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Quiescent T cells and HIV: an unresolved relationship

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Abstract

The ability of HIV to infect quiescent CD4⁺ T cells has been a topic of intense debate. While early studies suggested that the virus could not infect this particular T cell subset, subsequent studies using more sensitive protocols demonstrated that these cells could inefficiently support HIV infection. Additional studies showed that the kinetics of infection in quiescent cells was delayed and multiple stages of the viral life cycle were marred by inefficiencies. Despite that, proviral DNA has been found in these cells presenting them as a potential viral reservoir. Therefore, a better understanding of the relationship between HIV and quiescent T cells may lead to further advances in the field of HIV.

Keywords

Quiescent T cells; HIV resistance; HIV latency; Host restriction

Quiescent T cell biology

Human T lymphocytes are quite unique in their ability to remain at a quiescent state over long periods of time and only proliferate upon activation. The majority of T lymphocytes circulating in blood are at the G₀ state of the cell cycle [1-6]. Upon exposure to antigen, T cells clonally expand to deal with a potential foreign invader [1]. Even in this state, though, only a small fraction of T cells expands at a high rate. After stimulation, a large number of the clonally expanded lymphocytes will die, leaving a small fraction of non-dividing

memory cells, which live over long periods of time and are recruited only upon antigen re-exposure.

For the above reasons, T cell quiescence was viewed as a default state due to the absence of activation signals. Recent studies, however, have demonstrated that T cell quiescence is an actively maintained state [3, 5-13]. This is characterized by low metabolic rates, low levels of transcription, limited to basic housekeeping genes, small cell size and very long periods of survival [5, 6]. T cell quiescence prevents cellular damage from metabolism and uncontrolled expansion of T cells that could lead to lymphomas [5, 6]. A series of transcription factors have been implicated in establishing and maintaining this quiescent state. Kruppel-like factors (KLFs) and more specifically lung Kruppel-like factor (LKLF) have been shown to regulate and maintain quiescence [7, 8, 11]. Studies disrupting the expression of the transcription factor LKLF resulted in uncontrolled lymphocyte proliferation [7, 8]. Furthermore, overexpression of the protein in cell lines such as Jurkat T cells resulted in the induction of quiescence [11].

In addition to LKLF, there are other factors involved in the maintenance of quiescence. Forkhead Box class O (FOXO) factors have been identified as potential players in cell quiescence and cell death, especially FOXO1, 3 and 4 [14, 15]. These factors are shown to be active in resting cells and localized in the nucleus [10, 15-20]. Activation by cytokines such as IL-2 results in their inactivation and cell proliferation [10, 15-20]. In a recent study, FOXO1 knockout mice, displayed normal thymopoiesis, had elevated numbers of activated T cells but decreased levels of naïve T cells [21]. In addition, the expression of the IL-7 receptor was completely abrogated, suggesting that this receptor may be a target of FOXO1 [21].

Finally, Tob, another nuclear protein, has been recently shown to be involved in cellular quiescence [3, 13, 22, 23]. It belongs to a family of antiproliferative proteins known as APRO [3, 13, 22, 23]. The protein is expressed in anergic and naïve T cells and is downregulated upon stimulation of T cells. It seems to affect the thresholds of stimulation, since the knockdown of the protein leads to increased activation by TcR binding only [3, 13, 22]. Ectopic expression of the protein resulted in inhibition of CD3-CD28 mediated proliferation and abrogation of cytokine expression such as IL-2, IL-4 and IFN- [3, 13, 22]. Finally, Tob has been shown to enhance TGF- signaling pathways, thus, promoting cell quiescence [3, 13, 22].

In this review, we will examine the relationship between quiescent CD4 T cells and HIV. Quiescent T cell infection by HIV has been an interesting and controversial subject that has generated a number of high profile studies in the field. While HIV infection is not cell cycle-dependent [24-26], HIV cannot efficiently infect G₀ cells, as we will describe in the sections to follow. Despite the underlining inefficiencies, quiescent T cells have been shown in *in vitro* studies to harbor provirus, raising the possibility that they can be part of the viral reservoir. Therefore, due to the unique nature of quiescent cells, these reservoirs can potentially persist undetected over a long period of time with a very high survival rate. Consequently, a deeper understanding of the relationship between HIV and quiescent cells will provide us with better tools in dealing with the virus.

