RESTRICTION OF VIF-COMPETENT HIV-1 BY PHYSIOLOGICAL LEVELS OF APOBEC3G IN PRIMARY T-HELPER CELLS

by

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LIST OF ABBREVIATIONS

AID	=	Activation Induced Deaminse
AIDS	=	Acquired Immune Deficiency Syndrome
APC	=	Allophycocyanin
APOBEC1	=	apolipoprotein B mRNA editing enzyme, catalytic polypeptide 1
APOBEC3	=	apolipoprotein B mRNA editing enzyme, catalytic polypeptide like, 3
A3F	=	Human APOBEC3F
A3G	=	Human APOBEC3G
CCR5	=	Chemokine Receptor (C-C motif) 5
CXCR4	=	Chemokine Receptor (C-X-C motif) 4
DNA	=	Deoxyribonucleic acid
FACS	=	Fluorescence activated cell sorting
Foxp3	=	forkhead box P3
GAPDH	=	glyceraldehyde-3-phosphate dehydrogenase
GATA3	=	GATA Binding Protein 3
GFP	=	Green Fluorescent Protein
HDV	=	HIV-derived vector
HMM	=	High Molecular Mass A3G
HIV	=	Human Immunodeficiency Virus
IFN	=	Interferon

IL	=	Interleukin
LMM	=	Low Molecular Mass APOBEC3G
LTR	=	Long Terminal Repeat
PBMC	=	Peripheral Blood Mononuclear Cells
PE	=	Phycoerythrin
PEI	=	Polyethylenimine
PIC	=	Pre-Integration Complex
qPCR	=	Quantitative Real Time Polymerase Chain Reaction
RNA	=	Ribonucleic Acid
RT	=	Reverse Transcriptase
shRNA	=	Short hairpin Ribonucleic Acid
siRNA	=	Small interfering Ribonucleic Acid
Th	=	T Helper
Treg	=	Regulatory T- Cell
Tbet	=	T Box Expressed in T Cells
TCR	=	T Cell Receptor
STAT-3	=	Signal transducer and activator of transcription 3
Vif	=	Viral Infectivity Factor
VSV-G	=	Vesicular Stomatitis Virus glycoprotein

CHAPTER I

BACKGROUND AND RESEARCH OBJECTIVES

H.I.V.

As a virus, the Human Immunodeficiency Virus (H.I.V) is an obligate intracellular parasite. Machinery and resources provided by a host cell are required to complete its life cycle. As viruses and cells have co-evolved, viruses have changed to better utilize host machinery. Cells have also evolved innate defenses, separate from the immune system, to combat invading viral pathogens. This point is particularly evident in the replication of HIV-1 and the effects of host proteins in the APOBEC3 family, including primate APOBEC3G and APOBEC3F, on that replication.

HIV infects human cells known as CD4+ T lymphocytes and macrophages. These cells are important cell types in the robust immune defense against invading pathogens. The human immune system is comprised of innate and adaptive immunity. Innate immunity consists of responses to pathogens based on general pattern recognition mechanisms, but the magnitude or promptness of these responses does not increase with repeated exposure to a pathogen. Adaptive immunity refers to the recognition of pathogens by specific cells of the immune system, lymphocytes, and subsequent expansion of those pathogen specific cells. This does allow ‘immunologic memory” that improves and speeds recall responses. There are two main classes of lymphocytes, B-

cells and T- Cells. Each lymphocyte expresses a receptor that is specific for some antigen. B-cells, when activated by their antigen, secrete antibodies to bind and help neutralize the invading pathogen. There are two major types of T-cells, CD4+ and CD8+, so designated due to the major distinguishing receptor expressed on their surface. CD8+ cells, also known as cytotoxic T-lymphocytes, recognize and kill infected cells in order to clear the pathogen within. CD4+ T cells are known as helper T cells since they are responsible for providing stimulatory signals for the proper pathogen-specific set of B-cells and CD8+ T cells to be activated to optimal function. Macrophages are involved in both the innate and adaptive responses. [1]

Without CD4+ T cells, the cells of the adaptive immune system would lack the proper help and signals they need to mount the appropriate initial or recall response to a particular pathogen. The infection of CD4+ T cells by HIV-1 leads to an eventual decline in the CD4+ T cell number that leaves the infected individual vulnerable to pathogens that are normally easily recognized and cleared by the immune system of uninfected individuals. This vulnerability, known as an immune deficiency, is what leads to Acquired Immune Deficiency Syndrome, AIDS, and the death of HIV infected individuals by succumbing to opportunistic infections.

Early Steps of HIV Replication

HIV begins its replication process by entry into the target cell (Fig. 1-1).

To achieve entry the HIV envelope surface glycoprotein, gp120, first binds to the CD4 molecule on its host's target cell.

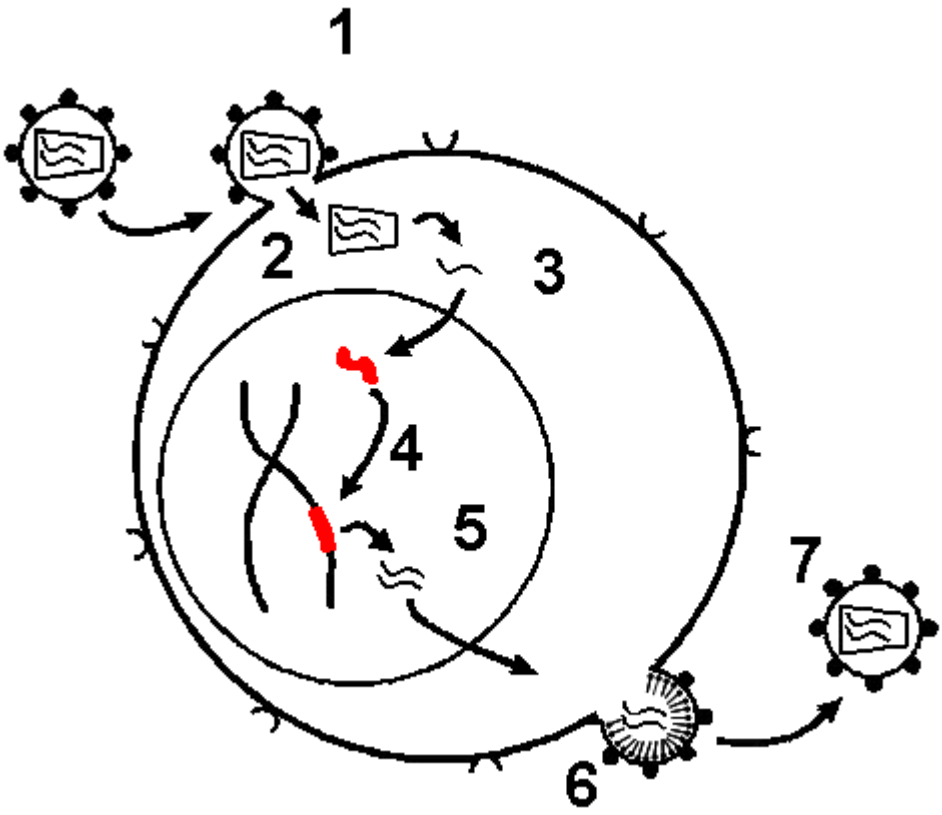


Figure 1-1. Overview of HIV Replication Cycle. HIV begins its replication by binding to CD4 and co-receptor either CXCR4 or CCR5 (1). After binding, fusion of the virion's lipid membrane and the cellular membrane occurs which allows the core of the virion containing the RNA genome to be released and uncoat (2). Reverse transcription occurs and the reverse transcribed DNA products (red) are translocated to the cellular nucleus as a pre-intergration complex (3). Once in the nucleus, the HIV integrase integrates the HIV DNA into the cellular chromosome in conjunction with cellular proteins (4). Viral RNAs are transcribed from the integrated DNA, translated in the cellular cytoplasm and these proteins translocate to the membrane of the cell (5). The congregated viral proteins bud out of the cell, eventually pinching off to form an immature virion (6). The viral protease cleaves proteins within the virion to form the viral core making the virion mature and infectious (7).

Adapted from *library.med.utah.edu/WebPath/TUTORIAL/AIDS/AIDS005.html* accessed on1/20/2009

It then undergoes a conformational change to engage either the CXCR4 or CCR5 co-receptor, and finally the gp41 transmembrane protein mediates fusion of virus and cell membranes [2] (Figure 1-2). HIV viruses can either bind the CXCR4 co-receptor or the CCR5 co-receptor or both and are referred to as X4 tropic, R5 tropic or dual tropic, respectively. After fusion, the viral core containing the diploid RNA genome is then released, and uncoats to release the viral ribonucleoprotein complex into the target cell cytoplasm. The uncoating process is not yet well characterized. It is known that it is a process that must be regulated to achieve full infectivity and can be affected by host cellular proteins [3,4]

The process of translating the diploid, single-stranded RNA genome into double-stranded DNA, known as reverse transcription, begins after/during uncoating. The process of reverse transcribing the HIV RNA genome begins with the reverse transcriptase (RT) recognizing a virion-packaged host transfer RNA ($tRNA_{lys3}$) bound to a site in the RNA genome known as the primer binding site (PBS) (Figure 1-3). The viral RT recognizes this duplex and begins transcribing 3' to 5' to form a complementary DNA strand (red in Figure 1-3) until it reaches the 5' end of the RNA genome (black in Figure 1-3). This stretch of DNA, known as strong stop DNA, is translocated to the 3' end of the RNA genome in what is known as the first strand transfer. The minus-strand, strong-stop DNA produced during the first steps of reverse transcription is known as an early RT product (Teal "ERT" in Figure 1-3).

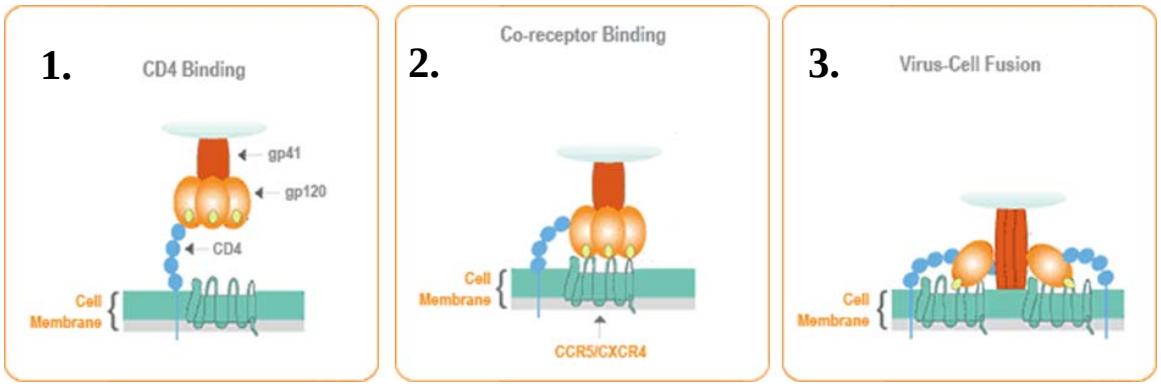


Figure 1-2 HIV Fusion. HIV gp120 recognizes and binds to the host target cell CD4 molecule (1). After a conformational change, gp120 then interacts with either the co-receptor CXCR4 or CCR5 (2). Subsequently, the HIV gp41 facilitates fusion of the viral and target cell membrane (3).

Adapted from *http://www.trofileassay.com/Viral_Entry* accessed on 02/04/09

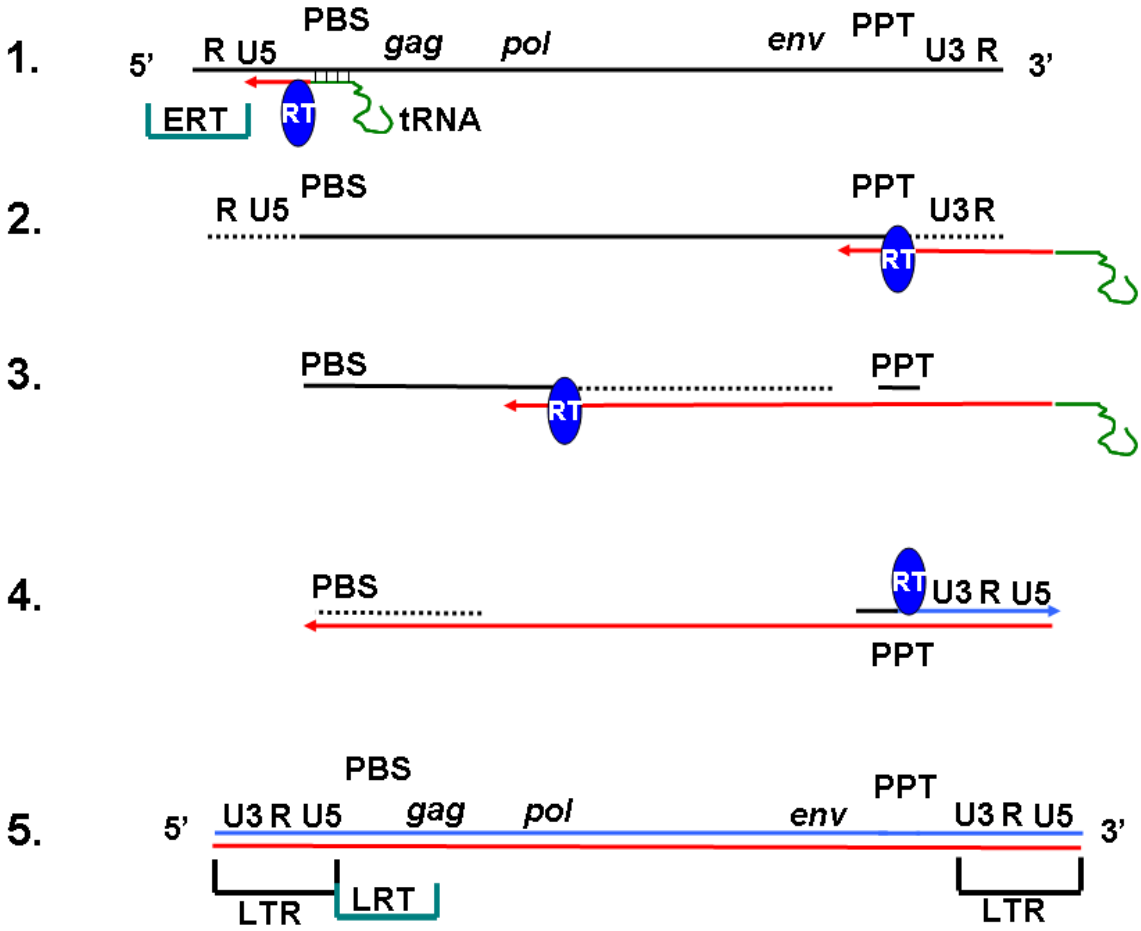


Figure 1-3 HIV Reverse Transcription. The packaged cellular transfer RNA (tRNA) binds to the primer binding site (PBS). This duplex is recognized by HIV reverse transcriptase (RT) and RT begins to polymerize a negative sense DNA strand (1). The –DNA strand is transferred to the 3' end of the HIV RNA genome and polymerization continues. The RNAse H activity of RT degrades the RNA genome as it polymerizes a DNA copy (2) The polypurine tract (PPT) is resistant to this degradation (3). RT recognizes the duplex of the PPT and the –DNA strand and begins to polymerize the +DNA strand (4). Following a second strand transfer from the 3' end to the 5'end, RT completes the +DNA strand resulting in two complementary long terminal repeats (LTR). qPCR based assays to quantify the amount of reverese transcription completed use primers that amplify early RT products (ERT) and late RT products (LRT)

RT continues to form a complementary (minus-strand) DNA (red, in Fig 1-3) by copying the genome RNA. The formation of the minus-strand DNA strand results in a RNA-DNA hybrid. The reverse transcriptase enzyme has a second enzymatic activity, RNAse H, which recognizes a hybrid RNA-DNA duplex as the polymerases slides down the RNA. It digests the RNA strand, transiently leaving a single-stranded, negative-sense DNA. Once this negative-sense, single-strand DNA is completed, the RT enzyme uses the negative-sense DNA strand as a template to construct a complementary positive strand. The positive strand synthesis begins to be primed from RNA left undigested at the central poly-purine tract (PPT) (black in step 4, Fig 1-3). The positive strand of DNA (blue in Figure 1-3) undergoes a second strand transfer to hybridize to the 3' end of the minus strand DNA before plus-strand DNA is completely copied, yielding a double stranded RT product. The late RT product reflects near completion of synthesis following second strand transfer (Teal "LRT" in Figure 1-3).

Once reverse transcription is complete, the DNA copy of the viral genome, a completed reverse transcription product, translocates to the nucleus of the cell in a structured protein/nucleic acid complex known as the pre-integration complex or PIC. The PIC is translocated to the nucleus most likely by interactions with host cell motor proteins and the cytoskeleton. Once at the nuclear envelope, the PIC, which is much larger than any nuclear pore, is moved across the nucleus in a manner that is poorly understood. Some studies have described interactions of virally encoded proteins interacting with importins and nucleoporins [5], but others argue the transportin family of proteins is involved

[6]. The integration of the completed RT product involves processing of the 3' ends of the viral DNA genome by integrase
as well as cutting the host chromosomal DNA. Subsequently the integrase protein joins the ends of the chromosomal DNA and the viral DNA and this break is repaired by host DNA repair machinery [7]

Not all HIV reverse transcription products, however, are able to fully complete the integration process to become a provirus. Some RT products that have entered the nucleus fail to integrate and become substrates for host DNA end-joining enzymes in the nucleus [8]. This process can result in homologous recombination of the long terminal repeats at the 5' and 3' end of the HIV genome (Figure 1-3) giving rise to a 1-LTR circle or joining of the 2 long terminal repeats resulting in a 2-LTR circle. The formation of these circular products is commonly used as a measure of nuclear entry and failed integration events [9] [10].

Late Steps of HIV Replication

Once integrated, the HIV-1 genome encodes six accessory genes, Nef, Vpu, Vpr, Tat, Rev and Vif in addition to the three structural genes, Gag, Pol and Env (Fig1-4). Transcription of the viral genes is driven by the proviral LTR and regulated by the accessory protein tat, one of the first genes to be translated [11]. Early transcripts of the viral genome remain in the nucleus and are processed by the host cellular splicing machinery.

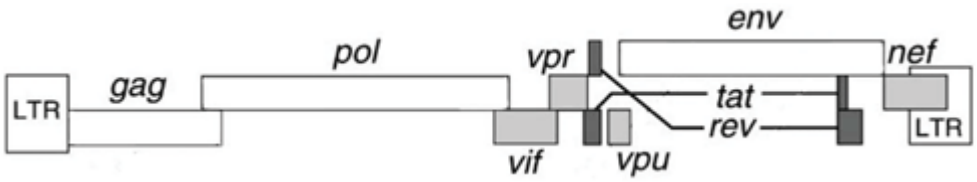


Figure 1-4 Genome of HIV. The HIV-1 genome consists of 3 structural genes *gag,pol* and *env*. *Gag* encodes for the matrix, capsid and nucleocapsid proteins. *Pol* encodes for the reverse transcriptase and integrase enzymes. *Env* encodes for the envelope protein. The genome also contains 6 accessory genes *vif, vpr,vpu,tat,rev* and *nef*.

