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Limited IL-6 production following infection with murine gammaherpesvirus 68

Brief Report

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Summary. Murine gammaherpesvirus 68 (γHV-68) was found to induce IL-6 secretion following *in vitro* infection of macrophages, but not cultured dendritic or epithelial cells. A detectable, but very limited IL-6 response was observed in the lungs and mediastinal lymph nodes following intranasal infection. Surprisingly, no detectable *in vivo* IL-6 production was observed in the spleen or sera of infected mice despite observable systemic leukocytosis. These studies demonstrate that endogenous IL-6 production contributes little to the host response, or to the viral-induced mononucleosis-like disease, due to the fact that limiting amounts of this cytokine are produced *in vivo* during γHV-68 infection.

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Like the human gammaherpesviruses, murine gammaherpesvirus 68 (γHV-68; species *Murid herpesvirus 4*, genus *Rhadinovirus*, family *Herpesviridae*) is an efficient pathogen [2, 9, 10, 14], requiring less than 100 plaque forming units of infectious virus given intranasally to cause a productive, acute infection [16]. The host response is insufficient to prevent the subsequent establishment of latency, which occurs within days of the initial infection and is a lifelong condition. Furthermore, the establishment of latency is accompanied by a mononucleosis-like disease, similar to that observed following infection with Epstein Barr virus [2, 5]. Therefore, when investigating the host response during infection with gammaherpesviruses, it is necessary to consider those events which protect the host from the acute infection, those events which limit establishment of latency, and those host responses which might not be protective, but actually contribute to virus-induced pathophysiology [6].

A previous report demonstrated that IL-6-deficient mice had no significant alteration in the viral burden or pathophysiological response following infection with γHV-68 when compared to wild-type mice [13]. This was surprising since IL-6 is a pleiotropic cytokine affecting such diverse events as B cell function, macrophage and dendritic cell maturation, and T helper type 2 cell differentiation [7]. Such activities can potentially contribute to the protective host response against viral infections [8]. Therefore, it would seem likely that if IL-6 is produced at any significant level during a particular viral infection, it would affect the host response. In the present study, we have addressed why IL-6 has so little effect during γHV-68 infection in mice. We were surprised to discover that IL-6 production in the first few days following infection was not significant, and that IL-6 production was undetectable later in the disease course during the establishment of viral latency.

Initially, we questioned whether a direct interaction between γHV-68 and cultured epithelial cells, macrophages, and dendritic cells resulted in the production of IL-6. For these studies, murine embryonic fibroblasts, NIH3T12 fibroblasts (CCL-164; ATTC), bone-marrow-derived macrophages [3], or dendritic cells [3, 11] were cultured in the presence of 10, 1, or 0.1 plaque forming units (pfu) per cell of infectious γHV-68, UV-killed virus, or 0.5 μg/ml of lipopolysaccharide (LPS) for varying periods of time. Surprisingly, primary cultures of mouse fibroblasts and the NIH 3T12 fibroblast cell line showed no detectable increases in IL-6 secretion into culture supernates over constitutive levels during the first 24 h following exposure to the virus (Fig. 1A) using an ELISA [1]. Similarly, cultured dendritic cells showed no virally-induced IL-6 secretion (Fig. 1B). Furthermore, it is unlikely that this lack of IL-6 secretion was due to post-transcriptional regulatory events, since mRNA expression in dendritic cells was consistently unaffected by exposure

