 7 ± 1 cells/µl) and unswitched naïve B cells (98 ± 15 vs 66 ± 10 cells/µl), all belonging to the preimmune/natural effector B cell compartment, resulted significantly higher in cKS patients than healthy controls ($P < 0.002$, $P < 0.0001$ and $P < 0.05$, respectively). The only $CD27^-$ B cell subset that was not expanded in cKS patients was represented by $CD27^-$, isotype-switched, memory B cells (14 ± 2 vs 17 ± 2 cells/µl).
Within $CD27^+$ cells, we defined switched memory, IgM memory and MZ-like cells. Among $CD27^+$ lymphocytes, only the subset of MZ-like B cells, a subpopulation that has been suggested to generate a preimmune repertoire was markedly expanded in cKS patients (42 ± 12 vs 17 ± 2 cells/µl*;* $P < 0.05$), while switched (19 ± 2 vs 24 ± 4 cells/µl) and IgM memory B cells (2 ± 0.3 vs 2 ± 0.4 cells/µl), all belonging to the antigen-experienced B cell compartment, did not change (Figure 9). Similar results were observed when the frequency of B cell subpopulations rather than their absolute count were considered.

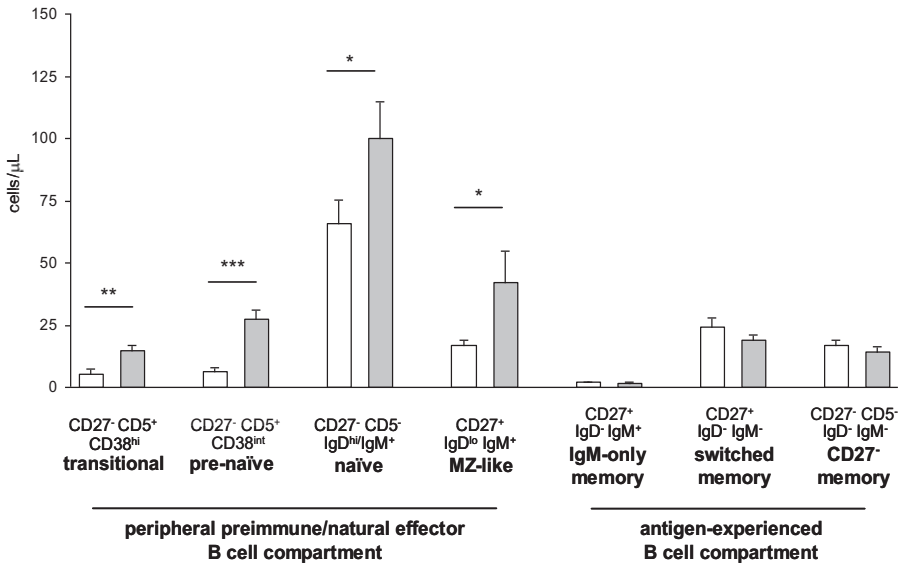


Figure 9: Absolute number of circulating B cell subsets in control subjects (white) and in cKSpatients (grey). Data presented as mean ± SE. The statistical significance was determined by the two-tailed Mann–Whitney test.

4.1.2.3 B cells spontaneous apoptosis and in vivo turnover in cKS patients and healthy controls

To futher understand the mechanisms possibly involved in the expansion of distinct B cell subsets in cKS patients, we examined two parameters that can impact on the size of cell populations and that can be measured in the periphery: cell apoptosis and cell turnover.
The level of spontaneous apoptosis of cultured B cells was detected by annexin V binding. We observed that apoptosis was significantly lower in cKS patients than in healthy donors ($P < .05$) (Figure 10 and 11).

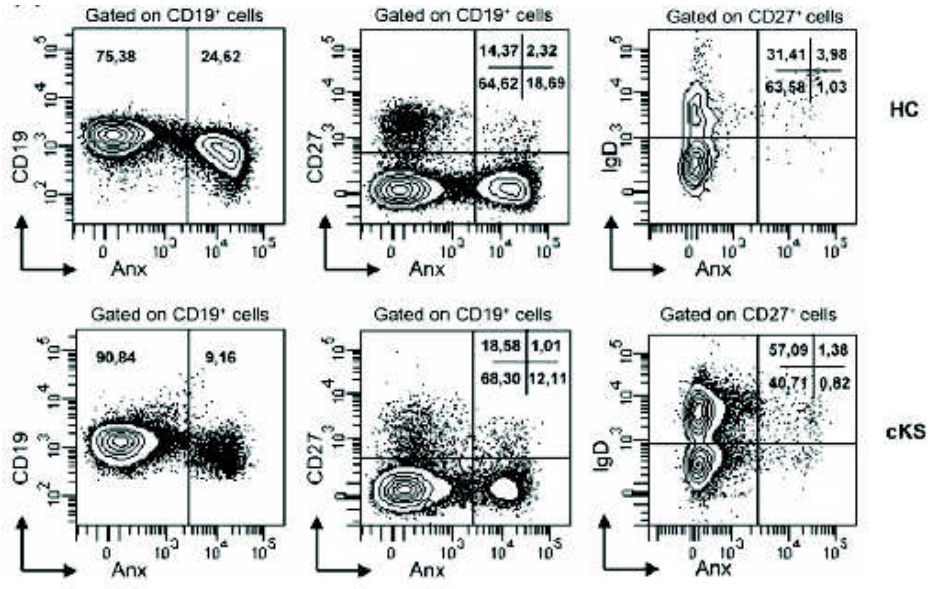


Figure 10: Representative flow cytometric analysis showing annexin V binding gated on total B cells, CD27-, MZ-like and switched memory B cells; comparison between one healthy control (HC) (upper row) and one cKS patients (lower row).

In Figure 11, we observed significantly lower apoptosis in $CD27^-$ B cells in cKS patients than healthy controls ($P < 0.05$), in particular in transitional, pre-naïve, naïve and $CD27^+$ IgD^{lo}, consisting in MZ-like B cells ($P < 0.02$). Accordingly, $CD27^+$ IgD^- cells that mainly consist of switched memory B cells did not vary between cKS patients and healthy controls.

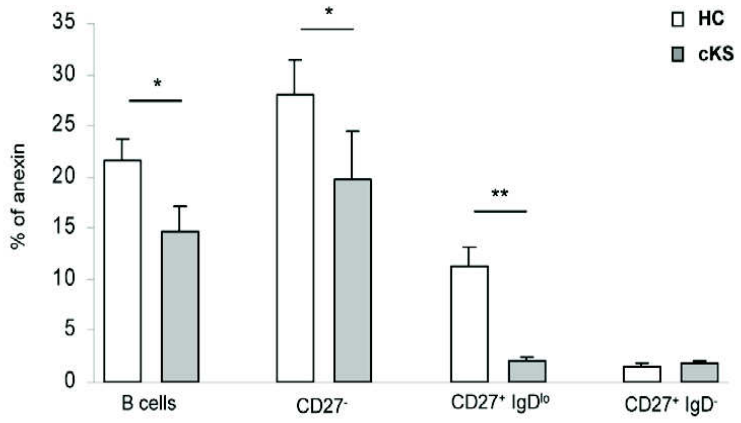


Figure 11: B cells from cKS patients (grey) showed significantly lower annexin V binding than B cells from HCs (white). Data shown as mean ± SE. P-values calculated using the Student t test for independent samples.