As concentrations of the protein Rev (encoded by a multiply-spliced early transcript) increase, longer transcripts that encode for polyproteins like Gag-Pol, Pol and Env and the full genome are translocated out of the nucleus in a Rev-dependent manner without splicing. Once these longer RNAs are translated, the structural proteins they encode self-assemble into virions at the surface of the infected cell and bud off. These particles are not yet infectious. After budding, the viral protease processes the Pol polyprotein into protease, reverse transcriptase and integrase, as well as cleaving the Gag-Pol polyprotein into matrix (MA) capsid (CA) and nucleocapsid (NC). This leads to formation of the internal conical core and a mature, infectious virion. Though the structural genes serve specific, fairly well-defined roles in the viral life cycle, each of the accessory gene products has been described to have numerous activities within the life cycle of the virus and their functional characterization is not yet complete. Only recently has the specific functional role of the viral infectivity factor, *vif,* been elucidated.

APOBEC3

It has been recognized for several years that certain immortalized CD4+T cell lines, and all primary CD4+ T cells, produced virions that were not infectious when infected with laboratory strains of HIV that had a deletion of the *vif* gene. [12,24]. As they produced virions that were not infectious, these cells were termed “non-permissive” for *vif*-deleted HIV-1. Cell types that produced equally infectious virions from wild type and *vif*-deleted HIV were termed “permissive” cells. More recent studies have revealed that the difference between the two cell

types responsible for this phenotype was that non-permissive cells expressed an enzyme first known as CEM15 and now called APOBEC3G [13].

APOBEC3G (A3G), which stands for apolipoporotein B mRNA editing enzyme, catalytic-like 3G, belongs to a larger family of proteins which all share a common active site motif important for their enzymatic activity of cytidine deamination [14]. This name was chosen because of homology to a previously described gene, APOBEC1, which is involved in editing the mRNA of apolipoporotein B in gut epithelial cells to regulate production of lipoproteins APOB100 or APOB48 [15]. APOBEC1 is the only family member described to act on RNA. Activation induced deaminase (AID) is another well described APOBEC family member involved in antibody maturation via cytidine deamination of nuclear DNA. The somatic hypermutation introduced into antibody genes by AID allows selection for antibodies with higher binding affinity to an antigen. APOBEC2 and APOBEC4 are also members of the larger APOBEC family though their functions are less well understood[16,17]. A3G's more closely related family members, APOBEC3A, B, C, DE, F, and H are variable gene duplications all residing consecutively on human chromosome 22. Each gene contains at least one cytidine deaminase active site. Although APOBEC3B, DE, G, and F contain two cytidine deaminase domains, only one domain shows enzymatic activity (the carboxy terminal domain in 3F and 3G, Fig1-5) [14,18].

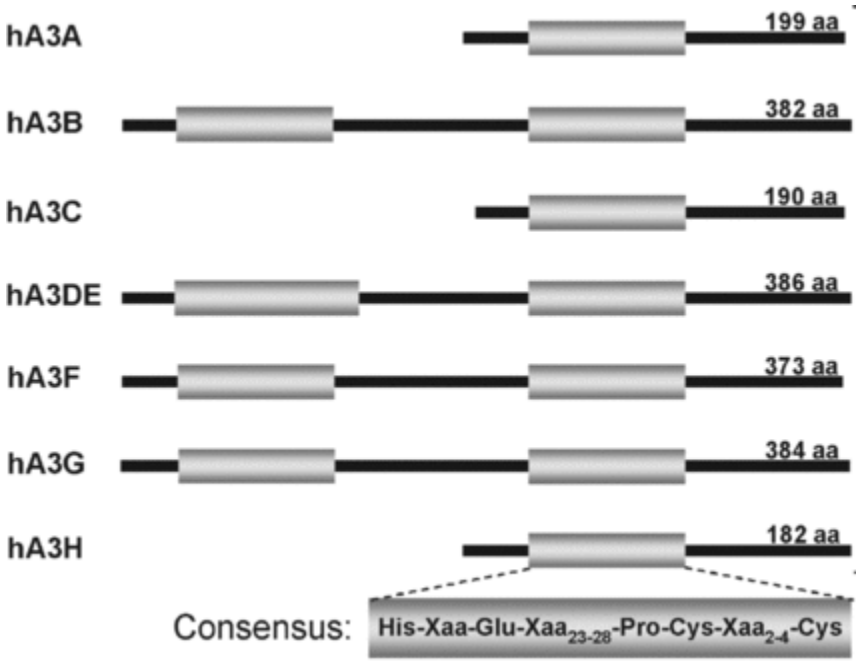


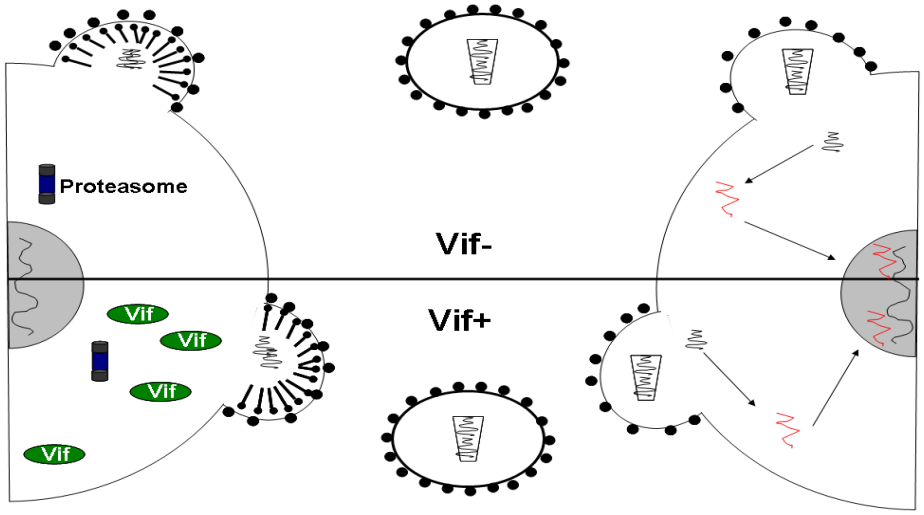
Figure 1-5 APOBEC3 Family: APOBEC3 proteins A-H are the result of gene duplications on human chromosome 22. Some the members contain two consensus deaminase domains, but others have only a single domain. The enzymatic activity has been determined to be in the C terminal of the two domains for A3G and A3F, with the N terminal domain having no cytidine deaminase enzymatic activity.

Adapted from Schumann, G.G. *Biochem. Soc. Trans.* APOBEC3 proteins: major players in intracellular defence against LINE-1-mediated retrotransposition (2007) 35, (637–642)

During formation of a nascent HIV virion in a non-permissive cell, A3G is packaged into the virion via interaction between its enzymatically inactive amino-terminal deaminase domain and the HIV nucleocapsid (encoded in the *gag* gene) [19-21]. Either the HIV genomic RNA or specific cellular RNAs may also be involved in packaging [22]. During or soon after reverse transcription in the subsequent target cell, A3G exerts its antiviral activity through several proposed mechanisms (Fig 1-6). The transiently single-stranded, negative-sense DNA is the substrate for A3G's cytidine deaminase enzymatic activity [23]. A3G binds to the single stranded DNA and deaminates cytosine residues, converting them to uracil. Adenine can hydrogen bond to uracil. Therefore, when the positive-sense, second strand of DNA is polymerized, adenine is base paired with the uracil. This base replacement is commonly referred to as G to A hypermutation because so many of the positive strand Gs in the HIV provirus are mutated to As by this process. This had been well documented in HIV-1 provirus sequences before the discovery of A3G [24,25]

Hypermutation of the viral genome during reverse transcription can reduce the infectivity of HIV-1 in several proposed ways [26]. First, DNA repair enzymes known as uracil glycosylayses may digest the uracil-containing DNA, degrading incomplete reverse transcripts. There is however no convincing evidence to date that uracil-containing incomplete reverse transcripts are degraded in the infected cell [27,28]. In addition, a second well-documented mechanism for a deaminase-mediated antiviral effect involves mutations in hypermutated, integrated genomes that result in malformed proteins or code for premature stop codons [23,29,30].

A. Permissive



B. Non-Permissive

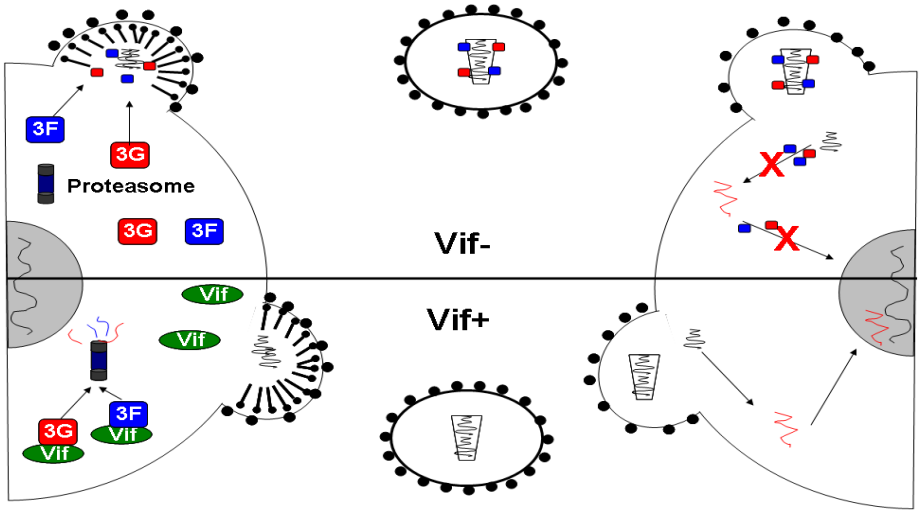


Figure 1-6 APOBEC3 Virion Packaged Effect: Permissive cells (A) do not express APOBEC3 proteins. A vif+ and vif- virus produced from permissive cells is therefore fully infectious when infecting a subsequent target cell. Non-permissive cells (B) do express APOBEC3 proteins. Vif- virions produced from non-permissive cells contain APOBEC3 proteins. These enzymes act in the target cell to interfere with the reverse transcription and integration. If vif is expressed in a non-permissive cell, it binds to the APOBEC3 proteins and targets them for degradation by the cellular proteosome. This reduces virion packaging of the enzymes and allows for normal reverse transcription and integration in the target cell.

There are conflicting reports about whether the *in vivo* level of A3G RNA in PBMCs correlates with HIV proviral genome hypermutation; one report shows a correlation supporting the theory that deaminase activity is involved in the reduction of HIV-1 infectivity [31] while another does not [32].

Besides these deaminase-dependent mechanisms, deaminase-independent mechanisms for A3G reduction of HIV infectivity have also been proposed. Studies have demonstrated that enzymatically inactive A3G packaged into the virion is still capable of reducing the infectivity of HIV-1 [33-35]. Additionally, cell-free reverse transcription assays have shown that A3G can reduce viral DNA synthesis with no apparent editing [36]. This finding is corroborated by results showing A3G's ability to block reverse transcription elongation and tRNA priming [37,38]. A3G has also been shown to affect integration independent of its deamination activity. Multiple reports have demonstrated that A3G packaged in a virion reduces the amount of HIV DNA that is integrated into the host genomes by binding to integrase or modifying the completed RT product [39,40]. These findings suggest that though A3G is a cytidine deaminase, that enzymatic activity may not be the only or the dominant mechanism it utilizes to reduce HIV infectivity.

HIV-1 has developed a mechanism to counteract the affects of APOBEC3G in the virion through the use of its accessory gene *vif*. When expressed, the viral infectivity factor (vif) binds to A3G [41,42] in the cytoplasm of producer cells and recruits Cullin5, elongin B and C in addition to other proteins involved in ubiquitin ligation [43]. This allows the A3G –vif complex to be

ubiquitinated and subsequently degraded, reducing the amount of A3G in the cell. The same mechanism has been described for reduction of A3F [44]. Vif's activity thereby limits the amount of A3G and A3F that can be packaged in the virion. Though *vif* genes with reduced activity can be indentified in patient samples, they generally represent only a minority of the circulating virus in an infected patient [45,46] except for rare cases of long-term non-progressors [47]

A second, vif insensitive, anti-HIV-1 activity of A3G has also been described. Resting CD4+ T cells are known to be refractory to HIV-1 infection [48,49]. However, removal of A3G by siRNA renders resting cells susceptible to infection [50]. Further studies by Chiu *et. al.* suggest that A3G can exist in two forms in the cytoplasm of a T-cell; a "low molecular mass", enzymatically active form and a higher order, "high molecular mass" enzymatically inactive ribonucleoprotein complex (Fig 1-7) [50]. Their results show that the cytoplasmic A3G in resting cells is of the enzymatically active form and restricts incoming HIV infection even if there is no APOBEC3 in the incoming virion. They conclude that activation of a T- cell moves the A3G to the high molecular mass form relieving the restriction on infection.

These studies show A3G to be a dynamic protein. However, there is little currently in the literature about the regulation of APOBEC3G, particularly in regards to its expression. Activation of a CD4+ T-cell alters the physical characteristics of the protein, but studies have also shown that some T cell activation signals also increase expression of A3G [51].

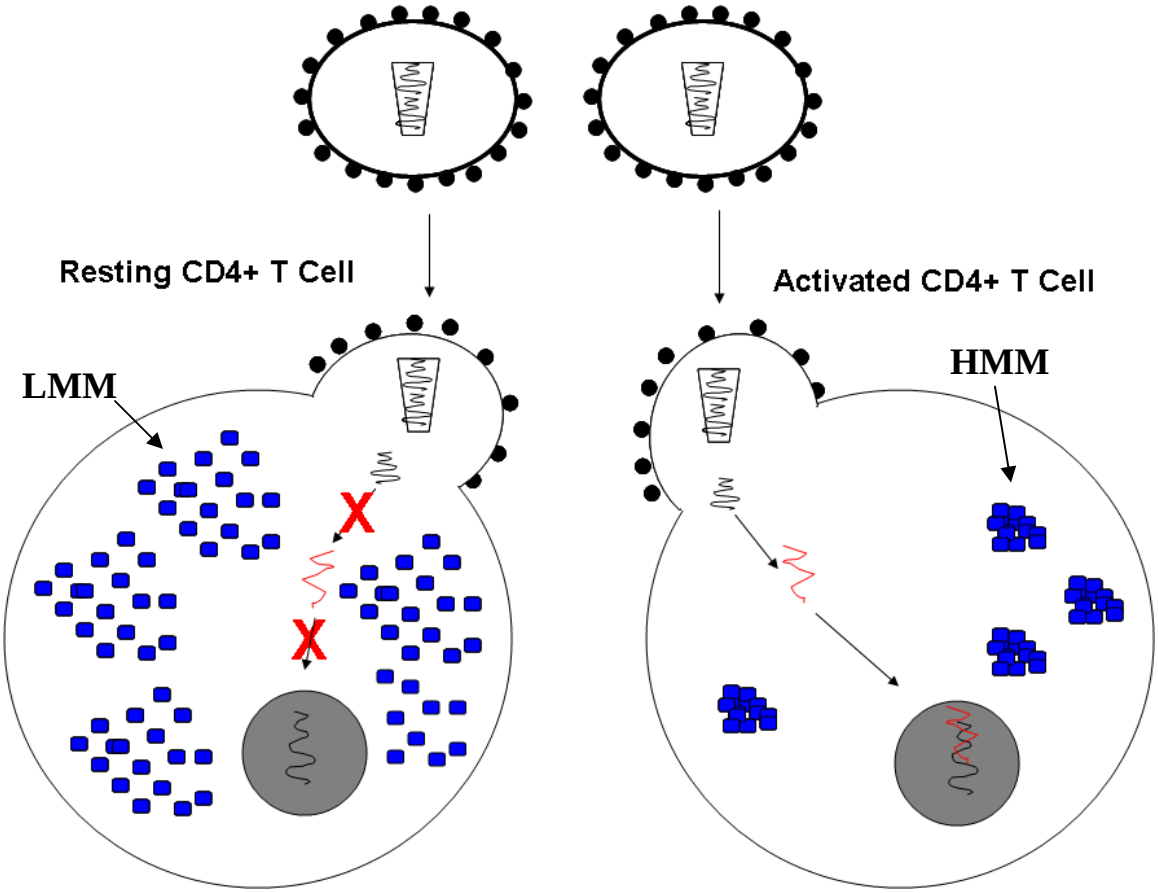


Figure 1-7 APOBEC3G Target Cell Effect: Resting CD4+ T cells are restrictive to HIV infection in part due to the enzymatically active low molecular mass (LMM) APOBEC3G. APOBEC3G restricts reverse transcription and integration. After activation of the cell HIV is able to replicate since the APOBEC3G has shifted to a enzymatically inactive high molecular mass form (HMM)

MAP kinase dependent pathways have been implicated in increases associated with some mitogens [52]. Promoter analysis has suggested that transcription factors Sp1 and Sp3 are also involved in regulating the basal transcription levels of A3G [53].

In addition, several studies have described that treatment with interferons can regulate expression of A3G in liver cells, and cells of the myeloid lineage [54,55] and resting primary CD4+ T cells [56]. Promoter analysis studies concluded that the induction of APOBEC3s by interferons was likely cell type and cell-status specific [53]. Additional studies have supported that conclusion. Peng *et.al*. showed that differentiation of myeloid cells to macrophages altered expression of APOBEC3G and renders the expression of A3G sensitive to interferon-alpha [54]. Similar findings were reported by Stopak *et.al*. in regards to myeloid differentiation; it was shown that specific cytokines can regulate the expression of APOBEC3G differently in different cell types [57]. These findings suggest that the differentiation state of a cell may affect how A3Gs expression is regulated in that cell.

CD4+ T Helper Subtypes

A T cell that has never come into contact with its cognate antigen is known as a naïve T cell. Interaction of a naïve T cell with its specific antigen initiates a differentiation pathway in the cell. Differentiation from a precursor to an eventual effector cell plays a key role in the human immune system and in HIV infection. CD4+ T lymphocytes can be further divided into subtypes based on the

differentiation pathway they take after activation. These subtypes include regulatory T cells (Treg), T helper 17 cells (Th17), T helper type 1 (Th1) and T helper type 2 (Th2). Tregs serve to regulate/down-modulate the immune response [58] Th17 cells produce IL-17 to combat extracellular bacteria and fungi and have been implicated in pathogenesis of autoimmune diseases [59]. However, the best described of the subsets to date are Th1 and Th2 cells (Fig 1-8).

Th1 cells differentiate from naïve CD4+ T cells when they come into contact with their cognate antigen and an abundance of cytokines IL-12 and IFN-γ. Th2 cells arise when a naïve cell contacts its antigen and IL-4 predominates [60-62]. The signals received from these cytokines during differentiation activate or inhibit, depending on the signal, specific transcription factors that are pivotal to the differentiation pathway choice. T box expressed in T cells (Tbet) is an important transcription factor in directing a naïve cell to a Th1 phenotype [63]. GATA3 is responsible for controlling differentiation to a Th2 phenotype [64].

The differentiation to subtypes has arisen to provide protection from diverse pathogens. Th1 cells produce predominantly IFN-γ when activated. This helps initiate a cell mediated response to intracellular pathogens by activating macrophages and other similar immune cell types, as well as acting in an autocrine loop to further stimulate Th1 cells. Th2 cells, however, produce many cytokines, predominantly IL-4, to activate B-cells as part of the adaptive humoral response to extracellular pathogens. [65].

