The Function of APOBEC3G in the Innate Immune Response against the HIV Infection of Primary Cells

Dissertation
zur Erlangung des Doktorgrades
der Naturwissenschaften

vorgelegt beim Fachbereich 14
Biochemische, Chemische und Pharmazeutische Wissenschaften
der Johann Wolfgang Goethe-Universität
in Frankfurt am Main

von

Anna Heinzelmann
aus Altoona, Pennsylvania, USA

Frankfurt am Main, 2008
(D30)

vom Fachbereich 14 Biochemische, Chemische und Pharmazeutische Wissenschaften der Johann Wolfgang Goethe-Universität als Dissertation angenommen.

Dekan: Prof. Dr. Harald Schwalbe

Erstgutachter: Prof. Dr. Volker Dötsch
Zweitgutachter: PD Dr. Hagen v. Briesen

Datum der Disputation: 30. März 2009

ACKNOWLEDGMENTS

I would like to thank the many people who supported me during the completion of my PhD.

To begin with, I would like to express my deep and sincere gratitude to my advisor, PD Hagen v. Briesen. His wide knowledge and logical way of thinking were a great value. His understanding, encouraging and personal guidance have provided a wonderful basis for the present thesis.

I wish to also express my warm thanks to Dr. Ursula Dietrich, who gave me the opportunity to work in her lab group. Her valuable advice and support were important for this work.

My sincere thanks also go to Prof Dr Volker Dötsch for the willingness to accept the supervision and assessment of this thesis.

During this work I have collaborated with many colleagues for whom I have great regard, and I wish to extend my warmest thanks to all those in the virology groups in the Georg-Speyer-Hause who have helped me with this work. A special thank you goes to Dr. Felix Hermann, Dr. Tsanan Giroglou, Dr. Roland Zahn and Dr. Sebastian Newrzela for their tireless discussions and brainstorming. For their technical and moral help during my thesis I would also like to thank Margot Landersz and the rest of the Dietrich group.

Last but not least, I owe my loving thanks to my husband Matthias Heinzelmann, without his encouragement and understanding it would have been impossible to finish this work. My special gratitude also goes to my family, especially my sister Karen Kubicko for reading and trying to understand my thesis and my parents for supporting me throughout my studies.

ZUSAMMENFASSUNG

Das HI-Virus stellt mit zahlreichen Neuinfektionen pro Jahr immer noch ein großes gesundheitliches und gesellschafliches Problem dar. Das Virus gehört der Familie der Retroviridae an und enthält entsprechend zwei einzelne Stränge RNA. Es greift zuerst die Zellen des Immunsystems an, allen voran die Makrophagen und die dendritischen Zellen, die als Reservoir funktionieren. Diese Zellen geben die Infektion weiter an die CD4+-T-Zellen, was zu deren Tod führt. Wenn die Konzentration der CD4+-T-Zellen unter 200 Zellen pro µl Blut fällt, wird laut CDC von AIDS gesprochen. Das Virus ist für eine hohe Mutationsrate bekannt, was die Entwicklung neuer Medikamente erschwert. Daher ist die ständige Entwicklung neuer, fortschrittlicher Medikamente wichtig. Um dies zu ermöglichen, muss man das Zusammenspiel zwischen Virus und Immunsystem, und hier insbesondere die Funktion zellulärer Cofaktoren, besser verstehen.

Der zelluläre Cofaktor APOBEC3G wurde in Makrophagen und dendritischen Zellen als antiviraler Faktor identifiziert (Sheehy et al. 2002). Als Cytidindeaminase verursacht APOBEC3G unter anderem G→A hypermutierte HIV-Genome, woraus nicht funktionierende Virus-Genome resultieren (Alce and Popik 2004; Schafer et al. 2004). Vif ist ein akzessorisches Protein von HIV, das für eine Infektion mancher Zellen, wie die der T-Zelllinie Jurkat, die kein APOBEC3G produziert, nicht notwendig ist. Es wird deshalb vermutet, dass Vif nur dazu dient, die antiviralen APOBEC-Proteine abzufangen und zum Proteasom zu führen, damit sie dort abgebaut werden können (Conticello et al. 2003; Marin et al. 2003; Sheehy et al. 2003; Stopak et al. 2003; Liu et al. 2005; Mehle et al. 2007). In Abwesenheit von Vif wird APOBEC3G in Virionen verpackt und wirkt erst bei der Infektion einer neuen Zelle. APOBEC-Proteine nutzen mehrere Mechanismen, um die Infektion mit HIV zu verhindern, allerdings sind diese noch nicht genauer untersucht worden (Newman et al. 2005; Holmes et al. 2007; Li et al. 2007; Noguchi et al. 2007). APOBEC3G kann in zwei unterschiedlichen Komplexen mit RNA gefunden werden: Dem HMM-Komplex (engl.: high molecular mass) und dem LMM-Komplex (engl.: low molecular mass) (Chiu et al. 2005). Der LMM-Komplex beinhaltet APOBEC3G in seiner aktiven Form, und es wird vermutet, dass der HMM-Komplex weitgehend inaktiv ist (Chiu et al. 2005).

APOBEC3G ist Bestandteil des angeborenen Immunsystems, wobei seine genaue Rolle in diesem Zusammenhang noch unerforscht ist. Auch die exakte Wirkungsweise mancher zellulärer Cofaktoren ist bisher nicht bekannt. So üben IFN-α und der bakterielles Lipopolysaccharid (LPS) einen starken Hemmeffekt auf die HIV-Infektion aus. Bei der Zugabe von IFN-α werden hunderte Gene hochreguliert, deren Effekt jedoch nicht alleine für die Hemmung der HIV-Infektion verantwortlich sein kann. Es wurde in Mikroarrayanalysen festgestellt, dass das APOBEC3G-Protein durch die Stimulierung mit IFN-α oder LPS in Makrophagen hochreguliert war (Bouazzaoui et al. 2006). Daher sollte in dieser Arbeit untersucht werden, ob ein Zusammenhang zwischen diesen zellulären Faktoren und der

HIV-Restriktion besteht. Dazu wurde die Expression von APOBEC3G in HIV-Zielzellen (Makrophagen, dendritische Zellen und CD34+-Vorläuferzellen) nach Stimulierung mit IFN-α oder LPS sowie die Steuerung der Expression von APOBEC3G in diesen Zellen untersucht. Des Weiteren wurde die Korrelation mit der Infektiösität der Zellen mit HIV-1 untersucht.

Zuerst konnte durch quantitative PCR bestätigt werden, dass die APOBEC3G-Expression nach der Zugabe von IFN-α oder LPS hochreguliert wurde. Dieses Ergebnis wurde in den von Monozyten abgeleiteten Makrophagen mehrerer Blutspender nachgewiesen. Es wurde auch gezeigt, dass die Überexpression von APOBEC3G direkt mit der Konzentration von IFN-α korrelierte. Danach wurde durch Mutationsanalyse der in diesen Zellen produzierten HIV-Genome die Funktionalität des APOBEC3G gezeigt. Dafür wurden die Überstände von HIV(ADA8) und HIV(ADA8)ΔVif infizierten Makrophagen gesammelt und damit HEK-293T Zellen infiziert, die CD4- und CCR5-Rezeptoren auf ihrer Oberfläche tragen. Die provirale DNA wurde dann im Bereich des Env-Gens sequenziert. Es hat sich in der Mutationsanalyse bestätigt, dass die Menge G→A hypermutierter Klone mit der Expression von APOBEC3G in den Makrophagen korrelierte und somit die Aktivität von APOBEC3G zu hypermutierten HIV-Genomen führte.

Um zu analysieren, welcher Zelltyp in der Makrophagen-Kultur für die hohe APOBEC3G-Expression verantwortlich war, wurden Makrophagen und dendritische Zellen getrennt ausdifferenziert. Zur Ausdifferenzierung von Monozyten zu Makrophagen wurden AB-Serum, GM-CSF und M-CSF verwendet, während durch Zugabe von GM-CSF und IL-4 dendritische Zellen hergestellt wurden. Die unterschiedlichen Zellen wurden ausdifferenziert und zur Bestätigung des Zelltyps wurden Oberflächenmarker bestimmt. Die Makrophagen hatten zwar alle dieselben Oberflächenproteine, unterschieden sich allerdings im Phänotyp. Die Expression von APOBEC3G wurde mit quantitativer PCR und Western Blot nachgewiesen. Es wurde gezeigt, dass die Expression in allen Zellen nach der Stimulation mit sowohl LPS als auch IFN-α hochreguliert wurde. Die Makrophagen, die mit GM-CSF ausgereift wurden, hatten eine wesentlich höhere Expression von APOBEC3G als die anderen Makrophagen. Eine Korrelation zwischen der Expression von APOBEC3G sowohl in GM-CSF-Makrophagen als auch in AB-Makrophagen und die Infizierbarkeit der jeweiligen Makrophagen wurde nachgewiesen.

Um zu zeigen, dass dieser Effekt auf APOBEC3G zurückzuführen ist, wurde die Proteinexpression direkt reguliert. In Makrophagen und primären Zellen sind lentivirale Vektoren bei der Einbringung von Genen am effizientesten. Daher wurde ein auf drei Plasmiden basierendes lentivirales System verwendet. Als Hüllprotein wurde VSV-G (pCMV-G) benutzt, damit die verwendeten Zelltypen infiziert werden konnten. Das Transferplasmid (pHR-SEW) wurde bei Bedarf modifiziert, um das APOBEC3G-Protein direkt manipulieren zu können. Zuerst wurden Vektoren mit dem eGFP-Protein hergestellt und in von Monozyten abgeleiteten Makrophagen getestet. Die Zellen waren sehr unterschiedlich transduzierbar.

Die Zellen mancher Spender wurden bis zu 80% transduziert, während solche von anderen gar nicht transduziert waren. In das Transferplasmid wurde eine APOBEC3G-IRES-eGFP-Sequenz unter einem SFFV-Promoter eingeführt. Die Virionenpartikel konnten nur mit der gleichzeitigen Zugabe von Vif produziert werden. Es konnte gezeigt werden, dass das APOBEC3G in Zelllinien hochreguliert worden ist. Allerdings konnten in Makrophagen keine weiteren Versuche gemacht werden, da diese nach der Transduktion abgestorben sind. Um das APOBEC3G-Protein in der Expression zu hemmen, wurde eine shRNA gegen APOBE3G hinter einen H1-Promoter im Transferplasmid kloniert. Zwei verschiedene shRNAs gegen APOBEC3G wurden danach eingesetzt, die in Zelllinien eine Hemmung der APOBEC3G-Expression von ungefähr 50-84% Protein erreicht hatten. Danach wurde gezeigt, dass eine HIV-Infektion von TZM-bl-Zellen durch exogenes APOBEC3G um 70% gehemmt wurde, was durch die Expression von shRNAs gegen APOBEC3G aufgehoben werden konnte.

Wegen der Schwierigkeiten mit den Makrophagen wurden in weiteren Versuchen menschliche blutbildende CD34+-Stammzellen benutzt. CD34+-Zellen können sich klonal replizieren und zu den verschiedenen Zellarten des Immunsystems ausreifen. Sie sind mit lentiviralen Vektoren einfach zu transduzieren. CD34+-Zellen von drei Spendern wurden am ersten Tag nach dem Auftauen mit lentiviralen Vektoren mit einem eGFP-Markergen transduziert und dann bis zum Erreichen einer bestimmten Zellzahl kultiviert. Nachdem genug Zellen vorhanden waren, wurden sie sowohl für Experimente mit den Zellen selbst als auch zum Ausreifen von Makrophagen benutzt. Die Zellen zeigten den typischen Phänotyp von Makrophagen, und zur genaueren Bestimmung wurden ihre Oberflächenmarker charakterisiert. Es hat sich bestätigt, dass das CD34-Protein zunächst anwesend war und nach der Ausdifferenzierung verschwand. Die HI-Virus-Rezeptoren CD4 und CCR5 waren die ganze Zeit auf den Zellen vorhanden. Nach der Transduktion mit verschiedenen lentiviralen Vektoren mit eGFP-Markergenen nahm die Menge der Zellen, die eGFP exprimiert hatten, unvermuteterweise mit der Zeit ab. Trotz dieses starken Rückgangs war die Expression von APOBEC3G in den Zellen, die mit dem APOBEC3G-Gen transduziert waren, immer sehr hoch. Allerdings hatte die shRNA gegen APOBEC3G, die in Zelllinien aktiv war, sowohl in CD34+-Zellen als auch in den von CD34+-Zellen abgeleiteten Makrophagen keine reduzierende Wirkung auf die APOBEC3G-Expression.

Als nächstes wurde die Infizierbarkeit der CD34+-Zellen analysiert. Erwartet wurde, dass die Infizierbarkeit in den Zellen mit dem zusätzlichen APOBEC3G geringer ist als in den Zellen mit shRNAs gegen APOBEC3G. Nach der Transduktion und Ausdifferenzierung wurden die Zellen von drei Spendern mit IFN-α oder LPS stimuliert und mit Wild-Typ-HIV-1 und Vif-defektem HIV-1 infiziert. Die CD34+-Zellen waren ursprünglich sehr gut infizierbar, nach Stimulierung mit IFN-α waren sie jedoch nicht mehr infizierbar. Die Stimulierung von LPS hat die Infizierbarkeit nicht stark beeinflusst. Die Zellen des ersten Spenders, die mit APOBEC3G transduziert worden waren, waren nur gering infizierbar, wobei die Zellen des

dritten Spender einen hohen Anstieg an p24 aufwiesen. Im Vergleich zu den anderen transduzierten Zellen desselben Spenders fiel die p24 Menge im Zeitablauf sehr schnell ab. Der Effekt der shRNA gegen APOBEC3G auf die HIV-Infektion war, wie aufgrund der Expressionsanalyse zu erwarten, sehr gering. Offenbar war die shRNA in diesen Zellen nicht aktiv. Lediglich die Zellen des ersten Spenders, die mit einem Vif-defektiven Virus infiziert wurden, waren nach der Transduktion mit shRNA gegen APOBEC3G wieder infizierbar, insbesondere auch diejenigen, die mit IFN-α stimuliert worden waren.

Die von CD34+-Zellen abgeleiteten Makrophagen wurden auch auf ihre HIV-Infizierbarkeit getestet. Die Infektion dieser Zellen war sensibler gegenüber LPS-Stimulierung, wobei die Zellen generell nach der Stimulierung durch IFN-α kaum infizierbar waren. Die Zellen der ersten zwei Spender haben auch nicht auf die Transduktion mit APOBEC3G reagiert. Die Infizierbarkeit dieser Kulturen wurde nicht gehemmt, allerdings konnten in den Zellen des dritten Spenders nur geringe Mengen an p24 nachgewiesen werden. Die shRNA gegen APOBEC3G hat in den von CD34+-Zellen abgeleiteten Makrophagen keines Spenders die erwartete Reaktion hervorgerufen.

Die ersten Versuche haben einen Zusammenhang zwischen der Stimulierung von IFN-α und der Expression von APOBEC3G bestätigt. Die antivirale Wirkung von IFN-α äußert sich in der Hemmung von sowohl der Integration als auch des Zusammenbaus des proviralen Genoms und des Abbaus der viralen RNA in Makrophagen (Kornbluth et al. 1989; Pitha 1994; Woelk et al. 2004). Diese Effekte stimmen teilweise mit der Wirkung von APOBEC3G überein. Es wurde auch gezeigt, dass die antivirale Wirkung von IFN-α auf die Anreicherung des aktiven APOBEC3G LMM-Komplexes in CD4+-T-Zellen zurückzuführen ist (Chiu et al. 2005; Chen et al. 2006). Es wäre interessant zu wissen, ob dies auch bei Makrophagen der Fall ist. Die Stimulierung und Wirksamkeit von APOBEC3G in den Makrophagen war, wie viele andere Eigenschaften, sehr spenderabhängig. Die Zellen der Spender, in denen wenig APOBEC3G vorhanden war, produzierten auch nur eine geringe Anzahl hypermutierter HIV-Genome. Die Ergebnisse eines Vergleichs zwischen Makrophagen, die mit GM-CSF und AB-Serum ausgereift worden sind, waren vergleichbar. Es wurde auch in früheren Arbeiten gezeigt, dass GM-CSF die Infektion von HIV hemmt, jedoch waren die genauen Gründe hierfür nicht klar (Kornbluth et al. 1989).

Der beste Weg, eine Eigenschaft endgültig einem Protein zuzuordnen, ist direkt die Expression dieses Proteins zu regulieren. Für diese Arbeit wurden lentivirale Vektoren verwendet, um das APOBEC3G-Protein zu regulieren. Sie sorgen für eine lang anhaltende Expression. Die effiziente Transduktion von Makrophagen war aber nicht möglich, da sie stark spenderabhängig war. Dies ist wahrscheinlich auf die fehlenden akzessorischen Proteine in den lentiviralen Vektoren zurückzuführen. Es war ohne die Zugabe von Vif unmöglich, das APOBEC3G-Gen in einem lentiviralen Vektor zu benutzen, da das hergestellte Protein selbst in den Virion verpackt wurde und die folgende Transduktion

hemmte. Die APOBEC3G-Expression wurde in TZM-bl-Zellen erfolgreich manipuliert. Bei der Transduktion mit Vektoren, die APOBEC3G beinhalteten, war die Expression hoch und die Infektion mit HIV entsprechend gering. Bei der zusätzlichen Expression von shRNA gegen APOBEC3G wurde diese Hemmung wiederum aufgehoben. Es zeigte sich, dass die shRNA gegen diese aktive Form von APOBEC3G wirkte. Allerdings ist der Anteil des Proteins, der aktiv war, nicht bekannt. Zudem muß man bedenken, dass Zelllinien sich sehr stark von primären Zellen unterscheiden.

Die CD34+-Stammzellen wurden auch mit APOBEC3G regulierenden lentiviralen Vektoren, die zusätzlich ein eGFP-Markergen enthielten, transduziert. Hier hat der Anteil von eGFP-exprimierenden Zellen stark nachgelassen. Dieses Phänomen kann einen von zwei möglichen Gründen haben. Zum einen könnte die transkriptionale Interferenz durch die Methylierung von DNA in der Nähe des Promotors und der Integrationsstelle eine Rolle gespielt haben. Transkriptionale Interferenz wird allerdings meist mit gamma-retroviralen Vektoren in Zusammenhang gebracht. Es ist auch denkbar, dass die untransduzierten Zellen einen Vorteil gegenüber den transduzierten Zellen hatten.

Obwohl der Anteil der Zellen, die eGFP exprimiert hatten, zurückgegangen ist, war die APOBEC3G-Expression sowohl in den CD34+-Zellen als auch in den von CD34+-Zellen abgeleiteten Makrophagen sehr hoch. Dies hat leider in den meisten Zellen nur eine schwache Auswirkung auf die HIV-Infektion gehabt. Die CD34+-Stammzellen waren sonst sehr gut infizierbar, was *in vivo* möglicherweise nicht immer der Fall ist. Ihre Infektion *in vitro* wurde mehrmals gezeigt (von Laer et al. 1990; Ruiz et al. 1998). Dass die hohe APOBEC3G-Expression nicht immer den erwünschten Effekt auf die Infektion hatte, könnte möglicherweise durch die LMM- und HMM-Komplexe verursacht worden sein. Diese Möglichkeit sollte näher untersucht werden.

Sowohl die CD34+-Zellen als auch die von CD34+-Zellen abgeleiteten Makrophagen haben nach der Stimulierung durch IFN-α oder, in geringerem Maß durch LPS, eine Erhöhung der APOBEC3G-Expression gezeigt. Dies übte einen Hemmeffekt auf die HIV-Infektion in den von CD34+-Zellen abgeleiteten Makrophagen aus, aber nicht in den CD34+-Zellen. Der Effekt von LPS könnte eine makrophagenspezifische Eigenschaft sein, da er auch in T-Zellen nicht gezeigt werden konnte (Rose et al. 2004).

Die vorliegende Arbeit hat gezeigt, dass APOBEC3G eine wichtige Rolle in der Immunität gegen HIV-1 spielt. Der Zusammenhang ist sowohl von Zelltyp als auch Spender abhängig. Interessanterweise wurden kürzlich Artikel über den Zusammenhang zwischen der Expression von APOBEC3G und der Geschwindigkeit ihrer Krankheitsprogression publiziert (Biasin et al. 2007; Jin et al. 2007; Land et al. 2008). Das bessere Verständnis dieser Zusammenhänge wäre eventuell eine Möglichkeit APOBEC3G therapeutisch einzusetzen.

Beispielsweise könnte durch Hemmung der Vif-APOBEC3G-Interaktion die Expression von aktivem APOBEC3G erhöht werden.

1 INTRODUCTION

1.1 Human Immunodeficiency Virus (HIV)

1.1.1 HIV/AIDS

It has been over 25 years since HIV/AIDS (Acquired Immune Deficiency Syndrome) was discovered in a group of homosexual men with opportunistic infections and today it is still a major problem. According to the World Health Organization (WHO), there are 39.5 million people estimated world wide living today with HIV/AIDS and 4.3 million new infections in 2006 (WHO 2007). HIV prevention strategies have not been as successful as hoped. In North American and Western Europe the rate of new infections has remained the same, and in some countries the rate of new infections is even increasing. This past year alone there has been a stronger commitment from governments through additional funding for preventative and alternative treatment strategies, underlining the necessity of a continued effort. (WHO 2007)

HIV is transmitted through body fluids including blood and semen, which puts intravenous drug users, people practicing unprotected sex, and babies during birth or who are nursed, at a high risk of acquiring the infection. After infection, some people have flu-like symptoms with swollen lymph nodes. People infected with HIV can be classified into different stages dependent on the symptoms as defined by the WHO in 1990 (revised in 2005). The classifications from the WHO do not require a CD4 cell count. Stage one is asymptomatic; stage two includes recurrent upper respiratory tract infections, moderate unexplained weight loss, and herpes zoster; stage three consists of severe weight loss, fever and unexplained chronic diarrhea for over a month, severe bacterial infections and pulmonary tuberculosis. Stage four is the final stage which is usually an indicator of having AIDS. It includes toxoplasmosis of the brain, HIV wasting syndrome, severe recurrent bacterial pneumonia, chronic herpes simplex infection, HIV encephalopathy, a yeast infection in the respiratory system and Kaposi's sarcoma. AIDS can develop anywhere from 2 – 15 years after infection with HIV and is also defined by the Center of Disease Control (CDC) as having less than 200 CD4 T helper cells/µl blood. The opportunistic infections which plague the victims during the last stage of infection include certain types of cancer, pneumonia and tuberculosis. These diseases are then the cause of death. (WHO 2007)

1.1.2 Taxonomy

The viral family Retroviridae was discovered in 1908 by Vilhelm Ellermann and Oluf Bang as an enveloped group of single stranded RNA (ssRNA) viruses with a DNA intermediate

product. Currently there are 7 genera in the family of the Retroviridae including lentivirus, spumavirus, gammaretrovirus, episilonretrovirus, alpharetrovirus, betaretrovirus, and deltaretrovirus. The genome consists of positive sense single stranded RNA which is transcribed to a DNA intermediate using a viral enzyme called reverse transcriptase, hence the name retrovirus. Retroviruses were the first viruses to be used in gene therapy and are still the most common vectors used today (Hu and Pathak 2000). (Modrow et al. 2003)

HIV falls into the Lentivirus genus of Retroviridae under the subgenus: “primate lentivirus group”, which is only shared by the Simian Immunodeficiency Virus (SIV) infecting monkeys. The genome consists of two identical plus strand RNA strands. Due to sequence analysis, HIV has been divided into different types and subtypes. HIV-1 is the most prevalent type of HIV, and has many genetic subtypes. Subtype B HIV-1 is found mainly in infected individuals in America and Europe. Types A and C are, however, the most prevalent in the world. In 1986 HIV-2 was found in AIDS patients in West Africa. It is thought to be less infectious than HIV-1. (Sander 1995-2004; Modrow et al. 2003; WHO 2007)

1.1.3 Morphology

The HIV virion is approximately 100nm in diameter and has a phospholipid envelope acquired from the cytoplasm membrane of the cell (Figure 1(Kendall 1999)) (Modrow et al. 2003). There are two glycoproteins located on the viral membrane, the transmembrane protein (gp41) where approximately 20 amino acids are anchored in the envelope and an external glycoprotein (gp120) which is bound to gp41. Both proteins are made from the same precursor protein, gp160, which is cleaved by cellular proteases and glycosylated to form the two different proteins. The matrix protein (MA) p17 is a trimer which forms a net-like structure and is bound to the inner side of the viral envelope, giving the virion its typical isometric structure. Inside the matrix is the cone shaped core of the virus which is made up of the capsid protein (CA) p24 and encircles two identical strands of RNA. The matrix and the capsid proteins are part of the group specific Gag protein p55 antigen. The virus particles contain two ssRNAs in plus orientation which interact with the nucleocapsid protein (NC) p7, which also falls under the polyprotein group Gag. The linker protein p9 anchors the capsid to the envelope. There are three important enzymes located in the virion: the reverse transcriptase (RT), the integrase (IN) p38, and the protease (PR) located inside the capsid (shown as a black ball on the RNA in figure 1). These three enzymes are produced as the fusion protein Gag-Pol is cleaved by the viral protease (Modrow et al. 2003). The reverse transcriptase is a heterodimer consisting of p51 and p66, which has different active sites including a RNA dependent DNA polymerase, a DNA dependent DNA polymerase, and an RNase H active site. The integrase is responsible for integration of the dsDNA into the cellular genome. The integrase also functions as a ligase and an endonuclease. The protease is a homodimer with two p10 proteins. The homodimer functions as an aspartate

protease splicing the proteins Gag and Pol into their different functional proteins. (Modrow et al. 2003)

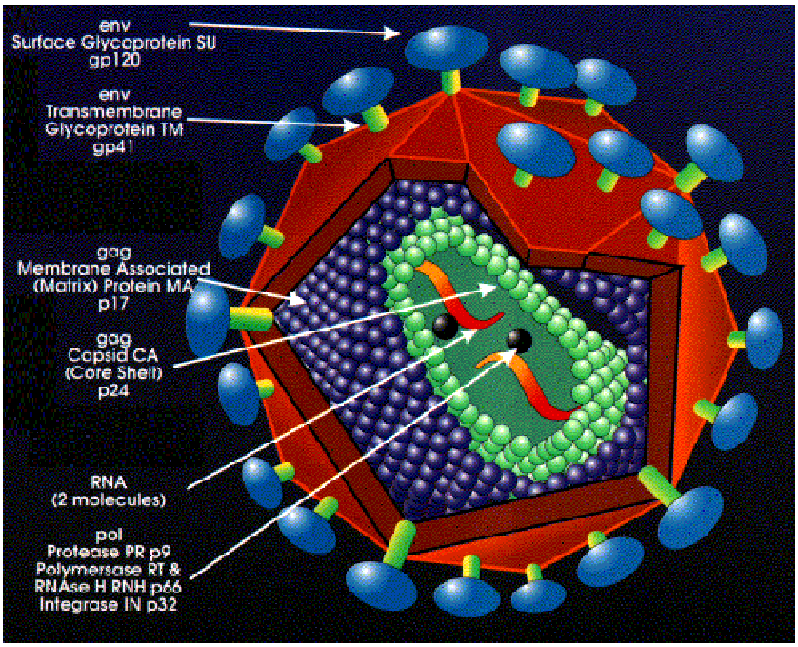


Figure 1. A mature HIV-1. The HIV is an enveloped virus containing the proteins gp120 and gp41 as envelope glycoproteins. The virus has a genome with two ssRNAs which are transcribed to cDNA via the viral reverse transcriptase. (Kendall 1999)

1.1.4 Genome

HIV-1 contains 2 single strand RNA genomes each approximately 9.7 kb long, which have a polyadenylated 3' end and a 5' CAP end resembling eukaryotic mRNA (Modrow et al. 2003). After integration of the proviral DNA, both ends have long terminal repeat (LTR) regions, which are made up of U3 (U as in unique), R (redundant) and U5 regions. On the RNA genome, the R region lies next to the Cap structure at the 5' end and has an identical sequence in the same orientation as on the 3' end where it lies next to the poly-A tail, used to stabilize the RNA, and the TAR (trans-activation response) element (Figure 2). The sequence needed for integration into the host is in the U3 and U5 region. The LTR region contains the cis-active elements such as the promoter and enhancer which control the gene expression of the virus. The primer binding (PB) region is situated directly following the U5 region and the loop contains the complementary sequence to the cellular tRNALys, which binds to this region and acts as a primer for the reverse transcriptase. Following the PB region is the Ψ (Psi) region, which is responsible for encapsidation of the viral RNA. (Modrow et al. 2003)

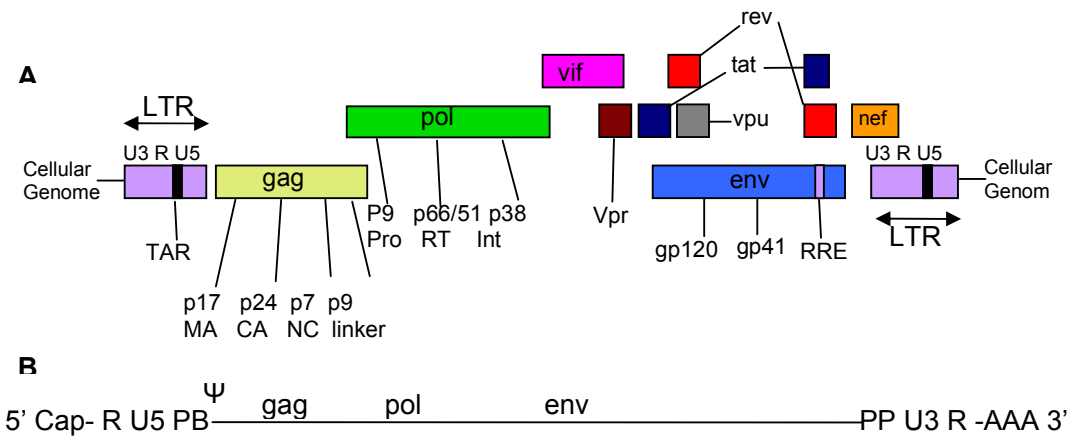


Figure 2. The HIV-1 genome. (A) The integrated proviral genome, with an LTR flanking both sides. (B) The viral RNA genome with the 5' Cap structure and the 3' poly-A sequence. (modified based on (Modrow et al. 2003))

After integration into the host's genome, the provirus codes for 16 proteins which are made from 10 transcripts. During transcription, three overlapping open reading frames are used. The use of overlapping open reading frames allows small genomes the possibility of synthesizing more proteins. (Modrow et al. 2003)

1.1.5 Viral Proteins

The gag gene codes for the capsid (CA, p24), the matrix proteins (MA, p17), the nucleocapsid (NCp7) and the linker protein p9. The proteins are made as a precursor protein, p55, which are cleaved with viral proteases. The three enzymes: the reverse transcriptase, integrase and protease are all made from the Pol gene. These proteins also start as a precursor protein, p160, which is coded by the same mRNA as p55 due to a frame shift. The hyper variable envelope (Env) gene codes for the viral envelope proteins gp120 and gp41. There are regulatory proteins which are also coded in this region in overlapping open reading frames. One such gene is the Tat protein (trans-activator of viral transcription) which binds to the TAR (trans-activation response) element on the RNA to upregulate transcription. The Rev protein (regulator of viral transport and splicing) also activates the transcription. This protein binds to the RRE (rev responsive element) on the viral RNA. This in turn allows unspliced and singly spliced mRNA to be exported out of the nucleus. (Modrow et al. 2003)

HIV also has many accessory proteins including Vif (viral infectivity factor), Vpr (viral protein rapid), Vpu (viral protein out), and Nef (negative factor). The Vif protein is conserved in most lentiviruses with the exception of the equine infectious anemia virus and is coded between the Pol and Env open reading frames (Yu et al. 2003). The Vpr protein open reading frame

overlaps with the Vif open reading frame and the first exon of the Tat gene. The Vpr protein is a very conserved 96 amino acid protein which has a variety of functions for the virus, making it important for viral reproduction. One of these functions includes the mediation of the nuclear import of the HIV-1 pre-integration complex, which has been found to be necessary in non-dividing cells, such as macrophages (Li et al. 2005). Vpr also inhibits the progression of the cells from the G2 phase and induces apoptosis in HIV-1 infected cells. The Vpu protein is a 9 kDa membrane protein and is only found in HIV-1 and SIVcpz. Vpu deficient viruses produce infectious viruses later or at a significantly lower amount. There are a few known reasons for this one of which is that Vpu induces the degradation of CD4 by the proteasome via the CD4-Vpu-h-βTrCP complex (Li et al. 2005). It also inhibits the cellular factor TASK-1, an acid-sensitive K+ channel present in some cells that enhances the release of progeny virions from infected cells (Hsu et al. 2004). The Nef protein is coded between the env gene and the 3' LTR region. The function of the Nef protein is associated with the modulation of the cellular receptors CD4, MHCI, MHCII, and CD28, the enhancement of viral infectivity and the interference with host cell signal transduction. It is one of the first detectable proteins in the infected cell and interacts with host cell signal transduction proteins to provide for long term survival of infected T cells and for destruction of non-infected T cells (by means of inducing apoptosis) (Rasola et al. 2001). (Modrow et al. 2003; Li et al. 2005)

1.1.6 Vif

Vif, another accessory protein of HIV-1, is a 192aa protein that is found in homohexamers in high concentrations in the cytoplasm of infected cells. The protein affects the infectivity of the virus, and for a long time the exact function of the Vif protein was unknown. It was known that the protein was needed to infect "non-permissive" cell types, such as macrophages or primary T cells, which otherwise made the virus progeny non-infective. It has now been determined that the function of Vif is to suppress the function of the antiviral cellular protein APOBEC3G by binding to the protein and inducing its ubiquitination and degradation by the proteasome. The antiviral activity of APOBEC3 is neutralized by Vif in two ways. First, Vif may use a proteasomal independent mechanism to degrade or block the APOBEC protein, which not much is known about, and the second method is through the proteasome. The Vif protein can bind directly to the cullin 5 (Cul5), elongin B (EloB), and elongin C (EloC) complex (Yu et al. 2003; Shirakawa et al. 2006). The interaction is directly between Vif's BC box motif and the EloC protein and between Vif's HCCH hydrophobic zinc finger motif to the Cul5 protein. After this interaction takes place and the Vif protein has bound to the APOBEC protein, the E3 ubiquitin ligase can attach a polyubiquitin chain on a lysine residue of the APOBEC protein. This results in the APOBEC protein being degradated by the 26S proteasome. However, the binding of the Vif protein to the APOBEC protein is species specific, even though there is only a single amino acid difference between the human APOBEC3G and the rhesus macaque APOBEC3G (Mariani et al. 2003; Bogerd et al. 2004;

Mangeat et al. 2004; Schrofelbauer et al. 2004). This binding is necessary for the degradation and is therefore a good target for new HIV-1 therapies. The exact binding domain for this interaction was recently determined by two different groups, the domain responsible for A3G interaction include the amino acids YRHHY (aa40-44) and the residues DRMR (aa14-17) are responsible for the A3F interaction (Mehle et al. 2007; Russell and Pathak 2007). (Sheehy et al. 2002; Sheehy et al. 2003; Pintard et al. 2004; Li et al. 2005; Xiao et al. 2006; Mehle et al. 2007)

1.1.7 Replication

The replication of HIV in the host cell begins with the absorption of the virus via binding of the C3-region of the gp120 protein onto the CD4 receptor (Modrow et al. 2003). This bond induces a conformational change in gp120 exposing areas including the V3-region of the protein, which can then interact with membrane proteins such as the CCR5 or CXCR4 receptors. Monocytotropic, otherwise known as R5, strains enter over the CCR5 protein. The lymphotropic or X4 strains enter over the CXCR4 receptor (Fotopoulos et al. 2002). The gp41 fusion protein can then be embedded into the cytoplasmic membrane and the viral membrane can then merge with the cytoplasmic membrane. (Modrow et al. 2003)

The capsid, accompanied by the viral genome, reverse transcriptase, protease and integrase, can then enter the cell. In the cytoplasm the RNA genome is then transcribed to dsDNA beginning with the 3' OH end of the tRNA molecule which binds to the 5' region of the RNA (Figure 3-I) (Fields et al. 2007). The 5' region is first transcribed, and then the elongated RNA jumps to the other side of the RNA, the complementary R region, and can completely elongate the minus strand DNA (Figure 3-II, III, and IV). The RNase H function of the reverse transcriptase then digests the RNA strand of the hybrid with exception of the RNA from the polypurine region (PP), which is protected from being digested and is used as a primer for the second strand of DNA (Figure 3-V). The DNA is now present as single stranded DNA for a short period of time (at this point the cellular protein APOBEC3G can attack the DNA). The DNA then forms a semi-circle and the end regions are copied (Figure 3-VI, VII, and VIII). The RNA then jumps a second time and the second DNA strand can be completed (Figure 3-IX and X). (Modrow et al. 2003)

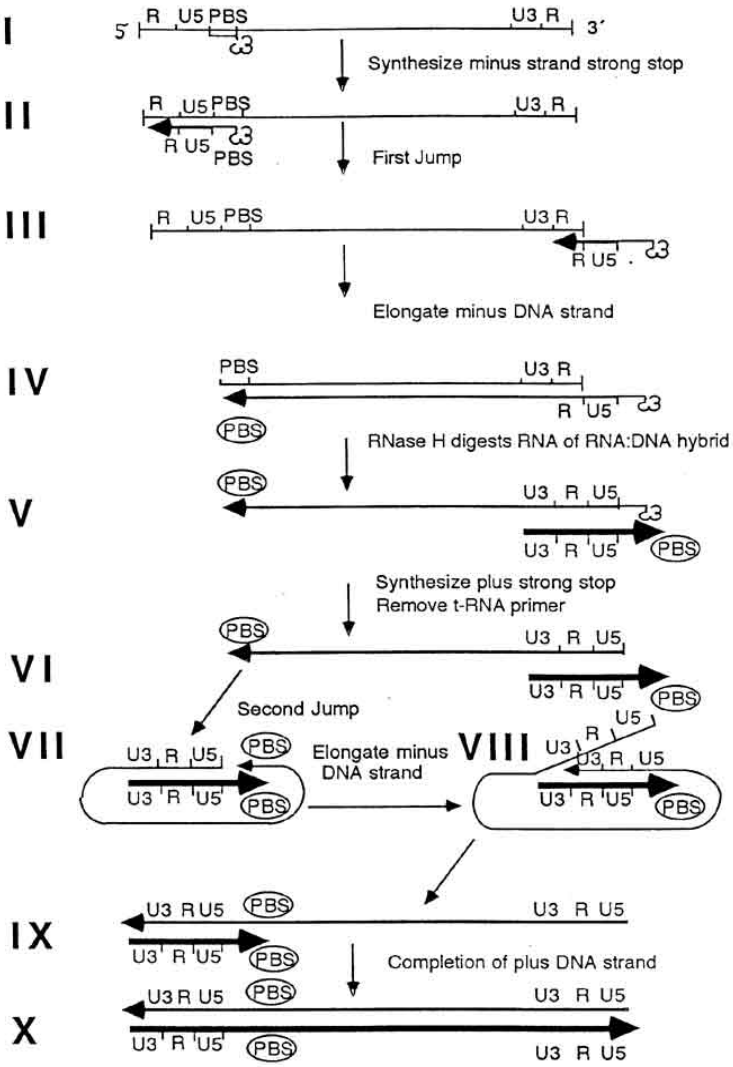


Figure 3. HIV- reverse transcription reaction. The first step is the binding of the tRNA onto the 5' PB region of the RNA. In step II the DNA synthesis begins, covering the PB, U5 and R regions of the 5' end. In the third step, the primer jumps to the other side, and in the fourth step the DNA minus strand is elongated. In step V, the RNA of the hybrid is degraded with the RHase-H activity of the reverse transcriptase. In step VI the tRNA is removed. In step VII, the DNA forms a circle and the U3, R and U5 region of the 3' end is synthesized. The primer then jumps to the other side in step VIII, and the plus strand DNA can be completed as shown in steps IX and X. (Fields et al. 2007)

The dsDNA is, at this point, still bound to the capsid, and can enter the nucleus with the partially disassembled capsid. Once in the nucleus, the DNA can be integrated into the host's DNA with the help of the enzyme integrase. After integration, the U3 region of the LTR binds the cellular transcription complex to transcribe the DNA with the RNA polymerase II. Once the protein Tat is made and enters the nucleus, it can bind to the TAR (trans-activation response) element resulting in a 100x increase in transcription. The Rev-protein is responsible for the change from early to late phase of infection, by allowing unspliced or single spliced mRNA to be exported from the nucleus. The single spliced mRNA is then

translated to the Env, Vif, Vpu and Vpr proteins, and unspliced mRNA can either be translated, or packaged into the viral particles. The Env protein, gp160, is synthesized in the endoplasmic reticulum and is then glycosylated on asparagine residues in the Golgi apparatus. On the way to the cytoplasmic membrane, gp160 is cleaved to gp120 and gp41 by the cellular protease. (Modrow et al. 2003)

The Gag and Gag/Pol precursor proteins are myristylated during synthesis, and transported to the cell membrane. Regulation and accessory proteins are also transported to the membrane. The two RNA strands of the genome are then associated with p55 through the two elements, Ψ and NC p7, which are necessary for the proper encapsidation of the viral RNA. Immature HIV particles are set free during budding and mature through the cleavage of the Gag and Gag/Pol precursor proteins by the viral protease. (Modrow et al. 2003)

1.2 The Immune System

Every organism is constantly challenged with pathogens which are eliminated via the immune system. The importance of the immune system becomes very apparent when confronted with genetic defects in the immune system or immune deficiency diseases such as AIDS. Most of these challenges to the immune system are counteracted by the innate immunity which acts as the first line of defense. Those which can not be disposed of with the innate immunity trigger the adaptive immune response. The adaptive immune response can then neutralize the infection and a memory of the infection is made so that the infection can be overcome more quickly the next time.