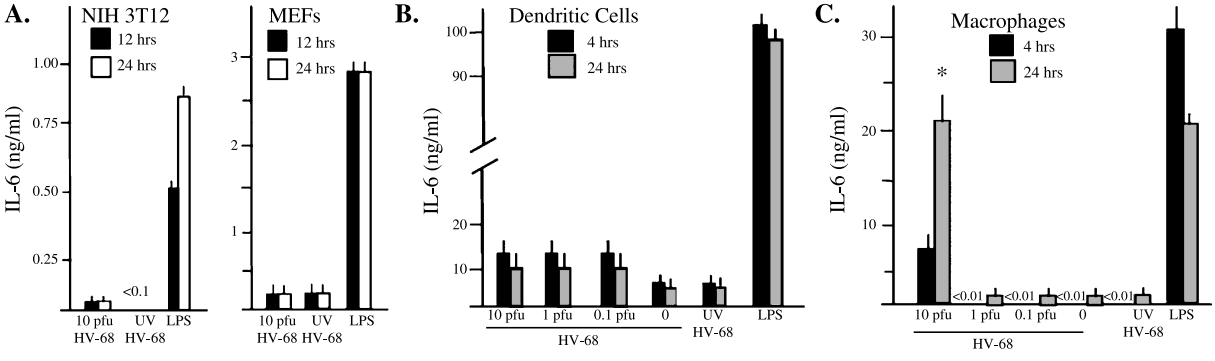

Fig. 1. γHV-68-induced expression by cultured cells. Murine embryonic fibroblasts (*MEFs*) and NIH3T12 fibroblasts (**A**) or bone marrow-derived dendritic cells, (**B**), or macrophages, (**C**), were exposed to varying numbers of plaque forming units of γHV-68 (HV-68), or cell lysates, (0), or UV-killed γHV-68, or LPS. ELISAs were performed with culture supernatants collected at the indicated times post-infection to quantify IL-6 secretion, and results are presented as mean values (±SEM). An asterisk indicates a statistically significant difference, ($p < 0.05$), when compared to uninfected cells

to γHV-68 (data not shown). Cultured macrophages, however, did respond to viral exposure when 10 pfu per cell were used. There was an approximate 11-fold increase in IL-6 mRNA expression in infected macrophages (data not shown). More importantly, infected macrophages secreted up to 20 ng/ml of IL-6 into culture supernates, which was similar to that observed for an LPS stimulus (Fig. 1C). Taken together, these results demonstrated that macrophages, and not cultured dendritic cells or fibroblasts, could express IL-6 in response to γHV-68.

The fact that cultured macrophages could respond to viral infection suggested that at least some cells that would initiate the host response following inoculation of the lungs might be capable of expressing IL-6. Further, the complexity of cell-cell interactions *in vivo* might provide additional stimuli for the induction of IL-6 following viral infection. Therefore, we questioned whether IL-6 expression could be detected *in vivo* following γHV-68 infection. Wild-type or IL-6-deficient mice were inoculated intranasally with γHV-68 [3], virus-free cell lysates, UV-inactivated γHV-68, or 100 μg of LPS. At varying times post-infection, mice were euthanized, and a sensitive, semi-quantitative RT-PCR was performed on RNA isolated from lung and mediastinal lymph nodes [1]. In addition, cDNA samples from the same tissues were subjected to real-time PCR [11]. No significant increases in IL-6 mRNA expression in the lungs or mediastinal lymph nodes of γHV-68 infected mice were consistently observed using either methodology (data not shown). To substantiate results from the mRNA analyses, a capture ELISA for IL-6 was performed on homogenates (T-PER, Pierce, Rockford, IL) [3] from lung (Fig. 2A and B) or mediastinal lymph nodes (Fig. 2C) following infection

