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## The Role of HIV Gp41 Mediated Fusion/Hemifusion in Bystander Apoptosis

Himanshu Garg and Robert Blumenthal\*

*Membrane Structure and Function Section, Center for Cancer Research, Nanobiology Program, National Cancer Institute, National Institutes of Health, Frederick, MD 21702 USA*

### Abstract

Mechanisms of HIV-mediated CD4+ T cell loss leading to immunodeficiency are amongst the most extensively studied yet unanswered questions in HIV biology. The level of CD4+ T cell depletion in HIV infected patients far exceeds the number of infected T cells suggesting an indirect mechanism of HIV pathogenesis termed bystander cell death. Evidence is accumulating that the HIV envelope glycoprotein (Env) is a major determinant of HIV pathogenesis and plays a critical role in bystander cell death. The complex structure and function of HIV Env makes the determination of the mechanism of Env mediated apoptosis more complex than previously thought. This review will examine the complex relationship between HIV Env phenotype, coreceptor expression and immune activation in determining HIV pathogenesis. We review data here corresponding to the role of HIV Env (hemi) fusion activity in HIV pathogenesis and how it interplays with other AIDS associated factors like chemokine receptor expression and immune activation.

### Keywords

HIV-1; Env; pathogenesis; fusion; hemifusion; apoptosis

### Envelope glycoprotein structure and function

The Envelope glycoprotein (Env) of HIV is arranged on the surface of the virus and virus-infected cells as a hetero-trimer. Each monomer is composed of a receptor-binding surface unit (gp120) and a fusogenic gp41 transmembrane unit [1]. The gp120 subunit binds to CD4 and a coreceptor either CXCR4 or CCR5 on T helper cells. Binding of HIV gp120 to CD4 triggers a complex sequence of events (Figure 1) involving several conformational changes in gp120 that result in exposure of coreceptor binding sites on gp120 and the N-terminal and C-terminal heptad repeat regions of gp41. Following engagement of gp120 with coreceptor, the gp41 heptad repeat domains interact with each other to form a six helix bundle catalyzing fusion of target and viral membranes [2]. Elucidation of mechanisms of fusion mediated by gp41 has been facilitated by high resolution determination of the gp41 core structure and by inhibition studies using peptides that mimic N or C-terminal heptad repeat sequences and interact with intermediate conformations of gp41 [3–7]. Although the function of HIV Env glycoprotein is to facilitate the entry of viral nucleocapsid into the target cell, its role in HIV pathogenesis is becoming increasingly evident [8].

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Address correspondence to: Robert Blumenthal, Center for Cancer Research, NCI-Frederick, P.O. Box B, Building 469, Room 152, Miller Drive, Frederick, MD 21702-1201. Phone: +1-301-846-5532, Fax: +1-301-846-5598. E-mail address: blumenthalr@mail.nih.gov.

## Factors effecting HIV gp41 mediated fusion and hemifusion

Studies of membrane fusion mediated by viral Envs have revealed a number of intermediate steps in the fusion cascade that occur prior to the opening of the large fusion pore that enables the transfer of the nucleocapsid [9]. These steps include hemifusion and the opening of small fusion pores [10]. Hemifusion is defined as a membrane fusion event characterized by the mixing of the outer leaflets of the lipid bilayer without progression to fusion pore formation [11]. In the case of influenza hemagglutinin-expressing cells that fuse with red blood cells, stable hemifusion intermediates have been identified [12–16]. In the case of HIV-1 Env mediated cell fusion lipid mixing has been observed which is followed by separation of cells and the process does not progress to syncytia formation [17–19]. Although the latter process would represent “stunted” fusion rather than hemifusion from a fusion purist point of view, we will stick for the purpose of this review to the operational definition of hemifusion as lipid mixing without contents mixing. The rate of fusion depends not only on the fusogenic activity of the fusion protein like gp41 but also on the expression levels of both the fusion protein as well as the cognate receptor/coreceptor [20;21]. Hence depending on the conditions present *in vitro* or *in vivo* a fusion protein may mediate more hemifusion versus fusion or vice versa. There also seems to be clear disconnect between cell to cell fusion and virus cell fusion. A number of HIV Envs that are capable of inducing virus cell fusion and efficient viral replication do not induce cell to cell fusion or syncytia formation. These include a majority of CCR5 tropic Envs and some experimentally-designed CXCR4 tropic Envs [22;23]. This suggests that virus replication may be independent of cell to cell fusion and that Env may play a role in HIV pathogenesis that is related to the cell to cell fusion capacity of Env proteins. Another factor to keep in mind is the levels of coreceptor expression. The majority of CCR5 tropic viruses are Non Syncytia inducing (NSI) based on the low levels of CCR5 expression on cell lines and also in primary cells compared to CXCR4 expression [24;25]. Hence differences between the fusion capacity of CXCR4 and CCR5 viruses maybe directly related to the surface expression of coreceptors.