HIV replication in quiescent CD4⁺ T cells

The ability of the human immunodeficiency virus (HIV) to infect quiescent CD4⁺ T cells generated a great deal of debate during the early years of studying the virus. Unlike other retroviruses, HIV replication is not dependent on cell division. HIV and other lentiviruses are characterized by their ability to infect non-dividing cells and establish a latent infection

[24-26]. Early reports suggested that HIV was able to bind to quiescent T cells, but failed to infect them unless they were previously activated [27-29].

Using more sensitive and quantitative PCR techniques, our group and others demonstrated that quiescent T cells were infectable by HIV [30-33]. However, disagreement arose regarding the levels and degree of infection efficiency. Our group demonstrated that there were no problems in viral entry [31, 32]. Furthermore, HIV did initiate reverse transcription in quiescent T cells, but this process was not completed efficiently. Thus, based on our data, there was the accumulation of labile, latent intermediate viral DNA species that could be rescued with stimulation [31, 32]. However, the ability to rescue productive infection decreased with time [32]. Other groups demonstrated that indeed quiescent T cells can be infected, but went further to show that there was completely reverse transcribed viral DNA. The full-length viral cDNA was localized in the cytosol over a prolonged period, produced virus and could integrate into the host genome following T cell activation [30, 33]. It was postulated that the viral cDNA failed to integrate due to a defect in nuclear transport or viral integration in quiescent T cells [30, 33]. Furthermore, studies by the Vitteta group, focused on the CD25⁻ and CD25⁺ T cell populations and their ability to be infected by the virus [34-36]. In separate studies, they showed that the CD25⁻ T cells, representing non-activated T cells, were not infectable by HIV, while the CD25⁺ T cells were able to support infection in the absence of any stimulation. However, when total human peripheral blood mononuclear cells (hPBMC) were infected, the CD25⁻ cells did have copies of viral DNA, suggesting either infection of activated cells that turned quiescent or a synergistic effect from other T cells. Furthermore, Tang et al. showed that while they could infect quiescent cells with the virus, they were not able to induce virus expression [37]. Meanwhile, studies by the Stevenson group showed that quiescent cells could be an inducible reservoir for HIV infection [38]. They saw high levels of extra-chromosomal viral DNA in HIV infected patients. Upon activation of these cells, these DNA species integrated in the host genome making them a potential viral reservoir. This was followed by a seminal study by the Siliciano group that showed the presence of integrated HIV in resting CD4 T cells [39]. Now, quiescent cells were important for HIV latency, however, it was, and still is, unclear if these cells were infected while in a quiescent state or infected while activated with subsequent return to quiescence.

Based on these early studies, it was evident that the life cycle of HIV in quiescent CD4 T cells was quite distinct from that of activated T cells and warranted further investigation. Subsequent studies in our laboratory helped further clarify earlier observations [40]. Using a cell cycle progression assay that could assess the levels of both cellular RNA and DNA synthesis, we were able to dissect the different stages of the G₁ phase of the cell cycle [40]. Using this assay, we were able to distinguish non-dividing T cells into two categories: (1) cells in the G₀/G_{1a} phase that is characterized by undetectable levels of DNA and RNA synthesis and (2) the G_{1b} phase that is characterized by high levels of RNA expression in the absence of DNA synthesis. We found that cells in the latter stage were permissive to HIV infection, while cells in the G₀/G_{1a} phase were truly quiescent and resistant to infection (Fig. 1) [40]. Therefore, the above data provided a foundation for the discrepancies seen among the different groups in terms of the permissiveness of quiescent T cells to HIV infection. Furthermore, this study showed that T cells need not be fully activated to support a productive infection. A series of concurrent and later studies showed very elegantly various ways by which quiescent T cells could be rendered permissive without being fully activated. This included the use of cytokines such as IL-4, IL-7, IL-15 [41-43], various chemokines as well as other receptor-signaling ligands [44-51]. In vivo and in vitro studies also underscored the importance of cell-cell contact and how that promoted infection of non-dividing T cells [47, 52]. The latter is quite important since quiescent T cells come into contact with other cells of the immune system in the densely packed lymphoid tissues. These

interactions can facilitate infection of quiescent cells. While the above studies did show the various ways that HIV allows infection, none fully addressed and explained the inefficiencies seen in the truly quiescent T cell population.