The cells that make up the immune response originate in the bone marrow, deriving from the precursors, the hematopoietic stem cells, this is shown in the classic diagram in figure 4. In the classic diagram, these stem cells then mature into stem cells with more limited differentiation potential: the myeloid progenitor or the common lymphoid progenitor. The lymphoid progenitor differentiates into lymphocytes and natural killer cells and the myeloid progenitor gives rise to a few different cell types, most notably monocytes and dendritic cells. This scheme has been recently modified to a “myeloid-based model”, where the lymphoid progenitor is replaced with a common lymphoid myeloid progenitor (Bell and Bhandoola 2008; Wada et al. 2008). In the new system macrophages can be derived from both pathways stemming out from the hematopoietic stem cells and these cells lose their potential to differentiate to macrophages at a much later time point than originally thought. The lymphocytes are responsible for the adaptive immune response and consist of B lymphocytes which later produce antibodies and T lymphocytes. T lymphocytes are made up of two classes, the cytotoxic T cells which kill cells infected with virus or helper T cells which activate other cells such as B cells. (Janeway 2004)

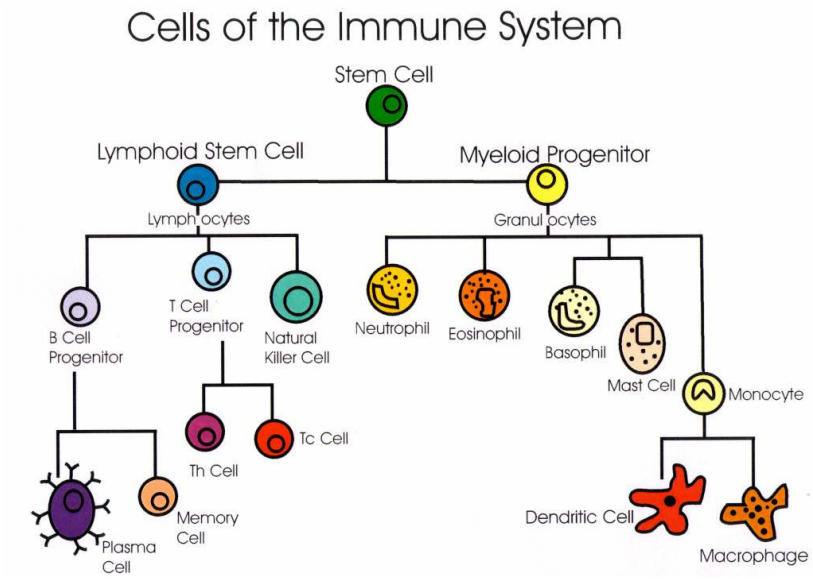


Figure 4. Classic scheme of haematopoiesis. The cells which make up the immune system all derive from one progenitor, the hematopoietic stem cell. http://www.omsusa.org/cellsis.jpg

1.2.1 CD34+ Hematopoietic Stem Cells

As per definition, stem cells are cells which can clonally replicate more stem cells as well as produce cells which can continue to differentiate (Weissman 2000). Hematopoietic stem cell can be easily put into this category due to their ability to give rise to more hematopoietic stem cells and to also produce the different cells of the blood (see Figure 4). They are responsible for the development and repopulation of all of the cells in the blood, which makes them the most important cells in hematopoietic tissue transplants. In humans these cells are positive for CD34 and Thy-1 and are negative for CD10, CD14, CD15, CD16, CD19 and CD20 (Shizuru et al. 2005). CD34+ cells can be isolated out of peripheral blood, umbilical cord blood, and bone marrow. It is controversial as to whether or not CD34+ cells can be infected with HIV-1 in HIV patients. There are some studies which show that rare infections in these cells can occur and it has been shown that the CD34+ cells isolated out of peripheral blood express the HIV receptors on their cell surface (von Laer et al. 1990; Kaczmarski et al. 1992; Ruiz et al. 1998). These cells can be easily transduced and because they can differentiate into a wide range of cells they make a good target for gene therapy (Gervaix et al. 1997; Demaison et al. 2002).

1.2.2 Macrophages

Monocytes circulate in the blood as precursor cells until they receive a signal, such as interferon γ, which leads to the spread of the cells into tissues and the differentiation of the monocytes into macrophages. Macrophages are much larger than monocytes and contain more organelles such as lysosomes. The differentiation of monocytes into macrophages can

also be done in vitro using serum and was shown to express the same marker as macrophages in tissues (Johnson et al. 1977; Andreesen et al. 1983). During differentiation, the macrophages have maturation antigens on the cell surface such as carboxypeptidase M also known as MAX.1 (Andreesen et al. 1986; Rehli et al. 1995), which is found in vivo mainly on peritoneal and pleural macrophages. Once resting macrophages take up pathogens they express MHC class II molecules on their surface where they can present peptides from degradated pathogens to T cells. Macrophages are also responsible for removing dead or dying cells, which produces a large amount of self antigens. In order to keep the risk of autoimmune diseases at a minimum, T cells are only stimulated when they also receive a co-stimulatory signal from the B7 receptor. Both of these signals are influenced by the stimulation of toll-like receptors (TLR) such as TLR4 for LPS.

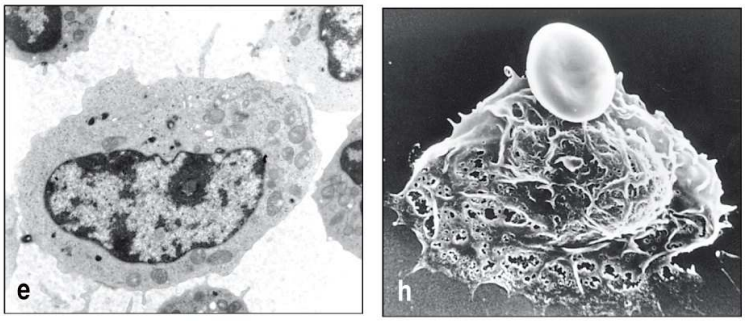


Figure 5. Macrophages. Shown here is a picture of a macrophage taken under a transmission electron microscope (left) and one while using a scanning electron microscope (right). Macrophages are specialized antigen presenting cells which phagocytose pathogens and present their antigen via the MHCII molecule on their cell surface. (Janeway 2004)

1.2.3 Dendritic Cells

Dendritic cells (DCs) are the main bridge between the innate and adaptive immunity. There are two different main types of dendritic cells, plasmacytoid dendritic cells and myeloid dendritic cells. The plasmacytoid DCs are a major producer of type I interferons (IFN) and tend to circulate in the blood. The myeloid DCs are the most common type and are responsible for the main function of dendritic cells: to present antigens to T cells, activating the adaptive immunity. DCs mature after being stimulated by pathogens, and present an increased amount of MHCII molecules on their cell surface along with co-stimulatory receptors for the activation of T-Cells, at the same time they loose the ability to take up pathogens. After being stimulated they migrate to the lymph nodes where they can interact with antigen-specific T cells for stimulation. The T cells are then stimulated and proliferate at high rates starting the first responses of the adaptive immune system. DCs are special MHCII presenting cells for a few reasons. First, they have the highest amount of MHCII molecules on their cell surface, which allows them to present foreign antigens more efficiently. The second reason is that they can also take up viruses and present them to the T cells without being infected. This is most likely the result of the affinity of the membrane

protein DC-SIGN to some viruses, such as HIV-1, SIV, and Ebola. DC-SIGN is also the receptor for ICAM-3 found on naïve T cells, and thus results in a trans infection of the T cells. HIV-1 has also evolved a way to take advantage of these means of infection, and the HIV-1 protein Nef upregulates the amount of DC-SIGN on the cell surface. (Su et al. 2003)

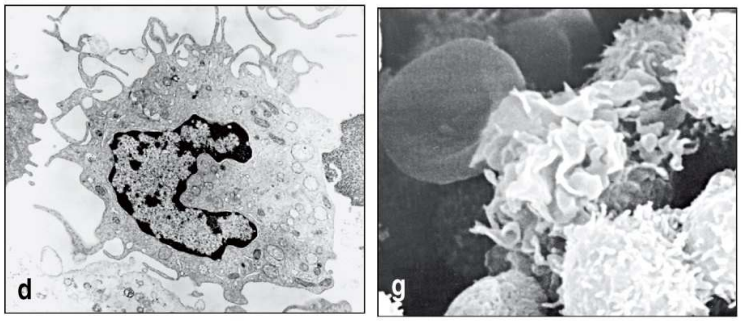


Figure 6. Dendritic cells. Shown here is a picture of a DC taken under a transmission electron microscope (left) and one while using a scanning electron microscope (right). Dendritic cells are specialized antigen presenting cells and have the highest concentration of MHCII molecules on their cell surface. They present the antigens specifically to T cells activating the adaptive immunity. (Janeway 2004)

1.2.4 Macrophages/Dendritic cells and HIV

The cells of the immune system are the main targets of the HIV-1 virus, the best known being the CD4+ T lymphocytes. Macrophages are a part of the innate immune system and as with most antigens, the first contact of the HIV virus is usually with macrophages or dendritic cells which engulf and are infected by the virus. When HIV-1 is transmitted through mucus membranes, macrophages and dendritic cells are infected first. They then present the antigen to the T-cells infecting them as well, this is demonstrated in figure 7 (Grouard and Clark 1997; Piguet and Steinman 2007). In CD4+ T-lymphocytes HIV-1 is dependent on proliferation and causes cell death, whereas macrophages and DCs can be infected as mature cells in a nonproliferating state (Nicholson et al. 1986; von Briesen et al. 1990; von Briesen et al. 1990). A main characteristic of macrophages and DCs is that they do not die from the infection, but instead serve as a reservoir for months to years within the tissue. DCs also have another possible mode of transmission, involving the binding of intact viral particles to the C-type lectin, DC-SIGN, which then transfers the virus to the CD4+ T cells (Piguet and Steinman 2007). With the infection of the T cells comes the infection of the lymphoid tissue. Viremia and the infection of the GALT (gut associated lymphoid tissue) follows. (Kuby 1997)

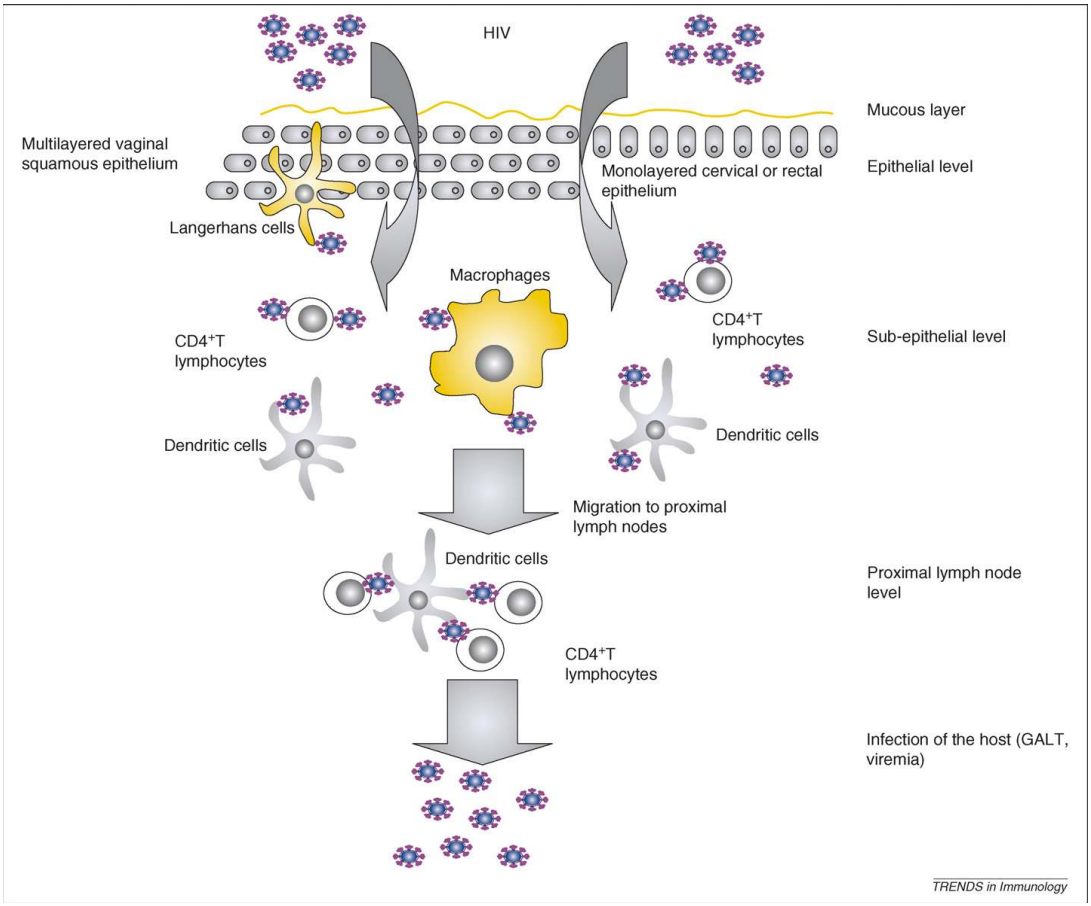


Figure 7. HIV-1 transmission. The transmission of HIV usually takes place over the mucosal membrane where it binds and infects dendritic cells and macrophages. It then infects CD4+ T cells in the sub-epithelial level. There is also the possibility that the DCs can capture virus without being infected via DC sign. This is then presented to CD4+ T cells which are in turn infected. After the infection enters the lymph nodes, the viral production increases and leads to host viremia and infection of the gut associated lymphoid tissue (GALT). (Piguet and Steinman 2007)

The viral progression in the macrophages and, most likely, DCs differ from that in the T cells. Figure 8 shows an overview of the differences between macrophages and T cells. This begins at the entry stage where the viral tropism makes it clear that the macrophages and DCs are preferably infected with CCR5 viruses. The RT reaction in macrophages takes 36-48 hrs compared to the few hrs it takes in T cells for unknown reasons. There are also differences in the regulation of the transcription in macrophages by means of other proteins including C/EBPβ, of which there are 2 isoforms, the small isoform inhibits the transcription. The small isoform can be induced by a few stimulants including GM-CSF, IFNβ or LPS (Honda et al. 1998; Verani et al. 2005). The viral release also differs in macrophages and T cells. Viral particles normally bud from the cell membrane at lipid rafts; however, the viruses in macrophages can also bud into the lumen of endocytic organelles. Another major difference which makes macrophages an important antiviral target is that the virus can accumulate in intracellular vacuoles where it can not be reached by antiviral drugs. This

enables the virus to persist in the cells and the macrophages act as a reservoir. (Verani et al. 2005)

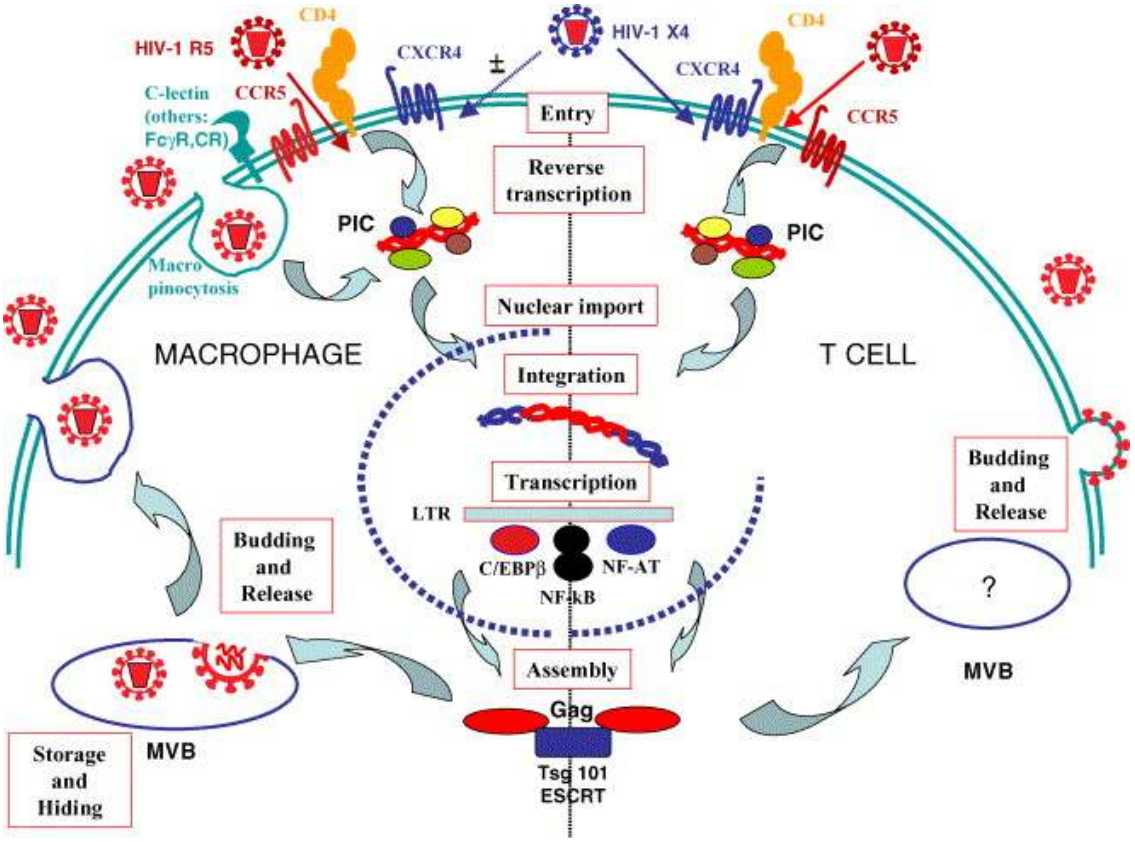


Figure 8. Comparison of the HIV-1 infection in macrophages and T cells. There are many steps in the viral infection of the cells that differ in macrophages and T cells. Some of these include viral entry and entry into the nucleus. (Verani et al. 2005)

1.2.5 Interferon alpha

Interferons (IFN) are pleiotropic cytokines which are split into three groups. Type I interferons is the largest group and consists of IFN-α, β, ε, ω, κ, and ζ all of which bind to the receptor heterodimer IFNAR-1/IFNAR2, type II interferons only consists of IFN-γ, and the third and new type of interferons consists of IFN-λ 1, 2 and 3. Interferon-α is a type I interferon, which can be coded by 13 different intronless genes. It plays a very important role in the innate immune response and can be produced by almost all cell types. However, the plasmacytoid dendritic cells (pDCs) are known to produce up to 1000-fold more IFN-α/β than other cells. Interferons regulate hundreds of genes in the cell, which can orchestrate antiviral, antitumor and other immunomodularly effects. Interferons also act as survival, maturation or differentiation factors in lymphoid tissues. (Theofilopoulos et al. 2005; Takaoka and Yanai 2006)

Toll-Like Receptor	Ligand	Location
TLR-1	Triacylated lipoprotein	Cell Surface
TLR-2	Tri/diacylated lipoprotein, peptidoglycan	Cell Surface
TLR-3	dsRNA	Interior mainly ER
TLR-4	LPS, VSV	Cell Surface
TLR-5	Flagellin, Zymosan	Cell Surface
TLR-6	Diacylated lipoprotein	Cell Surface
TLR-7	Unmethylated DNA, ssRNA	Interior (endosomal)
TLR-8	ssRNA	Interior mainly ER
TLR-9	CpG DNA	Interior mainly ER
TLR-10	unknown (closely related to TLR-1 and 6)	unknown
TLR-11	Uropathogenic bacteria	Cell Surface

Table 1. Toll-Like Receptor Ligands. The TLRs which activate IFN-α/β are highlighted in red. (Takeda and Akira 2004; Beutler 2005; Sandor and Buc 2005; Zhang et al. 2007)

IFNs are activated in large amounts during the body's response to pathogens. This can be mediated through Toll-like receptor (TLR) signaling. TLRs are found in all cells, and bind specific parts of pathogens in order to start the immune response. There are 11 different human TLRs, each of which recognizes a different ligand, and start the immune response (see Table 1). Of these TLRs, five are known to induce IFN production, TLR3, TLR4, TLR7, TLR8 and TLR9 using specific pathways (see Figure 9) (Zhang et al. 2007). The pathway from the TLR3 activates the MyD88 (Myeloid differentiation primary response gene 88)-independent pathway via TRIF which then activates NF-κB and the transcription factor IRF-3. The TLR4 can also activate this pathway with the help of TRAM or the MyD88-dependent TLR pathway with the help of TIRAP to activate IRF-7 or IRF-9. The IRFs and NF-κB proteins in turn activate the type I interferons IFN-α or IFN-β.

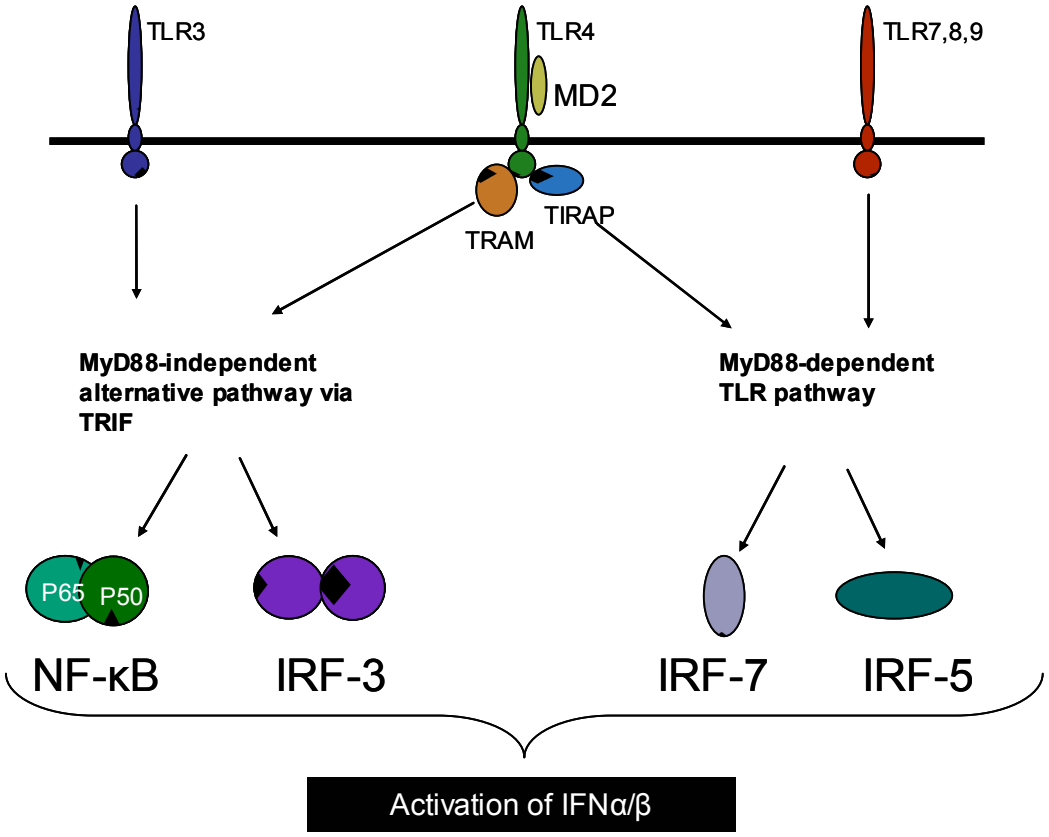


Figure 9. An overview of TLR signaling pathways leading to IFN-α/β activation. The activation of specific TLRs can in turn stimulate a MyD88- dependent or independent pathway causing the activation of IFN-α/β through NF-κB or an IRF protein. (modified based on (Edwards et al. 2007; Zhang et al. 2007))

When interferon-α is produced it in turn induces other antiviral proteins and other interferons. There are hundreds of proteins in the cell which are upregulated and turned on by IFN-α, regulated either through IFN-stimulated response element (ISRE) or the gamma activated sequence (GAS). The signaling process begins with IFN-α binding to the transmembrane glycoprotein IFNAR1/IFNAR2 heterodimer on the cellular surface (See figure 10). The extracellular part binds to the IFN and the cytoplasmic domains interact with members of the Janus family of tyrosine kinase (Jak) family. The kinase activity of the Jak kinases regulates phosphorylation of the Stat proteins 1, 2, 3 and 5. The Stat proteins can then form complexes such as the IFN-stimulated gene factor 3 (ISGF-3) consisting of a Stat1:2 heterodimer with protein p48 also called IRF-9. This complex can bind to promoter regions such as the ISRE for regulation. (Parmar and Platanias 2003; Takaoka and Yanai 2006)

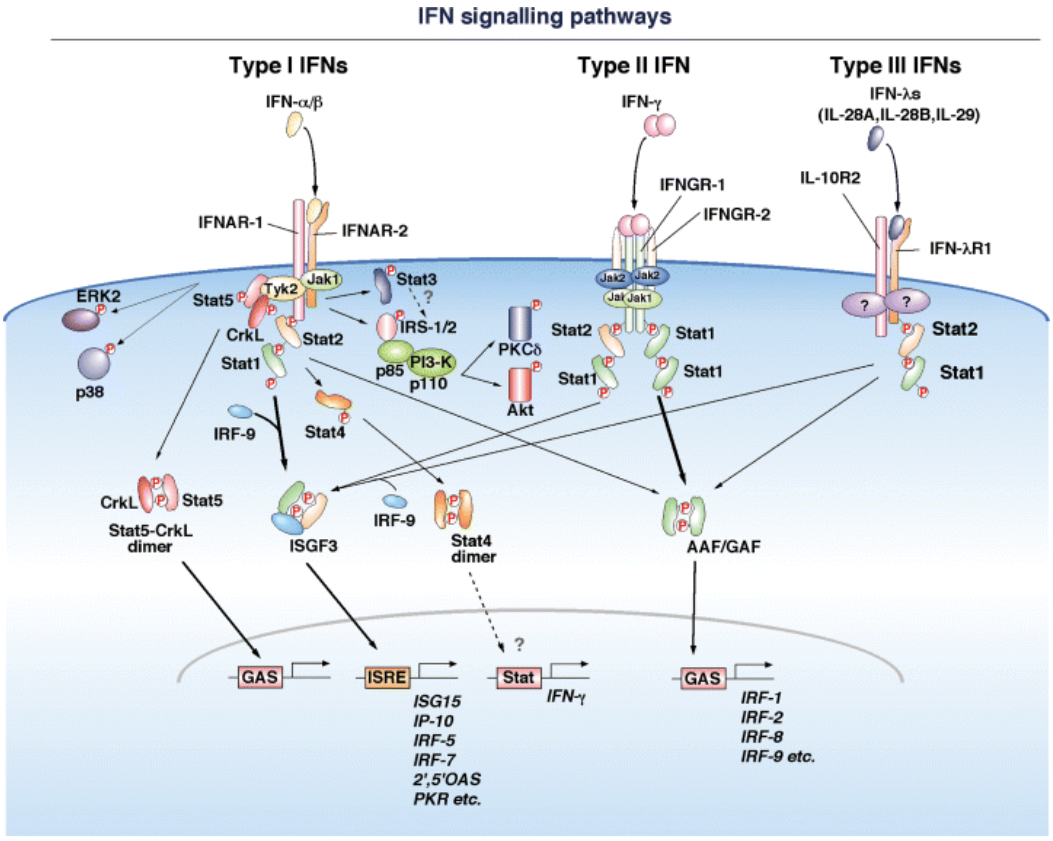


Figure 10. IFN signaling pathways. IFN-α plays a large role in the immune response to viral infections. The receptor is the type I interferon receptor and consists of two transmembrane subunits: IFNAR1 and IFNAR2 which induce the Stat signaling pathway. The activation of the Stat pathway turns on many genes containing specific activation signals such as GAS, ISRE or Stat. (Takaoka and Yanai 2006)

IFN-α down regulates a wide range of viruses by upregulating many different proteins, including the protein kinase R (PKR), oligoadenylate sythetase (OAS), adenosine deaminase (ADAR1), and recently noted, APOBEC3G. Due to the wide range of viruses that it affects, IFN-α is often used as a treatment for diseases such as hepatitis B infection. Currently there is a pharmacologically modified type of IFN-α: pegylated IFN-α which is licensed in the United States and Europe. Pegylated IFN-α is linked with polyethylene glycol (PEG) which is an uncharged polymer of various lengths. This composition leads to reduced immunogenicity, decreased sensitivity to proteolysis and it increases the serum half-life. (Parmar and Platanias 2003; Takaoka and Yanai 2006)

1.2.6 LPS

Lipopolysaccharide (LPS) is produced by Gram negative bacteria as part of the outer layer of their cell wall. It consists of Lipid A, a core polysaccharide which is made up of ketodeoxyoctonate, 7 carbon sugars, glucose, galactose, and N-acetyglycosamine, and an O-polysaccharide which is highly variable, but usually includes galactose, glucose, mannose

and rhaminose. The lipid A is very toxic to animals, but both the lipid and the polysaccharides are needed to cause an effect *in vivo*. LPS is an endotoxin, which means that it is bound to the bacteria and released in large amounts when the cells lyse. When this takes place the infected animal usually reacts with a fever, diarrhea, a rapid decrease in lymphocytes, leukocytes and platelets, and the organism goes into a general inflammatory state. If the dose is large enough death can occur due to hemorrhagic shock and tissue necrosis. (Brock et al. 2000)

Toll-like receptors bind and recognize different pathogen components, including LPS (see figure 11), lipoproteins, peptidoglycan, CpG DNA, double-stranded RNA, and bacterial flagellin (see section 1.2.5) (Akira and Takeda 2004). When the bacteria enter the body the LPS is bound by the LPS binding protein (LBP) in the serum. The LPS is then transferred to the cell surface protein, CD14, and then interacts with the Toll-like receptor-4 (TLR-4) (Figure 11). The TLR-4 activates the inflammatory response through the activation MAPK pathways and MyD88 pathways which then activate transcriptional factors such as NF-kB and interferon regulatory factors (IRF) which activate IFN-β and Rantes. (Guha and Mackman 2001)

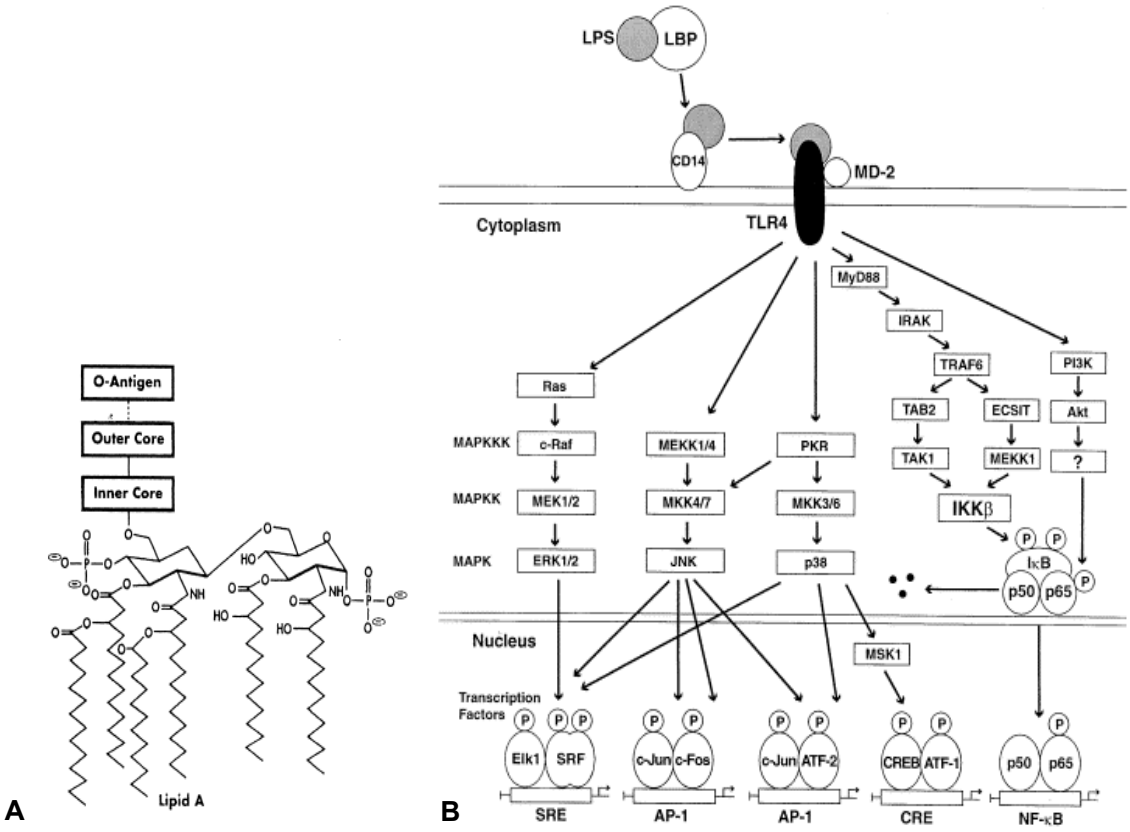


Figure 11. Signaling pathways of LPS in monocytes. (A) The structure of LPS (Guha and Mackman 2001). (B) The signaling pathway for LPS requires TLR-4, CD14 and MD-2. LPS is bound by LBP in the serum and the LPS is then transferred to the cell surface protein CD14. It then interacts with the TLR-4, which activates various MAPK pathways and the IKK pathway via MyD88. (Guha and Mackman 2001)

LPS protects human macrophages against HIV-1 infections. This has been attributed to the release of soluble factors, the C-C chemokines RANTES, MIP-1α, and MIP-1β which protect T cells and macrophages (Verani et al. 1997). IFN-α is also released by LPS stimulated macrophages; however this is not the main component responsible for HIV-1 suppression. (Verani et al. 2002; Verani et al. 2005)

1.2.7 APOBEC3G

In the year 2002 the group of Sheehy et al. published a paper in Nature showing that the reason for the inability to infect certain cells with a Vif deficient HIV-1 virus was the protein APOBEC3G (apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3G, formerly known as CEM15) (Sheehy et al. 2002). APOBEC3G is a cytidine deaminase in the APOBEC family, the same family as APOBEC1 (editor of the APO mRNA, hence the name), AID, and about 7 other APOBEC3 proteins (Navaratnam and Sarwar 2006). The APOBEC3 cluster is located on human chromosome 22 where it consists of 8 genes (Suspene et al. 2004; Holmes et al. 2007), which all contain the consensus sequence of His-X-Glu-X_{23-28}-Pro-Cys-X_{2-4}-Cys (see figure 12 (Holmes et al. 2007)) (Cullen 2006).

