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Kaposi's Sarcoma-Associated Herpesvirus Latency Locus Compensates for Interleukin-6 in Initial B Cell Activation

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Interleukin 6 (IL-6) is considered a proliferation and survival factor for B cells. To assess the role of IL-6 in Kaposi sarcoma-associated herpesvirus (KSHV) latency, KSHV latency locus-transgenic mice (referred to as latency mice) lacking IL-6 were evaluated. IL-6$^{-/-}$ latency mice had the same phenotypes as the latency mice, i.e., increased frequency of marginal zone B cells, hyperplasia, and hyperglobulinemia, indicating that the KSHV latency locus, which includes all viral microRNAs (miRNAs), can compensate for lack of IL-6 in premalignant B cell activation.

Aberrant interleukin 6 (IL-6) signaling is associated with tumorigenesis in preclinical and clinical models of lymphoma. Mice overexpressing IL-6 develop IgG1 plasmacytoma (1, 2), while IL-6 knockout (IL-6$^{-/-}$) mice exhibit a lower incidence of chemically induced liver cancer and resistance to pristane-induced plasmacytoma (3, 4). Anti-IL-6 (siltuximab) and anti-IL-6 receptor (tocilizumab) antibodies have clinical efficacy against multicentric Castleman's disease (MCD) (5–8). MCD is a preneoplastic hyperplasia of B cells, the plasmablastic variant of which is associated with Kaposi sarcoma-associated herpesvirus (KSHV). KSHV is also the etiologic agent of Kaposi sarcoma, primary effusion lymphoma (PEL) (9), and an IL-6-associated disorder called KSHV inflammatory cytokine syndrome (KICS) (10, 11). PELs produce IL-6 (12, 13), and an anti-IL-6 antibody inhibited growth of PELs both *in vitro* and *in vivo* (14, 15); however, some PEL cell lines, such as BCBL-1, do not express or depend on IL-6 (15, 16). KSHV encodes a viral IL-6 homolog which is expressed at various levels in PEL (17, 18). To understand the role of endogenous IL-6 in premalignant KSHV pathogenesis, we investigated KSHV transgenic mice without IL-6.

KSHV latency-associated nuclear antigen (LANA)-transgenic mice develop B cell hyperplasia, which is dependent on CD19 (19, 20). C57BL/6J KSHV latency locus-transgenic mice (referred to as latency mice), which in addition to LANA express all viral microRNAs (miRNAs), exhibit consistent expansion of the marginal zone (MZ) and plasma cells (PCs), as well as hypergammaglobulinemia (21). These mice were crossed to isogenic IL-6$^{-/-}$ knockout mice (B6;129S2-*Il6*^{tm1Kopf}/J). Genotyping was performed according to the supplier's protocol and as published elsewhere (Fig. 1A) (20). Splenocytes from 7- to 8-week-old mice were cultured with 100 ng/ml lipopolysaccharides (LPS) for 48 h, and IL-6 levels were measured by enzyme-linked immunosorbent assay (ELISA) (eBioscience). In response, cells from C57BL/6J and the latency mice secreted IL-6, while cells from IL-6$^{-/-}$ and IL-6$^{-/-}$ latency mice did not (Fig. 1B).

IL-6 plays important roles in immunoglobulin (Ig) secretion by sustaining long-lived PCs (reviewed in reference 22). IgG production is impaired in IL-6$^{-/-}$ mice (23–25), whereas IgG hyperglobulinemia is a consistent phenotype of the latency mice (Fig. 2A). To examine the genetic interaction between KSHV latency genes and lack of IL-6, we examined serum Ig levels by ELISA as