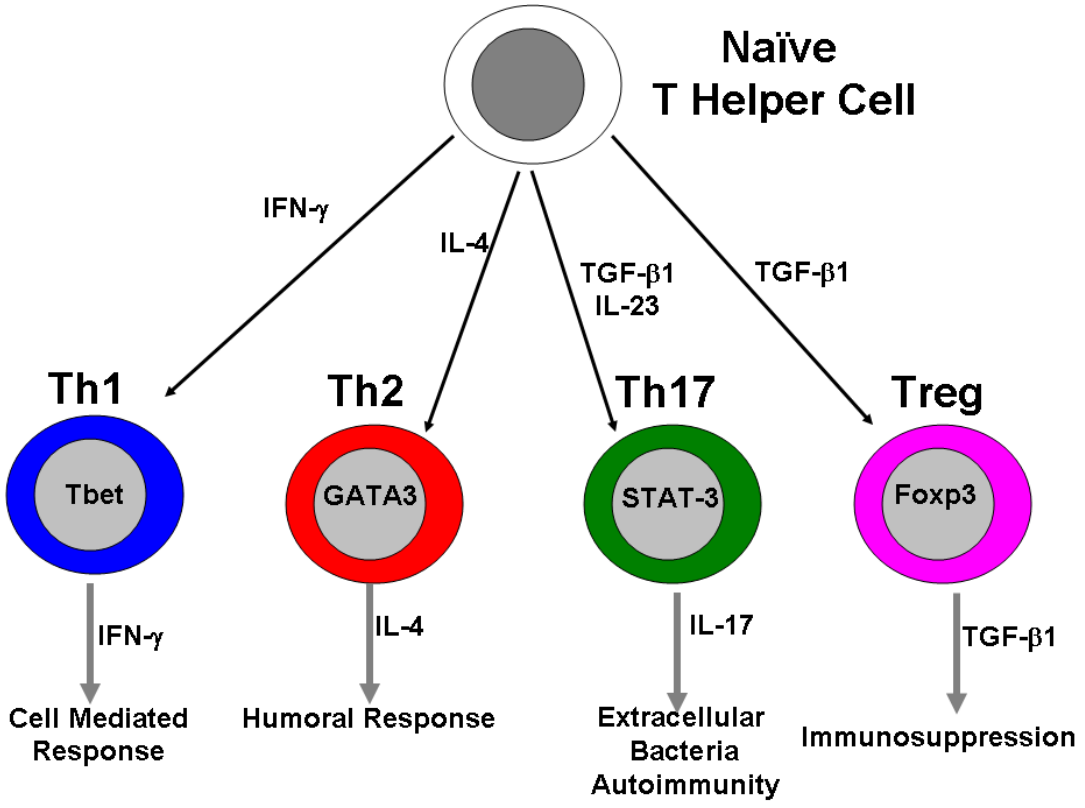


Figure 1-8 CD4+T Helper Subtypes: A naïve undifferentiated T Helper cell can differentiate to one of several phenotypes dependent upon the cytokines available in the milieu when the naïve cell encounters its cognate antigen. IL-4 drives cells to a Th2 phenotype. IL-12 initiates Th1 differentiation. TGF-β1 can drive cells towards a Treg phenotype and IL-23 can push a naïve cell towards a Th17. These differentiation pathways are controlled by the expression of specific master transcriptional regulators like GATA3, Tbet, STAT-3 and Foxp3.

Adapted from Tato, CM and O'Shea, JJ *Nature* **441**, 166 - 168 (11 May 2006);

As CD4+ cells, both Th1 and Th2 cells are susceptible to HIV infection. However, their different phenotypes contribute to a different capacity for infection by HIV. Infection by HIV requires binding to the CD4 receptor and a co-receptor CCR5 or CXCR4. Th1 cells express greater amounts of cell surface CCR5 than do Th2 cells. Therefore, a virus that is R5 tropic would preferentially bind a Th1 cell, relative to a Th2 cell [66]. Though the frequency of attachment of HIV to the two cell types may differ depending on the tropism of the virion, there are studies that demonstrate that the two cell types also differ in their ability to support post-entry steps in the HIV life cycle. Reports have shown that HIV-1 spreads better through cultures of Th2 cells than through cultures of Th1 cells and that this is not due to a difference in cell entry [66] Others have found that Th2 cells replicate CCR5 tropic virus to a greater level than do Th1 cells, despite the increased expression of CCR5 on Th1 cells [67]. Still, others have found equal replication in both cell types [68].

Research Objectives

Previous studies have demonstrated that A3G has the capability to restrict vif-deleted HIV replication through experiments in which the protein is over-expressed in transformed cell lines [13,35,41,42]. These, however, are not the conditions HIV encounters in human CD4+ T cells in infected individuals as transient transfection produces much more A3G than is present in transformed T cells and much more than seen in primary T cells [69,70]. These differences and the current lack of knowledge regarding A3G and A3F regulation prompted this

study to determine what factors regulate the expression of A3G in primary cells and if sufficient A3G could be produced in primary cells to restrict vif-competent HIV-1 replication.

The objectives of this study were to investigate: (1) what cellular factors control expression of A3G; (2) if sufficient A3G was expressed in some primary CD4+ T cells to overcome the activity of HIV vif and contribute to differences in infectious virus production from different cell types; and (3) whether sufficient cytoplasmic A3G remained in a restrictive form after cellular activation to restrict incoming infection with APOBEC3-negative virions. We have found that the transcription factors Tbet and GATA3 are involved in regulating expression of A3G along with, or in addition to, IFN-γ. This is corroborated by our finding that Th1 cells express more A3G than Th2 cells. We also find that the expression of A3G in Th1 cells is sufficient to overcome the effects of HIV-1 vif and reduce the infectivity of virions produced from Th1 cells relative to Th2 cells. This reduction in virion infectivity was not due to hypermutation of the virus. This study also demonstrates that Th1 cells are more capable of restricting incoming infection with APOBEC3-negative virions than are Th2 cells; this is seen even after cell activation and is due to their relatively greater expression of A3G. Interestingly, the restriction of HIV replication in Th1 cells also involves a relative increase in 2-LTR circle formation, which contrasts with the effect of virion-packaged A3G to reduce 2-LTR circle formation.

These findings provide novel insights into the regulation of an innate defense against this important pathogen and further demonstrate APOBEC3's

capability of restricting HIV despite the action of vif. Our results clearly show that the A3G content and antiviral activity depends on the differentiation state of the cell that is producing virions or being infected. These findings aid in gaining an understanding of A3G biology that adds a new dimension to our understanding of HIV-1 pathogenesis and may assist in future therapeutic development.

CHAPTER II

DIFFERENCES IN APOBEC3G EXPRESSION IN CD4+ T HELPER LYMPHOCYTE SUBTYPES MODULATE HIV-1 INFECTIVITY

Abstract

The cytidine deaminases APOBEC3G and APOBEC3F exert anti-HIV-1 activity that is countered by the HIV-1 vif protein. Based on potential transcription factor binding sites in their putative promoters, we hypothesized that expression of APOBEC3G and APOBEC3F would vary with T helper lymphocyte differentiation. Naïve CD4+ T lymphocytes were differentiated to T helper type 1 (Th1) and 2 (Th2) effector cells by expression of transcription factors, Tbet and GATA3, respectively, as well as by cytokine polarization. APOBEC3G and APOBEC3F RNA levels, and APOBEC3G protein levels, were higher in Th1 than Th2 cells. T cell receptor stimulation further increased APOBEC3G and APOBEC3F expression in Tbet- and control-transduced, but not in GATA3-transduced, cells. Neutralizing anti-interferon-γ antibodies reduced both basal and T cell receptor-stimulated APOBEC3G and APOBEC3F expression in Tbet- and control-transduced cells. HIV-1 produced from Th1 cells had more virion APOBEC3G, and decreased infectivity, compared to virions produced from Th2 cells. These differences between Th1- and Th2-produced virions were greater for viruses lacking functional *vif*, but also seen with *vif*-positive viruses. Over-expression of APOBEC3G in Th2 cells decreased the infectivity of virions

produced from Th2 cells and reduction of APOBEC3G in Th1 cells increased infectivity of virions produced from Th1 cells, consistent with a causal role for APOBEC3G in the infectivity difference. These results indicate that APOBEC3G and APOBEC3F levels vary physiologically during CD4+ T lymphocyte differentiation, that interferon-γ contributes to this modulation, and that this physiological regulation can cause changes in infectivity of progeny virions, even in the presence of HIV-1 vif.

Introduction

APOBEC3G (A3G) and APOBEC3F (A3F), two of several related cytidine deaminases, evolved to limit retrotransposition[71-73]. Although the HIV-1 accessory protein vif depletes A3G and A3F from the producer cell, A3G and A3F are packaged into *vif*-deleted HIV-1 and significantly impair virion infectivity [13,44,74]. IFN-α, and certain cytokines and mitogens, have been implicated in increasing A3G and A3F expression in certain cell types [52,54-57,75]. However, little more is known regarding the transcriptional regulation of APOBEC3s in CD4+ T lymphocytes [53]. We noted several potential binding sites for GATA family transcription factors[76], in addition to previously observed interferon-responsive elements[52,56,77], in the putative promoter regions of A3G and A3F. Since GATA3 is integral to the differentiation of naïve CD4+ T helper cells into Type 2 (Th2) effectors, we hypothesized that Type 1 (Th1) and Th2 effector lymphocytes differed in their expression of A3G and A3F.

After naive CD4+ T lymphocytes interact with their cognate antigen, IL-12 and interferon-γ IFN-γ signaling drive their differentiation to a Th1 effector phenotype. In contrast, IL-4 signaling after antigen recognition drives differentiation of naïve cells to a Th2 phenotype[60-62]. These subtypes of T helper cells produce distinct cytokine profiles after subsequent activation. Th1 cells, when activated, produce IFN-γ to activate cell-mediated immunity. Th2 cells, however, secrete IL-4 and other cytokines which augment humoral immune responses. The differentiation to a Th1 or Th2 phenotype is dependent on the regulated expression of two master transcriptional regulators, respectively: T Box expressed in T cells (Tbet) and GATA3 [63,64,78]. Relative differences in the ability of Th1 versus Th2 subtypes to produce infectious wild-type HIV-1 progeny have been reported previously in several studies and were not explained by differences in expression of chemokine co-receptors for HIV entry[66,79-81].

Although high level over-expression of A3G has been reported to decrease infectivity of *vif*-positive virions produced from cell lines *in vitro* [13,35,41,42], it is not known whether physiological increases in A3G or A3F can overcome the effect of vif in primary T cells. Reports conflict about whether differences in levels of A3G and A3F in lymphocytes *in vivo* are inversely associated with the level of wild-type HIV-1 RNA in plasma of untreated patients [82-84]. One of two reports has correlated provirus hypermutation attributable to A3G and A3F with plasma viral load, consistent with effects *in vivo* against at least some *vif*-positive viruses[31,32]. Since an effect of variation of levels of A3G and A3F in a physiologically relevant range on wild-type, *vif*-positive HIV-1 replication has not

yet been directly demonstrated, the present study aimed to define if such cellular differences occur during Th1 versus Th2 differentiation and may cause changes in HIV-1 infectivity that affect pathogenesis.

Materials and Methods

Cells

Blood was obtained from healthy volunteers under a protocol approved by the Vanderbilt Institutional Review Board. PBMCs were isolated using Ficoll Hypaque (Amersham Biosciences). CD4+ cells were isolated by negative selection through magnetic separation using autoMacs (Miltenyi Biotec, Auburn, CA) or Robosep (StemCell Technologies, Vancouver, BC. Canada). Naïve cells were subsequently purified by staining with CD45RO-FITC and CD25-PE (BD Pharmingen, San Jose, CA) followed by sorting on a FACSAria (Becton Dickinson, San Jose, CA). For activation and expansion, naïve cells were plated in wells coated with an anti-CD3 antibody (OKT3; American Type Culture Collection, Manassas, Virginia, United States) in RPMI with 10% FBS supplemented with 1μg/ml soluble anti-CD28 antibodies (BD Biosciences Pharmingen) and 50U/mL human rIL-2 (obtained from Dr. Maurice Gately, Hoffmann - La Roche Inc. through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH) [85]. DMEM with 10% FBS was used to culture TZM-bl cells (obtained from Dr. John C. Kappes, Dr. Xiaoyun Wu and

Tranzyme Inc. through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH)[86].

Transduction and T Helper cell differentiation

Naïve CD4+ T cells were differentiated by transduction with HIV derived lentiviral vectors expressing Tbet, GATA3, or a control vector at the time of activation [87,88]. The vectors express GFP alone (control), or the transcription factor and GFP, from an IRES. After infection and activation, cells were expanded for 10 days. Following expansion, cells were sorted on a FACSAria for GFP expression.

To achieve Th1 cell differentiation using cytokine polarization, naïve CD4+ T cells were plated on anti-CD3 (OKT3) coated plates in RPMI supplemented with anti-CD28 antibodies, 0.5μg/mL neutralizing anti-IL-4 antibody and 30ng/mL recombinant IL-12. For Th2 cell differentiation by cytokines, naïve cells were cultured in media supplemented with 2.5μg/mL neutralizing anti-IFN-γ antibody and 50ng/mL recombinant IL-4. Cytokines and neutralizing antibodies were obtained from R&D Systems, Minneapolis, MN. The cells were expanded for 10 days and differentiation was confirmed by intracellular cytokine staining for IL-4-PE and IFN-γ-APC (BD Pharmingen, San Jose CA.) as previously described [87], as well as surface staining for CXCR3-PE and CRTh2-APC (BD Pharmingen, San Jose CA) [89-91]. To increase APOBEC3G expression in cytokine polarized Th2 cells, differentiating cultures were transduced with an APOBEC3G-expressing HIV derived lentiviral vector at the time of activation. The vector was

constructed as other HIV derived lentiviral expression vectors previously described to express A3G and HSA as a marker of transduction [88]. To reduce APOBEC3G expression in cytokine polarized Th1 cells, fully differentiated Th1 cells were activated for 48hrs with CD3/CD28 coated beads (Invitrogen) in the presence of 5μg and 10μg anti-IFN-γ antibody (R&D Systems)

Real time PCR

Cytoplasmic RNA was isolated from cell pellets (Qiagen RNeasy, Valencia, CA). RNA was quantified by spectrophotometry on a GeneQuant Pro (Amersham Biosciences, Piscataway, NJ). RNA concentrations were normalized and TaqMan quantitative real-time RT-PCR was performed (Applied Biosystems Prism 7000 Sequence Detection System, Foster City, CA). Reverse transcription used A3G and A3F specific primers with the sequences 5'-GCGGCCTTCAAGGAAACC-3'and 5'-TTTTAAAGTGGAAGTAGAATATGTGTGGAT-3', respectively. The primer-probe set used for APOBEC3G real-time PCR was: forward: 5'-CTGCTGAACCAGCGCAGG-3' reverse: 5'-GCGGCCTTCAAGGAAACC-3' and probe: 5'-CTTTCTATGCAACCAGGCTCCACATAAAC-3'. The set for APOBEC3F was: forward: 5'-GCACCGCACGCTAAAGGA-3', reverse 5'-TTTTAAAGTGGAAGTAGAATATGTGTGGAT -3' and probe: 5'TTCTCAGAAACCCGATGGAGGCAATG-3'. Values are expressed as copies of

target per million copies of GAPDH or calculated as fold change using the delta-Ct method [92].

Western Blotting

Transduced or cytokine-derived T helper subtype cells were lysed in 50 mM HEPES, pH 7.4, 125 mM NaCl, 0.2% NP-40, 0.1 mM PMSF and EDTA-free protease inhibitor cocktail (CalBiochem, San Diego, CA). Protein concentrations were normalized based on results of a Bradford Assay (Bradford Assay reagent, Bio-Rad, Hercules, CA). Lysates were separated on a SDS-PAGE gel and proteins were subsequently transferred to a Trans-Blot nitrocellulose membrane (Bio-Rad, Hercules, CA). The membrane was then incubated with a polyclonal anti-APOBEC3G antibody [51], washed and probed with a goat anti-rabbit secondary antibody conjugated with Alexa Fluor 680 (Invitrogen Molecular Probes, Carlsbad, CA). Fluorescent signal was then measured using the Licor Odyssey system (LI-COR Biosciences, Lincoln, Nebraska). Membranes were subsequently probed with a monoclonal β-actin antibody (Sigma, St. Louis, MO) followed by a sheep anti-mouse secondary antibody conjugated with IR-Dye800 (Rockland Immunochemicals, Philadelphia, PA). APOBEC3G expression is expressed as fluorescent intensity (Relative Light Units, RLU) of APOBEC3G bands divided by the fluorescent intensity (RLU) of the □-actin band[93]. For quantification of virion packaged APOBEC3G, virions were concentrated by centrifugation of culture supernatants through a 20% sucrose cushion at 125,000 x *g* for 45 minutes and normalized for their p24 content with viral lysis buffer [50

mM Tris (pH 8.0), 40 mM KCl, 50 mM NaCl, 5 mM Na_2EDTA, 10 mM DTT and 0.1% (v/v) Triton X-100]. Lysates were blotted as described above with anit-APOBEC3G and an anti-HIV-1 capsid p24 antibody derived from the 183-H12-5C hybridomas (obtained from Dr. Bruce Chesebro and Dr. Hardy Chen through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH[94]) . Data are expressed as fluorescent intensity (RLU) of APOBEC3G bands divided by the fluorescent intensity (RLU) of the HIV-1 CA p24.

Viruses and infectivity

HIV-1 was produced by calcium phosphate transfection of 293T cells using NL4-3 (obtained from Dr. Malcom Martin through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH) [95] and vif-deleted NL4-3 (a gift from the Chris Aiken Laboratory, constructed by Hevey and Donehower)[96] After determination of the concentration of viral particles by HIV-1 CA p24 ELISA, 300ng of p24-equivalents of HIV-1 were spinoculated (300Xg, 30min) on $1X10^6$ Th1, Th2, or TH2-A3G cells that had been activated by anti-CD3/CD28 coated beads (Invitrogen Dynal, Carlsbad, CA) for 60 hours [97]. Twelve hours after infection, cultures were washed twice with PBS. The cells were then resuspended in RPMI media containing 10uM didanosine (Sigma, St. Louis, MO) and 25uM zidovudine (Sigma, St. Louis, MO) to limit virus spread. After another 12 hrs in culture, supernatant fluids were collected for p24 antigen ELISA. Equal p24 concentrations of viral supernatant were then used to infect

the TZM-bl indicator cells[86] and luciferase activity was determined in cell lysates 60 hours after infection (Bright-Glo Luciferase assay substrate, Promega, Madison, WI; TopCount scintillation counter, Packard/Perkin Elmer, Waltham, MA). Data are shown as RLU per nanogram p24 CA added.

Results

Master Transcriptional Regulators Modulate APOBEC3G and APOBEC3F Expression

Naïve CD4+ T cells from five individual HIV-1 negative donors were transduced with HIV-derived lentiviral vectors that expressed either GFP alone (control), or together with Tbet or GATA3. Expression of GATA3 and Tbet were found to have opposing effects on the expression of A3G and A3F mRNA by qRT-PCR (Figure 2-1A and 2-1B). Whereas expression of GATA3 reduced the level of A3G and A3F, Tbet significantly increased the levels of both enzymes. Based on these results, confirmation that this was a statistically and biologically significant effect was sought by studying Th1 versus Th2 differentiation using more physiological cytokine polarization.

