Pathogenesis of human cytomegalovirus in the immunocompromised

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Abstract

Human cytomegalovirus (HCMV) is a herpesvirus that infects 60% of adults in developed countries and over 90% in developing countries. Normally, it is controlled by a vigorous immune response so that symptoms are mild. However, if the immune system is compromised, HCMV can replicate to high levels and cause serious end-organ diseases. Substantial progress is being made in understanding the natural history and pathogenesis of cytomegalovirus infection and disease in the immunocompromised host. Serial measures of viral load defined the dynamics of HCMV replication and are now used routinely to allow intervention with antiviral drugs in individual patients. They are also used as pharmacodynamic readouts to evaluate prototype vaccines that may protect against HCMV replication and to define immune correlates of this protection. This novel information is informing the design of randomised controlled trials of new antiviral drugs and vaccines currently under evaluation. In this Review we will discuss immune responses to HCMV and countermeasures deployed by the virus, the establishment of latency and reactivation from it, exogenous reinfection with additional strains, pathogenesis, development of end-organ disease, indirect effects of infection, immune correlates of control of replication, current treatment strategies and the evaluation of novel vaccine candidates.

Introduction

Human cytomegalovirus (HCMV), also known as human herpesvirus 5, is the prototype member of the *Betahepesvirinae*. Like all herpesviruses, it establishes latency and persists for the life of the individual. Infection with HCMV is common throughout the globe.[1] The proportion of adults with specific IgG antibodies approximates to 60% in developed countries and over 90% in many developing countries.[1] Infection is more common in those from lower socio-economic groups and from non-Caucasian backgrounds.[2] Children born in the UK to women who have moved from high risk countries have the lowered risk of their adopted country.[2,3] The saliva and urine of young children are major sources of virus, especially for those with child-caring responsibilities.[4] HCMV is not highly contagious, with a basic reproductive number of approximately 1.7-2.4.[5-7] It can also be spread sexually, by transfusion of whole blood or by organ transplantation.[8,9] It is important to note that there are usually no symptoms associated with HCMV infection, except for occasional cases of infectious mononucleosis. This is

because a robust immune response to HCMV normally prevents the high viral loads required to cause the end-organ diseases (EOD) seen in immunocompromised hosts. However, despite the absence of overt symptoms, there is evidence that infected individuals may have long term adverse outcomes related to induction of a chronic inflammatory cell-mediated immune response to this apparently innocuous virus (indirect effects).[10]

The natural history of HCMV infection is complex, with three different subtypes of infection.[9] Primary infection occurs when an individual with no immunity against this virus becomes infected for the first time. Afterwards, the virus establishes latency from which it may reactivate (second type of infection). The third type of infection is called reinfection when contact with an infectious individual superinfects someone who has already been infected and despite their possession of natural immunity.[9] Any of these three subtypes of infection can complicate pregnancy, making HCMV the commonest cause of congenital infection.[11] It is also the most common and the most serious opportunistic infection after solid organ transplantation (SOT) or haematopoietic stem cell transplantation (SCT) and remains an important opportunistic infection in patients with HIV infection.[9,12,13]

In this review, we will not discuss infections in pregnancy, but will focus on HCMV infection in immunocompromised patients. Our aim is to integrate information about serial measures of viral load to explain features of pathogenesis, differences between distinct patient groups, show how active infection (although asymptomatic) is routinely monitored in selected patients and illustrate how prototype vaccines can be evaluated for efficacy. We will emphasise the evidence provided by double-blind randomised placebo-controlled clinical trials (DB PC RCTs) of active or passive immunotherapy specific for HCMV.

HCMV immune evasion and viral latency

Our understanding of the complex interaction of HCMV with the immune response has been informed, in part, by comparative analyses of established laboratory-adapted strains of HCMV (described in Box1) plus clinical isolates. with an overview of infection of cells shown in Figure 1. Less extensive variation in sequence also occurs naturally between HCMV strains and has the potential to impact pathogenesis and vaccine development. Note that individual genes of HCMV are numbered sequentially with the unique long, unique short or terminal repeat regions of the genome: thus, UL54 refers to the 54th gene in the unique long region.

Numerous studies have demonstrated that HCMV encodes countermeasures against the spectrum of immune responses.[14-16] This arsenal of immune-modulatory functions is likely a reflection of the natural history of the virus – whereby it has the capacity to establish lifelong infections of the host as well as re-infect people with an existing infection despite the presence of a substantial immune response – particularly an enlarged T cell compartment dominated by anti-HCMV T-cell responses that often exceeds 10%.[17]

Detailing the complexity of these immunological interactions is beyond the scope of this review and has been reviewed extensively elsewhere.[18-25] Suffice it to say that HCMV encoded gene functions target antigen presentation by MHC class I and class II molecules, utilise cytokine mimicry to exert paracrine functions against immune cells, and encode proteins that antagonise the range of innate immune responses directed against the virus. Despite this, HCMV infection or reactivation in the immune-competent is rarely a cause of morbidity, arguing that the surfeit of immune evasion mechanisms encoded by HCMV are imperative for long term persistence in the host but not sufficient to completely evade immune surveillance.

It is tempting to propose the long term solution for HCMV to evade immune responses is by hiding from them, rather than by running from them, as seen with HIV. This strategy of hiding from immune surveillance is exemplified by the virus spreading cell-to-cell within a sanctuary site of persistence. This is coupled with an ability to establish latency providing a mechanism by which the virus can go to ground if the immune system gains the upper hand, only to return later through reactivation if the immune system becomes impaired.

The ability to establish lifelong latent infections of the host is a defining characteristic of herpesvirus infections.[26] HCMV establishes latent infections in bone marrow haematopoietic progenitor cells.[27] Additionally, tissue endothelial cells may be another reservoir of latent/persistent infection and thus may contribute to HCMV pathogenesis in organ transplantation.[28] Latency can be defined as the persistence of the viral genome in an absence of lytic replication and virus production coupled with the retention of a capacity to reactivate when specific conditions are met. Typically, cellular differentiation and inflammation associated signalling appear to be important events required for reactivation in the myeloid lineage.[29-34] Specifically, differentiation of myeloid progenitors to macrophages (Mp) or dendritic cells (DCs) drives reactivation via the re-animation of a key viral gene locus required for lytic infection – the major immediate early (MIE) region.[27]

The activity of inflammatory cytokines as inducers of MIE gene expression and HCMV reactivation (e.g. TNF, IL-6) may be important in the process of organ transplantation which is associated with significant inflammation *(*FIG 2*).*[35-37] Indeed, one of the first reports of reactivation of naturally latent HCMV in vitro was achieved using the cocktail of cytokines released from allogeneically stimulated T cells.[29] Furthermore, sepsis increases the incidence of HCMV reactivation in immune-competent intensive care patients which could again be linked with the inflammation associated with bacterial complications.[38,39] Consequently, it is hypothesised that inflammation associated signalling is a key driver of reactivation. The fact that HCMV has evolved mechanisms to modulate host cell signalling during latency and reactivation would be consistent with this.

A common refrain is that immune-suppression is a key trigger of HCMV reactivation yet HCMV reactivation can be modelled in vitro in cell culture

systems. Thus a distinction between clinical reactivation and cellular reactivation is important. Clinical reactivation is the detection of viraemia in seropositive patients. Cellular reactivation is the re-initiation of viral replication in differentiated permissive cells. Importantly, understanding the relationship between these two events is likely crucial for understanding the basis of viraemia in immune-compromised HCMV seropositive individuals.

Studies of cells isolated from healthy immune-competent individuals have shown evidence of HCMV lytic gene expression in dendritic cells (cellular sites of HCMV reactivation) despite no evidence of viraemia. This argues that HCMV latency and reactivation is an ongoing event in the host – an event that is controlled by the prodigious immune response directed against it. Indeed, this constant exposure of HCMV to the immune system likely explains the immunological space devoted to control of the virus. Thus, in individuals with compromised immune systems (e.g. transplant), reactivation is still occurring but the loss of immune function allows the virus to reactivate unchecked, leading to viraemia and, ultimately, disease. Importantly, in many of these scenarios where we observe immune-suppression there is a concomitant inflammatory environment driven by co-infections or allogeneic T cell responses that exacerbate the situation due to their pro-viral roles in replication.

It is noteworthy that HCMV is often one of the first viral pathogens to reveal itself in transplant patients. This pre-eminent emergence is easily explained if we assume the virus is being controlled at the point of reactivation rather than at latency. Essentially, immune-suppression is akin to releasing the brake rather than representing the trigger. What remains to be understood is the relative contributions that different aspects of the innate and adaptive immune responses make towards the control of HCMV infection in vivo in different clinical settings of immune-suppression or immune-compromise. The first clues will come from studies of immune function in different patient cohorts and, specifically, identifying the loss of which elements of the response are responsible for pathogenesis (Fig. 2B).

Natural history and pathogenesis

We will review each patient group in turn. Note that they have different reasons for being immunocompromised (Box 2). Note also that each EOD is similar across different patient groups eg HCMV retinitis looks the same in all patients. Diagnosis requires evidence of symptoms at the affected site together with a biopsy showing histopathological changes of owl's eye inclusion bodies and/or immunocytological staining to demonstrate productively infected cells, except in the case of retinitis where the characteristic clinical appearance of haemorrhage in retinal vessels accompanied by exudate is accepted.[40]

Retinitis may be reported by the patient as a visual loss or "floaters" passing across the visual field, or may be asymptomatic and identified by clinical examination alone. This disease is attributed to lytic infection; a conclusion supported by clinical resolution with antiviral therapy (systemic ganciclovir

plus intravitreal foscarnet if the retinitis is immediately sight-threatening). Healing is by fibrosis, predisposing the patient to future retinal detachment as a cause of major vision loss. When antiretroviral therapy was introduced, some HIV patients developed immune recovery uveitis; an inflammatory response to the presence of HCMV antigens within the eye.[41] This condition may cause more visual disturbance to a patient than the underlying retinitis. Pneumonitis is typically seen soon after SCT. The viral load in bronchoalveolar fluid is high.[42] The pathology involves interstitial recruitment of lymphocytes increasing the distance that oxygen has to flow between the alveoli and blood vessels in the lung. There is evidence for an immunopathological contribution to this disease.[43]
The remaining EODs of hepatitis, gastrointestinal ulceration and rarer conditions such as nephritis or pancreatitis are assumed to be lytic in nature.

