



# Host and viral genes that control herpesvirus vasculitis

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The human vasculitides are idiopathic syndromes for which both autoimmune and infectious etiologies have been proposed. Depending on the syndrome, arteries and veins of all sizes can be affected. Signs of immune activation (inflammation, upregulated MHC Class II, expanded T-cell subsets, increased antibody titers) have been observed in several diseases, but antigens responsible for this activation have not been defined. While many postulate that these inflammatory diseases are autoreactive, infectious etiologies have also been proposed. Both bacterial and viral infections have been implicated (reviewed in references 1-3). The strongest viral candidates for human vascular disease are ones that establish long-term persistent infections, such as hepatitis B and hepatitis C viruses, human cytomegalovirus, Epstein-Barr virus, herpes simplex virus, and HIV (reviewed in references 3-5). However, it is difficult to prove causality because the presence of bacteria or virus may merely be bystander infection. In some cases (eg, CMV), latent infection of unaffected vessels further complicates evaluation of the etiology of human vasculitis and atherosclerosis.<sup>6,7</sup>

Evidence for infectious etiologies of atherosclerosis, arteritis, and coronary artery and transplant restenosis has also been reported<sup>8-9</sup> (reviewed in references 1-4 and 10-15). A number of studies have associated herpesvirus infection with human atherosclerosis (reviewed in references 10, 14, 16, and 17), and restenosis after angioplasty,<sup>18,19</sup> but proof of causality is lacking. Human cytomegalovirus (HCMV) infection has been more convincingly associated with rapidly progressive atherosclerotic-like lesions in cardiac transplant recipients.<sup>9,20,21</sup> In addition, active HCMV infection has been documented in inflammatory arteritis involving the human aorta.<sup>22-24</sup> Seroepidemiologic studies have suggested a modest association between HCMV infection and atherosclerosis,<sup>25-27</sup> although this is far from generally accepted.<sup>28,29</sup> HCMV and/or HSV nucleic acid or proteins have been detected in the aorta or in cells cultured from the aorta.<sup>5,6,14,23,30-33</sup> However, herpesvirus nucleic acid is found in normal as well as abnormal regions of the

aorta,<sup>6,34</sup> and no specific viral transcripts or proteins have been identified in classical atheromatous lesions.<sup>5,6,30,33</sup> Thus, studies of human disease remain inconclusive with respect to a direct role of herpesviruses in vascular pathology.

While a direct relationship between infection and atherosclerosis or vasculitides such as Takayasu arteritis remains unproven, it is nevertheless an attractive possibility that infection may provide the initial injury, or chronic antigenic stimulation, required for chronic vascular disease. Thus, it is possible that vasculitis and atherosclerosis are pathogenetically related in that each may be caused by or maintained by infection. Whether this is correct or not, it is true that immune activation in the wall of the great vessels occurs in both human vasculitis and atherosclerosis. Therefore, it is essential that we understand the fundamental immune mechanisms that are operative in the vascular wall.

To study infection-mediated vascular disease, different animal models have been developed. Infections with the bacteria *Chlamydia pneumoniae*,<sup>35,36</sup> the porcine RNA virus PRRSV,<sup>37-40</sup> a retrovirus,<sup>41</sup> and herpesviruses all cause vascular pathology. Many aspects of the human diseases are recapitulated in these models, so further animal studies may help elucidate mechanisms of infection-mediated vasculitis. Such results may improve management, and potentially prevention, of these important human diseases. In particular all three classes of herpesviruses ( $\alpha$ -,  $\beta$ -, and  $\gamma$ -herpesviruses) can cause vasculitis.

## ■ $\alpha$ -HERPESVIRUS-ASSOCIATED ARTERITIS

The initial observation that herpesviruses can cause vasculitis was reported by Paterson and Cottrill who demonstrated cosegregation of neurolymphomatosis (NL), or Marek's disease, with a vasculitic process, with some similarities to atherosclerosis, in chickens. Chickens with a low incidence of NL developed vasculitis after inoculation with tracheal washings from chickens with a high NL incidence. Churchill and Biggs later reported that a herpesvirus was the etiologic agent of Marek's disease. These reports were followed by an extensive set of experiments by Fabricant et al analyzing the vascular disease induced by Marek's disease virus (MDV) in chickens (reviewed in reference 42). Certain strains of quail also develop large vessel arteritis which may be linked to infection with an MDV-related herpesvirus.<sup>43,44</sup>