To estimate the in vivo turnover of B cells, we analyzed the expression of Ki67, a nuclear antigen that identifies recently divided cells. A representative flow cytometric analysis is shown in Figure 12. The proportion of Ki67 + cells was higher within $CD27^+$ than $CD27^-$ B cells, as expected. As shown in Figure 13, the percentage of Ki67+ cells among total B cells, as well as $CD27^-$ and $CD27^+$ B cells did not differ between cKS patients and healthy controls.

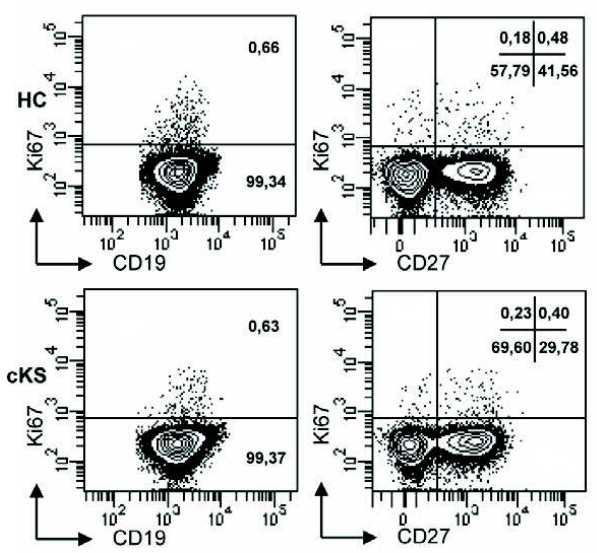


Figure 12: Representative flow cytometric analysis showing Ki67 binding gated on total B cells, CD27+ and CD27+ B cells, as indicated; comparison between one healthy control (upper row) and one cKS patient (lower row).

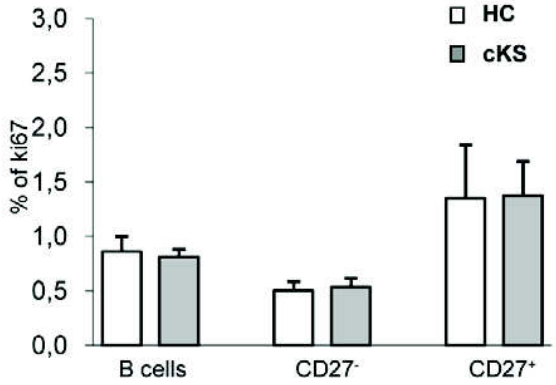
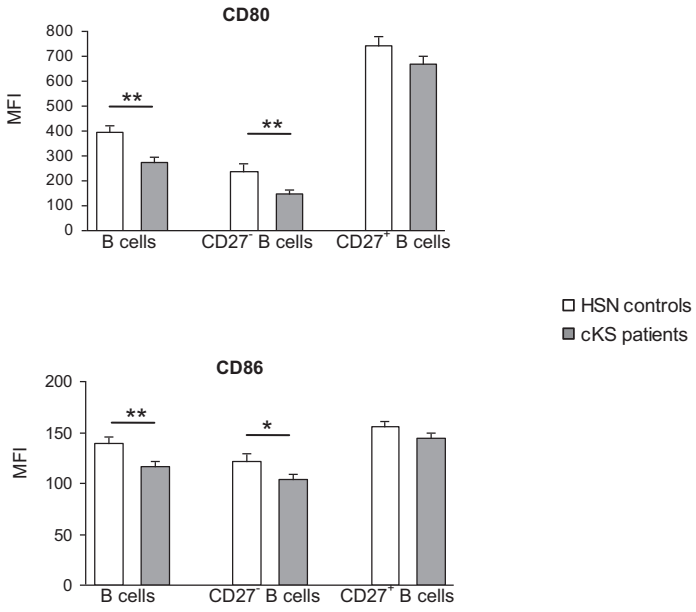


Figure 13: The proportion of Ki67+ cells within total B cells, CD272 and CD27+ B cells did not differ between cKS patients (grey) and healthy controls (white). Data shown as mean ± SE. P-values calculated using the Student t test for independent samples.

4.1.3 Expression of activation and maturation markers on B cells

To assess the activation state of B cell subsets, we investigated expression of various activation markers, including CD80, CD86, and HLA-DR. Moreover, we evaluated other cellular markers as CD20 expression, a putative calcium channel that is down-regulated upon B cell activation and that is expressed by the majority of human B-cell lymphomas and leukemias. All the data are expressed as MFI.

Expression of CD80 (264 ± 22 vs 385 ±29, cKS patients vs healthy controls; $P < 0.005$) and CD86 (111 ± 4 vs 142 ± 6; $P < 0.01$) was examined on CD19+ B cells, $CD27^-$ immature-naïve B cells (CD80: 145 ± 17 vs 239 ± 27, $P < 0.01$; CD86: 103 ± 5 vs 122 ± 7, $P < 0.05$), and $CD27^+$ memory B cells (CD80: 666 ± 33 vs 742 ± 35, P = ns; 144 ± 5 vs 156 ± 5; P = ns) and were lower in cKS patients compared with healthy controls; also the expression of HLA-DR molecules tended to be lower on B cells from KS patients than healthy donors (1627 ± 115 vs 1794 ± 177, P = ns), on $CD27^-$ cells (1779 ± 142 vs 1727 ± 196, P = ns) and on $CD27^+$ cells (1549 ± 106 vs 1860 ± 165, P = ns).
B lymphocytes from cKS patients expressed higher surface levels of the molecule CD20 even if we considered total B cells (8082 ± 556 vs 5292 ± 388; $P < 0.001$), $CD27^-$ cells (6478 ± 548 vs 4503 ± 343; $P < 0.001$) and $CD27^+$ cells (11202 ± 667 vs 6901 ± 548; $P < 0.001$), (Figure 14).



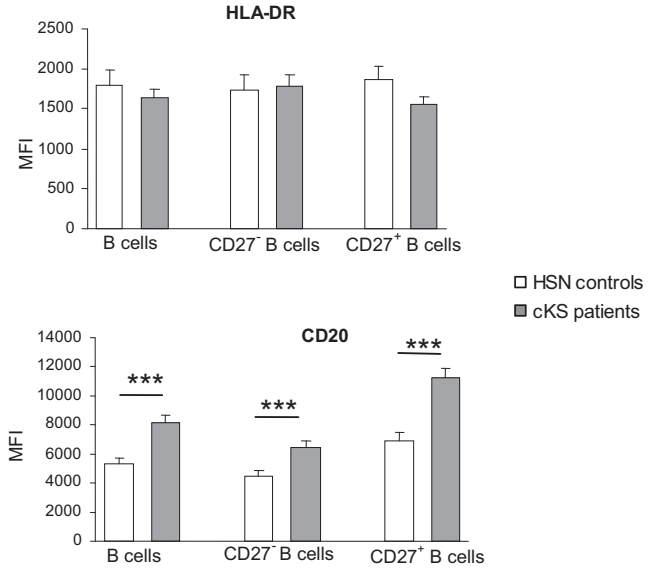
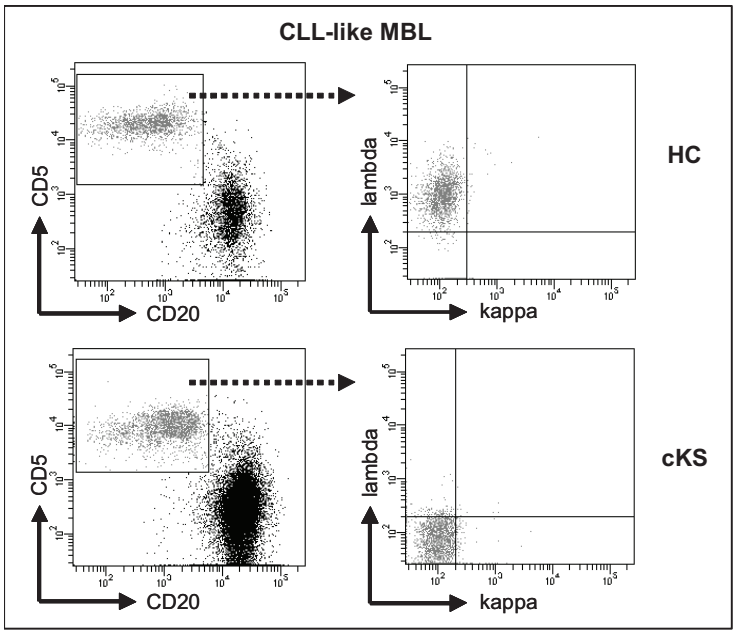