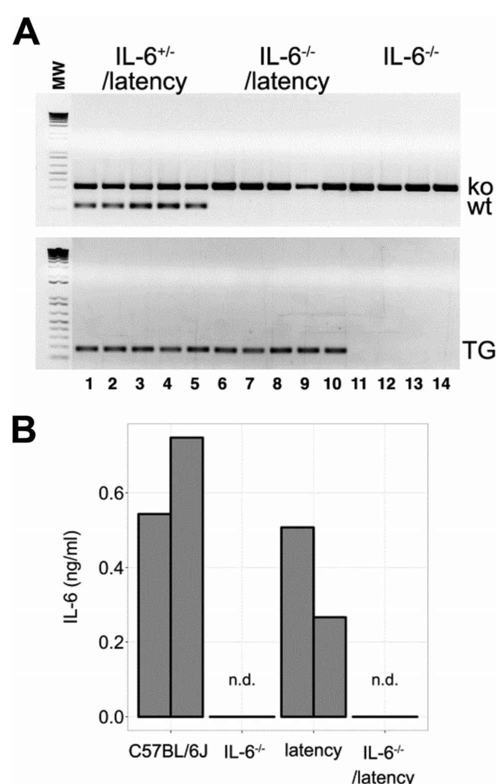

FIG 1 IL-6$^{-/-}$ latency transgenic mice. (A) Agarose gel of PCR products obtained with primers specific for IL-6$^{-/-}$ and a latency gene. (B) Level of IL-6 in supernatant from splenocytes which were cultured with 100 ng/ml LPS (from *Escherichia coli* 0111:B4; Invivogen). n.d., not detected.

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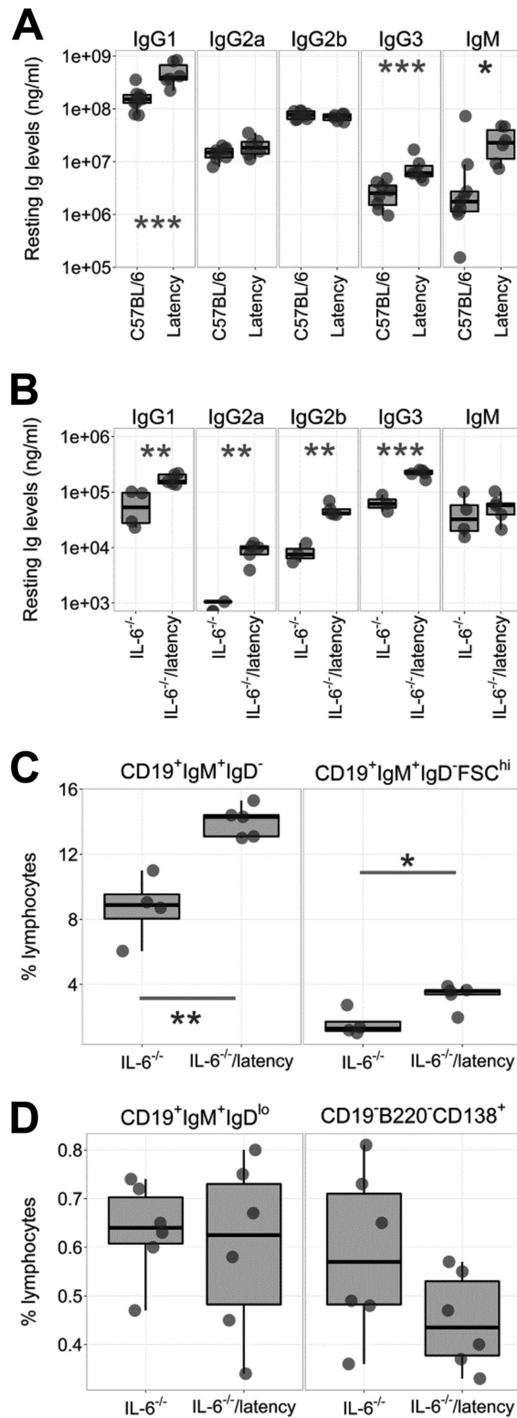

FIG 2 Phenotypes of IL-6$^{-/-}$ latency mice. Box plots show the 1st and 3rd quartiles, with the median indicated by the band. Whiskers extend to 1.5× the interquartile range. (A) Peripheral Ig levels were plotted from 9 C57BL/6J and 6 latency mice as determined by ELISA. This represents a meta-analysis. Some of the data points were previously reported (21). (B) Peripheral Ig levels were plotted from IL-6$^{-/-}$ and IL-6$^{-/-}$ latency mice as determined by ELISA (n = 5). (C) Splenic marginal zone B cells (CD19$^+$ IgM$^+$ IgD$^-$) and activated marginal zone B cells (CD19$^+$ IgM$^+$ IgD$^-$ FSC^{hi}) (n = 5). (D) Immature B cells (CD19$^+$ IgM$^+$ IgD^{lo}) and plasma cells (CD19$^-$ B220$^-$ CD138$^+$) in BM were plotted (n = 6). Data are frequencies, shown as a percentage of total lymphocytes. *, **, and ***, $P \leq 0.05$, $P \leq 0.005$, and $P \leq 0.0005$, respectively, by ANOVA.