***End-organ disease in SOTs*.** The very first cases of solid organ transplantation were complicated by HCMV pneumonitis, with high mortality.[44] The pioneer transplanters mitigated this by lowering doses of immunosuppressive drugs, but at the risk of allowing graft rejection to occur. Balancing the need for immune suppression (graft rejection) with a need for immune control (HCMV) benefited greatly from the development of less toxic immunosuppressive drugs coupled with the HCMV antiviral, ganciclovir and its prodrug valganciclovir. However, the problem of HCMV EOD still persisted. Cases of HCMV pneumonitis continued to occur without prior warning; although many patients had fever, this sign is so common post-transplant that it was not specific for HCMV. This virus was also detected histopathologically in biopsies from other organs, particularly the gastrointestinal tract, in patients with oesophagitis, gastritis or colitis. It was also recognised in the eye when patients complained of visual disturbances (retinitis). These conditions were grouped together as “end-organ diseases” with a poor outcome.

Key to advancing the treatment of HCMV in SOT was defining the natural history of HCMV infection. Infection with this virus was then seen to precede EOD and to rise to high levels of viral load before EOD occurred. This gave an opportunity for active infection to be treated before it caused EOD; termed pre-emptive therapy (PET).

A series of studies in the 1990s demonstrated that the highest viral loads in urine from renal transplant patients were found in the D+R- combination; that is, the donor is infected with HCMV and the recipient has no natural immunity to this virus.[45] Importantly, this subgroup also had the highest proportion meeting a case definition[40] of CMV EOD (signs and symptoms together with HCMV detected histopathologically in a biopsy of the affected organ). Importantly, multivariate statistical models were consistent with a high viral load causing EOD, rather than the alternative interpretation of the absence of natural immunity being responsible.[45,46] Although elements of immune function are important, their contribution is already captured by quantitative measures of viraemia.[46]

Figure 3A shows how the prevalence of IgG antibodies pre-transplant can group SOT into 4 subgroups. Post-transplant primary infection occurs in 80% of cases of D+R- transplantation whereas in the D-R+ subset only 40% of recipients reactivate latent CMV after transplantation (FIG 3B).[9] The most complicated group to study are the D+R+ subset who are at risk of either reactivation or reinfection and have an intermediate risk phenotype (55%).[9] Under the simple assumption that their risk of reactivation is the same as those in the D-R+ subgroup, the rate of reinfection must approximate to 15% (55%-40%). Where pairs of two renal transplant recipients (R+ and R-) received kidneys from the same seropositive donors, sequencing formally proved reinfection where viraemia came from the donor.[47] Furthermore, the risk of EOD was intermediate between that of the D+R- subgroup (high viral load, high risk of EOD) and the D-R+ subgroup (low viral load, low risk of EOD). In the future, sequencing of strains of HCMV offers the potential of diagnosing reinfection and differentiating it from reactivation; studies are underway in the more straightforward subgroup to interpret; D+R-.[48]

The better outcomes in the R+ populations overall clearly support a role for pre-existing immunity.[9] Comparison of the upper two panels of FIG 4 reveals that pre-existing natural immunity is moderately effective at preventing high peak viral loads, thereby explaining why EOD is much more common in the D+R- subgroup than the D+R+ subgroup. However, natural immunity is not able to prevent low-level replication, as illustrated in panel C of FIG 4 which represents reactivation of latent infection.

End organ disease in stem cell transplants. Evidence that viral load and natural immunity are an important component of EOD comes from studies of the other major transplant cohort – SCT.

The first notable difference is that the high-risk group here are the D-R+ cohort (FIG 3) and thus the opposite to SOTs.[12] This view is supported by the sero-epidemiology which suggests transmission from a D+ organ is rare in this setting.[49] This is likely a function of the number of stem cells donated coupled with the very low frequency of HCMV latent cells (<0.01%) predicted to be in the graft.[50] Additionally, in the D+ setting the graft also transfers HCMV cell mediated immunity to the recipient.[51] Thus it follows that the adoptive transfer of HCMV immunity without a substantial increase in risk of HCMV infection from a D+ donor leads to better outcomes than a D- graft into an R+ individual. Experimentally, immunisation of donors prior to marrow harvest can transfer immunity to recipients.[52]

Additionally, it is clear from FIG 3B that R+ SCT patients are more susceptible to EOD than are R+ SOT, because they have a higher incidence and greater severity of EOD. This is explained by SCT patients developing EOD at lower viral loads (FIG 4D) rather than the alternative of experiencing very high levels of viral replication.[46,53,54]

The advent of pre-emptive therapy to manage HCMV infection in transplantation. What became clear from studies of both SOT and SCT

cohorts is that viraemia is a robust biomarker to predict individuals most at risk of HCMV disease post-transplant.[55]

In both transplant groups, EOD had a non-linear relationship when plotted against viral load, such that disease was uncommon until a high viral load was reached, with SCTs being more susceptible than SOTs.[46,53] Importantly, all patients could be monitored and given antiviral drugs when a low threshold of viraemia was reached.[56] Such PET is highly effective at preventing EOD (FIG 5).[57] An alternative strategy of giving antiviral drugs prophylactically is also effective, but at the risk of delaying EOD until prophylaxis is stopped (FIG 5).[58,59] Both strategies are recommended in current clinical guidelines for the management of SOT and have served to reduce the number of patients experiencing EOD.[60,61] In a recent RCT, liver transplant patients randomised to be managed with PET had significantly less late onset EOD than did those managed with prophylaxis.[61] The concept that PET allows low level antigen presentation to the immune system is supported by studies of humoral and CMI post-transplant.[57] In other words, the low levels of viraemia that occur in patients monitored by pre-emptive therapy have a low risk of causing EOD yet are sufficient to stimulate the immune system to bring viraemia under control.

These quantitative studies characterised a series of parameters that can be used to define the severity of HCMV (proportion of patients with viraemia, duration of viraemia and peak viral load). These parameters are now sufficiently robust to be accepted by regulators as endpoints for RCTs.[55] Importantly, these surrogate markers of EOD also allow the continued study of HCMV natural history and pathogenesis without compromising patient treatment. For example, they revealed that the replication dynamics of primary HCMV infection in vivo are very similar to HIV – and much quicker than anticipated based on studies of the development of cytopathic effects in in vitro culture models.[62,63]

All of these observations were assimilated into a dynamic model of HCMV infection and EOD. Within hours of transplant, HCMV reactivates from the donor organ. This productive infection may be controlled by the local immune response (FIG 2). If it is not, HCMV appears in the blood, which is how the virus disseminates to multiple organs. Note that viraemia can be detected as a leucoviraemia or a plasma viraemia. Plasma viraemia (strictly plasma DNAemia) consists of short, fragmented portions of HCMV naked DNA.[64] Although it is clearly not infectious, plasma viraemia still acts as a good biomarker for taking decisions about pre-emptive therapy.[55,65] The physical state of the virus is not defined in the case of leukoviraemia. Separation techniques using magnetic beads followed by quantitative PCR for HCMV DNA revealed that polymorphonuclear leukocytes make the largest contribution to the overall viral load in blood, but that HCMV DNA and mRNA for a late transcript were also detected in monocytes, B cells and T cells, consistent with productive infection.[66] The response to ganciclovir was similar when HCMV DNA was measured in each of these fractions of peripheral blood.[65] Viraemia is not a guarantee of EOD because immune responses (FIG 2) at the level of each organ may be able to prevent blood-organ transmission of virus and/or the development of full-blown EOD. If these

immune responses are insufficient, HCMV may rise to high levels to cause EOD through a variety of potential pathological processes. For example, low levels of virus may not initiate a full replicative cycle (FIG 1) yet display HCMV antigens on cells to make them targets for immunopathological responses. There is some evidence for such responses in the lungs,[43] although a high viral load is also found in bronchoalveolar lavage fluid in patients with extensive, established pneumonitis.[42] Higher levels may lead to productive infection (FIG 1) with lysis of target cells; the retina would be a potential candidate for this, although it may also be followed by immune recovery uveitis which is an immunopathological condition.[41] In all cases, initial immunosuppression, which may include steroids, given to all SOT patients has an effect by increasing the viral load in the blood.[67] In contrast, steroids given to treat graft rejections in SOT (or graft-versus-host-disease; GVHD in SCT) increase the risk of EOD by lowering the viral load required to cause disease (that is, steroids remain statistically independent from high viral load as a risk factor for EOD in multivariate models).[46] In summary, there is evidence for both viral lysis and immunopathology contributing to EOD, but invasive samples from the affected site are only available from late stages of disease.

End organ disease in HIV infected individuals. HCMV retinitis presented as a major complication in the dawn of the AIDS epidemic with EOD most likely to occur in HCMV seropositive individuals (and thus a result of HCMV reactivation or reinfection).[68] Indeed, it is a startling clinical observation that retinitis accounts for 85% of EOD in HIV patients compared to only 1% in the transplant groups; with no proven explanation for this. A possible explanation is that damage to the blood-retina barrier due to HIV infection may facilitate HCMV viraemia gaining preferential access to that organ.

A major difference between these individuals and the transplant cohorts is the absence of a starting point equivalent to the date of transplant to indicate when risk of HCMV EOD increases. The major indicator is when the CD4+ T-cell count of HIV+ patients falls below 100/microlitre of blood. It is in these individuals that natural history studies showed (FIG 4E) that HCMV becomes detectable and rises to high levels in the blood, similar to those found in SOT.[69] Thereafter, individuals are at risk of developing EOD, but the tempo is altered; instead of preceding EOD by weeks (SOT) or days (SCT), HIV+ patients can have high HCMV viral loads for months before developing EOD.[70] One possible explanation that we can offer is that blood-organ barriers (apart from the retina) are better preserved in HIV patients than in the transplant groups.

