

Xenotropic murine leukemia virus-related virus: current research, disease associations and therapeutic opportunities

Despite the fact that xenotropic murine leukemia virus-related virus (XMRV) research is in its infancy, considerable attention has been focused on this recently discovered human retrovirus because of the surprising association of XMRV with two very different diseases: prostate cancer and myalgic encephalomyelitis/chronic fatigue syndrome. Like other retroviral infections, XMRV integrates into host-cell DNA and thus endures for a lifetime. Little is known about host-cell interactions, reservoirs of infection or the lifecycle of XMRV. Information on murine xenotropic viruses, as well as current research on cellular tropism and *cis*-acting glucocorticoid response elements, provides intriguing clues for viral persistence, mechanisms of pathogenesis and opportunities for XMRV as a diagnostic biomarker and therapeutic target in myalgic encephalomyelitis/chronic fatigue syndrome.

KEYWORDS: *cis*-acting elements ■ host-cell interactions ■ immune suppression ■ myalgic encephalomyelitis/chronic fatigue syndrome ■ prostate cancer ■ viral reservoirs ■ XMRV

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XMRV: a new human retrovirus?

Xenotropic murine leukemia virus-related virus (XMRV) was first identified in 2006 using microarray technology that could detect sequences of all known mammalian viruses [1]. DNA that hybridized to the virus microarray was detected in tumor-bearing prostate tissues of individuals homozygous for a variant of the *RNASEL* gene (R462Q). RNase L, a proapoptotic, antiviral enzyme that degrades viral and cellular ssRNA, is part of the type I interferon antiviral response pathway. The single nucleotide polymorphism R462Q is present in approximately 13% of the US and European populations and results in a threefold loss of RNase L activity [2]. Sequencing of the human genome and familial prostate cancer linkage studies revealed that a germline mutation in the *RNASEL* gene was the previously unidentified gene locus known only as hereditary prostate cancer 1 allele. This discovery prompted Robert Silverman and colleagues to hypothesize that men with hereditary prostate cancer harbored a novel virus infection that might be responsible, at least in part, for the apparently hereditary prostate cancer in these families. Silverman's group isolated retroviral sequences from several of the samples [1] and established that the sequences were only distantly related to the two previously known retroviruses, human T-lymphotropic virus (HTLV) and HIV, but were instead closely related to a type of retrovirus called xenotropic murine leukemia virus (X-MLV). As a result, the newly identified virus

was named XMRV. Infectious XMRV was not isolated directly from the cells; instead, Dong and colleagues constructed a molecular clone of XMRV and made a significant advance by demonstrating that it was a novel infectious gammaretrovirus [3]. Members of this retrovirus family contain only the structural genes *gag*, *pol* and *env*. These genes encode all of the proteins that make up the viral particle (FIGURE 1).

Simple retroviruses such as XMRV require infection into a proliferating cell to enter the nucleus and integrate into the host's genome. Dong *et al.* demonstrated that, like X-MLV, XMRV uses the XPR-1 receptor to enter cells [3]. XPR-1 is a transporter protein with multiple transmembrane domains and a possible role as a G-protein signaling molecule [4].

Kozak and colleagues have carried out extensive analyses of this family of receptors and recently identified variants that have altered host range and function [5]. Virus entry is mediated by a small number of critical amino acids in both the XPR-1 receptor and the envelope protein [6]. Examining XPR-1 variation and XMRV envelope variation will likely be key to understanding pathogenesis.