CD4+ T Helper Type 2 Lymphocytes Express Lower Levels of APOBEC3G and APOBEC3F than T Helper Type 1 Lymphocytes

Th1 and Th2 cells were differentiated *in vitro* by culturing naïve cells from nine individual donors in polarizing cytokines. Staining for Th1- and Th2-associated intracellular cytokines (IFN-γ and IL-4, respectively) and surface markers (CXCR3 and CRTh2, respectively) (Figure 2-2A), verified the

phenotypes of the cytokine-differentiated cells. Cytoplasmic RNA was isolated and the levels of A3G and A3F mRNA were determined relative to GAPDH expression by qRT-PCR. Th2 cells expressed significantly less A3G and A3F mRNA than Th1 cells (Figures 2-2B and 2-2C). Western blot analysis of the two helper cell subtypes revealed that Th2 cells also expressed lower levels of A3G

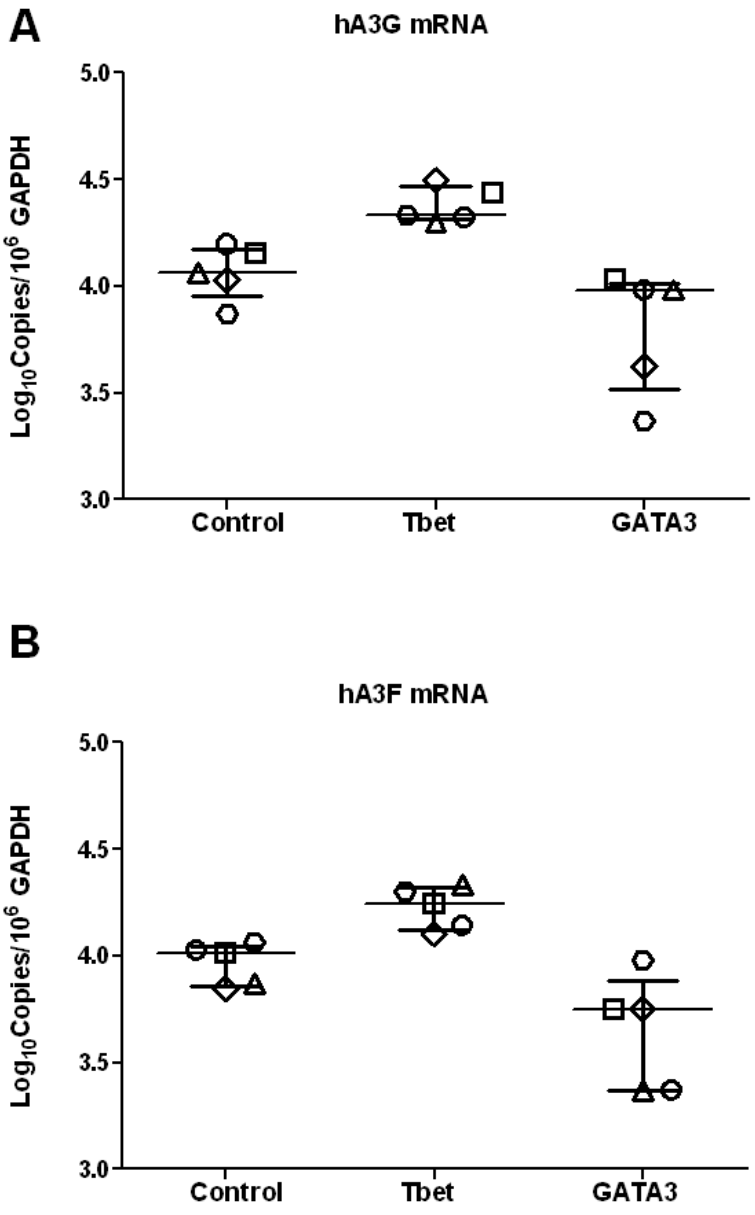


Figure 2-1. Tbet and GATA3 regulate A3G and A3F expression. Naïve CD4+ T cells were transduced with a Tbet or GATA3 expressing lentiviral vector. After sorting based on GFP marker gene expression, cytoplasmic RNA was isolated and used to determine mRNA levels of A3G (A) and A3F (B) by qRT-PCR. Data are expressed as copy number of A3G or A3F per 10^6 copies of GAPDH. Error bars represent median and interquartile range.

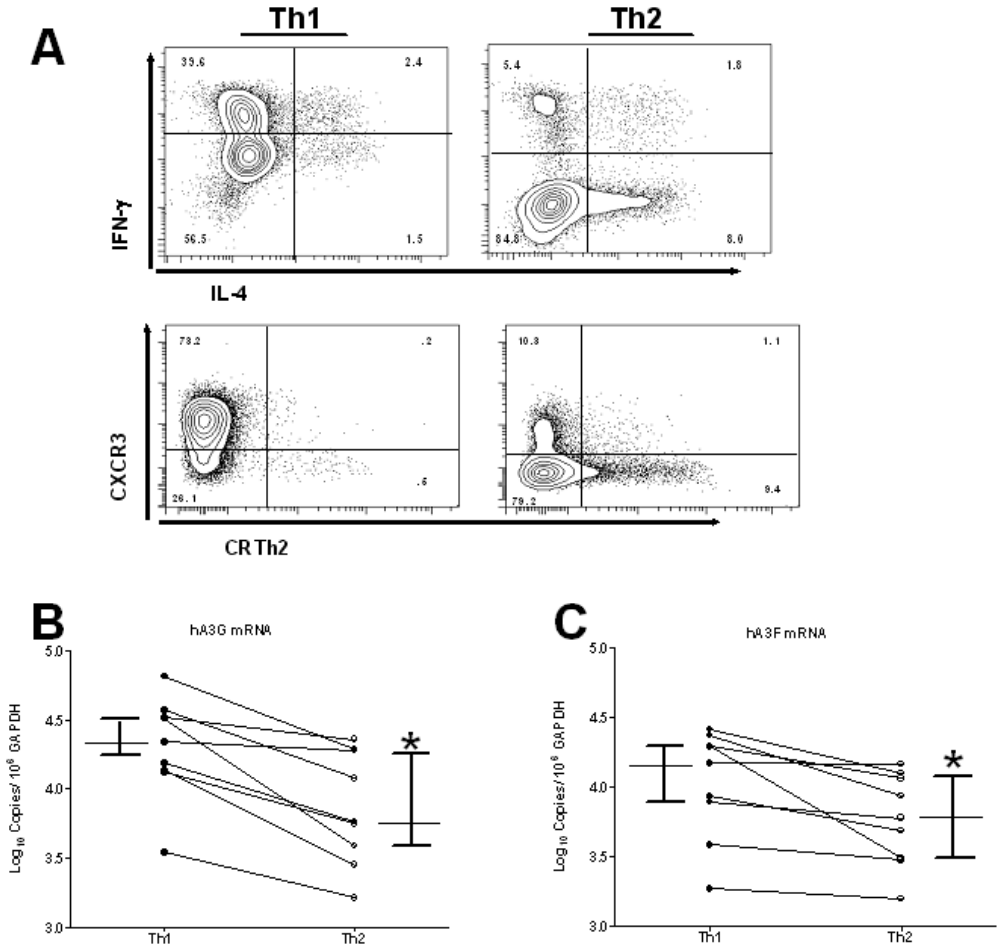


Figure 2-2A-C Th2 Cells Express Lower Levels of A3G and A3F than Th1. Naïve CD4+ T cells were derived to either a Th1 or Th2 phenotype using cytokines as described in Materials and Methods. The cells were then stained for intracellular cytokine production or surface markers (A) to confirm differentiation. Cytoplasmic RNA was isolated from the cells and used for qRT-PCR to determine the level of A3G (B) or A3F (C) mRNA. Error bars represent media and interquartile range(*p=0.0039).

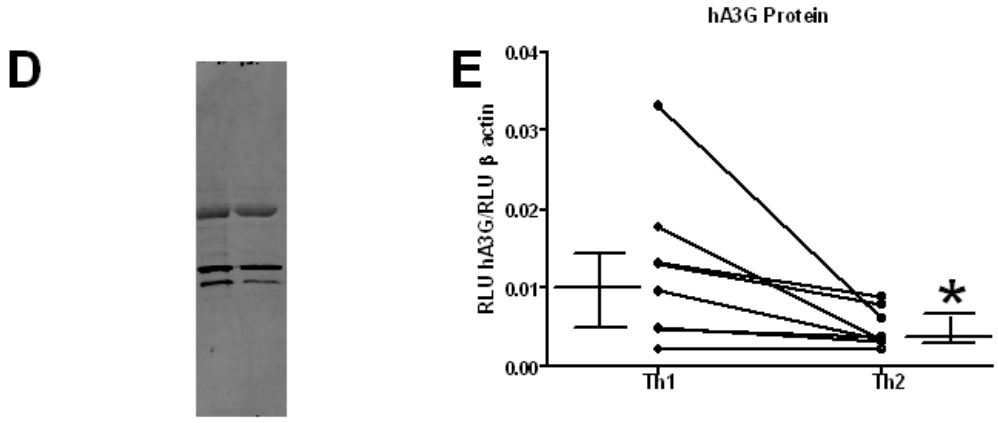


Figure 2-2D and E Th2 Cells Express Lower Levels of A3G and A3F than Th1. In vitro cytokine-derived Th1 or Th2 cells were also lysed and subjected to Western Blotting with a A3G specific antibody and levels of expression were quantified using a LICOR Odyssey system. A representative blot is shown (D) as well as the compilation of 8 individual donors (E) with quantities expressed as quantified intensity of A3G bands per quantified intensity of Beta-Actin bands of the same lane. Error bars represent median and interquartile range**(***p=0.0078).

protein than Th1 cells (Figure 2-2D and 2-2E). The statistically significant difference in median A3G mRNA levels, and in protein levels, between Th1 and Th2 cells was approximately 3-fold.

Interferon-γ Regulates Basal and TCR-stimulated Expression of APOBEC3s in Tbet-transduced cells

Since previous studies have observed that mitogen treatment increases A3G expression[52], we tested whether T Cell Receptor (TCR) stimulation would increase A3G and A3F expression in Tbet and GATA3 expressing T-cells. Levels of A3G and A3F RNAs increased after TCR stimulation of control vector- and Tbet-transduced cells, while this did not occur with TCR activation of GATA3-transduced cells (Fig. 2-3A). A defining characteristic of Th1 cells is their ability to produce IFN-γ upon activation, which then exerts autocrine effects[98]. It is also known that GATA3 diminishes IFN-γ expression. Therefore, the hypothesis that IFN-γ contributes to the observed increase in A3G and A3F expression after TCR stimulation was tested by performing TCR stimulation of control- and Tbet-transduced cells in the absence or presence of a neutralizing anti- IFN-γ antibody. The presence of neutralizing anti- IFN-γ antibody blocked the TCR-stimulated increased transcription of A3G and A3F, and reduced basal levels, in both control- and Tbet-transduced cells (Figure 2-3B and 2-3C). This suggests that IFN-γ contributes to maintaining the steady state level of A3G and A3F in Th1 cells, as well as in increasing expression after TCR activation.

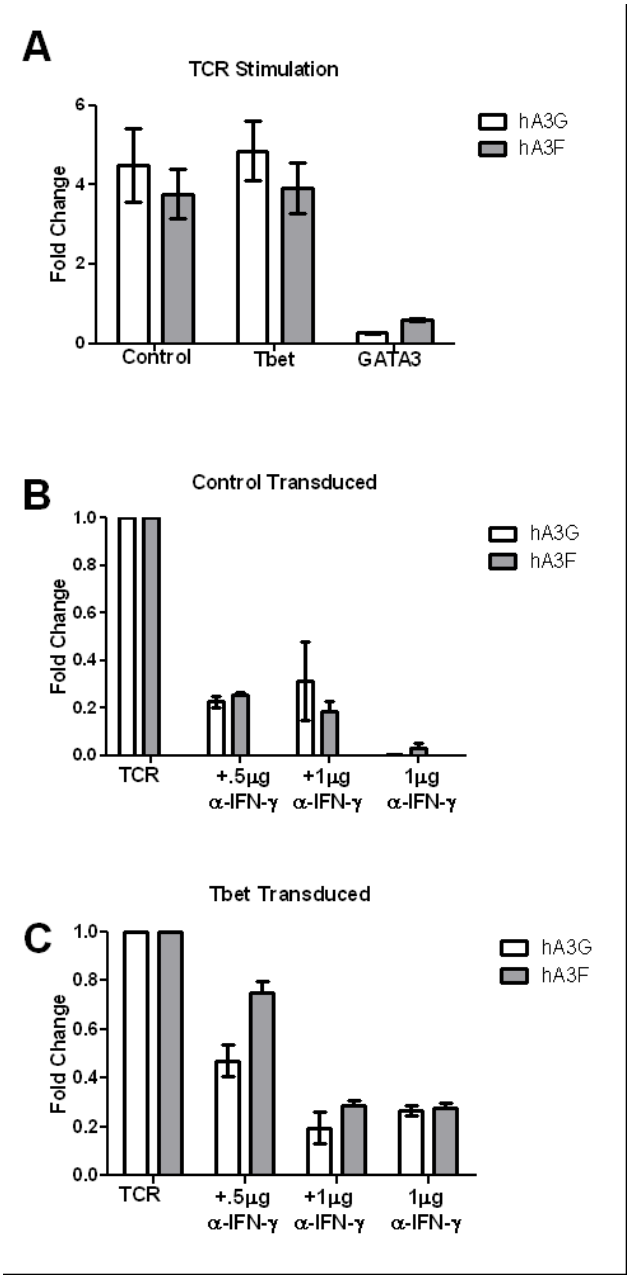


Figure 2-3.Interferon Gamma regulates expression of A3G and A3F in Tbet but not GATA3 Transduced Cells. Control, Tbet and GATA3 transduced cells were TCR stimulated with CD3/CD28 beads. Cytoplasmic RNA was then isolated to determine the fold change in mRNA expression by qRT-PCR (A). Control (B) and Tbet (C) transduced cells were TCR stimulated or left unstimulated in the presence of a neutralizing anti-interferon gamma antibody. The fold change in mRNA expression was again determined by qRT-PCR. Incubation of TCR stimulated cells with isotype control does not differ significantly from stimulation alone (data not shown). Error bars represent standard deviation from the mean.

Increased Infectivity of HIV-1 Produced from CD4+ T Helper Type 2 Compared to Type 1 Lymphocytes

We next tested whether the differential expression of APOBEC3s between Th1 and Th2 cells led to a difference in infectivity of HIV-1 virions produced from these cells. We infected TCR-activated, cytokine-derived T helper cells with *vif*-deleted or *vif*-competent HIV-1(NL4-3) produced from 293T cells (which do not express A3G or A3F). Infected cells were washed 12 hours after infection and new media containing reverse transcriptase inhibitors (didanosine and zidovudine) was added to prevent spread past the first-round infected cells. Twelve hours after the new media was added, the culture supernatant fluids were collected, normalized by Gag p24 capsid antigen concentrations, and used to infect the TZM-bl indicator cell line. Infectivity was determined by luciferase activity. Figure 2-4A quantitates infectivity of wild-type and *vif*-deleted viruses produced from Th1 and Th2 cells from one of nine donors studied. *Vif*-negative viruses produced from Th2 cells from this individual were five-fold more infectious than those produced from Th1 cells, whereas *vif*-competent virions from Th2 cells were three-fold more infectious than those produced from Th1 cells (Fig. 2-4A). The median infectivity of virions produced from Th1 cells of all nine donors studied was significantly less than that of viruses produced from all the different Th2 cells, whether vif was present or not (Fig. 2-4B and 2-4C). The magnitude of this difference varied across different individual donors' paired Th1 and Th2 cells, whether vif was present or not (Figs. 2-4B and 2-4C; each donor's Th1 and Th2 cells are linked by a line). A3G protein levels also varied, with Th1

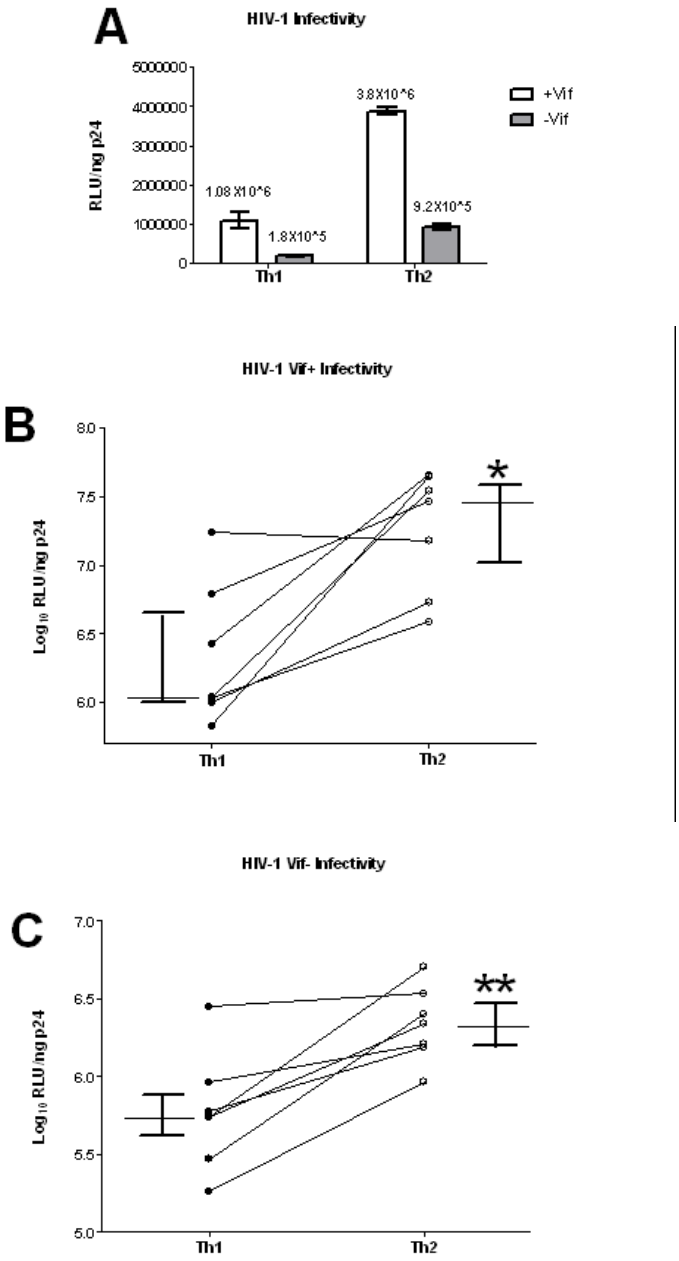


Figure 2-4A-C. Increased Infectivity of HIV-1 produced from Th2 cells compared to Th1 cells. *vif*-competent (+) or *vif*-deleted (-) HIV-1(NL4-3) was used to infect cultures of Th1 and Th2 cells as described in Materials and Methods. Infectivity of the virions produced was determined by infection of the TZM-bl indicator cell line and determination of luciferase activity. An example from an individual donor is shown (A). The experiment was repeated on a total of seven donors with both the *vif*-competent (B) and *vif*-deleted virus(C). Error bars represent median and interquartile range of difference in infectivity of virions produced from Th1 cells versus Th2 cells for vif-positive virus (B; *p=.031) and vif-negative virus (C; **p=.016).

