NOVEL ROLE OF APOBEC3G/F ENZYMES IN ADAPTIVE IMMUNITY

MAHDIS MONAJEMI

Novel role of APOBEC3G/F enzymes in adaptive immunity

By

Mahdis Monajemi

A thesis submitted to the school of Graduate Studies

In partial fulfillment of the requirements for the degree of

Master of Science

Graduate program in Immunology and Infectious disease, Faculty of Medicine

Memorial University of Newfoundland

November 2012

St. John's

Newfoundland

Abstract

Human immunodeficiency virus (HIV) utilizes our immune system to replicate in the body while having developed ways to evade it. However, immune cells have intrinsic barriers against HIV. Apolipoprotein B mRNA editing enzyme, catalytic polypeptide (APOBEC), most notably APOBEC3G (A3G) and APOBEC3F (A3F) are cytidine deaminases that act by mutating deoxycytidine (dC) to deoxyuridine (dU) in the (-) strand DNA that is generated upon reverse transcription of the viral genome. A3G/F are constitutively expressed pre-infection with HIV and act non-specifically throughout the viral DNA. For these reasons, they are considered anti-viral host restriction agents that participate in the initial innate immune response against HIV. Adaptive immunity is a more specific immune response that carries an immunological memory, and in the case of HIV, consists of anti-viral antibodies as well as cytotoxic $CD8^+$ T cells (CTL). Here, we hypothesize that beyond A3G/F role in innate immunity within infected cells, mutations by A3G/F modulate HIV recognition by the adaptive immune system through modifying peptide epitopes recognized by CTL. To test our hypothesis, we 1) identified all CTL epitopes restricted to each human leukocyte antigen (HLA) *in silico*, 2) identified the CTL epitopes and A3G/F hotspots therein that are mutable by A3G/F and simulated mutations in them, 3) measured the CTL responses of HLA-matched, HIV-infected individuals to peptides containing A3G/F mutations *ex vivo*, and 4) analyzed the frequencies of A3G/F hotspot motifs inside and outside of the CTL epitopes *in silico*. Our results reveal for the first time that rather than being beneficial for the adaptive immune system, mutations of the HIV genome by A3G/F may have the opposite effect by assisting

HIV to escape from CTL responses. Furthermore, we show that the HIV genome has adapted to position A3G/F hotspot motifs selectively in CTL epitopes to maximally subvert the action of A3G/F towards immune escape.

Appendix 1

Acknowledgment

I wish to express my sincere to my supervisor Dr. Mani Larijani and my co-supervisor Dr. Michael Grant for providing me this opportunity to complete my M.Sc. I am also thankful for their advice, facilities and funding during my research. I also would like to thank my committee member Dr. Ken Hirasawa. Financial support was provided by Memorial University and CIHR.

Appendix 2

Table of contents

Abstract	2-3
Acknowledgments	4
List of Tables	6
List of figures	6
HIV-infected cohort status table	7-10
Abbreviations	11-12
Chapter 1: Introduction	13-21
Co-authorship statement	22
Chapter 2: Emerging complexities of APOBEC3G action on immunity and viral fitness during HIV infection and treatment	23-42
Chapter 3: Positioning of APOBEC3G/F mutational hotspots favors reduced recognition of HIV by $CD8^+$ T cells	43-71
Summary	72-75
Bibliography	76-95

Appendix 3

List of Tables

Chapter 3:

Table 1. List of wild type and A3G/F-mutated epitopes used to measure the CD8+ T cells response of HLA-matched HIV-infected individuals. 67

Table 2. Analyzing enrichment of A3G/F hotspot inside/outside CTL epitopes in HIV genes. 68

List of Figures

Chapter 1:

Figure 1. Structure of HIV-1 21

Chapter 2:

Figure 1. The complexities of the pro- and anti-HIV actions of APOBEC3G 41

Chapter 3:

Figure 1. Delineation of the amino acid and DNA sequences of CTLepitopes. 61

Figure 2. Simulation of A3G/F-mediated mutations of CTL epitopes. 62

Figure 3. The response of CD8+ T cells from different HIV-infected individuals to HLA-B57-restricted wild type and A3G/F-mutated epitopes. 63

Figure 4. The response of CD8+ T cells from different HIV-infected individuals to HLA-A2-restricted wild type and A3G/F-mutated epitopes. 64

Figure 5. The response of CD8+ T cells from different HIV-infected individuals to HLA-B44-restricted wild type and A3G/F-mutated epitopes. 65

Figure 6. Analyzing enrichment of A3G/F hotspot inside/outside CTL epitopes in HIV genes. 66

Appendix 4

HIV-infected cohort status table

Patient ID	Viral load	HLA
1	1.6-5.49	A1,A2;B13,B18
3	1.6-4.9	A1,A2;B8,B44
7	1.6-5.6	A2,B44;Bw57
17	1.7-4.48	A3,A25;B7,B44
18	1.7-6.21	A2,A29;B44,Bw6
20	1.7-5.5	A1,A11;Bw57,Bw75
21	1.7-5.78	A2;Bw60,Bw62
26	1.6-4.75	A2,A26;B8,Bw55
27	1.6-4.84	A2,A3;B7,B27
30	1.6-4.53	A2,A11;B7,Bw62
35	1.6-4.77	A2,A30;B13,B44
44	1.6-4.67	A2,A11;B44
45	1.6-4.37	A1,A2;B44,Bw57
46	1.6-5.35	A1,A3;Bw53,Bw57
50	1.6-5.45	A2,A3;B35,BW67
51	1.6-5.7	A24,Aw34;B35,B41
55	1.7-5.14	A11,A28;B44,B51
57	1.6-4.95	A3,A29;B7,B44
60	4.57-5.88	A2,A3;B27,BW60
61	1.6	A3,A23;B49,B51

62	1.6-4	A2;B7,Bw60
63	2.01-5.61	A9;B18,B44
64	1.6-3.7	A2,A32;B44
67	1.6-5.67	A24,A29;B44,B48
68	2.7-4.41	A2,A29;Bw57,Bw62
69	1.7-3.18	A3,A28;B7,B8
70	1.7-5.41	A1,A2;B7,B44
71	1.6-4.37	A1,A2;B18,Bw57
75	1.7-5.28	A3,A24;B7,B44
76	1.7-4.83	A1,A9;B57,B49
77	2.1-5	A1,A3;B8,B44
78	1.69-5.88	A1,A29;B18,Bw41
79	1.6-2.6	A24,Aw34;B14,B35
81	1.7-5.72	A2;B7,Bw61
82	1.6-5.57	A3,A24;B7,B35
84	1.6-4.59	A3,A11;B7,B35
90	1.6-6.22	A2;B56, Bw62
98	1.6-5.73	A1,A2;B8,B49
103	1.6-4.9	A1;B8,B44
105	1.6-5.88	A2,A3;B7,B13
109	1.7-2.46	A2;B39
115	1.6-2.6	A1,A2;B44,Bw60
117	1.6-5.25	A2,A3;B7,B44

119	1.6-2.6	A1,A2;B8,Bw60
123	1.7-2.71	A1,A2;B8,Bw58
124	1.6-5	A3,A32;B7,B51
125	1.6-4.68	A2,A27;B14,B44
132	1.7-5.69	A2;Bw62
133	1.7-5	A1,A24;B37,B44
134	1.6-2	A2,A11;B44,B35
135	1.7	A2,A30;B13,Bw50
136	1.7-5.04	A11;B44,B51
138	1.6-1.7	A3,A28;Bw62
143	1.6-4.77	A26,A29;B38,B44
148	1.6-5.59	A1,A2;B8,B35
149	0-5.67	A1,A2;Bw57,Bw61
151	1.6-5.88	A2,A24;B13,B35
153	1.6-5.13	A2,A29;B44,Bw62
166	1.6-5.88	A2,A11;B57,B49
168		A2,A3;B15,B35
179	1.66-5.88	A1,A2;B7,B35
181	1.7-4.79	A1,A2;B27,B55
184	_	A1,A25;B44,B52
193	1.6-2.6	A2,A25;B44,B62
196	1.6-1.7	A2,A11;B7,B13
197	1.6-3	A2,A28;B15,B57
200	1.6-5.88	A3,A24;B39,B18

201	1.6-4.94	A2,A3;B7,B40
203	1.6-5.88	A28,A29;B44
209	1.6-6.2	A3,A11;B14,B40
210	1.6-4.67	A2;B40,B44
211	1.6-5.06	A2,A24;B40,B44
213	1.6-4.83	A2,A24;B51,B60
214	1-1.7	A2,A31;B40,B44
216	1.6-1.7	A2,A24;B50,B62
219	1.6-5.03	A11,A28;B14,Bw4,Bw6
224	1.6-5.02	A1,A3;B8,B18,Bw6
231	1.6-4.87	A2;B14,Bw27,Bw4,Bw6
233	1.7-4.19	A2,A24;B18,B44,Bw4,Bw6
234	0-5.8	A2,A24;Bw4,B18,B44
237	1.6	A2;B27,Bw4
242	1.78-4.98	A2,A24;B44,B55
244	3.81-6.3	A2,A24;B7,B35,Bw48
246	3.12	A3;B18,B35,B44,Bw48
247	1.6	A11,A24;B35,B52,Bw48
249	_	A1,A2;B27,B51
250	4.89	A2;B27;Bw48

Appendix 5

Abbreviation

Abbreviation	Full name
Ab	antibody
AID	activation induced deaminase
AIDS	acquired immunodeficiency syndrom
AP	alkaline phosphatase
APOBEC	apolipoprotein B mRNA editing enzyme, catalytic polypeptide
CTL	cytotoxic T lymphocyte
DC	dendritic cells
ELISA	enzyme-linked immunosorbent assay
FCS	fetal calf serum
HIV	human immunodeficiency virus
HMM	high molecular mass
HLA	human leukocyte antigen
ISED	influenza sequence & epitope database
LMM	low molecular mass
NK cells	natural killer cells
NCBI	national center for biotechnology information
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffer saline
PCR	polymerase chain reaction
PRR	pattern recognition receptor
RPMI	roswell Park Memorial Institute medium

RT	reverse transcriptase
SFC	spot forming cells
SIV	simian immunodeficiency virus
TLR	toll-like receptor
TRIM	tripartite motifs

Chapter 1

Introduction to retroviruses

Human immunodeficiency virus (HIV) is a lentivirus that belongs to the *retroviridae* family of viruses. Infection by HIV eventually causes acquired immunodeficiency syndrome (AIDS), a fatal disease characterized by weak immune system that was first discovered in 1981 [1]. The structure of a typical member of the *retroviridae* family is composed of genomic RNA in addition to Gag, Pol, and Env proteins that, after further processing, form other structural and functional proteins [2]. Some genes have been expanded in recently evolved members of this group, such as lentiviruses which encode for accessory proteins including Vif, Vpr, Vpu, Tat, Rev, and Nef whose main functions are to regulate replication, processing, gene expression, and assembly of viruses. HIV is a well-known member of this group discovered in 1983 by PCR (polymerase chain reaction) [2-4]. Another member of this group is simian immunodeficiency virus (SIV), which infects different primates but mainly Asian macaques. It has been suggested that cross-species transmission has caused progressive development of SIV into HIV [5]. There are two sub-types of HIV: HIV-1 and HIV-2. These two are different in structural and functional properties and cause immune responses that have distinct characteristics and require different therapeutic strategies [6, 7]. HIV-1 is the more popular and common type of the virus, having infected more than 40 million people worldwide [6]. Its structure is composed of a lipoprotein membrane that surrounds 72 glycoprotein complexes embedded in a lipid membrane including glycoprotein gp120 and a transmembrane protein gp41. Two other proteins (p17 and p24) surround and encapsulate the viral genome. HIV-1 genomic nucleic acid complex (Fig. 1)

is composed of RNA, nucleoprotein, reverse transcriptase (RT), integrase and a protease [8].

The life cycle of HIV

The HIV life cycle starts primarily with viral fusion to susceptible cells by binding the gp120 protein to CD4 on the target cells surface by non-covalent binding that leads to activation of co-receptors (CCR5 and CXCR4) on the cell surface and aids viral fusion to the target cell. This causes injection of a viral core complex into the host cell cytoplasm which is followed by viral un-coating in order to free the two copies of the HIV genomic RNA. HIV replicates in the infected cells by reverse transcription that generates double-stranded blunt-ended DNA (dsDNA) from single-stranded viral RNA [9, 4]. This process is catalyzed by viral reverse transcriptase (RT) complex that is composed of RT and a cellular tRNA3lys. TRNA3lys is used as primer to initiate reverse transcription [10]. Reverse transcriptase complex mediates the synthesis of a minus sense ssDNA template from the viral RNA. This complex also contains RNAase H activity to cleave RNA after formation of ssDNA and is used as a polymerase to generate double stranded DNA (dsDNA) from reverse transcribed ssDNA [10]. The dsDNA form of the viral genome is translocated to the nucleus and integrates into host chromosomal DNA by the integrase enzyme [11]. HIV utilizes host replication factors to express viral proteins that integrate into the budding virions with viral genomic RNA [11]. After further post- translational modifications and processing, proteins such as enzymes are assembled into viral particles. [12]. These assembled virions are infectious and possess the ability to infect other target cells.

HIV target cells, co-receptors, and pathogenesis

After primary infection by HIV, high level of virus in the body triggers the immune response. The asymptomatic stage of the disease may take several years during which the host immune system maintains low levels of the virus. The high cytotoxic activity of HIV enables it to destroy immune cells leading to the symptomatic phase of the disease and progression to AIDS [1]. One reason underlying reason for the effect of HIV on immune cells is that the key receptor for HIV is the CD4 surface marker on these cells [13]. HIV infects distinct cell types including HIV specific CD4+ T cells that are differentiating from naive to mature phenotypes. Moreover, HIV is able to replicate in differentiated monocytes, dendritic cells (DC) with up-regulated cell markers, and also in activated macrophages [14-16]. These cells represent the main antigen-presenting cells of the immune system and induce specific immune responses against antigens. The importance of DCs, macrophages, and monocytes in HIV infection is mainly because they capture HIV and mediate its transmission to CD4+ T cells and prolong HIV infection in the host [17]. CCR5 and CXCR4, the main co-receptors for HIV infection, are necessary for the interaction of the HIV-1 envelope glycoprotein gp120 with CD4 during binding of HIV to monocytes/macrophage-tropic and T-cell tropic HIV-1 viruses [13, 18]. Infection of CD4+ T cells by HIV cause a decline in cell number and cell markers. Fas-mediated apoptosis of infected cells, CD8+ cytotoxic T lymphocyte (CTL)-mediated destruction of infected cells, and down-regulation of CD4 co-stimulatory molecule on T cell surface [13, 19-21]. The final clinical outcome of these processes is that the depletion of CD4+ T cells in blood results in AIDS that increases the chance of opportunistic infections and tumors that are surmountable by a healthy immune system [22].

Innate immunity against HIV

After infection of susceptible cells by HIV, the immune response against the virus is activated. As with all infections, the initial response against HIV is through the innate immune pathways. Toll-like receptors (TLRs) are an important part of the innate immune response that belong to pattern recognition receptors (PRR) family and recognize specific motif in pathogens [23]. TLR 7/8 are important for HIV integration and are activated through the recognition of viral uridine-rich RNA [23]. TLR7/8 are mainly expressed in DCs and natural killer (NK) cells and lead to activation of immune pathways in these cells [24]. Interaction with pathogens activates these receptors and triggers a cell signaling cascade in the target cell. These cellular pathways induce or suppress the expression of genes that lead to inflammatory responses against the pathogen. After engagement of PRR by viral antigens, pro-inflammatory responses such as NF-κB and IFN are triggered in target cells [24]. This results in the production of cytokines and chemokines that are required to trigger other intracellular antiviral pathways, recruit immune cells to the site of infection and prime the adaptive immune response [24].

Intrinsic immunity against HIV

There is another layer of innate immunity against HIV that is caused by cellular factors including tripartite motifs (TRIM), tetherin, and apolipoprotein B mRNA-editing, enzyme-catalytic polypeptide-3 (APOBEC3) family members that belong to intrinsic immunity [27, 28, 29]. These host-restriction factors are the very first lines of defense

against HIV after its entry into target cells and are constitutively present in the target cells. TRIM are cellular proteins that have antiviral functions [27]. Different members of the TRIM family including Rhesus TRIM5 and TRIM1 affect viral uncovering by targeting capsid (CA) viral protein which then envelops the viral core complex and inhibits early or late viral replication [27]. TRIM22 and TRIM28 are other members of the family that interrupt viral reverse transcription and transcription processes [30, 31]. Another intracellular protein that restricts HIV infection is tetherin, which is a trans-membrane protein with an abnormal folding. It functions by changing into protease-sensitive tethers that retains viral particles on host cell membranes and inhibits their entrance to the target cells [28].

APOBEC3 family enzymes, especially A3G/F, are cytidine deaminases that act by substituting dC to dU in the negative strand of the viral genome during its replication in the cytoplasm of infected T cells. These mutations can lead to the degradation of viral DNA by uracil removal pathways, or alternatively result in dG to dA mutations in the coding (positive) strand which potentially incapacitate the coding ability of the viral genome [29, 32]. A3G/F have also been suggested to target HIV through deaminase independent mechanisms involving the physical binding of the enzyme to the viral genome and restriction of viral reverse transcription process, or restriction of integration into the host genome [29, 33].

A3G restricting factors and purifying selection

A3G is a kinetically rapid enzyme that has the capacity to induce tens to hundreds of mutations per viral replication [34]. Despite the theoretically high potential of mutation induction by A3G, there are a number of A3G restricting factors that limit its activity. For example, A3G is targeted for destruction by the virion infectivity factor (Vif) protein of HIV. It has been proposed that the level at which A3G edits the viral genome is partially controlled by the virus through Vif action [35-38]. It is possible that Vif is a regulatory factor that has evolved to control the induced rate of mutations in order to allow HIV to better adapt itself to external pressures [36]. In addition, entrapment of A3G/F in high molecular mass (HMM) complexes, composed of many different types of cellular proteins and RNA, limits the pool of active cytoplasmic A3G/F [40]. Also, after inducing mutations into the viral genome, other elements, such as DNA repair mechanisms, filter out some of these mutations induced within the HIV DNA sequence. In addition, viral sequences with high levels of mutations are sequentially eliminated as more fit viruses are selected for, at each progressive stage of the viral life cycle. This inherent selection for more fit and less mutated viruses throughout the viral lifecycle has been referred to as "purifying selection" [39]. Consequently, the numbers of the mutations persisting in the viral genome which are transmitted to the next viral generations are only a fraction of the initial level of mutations that A3G may have introduced in the parental viral genome.

Adaptive immunity against HIV

Following the initial innate immune restriction, adaptive immune response in the long term controls HIV replication. This task is mainly carried out by cytotoxic CD8+ T-cells (CTL) and also by neutralizing antibodies [41]. CD8+ T-cells recognize a complex of antigens and human leukocyte antigen (HLA) class I molecules on antigen-presenting cells. Various HLA types induce different levels of CTL response and are associated with slow versus fast disease progression [42]. HIV-specific CD8+ T-cells secrete perforin and granzymes A and B which mediate the cytolysis effect [43]. In addition to their cytotoxicity effect, they enhance the immune response by releasing IFN-γ and TNF-α/β [44]. During adaptive immune response, infected CD4+ T-cells that are the main target of HIV will be killed by CD8+ T-cells. Depletion of CD4+ T-cells and suppression of their generation leads to acute lymphopenia that causes AIDS progression.

Immune evasion by HIV

HIV is a highly mutation-tolerant virus with the ability to adapt to external pressures [45]. These pressures include antiviral drugs as well as the host immune system. The effect of mutations in the viral genome to escape from drug treatment and the immune response has been reported [46]. One of the mechanisms used by HIV to evade host immunity is through interruption of the CTL response [47], which can be caused by mutations in CTL epitopes [48-51]. Evolution of viral escape mutations inside epitopes is dependent on each individual type of HLA. For example, patients with HLA associated high CTL response develop specific CTL escape mutations in viral genome. These

mutations may impair viral replication and reduce viral load in the host. However, if this virus infects other individuals with different type of HLA and less immune pressure, the original genotype is regained that forms a fully replicative and competent virus [1, 52].

Hypothesis:

We hypothesize that beyond A3G/F recognized role in innate immunity, low level non-deactivating A3G/F-induced mutations modulate adaptive immunity by modifying peptide epitopes recognized by cytotoxic T cells. The following sections will discuss the dual role of APOBEC family member enzymes in HIV restriction and evolution (chapter 1). Experimental *ex vivo* and *in silico* data that support a prominent role for A3G/F induced mutations in the modulation of adaptive anti-viral immunity as well as in the evolution of the viral genomic sequence will be presented in chapter 2.

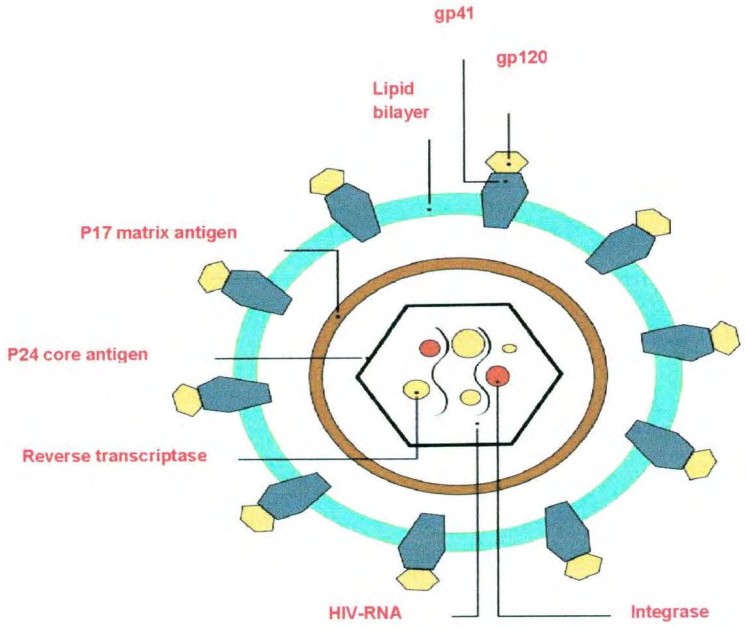


Figure 1. Structure of HIV-1. A lipid membrane including glycoprotein gp120 and a transmembrane protein gp41 surround HIV complex that are shown by the green circle and blue/brown hexagons. P17 and p24 proteins encapsulate the viral genome that are denoted by a brown circle and black hexagon. HIV-1 genomic nucleic acid complex is composed of RNA, nucleoprotein, reverse transcriptase (RT), integrase and protease that are represented by a black line and red/brown circles.

Appendix 6

Co-authorship statement

The online review article "Emerging complexities of APOBEC3G action on immunity and viral fitness during HIV infection and treatment" was published in the journal of Retrovirology (6.47 impact factor), one of the top journals in the field of virology. This article was published in April 2012 and has been already cited in the journal of Microbiology and Immunology. We selected this journal because of the specificity and in depth nature of the journal. I analyzed the literature and collected the materials for this article and presented some suggestions about the overall ideas discussed in the paper. An undergrad student who worked under my supervision helped me to write a manuscript for this paper that was further revised for publication by my supervisor Dr. Larijani and my co-supervisor Dr. Grant. This review discusses the ideas that are later supported in experimental research manuscript. I did all the experiments and analysis (*ex vivo* and *in silico*) for the second paper "Positioning of APOBEC3G/F mutational hotspots favors reduced recognition of HIV by $CD8^+$ T cells." The manuscript was prepared by me and was further edited by my supervisor (Dr. Larijani) and co-supervisor (Dr. Grant). It will be submitted very soon.

