Therapeutic manipulation of host cell death pathways to facilitate clearance of persistent viral infections

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ABBREVIATIONS

cART: Combination anti-retroviral therapy

cccDNA: Covalently closed circular DNA

cIAP: Cellular inhibitors of apoptosis

CLL: Chronic lymphocytic leukemia

CMV: Cytomegalovirus

EBV: Epstein Barr-virus

HBV: Hepatitis B virus

HCV: Hepatitis C virus

HDACi: Histone deactylase inhibitors

HIV: Human immunodeficiency virus

HSV: Herpes simplex virus

HTLV-1: Human T-leukemia virus 1

IL-7: Interleukin-7

LCMV: Lymphocytic choriomeningitis virus

PRR: Pattern recognition receptors

RIPK1: Receptor-interacting kinase 1

SAHA: Suberoylanilide hydroxamic acid

SMAC: Second mitochondria-derived activator of caspases

Tcm: T-central memory

TNF: Tumor necrosis factor

TNFR1: Tumor necrosis factor receptor 1

TRAIL: TNF-related apoptosis-inducing ligand

ABSTRACT

Most persistent viral infections can be controlled, but not cured, by current therapies. Abrogated anti-viral immunity and stable latently-infected cells represent major barriers to cure. This necessitates life-long suppressive anti-viral therapy. Achieving a cure for HIV, hepatitis B virus, Epstein Barr-virus and others, requires novel approaches to facilitate the clearance of infected cells from the host. One such approach is to target host cell death pathways, rather than the virus itself. Here we summarize recent findings from studies that have utilized therapeutics to manipulate host cell death pathways as a means to treat and cure persistent viral infections.

Introduction

Persistent viral infections are those that are not cleared from the host, primarily due to pathogen mediated immune escape or other immune failings [1,2]. Human immunodeficiency virus (HIV), hepatitis B and C virus (HBV, HCV), Epstein Barr-virus (EBV), herpes simplex virus (HSV), human T-leukemia virus 1 (HTLV-1) and cytomegalovirus (CMV) are among the most prevalent persistent viral diseases globally. A staggering 2 billion individuals have been infected with hepatitis B worldwide [3] and of these, 240 million have chronic active hepatitis. HBV infection was responsible for 1.45 million deaths in 2013, an increase from the year 1990 despite advances in treatment and vaccination [4]. Although mortality caused by HIV has decreased as a proportion of those infected, the total number of individuals living with HIV has steadily increased and reached 38.8 million in 2015 [5]. Epstein Barr-Virus is carried asymptomatically by 90% of the global population and causes malignancy in a small proportion of carriers [6]. Epstein Barr-Virus associated malignancy caused 140,000 deaths in 2010, representing 1.8% of all cancer deaths that year [7]. Aside from morbidity directly associated with these viruses, they contribute to increased incidences of co-morbidities [8-10], negatively impact the quality of life of sufferers and are a significant global economic burden. Novel therapeutic strategies are necessary if cures for these infections are to be found.

A major barrier to achieving the holy grail of HIV therapeutic interventions, that of a sterilizing cure, is the clearance of the latently infected cell. Latently infected cells are largely indistinguishable from uninfected cells, rendering host immune responses and therapeutic interventions ineffective in clearing the pathogen. This observation is no better underscored than in the context of HIV, where

antiretroviral therapy has turned this once deadly viral pathogen into a relatively manageable condition, with the advancement of combination antiretroviral therapy (cART). Antiretroviral compounds target numerous stages of the viral replication cycle with high efficacy and abort infection in cells newly exposed to the virus. However, these compounds do not eliminate the pro-viral DNA inserted into the host genome of surviving cells and the virus enters a transcriptionally silent 'latent' state. Moreover, a high mutational rate can allow the virus to acquire resistance to antiviral compounds under sub-optimal regimens [11]. Life-long and stringent ART administration is essential to prevent viral recrudescence (reviewed [12]). Hepatitis B virus also persists in the nucleus of infected cells in the form of a highly stable episome referred to as covalently closed circular DNA (cccDNA). Additionally, sub-genomic components of the virus can integrate into the host genome. The immune system is able to control infection in the majority of people infected with HBV. However, in 5-10% of cases (much higher in children) the viral episome and integrated sub-genomic elements remain transcriptionally active, coding for the production of viral antigens. Despite high serum levels of viral antigens, the immune system fails to efficiently respond and control infection[13]. Similar to HIV, people with active HBV infection require life-long antiviral therapy to suppress virus production but this therapy does not eliminate the nuclear viral templates [14]. In contexts such as these, novel therapeutic approaches and compounds are required to achieve a sterilizing cure. Targeting the host cell, rather than the virus is one such approach. Here we review what early progress has been made in attempts to examine the efficacy of therapeutics that target host cell death pathways to facilitate viral clearance from the host.