## Gp41 structure and functional domains

The gp41 is a complex transmembrane protein (Figure 2) that contains various well defined domains. The N terminal of the protein contains a hydrophobic fusion domain [26]. This is followed by N and C terminal heptad repeat (HR) regions that eventually form a six helix bundle which drives close membrane apposition and merging eventually leading to fusion pore formation [6;27]. The HR regions have been targets for development of numerous fusion inhibitors like T20 (Enfuvirtide) and C34 [7]. Although the HIV gp41 six helix bundle formation is the main driver of the fusion process, other gp41 domains may regulate fusion activity in numerous ways as indicated by studies on the effects of gp41 mutations on HIV Env mediated cell to cell or virus cell fusion [28]. The N-terminal and C-terminal heptad repeat regions are linked by an immunodominant loop region that plays a critical role in fusion. Mutations in the loop region of HIV have been shown to generate unique Envs that are restricted at the hemifusion step [19]. The tryptophan-rich membrane proximal ectodomain region (MPER) of HIV gp41 is also known to play a critical role in fusion. Mutation of more than one of the tryptophans in this region severely effects fusion activity [29;30]. Contrary to this replacement of tryptophans in this region with proline has been reported to enhance fusion activity [28]. This region is also highly investigated as a number of broadly neutralizing antibodies like 2F5 and 4E10 bind to this region and inhibit Env mediated fusion [31–33] making it an excellent target for vaccine development. The transmembrane region of gp41 is also important for Env activity as GPI anchored Env proteins fail to induce fusion [34] and a minimum length of transmembrane portion is critical for optimal Env function [35]. Interestingly the long cytoplasmic tail of gp41 not only regulates Env incorporation [36;37] but also regulates fusion [38]. The tail region of gp41 is known to contain 3 LLP (lentiviral

lytic peptides) regions that interact with the viral membrane [39] and regulate fusion as evident from truncation mutants [38].

## Env glycoprotein and bystander apoptosis

The amount of T cell depletion in HIV infected patients far exceeds the number of infected T cells suggesting an indirect mechanism of HIV pathogenesis termed bystander cell death [40; 41]. Many studies have shown that bystander cell death induced by HIV shows characteristics of apoptosis [42;43]. In lymph node section of HIV-infected patients and SIV infected animals it appears that the majority of apoptotic cells are uninfected cells found in close proximity to infected cells [44]. Since HIV Env is expressed on the surface of infected cells that can interact with CD4+ bystander cells, Env is regarded as the major culprit in causing bystander cell death. This premise is supported by numerous *in vitro* studies that show that Env expressed on infected/transfected effector cells can interact with bystander target cells and induce apoptosis [45–48]. The binding of gp120 to its receptor (CD4) and a coreceptor (CXCR4/CCR5) is critical for this apoptosis induction [49;50]. This is evident from the fact that inhibition of this interaction with either anti CD4 antibodies or CXCR4 antagonists abolishes Env mediated bystander death [51–53]. Recently it was shown that HIV gp41 fusion inhibitors like T20 and C34 can also inhibit bystander cell death in models where HIV Env expressing cells are cocultured with receptor and coreceptor expressing bystander cells [17;54–57]. The inhibition of HIV Env mediated bystander apoptosis by T20 has also been shown in *ex vivo* Thymic cultures [56] further validating this phenomenon. This suggests that HIV gp41 plays a significant role in Env mediated bystander death. Hence the process of Env mediated apoptosis is quiet complex and is likely to be initiated via binding of gp120 to CD4 and CXCR4 and culminates in gp41 mediated membrane hemifusion/fusion. The nature of downstream signaling events that trigger the apoptotic cascade remains to be determined.