The 'indirect effects' of HCMV infection. EOD associated with HCMV has been well described in a number of important patient populations. What is less clear is the associated "indirect effects" of HCMV infection and replication. The term was coined by Bob Rubin to describe the excess of conditions like accelerated atherosclerosis seen in cohort studies of heart transplant patients with active HCMV infection.[71] This condition was not unique to HCMV, but the virus increased its incidence. Potential mechanisms that could lead HCMV to

contribute to atherosclerosis include systemic inflammation, monocyte activation, T-cell stimulation and effects on the endothelium.[72]

The evidence for HCMV causing such phenomena comes from observations made in patients enrolled into DB PC RCTs. For example, accelerated atherosclerosis was significantly reduced by prophylaxis with ganciclovir in D+R- heart transplant patients.[73] Likewise, the incidence of biopsy-proven acute graft rejections after renal transplant was significantly reduced in an RCT of high-dose valaciclovir in the D+R- subgroup, but not in the seropositive recipients.[74] It is often said that the low levels of viraemia found in patients managed by PET must increase their risk of graft rejection. In fact, a meta-analysis by the Cochrane collaboration of the RCTs conducted to compare PET with prophylaxis show no differeces for graft rejection, graft survival or patient survival.[75]

In the SCT population, high mortality linked to HCMV serostatus is observed, even in the absence of overt HCMV-driven EOD.[12] Death in SCT patients is divided into relapse-related (i.e. recurrence of leukaemia) or transplant-related (e.g. opportunistic infections). Recipients who are HCMV seropositive have an increased transplant-related mortality which was reduced by acyclovir prophylaxis.[76,77] The interpretation of these observations was limited by the broad acting nature of acyclovir but subsequent studies with HCMV-specific letermovir significantly reduced mortality as a pre-defined secondary endpoint of the RCT.[78] Importantly, it was the ability of letermovir to prevent viraemia that conferred the statistical benefit of reduced mortality.[79]

As with SCT, the major indirect effect of HCMV infection in HIV infected individuals is death not explained by EOD.[12,79] Interestingly, it is HCMV viraemia and CD4 count, and not HIV loads, that are the correlates of mortality.[13] Consistent with this, systemic exposure to ganciclovir in patients experiencing their first episode of retinitis reported reduced mortality rates.[80] A meta-analysis of RCTs of acyclovir also showed significant reduction in mortality.[81]

Mechanisms by which HCMV has been shown to interact with HIV in vitro are transactivation of HIV DNA and pseudotype formation, both of which require the two viruses to infect a single cell.[82] Four other mechanisms that require the two viruses to infect neighbouring cells are stimulation of cytokine release, antigen presentation, upregulation of CD4 or co-receptor and induction of an alternative entry receptor for HIV.[82] The in vivo plausibility of these interactions was shown by detecting the nucleic acid of both viruses in over 50% of tissues sampled at autopsy.[83] However, no evidence was found to support the prediction that the HIV viral load should be increased in HIV positive individuals co-infected with HCMV (reviewed in[82]). Attention therefore moved to an alternative mechanism based on the induction by HCMV of an excess of immunocommitted CD8 T-cells as part of its contribution to the "immune risk phenotype" or "immunosenescence" that is associated with the excess of atherosclerosis experienced by HIV patients.[17,84] The possibility of a causal relationship was supported when the abundance of these HCMV-

specific CD8 T-cells was decreased significantly in a small RCT of valganciclovir.[84]

Overall, these observations are strikingly similar to those in the SOT and SCT populations, yet have been largely ignored by the HIV field despite similar results being reported every few years.[85] We continue to suggest that HIV and transplantation could both potentially benefit from collaboration to explore and compare potential mechanisms; for example, the observed excess of atherosclerosis in SOT could be explained by the excess of inflammatory T-cells that has been reduced in an RCT in HIV. This is important because the total amount of morbidity caused by the indirect effects of HCMV may exceed that currently attributed to EOD.[10] Furthermore, clinicians should be aware that silent HCMV infection may be predisposing a variety of their other patients to adverse outcomes including excess mortality in the general population, increased duration of ventilation when patients are admitted for intensive care following heart attacks, burns or sepsis and increased severity of Covid-19.[86-90] The important principle is that underlying HCMV infection induces a long term inflammatory bias that can contribute to other medical conditions without declaring its presence.

Immune correlates of control of HCMV replication

What is clear from our understanding of clinical HCMV infection is that pathogenesis is mainly observed in individuals with poor immune responses. That said, the precise component of the immune response responsible for protection is still unclear. For example, active HCMV infection is seen in patients with poor cell-mediated immunity measured against the major immediate early antigen or pp65 proteins – two immune-dominant antigens.[91] However, it has been difficult to define cut-off levels at baseline or at the end of prophylaxis to identify which SOT patients are not at risk of infection.[91] This is partly because of the fluctuating risk seen with time, as some patients require additional immunosuppression in the form of steroids.[46,67] It is also partly because clinicians wish to be informed preferentially about the highest risk patients, yet these are the D+R- subset where measurements of specific immunity in the recipient are undetectable.[91] Furthermore, studies of seropositive recipients often fail to differentiate between control of reactivation or reinfection. Recently, a single paper has produced substantial evidence focused on the D+R+ subset that immune responses to the major immediate early antigen detectable pre-transplant predict the risk of HCMV viraemia post-transplant.[92]

One potential issue with current strategies is that focus has often centred on measuring the quantity rather than the quality of the immune response. This may not simply be a numbers game – it may be more a question of having the right response rather than a large response. A heroic study by Sylwester and colleagues demonstrated that the T-cell response against HCMV is diverse and targeted against a breadth of HCMV proteins.[93] These observations have been substantiated in a number of smaller, more focused studies that essentially demonstrate the response is dynamic and broad.[17,94-97] Ongoing

studies are addressing whether infusion of HCMV specific T-cells into patients can provide protection.[98]

Antibody, NK cells and macrophages may theoretically contribute to a protective immune response and would be expected to interact and cooperate with T-cells to control HCMV replication. A very recent paper has assembled some of the immune functions that require collaborative contributions from more than one component of the immune system by studying viral proteins expressed at the surface of the infected cell and determining which could mediate antibody dependent cellular cytotoxicity[99] . Remarkably, these targets were not the major structural glycoproteins of the virus, but the proteins it deploys as immune evasins.[99] Future studies of this kind have the potential to give a more sophisticated assessment of the immune capability of individual patients at risk of HCMV infection.

Strategies to treat HCMV infection

One important outcome of comprehensive studies of the natural history and pathogenesis of HCMV is the provision of strong evidence that measuring viral load, and thus a requirement for PET, is a robust surrogate for measuring EOD.[55] This becomes particularly important for clinical trials seeking to test the anti-HCMV activity of novel compounds.

Three Phase 2 RCTs have been conducted in SCT using PET as the read-out to determine if novel antiviral drugs given prophylactically can control HCMV viraemia better than placebo.[100-102] All three were successful without causing bone marrow suppression and proceeded to Phase 3.

For the first Phase 3 study (maribavir), EOD was required as the primary endpoint. The drug failed to reduce this for two reasons: PET was allowed for patients in both arms and rescued those who had failed prophylaxis; the sponsors chose the lowest dose of drug instead of the highest non-toxic dose.[103] For the second (brincidofovir), PET was allowed as the primary endpoint, but a drug-free washout period was included after the end of prophylaxis. The drug initially suppressed the need for PET, but this difference then declined with time to leave no overall significant difference when compared to placebo.[104] The reason was an excess of GVHD in the drug arm which was treated with steroids that then precipitated CMV viraemia. Many of these clinically diagnosed cases were not true GVHD (which is classically diagnosed with diarrhoea, rash and abnormal liver function), but simply cases of diarrhoea caused as a known side effect of brincidofovir.[104] The third drug (letermovir) reduced PET significantly and was licensed for use.[78]

While these RCTs were in progress, regulators in the USA and EU progressively accepted that EOD was an undesirable and impractical endpoint and that the need for PET was now appropriate for Phase 3 studies.[55] The studies of future drugs should therefore now be more straightforward to conduct. Two other aspects of regulatory requirements for

Phase 3 now also need to be brought up to date. First, drugs for prophylaxis should be given immediately post-transplant rather than waiting for engraftment, which is a hangover from studying the bone marrow toxic ganciclovir. Second, there is no scientific rationale for requiring a washout period after prophylaxis ends before assessing whether the need for PET has been reduced. This is not a requirement for anti-HIV drugs and is another hangover from the original ganciclovir study. Thus, application of modern understanding of the natural history and pathogenesis of HCMV is rapidly improving clinical trial design.

Evaluation of novel vaccines

There is no doubt that the development of vaccines to protect against HCMV infection or disease will be complex. This virus can establish life long latency and immune individuals can experience repeat infections from endogenous (reactivation) or exogenous sources (reinfection) despite the host committing substantial immune resources against HCMV. An early RCT gave live attenuated Towne vaccine strain (see Box 1) or placebo to seronegative candidates awaiting renal transplantation.[105] Post-transplant, the incidence of HCMV infection and EOD were not reduced, but the severity of EOD was. The subsequent development of quantitative PCR allowed the viral load parameters described above to be used as pharmacodynamic readouts to determine if vaccines have activity against HCMV replication in these patient populations.

A vaccine consisting of gB with MF59 adjuvant given pre-transplant to SOT showed reduced post-transplant viral load parameters when compared to recipients of placebo.[106] The correlate of immune protection was the titre of IgG antibodies made against gB.[106] Subsequent detailed studies of responses against individual antigenic domains of gB proposed that antibodies against antigenic domain 2 helped protect seropositive recipients from viraemia.[107,108] Antibodies against the immunodominant antigenic domain 1 were not protective, consistent with the possibility that the presence of this domain represents another example of HCMV evading protective immune responses. These hypotheses should be tested formally in future RCTs.