An introduction to programmed cell death mechanisms

Programmed cell death is an essential mechanism for normal embryonic development, cellular homeostasis and immune regulation [15]. Apoptosis [16], programmed necrosis (necroptosis) [17] and pyroptosis [18] comprise three main pathways of programmed cell death. Apoptosis, the prototypic and

best defined programmed cell death pathway, is a non-inflammatory form of cell death that occurs following the activation of a family of cysteine-aspartic proteases known as caspases that facilitate ordered degradation of the cellular contents and death of the cell [19]. Unlike apoptosis, necroptosis and pyroptosis are highly inflammatory modes of cell death but will not be the focus of this review. Apoptosis, can be activated via two well defined and highly regulated pathways referred to as the intrinsic and extrinsic apoptotic pathways. The intrinsic apoptotic pathway, regulated by the pro- and anti-apoptotic Bcl-2 family of proteins, responds to cellular stress, including cytotoxic and genotoxic stimuli, growth factor deprivation and endoplasmic reticulum stress. Following a death stimulus, pro-apoptotic Bcl-2 family members, Bax and Bak, oligomerize and permeabilize the mitochondrial membrane allowing the release of cytochrome C which activates the initiator caspase, caspase 9, in the cytosol leading to cell death [20]. Upstream of Bax and Bak, pro-survival Bcl-2 family members, Bcl-2, Bcl-xL, Bcl-w, Mcl-1 and A1 function to inhibit the action of Bax and Bak. The pro-survival Bcl-2 family members themselves are inhibited by BH-3 only Bcl-2 proteins such as Bim and Bid. These anti- and pro- apoptotic Bcl2 family molecules differentially compete to regulate the process of apoptosis. The balance between the quantity of pro- and anti-apoptotic family members is governed by transcriptional and post-translational mechanisms and stoichiometry of these molecules ultimately determines the cell's fate [21]. The extrinsic apoptotic pathway is mediated by extracellular death receptor ligands, namely tumor necrosis factor (TNF), TNF-related apoptosis-inducing ligand (TRAIL) and FAS ligand [16]. Binding of these ligands to their cognate receptors can result in activation of downstream signaling and activation of the initiator caspase, caspase 8, and subsequent cell death. Dysregulation of these extrinsic and intrinsic apoptotic components can prevent physiological and homeostatic apoptosis, leading to various auto-immune disorders [22] and promoting survival of cancers (reviewed [23]). The action and expression of pro-survival Bcl-2 family proteins are often altered in hematological cancers [24]. These findings have prompted the development of compounds that block the action of these proteins in an attempt to sensitize cancers to apoptosis [25,26].