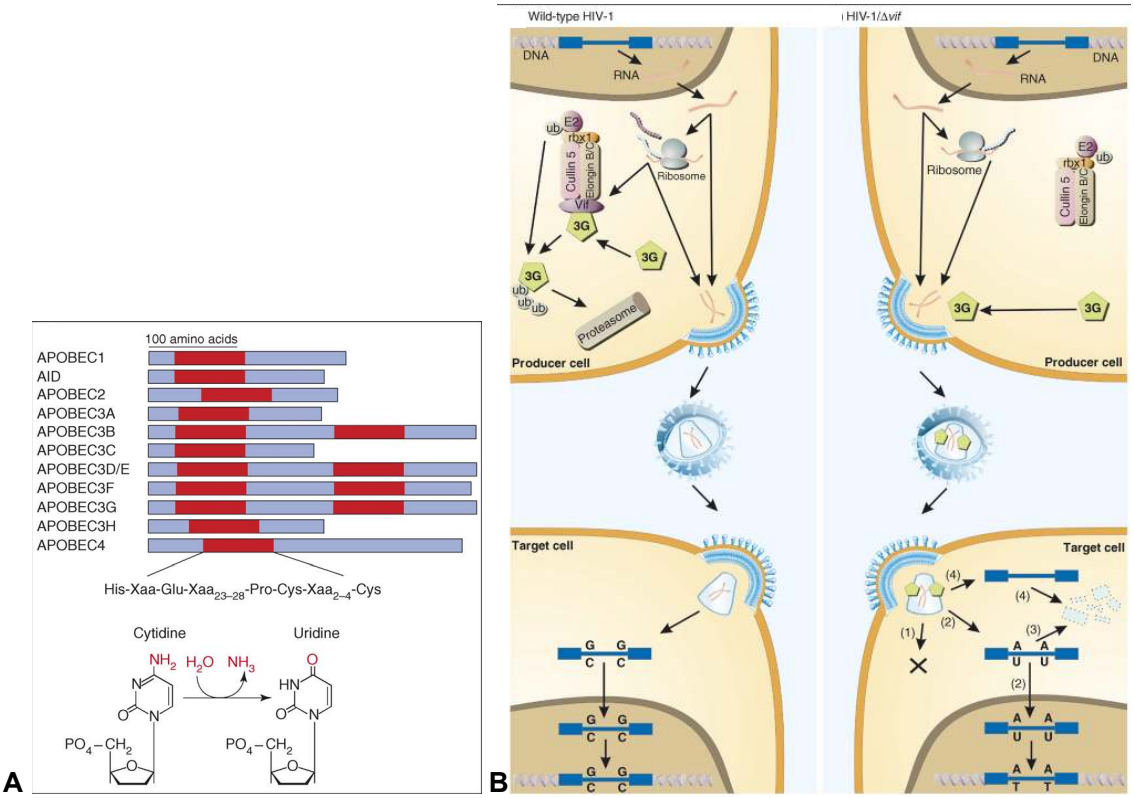


Figure 12. The proposed antiviral mechanism of APOBEC3G in HIV-1 infected cells. (A) The different APOBEC proteins and their conserved cytidine deaminase motifs, APOBEC3B, 3D/E, 3F, and 3G all have 2 cytidine deaminase motifs. Under the diagram of the genes is the chemical reaction for the cytidine deamination to uridine. (Holmes et al. 2007) (B) The left side shows the proposed pathway of APOBEC3G active in cells infected with wt HIV-1. The APOBEC3G is led to the proteasome with help from the viral Vif protein and is degraded. The right picture depicts the APOBEC3G pathway when infected with HIV-1 lacking the Vif protein. The APOBEC3G protein is packaged into the virions and restricts the viral production in the target cells. This is done by 4 proposed ways: 1 – an unknown mechanism that blocked the formation of the reverse transcripts, 2 – cytidine deamination of the ssDNA, 3 – mutated reverse transcripts could be recognized by the cellular repair pathways because of the presence of uracil and degraded, or 4 – a possible recruiting of a cellular endonuclease causing the HIV-1 reverse transcripts to be degraded independent of editing. (Holmes et al. 2007)

Some of the cell types which express APOBEC3G are monocytes, macrophages, DCs, and primary T cells. In HIV-1 infected cells the APOBEC3G protein is packaged into virions if the Vif protein is absent (Alce and Popik 2004; Schafer et al. 2004). The incorporation of APOBEC3G into the virion is possibly mediated through an interaction with the HIV-1 Gag nucleocapsid protein (Alce and Popik 2004; Cen et al. 2004; Douaisi et al. 2004). When these virions infect a new cell, the infection is downregulated as mentioned above. However, if the first cell is infected with a normal wild type HIV-1 virus with the viral protein Vif present, the antiviral activities of APOBEC3G can be thwarted (see figure 12 B). APOBEC3G produces G→A mutations on the viral plus strand DNA (Mangeat et al. 2003; Zhang et al. 2003). This happens through the deamination of cytidine to uracil on the minus strand DNA (see figure 12 A). Normally, DNA with uracil is detected by the uracil DNA glycosylase

(UNG), which can remove the uracil and leads to the degradation of the DNA. If the DNA escapes the UNG it can be transcribed into the plus strand DNA and the mutation, from a G in the RNA and now an A in the plus strand DNA, can be detected (Vartanian et al. 2003). It has also been found that APOBEC3G causes C→T mutations in regions of the genome where the minus strand DNA is single stranded for a longer period (Yu et al. 2004). These hypermutations were thought to be the only method of viral inhibition, however this has been since extended to other possible means, however the other methods which are used by APOBEC3G are not yet fully described (Newman et al. 2005; Holmes et al. 2007; Li et al. 2007; Noguchi et al. 2007). One possibility is that APOBEC3G also inhibits strand transfer during the RT reaction as described by Li et al. (Li et al. 2007). Other possibilities are a possible unknown mechanism that blocks the formation of the reverse transcripts or a possible recruiting of a cellular endonuclease causing the HIV-1 reverse transcripts to be degraded independent of editing (see figure 12) (Holmes et al. 2007).

The Vif protein can inhibit all of the effects from the APOBEC3G protein by binding to the APOBEC3G proteins and leading it into the 26S proteasome where it is degraded. This depletes the protein in the infected cells and can therefore not be packaged into the virions (Conticello et al. 2003; Marin et al. 2003; Sheehy et al. 2003; Stopak et al. 2003; Liu et al. 2005; Mehle et al. 2007). In addition to targeting APOBEC3G for degradation, Vif may also impair the translation of APOBEC3G in the cells (Stopak et al. 2003).

APOBEC3G can be found in two forms in cells: the high-molecular-mass (HMM) or low-molecular-mass (LMM) form (Chiu et al. 2005). The LMM form of APOBEC3G is found in resting CD4 T cells and monocytes where it is active in inhibiting HIV. The HMM form of APOBEC3G found in macrophages and active T cells is an RNA-protein complex and is not as active as the LMM form (Chiu et al. 2005). This form lacks deoxycytidine deaminase activity, but can be reduced to the active LMM form through addition of RNase A (Chiu et al. 2005). It was also suggested that the HMM complex is not efficiently packaged into the virions, however once packaged it becomes a HMM complex with the viral RNA (Soros et al. 2007). The APOBEC3G is then apparently reactivated by RNase H in the cell (Soros et al. 2007).

It has been recently shown that there is a correlation between CD4 T cell counts in HIV-1 infected individuals and the amount of G→A hypermutations caused by APOBEC3G (Biasin et al. 2007; Land et al. 2008). The amount of APOBEC3G was also discovered to be higher in long-term-non-progressors, in comparison to progressing HIV-1 individuals, which also showed a higher amount than healthy individuals (Jin et al. 2005).

1.3 Goal

More than 25 years have passed and there is still a lot to learn about HIV and its complex relationships with the immune system. There are many factors and pathways in the immune system which are not yet understood or known. A better understanding of the activation of certain pathways and factors may increase the chance of finding treatments for some diseases. In the last few years cellular proteins have been looked at closely in their relationship to HIV and many proteins have been found which were either necessary or detrimental for the infection. One such factor is APOBEC3G, which was shown to inhibit the replication of HIV after infection. The goal of this work was to determine the extent to which APOBEC3G is involved in the innate immune system and its response to HIV. To achieve this, the effect on APOBEC3G by LPS and IFN-α, two known stimulants of macrophages which downregulate HIV infections *in vitro,* was examined. These experiments also attempted to determine if the inhibition of HIV through LPS and IFN-α could be mainly attributed to APOBEC3G. For this, a lentiviral vector system to regulate the expression of APOBEC3G through stable expression of APOBEC3G or shRNAs against APOBEC3G was chosen. Other primary cells of the immune system, such as DCs and CD34+ cells, were also examined for their expression of APOBEC3G. The expression of the protein was then correlated to the ability of the respective cells to be infected by HIV.

2 MATERIALS

2.1 Chemicals

Chemical	Company
100x Bovine Serum Albumin (BSA)	Sigma-Aldrich
30% Acrylamide/Bis solution	BioRad
Acetone	Roth
Agar	Invitrogen (GIBCO)
Agarose	Q-Biogen
Ammonium persulfate (APS)	Merck
B-Mercaptoethanol	Merck
$CaCl_2$	Roth
Chloroquine	Sigma
Ethanol (99.9%)	Carl Roth GmbH
Ethidium Bromide	Carl Roth GmbH
potassium ferrocyanide	Serva
potassium ferricyanide	Serva
Glycerol	Roth
Glycine	Roth
HEPES	Applichem
HPLC water	Merck
Isopropanol (>99.7%)	Roth
Kaleidoscope Protein Marker	BioRad
L-Glutamine (200 mM in 0.85% NaCl solution)	Bio Whittaker
Methanol (>99.8%)	Roth
$MgCl_2$	Roth
NaCl	Roth
N,N,N',N'-Tetramethylendiamine (TEMED)	Merck
Powdered skim milk	Fluka
Sodium azide	Serva Feinbiochemica GmbH & Co. KG
Sodium Chloride (NaCl)	Roth
Sodium dodecyl sulfate (SDS)	Roth
Tris HCL (>99.9%)	Roth
Tween 20	Merck

2.2 Enzymes

Enzyme	Company
BglI	New England BioLabs
Biotherm^{tm} Taq Polymerase	NatuTec
EcoRI	New England BioLabs
EcoRV	New England BioLabs
HincII	New England BioLabs
HindIII	New England BioLabs
iQ^{tm} SYBR® Green Supermix	BIO-RAD
KpnI	New England BioLabs
PmeI	New England BioLabs
Quick Ligase	New England BioLabs
RNase-free DNase set	Qiagen
SmaI	New England BioLabs
SnaBI	New England BioLabs
SpeI	New England BioLabs
SpfI	New England BioLabs
Superscript III (200 U/µl)	Invitrogen
T4 DNA Ligase (4.0 Weiss Units)	Invitrogen

2.3 Antibodies

Antibody	Conjugate	Species	Catalog Number	Company
Annexin-V	APC		550475	BD Biosciences
Anti-ApoC17 (human APOBEC3G)		Rabbit	10082	Obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH from Dr. Klaus Strebel.
Anti Human CCR5	FITC	Mouse	555992	Beckman Coulter
Anti Human CD1a	PE	Mouse	IQp-126R	IQ Products
Anti Human CD4	PE	Mouse	555347	Beckman Coulter
Anti Human CD14	FITC	Mouse	MHCD1401	Beckman Coulter
Anti Human CD14	PE	Mouse		Beckman Coulter
Anti Human CD34	APC	Mouse	CD34-581-04	Caltag Laboratories
Anti Human CD64	PE	Mouse	IM3601	Beckman Coulter
Anti Human CD83	PE	Mouse	IM2218	Beckman Coulter

Anti Human CD209 (DC-SIGN)	PE	Mouse	A07407	Beckman Coulter
Anti-GAPDH		Mouse	MAB374	Chemicon International
AffiniPure Anti-Rabbit	Peroxidase	Goat	111-035-045	Dianova
AffiniPure Anti-Mouse	Peroxidase	Goat	115-035-003	Dianova

2.4 Kits

Kit	Company
Calcium Phosphate Transfection kit	Invitrogen
DNeasy® Tissue kit	Qiagen
ECL Plus Western Blotting Reagent Pack	GE Healthcare
E.Z.N.A. Plasmid Mini Kit II	Peq Lab
Innotest^{tm} HIV Antigen mAb	Innogenetics
NucleoBond PC 500	Macherey Nagel
NucleoSpin® Extract	Macherey Nagel
RNeasy® kit	Qiagen

2.5 Plasmids

Name	Description	Reference/Company
Vector pCR2.1 (25ng/µl)	TA cloning vector	Invitrogen
pBlueScript		Stratagene
pBlueA3G	APOBEC3G insert cut out of the pcDNA3.1A3G vector with PmeI and EcoRI, then brought into the BlueScript vector via EcoRI and EcoRV	
pcDNA3.1 human APOBEC3G-Myc-6xHis (pcDNA3.1A3G)	pcDNA3.1 vector with the human Apobec3G protein	This reagent was obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH from Dr. Klaus Strebel.

pcDNA hVif	Encodes the Vif protein of HIV-1 NL4-3 under the control of the CMV promoter. HVif is a partially codon-optimized version of the native vif gene. Codon optimization was achieved by changing the first 84 vif codons to conform to the reported codon usage of highly expressed human genes.	This reagent was obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: pcDNA-HVif from Dr. Stephan Bour and Dr. Klaus Strebel.
pNL(AD8)	full length HIV-1 ADA-M	donated by Simon Wain-Hobson at the Institut Pasteur, Paris
pNL(AD8)Dvif	full length HIV-1 ADA-MΔvif	donated by Simon Wain-Hobson at the Institut Pasteur, Paris
pSuper.basic	Vector system for expression of short interfering RNA	OligoEngin, WA USA
pSuper_Scram	Scramble siRNA oligonucleotides cloned into pSuper via the BglI and HindIII sites	See section 2.6 for the siRNA oligonucleotide sequence.
pSuper_APO960	APO960 siRNA oligonucleotides cloned into pSuper via the BglI and HindIII sites	See section 2.6 for the siRNA oligonucleotide sequence.
pSuper_APO471	APO471 siRNA oligonucleotides cloned into pSuper via the BglI and HindIII sites	See section 2.6 for the siRNA oligonucleotide sequence.
pSuper_siA3G1	A3G1 siRNA oligonucleotides cloned into pSuper via the BglI and HindIII sites	See section 2.6 for the siRNA oligonucleotide sequence(Pion et al. 2006).
pSuper_siA3G2	A3G2 siRNA oligonucleotides cloned into pSuper via the BglI and HindIII sites	See section 2.6 for the siRNA oligonucleotide sequence(Pion et al. 2006).
pSuper_siA3G3	A3G3 siRNA oligonucleotides cloned into pSuper via the BglI and HindIII sites	See section 2.6 for the siRNA oligonucleotide sequence(Pion et al. 2006).

pHR-SEW	Lentiviral SIN vector with an SFFV promoter and eGFP gene.	Kindly provided by Manual Grez, Georg-Speyer-Haus (Naldini et al. 1996; Zufferey et al. 1997; Demaison et al. 2002)
pCMVΔR8.91	Plasmid for the expression of the HIV proteins Gag-Pol and Rev	Kindly provided by Manual Grez, Georg-Speyer-Haus (Naldini et al. 1996)
pCMV-G	Plasmid for the expression of the VSV-G envelope under a CMV promoter and with a β-Globin intron	Kindly provided by Manual Grez, Georg-Speyer-Haus (Yee et al. 1994)
pSIEW::APO	pHR-SEW with the APOBEC3G protein from the pBlueAPO plasmid (KpnI, blunted and then digested with SpeI) cloned in front of an IRES - eGFP protein with a blunted SpfI site and the SpeI site.	This work
pSEW_SnaBI	pHR-SEW with a SnaBI digestion site in the 3'LTR	Kindly provided by Manual Grez, Georg-Speyer-Haus (Scherr et al. 2003)
pSEW_SnaBI:: siScram1	pHR-SEW_SnaBI with the siScram1 sequence under a H1 promoter blunt cloned from the SmaI and HincII sites into the SnaBI site from the pSuper_Scram plasmid	This work
pSEW_SnaBI:: siScram2	pHR-SEW_SnaBI with the siScram2 sequence under a H1 promoter blunt cloned from the SmaI and HincII sites into the SnaBI site from the pSuper_Scram plasmid	This work
pSEW_SnaBI::siAPO960	pHR-SEW_SnaBI with APO960 siRNA under a H1 promoter blunt cloned from the SmaI and HincII sites into the SnaBI site from the pSuper_Scram plasmid	This work
pSEW_SnaBI:: siAPO471	pHR-SEW_SnaBI with APO471 siRNA under a H1 promoter blunt cloned from the SmaI and HincII sites into the SnaBI site from the pSuper_Scram plasmid	This work

pSEW_SnaBI:: siA3G1	pHR-SEW_SnaBI with A3G1 siRNA under a H1 promoter blunt cloned from the SmaI and HincII sites into the SnaBI site from the pSuper_Scram plasmid	This work
pSEW_SnaBI:: siA3G2	pHR-SEW_SnaBI with A3G2 siRNA under a H1 promoter blunt cloned from the SmaI and HincII sites into the SnaBI site from the pSuper_Scram plasmid	This work
pSEW_SnaBI:: siA3G3	pHR-SEW_SnaBI with A3G3 siRNA under a H1 promoter blunt cloned from the SmaI and HincII sites into the SnaBI site from the pSuper_Scram plasmid	This work

2.6 Primers and Oligonucleotides

All primers and oligonucleotides were ordered from Thermo Fisher Scientific unless otherwise noted.

Random Primer (3µg/µl) Invitrogen

HIV env Primers 5'→ 3'

Env outer primers:
Ja19d: CAC AGT ACA ATC TAC ACA TG
7434R GTC TGG CCT GTA CCG TCA GCG

Env inner primers:
H1e6601d AAT GGC AGT CTA GCA GAA G
H1e7187r TCT CTT GTT AAT AGC AGC CC

APOBEC3 Primers 5'→ 3'
APOBEC3G 5P1 GCA ACC AGG CTC CAC ATA AAC ACG G
APOBEC3G 3P1 CGC AGC CCC TCC TGA CAT CTT CC

APOBEC3A_for TTC TTT GCA GTT GGA CCC GG
APOBEC3A_rev CTC ATC TAG TCC ATC CCA GG

APOBEC3F_for CAG CTG TGC TTT TGC CTG GTC ATC
APOBEC3F_rev TTA GGG GCA TGC AGG CTC AGG GAA CA

Normalization Primers 5'→ 3'

GAPDH.3P1 CGA CGC CTG CTT CAC CAC CTT CTT
GAPDH.5P1 CCG CGG GGC TCT CCA GAA CAT CA

EF-1α-3P1 AGG GCC ATC TTC CAG CTT TTT ACC AG
EF-1α-5P1 ATG TGT CTG TCA AGG ATG TTC GTC GTG G

MN51-3P1 AGG CGG TGG TGG GGG AGG CAG TG
MN51-5P1 CCT TCC CCA CCC AGG TTT ACA TCC C

siRNA Oligonucleotides (target sequence is in bold) 5'→ 3'

siA3G1_for GAT CCC C**GC ATC GTG ACC AGG AGT AT**T TCA AGA GA**A TAC TCC TGG TCA CGA TGC** TTT TTG GAA A
siA3G1_rev AGC TTT TCC AAA AA**G CAT CGT GAC CAG GAG TAT** TCT CTT GAA **ATA CTC CTG GTC ACG ATG C**GG G

siA3G2_for GAT CCC C**AA GCA ACC AGG CTC CAC AT**T TCA AGA GA**A TGT GGA GCC TGG TTG CTT** TTT TTG GAA A
siA3G2_rev AGC TTT TCC AAA AA**A AGC AAC CAG GCT CCA CAT** TCT CTT GAA **ATG TGG AGC CTG GTT GCT T**GG G

siA3G3_for GAT CCC C**AA CCG CAT CTA TGA TGA TC**T TCA AGA GA**G ATC ATC ATA GAT GCG GTT** TTT TTG GAAA
siA3G3_rev AGC TTT TCC AAA AA**A ACC GCA TCT ATG ATG ATC** TCT CTT GAA **GAT CAT CAT AGA TGC GGT T**GG G

siA3G-960_f GAT CCC C**CA ACC AGG CTC CAC ATA AA**T TCA AGA GA**T TTA TGT GGA GCC TGG TTG** TTT TTG GAA A
siA3G-960_r AGC TTT TCC AAA AA**C AAC CAG GCT CCA CAT AAA** TCT CTT GAA **TTT ATG TGG AGC CTG GTT G**GG G

siA3G-471_f GAT CCC C**GC ATC GTG ACC AGG AGT AT**T TCA AGA GA**A TAC TCC TGG TCA CGA TGC** TTT TTG GAAA
siA3G-471_r AGC TTT TCC AAA AA**G CAT CGT GAC CAG GAG TAT** TCT CTT GAA **ATA CTC CTG GTC ACG ATG C**GG G

siScram1_for AAA AGA TCT **TGG TCC ACA GGA CAC TCG T**TT CAA GAG A**AC GAG TGT CCT GTG GAC CA**T TTT AAG CTT AAA
siScram1_rev TTT AAG CTT AAA AA**T GGT CCA CAG GAC ACT CGT** TCT CTT GAA **ACG AGT GTC CTG TGG ACC A**AG ATC TTT T

siScram2_for GAT CCC C**AA CAT GTC ATG TGT CAC ATC T**TT CAA GAG A**AG ATG TGA CAC CTG ACA TGT T**TT TTT GGA AA
siScram2_rev AGC TTT TCC AAA AA**A ACA TGT CAT GTG TCA CAT CT**T CTC TTG AA**A GAT GTG ACA CAT GAC ATG TT**G GG

2.7 Cell Lines

Name	Species	Description	Medium	Reference/ATCC Number
CD34+ hematopoietic stem cells	Homo sapiens	Peripheral CD34+ stem cells from patients	RPMI (see section 3.1.4)	Kindly provided by Dr. Ulrike Köhl, Paediatric Haematology and Oncology, University Hospital, Frankfurt
HeLa-P4	Homo sapiens	human cervix carcinoma	DMEM Standard	(Clavel and Charneau 1994)
HEK 293 T (Human Kidney Cells)	Homo sapiens	Fetal kidney cells containing the SV40 T antigen	DMEM Standard	ATCC CRL-11268
HEK 293 T - CD4/CCR5 Cells	Homo sapiens	293T cells with CD4/CCR5 Receptors	DMEM Standard	As prepared by Chris Königs of the GSH (Konigs et al. 2007)
Te671	Homo sapiens	Rhabdomyosarcoma (Fetal muscle cells)	DMEM Standard	ATCC CRL-1573
TZM-bl	Homo sapiens	Derived from the HeLa cell line, this cell line expresses CD4 and CCR5 and contains the β-galactosidase genes under control of the HIV-1 promoter.	DMEM Standard	NIH cat. # 8129 This reagent was obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: TZM-bl from Dr. John C. Kappes, Dr. Xiaoyun Wu and Tranzyme Inc. (Platt et al. 1998; Derdeyn et al. 2000; Wei et al. 2002)

2.8 Bacteria

Name	Genotype	Company
One Shot® TOP 10 competent cells	F-mcrA Δ(mrr-hsdRMS-mcrBC) Φ80lacZΔM15 ΔlacX74 recA1 araD139 Δ(araleu)7697 galU galK rpsL (Strr) endA1 nupG	Invitrogen

2.9 Virus

Virus Name	Properties	Reference
$HIV\text{-}1_{D117III}$	Macrophagotropic (R5) HIV-1 virus Strain	Hagen von Briesen (Rubsamen-Waigmann et al. 1989)

2.10 Medium Supplements and Buffers

Name	Company
5x Sequencing Buffer	Applied Biosystems
10x PCR buffer without MgCl2	Applied Biosystems
10x Ligation buffer	Invitrogen
Ampicillin	Roth GmbH
Annexin-V binding buffer	0.01 M HEPES, pH 7.4; 0.14 M NaCl; 2.5 mM $CaCl_2$
Big Dye Terminator Premix	Applied Biosystems
BioTherm™ 10x Buffer	NatuTec
Bovine Serum Albumin	PAA
Bovine Serum Albumin (100x)	New England Biolabs
Buffy Coat – Fresh from healthy blood donor	Blood Bank, DRK
dNTP Set (100 mM of dATP, dCTP, dTTP, dGTP)	Peq Lab
FACS Buffer	PBS with 3% FCS
FACS clean	BD Biosciences
FACS flow	BD Biosciences
Fetal calf serum (FCS)	PAN
Fetal calf serum (FCS) low endotoxin	Biochrom
Ficoll serperating solution (density 1.007)	PAN
Formamide	Applied Biosystems
G418	CALBIOCHEM
Human AB-Group Serum	PAN
Hygromycine	MB biomedicals

Interferon-α (cat. #111-40-128)	ImmunoKontact (AMS Biotechnology GMBH)
IPTG	Roth GmbH
L-glutamine	Biochrom
Leukin/Sargramostim (GM-CSF)	Berlex
Lipofectamine™ 2000	Invitrogen
Lipopolysaccharide (LPS) (*Salmonella abortus equi*)	Gift from Dr. Galanos, MPI for Immunobiology, Freiburg
PBS, Dubecco's(phosphate buffered saline, without Ca++, Mg++)	PAA
Plumozyme (DNase)	Genentech, Inc.
Penicillin/Streptomycine	Biochrom
MEM-vitamins (100x)	Life Technologies
MEM non-essential amino acids	Life Technologies
MgCl2	Applied Biosystems
NEBuffer 1	New England Biolabs
NEBuffer 2	New England Biolabs
NEBuffer 3	New England Biolabs
Recombinant human Flt-3 Ligand (cat. #308-FKN)	R&D Systems
Recombinant human Interleukin-4 (cat. #hIL4-25)	Strathmann Biotech
Recombinant human Interleukin-3 (cat. #203-IL-050/CF)	R&D Systems
Recombinant human Interleukin-6 (cat. # 206-IL-050/CF)	R&D Systems
Recombinant human M-CSF (cat. #216-MC)	R&D Systems
Recombinant human SCF (cat. #300-07)	PeproTech Inc.
Recombinant human Thrombopoietin (TPO) (cat. #300-18)	PeproTech Inc.
RNA guard RNase Inhibitor	Amersham Pharmazia Biotech
Sodium pyruvate	Life Technologies
X-Gal	Roth GmbH

2.11 Cell Culture and Bacteria Medium

Medium	**Company**
Dubecco's modified Eagle Medium (DMEM)	Invitrogen (GIBCO)
RPMI 1640 medium	Biochrom
LB Medium	Invitrogen (GIBCO)

Medium Name	Concentration	Ingredients
AB Macrophage Medium	1x	RPMI 1640
	100 U/ml	Penicillin
	100 µg/ml	Streptomycin
	2 mM	L-glutamine
	4%	human AB-group Serum
	1x	MEM non-essential amino acids (100x)
	1:250	MEM vitamin solution
	1mM	sodium pyruvate (100 mM)
CD34 Proliferation Medium (Liu et al. 2002)	1x	IMDM
	10 %	FCS (low endotoxin)
	0.1 mM	β-Mercaptoethanol
	2 mM	L-glutamine
	100 U/ml	Penicillin
	100 µg/ml	Streptomycin
	25ng/ml	Flt3-L
	10 ng/ml	SCF
	10 ng/ml	IL-3
	10 ng/ml	IL-6
CD34 pre-differentiation Medium (Liu et al. 2002)	1x	IMDM
	10 %	FCS (low endotoxin)
	0.1 mM	β-Mercaptoethanol
	2 mM	L-glutamine
	100 U/ml	Penicillin
	100 µg/ml	Streptomycin
	25ng/ml	Flt3-L
	10 ng/ml	SCF
	10 U/ml	TPO
DC Medium	1x	RPMI 1640
	10 %	FCS (low endotoxin)
	2 mM	L-glutamine
	100 U/ml	Penicillin
	100 µg/ml	Streptomycin
	800 U/ml	G-MCSF

	40 U/ml	IL-4
DMEM 293T CD4/CCR5 Medium	1x	DMEM
	5 %	FCS
	2 mM	L-glutamine
	100 U/ml	Penicillin
	100 µg/ml	Streptomycin
	0.5 µg/ml	G418
	0.05 µg/ml	Hygromycine
DMEM Standard Medium	1x	DMEM
	5 %	FCS
	2 mM	L-glutamine
	100 U/ml	Penicillin
	100 µg/ml	Streptomycin
GM-CSF Macrophage Medium	1x	RPMI 1640
	10 %	FCS (low endotoxin)
	100 U/ml	Penicillin
	100 µg/ml	Streptomycin
	2 mM	L-glutamine
	500 U/ml	GM-CSF
M-CSF Macrophage Medium	1x	RPMI 1640
	10 %	FCS (low endotoxin)
	100 U/ml	Penicillin
	100 µg/ml	Streptomycin
	2 mM	L-glutamine
	100 ng/ml	M-CSF
LB Medium	5 g	Yeast Extract
	10 g	Trypton
	15 g	Agar (only for Agar plates)
	Fill to 1 liter	Distilled Water
		Set to pH 7.0 and autoclaved

2.12 Hardware and Other Supplies

Name	Company
24-well plates	Costar
96-Well 0.2 ml Thin-Wall PCR Plates	BIO-RAD
Amicon Ultra-4 30kD	Millipore
T3 Thermocycler	BioMetra
ECL plus hyperfilm	Amersham Biosciences
ELISA- automatic washer	Biotek Instruments GmbH
FACSCaliber	BD Biosciences
FACS tubes	Sarstedt
Falcon tubes, 50 ml	Becton Dickinson
Falcon tubes, 15 ml	Becton Dickinson
Fuji medical xRay 100NIF 18x24 Super RX	Allmedt
Gene Qunant II RNA/DNA Calculator	Amersham Pharmacia Biotech
i-cycler thermal cycler	Bio-Rad
Mini-PROTEIN Electrophoresis system	Bio-Rad
NanoDrop 1000	Thermo Fisher Scientific
Optimax type TR X-Ray film processor	Protec Medizin Technik GmbH
Photometer Spectomax 340	MWG Biotech
Polystar 350 DMS, sealing machine	Rische & Herfurth
Sequencer 3100 Avant	Applied Biosystems
Sterile filter Millex-GP 0.22µm PES 33mm	Millipore
TC flask (50 ml)	Greiner
TC flask (250 ml)	Greiner
Teflon foil (Biofolie 25)	Heraeus
Trans-Blot® SD Semi-Dry Cell	Bio-Rad
L8-M Ultracentrifuge	Beckman Coulter
Ultracentrifuge tubes	Beckman Coulter

3 METHODS

3.1 Cell culture

3.1.1 PBMC isolation

Fresh buffy coat blood from healthy HIV-1-seronegative blood donors obtained from the DRK-Blutspendedienst, Frankfurt was filled into two 50ml falcon tubes (per tube approximately 30-35 ml). The blood was then centrifuged at 2000 rpm for 10 min, the plasma was then carefully removed, and the tube was filled to 50 ml with PBS. For each buffy coat four gradients were prepared using 25 ml Ficoll separating solution (density 1.007) from PAA each. The gradients were then carefully overlaid with 25 ml of the blood/PBS solution and centrifuged at 2000 rpm for 30 min without using the brake. The white interphase (PBMCs) was then extracted and filled into another 50 ml tube and washed twice with PBS.

3.1.2 Macrophage differentiation from PBMCs

The PBMCs were cultivated in hydrophobic Teflon bags for 7 days in AB macrophage medium (described in section 2.11) or GM-CSF macrophage medium (see section 2.11). After the cultivation in Teflon bags, the cells were centrifuged at 1000 rpm and the supernatant was discarded. The cells were resuspended in fresh medium and viable cells were counted by trypan blue exclusion. The cells were plated at $4x10^5$ (24-well plate) or $2x10^6$ (75 cm2 flask) cells/well and were incubated for 1 hour. After 1 hr, the macrophages were isolated by adherence. The nonadherent cells were removed by repeated washing with RPMI medium. The cells were then further cultured with fresh AB macrophages medium or complete RPMI containing GM-CSF and the medium was changed every 3^{rd} or 4^{th} day. The adherent cell layer consisted of over 95% macrophages as determined by morphology and FACS analysis.

3.1.3 Dendritic cell differentiation from PBMCs

PBMCs could also be differentiated into dendritic cells (DCs) using GM-CSF and IL-4. In this case, the cells isolated from the buffy coat were resuspended in RPMI medium and set to $1.5x10^8$ cells/ml. 250ml culture flasks were used to cultivate 20 ml of the cell suspension and incubated at 37˚C for 1 hr. The nonadherent cells were then removed by washing gently with PBS 3 times. The adherent cells were cultivated in complete DC medium (see section 2.11). After 3 days of cultivation, 5 ml of the medium was replaced with fresh DC medium. Six days following isolation the cells were differentiated to immature DCs (iDCs). In order to obtain mature DCs (mDCs), the iDCs could be stimulated with 100 ng/ml LPS for 48hrs.

3.1.4 CD34+ Cultivation

Frozen CD34+ cells were quickly thawed in a 37˚C water bath. The cells were then diluted 1:3 in cold PBS with 1% BSA, 7.5 mM EDTA, and 0.025 mg/ml Pulmozyme (a DNase to reduce clumping of the cells) and incubated for 5 min at 4˚C. The cells were centrifuged for 10 min at 1400 rpm to remove the DMSO and were resuspended in warm PBS with 1% BSA. The cells were centrifuged for 10 min at 1000 rpm. The pellet was resuspended in CD34 Proliferation Medium (see section 2.11) and the cell count and viability was determined with tryphan blue exclusion. The cells were stained using different antibodies (see section 2.3) against cell markers and FACSed to determine their purity (see section 3.2).

The CD34+ cells were seeded in 24 well Costar plates and transduced with lentiviral vectors at an MOI of 20 (see section 3.4). They were kept in CD34 proliferation medium (described in section 2.11) at 1-3x10^5 cells/ml for 1-2 weeks depending on the cell count. After this time the CD34+ cells were used for testing or further cultivated in CD34 pre-differentiation medium (described in section 2.11) at 4x10^5 cells/ml in 24 well plates and then differentiated. (Liu et al. 2002)

3.1.5 Macrophage differentiation from CD34+ cells

For the differentiation of the transduced CD34+ cells into macrophages, the CD34+ cells were cultured in the pre-differentiation medium (section 2.11) for 4 days. They were then washed with PBS and centrifuged at 1400 rpm for 10 min and set to 4x10^5 cells/ml for cultivation in M-CSF macrophage medium (described in section 2.11) for another 4 days. During this time most of the cells adhered to the bottom of the well. After cultivation in the M-CSF macrophage medium, the medium was removed and the cells in suspension were again washed with PBS and centrifuged for 10 min at 1400 rpm. The wells were rinsed once with PBS and the cells in suspension were resuspended in AB macrophage medium and were distributed among the wells. These cells could then be used for further tests such as gene expression analysis and HIV infectivity.

3.1.6 Cultivation of cell lines

The 293T cell line was cultured in 293T medium (described in section 2.11). The 293T CD4/CCR5 cell line was cultured in 293T CD4/CCR5 medium (described in section 2.11). Cells were incubated at 37°C in 7% carbon dioxide (CO2) atmosphere. The cells were split two times a week at a ratio of 1:10 or 1:20 depending on their confluence.