Unlike HIV and HTLV-1, XMRV integrates preferentially into the start sites of genes and CpG islands [7]; sites critical to epigenetic regulation of gene expression through the DNA methylation machinery. Recently, analysis of infected prostate cancer cells has revealed that most XMRV proviruses were found to be correctly inserted into human chromosomes and

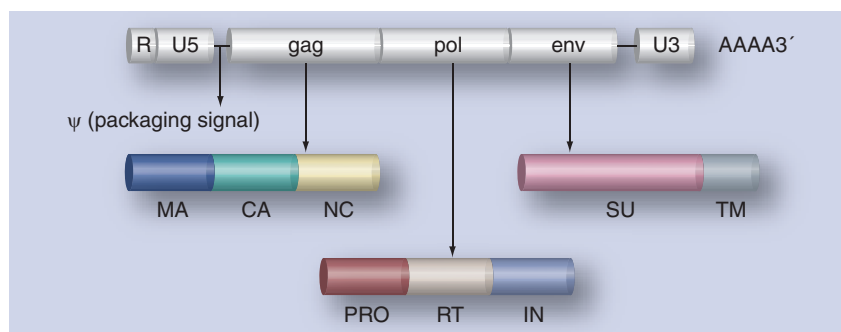


Figure 1. Genomic structure of a gammaretrovirus. The gag gene encodes MA, CA and NC. The env gene encodes TM and SU, and the pol gene encodes PRO, RT and IN.

CA: Capsid; IN: Integrase; MA: Matrix; NC: Nucleocapsid; PRO: Protease; RT: Reverse transcriptase; SU: Surface unit of the envelope; TM: Transmembrane domain of envelope.

were flanked by a four base pair repeat of human DNA [8]. These reports suggest that XMRV might play a role in familial prostate cancer, which is known to involve inflammation and immune dysregulation. DNA hypermethylation of immune response and tumor suppressor genes provide key biomarkers of early prostate disease [9].

Clinical & immunological aspects of myalgic encephalomyelitis/chronic fatigue syndrome

Myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS) is a disease characterized by severe chronic fatigue lasting at least 6 months and accompanied by symptoms such as impairment in short-term memory or concentration, sleep disturbance, sore throat, tender lymph nodes, and muscle and joint pain [10]. ME/CFS is thought to have an incidence of at least 17 million people worldwide [11]. The diagnosis of ME/CFS is a diagnosis of exclusion of other diseases. Currently, clinicians rely on two main diagnostic criteria: the 1994 CDC Fukuda Criteria for CFS [12] and the 2003 Canadian Consensus Criteria (CCC) for ME/CFS [13]. A number of clinicians prefer the CCC because of its requirement for postexertional malaise, a hallmark of ME/CFS. Unfortunately, because some of the symptoms required for the Fukuda criteria overlap with depression, and the Fukuda criteria do not require exercise intolerance for a diagnosis of ME/CFS, the composition of the patient populations studied by different clinicians may be at variance. Given the heterogeneity of the disease presentation, the existence of CFS subgroups have been proposed [14]; molecular biomarkers of clinical disease could aid greatly as diagnostics and in therapeutic strategies.

Decades of research into the pathophysiology of ME/CFS has characterized the disease as a multisystem disorder, manifested by low natural killer (NK) cell numbers and function, innate immune activation and antiviral enzyme (RNase L) dysfunction. Klimas *et al.* reported a significant expansion of CD26⁺-activated T cells in CFS subjects [15]. This multifunctional molecule plays a major role in the regulation, development, maturation and migration of T helper and NK cells as well as in B-cell immunoglobulin switching [16]. Moreover, abnormal expression of CD26⁺ is found in autoimmune diseases [17]. Numerous publications document dysregulation of cytokines, chemokines and growth factors including altered amounts of TGF- β , IFN- α and upregulation of IL-6 and the CXC chemokines IL-8, MIP-1 α and MIP-1 β [18]. Clinical features of CFS include immunological, neurological and autoimmune abnormalities, all of which are known to be associated with HTLV and HIV infection [19]. While there have been previous intriguing reports of retroviruses in CFS patients' cells [20], these prior experiments had not resulted in definitive identification of a particular retrovirus common to most individuals with CFS. The striking immune dysfunction in CFS as well as the documented neurological defects inspired us to consider a possible role of a retroviral infection in the pathophysiology of ME/CFS. The observation that XMRV sequences were found almost entirely in tumors of prostate cancer patients harboring a defect in RNase L led us to hypothesize in the fall of 2008 that XMRV might play a role in ME/CFS, given an RNase L dysfunction that was also known in CFS [21,22].