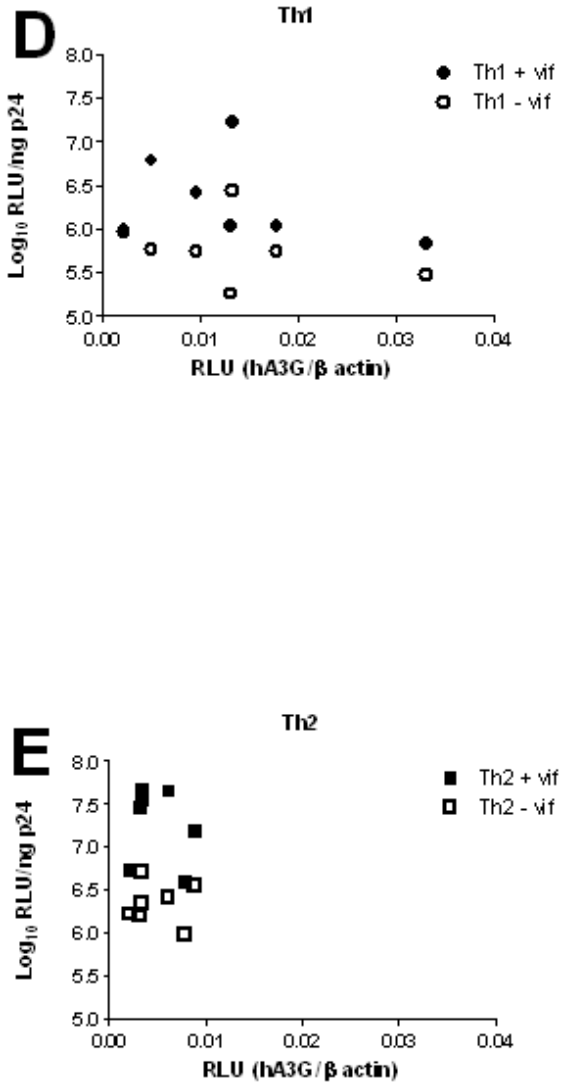


Figure 2-4D and E. Increased Infectivity of HIV-1 produced from Th2 cells compared to Th1 cells. To test for correlation individual donor cells' levels of A3G protein expression were plotted against infectivity of the *vif*-competent and *vif*-deleted virions produced from that individual's Th1 cells (D) and Th2 cells (E).

cells having higher levels and a broader range of A3G protein than Th2 cells (comparing X-axis in Figures 4 2-4D and 2-4E). Despite the small number of subjects and variability of the assays, there was a suggestion of an inverse correlation between A3G protein expression and infectivity of virions produced from Th1 (+vif r =-.16, -vif r=-.18; all p >0.05) or Th2 (+vif r = -.28, -vif r= -.01; all p>0.05). We amplified a pol gene fragment from the TZM-bl cells infected for 60 hours with Th1- or Th2-produced *vif*-negative virions to quantify if effects of cytidine deamination differed by cell type. Hypermutation was not seen in HIV pol DNA amplified from cells infected with virus produced from either cell type (data not shown), using either population sequencing subsequent to standard PCR or 3D PCR[99]. Although these data are consistent with direct effects of A3G and A3F on infectivity of *vif*-competent HIV as well as *vif*-defective HIV-1, it is possible that other variables may affect infectivity of virions produced from these cytokine-polarized cells.

Changes in APOBEC3G cause differences in infectivity of Th1- versus Th2-produced HIV-1

To confirm a causal role for A3G in the observed virion infectivity differences we modulated expression of A3G in Th1 and Th2 cells by increasing expression in Th2 cells and decreasing expression in Th1 cells. We increased expression of A3G in cytokine-derived Th2 cells by transduction with a A3G-expressing lentiviral vector or an “empty” control vector for comparison. Transduction of Th2 cells with the A3G-expressing vector increased A3G levels 4 fold over Th1 cells and 7 fold over Th2 cells (data not shown). After expansion,

the unsorted population of A3G vector-transduced Th2 cells (Th2-A3G), as well as Th1 and Th2 cells, were infected. The *vif*-deleted virions produced from Th1, Th2 and Th2-A3G cells were concentrated and the relative levels of virion packaged A3G were determined by Western blotting. Figure 2-5A demonstrates that *vif*-deleted virions produced from Th1 cells contain more A3G than virions produced from Th2 cells. Th2-A3G cells produced virions with more packaged A3G than Th2 cells (Fig. 2-5A). Transduction with the empty vector (Th2-Empty) caused no increase in cellular or virion A3G levels, relative to untransduced Th2 cells (data not shown). Virions produced from the Th2-A3G cells were significantly less infectious than those produced from the Th2 cells transduced with the "empty" control vector ("Th2-Empty") (Fig 2-5B). There was an inverse correlation between virion (and cellular) A3G levels by western blot and virion infectivity.

Neutralizing anti-IFN-γ antibody was used to decrease expression of A3G in Th1 cells (as seen in Fig 2-3). Incubation with neutralizing anti-IFN-γ antibody, concurrent with activation, reduced the expression of A3G in Th1 cells nearly 2 fold (relative to Th1 cells incubated with an isotype control antibody) (Fig 2-5C, open bars). Virions produced from Th1 cells with reduced A3G had increased infectivity (Figure 2-5C, closed bars). Taken together, these data indicate that variation in infectivity of virions produced from cells is related to differences in A3G expression.

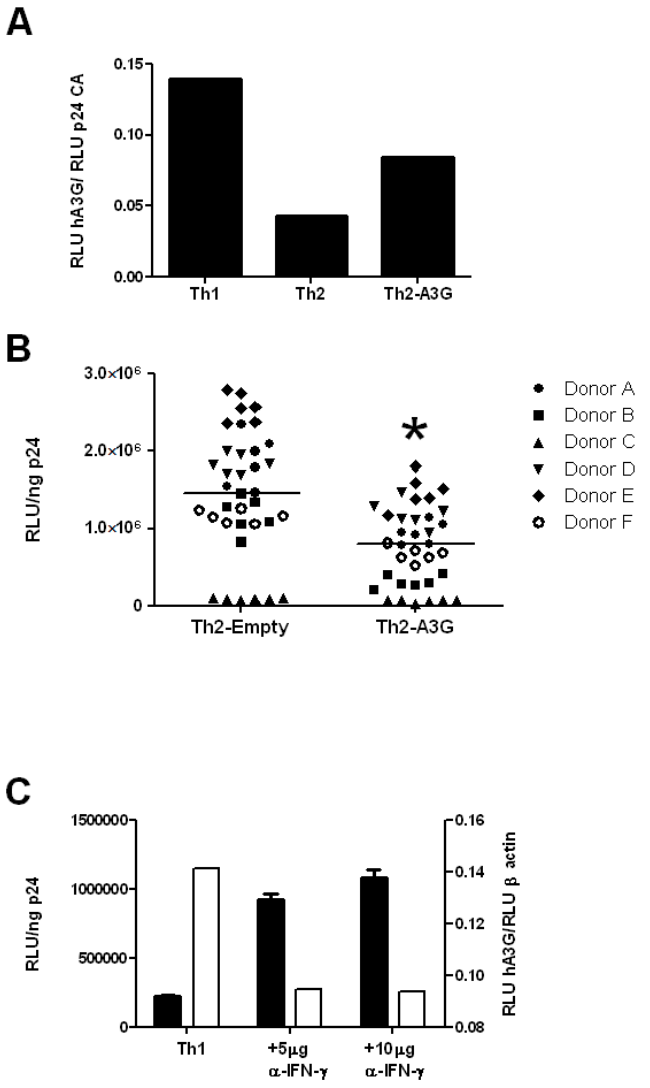


Figure 2-5. Evidence supporting a direct effect of A3G on relative infectivity of HIV-1 virions produced from Th1 and Th2 cells. In vitro cytokine-derived Th2 cells were transduced with a control lentiviral vector (Th2-Empty) or a vector expressing A3G (Th2-A3G). Protein levels of A3G packaged into virions produced from Th1, Th2 and A3G-transduced Th2 cells were determined by Western Blotting on the LICOR Odyssey system (Relative Light Units (RLU) of A3G as normalized by HIV-1 p24 antigen) Shown is a representative blot of three experiments with similar trends. (A). The infectivity of *vif*-deleted HIV-1(NL4-3) virions produced from those cells from 6 donors was determined using the TZM-bl indicator cell line. Error bars represent median and interquartile range (*p=.03). (B) In vitro cytokine-derived Th1 cells were activated and incubated with either an isotype control or neutralizing IFN-γ antibodies for 48 hours. Protein concentrations were determined by Western blot (C,open bars). The infectivity of *vif*-deleted HIV-1(NL4-3) virions produced from those cells was determined using the TZM-bl indicator cell line.(C, closed bars)

Discussion

In this study, we have shown that the expression and anti-HIV function of A3G and A3F vary with naïve CD4+ T helper cell differentiation to Th1 and Th2 effector cells. Cytokine polarization of naïve cells into Th1 and Th2 effectors had similar effects to transduction of naïve cells with Tbet or GATA3. In both cases, decreased expression of A3G and A3F was seen in Th2 cells relative to Th1 cells. These complementary methods demonstrate that the differences observed in the Tbet and GATA3 transduced cells were due to transcription regulated by those factors and not an artifact of over-expression. Such an opposing effect of differentiation on expression of A3G and A3F is consistent with earlier findings of opposing effects on the expression of several other genes in these two T helper subtypes [63,100]. This A3G and A3F expression difference between Th1 and Th2 cells affected wild type, as well as *vif*-deleted, HIV-1 infectivity.

Expression of Tbet in naïve helper cells has been shown to lead to production of IFN-γ [63]In turn, that IFN-γ can act in an autocrine manner on Th1 cells[98]. Extracellular neutralization of IFN-γ secreted by Tbet-transduced and control-transduced cells blocked basal and TCR-stimulated A3G and A3F expression. This is consistent with an autocrine effect of IFN-γ regulating A3G and A3F expression. GATA3 is known to inhibit the production of IFN-γ [101] and no effect was observed with neutralizing anti-IFN-γ antibody or TCR stimulation of GATA3-transduced cells. This may be due to a GATA3-mediated block to production of IFN-γor a direct effect of GATA3 binding to the A3G and A3F promoters. These possibilities remain to be directly tested.

We verified that the difference in expression in cytokine-derived T helper cells led to a biological difference: infectivity of HIV-1 virions produced from Th1 and Th2 effectors varied inversely with their relative levels of cellular and virion A3G and A3F. Removal of vif resulted in reduced infectivity of virions produced from both cell types. The greater reduction of infectivity of virions produced from the Th1 cells is consistent with the relative greater APOBEC3 levels in those cells. Over-expression of A3G in Th2 cells reversed the relative decrease in virion A3G and the consequent relative increase in infectivity of virions produced from Th2 cells. The magnitude of the effect of the ectopically-expressed A3G is likely underestimated here, as not every cell in this population is expressing the transduced A3G. In addition, reduction of A3G in Th1 cells also correlated with an increase in infectivity. We used neutralizing anti-IFN-γ antibody to decrease A3G expression in Th1 cells because shRNA against A3G or nucleofection (for introduction of siRNA against A3G) proved toxic to *in vitro*-derived Th1 cells, which are more prone to cell death than other cultured T cells [102-104].These results are consistent with the variation in virion infectivity being caused, at least in part, by the differences in cellular and therefore virion A3G, rather than other effects of the cytokine derivation.

In this study, we observed reduction of infectivity associated with increased amounts of readily detectable virion A3G without identification of any G-to-A hypermutation. Although A3G and A3F are cytidine deaminases, there is extensive evidence that A3G also reduces HIV infectivity through other mechanisms that may be the major contributor to A3G's inhibition of reverse

transcription[32-35]. Previous studies that have observed A3G-related hypermutation *in vitro* differed from the short term virus replication allowed here, and instead used prolonged serial passage of HIV in transformed cell lines over-expressing A3G[30,105]. Therefore, it is likely that the difference in infectivity based on cell source of virus observed here is due to the other antiviral activities of A3G that are not measured by hypermutation.

A major issue concerning the role of A3G and A3F in HIV-1 pathogenesis is the question of whether *in vivo* variation in these cellular restriction factors affects replication of wild type (eg, *vif*-competent) HIV-1. Although high level over-expression of A3G does impair replication of wild type HIV-1 in cell lines [13], more recent studies have not conclusively determined if there is a correlation between the variation in cellular A3G expression observed across HIV-infected individuals' peripheral blood mononuclear cells and the plasma viral load in these subjects [31,82,83]. The present results clearly indicate that physiological variations in A3G levels in primary cells are inversely correlated with A3G content and infectivity of wild type virions. This more direct measure of biological relevance observed here supports the conclusions of earlier reports showing that greater A3G activity was associated with lower viral load set-point[31], and suggests that continued investigation of the effect of APOBEC3 restriction factors on *vif*-competent HIV-1 pathogenesis *in vivo* is warranted.

The present results are also consistent with earlier reports that HIV-1 spreads better through cultures of Th2 cells than Th1 cells [80]. This effect was most apparent in the prior studies using CXCR4 (X4) tropic viruses[81], such as

the viruses used here, and not explained by differences in expression of that co-receptor between Th1 and Th2 cells. The present results suggest, however, that virions produced from Th2 cells may be relatively more infectious than those produced from Th1 cells because of their relatively lower A3G content. In an earlier study [81], CCR5-tropic HIV replicated equally well in Th1 and Th2 cells. Th1 cells express higher levels of CCR5 coreceptor than Th2 cells[63,66]. Indeed, X4-tropic viruses were chosen for study here to minimize possible difficulty in interpretation of opposing effects of both increased CCR5 co-receptor expression and increased A3G expression in Th1 cell cultures, though further investigation into how co-receptor tropism affects infectivity is certainly warranted. Moreover, the wide inter-individual variation in A3G and A3F expression in our results (a 14 fold range in A3G protein expression in Th1 cells and a four fold range in Th2 cells) suggests that there may be polymorphisms in the regulatory regions of the APOBEC3 promoters [106], or in factors that can modulate A3G and A3F expression or function. We hypothesize that this variation in A3G and A3F may contribute to the wide variation of progression time to AIDS among different patients. The Th1/Th2 cell balance may also vary across individuals based on several factors. Autoimmunity may lead to a Th1 cell skewing and parasitic infections may cause aTh2 cell predominance. Our findings suggest that a shift in this balance prior to, or during, HIV-1 infection may lead to compounded pathogenic effects. Decreased relative expression of A3G and A3F in Th2 cells may lead to a greater rate of decrease in that cellular pool, decreasing CD4+ help to B cells for antibody production. Also, an individual's

variation in Th1/Th2 balance may lead to differences in HIV-1 genetic variation due to A3G- and A3F-mediated sub-lethal cytidine deamination of viral genomes over repeated cycles of infection [107].

The present study indicates that the regulation of expression of A3G and A3F, and their functional effect on HIV-1 infectivity, depends on the cytokine-regulated differentiation state of CD4+ T helper cells. Further molecular characterization of signals that modulate A3G and A3F expression will be needed. The current results provide compelling evidence that increasing A3G in primary T cells impairs HIV-1 replication despite the presence of Vif. This validates inducing higher A3G expression as a novel strategy for prevention of infection and/or treatment of the *vif*-positive viruses present in infected humans.

CHAPTER III

CYTOPLASMIC APOBEC3G RESTRICTS INCOMING VIF POSITIVE HIV-1 AND INCREASES 2-LTR CIRCLE FORMATION IN ACTIVATED T HELPER SUBTYPE CELLS

Abstract

Cytoplasmic APOBEC3G (A3G) blocks wild type HIV-1 infection in resting blood CD4+ T lymphocytes. It is not known if cytoplasmic APOBEC3G has residual activity in activated T cells, even though virion-packaged APOBEC3G does restrict HIV-1 in activated T cells. Because APOBEC3G expression is greater in activated CD4+ T helper type 1 (Th1) than T helper type 2 (Th2) lymphocytes, we hypothesized that residual target cell restriction of incoming Vif-positive virions that lack APOBEC3G would be greater in Th1 than Th2 lymphocytes. Infection of activated Th1 cells with APOBEC3-negative virions did result in decreased amounts of early and late reverse transcription products, and integrated virus, relative to activated Th2 cells. Two-LTR circles, which are formed in the nucleus when reverse transcripts do not integrate, were increased after APOBEC3-negative virus infection of activated Th1 cells, relative to infection of activated Th2 cells. In contrast, 2-LTR circle forms were decreased after infection of APOBEC3G negative cells with APOBEC3G-containing virions relative to APOBEC3G-negative virions, and with Th1 cell-produced virions relative to Th2 cell-produced virions. Increasing APOBEC3G in Th2 cells and

decreasing APOBEC3G in Th1 cells modulated the target cell phenotypes, indicating causation by APOBEC3G. The comparison between activated Th1 and Th2 cells indicates that cytoplasmic APOBEC3G in activated Th1 cells partially restricts reverse transcription and integration of incoming Vif-positive, APOBEC3G-negative HIV-1. The differing effects of cytoplasmic and virion-packaged APOBEC3G on 2-LTR circle formation indicate a difference in their antiviral mechanisms.

Introduction

Two separate A3G activities that restrict HIV-1 have been described. A3G packaged into virions released from a producer cell exerts antiviral effects during both reverse transcription and integration in the subsequent target cell [13,23]. This A3G activity is antagonized by HIV-1 vif limiting packaging in the producer cell [43]. Endogenous cytoplasmic A3G in resting CD4+ T cells can also restrict the replication of an incoming APOBEC3G-free HIV-1 virion; this occurs even if the virus encodes a functional vif gene [50]. Chiu, et al. showed that siRNA knock-down of this endogenous A3G, which is in a “low molecular mass” form in resting T cells, removed this restriction. Cellular activation or specific cytokine signaling moved A3G from the “low molecular mass” form to a “high molecular mass” complex and abrogated this antiviral effect [50,57]. The phenomenon of cytoplasmic A3G transitioning to a higher order complex and losing the ability to restrict incoming HIV-1 replication was also demonstrated when monocytes differentiated to macrophages and immature dendritic cells matured

[57,108,109].

The CD4+ T cell is a major target of HIV-1. There are several subtypes of CD4+ T-cells including T Helper Type 1 (Th1), T Helper Type 2 (Th2) [110]. In the previous chapter, we demonstrated that these two CD4+ T cell subtypes differ in the level of expression of A3G and in the infectivity/A3G content of HIV-1 virions produced from them [111]. Given this difference in cellular A3G expression, we tested whether Th1 target cells would better restrict replication of incoming HIV-1 than would Th2 cells. The comparison indicates that endogenous A3G can also play a role in partially restricting HIV-1 infection in activated CD4+ T cells.

Materials and Methods

Human Subjects

Peripheral blood was obtained with informed consent from healthy volunteers, under a protocol approved by the Vanderbilt Institutional Review Board.

Cells

Peripheral blood mononuclear cells were isolated from blood using Ficoll-Hypaque (Amersham Biosciences) gradients. CD4+ cells were isolated by negative selection through magnetic separation using autoMacs (Miltenyi Biotec, Auburn, CA) or Robosep (StemCell Technologies, Vancouver, BC. Canada). Naïve cells were subsequently purified by staining with CD45RO-FITC and CD25-PE (BD Pharmingen, San Jose, CA) followed by sorting on a FACSAria

(Becton Dickinson, San Jose, CA). Naïve CD4+ T cells were differentiated to Th1 cells using anti-CD3 (OKT3; ATCC)-coated plates in RPMI supplemented with anti-CD28 antibodies (BD Biosciences Pharmingen), 0.5μg/mL neutralizing anti-IL-4 antibody and 30ng/mL recombinant IL-12. For Th2 cell differentiation, naïve cells were cultured on the same coated plates in media supplemented with 2.5μg/mL neutralizing anti-IFN-γ antibody and 50ng/mL recombinant IL-4. Cytokines and neutralizing antibodies were obtained from R&D Systems, Minneapolis, MN. The cells were expanded for 10 days and differentiation was confirmed by intracellular cytokine staining for IL-4-PE and IFN-γ-APC (BD Pharmingen), as well as surface staining for CXCR3-PE and CRTh2-APC (BD Pharmingen) [87,89,91]. To increase APOBEC3G expression in cytokine-polarized Th2 cells, differentiating cultures were transduced with an APOBEC3G-expressing, HIV-derived lentiviral vector at the time of activation. The vector expressed APOBEC3G, and human serum albumin (HSA) as a marker of transduction [88,111]. To reduce APOBEC3G expression in cytokine-polarized Th1 cells, fully differentiated Th1 cells were activated for 48hrs with anti-CD3/CD28 coated beads (Invitrogen) in the presence of 10μg anti-IFN-γ antibody [111] (R&D Systems).