3.1.7 Splitting Adherent Cells

Cells were allowed to grow in monolayers to a confluence of approximately 80%. They were then washed twice with PBS, and the cells were trypsinized with 1 ml trypsine per 250 ml culture flask. When the cells released from the flask (after approximately 1-2 min) they were then resuspended in 10 ml fresh medium. One tenth of this cell suspension was used to further cultivate the cells, which were split two times a week. Most cells were kept in culture for 10-20 passages.

3.1.8 Splitting Suspension Cells

Suspension cells were resuspended every three to four days and one tenth of the cellular suspension was added to fresh medium for further cultivation.

3.1.9 Freezing/Thawing Cells

Cells to be frozen were centrifuged at 1400 rpm for 10 min and resuspended at a concentration of $1x10^6$ cells/ml in 10% DMSO and 90% FCS. The cells were slowly frozen (in Styrofoam boxes) at -80ºC in 1 ml aliquots in liquid nitrogen safe cups for 24 – 48 hrs. After the initial freezing periods, the cells were moved to liquid nitrogen gas phased freezers.

Frozen cells were thawed quickly in 37ºC water baths and mixed with warm medium. The cells were then centrifuged to remove the DMSO used for freezing and resuspended in fresh medium. Suspension cells were washed twice. Adherent cells were seeded after the first washing and allowed to adhere to the flask for 1 hr, at which time the medium was removed and fresh medium was added to the cells.

3.2 FACS Analysis

Flow cytometry or FACS (Fluorescence Activated Cell Sorting) is based on different cellular properties which are detected by a laser. The cells are passed through a capillary in a thin stream of fluid where laser beams cause the light to scatter and fluorescent dyes to emit light at various frequencies. Based on the scattered light, data is produced including the forward scatter which shows the approximate cell size or the sideward scatter showing the granularity of the cells. The cells can be stained with the help of antibodies conjugated with a dye binding to molecules on the cell surface. This is extremely helpful when determining the cell type. Another application for FACS analysis is to measure the transduction efficiency of gene expression in cells transduced with a construct expressing eGFP (enhanced green fluorescent protein).

For the experiments, a minimum of $1x10^5$ cells were transferred to a FACS tube and centrifuged for 5 min at 1500 rpm. Cells expressing eGFP could be directly resuspended in

FACS buffer and analyzed without staining. To stain certain cellular markers, a conjugated antibody directed at the target was diluted as described by the manufacturer and added to the cells (see section 2.3). The cells were then incubated for 30 min at room temperature and washed. To wash the cells, 2 ml of FACS buffer (PBS with 3% FCS) was added and centrifuged for 5 min. This step was repeated 3 times in total. The cells were resuspended in 500 µl FACS buffer with 1.5% formaldehyde. The samples were then analyzed by FACS using the FACSCaliber and the Cell Quest Pro software from Beckham Dickenson.

3.3 Annexin-V Staining

During apoptosis, the membrane phospholipid phosphatidylserine translocates from the inner to the outer part of the plasma membrane. When this happens, high affinity binding sites become available for Annexin-V making it possible to determine if the cells are undergoing the early stages of apoptosis. This is relatively easy to confirm using Annexin-V; however the protocol is slightly different than normal antibody staining procedures. The cells (approximately $1x10^5$ cells) are washed twice with cold PBS and then resuspended in Annexin-V binding buffer (0.01 M HEPES, pH 7.4; 0.14 M NaCl; 2.5 mM $CaCl_2$). Then 5 µl of APC conjugated Annexin-V was added to the cell suspension and was mixed then incubated for 15 min at room temperature in the dark. The cells were then kept on ice and analyzed with FACS immediately.

3.4 Lentiviral Vectors (LVV)

Transduction is the transfer of genetic material into cells via viral or phage particles. Lentiviral vectors are important delivery systems especially due to their ability to sustain the expression over several months and to transduce both dividing and non-dividing cells. The system is based on the HIV virus, with modifications for safety. Only the viral proteins which are necessary for the production of the vectors are present in the 3 plasmids used to produce the vectors. The transfer vector (for example: pSEW and pSEW derived vectors), containing the gene to be brought into the cells, is the only vector which contains the packaging signal Ψ, meaning this is the only plasmid packaged into the viral particles. It also contains a SIN (self-inactivating) LTR, which means that there is a deletion in the 3' LTR region, removing the LTR promoter activity and reducing the threat of recombination. The packaging construct (for example: pCMVΔR8.91) has the Gag-Pro-Pol, Tat, Rev, RRE proteins controlled under a CMV promoter. The third plasmid (for example: pCMV-G) contains the envelope protein, in this case from the VSV virus which enables the transduction of most cells.

3.4.1 Lentiviral vector production

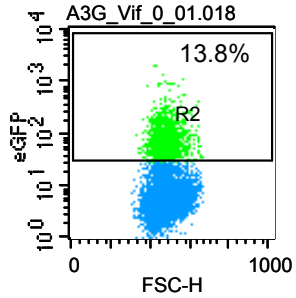
Production of the lentiviral vectors was achieved with calcium phosphate transfection of the three plasmids (pSEW or pSEW derived vectors, pCMVΔR8.91, and pCMV-G) into HEK-293T cells. Calcium phosphate transfection brings the DNA into the cells via precipitates of the plasmid DNA formed by its interaction with the calcium ions which are taken up by the cell through endocytosis. The HEK-293T cells are plated the day before in 10 cm culture dishes with $6x10^6$ cells per dish. On the day of the transfection, 25 mM Chloroquine is added to the cells at a dilution of 1:1000. The DNA is then mixed together as follows: 7.5 µg transfer vector (the pSEW derived plasmids), 12.5 µg packaging construct (pCMVΔR8.91), and 2 µg of the envelope plasmid (pCMV-G) and filled to 450 µl with sterile water (an additional plasmid, a eukaryotic vector containing the Vif protein (1 µg) was also added to the packaging of the LLVs which contain the gene for APOBEC3G). Then 50 µl of 2.5 mM $CaCl_2$ was added to the DNA mixture. The DNA/$CACl_2$ solution was then added dropwise to 500 µl 2x HEPES buffer while vortexing. The mixture was incubated for 20 min at room temperature and then 1 ml was added to each plate. The cells were incubated under normal cell culture conditions for 6-8 hrs after which time the medium was changed. The supernatants were collected 24, 36 and 48 hrs after transfection and directly filtered with a syringe top sterile filter (0.22µm). The filtered supernatant was pooled and kept at 4˚C until being concentrated and frozen at -80ºC. (see appendix for the plasmid maps)

3.4.2 Concentrating lentiviral vectors

Lentiviral vectors were concentrated using the ultracentrifuge. For this, 34 ml of the culture supernatant was filled into each tube. This was then centrifuged for 2 hrs at 19500xg and the supernatant was discarded. The pellet was resuspended overnight at 4˚C in the medium left in the tube (approximately 500 µl) and was then aliquoted, frozen and titrated.

3.4.3 Titration of lentiviral vectors

To determine the amount of lentiviral particles are in a milliliter, the viral stock was titrated. For the titration, $5x10^4$ cells per well were seeded in a 24 well plate one day before titrating the viruses. A dilution series of the lentiviral vectors was done, in the case of the concentrated vectors they were diluted as follows: 1:100,000; 1:10,000, 1:5000; 1:1000; 1:500; 1:100. The unconcentrated lentiviral vectors were diluted 1:10,000; 1:1000; 1:500; 1:100; 1:500; 1:100 in medium. The plates were centrifuged for 1 hr at 2000 rpm at 31ºC and incubated with standard cell conditions. After 48 hrs the cells could be analyzed by FACS and the titer calculated. The titer was calculated with cultures which had less than 20% positive cells (to reduce the double positives) as follows (see figure13 for an example):



Titer [infectious particles/ml] = (positive transduced cells (%)/100) x seeded cell number x dilution factor

Figure 13. FACS analysis of a titration with LVVs. For this analysis, using a dilution of 1:100,000 13.8% of the cells were eGFP positive, meaning they were transduced with the LVV. Putting this into the formula it is: Titer = 13.8%/100 x 50,000 cells seeded x 100,000. Using this formula, the titer is 6.9x10^8 infectious particles /ml.

With the titer of the virus stock, the amount of virus needed for transducing cells can be determined. The MOI, also known as multiplicity of infection, is defined as being the amount of infectious particles per cell. An MOI of 1 is the same as having one virus per cell. The MOI needed to infect cells to the extent that on average only one virus enters each cell is different for each cell type. The MOI can be determined with the following formula:

MOI [infectious particles/cell] = titer [infectious particles/ml] x volume / cell number

The optimal MOI for certain cells can be determined by plotting the percent of positive cells against the MOI. In this case 50-100% positive HUT78 cells were required therefore the optimal MOI was approximately 5. This is shown in Figure14.

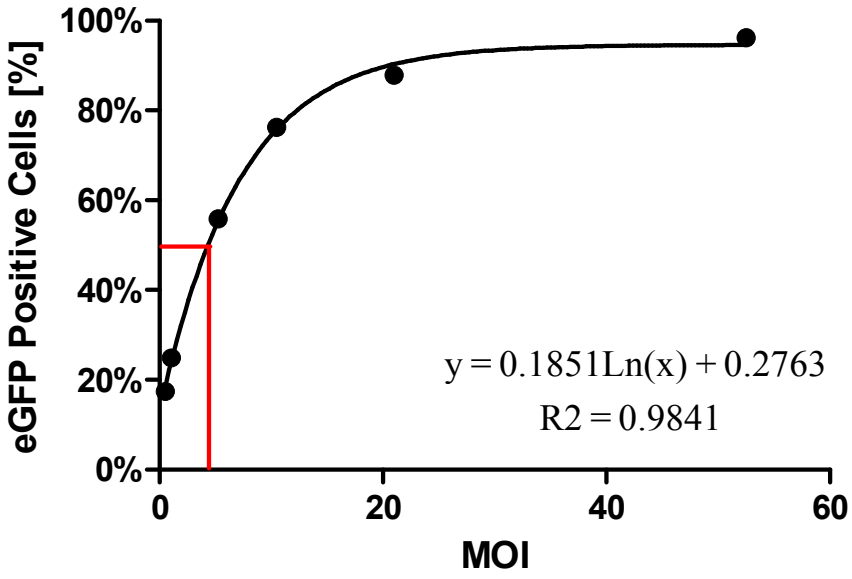


Figure 14. Determining the optimal MOI to transduce cell lines. In this case LVVs titered on Te671 cells were tested to determine the optimal MOI to use in HUT78 cells.

3.4.4 Transduction

The cells are transduced by quickly thawing the concentrated or unconcentrated LVVs and adding the correct MOI to the cells. The cells are then centrifuged for 1hr at 2000 RPM and incubated over night in cell culture conditions. After at least 12 hrs, the medium is changed. The transduction efficiency can be analyzed after 48 hrs by means of FACS (see section 3.2).

3.5 Transfection

3.5.1 Lipofectamin Transfection

Transfection is a way of bringing plasmids into eukaryotic cells by means of reagent. In this case, Lipofectamine™ 2000 (LF2000) was used. Lipofectamine™ is a cationic lipid that provides a high transfection efficiency. When the plasmid is incubated with LF2000 and DMEM (or another serum-free medium), micelles form around the plasmid. These plasmid containing micelles can then cross the cellular membrane.

For transfection, 293T cells (used because of their high transfection efficiency) were plated the day before with $2x10^5$ cells per well in a 24 well plate in DMEM with 10% FCS, 2% L-glutamine, and 1% penicillin/streptomycin. On the morning of the transfection the medium is changed to 500 µl DMEM without serum or antibiotics. Per sample, 0.8 µg plasmid was used and diluted in 50 µl of DMEM. Separately, 2.0 µl LF2000 was also diluted in 50 µl of DMEM. The latter was mixed gently and incubated for 5 min at room temperature. The plasmid was then added to the LF2000 solution and gently mixed, then incubated for 20 min at room temperature. After the 20 min, 100 µl of the DNA-LF2000 complex was added to each well and gently mixed. The medium was changed to DMEM with 10% FCS, 2% L-glutamine, and 1% penicillin/streptomycin after 6 hrs.

3.5.2 Calcium Phosphate Transfection

Calcium phosphate transfection is achieved because the precipitates of the plasmid DNA formed by its interaction with the calcium ions that enter the cell by endocytosis. The HEK-293T cells are plated the day before in 10 cm culture dishes with $6x10^6$ cells per dish. The day of the transfection, 25 mM Chloroquine is added to the cells at a dilution of 1:1000. The DNA is then mixed together as follows: 25 µg vector and filled to 450 µl with sterile water. Then 50 µl of 2.5 mM $CaCl_2$ was added to the DNA mixture. The DNA/$CaCl_2$ solution was then added dropwise to 500 µl 2x HEPES buffer while vortexing. The mixture was incubated for 20 min at room temperature and 1 ml was added to each plate. The cells were incubated at normal cell culture conditions for 6-8 hrs before changing the medium. Clonal Viruses were produced in the same way and frozen unconcentrated in 1 ml aliquots at -80ºC.

3.6 Titration of Viral Stocks

Viral stocks can be titrated to determine the amount of infectious particles/ml or titer of the stock. This is necessary to determine the amount of viral stock needed in order to infect cells with an equal amount of viral particles. For this test, TZM-bl cells were used as an indicator cell line, as they express both CD4 and CCR5 and contain the β-galactosidase genes under control of the HIV-1 promoter. The day before infection, $3.5x10^3$ cells were plated per well in a 96 well plate in 100 µl DMEM standard medium (see section 2.11). On the day of infection a dilution series of the viral stocks was performed from 1 µl of viral stock to 100 µl in a total volume of 100 µl. The medium was removed from the wells and replaced with the dilution series and the cells were cultured under normal culture conditions for 2-3 days. After this time the cells were washed carefully with PBS and fixed with 100 µl of an Acetone/Methanol (1:1) solution for 5 min. The cells were then washed a second time with PBS. After washing 200 µl substrate solution (per well: 200 µl - PBS, 3 µl - 3 mM potassium ferrocyanide, 3 µl - 3 mM potassium ferricyanide, 0.2 µl - 1 mM $MgCl_2$ and 2.5 µl - 0.5 mg/ml x-gal) was added to each well. The cells were incubated for 1-2 hrs at cell culture conditions with the substrate solution and then analyzed under the light microscope. The blue syncytia were counted and the titer was determined using the formula in figure 13 (in this case: Titer [infectious particles/ml] = blue stained syncytia x dilution factor).

3.7 Stimulating and infecting cells

For mutation analysis, monocytes derived macrophages were infected using HIV-1 ADA-M and HIV-1 ADA-MΔvif virus. These virus strains were produced through calcium phosphate transfection as described in section 3.5.2 and never passaged on cells. This was to reduce the risk of random mutations from continued growth on cells, allowing us to assume that any mutations found in the viral genome after infection were as a result of these infections. For infection with HIV-1ADA-M and HIV-1ADA-MΔvif, the macrophages were plated at $4x10^5$ in wells of a 24 well plate. The macrophages were then infected with 0.005 MOI of HIV-1 ADA-M or HIV-1 ADA-MΔvif viruses. The medium was changed every 3-4 days. After 10 days, the cells were stimulated with 100 ng/ml LPS, 30 U/ml IFN-α, or were left unstimulated. On day 11 and day 12 after infection the supernatants were harvested for further infection of indicator cells for mutation analysis (see section 3.7).

To determine the infectability of the monocytes derived macrophages or CD34+ derived macrophages, the cells were stimulated as soon as they were differentiated with either 100 ng/ml LPS or 100 U/ml IFN-α (unless otherwise noted). After 8 hrs of stimulation, the cells were infected with 0.005 MOI of HIV-1 ADA-M or HIV-1 ADA-MΔvif virus, cells were lysed for RNA isolation at various times, as noted in the results. After 24 hrs of infection, the cells

were washed twice with medium and a p24 sample was taken. Further p24 samples were taken throughout the infection and the medium was changed every 3-4 days.

CD34+ cells were stimulated after culturing in labor for several weeks to increase the amount of cells. They were stimulated with either 100 ng/ml LPS or 100 U/ml IFN-α in proliferation medium (as described in section 2.11). As above, 8 hrs after stimulation the cells were infected with 0.005 MOI of HIV-1 ADA-M or HIV-1 ADA-MΔvif virus. After 24 hrs only one half of the medium was replaced and a p24 sample was taken. The cells were lysed for RNA isolation 24 hrs after stimulation.

3.8 Infection of a Cell Line for Mutation Analysis

The indicator cells line, 293T CD4/CCR5, produces no or very little APOBEC3G. The 293T CD4/CCR5 were plated in a 24 well plate with $7x10^5$ cells per well. For infection, the medium was removed from the cells. The cells were infected with 1 ml of unconcentrated supernatants from infected macrophages (see section 3.7). The plate was then centrifuged for 1 hour at 1300 rpm and 37˚C in order to increase the concentration of viruses near the cells. After 1 hour, the plate was removed from the centrifuge and kept at normal cell conditions in the incubator for another 3 hrs. After incubation, 50 U of DNase I was added to the infection. The cells were incubated for an additional 2 hrs to remove excess DNA from the supernatant. It was unnecessary to deactivate the DNase I prior to DNA purification. The five hour incubation time was chosen in order to analyze the first reverse transcription reaction of the HIV virus in the cells. The APOBEC3G which was packaged into the virions during production in the macrophages could interact with the single stranded DNA in the indicator cells during the reverse transcription and produce possible mutations (Vartanian et al. 2003; Zhang et al. 2003; Yu et al. 2004).

3.9 P24 ELISA Assay

The p24 ELISA test is an indirect assay to test the amount of virus; it can not distinguish defective particles from intact particles and therefore can not be used to test the infectivity of the virus supernatant. To test the infectivity, titration or other direct methods should be used.

For these experiments, the Innotest^{tm} HIV Antigen mAb from Innogenetics was used. To prepare the samples, 72 µl of sample was added to 8 µl of NP40 (which inactivates the sample). The samples were then diluted (1:10, 1:100, 1:1,000, and 1:10,000) by adding 14 µl from the sample/NP40 mixture to 126 µl PBS. At this point the samples can be removed from the L3 laboratory and taken to another laboratory. A standard series was always done with 0, 18.9, 101, and 178 pg/ml p24. In order to get these amounts, the negative control of HIV antigen free serum with 0.1% sodium azide for conservation provided in the kit was used. For the other p24 standard concentrations: 15 µl of the standard was added to 225 µl

PBS, 80 µl standard was added to 160 µl PBS and 140 µl standard was added to 97 µl PBS respectively. From each sample, 100 µl was added into the wells supplied. The wells were coated with human polyclonal antibodies against HIV. The conjugation mix 1 containing monoclonal biotinylated murine anti-p24 antibodies, was prepared using the amounts specified in the kit. The conjugation mix 1 was then vortexed and 100 µl was added to each well and then incubated for 1 hour at 37˚C. The wells were then washed with an automatic washer 5 times with 300 µl 1x wash solution. The conjugation mix 2 containing a peroxidase conjugated streptavidin was then prepared using the amounts specified. The solution was vortexed and 200 µl was given to each well. They were then incubated for 30 min at 37˚C. After the incubation, the wells were washed five times with 300 µl wash solution with the automatic washer. The substrate mix containing tetramethylbenzidine in dimethylsulfoxide, was then prepared as specified and vortexed. Then 200 µl of the substrate mix was added to each well, and it was incubated in the dark for 30 min. The reaction was then stopped with 50 µl H2SO4. The extinction was then measured with the photometer Spectomax 340 from MWG Biotech and the computer software Soft Max Pro using the wavelengths 450 nm and 650 nm. To analyze the results, the extinction from 650 nm was subtracted from the extinction of the wavelength at 450 nm. Then the amount of p24 was formulated using the standard curve to calculate the results in pg p24/ml.

3.10 RNA Isolation

For the RNA isolation and purification, the RNeasy kit from Qiagen was used. Before use, four volumes of 96-100% ethanol were given to the RPE wash buffer, and 10 µl of β-mercaptoethanol was added per 1 ml of lysis buffer RLT which expires 1 month after made. The macrophages, which were plated with $4x10^5$ cells per well, were washed once with PBS and then 250 µl of lysis buffer RLT was given to the cells and mixed well with the pipette. For infected cells, this was done in the L3 laboratory and then brought out after lysis or frozen at -80˚C. One volume of 70% RNase free Ethanol (98% Ethanol mixed with RNase free water) was then added to the samples. This mixture was then pipetted onto the columns provided by Qiagen and centrifuged at 10,000 rpm for approximately 15 seconds in order to bind the RNA to the filter. After centrifuging, the flow through was discarded and 350 µl of wash buffer RW1 was added. This was centrifuged again at 10,000 rpm for 15 seconds and the discharge was again thrown out. Then the DNA left over on the column was digested for 15 min at room temperature using 60µl RNase-free DNase I mixed 1:7 with the buffer provided from Qiagen. After the 15 minute incubation, 350 µl of wash buffer RWI was again added to the column and centrifuged at 10,000 rpm for approximately 15 seconds and the discharge was discarded. The column was then washed twice with 500 µl RPE separated by a centrifugation step at 10,000 rpm for 15 seconds where the discharge was again discarded. After the second wash step with the RPE, the columns were centrifuged at maximum speed for 2 min and the 2 ml collection tubes were discarded. The columns were placed in new 1.5 ml collection tubes and 30 µl RNase free water was added directly to the filter. The columns

were then centrifuged at 10,000 rpm for 1 minute to elute the RNA. The RNA could then be transcribed to cDNA via an RT PCR (see section 3.13.1) or stored at -20 or -80˚C.

3.11 Protein Isolation

Protein for Western blot analysis was isolated by removing the cell medium either directly in the well or centrifuging the cells in 1.5ml cups. The cells were then lysed by adding 50 µl of 3x sample buffer (0.5 M Tris-HCl, pH 6.8; 25% Glycerine; 10% (w/v) SDS; 0.5% (w/v) bromophenol blue – 5% β-Mercaptoethanol was added directly before use) and then heated to 98˚C for 4 min. The protein solution was then immediately used for SDS gels and Western blot analysis.

3.12 Western Blot analysis

SDS-page gels were cast in mini gel casting chambers from the Mini-PROTEIN Electrophoresis system from Bio-Rad with 12% resolving and 4% stacking gels. The ingredients were mixed together and the APS and TEMED were added shortly before pouring the gels into the casting chambers with 1 mm glass plates. The resolving gel was poured first, allowed to become solid and then the stacking gel was poured and the comb inserted.

12% Resolving Gel		**4% Stacking Gel**	
3.4 ml	dH2O	6.1 ml	dH2O
4.0 ml	30% Acrylamide	1.3 ml	30% Acrylamide
2.5 ml	1.5 M Tris-HCl, pH 8.8	2.5 ml	0.5 M Tris-HCl, pH 6.8
0.1 ml	10% w/v SDS	0.1 ml	10% w/v SDS
50 µl	10% APS	50 µl	10% APS
5 µl	TEMED	10 µl	TEMED

The gels were built into the electrophoresis gel chamber, also from the Mini-PROTEIN Electrophoresis system from Bio-Rad, containing Tris running buffer (25 mM Tris-HCl; 192 mM glycine and 1% SDS). The samples, prepared as described in section 3.11, and the Kaleidoscope protein marker were added to the wells. The gel was run at 150 Volts for 1.5 hrs. The gels were then removed from the glass plates and blotted with the Trans-Blot® SD Semi-Dry Cell from Bio-Rad. For this, all components were soaked in blotting buffer (25 mM Tris-HCl, 192 mM glycine, and 10% Methanol) and the nitrocellulose paper was laid upon 2 pieces of thick blotting paper. The gel was smoothed out on the nitrocellulose and 2 pieces of thick blotting paper were laid on top and air bubbles were rolled out. The membrane was blotted at 15 Volts for 1 hr.

After the proteins were successfully blotted onto the nitrocellulose paper, it was blocked in blocking buffer (PBS with 0.05% Tween, 1% non-fat powdered milk, and 1% BSA) overnight at 4˚C. The blot was then incubated for 1 hr at room temperature with the first antibody (anti-ApoC17 or the anti-GAPDH antibody were diluted 1:10,000 in blocking buffer). The membrane was then washed for 20 min three times in blocking buffer and then incubated with the second antibody (either anti-rabbit HRP (for the anti-ApoC17 antibody) or anti-mouse HRP antibody (for anti-GAPDH antibody) diluted 1:10,000 in blocking buffer) for 1 hr at room temperature. The membrane was then washed two times for 20 min in blocking buffer and then two times in PBS with 0.05% Tween for 20 min.

The Western blotting detection reagent, ECL Plus Western Blotting Reagent Pack, was then applied to the blot as per manufacturer's instructions. The blot could then be developed on ECL plus hyperfilm or Fuji medical xRay film using the Optimax X-Ray film processor.

3.13 PCR

Polymerase chain reaction (PCR) is used to amplify even small amounts of DNA. For the reaction, primers, which are pieces of DNA approximately 20-30 bp long, a polymerase, dNTPs, and buffer, are needed. The PCR then goes through different steps, beginning with denaturation at approximately 95ºC so that the DNA is single stranded. The primers can then anneal to the single stranded DNA at a temperature dependant on the melting temperature of the primers. The polymerase can then elongate the DNA strands and the DNA is through the repeating cycles exponentially amplified. This method has a variety of applications, including the production of cDNA from RNA with a RT-PCR, the quantification of mRNA with the RTQ-PCR, and the amplification of extremely small amounts of DNA with a nested PCR.

3.13.1 Reverse Transcription Assay (RT-PCR)

RT-PCR is used to make complementary DNA (cDNA) out of mRNA. For the RT-PCR, RNA isolated from macrophages was photometrically measured with a dilution of 1:50 using the Gene Quant II RNA/DNA calculator from Pharmacia Biotech or undiluted in the Nanodrop. In order to have equal concentrations in each PCR reaction, 0.5 µg RNA was diluted with RNase free water for a total volume of 15 µl in 200 µl tubes. The RNA mixture was then heated for 5 min to 75˚C in order to melt any RNA secondary structures. A master mix with 2 µl 10x PCR Buffer without MgCl2, 1 µl 10mM dNTPs, 1 µl 100 ng/µl Random Primer, 4 µl MgCl2, 0.5 µl RNase Inhibitor, 0.25 µl Superscript III, and 1.25 µl RNase free water per sample was made. From the master mix, 10 µl was added to the RNA to make a total of 25 µl. The PCR tubes where then put into the PCR T3 Thermocycler and run through the program 25˚C for 10 min where the primers have enough time to hybridize to the RNA, 44˚C for 60 min for the reverse transcriptase to translate the RNA to DNA, and then 95˚C for 5

min. The cDNA was stored at 4˚C until further use in an RTq-PCR and at -20˚C for prolonged storage.

3.13.2 Real Time Quantitative PCR (RTq-PCR)

Another application of the PCR method, the real time quantitative PCR (RTq-PCR), can be used to quantify the amount of mRNA, and therefore the expression of the targeted gene in the cell. This method was used to measure the relative amount of a target gene expressed by the cells in comparison to a standard mRNA made by the cell, EF-1α. EF-1α is made at a constant amount and is therefore an important control of the mRNA amount put into the reactions. SYBR Green from Bio-Rad was used to detect the amount of dsDNA products made by being randomly built into the dsDNA as it was produced. This then produces a measurable amount of fluorescence which could be detected. The target primers, for example: APOBEC5P1 and APOBEC3P1 were used and the results were normalized with EF-1α primers EF-1α-3P1 and EF-1α-5P1 to be certain that the amount of cDNA in the reaction was always the same. The cDNA from the RT-PCR was diluted 1:10 with HPLC water. The master mix containing 12.5 µl Bio-Rad SYBR Green supermix, 0.5 µl from primer 1, 0.5 µl from primer 2, and 6.5 µl HPLC water per reaction was distributed 20µl per well into a 96 well PCR plate, and then 5 µl of the diluted cDNA was added to each well with triplicates. A PCR plate cover was placed on the PCR plate and then the plate was put into the i-cycler thermal cycler. The program was run at 95˚C for 3 min in order to denature the cDNA, 95˚C for 30 seconds, 59˚C for 30 seconds so that the primers can bind to the cDNA, then 30 seconds at 72˚C, which is the optimal temperature for the polymerase to work. At this point, the fluorescence was measured. To see if there were other contaminations or unspecific binding of the primers in the PCR a melt curve was also done by increasing 0.5˚C every 10 seconds 100 times starting at 55˚C. The melt curve (see figure 16) shows at what temperature the PCR products melt. If there are other peaks, then there are contaminations or unspecific binding of the primers.

The results from the RTq-PCR are in a graph which shows the amount of fluorescence measured related to the number of cycles (see figure 15). The number of cycles completed where the amount of fluorescence is substantially higher than the background can be used to further calculate the relative amount of expression.

The relative expression of the gene can be analyzed in different ways. Q-gene, an Excel based software application, was used to analyze the data (Muller et al. 2002; Simon 2003). The program has two different means of calculating the results; in this case the mean normalized expression was used. As a normalizing gene EF-1α was used. The program then calculates the mean normalized expression automatically.

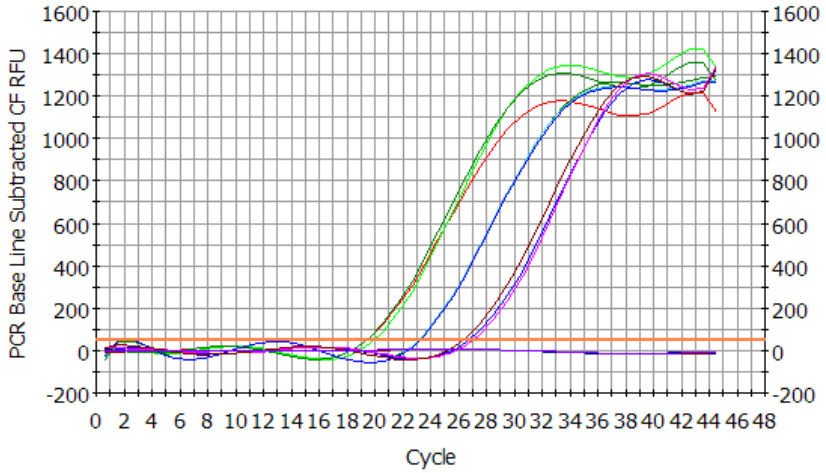


Figure 15. PCR amplification versus cycle number. The horizontal orange line represents the threshold where the amount of fluorescence measured is substantially higher than the background. In this case cDNA from cells producing different amounts of APOBEC3G was use with APOBEC3G primers. The line furthest to the left has the highest APOBEC3G production. The curve on the right had the lowest expression of APOBEC3G mRNA and the negative control did not go above the threshold.

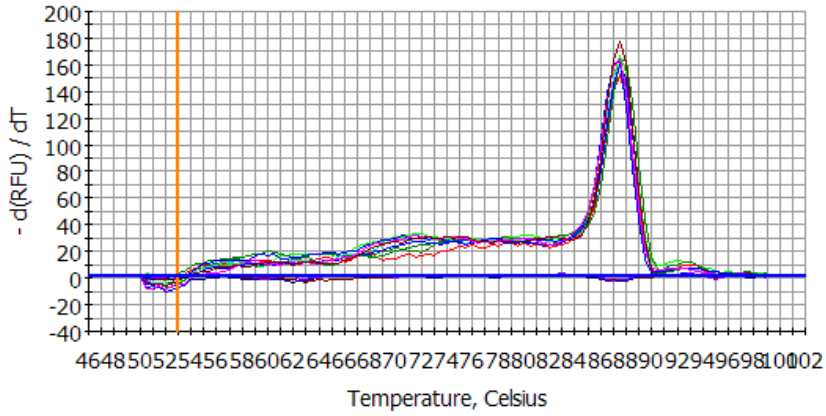


Figure 16. Melt Temperature curve. This is the melt curve from the experiment shown in figure 15. It shows the PCR products which were amplified. If other peaks are present, it means that there are contaminations or the primer was bound unspecifically.

3.13.3 Nested env PCR Amplification

Nested PCRs are used when the amount of DNA in a solution is very low. Using this method, the PCR product from the first PCR is amplified a second time using primers inside the first set of primers. The DNA purified as indicated in section 3.14 from the indicator cell line was used. Due to the fact that there was such a low concentration of virus in the supernatants from the macrophages, it must be assumed that there will be little DNA made in the reverse transcription reaction of the virus in the indicator cells. In order to work with such small amounts of DNA, a nested PCR was used, so that even a single copy of proviral

DNA would provide enough DNA after amplification to further work with it. This is done by first amplifying a larger piece of DNA with the outer primers: Ja19d and 7434R. Then the inner primers: H1e6601d and H1e7187r amplified a smaller piece of DNA from the V3 region of the env gene of the already amplified DNA.

The master mix for the first PCR consisted of 5 µl Biotherm 10x Buffer, 1 µl dNTPs (10 mM), 1 µl primer Ja19d (10 pM), 1 µl primer 7434r (10 pM) and 0.5 µl Biotherm Taq Polymerase per sample and 10 µl DNA was added to each reaction. A negative and positive control was always amplified with the samples and the reactions were filled to 50 µl with HPLC water. The samples were then put into the PCR thermal cycler with the program: 94°C for 3 min. in order to denature the DNA, then the following was repeated 35 times: 94°C for 30 sec, 55°C for 30 sec, and 72°C for 30 sec, following which, the same was kept at 72°C for 10 min and then paused at 4°C.

For the second PCR, 1 µl of amplified DNA from the first PCR was used. The master mix for the second PCR contained: 5 µl Biotherm 10x buffer, 1 µl dNTPs (10mM), 1 µl primer H1e6601d (10 pM), 1 µl primer H1e7187r (10pM), 0.5 µl Biotherm Tag Polymerase, and 40.5 µl HPLC water per sample. The second PCR used the same program as in the first PCR explained above. The DNA was then stored at 4ºC for short-term storage, or at -20ºC for long-term storage. The DNA from this PCR was then cloned into a TA cloning vector as explained in sections 3.18. The plasmid DNA was isolated (section 3.20) and sequenced (section 3.22) for mutation analysis.

3.14 DNA purification

The DNA from indicator 293T CD4/CCR5 cells was used to analyze mutations in the HIV genome. After a total of 5 hrs, the medium was removed from the cells, and the cells were washed with approximately 200 µl PBS. The cells were then lysed using 20 µl protease K and 200 µl buffer AL from the DNeasy tissue kit (Qiagen). This was then quickly and thoroughly resuspended by pipetting and transferred to 1.5 ml tubes and vortexed 10 seconds each. The samples were then incubated at 70˚C for 10 min. Then 200 µl of 96-100% ethanol was added to the sample and the sample was vortexed. The samples were then pipetted onto the DNeasy mini spin column provided and centrifuged at 8000 rpm for 1 minute. The 2 ml collection tube was discarded and the column was placed in a new collection tube. To wash the column, 500 µl buffer AW1 was added to each sample and they were centrifuged again for 1 minute at 8,000 rpm. The collection tube was again discarded and the column was placed in a new collection tube. The column was then washed with 500 µl buffer AW2 and centrifuged for 3 min at 14,000 rpm to dry the membrane. The collection tube was then very carefully discarded and the column was placed in a clean 1.5 ml tube for collection of the DNA. To elute the DNA, 150 µl of buffer AE was pipetted directly onto the DNeasy membrane. The samples were incubated for 1 min at room temperature and then

centrifuged for 1 minute at 8,000 rpm to elute the DNA. The DNA was then kept at 4˚C for short term storage or at -20˚C for long term storage.

3.15 PCR Product Purification

The PCR products had to be purified so that the salts, primer, and other reagents from the PCR could be removed before cloning the DNA. These other products could interfere with further analysis including sequencing the DNA. This step was done using NucleoSpin® Extract. The sample, which was 50 µl, was diluted with 4 volumes of NT2 in order to adjust the DNA binding conditions. This was then pipetted onto the supplied silica matrix columns which were in 2 ml collection tubes and centrifuged at 13,000 rpm for 1 min. in order to bind the DNA. The silica membrane was washed once with 600 µl NT3 and centrifuged at 13,000 rpm for 1 min and then with 200 µl NT3 again for 2 min at full speed to dry the membrane. The DNA was then eluted into a new 1.5 µl collection tube using 25 µl NE buffer and centrifuging 1 min at 13,000 rpm.

3.16 Digestion of DNA with Restriction Enzymes

DNA was digested in 10 or 30 µl reactions. The 10 µl reactions were completed with approximately 1 µg DNA and the 30 µl reactions were for larger amounts of up to 5 µg DNA. Each reaction had 1x buffer (either NEBuffer 1, 2 or 3) and if needed 1x BSA (100x stock solution). The respective restriction enzyme was added to the reaction at 1 Unit per 1 µg DNA and the reaction was filled to 10 or 30 µl with water. Most enzymes required incubation at 37ºC and were incubated for 3 hrs. The specific protocol depends on the enzyme being used and the requirements as stated on the data sheets provided or on the New England Biolabs website (www.NEB.com).