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These studies demonstrated that infection of genetically susceptible newborn chickens resulted in inflammatory lesions in coronary arteries, aortas, and major arterial branches. Additionally, the fatty proliferative lesions contained intimal and medial foam cells, cholesterol clefts, extracellular lipid, and calcium deposits. Adventitial inflammatory infiltrates as well as mononuclear cells in the intima were evident. High-cholesterol feeding synergized with viral infection resulting in increased fatty proliferative lesions. Uninfected controls did not have lesions, regardless of diet. Gross arterial lesions were visible after 3 months postinfection, and persisted for at least 4 months. Microscopic lesions were discernible one month postinfection. There was no change in incidence of diseases related to cholesterol levels in the diet. Antibody-mediated injury seems unlikely as IgG and C3 deposits in the lesions were not detectable by immunohistochemistry.

Immunofluorescence revealed viral antigen in a few smooth muscle cells (SMCs) of the arterial media, providing evidence of direct MDV infection within arterial lesions. Infection was demonstrated in SMCs within plaques by *in situ* DNA hybridization. The authors argue that latent infection may result in SMC proliferation, thereby resulting in atherosclerotic-like proliferative plaques. It should be noted that latent and lytic infections were not formally distinguished in these studies.

#### ■ $\beta$ -HERPESVIRUS-ASSOCIATED ARTERITIS

Murine CMV (MCMV) or rat CMV (RCMV) infection are widely used as model systems for studying aspects of HCMV infection. Given the interest in HCMV as a possible agent in human vascular disease, several groups have used the murine system to test whether CMV infection can result in vasculitis. Persoons et al<sup>45</sup> demonstrated small-vessel pathology following local (subcutaneous in the foot pad) and generalized (intraperitoneal) infection of irradiated rats with RCMV. This work builds on a large body of work with RCMV in a transplant model<sup>46,47</sup> of large-vessel arteritis. MCMV infection can cause vascular disease, as seen by infection of mice at one to two weeks of age, as well as of wild-type and immunocompromised adult mice. Dangler et al<sup>48</sup> infected suckling BALB/c and C57BL/6 mice at 7–15 days of age with near-lethal doses of MCMV intraperitoneally (*i.p.*), and demonstrated very significant arteritic lesions at the base of the aorta. We infected adult (6–8 weeks old) wild-type 129 mice as well as IFN $\gamma$ R<sup>-/-</sup> mice and found significant arteritis in the same locale (Figure 1).<sup>49</sup> Berencsi et al<sup>50</sup> found arteritis after MCMV infection of irradiated adult BALB/c mice. For each group, aortic inflammation developed and was characterized by mononuclear cells in the intima and adventitia, although the adventitial inflammation was often most severe, with few infiltrates in the media. Dangler et al<sup>48</sup> demonstrated such pathology at 8 and 15–16 weeks postinfection, with increased penetrance when younger mice were infected. We found that, although lesions were present in wild-type mice at 28 and 56 days postinfection, they resolved by 84 days postinfection. In contrast, IFN $\gamma$ R<sup>-/-</sup> mice had significant arteritis as late as 154 days postinfection.<sup>49</sup>

Immunohistochemistry for CD3, CD8, and CD4 re-

sulted in staining predominantly in the adventitial infiltrate, with only scattered positive cells in the medial and intimal infiltrates.<sup>48</sup> Although the media contained the least infiltration, we demonstrated medial infection by the presence of cytomegalic nuclear inclusion bodies and MCMV antigen in SMC of the elastic media.<sup>49</sup> It is therefore likely that the continued inflammatory response is directed against viral antigens. The prevalence and severity of aortic lesions in MCMV induced vasculitis were shown to be independent of diet.<sup>48,50</sup> However, an increase in serum LDL-cholesterol levels with MCMV infection was reported,<sup>50</sup> as well as increased aortic lipid deposition in infected versus uninfected mice fed a high fat diet.<sup>48</sup>

These studies demonstrate that CMV infection can cause aortic inflammation and that disease can persist for many weeks after infection. The presence of nuclear inclusions and detection of viral antigen suggest continued viral gene expression and replication, thereby providing stimulation for chronic disease. As with MDV, dietary factors can influence lipid deposition in the arterial wall, but do not affect the vascular inflammation *per se*.