To test that antibodies were a mechanistic correlate of protection, one of us (PG) suggested to Genentech that they should evaluate placebo-controlled infusion of preformed monoclonal antibodies specific for HCMV at the time of D+R- renal transplantation.[109] The company conducted an RCT in 120 patients and demonstrated significant interruption of transmission of HCMV from donor to recipient.[110] This approach of using active and passive immunisation serially and in tandem in SOT should be applied to the evaluation of novel vaccines in the future.[111]

Disappointing results were recently presented orally with a DNA plasmid vaccine in SCT that appeared to be poorly immunogenic and did not reduce the need for PET. When these Phase 3 results are published, it will be

important to determine if the change from immunising donors in the encouraging Phase 2 study was important.[112]

Two more HCMV vaccines have proceeded to Phase 2 studies. Hookipa have a modified lymphocytic choriomeningitis virus construct that expresses gB and another that expresses pp65. Coadministration produced good humoral and cell-mediated responses and the results of a Phase 2 study in seronegative renal transplant patients is awaited.[113] Positive results from this study could lead to RCTs in women of childbearing age at risk of primary infection. Merck have engineered 2 proteins within an Ad169 strain modified to express the pentameric complex by fusing two viral proteins (IE1/2 and pUL51) to the destabilizing domain of FK506-binding protein 12. This fusion sends these essential proteins to be digested in the proteasome unless an exogenous chemical is present.[114] The resulting genetically inactivated whole virus strain is being studied in seronegative women of childbearing age but could easily be applied to the immunocompromised in the future. When the results of these two studies are published, it will be possible to review the evidence for reduced primary infection, examine the immune correlates of protection and make recommendations for whether either or both products should proceed to Phase 3 studies. These will be larger versions of the current Phase 2 studies, with at least 30,000 seronegative women required. The primary endpoint will also change from primary infection in the women to congenital infection in their neonates. We recommend that such studies in women and SOT should proceed in parallel because of the similarities of HCMV in both patient populations.[115] Meanwhile, the same and/or different vaccines should be studied for their ability to “boost” or “improve” the natural immune response to HCMV so that the incidence of reactivations or reinfections can be reduced. The SOT population routinely monitored by PCR and managed by pre-emptive therapy represents an ideal population to study. We also recommend that studies of active immunisation should proceed hand in hand with studies of passive immunotherapy using monoclonal antibodies with defined reactivity against specific proteins of HCMV; the SOT population acts effectively as a human challenge model to facilitate such studies.

Conclusions and open questions

We have shown how a virus that does not declare its presence by producing specific symptoms can nevertheless be monitored prospectively to define quantitative parameters of replication. These measures can be deployed for pre-emptive therapy to reduce EOD and to define immune corelates of control. By giving a prototype vaccine or placebo pre-transplant, the viral load parameters can be used as pharmacodynamic readouts of successful protection. Passive transfer of monoclonal antibodies or T-cells can then be used to both confirm the immune correlate and establish a medically acceptable new treatment. Clinical cohorts continue to report reduced survival of graft and/or allograft patient in subgroups at risk of active HCMV infection, [12,116] so the goal should be to return these parameters to the values found in the D-R- subgroup. Although HCMV represents a complex target, we are

optimistic that serial rounds of iterative studies will finally bring this important and under-recognised human pathogen under control.

Figure 1. Overview of entry of human cytomegalovirus into target cells and contribution to the establishment of latency in non-permissive myeloid cells.

A) Virus-encoded glycoproteins on the surface of the virion engage with receptors on the surface of cells and can drive entry by multiple processes in a cell type dependent manner. In fibroblasts, glycoproteins H (gH), L (gL) and O (gO) form a trimer that binds to platelet-derived growth factor alpha and co-receptors. This binding triggers glycoprotein B (gB) to fuse directly with the plasma membrane at neutral pH. In permissive epithelial and endothelial cells, gH and gL form a pentameric complex with three other proteins encoded within the ULb' region (UL128/UL130/UL131). This pentameric complex binds to neuropilin 2 and triggers pH dependent endocytosis. The fusion activity of gB becomes relevant for escape from the endosome. For both cell types, once the capsid and associated tegument proteins are released into the cytoplasm they move independently to the nucleus where virion DNA interacts with the nuclear pore complex to transition into the nucleus. Infection of myeloid cells (including potential sites of latency) involves macropinocytosis. In myeloid cells where HCMV establishes latency (i.e. CD34+ cells) activation of EGFR and integrin-mediated src kinase signalling via gB and pentamer, respectively is required for the correct trafficking of HCMV to the nucleus via recycling endosomes. B&C) The establishment of latency is dependent on effective silencing of major immediate early (MIE) gene expression. In CD34+ cells this is likely a combination of host and viral encoded events including a failure of virion transactivators (e.g. pp71) to enter the nucleus coupled with a host environment of high levels of transcriptional repressors of the MIE promoter (MIEP). The result is establishment of a repressive chromatin phenotype driving MIEP silencing (B) which is maintained by viral UL138 gene expression. Cellular differentiation to a dendritic cell (DC) promotes re-animation of the MIE locus through the activity of host chromatin remodelling enzymes. This process is responsive to inflammatory cytokine signalling through ERK and SFK signalling pathways.

Figure drawn from information provided in reference[117]

Figure 2 Viral and host function act at multiple stages of latency and reactivation.

A) In healthy individuals, a robust innate and adaptive immune response restricts HCMV reactivation and replication. HCMV counters this with an armoury of measures to disable all arms of the immune response. Recognition by CD8 T cells is limited by class I MHC down-regulation and prevention of antigen loading and presentation at the cell surface. Similarly,

class II MHC presentation to CD4 T cells is prevented by similar strategies including the expression of a viral IL-10 homologue that promotes class II down-regulation. Loss of MHC class I can potentially activate NK cell recognition and killing via the 'missing self hypothesis' thus HCMV promotes the expression of an HLA-E inhibitory receptor as well as a number of gene products that disable NK activating receptors and up-regulate NK inhibitory receptors. The IFN response is disabled both upstream and downstream of HCMV. Specifically, HCMV gene products interfere with DNA sensing pathways to prevent activation including inhibitors of IFI16 (pp65/US28) and cGAS/STING (UL31 and pp71). IFN signalling is also disabled via an interaction of IE72 with the STAT transcription factor. HCMV also modulates the bio-activity of cytokines through expression of beta-chemokine receptors that bind and sequester host cytokines. Additionally, HCMV encodes a number of alpha chemokines which mimic CXCL1 and CXCL2 activity to modulate the recruitment to, and activity of, immune cells at the site of infection. B) Potential roles for immune-suppression in HCMV infection and reactivation. HCMV establishes latency in CD34+ progenitor cells. Myeloid/DC progenitor (a) differentiation into macrophages or DCs promotes cellular reactivation (b), production of infectious virus and subsequent infection and replication in multiple permissive tissue cells (c). CMV-specific T cells can recognise cellular reactivation (d) or disseminated infection (e). Additionally, B cells produce neutralising antibodies (f) or non-neutralising antibodies that likely recognise viral cell surface antigens on reactivating cells (b) or newly infected cells (c). This will promote the recruitment of antibody dependent effector functions (g,h) to target the infected cells. A second site of viral persistence is hypothesised to be tissue resident endothelial cells (i) although whether they are seeded via differentiation from a latently infected CD34+ progenitor or by direct infection in tissue is unknown. Hypothetically, these latently infected endothelial cells are activated and thus can be recognised by T cell (j) and B cell (k,l) mediated immune responses. In the context of immune suppression, pre-existing T cell responses will be reduced which, in seropositives, reduced control of both cellular and clinical reactivation. In seronegatives experiencing primary infection (who have no latent HCMV reservoir in CD34+ cells), the major impact of immune suppression will be a reduction in the generation of new T and B cell responses reducing control of replication in permissive cells (e,f,h). These processes are likely exacerbated through inflammation (allogeneic T cells or co-infection) which enhance cellular reactivation and replication in seropositives (m,n) and viral replication in seronegative infected individuals (n).

Figure 3. Baseline prevalence of HCMV antibodies in different populations and incidence of infection once they become immunocompromised.

A. The prevalence of prior HCMV infection is high (90%) in those with HIV infection and intermediate (60%) in those awaiting SCT. The SOT group can be divided further according to prevalence of antibodies in the donor as well as the recipient.

B. Once they become immunocompromised through transplant or because the CD4 count in HIV positives declines below 100/microlitre, they are at risk of HCMV viraemia detected by PCR. In SOT, the risk is highest in those with

primary infection (D+R-), intermediate in those at risk of reactivation or reinfection (D+R+) and lowest in those at risk of reactivation only (D-R+). This illustrates that pre-existing natural immunity against HCMV provides substantial protection against exogenous (reinfection) and endogenous (reactivation) sources of virus. Note that the incidence of EOD declines in parallel with reduced detection of viraemia. In SCT, the risk of viraemia and EOD is as high as in D+R- SOT, despite the SCT patients being R+. Comparison of SCT with D-R+ SOT shows that reactivation dominates R+ SCT recipients and that ablation of their bone marrow greatly reduces immunity acquired in the past. The incidence of both viraemia and EOD is intermediate in HIV positive individuals.
Figure drawn from information provided in references[9,13,49,69,118-120]

Figure 4. Distribution of peak viral loads found in three HCMV subgroups of SOT, SCT and those with HIV.
The peak viral loads approximate to a normal distribution in the D+R- SOT subgroup (panel A). In contrast, the distribution is shifted strongly to the left in the D+R+ subgroup where the recipient has natural immunity pre-transplant (panel B). Natural immunity does not prevent low viral loads resulting from reactivation (panel C) or either reactivation or reinfection (panel B). Following SCT (panel D) the peak viral loads are relatively low, yet these patients have a high risk of EOD (figure 3). This shows that SCT patients are susceptible to a low viral load that would be unlikely to cause EOD in SOT. Patients with HIV (panel E) have a high viral load distribution, similar to that seen after D+R- SOT.
Figure drawn from references[9,49,69,118-121]

Figure 5. Two distinct strategies used to reduce CMV disease in allograft recipients.
In the case of prophylaxis (upper panel), an antiviral drug is given from the time of transplant (as soon as the patient can tolerate oral medication) for a fixed period of time, with clinical trials for SOT supporting durations of either 100 or 200 days.[58,122] This strategy is effective in preventing EOD, but patients are at risk once again after prophylaxis is stopped, including with strains of CMV resistant to the drug used for prophylaxis (late onset disease).[123]
In the case of pre-emptive therapy (lower panel), patients are monitored frequently to determine if HCMV DNA is detectable by polymerase chain reaction. Those with low viral loads continue to be monitored, but those with a viral load above a defined threshold are given antiviral therapy until two consecutive blood tests can no longer detect HCMV DNA. Patients continue to be monitored and may require a subsequent episode of pre-emptive therapy.
Humoral and cell mediated responses are superior in SOT managed using pre-emptive therapy and late onset disease is uncommon.[57,61]
For SCT, valganciclovir prophylaxis cannot be used because of bone marrow toxicity of the drug. Letermovir is safe enough to be used for prophylaxis[78] and is combined with pre-emptive therapy.