3.17 Ligation

Digested DNA or PCR products could be bound together via ligation. This was generally performed using the quick ligase. Approximately 50 ng of vector plasmid was mixed with 3 times that of the insert, and the reaction was filled to 10 µl which was diluted with the 2x quick ligase buffer to 20 µl. The reaction was briefly centrifuged and incubated at room temperature for 30 min immediately following the ligation. It was then cooled on ice and used to transform bacteria (see section 3.19).

3.18 TA Cloning

In order to sequence the DNA for mutation analysis, the DNA was ligated into the vector pCR2.1 and then transformed into One Shot® E. coli Top 10 cells. A blue-white-selection was used to detect the bacteria colonies with an insert. This method, also called α-

complementation, is based on the disruption of the lacZ' gene, which in this case consists of only the N-terminal α-fragment of the β-Galactosidase, which is alone not fully functional. If the β-Galactosidase is brought together with the also non-functional C-terminal ω-fragment then the activity is restored, and the colony is blue after incubation with IPTG and X-Gal. If the DNA fragment is inserted into the vector, then the insert interrupts the lacZ' gene and the colonies remain white.

A ligation was preformed using solutions from Invitrogen (Life Technology). For the ligation, 1 µl 10x ligation buffer, 1.5 µl from the vector pCR2.1 (25 ng/µl), 6.5 µl of the fresh purified PCR product (from section 3.15), and 1 µl of T4 DNA ligase (4.0 Weiss Units) was used for each reaction, giving a total volume of 10 µl. The ligation reaction was then incubated at 16ºC for 4 hrs in a thermal cycler and then cooled to 4ºC. During this reaction, the lid was not heated and was left open. After the ligation, the plasmid was brought into E. coli bacteria as described in section 3.19.

3.19 Transformation into E. coli Bacteria

For the transformation, One Shot® E. coli "Top 10" chemically competent cells were thawed for 20 min on ice and 50 µl of the bacteria were then carefully pipetted into each reaction tube needed on ice and not moved. Then 10 µl of the ligation was pipetted into the cells and gently stirred. The cells were incubated for 30 min on ice. The reaction was then heat-shocked for 30 sec in a 42ºC water bath, removed and incubated on ice again for 2 min. After this time, 500 µl LB medium was added to each vial and shaken at 37ºC for approximately 45 min.

After the incubation time, the bacteria were centrifuged for 5 min at 7,000 rpm and 450 µl of the supernatant was discarded. The remaining 50 µl was then used to resuspend the cells. The bacteria were plated onto the LB/amp agar plates optionally with IPTG and X-Gal if blue-white selection is possible. The agar plates were incubated at 37ºC overnight. The next day bacterial colonies were picked and grown in LB/amp medium overnight. It can be assumed that each colony derived from a single bacteria and that it is therefore a single clone. The plasmid DNA was then isolated as described in section 3.20.

3.20 Preparation of Plasmid DNA (mini)

Plasmids are small circular pieces of DNA that are not built into the bacterial genome. For this reason, they can be easily isolated and purified. The mini prep kit: E.Z.N.A. Plasmid Mini Kit II from PeqLab was used to purify the plasmids out of the bacteria after transformation. This is done with a silica column based system. To begin with 1.5 – 2 ml of bacteria from an overnight culture was centrifuged for 1 min at 10,000 rpm. The supernatant was discarded and the bacterial cells were lysed with 250 µl of solution I / with RNase A. The sample was

thoroughly mixed by vortexing, and 250 µl of solution II was given to the samples and was mixed by inverting 4-6 times until a clear lysate appears. The lysate was neutralized by adding 350 µl of solution III and inverting to mix the solution and then centrifuged for 5 min at 13,000 rpm to separate the white precipitate. The supernatant was then pipetted onto the columns provided and centrifuged at 13,000 rpm for 1 min. The samples were then washed with 500 µl HB buffer and centrifuged 1 min at 13,000 rpm. They were then washed with 750 µl DNA wash buffer with ethanol and centrifuged 1 min at 13,000 rpm. The membrane was dried by centrifuging 1 min using maximum speed. After which, the DNA was eluted with 50 µl TE-Buffer and centrifuged 1 min at 13,000 rpm. The plasmid DNA was stored at -20˚C.

3.21 Preparation of Plasmid DNA (maxi)

In order to prepare larger amounts of plasmid DNA out of bacterial cultures, the maxi prep kit from Macherey-Nagel, NucleoBond AX 500, was used. This kit is also based on a silica anion-exchange resin in prepacked columns, but made to purify up to 500 µg plasmid DNA. For the max prep, 100 ml of an overnight bacterial culture was centrifuged for 20 min at 6,000 x g and 4ºC. The supernatant was discarded and the pellet was resuspended in 12 ml Buffer S1 with RNase A. After fully resuspending the culture, 12 ml of Buffer S2 was added to the suspension and it was mixed gently by inverting the tube 6-8 times. The mixture was incubated at room temperature for 2-3 min after which cold Buffer S3 was added to the suspension, and it was again gently mixed by inversion. At this time the column was equilibrated with 6 ml of Buffer N2 and the lysate was clarified through a folded filter. The clarified lysate was loaded onto the column and allowed to flow through. The column was then washed with 32 ml of Buffer N3 and eluted with 15 ml of Buffer N5. After the elution, the plasmid DNA was precipitated with 11 ml isopropanol and centrifuged for 30 min at 15,000 x g at 4ºC. The supernatant was carefully discarded and the DNA pellet was washed with 5 ml of 70% ethanol and centrifuged for 10 min at 15,000 x g. After centrifuging, the ethanol was also discarded and dried in a sterile work bench for 1 hour. The plasmid DNA was reconstituted with 100 µl sterile deionized H_2O and the concentration was set to 1 µg/µl. The plasmid DNA was stored at -20˚C.

3.22 Sequencing

The sequencing was done by Margot Landersz in the molecular virology department at the Georg-Speyer-Haus. For each sequence reaction for mutation analysis, 3 µl of the mini prep DNA was mixed with 12 µl HPLC water and 1 µl H1e6601d primer (10 pmol/µl). Premix Big Dye Terminator containing the fluorescently marked ddNTPs, dNTPs and polymerase was used for the reaction. The reactions were amplified in the thermal cycler with the following program: 25 cycles of 96°C for 10 sec (denaturing step), 53°C for 5 sec (binding of the primer onto the DNA) and 60°C for 4 min (extension of the DNA strand). The reaction was then cooled to 4°C. The sequencing products were analyzed on an automated sequencer

(ABI3100). Sequences were aligned using the clustal method in MultiAlign from DNAstar. Other plasmid DNA was sequenced using the same method and with 0.5-1 µg DNA.

3.23 Mann-Whitney U test statistic

The two tailed P value was calculated using the Mann-Whitney U test. The Mann-Whitney U test (also known as the Wilcoxon rank-sum test) is a non-parametric statistic that does not assume that the populations follow Gaussian distributions. But it does assume that the shape of the two distributions is identical. The test works by ranking all the values from low to high, and comparing the mean rank in the two groups. The P value was calculated using the Mann-Whitney U test calculator at http://elegans.swmed.edu/~leon/stats/utest.html (Avery 2007). A two-tailed P value which is greater than or equal to 0.05 means that the two datasets are not significantly different, if the P value is less than 0.05 then difference in the two datasets is marginally significant, if the P value is less than 0.01 then there is a significant difference, and if the P value is less than 0.001 then the difference is highly significant. (Heath 1995)

4 RESULTS

4.1 APOBEC3G is upregulated in macrophages stimulated with IFN-α or LPS

It has been well known and documented that stimulation of macrophages with IFN-α or LPS results in an antiviral state (Kornbluth et al. 1989; Pitha 1994). There are hundreds of genes which are activated during this stimulation, but the genes responsible for the antiviral response have not yet been completely elucidated. Microarray analysis of macrophages stimulated with IFN-α or LPS done previously at the Georg-Speyer-Haus showed many of the genes which were upregulated, including APOBEC3G (Bouazzaoui et al. 2006). Around the time of these experiments, the first paper from Sheehy et al. (Sheehy et al. 2002) was published showing that APOBEC3G was responsible for the HIV-1 non-permissive cell type in T-cell lines. The antiviral effect against Vif defective HIV-1 viruses could be partially attributed to the G→A hypermutations of the HIV genome during the reverse transcription (RT) reaction.

The goal was to see if APOBEC3G was one of the proteins responsible for the antiviral state in macrophages. To begin with, the expression of APOBEC3G was tested in stimulated cells. Macrophages from 5 different blood donors were differentiated from PBMCs isolated out of fresh buffy coats with AB serum in Teflon bags for 7 days, after which time they were seeded and isolated by adherence. The day after seeding the macrophages, they were stimulated with IFN-α or LPS and RNA was isolated. The cDNA was then used to analyze the APOBEC3G expression in RT-qPCR. The results were then analyzed to get the mean normalized expression of the gene.

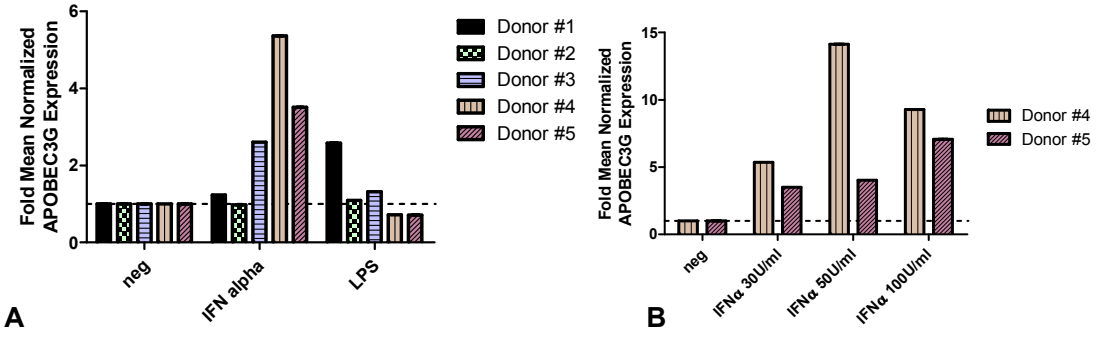


Figure 17. <u>Expression of A3G in AB Macrophages.</u> Macrophages were isolated and differentiated from 5 different blood donors. The cells were then stimulated with either IFN-α or LPS and the expression of APOBEC3G was measured using RTq-PCR. (A) The expression of APOBEC3G in 4 of 5 blood donors increased up to six-fold after 24 hrs of stimulation with IFN-α. (B) The expression of APOBEC3G in the macrophages from 2 of the blood donors increased with increasing IFN-α concentration.

As shown in figure 17, the expression of APOBEC3G increases after the macrophages are stimulated with IFN-α or LPS for 24hrs. Depending on the blood donor, the increase ranges from 1.4 to 5 times the expression when stimulated with IFN-α compared to the expression of the control macrophages. When stimulated with LPS, the expression of APOBEC3G reached up to 1.5 fold that of the unstimulated macrophages (see figure 17 A). The IFN-α response was also shown to be concentration dependant. When the macrophages were stimulated with an increasing amount of IFN-α: 30, 50, and 100 U/ml, the expression of APOBEC3G also increased to five-, fourteen-, and nine-fold respectively in donor #4 and three-, four- and seven-fold in donor #5 (see figure17 B). These results show that APOBEC3G expression is induced in both IFN-α and LPS stimulated macrophages. The upregulation shown in the IFN-α stimulated macrophages is concentration dependant.

4.2 The stimulated form of APOBEC3G is active

It is known that APOBEC3G can cause mutations in the HIV-1 genome, and that the protein is normally blocked by the viral protein Vif. To be certain that the interferon induced APOBEC3G was also active in the cells more testing was necessary. One way of analyzing the activity of APOBEC3G in cells is to analyze the G→A hypermutations caused by APOBEC3G in HIV-1. The G→A mutations should be clearly seen in a Vif defective virus. To test this, macrophages were infected with either clonal HIV-1 (ADA8) virus or HIV-1 (ADA8)dVif virus. The concentrated supernatants were then used to infect 293T CD4/CCR5 indicator cells. After 5 hrs of infection, the DNA from the cells was isolated and the V3 region of the HIV-1 Env gene was amplified and cloned into pCR2.1 vector where it could then be analyzed by sequencing. Twenty clones of each type were used for sequence analysis and the percentage of mutations found in a certain base pair was recorded.

As shown in figure 18, the amount of mutations found in the HIV-1 (ADA8) genome is minimal or zero (figure 18A), however the mutations clearly increase in the genome of the Vif defective HIV-1. The percentage of mutations increases from near 0% up to 2 or 3% depending on the blood donor and the stimulant added to the medium. These results suggest that the mutations are made by an active APOBEC3G protein in the cells. Supporting this hypothesis is the observation that the mutations also increase with the addition of the stimulants to almost the same extent as the corresponding donor shows an increase of APOBEC3G in the cells stimulated with IFN-α or LPS (see figure 17). For example, donor #1 shows an increase in APOBEC3G expression in respect to the unstimulated cells of 1.2x for IFN-α stimulated cells or 2.5x for LPS stimulated cells. This shows a certain degree of concurrence to the mutation rate which was respectively 2.34x and 7x the amount of mutation of the viruses from the unstimulated cells. The same general concurrence was found in both other donors: donor #2 shows a reduction in APOBEC3G expression to 0.98x in IFN-α stimulated cells and 1x in LPS stimulated cells. Mutations also decreased respectively to 0.22x or 0.75x in comparison with the control and donor #3's

APOBEC3G expression increased to 2.6x and1.3x while the mutations also increased to 1.8x and 1.55x more than in the control sequences.

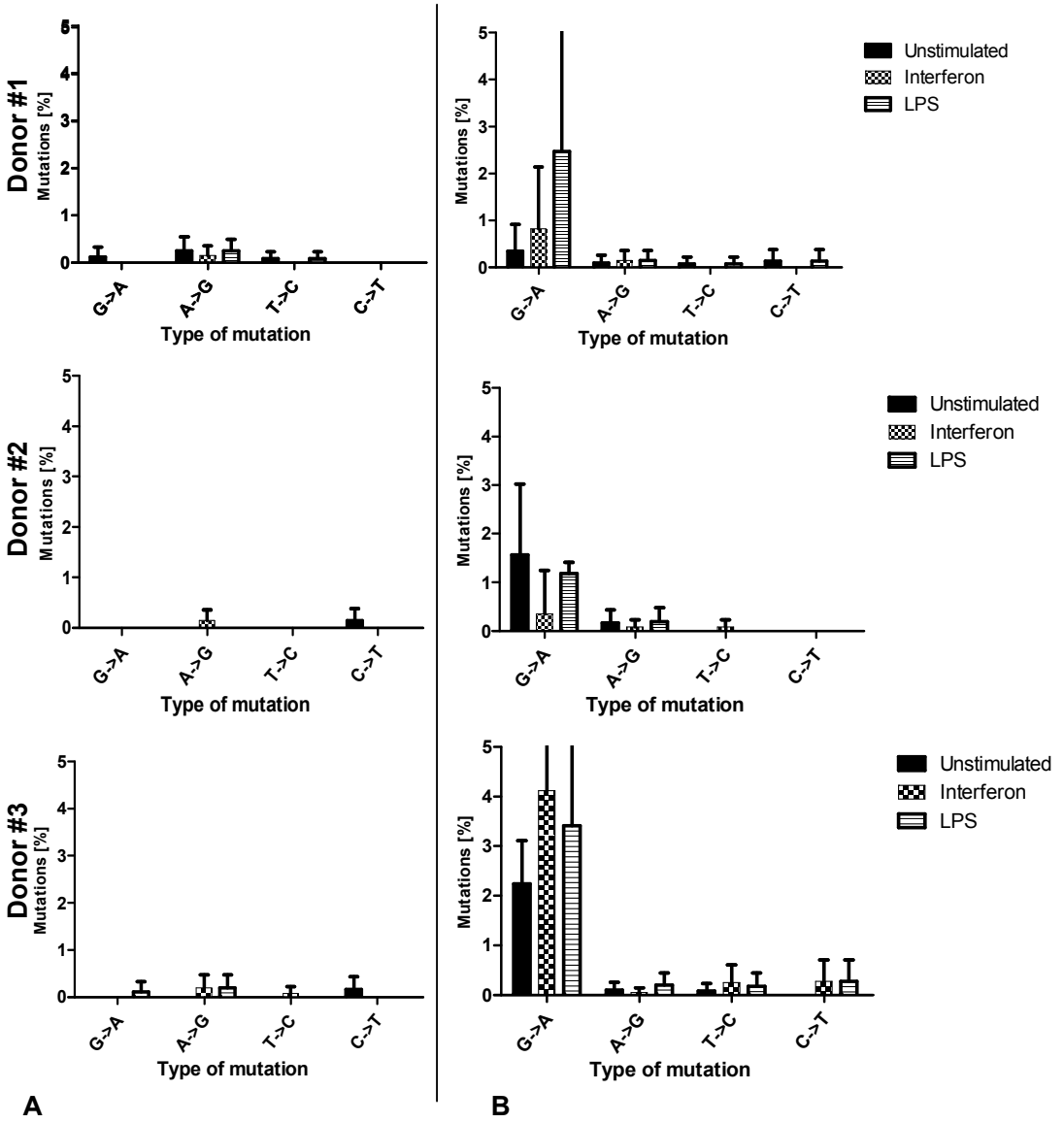


Figure 18. <u>Transition mutations in the HIV Genome.</u> The percentage of transition mutations found in the HIV-1 genome produced in macrophages of 3 different blood donors varied with donor, type of stimulant, and virus. (A) The HIV-1(AD8) viruses did not have as many mutated clones as the (B) HIV-1 (AD8)dVif viruses.

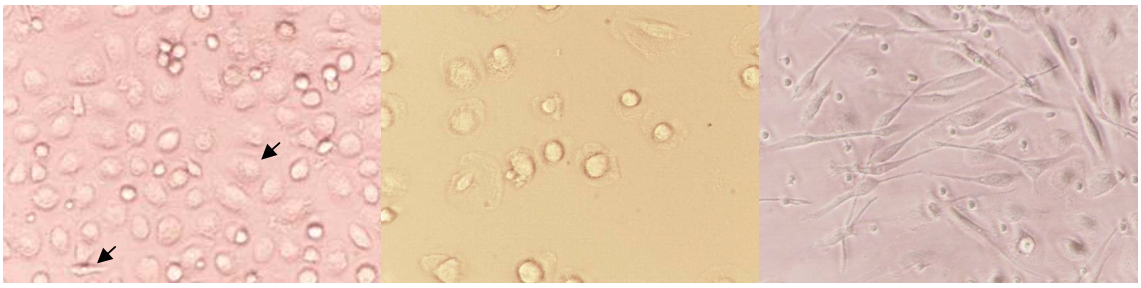
4.3 Monocyte differentiation has an effect on the APOBEC3G expression

Laboratories use an array of different methods to differentiate monocytes into macrophages in addition, monocytes can also be differentiated into DCs (Gluckman et al. 1997; O'Doherty

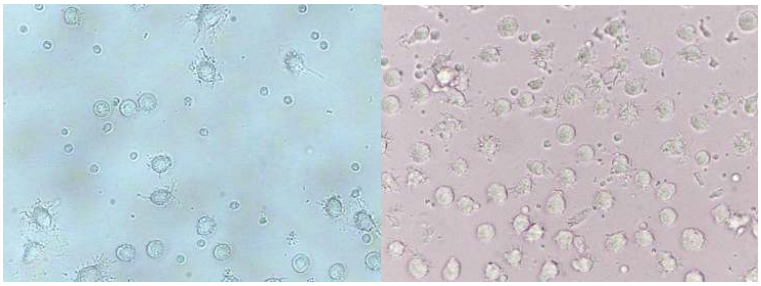
et al. 1997; Mallon et al. 1999). The expression of genes in these cells is very different. The results of the tests done with the macrophages could possibly differ depending on their cultivation method. When macrophages are differentiated with AB serum, there are both round and elongated cells, however, when differentiated with GM-CSF or M-CSF, the macrophages are, for the most part, either round or elongated (De Nichilo and Burns 1993; Lemaire et al. 1996) (see figure 19).

In this experiment the goal was to test if the different forms of differentiation had an effect on the cell type, phenotype, receptors, and gene expression. A main concern was a DC contamination in the macrophages differentiated with AB serum due to the fact that the cells did not have identical morphology. Samples were also received samples from another lab where the results from the expression analysis of APOBEC3G differed from the previous results. To test the phenotypes of the differentiations, monocytes were isolated out of healthy blood donors and differentiated in 5 different ways. The AB macrophages were differentiated for 7 days in a Teflon bag with the AB macrophage medium, the M-CSF macrophages were differentiated in a Teflon bag with M-CSF macrophage medium, and the GM-CSF macrophages were also differentiated in a Teflon bag with GM-CSF macrophage medium. After the 7 days of differentiation, the macrophages were counted and plated in 24 well Costar cell culture plates, with a cell count of $4x10^5$ cells per well.

The immature DCs were differentiated as described in the methods section 3.1.3 and the mature DCs were differentiated from the immature DCs by adding 100 ng/ml LPS to the culture. The cells were characterized by FACS staining for specific cellular markers. The results in figure 19 show that all of means of differentiation produced macrophages and DCs which displayed the correct markers for their cellular type. The one noticeable difference in surface proteins is that a small amount of the GM-CSF macrophages also have the CD1a receptor which is normally found on DCs (Patterson et al. 1999; Robinson et al. 1999). The phenotype shown is what was expected: there were both round and elongated cells found in the AB macrophages. Arrows in the picture of the AB macrophages (figure 19) show the elongated and round type of macrophages (Wijffels et al. 1993; Yu et al. 2008). The GM-CSF macrophages were primarily elongated macrophages and the M-CSF macrophages were generally round (De Nichilo and Burns 1993; Wijffels et al. 1993; Lemaire et al. 1996). Both the mature and immature DCs were suspension cells, unlike the macrophages, which were very adhesive to the surface of the culture plates and had dendritic-like elongations. The immature DCs had no macrophage markers on their cellular surface and had a high amount of the CD1a receptor on the surface, showing that they did indeed differentiate to DCs. The mature DCs had reduced CD1a receptor expression and an increase in CD83 receptors indicating the differentiation was successful (Santin et al. 1999).



	AB Macrophages	M-CSF Macrophages	GM-CSF Macrophages
CD14	++	++	++
CD64	++	++	++
CD1a	-	-	+
CD83	-	-	-



	Immature DCs	Mature DCs
CD14	-	-
CD64	-	-
CD1a	++	+
CD83	+	++

Figure 19. <u>Differentiation of monocytes.</u> Monocytes from one blood donor were differentiated in 5 different ways to macrophages and to DCs. The cells were stained with differentiation markers and analyzed by FACS. All three macrophages had a high expression of CD14 and CD64 on the cell surface; however the phenotype was a little different. The macrophages differentiated with M-CSF were more round and those differentiated with GM-CSF were more elongated. The dendritic cells were in suspension and had dendritic arms which were more pronounced when matured.

In order to see if there was an effect in the APOBEC3G expression when the cells were differentiated differently, the expression of APOBEC3G in these cells was examined. The AB macrophages, the GM-CSF macrophages, and the dendritic cells were stimulated 24 hrs after plating with either IFN-α or LPS. After 24 and 48 hrs the RNA was isolated for further testing. The cDNA was used in an RTq-PCR to analyze the expression of APOBEC3G. Cells were also lysed 24 and 48 hrs after stimulation for Western blot analysis, which was stained with an APOBEC3G antibody.

The results can be seen in figure 20. In comparison to the other forms of differentiation, the macrophages differentiated with AB serum had the lowest expression of APOBEC3G. The macrophages differentiated with GM-CSF had five times as much APOBEC3G mRNA in the

unstimulated macrophages, and they were also more susceptible to LPS stimulation (figure 20 A). The different dendritic cells also showed a higher expression of APOBEC3G. However, the mature DCs were already treated with LPS in order mature them. RTq-PCRs are more sensitive than Western blots and the amount of APOBEC3G protein which is found in the cells or is not degraded before being separated in SDS-page is very low. The APOBEC3G protein was most clearly seen in the GM-CSF macrophages where the upper band in the Western blot (figure 20 B), located at 45 kDa, is the APOBEC3G protein. The Western blot for the AB macrophages and the immature DCs showed much less protein made but the same tendency. The lower band could possibly be degraded APOBEC3G protein.

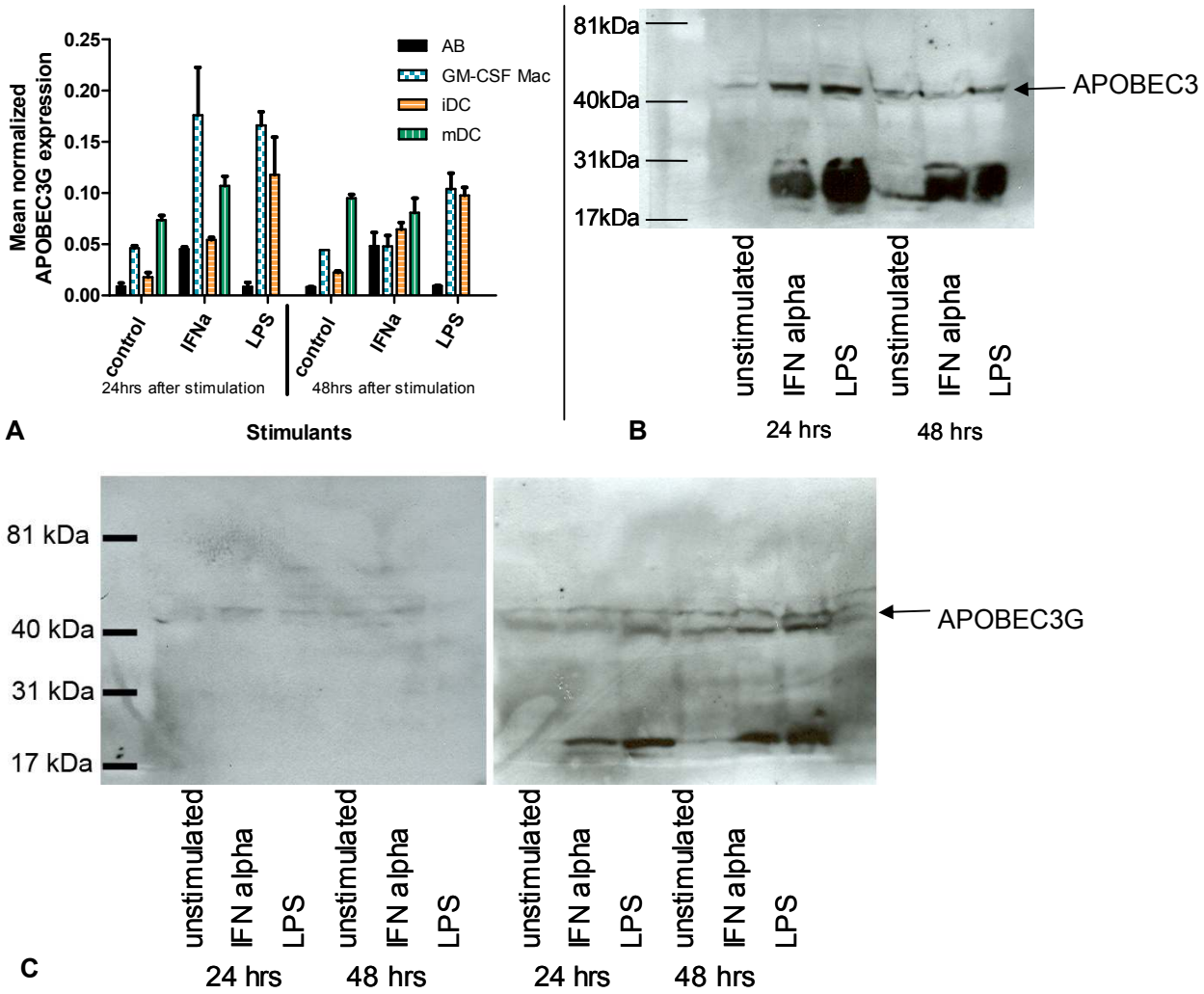


Figure 20. <u>The expression of APOBEC3G in monocytes derived macrophages and DCs.</u> (A) RTq-PCR of APOBEC3G normalized with the EF1 protein in the different cell types shows the highest expression of APOBEC3G in the GM-CSF macrophages. (B) Western blot analysis of the GM-CSF macrophages stimulated with IFN-α or LPS and stained with a APOBEC3G antibody showed a clear upregulation of the protein after stimulation. (C) The Western blot analysis of the AB macrophages (left) and the immature DCs (left) showed much less APOBEC3G protein than in the GM-CSF macrophages.

In order to determine if the increase in APOBEC3G protein also reduced the infectivity of HIV-1 in the GM-CSF macrophages, they were infected and compared to the AB macrophages. The macrophages were infected with 0.005 MOI HIV-1 (ADA8) or HIV-1 (ADA8) dVif virus after 8 hrs of stimulation. Samples for a p24 ELISA were taken from the fresh medium starting at 24 hrs to make sure that there was no contaminating p24 protein from the infection present. Samples were then taken every 2 days after the infection and used in the p24 antigen test. The results can be seen in figure 21. The infection in the AB macrophages (figure 21 A) or the GM-CSF macrophages (figure 21 B) was minimal in the cells stimulated with IFN-α. The infection with the HIV-1(ADA8)dVif virus was lower than that of the HIV-1(ADA8) virus, as expected when infecting macrophages with a defective Vif virus. This effect is much more pronounced in the GM-CSF macrophages which also showed a higher expression of APOBEC3G.

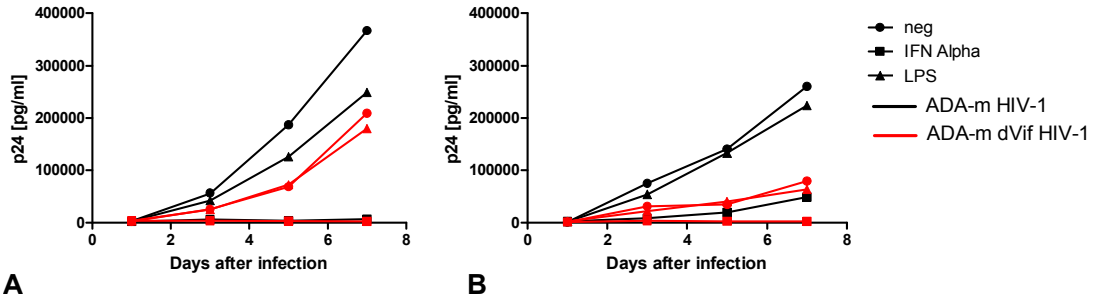


Figure 21. <u>The course of infection in AB macrophages vs. GM-CSF macrophages.</u> The viral infection of the AB and GM-CSF macrophages was followed with p24 antigen tests at different time points in the infection. (A) The infection in AB macrophages with either HIV-1 (ADA8) or HIV-1 (ADA8)dVif and stimulated with IFN-α or LPS. (B) The viral infection in GM-CSF macrophages where the reduction in viral production in the stimulated cells was more pronounced than in the AB macrophages.

4.4 Direct regulation of APOBEC3G in macrophages.

The expression of many genes is altered when cells are stimulated with IFN-α or LPS. For this reason it is difficult to point to only one gene being responsible for reduced infections in the cells. Therefore, the goal was to regulate only the APOBEC3G gene in macrophages to examine the effects on the HIV-1 infection. The experiment would ideally be as follows: macrophages stimulated with IFN-α or macrophages with APOBEC3G under a strong promoter would not be infected easily. The addition of shRNA against APOBEC3G would again increase the infection.

4.4.1 Transduction of macrophages

Macrophages cannot be efficiently transfected for various reasons. The transduction through viral vectors is therefore a better choice, of which only lentiviral vectors are appropriate because of their ability to infect no replicating cells. Multiple vectors were tested for their efficiency of infecting macrophages and the lentiviral vectors packaged with the plasmids:

pCMVΔR8.91, pCMV-G and pHR-SEW were found to be most efficient in transducing the macrophages. Even though these LVVs were the most efficient, the transduction efficiency is very dependant on the donor, as can be seen in figure 22. For this experiment, LVVs containing eGFP (packaged with the plasmids: pCMVΔR8.91, pCMV-G, and pHR-SEW) were titered on Te671 cells and increasing MOIs were used to infect macrophages from three different blood donors. The results show that some blood donors are not transducable with this vector (donor #2), while other donors (donor #1) are highly transducable. From this data it was determined that an MOI of more than 20 does not necessarily produce more transduced cells; therefore, further transductions were done with MOI 20.

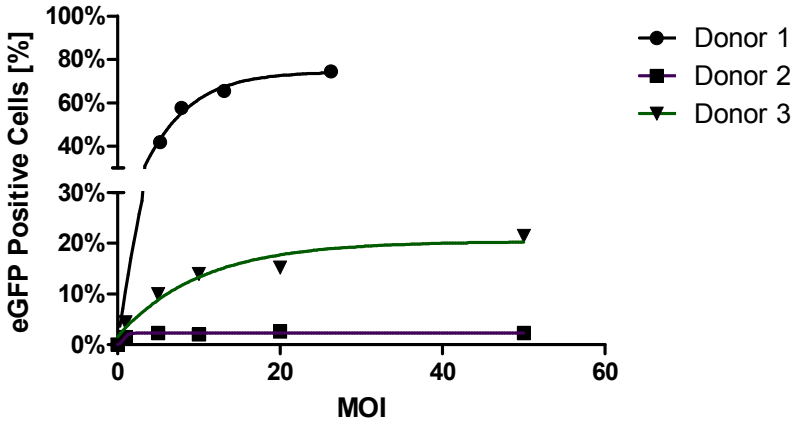


Figure 22. Macrophages transduced with eGFP LLVs. The transduction of macrophages from 3 donors with lentiviral vectors containing eGFP at increasing MOIs showed a very different transduction efficiency for each donor.

4.4.2 Packaging of APOBEC3G

Before transducing cells with the lentiviral vectors, the vectors must be efficiently packaged to produce titers high enough to transduce macrophages. The APOBEC3G gene was cloned into the transfer vector pHR-SIEW (the pHR-SEW plasmid modified to include an IRES in front of the eGFP sequence) in front of the IRES sequence making the vector pHR-SIEW::APO. This plasmid was used with pCMVΔR8.91 and pCMV-G to package lentiviral vectors containing the APOBEC3G gene in HEK-293T cells. The titer of these LVVs were constantly low in comparison to other vectors (see Figure 23 A) as determined on Te671 cells. It was hypothesized that the reason for this extreme decrease in titer was that the exogenous APOBEC3G protein from the transfer vector was packaged into the viral particles and inhibited the transduction of the lentiviral vectors in the target cells. The three vector system used for the packaging of LVVs does not contain HIV-1 accessory proteins such as the Vif protein. This theory was tested by adding a eukaryotic expression plasmid containing humanized Vif protein (pCDNA_hVif) to the packaging of the LVVs to remove the APOBEC3G protein. The results can be seen in the FACS analysis in figure 23 B. Both pictures A and B show the amount of eGFP producing Te671 cells transduced with the LVVs

containing APOBEC3G-IRES-eGFP. Picture A shows a dilution of 1:2000 of concentrated LVV packaged without the Vif protein and picture B shows a 1:2000 dilution packaged with the Vif protein. The titer of the LVV in figure 23 A was $1x10^6$ viral particles/ml and in B it was $7x10^8$ viral particles/ml, which is a 700 fold increase in titer through the addition of the Vif protein during packaging. These results support the hypothesis that the APOBEC3G protein inhibited the LVV transduction. The addition of the Vif protein to the packaging of the viruses allowed us to package the LVVs at a much high titer for further experiments.

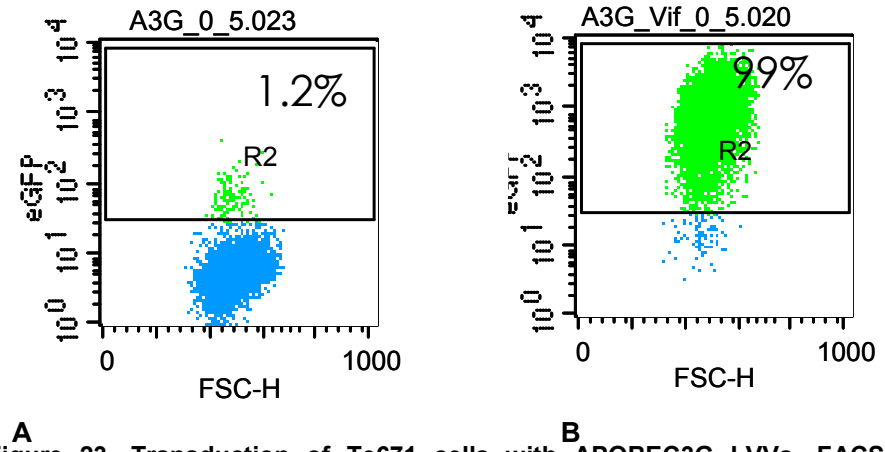


Figure 23. Transduction of Te671 cells with APOBEC3G LVVs. FACS analysis of LVVs containing APOBEC3G packaged in HEK 293T cells (A) without the Vif protein or (B) with the addition of the Vif protein and used to transduce Te671 cells. The addition of the Vif protein in the packaging cell line increased the viral titer 700-fold.