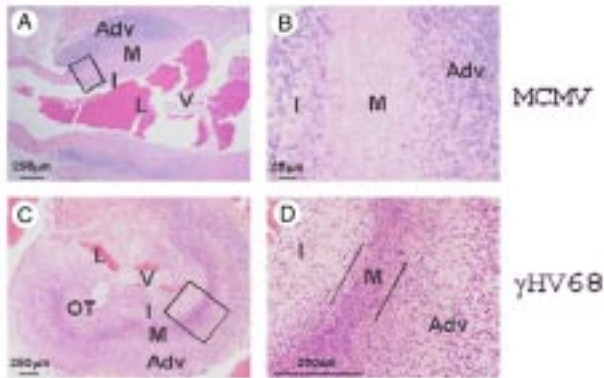
#### ■ $\gamma$ -HERPESVIRUS-ASSOCIATED ARTERITIS

Recognizing the importance of understanding mechanisms of immunity to  $\gamma$ -herpesvirus infection, we and others have been studying murine  $\gamma$ -herpesvirus 68 ( $\gamma$ HV68 or MHV-68), which is closely related at the genomic level to EBV and KSHV.<sup>51–53</sup> While  $\gamma$ HV68 is clearly distinct from EBV and KSHV in important ways, this model has many advantages, including the availability of mutant mouse strains and the ease of generating viral mutants. Conservation of multiple nonessential genes (eg, *v-cyclin*, *v-bcl-2*, K3 [MHC regulation], *regulator of complement activation*) between  $\gamma$ HV68 and EBV and/or KSHV, together with the fact that these viruses are associated with induction of lymphomas, makes  $\gamma$ HV68 a relevant model for understanding aspects of EBV and KSHV pathogenesis and immunity.

$\gamma$ HV68, a natural pathogen of wild rodents,<sup>54,55</sup> is capable of infecting both outbred and inbred mice. Following inoculation with  $\gamma$ HV68, acute productive replication occurs in multiple organs, and is cleared 9 to 15 days postinfection.<sup>56–58</sup>  $\gamma$ HV68 establishes latent infection in macrophages (M $\phi$ s) and B cells in the peritoneum,<sup>59</sup> and in B cells and dendritic cells (DCs) in the spleen.<sup>60,61</sup> Chronic  $\gamma$ HV68 infection is associated with three diseases: (i) lymphomas and lymphoproliferative disease (reference 62 and unpublished data), (ii) severe large-vessel arteritis,<sup>63–65</sup> and (iii) splenic fibrosis.<sup>64,66</sup>

We found that  $\gamma$ HV68 caused death of infected IFN $\gamma$ R<sup>-/-</sup> mice over weeks to months. Based on our experience with MCMV, and the tropism of the human  $\gamma$ -herpesvirus KSHV for vascular tissues, we investigated the possibility that  $\gamma$ HV68 also caused severe arteritic lesions. We found that IFN $\gamma$ R<sup>-/-</sup> mice were profoundly susceptible to induction of arteritis by infection with either MCMV or  $\gamma$ HV68 (Figure 1).<sup>64</sup>

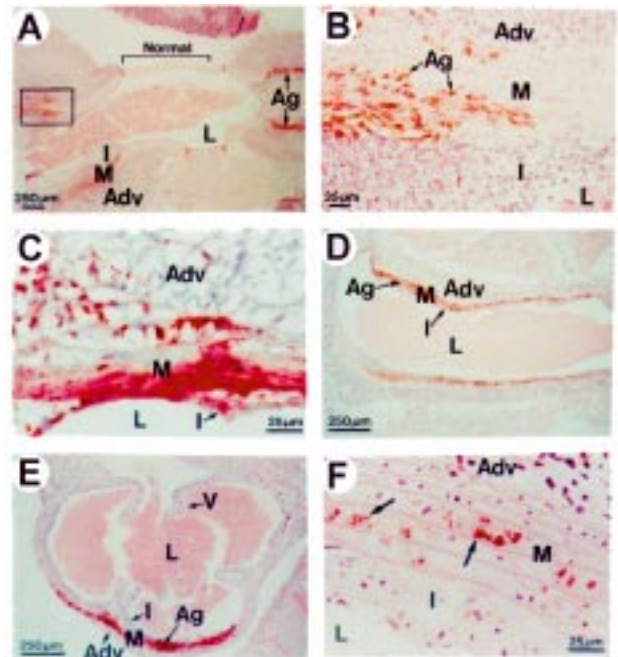
Several things were striking about the lesions we observed. First, they were restricted to the great elastic arteries and manifested as skip lesions (Figure 2A) with a