Figure 14: Expression of activation markers on B cells in control subjects (white) and in cKS patients (grey). Data presented as mean ± SE. The statistical significance was determined by the two-tailed Mann–Whitney test.

4.1.4 Frequency of monoclonal B cell expansion

Due to the involvement of HHV-8 infection in lymphoproliferative disorders, we further characterized B cells to analyse the frequency of monoclonal B cell expansions in cKS patients.
We analyzed the immunophenotype of B cells and evaluated the k/λ light chain ratio in cKS patients and healthy controls, according to the diagnostic criteria proposed for monoclonal B lymphocytosis (MBL). In particular, CLL-like MBL was defined based on the distinct $CD5^{bright}CD20^{dim}$ expression pattern on $CD19^{+}$ B cells. Atypical CLL-like and non-CLL-like MBL were defined based on the occurrence of an unbalanced k/λ ratio (more than 3:1 or less than 1:3) within $CD5^{+}$ and $CD5^{-}$ B cells, respectively.
On the base of this strategy, we identified MBL in 4 of 43 healthy controls (9.3%; 1 CLL-like, 2 atypical CCL-like and 1 non-CLL-like MBL. Moreover, we identified MBL in 2 of 47 cKS patents (4.3%; 1 CLL-like and 1 non-CLL-like MBL). k light chain restriction was observed in 2 MBL clones (2 HCs), λ light chain restriction in 2 clones (1 healthy control and 1 cKS patient), while negativity for both light chains was evident in the remaining 2 cases (Figure 15).



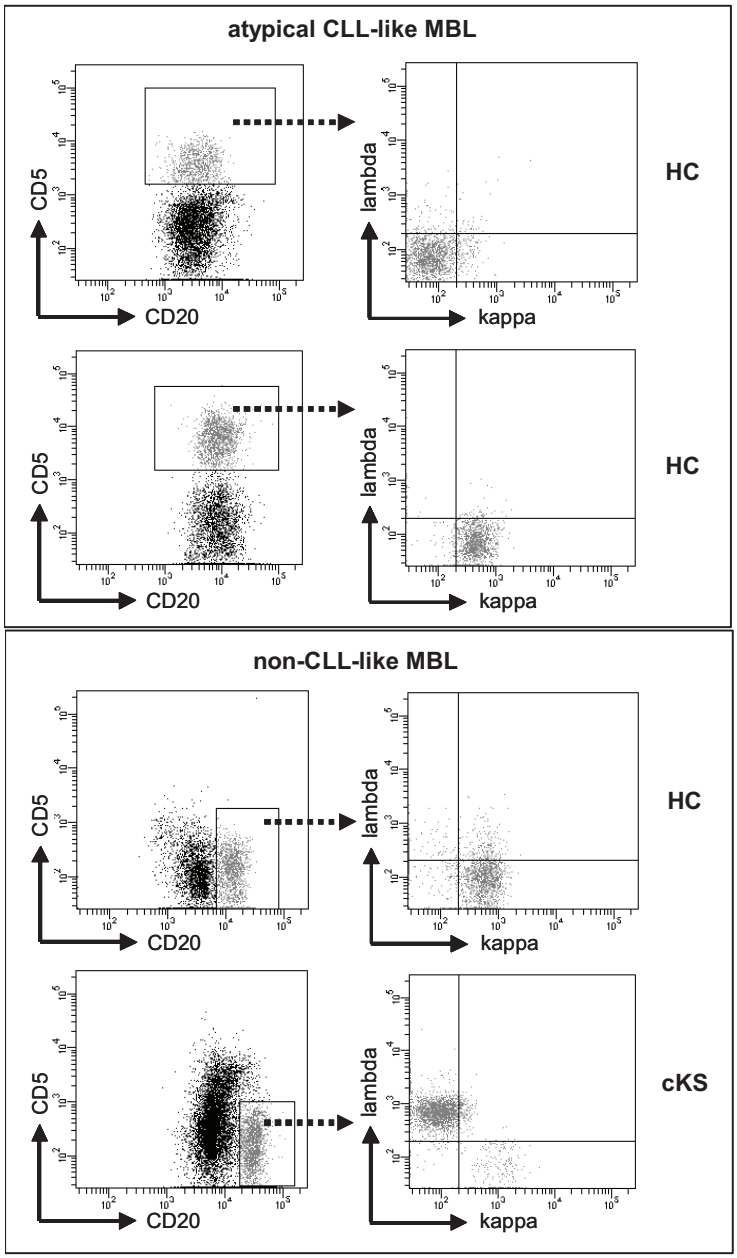


Figure15: Representative flow cytometric analysis showing CLL-like MBL, atypical CCL-like and non-CLL-like MBL; comparison between one HC (upper row) and one cKS patient (lower row).

4.1.5 IL-6, IL-7 and BAFF plasmatic levels

To evaluate whether the changes in B cells observed in cKS patients could be affected by cytokines involved in the growth and differentiation of B lymphocytes, we measured the plasmatic levels of IL-6, IL-7 and BAFF in our samples.
Low levels of IL-6 were found in 67% of the cKS patients (mean ± SE: 7.9 ± 1.7 pg/ml) and 30% of the healthy controls (4.9 ±1.9 pg/ml) and did not significantly differ between groups. Also the plasmatic levels of IL-7, detected in all the samples, showed no differences between cKS patients and controls (8.6 ± 1.0 and 7.1 ± 0.7 pg/ml, respectively). Also, Baff did not change between cKS patients and controls (0.91 ± 0.05 and 1.17 ± 0.09 ng/ml, respectively). No correlation was observed between the number of B cells or their subsets and the plasmatic levels of cytokines.