Association of XMRV with ME/CFS

HIV and HTLV-1 retrovirus infections are detected clinically by:

- Serological detection of antibodies to viral proteins in blood
- Detection of viral proteins by immunoblot
- Detection of proviral DNA by PCR of DNA or detection of virus RNA by reverse transcription-PCR
- Isolation of virus from cell culture

In our initial publication in *Science* [23] and follow-up papers [24,25], we demonstrated that each of these detection methods could be used to detect XMRV in the blood of infected individuals [21]. In addition, for the first time, we demonstrated that infectious XMRV is present in infected individuals. We observed that

infectious XMRV could be transmitted from activated lymphocytes, from isolated T and B cells (cell-to-cell) and from plasma (cell-free) from infected individuals to primary lymphocytes and to permissive cell lines. Moreover, we demonstrated that infected individuals also have antibodies specific for this type of retrovirus. The existence of antibodies reveals that XMRV can elicit an immune response, thus opening the door for immune-based therapies in XMRV-infected individuals. In contrast to the Urisman *et al.* [1] prostate cancer study, we did not find an association of the *RNASEL* R462Q variant with CFS patients harboring XMRV [23].

Prior to the initiation of our study, the only method for sensitive detection of XMRV was by PCR from DNA as developed by Urisman and colleagues [1]. XMRV had not previously been shown to be infectious or bloodborne. Our finding of XMRV in 67% of 101 patients, predominantly by nested PCR of *gag* from cDNA of leukocytes [24], firmly associated XMRV with a second disease, ME/CFS. Moreover, detection of XMRV in 3.75% of geographically matched healthy controls raises significant concerns for the public health.

The discovery of XMRV has opened up a new area of research with many unanswered questions:

- What is the prevalence of XMRV in the human population?
- Is XMRV a direct cause of one or both of these diseases or does it contribute to their development or progression?
- How is XMRV transmitted?
- What are the tissue reservoirs of XMRV?
- Does XMRV affect innate and/or adaptive immune responses?
- What is the key immune cell target?
- Is it present in other immune-compromised individuals?
- Does XMRV play a role in malignancy or other neuroimmune illnesses?

Incidence of XMRV in prostate cancer & CFS

A significant advance in the association of XMRV with prostate cancer was reported by the laboratory of Ila Singh at the University of Utah [26]. Although the original finding of XMRV was in the stromal infiltrates of prostate tumors [1], Schalberg *et al.* [26] detected XMRV

in malignant prostatic epithelia, primarily in patients with higher Gleason scores, which are associated with more aggressive disease and poor prognosis. XMRV DNA was found in 6% of prostate cancers and XMRV protein in 23% of those tumors. In contrast to the Urisman *et al.* report [1], but consistent with our CFS XMRV study, Schalberg *et al.* did not find a correlation of the R462Q variant and infection with XMRV [26]. This does not mean that there are not other mechanisms by which dysfunctional RNase L can contribute to the inability of this antiviral pathway to control XMRV infection in human disease.

In contrast to the US reports of XMRV in prostate cancer and ME/CFS patients, two independent groups in Germany, two groups in the UK and one group in The Netherlands have reported observing little (1/150 [27]) or no (0/589 [28], 0/186 [29], 0/299 [30], 0/32 [31]) XMRV in prostate cancer or ME/CFS, respectively. These differences could reflect geographic differences in the prevalence or sequence of XMRV or differences in the assays used to detect XMRV.

None of these studies of prostate tumors or CFS patients that failed to detect XMRV used multiple methods, which were necessary in order to observe XMRV infection in the majority of ME/CFS patients [23]. All used the least sensitive method of detection, which is PCR on DNA from nonreplicating blood leukocytes. Better understanding of the incidence of XMRV in disease and its prevalence worldwide will require the generation of standardized reagents, such as monoclonal antibodies generated against XMRV proteins, the development of sensitive, specific and well-validated XMRV detection assays and subsequent large-scale clinical studies.