TABLE 1 MZ frequency of the IL-6$^{-/-}$, IL-6$^{-/-}$ latency, latency, and C57BL/6 mice[a]

		IL-6$^{-/-}$			IL-6$^{-/-}$ latency[b]				Latency[b]				C57BL/6				
Class	Marker	%	SD	n	%	SD	n	P (vs IL-6$^{-/-}$)	%	SD	n	P (vs IL-6$^{-/-}$ latency)	%	SD	n	P (vs IL-6$^{-/-}$)	P (vs latency)
MZ	CD19$^+$ IgM$^+$ IgD$^-$	8.7	2.0	4	14.0	1.0	5	0.001	15.8	2.2	9	NS	11.8	3.1	8	NS	0.01
Activated MZ	CD19$^+$ IgM$^+$ IgD$^-$ FSC^{hi}	1.6	0.8	4	3.3	0.8	5	0.01	4.5	1.5	9	NS	2.6	1.1	8	NS	0.01

[a] The background of the latency, IL-6$^{-/-}$, and IL-6$^{-/-}$ latency mouse was C57BL/6. Splenic cells were analyzed using flow cytometry. Values are the percentages in total lymphocytes. MZ, marginal zone B cells; SD, standard deviation; n, number of animal analyzed; NS, not significant.

[b] This represents a meta-analysis. Frequencies of MZ and activated MZ from the latency and C57BL/6 mice were previously reported (21).