Restriction factors

Based on the earlier reports, it was evident that truly quiescent T cells did not support HIV infection. As stated earlier, these cells are characterized by their low metabolic rates and low levels of transcription. HIV, like any other virus, relies on cellular resources to replicate efficiently. Thus, it is very reasonable to infer that the lack of cellular substrates or raw materials can have a detrimental effect on viral replication. We examined the effect of nucleosides, an important substrate for transcription, on the infection of quiescent T cells [53]. Pretreatment of quiescent T cells with nucleosides improved reverse transcription in these cells. However, it did not rescue productive infection. This suggested that the presence of inhibitory factors or the absence of supportive processes were responsible for this phenotype. It was possible, as was shown earlier by Stevenson et al., that defects in nuclear transport prevented newly synthesized viral DNA to enter the nucleus and integrate [30]. Later studies by the O'Doherty group did support the earlier conclusions by showing that nucleoside treatment increased the amount of integrated provirus, along with an increase in 2-LTR circle formation, but the defect in expression of viral genes was still present [54]. Therefore, the focus was shifted on to cellular restriction factors, which we will outline below.

Murr1

The advances in siRNA technology enabled researchers to identify the role of a number of genes, using siRNA-mediated knockdown. This was also used to study the effect of cellular factors on HIV replication. Ganesh et al. used this technology to identify a potential HIV restriction factor in quiescent T cells [55]. Murr1 is a protein involved in copper regulation and inhibition of NF- κ B activity. The protein blocked I- κ B degradation by the proteasome, leading to decreased NF- κ B activity [55]. When knocked down in cell lines, it decreased levels of I- κ B- and enhanced NF- κ B activity. In addition, the authors found that Murr1 was highly expressed in T cells. Using nucleofection, they introduced Murr1 siRNA to quiescent CD4 T cells and monitored HIV infection. The knockdown resulted in increased Gag expression, suggesting the Murr1 may regulate HIV infection in quiescent CD4 T cells [55]. While the authors did show that the cells were not activated (based on T cell activation marker expression CD25, CD69 and HLA-DR), the process of nucleofection may have had an impact on the infection. However, no additional studies were performed to further elucidate the role of this protein.

APOBEC3G

Apolipoprotein B mRNA editing catalytic peptide like 3G (APOBEC3G) has been shown to have strong antiviral activity and been classified as an innate antiviral factor. APOBEC3G (A3G) is a cellular cytidine deaminase expressed in T cells and was initially found to be a potent antiviral factor against *vif* deficient HIV-1 [56-67]. The protein was found to cause severe hypermutation of the HIV genome preventing viral replication. However, the protein is sequestered by *vif* and degraded by the proteasome, preventing such damage to take place. Additional studies showed that A3G is found in a catalytically active low molecular mass form in quiescent T cells and in an inactive high molecular mass form in activated T cells, resulting in the discrepancy in permissivity between cellular activation states [68].

A3G knockdown experiments in quiescent T cells initially indicated that the restriction to infection in quiescent T cells is abrogated in the absence of A3G. By nucleofecting an

siRNA against A3G into quiescent T cells, Chiu et al. [68] demonstrated that these cells could be infected once A3G is eliminated, even though there was no apparent indication of cellular activation. However, two groups have independently published results that would indicate otherwise. Using identical techniques with the same, and two additional, siRNAs against A3G, Kamata et al. could not reproduce the results of these experiments [69]. Further, Santoni et al. knocked down A3G using both stable shRNA and ectopic *vif* expression in activated T cells, then allowed them to return to a resting state before infecting them with HIV-1. They found no difference in infection between cells with, and those without, A3G nor did they find a correlation between viral restriction and high versus low molecular mass A3G complexes [70]. Therefore, while A3G does affect viral replication by modifying the sequence of the viral genome during reverse transcription in activated cells, it seems that it is not the sole factor responsible for the block seen in quiescent T cells.

JNK and Pin1

Recent studies by Manganaro et al. focus on the lack of a cellular protein rather than the presence of a restriction factor that may be responsible for the block to productive infection in quiescent T cells. In a series of experiments, the authors demonstrated that c-Jun N-terminal kinase (JNK) phosphorylates viral integrase, which in turn interacts with the peptidyl prolyl-isomerase enzyme Pin1 causing a conformational change in integrase [71]. The effect of both cellular factors increases the stability of integrase and allows the viral integration process to take place. Since quiescent T cells do not express JNK, the process is not efficient in these cells [71]. These findings do provide support for the earlier studies that suggested the presence of a preintegrated viral DNA in resting cells that acts as an inducible reservoir [30, 33]. However, they do not explain the recent findings by our group as well as others (discussed in the next section) that show integration occurring in quiescent T cells and that the defects seen in these cells are prior to that event in the viral life cycle [72-75].