4.1.6 Changes in B cells from cKS patients and HSP controls do not correlate with HHV-8 load

We analyzed possible correlations between the number of B cells or their subsets and the viral gEq measured in plasma. No correlation was observed between the count of either total, $CD27^-$, $CD27^+$ B cells or their subsets and plasmatic HHV-8 gEq. To investigate whether B cell infection in correlated with the expansion of B cells and their subsets, we selected 7 cKS patients and 6 HSP controls with B cell count either over the 75^{th} percentile or under the 25th percentile calculated for each group, and determined the presence of HHV-8 gEq in their PBMCs and isolated B cell and non-B fractions. B cells from 6 of 7 cKS patients and from 3 of 6 HSP controls were found to harbor HHV-8 DNA sequences (6–475 HHV-8 gEq/10^5 cells). HHV-8 DNA was detected in B cells from 5 of 7 subjects with B cell expansion and from 4 of 6 subjects without expansion; moreover, there is no difference in the number of HHV-8 gEq of individuals with or without B cell expansions (Table 4). Indeed we found no correlation between B cell viral infection and B cell changes. Also, no correlation was observed between anti- HHV-8 antibody titres and B cell changes.

Table 4. HHV-8 DNA in isolated peripheral blood mononuclear cells, B cells and non-B cells from patients wit and HHV-8 seropositive healthy controls according to their changes in B cell count

				B cell count		gEq/10^5cells		
patient	**gender**	**age**	**KS stage[b]**	**percentile**	**10^3/mmc**	**PBMC**	**nonB**	**B**
KS 5	M	51	1A	<25th percentile	491,16	<5	<5	5
KS 4	M	69	1B	>75th percentile	355,41	11	<5	6
KS 1	M	69	2A	>75th percentile	302,59	244	98	475
KS 2	M	66	2A	>75th percentile	277,78	< 5	< 5	10
KS 3	M	74	1A	>75th percentile	90,83	< 5	< 5	15
KS 7	M	75	1B	<25th percentile	69,38	< 5	< 5	< 5
KS 6	M	62	3A	<25th percentile	43,88	<5	< 5	20
HHV-8+ 1				>75th percentile	297,79	< 5	< 5	< 5
HHV-8+ 3				>75th percentile	249,67	19	< 5	15
HHV-8+ 2				>75th percentile	244,16	< 5	< 5	< 5
HHV-8+ 4				<25th percentile	144,66	< 5	< 5	11
HHV-8+ 5				<25th percentile	125,46	27	<5	294
HHV-8+ 6				<25th percentile	107,26	< 5	< 5	< 5

4.1.7 Absolute number of total B cells and B cell subpopulation in HHV-8 seropositive controls

To investigate whether the changes observed in circulating B cells from cKS patients were dependent on HHV-8 infection or whether they were related to the presence of the tumor, we included in our study ten age- and sex-matched HHV-8 seropositive (HSP) controls without KS. We analyzed the number of B cells and their subsets and the state of activation of B cells in HSP subjects compared with healthy controls and cKS patients. As reported in Table 5, HSP controls showed changes in B cells that were similar to those observed in cKS patients. In particular, HSP had higher number of total B cells than healthy controls (P<0.01); similarly to what we observed in cKS patients, the expansion of B cells in HSP controls was restricted to the $CD27^-$ population, that was increased in HSP compared with healthy controls (P<0.002). As observed in cKS patients, HSP controls showed expanded transitional, pre-naı¨ve, naı¨ve and MZ-like B cells, while the number of IgM-only, switched and $CD27^-$ memory B cells was unaffected.
We further investigate B cells phenotype in HSP controls; we analyzed B cell expression of CD80 and CD86 and observed that MFI of both costimulatory molecules was significantly lower on B cells from HSP than healthy controls (P<0.02 and P<0.05 for CD80 and CD86, respectively).

Table 5: Analysis of B cells from healthy HHV-8-seropositive (HSP) compared with HHV-8-seronegative controls and cKS patients.

	healthy controls n=43	cKS patients n=47	HHV8+ seropositive controls n=10	p values HSP healthy controls	p values HSP vs KS
B cells (cells/μL)	128,53 ± 12,85	234,41 ± 35,28	187,19 ± 19,26	0,0100	ns
CD27- B cells (cells/μL)	85,56 ± 9,11	172,38 ± 24,30	140,6 ± 17,13	0,0020	ns
CD27+ B cells (cells/μL)	42,97 ± 5,79	58,54 ± 12,21	50,37 ± 9,12	ns	ns
Preimmune pools					
transitional (cells/μL)	5,29 ± 2,03	14,67 ± 2,03	8,39 ± 1,56	0,0010	ns
pre-naive (cells/μL)	6,58 ± 1,35	27,15 ± 3,73	15,46 ± 2,89	0,0100	ns
naive (cells/μL)	65,7 ± 9,6	98,24 ± 14,87	88,93 ± 12,81	0,0500	ns
MZ-like (cells/μL)	16,59 ± 2,38	42,12 ± 12,45	22,39 ± 4,17	0,0500	ns
Antigen-experienced pools					
IgM-only memory (cells/μL)	1,96 ± 0,38	1,68 ± 0,26	1,86 ± 0,42	ns	ns
switched memory (cells/μL)	24,35 ± 3,54	14,28 ± 2,17	25,5 ± 5,69	ns	ns
$CD27^-$ memory (cells/μL)	16,95 ± 2,15	19,03 ± 2,23	13,11 ± 2,43	ns	ns
B cell immunophenotype					
CD80 (MFI)	394,28 ± 29,15	273,24 ± 22,94	272,50 ± 35,86	0,0200	ns
CD86 (MFI)	139,02 ± 6,31	116,73 ± 4,83	113,78 ± 9,63	0,0500	ns

4.2 Efficacy and safety of seasonal influenza vaccination in cKS patients

4.2.1 Clinical characteristics of vaccinated cKS patients

Forty-six cKS patients and 44 healthy controls were enrolled into the study. Their demographic characteristics at enrollment are reported in Table 6. KS staging was performed in accordance with our classification that takes into account the prevalent type of lesions, localization, clinical behavior, evolutive pattern, and presence of complications [42, 265]. All patients were positive for anti-HHV-8 antibody and negative for human immunodeficiency virus (HIV). Patients in systemic chemotherapy were excluded. None of the applicants had acute febrile illness or signs of severe acute illness at the time of vaccination, a history of anaphylaxis due to vaccine components, or other inappropriate condition to receive vaccination, nor they had suffered from autoimmune and cancer disease other than KS. All patients and controls reported receiving influenza vaccine in the previous year.

Table 6. Baseline characteristics of study participants

Characteristic	healthy controls (n = 44)	cKS patients (n = 46)
Male sex	36 (82)	38 (85)
Age	74 ± 5	74 ± 6
KS stage		
I: maculo-nodular		
A: slow		18
B: rapid		14
II: infiltrative		
A: slow		3
B: rapid		6
III: florid		
A: slow		1
B: rapid		2
IV: disseminated		
A: slow		0
B: rapid		2

NOTE. A = slow evolution; B = rapid evolution.

4.2.2 Immunogenicity

Serum samples were assessed for HAI antibodies to each hemagglutinin of the H1N1, H3N2 and B strains contained in the vaccine, using a standard microtiter assay HAI antibody titers.

Titers below the detection limit (i.e. <1:10) were assigned a value of 1:5. Geometric mean titers (GMTs) and seroprotection rates (defined as HI titers >1:40) were the main outcome measures. Seroconversion was defined by a postvaccination HI titer of at least 1:40 combined with at least a four-fold increase in titer in accordance to European and international guidance.