Chapter 2

Emerging complexities of APOBEC3G action on immunity and viral fitness during HIV infection and treatment

Mahdis Monajemi[1†], Claire F Woodworth[2†], Jessica Benkaroun[2], Michael Grant[3*] and Mani Larijani[3*]

Abstract

The enzyme APOBEC3G (A3G) mutates the human immunodeficiency virus (HIV) genome by converting deoxycytidine (dC) to deoxyuridine (dU) on minus strand viral DNA during reverse transcription. A3G restricts viral propagation by degrading or incapacitating the coding ability of the HIV genome. Thus, this enzyme has been perceived as an innate immune barrier to viral replication whilst adaptive immunity responses escalate to effective levels. The discovery of A3G less than a decade ago led to the promise of new anti-viral therapies based on manipulation of its cellular expression and/or activity. The rationale for therapeutic approaches has been solidified by demonstration of the effectiveness of A3G in diminishing viral replication in cell culture systems of HIV infection, reports of its mutational footprint in virions from patients, and recognition of its unusually robust enzymatic potential in biochemical studies *in vitro*. Despite its effectiveness in various experimental systems, numerous recent studies have shown that the ability of A3G to combat HIV in the physiological setting is severely limited. In fact, it has become apparent that its mutational activity may actually enhance

viral fitness by accelerating HIV evolution towards the evasion of both anti-viral drugs and the immune system. This body of work suggests that the role of A3G in HIV infection is more complex than heretofore appreciated and supports the hypothesis that HIV has evolved to exploit the action of this host factor. Here, we present an overview of recent data that bring to light historical overestimation of A3G's standing as a strictly anti-viral agent. We discuss the limitations of experimental systems used to assess its activities, as well as caveats in data interpretation.

The role of APOBEC3G in HIV restriction

APOBEC3G (A3G) is a recently discovered primate-specific member of the apolipoprotein B mRNA-editing enzyme, catalytic polypeptide-like editing complex family of cytidine deaminase enzymes with potential to inhibit propagation of the HIV [1,2]. Eleven members of the APOBEC family are found in humans and includes activation-induced cytidine deaminase (AID), APOBEC1, APOBEC2, APOBEC3A-H, and APOBEC4 [3,4]. These enzymes convert deoxycytidine (dC) to deoxyuridine (dU) in single stranded DNA (ssDNA) or RNA of human and viral genomes, thereby affecting a variety of physiological functions [5–7]. A3G was discovered through the study of heterokaryons generated between cells permissive and non-permissive to infection by virion infectivity factor (Vif)-deficient HIV that were used to determine the action of the HIV protein Vif [1,8,9]. A3G is primarily expressed in CD4+ T lymphocytes, macrophages, and dendritic cells, which are all the natural targets of HIV infection [2,10–14], although expression in other tissues may be induced by interferon(s) [15–18]. A3G mutates dC in nascent viral minus strand DNA generated by reverse transcription [17–24]

and preferentially deaminates dC in signature trinucleotides (CCC, TCC) often referred to as hotspots [6,19–21]. The resulting dUs can trigger DNA degradation through the action of DNA repair pathways, such as those involving uracil DNA glycosylase and apurinic-apyrimidinic endonuclease [25,26]. For viral genomes that evade destruction, the consequent deoxyguanosine (dG) to deoxyadenosine (dA) substitutions in plus strand DNA can alter reading frames, introduce premature translation termination codons, and/or produce mutated viral proteins [7,20–25]. In addition, A3G can disrupt propagation of HIV by binding viral RNA, interfering with the DNA strand transfer acrobatics of reverse transcription, physically blocking reverse transcriptase (RT), and obstructing integration into the host cell genome [24,26–31]. A3G has been shown to block RT activity by decreasing tRNA priming, competing for binding to templates, restricting strand transfer during reverse transcription, and direct binding [28,32,33]. Beyond the reverse transcription stage, incorporation of dU into minus strand DNA of the HIV genome has been shown to interfere with synthesis of the complementary plus strand [23]. These findings initially led to the notion that A3G can inhibit viral propagation through pathways dependent or independent of its deamination activity. However, many studies supporting deaminase-independent activities utilized A3G overexpression. It has recently been appreciated that with low level A3G expression, which may be a more accurate representation of the physiological case, deaminase activity is required for viral restriction [34–39]. While the relative contribution of deamination independent activities to viral restriction remains contentious, these may prove more relevant to the action of A3G in restricting endogenous non-long terminal repeat retrotransposons, such as long and short interspersed nuclear elements [40–45]. The anti-retroelement activity of A3G may

represent a host strategy to protect its genome from the deleterious effects of transposable elements. A possible mechanism could involve the binding of A3G to retroelements resulting in blockage of their mobility [46].

The recent expansion of a single APOBEC3 gene in mice to seven (APOBEC3A-H) in primates and the relatively high divergence within APOBEC3 enzymes in primates are evidence for immense evolutionary pressure on the locus suggested to possibly be concomitant with the emergence of modern lentiviruses [3,4,47,48]. Conversely, the finding that the accelerated rate of A3G divergence predates modern lentiviruses, together with the lack of a clear correlation between human A3G polymorphisms and the progression of acquired immunodeficiency syndrome (AIDS), suggest that lentiviral pressure may be, at best, only partially responsible for expansion of the APOBEC3 locus [49–52]. This manner of growth in host defence capacity can reciprocally drive co-evolution of highly adaptable viruses. In this regard, we highlight an emerging body of evidence suggesting that the activity of A3G may be partially subverted by HIV for its survival benefit. These data support a more complex scenario in which the initial perception of A3G as a strictly anti-viral agent may have been naïve.

Viral and cellular factors limiting APOBEC3G effectiveness

The view of A3G as a potent intrinsic anti-viral factor was largely borne out of findings of high levels of dG to dA hypermutated virus sequences in di- and tri-nucleotide motifs targeted by A3G [53–57]. In stark contrast, the previously recognized mutational machinery of HIV, RT, only introduces approximately one mutation per viral genome during a replication cycle [58]. Supporting the potency of A3G as a mutagenic agent is a

wealth of biochemical data showing that it is a highly processive enzyme able to mediate multiple mutations on a given stretch of ssDNA. Accordingly, A3G significantly diminishes viral propagation in several cell culture experimental systems of HIV infection [7,20,22,42,47].

To counteract these activities, lentiviruses have evolved several strategies, primarily in the form of auxiliary proteins such as Vif, which binds and targets newly synthesized A3G for degradation via an ubiquitin-dependent proteosomal pathway [59–69]. A3G is packaged into virions in infected virus-producing cells and it has been shown that it is largely this virion-packaged fraction of A3G rather than the pool of cytoplasmic A3G that is most active on the viral genome in newly infected cells [70–74]. The number of A3G molecules incorporated into each virion is dependent on the level of A3G expression in the producer cell [75]. On average, 3 to 11 molecules of A3G are sufficient for effective viral restriction in the target cell [76]. Besides lowering A3G levels through degradation, Vif has also been suggested to directly interfere with A3G encapsidation and may impair its translation [66,74,77–80]. Vif utilizes other co-factors present in the target cell to ubiquitinate A3G and it was recently shown that Core binding factor (CBF)-β, a cellular transcription factor, is required for Vif-mediated degradation of A3G [81,82]. As a result, when Vif is present, the mutation levels induced by A3G and its effectiveness in viral restriction are diminished.

That Vif is essential for HIV replication in A3G expressing cells, and that the sole function of Vif was thought to be A3G inactivation, lent credence to the notion that A3G is a potent restrictor of HIV propagation [83]. On the other hand, it is now appreciated that even in the presence of Vif, A3G can still cause sub-lethal levels of dG to dA

mutations [19,84]. It is possible that the preferential targeting of newly synthesized A3G by Vif leaves a fraction of previously synthesized A3G intact [85]. In addition, it appears that Vif expression does not completely abolish A3G activity and the correlation between the levels of viral infectivity and A3G inhibition by Vif is not absolute [62,78]. Other functions for Vif and Vif mediated ubiquitination, besides A3G degradation, are also coming to light. For instance, along with the auxiliary protein Vpr, Vif can induce G2 cell-cycle arrest, which may contribute to CD4+ T lymphocyte depletion [86–89]. Vif thus mediates several functions that are independent of its interaction with A3G and is a variable negative regulator of A3G activity rather than a complete inhibitor.

A3G action is further limited by its entrapment in high-molecular-mass ribonuclear complexes (HMM) that may reach mega daltons in size, mediated by non-specific binding of cellular and/or viral RNA and proteins [12,71,90–97]. The shuttling of A3G into newly synthesized virions depends on binding viral RNA and/or proteins [98–101]. The requirement for high affinity interactions with RNA/DNA substrates may explain the evolution of A3G (and other APOBEC enzymes, e.g. AID) to contain an unusually high number of charged residues on its surface [102–104]. Ironically, this same attribute, necessary to enact the anti-viral function of A3G, may also be a key contributor to limiting its antiviral function through HMM formation. Reversion of HMM to low-molecular-mass (LMM) A3G can be experimentally mediated by treatment with RNase A/H [70,71,105]. The RNase H activity of RT is thought to release viral RNA-bound A3G, allowing it to act on the proximal minus strand DNA during its synthesis [2,19,75]. Enzymatically active A3G able to be incorporated into newly synthesized virions is strictly found outside of the HMM complexes in the LMM fraction [73,106]. The LMM

form primarily resides in peripheral blood-derived resting CD4+ T cells and monocytes [12]. However, upon activation of CD4+ T cells or differentiation of monocytes into macrophages, a higher proportion of A3G is shuttled to HMM complexes [2,91]. Although this was suggested to be a mechanism that restricted the infection of resting T cells by HIV, subsequent knockout studies of LMM A3G in resting CD4+ T cells did not render these cells permissive to HIV infection, thus indicating that the difference in the LMM versus HMM-bound proportion of A3G is not the sole mechanism for resistance of resting CD4+ T cells to HIV infection [107,108]. Beyond the induction of HMM formation by HIV through cellular activation processes, Vif has been shown to directly promote HMM production [109]. Remarkably complex co-evolution is evident considering the intimate linkage between HIV infection and HMM formation and the notable level of mechanistic integration between A3G function and the viral replication machinery. The RNase H activity of RT is at once both necessary and detrimental to viral propagation due to its role in the release of active A3G.

The complexities surrounding regulation of Vif activity and HMM formation notwithstanding, it is clear that both result in diminished A3G efficacy. It is possible that mutations introduced by A3G only succeed in restricting viral replication at a sub-optimal level and conversely may assist the virus by generating sequence variation [35,39,84]. Consequently, an alternative view that A3G activity can contribute to viral fitness has recently gained strong support. In the following sections, we highlight evidence for the pro-viral activities of A3G. At the same time, we discuss caveats of experimental systems and data interpretation that must henceforth be considered in development of a revised and better-informed picture of A3G function.

The role of APOBEC3G in generation of anti-viral drug resistant HIV

Gain of resistance to drug(s) used in the treatment of HIV is a major determinant of viral evolution during the course of disease. To date, almost a hundred drug resistance mutation sites have been identified in the HIV genome [110]. These induce resistance to common anti-HIV drugs acting as nucleoside/nucleotide analogue RT inhibitors, such as 2',3'-dideoxy-3'-thiacytidine (3TC), abacavir (ABC), and 2',3'-dideoxyinosine (DDI), as well as non-nucleoside/nucleotide analogue RT inhibitors, including Nevirapine (NVP), Delavirdine (DLV), and Efavirenz (EFV) [110]. Drug resistance mutations function directly by altering drug targets or indirectly by modifying pathways that contribute to drug escape. Many drug resistance mutations have been shown to reside in A3G hotspots [111].

A bioinformatics study assessed the probability of A3G mutations in known drug resistance sites taking into consideration the double-crested gradient of A3G induced mutational levels throughout the HIV genome. Out of 52,000 G to A mutations, only 695 (1.3%) were located in drug resistance sites [112]. In this context, the investigators reported a modest correlation between A3G activity and the generation of drug resistance mutations relative to the overall footprint of A3G on the HIV genome [19,112,113]; however, recent experimental evidence more strongly implicates A3G in the generation of drug resistance mutations. For example, the very common M184I (V) mutation of RT that causes resistance to 3TC and, to a lesser extent, ABC and DDI, is located in an A3G hotspot (TCCAT to TCUAT) and is produced by A3G *in vitro* during HIV replication in cell culture systems [114]. Intriguingly, this was observed in the absence of 3TC in as many as 40% of sequenced proviruses, reflecting a pre-treatment pool of resistant viruses

poised for propagation after drug exposure [29,115–118]. Because this mutation may in fact reduce viral replication fitness in the absence of 3TC [119–122], it is likely that this measurement actually underestimates the role of A3G in the generation of this mutation. In support of this notion, the M184I mutation emerges at significantly higher rates when the virus is grown in A3G-expressing as compared to A3G non-expressing host cells, indicating that A3G activity is the major source of this mutation [123]. This is a striking example of the parallel role of A3G in simultaneously aiding host and virus: in the same manner that it acts as a pre-existing innate immune factor that fortifies host defenses prior to viral exposure, A3G boosts the inherent ability of HIV to gain resistance even before drug treatment. That this mutation is associated with a decline in viral fitness may indicate that drug resistance presents a significant source of pressure in viral evolution resulting in the gain or maintenance of A3G hotspots in key positions in the viral genome.

If the contribution of A3G action to drug resistance and survival of HIV is a biologically considerable one, the evolution of HIV during disease could involve active relaxation of A3G inhibition. Indeed, direct evidence for this phenomenon was provided by the prevalence of the Vif K22H mutation in patients failing drug treatment, as compared to treatment-naïve patients [124,125]. Vif K22 is a key residue for interaction with A3G, and Vif K22H exhibits reduced effectiveness in neutralizing A3G [115]. *Ex vivo* infection of peripheral blood mononuclear cells (PBMCs) with viral stocks harboring various other Vif mutations that are unable to deactivate A3G (e.g. Vif K22E) yielded a significant increase in the generation of M184I mutants [114]. In addition, several drug resistance mutations, including M184I in RT and G16E/M36I in the protease, are significantly more common in patients harbouring elevated relative levels of K22H-

mutated viruses [125]. Like the M184I mutation, both G16E and M36I mutation sites are located in A3G hotspots. Thus, not only does HIV benefit from spontaneous pre-drug treatment A3G induced mutations in a passive, somewhat random manner, it appears that resistance sites for some of the most commonly used drugs arose in A3G hotspots. This in no way implies viral sentience, but merely indicates a selective advantage derived from the overlap of sites more susceptible to mutation (A3G hotspots) being able to confer drug resistance.

The contribution of APOBEC3G to the evasion of adaptive immunity by HIV

Restrictions imposed on the activity of A3G by Vif and HMM limit its effectiveness as an innate immune agent. However, following the first weeks of HIV infection, development of B and T cell mediated adaptive immunity partially controls viremia [116–118,126]. A central facet of the adaptive immune response is elimination of infected target cells by cytotoxic T cells (CTL), as highlighted by the close inverse association between robustness of the CTL response with viremia levels and disease progression [127–129]. Thus, evasion of the CTL response is thought to be a powerful driving force for the evolution of HIV during disease, as confirmed by several studies showing the prevalence of CTL escape in HIV infection [130,131]. CTL evasion may result from alterations in CTL access to infected cells. For instance, the auxiliary HIV protein Nef modulates class I MHC expression to decrease the recognition and killing of infected cells [132]. Alternatively, CTL evasion may result from alterations in the interactions between the CTL and infected target cell. Mutations in CTL recognition

epitopes have been shown to mediate CTL evasion through modulating the efficacy of CTL activation [133–135].

It is possible that HIV can exploit the limited non-lethal action of A3G to generate CTL escape mutants. In support of this model, a study examining CTL escape during early infection found that approximately a third of the rapidly mutating sites mediating CTL escape were embedded in A3G hotspots, with more highly mutating sites being relatively enriched in A3G hotspots [136]. Twenty-four rapidly diversifying sites were identified at which G to A mutations were 2–3 fold more frequent than the overall G to A mutation rate across the entire HIV genome (29 versus 12%). Fourteen of these sites were located in or near CTL epitopes. These data suggest that it may be advantageous towards immune escape for HIV to maintain A3G hotspots in areas where mutations can affect processing, presentation or recognition of T cell epitopes, or conversely to establish T cell epitopes near A3G hotspots.

In contrast, another study reported that A3G mutations enhance the virus-specific CTL response through the introduction of premature stop codons into the HIV genome that cause the generation of truncated or misfolded proteins [137]. In this study, Vif+ or Vif- HIV was produced in the presence or absence of A3G in a cell line and subsequently used to infect PBMCs followed by assessing their susceptibility to MHC-matched peptide specific CTL clones. It is possible that the finding of enhanced target cell killing as a result of A3G activity reflects an inherent bias of the specificity of the CTL clones examined. In addition, given the numbers, diversity, and relative scarcity of CTL specific for each particular peptide in vivo, the general biological relevance of this work remains to be determined. Therefore, although A3G appears to play a role beyond innate

immunity and modulate adaptive immunity, further work is required to elucidate the nature and extent of this activity.

Manipulation of APOBEC3G effectiveness: implications and challenges for the design of therapeutic approaches

To date, multiple avenues have been suggested and/or pursued towards exploitation of A3G as an antiviral therapy. These approaches include the development of small molecules that inhibit the interaction between Vif and A3G and/or inhibit interactions with cellular factors that act downstream of Vif, enhancement of LMM formation over HMM formation, and increasing A3G levels by treatment with interferons or gene-therapy delivery of A3G along with the restriction factor Trim 5α [70,138–140]. Strategies to down-regulate the action of Vif and HMM that were initially suggested as therapeutic approaches have recently been questioned in light of the increasingly apparent pro-viral activities of A3G [35,141]. Disturbing the Vif-APOBEC interaction presents a delicate challenge because subtle adjustments to Vif activity have been shown to modulate levels of A3G activity. For instance, naturally occurring patient-derived virions harbouring Vif mutations selectively exhibit viral genome sequence variations consistent with survival advantage under their environmental pressures [25,54,125]. Incomplete Vif inhibition might increase effective A3G concentrations and in so doing actually accelerate viral evolution by only modestly increasing non-lethal mutation rates [22,111]. On the other hand, complete Vif inhibition may result in A3G activity levels high enough to tip the balance towards immunity through mutation loads capable of disabling viral replication. The isolation of viral sequences harboring Vif mutants significantly

diminished in their ability to neutralize A3G challenges this scenario as it brings into question the ability of A3G to fully abrogate the propagation of Vif-deficient viruses [124,125]. Furthermore, it may also be important to consider the involvement of Vif inactivation in the generation of drug resistance as a cautionary note against therapies designed for complete elimination of Vif activity.

Studies of the effect of A3G expression levels on HIV disease progression rates in both humans and other primates have yielded conflicting results. One investigation reported an inverse correlation between A3G expression levels and disease progression [142,143], while another noted no such association [144]. A third study conducted on SIV-infected rhesus macaques reported an inverse correlation between A3G expression levels and disease progression [145]. Further work will be required to conclusively define any association between A3G expression patterns or levels and HIV disease progression. In addition, it is not clear whether A3G expression levels can influence the relative extent of its pro- versus anti-viral activities.

If indeed there is any correlation, it remains to be determined where the threshold level of A3G activity lies and whether it varies during the course of infection. Whatever the pivotal point, the underlying premise that regulating A3G activity by modulating Vif/HMMs can alter viral mutation levels to the detriment of HIV may be flawed in viewing HIV as an acquiescent canvas for mutational activity. Examination of the spectrum of A3G induced mutations during the viral life cycle paints a different picture in which there is a high level of mutation in viral DNA, an intermediate level in cellular RNA, and a low level in viral RNA. Non-advantageous or detrimental mutations are serially filtered out during the transcription, nuclear-cytoplasmic transport, translation and

assembly phases of the viral life cycle, resulting in a pool of virions emerging from the host cell that bear a suppressed footprint of total A3G mutational activity, a process termed purifying selection [146]. Although at first glance it ought to decrease viral variation, purifying selection is balanced in favour of HIV by other diversification processes, such as recombination between mutated and wild-type viral genomes [147,148]. This is a sophisticated mechanism of protection for the virus as it enhances the potential for beneficial mutations to propagate quickly and represents a heretofore unappreciated layer of complexity when considering therapeutic strategies centered around modulating the activity and/or levels of A3G.

The relative contribution of APOBEC3G in the context of other viral factors to HIV evolution

Formulating therapeutic strategies also requires a careful assessment of the relative contributions of non-A3G factors to the sequence variation of HIV. In general, retroviral genomes are prone to a high frequency of mutation [149–153]. The elevated error rate of the HIV RT, alterations in the nucleoside triphosphate (NTP) and deoxynucleotide triphosphate (dNTP) levels that affect polymerase accuracies, and the lack of proofreading machinery during viral genome replication all contribute to the highly mutagenic nature of viral genomes [58,151]. In addition to mutations, HIV exhibits a notably high rate of genomic recombination amongst retroviruses, possibly due to its cellular transmission properties resulting in frequent co-infection by genetic variants [154–157]. Unlike in humans, recombination in retroviruses does not result from

breakage and rejoining of DNA, but is instead mediated by the ability of RT to switch templates between the two encapsidated proviral RNAs [158–160].

Distinguishing between the actions of RT versus A3G is essential in determining the relative contribution of each to HIV pathogenesis given that its genome is predominated by dA nucleotides, and dG to dA changes are key to the generation of many drug resistance variants [111,161,162]. Prior to the discovery of A3G, RT was viewed as the main generator of genetic diversification in the HIV genome throughout the course of infection; however, both RT and A3G most frequently induce dG to dA transition mutations on the plus viral DNA strand [163]. Although a degree of uncertainty arises in assigning the source of hypermutations in the HIV genome, the fact that A3G preferentially deaminates dC nucleotides in signature trinucleotide hotspots (CCC, TCC) can be used to assign mutations [6,19–21]. In contrast to RT, which is capable of introducing one to two mutations in each viral genome per replication cycle [58], A3G is a highly processive and robust deaminase enzyme [164]. The rate of dG to dA hypermutation found in HIV genomes is approximately 1000 fold higher than RT alone would be expected to introduce [71]. Furthermore, A3G expressing cells support significantly more HIV hypermutation than their A3G-deficient counterparts. While the impact of A3G on mutational load is tempered during wild-type HIV infection by factors such as Vif and HMM, and potentially obscured from the circulating virus pool by purifying selection, these and other observations provide evidence that A3G can and does make substantial contributions to HIV sequence variation [123]. Somewhere between the unfettered A3G activity that causes a lethal mutational load and complete A3G inhibition by Vif and other cellular factors lies a level of activity with the potential to favor drug

resistance, immune escape and viral fitness. Given the demonstrated ability of HIV to adapt to its host, it would be surprising if adaptations deriving benefit from some level of A3G activity have not occurred.

Conclusions

Figure 1 illustrates the various topics discussed herein with respect to the dual role of A3G in aiding the host or virus. As shown, there are clear instances when HIV can take advantage of A3G induced mutation across a range of activity levels. Low mutation rates do not inactivate the viral genome and may in fact contribute to both drug resistance and immune escape. Conversely, HIV genomes suffering high mutation rates may be filtered out during viral replication to favor viral progeny with better fitness. Therefore, an ominous picture emerges wherein regardless of the action of A3G, HIV gradually gains the upper hand as a result of its fast replication rate and purifying selection processes that allow it to essentially optimize A3G mutation loads in progeny virions and better adapt to host defences and other selective pressures.