Possible pathogenesis of XMRV: using clues from other retroviral infections, is chronic innate immune activation harmful during retroviral infection?

Innate immune responses to viral infections led by macrophages, NK cells, plasmacytoid dendritic cells (pDCs) and $\gamma\delta$ -T cells are usually initiated within hours of the first encounter with the virus. NK lymphocytes are among the first responders to viral infections, killing virus-infected cells and reducing initial viral production [32]. These non-antigen-specific responses limit viral replication for a short period of time and are normally replaced by an adaptive, antigen-specific response within days

after infection, ultimately resulting in the efficient clearance or control of the infectious agent. However, in retroviral infections, continuous replication occurs throughout life. Chronic viral infections survive directly and/or indirectly by affecting the innate and adaptive immune responses. ME/CFS patients typically have low NK function [33], may have lower than normal numbers of NK cells [34] and often have concurrent β - and γ -herpes virus infections [35]. NK cells in humans are essential for control of herpes viruses [36]. These observations suggest that CFS may be an acquired immune deficiency.

We do not yet know whether XMRV causes the immune deficiency or exploits it. However, we hypothesize that chronic stimulation and/or XMRV infection of cells of the innate immune system could lead to a phenotypic immune suppression. For example, constant activation of CD26⁺ T cells against XMRV could render the adaptive response poorly able to respond to other pathogens and autoantigens. A detrimental effect of retroviral infection on the adaptive response was first proposed by Gene Shearer and colleagues with regard to HIV [37]. In natural hosts of HIV/simian immunodeficiency virus, which are resistant to disease, the virus does not activate pDCs or T cells. Thus, T-cell functional impairment is not observed despite chronically high levels of viral replication. However, in HIV/simian immunodeficiency virus infection of susceptible hosts, elevated expression of markers associated with pDC activation, such as type I IFN and indoleamine 2,3-dioxygenase, is maintained during the chronic phase, concomitant with the lack of an efficient adaptive anti-HIV response [38]. Thus, because of their chronic nature, HIV-1 and HTLV-1 infection result in prolonged continuous stimulation of the innate immune system. We therefore suggest that uncontrolled innate immune activation during XMRV infection may lead to a dysregulated adaptive immune response, characterized by functionally impaired T cells and increased expression levels of phenotypic markers of activation and inflammatory cytokines and chemokines. A dysfunctional adaptive immune system is associated with neurological diseases that are prevalent in retroviral infections in animals and humans.

HIV and HTLV are known to cause immune deficiencies and an elevated rate of lymphoma, the development of Kaposi's sarcoma and T-cell leukemia/lymphoma, respectively [39]. Levine *et al.* reported NK cell dysfunction in a family with CFS. Five of six siblings and three other

family members developed CFS as adults [40]. All of the affected family members had persistently low NK cell activity, while unaffected family members had intermediate NK cell function compared with healthy controls. Two of the offspring of the six siblings in this CFS-affected family developed pediatric malignancies. Further studies are needed to determine whether CFS patients with persistent viral infections might have increased risk of cancer.

We hypothesize that XMRV infection of B, T, NK and other immune cells could cause chronic inflammation and immune deficiency, resulting in an inability to mount an effective immune response to cancerous cells or opportunistic infections, as seen in CFS. A potential therapeutic target is revealed by the *cis*-acting glucocorticoid response elements characterized recently in Stephen Goff's laboratory [41]. When cell lines were evaluated for their ability to sustain robust replication of XMRV, the hormone-responsive prostate cancer cell line LNCaP, produced from the lymph node of a 62-year-old man with metastatic prostate cancer more than two decades ago, was identified as a useful indicator line for the presence of XMRV. Significantly, LNCaP cells are defective in the RNase L and Jak-Stat interferon responsive antiviral pathways. Androgens were also found to increase XMRV replication in these cell lines [42]. Interestingly, prostate cancer and ME/CFS both have inflammatory and stress-related clinical and epidemiological components.