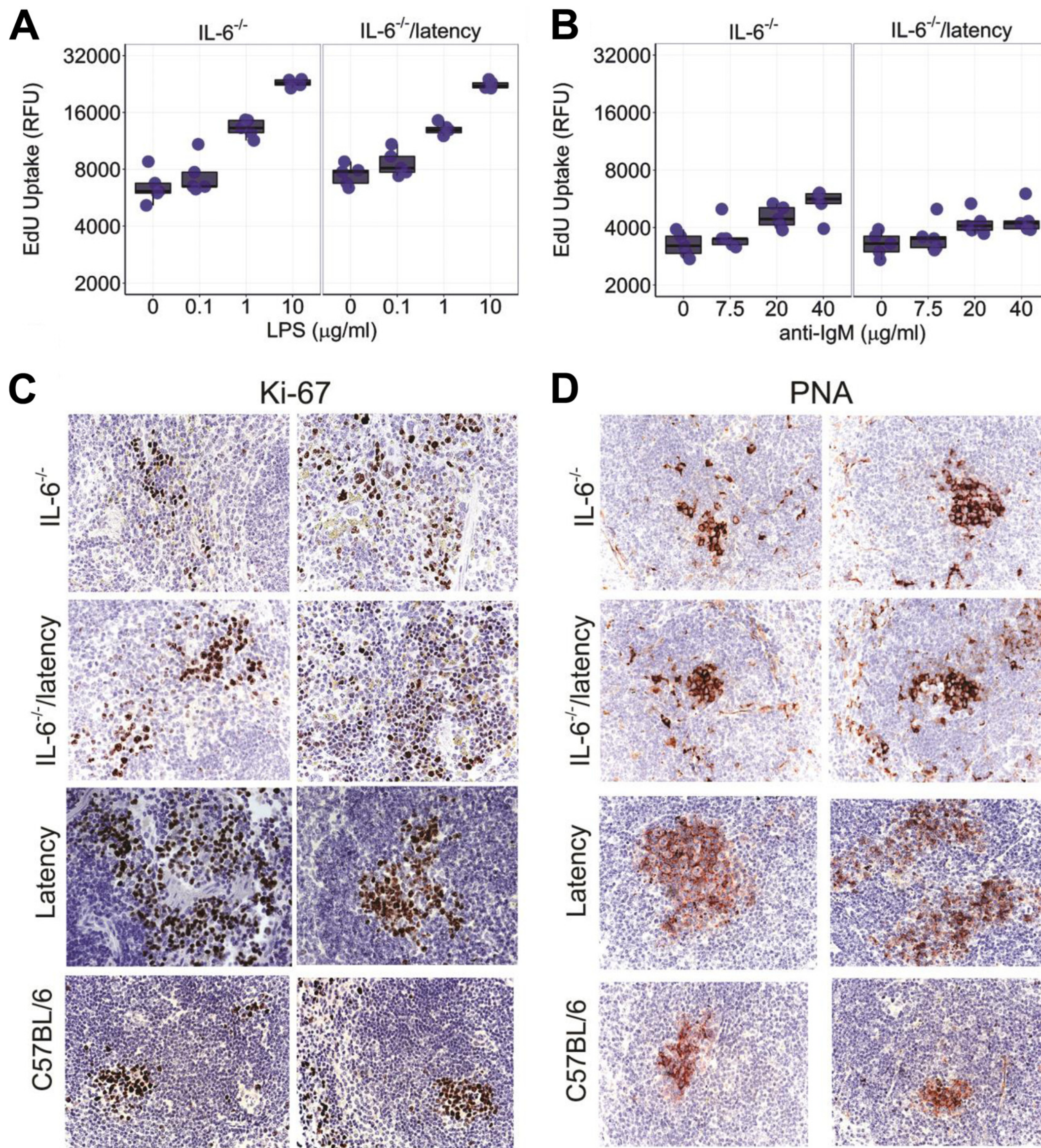

FIG 3 The degree of *ex vivo* proliferation of B cells was plotted in response to LPS (A) or anti-IgM antibody (B). The box plots ($n = 5$) show the 1st and 3rd quartiles, with the median indicated by the band. Whiskers extend to 1.5× the interquartile range. Significance levels were determined by ANOVA. Immunostaining of spleen sections (5 IL-6$^{-/-}$ or IL$^{-/-}$ latency mice; 3 C57BL/6 or latency mice) with Ki-67 (C) and PNA (D). Representative images are shown. Magnification, ×400.

described previously (21). Total IgG1, IgG2a, IgG2b, and IgG3 levels were higher in IL-6$^{-/-}$ latency than IL-6$^{-/-}$ mice (Fig. 2B). This demonstrates that KSHV latent genes (and miRNAs) in B cells can compensate for the absence of IL-6 in B cell maturation.

To assess the effect of IL-6 on B cell development, cells were isolated from the spleen or bone marrow (BM) of 7- to 11-week-old IL-6$^{-/-}$ and IL-6$^{-/-}$ latency mice and analyzed by flow cytometry. IL-6$^{-/-}$ latency mice displayed the same phenotypes as the latency mice, specifically, increased frequencies of MZ cells (CD19$^+$ IgM$^+$ IgD$^-$) and activated MZ B cells (CD19$^+$ IgM$^+$

TABLE 2 *Ex vivo* proliferation of splenic B cells from IL-6$^{-/-}$, IL-6$^{-/-}$ latency, latency, and C57BL/6 mice[a]

	IL-6$^{-/-}$	IL-6$^{-/-}$ latency		Latency[b]		C57BL/6[b]		
Treatment	Slope	Slope	*P* (vs IL-6$^{-/-}$)	Slope	*P* (vs IL-6$^{-/-}$ latency)	Slope	*P* (vs IL-6$^{-/-}$)	*P* (vs IL-6$^{-/-}$ latency)
LPS	1,474.0	1,348.9	NS	1,629.5	NS	910.4	NS	NS
Anti-IgM	53.8	29.4	NS	229.3	0.001	158.1	0.04	0.007

[a] The background of the latency, IL-6$^{-/-}$, and IL-6$^{-/-}$ latency mice was C57BL/6. The degree of *ex vivo* proliferation of splenic B cells was analyzed by ANOVA. Five mice per genotype were analyzed. The regression line slope and its 95% confidence interval were calculated based on *t* distribution for each genotype. NS, not significant.
[b] This represents a meta-analysis. The *ex vivo* response to LPS and anti-IgM of the latency and C57BL/6 mice was previously reported (21).