On the other hand, interpretation of studies examining the effect of A3G on the drug resistance and CTL escape mutations in the HIV genome is subject to a major caveat. To date, studies identifying CTL escape or drug resistance mutations have been conducted using two general approaches: firstly, by searching for such mutations in clinical isolates, and secondly, by analyzing mutations in cell culture infection systems where A3G is expressed. We suggest that these types of studies are inherently biased towards generating the observed results and missing the bigger picture. In the first case, the virus pool obtained from infected individuals will inevitably be enriched for CTL and

drug escape mutants as these have a replication advantage wherein virions harbouring CTL or drug target motifs modified by A3G in a way that supports the opposite outcome (i.e. enhanced CTL recognition or increased drug susceptibility) would have been efficiently eliminated. Therefore, A3G could potentially create new or more immunogenic epitopes or epitopes with increased drug susceptibility that have not yet been characterized. Furthermore, any suggestion that CTL escape or drug resistance is merely serendipitous neglects the point that a limited number of high quality escape and resistant epitopes are selected, as opposed to a large quantity of epitopes with low immune evading potential or substantial negative effects on viral fitness. HIV features a very economical propagation process in that the cost of having some genes manipulated is in-turn compensated for by a net effect favouring evasion of composite selective pressure. In the case of cell culture systems examining the role of A3G in generating drug resistance variants, the same caveat stands. That is, multiple drug resistance mutations have been identified and well characterized due to their prominence in patients. A3G induced mutations that may conversely enhance drug susceptibility have not been identified because of their scarcity caused by more rapid elimination. Accordingly, we suggest that instances where A3G may in fact bestow the upper hand upon the host by generating mutations that enhance CTL recognition or drug susceptibility have likely been underestimated because of their inevitable transience. It is probable that the pro- and anti-viral activities of A3G are not mutually exclusive and that, at different points throughout HIV infection and in different patients, both scenarios unfold. However, the principles of purifying selection are active at the level of individual cells and that of the entire host organism, which buries the evidence of maladaptive A3G imposed mutations

beneath an avalanche of fast replicating adapted variants. While new experimental approaches are required to identify the relative proportion of both categories of A3G induced mutations in an unbiased manner, the final outcome following multiple selection processes will determine the global impact of A3G mutations. Even if thousands of A3G induced mutations favouring the host occur for a single mutation that favours HIV, the net advantage will be to HIV as long as one favourable mutation becomes incorporated into the circulating viral pool. Thus, the overall context within which A3G acts is probably just as relevant as the ratio of pro- versus anti-viral mutations. Resolution of this bigger picture will be critical in order to guide future therapeutic strategies centered on altering A3G activity.


Cytotoxic
CD8+ T-cell
Infected
CD4+ T-cell
drug
more fit HIV
more fit HIV
more fit HIV

Figure 1. The complexities of the pro- and anti-HIV actions of APOBEC3G. Cross section into the cytoplasm of an infected CD4+ T cell is shown, with A3G (yellow) bound to the minus strand ssDNA of the viral genome (white). Virus is shown as green (fit virus) or red (unfit virus) circles forming within and budding out of the infected T cell. Each viral particle contains two copies of the RNA genome and multiple copies of A3G (rods). On the outside of the infected T cell, a cytotoxic CD8+ T cell (CTL) is shown recognizing a viral epitope in the context of MHC class I on the surface of the infected T cell. Arrows depict several possible outcomes of A3G action: (1) the classic mode of A3G action as an innate host defense agent whereby it generates mutations in the viral genome resulting in less fit or deactivated virions (red); (2) some low level mutations by A3G that may result in the production of more fit virions (green); (3) A3G may induce mutations in the viral genome that result in drug resistance, as shown by the emergence of more fit virions (green) through the pool of cytoplasmic drug (yellow dots); (4) the process of purifying selection wherein a heavy mutation load on the viral genome is filtered out throughout various stages in the viral life-cycle, resulting in selection for a final pool of viruses with low level mutations that may enhance viral fitness; (5) the mutations generated by A3G may result in the alteration of MHC class I-restricted viral peptide epitopes such that recognition by CTL is abrogated (A3G-mutated CTL escape epitopes that result in the cloaking of the infected cell from the CTL response are shown in green while wild-type CTL epitopes that result in the recognition and killing of the infected cell are shown in red); (6) the virion infectivity factor (Vif) of HIV (purple) binds cytoplasmic A3G marking it for degradation; and (7) cytoplasmic A3G is trapped in high-molecular-mass (HMM) ribonuclear complexes and consequently rendered ineffective.

Chapter 3

Positioning of APOBEC3G/F mutational hotspots favors reduced recognition of HIV by $CD8^+$ T cells.

Monajemi M, Woodworth C, Zipperlen K, Gallant M, Grant M[#], and Larijani M[#]

Immunology and Infectious Diseases Program, Division of Biomedical Sciences, Faculty of Medicine, Memorial University of Newfoundland

[#]To whom correspondence should be addressed:

Mani Larijani, Division of Biomedical Sciences, Faculty of Medicine, Health Sciences Center, MUN, 300 Prince Phillip Dr., St. John's, NL, Canada A1B 3V6, mlarijani@mun.ca, or Michael Grant, Division of Biomedical Sciences, Faculty of Medicine, Health Sciences Center, MUN, 300 Prince Phillip Dr., St. John's, NL, Canada A1B 3V6, mgrant@mun.ca

Abstract

Through their constitutive expression in cell types targeted by human immunodeficiency virus (HIV) and non-specific editing of DNA, the deoxycytidine deaminates APOBEC3G (A3G) and APOBEC3F (A3F) have been considered agents of innate immunity. Recent bioinformatic and mouse model studies have hinted that HIV mutations mediated by these enzymes can potentially enhance or diminish adaptive immunity. However, their impact on the adaptive immune response within HIV infected individuals has not been evaluated. We studied *ex vivo* activation of $CD8^+$ T cells from HIV-infected individuals by HIV

peptide epitopes bearing A3G/F mutations. We found that A3G/F mutations within epitopes selectively diminished the $CD8^+$ T cell response. This effect was more pronounced for epitopes restricted to HLA alleles associated with slower disease progression (due to their propensity for presentation of immunodominant CTL epitopes). If this observation reflects an appreciable *in vivo* effect, it indicates the adaptation by HIV to enrich A3G/F mutational hotspots in portions of its genome that encode highly immunogenic CTL epitopes would favor viral escape. Indeed, we found the most frequently mutated A3G motif (CCC) is enriched up to 6-fold within viral genomic sequences encoding immunodominant epitopes from Gag, Pol and Nef. Within each gene, A3G/F hotspots are more abundant in sequences encoding epitopes restricted to HLA alleles that present immunodominant epitopes and are common. Thus, A3G/F mutation of HIV mediates CTL escape in a manner more pronounced for the most immunogenic epitopes, as reflected in adaptation of the viral genome.

Introduction:

APOBEC3G (A3G) and APOBEC3F (A3F) mutate deoxycytidine (dC) to deoxyuridine (dU) in the reverse transcribed nascent DNA of human immunodeficiency virus (HIV) [1-5]. These host restriction factors have classically been considered innate immune agents because they act indiscriminately on cytoplasmic single-stranded DNA and are constitutively expressed in $CD4^+$ T lymphocytes, macrophages, and dendritic cells [6-12]. A3G/F deaminate dC in trinucleotide mutational hotspots (CCC and less often TCC for A3G; TTC for A3F) in the minus strand registering as deoxyguanosine

(dG) to deoxyadenosine (dA) substitutions in the plus strand DNA potentially alter reading frames, terminate translation, and produce mutated proteins [13-17].

In the physiological situation, the effectiveness of A3G/F is restricted due to several factors. Firstly, the HIV auxiliary protein viral infectivity factor (Vif) binds and targets A3G/F for degradation [18-21]. Secondly, entrapment of A3G/F in high molecular mass (HMM) ribonuclear complexes inactivates them [22-25]. Thirdly, mutation levels in synthesized virions appear to be actively reduced as highly mutated genomes are eliminated at successive stages of viral replication [26]. Thus, the majority of viral genomes subject to A3G/F activity suffer sublethal mutation loads.

Adaptive responses in the form of anti-viral antibodies and cytotoxic T cells (CTL) follow innate immunity to contain viral propagation [27]. Robust anti-viral CTL responses occur in patients exhibiting slower disease progression and lower viremia [28-33]. Mutations in CTL recognition epitopes of HIV favour immune evasion by reducing CTL activation [34-40]. A3G/F are intrinsic anti-viral barriers that may function early in infection. Once the CTL response escalates to effective levels, A3G/F action may actually aid HIV by mutating its CTL epitopes towards immune evasion [41, 42].

One study found approximately a third of rapidly diversifying regions of HIV mediating CTL escape were embedded in A3G hotspots, with more highly mutating sites being relatively enriched in A3G hotspots [42]. Sequenced viral genomes derived from infected individuals are inherently biased towards displaying escape mutations because those that enhance CTL recognition are eliminated. Therefore, it remains unclear whether A3G/F mutations within CTL epitopes can equally enhance the CTL response. A second study measured the response of MHC-matched CTL clones bearing transgenic peptide-

specific T cell receptors (TCR) to peripheral blood mononuclear cells (PBMCs) infected by HIV produced in A3G-expressing or deficient cells. A3G mutations enhanced the CTL response through the introduction of stop codons resulting in truncated proteins [43]. Although this study indicates that A3G can modulate the CTL response in principle, the use of peptide-specific CTL clones may not reflect the *in vivo* scenario where specific CTLs are diverse and relatively scarce.

We sought to address the role of A3G/F in modulating adaptive immunity in HIV-infected individuals with the hypothesis that low-level mutations by A3G/F modify CTL recognition epitopes *in vivo*. We considered A3G/F mutations in CTL epitopes throughout the HIV genome and measured their effect on recognition by $CD8^+$ T cells from HIV-infected individuals with appropriate HLA. We report that CTL activity is generally diminished by A3G/F-induced mutations and that this effect varies with epitope immunogenicity. *In silico* analysis revealed greatest enrichment of A3G/F hotspot motifs in more immunogenic CTL epitopes. Our data suggest that A3G/F mutations primarily lead to CTL-escape and that HIV has evolved strategically-placed A3G/F hotspots to maximize this effect.

Material and Methods

In Silico demarcation and simulation of A3G/A3F hotspot mutations in CTL epitopes.

The 9229 nucleotide-long, plus-sense strand of HIV isolate BRU was retrieved from the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.ie). Isolate BRU was selected due to its representation of clade B HIV,

which is most prevalent in western countries [44]. Portions of the HIV genome encoding CTL epitopes were identified using the HIV Molecular Immunology Database (http://www.hiv.lanl.gov/content/immunology/tables/ctl_summary.html).A3G/F mutations on the 5' dG hotspots in the CTL epitope-coding genomic DNA were simulated and translated to generate mutated epitopes using DNASTAR software (DNASTAR, Madison, WA). For epitopes with multiple mutational hotspots or those with potential for the generation of novel hotspots, as a result of a first-round mutation on an existing hotspot, multiple mutant (mut) versions were considered. Wild-type (wt) and mutant (mut) forms of all selected epitopes were synthesized and used at 90% purity (Peptide 2.0 Inc., Chantilly, VA).

Measurement of CTL Response

HIV-infected study participants were recruited through the Newfoundland and Labrador Provincial HIV Clinic in St. John's, Newfoundland, and selected on the basis of their HLA class I types. Cryopreserved PBMC served as responder $CD8^+$ T cells from HLA-matched patients. The ELISPOT assay used to measure the frequency of HIV-specific CD8+ T cells was described elsewhere [45]. Briefly, 2×10^5 PBMC and 0.1 μg/ml peptide were added to duplicate wells for each PBMC:peptide combination and the plates were incubated overnight. PBMC were stimulated with 5 μg/mL phytohemagluttinin (MP Biomedical, Solon, OH) as a positive control or incubated in medium alone as a negative control. Plates were incubated for 18-22 hours at 37° C, the cells were removed and 100 μl/well of 1:1000 dilution of biotinylated IFN-γ Ab (Mabtech, Florence, Italy) was added

at room temperature for 2 hours. This was followed by 100 µl/well of 1:1000 dilution of streptavidin-alkaline phosphatase (Mabtech, Florence, Italy). After1 hour incubation, 100 µl/well of chromogenic AP substrate was added for 30 minutes at room temperature. IFN-γ spot forming cells (SFC) were counted the following day using an Immunoscan Reader (Cellular Technology Limited, Shaker Heights, OH). The average of duplicate wells was obtained and the number of background spots in the negative control well subtracted. The total number of SFC in each well was expressed as IFN-γ SFC extrapolated to SFC/10^6 PBMC tested. Even though the background frequency of IFN-producing un-stimulated CTL was negligible (typically < 10 in 10^6 PBMC), we only considered a frequencies of >50 SFC/10^6 PBMC as positive responses. In all, 77 subjects were tested and 27 had positive responses.

***In silico* analysis of A3G/F hotspot frequencies in CTL epitopes**

The number of hotspots (for A3G: GGG, GGT or GGA; for A3F: GAA) inside *vs.* outside CTL epitopes in each HIV gene was determined by scanning the retrieved BRU sequence. The frequencies of hotspot occurrence were normalized to the number of total nucleotides. This frequency was calculated both for all A3G/F hotspots and only for the most frequently targeted A3G hotspot (GGG). Subsequently, the ratio (R) of hotspot frequency inside to outside epitopes was calculated for each gene. In order to evaluate differences in R between various genes, an index was generated by determining the average of the R of all genes and dividing the R of each gene by that average. Thus, an index of >1 indicates an above average ratio of A3G/F hotspots inside to outside CTL

epitopes. We further conducted the same type of analysis within each gene in order to compare hotspot frequencies between epitopes restricted to different HLA alleles (A1, A2, A3, A11, A24, B7, B8, B35, B40, B44, B53, B57).

Statistical Analysis

Bar graphs showing SFC frequencies represent the average of duplicate tests. Frequencies of responding cells are expressed as spot-forming cells (SFC) per million PBMC (after subtracting background spots in negative control wells). ELISPOT responses of HIV-infected individuals that are more than two fold above those of negative control wells are considered as positive responses. Also, a cutoff value of 50 SFC/10^6 PBMC is considered as positive response for ELISPOT assay, which is based on spot frequencies greater than 2 SD above the mean [70].

Results

Demarcation of A3G/F hotspots within genomic sequences of CTL epitopes and simulation of A3G/F-induced mutations

To test the hypothesis that A3G/F-mediated mutations modulate $CD8^+$ T cell CTL recognition in HIV infected individuals, we examined CTL epitopes in all HIV proteins using the experimentally-verified HIV molecular immunology database (http://www.hiv.Lnl.gov/content/immunology/tables/ctl_summary.html) as shown in Figure 1 (see also Supplementary Figures 1 and 2). We analyzed a number of epitopes restricted to HLA-A2, B44, B57 and B35. Strong CTL responses to B57-restricted and to a lesser degree B-35 restricted epitopes correlate inversely with disease progression whilst B35 is

associated with rapid disease progression [46-57]. A2 and B44 are common in the population and present numerous HIV epitopes [28, 58]. We examined CTL epitopes in Gag, Pol and Nef, which comprise the most prominently recognized HIV proteins [59-61]. Of 257 epitopes in the database restricted to these HLA types, the genomic sequences of 123 were identifiable in BRU (Supplementary Figure 2). Of these, 98 epitopes (80%) contained ≥1 A3G/F hotspot in which A3G/F mutations were simulated and translated *in silico*. Epitopes for which the only possible mutation led to a stop codon or non-conservative changes in HLA anchor residues were excluded. Using these criteria, we selected 27 wild-type (wt) epitopes and synthesized 64 derivative mutants there of (Table 1). Of these, 7 contained a single A3G or A3F hotspot yielding 1 mut each (example shown in Figure 2-A). The other 20 yielded multiple muts, either because they contained >1 A3G/F hotspot (multiple hotspots separated by >3 nucleotides and were considered independently; example shown in Figure 2-B) or because from the initial A3G/F mutation a new hotspot arose that could be a A3G/F substrate in the same or subsequent round(s) of viral replication (example shown in Figure 2-C). Considering these possibilities, an epitope can potentially yield a large number of muts. For instance, an epitope containing the hexanucleotide GGGGAA yielded 24 possible muts. The selected wt and mut epitopes thus considered (Table 1) were synthesized and used to evaluate the HIV-specific $CD8^+$ T cell responses of HLA-matched patients by ELISPOT.

A3G/F-simulated mutations diminish CTL recognition of HLA-B57-restricted epitopes

We examined the frequency of IFN-γ producing $CD8^+$ T cells of HLA matched HIV infected individuals against 3 wt epitopes in Gag. Among Gag epitopes restricted to HLA-B57, TSTLQEQIGW and 1 mut were tested in 5 HLA-$B57^+$ subjects (P7, P45, P71, P166, P197). Of these, only P197 responded, showing a 3-fold higher response to wt over the mut (280 *vs.* 85 SFC/10^6), (Figure 3-A). KAFSPEVIP and 1 mut were tested in the same 5 subjects (P7, P45, P71, P166, P197); three (P71, P166, P197) responded to the wt (P71: 397, P166: 50, P197: 90 SFC/10^6), while none responded to the mut (Figure 3-B). AISPRTLNAW and 1 mut were examined in 8 patients (P7, P20, P45, P71, P76, P166, P185, P197), of which 4 (P7, P71, P166, P197) responded; P71 and P197 exhibited responses to wt that were 3.27- and 1.4-fold higher than the mut, respectively (P71: 895 *vs.* 273; P197: 160 *vs.* 112 SFC/10^6 PBMC); P7 and P166 responded only to wt (P7: 815; P166: 386 SFC/10^6 PBMC), (Figure 3-C). The wt Nef epitope GPGVRYPLTFGWCY and 7 muts were tested in 6 patients (P20, P45, P68, P76, P166, P185); only P68 responded and only to the wt (180 SFC/10^6 PBMC), (Figure 3-D). The wt Pol epitope PIVLPEKDSW and 1 mut were tested in 2 patients but did not induce a response. Taken together, 9 patients responded only to wt epitopes while 3 responded to both wt and mut, for which the response to wt epitopes was 1.4- to 3-fold higher.

A3G/F-simulated mutations diminish CTL recognition of HLA-A2-restricted epitopes

We examined 2 types of HLA-A2-restricted epitopes of Gag. Among them, FLGKIWPS and 3 muts were tested in 9 patients (P3, P18, P30, P35, P64, P71, P78, P98, P234), of which 5 responded and only to wt (P3: 152; P64: 88; P71: 60; P78: 85; P98: 142 SFC/10^6), (Figure 4-A); YVDRFYKTL and 1 mut were tested in 10 patients but none responded. 2 Nef epitopes were examined. The immunodominant FLKEKGGLEGL epitope and 15 muts were tested in 30 patients, of which 4 responded to wt (P62: 72; P201: 1145; P211: 1512; P213: 231; P213': 390 SFC/10^6 PBMC); of these, 1 patient responded to a single mutant with a 40% response, relative to that of wt (P211: 1512 *vs.* 910 SFC/10^6 PBMC), (Figure 4-B to E). The Nef epitopes VLEWRFDSRL and PLTFGWCYKLV, each with 1 mut, were tested in 10 and 13 patients respectively, but no response was detected. 9 Pol epitopes were tested and 4 induced a response. IYQYMDDLYV and 1 mut elicited a relatively comparable response; however, unlike most muts that harbor non-conservative alterations, A3G/F action on this epitope induces a conservative M to I mutation. This epitope was tested in 20 different patients, of which 3 responded (for wt *vs.* mut: P35: 147 *vs.* 92; P35': 190 *vs.* 160; P214: 225 *vs.* 90; P233: 570 *vs.* 495 SFC/10^6 PBMC), (Figure 4-F); ILKEPVHGV and 1 mut were tested in 17 patients, out of which 3 responded comparably to wt and mut (for wt *vs.* mut: P35: 2675 *vs.* 2442; P35': 1380 *vs.* 1305; P71: 90 *vs.* 88; P105: 305 *vs.* 405 SFC/10^6 PBMC), (Figure 3-G). LVGPTPVNII with 1 mut were tested in 8 patients, out of which 2 responded only to wt (P117: 62; P214: 300 SFC/1×10^6 PBMC), (Figure 4-H); ALVEICTEM and 1 mut were tested in 11 patients and only elicited a response to wt in 1

(P43: 157 SFC/10^6 PBMC), (Figure 4-I). 5 other Pol epitopes (LLWKGEGAV, VIYQYMDDL, VLVGPTPVNI, LLRWGLTTPDKK and VLDVGDAYFSV) and muts thereof did not produce a response in 38 patients. Altogether, for the 6 A2-restricted epitopes of Gag, Nef and Pol that induced responses, 4 induced a greater response in wt whilst 2 induced a comparable response in wt and muts in all patients. Thus, A3G/F-induced mutations in A2-restricted epitopes diminished the CTL response in 67% and did not affect the response in 33% of cases.

A3G/F-simulated mutations reduce the CTL response to the majority of HLA-B44-restricted epitopes

Two B44-restricted epitopes in Gag were examined. Among them, LSEGATPQDL and 3 muts were tested in 16 patients, of which 5 responded to wt (P35: 4245; P57: 712; P67: 775; P133: 1137; P242: 610 SFC/10^6 PBMC). Amongst these, 2 responded to muts (P35: 3645; P57: 192 SFC/10^6 PBMC to G to R mut), (Figure 5-A); RDYVDRFYKTL and 1 mut were tested in 6 patients but no response was detected. The Nef epitope KEKGGLEGL and 5 muts were tested in 5 patients (P55, P67, P103, P118, P210), 4 of which responded to wt only (P55: 312; P67: 62; P103: 705; P118: 240 SFC/10^6 PBMC), (Figure 5-B). Overall, 9 patients responded to 2 types of epitopes restricted to B44. Of these, 7 responded only to wt whilst 2 responded to both wt and mut and in all cases, the response to wt was greater.

Analysis of A3G/F hotspot frequency inside CTL epitopes

The *ex vivo* reduction of CTL response by A3G/F mutations suggests that HIV would adapt to maximize A3G/F hotspots in CTL epitopes, and that A3G/F hotspots may be more frequent in more immunogenic or broadly presented epitopes. To test this hypothesis, we compared the ratio (R value) of A3G/F hotspot frequency inside to outside CTL epitopes between different HIV genes, after normalizing them for sequence length. We considered the frequencies of all A3G/F hotspots. Because A3G mutates HIV ~10-fold more potently than A3F and GGG is its preferentially targeted hotspot [13, 24, 62, 63], we also considered the frequency of GGG independently (Table 2). Relative enrichment of hotspots inside vs. outside CTL motifs for each gene is defined by an index of the R value of each gene to relative the average R. Considering the index values for all hotspots, as well as GGG alone, Gag, Pol and Nef exhibited the highest indices compared to other genes (for GGG: 1.3, 2.1 and 2.0; for all hostpots: 1.3, 1.4 and 1.1, respectively), (Figure 6-A).

We next addressed the suggestion that enrichment of A3G/F hotspots amongst various epitopes in each gene correlates with immunogenicity as defined by the commonness of its recognition by CTL. We divided the epitopes on the basis of their restricting HLA (A1, A2, A3, A11, A24, B7, B8, B35, B44, B57). For each gene, we quantitated hotspots inside *vs.* outside epitopes restricted to individual HLA alleles. The pattern of hotspot enrichment with respect to HLA-restriction was not consistent amongst genes (Figure 6-b). However, for genes with the most experimentally verified CTL epitopes (Gag, Pol, Env, Nef), the indices for HLA-B57 were >1, with GGG being over-represented by 1.7- to 4.9-fold. In contrast, the frequency of GGG motifs in HLA-B35 is

>2-fold lower than that of other HLA-B alleles. The most striking enrichment was observed in Nef, where all A3G/F hotspots and GGG were respectively 5.7-, 17.6-fold enriched in B44-restricted as compared to B35-restricted epitopes. Nef contains a single B44-restricted epitope that induces robust CTL responses in a broad portion of the population [28]. We noted that its coding sequence contains a GAA tract, a GGGGGA tract, a GGAA tract and a GGA tract, providing 9 hotspots for first round mutations by A3G/F and >20 muts considering multiple replication rounds. We also noted a higher frequency of GGG motifs in A3-restricted epitopes of Gag and Pol (2.1- to 2.3-fold enriched over epitopes restricted to other HLAs). These results may reflect the higher number of CTL epitopes in these gene segments in combination with the prevalence of the A3 allele in the population.

In order to test if there is selective A3G/F hotspot enrichment within CTL epitopes, we considered antibody recognition epitopes within Env as a control. We compared the R values of A3G/F hotspots inside to outside CTL epitopes of each gene to the R value of hotspots inside to outside antibody recognition epitopes in Env. The R value for antibody epitopes in Env is 0.71 and 0.9 for all A3G/F hotspots and GGG, respectively. These are 1.3- to 3-fold lower than the R values for CTL epitopes of Pol (1.06, 1.63), Gag (1.16, 2.60) and Nef (0.89, 2.45), but up to 7-fold higher than the R values found in the CTL motifs of other HIV genes (Vif, Vpr, Tat, Rev,Vpu). The average R value of GGG inside to outside CTL epitopes in all genes is 2-fold higher than its counterpart for Ab epitopes across all genes (Figure 6-c). Collectively, this analysis reveals two levels of enrichment in A3G/F hotspots that is of significant for adaptive immunity: first, enrichment within CTL epitopes of Gag, Pol and Nef, and second,

relative enrichment in epitopes within each gene restricted to HLAs that present more immunogenic epitopes at an individual or population level.