Viruses and Infection

VSV-G-pseudotyped, GFP-expressing HIV-1 was produced by polyethylenimine (PEI) transfection [112] of 293T cells with a NL4-3 recombinant with GFP replacing nef as well as phCMV-VSV-G. Prior to infection, viral

supernatants were treated with 100u DNaseI (Bio-Rad) for one hour. Cells were activated for 48 hours with anti-CD3/CD28 antibody coated beads (Invitrogen), then treated with virus (400ng of p24) for 4hrs, washed and resuspended in fresh RPMI media with 10%FBS, 1%Penicillin/Streptomycin and 50U/ml IL-2. Cells were fixed in 2% paraformaldehyde 48 hours post infection and analyzed for the percentage of cells expressing GFP on a FACSAria cell sorter/cytometer (Becton Dickinson).

Virions with or without packaged A3G were produced by PEI transfection of 293T cells with a vif-deleted NL4-3 provirus construct (15 ng of DNA) either with or without an A3G expression plasmid (3 ng). Th1 and Th2 produced virions were generated as previously described [111].

Polymerase chain reactions

Infected T helper cells were collected at 2 4, 18, 24, and 48 hours after infection, and washed in PBS. Cellular DNA was isolated using the DNeasy kit (Qiagen). DNA was quantified by spectrophotometry on a GeneQuant Pro (Amersham Biosciences) and normalized prior to use in qPCR assays on an ABI Prism 7000 (Applied Biosystems). To detect early RT products the primers used were 5'-GTGCCCGTCTGTTGTGTGAC-3' and 5'-GGCGCCACTGCTAGAGATTT-3', in conjunction with a probe 5'-(FAM)-CTAGAGATCCCTCAGACCCTTTTAGTCAGTGTGG-(TAMRA)-3'. Late RT products were detected using primers 5'-TGTGTGCCCGTCTGTTGTGT-3' and

5'-GAGTCCTGCGTCGAGAGAGC-3' with probe 5'-(FAM)-CAGTGGCGCCCGAACAGGGA-(TAMRA)-3' [113,114]. For 2-LTR quantification, DNA isolated at 48 hours after infection was normalized and used in qPCR . The forward primer was (MH535) 5'-AACTAGGGAACCCACTGCTTAAG-3', the reverse primer was (MH536) 5'-TCCACAGATCAAGGATATCTTGTC-3', and the probe was (MH603) 5'-(FAM) ACACTACTTGAAGCACTCAAGGCAAGCTTT-(TAMRA)-3'[114]. For quantification of GFP DNA following reverse transcription, the forward primer used was 5'AAGCTGACCCTGAAGTTCATCTG-3', the reverse primer was 5'-TTGAAGAAGTCGTGCTGCTTCAT-3' and the probe was 5' (FAM)-ACCGGCAAGCTGC-(MGB NFQ) 3' [115].

Cellular Fractionation

Resting cells, or cells that were activated by anti-CD3/CD28 antibody for 48 hours, were lysed **(**50 mM HEPES, pH 7.4, 125 mM NaCl, 0.2% NP-40, 0.1 mM PMSF and EDTA-free protease inhibitor cocktail (CalBiochem, San Diego, CA) and subjected to ultracentrifugation as previously described (125,000 X g, TLA 55 rotor, Beckman Coulter) [109]. Equal volumes of supernatant and pellet were subjected to Western blotting using polyclonal anti-APOBEC3G antibody [51], with a goat anti-rabbit secondary antibody conjugated with Alexa Fluor 680 (Invitrogen Molecular Probes, Carlsbad, CA). Band intensity was quantified using the Odyssey (LI-COR Biosciences, Lincoln, Nebraska). Values

are expressed as the percent relative light units (RLUs) of supernatant band, relative to total RLUs for both supernatant and pellet.

Cellular lysates of activated Th1 and Th2 cells were subjected to centrifugation through a 4%-40% sucrose gradient as previously described [116]. Gradients were centrifuged overnight at 32,000 RPM in a SW-41 rotor (Beckman), and then 12 one milliliter fractions were collected, precipitated by trichloroacetic acid, resuspended in SDS sample buffer, and subjected to Western blotting. Values are presented as the percent RLUs in each fraction relative to the total RLUs for each sample.

Results

HIV-1 infection of, and integration into, CD4+ T helper type 1 cells relative to T helper type 2 cells.

Following 48 hours of T cell receptor activation by anti- CD3/CD28 antibody, cytokine-polarized Th1 and Th2 cell cultures were infected with VSV-G pseudotyped, vif-competent HIV-1 expressing GFP [117-119]. Forty-eight hours after infection, the percentage of cells with integrated HIV genomes was determined by GFP expression using FACS analysis. In cells from each of three donors, infection of Th2 cells yielded greater GFP signal than did infection of Th1 cells (Figure 3-1A).

Since the block to reverse transcription in resting CD4+ T cells has been attributed to the “low molecular mass” form of A3G [50], we separated A3G forms

in resting and activated Th subtype cells by two methods. Sucrose gradient density centrifugation was validated previously to separate A3G forms [116]. Density gradients of activated Th1 cell lysates confirmed that 39% of the total A3G in activated Th1 cells was in the two lower density fractions, and 23% was in the two highest density fractions (Figure 3-1B). There was 1.5-fold more lower-density A3G in activated Th1 cells than activated Th2 cells (data not shown). A second method of characterizing A3G forms was also used. “Low molecular mass” A3G has previously been shown to remain in the supernatant after ultracentrifugation [109]. After ultracentrifugation, activated Th1 cell lysates had less supernatant A3G than did resting Th1 cells. However, a substantial proportion of A3G remained in the supernatant of activated Th1 cell lysates after ultracentrifugation. A similar difference was seen after ultracentrifugation of resting versus activated Th2 cell lysates, although the amount of total and supernatant A3G was lower in the Th2 than Th1 lysates (data not shown).

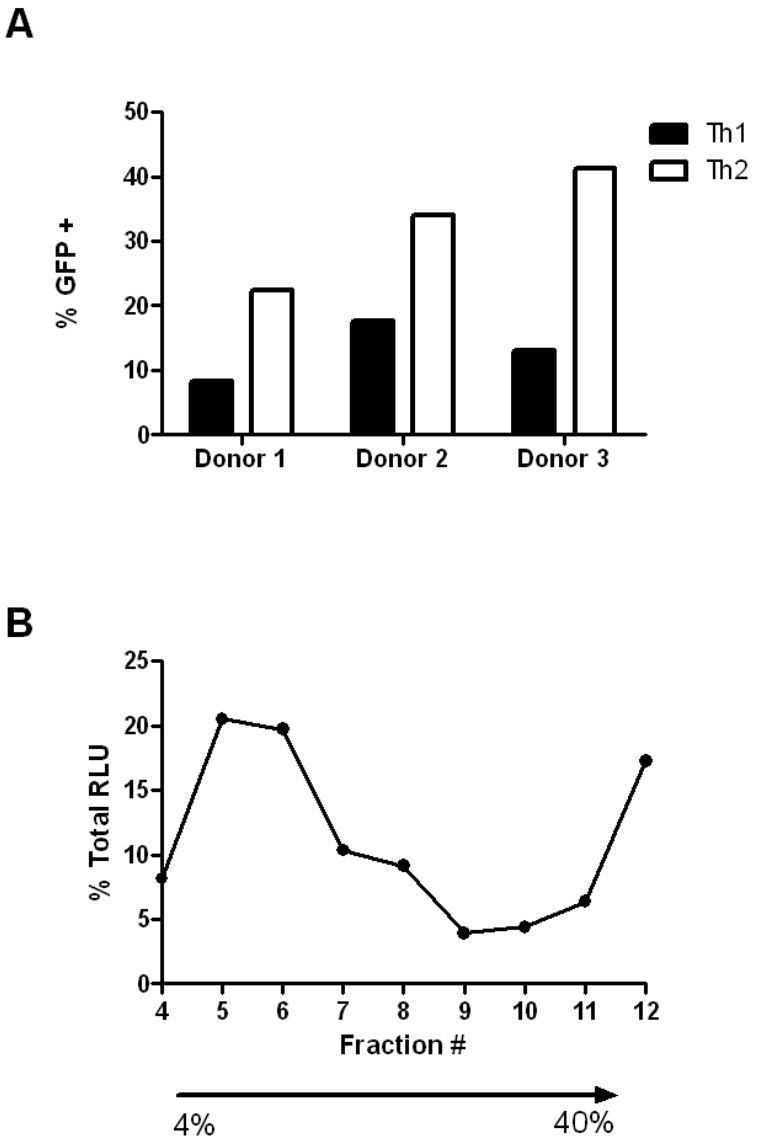


Figure 3-1. HIV-1 infection / integration is decreased in CD4+ T helper type 1, relative to T helper type 2, lymphocytes. (A) Cytokine polarized Th1 and Th2 cells derived from three individual donors' naïve cells were activated with anti- CD3/CD28 antibody for 48 hours, then infected with VSV-G, vif-positive HIV-GFP. Virus was washed off after 4 hours and GFP positive cells, reflecting cells with integrated provirus, were counted 48 hours post infection. A smaller percentage of the Th1 cell population is GFP+ than the Th2 cell population.
(B) Th1 cells were activated with anti- CD3/CD28 antibody for 48 hours then lysed and subjected to sucrose gradient separation as described in Materials and Methods. Subsequent to activation some A3G still remains in lower density fractions (fractions 4, 5, and 6).

Reverse transcription in CD4+ T helper type 1, relative to T helper type 2, lymphocytes.

Given the reduced amount of GFP from integrated virus in Th1 cells, we next determined at which step of infection this restriction was occurring relative to Th2 cells. A qPCR assay for early and late reverse transcription products showed that the two cell types differed in their ability to support reverse transcription (Figure 3-2). Figures 3-2A and 3-2B indicate that Th1 cells lag behind Th2 cells from one donor in the amount of both early and late RT products formed. There was a significant difference at a single time point (18 hours after infection) across multiple donors in both early and late RT products (Figures 3-2C and 3-2D), as well as a significant difference between Th1 and Th2 early and late RT products from 3 replicate infections of a single donor's cells (Figure 3-2E).

2-LTR circle formation in CD4+ T helper type 1, relative to T helper type 2, lymphocytes.

The measurement of 2-LTR circles formed following HIV-1 infection serves as a marker for nuclear entry of reverse transcripts and abortive integration events [9]. We studied whether 2-LTR circle formation differed after A3G-free virion infection of activated Th1 versus Th2 cells. Infection of activated Th1 cells with A3G-free virions led to significantly more 2-LTR circles than did infection of activated Th2 cells (Figure 3-3A). This is consistent with relatively more reverse transcripts not integrating and forming abortive 2-LTR circle forms in activated Th1 than Th2 cells. Previous studies reported, however, that A3G packaged in the virion reduced 2-LTR circle formation as well as decreasing

integration, consistent with impaired host cell-mediated 2-LTR circle formation and integration [39,120]. Therefore, we also evaluated effects of virion-packaged A3G on 2-LTR circle formation.

To study virion packaged A3G, TZM-bl cells, which do not contain A3G, were infected with vif-deleted virions that did, or did not, contain A3G. These were produced by transfection of 293T cells, with or without a co-transfected A3G expression plasmid. Infection with vif-deleted viruses containing A3G led to fewer 2-LTR circles, relative to TZM-bl cells infected with A3G-free virions. (Figure 3-3B). Fewer 2-LTR circles were also seen in TZM-bl cells infected with Th1 cell-produced virions than in those infected with Th2 cell-produced virions (Figure 3-3C), consistent with the previously documented greater packaging of A3G in Th1 cell-produced virions [111]. Thus, virion-packaged A3G and cytoplasmic A3G have different effects on 2-LTR circle formation.

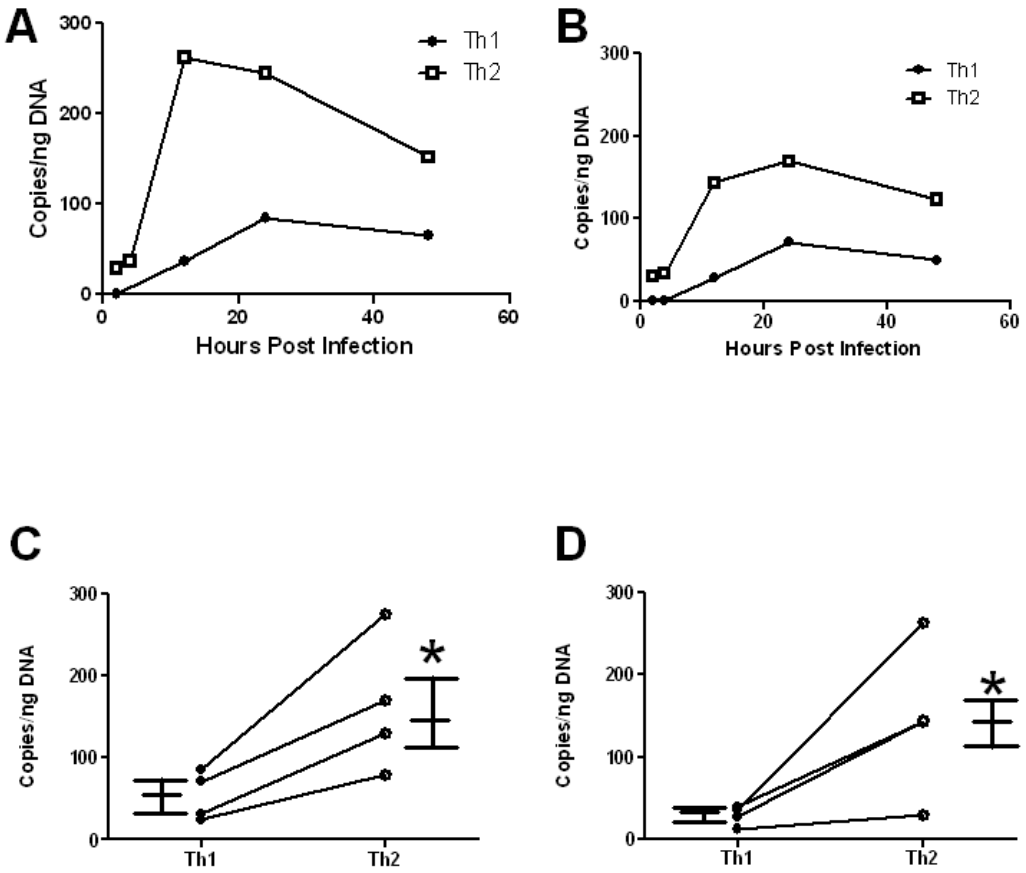


Figure 3-2A-D. Reverse transcription is decreased in CD4+ T helper type 1, relative to T helper type 2, lymphocytes.
(A and B) Following infection with VSV-G, vif-positive HIV-GFP, DNA from Th1 and Th2 cells was isolated and assayed by qPCR for early (A) and late (B) RT products. DNA was collected at 2, 4, 18, 24 and 48 hours post infection from a single donor's cells. Th1 cells produced fewer reverse transcription products over time than did the Th2 cells. (C and D) Reverse transcription products were also measured after VSV-G, vif-positive HIV-GFP infection of Th1 and Th2 cells derived from multiple donors' naïve cells at a single time point,18 hours post infection (C, early RT products, and D, late RT products; medians and interquartile range are indicated; *p≤ .02, Mann Whitney U Test). Th1 cell RT products were decreased relative to Th2 cell RT products.

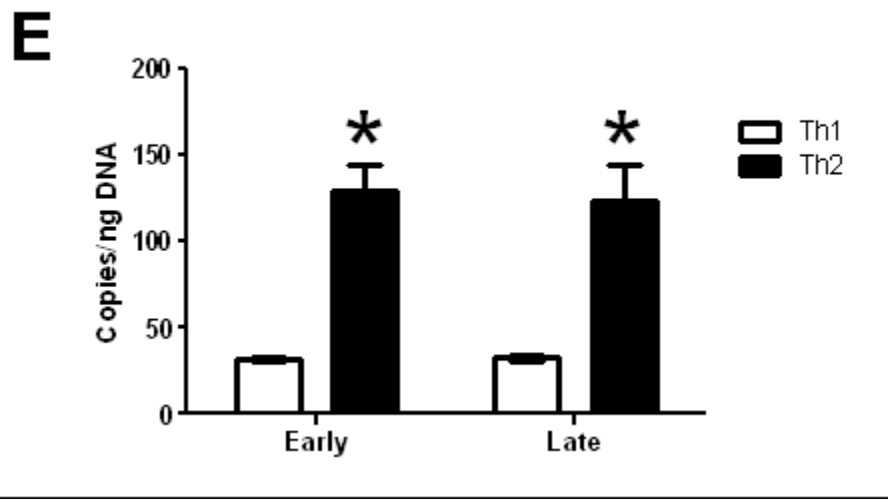


Figure 3-2E. Reverse transcription is decreased in CD4+ T helper type 1, relative to T helper type 2, lymphocytes. (E)Early and late RT products were also assayed after triplicate VSV-G, vif-positive HIV-GFP infections of Th1 and Th2 cells derived from a single donor's naïve cells at 18 hours post infection (E, mean and standard error are indicated). Th1 cells had fewer early and late reverse transcription products at 18 hours following infection.(*p≤ .02, Mann Whitney U Test).