The co-primary immunogenicity outcomes were: a) the proportion of subjects with antibody titer ≥1:40 (seroprotection rate); b) the proportion of subjects with either a prevaccination titer ≤1:10 and a postvaccination titer ≥1:40 or a prevaccination titer ≥1:40 and a minimum 4-fold increase in postvaccination titer (seroconversion rate); c) the geometric mean titer (GMT); and d) the geometric mean of the within-subject ratios of pre- and postvaccination reciprocal antibody titers (GMT increase). Antibody responses were measured at baseline, 4- and 12 weeks after vaccination.

Among both cKS patients and controls, seasonal influenza vaccine led to significant increases in HAI antibodies to all three strains, with an increase in the antibody level against H1N1, H3N2 and B strains of ≥ 4-fold (P < 0.001), (H1N1: 9.6-fold vs 7,5-fold; H3N2: 4.4-fold vs 6.0-fold; B 8.2-fold vs 15.5-fold, cKS patients vs healthy controls, respectively).

At baseline, seroprotection rate was comparable between cKS patients and healthy controls for H1N1 (57% vs 59%) and H3N2 (39% vs 55%) but not for B: cKS patients showed higher rate (48% vs 27%) of seroprotection. After 4- and 12 weeks post vaccination, seroprotection rate was comparable between cKS patients and healthy controls for all the 3 antigens (H1N1: 87% vs 77%; H3N2: 65% vs 82%; B: 83% vs 77%).

Also, seroconversion rate was similar in cKS patients and healthy controls at any time point and for each antigen (4 weeks: H1N1: 74% vs 73%; H3N2: 43% vs 68%; B: 65% vs 73%; 12 weeks: 57% vs 50%; H3N2: 26% vs 36%; B: 43% vs 59%).

The GMT was similar in cKS patients and healthy controls at any time point for H1N1 antigen (baseline: 25.5 vs 28.3; 4 weeks: 244.0 vs 212.5; 12 weeks: 114.9 vs 113.1) but was lower at 4 and 12 weeks in cKS patients for H3N2 antigen (P<0.05 and P<0.02, respectively) (baseline: 17.7 vs 32.1; 4 weeks: 77.6 vs 193.3; 12 weeks: 47.9 vs 106.2) and was higher at baseline and at 4 weeks in cKS patients for B antihen (P<0.05 and P<0.02, respectively) (baseline: 27.9 vs 11.3; 4 weeks: 229.7 vs 175.9; 12 weeks: 122.0 vs 109.6) (Table 7).

Among cKS patients, no correlation was observed between immunogenicity parameters (seroprotection rate, seroconversion rate, GMT or GMT increase) and either age, cKS stage or HHV-8 viremia. No correlation was observed between pre- and post-vaccination HAI antibodies and any B cell subset.

Table 7. Antibody Titers against Seasonal A/H1N1, A/H3N2 and B Virus before and 4 and 12 Weeks after Receipt of Seasonal Vaccine in cKS Patients Compared with Healthy Controls.

Virus, antibody	healthy controls (n = 44)	cKS patients (n = 46)
A/Brisbane/59/2007 (H1N1)		
Seroprotection rate		
Prevaccination	59	57
4 weeks after vaccination	77	87
12 weeks after vaccination	77	87
Seroconversion rate		
4 weeks after vaccination	73	74
12 weeks after vaccination	50	57
GMT [fold increase]		
Prevaccination	28.3	25.5
4 weeks after vaccination	212.5 [7.5][a]	244.0 [9.6][a]
12 weeks after vaccination	113.1 [4.0][a]	114.9 [4.5][a]
A/Brisbane/10/2007 (H3N2)		
Seroprotection rate		
Prevaccination	55	39
4 weeks after vaccination	82	65
12 weeks after vaccination	82	65
Seroconversion rate		
4 weeks after vaccination	68	43
12 weeks after vaccination	36	26
GMT [fold increase]		
Prevaccination	32.1	17.7
4 weeks after vaccination	193.3 [6.0][a]	77.6 [4.4][a,b]
12 weeks after vaccination	106.2 [3.3][a]	47.9 [2.7][a,c]
B/Florida/4/2006		
Seroprotection rate		
Prevaccination	27	48[d]
4 weeks after vaccination	77	83
12 weeks after vaccination	77	83
Seroconversion rate		
4 weeks after vaccination	73	65
12 weeks after vaccination	59	43
GMT [fold increase]		
Prevaccination	11.3	27.9[b]
4 weeks after vaccination	175.9 [15.5][a]	229.7 [8.2][a,c]
12 weeks after vaccination	109.6 [9.7][a]	122.0 [4.4][a]

NOTE. GMT, geometric mean titer

[a] Wilcoxon signed-rank test, for intragroup postvaccination to prevaccination comparison of GMT: $P < 0.001$

[b] Mann-Whitney U test, for intergroups comparison of GMT: $P < 0.05$

[c] Mann-Whitney U test, for intergroups comparison of GMT: $P < 0.02$

[d] χ^2 test, for intergroup comparison of seroprotection and seroconversion rates: $P < 0.05$

4.2.3 Safety

We evaluated reported symptoms and we found no serious adverse events following influenza vaccination. Similar frequencies of solicited local and systemic reactions to vaccine were reported in the diary card by cKS patients and controls. We considered both local and systemic reactions. The most frequent local reaction reported by both cKS patients and controls was pain at the injection site (33% and 36%, respectively) with or without redness and swelling. Headache and malaise were the most frequently reported systemic reactions, reported by 20% and 10% of subjects, respectively. Mild elevation of body temperature (37.8°C) was only reported by one cKS patient 2 days after vaccination (Table 8).

Table 8: Reported symptoms after vaccination

	healthy controls n=44	cKS patients n=46
Local reaction		
Pain at the injection site	36% (16/44)	33% (15/46)
Systemic reactions		
Headache and malaise	10% (4/44)	20% (9/46)
Mild elevation of body temperature	0% (0/44)	4% (2/46)

4.2.4 HHV-8 virologic rebound

At baseline, 5 cKS patients (11.6 %) had copy number of HHV-8 genome above 250 gEq/ml. Following influenza vaccination, a virologic rebound, defined as an increase of at least 3-fold from baseline [284] was observed in one of these patients and in 3 cKS patients who had undetectable HHV-8 DNA before immunization. Virologic rebound was observed 4 weeks after vaccination in 2 cases and 12 weeks after vaccination in the other 2 cases. None of the 4 patients showing virologic rebound experienced a progression of KS lesions within 12 months starting from vaccine administration.

4.2.5 Anti-HHV-8 titers

At baseline, all cKS patients had positive anti-HHV-8 antibodies, with titers 1:512 or higher. By contrast, all healthy controls were HHV-8 seronegative. Following vaccination, 11 cKS patients (25.0%) showed an increase of anti-HHV-8 antibodies titer from baseline: 3 patients showed a transient increase 4 weeks after vaccination that reverted after 12 weeks; 2 patients showed an increase after 4 weeks that persisted 12 weeks after vaccination; 6 patients showed an increase 12

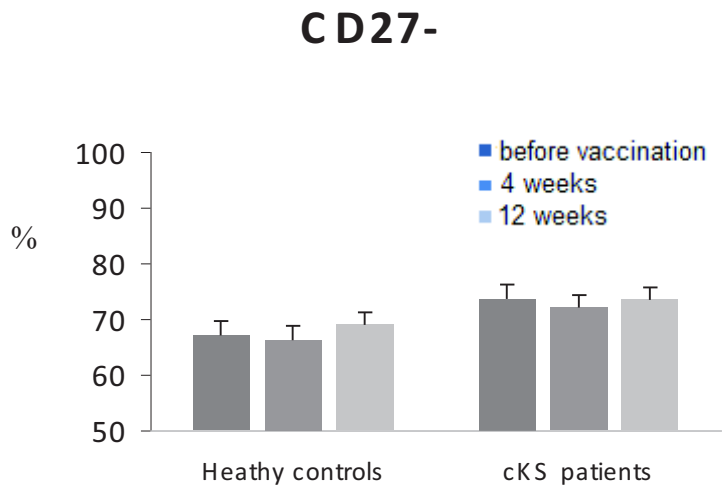
weeks after vaccination. None of the patients with post- to pre-vaccination increase of anti-HHV-8 antibodies showed HHV-8 virologic rebound.