4.4.3 Transduction of APOBEC3G LVVs

To test the APOBEC3G-IRES-eGFP LVVs, two cell lines, HEK 293T and HeLa cells, were transduced with the LVVs at an MOI of 5. These two cell lines have good transduction efficiencies in comparison to other cells and produce no or very low amounts of endogenous APOBEC3G protein, therefore, the increase in protein level should be easily seen. After three days, the cells were lysed and their mRNA was isolated using the RNeasy kit from Qiagen. The mRNA in the samples was set to the same concentration and used in the RT PCR to make cDNA, which was then diluted 1:10 with water. The diluted cDNA was used to determine the expression of APOBEC3G in the cells by means of RTq-PCR using primers against APOBEC3G and EF1 as a normalizing control. The results, shown in figure 24(A), show the large increase in the mRNA production of APOBEC3G. Cells in another well, which were treated the same, were lysed with 3x sample buffer. The samples were loaded onto an SDS-Page gel which was then used for a Western blot. The Western blot was stained with the anti-APOBEC3G C17 antibody and the anti rabbit HRP secondary antibody. The membrane was stripped after staining and restained with anti-GAPDH antibody and the anti-mouse HRP secondary antibody. The results, shown in figure 24 (B), show the increase in

the protein production in both cell lines, meaning that the LVVs were successful in producing the protein in the cells.

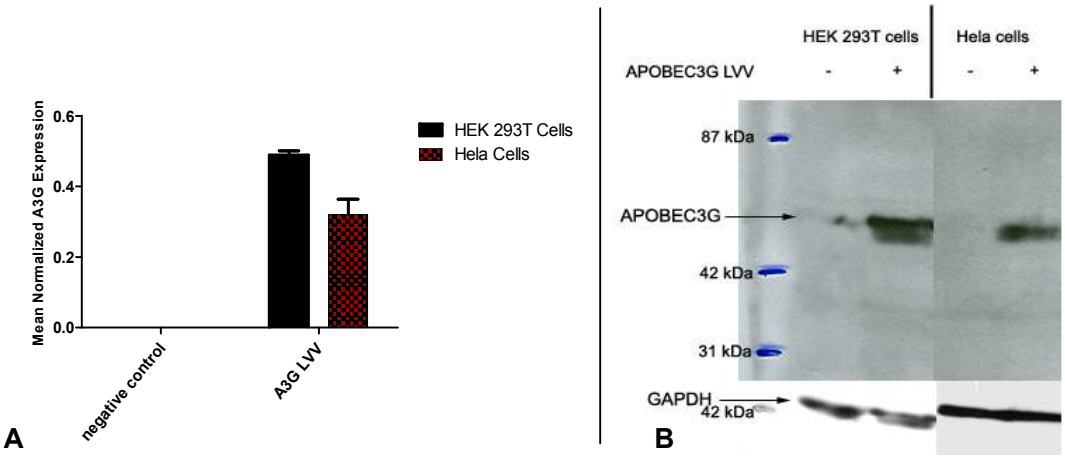


Figure 24. Expression of exogenous APOBEC3G. The cell lines, HEK 293T and HeLa were used to test the APOBEC3G LVVs. The cells were transduced with the vectors and then lysed. A. The RTq-PCR from the cell lines showing the increase in APOBEC3G mRNA. B. The Western blot was stained with the A3G C17 antibody, stripped and stained a second time with a GAPDH antibody as load control. The increase in the APOBEC3G protein is very clear.

The A3G lentiviral vectors were then used to transduce macrophages. When the macrophages were transduced with the vectors, the cells became rounded and very granulated. After a maximum of 48 hrs, the cells were no longer fit enough to be used in further experiments. The reason for this could possibly be related to the non-replicating state of macrophages. To be certain that the cells were dying and not only changing morphology due to the protein, they were stained with an Annexin-V antibody to determine if they were undergoing apoptosis after transduction with either LVVs containing only eGFP or containing APOBEC3G-IRES-eGPF. The amount of Annexin-V on the surface of the cells transduced with the LVVs containing APOBEC3G is almost 3 times that of in the cells containing only eGFP (see table 2).

	Annexin-V
eGFP lentiviral vector (pSEW)	12.0%
APOBEC3G-IRES-eGFP LVV (pSIEW::A3G)	44.7%

Table 2. Annexin-V expression. The percentage of macrophages expressing Annexin-V on their surface after transduction with lentiviral vectors containing either APOBEC3G or eGFP as determined by FACS analysis. The amount of Annexin-V was higher on the cells transduced with vectors containing APOBEC3G.

4.4.4 Downregulation of APOBEC3G via LVV containing shRNA

In order to fully determine if APOBEC3G was responsible for the noninfectious state of macrophages stimulated with IFN-α, the APOBEC3G gene needs to be knocked down. The sequences for three different shRNAs were taken from a paper by Pion et al. (Pion et al. 2006) and tested in the lentiviral vector system used. To test the shRNA LVVs, cells lines were used that had high transduction efficiencies, in this case 293T cells and HeLa cells. Neither cell line produces endogenous APOBEC3G. In order to increase the APOBEC3G production, both cell lines were transduced with LVV containing A3G-IRES-eGFP at an MOI of 4. The cells were then transduced with the control vectors containing scramble shRNA or a combination of the three shRNAs (MOI of 5 for each vector). The cDNA was then used in an RTq-PCR to examine the expression of the APOBEC3G gene. The results can be seen in figure 25. They show that whereas the scramble shRNA at different MOIs showed no effect, the effect of the A3G shRNAs drastically reduced the expression of the APOBEC3G mRNA. The effect of the shRNA A3G3 alone seemed to increase the expression and in combination with the other 2 shRNAs reduced the expression as much as with the combination of shRNA A3G1 and A3G2. Therefore the shRNA A3G3 was left out from further experiments and the combination of the siRNAs A3G1 and A3G2 was used.

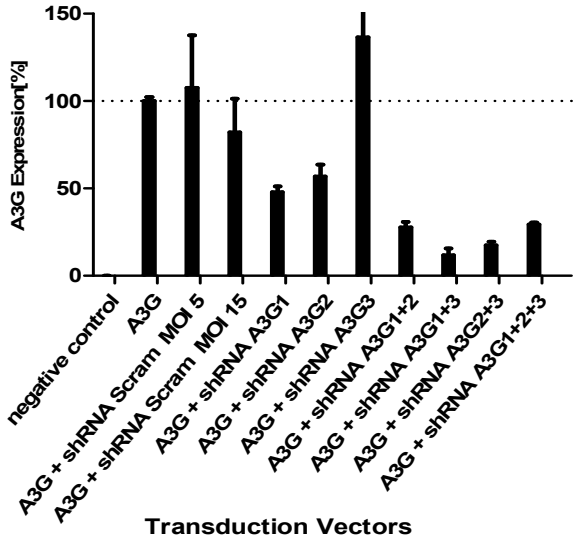


Figure 25. <u>APOBEC3G expression in 293T cells transduced with shRNA.</u> HEK-293T cells were transduced with APOBEC3G LVVs followed by LVVs containing shRNAs either against APOBEC3G or a scramble shRNA.

This test was repeated in HELA cells and 293T cells with MOIs of 4 for each vector to verify these results. In addition to the mRNA expression (figure 26 A), the protein expression was examined by Western blot analysis (figure 26 B). This shows a downregulation of approximately 50% of the mRNA production. The Western blot was probed with the Anti-ApoC17 antibody, stripped and stained again with GAPDH antibody as a load control. The

Western blot also shows a strong downregulation of the APOBEC3G protein in the cells with the A3G1 and A3G2 shRNAs; however this was not a complete knockout of the expression. Most likely due to the low transduction efficiency of macrophages, the introduction of shRNAs in the macrophages showed no effect on the APOBEC3G expression (data not shown).

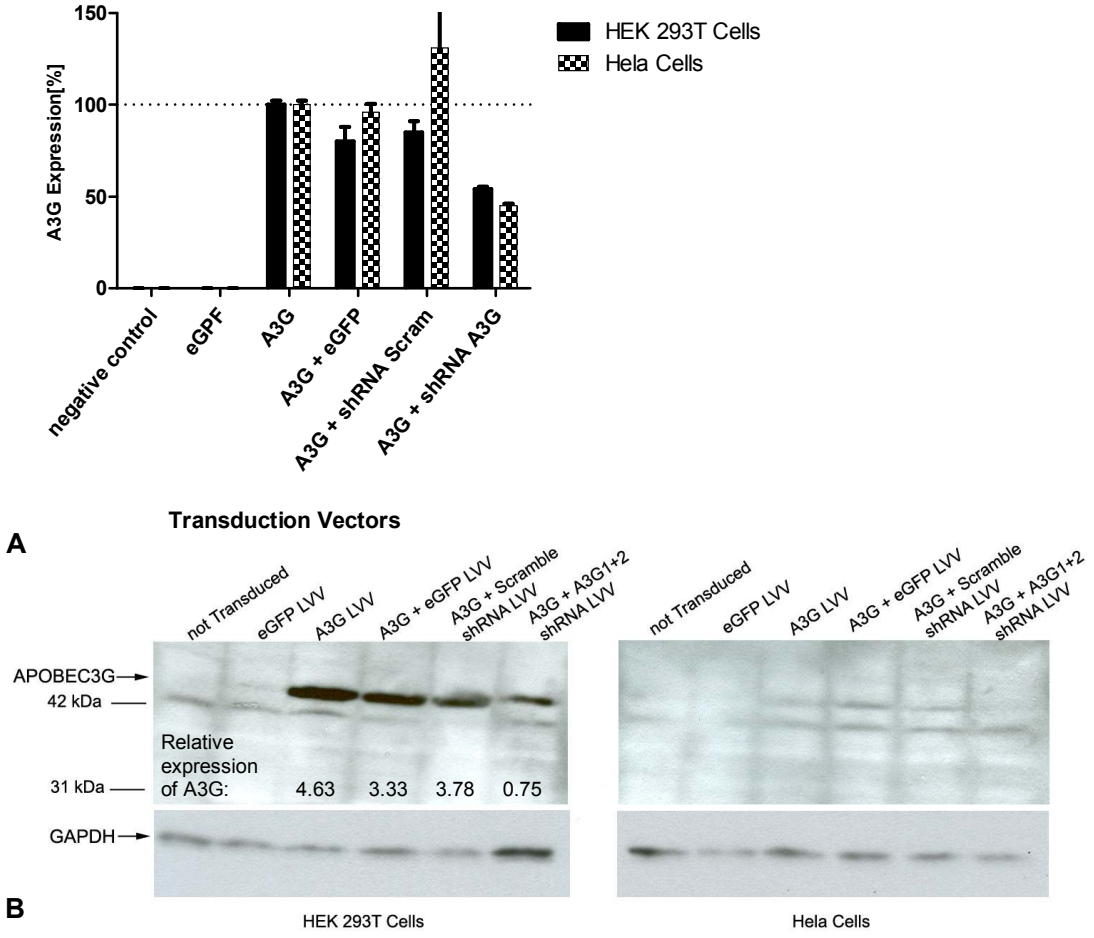


Figure 26. Expression of APOBEC3G in cells containing shRNA. (A) The percent APOBEC3G mRNA expression in HEK 293T and HeLa cells transduced with LVVs containing APOBEC3G followed by shRNA against APOBEC3G or a scramble sequence as determined by RT qPCR. (B) The Western blot showing the protein expression of the cells used to test the shRNA LVVs. The blot from the HEK-293T cells was quantified relative to GAPDH and showed that the shRNA caused an 84% decrease in A3G protein.

4.5 The effect of direct APOBEC3G regulation on the HIV-1 infection of TZM-bl cells.

The previous studies were necessary to test the effect of the lentiviral vectors on the expression of APOBEC3G in the cells. To fully test the lentiviral vectors and their intended affect on the HIV-1 infection, the HIV sensitive cell line, TZM-bl, was transduced with the

vectors and then infected. TZM-bl cells do not produce endogenous APOBEC3G. The cells transduced only with the lentiviral vectors containing APOBEC3G should show a decrease in infectivity. The cells transduced with the LLVs containing shRNA in addition to the vectors containing APOBEC3G should show an increase in infectivity, ideally to the extent of the cells not transduced at all (Sheehy et al. 2002). For this experiment, TZM-bl cells were transduced with LVVs with an MOI of 2 for each LVV containing eGFP, APOBEC3G, scramble shRNA or 2 different shRNA against APOBEC3G (siA3G1 and siA3G2). Following the transductions, the cells were infected with 0.005 MOI of HIV-1 (AD8)dVif virus. After three days of incubation, the cells were lysed and the mRNA expression of APOBEC3G was analyzed via RT qPCR. The results (figure 27 A) show that the lentiviral vectors had the intended effect on the expression. The cells not transduced with LVVs containing APOBEC3G produced no APOBEC3G. The cells transduced with the LVVs containing APOBEC3G expressed high levels of that protein, which decreased when the cells were transduced additionally with LLV containing shRNA against APOBEC3G. The infection was also followed by means of p24 expression in the supernatant. The infection of the cells transduced with the APOBEC3G LVVs decreased in comparison to the cells which were untransduced (figure 27 B). The cells which were cotransduced with LVVs containing APOBEC3G and shRNA against APOBEC3G showed a recovery of the infection. These results had been expected due to the results from the regulation of the APOBEC3G.

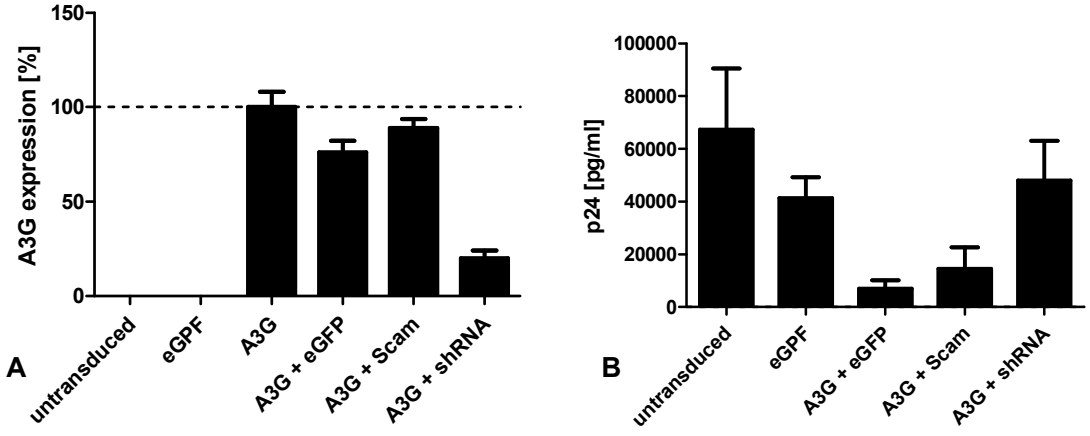


Figure 27. <u>The expression of A3G and the infection in transduced TZM-bl cells.</u> (A) The mRNA expression of APOBEC3G after transduction of the cells. The expression from the APOBEC3G LVVs was reduced in the cotransduction with shRNA against APOBEC3G. (B) The p24 expression in the TZM-bl cells infected with dVif HIV-1. The downregulation of the infection by A3G was rescued by the cotransduction with a LVV containing shRNA against APOBEC3G.

4.6 CD34+ Cells

Due to the limitation from the macrophages, CD34+ cells were used in the following experiments. CD34+ cells are hematopoietic stem cells which can multiply in addition to

being differentiated into different cells of the immune system such as macrophages and dendritic cells. They can also be rather easily transduced in culture. CD34+ cells were transduced and differentiated into MO, as CD34+ cells are more easily transducable than mature MO.

4.6.1 Differentiation of CD34+ cells into macrophages

The differentiation of CD34+ cells into macrophages has been documented before. For these experiments the cultivation method published in Liu et al was used (Liu et al. 2002) to rapidly proliferate the CD34+ cells. This included keeping them in CD34 proliferation medium for one to two weeks, until they reached the desired cell count. After there were enough cells to complete the experiments, the cells were centrifuged and washed with PBS once, then further cultivated in CD34 pre-differentiation medium for 4 days. Half of these CD34+ cells (figure 28 A) were used for further experiments and the rest were differentiated to macrophages. After differentiation to macrophages, most of the cells phenotypically resembled macrophages (see figure 28 B).

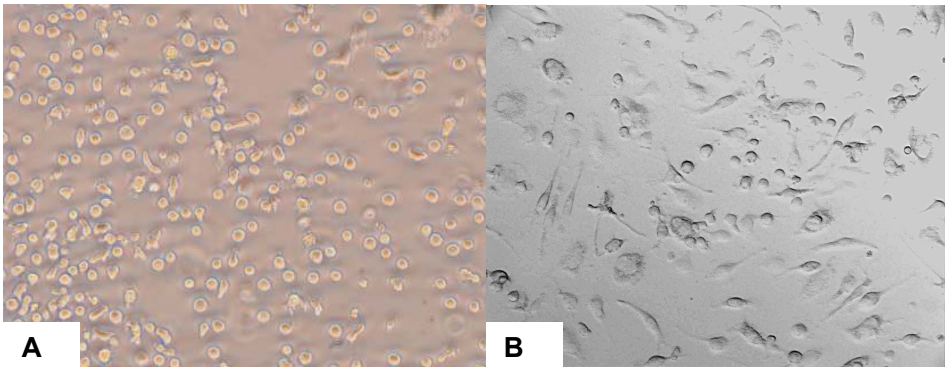


Figure 28. Differentiation of CD34+ cells into Macrophages. (A) The CD34+ cells after 1 week of culture. (B) The CD34+ derived macrophages after being differentiated.

During these weeks of proliferation and differentiation the cells were stained and analyzed by FACS analysis at different times depending on the step of differentiation and the cell count. The cells were stained with an array of conjugated antibodies against cells surface markers to more accurately determine the phenotype. Of the freshly thawed cells, 90-100% were CD34 positive (see table 3). The cells stayed positive for at least a week after cultivation in proliferation medium. After switching to pre-differentiation medium, the amount of CD34+ cells was reduced to about 35%, and after differentiation, the macrophages had less than 2% cells with CD34 marking and 6% CD4 positive cells, which is normal for differentiated macrophages (Kazazi et al. 1989; von Laer et al. 1990; Ruiz et al. 1998). The cultivation of the CD34+ cells under these conditions had a variety of different cellular markers while continuing to proliferate clonally, including an increase of CD14 from 5% to 65% after a few weeks as well as an increase of the CD4 marker from under 10% to 40%

(Muench et al. 1994; Ruiz et al. 1998). After one week of cultivation there were approximately 65% CD14+ cells and 70% CD64+ cells. These were the expected results as the cells differentiated into macrophages.

Antibody against	1st day in culture	After 1 week in proliferation medium	Pre-differentiation medium	Macrophages medium
CD34	> 90%	> 90%	35%	< 2%
CD14	5%	65%	65%	35%
CD64	3%	70%	80%	55%
CD4	< 10%	40%	40%	6%
CCR5	< 5%	35%	45%	20%

Table 3. Characterization of CD34+ cells differentiation to macrophages. The results from FACS analysis at different stages of CD34+ cell cultivation and differentiation showed a high amount of CD34 surface protein at the beginning which decreased to under 2% after being differentiated.

4.6.2 Transduction of CD34+ Cells

In order to support the theories (if APOBEC3G is expressed in the cells, there a reduction in the infection and if shRNA against APOBEC3G is added the infection should increase), it was necessary to transduce CD34+ cells. CD34+ cells are rather easily transduced with lentiviral vectors (Uchida et al. 1998) (Demaison et al. 2002). To assure the continued expression of the genes in the cells, the eGFP expression was monitored after transduction. The CD34+ cells were transduced with 4 different LVVs (containing eGFP in addition to scramble shRNA, APOBEC3G shRNA, A3G or only eGFP) 24 hrs after taking them into culture at an MOI of 10. The eGFP expression was monitored via FACS during the cultivation of the cells. The results can be seen in figure 29, where the percentage of the cells expressing eGFP rapidly decreased after transduction. The eGFP expression in the cells from the different donors decreased to different extents, some starting with a transduction efficiency of 60% reduced down to 20% or lower, some decreasing to 60% from 80%.

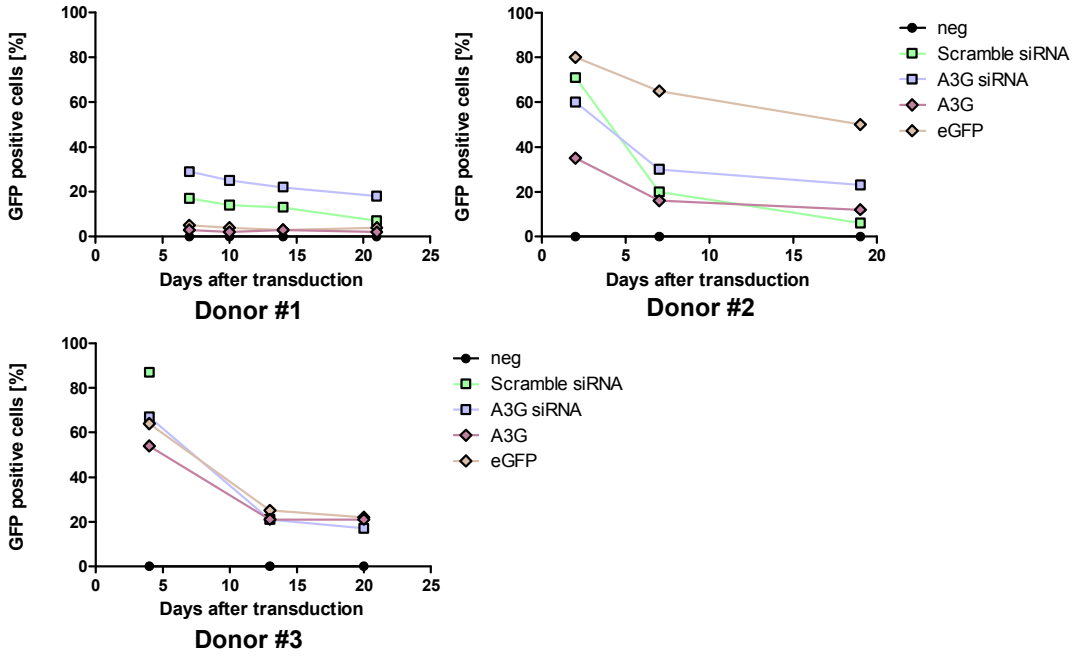


Figure 29. The percentage expression of eGFP in CD34+ cells transduced with LVVs containing eGFP over time. The amount of positive cells drops rapidly in each of the donors.

To analyze the effect of the lentiviral vectors on the cells, CD34+ cells or CD34+ derived macrophages were plated and stimulated. The cells transduced with LVVs containing shRNA against APOBEC3G should show a downregulation of APOBEC3G and those transduced with APOBEC3G LVVs should show an upregulation of APOBEC3G, while the controls should show no change.

The results can be seen in figures 30 and 31. In all three donors the cells transduced with the LVV containing APOBEC3G also showed an increase in APOBEC3G expression whereas the eGFP transduced cells showed no clear effect. The cells transduced with scramble shRNA showed a slight upregulation of APOBEC3G, but the cells with the shRNA against APOBEC3G showed almost no effect on the APOBEC3G expression.

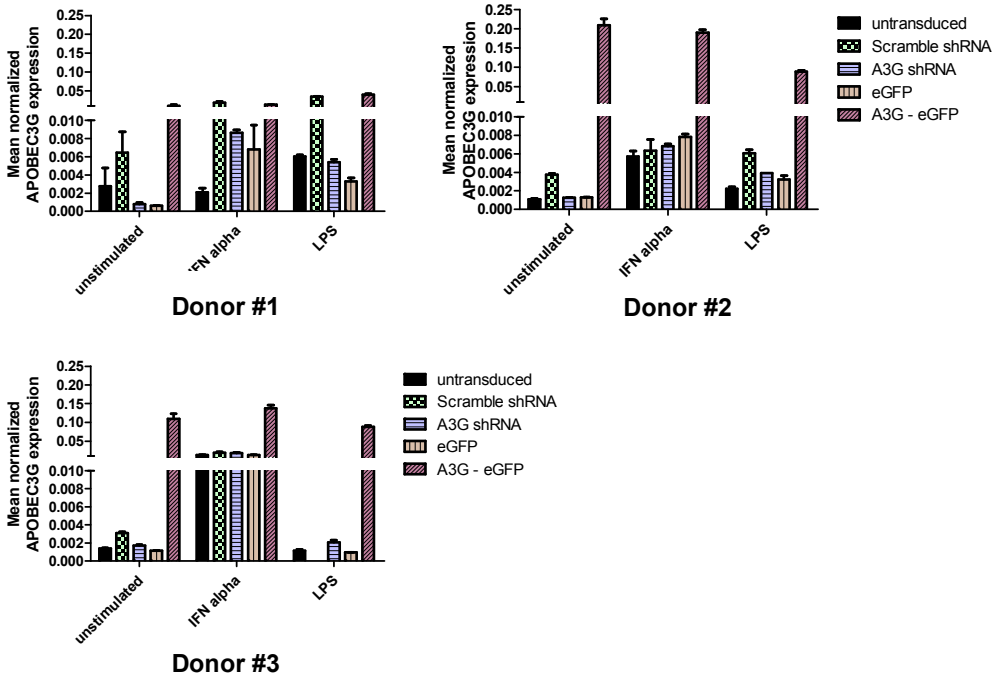


Figure 30. The expression of APOBEC3G mRNA in CD34+ cells after 24 hours of stimulation. The CD34+ cells showed an increase in APOBEC3G expression in IFN-α stimulated cells. The cells transduced with A3G-eGFP lentiviral vectors also showed a very high increase in APOBEC3G. The shRNA did not have any major effect on the expression.

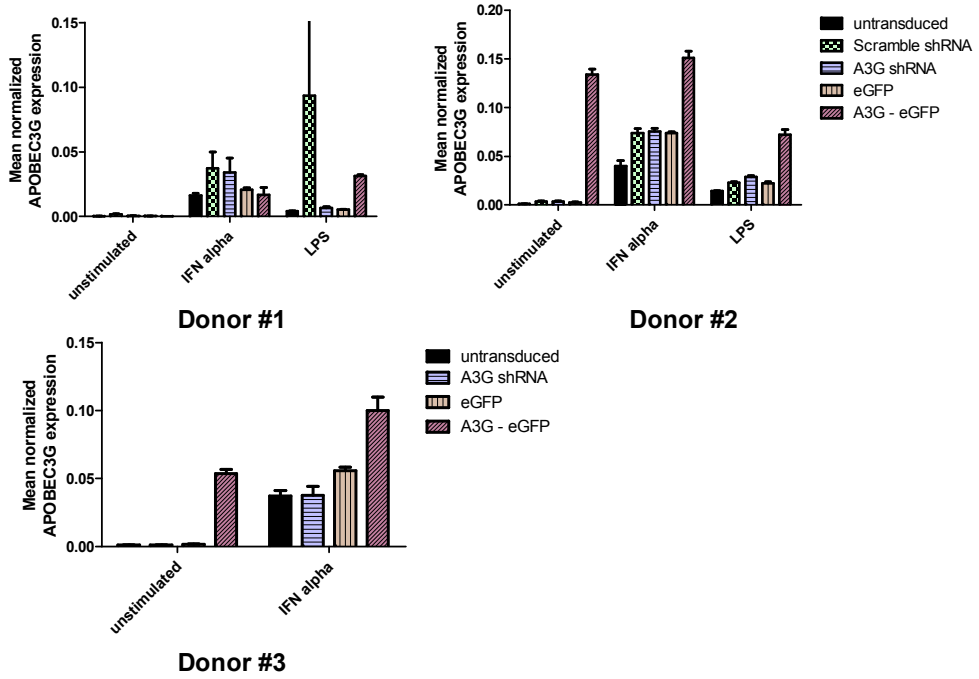


Figure 31. The expression of APOBEC3G mRNA in CD34 derived macrophages after 24 hrs of stimulation. The CD34+ derived Macs showed an increase in APOBEC3G expression in IFN-α stimulated cells. The cells transduced with A3G-eGFP lentiviral vectors also showed a very high increase in APOBEC3G. As in the CD34+ cells, the shRNA did not have any major effect on the expression of APOBEC3G.

4.6.3 Is IFN-α upregulated in the LVV transduced cells?

As seen in the previous experiments, IFN-α induces the expression of APOBEC3G. There have also been some papers showing that short RNAs can induce IFN-α in cells (Reynolds et al. 2006; Schlee et al. 2006; Zamanian-Daryoush et al. 2008). To determine if this is what was happening, the IFN-α expression was indirectly analyzed in the cells. To determine if the upregulation of the APOBEC3G in the cells transduced with shRNA was possibly induced by IFN-α, the expression of the known IFN-α induced protein, OAS1, was analyzed with RT qPCR.

For these experiments, the same cDNA used in the previous section was used, and primers against OAS1 were used and normalized with EF1 primers. The results can be seen in figures 33 and 34. The first donor showed a very strong induction of IFN-α in the cells transduced with scramble siRNA (siScram1) sequence which was marginally significant ($P_{two\text{-}tailed} \leq 0.05$), therefore the scramble sequence was changed. The new scramble shRNA (shScram2) sequence was used in the transduction of the following 2 CD34 donors. The new scramble shRNA did not show a significant upregulation of the IFN-α induced proteins ($P_{two\text{-}tailed} \geq 0.05$) or APOBEC3G ($P_{two\text{-}tailed} \geq 0.05$) (see figures 30 and 31). The other vectors showed no significant effect on the IFN-α expression ($P_{two\text{-}tailed} \geq 0.05$).

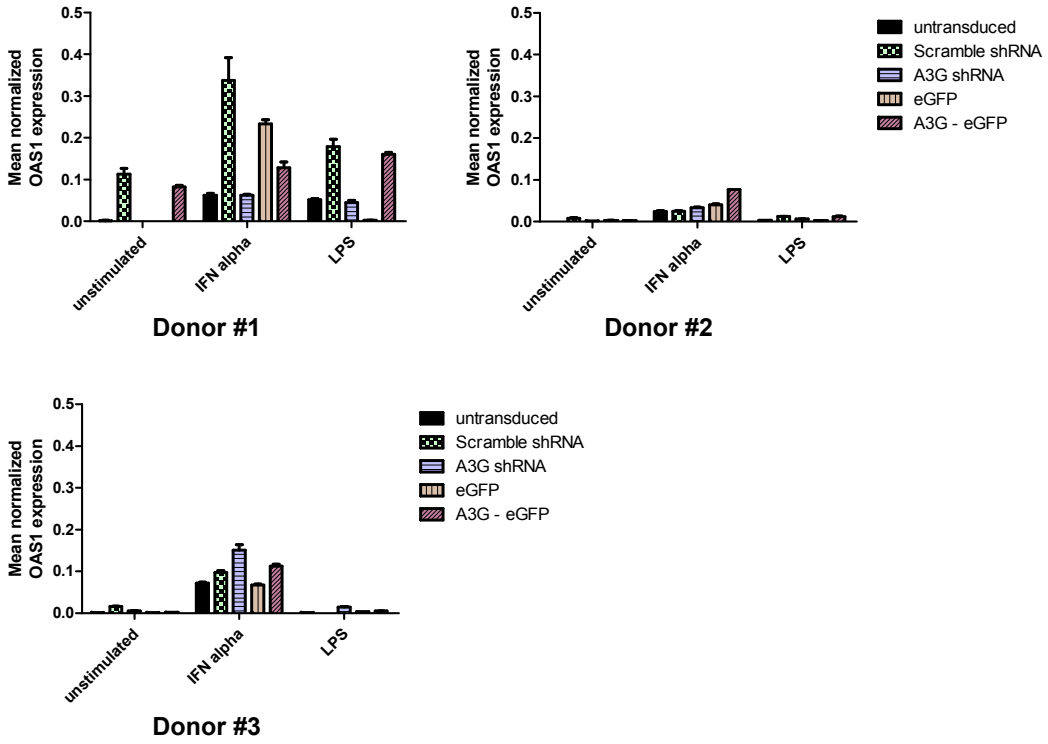


Figure 32. <u>The expression of the IFN-α regulated protein OAS1 in CD34+ cells.</u> The cells transduced with the siScam1 sequence in donor #1 had an increase in OAS1 protein, which is an indication for IFN-α stimulation. The cells from the other 2 donors were transduced with a different scramble shRNA sequence (siScram2).

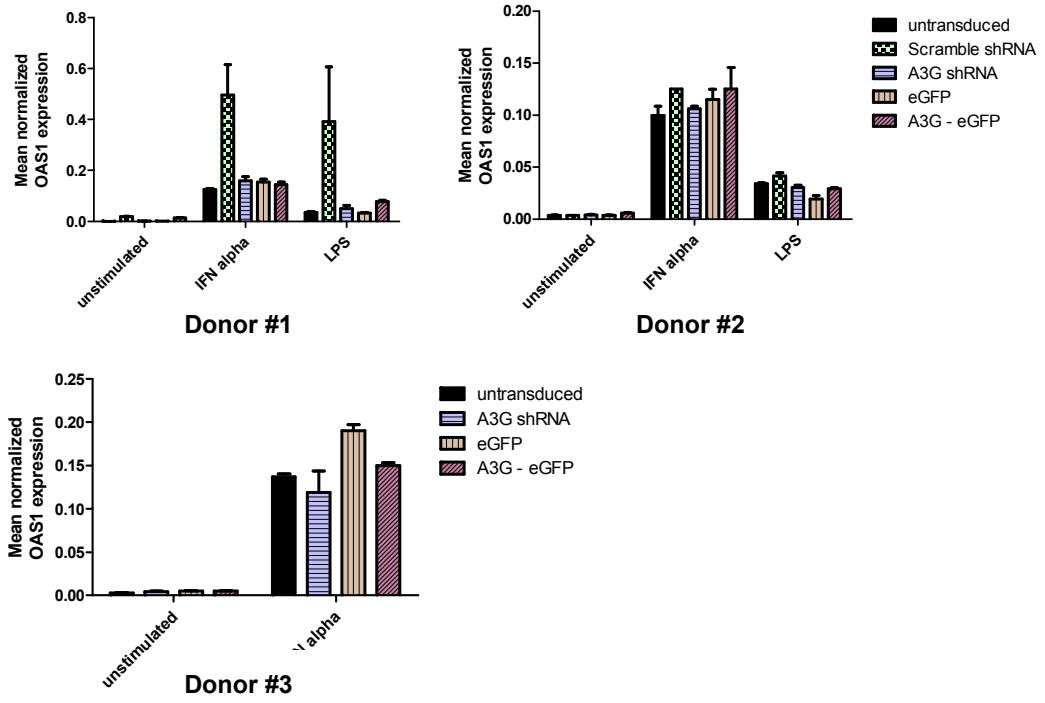


Figure 33. The expression of the IFN-α regulated protein OAS1 in CD34 derived macrophages. As in the CD34+ cells, the macrophages transduced with the scramble shRNA (siScram1) sequence in donor #1 had an increase in OAS1 protein, which is an indication for IFN-α stimulation. The new scramble sequence (siSram2) used in the other 2 donors did not show this effect.

4.6.4 The expression of APOBEC3F

There are many other proteins in the APOBEC family, a few of which, for example APOBEC3F, also have an HIV-1 antiviral effect (Liddament et al. 2004; Zheng et al. 2004; Holmes et al. 2007). APOBEC3F has also been shown to be upregulated when stimulated with IFN-α (Bonvin et al. 2006), therefore the expression of the APOBEC3F mRNA in the cells using the cDNA which was used in the previous tests was tested. The primers were directed at A3F and the results were normalized with the EF1 primers.

The results are shown in figure 34 for the CD34+ cells and figure 35 for the CD34 derived macrophages. The expression of the A3F mRNA also increased with the stimulation of IFN-α. It was not affected by the addition of the shRNA against A3G and only slightly if at all affected by the addition of the A3G gene. This showed that although the protein might be increased in the cells stimulated with IFN-α and could have an effect on the infectivity of the cells in this state; it should not interfere with the direct regulation of the APOBEC3G protein in the cells.