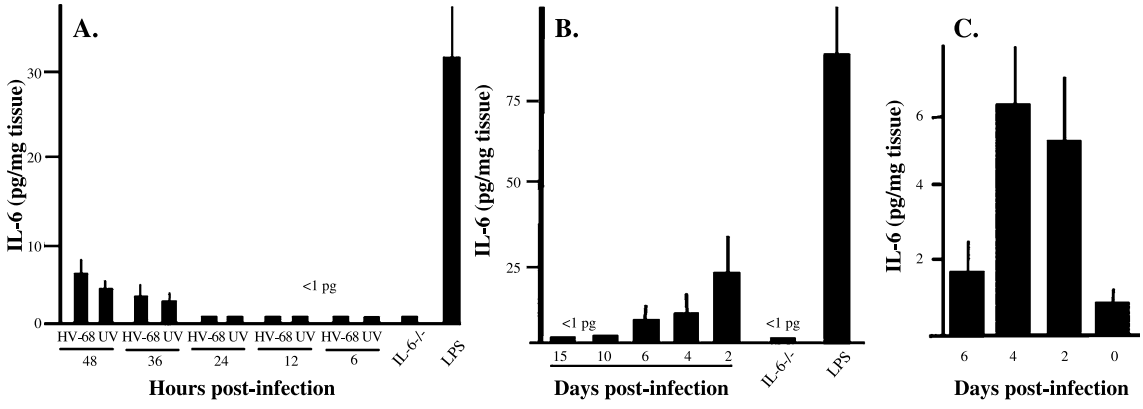

Fig. 2. γHV-68-induced IL-6 production in the lungs and lymph nodes of infected mice. Groups of C57BL/6 mice (N = 3 per group) were exposed to UV-killed γHV-68 (UV), or infected with γHV-68 (HV-68). Control mice were uninfected, (0), or were deficient for IL-6, (IL-$6^{-/-}$), or were inoculated with LPS intranasally. At the indicated times post-infection, two different portions of lungs from each animal (**A** and **B**) or the mediastinal lymph nodes (**C**) were extracted, weighed, and immediately homogenized. A capture ELISA was used to quantify levels of IL-6 present in lung homogenates. Results are presented as mean values (±SEM) of two separate studies. There were no statistically significant differences when comparing IL-6 levels in tissues from infected versus uninfected mice