4.2.6 ANA titers

The percentage of subjects positive for ANA before vaccination were similar among cKS patients and controls (15.2% and 22.7%, respectively; Fisher's exact tests: *P* = ns). Among cKS patients, 5 subjects who were initially negative for ANA became positive after immunization (4 subjects after 4 weeks, 1 subject after 12 weeks). Among controls, 2 subjects became positive (1 after 4 weeks, 1 after 12 weeks). In all but 1 subject, positive ANA was detected 12 weeks after vaccination. Moreover, 1 patient and 1 control that were initially positive for ANA showed an increased titer after vaccination. The post- to pre-vaccination changes in the level of ANA did not differ significantly between cKS and healthy controls. No correlation was observed between pre- and post-vaccination ANA titers and any B cell subset.

4.2.7 B cell subpopulation homeostasis after influenza vaccination

We analyzed B cells subpopulations at 4 and 12 weeks post vaccination to determine possible changes in their frequency. cKS patients showed a higher frequency of $CD27^-$ B cells than healthy controls, as previously assessed, in all time points; the frequency of $CD27^-$ and $CD27^+$ B cells did not vary in all the time points, both in healthy control group and in cKS patient group (Figure 16).



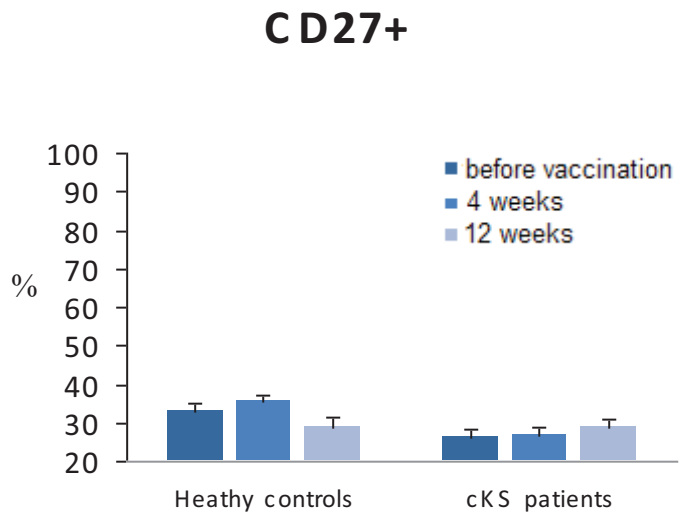


Figure 16: Frequency of CD27- and CD27+ circulating B cells in control subjects and in cKS patients at three time points (dark blue= before vaccination; blue= 30 days after vaccination; light blue= 60 days after vaccination) . Data presented as mean ± SE.

We further analyzed B cells subpopulations; as expected, the frequency of preimmune/natural effector compartment was higher in cKS patients compared to healthy controls at all the three time points. Generally, we did not observed any change in the frequency of B cell subpopulations before and after vaccination, both in healthy controls and cKS patients, with the exception of transitional B cells in cKS patients; indeed, we observed a slight decrease in the frequency at 4 and 12 weeks after vaccination compared to the frequency before influenza vaccination (6,7% vs 3,6% vs 3,1; $P<0.05$) (Figure 17).

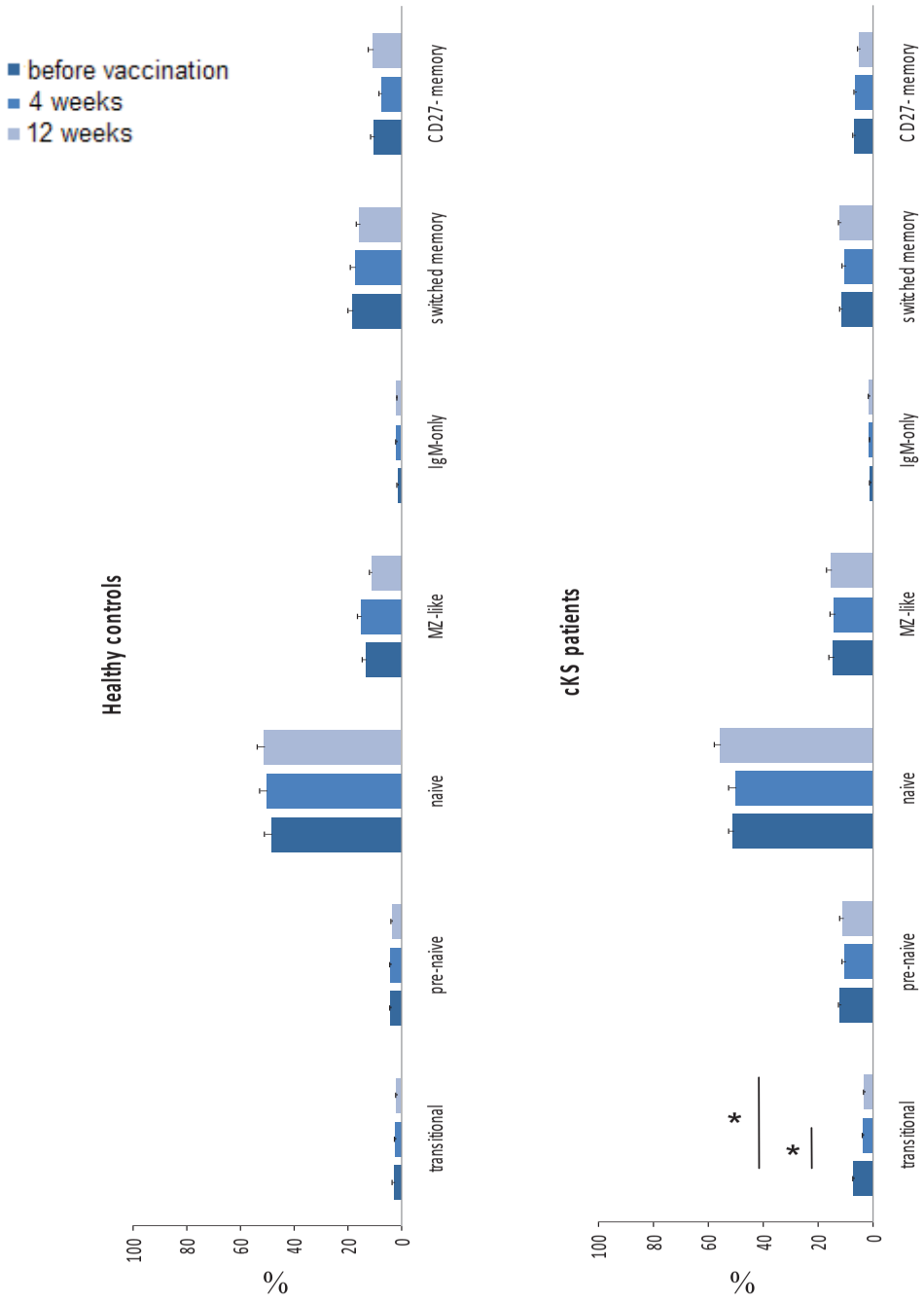


Figure 17: Frequency of in control subjects and in cKS patients at three time points (dark blue= before vaccination; blue= 30 days after vaccination; light blue= 60 days after vaccination) . Data presented as mean ± SE.