Programmed cell death in persistent viral infections

Apoptotic mechanisms are relied upon by the host for clearance of viral pathogens; shutting down the 'factory' is an effective means to cease all cellular production, including that of the virus [27]. Many aspects of innate and adaptive cellular immunity primarily rely on death ligands and intrinsic apoptotic signals to activate caspases, destroying the cell and the pathogen within [28]. Apoptotic bodies also provide a source of viral antigen for presentation after phagocytosis by macrophages and dendritic cells [29,30], potentially eliciting an immune response to promote viral clearance [31]. The contribution of necroptosis and pyroptosis to the pathophysiology of infection is reviewed elsewhere [32,33]. Considering the virus' reliance on a functional host cell for its lifecycle, and the host's reliance on programmed cell death for viral clearance, it is not surprising that many viruses have evolved mechanisms to actively subvert, delay or otherwise manipulate apoptosis to persist within the host (reviewed [34]). Virally mediated modulation of host cell death pathways is often critical for the virus to establish latency, as is the case for many herpesviruses, poxviruses and cytomegaloviruses [35,36]. Viral homologues of host pro-survival proteins such as vBcl-2 [37] and vFLIP [38], have been implicated in this phenomenon. Viral proteins such as the human T-leukemia virus 1 transcriptional transactivator, Tax, modulate host cell proteins involved in programmed cell death to persist within host cells and promote survival of transformed cells [39]. The Tax protein prevents apoptosis by activating pro-survival NF-kB, AKT and interleukin-2 pathways in infected cells [40]. EBV is associated with the development of Burkitt's and Hodgkin's lymphoma [41] by way of genomic instability and cell cycle manipulation by virally encoded proteins [42]. The causative link between some persistent viruses and cancer highlights the degree to which these viruses subvert and dysregulate host cell survival and cell death pathways to avoid clearance. The potential exists to therapeutically target intrinsic host cell death pathways that interfere with this viral pathological mechanism and induce or re-establish a death signal within infected cells. Alternatively, harnessing the pro-inflammatory milieu surrounding infected cells to

encourage their death by manipulating the extrinsic apoptotic pathway could conceivably facilitate pathogen clearance. Depriving virally infected cells of critical anti-apoptotic proteins using targeted therapeutics may be a complementary approach to current antiviral strategies and circumvents complications associated with viral resistance against antiviral compounds. The development of anti-cancer therapeutics targeting programmed cell death pathways presents an opportunity to tap into this source of cell death inducing compounds and repurpose those that demonstrate efficacy in persistent viral infections. A particular focus on compounds that are already in the clinic or undergoing clinical trials for cancer and inflammatory conditions is warranted on the basis that they can be progressed to clinical use more quickly for viral infections if efficacy is demonstrated.

Therapeutically targeting intrinsic apoptotic host cell pathways to promote viral pathogen clearance

The shock and kill approach to HIV eradication postulates that cells harboring latent virus should die, as actively infected cells do, if reactivated by latency reversing agents such as histone deactylase inhibitors (HDACi). Although initial results in human trials were promising with respect to viral transcription, no meaningful impact on integrated HIV DNA was observed by utilizing this approach [43]. It is evident that some latently infected cells are refractory to death following reactivation, explaining the lack of contraction of the latent pool. The latent HIV pool resides predominantly within the central memory CD4 T cell (Tcm) compartment likely in part due to their innate ability to resist apoptosis, a property crucial to long lived cells [44,45]. Memory T-cells express higher levels of Bcl-2 relative to pro-apoptotic proteins, possibly promoting survival following reactivation and contributing to viral recrudescence following cessation of ART [46]. Indeed, CD4 T-cells transduced with Bcl-2

become long-lived in culture [47] and memory T cells rely on the interleukin-7 (IL-7) cytokine for upregulation of pro-survival Bcl-2 members to survive [48]. The role of Bcl-2 for infected cell survival was confirmed in an in-vitro model of persistent HIV infection (J-Lat 10.6 model) [49]. Venetoclax, a clinically relevant Bcl-2 antagonist (fig 1) may sensitize these cells to apoptosis upon HIV reactivation particularly as they are addicted to pro-survival Bcl-2 to counteract pro-death proteins[46,50,51]. This supports the claim for potential clinical use of venetoclax for the treatment of HIV in a setting where ART will continue to be standard of care. Bcl-2 antagonism by venetoclax inhibits proliferation and induces death of chronically HIV-infected cells in this setting, establishing Bcl-2 as necessary for the survival of latently infected cells. Venetoclax is an FDA approved compound currently employed in the clinic for the treatment of chronic lymphocytic leukemia (CLL) [52] It is potentially many steps closer to clinical use in HIV on the virtue of having already passed pharmacology, drug-drug interaction and safety profile testing in clinical trials for the treatment of CLL. This may permit rapid repurposing and implementation of venetoclax in the clinic for HIV in combination with current antivirals, however more robust pre-clinical studies are necessary.