Hypothesis of XMRV-induced neuroimmune pathogenesis

Nothing is known about the events following acute infection by XMRV nor about the time course of infection. Our hypothesis, shown schematically in FIGURE 2, is that the acute infection is met with a strong immune response (gray line), which decreases the viral load (green line) and perhaps silences the active infection (pink line), but establishes reservoirs in long-lived immune monocyte/macrophages [43], T- and B-cell populations [44] and dendritic cells [44]. Stress hormones and inflammation (γ -axis) could possibly activate XMRV expression increasing reservoirs and dysfunction of infected cell subsets. Over time, we propose that chronic XMRV expression, increasing viral reservoirs and chronic inflammation could lead to immune deficiency and, ultimately, increases in the presence of envelope proteins (pink line) lead to neurodegeneration and development of disease (blue line).

A question of balance

HIV-1 infection affects and is affected by the balance between the host and other microbes. These microbes, and new ones that breach host defenses once the efficiency of the immune system is impaired, are called co-pathogens and play an important role in HIV disease. These co-pathogens greatly affect HIV transmission and disease progression. Their role is emphasized by the Centers for Disease Control and Prevention's definition of AIDS: "HIV-1 infection and a CD4 T-cell count below 200 cells/ μ l or, alternatively, the presence of at least one of 25 AIDS-defining conditions, the great majority of which are sustained by other microbes" [45]. Herpes viruses are among the most frequent co-pathogens establishing infection with HIV-1, resulting in a vicious cycle in which each virus facilitates the replication, shedding and transmission of the other. Intersecting epidemics are known in which the disease caused by one virus facilitates the transmission and pathogenesis of the other. An example of this is the herpes simplex virus, which may have contributed to the spread of HIV in Africa, where genital herpes has been estimated to account for 30% of HIV infections [34].

Similar intersecting epidemics can be hypothesized for the spread of XMRV. Chronic herpes virus infections such as Epstein-Barr virus, human herpes virus-6 and cytomegalovirus are documented among significant proportions of ME/CFS patients. Should XMRV infection be shown to be necessary for the development of ME/CFS, diseases caused by herpes virus may ultimately be defined as XMRV-associated neuroimmune disease codefining illnesses. Acyclovir, a well-known inhibitor of herpes viral DNA polymerase, can also inhibit reverse transcriptase after it has been converted into its 5'-triphosphate derivative [45]. Such dual-targeted therapeutics are promising new strategies for treatment of HIV-infected patients with herpes simplex virus-2 co-infections and may also be of value for XMRV-infected patients [46].

Owing to its recent discovery, little is known about XMRV; however, much is known about the closely related xenotropic murine retroviruses (X-MLVs) and other members of the genus *Gammaretrovirus*. The lifecycle and the diseases associated with these viruses provide important lessons for the study of XMRV. Although XMRV is the first gammaretrovirus discovered to infect humans, in other mammals, members of this genus are known to be associated primarily with leukemia and lymphomas but also with other malignancies, immune deficiencies and