## Signaling in HIV Env mediated apoptosis

Bystander cell death mediated via HIV Env shows classical signs of apoptosis. The apoptotic cascade initiated by HIV Env has been shown to involve caspase-3 activation [58], mitochondrial depolarization [53;59] as well as reactive oxygen species production [57]. The characteristics of apoptosis have also been reported in PBMCs from HIV infected individuals [60]. However the mechanism via which Env mediates these apoptotic features remains debated [8]. It is clear that this process is independent of Fas and TNF signaling [58;61]. As gp120 binds CD4 and CXCR4/CCR5 prior to fusion mediated by gp41, the role of signaling via either of these receptors becomes evident. Studies by Biard-Piechaczky et al using cells expressing cytoplasmic tail truncated form of CD4 showed that these cells still undergo Env mediated apoptosis [52] indicating that CD4 signaling may not be required for this phenomenon. However recent studies by Py et al suggest that the Siva protein interaction with CD4 may play a role in Env mediated apoptosis [62]. G protein dependent signaling via CXCR4 is also not likely to be involved in Env mediated apoptosis based on studies with pertussis toxin mediated inhibition of G protein [52;63]. However the role of G protein independent signaling via CXCR4 cannot be ruled out. The inhibition of HIV Env mediated bystander cell death by gp41 inhibitors like T20 and C34 suggest that the signaling event may in fact be initiated by gp41. In an attempt to understand this signaling mechanism we have shown that this process involves early caspase 3 activation that is propagated via a mitochondrial amplification loop [57]. Interestingly this apoptotic signaling via gp41 is inhibited by HIV protease inhibitor nelfinavir via its action on mitochondrial ANT transport system [64]. Other groups have also confirmed that nelfinavir inhibits HIV Env mediated apoptosis [65] establishing the critical role of mitochondria in HIV mediated apoptosis. This is further strengthened by findings that nelfinavir may have beneficial effects beyond virus suppression in HIV infected individuals by suppressing mitochondria mediated apoptosis [66].

## Relationship between Env fusion and HIV pathogenesis

The fusogenic activity of HIV Env has long been associated with HIV pathogenesis both *in vitro* and *in vivo*. In clinical studies the finding of a highly fusogenic Syncytia inducing (SI) virus versus a Non Syncytia inducing (NSI) virus was associated with poor prognosis [67; 68]. The classification of SI versus NSI is largely based on the syncytia formation by virus in MT2 cell lines [25]. This has often been correlated to coreceptor usage as CXCR4 utilizing viruses are most often SI while CCR5 viruses are NSI [69]. Furthermore, studies in chimeric SHIV containing HIV Env Rev Tat and Nef genes showed that passage of a non pathogenic chimera SHIV 89.6 resulted in a pathogenic SHIV89.6P which showed rapid CD4 loss in Rhesus Macaques [70;71]. The increased pathogenesis of SHIV89.6P could be mapped to the Env glycoprotein and correlated with fusion activity [72]. The role of SI phenotype in depletion of CD4+ T cells has also been demonstrated in various SCIDhu mouse models [73–75] as well as lymphnode [76;77] and thymus histocultures [56;78]. This suggests that a direct correlation between the fusogenic potential of HIV gp41 and CD4 T cell loss exists.

### Gp41 mediated syncytial apoptosis

HIV Env expressing cells cocultured with CD4 and CXCR4/CCR5 expressing target cells undergo cell fusion. Cells undergoing fusion mediated by HIV gp41 form multinucleated syncytia that after a relatively short period 48–72h undergo apoptosis [79–83]. This method of apoptosis has been well characterized by Perfettini et al [84] and has been shown to involve p38 MAPK and mTOR mediated activation of p53. Under this model p53 dependent expression of bax and puma leads to mitochondrial depolarization and apoptosis [84;85]. In other studies with a slightly different model it has been shown that Env expressing cells on the verge of undergoing apoptosis can fuse with neighboring cells and transmit the lethal signal in a contagious fashion [86]. However the role of post-fusion hypotheses for HIV pathogenesis *in vivo* remains uncertain since little or no syncytia are observed in the lymph nodes of HIV infected individuals or SHIV infected Macaques [87].