If the viral load fails to respond to treatment with at least a one log reduction over two weeks, refractory HCMV infection is diagnosed. This may be due to poor host responses and/or the selection of strains resistant to the antiviral drug being administered. At present, foscarnet is commonly used off-label to treat ganciclovir resistant strains of HCMV, but has severe side-effects. Phase 2 results of maribavir are encouraging[124] with Phase 3 RCT results expected in 2021.

Box 1 HCMV strains and their genomes

HCMV was first grown in 1957 using the then new technology of cell culture.[125] It was difficult to propagate serially, but laboratory-adapted strains such as Ad169 (cultured from adenoids) or Towne (named after a patient) released more cell-free virus into the extracellular fluid and were shared widely among researchers. The Nobel laureate and co-discoverer of HCMV, Dr Thomas Weller, anticipated the possibility that this laboratory adaptation may have selected for genetic changes.[126,127] In 1990, Cha and colleagues confirmed this when they reported that a 20kb segment was missing from some strains (designated the ULb' region).[128] The 19 genes encoded within ULb' were clearly dispensable for growth in fibroblast cell culture. Thus their retention in wild type strains argued they played crucial roles during infection in vivo.

One aspect associated with the ULb' region was viral tropism and entry. A trimer of proteins (glycoprotein H (gH), glycoprotein L (gL) and glycoprotein O) form a complex on the surface of the virion that binds to platelet derived growth factor receptor alpha and co-receptors on the surface of fibroblasts.[129] Entry into the fibroblasts then occurs via fusion at the plasma membrane at neutral pH (FIG 1).[130] In contrast, gH and gL can also bind to 3 distinct small proteins (encoded within the ULb' region) to form a pentameric complex.[131] This complex binds to a number of receptors including CD147 and neuropilin 2 receptor on epithelial and endothelial cells facilitating entry by means of a pH dependent endocytic pathway (FIG 1).[117,132,133] What has become clear from the ability to reconstruct the wild type HCMV genome using bacterial artificial chromosome (BAC) technology is that HCMV strains that express the pentameric complex propagate in a highly cell-cell fashion but, when the pentamer is absent (as in Ad169 or Towne laboratory strains), a high proportion of cell-free virus is produced.[134]

We now know (via whole genomic sequencing), that HCMV has the largest genome of any virus known to infect humans, (235-250kb double-stranded DNA).[135-137] HCMV encodes ~170 canonical open reading frames although non-canonical ORFs may increase this coding capacity 5 fold.[138,139] Despite the size of the genome it is still notable that as much as 70% of the viral genome (including the example of ULb') is dispensable for growth in vitro.[140] While it is understandable that the early pioneers utilised the most tractable strains to work with in vitro, scientific experimentation remains a trade-off between authenticity (are we measuring a laboratory phenomenon?) versus tractability. What is now clear is that many of the genes encoded within ULb' are involved in cell tropism and immune evasion explaining why they became

dispensable in vitro. Indeed, HCMV encodes more genes for immune evasion than it does to produce the virus particle itself.

These genetic differences in strains of HCMV need to be borne in mind when considering whether particular vaccines can protect against primary infection, reinfection or reactivation. At present, information from clinical trials is only available for gB. The gB/MF59 vaccine is based on the Towne strain that has gB1 (out of 4 possible genotypes[141]). There is some evidence that women immunised with gB/MF59 had better protection against primary infection with natural strains bearing gB1 than against viruses with other gB genotypes[142]. The plasmid vaccine[112,143] is based on Ad169 (gB2) as are the Hookipa[113] and Merck[114] vaccines.

In addition to mutations selected in vitro (such as the UL/b' example), variation occurs naturally in vivo. Whole genomic sequencing direct from clinical samples is starting to describe the level of variation in terms of genotypes of numbered genes and document that not all circulating strains encode exactly the same repertoire of genes.[144-146] The extensive variation seen has the potential to affect virus pathogenesis in vivo, but no definitive studies have yet been reported.

Box 2 Overview of the different immunocompromised groups

The 3 groups of patients considered here are all immunocompromised, but for different reasons.

Candidates for SOT become immunocompromised because of the drugs given to suppress cell-mediated immunity which would otherwise cause graft rejection. The immunocompromised state is most profound immediately after transplant and frequently facilitates HCMV viraemia, but moderates as these drugs are reduced in dose over weeks. Graft rejection episodes are treated with methylprednisolone which increases the risk associated with any given viral load and maintains immunocompromise for weeks to months. Memory humoral immunity remains relatively intact, but responses to new antigens (seen for HCMV in D+R- SOT) are blunted by poor T-help. Risk factors for profound immunocompromise include: graft rejection and the immunosuppression required to control it.

Stem cell transplant patients become immunocompromised when their bone marrow is ablated by chemotherapy to make room for donor marrow to engraft. They remain profoundly immunocompromised until this occurs, starting from 2-3 weeks after the procedure, but taking months to reach sufficient immunity to protect against HCMV. Evidence from cohort studies supports recovery of CD8 T-cells[147-149] and CD4 T-cells[149,150] contributing to reduced CMV EOD. They are also consistent with case-series from several investigators using methodological improvements on the original case series[98] of adoptive transfer of T-cells of various specificities,[151,152] but we are not aware of a single DB PC RCT that has formally proven the safely and efficacy of adoptive transfer. The appearance of graft versus host disease, coupled with its treatment, adds further immunocompromise. Humoral immunity to recall antigens (like HCMV reactivating from R+ recipients) remains relatively

intact because plasma cells are resistant to the chemotherapy. To illustrate how long it takes to establish a new immune system, patients are typically not given vaccines based on live attenuated viruses until 12 months post-transplant (24 months for those with graft versus host disease). Risk factors for profound immunocompromise include: allogeneic rather than autologous transplant; unrelated rather than sibling donor; small number of bone marrow cells transferred (cord blood or T-cell depleted transplants); haploidentical rather than fully HLA-matched donor.

Individuals with HIV are immunocompromised because active HIV infection depletes CD4 T-cells. Once the CD4 count in peripheral blood declines from 1000/microlitre to 100/microlitre, patients become at risk of HCMV EOD. There is no formal evidence of the specific immune functions whose decline has allowed active infection to occur, but they are assumed to be CD4 and CD8 T-cells, by analogy with SCT patients discussed above. Maintaining the CD4 count above 100/microlitre through lifelong antiretroviral therapy has virtually abolished CMV EOD in those with HIV infection. Risk factors for profound immunocompromise include: non-availability of antiretroviral therapy (due to cost, health care access or patient choice).

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Author contributions

Both authors researched data for the article. PG wrote the first draft which was revised by MR. Both authors contributed substantially to the discussion of content, reviewed the text and edited to form the final manuscript.