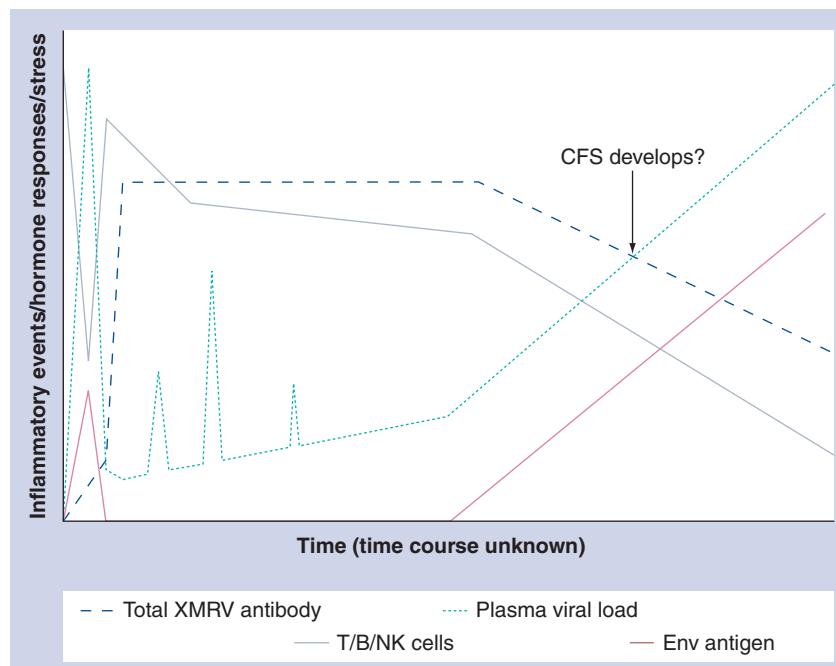


Figure 2. Schematic of hypothesis of xenotropic murine leukemia virus-related virus-induced pathogenesis.

CFS: Chronic fatigue syndrome; NK: Natural killer; XMRV: Xenotropic murine leukemia virus-related virus.

neurological diseases. Following gammaretrovirus infection, leukemogenesis is thought to occur primarily by insertional mutagenesis. Although XMRV integration sites have been examined only in a small number of prostate cancer tissue samples from XMRV-infected individuals, to date, these samples did not show evidence of a common integration site near known oncogenes or tumor suppressor genes [7,8]. However, a number of the proviruses were integrated into transcriptionally active regions and several of the samples contained XMRV insertions near cancer-related genes and cancer breakpoints. Since XMRV has been found in stromal as well as tumor tissue, more studies are needed to determine whether XMRV is causative in cancer and, if so, whether XMRV directly promotes prostate cancer by insertional mutagenesis or has an indirect effect through suppression of the immune system.

Therapeutic opportunities

Can antiretroviral therapy impair XMRV replication *in vivo*, decrease or prevent the spread between individuals or be of clinical benefit? The answers to these questions depend on the mechanism(s) of XMRV replication and the mode of virus transmission. At this time, the level of XMRV replication in infected individuals, the cell types infected and the resultant pathogenesis of this infection are not understood.

Human T-lymphotropic virus-1 replicates primarily through expansion of previously infected cells through the expression of viral oncogenic proteins. Antivirals that directly target retroviruses have been primarily developed and tested against HIV-1, although some appear to have a beneficial effect in HTLV-1-infected individuals. A recent study has demonstrated the effectiveness of azido-deoxythymidine against XMRV replication in cell culture [47], and two additional studies have identified the integrase inhibitor raltegravir and the nucleoside transcriptase inhibitor tenofovir disoproxil fumarate as potent inhibitors with synergistic function [48,49]. An obvious benefit of exploiting HIV-1 antivirals for possible treatment of an XMRV infection is that they are already tested and approved for use in humans.

The utility of drugs that impair virus replication will be more pronounced if robust replication of XMRV is intrinsic to its pathogenesis. If XMRV prevalence in humans is found to be causally associated with disease, the extensive experience in HIV-1 antiviral drug development that exists in the biomedical research community

and pharmaceutical industry can be leveraged to develop specific inhibitors. At the moment, the use of antiretroviral drugs in persons with CFS or prostate cancer are biological experiments rather than a clinically tested therapy.

Conclusion

There are now three well-documented human exogenous retroviruses: HIV, HTLV (both complex retroviruses) and XMRV (a simple retrovirus). All human retroviruses are associated with both cancer and neurological disease. There are variants of HIV and HTLV with different pathogenic profiles; likewise there could be variants of XMRV that might contribute to the divergent disease profiles seen in prostate cancer and ME/CFS. The association of XMRV with two disparate conditions, prostate cancer and CFS, raises the possibility that this virus may be both widespread and a public health concern, especially given that the presence of infectious virus in human blood has already been demonstrated [23]. Recently, several countries have excluded blood donation from individuals with a CFS diagnosis.