### Gp41 hemifusion mediated apoptosis

HIV gp41 mediates efficient fusion in a variety of cell lines expressing receptor and coreceptor. However, as mentioned above, in a number of situations the fusion process may not result in complete fusion of cells and maybe interrupted at the hemifusion step. This probably happens more often than thought especially *in vivo* where virus induced syncytia are rarely seen in infected patients. This process of hemifusion could be detrimental to the target cells forcing them to undergo apoptosis as observed by Blanco et al [17]. We and others have shown that *in vitro* the single cells dying after coculture of Env expressing cells with target cells have taken up a membrane dye from the effector cells [17;57]. These cells undergo classical apoptosis characterized by caspase 3 activation and mitochondrial dysfunction [57]. The inhibition of both membrane dye transfer and apoptosis by gp41 inhibitors like C34 suggests a direct role of gp41 in this process and forms the basis of the hemifusion hypothesis. To address this issue directly we have used a mutational approach [18] to show that mutations in the gp41 fusion domain that abolish fusion activity also inhibit apoptosis induction. Further support of the role of gp41 mediated hemifusion in bystander apoptosis comes from our demonstration that an Env glycoprotein mutant D589L that is restricted at the hemifusion step [19] mediates apoptosis in bystander cells in the absence of cell to cell fusion. The mechanism of apoptosis mediated by hemifusion restricted mutant is identical to wild type Env, based on the inhibition by nelfinavir and caspase 3 inhibitors, strengthening the hypothesis that gp41 mediated hemifusion is both required and sufficient for apoptosis induction. This hemifusion induced single cell death mediated by HIV Env has been aptly named the “Kiss of Death” [8] and its role in HIV pathogenesis is becoming increasingly evident.

## Viral Synapse and bystander cell death

Recent evidence suggests that the transmission of HIV occurs more efficiently via cell to cell contact than free virus [88]. This cell contact dependent transmission of virus occurs across a viral synapse, which is similar to an immunological synapse, is formed between the infected and uninfected cell [89]. Upon contact of infected cell with uninfected cell there is a polarization of both HIV receptor and coreceptor on target cell as well as the budding of virus at the cell to cell interface [90;91]. Various cellular and viral proteins are known to facilitate the viral synapse formation including adhesion molecules like LFA-1, ICAM-1, ICAM-3 [92], tetraspanin [91], actin and tubulin cytoskeletal proteins [93], and Env glycoprotein interaction with CD4 and CXCR4 [94]. The formation of this cell to cell contact interface involving the Env glycoprotein further supports the importance of cell surface expressed HIV Env in mediating bystander cell death. It is important to note that this viral synapse formation can not only effect virus replication by enhancing virus transmission but may also mediate apoptosis via gp41 mediated events. In fact we have shown that replication of viruses that induce cell to cell fusion is slower than viruses that don't [18]. Furthermore, inhibition of bystander apoptosis via caspase inhibitors results in faster replication of SI viruses in T cells as shown by us and others [18;95]. Hence while virus transmission across the viral synapse facilitates virus replication it may also come at the cost of bystander cell death in the case of pathogenic viruses.

## Enfuvirtide resistant mutants and HIV pathogenesis

The hypothesis that HIV gp41 fusion/hemifusion activity correlates with pathogenesis suggests that drugs targeting gp41 function may alter HIV pathogenesis. In this context, it has been suggested that Enfuvirtide therapy may have beneficial effects by directly inhibiting gp41 mediated bystander cell death [96]. The effect of Enfuvirtide on bystander cell death is not restricted to direct inhibition of gp41 function. In a recent clinical study Aquaro et al [97] showed that certain resistant viruses emerging during Enfuvirtide therapy are associated with CD4 increase in patients even after virological failure. These mutations are specifically in the gp41 HR1 region and are known to effect gp41 fusion activity [98]. These findings were confirmed by Melby et al [99] and showing that a mutation specifically at position V38 (V549 based on Env numbering) are associated with increase in CD4 recovery in patients after virological failure. Similar findings have recently been reported by Svicher et al [100]. These findings not only support the hypothesis that gp41 mediated fusion/hemifusion are critical for HIV pathogenesis but also that targeting Env mediated fusion by inhibitors of gp41 should inhibit both virus replication and Env-mediated bystander cell death. Furthermore treatment strategies can be designed to select Enfuvirtide resistant mutants that seem to be less pathogenic via mutations in gp41 that effect fusion activity.