Competing interests

The authors declare no competing interests

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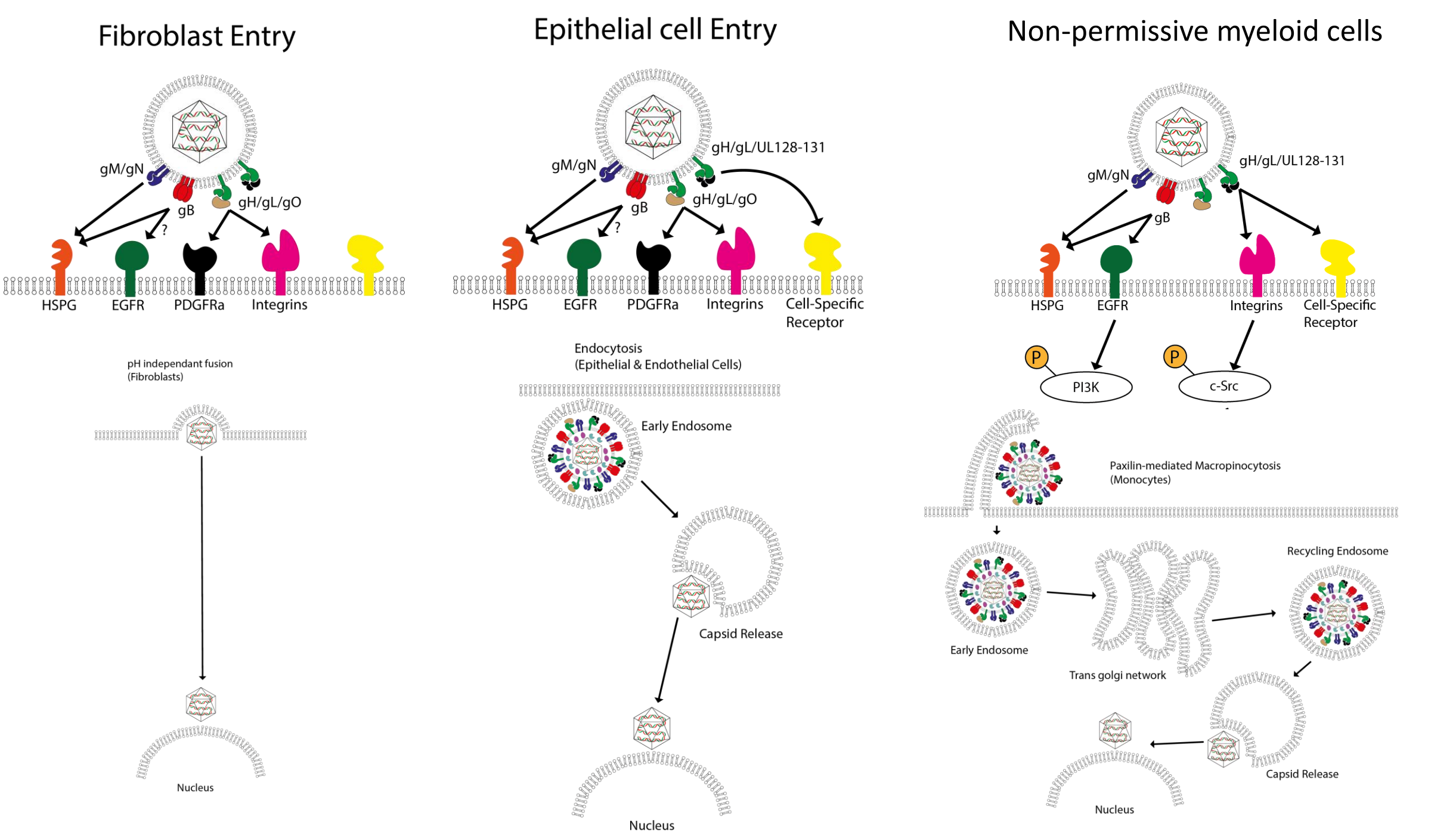
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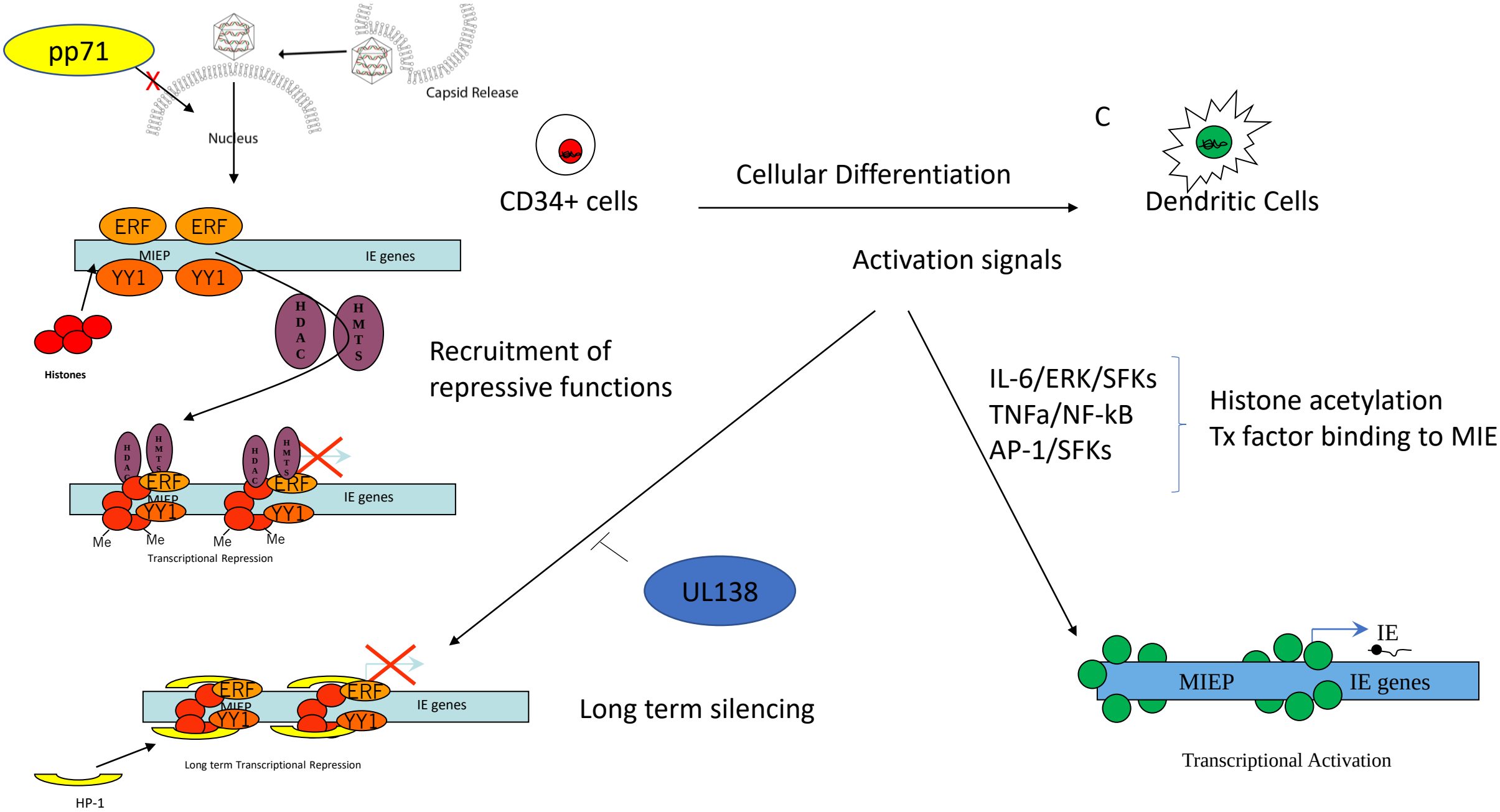
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UL138
Long term Transcriptional Repression
HP-1
Long term silencing
CD34+ cells
Cellular Differentiation
C
Dendritic Cells
Activation signals
IL-6/ERK/SFKs
TNFa/NF-kB
AP-1/SFKs
Histone acetylation
Tx factor binding to MIE
IE
MIEP
IE genes
Transcriptional Activation

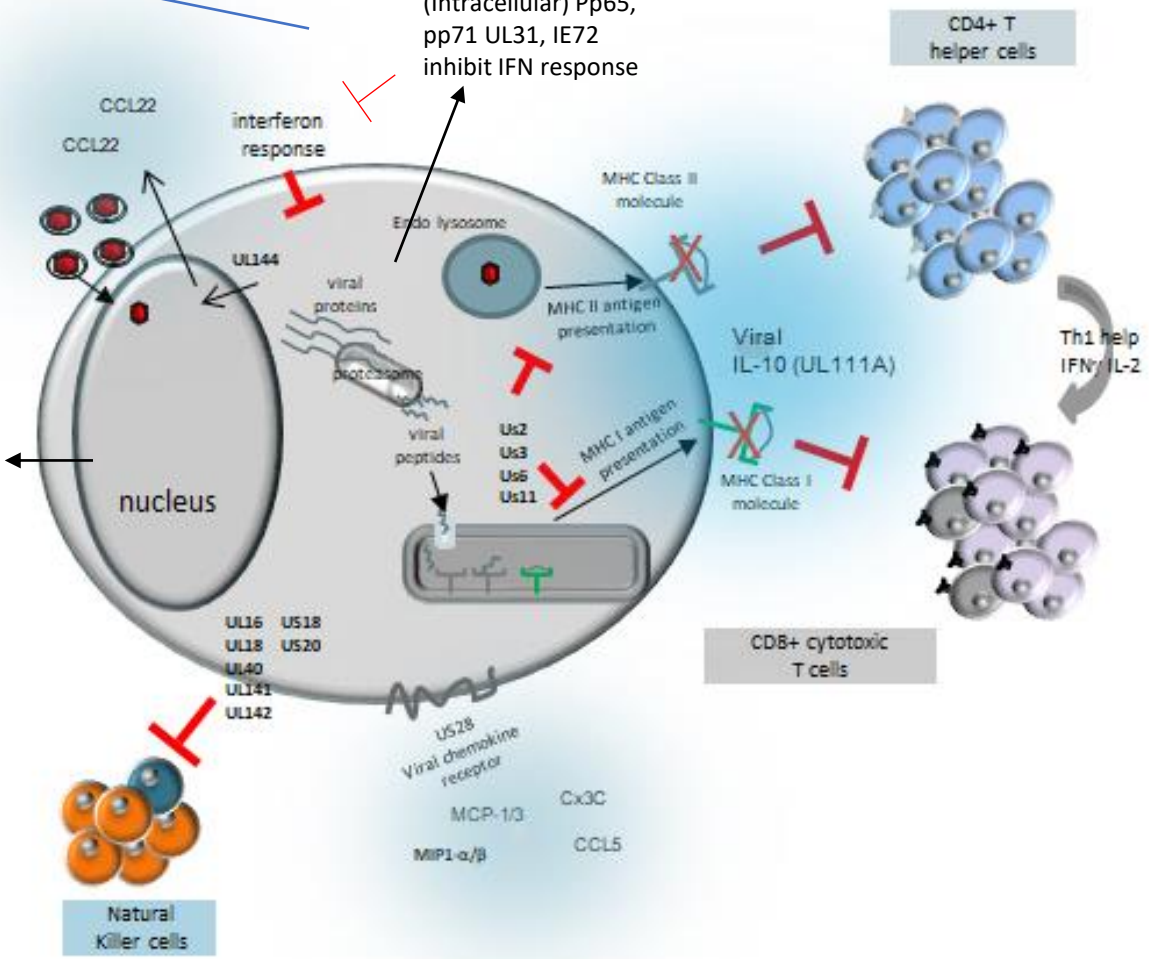
What can be shown is
That incoming viral DNA
Interacts with cGAS in the cytoplasm
And that UL31 is a protein that promotes the dissociation of DNA from cGAS to prevent signalling.