Different cellular niches evidently rely on different pro- and anti-apoptotic regulators to dictate their survival or death. Macrophages are susceptible to HIV infection, resistant to HIV cytopathic effects and are therefore long lasting reservoirs of the virus [53]. The anti-apoptotic Bcl-xL and Mcl-1 as well as cellular inhibitor of apoptosis proteins (cIAPs) contribute to macrophage resistance to HIV-Vpr-induced apoptosis [54]. Knock down of Bcl-xL and Mcl-1 as well as antagonism of cIAPs by a second mitochondrial activator of caspases (SMAC) mimetic compound sensitized infected macrophages to apoptosis [54]. Therapeutic antagonism of multiple anti-apoptotic members may be necessary to kill infected cells and facilitate clearance of HIV from multiple cellular reservoirs.

The ability of a cell to detect intracellular viral pathogens is a critical innate host defense mechanism, allowing cells to respond to danger via pro-inflammatory interferon production to prevent the spread of viral progeny [55]. Toll-like-receptors and RIG-I-like receptors belong to a family of pattern recognition receptors (PRRs) that detect pattern recognition receptors within the cell and mediate these host responses [56] and may also induce apoptotic cell death as means to combat infection [57,58]. Persistent viruses however, employ mechanisms to evade these modes of detection [59]. HIV encodes a protease that directs RIG-I to the lysosome for degradation, thus suppressing the cells ability to detect the virus and promoting the establishment of viral latency [60]. A recent study demonstrated that the FDA-approved retinoic acid derivative, acitretin (fig 1), induced RIG-I expression permitting increased intracellular detection of virus, and subsequent Bax dependent apoptosis of infected cells in-vitro [61]. They showed enhanced killing of latently infected cells by acitretin in combination with the histone deacetylatase inhibitor, suberoylanilide hydroxamic acid (SAHA) or vorinostat, a latency reversing agent. Critically, this was observed in the context of antiretroviral therapy suppression and using clinically achievable concentrations of acitretin, granting clinical applicability to this finding. Acitretin is used in the clinic for the treatment of severe psoriasis and which opens the possibility of rapid repurposing and implementation into combination HIV treatments.

Therapeutically targeting death ligand induced apoptosis to promote viral pathogen clearance

Cells may respond differently to the same inflammatory stimulus, a critically important feature for a balanced immune response. The TNF signaling pathway may result in two possible outcomes, pro-survival and pro-death, depending on a multitude of extra-cellular and intra-cellular factors (reviewed [62]). The pro-survival route of the TNF signaling pathway ultimately leads to NF-kB and MAP kinase activation, and a pro-inflammatory/pro-proliferative cell state ensues. The pro-death pathway results

in caspase dependent apoptotic cell death, or in context of caspase inhibition, necroptotic cell death [63]. The TNF pathway is tightly regulated by a multitude of scaffold proteins, kinases, E3 ubiquitin ligases and de-ubiquitin ligases. Cellular inhibitor of apoptosis proteins are one such E3 ubiquitin ligase protein family and function at a critical intersection of the tumor necrosis factor receptor 1 (TNFR1) signaling pathway [64] to facilitate an inflammatory cell state and cell survival. In order for the pro-survival route of the TNF pathway to proceed, receptor-interacting protein kinase (RIPK1) is recruited to the receptor complex and ubiquitinated by cIAP 1 and 2. These ubiquitin chains form a scaffold for recruitment of downstream kinases and subsequent NF-kB activation. A multitude of pro-survival factors are transcribed by NF-kB such as Bcl-2 and c-Flip that ensure survival of the cell. In the absence of cIAPs however, RIPK1 is no longer ubquitinated and associates with the adaptor protein, FADD, and caspase 8, resulting in caspase 8 activation and cell death [65]. Our group recently demonstrated that cIAPs are critical for maintenance of hepatitis B virus in an immunocompetent mouse model of hepatitis B infection [66]. Gene targeted mice lacking cIAP1 and cIAP2 rapidly cleared virally-infected hepatocytes compared to wildtype animals in a TNF-dependent manner. Cells that are most permissive to viral infection have high TNF-NFκB activity [67] and TNF/TNFR1 upregulation on hepatocytes in this pre-clinical model provides ample fuel for driving increased cell death and pathogen clearance in the absence of cIAPs. Importantly, non-specific killing of uninfected cells was not observed. Cellular inhibitor of apoptosis proteins are naturally inhibited by the SMAC protein that is released by the mitochondria during apoptosis [68]. A positive loop is therefore formed, ensuring the demise of the cell. The SMAC protein possesses the ability to sensitize tumor cells to death, and hence has been the focus of development of compounds that mimic its function [26]. One such drug, birinapant (fig 1), is a bivalent SMAC mimetic compound that potently inhibits cIAPs and has shown efficacy in tumor models [69]. Subsequent to the initial finding of increased hepatitis B viral clearance in cIAP knockout mice, we demonstrated that antagonizing cIAPs by administration of birinapant, permitted specific TNF mediated killing of hepatitis B-virus infected hepatocytes [70]. Furthermore, birinapant acts synergistically with the nucleoside analog, entecavir, to promote synergistic clearance