Discussion

The character of the anti-HIV CTL response plays a key role in controlling viremia as evidenced by the fact that susceptibility to disease progression is associated with variations in an HIV-infected individual's class I HLA genotype [46, 48, 64-67]. A3G induced mutations that generate truncated peptides have the potential to improve the CTL response against HIV-infected cells *in vitro* [43]. In contrast, another study proposed that A3G hotspots in CTL escape sites can result in the reduction of CTL response [42]. Our research indicates that A3G/F may in fact benefit HIV by contributing to drug resistance and other contextual fitness-inducing mutations [41, 68]. We reasoned that the role of A3G/F in the CTL response is also likely to include both pro- and anti-viral outcomes at different times during HIV infection. We hypothesized that A3G/F may be able to mediate mutations in CTL epitopes that strengthen immune recognition, but that this activity goes unobserved due to the rapid elimination of viral genomes harboring these mutations. Instead, this anti-viral role of A3G/F may reveal itself by examining patients' CTL response to A3G/F-mutated CTL epitopes, as some CTL would have likely differentiated into rare, but lingering memory cells. We show that A3G/F mutations can indeed modulate adaptive immunity in HIV-infected individuals by decreasing CD8+ T cell recognition of common CTL epitopes. The response of $CD8^+$ T cells from HLA-matched HIV-infected individuals was almost always reduced as a result of A3G/F mutations in epitopes restricted to A2, B44, and B57 HLA alleles. In order to ascertain

whether this trend was a product of our experimental procedures, we tested several epitopes and muts thereof that are restricted to HLA-B35. Interestingly, we found that for HLA-B35, which associates with rapid disease progression, A3G/F mutated epitopes enhanced the CTL response (Supplementary Figure 3).

Previous studies of the $CD8^+$ T cell response in HIV-infected cohorts to epitope variants adapted at the population level found that more than half of the muts retained their ability to stimulate $CD8^+$ T cells [29]. Because some of the epitopes we tested were hypothetical variants, our observation that A3G/F-induced mutations specifically alter CTL epitopes so as to diminish CTL recognition is unlikely to be due entirely to simulating mutations *in silico*. Instead, our observations can be explained by the fact that the majority of A3G/F-induced mutations are non-conservative and are thus likely to change the epitope conformation. Alternatively, CTL recognition of A3G/F hotspot-containing epitopes may be less tolerant of mutations compared to other epitopes. Either possibility suggests that the viral genome has evolved to place and/or maintain A3G/F hotpots in positions that exploit A3G/F activity to escape CTL. This is also supported by our observation of A3G/F hotspot enrichment inside genomic sequences that encode CTL epitopes. Within each HIV gene, A3G/F hotspots were enriched in sequences encoding epitopes restricted to common HLA alleles or HLA alleles that present more immunogenic epitopes and were conversely underrepresented in HLA alleles that do not present immunodominant epitopes. This finding further supports a model where the CTL response at the population level is an appreciable force in directing the evolution of A3G/F hotspot placement throughout the viral genome. Indeed, had we observed the opposite, that A3G/F-mutated epitopes elicit a higher or even similar CTL response in

immunogenic epitopes, it would have suggested that the viral genome lacks sufficient plasticity to adapt to utilize A3G/F to escape CTL, or that the CTL response is not a crucial determinant of viral genome evolution.

Several non-mutually exclusive mechanisms can explain the reduction of CTL response as a result of A3G/F mutations. First, it may be caused by decreased availability of mut epitopes due to elimination of viral genomes harboring A3G/F mutations or the ineffectiveness of A3G/F action *in vivo.* Second, it is possible that some simulated A3G/F mutations did not occur in tested patients or that they were short-lived because they were rapidly replaced by other mutations that increased fitness in the face of stronger non-CTL pressures (e.g. drug treatment). These possibilities are counteracted by frequent detection of mutations at A3G/F hotspots within sequenced viral genomes from HIV-infected individuals. Even if the occurrence of the mutation elicited a response in as low as ~1 in 10,000 $CD8^+$ T cells, it should be detectable by ELISPOT. Furthermore, our observation that A3G/F muts enhanced the response to several epitopes (e.g. B-35 restricted epitopes) argues against these being the sole mechanisms. Third, it is possible that there are in fact CTL that would respond to the mut epitope, but that their frequency is below the limit of detection by ELISPOT (~ 1 in 10,000 CTL). If this is the case, a revised assay including longer incubations and different culture conditions that stimulate for the expansion of these rare CTLs should reveal responder CTL specific for muts. Finally, it is possible that our observations are not due to the lack of A3G/F-mutated epitopes or responder CTL, but that indeed A3G/F mutates epitopes with the specific outcome of diminishing the CTL response. Several lines of evidence support this model. First, we excluded A3G/F mutations that may affect HLA-binding anchor residues in epitopes so that our

observations cannot be explained entirely by failure of epitope presentation. Second, we showed that it is possible for A3G/F mutation to enhance CTL recognition in our experimental system. Third, our *in silico* analysis has revealed selective enrichment of A3G/F hotspots in immunogenic CTL with a HLA association, suggesting that our observation is reflective of an *in vivo* effect that is sufficiently relevant to have left its evolutionary footprint on the viral genome.

The molecular basis for our results may be that A3G/F mutations decrease the affinity between the TCR/epitope/MHC complex. Alternatively, a distinct CTL pool may recognize mut epitopes less avidly. Whatever the case may be, our results show that A3G/F mutations can play a role in adaptive immunity against HIV by diminishing the CTL response. This contrasts a recent report that A3G mutations promote the CTL response through introducing premature stop codons leading to the generation of truncated proteins [43]. Although this study demonstrated the potential of A3G/F to generate more immunogenic epitopes through an indirect mechanism, we report here that the opposite occurs in HIV-infected individuals thorough the direct action of A3G/F on $CD8^+$ T cell epitopes. In said study, an *in vitro* infection system was utilized with Vif+ or Vif- virus and CTL clones bearing transgenic receptors, while our study probed the CTL response of PBMC obtained from HIV infected individuals. Our results may be more reflective of the physiological setting that accounts for the diversity of individual patients with respect to differences in HLA make up, infection and drug treatment stages.

A recent study shows that the generation of "neo-epitopes", possibly to act as CTL decoys, is a strategy for viral escape from the host immune system [69]. Nef has been shown to frequently deploy decoy epitopes, possibly because it is less important for the

viral life cycle and hence more tolerant of mutations. HLA-A24 is a frequent presenter of these decoy epitopes and we note that interestingly, the high response to A3G/F-mutated B35 restricted epitopes was observed in patients who also carried the A24 allele. Thus, it is possible that mutations in B35-restricted epitopes enhance their immunogenicity when presented by A24. Alternatively, since CTL responses to B35-restriced epitopes have been shown to not be as effective as responses to epitopes presented by B57 and other HLAs in combating viral loads, our observation may represent a role for A3G/F in the generation of decoy epitopes, in addition to its role in suppressing the recognition of immunodominant epitopes. Further work is required to shed light on the full role of A3G/F on the CTL response, as well as on the molecular and cellular pathways through which these DNA-mutating enzymes influence adaptive immunity.

Acknowledgments

This work was supported by a CIHR operating grant (MOP111132) to ML, CIHR operating grants (HOP-111725, HOP-93428) and catalyst grant (HC1-112568) to MG, the Research and Development Corporation (Newfoundland), Wallace Ingram Operating Award (Memorial University). ML is supported by a CIHR New Investigator award.

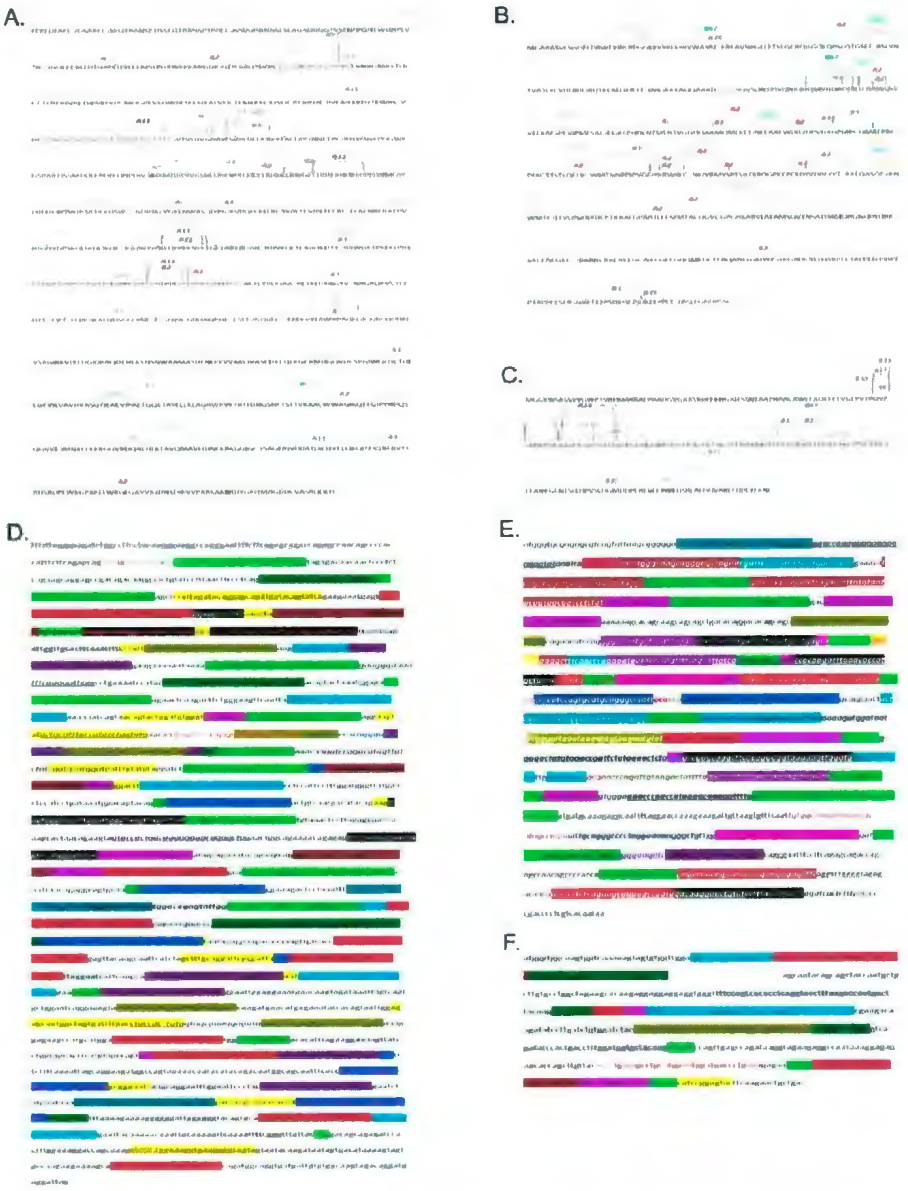


Figure 1. Delineation of the amino acid and DNA sequences of CTL epitopes. Panels A, B and C show the locations of CTL epitopes in the Pol, Gag and Nef polypeptides respectively of HIV-1 BRU isolate. The brackets indicate epitopes on the peptide sequence. Epitopes restricted to different HLA alleles are shown in different colors and the restricting HLA is indicated above the bracket. Broken lines display epitopes with no A3G/F hotspots and continuous lines show epitopes that harbor A3G/F hotspots. Panels D, E and F show the location of the viral genomic sequences that encode CTL epitopes in Pol, Gag and Nef, respectively in the HIV-1 BRU isolate plus-sense strand. Color delineates the sequences encoding CTL epitopes from the surrounding DNA. Colors do not otherwise correspond to any common features amongst genes or epitopes.

Figure 2. Simulation of A3G/F-mediated mutations in CTL epitopes. Wild type epitopes and their encoding DNA sequence are shown. The A3G/F targeting hotspots in the plus-sense epitope encoding sequence are colored blue and underlined. The simulated A3G/F-mediated G to A mutation in the viral genomic sequence and the resulting amino acid mutation in the CTL epitope are shown below in red. **A.** Example of an epitope with a single possible mutation. **B.** Example of an epitope with multiple independent mutations. Hotspots separated by >3 nucleotides were considered as independent and mutant epitopes bearing either mutation or combinations of multiple mutations were considered. **C.** Example of an epitope with multiple sequential mutations. For hotspots where an initial A3G/F-mediated mutation can generate a new A3G/F hotspot that can be acted upon in the same or a subsequent viral replication cycle, mutant epitopes bearing each individual mutation or combinations of mutations were considered.

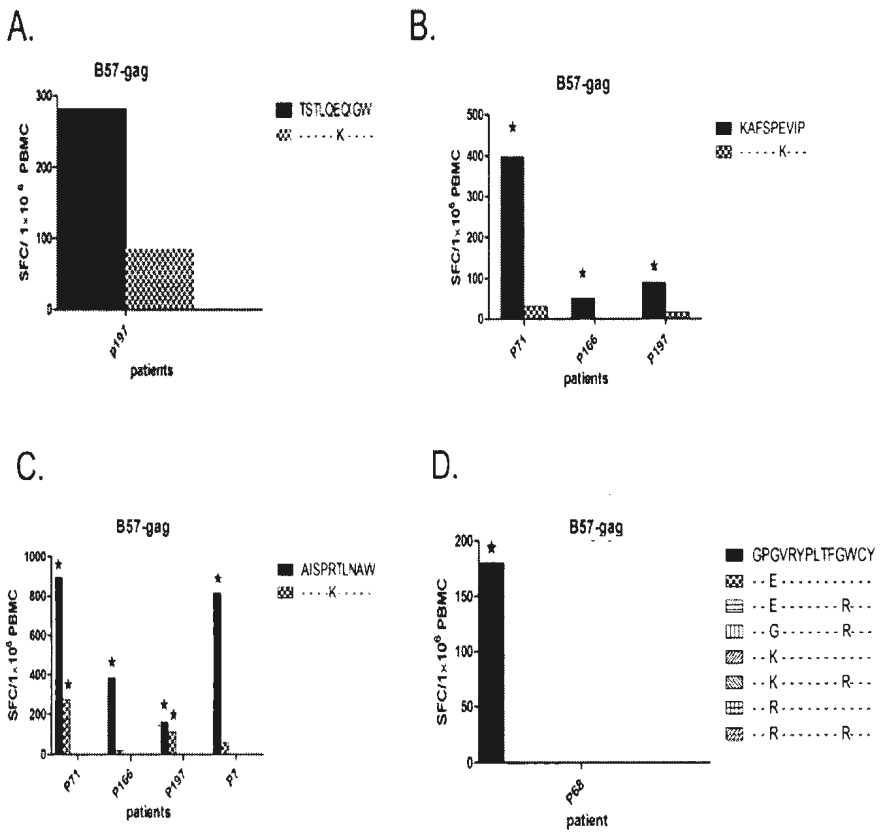


Figure 3. The response of CTL from different HIV-infected individuals to HLA-B57-restricted wild type and A3G/F-mutated epitopes. HIV-infected individual PBMC response to wild type and mutant epitopes are shown on the Y-axis, as SFC (Spot Forming Cell) /10^6 PBMC. X-axis displays different HIV infected individuals (patients). **A.** Induction of IFN-ɣ is compared between wild type and a mutant form of the B57-restriced Gag epitope TSTLQEQIGW in P197. **B.** Induction of IFN-ɣ is compared between wild type and 2 mutant forms of the B57-restriced Gag epitope KAFSPEVIP in P71, P166, P197. **C.** Induction of IFN-ɣ is compared between wild type and a mutant form of the B57-restriced Gag epitope AISPRTLNAW in P71, P166, P197, P7. **D.** Induction of IFN-ɣ is compared between wild type and 7 mutant forms of the B57 restricted Nef epitope GPGVRYPLTFGWCY in P68. Asterisk indicates the responses greater than 50 SFC/10^6 PBMC.

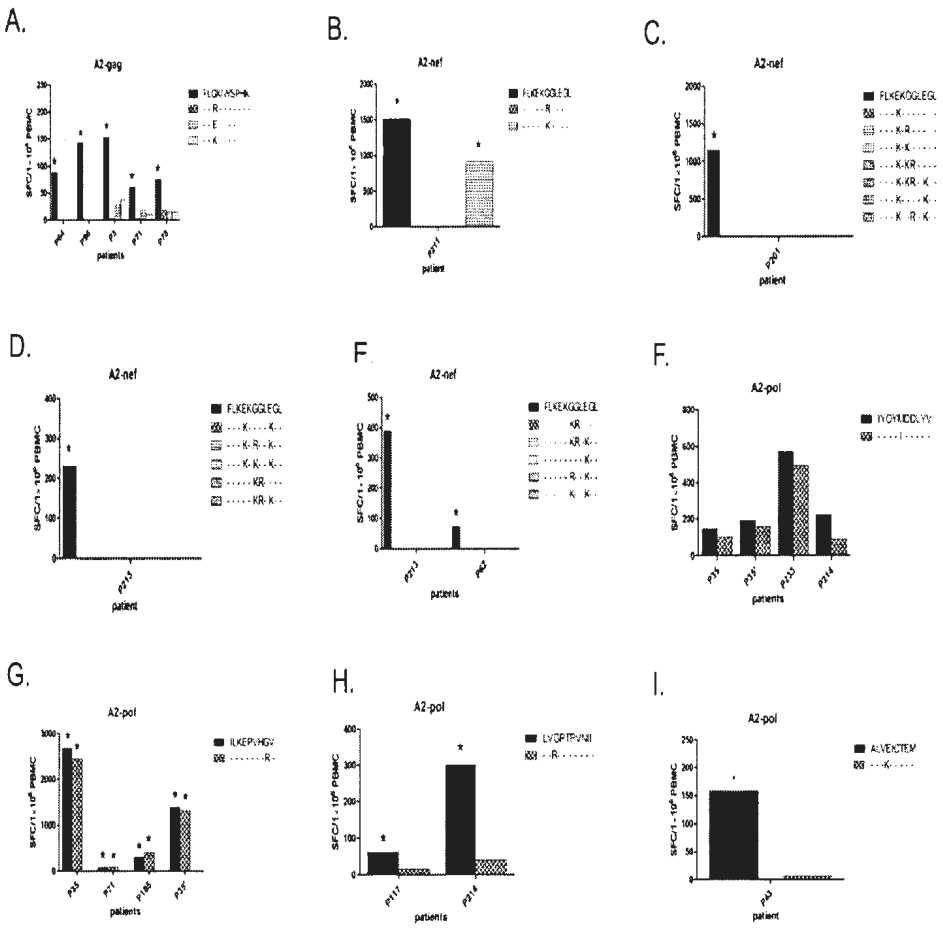


Figure 4. The response of CTL from different HIV infected individuals to HLA-A2-restricted wild type and A3G/F-mutated epitopes. HLA-matched, HIV-infected individuals PBMC response to wild type and mutant epitopes are shown on the Y-axis, as SFC (Spot Forming Cell) /10^6 PBMC. X-axis displays different HIV-infected individuals (patients). **A.** Induction of IFN-ɣ is compared between wild type and 3 mutant forms of the HLA-A2 restricted Gag epitopes FLGKIWSPHK in P64, P98, P3, P71, P78. **B-E.** Induction of IFN-ɣ is compared between wild type and 15 mutant forms of the HLA-A2 restricted Nef epitope FLKEKGGLEGL in P62, P201, P211, P213. **F.** Induction of IFN-ɣ is compared between wild type and a mutant form of the HLA-A2 restricted Pol epitope IYQYMDDLYV in P35, P214, P233. **G.** Induction of IFN-ɣ is compared between wild type and a mutant form of the HLA-A2 restricted Pol epitope ILKEPVHGV in P35, P71, P105. **H.** Induction of IFN-ɣ is compared between wild type and a mutant form of the HLA-A2 restricted Pol epitope LVGPTPVNII in P117, P214. **I.** Induction of IFN-ɣ is compared between wild type and a mutant form of the HLA-A2 restricted Pol epitope ALVEICTEM in P43. Asterisk indicates the responses greater than 50 SFC/10^6 PBMC.

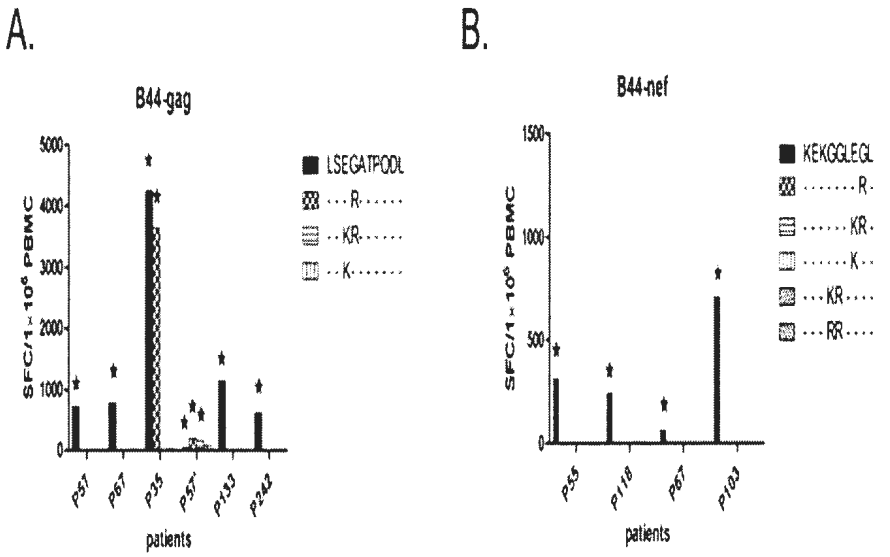


Figure 5. The response of CTL from different HIV infected individuals to HLA-B44-restricted wild type and A3G/F-mutated epitopes. HLA matched, HIV-infected individuals PBMC response to wild type and mutant epitopes are shown on Y-axis, as SFC (Spot Forming Cell)/10^6 PBMC. X-axis displays different HIV-infected individuals (patients). **A.** Induction of IFN-ɣ is compared between wild type and 3 mutant forms of the HLA-B44 restricted Gag epitope LSEGATPQDL in P35, P57, P67, P133, P242 . **B.** Induction of IFN-ɣ is compared between wild type and 6 mutant forms of the HLA-B44 restricted Nef epitope KEKGGLEGL in P55, P67, P103, P118. A response greater than 50 SFC/10^6 PBMC is indicated by *.