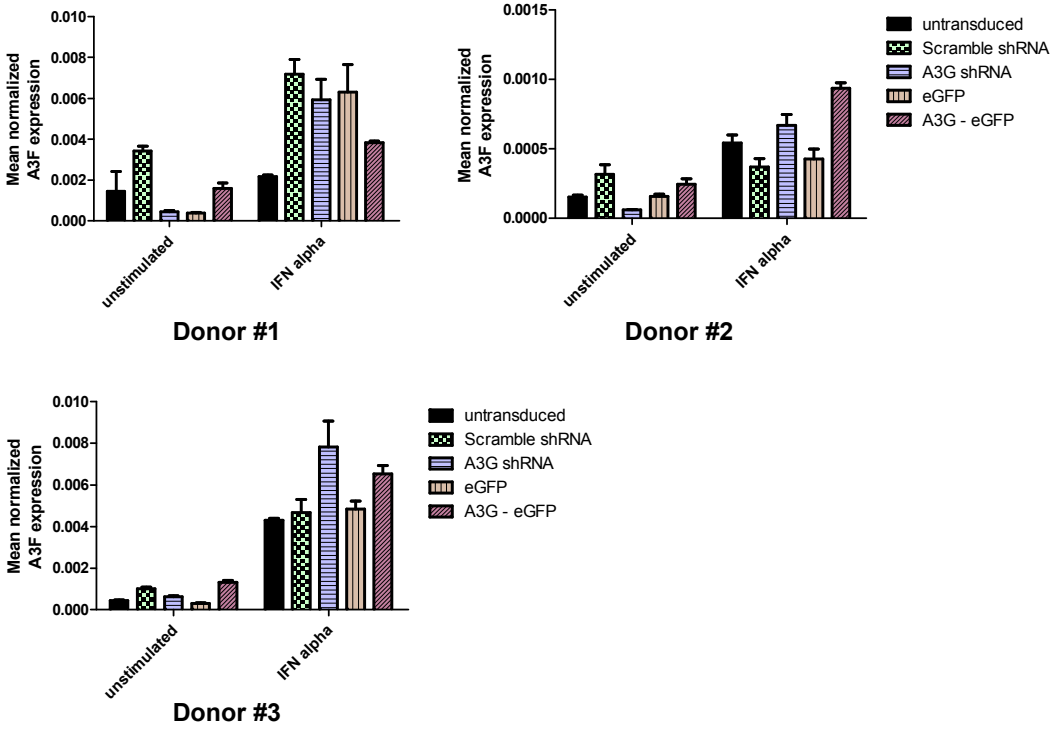


Figure 34. <u>The expression of APOBEC3F in CD34+ cells.</u> The expression of the A3F mRNA was upregulated in the IFN-α stimulated cells; however the LVVs had no noticeable affect on this protein.

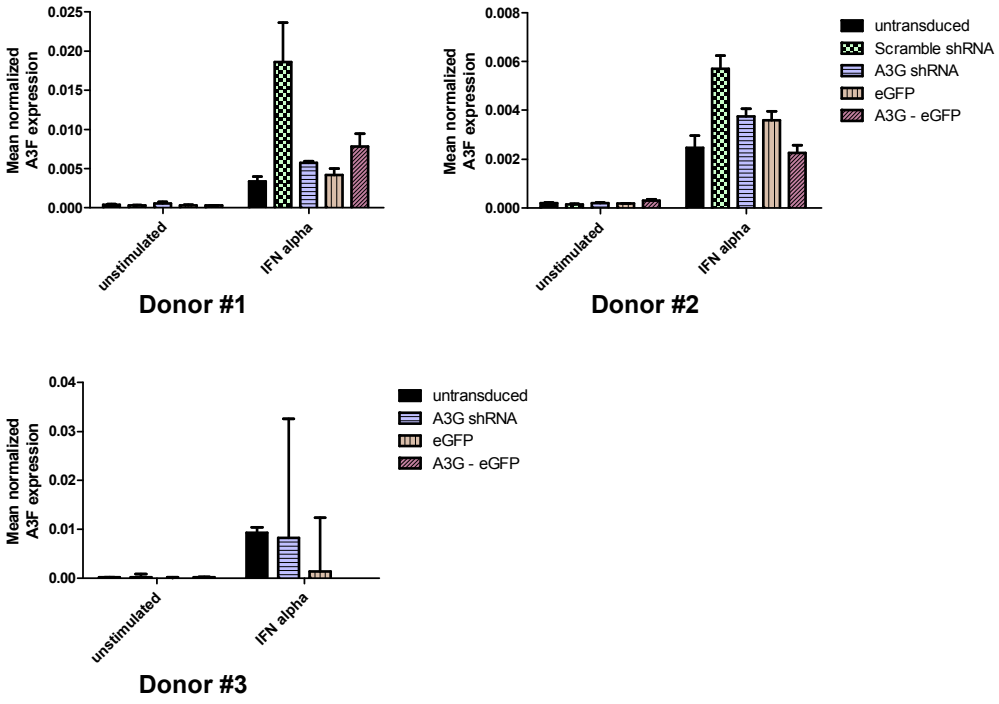


Figure 35. <u>The expression of APOBEC3F in CD34 derived macrophages.</u> The expression of the A3F protein was upregulated after stimulation with IFN-α. However there was no effect from the LVVs transduced into the cells. This shows that the LVVs were specific for their target.

4.6.5 Infection of the CD34+ cells

Although the cells stained for CD34 after cultivation (see table 3), the cells also carried the surface proteins CD4 and CCR5 which made them susceptible to HIV-1 infections in this particular case. The infection of CD34+ cells is somewhat controversial *in vivo*, however the infection *in vitro* has been shown and the HIV-1 receptors on the CD34+ cells were also previously characterized (von Laer et al. 1990; Ruiz et al. 1998; Yurasov et al. 1999; Majka et al. 2000). The effect of the APOBEC3G protein on the infection of CD34+ cells was tested to see if the results showed the same effect as in the macrophages. On the day they were stimulated and lysed for RNA analysis, they were also infected with 0.005 MOI HIV-1 (AD8) or HIV-1 (AD8)dVif virus. After 24 hrs, half of the medium was removed and replaced with fresh medium and p24 samples were taken. Further p24 samples were taken throughout the infection and half of the medium was replaced after the samples were taken. The results shown in figure 36 are the untransduced cells that are representative of the results from all of the CD34+ cells (all results are shown in the appendix). The cells stimulated with IFN-α produced little or no virus while the cells stimulated with LPS produced only a little less than that of the nonstimulated cells. The results from the cells infected with HIV-1(AD8) and HIV-1(AD8)dVif were very similar in this case.

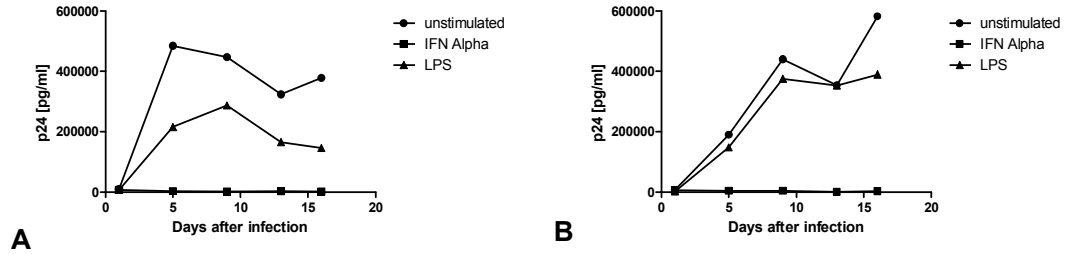


Figure 36. <u>Infection of CD34+ cells.</u> This is a representative example of the p24 expression in CD34+ cells infected with either (A) HIV-1 (AD8) or (B) HIV-1 (AD8) dVif virus. The cells stimulated with IFN-α showed no active infection.

The cells were transduced with 4 different lentiviral vectors after being thawed during the first few days of cultivation. The infection, more so with the HIV-1 (ADA8) dVif virus, should be downregulated in the cells transfected with A3G lentiviral vectors and upregulated in the cells transduced with lentiviral vectors containing shRNA against A3G. The results of the p24 analysis from these experiments were not quite as convincing as had been anticipated (see figure 37). There was no constant trend in the p24 amounts dependant on the different vectors, however some of the results did match what was expected. Notably, the results from donor #1 where the A3G shRNA transduced cells were infected with HIV-1(AD8)dVif virus showed an increase in the HIV-1 infection corresponding to the decrease in APOBEC3G expression in this donor. The effect is also very apparent in the same cells stimulated with IFN-α, which normally shows no infection and in this case the infection was

at the same level as the unstimulated cells (see appendix 7.3.1.1). Donor #3 showed a decrease in infection in the cells transduced with APOBEC3G.

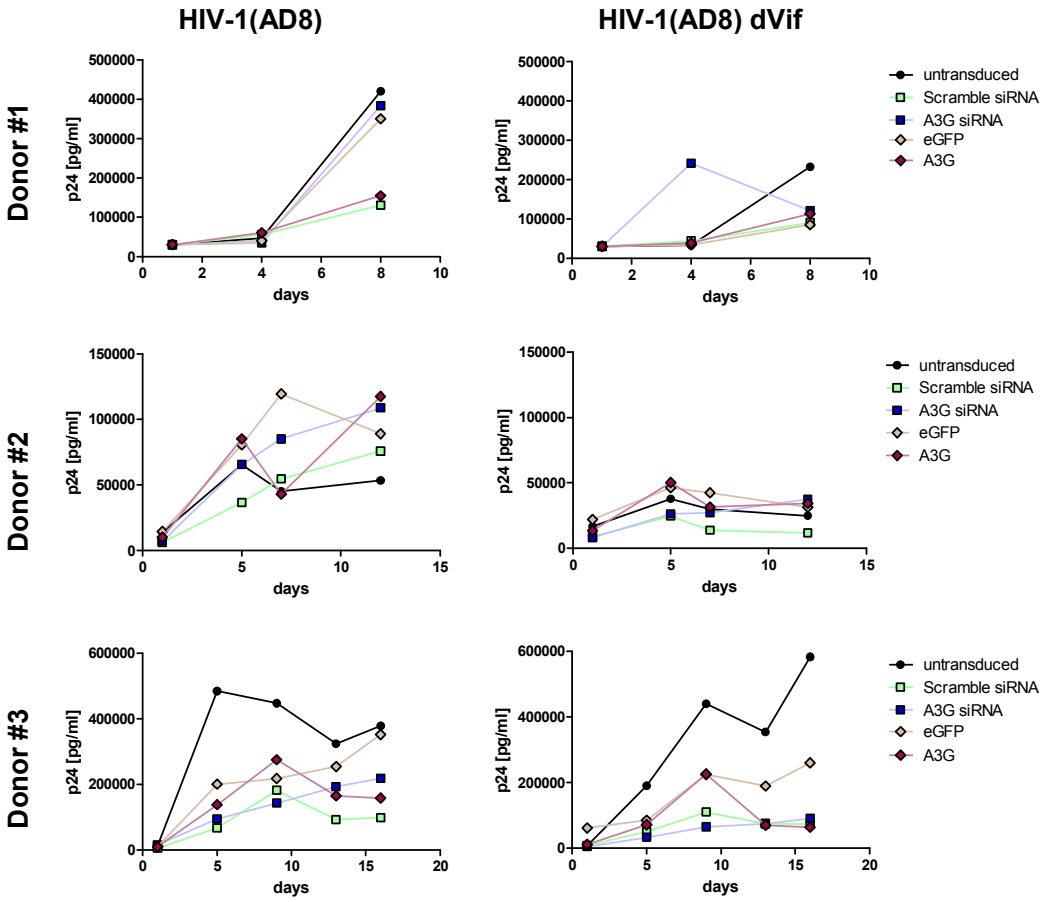


Figure 37. <u>The p24 amount in the supernatant of infected CD34+ cells</u>. The results from 3 different donors transduced with 4 different vectors and infected with either HIV-1 (ADA8) (right) or HIV-1 (ADA8) dVif virus (left).

4.6.6 Infection of the CD34 derived macrophages

To see if the different transduction vectors had the effect on the CD34 derived macrophages, this was also analyzed. The macrophages were derived from the transduced and untransduced CD34+ cells described above. They were then differentiated and characterized (see table 3). After differentiation, the CD34 derived macrophages were stimulated and then infected with an MOI of 0.005 with HIV-1 (ADA8) or HIV-1 (ADA8)dVif virus. The cells were washed once with fresh AB medium 24 hrs after infection and the medium was replaced with fresh AB medium with the perspective stimulants. The infection of the stimulated and unstimulated CD34 derived macrophages looked similar to CD34+ infected cells. However the effect of LPS on the infectivity of the cells was much higher in the macrophages where the p24 amounts were similar to that of the IFN-α stimulated cells. In

comparison, the p24 amounts in the LPS stimulated CD34+ cells were closer to the unstimulated cells (see figures 36 and 38, see also appendix). The infection with the HIV-1 (ADA8)dVif virus was also not as strong in the macrophages as it was in the CD34+ cells.

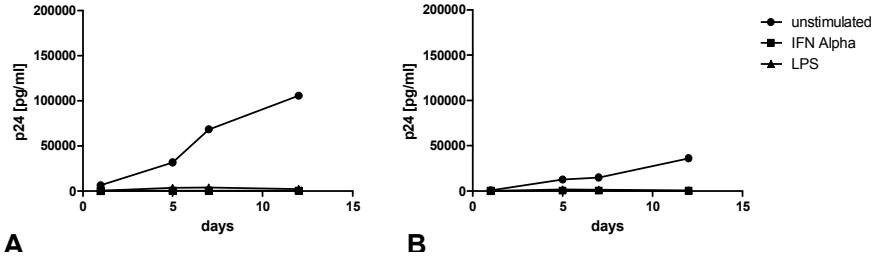


Figure 38. <u>Infection of CD34 derived macrophages.</u> This is a representative example of the p24 expression in CD34 derived macrophages infected with either (A) HIV-1 (AD8) or (B) HIV-1 (AD8) dVif virus. The cells stimulated with IFN-α or LPS showed no active infection and unlike in the CD34+ cells, the infectivity in the HIV-1(AD8) dVif virus was also reduced.

The CD34 derived macrophages were differentiated from the CD34+ cells which were transduced with the different vectors to up- or downregulate APOBEC3G in the cells. The expected results would be that the cells transduced with shRNA against APOBEC3G would show a higher infectivity than the normal macrophages. The cells transduced with the APOBEC3G vector should not be as infective as the other cells. The results should be more pronounced in the cells infected with HIV-1(AD8) dVif virus. The p24 results, shown in figure 39, showed no convincing trend. The results from the 3rd donor were as expected, the cells transduced with APOBEC3G were unable to be infected well as in the CD34+ cells from this donor (see figure 39). This was also the case for the A3G transduced cells from donor #1. The cells which were transduced with shRNA against A3G from donor #3 showed a higher infection, at least in those cells infected with the HIV-1(AD8)dVif virus than the untransduced cells. However, the cells stimulated with IFN-α did not show an increase in infection as it did in the CD34+ cells (see appendix section 7.3.2.3).

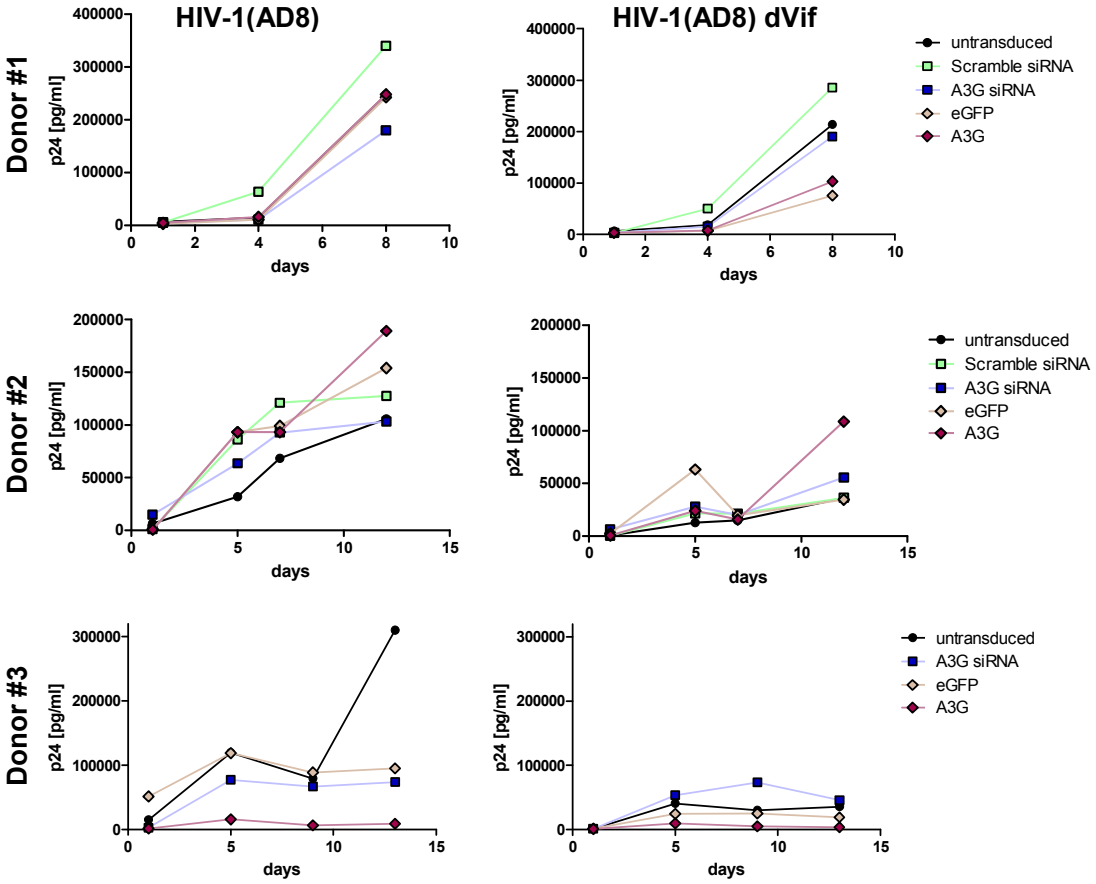


Figure 39. <u>The p24 amount in the supernatant of infected CD34 derived macrophages</u>. The results from 3 different donors transduced with 4 different vectors and infected with either HIV-1 (ADA8) (right) or HIV-1 (ADA8) dVif virus (left).

5 DISCUSSION

The goal of this work was to determine if APOBEC3G was the major factor in the antiviral state of IFN-α or LPS stimulated macrophages. This turned out to be complicated for various reasons including the differences in primary cell donors, external effects on the cells, and difficulty in directly regulating the protein in the cells. Various approaches were taken to circumvent these problems in order to find conclusive answers.

5.1 APOBEC3G in monocyte derived macrophages

In this study it was shown that APOBEC3G mRNA was upregulated in macrophages stimulated with either IFN-α or LPS (figure 17). IFN-α and LPS have been long known to have an antiviral effect on macrophages (Kornbluth et al. 1989). The upregulation of APOBEC3G after stimulation has been recently shown in a few different cells type, including resting CD4+ T-cells, hepatocytes and plasmacytoid DCs (Bonvin et al. 2006; Chen et al. 2006; Peng et al. 2006; Sarkis et al. 2006; Tanaka et al. 2006; Wang et al. 2008). After stimulation with IFN-α, hundreds of wide ranged antiviral genes are activated. In macrophages, IFN-α was found to inhibit provirus formation, integration and induce degradation of the viral RNA (Kornbluth et al. 1989; Pitha 1994) (Woelk et al. 2004). These effects of IFN-α on HIV-1 are similar to those found for APOBEC3G: specifically the degradation of the viral RNA which is postulated to take place after the hypermutation of the viral DNA through APOBEC3G. There are two complexes that form with APOBEC3G: the high-molecular-mass (HMM) and the low-molecular-mass (LMM) ribonucleoprotein complex rendering them either enzymatically active or inactive (Chiu et al. 2005; Soros et al. 2007). It was shown that the presence of the LMM form in resting CD4+ T-cells can be attributed to the wild type (wt) HIV-1 replication block in these cells (Chiu et al. 2005). This form of APOBEC3G complex was also found in monocytes and could be responsible for the antiviral state of these cells (Chiu et al. 2005). Macrophages on the other hand usually contain the HMM form of the APOBEC3G complex and they are considered to be more easily infected. It has also been demonstrated that part of the antiviral activity of IFN-α could be attributed to the enhancement of the A3G LMM ribonucleoprotein complex in resting CD4+ T-cells (Chiu et al. 2005; Chen et al. 2006).

The results showed that the degree to which APOBEC3G was upregulated in monocytes derived macrophages was dependant on the blood donor (figure 17 A) and the stimulation with IFN-α showed a concentration dependant upregulation of APOBEC3G (figure 17 B). This at least proved that APOBEC3G was found in an increased amount in the macrophages which were in an antiviral state provided by IFN-α or LPS. At this point, although there are most likely other antiviral mechanisms provided by APOBEC3G (Iwatani et al. 2007; Li et al. 2007), the only well tested mechanism is the hypermutation of the HIV-1 genome during the RT reaction. This occurs when the DNA is present as a single strand and the results can be

easily analyzed through sequence analysis. It was therefore shown that the APOBEC3G protein was also active in these cells by means of mutation analysis where the rate of mutations in the viral DNA increased in cells stimulated with IFN-α or LPS (figure 18). The error bars for the mutation analysis (figure 18) are very high due to the presence of both hypermutated clones and clones that are not mutated at all. In addition to this, the reaction of the cells to IFN-α or LPS was very much donor dependant. Some blood donors had very little upregulation of the protein, while others had a strong upregulation and this resulted in more or less hypermutated clones. Mutations have been known to appear in PBMCs in HIV-1 infected patients (Janini et al. 2001). This could be due to the APOBEC3G activity in these cells, however this theory would need to be tested further. It is not thought that LPS induces the antiviral state in macrophages indirectly though IFN-α stimulation, but through a separate pathway (Kornbluth et al. 1989).

In addition to IFN-α and LPS, GM-CSF stimulated macrophages also show a reduced infection (Kornbluth et al. 1989). The macrophages were differentiated with medium containing AB serum, however this does not produce a completely homogenous culture. Due to this, there were questions as to the possibility of a dendritic cell contamination or perhaps other subcultures of cells within the population. It was shown that each way of differentiating monocytes into macrophages produced $CD14^+$, $CD64^+$, $CD1a^-$, and $CD83^-$ cells, but that the morphology was somewhat different (see figure 19). The dendritic cells were $CD14^-$, $CD64^-$, $CD1a^+$, and $CD83^+$ and produced mainly suspension cells. The evidence clearly shows that the macrophages differentiated with AB serum were indeed CD14+ macrophages and not a mix with DCs. Due to the limited amount of cells isolated from one blood donor, there were not enough to do further tests with the isolated cells. However, the expression of APOBEC3G was tested where possible. It was very clear that the amount of APOBEC3G was highest in the macrophages differentiated with GM-CSF (figure 20). The increase in APOBEC3G in the GM-CSF macrophages also correlated with the infection in the GM-CSF macrophages in comparison to the AB macrophages. This decrease in HIV-1 infection in GM-CSF stimulated macrophages was also shown in an earlier work by Kornbluth et. al. (Kornbluth et al. 1989), however before suggesting that APOBEC3G is the cause for this, it would need to be tested more extensively. There was not a large difference in the expression of APOBEC3G in the unstimulated DCs in comparison to the AB macrophages. The mature DCs were matured using LPS, which most likely caused the high expression of APOBEC3G in this set of cells.

5.2 Direct regulation of APOBEC3G in cells

The only way to prove that APOBEC3G was responsible for the antiviral state was to directly regulate the APOBEC3G expression in the cells. The problem here is choosing the correct method to bring the DNA of up- or downregulating constructs into the cells. Macrophages can take up naked DNA, however this is said to activate immunological proteins which

degrade the DNA in endosomes (Stacey et al. 1996; Burke et al. 2002; Hacker et al. 2002). Liposomes, lipoplexes, and cationic compounds such as Lipofectamine™ are capable of transfecting macrophages at a rather low efficiency and the expression only lasts a short time (Burke et al. 2002). There is a relatively new technology, the nucleofector system from Amexa which came out in 2005. This system can transfect macrophages with DNA at an efficiency of approximately 60% with 80% cell survival, however the survival rate decreases after 48 hrs (Van De Parre et al. 2005). These shortcomings can apparently be largely avoided by the use of mRNA instead of DNA in the transfection (Van De Parre et al. 2005). A system which provided a long term stable expression of the genes was needed, which is why a viral vector system was used. Retroviral (with the exception of lentiviral) vectors can not transduce non replicating cells, most likely because they can not enter the nucleus (Burke et al. 2002). A lentiviral vector system comprising of 3 plasmids with only the transfer plasmid, containing the target gene and the packaging signal, packaged into the virions was used. Lentiviruses can infect macrophages at a relatively good efficiency; however the lentiviral vector system lacks the accessory proteins used by the viruses for a truly efficient transduction. Vpr for one is believed to be necessary for the nuclear import of the pre-integration complex, which is necessary for non dividing cells such as macrophages (Li et al. 2005). It was shown that although it was possible to successfully transduce macrophages with lentiviral vectors, the efficiency differed drastically between donors (figure 22). This made an efficient transduction of the macrophages impossible without sorting the cells afterwards, but due to the amount of cells needed and the amount transduced, this was also not possible.

It was also shown that during the packaging of APOBEC3G, the exogenous APOBEC3G from the plasmid inhibited the transduction of the lentiviral vectors (figure 23). This problem was solved when adding a separate eukaryotic Vif expression vector to the packaging, which presumably kept the APOBEC3G protein from being packaged into the lentiviral vectors. The target cells could then be transduced without the interference of the APOBEC3G protein during the RT reaction. The cell lines transduced with APOBEC3G were shown to produce high levels of APOBEC3G (figure 24). This showed that the expression vector is functional. However, the macrophages transduced with APOBEC3G were repeatedly not able to be used for further experiments. The results suggest that this may be due to apoptosis (table 2); however the reasons for this and the pathways that lead to this would have to be examined further before this is certain.

RNA interference (RNAi) is a mechanism where small RNAs bind to a target sequence to inhibit the translation of a gene. The use of RNAi has become a standard method used to downregulate genes in order to conclusively determine if a protein is responsible for a cellular process. Silencing of genes with RNAi can be done via different pathways. Cellular micro RNAs (miRNAs) tend to inhibit mRNA translation while sequence specific short hairpin RNAs (shRNA) or small interfering RNA (siRNA) tend to cleave and degrade the mRNA

(Rana 2007). When using shRNAs it is important to show that the protein is downregulated. It was therefore important to show that the shRNA used against APOBEC3G also degraded the mRNA in the cells to determine if mRNA expression analysis was adequate for the study. It was shown that shRNA could be used to downregulate APOBEC3G mRNA and APOBEC3G protein in cell lines (figures 25 and 26) at an efficiency of 50-70% in HEK-293T and HeLa cells. The difference in silencing efficiency could be a result of a lower MOI. The transduction efficiency of each vector could not be separately determined because they all used eGFP as a reporter gene.

As noted above, APOBEC3G can be found in two different complex forms, either LMM or HMM, where only the LMM form is active, and it was not certain what forms would be present or affected when the genes were regulated. To determine if the cells transduced with either the APOBEC3G gene or the shRNA against APOBEC3G had the desired effect on the infection, an infectable cell line, TZM-bl cells, were selected to test the infection on these transduced cells. For this experiment the cells were transduced with both the APOBEC3G vector and the vector containing shRNA against APOBEC3G. The expression results clearly showed a strong expression of the APOBEC3G mRNA in the cells transduced with the LVV containing APOBEC3G. The cotransduced cells with shRNA against the APOBEC3G gene showed a downregulation of 80%. The infection of the cells was clearly suppressed in the cells transduced with APOBEC3G, which was recovered in the cotransduction with shRNA against the APOBEC3G. This showed that the APOBEC3G was active in the TZM-bl cells, and therefore most likely in the LMM complex. These results were very promising for further experiments in primary cells.

5.3 APOBEC3G in CD34 cells

Due to the low transduction efficiency in macrophages, hematopoietic stem cells were used, otherwise known as CD34+ cells. CD34+ cells can be transduced with a transduction efficiency of 70-100% (Gervaix et al. 1997). As the name suggests, CD34+ cells are the precursor cells for all of the cells in the hematopoietic system, including macrophages and can therefore be differentiated into macrophages (Huang and Terstappen 1994; Szabolcs et al. 1996). It was demonstrated that the CD34+ cells could be differentiated into macrophages using the method selected (figure 28 and table 3) and that the CD34+ cells still expressed CD34 after weeks in culture. In the donors tested, the CD34+ cells also stained positive for CD4 and CCR5 during their cultivation. The transduction of the CD34+ cells was a success with a high amount of GFP positive cells after transduction (figure 29). The problem that arose was that the amount of positive cells drastically sank within a week after transduction. This could be explained a few ways. The first possibility is that this could be silencing. Transcriptional silencing is caused by methylation of DNA which takes place around the promoter and near the integration site, making the gene inaccessible for transcription. This is a major problem usually associated with gamma retroviral vectors.

Lentiviral vectors are said to be less susceptible to transcriptional silencing (Mountain 2000; Burke et al. 2002). The second possibility is that the untransduced cells had a selection advantage in comparison to the transduced cells. It would need to be more thoroughly tested to determine which of these possibilities could be more accurate. If it is true that the untransduced cells had a selection advantage, sorting of the cells to obtain 100% eGFP positive cells would be a way to get around this problem.

The APOBEC3G mRNA expression was then analyzed in the cells. CD34+ cells generally have a very low endogenous expression of APOBEC3G. The first donor had a very strong increase in the APOBEC3G expression in the cells transduced with LVVs containing scramble shRNA. It was decided that this was most likely caused by the stimulation of IFN-α with the shRNA sequence (Reynolds et al. 2006; Schlee et al. 2006). This can happen with dsRNA sequences which are over 23 bps long or have GU-rich sequences (Judge et al. 2005). In order to test this, it was indirectly shown that IFN-α was upregulated in the cells transduced with the LVVs containing scramble shRNA (figure 32). A different scramble sequence was then used as a control shRNA, which did not stimulate IFN-α as much if at all. One thing that is notable is that the LPS did not stimulate the IFN-α stimulated protein OAS1. This most likely means that IFN-α is not stimulated indirectly through LPS. This corresponds with the findings from Kornbluth et al. (Kornbluth et al. 1989). It was shown in one paper that MAPK activated APOBEC3G in maturing CD4+ T cells (Stopak et al. 2007). When LPS binds to the TLR-4 the MAPK pathway is activated, which could possibly explain the induction of APOBEC3G in LPS stimulated cells. The regulation of APOBEC3G through MAPK seems to be complicated. The results also show that although APOBEC3G is upregulated, it is also found in the inactive HMM complex, which was shown to be induced during mitogen activation in stimulated T cells, making the activated T cells susceptible to HIV-1 infection (Chiu et al. 2005; Stopak et al. 2007).

For both the CD34+ cells and the CD34 derived macrophages, the APOBEC3G expression increased to a very high amount in the APOBEC3G transduced cells although the amount of eGFP positive cells reduced to under 20% in the FACS results from the macrophages (figures 30 and 31). This could be due to a reduced eGFP expression caused by the eGFP gene following an IRES sequence. This could make it difficult to distinguish the cells which only produced a little amount of eGFP from the untransduced ones. It could also be because the APOBEC3G is expressed at a very high amount in those few cells, which causes the average amount of the mRNA in the cells to increase a substantial amount. It was also expected that the APOBEC3G mRNA would be downregulated in the cells transduced with the shRNA against APOBEC3G. However, the expression of APOBEC3G in the cells transduced with the shRNA against APOBEC3G was not downregulated as expected, with the exception of the CD34+ cells from donor #1 which was slightly lower than the already low endogenous expression of APOBEC3G (figure 30 and 31). There was a minimum of 20% of the cells expressing eGFP during the culture of the cells transduced with LVV

containing shRNA against APOBEC3G (figure 29). If the effect was too small to see in the unstimulated cells, the effect should have at least been shown in the cells stimulated with IFN-α. There was a slight downregulation of APOBEC3G in some of the cells especially when compared to the cells transduced with scramble shRNA (see appendix for complete set of results). The reason for this is difficult to pinpoint, the shRNAs were tested to work before, however it could possibly be a CD34+ cell specific reaction to the shRNAs. This effect would need to be independent of OAS1, because OAS1 was tested and not upregulated in the experiments with the shRNA against APOBEC3G, however there have been other experiments where shRNA transduction into CD34+ cells has worked to downregulate the protein, so this is an unlikely explanation (Scherr et al. 2003; Li et al. 2005; Samakoglu et al. 2006; Jeanson-Leh et al. 2007). One possibility is a different splice form of APOBEC3G present in these cells, which could be missing the sequence for the shRNA, rendering it useless. There are splice patterns present which predict the possibility of ten different splicing products, but none have been experimentally shown (Safran et al. 2008).

The expression of another APOBEC gene that is closely related to APOBEC3G: APOBEC3F was tested to show that these transgenes were specific for APOBEC3G. APOBEC3F is also said to have antiviral activity in the cells and also causes G→A mutations preferring 5' –TC over the 5' -CC dinucleotide hypermutation which APOBEC3G usually mutates (Hache et al. 2005). The expression of APOBEC3F was very low and did not seem to change depending on the transduction vector, with the exception of the cells from the first donor which were transduced with scramble shRNA, but this probably has more to do with the induction of IFNA-α in those cells. These results showed that the transduction vectors acted specifically on APOBEC3G. These experiments also showed that the APOBEC3F mRNA was upregulated when the cells were stimulated with IFN-α (figures 34 and 35). This came as no surprise and was shown previously by Jost et al. and Bonvin et al. in other cell types (Bonvin et al. 2006; Jost et al. 2007).

It was then shown that the CD34+ cells were easily infected and the p24 amounts quickly increased exceeding 400,000 pg/ml of p24 (figure 36). It has been highly debated if infection of CD34+ cells takes place *in vivo*, however as demonstrated in table 3, the HIV-1 receptors were present on the cells allowing an infection *in vitro* to take place (von Laer et al. 1990; Ruiz et al. 1998). When the CD34+ cells were stimulated with IFN-α, they were unable to sustain an infection; however stimulation with LPS showed practically no effect on the infection (see appendix section 7.3.1). The infection in LPS stimulated cells was generally similar to the unstimulated CD34+ cells. This is very different from the results with the macrophages, where after stimulation with LPS, the infection stayed at the same levels as the cells stimulated with IFN-α (figure 38). This is not due to the lack of CD14 receptors on the surface of the CD34+ cells. As shown in table 3, 65% of the cells were CD14 positive. LPS was shown to have an effect on CD34+ cells previously, possibly causing an increase of apoptosis in these cells and the TLR-4 was also shown to be present (Maratheftis et al.

2007). This could be a cell specific activation in macrophages. Other studies that would support this have showed no effect on the APOBEC3G expression after stimulation with LPS in other cell lines such as T cells (Rose et al. 2004).

In regards to the infection of the transduced cells, what was expected, at least in the dVif HIV-1 virus, was the inhibition of the infection in the cells transduced with the APOBEC3G LVVs. In the first donor, the CD34+ cells transduced with the APOBEC3G vector were infected to the same degree as the cells containing scramble shRNA (which had induced IFN-α), which means both were heavily downregulated (see figure 37). The CD34+ cells from the same donor also showed an increase in the infection with dVif HIV-1 in the cells transduced with shRNA against APOBEC3G at the very beginning, corresponding to the slight decrease in the endogenous APOBEC3G expression in these cells. The CD34+ cells from the second donor were generally not as infectable as the other donors. However, in the wild type (wt) HIV infection the cells which had shRNA against APOBEC3G also had an increase in infection in comparison to the cells with scramble shRNA. The cells transduced with APOBEC3G, which also showed a very high expression of the mRNA, did not show a large effect on the infection. The infection was very low on one of the days measured, however this could be an artifact since the infection was high on the last day measured and the cells infected with the dVif virus were not infected enough to see a difference. The CD34+ cells from the third donor were highly infectable. Those cells transduced with APOBEC3G had a definite decrease in infection in comparison to the other cells, if the sample day 9 is looked at as an artifact. In order to have conclusive results more CD34+ cell donors would need to be analyzed.

The CD34 derived macrophages were differentiated from the transduced and untransduced CD34+ cells from the donors mentioned above. They were more sensitive to the stimulation from LPS, which proved to be as antiviral as the IFN-α in the macrophages. The infection with the dVif virus was generally a lot lower than that of the wt virus showing that the macrophages contain factors which somewhat inhibit this infection. The results from the infection in the macrophages from the first donor were not expected (figure 39). Although the cells containing the APOBEC3G gene had a reduced infection with the dVif virus, the control with only the eGFP gene showed the same effect. In addition the scramble shRNA and the shRNA against APOBEC3G both showed a rather strong infection. This was not expected for many reasons. The infection in the CD34+ cells from the same donor did somewhat correlate with what was expected from the vectors (see figure 37), and generally the same results would be expected in the macrophages derived from these cells. In the second blood donor the infection in the CD34 derived macrophages was similar to one another as it was in the CD34+ cells from this donor. The third donor had a high infection in the untransduced macrophages which was inhibited in the cells transduced with APOBEC3G, this was even more apparent in those cells infected with the dVif HIV-1 virus. The cells transduced with shRNA against APOBEC3G were more infectable with the wt virus and the infection was

also higher in the dVif HIV infection, showing that with this donor there was a noticeable effect from the shRNA against APOBEC3G. These are the type of results that were expected. Notable is that the infection was not recovered with the addition of the shRNA against APOBEC3G in any of the cells which were stimulated with IFN-α (see appendix, section 7.3.2). This is very different than the results found by Peng et al. and Wang et al., where they showed that siRNA against APOBEC3G also reversed the HIV-1 suppression in macrophages stimulated with IFN-α (Peng et al. 2006; Wang et al. 2008). As shown earlier, the expression of the mRNA was not reduced although there was such a high percent of macrophages which were eGFP positive. As with the CD34+ cells, more cell donors would have to be tested to have conclusive results.

The results of this work showed that APOBEC3G plays an important role in the response of the immune system to HIV-1. It was shown that the relationship is very complicated and is cell type as well as donor dependant. One interesting idea would be to determine the form in which APOBEC3G is found in these cell types and if this differs when it is exogenous APOBEC3G or the expression is stimulated through IFN-α. It is very important to determine the other mechanisms of induction of this protein and the conditions under which it is in the HMM (inactive) form. There have been recent papers suggesting that the level of expression of APOBEC3G in individuals can determine their progression to AIDS (Biasin et al. 2007; Jin et al. 2007; Land et al. 2008). Perhaps with a better understanding of these mechanisms, the expression of APOBEC3G could be enhanced enough to slow down the progression of HIV in patients.