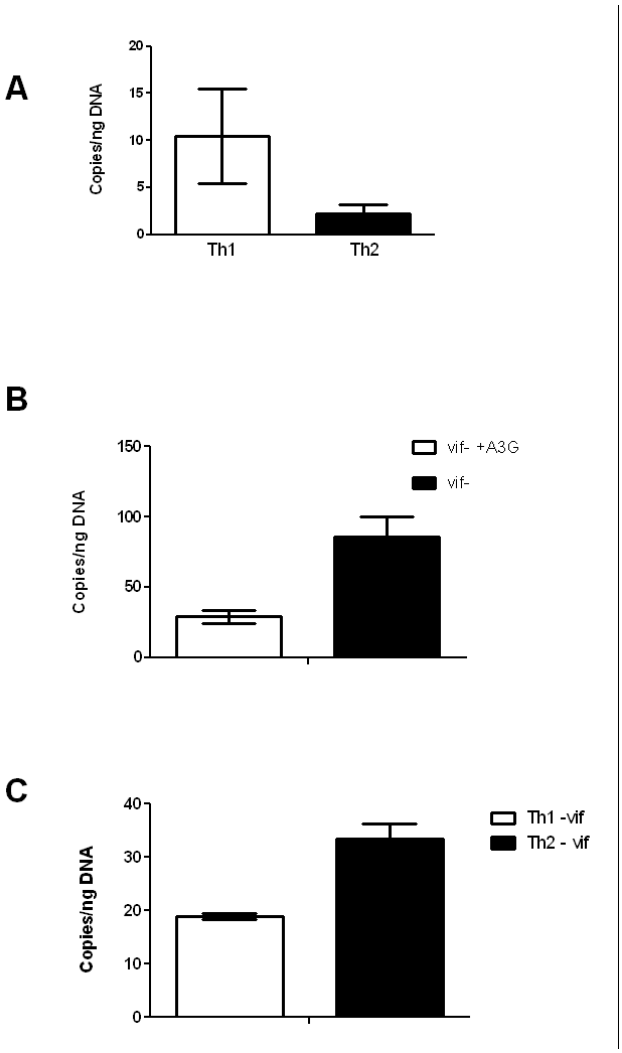


Figure 3-3. 2-LTR circle formation is increased in CD4+ T helper type 1, relative to T helper type 2, lymphocytes after vif-positive HIV-1 infection.
Increased cytoplasmic A3G led to increased 2-LTR circle formation (A), while increased virion-packaged A3G caused increased 2-LTR circle formation (B and C).(A) Th1 and Th2 cells derived four individual donors were infected with VSV-G, vif-positive HIV-GFP. Forty-eight hours after infection, DNA from infected Th1 and Th2 cells was assayed by qPCR for 2-LTR circles, which were found to be increased in Th1, relative to Th2, cells. Means and standard errors of the four donors are shown (*****p< .03, Mann Whitney U Test). (B) TZM-bl cells, which lack APOBEC3G, were infected with vif-deleted virus with or without packaged A3G. Forty-eight hours after infection, DNA was isolated and assayed for 2-LTR circle formation by qPCR. Virions containing A3G produced fewer 2-LTR circles. Means and standard errors of duplicate experiments are shown. (C) TZM-bl cells were infected with vif-deleted HIV-1 produced from Th1 or Th2 cells. Th1-produced virions led to fewer 2-LTR circles.

APOBEC3G expression modulates reverse transcription, 2-LTR circle formation, and integration

To determine whether the difference in cytoplasmic A3G between Th1 and Th2 lymphocytes causes the observed difference in reverse transcription, 2-LTR circle formation and integration between these cells when infected, the expression level of A3G was altered in each cell type. Retroviral transduction of A3G was used to increase A3G expression in Th2 cells, as previously reported [111]. Since the A3G-transducing vector was derived from HIV and the virus used for subsequent infection expresses GFP in place of nef, GFP DNA was quantified rather than using the early and late HIV-1 reverse transcription product primers. The amount of reverse transcribed GFP DNA was reduced in Th2 cells over-expressing A3G relative to control vector-transduced Th2 cells (Figure 3-4A and 3-4B). The formation of 2-LTR circles was also increased after infection in Th2 cells over-expressing A3G relative to control vector-transduced cells (Figure 3-4C). A3G over-expression in Th2 cells also reduced the amount of integrated genomes as measured by GFP production (Figure 3-4D).

As further confirmation of the role of endogenous cytoplasmic A3G in modulating reverse transcription, 2-LTR circle formation and integration, Th1 cells were incubated with a neutralizing anti-IFN-γ antibody previously documented to reduce A3G expression in Th1 cells [111], or an isotype control antibody. Infection of Th1 cells treated with the neutralizing anti-IFN-γ antibody with GFP-expressing HIV-1 led to increased early and late RT products (Figure 3-5A), reduced 2-LTR circle formation (Figure 3-5B), and an increase in

integrated genomes based on increased GFP expression (Figure 3-5C), relative to the isotype antibody control.

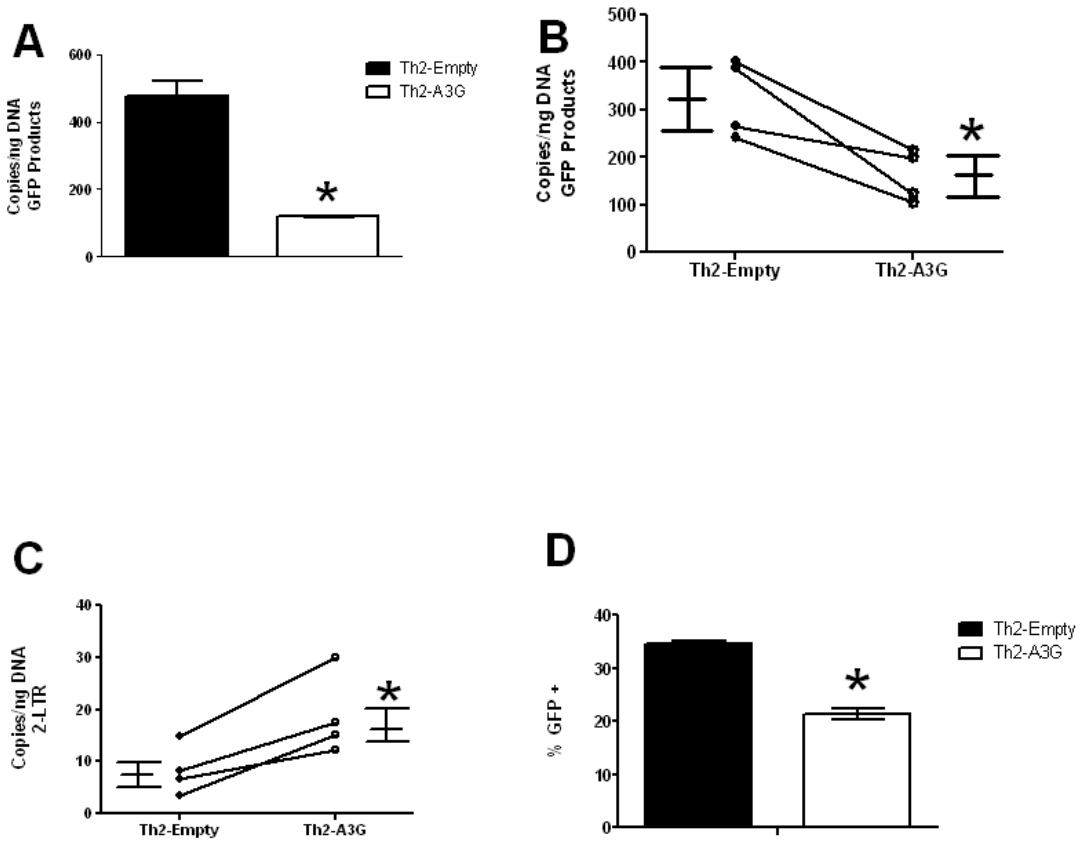


Figure 3-4. Over-expression of cytoplasmic A3G in Th2 cells reduces reverse transcription products and increases 2-LTR circle formation.
Th2 cells were transduced with an A3G-expressing or control retrovirus vector, activated for 48 hours, and then infected with VSV-G, vif-positive HIV-GFP. The increased expression of A3G decreases the formation of reverse transcription products, increases the formation of 2-LTR circles and reduces amount of integrated proviruses as measured by GFP expression. (A) DNA was isolated at 18 hours after infection and used in qPCR assays to analyze GFP reverse transcription product formation in triplicate from one donor's cells. Over-expression of A3G in Th2 cells decreased reverse transcription products measured by amplifying the GFP sequences that replaced the nef open reading frame (means and standard errors are shown; *****p≤ .02, Mann Whitney U Test). (B) GFP reverse transcription products measured as in (A) from matched A3G- and control (Th2-empty) vector-transduced Th2 cells derived from 4 different donors' naïve cells. (C) DNA was isolated at 48 hours after infection to analyze 2-LTR circle formation. Over-expression of A3G in Th2 cells increased 2-LTR circles (means and interquartile ranges are shown; *****p≤ .02, Mann Whitney U Test). (D) Cells were also analyzed 48 hours post infection for GFP expression. A3G over-expression in Th2 cells decreased GFP expression from integrated provirus (mean and standard error are shown; *****p≤ .02, Mann Whitney U Test)

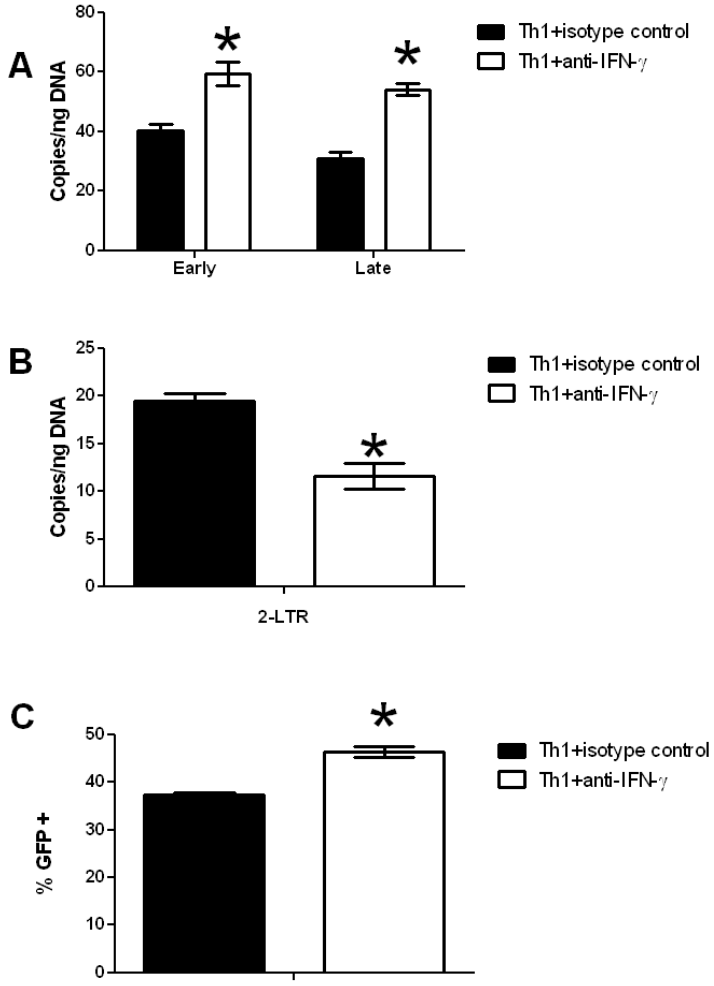


Figure 3-5. Reduction of cytoplasmic A3G in Th1 cells increases reverse transcription products and decreases 2-LTR circle formation.

Th1 cells were activated for 48 hours in conjunction with neutralizing anti-IFN-γ antibody, which decreases A3G expression, or an isotype antibody control. The cells were infected with VSV-G, vif-positive HIV-GFP and DNA was isolated at 18 or 48 hours post infection. Decreased expression of A3G in Th1 cells leads to increased reverse transcription products, decreased 2-LTR circles and increased integration, relative to the isotype antibody control.(A) Early and late RT products were determined 18hours post infection. Both early and late RT products were increased in the presence of the A3G-reducing anti-IFN-γ antibody (means and standard errors are shown**; ***p≤ .02, Mann Whitney U Test). (B) 2-LTR circles were quantified 48 hours post infection. Two-LTR circles were decreased in the presence of the A3G-reducing anti-IFN-γ antibody (means and standard errors are shown**; ***p≤ .05, Mann Whitney U Test). (C) GFP expression from integrated proviruses in live cells was analyzed 48 hours post infection. Integrated copies of HIV were increased in the presence of the A3G-reducing anti-IFN-γ antibody (means and standard errors are shown**; ***p≤ .02, Mann Whitney U Test).

Discussion

Comparison of HIV-1 infection of activated Th1 and Th2 lymphocytes in the present study provides some of the first documentation that cytoplasmic A3G partially restricts incoming A3G-negative HIV-1 virions at the level of reverse transcription and integration in activated T lymphocytes. The effects of cytoplasmic A3G on incoming A3G-negative virions, the “target cell effects” of A3G, have previously been characterized in resting T cells, monocytes, and immature dendritic cells [50,108,109] . T cell activation has been hypothesized to completely abrogate this target cell block. However, the present comparison of different types of activated T helper cells has now revealed relatively less reverse transcription, less integration, and more 2-LTR circle formation in activated Th1 than activated Th2 cells. Modulating the levels of expression of A3G in these two cell types alters each of these phenotypes and strongly supports the causal role for A3G in the relative restriction in Th1 cells observed here. When Th2 cells that express relatively lower levels of A3G were transduced with a vector that over-expresses A3G, they become more restrictive to reverse transcription and integration. When A3G expression was reduced in Th1 cells, they were rendered less restrictive to reverse transcription and integration. In addition to supporting that A3G is responsible for the observed reduction in replication of vif-positive, A3G-negative virus in activated Th1 cells noted here, these results add to prior data indicating that A3G activity in CD4+ T lymphocytes is regulated by IFN-γ [111] as well as other cytokines [57].

Variation in cytoplasmic A3G and virion-packaged A3G unexpectedly differed in effects on 2-LTR circle formation. Earlier studies have observed that increasing virion-packaged A3G decreased 2-LTR circle formation [39,120]. We confirmed this in a comparison of A3G-positive and A3G-negative virion infections of A3G-negative cells, as well as in a comparison of infections of A3G-negative cells with virions produced from activated Th1 cells (e.g.; with greater virion A3G content) and virions produced from activated Th2 cells (e.g.; containing relatively less virion A3G). In sharp contrast, A3G-negative virion infection of activated Th1 cells led to increased 2-LTR circle formation compared to infection of activated Th2 cells. This opposite effect on 2-LTR circle formation indicates that cytoplasmic A3G has a different effect than A3G packaged in the virion. Based on our data, and earlier studies analyzing virion-packaged A3G, we hypothesize that virion-packaged A3G may have a different association with one or more components of the pre-integration complex than does cytoplasmic A3G, thereby having a different effect on the ability of cellular enzymes to process ends of the reverse transcribed DNA and/or on the activities of the pre-integration complex [39,40,120]. It is also possible that virion-packaged APOBEC3G interferes with nuclear entry of the pre-integration complex, while cytoplasmic APOBEC3G does not.

Prior studies have implicated a “low molecular mass” form of A3G in mediating the resting T cell block to incoming A3G-negative virions. The results of the present study indicate that some A3G persists in activated Th1 and Th2 cell lysate supernatants after ultracentrifugation and in lower density fractions of

sucrose density gradients. We hypothesize that the remnant “low molecular mass” forms mediate this relatively greater restriction we have identified in activated Th1 cells compared to Th2 cells. Further work will be needed to confirm or refute this hypothesis by further characterizing and selectively affecting the remnant “low molecular mass” A3G in activated Th1 cells. If this hypothesis is supported, it will also be of interest for future studies to determine the minimum concentration of “low molecular mass” A3G required for restriction.

The results reported here indicate that wild-type HIV-1 virions that infect Th2 cells are more likely to successfully replicate than those infecting Th1 cells. Our earlier observations also indicated that Th2 cells that become infected will subsequently produce virions that are more infectious due to reduced virion packaging of A3G. These data may explain earlier studies demonstrating better replication of HIV in Th2 cells [67,79,81,121] and suggest new work to characterize the role of Th2 cells during HIV pathogenesis. This study also adds further support to the important role of A3G in HIV pathogenesis through multiple different restricting mechanisms. Further characterization of these mechanisms may lead to novel approaches to improving A3G effects as a potential therapeutic or preventive strategy.

CHAPTER IV

CONCLUSIONS AND FUTURE DIRECTIONS

APOBEC3G is a cellular protein that is a potent innate defense against endogenous retrotransposition and exogenous pathogens that require reverse transcription, including HIV-1 and HBV. A deeper understanding of the biology of this defense mechanism in human T cells will provide a greater insight into the pathogenesis of HIV-1. Given poor progress in HIV vaccine development and a continuing need for new therapeutics due to antiretroviral drug resistance, these insights may also suggest potential avenues for novel therapeutic or preventive approaches. There are several strategies that could utilize APOBEC3G as a therapeutic. For instance, targeted therapies may block the vif-mediated degradation of APOBEC3G, overcome vif's effect by increasing cellular levels of APOBEC3G, increase specific packaging of A3G in virions, or maximize antiviral activity by blocking the formation of large inactive complexes of APOBEC3G. Development of these strategies depends on greater understanding of APOBEC3 biology. The goals of this project were to identify if T helper cell differentiation modulated this defense mechanism. Specifically, I aimed to determine if increases in expression of APOBEC3s within a physiological range could augment anti-HIV effects in the presence of vif. The implications of this work for understanding evolutionary mechanisms for APOBEC3 expression differences with T cell differentiation, further characterizing early steps in HIV

replication, expanding knowledge of HIV pathogenesis, and exploring basic mechanisms of transcriptional regulation during T cell differentiation in greater depth will be described.

APOBEC3 expression and T Helper Cell Differentiation

Differentiation of CD4+ T cells from a naïve to an effector or memory phenotype involves vast differences in gene expression profiles[65]. These differentiation pathways are regulated by broadly-acting transcription factors including Tbet and GATA3. Tbet is a master transcription factor important for the differentiation of a naïve T cell to a Th1 phenotype, whereas GATA3 plays a primary role in differentiation to a Th2 phenotype [60-62]. Based on observations that the putative promoter regions of APOBEC3G and APOBEC3F harbored binding sites for GATA family members, we hypothesized that expression of APOBEC3G would vary with T cell differentiation. Indeed, we found that expression of GATA3 reduced expression of APOBEC3G relative to control transduced cells. Interestingly, expression of Tbet increased the amount of APOBEC3G message in primary T-cells.

Our findings that Tbet and GATA3 affect regulation of APOBEC3s expression led us to hypothesize that the difference in APOBEC3s expression occurred with physiological T helper cell differentiation. Following *in vitro* cytokine derivation of T helper cells, we found that Th1 cells expressed greater levels of APOBEC3G and APOBEC3F than Th2 cells. This is the first evidence of A3 variation in differentiated primary T cells.

Given our findings about APOBEC3 expression in the Th1 and Th2 helper subtypes, it is of interest to extend this study in the future to characterize the relative APOBEC3 expression levels in other T helper subtypes. Th17 cells provide immune protection at mucosal sites, particularly against bacterial and fungal pathogens. [59] This defense is decimated by HIV-1 infection, allowing ongoing translocation of gut flora to the blood stream that results in massive, persistent immune activation [122]. This immune activation is the likely cause of most of the CD4+ T cell loss during HIV-1 infection, and is not completely stopped by antiretroviral therapy [123]. The regulatory T cell (Treg) subset provides a way to down-modulate and stop immune responses following clearance of the offending agent. The loss of those cells following HIV infection [124,125] could also result in unregulated immune activation that contributes to T cell loss. Given that HIV-1 can replicate robustly in both Treg and Th17 cells, we hypothesize that expression/function of APOBEC3s may be at a relatively low level. If so, the potential ability to therapeutically increase protection from APOBEC3s in these cells may offer an opportunity to increase mucosal integrity, maintain immune regulation, dampen abnormal T cell activation, and subsequently decrease T cell loss in HIV infection. Understanding the expression and regulation of APOBEC3s in these additional subtypes would further aid our understanding of APOBEC3 biology and HIV pathogenesis. The analysis of APOBEC3 expression in these cell types can be done using an approach similar to the one used here: *in vitro* deriving or directly sorting these other subtypes, and then analyzing them for their APOBEC3 protein content, infectivity of HIV

virions produced from them as well as susceptibility to HIV infection.