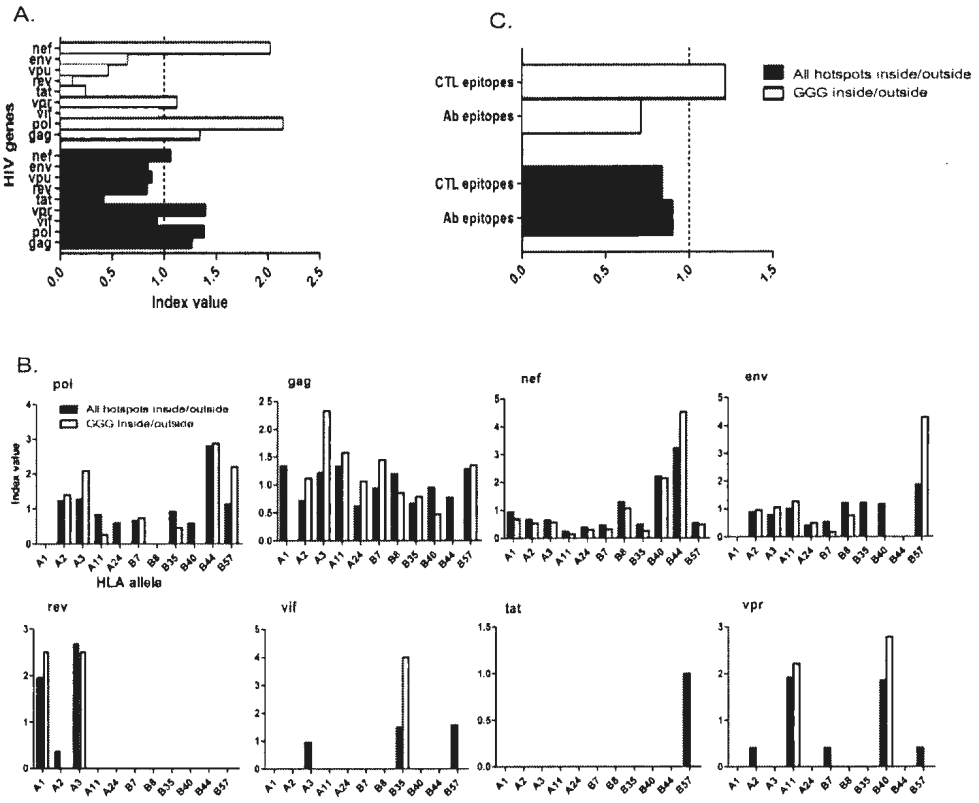


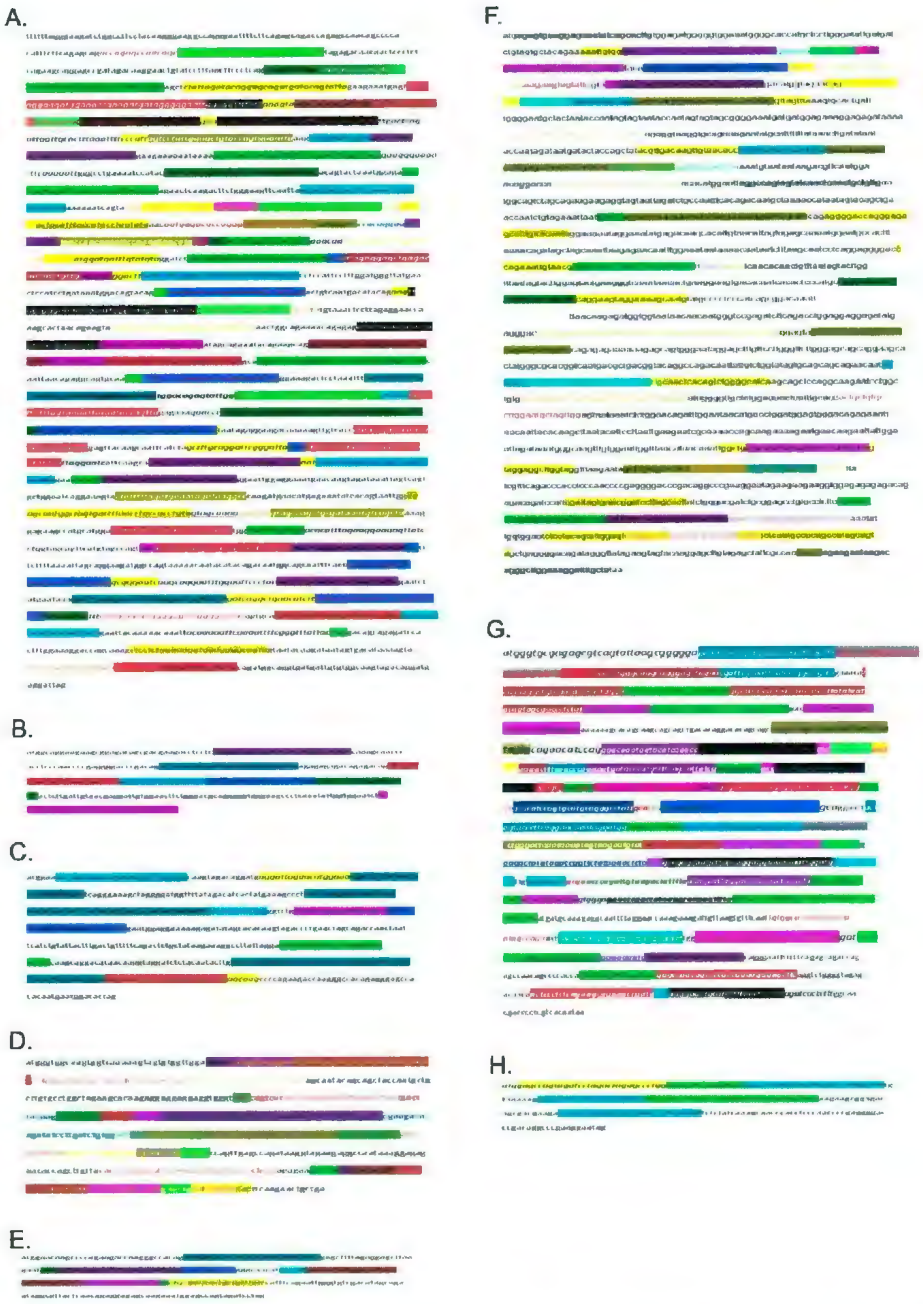
Figure 6. Analyzing enrichment of A3G/F hotspot inside/outside CTL epitopes in HIV genes. A. For each HIV gene, the frequency of A3G/F hotspots (either all hotspots: GGG, GGA, GGT, GAA, or GGG alone) was calculated in regions that encode for CTL epitopes and regions that do not encode for CTL epitopes. Normalized frequencies were calculated by taking into account the total length of the sequence. For each HIV gene, the normalized frequency of A3G/F hotspots inside sequences that encode CTL motifs was divided by the normalized frequency of A3G/F hotspots in sequences that do not encode CTL epitopes to generate a Ratio (R-value). The average of R-values was determined and each R-value was divided by the average to obtain the index value and allow for determination of the relative enrichment of hotspots within the CTL epitopes of each gene, as compared to the average ratio of hotspots inside to outside CTL epitopes. **B.** The same analysis as panel A was conducted within each HIV gene to compare the relative enrichment of A3G/F hotspots in sequences that encode CTL epitopes restricted to different HLA alleles. **C.** Comparison of A3G/F enrichment in CTL epitopes *vs.* the portions of the Env gene that encode antibody recognition epitopes. Average R-value for the CTL epitopes of all genes (average of all R values from panel A) was compared to the R value of A3G/F hotspots inside:outside sequences encoding antibody recognition motifs within Env. The Index value=1 is shown as a scale, since an index >1 indicates relative enrichment.

Table 1. List of wild type and A3G/F-mutated epitopes used to measure the CTL response of HLA-matched, HIV-infected individuals. Wild type epitopes and their A3G/F-mutated versions used to test the CTL response of HLA-matched, HIV-infected individuals by ELISPOT are shown. The polypeptide of origin for each epitope, its restricting HLA allele and the viral genomic sequence encoding the epitope in HIV-1 BRU isolate are shown.

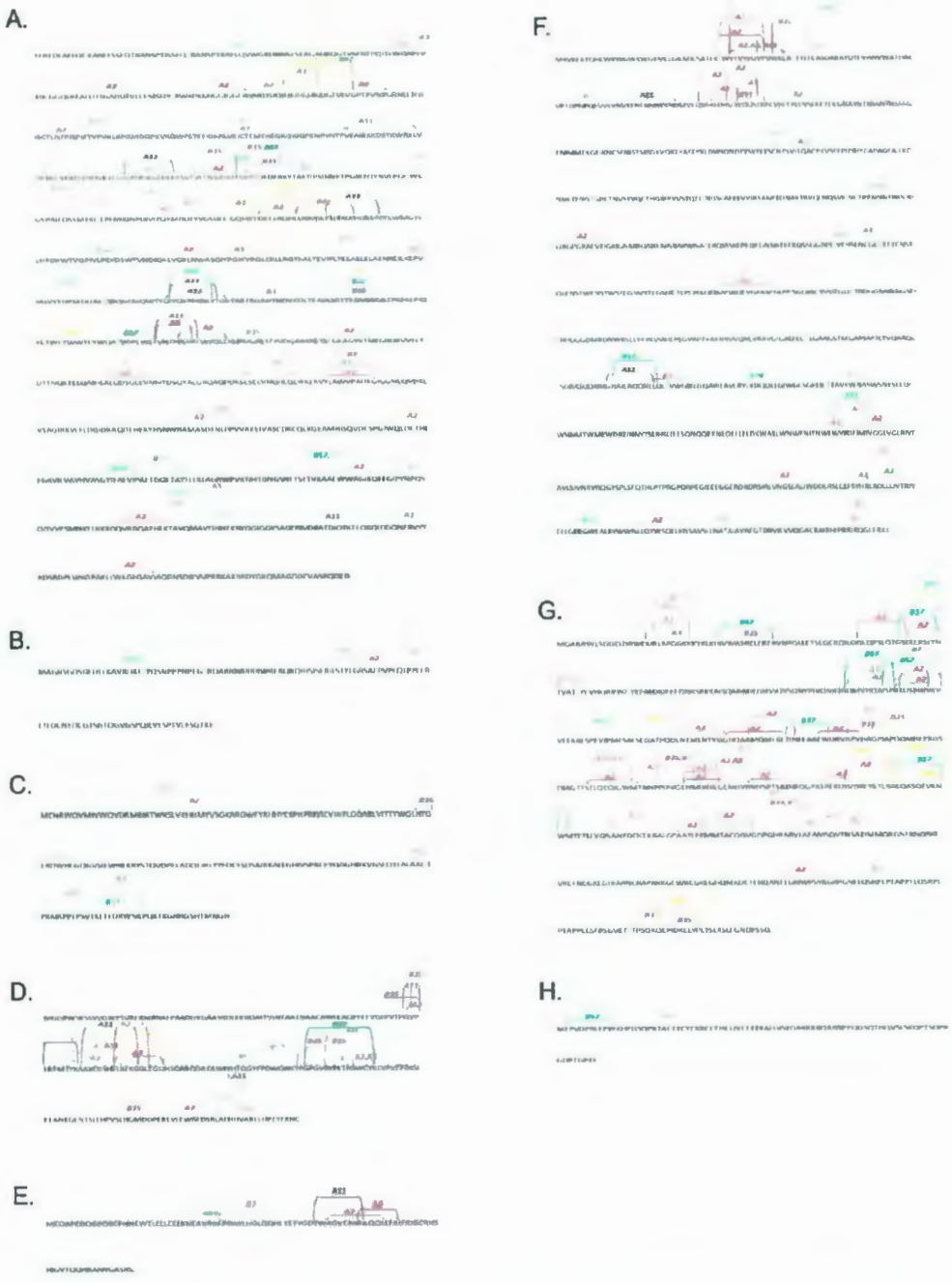
Epitope	Wt/Mut	HLA	Gene	DNA sequence
AISPRTLNAW	Wt	B57	Gag	gccatatcacctagaacttaaatgccatgg
AISPKTLNAW	Mut	B57	Gag	gccatatcacctaaaacttaaatgccatgg
KAFSPEVIP	Wt	B57	Gag	aaggctttcagcccagaagtgatacccc
KAFSPKVIP	Mut	B57	Gag	aaggctttcagcccaaaagtgatacccc
TSTLQEQIGW	Wt	B57	Gag	actagtacccttcaggaaccaaatagaatgg
TSTLQKQIGW	Mut	B57	Gag	actagtacccttcaaaaaccaaatagaatgg
GPGVRYPLTFGWCY	Wt	B57	Nef	ggggcaggggtcagatatccactgaccttggatggctgctac
GPEVRYPLTFGWCY	Mut	B57	Nef	gaggccagaggtcagatatccactgaccttggatggctgctac
GPEVRYPLTFRWCY	Mut	B57	Nef	ggaggccagaggtcagatatccactgaccttggatggctgctac
GPGVRYPLTFRWCY	Mut	B57	Nef	gaaggccagaggtcagatatccactgaccttggatggctgctac
GPKVRYPLTFGWCY	Mut	B57	Nef	gaggccaaaagtcagatatccactgaccttggatggctgctac
GPKVRYPLTFRWCY	Mut	B57	Nef	gaggccaaaagtcagatatccactgaccttgaatggctgctac
GPRVRYPLTFGWCY	Mut	B57	Nef	gaggccaagggtcagatatccactgaccttggatggctgctac
GPRVRYPLTFRWCY	Mut	B57	Nef	gaggccaagggtcagatatccactgaccttgaatggctgctac
PIVLPEKDSW	Wt	B57	Pol	cctatagtgctgccagaaaaaagacagctgg
PIVLPKKDSW	Mut	B57	Pol	cctatagtgctgccaaaaaaaagacagctgg
FLGKIWPSHK	Wt	A2	Gag	tttttaggaaagatctggccttcctacaagaag
FLRKIWPSHK	Mut	A2	Gag	tttttaaggaagatctggccttcctacaaggg
FLEKIWPSHK	Mut	A2	Gag	tttttaggaaagatctggccttcctacaag
FLKKIWPSHK	Mut	A2	Gag	tttttaaaaaagatctggccttcctacaaggg
YVDRFYKTL	Wt	A2	Gag	tatgtagaccgattctatagaaactcta
YVDQFYKTL	Mut	A2	Gag	tatgtagaccgattctatagaaactcta
PLTFGWCYKLV	Wt	A2	Nef	ccactgacctttggatggtgctacaagctagtg
PLTFRWCYKLV	Mut	A2	Nef	ccactgacctttgaatggtgctacaagctagtg
VLEWRFDSRL	Wt	A2	Nef	gtgttgaggtgggaggtttgacagccgccta
VLEWKFDSRL	Mut	A2	Nef	gtgttgaggtgaaaggtttgacagccgccta
FLKEKGGLEGL	Wt	A2	Nef	tttttaaagaaaaggggggactggaagggcta
FLKEKRGLEGL	Mut	A2	Nef	tttttaaagaaaaaaggggactggaagggcta
FLKEKKGLEGL	Mut	A2	Nef	tttttaaaagaaaaaaggggactggaagggcta
FLKKKGGLEGL	Mut	A2	Nef	tttttaaaaaaaaaggggggactggaagggcta
FLKKKRGLEGL	Mut	A2	Nef	tttttaaaaaaaaaaggggactggaagggcta
FLKKKKGLEGL	Mut	A2	Nef	tttttaaaaaaaaaaaggggactggaagggcta
FLKKKRRLEGL	Mut	A2	Nef	tttttaaaaaaaaaaaggggactggaagggcta
FLKKKRRLKGL	Mut	A2	Nef	tttttaaaaaaaaaaaggggactaaaagggcta
FLKKKGGLKGL	Mut	A2	Nef	tttttaaaaaaaaaaggggactggaagggcta
FLKKKRGLKGL	Mut	A2	Nef	tttttaaaaaaaaaaggggactaaaagggcta
FLKKKKGLKGL	Mut	A2	Nef	tttttaaaaaaaaaaggggactaaaagggcta
FLKEKKRLEGL	Mut	A2	Nef	tttttaaaagaaaaaaggggactggaagggcta
FLKEKKRLKGL	Mut	A2	Nef	tttttaaaagaaaaaaggggactaaaagggcta
FLKEKGGLKGL	Mut	A2	Nef	tttttaaaagaaaaaaggggactaaaagggcta
FLKEKRGLKGL	Mut	A2	Nef	tttttaaaagaaaaaaggggactaaaagggcta
FLKEKKGLKGL	Mut	A2	Nef	tttttaaaagaaaaaaggggactaaaagggcta
ALVEICTEM	Wt	A2	Pol	gcattagtagaaatttgtacagaaaatgga
ALVKICTEM	Mut	A2	Pol	gcattagtaaaaatttgtacagaaatgga
LLWKGEGAV	Wt	A2	Pol	ctcctctggaaaggtgaaggggcagta
LLWKSEGAV	Mut	A2	Pol	ctcctctggaaaagtgaaggggcagta
LLWKSERAV	Mut	A2	Pol	ctcctctggaaaagtgaaaggaccagta
LLWKSEEAV	Mut	A2	Pol	ctcctctggaaaagtgaagaggcagta
ILKEPVHGV	Wt	A2	Pol	attctaaagaaccagtacatggagtgtat
ILKEPVHRV	Mut	A2	Pol	attctaaagaaaccagtacatagagtgtat
VIYQYMDDL	Wt	A2	Pol	gttatctatcaatacatggatgatttg
VIYQYIDDL	Mut	A2	Pol	gttatctatcaatacatagatgatttg
VLVGPTPVNI	Wt	A2	Pol	gtattagtaggacctacacctgtcaacata
VLVRPTPVNI	Mut	A2	Pol	gtattagtaggacctacacctgtcaacata
IYQYMDDLYV	Wt	A2	Pol	atctatcaatacatggatgatttgtatgta
IYQYIDDLYV	Mut	A2	Pol	atctatcaatacatagatgatttgtatgta
LLRWGLTTPDKK	Wt	A2	Pol	ctgttaggtggggacttaccacaccagacaaaaaa
LLKWGLTTPDKK	Mut	A2	Pol	ctgttaggtggggacttaccacaccagacaaaaaa
LVGPTPVNII	Wt	A2	Pol	ttagtaggacctacacctgtcaacataatt
LVRPTPVNII	Mut	A2	Pol	ttagtaggacctacacctgtcaacataatt
VLDVGDAYFSV	Wt	A2	Pol	gtactggatgtgggtgatgcatatttttcagtt
VLDVSDAYFSV	Mut	A2	Pol	gtactggatgtaagtgatgcatatttttcagtt
LSEGATPQDL	Wt	B44	Gag	ttatcagaaggagccaccccacaagattta
LSERATPQDL	Mut	B44	Gag	ttatcagaaagagccaccccacaagattta
LSKRATPQDL	Mut	B44	Gag	ttatcaaaaagagccaccccacaagattta
LSKGATPQDL	Mut	B44	Gag	ttatcaaaggagccaccccacaagattta
RDYVDRFYKTL	Wt	B44	Gag	agagactattgtagaccgattctatagaaactcta
RDYVDQFYKTL	Mut	B44	Gag	agagactatgtagaccagttctatagaaactcta
KEKGGLEGL	Wt	B44	Nef	aaagaaaaggggggactggaagggcta
KEKGGLERL	Mut	B44	Nef	aaagaaaaggggggactggaaagggcta
KEKGGLKRL	Mut	B44	Nef	aaagaaaaggggggactaaaagggcta
KEKGGLKGL	Mut	B44	Nef	aaagaaaaggggggactggaaagggcta
KEKKRLEGL	Mut	B44	Nef	aaagaaaaaaggggactggaagggcta
KEKRRLEGL	Mut	B44	Nef	aaagaaaaggaggaggactggaagggcta
PPIPVGEIY	Wt	B35	Gag	ccacctatcccagtaggagaaatttat
PPIPVREIY	Mut	B35	Gag	ccacctatcccagtaggagaaatttat
PPIPVRKIY	Mut	B35	Gag	ccacctatcccagtaagaaaaatttat
PPIPVKKIY	Mut	B35	Gag	ccacctatcccagtaaaaaaaatttat
PPIPVGKIY	Mut	B35	Gag	ccacctatcccagtaggaaaaatttat
PPIPVEKIY	Mut	B35	Gag	ccacctatcccagtaggaaaaatttat
TVLDVGDAY	Wt	B35	Pol	acagtactggatgtgggtgatgcatat
TVLDVSDAY	Mut	B35	Pol	acagtactggatgtaagtgatgcatat
GPGVRYPLTF	Wt	B35	Nef	gaggccaggggtcagatatccactgaccttt
GPRVRYPLTF	Mut	B35	Nef	gaggccaagggtcagatatccactgaccttt
GPEVRYPLTF	Mut	B35	Nef	gaggccagaggtcagatatccactgaccttt
GPKVRYPLTF	Mut	B35	Nef	gaggccaaaggtcagatatccactgaccttt
YPLTFGWCY	Wt	B35	Nef	tatccactgaccttggatggctgctac
YPLTFRWCY	Mut	B35	Nef	tatccactgaccttgaatggctgctac
VPLDEDFRKY	Wt	B35	Pol	gttccttagatgaagacttcaggaagtat
VPLDKDFRKY	Mut	B35	Pol	gttccttagatgaagacttcaggaagtat
VPLDEDFKKY	Mut	B35	Pol	gttccttagatgaagacttcaaaaagtat
VPLDKDFKKY	Mut	B35	Pol	gttccttagatgaagacttcaaaaagtat

Table 2. Analyzing enrichment of A3G/F hotspot inside/outside CTL epitopes in HIV genes. For each HIV gene, the frequency of A3G/F hotspots (either all hotspots: GGG, GGA, GGT, GAA, or GGG alone) was calculated in regions that encode for CTL epitopes and regions that do not encode for CTL epitopes. Normalized frequencies were calculated by taking into account the total length of the sequence. For each HIV gene, the normalized frequency of A3G/F hotspots inside sequences that encode CTL motifs was divided by the normalized frequency of A3G/F hotspots in sequences that do not encode CTL epitopes to generate a Ratio (R-value). The average of R-values was determined and each R-value was divided by the average to obtain the index value and allow for determination of the relative enrichment of hotspots within the CTL epitopes of each gene, as compared to the average ratio of hotspots inside to outside CTL epitopes. An index value >1 indicates relative enrichment.

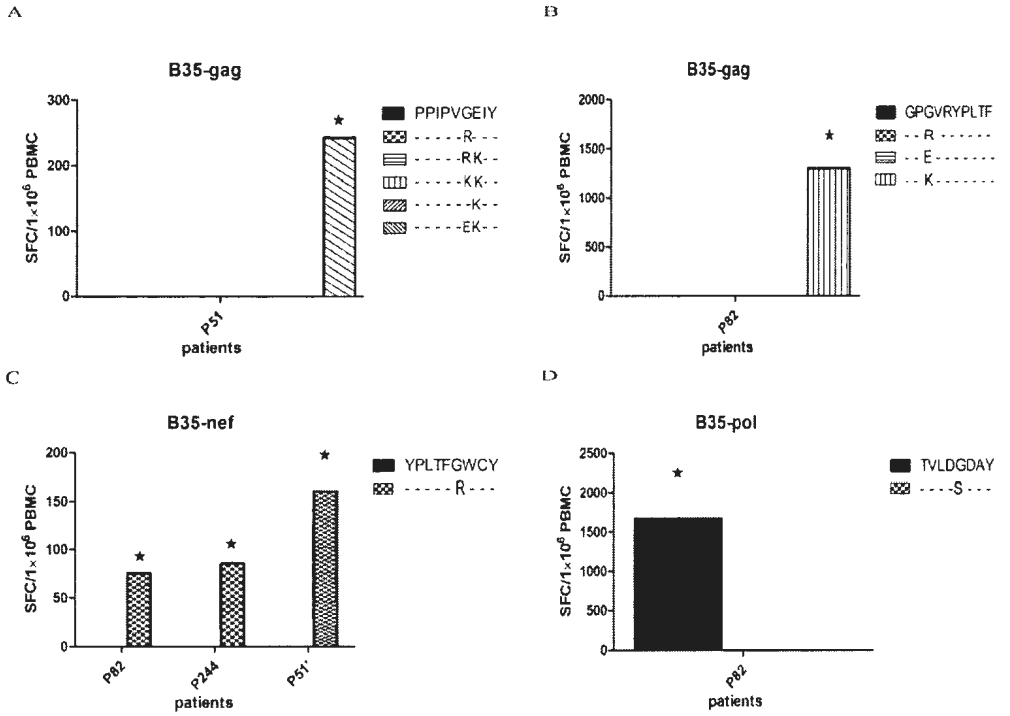
Genes	Normalized number of hotspots inside CTL-epitopes	Normalized number of GGG hotspots inside CTL-epitopes	Normalized number of hotspots outside CTL-epitopes	Normalized number of GGG hotspots outside CTL-epitopes	Ratio of all hotspots Inside:outside epitopes	Index value	Ratio of GGG hotspots Inside:outside epitopes	Index value
Vpr	0.1041	0.0277	0.0884	0.0204	1.17	1.3948	1.36	1.119
Pol	0.0924	0.023	0.0794	0.00883	1.16	1.3829	2.60	2.139
Gag	0.0864	0.0233	0.0813	0.0143	1.06	1.2637	1.63	1.341
Vif	0.0774	0.0202	0.0992	0.0177	0.78	0.9299	1.14	0.938
Nef	0.0839	0.0272	0.0944	0.0111	0.889	1.0598	2.45	2.016
Env	0.070	0.0176	0.0991	0.0226	0.706	0.8416	0.779	0.641
Rev	0.094	0.00556	0.1345	0.0409	0.699	0.8333	0.136	0.111
Tat	0.0363	0.00	0.1041	0.0208	0.349	0.4160	<0.291	0.239
Vpu	0.0701	0.00	0.952	0.0317	0.736	0.8774	<0.553	0.455



Supplementary Figure 1. Map of all HIV-1 BRU isolate CTL epitopes across the viral proteome. HIV proteins are shown individually (**A:** Pol, **B:** Rev, **C:** Vif, **D:** Nef, **E:** Vpr, **F:** Env, **G:** Gag, **H:** Tat). The brackets indicate epitopes on the peptide sequence. Epitopes restricted to different HLA alleles are shown in different colors and the restricting HLA is indicated above the bracket. Broken lines display epitopes with no A3G/F hotspots and continuous lines show epitopes that harbor A3G/F hotspots.