Pp71 should be shown in the nucleus and show pp71 promotes Daxx degradation and ATRX re-localisation

IE72 is then produced de novo from the viral genome and targets nuclear PML for degradation

information

(Intracellular) Pp65, pp71 UL31, IE72 inhibit IFN response



Viral alpha chemokines: Ul128, UL146 and UL147

Neutrophil recruitment & activation

Would just need to show neutrophil cells being recruited

Figure 2A

Latency, reactivation, infection, immune control and immune suppression

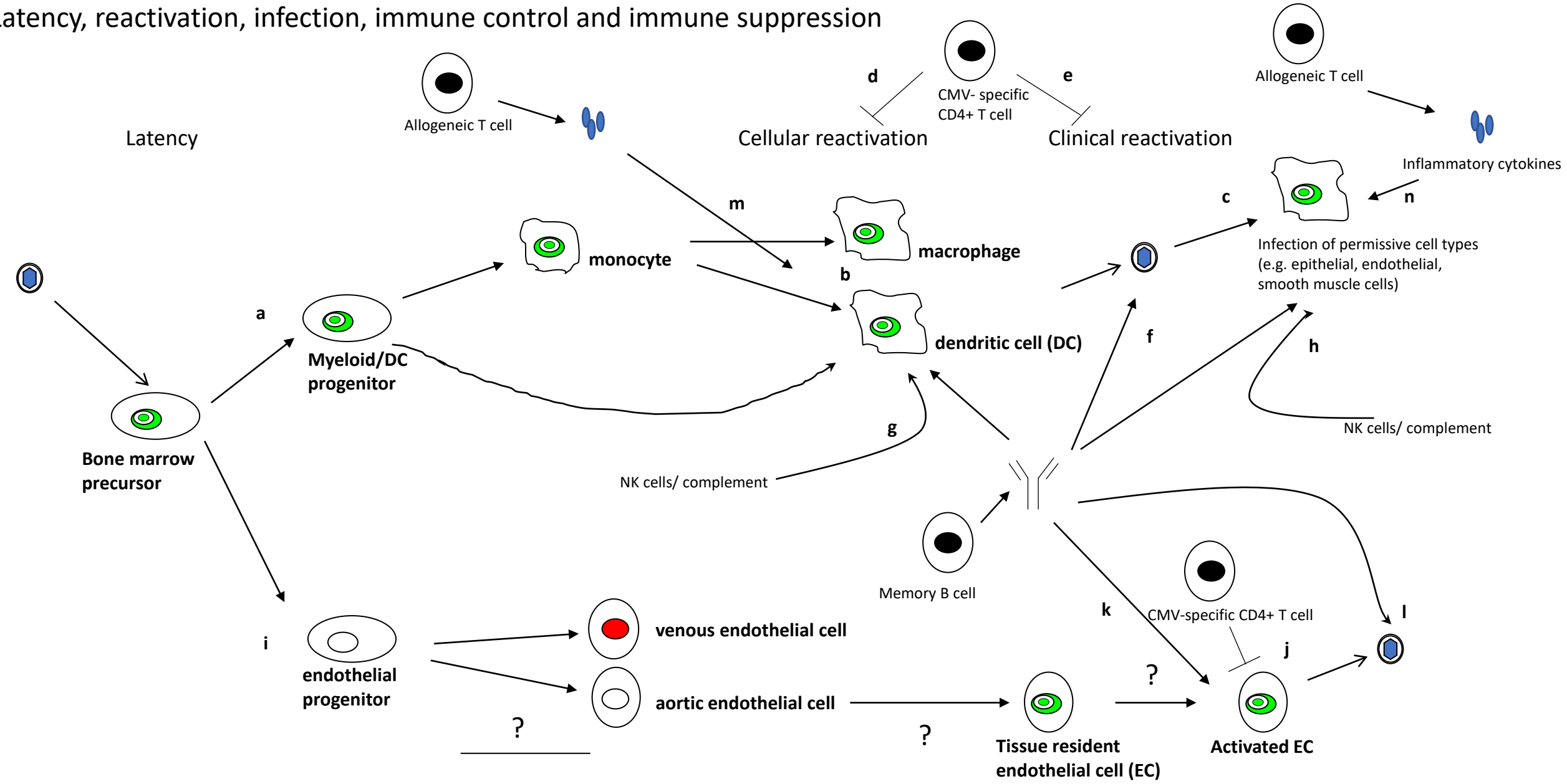
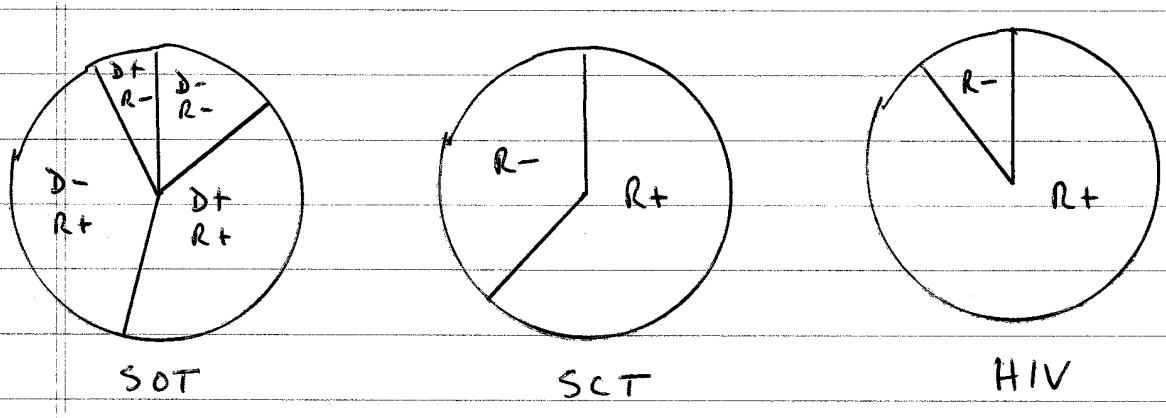


Figure 2B

Fig 3.