of hepatitis B virus surface antigen. This synergy is proof of concept for combining cell death inducing compounds with current antiviral therapies for more effective therapeutic treatment of persistent viral infections.

Potential for combinatorial therapeutic approaches targeting cell death pathways

Combination therapies that target multiple critical proteins in cell signaling and cell death pathways may be more effective at clearing a persistent virus than using any one compound alone. This is certainly the case in the context of cancer therapy [71]. Recent work has shown that inhibition of p38, a mitogen-activated protein kinase, or its downstream kinase MK2, resulted in increased production of TNF in the presence of a SMAC mimetic compound [72]. This increased TNF production provides the fuel for TNF mediated cell death in the presence of IAP inhibitors. Critically, p38 and MK2 inhibition (fig 1) enhanced SMAC mimetic mediated killing in-vitro and combination of clinical IAP and p38 inhibitors was showed to be therapeutically effective in an in-vivo model of adult myeloid leukemia. P38 inhibitors are currently under investigation for the treatment of various autoimmune diseases [73], but their progress is hampered by dose-limiting toxicities. MK2 however has fewer toxicities and is anticipated to be a more clinically viable target. MK2 was recently discovered to act as a checkpoint in the life-death axis of the TNFR pathway and functions to inhibit TNF mediated cell death by preventing RIPK1 from associating with FADD and caspase 8 and inducing cell death [74]. The intriguing possibility exists for the use of p38 or MK2 inhibitors to enhance the death of virally infected cells by SMAC mimetics for the treatment of persistent viral infections such as hepatitis B.

As eluded to earlier, apoptotic death of infected cells augments an immune response by providing a source of exogenous viral antigen for antigen presenting cells following phagocytosis of apoptotic

bodies [29] [30]. This permits more effective priming of naive lymphocytes that are specific for viral antigen. The possibility exists of combining cell death-inducing therapeutics with existing immunotherapies such as administration of exogenous IL-7[75,76], immune checkpoint inhibitors [77] or adoptive transfer of virus specific T-cells [78] to augment pathogen clearance. Similar approaches are of interest for the treatment of cancers[79]. For example, Interleukin-7 is a critical cytokine required for normal development and homeostasis of lymphocytes [80] and its administration into lymphocytic choriomeningitis virus (LCMV) infected mice has been shown to substantially enhance the number of functional virus-specific CD8 T cells[81,82]. Combining exogenous IL-7 with cell death inducing therapeutics may act synergistically to kill infected cells by attacking from potentially three different angles: 1) induced cell death of infected cells directly by cell death inducing therapeutics, 2) increased presentation of viral antigen to existing virus specific T-cells by increased numbers of phagocytosed apoptotic bodies and, 3) augmented lymphocyte responses by expanding functional virus specific lymphocyte populations by administration of exogenous IL-7. Indeed, latently HIV-infected cells are killed more efficiently when reactivated and co-incubated with pre-stimulated autologous CD8 T-cells from an elite controller [83].