5. Discussion

B cells represent the major reservoir of HHV-8, which is the etiologic agent of KS. HHV-8 is a good potential candidate as B cell disregulating agent, because it is able to influence the fate and function of B cells through a number of viral proteins that affect B cell development, allow infected B cells to escape from the control of the immune system, and trigger clonal B cell proliferation by affecting B cell cycle check-points and by mimicking cell signals that control cell proliferation [246,247,248]. In order to better understand the role of B cells in chronic HHV-8 infection and associated malignancies and whether HHV8-infection may be associated with B cell abnormalities, in the first part of our study we characterized circulating B cells in patients with cKS by 6-color flow cytometric assay. Moreover, to ascertain whether B cell alteration occurring in cKS patients were secondary to HHV-8 infection per se, we included a group of healthy HHV-8 seropositive (HSP) controls not affected by KS.

The markers used in the flow cytometric enable the analysis of B cells belonging to either preimmune/natural effector compartment or the antigen-experienced compartment. Within the preimmune/natural effector compartment, transitional, pre-naı¨ve and naı¨ve B cells were identified as cells lacking the expression of CD27 and further defined by the expression of CD38, CD5 and/or Ig isotypes [249]. MZ-like B cells were identified as $CD27^+$ $IgD^{lo}IgM^+$ [250]; these cells are a unique population with attributes of both naı¨ve and memory B cells [251]. Within the antigen-experienced T helper-dependent compartment, memory $CD27^+$ B cells, including IgM-only and switched memory B cells, as well as a subset of $CD27^+IgD^+$ memory B cells [252] were identified.

Our results indicated that cKS patients have a significantly higher number of circulating B cells than healthy controls. Also, analysis of circulating B cells in HSP showed similar levels compared with cKS patients.

Moreover, the expansion of B cells in cKS patients and HSP was evident in the preimmune/natural effector compartment: we observed increased number of transitional, pre-naı¨ve, naı¨ve and MZ B cells, although we did not observe an increased frequency of monoclonal B cell expansions in our patients, while no difference was observed in the antigen-experienced T helper-dependent compartment.

Such B cell perturbation has been reported in other conditions characterized by viral chronic infections and autoimmune diseases. In particular, an increase of $CD27^-$, naïve B lymphocytes not accompanied by expanded memory cells and lacking of B cell activation has been studied in patients with chronic C hepatitis [253]. An expansion of circulating immature transitional B cells has been described in individuals infected with either hepatitis C virus (HCV) or human immunodeficiency virus (HIV) [254,255]. These observations may suggest that diverse viral infections, all characterized by frequent chronicization, share the ability to perturb the homeostasis of B lymphocytes by promoting the accumulation of non-memory B cell subsets. Whether the virus-induced changes of B cells may represent a common strategy acted by diverse viruses to elude the control of B cell-mediated immune responses remains an intriguing possibility that may deserve to be further investigated. Expansion of transitional and MZ like B cells compartment was described in autoimmune disease such as Sjogren Syndrome [256]. We measured the level of autoantibodies and BAFF level in cKS patients and HSP, that are often correlated with the expansion of transitional and MZ B cells in autoimmune diseases, but we didn't observed increased levels compared to

healthy controls. We can hypotize a local increase of BAFF in lymphopoietic organs, not measurable in the serum; other factors known to affect B cell maturation IL-5 and IL-21 [285, 286], were not investigated in this study.
We further analyzed the expression of activation markers on B cell subpopulations. Our results demonstrated that B cells in cKS patients expressed lower levels of activation markers CD80 and CD86 either on $CD27^-$ or $CD27^+$ B cells, suggesting that there is no correlation between the expansion observed in the preimmune/natural effector compartment and the state of activation observed in the total B cell population of HHV-8 infected subjects. Also, the surface expression of CD20, a putative calcium channel that is downregulate upon B cell activation and that is expressed by the majority of human B cell lymphomas and leukemias [257,258], on the contrary is higher on both $CD27^-$ and $CD27^+$ B cells of cKS patients and HSP compared to healthy controls, according to their state of low activation.
Moreover, we analyzed alternative mechanisms that possibly could promote the accumulation of B cells in the preimmune/natural effector compartment: spontaneous apoptosis and *in vivo* turnover. The most important finding was the increased resistance to spontaneous apoptosis that is specific of the preimmune/natural effector compartment while we didn't observe any difference in the rate of proliferation between the two compartments.
It is well known that multiple HHV-8 gene products are able to promote survival of infected cells by increasing resistance to apoptosis, including latency-associated nuclear antigen (LANA), viral Flice-inibitory protein (v-Flip) and the viral chemokine ligands v-CCL1 and v-CCL2 [259] but the hypothesis of direct effects of HHV-8 is unlikely due to the low frequency of infected cells.
So we hypothesized that HHV-8 infection may perturb the homeostasis of B cells in an indirect way, maybe through some mediators that could affect proliferation, differentiation, traffiking and lifespan. Among other factors, IL-6 of either viral or human origin may represent a good candidate factor, as it is a well defined B cell growth factor [260] centrally involved in KS. Its antiapoptotic effects on B cells were proven [261,262] and it is expressed by a multitude of cell types upon HHV-8 infection such as lymphocytes [263,264]. Also, we previously demonstrated that dendritic cells from cKS patients produce higher amounts of IL-6 than controls [265]. The lack of increased human-IL-6 levels in our patients and the lack of correlation between circulating IL-6 and B cell changes cannot rule out either the involvement of v-IL-6 or a local effect exerted by IL-6 within lymphoid tissues.
Cell fate during early B-cell development is also influenced by IL-7, which have a central role in controlling the survival, proliferation and differentiation of B cells [266]; also, increased serum level of IL-7 reactive to $CD4^+$-T cells lymphopenia was proposed to explain the disturbance of peripheral blood B cell homeostasis during pathological condition such as HCV infection and HIV infection. We measured IL-7 in the plasma of cKS patients and HSP but we did not observed increased level, suggesting that this factor did not impact on B cell alteration observed in our cKS patients.
In conclusion, in this first part of the study, we provide evidence that HHV-8 chronic infection induces an expansion of B cells in the preimmune/natural effector compartment but further insight might be gained by additional experiments designed to understand the exact mechanism leading expansion on immature B cells in cKS patients.