Supplementary Figure 2. Map of the viral genomic sequences that encode CTL epitopes in the HIV-1 BRU isolate plus-sense strand. The sequence of each gene is shown individually (**A:** Pol, **B:** Rev, **C:** Vif, **D:** Nef, **E:** Vpr, **F:** Env, **G:** Gag, **H:** Tat). Color delineates the sequences encoding CTL epitopes from the surrounding DNA. Colors do not otherwise correspond to any common features amongst genes or epitopes.



Supplementary Figure 3. The IFN-ɣ response to HLA-B35-restricted epitopes harboring A3G/F-mediated mutations. HLA-matched, HIV-infected individuals PBMC response was measured by ELISPOT to wild type and mutant epitopes in Pol, Gag and Nef. X-axis displays different HIV infected individuals (patients). **A.** Induction of IFN-ɣ is compared between the wild type form and 5 mutant versions of the Gag epitope PPIPVGEIY in P51. **B.** Induction of IFN-ɣ is compared between the wild type form and 3 mutant versions of the Gag epitope GPGVRYPLTF in P82. **C.** Induction of IFN-ɣ is compared between the wild type form and a mutant version of the Nef epitope YPLTFGWCY in P82, P51', P244. **D.** Induction of IFN-ɣ is compared between the wild type form and a mutant version of the Pol epitope TVLDGDAY in P82. Multiple other B35-restricted epitopes were tested but did not yield a response. Asterisk indicates the responses greater than 50 SFC/10^6 PBMC.

Summary:

Human immunodeficiency virus (HIV) utilizes our immune system to replicate in the body and has developed ways to overcome it. However, our immune cells have intrinsic barriers against HIV. An emerging set of these antiviral factors are the APOBEC enzymes, most notably APOBEC3G (A3G) and APOBEC3F (A3F). These cytidine deaminase enzymes act by introducing C to U mutations in the negative strand of the viral genome during its replication in the cytoplasm of infected T cells. These mutations can lead to the degradation of viral DNA by uracil-removal pathways, or alternatively result in G to A mutations in the coding (positive) strand, which can inactivate the virus. Because they are constitutively expressed pre-infection with HIV, and act non-specifically throughout the viral DNA, APBOECS have been considered agents of the innate immune response.

We hypothesized that beyond their role in innate immunity, mutations by A3G or A3F modulate the adaptive immune system by modifying peptide epitopes recognized by cytotoxic CD8+ T (CTL) cells. To test our hypothesis, we proceeded with three goals:

1) *In silico* identification of all CTL epitopes for each HLA throughout the HIV genome

2) Measuring the CTL responses of HIV+ patients to peptides containing A3G/F simulated mutations

3) *In silico* analysis of A3G/F hotspot motifs inside and outside of the CTL epitopes.

Thus, we simulated A3G mutations throughout the HIV genome *in silico* and catalogued those situated in CTL epitopes in the context of different class I MHC molecules and genes. We selected B57, A2, and B44 because they are common HLA and the degree of CTL response to their epitopes correlates inversely with disease progression. As a control, we selected HLA-B35 as it positively correlates with the progression of AIDS. Also, we initially selected epitopes of *gag*, *pol* and *nef* genes since they represent prominent CTL epitopes and a strong CTL response to epitopes in *gag* and *nef* correlates with viral containment by the immune system. We then used synthetic A3G mutant peptides and their wild-type (WT) counterparts to stimulate the CTL response in PBMCs of HLA-matched patients and compared the *ex vivo* response of CTL to peptide epitopes bearing A3G/F mutations with WT epitopes. Using the ELISPOT assay, we found that the vast majority of mutant epitopes elicit decreased IFN-γ production by CTL, in comparison to WT. Epitopes, including WT and mutant forms were tested in HIV patients. Notably, the gap between the CTL response to the WT and mutant epitopes are higher among the epitopes for which a strong CTL response correlates inversely with disease progression. However, this scenario is different in the case of HLA-B35 with a direct effect on disease progression. While there is no CTL response to the WT form of these epitopes, except in *pol*, the response to mutant epitopes restricted to HLA-B35 located in *gag* and *nef* is increased. Our results indicate that HIV may use APOBEC induced mutations as a mechanism of CTL escape. Furthermore, our findings suggest that the HIV genome may be enriched in APOBEC hotspot motifs in regions encoding for CTL epitopes.

To test this prediction, we compared A3G/F hotspots inside and outside CTL epitopes. We located A3G/F mutational hotspots in all epitopes. After normalizing them to the number of nucleotides in the different epitopes, we calculated the index number by determining the average of total R values of all genes or HLAs and dividing the R value of each gene or HLA by the average total R value. We compared the index number of hotspots in CTL epitopes between different genes and HLAs and found that indeed there are higher number of A3G/F specific motifs inside CTL epitopes versus outside them in *gag, nef,* and *pol* in comparison to the other viral genes. Also, A3G/F hotspots are more enriched inside CTL epitopes versus outside, especially in epitopes restricted to HLA-B57 rather than HLA-B35. HLA types that are prevalent and immunodominant in the human population (HLA-B44, HLA-A3), and immunodominant in terms of presentation of HIV epitopes, show higher hotspot enrichment compared to other HLAs. As a negative control, we checked the number of A3G/F hotspots inside and outside antibody aligned epitopes. There was no hotspot accumulation inside Ab epitopes. Our results indicate that rather than being beneficial for the adaptive immune system, mutations of the HIV genome by A3G and A3F may have the opposite effect by assisting HIV to escape from CTL responses.

Previous studies report that CTL response is improved through A3G mutations leading to formation of stop codons that generate truncated proteins in cultured HIV infected cells. In contrast, we report here that direct action of A3G/F on CTL epitopes have reverse effect in HIV-infected individuals. Also, another study indicates the role of "neo-epitopes" as CTL decoys, which is an approach for viral escape from the host

immune system. "Neo-epitopes" are primarily restricted to HLA-A24 epitopes located in Nef. This is consistent with our data showing an improved CTL response to A3G/F-mutated HLA-B35 restricted epitopes in patients with HLA-A24. Thus, our data may reflect the effect of A3G/F in both suppressing the recognition of immunodominant epitopes and the generation of decoy epitopes. Further investigation is needed to gain better insight into the mechanisms underlying the differences in CTL response between WT and mutant epitopes. Uncovering the role of specific subsets of CTLs (e.g. memory T cells) will aid in elucidating this mechanism. It will also be essential to understand how A3G/F induced mutations affect CTL response through studying the downstream molecular and cellular signaling pathways.

Appendix 7
Bibliography

Chapter 1

1. C Hoffmann, J Rockstroh, B Seba, Kamps et al., HIV medicine. 2005.

2. Levy, J.A., HIV and the Pathogenesis of AIDS. 2007: ASM Press Washington, DC.

3. Luciw, P.A., et al., Molecular cloning of AIDS-associated retrovirus. Nature, 1984. 312(5996): p. 760-3.

4. Frankel, A.D. and J.A.T. Young, HIV-1: fifteen proteins and an RNA. Annual review of biochemistry, 1998. 67(1): p. 1-25.

5. Hahn, B.H., et al., AIDS as a zoonosis: scientific and public health implications. Science, 2000. 287(5453): p. 607-614.

6. Barroso, H., et al., Evolutionary and structural features of the C2, V3 and C3 envelope regions underlying the differences in HIV-1 and HIV-2 biology and infection. PloS one. 6(1): p. e14548.

7. Zhang, M., et al., The role of recombination in the emergence of a complex and dynamic HIV epidemic. Retrovirology, 2010. 7(1): p. 25.

8. Gelderblom, H.R., Fine structure of HIV and SIV. Los Alamos National, 1997.

9. Greene, W.C., et al., Novel targets for HIV therapy. Antiviral research, 2008. 80(3): p. 251-265.

10. Nielsen, M.H., F.S. Pedersen, and J. Kjems, Molecular strategies to inhibit HIV-1 replication. Retrovirology, 2005. 2(1): p. 10.

11. Richard, S. and W. Mark, The role of unintegrated DNA in HIV infection. Retrovirology. 8.

12. Gheysen, D., et al., Assembly and release of HIV-1 precursor Pr55< sup> gag</sup> virus-like particles from recombinant baculovirus-infected insect cells. Cell, 1989. 59(1): p. 103-112.

13. Veazey, R.S., P.A. Marx, and A.A. Lackner, The mucosal immune system: primary target for HIV infection and AIDS. Trends in immunology, 2001. 22(11): p. 626.

14. Smed-SÃ¶rensen, A., et al., HIV-1-infected dendritic cells up-regulate cell surface markers but fail to produce IL-12 p70 in response to CD40 ligand stimulation. Blood, 2004. 104(9): p. 2810-2817.

15. Izmailova, E., et al., HIV-1 Tat reprograms immature dendritic cells to express chemoattractants for activated T cells and macrophages. Nature medicine, 2003. 9(2): p. 191-197.

16. Yu, W., et al., Induction of the HIV-1 Tat co-factor cyclin T1 during monocyte differentiation is required for the regulated expression of a large portion of cellular mRNAs. Retrovirology, 2006. 3(1): p. 32.

17. Wu, L. and V.N. KewalRamani, Dendritic-cell interactions with HIV: infection and viral dissemination. Nature Reviews Immunology, 2006. 6(11): p. 859-868.

18. Guergnon, J. and C. CombadiÃ¨re, Role of chemokines polymorphisms in diseases. Immunology Letters. 145(1): p. 15-22.
19. Boirivant, M., et al., HIV-1 gp120 accelerates Fas-mediated activation-induced human lamina propria T cell apoptosis. Journal of clinical immunology, 1998. 18(1): p. 39-47.

20. Greenberg, M.E., et al., Co-localization of HIV-1 Nef with the AP-2 adaptor protein complex correlates with Nef-induced CD4 down-regulation. The EMBO journal, 1997. 16(23): p. 6964-6976.

21. Gougeon, M.L., et al., T cell apoptosis in HIV infection: mechanisms and relevance for AIDS pathogenesis. Results Probl Cell Differ, 1998. 24: p. 233-48.

22. Siliciano, R.F., Acquired immune deficiency syndrome (AIDS). eLS, 2006.

23. Barton, G.M. Viral recognition by Toll-like receptors. in Seminars in immunology. 2007. Elsevier.

24. Trine, M., et al., Innate immune recognition and activation during HIV infection. Retrovirology.

25. Biasin, M., M. Clerici, and L. Piacentini, Innate immunity in resistance to HIV infection. Journal of Infectious Diseases. 202(Supplement 3): p. S361-S365.

26. Servet, C., L. Zitvogel, and A. Hosmalin, Dendritic cells in innate immune responses against HIV. Current molecular medicine, 2002. 2(8): p. 739-756.

27. Nisole, S., J.P. Stoye, and A. SaÃ¯b, TRIM family proteins: retroviral restriction and antiviral defence. Nature Reviews Microbiology, 2005. 3(10): p. 799-808.

28. Perez-Caballero, D., et al., Tetherin inhibits HIV-1 release by directly tethering virions to cells. Cell, 2009. 139(3): p. 499-511.

29. Holmes, R.K., M.H. Malim, and K.N. Bishop, APOBEC-mediated viral restriction: not simply editing? Trends in biochemical sciences, 2007. 32(3): p. 118-128.

30. Barr, S.D., J.R. Smiley, and F.D. Bushman, The interferon response inhibits HIV particle production by induction of TRIM22. PLoS pathogens, 2008. 4(2): p. e1000007.

31. Wolf, D. and S.P. Goff, TRIM28 mediates primer binding site-targeted silencing of murine leukemia virus in embryonic cells. Cell, 2007. 131(1): p. 46-57.

32. Simpson, L., 22 RNA Editing—An Evolutionary Perspective. Cold Spring Harbor Monograph Archive, 1999. 37: p. 585-608.

33. Guo, F., et al., Inhibition of-primed reverse transcription by human APOBEC3G during human immunodeficiency virus type 1 replication. Journal of virology, 2006. 80(23): p. 11710-11722.

34. Chelico, L., et al., APOBEC3G DNA deaminase acts processively 3â€²â†' 5â€² on single-stranded DNA. Nature structural & molecular biology, 2006. 13(5): p. 392-399.

35. Sadler, H.A., et al., APOBEC3G contributes to HIV-1 variation through sublethal mutagenesis. Journal of virology. 84(14): p. 7396-7404.

36. Yu, Q., et al., Single-strand specificity of APOBEC3G accounts for minus-strand deamination of the HIV genome. Nature structural & molecular biology, 2004. 11(5): p. 435-442.

37. Goncalves, J. and M. Santa-Marta, HIV-1 Vif and APOBEC3G: Multiple roads to one goal. Retrovirology, 2004. 1(1): p. 28.

38. Kao, S., et al., The human immunodeficiency virus type 1 Vif protein reduces intracellular expression and inhibits packaging of APOBEC3G (CEM15), a cellular inhibitor of virus infectivity. Journal of virology, 2003. 77(21): p. 11398-11407.

39. Russell, R.A., et al., APOBEC3G induces a hypermutation gradient: purifying selection at multiple steps during HIV-1 replication results in levels of G-to-A mutations that are high in DNA, intermediate in cellular viral RNA, and low in virion RNA. Retrovirology, 2009. 6(1): p. 16.

40. Chiu, Y.L., Biochemical fractionation and purification of high-molecular-mass APOBEC3G complexes. Methods Mol Biol, 2011. 718: p. 185-206.

41. Harrer, T., et al., Strong cytotoxic T cell and weak neutralizing antibody responses in a subset of persons with stable nonprogressing HIV type 1 infection. AIDS research and human retroviruses, 1996. 12(7): p. 585-592.

42. Pereyra, F., et al., The major genetic determinants of HIV-1 control affect HLA class I peptide presentation. Science (New York, NY), 2010. 330(6010): p. 1551.

43. Fujiwara, M., et al., Patterns of cytokine production in human immunodeficiency virus type 1 (HIV-1)-specific human CD8+ T cells after stimulation with HIV-1-infected CD4+ T cells. Journal of virology, 2005. 79(19): p. 12536-12543.

44. Jassoy, C., et al., Human immunodeficiency virus type 1-specific cytotoxic T lymphocytes release gamma interferon, tumor necrosis factor alpha (TNF-alpha), and TNF-beta when they encounter their target antigens. Journal of virology, 1993. 67(5): p. 2844-2852.

45. Preston, B.D., B.J. Poiesz, and L.A. Loeb, Fidelity of HIV-1 reverse transcriptase. Science (New York, NY), 1988. 242(4882): p. 1168.

46. Phillips, R.E., et al., Human immunodeficiency virus genetic variation that can escape cytotoxic T cell recognition. Nature, 1991. 354(6353): p. 453.

47. Allen, T.M., et al., Selective escape from CD8+ T-cell responses represents a major driving force of human immunodeficiency virus type 1 (HIV-1) sequence diversity and reveals constraints on HIV-1 evolution. J Virol, 2005. 79(21): p. 13239-49.

48. Cardinaud, S., et al., CTL Escape Mediated by Proteasomal Destruction of an HIV-1 Cryptic Epitope. PLoS pathogens. 7(5): p. e1002049.

49. Le Gall, S., P. Stamegna, and B.D. Walker, Portable flanking sequences modulate CTL epitope processing. Journal of Clinical Investigation, 2007. 117(11): p. 3563.

50. Zimbwa, P., et al., Precise identification of a human immunodeficiency virus type 1 antigen processing mutant. Journal of virology, 2007. 81(4): p. 2031-2038.

51. Ossendorp, F., et al., A single residue exchange within a viral CTL epitope alters proteasome-mediated degradation resulting in lack of antigen presentation. Immunity, 1996. 5(2): p. 115-124.

52. Friedrich, T.C., et al., Reversion of CTL escape–variant immunodeficiency viruses in vivo. Nature medicine, 2004. 10(3): p. 275-281.

Chapter 2

1. Sheehy AM, Gaddis NC, Choi JD, Malim MH: Isolation of a human gene that inhibits HIV-1 infection and is suppressed by the viral Vif protein.

2. Chiu YL, Soros VB, Kreisberg JF, Stopak K, Yonemoto W, Greene WC: Cellular APOBEC3G restricts HIV-1 infection in resting CD4+ T cells. Nature 2005,435:108–114.

3. Conticello SG, Thomas CJ, Petersen-Mahrt SK, Neuberger MS: Evolution of the AID/APOBEC family of polynucleotide (deoxy)cytidine deaminases. Mol Biol Evol 2005, 22:367–377.

4. Jarmuz A, Chester A, Bayliss J, Gisbourne J, Dunham I, Scott J, Navaratnam N: An anthropoid-specific locus of orphan C to U RNA-editing enzymes on chromosome 22. Genomics 2002, 79:285–296.

5. Conticello SG: The AID/APOBEC family of nucleic acid mutators. Genome Biol 2008, 9:229.

6. Beale RC, Petersen-Mahrt SK, Watt IN, Harris RS, Rada C, Neuberger MS: Comparison of the differential context-dependence of DNA deamination by APOBEC enzymes: correlation with mutation spectra in vivo. J Mol Biol 2004, 337:585–596.

7. Harris RS, Bishop KN, Sheehy AM, Craig HM, Petersen-Mahrt SK, Watt IN, Neuberger MS, Malim MH: DNA deamination mediates innate immunity to retroviral infection. Cell 2003, 113:803–809.

8. Simon JH, Gaddis NC, Fouchier RA, Malim MH: Evidence for a newly discovered cellular anti-HIV-1 phenotype. Nat Med 1998, 4:1397–1400.

9. Madani N, Kabat D: An endogenous inhibitor of human immunodeficiency virus in human lymphocytes is overcome by the viral Vif protein. J Virol 1998, 72:10251–10255.

10. Peng G, Greenwell-Wild T, Nares S, Jin W, Lei KJ, Rangel ZG, Munson PJ, Wahl SM: Myeloid differentiation and susceptibility to HIV-1 are linked to APOBEC3 expression. Blood 2007, 110:393–400.

11. Pido-Lopez J, Whittall T, Wang Y, Bergmeier LA, Babaahmady K, Singh M, Lehner T: Stimulation of cell surface CCR5 and CD40 molecules by their ligands or by HSP70 up-

regulates APOBEC3G expression in CD4(+) T cells and dendritic cells. J Immunol 2007, 178:1671–1679.

12. Stopak KS, Chiu YL, Kropp J, Grant RM, Greene WC: Distinct patterns of cytokine regulation of APOBEC3G expression and activity in primary lymphocytes, macrophages, and dendritic cells. J Biol Chem2007, 282:3539–3546.

13. Koning FA, Newman EN, Kim EY, Kunstman KJ, Wolinsky SM, Malim MH: Defining APOBEC3 expression patterns in human tissues and hematopoietic cell subsets. J Virol 2009, 83:9474–9485.

14. Pion M, Granelli-Piperno A, Mangeat B, Stalder R, Correa R, Steinman RM, Piguet V: APOBEC3G/3 F mediates intrinsic resistance of monocytederived dendritic cells to HIV-1 infection. J Exp Med 2006, 203:2887–2893.

15. Bonvin M, Achermann F, Greeve I, Stroka D, Keogh A, Inderbitzin D, Candinas D, Sommer P, Wain-Hobson S, Vartanian JP, Greeve J: Interferoninducible expression of APOBEC3 editing enzymes in human hepatocytes and inhibition of hepatitis B virus replication. Hepatology 2006, 43:1364–1374.

16. Komohara Y, Suekane S, Noguchi M, Matsuoka K, Yamada A, Itoh K: Expression of APOBEC3G in kidney cells. Tissue Antigens 2007, 69:95–98.

17. Peng G, Lei KJ, Jin W, Greenwell-Wild T, Wahl SM: Induction of APOBEC3 family proteins, a defensive maneuver underlying interferon-induced anti-HIV-1 activity. J Exp Med 2006, 203:41–46.

18. Tanaka Y, Marusawa H, Seno H, Matsumoto Y, Ueda Y, Kodama Y, Endo Y, Yamauchi J, Matsumoto T, Takaori-Kondo A, et al: Anti-viral protein APOBEC3G is induced by interferon-alpha stimulation in human hepatocytes. Biochem Biophys Res Commun 2006, 341:314–319.

19. Yu Q, Konig R, Pillai S, Chiles K, Kearney M, Palmer S, Richman D, Coffin JM, Landau NR: Single-strand specificity of APOBEC3G accounts for minusstrand deamination of the HIV genome. Nat Struct Mol Biol 2004, 11:435–442.

20. Lecossier D, Bouchonnet F, Clavel F, Hance AJ: Hypermutation of HIV-1 DNA in the absence of the Vif protein. Science 2003, 300:1112.

21. Mangeat B, Turelli P, Caron G, Friedli M, Perrin L, Trono D: Broad antiretroviral defence by human APOBEC3G through lethal editing of nascent reverse transcripts. Nature 2003, 424:99–103.

22. Zhang H, Yang B, Pomerantz RJ, Zhang C, Arunachalam SC, Gao L: The cytidine deaminase CEM15 induces hypermutation in newly synthesized HIV-1 DNA. Nature 2003, 424:94–98.

23. Klarmann GJ, Chen X, North TW, Preston BD: Incorporation of uracil into minus strand DNA affects the specificity of plus strand synthesis initiation during lentiviral reverse transcription. J Biol Chem 2003, 278:7902–7909.

24. Mbisa JL, Barr R, Thomas JA, Vandegraaff N, Dorweiler IJ, Svarovskaia ES, Brown WL, Mansky LM, Gorelick RJ, Harris RS, et al: Human immunodeficiency virus type 1 cDNAs

produced in the presence of APOBEC3G exhibit defects in plus-strand DNA transfer and integration. J Virol 2007, 81:7099–7110.

25. Simon V, Zennou V, Murray D, Huang Y, Ho DD, Bieniasz PD: Natural variation in Vif: differential impact on APOBEC3G/3 F and a potential role in HIV-1 diversification. PLoS Pathog 2005, 1:e6.

26. Newman EN, Holmes RK, Craig HM, Klein KC, Lingappa JR, Malim MH, Sheehy AM: Antiviral function of APOBEC3G can be dissociated from cytidine deaminase activity. Curr Biol 2005, 15:166–170.

27. Anderson JL, Hope TJ: APOBEC3G restricts early HIV-1 replication in the cytoplasm of target cells. Virology 2008, 375:1–12.

28. Li XY, Guo F, Zhang L, Kleiman L, Cen S: APOBEC3G inhibits DNA strand transfer during HIV-1 reverse transcription. J Biol Chem2007, 282:32065–32074.

29. Navarro F, Bollman B, Chen H, Konig R, Yu Q, Chiles K, Landau NR: Complementary function of the two catalytic domains of APOBEC3G. Virology 2005, 333:374–386.

30. Luo K, Wang T, Liu B, Tian C, Xiao Z, Kappes J, Yu XF: Cytidine deaminases APOBEC3G and APOBEC3F interact with human immunodeficiency virus type 1 integrase and inhibit proviral DNA formation. J Virol 2007, 81:7238–7248.

31. Mbisa JL, Bu W, Pathak VK: APOBEC3F and APOBEC3G inhibit HIV-1 DNA integration by different mechanisms. J Virol 2010, 84:5250–5259.