Executive summary

Xenotropic murine leukemia virus-related virus: a new human retrovirus

- The isolation of transmissible xenotropic murine leukemia virus-related virus (XMRV) from myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS) patients and phylogenetic analysis of full-length clones, both from natural isolates and from molecular clones from prostate cancer tissues, establishes XMRV as a new human retrovirus.

Clinical & immunological aspects of ME/CFS

- ME/CFS is a heterogeneous disease currently diagnosed through exclusion. Chronic immune activation, increases in inflammatory cytokines and chemokines, and dysregulation of the type I interferon and RNase L antiviral pathway are hallmarks of the disease in some patient populations. Currently, there is a lack of molecular biomarkers of disease subgroups. XMRV may provide a biomarker for stratification of patient populations for therapeutic interventions.

Association of XMRV with ME/CFS

- XMRV was detected in ME/CFS patient blood samples by:
 - Serological detection of antibodies to XMRV envelope protein
 - Detection of viral proteins on immunoblots
 - Detection of proviral DNA by PCR of DNA
 - Detection of XMRV RNA by reverse transcription-PCR
 - Isolation of XMRV from the cultured leukocytes of ME/CFS patients

Incidence of XMRV in prostate cancer & ME/CFS

- While XMRV has been detected in prostate tumor tissue and blood samples from ME/CFS patients in several studies, there have also been reports that have failed to detect XMRV in either prostate tumors or in ME/CFS patients. The negative results may reflect the heterogeneity of patient populations, differences in the geographical distribution of XMRV and/or differences in the sensitivities of the assays used to detect XMRV.

Possible mechanisms of pathogenesis of XMRV

- As in other human retroviral infections, XMRV viral proteins, ENV and GAG, may directly or indirectly activate the immune system through chronic stimulation of cytokine and chemokine production in the tissues. Variation in the XPR-1 receptor and envelope protein, through dysregulation of cellular signaling pathways, may also play a significant role in the pathogenesis of XMRV.

Therapeutic opportunities

- Antiretrovirals have recently been demonstrated to exert potent and synergistic inhibition of XMRV replication *in vitro*. Clinical trials are needed in XMRV-infected populations in order to determine the efficacy of antiretrovirals. Moreover, there will likely be a need to develop less toxic XMRV-specific inhibitors such as entry inhibitors. The potential involvement of immune activation of cytokines, chemokines and cellular signaling pathways affords the opportunity to design clinical trials to test small molecules known to block the activation of these pathways. Combined anti-inflammatory and antiretroviral therapies may be valuable in the treatment of XMRV infection.

In order to ensure the safety of the blood supply, sensitive nucleic acid and serologic tests are needed for high-throughput screening and for the development of therapeutics for clinical testing.

Future perspective

If future studies support a role for XMRV as a biomarker and/or therapeutic target in prostate cancer and/or ME/CFS, treatment strategies could significantly impact both diseases. Currently, most early-stage prostate cancers are not treated because most do not progress to metastatic tumors. A biomarker for determining the difference between indolent and life-threatening tumors is a critical need. If XMRV proves to be associated with the more aggressive cancers, then XMRV-infected patients may need to be treated more aggressively. Presently, there are no effective treatments for ME/CFS. The advent of highly active antiretroviral therapy [49] for HIV-1 infection and steroid suppression

of chronic immune activation in HTLV-1-associated myelopathy [50] has markedly reduced neurological morbidity and mortality. Based on the known efficacy of highly active antiretroviral therapy for other retroviral infections, we are thus cautiously optimistic that, within a few years, therapeutic intervention could be developed that targets XMRV or the immune response to XMRV and thus could treat or prevent XMRV-associated disease.

Financial & competing interests disclosure

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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