## Coreceptor expression

The role of a specific type of coreceptor in HIV pathogenesis remains much debated. It is clear from various studies that switch of coreceptor usage by HIV from CCR5 to CXCR4 precedes the rapid decline in CD4 cells and AIDS development in numerous cases [67]. The differences in CXCR4 and CCR5 expression levels maybe related to the relative pathogenesis of these viruses [101]. Also the increased fusogenicity of CXCR4 viruses is suggested to be a factor in the pathogenesis of these viruses [68;76]. However in 50% of patients there is no switch in coreceptor and although the presence of CXCR4 tropic virus has been associated with poor prognosis in patients, it is evident that coreceptor switch may not be required for progression to AIDS [77]. Nevertheless, a selection of more pathogenic CCR5 utilizing HIV is possible and in recent studies by Oliveri et al [102] show that CCR5 tropic viruses isolated from patients early during disease are different from those isolated at the development of AIDS. More

specifically, the Env fusion activity could be correlated with CD4 cell loss for CCR5 viruses as well. *In vitro* experiments have also suggested that the loss of CCR5 cells via R5 Tropic Env is mediated by gp41 [103]. The role of CCR5 in HIV infection and pathogenesis *in vivo* is supported by resistance to HIV infection in CCR5 $\Delta$ 32 homozygous populations [104;105]. On the other hand CCR5 $\Delta$ 32 heterozygous population has low levels of CCR5 expression making them susceptible to HIV infection but delayed progressing to AIDS [106;107]. In fact in a study by Scoggins et al in SCID-hu mice reconstituted with CCR5 $\Delta$ 32 heterozygous thymus grafts were resistant CCR5 virus mediated CD4 cell loss even in the presence of virus replication [108]. The role of CCR5 expression is further complicated by the polymorphism in CCR5 promoter region. Epidemiological studies suggest that the rate of disease progression in HIV infected individual correlates with CCR5 promoter polymorphism [109–112]. This promoter polymorphism in turn has been associated with surface expression levels of CCR5 [113]. The importance of CCR5 expression levels is further emphasized by the role of physiological levels of CCR5 expression on Env mediated fusion and virus replication [20; 114]. Whether increased surface expression of CCR5 accounts for the Env hemifusion mediated apoptosis phenotype in certain R5 virus infected patients remains to be determined.

## Immune activation and HIV pathogenesis

As HIV selectively targets CD4<sup>+</sup> cells of the immune system, it is not surprising that it has complex immune manifestations. Whether HIV induced CD4 T cell loss is also immune mediated remains to be determined. Nevertheless, chronic immune activation remains a hallmark of pathogenic HIV infections [115;116]. The activation of CD4<sup>+</sup> T cells as determined by surface expression of activation markers like Ki67, HLA-DR, CD25 and CD38 [117; 118] have been associated with HIV disease progression. In fact immune activation is a better predictor of CD4 apoptosis than plasma viremia [119–122]. Although it is widely accepted that pathogenic HIV infections lead to chronic immune activation it is not clear what mediates this immune activation and whether it is a cause or consequence of CD4 T cell loss. HIV infection of resting T cells results in latent infection and immune activation drives the virus into productive replication. Also immune activation leads to an up regulation of coreceptors, both CXCR4 and CCR5, that not only facilitate virus infection [123] but may also enhance Env mediated apoptosis. Biancotto et al [124] have recently shown that infection of *ex vivo* human lymphoid tissue with HIV-1 leads to a unique pattern of T cell activation, characterized by CD25<sup>+</sup>/HLADR<sup>+</sup> cells that facilitate virus replication. These studies underscore the fact that immune activation is virus mediated, although the mechanism is not clear. Recent studies by Rawson et al [125] demonstrate that cross presentation of caspase cleaved self antigens generated by T cells undergoing apoptosis may induce systemic immune activation providing a mechanistic understanding of immune activation. It is thus conceivable that during infection with pathogenic HIV-1, Env mediated apoptosis generates caspase cleaved antigens that in turn induce immune activation and perpetuates a vicious cycle that involves increased virus infection and replication at the cost of bystander cell death.