32. Guo F, Cen S, Niu M, Yang Y, Gorelick RJ, Kleiman L: The interaction of APOBEC3G with human immunodeficiency virus type 1 nucleocapsid inhibits tRNA3Lys annealing to viral RNA. J Virol 2007, 81:11322–11331.

33. Wang X, Ao Z, Chen L, Kobinger G, Peng J, Yao X: The Cellular Antiviral Protein APOBEC3G Interacts with HIV-1 Reverse Transcriptase and Inhibits Its Function during Viral Replication. J Virol 2012, 86:3777–3786.

34. Browne EP, Allers C, Landau NR: Restriction of HIV-1 by APOBEC3G is cytidine deaminase-dependent. Virology 2009, 387:313–321.

35. Smith HC: APOBEC3G: a double agent in defense. Trends Biochem Sci 2011, 36:239–244.

36. Miyagi E, Opi S, Takeuchi H, Khan M, Goila-Gaur R, Kao S, Strebel K: Enzymatically active APOBEC3G is required for efficient inhibition of human immunodeficiency virus type 1. J Virol 2007, 81:13346–13353.

37. Schumacher AJ, Nissley DV, Harris RS: APOBEC3G hypermutates genomic DNA and inhibits Ty1 retrotransposition in yeast. Proc Natl Acad Sci U S A 2005, 102:9854–9859.

38. Schumacher AJ, Hache G, Macduff DA, Brown WL, Harris RS: The DNA deaminase activity of human APOBEC3G is required for Ty1, MusD, and human immunodeficiency virus type 1 restriction. J Virol 2008, 82:2652–2660.

39. Holmes RK, Koning FA, Bishop KN, Malim MH: APOBEC3F can inhibit the accumulation of HIV-1 reverse transcription products in the absence of hypermutation. Comparisons with APOBEC3G. J Biol Chem 2007, 282 (4):2587–2595. Epub 2006 Nov 22.

40. Stenglein MD, Harris RS: APOBEC3B and APOBEC3F inhibit L1 retrotransposition by a DNA deamination-independent mechanism. J Biol Chem 2006, 281:16837–16841.

41. Muckenfuss H, Hamdorf M, Held U, Perkovic M, Lower J, Cichutek K, Flory E, Schumann GG, Munk C: APOBEC3 proteins inhibit human LINE-1 retrotransposition. J Biol Chem 2006, 281:22161–22172.

42. Turelli P, Mangeat B, Jost S, Vianin S, Trono D: Inhibition of hepatitis B virus replication by APOBEC3G. Science 1829, 2004:303.

43. Bogerd HP, Wiegand HL, Hulme AE, Garcia-Perez JL, O'Shea KS, Moran JV, Cullen BR: Cellular inhibitors of long interspersed element 1 and Alu retrotransposition. Proc Natl Acad Sci U S A 2006, 103:8780–8785.

44. Esnault C, Heidmann O, Delebecque F, Dewannieux M, Ribet D, Hance AJ, Heidmann T, Schwartz O: APOBEC3G cytidine deaminase inhibits retrotransposition of endogenous retroviruses. Nature 2005, 433:430–433.

45. Esnault C, Millet J, Schwartz O, Heidmann T: Dual inhibitory effects of APOBEC family proteins on retrotransposition of mammalian endogenous retroviruses. Nucleic Acids Res 2006, 34:1522–1531.

46. Chiu YL, Witkowska HE, Hall SC, Santiago M, Soros VB, Esnault C, Heidmann T, Greene WC: High-molecular-mass APOBEC3G complexes restrict Alu retrotransposition. Proc Natl Acad Sci U S A 2006, 103:15588–15593.

47. Zhang J, Webb DM: Rapid evolution of primate antiviral enzyme APOBEC3G. Hum Mol Genet 2004, 13:1785–1791.

48. Wedekind JE, Dance GS, Sowden MP, Smith HC: Messenger RNA editing in mammals: new members of the APOBEC family seeking roles in the family business. Trends Genet 2003, 19:207–216.

49. Do H, Vasilescu A, Diop G, Hirtzig T, Heath SC, Coulonges C, Rappaport J, Therwath A, Lathrop M, Matsuda F, Zagury JF: Exhaustive genotyping of the CEM15 (APOBEC3G) gene and absence of association with AIDS progression in a French cohort. J Infect Dis 2005, 191:159–163.

50. Sawyer SL, Emerman M, Malik HS: Ancient adaptive evolution of the primate antiviral DNA-editing enzyme APOBEC3G. PLoS Biol 2004, 2: E275.

51. Bizinoto MC, Leal E, Diaz RS, Janini LM: Loci polymorphisms of the APOBEC3G gene in HIV type 1-infected Brazilians. AIDS Res Hum Retroviruses 2011, 27:137–141.

52. An P, Bleiber G, Duggal P, Nelson G, May M, Mangeat B, Alobwede I, Trono D, Vlahov D, Donfield S, et al: APOBEC3G genetic variants and their influence on the progression to AIDS. J Virol 2004, 78:11070–11076.

53. Kieffer TL, Kwon P, Nettles RE, Han Y, Ray SC, Siliciano RF: G->A hypermutation in protease and reverse transcriptase regions of human immunodeficiency virus type 1 residing in resting CD4+ T cells in vivo. J Virol 2005, 79:1975–1980.

54. Pace C, Keller J, Nolan D, James I, Gaudieri S, Moore C, Mallal S: Population level analysis of human immunodeficiency virus type 1 hypermutation and its relationship with APOBEC3G and vif genetic variation. J Virol 2006, 80:9259–9269.

55. Piantadosi A, Humes D, Chohan B, McClelland RS, Overbaugh J: Analysis of the percentage of human immunodeficiency virus type 1 sequences that are hypermutated and markers of disease progression in a longitudinal cohort, including one individual with a partially defective Vif. J Virol 2009, 83:7805–7814.

56. Land AM, Ball TB, Luo M, Pilon R, Sandstrom P, Embree JE, Wachihi C, Kimani J, Plummer FA: Human immunodeficiency virus (HIV) type 1 proviral hypermutation correlates with CD4 count in HIV-infected women from Kenya. J Virol 2008, 82:8172–8182.

57. Gandhi SK, Siliciano JD, Bailey JR, Siliciano RF, Blankson JN: Role of APOBEC3G/ F-mediated hypermutation in the control of human immunodeficiency virus type 1 in elite suppressors. J Virol 2008, 82:3125–3130.

58. Preston BD, Poiesz BJ, Loeb LA: Fidelity of HIV-1 reverse transcriptase. Science 1988, 242:1168–1171.

59. Yu X, Yu Y, Liu B, Luo K, Kong W, Mao P, Yu XF: Induction of APOBEC3G ubiquitination and degradation by an HIV-1 Vif-Cul5-SCF complex. Science 2003, 302:1056–1060.

60. Santa-Marta M, da Silva FA, Fonseca AM, Goncalves J: HIV-1 Vif can directly inhibit apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like 3 G-mediated cytidine deamination by using a single amino acid interaction and without protein degradation. J Biol Chem2005, 280:8765–8775.

61. Khan MA, Aberham C, Kao S, Akari H, Gorelick R, Bour S, Strebel K: Human immunodeficiency virus type 1 Vif protein is packaged into the nucleoprotein complex through an interaction with viral genomic RNA. J Virol 2001, 75:7252–7265.

62. Goncalves J, Santa-Marta M: HIV-1 Vif and APOBEC3G: multiple roads to one goal. Retrovirology 2004, 1:28.

63. Sheehy AM, Gaddis NC, Malim MH: The antiretroviral enzyme APOBEC3G is degraded by the proteasome in response to HIV-1 Vif. Nat Med 2003, 9:1404–1407.

64. Mehle A, Strack B, Ancuta P, Zhang C, McPike M, Gabuzda D: Vif overcomes the innate antiviral activity of APOBEC3G by promoting its degradation in the ubiquitin-proteasome pathway. J Biol Chem 2004, 279:7792–7798.

65. Goila-Gaur R, Strebel K: HIV-1 Vif, APOBEC, and intrinsic immunity. Retrovirology 2008, 5:51.

66. Stopak K, de Noronha C, Yonemoto W, Greene WC: HIV-1 Vif blocks the antiviral activity of APOBEC3G by impairing both its translation and intracellular stability. Mol Cell 2003, 12:591–601.

67. Strebel K, Luban J, Jeang KT: Human cellular restriction factors that target HIV-1 replication. BMC Med 2009, 7:48.

68. Conticello SG, Harris RS, Neuberger MS: The Vif protein of HIV triggers degradation of the human antiretroviral DNA deaminase APOBEC3G. Curr Biol 2003, 13:2009–2013.

69. Kao S, Akari H, Khan MA, Dettenhofer M, Yu XF, Strebel K: Human immunodeficiency virus type 1 Vif is efficiently packaged into virions during productive but not chronic infection. J Virol 2003, 77:1131–1140.

70. Soros VB, Greene WC: APOBEC3G and HIV-1: strike and counterstrike. Curr Infect Dis Rep 2006, 8:317–323.

71. Svarovskaia ES, Xu H, Mbisa JL, Barr R, Gorelick RJ, Ono A, Freed EO, Hu WS, Pathak VK: Human apolipoprotein B mRNA-editing enzyme-catalytic polypeptide-like 3 G (APOBEC3G) is incorporated into HIV-1 virions through interactions with viral and nonviral RNAs. J Biol Chem 2004, 279:35822–35828.

72. Alce TM, Popik W: APOBEC3G is incorporated into virus-like particles by a direct interaction with HIV-1 Gag nucleocapsid protein. J Biol Chem 2004, 279:34083–34086.

73. Ma J, Li X, Xu J, Zhang Q, Liu Z, Jia P, Zhou J, Guo F, You X, Yu L, et al: The cellular source for APOBEC3G's incorporation into HIV-1. Retrovirology 2011, 8:2.

74. Mariani R, Chen D, Schrofelbauer B, Navarro F, Konig R, Bollman B, Munk C, Nymark-McMahon H, Landau NR: Species-specific exclusion of APOBEC3G from HIV-1 virions by Vif. Cell 2003, 114:21–31.

75. Wissing S, Galloway NL, Greene WC: HIV-1 Vif versus the APOBEC3 cytidine deaminases: an intracellular duel between pathogen and host restriction factors. Mol Aspects Med 2010, 31:383–397.

76. Xu H, Chertova E, Chen J, Ott DE, Roser JD, Hu WS, Pathak VK: Stoichiometry of the antiviral protein APOBEC3G in HIV-1 virions. Virology 2007, 360:247–256

77. Mercenne G, Bernacchi S, Richer D, Bec G, Henriet S, Paillart JC, Marquet R:HIV-1 Vif binds to APOBEC3G mRNA and inhibits its translation. Nucleic Acids Res 2010, 38:633–646.

78. Kao S, Khan MA, Miyagi E, Plishka R, Buckler-White A, Strebel K: The human immunodeficiency virus type 1 Vif protein reduces intracellular expression and inhibits packaging of APOBEC3G (CEM15), a cellular inhibitor of virus infectivity. J Virol 2003, 77:11398–11407.

79. Opi S, Kao S, Goila-Gaur R, Khan MA, Miyagi E, Takeuchi H, Strebel K: Human immunodeficiency virus type 1 Vif inhibits packaging and antiviral activity of a degradation-resistant APOBEC3G variant. J Virol 2007, 81:8236–8246.

80. Kao S, Goila-Gaur R, Miyagi E, Khan MA, Opi S, Takeuchi H, Strebel K: Production of infectious virus and degradation of APOBEC3G are separable functional properties of human immunodeficiency virus type 1 Vif. Virology 2007, 369:329–339.

81. Jager S, Kim DY, Hultquist JF, Shindo K, LaRue RS, Kwon E, Li M, Anderson BD, Yen L, Stanley D, et al: Vif hijacks CBF-beta to degrade APOBEC3G and promote HIV-1 infection. Nature 2011, 481:371–375.

82. Zhang W, Du J, Evans SL, Yu Y, Yu XF: T-cell differentiation factor CBF-beta regulates HIV-1 Vif-mediated evasion of host restriction. Nature 2011, 481:376–379.

83. Hamilton CE, Papavasiliou FN, Rosenberg BR: Diverse functions for DNA and RNA editing in the immune system. RNA Biol 2010, 7:220–228.

84. Sadler HA, Stenglein MD, Harris RS, Mansky LM: APOBEC3G contributes to HIV-1 variation through sublethal mutagenesis. J Virol 2010, 84:7396–7404.

85. Goila-Gaur R, Khan MA, Miyagi E, Strebel K: Differential sensitivity of "old" versus "new" APOBEC3G to human immunodeficiency virus type 1 vif. J Virol 2009, 83:1156–1160.

86. Sakai K, Dimas J, Lenardo MJ: The Vif and Vpr accessory proteins independently cause HIV-1-induced T cell cytopathicity and cell cycle arrest. Proc Natl Acad Sci U S A 2006, 103:3369–3374.

87. Wang J, Shackelford JM, Casella CR, Shivers DK, Rapaport EL, Liu B, Yu XF, Finkel TH: The Vif accessory protein alters the cell cycle of human immunodeficiency virus type 1 infected cells. Virology 2007, 359: 243–252.

88. Wang J, Shackelford JM, Selliah N, Shivers DK, O'Neill E, Garcia JV, Muthumani K, Weiner D, Yu XF, Gabuzda D, Finkel TH: The HIV-1 Vif protein mediates degradation of Vpr and reduces Vpr-induced cell cycle arrest. DNA Cell Biol 2008, 27:267–277.

89. DeHart JL, Bosque A, Harris RS, Planelles V: Human immunodeficiency virus type 1 Vif induces cell cycle delay via recruitment of the same E3 ubiquitin ligase complex that targets APOBEC3 proteins for degradation. J Virol 2008, 82:9265–9272.

90. Chiu YL, Greene WC: APOBEC3G: an intracellular centurion. Philos Trans R Soc Lond B Biol Sci 2009, 364:689–703.

91. Kreisberg JF, Yonemoto W, Greene WC: Endogenous factors enhance HIV infection of tissue naive CD4 T cells by stimulating high molecular mass APOBEC3G complex formation. J Exp Med 2006, 203:865–870.

92. Chiu YL, Greene WC: The APOBEC3 cytidine deaminases: an innate defensive network opposing exogenous retroviruses and endogenous retroelements. Annu Rev Immunol 2008, 26:317–353.

93. Wichroski MJ, Robb GB, Rana TM: Human retroviral host restriction factors APOBEC3G and APOBEC3F localize to mRNA processing bodies. PLoS Pathog 2006, 2:e41.

94. Kozak SL, Marin M, Rose KM, Bystrom C, Kabat D: The anti-HIV-1 editing enzyme APOBEC3G binds HIV-1 RNA and messenger RNAs that shuttle between polysomes and stress granules. J Biol Chem 2006, 281:29105– 29119.

95. Chiu YL: Biochemical fractionation and purification of high-molecularmass APOBEC3G complexes. Methods Mol Biol 2011, 718:185–206.

96. Gallois-Montbrun S, Kramer B, Swanson CM, Byers H, Lynham S, Ward M, Malim MH: Antiviral protein APOBEC3G localizes to ribonucleoprotein complexes found in P bodies and stress granules. J Virol 2007, 81:2165–2178.

97. Strebel K, Khan MA: APOBEC3G encapsidation into HIV-1 virions: which RNA is it? Retrovirology 2008, 5:55.

98. Khan MA, Goila-Gaur R, Opi S, Miyagi E, Takeuchi H, Kao S, Strebel K: Analysis of the contribution of cellular and viral RNA to the packaging of APOBEC3G into HIV-1 virions. Retrovirology 2007, 4:48.

99. Khan MA, Kao S, Miyagi E, Takeuchi H, Goila-Gaur R, Opi S, Gipson CL, Parslow TG, Ly H, Strebel K: Viral RNA is required for the association of APOBEC3G with human immunodeficiency virus type 1 nucleoprotein complexes. J Virol 2005, 79:5870–5874.

100. Wang T, Tian C, Zhang W, Luo K, Sarkis PT, Yu L, Liu B, Yu Y, Yu XF: 7SL RNA mediates virion packaging of the antiviral cytidine deaminase APOBEC3G. J Virol 2007, 81:13112–13124.

101. Onafuwa-Nuga AA, Telesnitsky A, King SR: 7SL RNA, but not the 54-kd signal recognition particle protein, is an abundant component of both infectious HIV-1 and minimal virus-like particles. RNA 2006, 12:542–546.

102. Shandilya SM, Nalam MN, Nalivaika EA, Gross PJ, Valesano JC, Shindo K, Li M, Munson M, Royer WE, Harjes E, et al: Crystal structure of the APOBEC3G catalytic domain reveals potential oligomerization interfaces. Structure 2010, 18:28–38.

103. Chen KM, Harjes E, Gross PJ, Fahmy A, Lu Y, Shindo K, Harris RS, Matsuo H: Structure of the DNA deaminase domain of the HIV-1 restriction factor APOBEC3G. Nature 2008, 452:116–119.

104. Holden LG, Prochnow C, Chang YP, Bransteitter R, Chelico L, Sen U, Stevens RC, Goodman MF, Chen XS: Crystal structure of the anti-viral APOBEC3G catalytic domain and functional implications. Nature 2008, 456:121–124.

105. Huthoff H, Autore F, Gallois-Montbrun S, Fraternali F, Malim MH: RNAdependent oligomerization of APOBEC3G is required for restriction of HIV-1. PLoS Pathog 2009, 5:e1000330.

106. Wang X, Dolan PT, Dang Y, Zheng YH: Biochemical differentiation of APOBEC3F and APOBEC3G proteins associated with HIV-1 life cycle. J Biol Chem 2007, 282:1585–1594.

107. Kamata M, Nagaoka Y, Chen IS: Reassessing the role of APOBEC3G in human immunodeficiency virus type 1 infection of quiescent CD4+ Tcells. PLoS Pathog 2009, 5:e1000342.

108. de Sio FR Santoni, Trono D: APOBEC3G-depleted resting CD4+ T cells remain refractory to HIV1 infection. PLoS One 2009, 4:6571.

109. Goila-Gaur R, Khan MA, Miyagi E, Kao S, Opi S, Takeuchi H, Strebel K: HIV-1 Vif promotes the formation of high molecular mass APOBEC3G complexes. Virology 2008, 372:136–146.

110. Johnson VA, Calvez V, Gunthard HF, Paredes R, Pillay D, Shafer R, Wensing AM, Richman DD: 2011 update of the drug resistance mutations in HIV-1. Top Antivir Med 2011, 19:156–164.

111. Berkhout B, de Ronde A: APOBEC3G versus reverse transcriptase in the generation of HIV-1 drug-resistance mutations. AIDS 2004, 18:1861–1863.

112. Jern P, Russell RA, Pathak VK, Coffin JM: Likely role of APOBEC3G-mediated G-to-A mutations in HIV-1 evolution and drug resistance. PLoS Pathog 2009, 5:e1000367.

113. Suspene R, Rusniok C, Vartanian JP, Wain-Hobson S: Twin gradients in APOBEC3 edited HIV-1 DNA reflect the dynamics of lentiviral replication. Nucleic Acids Res 2006, 34:4677–4684.

114. Mulder LC, Harari A, Simon V: Cytidine deamination induced HIV-1 drug resistance. Proc Natl Acad Sci U S A 2008, 105:5501–5506.

115. Dang Y, Wang X, Zhou T, York IA, Zheng YH: Identification of a novel WxSLVK motif in the N terminus of human immunodeficiency virus and simian immunodeficiency virus Vif that is critical for APOBEC3G and APOBEC3F neutralization. J Virol 2009, 83:8544–8552.

116. Borrow P, Lewicki H, Hahn BH, Shaw GM, Oldstone MB: Virus-specific CD8+ cytotoxic T-lymphocyte activity associated with control of viremia in primary human immunodeficiency virus type 1 infection. J Virol 1994, 68:6103–6110.

117. Rinaldo C, Huang XL, Fan ZF, Ding M, Beltz L, Logar A, Panicali D, Mazzara G, Liebmann J, Cottrill M, et al: High levels of anti-human immunodeficiency virus type 1 (HIV-1) memory cytotoxic T-lymphocyte activity and low viral load are associated with lack of disease in HIV-1-infected long-term nonprogressors. J Virol 1995, 69:5838–5842.

118. Koup RA, Safrit JT, Cao Y, Andrews CA, McLeod G, Borkowsky W, Farthing C, Ho DD: Temporal association of cellular immune responses with the initial control of viremia in primary human immunodeficiency virus type 1 syndrome. J Virol 1994, 68:4650–4655.

119. Duffy S, Shackelton LA, Holmes EC: Rates of evolutionary change in viruses: patterns and determinants. Nat Rev Genet 2008, 9:267–276.

120. Domingo E, Holland JJ: RNA virus mutations and fitness for survival. Annu Rev Microbiol 1997, 51:151–178.

121. Frost SD, Nijhuis M, Schuurman R, Boucher CA, Brown AJ: Evolution of lamivudine resistance in human immunodeficiency virus type 1-infected individuals: the relative roles of drift and selection. J Virol 2000, 74:6262–6268.

122. Moya A, Holmes EC, Gonzalez-Candelas F: The population genetics and evolutionary epidemiology of RNA viruses. Nat Rev Microbiol 2004, 2:279–288.

123. Kim EY, Bhattacharya T, Kunstman K, Swantek P, Koning FA, Malim MH, Wolinsky SM: Human APOBEC3G-mediated editing can promote HIV-1 sequence diversification and accelerate adaptation to selective pressure. J Virol 2010, 84:10402–10405.

124. Yebra G, Holguin A: Mutation Vif-22 H, which allows HIV-1 to use the APOBEC3G hypermutation to develop resistance, could appear more quickly in certain non-B variants. J Antimicrob Chemother 2011, 66:941–942.

125. Fourati S, Malet I, Binka M, Boukobza S, Wirden M, Sayon S, Simon A, Katlama C, Simon V, Calvez V, Marcelin AG: Partially active HIV-1 Vif alleles facilitate viral escape from specific antiretrovirals. AIDS 2010, 24:2313–2321.

126. Harrer T, Harrer E, Kalams SA, Elbeik T, Staprans SI, Feinberg MB, Cao Y, Ho DD, Yilma T, Caliendo AM, et al: Strong cytotoxic T cell and weak neutralizing antibody responses in a subset of persons with stable nonprogressing HIV type 1 infection. AIDS Res Hum Retroviruses 1996, 12:585–592.

127. Shankar P, Russo M, Harnisch B, Patterson M, Skolnik P, Lieberman J: Impaired function of circulating HIV-specific CD8(+) T cells in chronic human immunodeficiency virus infection. Blood 2000, 96:3094–3101.

128. Heintel T, Sester M, Rodriguez MM, Krieg C, Sester U, Wagner R, Pees HW, Gartner B, Maier R, Meyerhans A: The fraction of perforin-expressing HIV-specific CD8 T cells is a marker for disease progression in HIV infection. AIDS 2002, 16:1497–1501.

129. Ogg GS, Jin X, Bonhoeffer S, Dunbar PR, Nowak MA, Monard S, Segal JP, Cao Y, Rowland-Jones SL, Cerundolo V, et al: Quantitation of HIV-1-specific cytotoxic T lymphocytes and plasma load of viral RNA. Science 1998, 279:2103–2106.

130. Yusim K, Kesmir C, Gaschen B, Addo MM, Altfeld M, Brunak S, Chigaev A, Detours V, Korber BT: Clustering patterns of cytotoxic T-lymphocyte epitopes in human immunodeficiency virus type 1 (HIV-1) proteins reveal imprints of immune evasion on HIV-1 global variation. J Virol 2002, 76:8757–8768.

131. Jones NA, Wei X, Flower DR, Wong M, Michor F, Saag MS, Hahn BH, Nowak MA, Shaw GM, Borrow P: Determinants of human immunodeficiency virus type 1 escape from the primary CD8+ cytotoxic T lymphocyte response. J Exp Med 2004, 200:1243–1256.

132. Tomiyama H, Fujiwara M, Oka S, Takiguchi M: Cutting Edge: Epitope-dependent effect of Nef-mediated HLA class I down-regulation on ability of HIV-1-specific CTLs to suppress HIV-1 replication. J Immunol 2005, 174:36–40.