**Figure 1.** Arteritis induced by both MCMV and  $\gamma$ HV68. (A) Aorta from an  $IFN\gamma R^{-/-}$  mouse sacrificed 154 days after infection with  $10^4$  PFU MCMV. (B) High-power view of the boxed region shown in A. (C) Base of the aorta from a  $\gamma$ HV68-infected  $IFN\gamma R^{-/-}$  mouse that died 5.6 weeks after infection. (D) Higher-power view of the boxed region shown in C. Reprinted from *International Journal of Cardiology*; 75(suppl 1). Dal Canto AJ, Virgin HW IV. Animal models of infection-mediated vasculitis: implications for human disease, pp. S37-S45. Copyright 2000, with permission from Elsevier Science.

high frequency of involvement of the base of the aorta. Second, the pathology had similarities to both atherosclerotic lesions and to lesions of Takayasu's arteritis, including extensive deposition of lipid in the lesions. Third,  $\gamma$ HV68 antigen was found in the SMC of the elastic media for months after infection (Figure 2B, D, E, and F).<sup>64</sup> Remarkably, this was the same distribution seen for MCMV.<sup>49</sup> Fourth, in addition to deficiency of  $IFN\gamma$ , B cell deficiency and MHC Class II and therefore CD4 T cell deficiency (Figure 2D)<sup>64</sup> predisposed to lesion development. However, mice lacking most CD8 T cells ( $\beta 2$ microglobulin $^{-/-}$ ,  $\beta 2m^{-/-}$ ) mice did not.<sup>64</sup> This latter result was surprising since it is generally considered that CD8 T cells are important components of the host defense against herpesvirus infection. However, it was consistent with the finding that CD8 T cells may play an immunopathologic role during  $\gamma$ HV68 infection.<sup>66</sup> Lastly, we found that young mice without immunodeficiency were susceptible to arteritic lesions (Figure 2E and F).<sup>64</sup> Since infection with herpesviruses often occurs in young hosts sets up a situation in which secondary genetic factors might predispose to atherosclerosis or vasculitis. This was similar to findings by Dangler et al for MCMV showing that younger mice are more susceptible to MCMV induced arteritis than adult mice.<sup>48</sup>

These initial observations begged several basic questions. Were arteritic lesions secondary to persistent lytic replication, or latent infection? What was the basis for the tropism of the virus to the media of the great elastic arteries? Was tropism due to a specific interaction between a viral protein and cells of the elastic media or due to some intrinsic property of the arteries themselves? This was particularly of interest since MCMV localized



**Figure 2.** Immunohistochemical localization of  $\gamma$ HV68 antigen and lipid deposition in  $\gamma$ HV68-induced arteritic lesions. For all panels, L, lumen; I, intima; M, media; Adv, adventitia; and Ag,  $\gamma$ HV68 antigen. (A) Longitudinal section of the aorta from a  $\gamma$ HV68-infected  $IFN\gamma R^{-/-}$  mouse that died 4 weeks after infection stained with anti- $\gamma$ HV68 antibody. Note skip lesions in the aorta, and the correlation between inflammatory arteritis and regions containing viral antigen (brown color). The bracket indicates a region of normal aortic wall between two arteritic lesions. (B) High-power view of the boxed region in (A) demonstrating the nature of the inflammatory process occurring in  $IFN\gamma R^{-/-}$  mice infected with  $\gamma$ HV68. Note staining for viral antigen (brown color) in elongated cells within the media of the vessel. (C) Oil Red O-stained frozen section from the base of the aorta of a  $\gamma$ HV68-infected  $IFN\gamma R^{-/-}$  mouse fed a high-fat diet that died 8 weeks after infection. Lipid deposition, demonstrated by red staining, is evident in subendothelial and medial regions. (D) Longitudinal section of pulmonary artery from a  $\gamma$ HV68-infected B-cell-deficient mouse that was sacrificed 13 weeks after infection. Note the inflammation in both the intima and adventitia as well as viral antigen in the media (brown color). (E) Aorta from a normal pup that died 9 days after infection (day 16 of life). Viral antigen was detected within the media (brown color), and intimal inflammation is present. (F) Aorta from a pup that died 19 weeks after infection with  $\gamma$ HV68. Note adventitial inflammation, intimal thickening, and the presence of viral antigen (brown color, examples indicated by arrows) in the media. From *Nature Medicine* 1997; 3:1346–1353.

to the same site generating similar lesions,<sup>49</sup> suggesting that viral tropism for the elastic arteries might be due to properties of the elastic media rather than specific tropism determinants of a given virus. Did normal adult mice also develop arteritis, or was this a phenomenon only observed in the artificial situation of immunodeficiency? Where and how did  $IFN\gamma$  act to protect against chronic arteritis? What were the specific roles of CD4 T

cells, CD8 T cells, and B cells in protection against or induction of arteritis? What components of the virus contributed to the capacity to induce arteritis?