## Conclusions and future directions

Although HIV Env glycoprotein structure and function have been studied extensively, the role it plays in HIV pathogenesis remains enigmatic. There seems to be a lack of consensus on whether HIV Env mediates bystander cell death and if so via what mechanism. In this review, we have provided evidence in favor of a role of the fusion activity of Env glycoprotein gp41 subunit in HIV pathogenesis. While the correlation between HIV Env fusogenicity and pathogenesis has long existed, it has been unclear as to the mechanism of this relation especially in terms of single cell death. Recent evidences suggest that gp41 mediated bystander cell death in single cells can be explained by the phenomenon of hemifusion. Application of the hemifusion hypothesis seems to put a consensus on the variety of observations over the years

related to HIV pathogenesis. In this model, a complex interplay between HIV fusion activity, type and amount of coreceptor expression and immune activation play an intertwining role. Based on published studies, we can propose a model of HIV pathogenesis that tends to encompass all the above mentioned issues in a simple model (Figure 3). Based on recent evidence that pathogenesis mediated by Enfuvirtide resistant Env gp41 subunit mutants is reduced; we are for the first time able to appreciate the role of Env mediated fusion in HIV pathogenesis in clinical settings. However future studies need to address the direct role of gp41 in HIV pathogenesis via *in vivo* and *in vitro* models. Some questions that remain unanswered include whether CCR5 polymorphism effects bystander apoptosis mediated by CCR5 Env, whether immune activation is mediated by apoptotic cells generated by Env mediated bystander cell death and whether the reduced pathogenesis of Enfuvirtide resistant mutants is due to lack of Env mediated apoptosis inducing capacity. Also further analysis of the cellular and biochemical changes occurring during this “kiss of death” phenomenon need to be examined in detail.