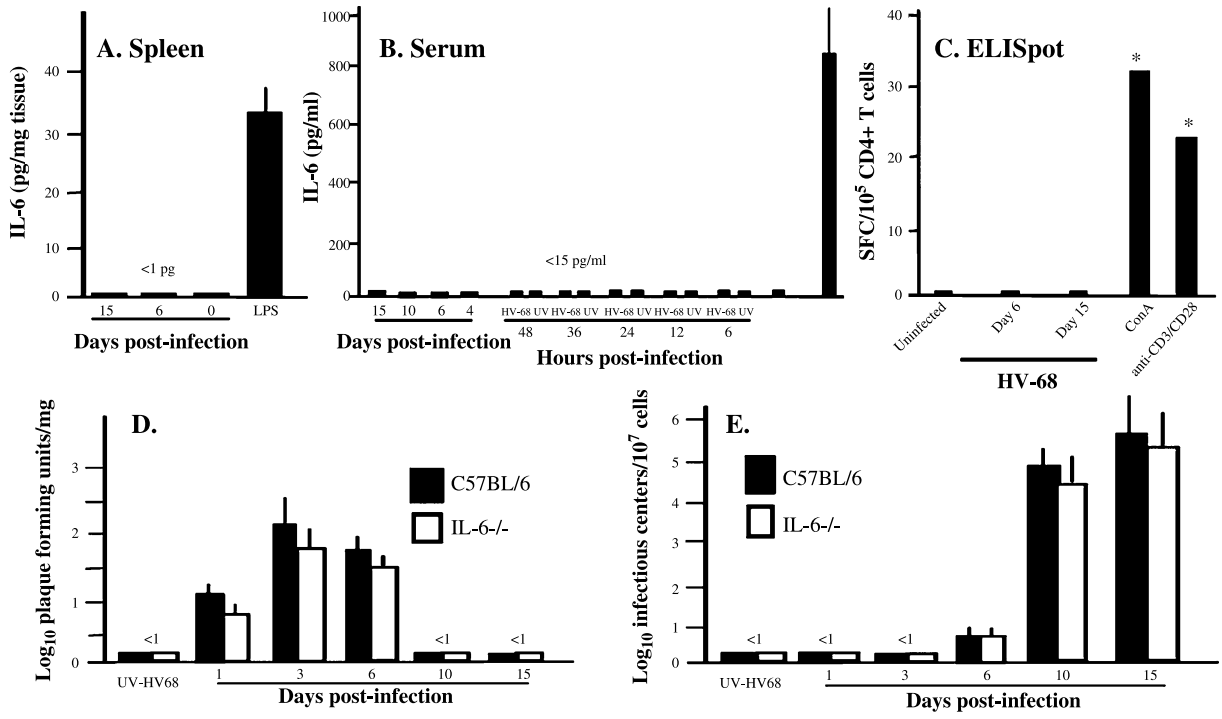

Fig. 3. γHV-68-induced IL-6 in the spleens and sera of infected mice. Groups of C57BL/6 mice (N = 3 per group) were exposed to UV-killed γHV-68, (UV), or infected with γHV-68 (HV-68). Control mice were uninfected (0), or were deficient for IL-6 (IL-$6^{-/-}$), or were inoculated with LPS intraperitoneally. At the indicated days post-infection, spleen tissue was removed, weighed, and serum was extracted. A capture ELISA was used to quantify levels of IL-6 present in spleen homogenates (**A**) or sera (**B**). Results are presented as mean values (±SEM) of two separate studies. There were no statistically significant differences when comparing IL-6 levels in spleen tissue or sera from infected versus uninfected mice. To quantify *ex vivo* production of IL-6 by splenocytes from γHV-68-infected and naïve mice, spleens were removed and splenic CD4+ T cells were isolated. ELISpot analyses were performed on these CD4+ T cells to quantify IL-6 spot-forming cells (**C**). As a positive control, CD4+ splenocytes were incubated for 5 days with 1 μg/ml of concanavalin A (*ConA*), or with anti-CD3/anti-CD28 coated beads (Dynal, Brown Deer, WI). Results are presented as spot-forming cells per 1×10^5 CD4+ T cells. This study was performed twice with similar results. There were no statistically significant differences when comparing the number of IL-6 spot-forming cells from CD4+ T cells isolated from infected versus uninfected mice. For quantification of acute (**D**) and latent (**E**) viral burdens in the lungs and spleens of IL-6-deficient (IL-$6^{-/-}$) and wild-type C57BL/6 mice, groups of mice (N = 3 per group) were infected with γHV-68 or exposed to UV-killed γHV-68. At the indicated days post-infection, tissue was removed to quantify viral burden. Infectious virus was quantified using homogenized lung tissue in a plaque-forming cell assay. Latent virus was quantified using splenic leukocytes in an infectious centers assay. Results are presented as mean values of triplicate determinations (±SEM). These studies were performed twice with similar results. There were no statistically significant differences when comparing lytic and latent viral burdens between wild-type and IL-6-deficient mice

of groups of wild-type mice. While limited increases in IL-6 protein content in these tissue homogenates were suggested (Fig. 2), the differences were not statistically significant when comparing IL-6 levels in infected versus uninfected mice. Conversely, wild-type mice receiving intranasal LPS were found to have relatively high levels of IL-6 in lung tissue (Fig. 2A and B). In order to ensure that the absence of IL-6 in infected mice was not due to protein degradation following tissue homogenization, duplicate lung tissues were removed and spiked with recombinant murine IL-6 prior to homogenization. Following processing as described above, the amount of recombinant IL-6 in these processed tissues was determined. Greater than 95% of the recombinant IL-6 originally added to the tissues could be detected using this extraction procedure [3].

A hallmark of γHV-68 infection is the development of a mononucleosis-like disease, accompanied by splenomegaly and leukocytosis. If virus-induced IL-6 production contributed to this inflammatory response, expression of IL-6 would be expected in peripheral tissues. To address this possibility, IL-6 mRNA and protein expression in the spleen and sera were quantified in groups of mice following γHV-68 infection. No significant increases in splenic IL-6 mRNA expression (data not shown), or IL-6 protein production in spleens (Fig. 3A) or sera (Fig. 3B) of groups of infected mice, were observed at any time during the course of γHV-68 infection. Conversely, mice exposed to LPS could readily respond to this stimulus by secreting IL-6 (Fig. 3A and B).