Over the past 3 years we have addressed a number of these questions. Our major findings have been that: (i) persistent viral replication in the SMC of the media of the great elastic arteries is responsible for arteritic lesions,<sup>65</sup> (ii) the tropism of virus for the media of the great elastic arteries is explained by the finding that the media of the great elastic arteritis is an immunoprivileged site,<sup>63</sup> (iii) the M3 protein of  $\gamma$ HV68 is a high affinity chemokine binding protein of novel structure that regulates virus induced inflammation but not arteritis (article submitted and references 67 and 68), and (iv) two genes in  $\gamma$ HV68, one encoding a homolog of host bcl-2 proteins and the other encoding a homolog of host D-type cyclins, are important for both persistent replication and induction of arteritis (submitted).

### ■ ROLE OF PERSISTENT REPLICATION

We have shown using immunofluorescence, in situ hybridization, and electron microscopy that the virus infects, replicates, and kills vasculature SMC of the arterial media during chronic infection.<sup>63,65</sup> Studies using the antiviral drug cidofovir show that replication is required for maintenance of arteritic lesions.<sup>65</sup> Interestingly, we found that persistence of virus was specific for the media of the great elastic arteries. These studies led to the identification of the media of the great elastic arteries as a novel immunoprivileged site.<sup>63</sup> The mechanism(s) responsible for the immunoprivilege are not defined. We found that lymphocytes and macrophages, but not neutrophils, are excluded from the vascular media, consistent with an anatomic blockade to entry and/or a viral protein that modulates inflammation at this site.<sup>63,68</sup> The possibility that a viral protein might prevent lymphocyte entry into the media of the great vessels led to studies of the  $\gamma$ HV68 M3 chemokine binding protein. This protein is a high affinity chemokine scavenger of novel structure (unpublished and references 67 and 68). We found that it is not involved in vasculitis induction by  $\gamma$ HV68, but is involved in encephalitis induced by the virus (submitted).

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### ■ ROLE OF IFN $\gamma$

We showed that normal adult mice develop arteritis. However, transient depletion of IFN $\gamma$  increased the incidence of arteritic lesions, and chronic depletion of IFN $\gamma$  increased the severity of arteritic lesions.<sup>63</sup> Using bone marrow transplantation we found that IFN $\gamma$  responsiveness of somatic cells is critical for controlling arteritis, but that hematopoietic cell IFN $\gamma$  responses regulate the nature of the pathology. This latter finding suggests that IFN $\gamma$  may limit immunopathologic injury to the wall of the great vessels.<sup>63</sup> We have also found that IFN $\gamma$  protects cultured primary aortic SMC from infection, making it likely that IFN $\gamma$  acts both by direct control of viral replication in SMC and by immunoregulatory mechanisms.<sup>63</sup>

### ■ ROLE OF VIRAL GENES

Since persistent replication in SMC of the media is required for vasculitis,<sup>65</sup> and the v-bcl-2 and v-cyclin are important for persistent replication in IFN $\gamma$ -/- mice (submitted), we evaluated the role of the v-bcl-2 and v-cyclin in arteritis. We isolated the appropriate mutants and found that the v-bcl-2 and v-cyclin are critical for both persistent replication and virulence in IFN $\gamma$ -/- mice which develop arteritis. The incidence of arteritis is significantly decreased in IFN $\gamma$ R-/- mice infected with v-bcl-2 mutant or v-cyclin mutant virus as compared to wild-type virus (submitted). This is the first work identifying viral genes important for induction of chronic vascular pathology.

### ■ CONCLUSION

Studies in the MCMV and  $\gamma$ HV68 systems are beginning to unravel the immunologic mechanisms responsible for protection of the great elastic arteritis from viral infection. During the course of this work the vulnerability of the elastic media to chronic infection has become clear. This has implications for human disease, raising the possibility that a number of pathogens may persistently replicate in this immunoprivileged site. Identification of specific host (IFN $\gamma$ ) and virus (v-bcl-2 and v-cyclin) genes that are involved in vasculitis provides a basis for further studies on mechanisms responsible for chronic arterial injury.

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