A. Prevalence of IgG antibodies



B. Incidence of infection and disease

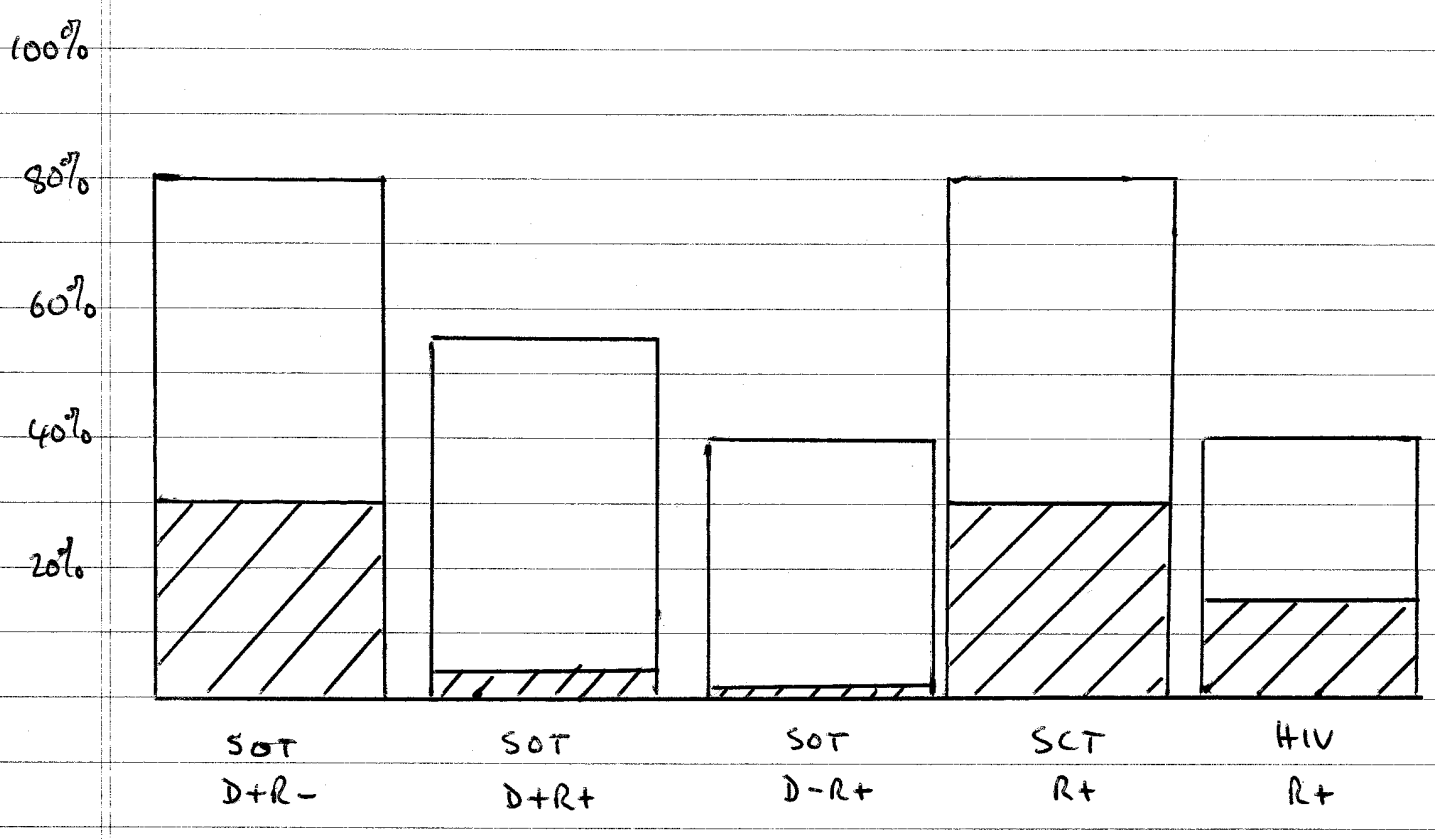
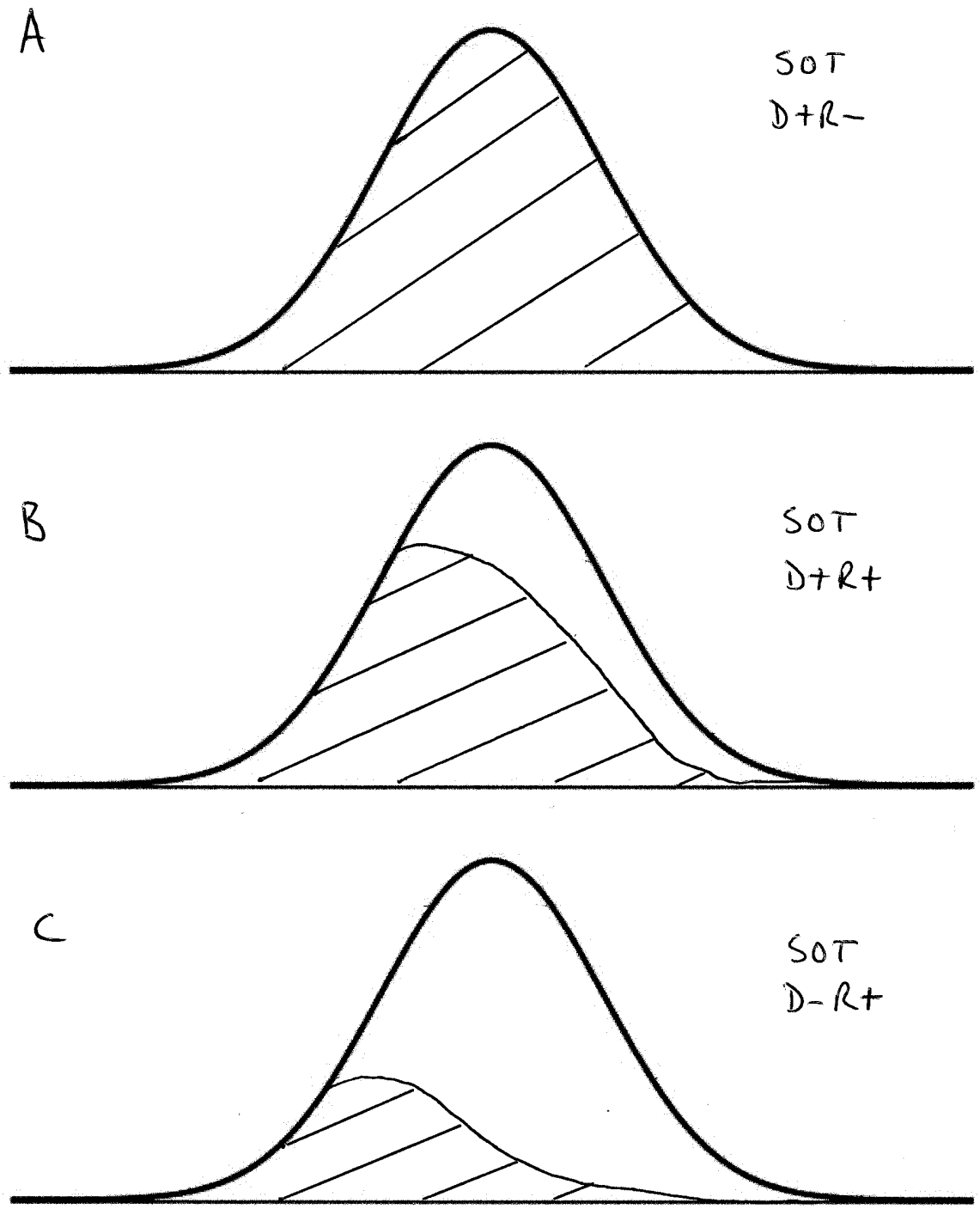
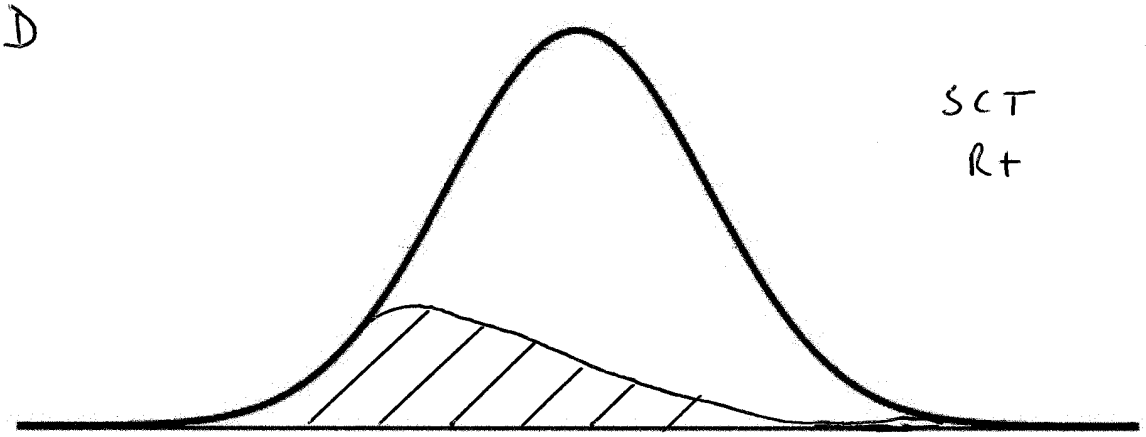


Fig 4



D



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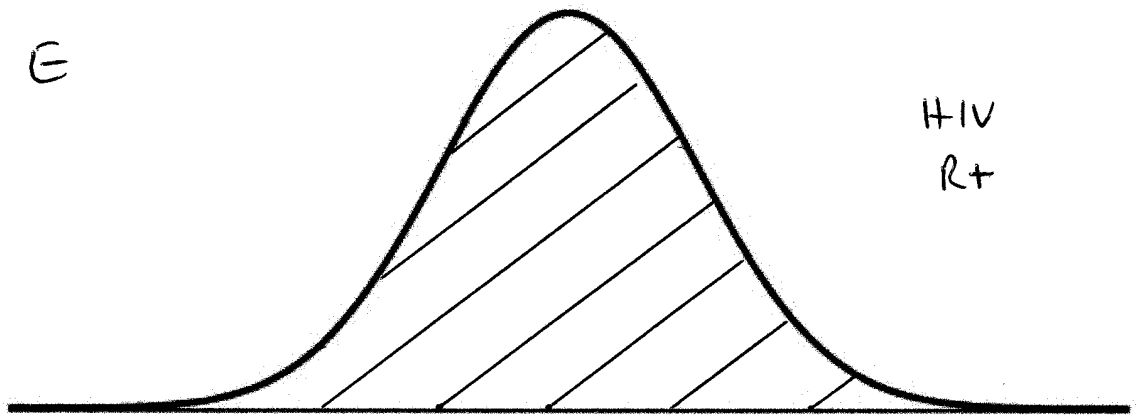

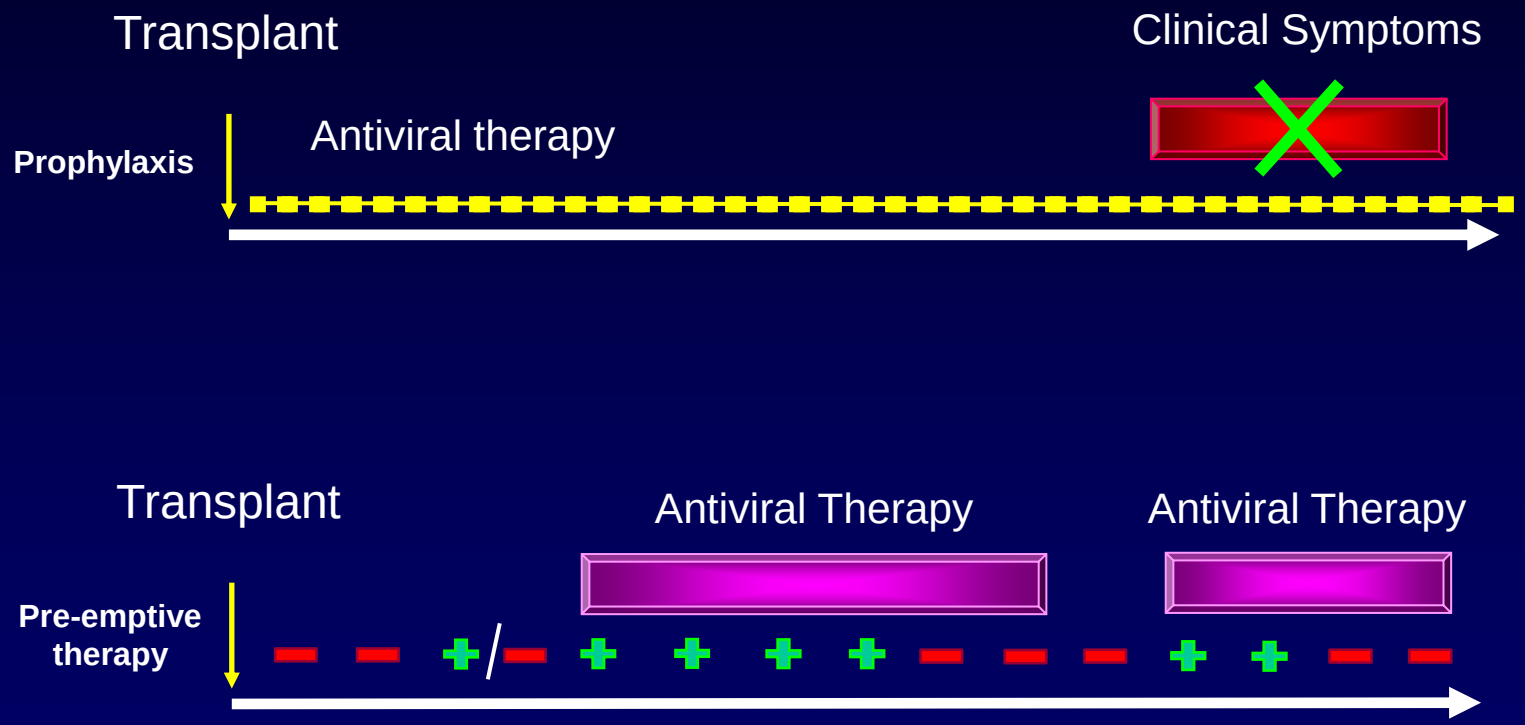

◇ = surveillance blood PCR