To further demonstrate the lack of IL-6 production *in vivo* following γHV-68 infection, we employed ELISpot analyses. For these *ex vivo* studies, CD4+ T lymphocytes were negatively selected by magnetic activated cell sorting (Miltenyi Biotec, Auburn, CA) from wild-type mice 6 or 15 days post-infection. Cells were plated onto Multiscreen IP plates (Millipore) coated with an anti-IL-6 capture antibody, and incubated for 48 h at 37 °C, 5% CO_2, 99% humidity. Following washing, wells were incubated with a biotinylated anti-IL-6 antibody, and then streptavidin-alkaline phosphatase. After washing, 100 μl BCIP/NBT substrate was added to each well, and plates were incubated at room temperature for 5 min for color development. Spot development was stopped by rinsing plates with distilled water, and plates were blotted and dried overnight prior to image analysis using an ImmunoSpot analyzer with dedicated software (Cellular Technologies, Ltd. Becton Dickinson). There was no significant difference in the number of IL-6 spot-forming cells in uninfected versus γHV-68 infected mice (Fig. 3C). The lack of spot-forming cells was not due to the inability of these cells to produce IL-6 since concanavalin A or anti-CD3/CD28 stimulation induced significant numbers of spot-forming cells (Fig. 3C). Taken together, these results (Fig. 3) clearly demonstrate that no significant IL-6 production occurs in the spleen or is present in the sera throughout the course of γHV-68 infection.

Based on these studies, we concluded that IL-6 does not play an important role in the host response against γHV-68 since it is not made in sufficient quantities *in vivo* to have any significant biological effect. To confirm this fact, and to extend results of a previous investigation [13] to earlier time points, groups of IL-6 deficient (IL-$6^{-/-}$) and syngeneic C57BL/6 mice were infected with γHV-68.

At the indicated times post-infection, lytic virus (Fig. 3D) and latent virus (Fig. 3E) were quantified [3]. No significant differences were observed when comparing IL-$6^{-/-}$ and IL-$6^{+/+}$ mice. These results further confirm the notion that IL-6 does not contribute to the host response during γHV-68 infection.

IL-6 would seem to be a likely candidate for modulating immunity following γHV-68 infection. Significant amounts of this cytokine can be secreted by epithelial cells, B lymphocytes, macrophages, and dendritic cells, which is also the list of cells that can be infected by this virus [4, 15]. Thus, it seems difficult to understand how such an important cytokine could have no significant effect on the host response against this leukotrophic gammaherpesvirus. Based on the studies shown here, the explanation for such an observation appears to be a limited production of biologically significant levels of IL-6 during the course of γHV-68 infection. At present it is not clear if γHV-68 directly or indirectly manipulates IL-6 production as a defense against any beneficial effect this cytokine might contribute. The possibility exists that this gammaherpesvirus limits its expression of IL-6 *in vivo* as one mechanism to evade the protective host response.

While a previous study [12] demonstrated IL-6 production by splenocytes isolated from γHV-68-infected mice following 3 days of *in vitro* re-stimulation with virus, it is clear from the work presented here that such IL-6 expression does not occur *in vivo* (Fig. 3). Furthermore, while IL-6 was detected in bronchial lavage fluid after it had been concentrated 20 fold at 10 days post-infection [12], it was clear that no IL-6 was present at day 15 post infection [12] (Fig. 3). Despite the use of a variety of methodologies in the present study, we were unable to detect any significant IL-6 production *in vivo* following γHV-68 infection when compared to uninfected mice. These findings provide an explanation as to why IL-6-deficient animals [13] (Fig. 3D and E) have no significant alteration in their susceptibility to viral infection. Stated simply, infection with γHV-68 does not result in the *in vivo* production of levels of IL-6 that have any biologically significant effect on the host response or on virally-induced pathophysiology.

Acknowledgements

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