In summary, while it is accepted that there is a plethora of cellular factors that can enhance or restrict HIV replication in quiescent CD4 T cells, it is becoming more evident that the concerted action of multiple events is responsible for the phenomena seen in quiescent CD4 T cells.

Quiescent CD4 T cells: an inefficient but potential HIV reservoir

The development of more sensitive PCR techniques as well as cell purification assays led to a more in depth examination of HIV infection in quiescent T cells by our group and others. A series of elegant studies by the Siliciano group shed more light on the infection of quiescent T cells by HIV. Pierson et al., using a linker-mediated PCR assay, measured the rate of reverse transcription and degradation of the non-integrated linear viral DNA [76]. More specifically, quiescent T cells could support reverse transcription at a much slower rate than that seen in activated cells; it took 2–3 days for the process to complete, whereas in activated cells reverse transcription is completed within 4–6 h. The linear piece of DNA had a half-life of about one day. This in conjunction with the slow rate of reverse transcription severely compromised the ability of the virus to establish a productive infection. These observations were followed by a second study showing that the linear non-integrated viral DNA was integration competent [77]. These studies confirmed our earlier reports and raised the possibility that integration may occur in quiescent T cells.

Furthermore, Swiggard et al. showed that while reverse transcription was decreased in quiescent T cells, full-length HIV cDNA accumulated over time was stable for approximately 3 days and partial viral transcripts were degraded [74, 78]. However, the use of an alternative method of infection, spinoculation [79], raised the possibility of partial cell activation that may have improved the stability of the full-length viral cDNA.

Meanwhile, the development of a sensitive and quantitative assay allowed detection of low levels of integration in HIV infected cells [80], which proved very useful in the studies outlined below. Using this assay and spinoculation as the infection method, the O'Doherty group showed that quiescent CD4 T cells could be infected by HIV and detected integrated virus in these cells [78, 81]. In addition, the authors were able to induce expression of virus following stimulation of infected quiescent cells with IL-7 or anti-CD3/anti-CD28 co-stimulation. The results from this study demonstrated that a latent infection could be established in quiescent CD4 T cells. However, despite these promising results, the data revealed the major deficiencies previously seen in quiescent T cells, significant delays in kinetics and lower levels of infection compared to stimulated cells. These deficiencies were in part masked by the use of spinoculation as a method of infection.

Our group took a more comprehensive look at quiescent T cell infection by HIV [73]. Using quantitative real-time PCR assays and the integration assay developed by O'Doherty, we examined the kinetics of infection in quiescent CD4 T cells and compared them with activated T cells [73]. We examined several parameters of the viral life cycle. Entry was comparable between the two cell types. However, initiation of reverse transcription was 30-fold lower in quiescent T cells. Interestingly, there was some completion of reverse transcription and was delayed by 16 h compared to activated cells. Integration occurred with the same efficiency between the two groups, but it was delayed by 24 h in quiescent CD4 T cells. In addition to the above, quiescent T cells expressed multiply spliced viral RNA at significantly lower levels than stimulated cells, and this expression initiated 48 h later. Despite the expression of viral mRNA, there was no detectable Gag protein expression. Furthermore, the levels of integrated HIV copies remained unchanged for 5 days, suggesting that these cells did not die after infection but were latently infected. Interestingly, this inefficient infection process of quiescent T cells was not rescued with immediate stimulation [73].

The above studies revealed that quiescent CD4 T cells can get infected by HIV, albeit very inefficiently, and unlike what was reported in early studies, can harbor integrated HIV. Did this suggest that quiescent CD4 T cells can get latently infected and be part of the long-term viral reservoir? Integrated virus was found in resting cells of HIV infected individuals, but this was attributed to infection of previously activated T cells that return to a resting state [82]. There was no indication that these cells were infected while quiescent. Furthermore, the presence of viral mRNA but the lack of detectable viral protein in quiescent T cells was quite intriguing [73]. This raised the question of whether integration in quiescent T cells is distinct from that seen in their stimulated counterparts. HIV preferentially integrates into actively transcribing genes. Since T cell quiescence is an actively maintained state, it could be inferred that a distinct distribution of integration sites could explain our observations. Others and we examined the distribution of integration sites in quiescent CD4 T cells [72, 75]. Based on our data, integration in both activated and quiescent CD4 T cells occurred in similar sites, mostly genes that were not affected by cell state such as housekeeping genes [72]. The majority of integration sites were hosted on transcriptionally active genes. The orientation of integrants between the two cells was similar, as were the chromosomal locations of the integrated proviruses. However, in quiescent CD4 T cells, we observed increased levels of LTR end attrition. Quiescent cells had higher levels of abnormal LTR-host junctions, 2-LTR circles with both normal and abnormal junctions [72]. It seems that the delays seen in quiescent cells had a detrimental effect on the ends of the viral cDNA. Similar patterns of integration were seen in studies by Brady et al. However, in their studies, HIV integration patterns were somewhat different between stimulated and quiescent T cells [75]. HIV appeared to integrate in less transcriptionally active regions in quiescent cells when compared to stimulated cells, but the observed differences were modest.