In the second part of the study, we investigated whether B cell alterations observed in cKS patients may alter the response to influenza vaccination, in terms of clinical efficacy, humoral response, safety and B cell homeostasis. The majority of cKS patients usually undergo seasonal influenza vaccination due to the fact that cKS mainly occurs in the elderly, when influenza vaccination is recommended, as people aged 65 and older are at a higher risk of debilitating complications, hospitalizations and deaths from influenza [267; 268; 256].
It is well known that aging affects both humoral immune response and cell-mediated immunity quantitatively and qualitatively [269]; influenza virus should stimulate an antiviral response in both B and T lymphocytes resulting in humoral and cell-mediated immunity, respectively. Virus-activated T-cells, through cytokine mediators, stimulate B-cells to differentiate and produce antibodies specific for a particular vaccine strain and antibody responses to influenza vaccination have largely been evaluated by the hemagglutination inhibition assay (HAI).
We evaluated the immune response to influenza vaccine in cKS patients and we observed a significant increase in HAI titers against the three viral strains in both patients and controls; we observed some differences in antibody levels between the two groups, maybe due to the small sample size, but we can assert that the immunogenicity after vaccination was adeguate in both patients and controls, according to the European Agency for Evaluation of Medical Products (EMEA) criteria for adults ≥ 60 years of age.
Influenza vaccination often occurs in September through November while influenza virus exposures occur well through the end of winter. Therefore, we assessed the prolonged effectiveness of influenza vaccine by measuring the seroprotection against all three antigens after 3 months from the vaccination and we observed that seroprotection persisted, demostrating a lasting response to vaccination as already demonstrated [270].
According to the serologic responses that were similar in cKS patients and controls, during the influenza season an equal number of cKS patients and controls (4 and 4 corresponding to 8.7 and 9.1% of subjects, respectively) reported influenza-like illness episodes, defined as temperature ≥37.8°C plus cough or sore throat [272]. All the episodes were registered in a a diary card containing a list of solicited adverse events and their grades during the course of the study. Due to the fact that we enrolled subjects from all the regions of Italy, it was quite impossible to documente any adverse event that would be consistent with either influenza infection or influenza-like illness by using methods for virus detection such as by an influenza-positive nasopharyngeal wash [271].
All the patients and controls who experienced influenza-like illness had achieved postvaccination seroprotection to at least one viral strain. This is in accordance with previous reports that a HAI titer ≥ 40, generally considered predictive of protection in adults, may not provide the same level of protection from influenza infection in the elderly [273, 274, 275].
The rate and intensity of reported solicited local and systemic reactions to vaccination were similar in cKS patients and controls; the most frequent local reaction was pain at the injection site while the most frequent sistemic reaction was headache with malaise. This data are in accordance with other studies using virosomal influenza vaccines [273].

At baseline, we observed an expansion of transitional and other preimmune B cells, that represent B cell developmental stages in which autoreactive B cells occur more frequently [256]; to assess if a stimulation such as vaccination could lead to a autoantibodies production, we measured anti-ANA antibodies in the serum. Influenza vaccination induced the production of new autoantibodies in a higher, although not significant, proportion of cKS patients (10.9%) than controls (4.5%). Importantly, newly synthesized autoantibodies after vaccination had no apparent clinical significance as no subject among patients and controls developed clinical signs of autoimmune disease; our observation are in line with a previous work, in which patients affected by Sistemic Lupus Erythematosus were vaccinated, the safety of the influenza vaccination was demonstrated and no correlation with the worsening of the disease was found, although a transient increase of ANA levels after vaccination was observed in some patients [276].
To futher investigate the safety of influenza vaccination in our cKS patients, we also evaluated vaccination-induced HHV-8 virologic rebound in the serum. It is already demonstrated in HIV infected patients that vaccination leads to an increase il plasma virus [277, 278] but this has been shown to be a transient phenomenon and did not have any clinical significance [277]. We observed an increase of HHV-8 DNA in 4 patients (8.7%), a lower frequency compared to HIV infected patients.
Vaccination-induced HIV rebound was suggested to be a consequence of the activation of the immune system, which is a normal response to antigenic stimulation, and may enhance viral replication due to the critical role played by cellular activation in the initiation and propagation of HIV infection; also, Fauci proposed that inappropriate immune activation and elevated secretion of certain cytokines may compound the pathogenic process in HIV infected patients [279]. In our cKS patients immune activation from influenza vaccination may promote HHV-8 rebound through the production of inflammatory cytokines, which have been suggested to play an important role in the initiation and progression of KS through reactivation of HHV-8 [280, 281]. It is important to note, however, that in no patient virologic rebound was associated with progression of KS lesions.
Finally, we monitored whether a perturbation in the frequency of B cell subpopulations may occur after vaccination. We observed that cKS patients showed a higher frequency of immature/preimmune B cells than healthy controls in all the time points, as previously determined, but we observed a slight decrease in the average of transitional B cells in cKS patients after vaccination. Transitional B cell decrease was observed in other infectious diseases such as parasitic disease; reduced lymphopoiesis and enhanced apoptosis were the events evoked to explain this observation [282]. We can hypothize that antigenic stimuli such as new infection or influenza vaccination can perturbe temporarily the frequency of transitional B cells through an excess production of pro-inflammatory citokines that negatively affects this lymphoid compartment [283]. Our study has some limitations: the last time point was 3 months after vaccination; anyway, the slight decrease in transitional B cell frequency was not associated with progression of the disease.
To our knowledge, this is the first report concerning the effects of influenza vaccination on cKS patients and we demonstrated the safety and immunogenicity induced by influenza vaccination in cKS patients. This is relevant because annual influenza vaccination may be particularly recommended for cKS patients considering their advanced age and comorbidity.

5.1 Conclusion

In conclusion in this study we report, for the first time to our knowledge, that HHV-8 chronic infection promotes a perturbation of peripheral B cell homeostasis characterized by expansion of B cells of the preimmune/natural effector compartment, in patients with cKS without clinical signs of humoral immune deficiency. This novel observation may broaden our understanding of the complex interplay of viral and cellular factors leading HHV-8-infected individuals to develop either KS or lymphoproliferative malignancies. The discrepancy between the high number of HHV-8 infected subjects and the lower rate of subjects developing an HHV8- related disease suggests that immune control of the virus is lost in these subjects. It could be interesting to monitor a cohort of HHV-8-infected subjects that will and will not develop primary effusion lymphoma and the plasmablastic form of multicentric Castleman's disease with the aim to broaden the comprehension of the mechanisms of viral tumorigenesis. Perspectively, long-term follow-up analysis of peripheral B cell changes in cKS patients will greatly help the comprehension of the mechanisms of viral tumorigenesis and possibly enable the development of efficient targeted therapies and novel treatment approaches. Moreover, the availability of a cell culture system an/or an animal model will extend the studies of KS pathogenesis and also the evaluation of antiviral agents and pathogenesis-based therapies able to block viral products interfering with the cellular defense mechanisms such as cell cycle shutdown and apoptosis.

Moreover, we demonstrated that influenza vaccination is safe and immunogenic on cKS patients compared to age- and sex-matched controls. It is well established that there is a complex regulatory network that actively inhibits immune responses during chronic viral infections such as HIV, HCV, or HBV infection so that we suggest that this study could be extended to evaluate the immune response to influenza vaccination in other subjects affected by chronic infection, to better determine similarities and differences of humoral response in chronic inflammation diseases.

Generally, HHV-8 infection is considered harmless or unimportant but it plays a role in shaping the normal immune response and induce diseases in rare individuals.

Future studies need to specifically focus on defining the mechanistic details of the disease and immunologic responses to viruses, so that we can intervene appropriately.

6. References

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