133. Barouch DH, Letvin NL: Viral evolution and challenges in the development of HIV vaccines. Vaccine 2002, 20(Suppl 4):A66–A68.

134. Borrow P, Lewicki H, Wei X, Horwitz MS, Peffer N, Meyers H, Nelson JA, Gairin JE, Hahn BH, Oldstone MB, Shaw GM: Antiviral pressure exerted by HIV-1-specific cytotoxic T lymphocytes (CTLs) during primary infection demonstrated by rapid selection of CTL escape virus. Nat Med 1997, 3:205–211.

135. Geels MJ, Cornelissen M, Schuitemaker H, Anderson K, Kwa D, Maas J, Dekker JT, Baan E, Zorgdrager F, van den Burg R, et al: Identification of sequential viral escape mutants associated with altered T-cell responses in a human immunodeficiency virus type 1-infected individual. J Virol 2003, 77:12430–12440.

136. Wood N, Bhattacharya T, Keele BF, Giorgi E, Liu M, Gaschen B, Daniels M, Ferrari G, Haynes BF, McMichael A, et al: HIV evolution in early infection: selection pressures, patterns of insertion and deletion, and the impact of APOBEC. PLoS Pathog 2009, 5:e1000414.

137. Casartelli N, Guivel-Benhassine F, Bouziat R, Brandler S, Schwartz O, Moris A: The antiviral factor APOBEC3G improves CTL recognition of cultured HIV-infected T cells. J Exp Med 2010, 207:39–49.

138. Sakuma R, Noser JA, Ohmine S, Ikeda Y: Inhibition of HIV-1 replication by simian restriction factors, TRIM5alpha and APOBEC3G. Gene Ther 2007, 14:185–189.

139. Cen S, Peng ZG, Li XY, Li ZR, Ma J, Wang YM, Fan B, You XF, Wang YP, Liu F, et al: Small molecular compounds inhibit HIV-1 replication through specifically stabilizing APOBEC3G. J Biol Chem 2010, 285:16546–16552.

140. Chen H, Wang LW, Huang YQ, Gong ZJ: Interferon-alpha Induces High Expression of APOBEC3G and STAT-1 in Vitro and in Vivo. Int J Mol Sci 2010, 11:3501–3512.

141. Pillai SK, Wong JK, Barbour JD: Turning up the volume on mutational pressure: is more of a good thing always better? (A case study of HIV-1 Vif and APOBEC3). Retrovirology 2008, 5:26.

142. Vazquez-Perez JA, Ormsby CE, Hernandez-Juan R, Torres KJ, Reyes-Teran G: APOBEC3G mRNA expression in exposed seronegative and early stage HIV infected individuals decreases with removal of exposure and with disease progression. Retrovirology 2009, 6:23.

143. Jin X, Brooks A, Chen H, Bennett R, Reichman R, Smith H: APOBEC3G/ CEM15 (hA3G) mRNA levels associate inversely with human immunodeficiency virus viremia. J Virol 2005, 79:11513–11516.

144. Cho SJ, Drechsler H, Burke RC, Arens MQ, Powderly W, Davidson NO: APOBEC3F and APOBEC3G mRNA levels do not correlate with human immunodeficiency virus type 1 plasma viremia or CD4+ T-cell count. J Virol 2006, 80:2069–2072.

145. Mussil B, Sauermann U, Motzkus D, Stahl-Hennig C, Sopper S: Increased APOBEC3G and APOBEC3F expression is associated with low viral load and prolonged survival in simian immunodeficiency virus infected rhesus monkeys. Retrovirology 2011, 8:77.

146. Russell RA, Moore MD, Hu WS, Pathak VK: APOBEC3G induces a hypermutation gradient: purifying selection at multiple steps during HIV-1 replication results in levels of G-to-A mutations that are high in DNA, intermediate in cellular viral RNA, and low in virion RNA. Retrovirology 2009, 6:16.

147. Hu WS, Temin HM: Retroviral recombination and reverse transcription. Science 1990, 250:1227–1233.

148. Zhang J, Temin HM: Rate and mechanism of nonhomologous recombination during a single cycle of retroviral replication. Science 1993, 259:234–238.

149. Temin HM: Mixed infection with two types of Rous sarcoma virus. Virology 1961, 13:158–163.

150. Holland JJ, De La Torre JC, Steinhauer DA: RNA virus populations as quasispecies. Curr Top Microbiol Immunol 1992, 176:1–20.

151. Mansky LM, Temin HM: Lower in vivo mutation rate of human immunodeficiency virus type 1 than that predicted from the fidelity of purified reverse transcriptase. J Virol 1995, 69:5087–5094.

152. Coffin JM: Genetic diversity and evolution of retroviruses. Curr Top Microbiol Immunol 1992, 176:143–164.

153. Katz RA, Skalka AM: Generation of diversity in retroviruses. Annu Rev Genet 1990, 24:409–445.

154. Jetzt AE, Yu H, Klarmann GJ, Ron Y, Preston BD, Dougherty JP: High rate of recombination throughout the human immunodeficiency virus type 1 genome. J Virol 2000, 74:1234–1240.

155. Charpentier C, Nora T, Tenaillon O, Clavel F, Hance AJ: Extensive recombination among human immunodeficiency virus type 1 quasispecies makes an important contribution to viral diversity in individual patients. J Virol 2006, 80:2472–2482.

156. Chen J, Powell D, Hu WS: High frequency of genetic recombination is a common feature of primate lentivirus replication. J Virol 2006, 80: 9651–9658.

157. Onafuwa-Nuga A, Telesnitsky A: The remarkable frequency of human immunodeficiency virus type 1 genetic recombination. Microbiol Mol Biol Rev 2009, 73:451–480. Table of Contents.

158. Jung A, Maier R, Vartanian JP, Bocharov G, Jung V, Fischer U, Meese E, Wain-Hobson S, Meyerhans A: Recombination: Multiply infected spleen cells in HIV patients. Nature 2002, 418:144.

159. Nikolenko GN, Svarovskaia ES, Delviks KA, Pathak VK: Antiretroviral drug resistance mutations in human immunodeficiency virus type 1 reverse transcriptase increase template-switching frequency. J Virol 2004, 78:8761–8770.

160. Malim MH, Emerman M: HIV-1 sequence variation: drift, shift, and attenuation. Cell 2001, 104:469–472.

161. Van Hemert FJ, Berkhout B: The tendency of lentiviral open reading frames to become A-rich: constraints imposed by viral genome organization and cellular tRNA availability. J Mol Evol 1995, 41:132–140.

162. Berkhout B, van Hemert FJ: The unusual nucleotide content of the HIV RNA genome results in a biased amino acid composition of HIV proteins. Nucleic Acids Res 1994, 22:1705–1711.

163. Berkhout B, Das AT, Beerens N: HIV-1 RNA editing, hypermutation, and error-prone reverse transcription. Science 2001, 292:7.

164. Chelico L, Pham P, Goodman MF: Mechanisms of APOBEC3G-catalyzed processive deamination of deoxycytidine on single-stranded DNA. NatStruct Mol Biol 2009, 16:454–455. author reply 455–456.

Chapter 3

1. Sheehy AM, Gaddis NC, Choi JD, Malim MH. Isolation of a human gene that inhibits HIV-1 infection and is suppressed by the viral Vif protein. *Nature* 2002,418:646-650.

2. Harris RS, Petersen-Mahrt SK, Neuberger MS. RNA editing enzyme APOBEC1 and some of its homologs can act as DNA mutators. *Mol Cell* 2002,10:1247-1253.

3. Wedekind JE, Dance GS, Sowden MP, Smith HC. Messenger RNA editing in mammals: new members of the APOBEC family seeking roles in the family business. *Trends Genet* 2003,19:207-216.

4. Bishop KN, Holmes RK, Sheehy AM, Davidson NO, Cho SJ, Malim MH. Cytidine deamination of retroviral DNA by diverse APOBEC proteins. *Curr Biol* 2004,14:1392-1396.

5. Liddament MT, Brown WL, Schumacher AJ, Harris RS. APOBEC3F properties and hypermutation preferences indicate activity against HIV-1 in vivo. *Curr Biol* 2004,14:1385-1391.

6. Chiu YL, Soros VB, Kreisberg JF, Stopak K, Yonemoto W, Greene WC. Cellular APOBEC3G restricts HIV-1 infection in resting CD4+ T cells. *Nature* 2005,435:108-114.

7. Peng G, Lei KJ, Jin W, Greenwell-Wild T, Wahl SM. Induction of APOBEC3 family proteins, a defensive maneuver underlying interferon-induced anti-HIV-1 activity. *J Exp Med* 2006,203:41-46.

8. Peng G, Greenwell-Wild T, Nares S, Jin W, Lei KJ, Rangel ZG, *et al.* Myeloid differentiation and susceptibility to HIV-1 are linked to APOBEC3 expression. *Blood* 2007,110:393-400.

9. Pido-Lopez J, Whittall T, Wang Y, Bergmeier LA, Babaahmady K, Singh M, Lehner T. Stimulation of cell surface CCR5 and CD40 molecules by their ligands or by HSP70 up-regulates APOBEC3G expression in CD4(+) T cells and dendritic cells. *J Immunol* 2007,178:1671-1679.

10. Stopak KS, Chiu YL, Kropp J, Grant RM, Greene WC. Distinct patterns of cytokine regulation of APOBEC3G expression and activity in primary lymphocytes, macrophages, and dendritic cells. *J Biol Chem* 2007,282:3539-3546.

11. Koning FA, Newman EN, Kim EY, Kunstman KJ, Wolinsky SM, Malim MH. Defining APOBEC3 expression patterns in human tissues and hematopoietic cell subsets. *J Virol* 2009,83:9474-9485.

12. Pion M, Granelli-Piperno A, Mangeat B, Stalder R, Correa R, Steinman RM, Piguet V. APOBEC3G/3F mediates intrinsic resistance of monocyte-derived dendritic cells to HIV-1 infection. *J Exp Med* 2006,203:2887-2893.

13. Yu Q, Konig R, Pillai S, Chiles K, Kearney M, Palmer S, *et al.* Single-strand specificity of APOBEC3G accounts for minus-strand deamination of the HIV genome. *Nat Struct Mol Biol* 2004,11:435-442.

14. Lecossier D, Bouchonnet F, Clavel F, Hance AJ. Hypermutation of HIV-1 DNA in the absence of the Vif protein. *Science* 2003,300:1112.

15. Mangeat B, Turelli P, Caron G, Friedli M, Perrin L, Trono D. Broad antiretroviral defence by human APOBEC3G through lethal editing of nascent reverse transcripts. *Nature* 2003,424:99-103.

16. Zhang H, Yang B, Pomerantz RJ, Zhang C, Arunachalam SC, Gao L. The cytidine deaminase CEM15 induces hypermutation in newly synthesized HIV-1 DNA. *Nature* 2003,424:94-98.

17. Klarmann GJ, Chen X, North TW, Preston BD. Incorporation of uracil into minus strand DNA affects the specificity of plus strand synthesis initiation during lentiviral reverse transcription. *J Biol Chem* 2003,278:7902-7909.

18. Yu XF. Innate cellular defenses of APOBEC3 cytidine deaminases and viral counter-defenses. *Curr Opin HIV AIDS* 2006,1:187-193.

19. Santa-Marta M, Aires da Silva F, Fonseca AM, Rato S, Goncalves J. HIV-1 Vif protein blocks the cytidine deaminase activity of B-cell specific AID in E. coli by a similar mechanism of action. *Mol Immunol* 2007,44:583-590.

20. Sheehy AM, Gaddis NC, Malim MH. The antiretroviral enzyme APOBEC3G is degraded by the proteasome in response to HIV-1 Vif. *Nat Med* 2003,9:1404-1407.

21. Mehle A, Strack B, Ancuta P, Zhang C, McPike M, Gabuzda D. Vif overcomes the innate antiviral activity of APOBEC3G by promoting its degradation in the ubiquitin-proteasome pathway. *J Biol Chem* 2004,279:7792-7798.

22. Chiu YL, Greene WC. APOBEC3G: an intracellular centurion. *Philos Trans R Soc Lond B Biol Sci* 2009,364:689-703.

23. Wichroski MJ, Robb GB, Rana TM. Human retroviral host restriction factors APOBEC3G and APOBEC3F localize to mRNA processing bodies. *PLoS Pathog* 2006,2:e41.

24. Chiu YL. Biochemical fractionation and purification of high-molecular-mass APOBEC3G complexes. *Methods Mol Biol* 2011,718:185-206.

25. Gallois-Montbrun S, Kramer B, Swanson CM, Byers H, Lynham S, Ward M, Malim MH. Antiviral protein APOBEC3G localizes to ribonucleoprotein complexes found in P bodies and stress granules. *J Virol* 2007,81:2165-2178.

26. Russell RA, Moore MD, Hu WS, Pathak VK. APOBEC3G induces a hypermutation gradient: purifying selection at multiple steps during HIV-1 replication results in levels of G-to-A mutations that are high in DNA, intermediate in cellular viral RNA, and low in virion RNA. *Retrovirology* 2009,6:16.

27. Koup RA, Safrit JT, Cao Y, Andrews CA, McLeod G, Borkowsky W, *et al.* Temporal association of cellular immune responses with the initial control of viremia in primary human immunodeficiency virus type 1 syndrome. *J Virol* 1994,68:4650-4655.

28. Perez CL, Larsen MV, Gustafsson R, Norstrom MM, Atlas A, Nixon DF, *et al.* Broadly immunogenic HLA class I supertype-restricted elite CTL epitopes recognized in a diverse population infected with different HIV-1 subtypes. *J Immunol* 2008,180:5092-5100.

29. Almeida CA, Bronke C, Roberts SG, McKinnon E, Keane NM, Chopra A, *et al.* Translation of HLA-HIV associations to the cellular level: HIV adapts to inflate CD8 T cell responses against Nef and HLA-adapted variant epitopes. *J Immunol*,187:2502-2513.

30. Borrow P, Lewicki H, Hahn BH, Shaw GM, Oldstone MB. Virus-specific CD8+ cytotoxic T-lymphocyte activity associated with control of viremia in primary human immunodeficiency virus type 1 infection. *J Virol* 1994,68:6103-6110.

31. Rinaldo C, Huang XL, Fan ZF, Ding M, Beltz L, Logar A, *et al.* High levels of anti-human immunodeficiency virus type 1 (HIV-1) memory cytotoxic T-lymphocyte activity and low viral load are associated with lack of disease in HIV-1-infected long-term nonprogressors. *J Virol* 1995,69:5838-5842.

32. Harrer T, Harrer E, Kalams SA, Elbeik T, Staprans SI, Feinberg MB, *et al.* Strong cytotoxic T cell and weak neutralizing antibody responses in a subset of persons with stable nonprogressing HIV type 1 infection. *AIDS Res Hum Retroviruses* 1996,12:585-592.

33. Shankar P, Russo M, Harnisch B, Patterson M, Skolnik P, Lieberman J. Impaired function of circulating HIV-specific CD8(+) T cells in chronic human immunodeficiency virus infection. *Blood* 2000,96:3094-3101.

34. Borrow P, Lewicki H, Wei X, Horwitz MS, Peffer N, Meyers H, *et al.* Antiviral pressure exerted by HIV-1-specific cytotoxic T lymphocytes (CTLs) during primary infection demonstrated by rapid selection of CTL escape virus. *Nat Med* 1997,3:205-211.

35. Barouch DH, Letvin NL. Viral evolution and challenges in the development of HIV vaccines. *Vaccine* 2002,20 Suppl 4:A66-68.

36. Jones NA, Wei X, Flower DR, Wong M, Michor F, Saag MS, *et al.* Determinants of human immunodeficiency virus type 1 escape from the primary CD8+ cytotoxic T lymphocyte response. *J Exp Med* 2004,200:1243-1256.

37. Leslie AJ, Pfafferott KJ, Chetty P, Draenert R, Addo MM, Feeney M, *et al.* HIV evolution: CTL escape mutation and reversion after transmission. *Nat Med* 2004,10:282-289.

38. Leslie A, Kavanagh D, Honeyborne I, Pfafferott K, Edwards C, Pillay T, *et al.* Transmission and accumulation of CTL escape variants drive negative associations between HIV polymorphisms and HLA. *J Exp Med* 2005,201:891-902.

39. Geels MJ, Cornelissen M, Schuitemaker H, Anderson K, Kwa D, Maas J, *et al.* Identification of sequential viral escape mutants associated with altered T-cell responses in a human immunodeficiency virus type 1-infected individual. *J Virol* 2003,77:12430-12440.

40. O'Connor DH, Allen TM, Vogel TU, Jing P, DeSouza IP, Dodds E, *et al.* Acute phase cytotoxic T lymphocyte escape is a hallmark of simian immunodeficiency virus infection. *Nat Med* 2002,8:493-499.

41. Monajemi M, Woodworth CF, Benkaroun J, Grant M, Larijani M. Emerging complexities of APOBEC3G action on immunity and viral fitness during HIV infection and treatment. *Retrovirology*,9:35.

42. Wood N, Bhattacharya T, Keele BF, Giorgi E, Liu M, Gaschen B, *et al.* HIV evolution in early infection: selection pressures, patterns of insertion and deletion, and the impact of APOBEC. *PLoS Pathog* 2009,5:e1000414.

43. Casartelli N, Guivel-Benhassine F, Bouziat R, Brandler S, Schwartz O, Moris A. The antiviral factor APOBEC3G improves CTL recognition of cultured HIV-infected T cells. *J Exp Med* 2010,207:39-49.

44. Alizon M, Wain-Hobson S, Montagnier L, Sonigo P. Genetic variability of the AIDS virus: nucleotide sequence analysis of two isolates from African patients. *Cell* 1986,46:63-74.

45. Julia P, Katrin Z, Natasha H, Maureen G, Michael G. Human immunodeficiency virus type I-specific CD8+ T cell subset abnormalities in chronic infection persist through effective antiretroviral therapy. *BMC Infectious Diseases*,10.

46. Brennan CA, Ibarrondo FJ, Sugar CA, Hausner MA, Shih R, Ng HL, *et al.* Early HLA-B*57-Restricted CD8+ T Lymphocyte Responses Predict HIV-1 Disease Progression. *J Virol.*

47. Cao K, Hollenbach J, Shi X, Shi W, Chopek M, Fernandez-Vina MA. Analysis of the frequencies of HLA-A, B, and C alleles and haplotypes in the five major ethnic groups of the United States reveals high levels of diversity in these loci and contrasting distribution patterns in these populations. *Hum Immunol* 2001,62:1009-1030.

48. Altfeld M, Kalife ET, Qi Y, Streeck H, Lichterfeld M, Johnston MN, *et al.* HLA Alleles Associated with Delayed Progression to AIDS Contribute Strongly to the Initial CD8(+) T Cell Response against HIV-1. *PLoS Med* 2006,3:e403.

49. Flores-Villanueva PO, Yunis EJ, Delgado JC, Vittinghoff E, Buchbinder S, Leung JY, *et al.* Control of HIV-1 viremia and protection from AIDS are associated with HLA-Bw4 homozygosity. *Proc Natl Acad Sci U S A* 2001,98:5140-5145.

50. Rohowsky-Kochan C, Skurnick J, Molinaro D, Louria D. HLA antigens associated with susceptibility/resistance to HIV-1 infection. *Hum Immunol* 1998,59:802-815.

51. Pelak K, Goldstein DB, Walley NM, Fellay J, Ge D, Shianna KV, *et al.* Host determinants of HIV-1 control in African Americans. *J Infect Dis*,201:1141-1149.

52. Migueles SA, Sabbaghian MS, Shupert WL, Bettinotti MP, Marincola FM, Martino L, *et al.* HLA B*5701 is highly associated with restriction of virus replication in a subgroup of HIV-infected long term nonprogressors. *Proc Natl Acad Sci U S A* 2000,97:2709-2714.

53. Lambotte O, Boufassa F, Madec Y, Nguyen A, Goujard C, Meyer L, *et al.* HIV controllers: a homogeneous group of HIV-1-infected patients with spontaneous control of viral replication. *Clin Infect Dis* 2005,41:1053-1056.

54. Carrington M, Nelson GW, Martin MP, Kissner T, Vlahov D, Goedert JJ, *et al.* HLA and HIV-1: heterozygote advantage and B*35-Cw*04 disadvantage. *Science* 1999,283:1748-1752.

55. Fellay J, Shianna KV, Ge D, Colombo S, Ledergerber B, Weale M, *et al.* A whole-genome association study of major determinants for host control of HIV-1. *Science* 2007,317:944-947.

56. Emu B, Sinclair E, Hatano H, Ferre A, Shacklett B, Martin JN, *et al.* HLA class I-restricted T-cell responses may contribute to the control of human immunodeficiency virus infection, but

such responses are not always necessary for long-term virus control. *J Virol* 2008,82:5398-5407.

57. Poropatich K, Sullivan DJ, Jr. Human immunodeficiency virus type 1 long-term non-progressors: the viral, genetic and immunological basis for disease non-progression. *J Gen Virol*,92:247-268.

58. Sette A, Sidney J. Nine major HLA class I supertypes account for the vast preponderance of HLA-A and -B polymorphism. *Immunogenetics* 1999,50:201-212.

59. Brumme ZL, John M, Carlson JM, Brumme CJ, Chan D, Brockman MA, *et al.* HLA-associated immune escape pathways in HIV-1 subtype B Gag, Pol and Nef proteins. *PLoS One* 2009,4:e6687.

60. Novitsky V, Cao H, Rybak N, Gilbert P, McLane MF, Gaolekwe S, *et al.* Magnitude and frequency of cytotoxic T-lymphocyte responses: identification of immunodominant regions of human immunodeficiency virus type 1 subtype C. *J Virol* 2002,76:10155-10168.

61. Karlsson AC, Iversen AK, Chapman JM, de Oliviera T, Spotts G, McMichael AJ, *et al.* Sequential broadening of CTL responses in early HIV-1 infection is associated with viral escape. *PLoS One* 2007,2:e225.

62. Bishop KN, Holmes RK, Malim MH. Antiviral potency of APOBEC proteins does not correlate with cytidine deamination. *J Virol* 2006,80:8450-8458.

63. Wang X, Dolan PT, Dang Y, Zheng YH. Biochemical differentiation of APOBEC3F and APOBEC3G proteins associated with HIV-1 life cycle. *J Biol Chem* 2007,282:1585-1594.

64. Betts MR, Krowka JF, Kepler TB, Davidian M, Christopherson C, Kwok S, *et al.* Human immunodeficiency virus type 1-specific cytotoxic T lymphocyte activity is inversely correlated with HIV type 1 viral load in HIV type 1-infected long-term survivors. *AIDS research and human retroviruses* 1999,15:1219-1228.

65. Huang XL, Fan Z, Kalinyak C, Mellors JW, Rinaldo Jr CR. CD8+ T-cell gamma interferon production specific for human immunodeficiency virus type 1 (HIV-1) in HIV-1-infected subjects. *Clinical and diagnostic laboratory immunology* 2000,7:279-287.

66. Altfeld M, Rosenberg ES, Shankarappa R, Mukherjee JS, Hecht FM, Eldridge RL, *et al.* Cellular immune responses and viral diversity in individuals treated during acute and early HIV-1 infection. *J Exp Med* 2001,193:169-180.

67. Letvin NL, Walker BD. HIV versus the immune system: another apparent victory for the virus. *Journal of Clinical Investigation* 2001,107:273-275.

68. Smith HC. APOBEC3G: a double agent in defense. *Trends Biochem Sci* 2011,36:239-244.

69. Almeida CA, Roberts SG, Laird R, McKinnon E, Ahmad I, Keane NM, *et al.* Exploiting knowledge of immune selection in HIV-1 to detect HIV-specific CD8 T-cell responses. *Vaccine*,28:6052-6057.

70. Zichun Wang, Benjamin Metcalf1, Ruy M. Ribeiro3, Harold McClure2, and Amitinder Kaur1. Th-1-Type Cytotoxic $CD8^+$ T-Lymphocyte Responses to Simian Immunodeficiency Virus (SIV) Are a Consistent Feature of Natural SIV Infection in Sooty Mangabeys. *JVI*, 80:2771-2783.