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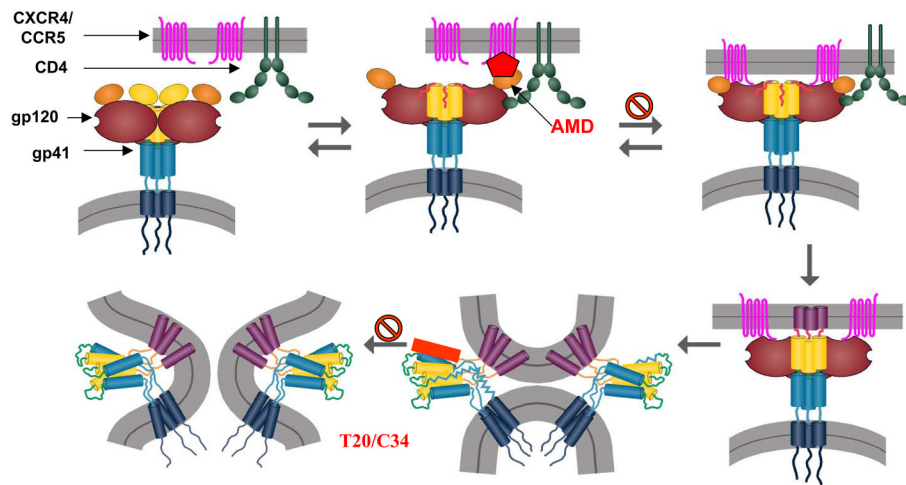
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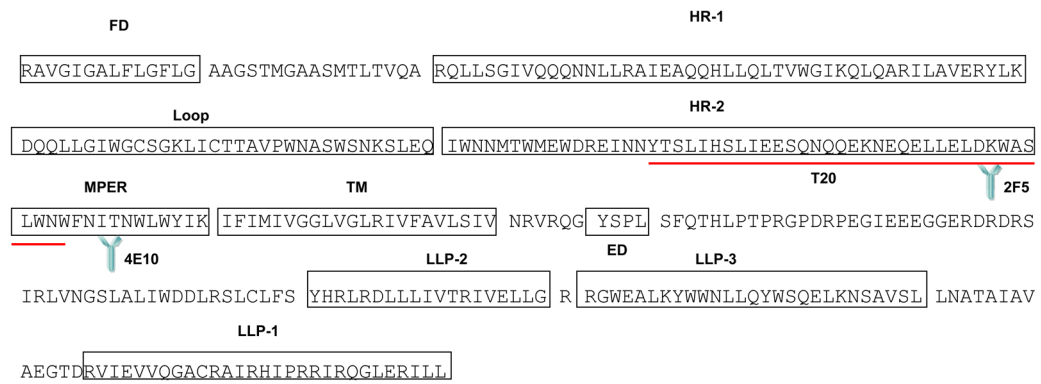
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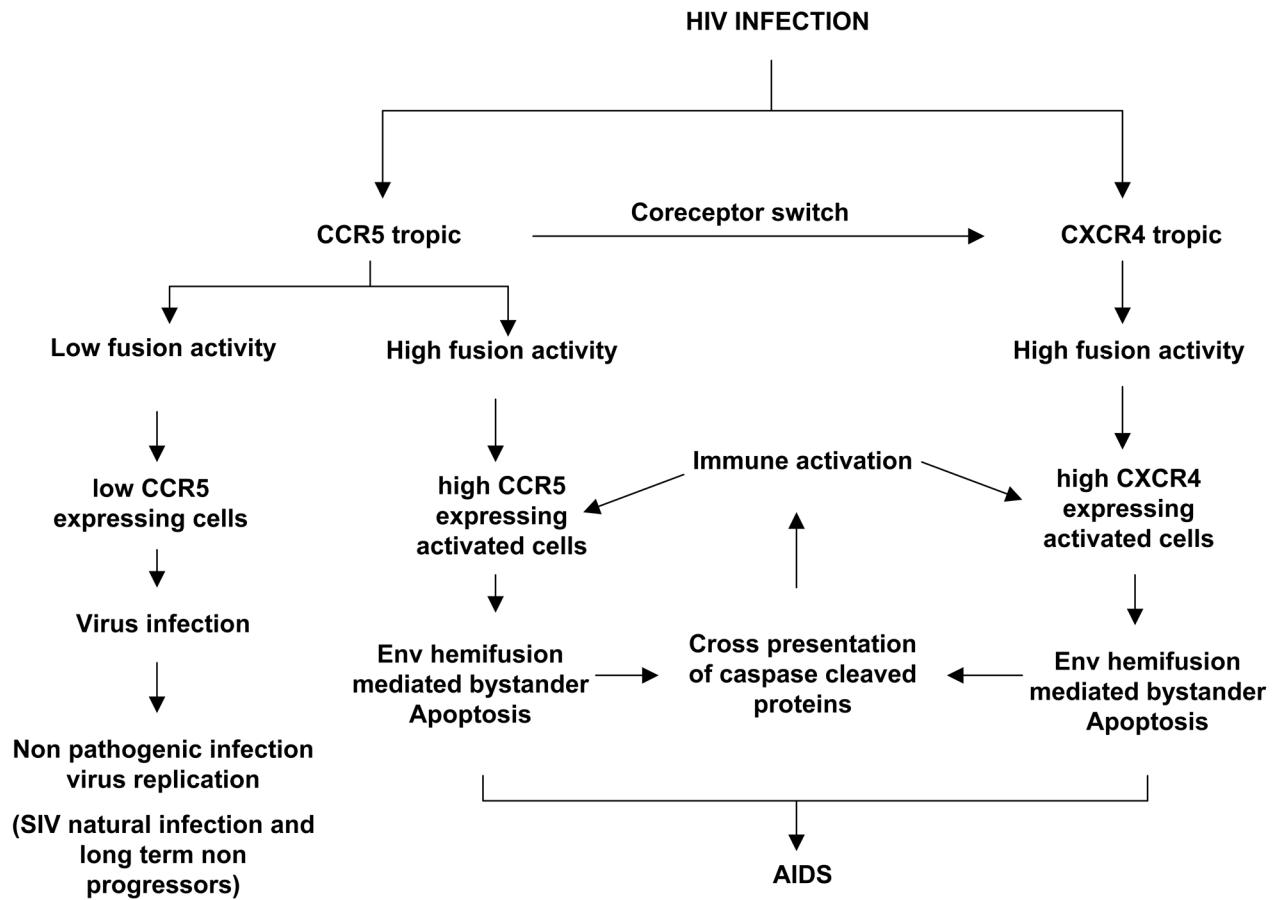


**Figure 1.** Schematic diagram of sequence of events involved in HIV Env mediated fusion. Binding of gp120 to receptor and coreceptor induces conformational changes that allow the exposure of gp41 Heptad Repeat (HR) regions. Interaction of HR1 and HR2 regions of gp41 results in six helix bundle formation resulting in fusion. The binding of CXCR4 antagonist AMD3100 and gp41 fusion inhibitors T20 and C34 are also shown.



**Figure 2.** Sequence of HIV-1 gp41 showing different domains. FD (Fusion Domain), HR (Heptad Repeat), MPER (Membrane Proximal Ectodomain Region), TM (Transmembrane Domain), ED (Endocytosis Domain), LLP (Lentiviral Lytic Peptide). Binding sites for neutralizing antibodies 2F5 and 4E10 are also shown along with the sequence of fusion inhibitor T20 (Enfuvirtide)





**Figure 3.**  
Simplified model of HIV gp41 hemifusion mediated pathogenesis.