The most important aspect of these studies is that *in vitro* quiescent CD4 T cells can be latently infected with HIV and can be a potential reservoir of virus. However, the question remains whether this can occur *in vivo*. Two recent studies have underscored the importance of resting cells in maintaining this stable viral reservoir. Nishimura et al. infected rhesus macaques with SIV or X4-SHIV and examined patterns of infection 10 days later [83]. They found that SHIV preferentially infected resting CD4 T cells and observed a high frequency of integrated viral DNA. Furthermore, they were able to detect *ex vivo* viral release from resting T cells without any activation [83]. However, it is possible that these resting cells were infected in a more activated state and returned to a resting state later. Chomont et al. focused more on the memory T cell populations, the central memory and transitional memory CD4 T cells [84]. The central memory T cells are characterized by low levels of cell division. The transitional memory cells on the other hand are actively dividing cells. The authors found that the provirus was harbored in both cell types, however, the central memory cells were the long-lasting reservoir due to the low levels of cell proliferation and their quiescent nature [84]. On the other hand, the transitional memory cells driven by homeostatic proliferation are at lower levels in viremic individuals with low CD4 counts, but they keep maintaining the levels of viral expression and spread [84]. However, these *in vivo* studies have not yet addressed the issue of whether these cells were infected when quiescent. They do underscore, however, the importance and contribution to HIV infection of non-dividing, latently infected T cells.

Conclusions

The permissiveness of quiescent CD4 T cells to HIV infection has been quite controversial. However, the development of more quantitative and sensitive techniques has provided us with some concrete answers as to the nature of HIV infection in this cell type. It is now clear that:

- i. Quiescent CD4 T cells can get infected by HIV but very inefficiently; defects are observed at the early stages of infection.
- ii. HIV infected quiescent T cells can harbor integrated virus in actively transcribing genes, making them a potential reservoir.
- iii. Host restriction factor impact is not discounted, but it seems to be at best incremental.

Thus, what are the next steps? We still have not elucidated why initiation of reverse transcription is low in these cells. There have been suggestions of poor uncoating in quiescent cells that can prove detrimental to infection, but these studies are very limited due to the inherent difficulties studying the viral capsid [85]. The lack of cellular factors can play a role as some studies have suggested and so does the presence of cellular restriction factors. On the other hand, these inefficiencies could be due to quiescent T cell physiology.

In addition, we need to further examine quiescent CD4 T cells as a viral reservoir. Epigenetic changes of the LTR ends, the sequence state of the entire integrated virus and the state of the viral mRNA produced can provide us with answers regarding the lack or the undetectable levels of viral production in this cell type. This may reveal some unique aspects of HIV infection in quiescent T cells that may be seen *in vivo*.

In summary, there are still many unanswered questions on the mechanisms of HIV infection in quiescent CD4⁺ T cells. While the bulk of our current knowledge comes from *in vitro* studies, the rapid development of many *in vivo* models may further expand our knowledge on this aspect of HIV biology. The identification of the nature of the block in quiescent T

cells and the confirmation that their *in vitro* infection can occur *in vivo* can lead to significant advances in the eradication of the virus.

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Fig. 1. Identification of the different cell cycle phases following CD4 T cell stimulation. Quiescent CD4 T cells are purified and stimulated for 3 days using anti-CD3/anti-CD28. This is a representative figure of T cell cycle progression. Cells are harvested and stained with 7AAD (y-axis, lin fluorescence) (DNA) and Pyronin Y (x-axis, lin fluorescence) (RNA) and analyzed by flow cytometry. The quadrants are set based on n-butyrate (G_{1a} arrest) and aphidocolin (G_{1b} arrest) treatment of these cells