TABLE 3 *In vivo* phenotypes[a]

Tissue	Phenotype	IL-6$^{-/-}$		IL-6$^{-/-}$ latency			Latency[b]			
		No. of mice	Rate (%)	No. of mice	Rate (%)	*P* (vs IL-6$^{-/-}$)	No. of mice	Rate (%)	*P* (vs IL-6$^{-/-}$)	*P* (vs IL-6$^{-/-}$ latency)
Spleen	Lymphoma	0	0.0	1	3.0	1	8	16.0	0.093	0.076
	Lymphoid hyperplasia	6	31.6	9	27.3	1	13	26.0	1	1
	Normal	13	68.4	23	69.7		29	58.0		
	Severe EMH	2	10.5	3	9.1	1	11	22.0	0.491	0.147
Liver	Severe EMH	5	26.3	2	6.7	0.085	NA			
	Total no. of mice	19		33			50			
MLN	Lymphoma	2	14.3	0	0.0	0.333	NA			
	Lymphoid hyperplasia	12	85.7	20	95.2	1	NA			
	Normal	0	0.0	1	4.8		NA			
	Total no. of mice	14		21			NA			

[a] Data were analyzed using ANOVA. A *P* value of ≤0.05 was regarded as significant. NA, data not available.
[b] This represents a meta-analysis. The phenotype of the latency mice was previously reported (21).

IgD^{-} FSC^{hi}) in spleen (Fig. 2C; Table 1). Frequencies of mature B cells and plasma cells (PC) were not significantly different. This held true for spleen (data not shown) and BM (Fig. 2D). This suggests that the KSHV latency-associated hyperplasia of naive, pre-GC B cells was not dependent on IL-6.

Ex vivo hyperresponsiveness to B cell stimuli is a distinct phenotype of KSHV latency mice (21). To test the hypothesis that this phenotype was dependent on IL-6, splenic B cells from 5- to 6-week-old IL-6$^{-/-}$ and IL-6$^{-/-}$ latency mice were purified by negative selection and cultured with LPS or anti-IgM antibody. Proliferation was measured using a Click-iT EdU assay (Invitrogen). The Toll-like receptor (TLR)-driven responsiveness to LPS persisted in the absence of IL-6 (Fig. 3A; Table 2); however, the B cell receptor (BCR)-driven responsiveness was damped in the absence of IL-6 (Fig. 3B; Table 2). This is consistent with a mechanism whereby BCR-induced B cell proliferation was aided by an IL-6 feedback loop but TLR-induced proliferation was not.

To document the *in vivo* phenotype of IL-6$^{-/-}$ latency mice, formalin-fixed, paraffin-embedded spleen sections were prepared and evaluated by immunohistochemistry for two established proliferation markers, Ki-67 and peanut agglutinin (PNA). We could not find a difference in staining degree or intensity between IL-6$^{-/-}$ and IL-6$^{-/-}$ latency mice; however, the staining was stronger in tissues from latency mice than in either C57BL/6 or IL-6$^{-/-}$ latency mice (Fig. 3C and D).

The rates of lymphoma and splenic lymphoid hyperplasia as ascertained by hematoxylin and eosin (H&E) stain showed no difference (Table 3). Mesenteric lymph nodes (MLNs) are chronically stimulated by gut microbiota. MiR-155 knockout mice exhibit a lower frequency of germinal center (GC) B cells in MLNs (26). KSHV encodes miR-K12-11, which is an ortholog of miR-155 and rescued the miR-155 deficiency-associated phenotype in MLNs (21, 27). IL-6$^{-/-}$ latency mice, which express miR-K12-11, had the same rate of lymphoid hyperplasia as the latency mice (Table 3). Another phenotype of KSHV latency mice is severe extramedullary hematopoiesis (EMH). The rates of EMH in spleen and liver were not dependent on the presence of IL-6 (Table 3).

B cell hyperplasia in spleen and proliferation in lymph nodes, as scored here, are complex and progressive phenotypes that develop over months and are subject to a multitude of compensatory and counterbalancing mechanisms in the animal. Clearly, IL-6 is needed for maximal B cell function and sustained proliferation during normal development and in preneoplastic scenarios, such as MCD. However, many mechanisms are known to relieve the dependence on IL-6 in disease. Augmented NF-κB signaling was found in an IL-6-independent variant of multiple myeloma (28). Activation of STAT3 (signal transducers and activators of transcription 3), an important intermediate of IL-6 signaling, was observed in IL-6-independent plasmacytomas (29). The genetic experiment presented here suggests that KSHV latent genes, too, can compensate for IL-6 in the early stages of B cell activation and development. The miRNAs are known for their profound effects on cell lineage development and differentiation. The KSHV miRNAs, most likely, evolved to foster initial infection and latent persistence in naive B cells and eventual, preferential expansion of infected cells. One mechanism to facilitate this "goal" would be to compensate for limiting host activators, such as IL-6.

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