Concluding remarks

Therapeutically targeting host cell death pathways for the treatment of persistent viruses is a promising novel approach to achieve a sterilizing cure. Reliance of persistent viruses on cell survival is evidenced by the many pathological mechanisms employed by viruses to subvert and manipulate host programmed cell death. Interfering with host apoptotic pathways and inducing specific cell death of infected cells aims to facilitate clearance of the viral pathogen. Targeting the host, rather than the virus itself, substantially mitigates the likelihood of acquired resistance as seen for compounds that

exclusively target the viral pathogen. Several therapeutics are being developed to target the apoptotic pathways, primarily to treat cancer and autoimmune disease. This drug development provides an opportunity to repurpose those drugs that demonstrate efficacy in clearing persistent virus by inducing specific killing of infected cells. Proof of principal demonstrated by pre-clinical studies provide grounds for further investigation. Achieving a functional and sterilizing cure for any persistent viral pathogen will likely require a combinatorial therapeutic approach with existing antivirals. Compounds that target host apoptosis are increasingly becoming viable candidates as part of this approach.

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JC wrote the manuscript. SP made the figure. Editorial and intellectual input from CA and MP.

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CONFLICT OF INTEREST DISCLOSURE

JC and SP are post-graduate students and CA and MP are employees of the Walter and Eliza Hall Institute of Medical Research that receives milestone and royalty payments related to venetoclax.

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Figure 1. Therapeutic interventions that augment apoptosis can be utilized to kill infected cells.

The extrinsic apoptotic pathway is induced by death receptors of the TNF superfamily, including TNFR1 (top left). Binding of TNF to TNFR1 induces the formation of complex I, that consists of TRADD, RIPK1, Caspase 8, TRAF2 and the E3 ubiquitin ligases cIAP1, cIAP2. cIAPs generate ubiquitin chains attached to components of complex I, including RIPK1 (top left). Through a cascade of phosphorylation events, this leads to liberation of NF-kB that translocates to the nucleus and induces the production of inflammatory mediators and pro-survival proteins including cFLIP, BCL-2 family members and TNFR1 (bottom left). Inhibition of cIAPs by SMAC mimetic compounds (i.e. birinapant) prevents ubiquitination of RIPK1 which then associates with caspase 8 and FADD to form complex II (middle). Unless inhibited by sufficient levels of cFLIP, complex II results in caspase dependent apoptosis. Virally infected cells that upregulate TNFR1 may be sensitized to apoptosis following treatment with birinapant. The MAPK pathway can be activated by oxidative stress and other signals (top middle) and functions to activate a host of transcription factors involved in cell cycle regulation, differentiation, inflammation and apoptosis. Inhibition of downstream MAP kinases such as P38 MAPK (not shown) and MK2 (MK2i) can enhance the production of autocrine TNF when in the presence of SMAC mimetics and enhance the killing capacity of therapeutics such as

birinapant. MK2 also inhibits complex II formation and apoptosis by phosphorylating RIPK1. HIV RNA in the cyotosol of HIV infected cells can be detected by the RNA sensor RIG-I and may lead to Bax dependent apoptosis of infected cells (bottom middle). Acetretin is a therapeutic that has been shown to induce the expression of HIV RNA in latently infected cells as well as inducing the expression of greater amounts of RIG-I (bottom middle). Higher levels of RIG-I lead to greater detection of HIV RNA and induced apoptosis. The intrinsic apoptotic pathway can be triggered by a number of stimuli that include stress, cytokine withdrawal and cytotoxic agents (top right) and results in Bax and Bak oligomerization on the mitochondrial membrane. Bax/Bak oligomerization results in pore formation, membrane disruption, release of cytochrome c, activation of the apoptosome and subsequent apoptosis via cleavage of downstream caspases (left). Pro- and anti-apoptotic Bcl-2 family members differentially compete to regulate the oligomerization of Bax and Bak to determine the cell's fate. Anti-apoptotic Bcl-2 proteins (i.e. Bcl-2 and Mcl-1) act to inhibit Bax/Bak oligomerization by direct binding. Pro-apoptotic BH3-only Bcl-2 family members (i.e. Bim and Bid) may inhibit anti-apoptotic members or activate Bax/Bak directly. Venetoclax is a BH3-mimetic that specifically antagonizes the pro-survival protein Bcl-2 and induces apoptosis in cells that are reliant on Bcl-2 for survival.