Future work that will also be worthy of consideration includes characterizing the biological selective pressure underlying why different T cell differentiation states differ in their expression of APOBEC3G. Several studies have shown that APOBEC3s have evolved to limit retrotransposition. This suggests the hypothesis that greater APOBEC3G in Th1 cells may be associated with greater retrotransposition activity in those cells. Evaluating both relative expression levels and mobility of transposable elements in the two cell types could test the hypothesis that expression of APOBEC3s is increased as a physiological response to relatively greater retrotransposition. To perform this evaluation, the amount of retrotransposon RNA (LINE, alu, hY [126] in activated Th1 and Th2 cells will be determined by qRT-PCR from whole cell lystates, as well as from A3G immunoprecipitates. This will gauge the relative total amounts of retrotransposon expression as well as what relative quantity is sequestered in A3G complexes in each of the two cell types. Retrotransposition will also be experimentally decreased using siRNA specific to the LINE-1 RT [127]. The hypothesized result is that A3G levels in the Th1 and Th2 cells will be reduced if not needed for retrotransposon defense. Interestingly, treatment of cells with HIV reverse transcriptase inhibitors also demonstrated a reduction in LINE-1 activity [128]. Therefore, it would also be interesting to test if RT inhibitor treatment reduces retrotransposition in these T cell subsets and if that treatment decreases APOBEC3 expression. If this hypothesis is supported, it may lead to a better understanding of how to prevent retrotranposition mediated malignancy[129-

131]. This would have broader implications in understanding effects of antiretroviral therapy on T cells, and may bear on the difficult problem of HIV persistence despite currently optimal antiretroviral therapy.

Mechanisms of Virion Packaged APOBEC3G HIV Replication Restriction

The higher levels of APOBEC3G and APOBEC3F in Th1 than Th2 cells were shown here to have important functional antiviral effects. We found that this expression difference led to a difference in infectivity of virions produced by these cells following infection with a CXCR4 tropic lab strain of HIV-1 [Fig 2-4]. This decreased virion infectivity occurred even if vif was present in the HIV-producing cell. This demonstrates that physiological levels of APOBEC3G can overcome the countermeasure of HIV-1 vif and partially decrease virion infectivity. This is the first demonstration of restriction of vif-positive HIV-1 by APOBEC3s in primary cells.

The concept of increasing expression of APOBEC3G to reduce infectivity of HIV-1 is inviting as a therapeutic approach, but has a potentially significant limitation. As these proteins are cytidine deaminases, there exists the concern that their over-expression may lead to deamination. Such an increase in expression may lead to chromosomal mutation and oncogenesis. Though some APOBEC family members do translocate to the nucleus where they may affect host chromosomes, A3G and A3F maintain cytoplasmic localization, even when over-expresssed [132] Nevertheless, the possibility of mutagenic effects on either nuclear or mitochondrial DNA will need to be thoroughly evaluated as part of

future studies intended to further explore increasing APOBEC3s by cytokines or other interventions.

The originally described mechanism of restriction by virion packaged APOBEC3G was by its enzymatic activity of cytidine deamination. In our study, we were unable to identify evidence of cytidine deamination, even though we were observing an APOBEC3G-mediated reduction in infectivity. This finding argues that there are other mechanisms of restriction beyond the deaminase enzymatic activity of APOBEC3G. The results of other recent studies directly addressing this question support this conclusion [32,36,39,120]. These studies suggest that infectivity can be reduced by APOBEC3G's ability to interfere with the process of reverse transcription and subsequent integration, and that cytidine deaminase activity is not essential for antiviral activity. Indeed, further understanding of non-deaminase mediated mechanisms is the preferred focus for future work, as if those mechanisms can be exploited further therapeutically this would help to avoid potential cellular mutagenesis as a potential toxicity.

Though theses studies provide strong evidence for the mechanism of non-deaminase mediated APOBEC3G restriction of replication, there remains a need to further characterize these non-deaminase mediated mechanisms. APOBEC3G was shown to reduce infection by diminishing the synthesis of viral DNA [39,40,120]. Some data suggest a mechanism for this effect on reverse transcription involves reduction in processing of the tRNA primer [39], or reduction in tRNA primer binding [38]. However, no specific interactions of APOBEC3G with reverse transcriptase have been demonstrated. As A3G has

been shown to bind integrase in the virion [40], it may also bind directly to RT. A3G is an RNA binding protein, so there is also a possibility that it may directly bind the tRNA primer. This can be tested by immunoprecipitation of viral lysates to detect an interaction between A3G and RT and/or the tRNA primer as well as immunoprecipitation of permissive cells infected with A3G-containing virus as the association may not occur until the process of reverse transcription begins in the target cell. This interaction may certainly be bridged by the HIV nucleocapsid, as A3G has been demonstrated to bind nucleocapsid or an RNA bridge, a possibility which would be tested by treating the lysates to be immunoprecipitated with RNase prior to precipitation.

In addition to demonstrating reduction of viral DNA synthesis as a non-deaminase mechanism of HIV infectivity reduction, these studies have also demonstrated that virion packaged A3G results in a reduction in viral DNA integration [39,40,120] Interestingly, the reduction in integration is independent of the reduction in reverse transcription products. It is surprising that both a reduction in integration and 2-LTR circles is observed. Two-LTR circles, a measure of aborted integration, would normally be expected to increase as integration decreases. However, one of these studies provides some evidence that this reduction in integration occurs due to APOBEC3G modifying the terminal ends of the completed reverse transcription product, thereby lowering the efficiency of 2-LTR circle formation [39]. We demonstrate that infection of A3G-negative (permissive) cells with Th1 cell-produced virions forms fewer 2-LTR circles than does infection with Th2 cell-produced. This reduction in 2-LTR

circles has also been shown by others to result from HIV replication in the face of exosome-packaged A3G[133]. Though modification of the viral DNA ends may reduce 2-LTR circle formation, a reduction in nuclear translocation and import of unmodified, completed reverse transcripts can also lead to this result.

To investigate the possibility of APOBEC3G-dependent reduction in nuclear translocation or import, we would begin by infecting permissive cells with virions that contain a GFP-tagged integrase protein (using vpr trans-incorporation as described by Christ *et. al*.[6]) and either do, or do not, contain APOBEC3G. The GFP level in the nuclei of infected cells will be quantified. In addition, integration events (eg, as measured by alu-PCR), 1-LTR circles and 2-LTR circles would be measured from each infection to evaluate the total amount of viral DNA that was translocated to the nucleus. If these studies demonstrate equal nuclear entry of reverse transcripts from the A3G-containing and A3G-negative virions, then the conclusion that modification of the ends of the reverse transcription product is the only mechanism by which integration and 2-LTR circle formation are reduced would be supported.. If lower amounts of GFP are observed in the nucleus from the A3G-containing virions relative to the A3G-deficient virions, this would support a role for decreased nuclear import in the reduction of 2-LTR circles and integration events. Nuclear import machinery components that have been implicated as interacting with integrase or other PIC components will also be examined to determine if APOBEC3G interferes with the interaction between an import protein and integrase/PIC. These experiments may

lead to further investigation into APOBEC3G's interaction with the PIC and/or proteins involved in PIC nuclear transport.

Cytoplasmic APOBEC3 Restriction of HIV Replication

It has long been accepted that resting CD4+ T cells are refractory to infection by HIV-1 [49]. Previous studies of APOBEC3G have shown that a low molecular mass form of APOBEC3G in the cytoplasm of resting CD4+ T cells is responsible for this essentially complete block to the early phase of HIV-1 replication [50]. We hypothesized that there is residual APOBEC3G left in a low molecular mass form after T cell activation, and that this residua may lead to some partial restriction of HIV even in activated cells. Using the CD4+ T helper subtypes shown here to have differing expression of APOBEC3G as a model, we tested whether differences in APOBEC3G in primary CD4+ T cells would lead to differences in degree of partial restriction of early replication events even in activated cells. Such a partially restricting effect had not previously been hypothesized, and could not be identified by comparison of activated cells (which do support replication) to resting cells (which do not support replication).

We found that, even after activation, Th1 cells were more restrictive to incoming HIV-1 replication than Th2 cells, in an APOBEC3G dependent manner. This restriction was demonstrated by a reduction in early and late reverse transcription products and integrated proviruses. We also showed that residual APOBEC3G remained in lower density fractions in activated Th1 cells using sucrose density gradient centrifugation [Fig 3-1]. Therefore, the proposed

transition from restricting low molecular mass APOBEC3G to a non-restricting high molecular mass complex of A3G is not an all-or-nothing event. If sufficient APOBEC3G is expressed in the cytoplasm of a target cell, a portion will remain actively restrictive of incoming HIV-1 even after activation.

These results also raise the question of how cytoplasmic APOBEC3G is affecting this restriction mechanistically. The initial study describing the APOBEC3G-mediated block in resting T cells found no hypermutation of reverse transcription products [50]. This suggests, as discussed earlier for virion-packaged APOBEC3G, that the mechanism of restriction is cytidine deaminase-independent. There are multiple effects of virion packaged APOBEC3G on early steps of HIV replication that have been described previously. We found many of those same restrictions in early and late RT product formation and integration in our study of the target cell-expressed APOBEC3G. This suggests that virion packaged A3G and target cell expressed A3G likely act in very similar manners. However, an additional result in our work suggests that yet another mechanism of restriction may remain to be characterized.

Interestingly, we observed an increase in 2-LTR circles after APOBEC3G-negative virion infection of activated T cells with more APOBEC3G, relative to target cells with less APOBEC3G. This contrasts with the reduction in 2-LTR circles seen with virion-packaged APOBEC3G and has not been reported before. Virion-packaged APOBEC3G is hypothesized to reduce 2-LTR circles by altering the ends of completed reverse transcript product and/or interacting with the PIC or nuclear import machinery to reduce nuclear import [39,40]. However, our

result suggests that cytoplasmic target cell APOBEC3G will not do either of these activities that will interfere with 2-LTR circle formation, while still having antiviral mechanism(s) in common with virion-packaged APOBEC3G.

To begin to explore the differences and similarities between the mechanism of action of cytoplasmic and virion-packaged A3G, experiments are suggested similar to those already described in this section investigating the role of virion-packaged A3G restriction. As virion A3G has been shown to bind integrase, we would immunoprecipitate integrase from a non-permissive target cell infected with A3G-free HIV to determine if the cytoplasmic APOBEC3G can bind to integrase. If virion A3G is found to prevent binding of nuclear import components to the PIC/integrase, we would hypothesize that cytoplasmic A3G may not have that effect. Similar studies to those proposed above to evaluate nuclear import would be performed by infecting A3G-expressing cells with A3G-free virus. Comparing those results to the results of an infection of permissive cells with an A3G containing virion, will test the hypothesis that virion A3G has an effect on 2-LTR circles that is not seen with cytoplasmic A3G.

In vivo, a spreading HIV infection will encounter both the virion packaged and target cytoplasmic mechanisms of A3G restriction. This study has evaluated these two restriction mechanisms independently. It is of interest then to ask if the two mechanisms can act synergistically or even in opposition. This question could be initially investigated by performing infections under three conditions. One condition would be infecting permissive cells with an A3G containing virus. The second condition would involve infecting the same permissive cell line that is

transiently transfected with A3G with an A3G-free virus. The third condition would be infecting the A3G expressing line with an A3G containing virus. Evaluation of the formation of RT products, 2-LTR circles, integration events and subsequent virion release would determine under which condition the A3G was having the greatest effect on replication. We hypothesize that the third condition would result in the greatest reduction in replicative capacity. There is a precedent for this, as such synergistic effects of virion and cytoplasmic APOBEC3G were recently reported in studies of mouse mammary tumor virus [134].

Implications of APOBEC3 Expression in HIV-1 Pathogenesis

One of the major unanswered questions in pathogenesis is what factors favor a particular dominant virus co-receptor tropism. Initial infection by HIV is generally caused by a CCR5 tropic virus [135]. However, recent data from clinical trials of a CCR5 antagonist indicate that a substantial proportion of patients previously thought to have only CCR5 virus also have a small proportion of dual/mixed-tropic virus detectable in plasma [136]. In addition, the tropism dominating in the circulating virus population shifts in about 50% of subjects to sole CXCR4 use during the course of infection and progression to AIDS. This ‘tropism shift’ to sole CXCR4 use is associated with a change to a more rapid decline in CD4+ T cells in peripheral blood; it is hypothesized and not proven that the ‘tropism shift’ causes the increased pace of immunodeficiency progression [135] The factors that favor selection of subpopulations of dual/mixed virus or emergence of a ‘tropism shift’ are not defined. Our results suggest novel

hypotheses that may contribute to better understanding of these aspects of pathogenesis.

Vicenzi, et al 2002 found that dual tropic viruses (and X4 tropic viruses)replicated much better in Th2 than Th1 cells, and that X4 tropic viruses replicated less well than the dual tropic viruses in both these cells [137]. These findings were not explained by differences in virus entry and were established in that earlier work to be due to an effect on an early post-entry step of replication. Given that we identified differences in virion infectivity and early steps of HIV replication in Th1 versus Th2 cells using CXCR4-tropic virus, we hypothesize that such differences may be greater with dual-tropic viruses and less with CCR5-tropic viruses. If this is found, it would suggest differences in early steps of replication based on which co-receptor is used for virus entry. This has the potential for adding new insights into virus entry and/or the role of signaling in post-entry steps of HIV replication.

The current work may also suggest that differences in A3G, or changes in the balance of cells expressing high levels of A3G, may be involved in shifts in virus tropism that have major impact on pathogenesis. The evidence in this study leads to the hypothesis that sub-populations of dual tropic virus (or a shift to sole CXCR4 tropic virus) may occur in individuals with a relatively lower Th1/Th2 cell ratio. Those who shift tropism may be individuals who have relatively more Th2 host cell persistence that favors replication of the viruses that replicate better in those cells. It is also possible that there are differences across individuals in the level of APOBEC3G in Th2 cells and that the more immuno-depleting dual- or

CXCR-4 viruses are favored in virus populations of those who have relatively higher APOBEC3G levels in Th2 cells that allow longer persistence of those cells.

Th2 cells are important activators of the humoral immune response by providing IL-4 and stimulatory signals to B-cells. A rapid decline in Th2 cell number could lead to reduction in help to B- cells and thereby reduce an infected individual's ability to produce effective and mature antibodies against opportunistic infections and HIV. This role for APOBEC3G in supporting neutralizing antibody production has been suggested by APOBEC3 knockout studies in mice [138]. The importance of A3G in Th2 protection, and providing help to B cells, could be tested in an *in vitro* model of B-cell help. After HIV infection of Th2 cells that differ in their A3G levels, their ability to activate autologous B-cells could be tested to gauge the ability of APOBEC3G to protect the T-cell help provided by Th2 cells. This A3G-mediated assistance to broader immune function may also be analyzed by determining if a correlation exists between expression levels of A3G in Th2 cells and an individual's ability to produce broadly neutralizing antibodies to HIV or opportunistic infections. There are relatively few patients who produce broadly neutralizing anti-HIV antibodies and it is of interest to understand the factors that contribute to that phenotype. One hypothesis is that the Th2 cells in those subjects may be relatively better protected from HIV by relatively higher A3G levels than are seen in the Th2 cells of most subjects. If a positive correlation exists it would give credence to the idea that increased expression of A3G provides support to the immune system as a

whole as well as the cell it is expressed in by allowing that cell to better withstand the assault from HIV infection and continue to perform its helper functions. If so, research into the genomics and proteomics of increasing A3G expression in Th2 cells may become very promising to develop an adjunct to vaccination for both prevention and treatment of HIV infection.

Transcriptional Regulation of APOBEC3 During T cell Differentiation

The results of this study demonstrate that GATA3 and Tbet master transcription factors are inherently involved in regulating the expression of APOBEC3G and APOBEC3F. The specific role the two transcription factors play in regulating APOBEC3G and APOBEC3F expression however is open for further investigation. Though a database binding site search suggests binding sites for GATA3, it is unclear from our data whether direct binding to the APOBEC3G or APOBEC3F promoters by either transcription factor is responsible for their effect on expression. Given the observed lack of increase in APOBEC3G and APOBEC3F RNA following TCR stimulation of GATA3 transduced cells, it is possible that GATA3 is directly binding to the promoter regions and blocking their transcription. This hypothesis could be tested by TCR stimulating Th2 cells and performing chromatin immunoprecipitation using a GATA3 specific antibody and amplifying DNA regions with PCR primers specific for the A3G or A3F promoter. If GATA3 is found to be associated with the promoter region, TCR stimulation of Th2 cells that are reduced in GATA3 activity either by siRNA knockdown or expression of a GATA3 dominant negative [139]

and subsequent evaluation of A3G levels would further suggest that GATA3 is reducing expression of A3G by direct binding to the promoter region.

There is no suggested binding site for Tbet in the potential promoter regions; this neither suggests nor denies direct binding by Tbet. Our finding that neutralization of IFN-γ reduces basal and TCR stimulated levels of APOBEC3G in Tbet transduced and Th1 cells elucidates one role of Tbet in APOBEC3G regulation, as a major target of transcription regulated by Tbet is IFN-γ. Therefore, our data suggests that Tbet driven transcription of IFN-γ regulates the expression of APOBEC3G in Th1 cells. This conclusion is supported by our observation of potential interferon stimulated response elements in the putative promoter regions as well as others results demonstrating APOBEC3G can be regulated by interferons [53,54,56]. Investigations into finer detail of actual binding sites of GATA3, Tbet or interferon stimulated transcription factors in the APOBEC3G or APOBEC3F promoter regions by promoter driven reporter assays or chromatin immunoprecipitation are required to address these issues. Based on the current data, we would expect these experiments to find that GATA3 directly binds to the promoter to block transcription from interferon regulated elements that are stimulated by Tbet-driven IFN-γ.

In our analysis of the expression level of APOBEC3G and APOBEC3F in Th1 and Th2 cells, we observed wide donor-to-donor variation in expression of APOBEC3G and APOBEC3F. There are several possible explanations for this, including variation in expression or activity of upstream regulatory factors such as Tbet or GATA3. Another explanation, that would be an important area of future

research, is whether there are polymorphisms in the promoters of APOBEC3G and APOBEC3F. Polymorphisms associated with rapid or slowed CD4+ loss in non-coding regions of APOBEC3G have already been described [106,140]. Polymorphisms in the regulatory regions of these genes may alter expression and subsequently disease progression. Polymorphisms in the regulatory regions may be those already suggested by An et. al. Screening for identification of additional polymorphisms is also of interest [106,141] If experimentation has identified specific binding sites for GATA3, Tbet or IFN regulated transcription factors, then a search for polymorphisms in those regions would be simpler and more directed. Given the amount of polymorphisms identified in A3G in previous studies and the variation in expression, it is likely that polymorphisms exist in the promoter regions of APOEBC3G and APOBEC3F. Though these polymorphisms may be identified, they may not fully explain donor to donor variation as it is likely that the regulation of A3G transcription and translation is controlled by many different factors

Conclusion

This study indicates novel regulation of expression of the innate defenses APOBEC3G and APOBEC3F with T cell differentiation. This study also adds to the understanding of T helper cell biology and, most importantly, demonstrates that physiological levels of A3G in both virions and target cell cytoplasm are capable of restricting vif+ HIV. This last finding leads then to the conclusion that boosting APOBEC3G levels above the threshold of vif activity, or even partially

interfering with the activity of vif, will have an overall antiviral effect. This is an important step in understanding APOBEC3's restriction of reverse-transcribing pathogens as well as knowledge that may lead to therapeutics targeted at this system. This work has implications that suggest new antiviral mechanisms of APOBEC3s, new insights into HIV nuclear import, and promising new approaches to studying HIV pathogenesis and T helper cell differentiation.

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