6 REFERENCES

Akira, S. and K. Takeda (2004). "Toll-like receptor signalling." Nat Rev Immunol **4**(7): 499-511.

Alce, T. M. and W. Popik (2004). "APOBEC3G is incorporated into virus-like particles by a direct interaction with HIV-1 Gag nucleocapsid protein." J Biol Chem.

Andreesen, R., K. J. Bross, J. Osterholz and F. Emmrich (1986). "Human macrophage maturation and heterogeneity: analysis with a newly generated set of monoclonal antibodies to differentiation antigens." Blood **67**(5): 1257-64.

Andreesen, R., J. Picht and G. W. Lohr (1983). "Primary cultures of human blood-born macrophages grown on hydrophobic teflon membranes." J Immunol Methods **56**(3): 295-304.

Avery, L. (2007, Jan 7, 2007). "Mann-Whitney U Test." http://elegans.swmed.edu/~leon/stats/utest.html, from http://elegans.swmed.edu/~leon/stats/utest.html.

Bell, J. J. and A. Bhandoola (2008). "The earliest thymic progenitors for T cells possess myeloid lineage potential." Nature **452**(7188): 764-7.

Beutler, B. (2005). "The Toll-like receptors: analysis by forward genetic methods." Immunogenetics **57**(6): 385-92.

Biasin, M., L. Piacentini, S. Lo Caputo, Y. Kanari, G. Magri, D. Trabattoni, V. Naddeo, L. Lopalco, A. Clivio, E. Cesana, F. Fasano, C. Bergamaschi, F. Mazzotta, M. Miyazawa and M. Clerici (2007). "Apolipoprotein B mRNA-editing enzyme, catalytic polypeptide-like 3G: a possible role in the resistance to HIV of HIV-exposed seronegative individuals." J Infect Dis **195**(7): 960-4.

Bogerd, H. P., B. P. Doehle, H. L. Wiegand and B. R. Cullen (2004). "A single amino acid difference in the host APOBEC3G protein controls the primate species specificity of HIV type 1 virion infectivity factor." Proc Natl Acad Sci U S A **101**(11): 3770-4.

Bonvin, M., F. Achermann, I. Greeve, D. Stroka, A. Keogh, D. Inderbitzin, D. Candinas, P. Sommer, S. Wain-Hobson, J. P. Vartanian and J. Greeve (2006). "Interferon-inducible expression of APOBEC3 editing enzymes in human hepatocytes and inhibition of hepatitis B virus replication." Hepatology **43**(6): 1364-74.

Bouazzaoui, A., M. Kreutz, V. Eisert, N. Dinauer, A. Heinzelmann, S. Hallenberger, J. Strayle, R. Walker, H. Rubsamen-Waigmann, R. Andreesen and H. von Briesen (2006). "Stimulated trans-acting factor of 50 kDa (Staf50) inhibits HIV-1 replication in human monocyte-derived macrophages." Virology **356**(1-2): 79-94.

Brock, T. D., J. Parker, J. M. Martinko and M. T. Madigan (2000). Brock biology of microorganisms. Englewood Cliffs, N.J., Prentice Hall.

Burke, B., S. Sumner, N. Maitland and C. E. Lewis (2002). "Macrophages in gene therapy: cellular delivery vehicles and in vivo targets." J Leukoc Biol **72**(3): 417-28.

Cen, S., F. Guo, M. Niu, J. Saadatmand, J. Deflassieux and L. Kleiman (2004). "The interaction between HIV-1 Gag and APOBEC3G." J Biol Chem.

Chen, H., C. E. Lilley, Q. Yu, D. V. Lee, J. Chou, I. Narvaiza, N. R. Landau and M. D. Weitzman (2006). "APOBEC3A is a potent inhibitor of adeno-associated virus and retrotransposons." Curr Biol **16**(5): 480-5.

Chen, K., J. Huang, C. Zhang, S. Huang, G. Nunnari, F. X. Wang, X. Tong, L. Gao, K. Nikisher and H. Zhang (2006). "Alpha interferon potently enhances the anti-human immunodeficiency virus type 1 activity of APOBEC3G in resting primary CD4 T cells." J Virol **80**(15): 7645-57.

Chiu, Y. L., V. B. Soros, J. F. Kreisberg, K. Stopak, W. Yonemoto and W. C. Greene (2005). "Cellular APOBEC3G restricts HIV-1 infection in resting CD4+ T cells." Nature **435**(7038): 108-14.

Clavel, F. and P. Charneau (1994). "Fusion from without directed by human immunodeficiency virus particles." J Virol **68**(2): 1179-85.

Conticello, S. G., R. S. Harris and M. S. Neuberger (2003). "The Vif protein of HIV triggers degradation of the human antiretroviral DNA deaminase APOBEC3G." Curr Biol **13**(22): 2009-13.

Cullen, B. R. (2006). "Role and mechanism of action of the APOBEC3 family of antiretroviral resistance factors." J Virol **80**(3): 1067-76.

De Nichilo, M. O. and G. F. Burns (1993). "Granulocyte-macrophage and macrophage colony-stimulating factors differentially regulate alpha v integrin expression on cultured human macrophages." Proc Natl Acad Sci U S A **90**(6): 2517-21.

Demaison, C., K. Parsley, G. Brouns, M. Scherr, K. Battmer, C. Kinnon, M. Grez and A. J. Thrasher (2002). "High-level transduction and gene expression in hematopoietic repopulating cells using a human immunodeficiency [correction of imunodeficiency] virus type 1-based lentiviral vector containing an internal spleen focus forming virus promoter." Hum Gene Ther **13**(7): 803-13.

Derdeyn, C. A., J. M. Decker, J. N. Sfakianos, X. Wu, W. A. O'Brien, L. Ratner, J. C. Kappes, G. M. Shaw and E. Hunter (2000). "Sensitivity of human immunodeficiency virus type 1 to the fusion inhibitor T-20 is modulated by coreceptor specificity defined by the V3 loop of gp120." J Virol **74**(18): 8358-67.

Douaisi, M., S. Dussart, M. Courcoul, G. Bessou, R. Vigne and E. Decroly (2004). "HIV-1 and MLV Gag proteins are sufficient to recruit APOBEC3G into virus-like particles." Biochem Biophys Res Commun **321**(3): 566-73.

Edwards, M. R., L. Slater and S. L. Johnston (2007). "Signalling pathways mediating type I interferon gene expression." Microbes Infect **9**(11): 1245-51.

Fields, B. N., D. M. Knipe and P. M. Howley (2007). Fields virology. Philadelphia, Wolters Kluwer/Lippincott Williams & Wilkins.

Fotopoulos, G., A. Harari, P. Michetti, D. Trono, G. Pantaleo and J. P. Kraehenbuhl (2002). "Transepithelial transport of HIV-1 by M cells is receptor-mediated." Proc Natl Acad Sci U S A **99**(14): 9410-4.

Gervaix, A., L. Schwarz, P. Law, A. D. Ho, D. Looney, T. Lane and F. Wong-Staal (1997). "Gene therapy targeting peripheral blood CD34+ hematopoietic stem cells of HIV-infected individuals." Hum Gene Ther **8**(18): 2229-38.

Gluckman, J. C., B. Canque, F. Chapuis and M. Rosenzwajg (1997). "In vitro generation of human dendritic cells and cell therapy." Cytokines Cell Mol Ther **3**(3): 187-96.

Grouard, G. and E. A. Clark (1997). "Role of dendritic and follicular dendritic cells in HIV infection and pathogenesis." Curr Opin Immunol **9**(4): 563-7.

Guha, M. and N. Mackman (2001). "LPS induction of gene expression in human monocytes." Cell Signal **13**(2): 85-94.

Hache, G., M. T. Liddament and R. S. Harris (2005). "The retroviral hypermutation specificity of APOBEC3F and APOBEC3G is governed by the C-terminal DNA cytosine deaminase domain." J Biol Chem **280**(12): 10920-4.

Hacker, G., V. Redecke and H. Hacker (2002). "Activation of the immune system by bacterial CpG-DNA." Immunology **105**(3): 245-51.

Heath, D. (1995). An introduction to experimental design and statistics for biology. London ; Bristol, Penn., UCL Press.

Holmes, R. K., F. A. Koning, K. N. Bishop and M. H. Malim (2007). "APOBEC3F can inhibit the accumulation of HIV-1 reverse transcription products in the absence of hypermutation. Comparisons with APOBEC3G." J Biol Chem **282**(4): 2587-95.

Holmes, R. K., M. H. Malim and K. N. Bishop (2007). "APOBEC-mediated viral restriction: not simply editing?" Trends Biochem Sci **32**(3): 118-28.

Honda, Y., L. Rogers, K. Nakata, B. Y. Zhao, R. Pine, Y. Nakai, K. Kurosu, W. N. Rom and M. Weiden (1998). "Type I interferon induces inhibitory 16-kD CCAAT/ enhancer binding protein (C/EBP)beta, repressing the HIV-1 long terminal repeat in macrophages: pulmonary tuberculosis alters C/EBP expression, enhancing HIV-1 replication." J Exp Med **188**(7): 1255-65.

Hsu, K., J. Seharaseyon, P. Dong, S. Bour and E. Marban (2004). "Mutual functional destruction of HIV-1 Vpu and host TASK-1 channel." Mol Cell **14**(2): 259-67.

Hu, W. S. and V. K. Pathak (2000). "Design of retroviral vectors and helper cells for gene therapy." Pharmacol Rev **52**(4): 493-511.

Huang, S. and L. W. Terstappen (1994). "Lymphoid and myeloid differentiation of single human CD34+, HLA-DR+, CD38- hematopoietic stem cells." Blood **83**(6): 1515-26.

Iwatani, Y., D. S. Chan, F. Wang, K. S. Maynard, W. Sugiura, A. M. Gronenborn, I. Rouzina, M. C. Williams, K. Musier-Forsyth and J. G. Levin (2007). "Deaminase-independent inhibition of HIV-1 reverse transcription by APOBEC3G." Nucleic Acids Res **35**(21): 7096-108.

Janeway, C. (2004). Immunobiology, Garland Science.

Janini, M., M. Rogers, D. R. Birx and F. E. McCutchan (2001). "Human immunodeficiency virus type 1 DNA sequences genetically damaged by hypermutation are often abundant in patient peripheral blood mononuclear cells and may be generated during near-simultaneous infection and activation of CD4(+) T cells." J Virol **75**(17): 7973-86.

Jeanson-Leh, L., J. Blondeau and A. Galy (2007). "Optimization of short hairpin RNA for lentiviral-mediated RNAi against WAS." Biochem Biophys Res Commun **362**(2): 498-503.

Jin, X., A. Brooks, H. Chen, R. Bennett, R. Reichman and H. Smith (2005). "APOBEC3G/CEM15 (hA3G) mRNA levels associate inversely with human immunodeficiency virus viremia." J Virol **79**(17): 11513-6.

Jin, X., H. Wu and H. Smith (2007). "APOBEC3G levels predict rates of progression to AIDS." Retrovirology **4**: 20.

Johnson, W. D., Jr., B. Mei and Z. A. Cohn (1977). "The separation, long-term cultivation, and maturation of the human monocyte." J Exp Med **146**(6): 1613-26.

Jost, S., P. Turelli, B. Mangeat, U. Protzer and D. Trono (2007). "Induction of antiviral cytidine deaminases does not explain the inhibition of hepatitis B virus replication by interferons." J Virol **81**(19): 10588-96.

Judge, A. D., V. Sood, J. R. Shaw, D. Fang, K. McClintock and I. MacLachlan (2005). "Sequence-dependent stimulation of the mammalian innate immune response by synthetic siRNA." Nat Biotechnol **23**(4): 457-62.

Kaczmarski, R. S., F. Davison, E. Blair, S. Sutherland, J. Moxham, T. McManus and G. J. Mufti (1992). "Detection of HIV in haemopoietic progenitors." Br J Haematol **82**(4): 764-9.

Kazazi, F., J. M. Mathijs, P. Foley and A. L. Cunningham (1989). "Variations in CD4 expression by human monocytes and macrophages and their relationships to infection with the human immunodeficiency virus." J Gen Virol **70 (Pt 10)**: 2661-72.

Kendall, N. S. (1999). HIV Structure.

Konigs, C., A. Pustowka, J. Irving, C. Kessel, K. Klich, V. Wegner, M. J. Rowley, I. R. Mackay, W. Kreuz, C. Griesinger and U. Dietrich (2007). "Peptide mimotopes selected with HIV-1-blocking monoclonal antibodies against CCR5 represent motifs specific for HIV-1 entry." Immunol Cell Biol **85**(7): 511-7.

Kornbluth, R. S., P. S. Oh, J. R. Munis, P. H. Cleveland and D. D. Richman (1989). "Interferons and bacterial lipopolysaccharide protect macrophages from productive infection by human immunodeficiency virus in vitro." J Exp Med **169**(3): 1137-51.

Kuby, J. (1997). Immunology, W.H. Freeman and Company.

Land, A. M., T. B. Ball, M. Luo, R. Pilon, P. Sandstrom, J. E. Embree, C. Wachihi, J. Kimani and F. A. Plummer (2008). "HIV-1 Proviral Hypermutation Correlates with CD4 Count in HIV Infected Women from Kenya." J Virol.

Lemaire, I., H. Yang, W. Lauzon and N. Gendron (1996). "M-CSF and GM-CSF promote alveolar macrophage differentiation into multinucleated giant cells with distinct phenotypes." J Leukoc Biol **60**(4): 509-18.

Li, L., H. S. Li, C. D. Pauza, M. Bukrinsky and R. Y. Zhao (2005). "Roles of HIV-1 auxiliary proteins in viral pathogenesis and host-pathogen interactions." Cell Res **15**(11-12): 923-34.

Li, M. J., J. Kim, S. Li, J. Zaia, J. K. Yee, J. Anderson, R. Akkina and J. J. Rossi (2005). "Long-term inhibition of HIV-1 infection in primary hematopoietic cells by lentiviral vector delivery of a triple combination of anti-HIV shRNA, anti-CCR5 ribozyme, and a nucleolar-localizing TAR decoy." Mol Ther **12**(5): 900-9.

Li, X. Y., F. Guo, L. Zhang, L. Kleiman and S. Cen (2007). "APOBEC3G inhibits DNA strand transfer during HIV-1 reverse transcription." J Biol Chem **282**(44): 32065-74.

Liddament, M. T., W. L. Brown, A. J. Schumacher and R. S. Harris (2004). "APOBEC3F properties and hypermutation preferences indicate activity against HIV-1 in vivo." Curr Biol **14**(15): 1385-91.

Liu, A., M. Takahashi, M. Narita, Z. Zheng, N. Kanazawa, T. Abe, K. Nikkuni, T. Furukawa, K. Toba, I. Fuse and Y. Aizawa (2002). "Generation of functional and mature dendritic cells from cord blood and bone marrow CD34+ cells by two-step culture combined with calcium ionophore treatment." J Immunol Methods **261**(1-2): 49-63.

Liu, B., P. T. Sarkis, K. Luo, Y. Yu and X. F. Yu (2005). "Regulation of Apobec3F and human immunodeficiency virus type 1 Vif by Vif-Cul5-ElonB/C E3 ubiquitin ligase." J Virol **79**(15): 9579-87.

Majka, M., T. Rozmyslowicz, J. Ratajczak, A. Dobrowsky, Z. Pietrzkowski, G. N. Gaulton, A. Janowska-Wieczorek and M. Z. Ratajczak (2000). "The limited infectability by R5 HIV of CD34(+) cells from thymus, cord, and peripheral blood and bone marrow is explained by their ability to produce beta-chemokines." Exp Hematol **28**(12): 1334-42.

Mallon, D. F., A. Buck, J. C. Reece, S. M. Crowe and P. U. Cameron (1999). "Monocyte-derived dendritic cells as a model for the study of HIV-1 infection: productive infection and phenotypic changes during culture in human serum." Immunol Cell Biol **77**(5): 442-50.

Mangeat, B., P. Turelli, G. Caron, M. Friedli, L. Perrin and D. Trono (2003). "Broad antiretroviral defence by human APOBEC3G through lethal editing of nascent reverse transcripts." Nature **424**(6944): 99-103.

Mangeat, B., P. Turelli, S. Liao and D. Trono (2004). "A single amino acid determinant governs the species-specific sensitivity of APOBEC3G to Vif action." J Biol Chem **279**(15): 14481-3.

Maratheftis, C. I., E. Andreakos, H. M. Moutsopoulos and M. Voulgarelis (2007). "Toll-like receptor-4 is up-regulated in hematopoietic progenitor cells and contributes to increased apoptosis in myelodysplastic syndromes." Clin Cancer Res **13**(4): 1154-60.

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

Marin, M., K. M. Rose, S. L. Kozak and D. Kabat (2003). "HIV-1 Vif protein binds the editing enzyme APOBEC3G and induces its degradation." Nat Med **9**(11): 1398-403. Epub 2003 Oct 5.

Mehle, A., H. Wilson, C. Zhang, A. J. Brazier, M. McPike, E. Pery and D. Gabuzda (2007). "Identification of an APOBEC3G Binding Site in HIV-1 Vif and Inhibitors of Vif-APOBEC3G Binding." J Virol.

Modrow, S., D. Falke and U. Truyen (2003). Molekulare Virologie, Spektrum Akademischer Verlag GmbH Heidelberg/Berlin.

Mountain, A. (2000). "Gene therapy: the first decade." Trends Biotechnol **18**(3): 119-28.

Muench, M. O., J. Cupp, J. Polakoff and M. G. Roncarolo (1994). "Expression of CD33, CD38, and HLA-DR on CD34+ human fetal liver progenitors with a high proliferative potential." Blood **83**(11): 3170-81.

Muller, P. Y., H. Janovjak, A. R. Miserez and Z. Dobbie (2002). "Processing of gene expression data generated by quantitative real-time RT-PCR." Biotechniques **32**(6): 1372-4, 1376, 1378-9.

Naldini, L., U. Blomer, P. Gallay, D. Ory, R. Mulligan, F. H. Gage, I. M. Verma and D. Trono (1996). "In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector." Science **272**(5259): 263-7.

Navaratnam, N. and R. Sarwar (2006). "An overview of cytidine deaminases." Int J Hematol **83**(3): 195-200.

Newman, E. N., R. K. Holmes, H. M. Craig, K. C. Klein, J. R. Lingappa, M. H. Malim and A. M. Sheehy (2005). "Antiviral function of APOBEC3G can be dissociated from cytidine deaminase activity." Curr Biol **15**(2): 166-70.

Nicholson, J. K., G. D. Cross, C. S. Callaway and J. S. McDougal (1986). "In vitro infection of human monocytes with human T lymphotropic virus type III/lymphadenopathy-associated virus (HTLV-III/LAV)." J Immunol **137**(1): 323-9.

Noguchi, C., N. Hiraga, N. Mori, M. Tsuge, M. Imamura, S. Takahashi, Y. Fujimoto, H. Ochi, H. Abe, T. Maekawa, H. Yatsuji, K. Shirakawa, A. Takaori-Kondo and K. Chayama (2007). "Dual effect of APOBEC3G on Hepatitis B virus." J Gen Virol **88**(Pt 2): 432-40.

O'Doherty, U., R. Ignatius, N. Bhardwaj and M. Pope (1997). "Generation of monocyte-derived dendritic cells from precursors in rhesus macaque blood." J Immunol Methods **207**(2): 185-94.

Parmar, S. and L. C. Platanias (2003). "Interferons: mechanisms of action and clinical applications." Curr Opin Oncol **15**(6): 431-9.

Patterson, S., S. P. Robinson, N. R. English and S. C. Knight (1999). "Subpopulations of peripheral blood dendritic cells show differential susceptibility to infection with a lymphotropic strain of HIV-1." Immunol Lett **66**(1-3): 111-6.

Peng, G., K. J. Lei, W. Jin, T. Greenwell-Wild and S. M. Wahl (2006). "Induction of APOBEC3 family proteins, a defensive maneuver underlying interferon-induced anti-HIV-1 activity." J Exp Med **203**(1): 41-6.

Piguet, V. and R. M. Steinman (2007). "The interaction of HIV with dendritic cells: outcomes and pathways." Trends Immunol **28**(11): 503-10.

Pintard, L., A. Willems and M. Peter (2004). "Cullin-based ubiquitin ligases: Cul3-BTB complexes join the family." Embo J **23**(8): 1681-7.

Pion, M., A. Granelli-Piperno, B. Mangeat, R. Stalder, R. Correa, R. M. Steinman and V. Piguet (2006). "APOBEC3G/3F mediates intrinsic resistance of monocyte-derived dendritic cells to HIV-1 infection." J Exp Med **203**(13): 2887-93.

Pitha, P. M. (1994). "Multiple effects of interferon on the replication of human immunodeficiency virus type 1." Antiviral Res **24**(2-3): 205-19.

Platt, E. J., K. Wehrly, S. E. Kuhmann, B. Chesebro and D. Kabat (1998). "Effects of CCR5 and CD4 cell surface concentrations on infections by macrophagetropic isolates of human immunodeficiency virus type 1." J Virol **72**(4): 2855-64.

Rana, T. M. (2007). "Illuminating the silence: understanding the structure and function of small RNAs." Nat Rev Mol Cell Biol **8**(1): 23-36.

Rasola, A., D. Gramaglia, C. Boccaccio and P. M. Comoglio (2001). "Apoptosis enhancement by the HIV-1 Nef protein." J Immunol **166**(1): 81-8.

Rehli, M., S. W. Krause, L. Schwarzfischer, M. Kreutz and R. Andreesen (1995). "Molecular cloning of a novel macrophage maturation-associated transcript encoding a protein with several potential transmembrane domains." Biochem Biophys Res Commun **217**(2): 661-7.

Reynolds, A., E. M. Anderson, A. Vermeulen, Y. Fedorov, K. Robinson, D. Leake, J. Karpilow, W. S. Marshall and A. Khvorova (2006). "Induction of the interferon response by siRNA is cell type- and duplex length-dependent." Rna **12**(6): 988-93.

Robinson, S. P., S. Patterson, N. English, D. Davies, S. C. Knight and C. D. Reid (1999). "Human peripheral blood contains two distinct lineages of dendritic cells." Eur J Immunol **29**(9): 2769-78.

Rose, K. M., M. Marin, S. L. Kozak and D. Kabat (2004). "Transcriptional regulation of APOBEC3G, a cytidine deaminase that hypermutates human immunodeficiency virus." J Biol Chem **279**(40): 41744-9.

Rubsamen-Waigmann, H., W. R. Willems, U. Bertram and H. von Briesen (1989). "Reversal of HIV-phenotype to fulminant replication on macrophages in perinatal transmission." Lancet **2**(8672): 1155-6.

Ruiz, M. E., C. Cicala, J. Arthos, A. Kinter, A. T. Catanzaro, J. Adelsberger, K. L. Holmes, O. J. Cohen and A. S. Fauci (1998). "Peripheral blood-derived CD34+ progenitor cells: CXC chemokine receptor 4 and CC chemokine receptor 5 expression and infection by HIV." J Immunol **161**(8): 4169-76.

Russell, R. A. and V. K. Pathak (2007). "Identification of two distinct human immunodeficiency virus type 1 Vif determinants critical for interactions with human APOBEC3G and APOBEC3F." J Virol **81**(15): 8201-10.

Safran, M., I. Dalah, N. Rosen, G. Stelzer, A. Harel, M. Shmoish, T. I. Stein, E. Matusevich, Y. Perlman, O. Shmueli, A. Madi, A. Sirota, Y. Sole, E. Brewster, G. Celniker, J. Alexander and D. Lancet (2008). GeneCard for protein-coding APOBEC3G - GC22P037762 (http://www.genecards.org/cgi-bin/carddisp.pl?gene=APOBEC3G). W. I. o. Science, Weizmann Institute of Science. **2008**.

Samakoglu, S., L. Lisowski, T. Budak-Alpdogan, Y. Usachenko, S. Acuto, R. Di Marzo, A. Maggio, P. Zhu, J. F. Tisdale, I. Riviere and M. Sadelain (2006). "A genetic strategy to treat sickle cell anemia by coregulating globin transgene expression and RNA interference." Nat Biotechnol **24**(1): 89-94.

Sander, D. (1995-2004). "The Big Picture Book of Viruses." http://www.virologynet/Big_Virology/BVretro.html, from http://www.virologynet/Big_Virology/BVretro.html.

Sandor, F. and M. Buc (2005). "Toll-like receptors. I. Structure, function and their ligands." Folia Biol (Praha) **51**(5): 148-57.

Santin, A. D., P. L. Hermonat, A. Ravaggi, M. Chiriva-Internati, M. J. Cannon, J. C. Hiserodt, S. Pecorelli and G. P. Parham (1999). "Expression of surface antigens during the differentiation of human dendritic cells vs macrophages from blood monocytes in vitro." Immunobiology **200**(2): 187-204.

Sarkis, P. T., S. Ying, R. Xu and X. F. Yu (2006). "STAT1-independent cell type-specific regulation of antiviral APOBEC3G by IFN-alpha." J Immunol **177**(7): 4530-40.

Schafer, A., H. P. Bogerd and B. R. Cullen (2004). "Specific packaging of APOBEC3G into HIV-1 virions is mediated by the nucleocapsid domain of the gag polyprotein precursor." Virology **328**(2): 163-8.

Scherr, M., K. Battmer, A. Ganser and M. Eder (2003). "Modulation of gene expression by lentiviral-mediated delivery of small interfering RNA." Cell Cycle **2**(3): 251-7.

Schlee, M., V. Hornung and G. Hartmann (2006). "siRNA and isRNA: two edges of one sword." Mol Ther **14**(4): 463-70.

Schrofelbauer, B., D. Chen and N. R. Landau (2004). "A single amino acid of APOBEC3G controls its species-specific interaction with virion infectivity factor (Vif)." Proc Natl Acad Sci U S A **101**(11): 3927-32.

Sheehy, A. M., N. C. Gaddis, J. D. Choi and M. H. Malim (2002). "Isolation of a human gene that inhibits HIV-1 infection and is suppressed by the viral Vif protein." Nature **418**(6898): 646-50.

Sheehy, A. M., N. C. Gaddis and M. H. Malim (2003). "The antiretroviral enzyme APOBEC3G is degraded by the proteasome in response to HIV-1 Vif." Nat Med **9**(11): 1404-7. Epub 2003 Oct 5.

Shirakawa, K., A. Takaori-Kondo, M. Kobayashi, M. Tomonaga, T. Izumi, K. Fukunaga, A. Sasada, A. Abudu, Y. Miyauchi, H. Akari, K. Iwai and T. Uchiyama (2006). "Ubiquitination of APOBEC3 proteins by the Vif-Cullin5-ElonginB-ElonginC complex." Virology **344**(2): 263-6.

Shizuru, J. A., R. S. Negrin and I. L. Weissman (2005). "Hematopoietic stem and progenitor cells: clinical and preclinical regeneration of the hematolymphoid system." Annu Rev Med **56**: 509-38.

Simon, P. (2003). "Q-Gene: processing quantitative real-time RT-PCR data." Bioinformatics **19**(11): 1439-40.

Soros, V. B., W. Yonemoto and W. C. Greene (2007). "Newly synthesized APOBEC3G is incorporated into HIV virions, inhibited by HIV RNA, and subsequently activated by RNase H." PLoS Pathog **3**(2): e15.

Stacey, K. J., M. J. Sweet and D. A. Hume (1996). "Macrophages ingest and are activated by bacterial DNA." J Immunol **157**(5): 2116-22.

Stopak, K., C. de Noronha, W. Yonemoto and W. C. Greene (2003). "HIV-1 Vif blocks the antiviral activity of APOBEC3G by impairing both its translation and intracellular stability." Mol Cell **12**(3): 591-601.

Stopak, K. S., Y. L. Chiu, J. Kropp, R. M. Grant and W. C. Greene (2007). "Distinct patterns of cytokine regulation of APOBEC3G expression and activity in primary lymphocytes, macrophages, and dendritic cells." J Biol Chem **282**(6): 3539-46.

Su, S. V., K. B. Gurney and B. Lee (2003). "Sugar and spice: viral envelope-DC-SIGN interactions in HIV pathogenesis." Curr HIV Res **1**(1): 87-99.

Suspene, R., P. Sommer, M. Henry, S. Ferris, D. Guetard, S. Pochet, A. Chester, N. Navaratnam, S. Wain-Hobson and J. P. Vartanian (2004). "APOBEC3G is a single-stranded DNA cytidine deaminase and functions independently of HIV reverse transcriptase." Nucleic Acids Res **32**(8): 2421-9. Print 2004.

Szabolcs, P., D. Avigan, S. Gezelter, D. H. Ciocon, M. A. Moore, R. M. Steinman and J. W. Young (1996). "Dendritic cells and macrophages can mature independently from a human bone marrow-derived, post-colony-forming unit intermediate." Blood **87**(11): 4520-30.

Takaoka, A. and H. Yanai (2006). "Interferon signalling network in innate defence." Cell Microbiol **8**(6): 907-22.

Takeda, K. and S. Akira (2004). "TLR signaling pathways." Semin Immunol **16**(1): 3-9.

Tanaka, Y., H. Marusawa, H. Seno, Y. Matsumoto, Y. Ueda, Y. Kodama, Y. Endo, J. Yamauchi, T. Matsumoto, A. Takaori-Kondo, I. Ikai and T. Chiba (2006). "Anti-viral protein APOBEC3G is induced by interferon-alpha stimulation in human hepatocytes." Biochem Biophys Res Commun **341**(2): 314-9.

Theofilopoulos, A. N., R. Baccala, B. Beutler and D. H. Kono (2005). "Type I interferons (alpha/beta) in immunity and autoimmunity." Annu Rev Immunol **23**: 307-36.

Uchida, N., R. E. Sutton, A. M. Friera, D. He, M. J. Reitsma, W. C. Chang, G. Veres, R. Scollay and I. L. Weissman (1998). "HIV, but not murine leukemia virus, vectors mediate high efficiency gene transfer into freshly isolated G0/G1 human hematopoietic stem cells." Proc Natl Acad Sci U S A **95**(20): 11939-44.

Van De Parre, T. J., W. Martinet, D. M. Schrijvers, A. G. Herman and G. R. De Meyer (2005). "mRNA but not plasmid DNA is efficiently transfected in murine J774A.1 macrophages." Biochem Biophys Res Commun **327**(1): 356-60.

Vartanian, J. P., P. Sommer and S. Wain-Hobson (2003). "Death and the retrovirus." Trends Mol Med **9**(10): 409-13.

Verani, A., G. Gras and G. Pancino (2005). "Macrophages and HIV-1: dangerous liaisons." Mol Immunol **42**(2): 195-212.

Verani, A., G. Scarlatti, M. Comar, E. Tresoldi, S. Polo, M. Giacca, P. Lusso, A. G. Siccardi and D. Vercelli (1997). "C-C chemokines released by lipopolysaccharide (LPS)-stimulated human macrophages suppress HIV-1 infection in both macrophages and T cells." J Exp Med **185**(5): 805-16.

Verani, A., F. Sironi, A. G. Siccardi, P. Lusso and D. Vercelli (2002). "Inhibition of CXCR4-tropic HIV-1 infection by lipopolysaccharide: evidence of different mechanisms in macrophages and T lymphocytes." J Immunol **168**(12): 6388-95.

von Briesen, H., R. Andreesen and H. Rubsamen-Waigmann (1990). "Systematic classification of HIV biological subtypes on lymphocytes and monocytes/macrophages." Virology **178**(2): 597-602.

von Briesen, H., C. Meichsner, R. Andreesen, R. Esser, E. Schrinner and H. Rubsamen-Waigmann (1990). "The polysulphated polyxylan Hoe/Bay-946 inhibits HIV replication on human monocytes/macrophages." Res Virol **141**(2): 251-7.

von Laer, D., F. T. Hufert, T. E. Fenner, S. Schwander, M. Dietrich, H. Schmitz and P. Kern (1990). "CD34+ hematopoietic progenitor cells are not a major reservoir of the human immunodeficiency virus." Blood **76**(7): 1281-6.

Wada, H., K. Masuda, R. Satoh, K. Kakugawa, T. Ikawa, Y. Katsura and H. Kawamoto (2008). "Adult T-cell progenitors retain myeloid potential." Nature **452**(7188): 768-72.

Wang, F. X., J. Huang, H. Zhang, X. Ma and H. Zhang (2008). "APOBEC3G upregulation by alpha interferon restricts human immunodeficiency virus type 1 infection in human peripheral plasmacytoid dendritic cells." J Gen Virol **89**(Pt 3): 722-30.

Wei, X., J. M. Decker, H. Liu, Z. Zhang, R. B. Arani, J. M. Kilby, M. S. Saag, X. Wu, G. M. Shaw and J. C. Kappes (2002). "Emergence of resistant human immunodeficiency virus type 1 in patients receiving fusion inhibitor (T-20) monotherapy." Antimicrob Agents Chemother **46**(6): 1896-905.

Weissman, I. L. (2000). "Stem cells: units of development, units of regeneration, and units in evolution." Cell **100**(1): 157-68.

WHO, W. H. O. (2007) "WHO and HIV/AIDS." **Volume**, DOI:

Wijffels, J. F., Z. de Rover, G. Kraal and R. H. Beelen (1993). "Macrophage phenotype regulation by colony-stimulating factors at bone marrow level." J Leukoc Biol **53**(3): 249-55.

Woelk, C. H., F. Ottones, C. R. Plotkin, P. Du, C. D. Royer, S. E. Rought, J. Lozach, R. Sasik, R. S. Kornbluth, D. D. Richman and J. Corbeil (2004). "Interferon gene expression following HIV type 1 infection of monocyte-derived macrophages." AIDS Res Hum Retroviruses **20**(11): 1210-22.

Xiao, Z., E. Ehrlich, Y. Yu, K. Luo, T. Wang, C. Tian and X. F. Yu (2006). "Assembly of HIV-1 Vif-Cul5 E3 ubiquitin ligase through a novel zinc-binding domain-stabilized hydrophobic interface in Vif." Virology **349**(2): 290-9.

Yee, J. K., A. Miyanohara, P. LaPorte, K. Bouic, J. C. Burns and T. Friedmann (1994). "A general method for the generation of high-titer, pantropic retroviral vectors: highly efficient infection of primary hepatocytes." Proc Natl Acad Sci U S A **91**(20): 9564-8.

Yu, Q., D. Chen, R. Konig, R. Mariani, D. Unutmaz and N. R. Landau (2004). "APOBEC3B and APOBEC3C are potent inhibitors of simian immunodeficiency virus replication." J Biol Chem **279**(51): 53379-86.

Yu, Q., R. Konig, S. Pillai, K. Chiles, M. Kearney, S. Palmer, D. Richman, J. M. Coffin and N. R. Landau (2004). "Single-strand specificity of APOBEC3G accounts for minus-strand deamination of the HIV genome." Nat Struct Mol Biol **11**(5): 435-42. Epub 2004 Apr 18.

Yu, W., J. Chen, Y. Xiong, F. J. Pixley, X. M. Dai, Y. G. Yeung and E. R. Stanley (2008). "CSF-1 receptor structure/function in MacCsf1r-/- macrophages: regulation of proliferation, differentiation, and morphology." J Leukoc Biol.

Yu, X., Y. Yu, B. Liu, K. Luo, W. Kong, P. Mao and X. F. Yu (2003). "Induction of APOBEC3G ubiquitination and degradation by an HIV-1 Vif-Cul5-SCF complex." Science **302**(5647): 1056-60. Epub 2003 Oct 16.

Yurasov, S. V., M. Pettoello-Mantovani, C. A. Raker and H. Goldstein (1999). "HIV type 1 infection of human fetal bone marrow cells induces apoptotic changes in hematopoietic precursor cells and suppresses their in vitro differentiation and capacity to engraft SCID mice." AIDS Res Hum Retroviruses **15**(18): 1639-52.

Zamanian-Daryoush, M., J. T. Marques, M. P. Gantier, M. A. Behlke, M. John, P. Rayman, J. Finke and B. R. Williams (2008). "Determinants of cytokine induction by small interfering RNA in human peripheral blood mononuclear cells." J Interferon Cytokine Res **28**(4): 221-33.

Zhang, H., B. Yang, R. J. Pomerantz, C. Zhang, S. C. Arunachalam and L. Gao (2003). "The cytidine deaminase CEM15 induces hypermutation in newly synthesized HIV-1 DNA." Nature **424**(6944): 94-8.

Zhang, S. Y., E. Jouanguy, V. Sancho-Shimizu, H. von Bernuth, K. Yang, L. Abel, C. Picard, A. Puel and J. L. Casanova (2007). "Human Toll-like receptor-dependent induction of interferons in protective immunity to viruses." Immunol Rev **220**(1): 225-236.

Zheng, Y. H., D. Irwin, T. Kurosu, K. Tokunaga, T. Sata and B. M. Peterlin (2004). "Human APOBEC3F Is Another Host Factor That Blocks Human Immunodeficiency Virus Type 1 Replication." J Virol **78**(11): 6073-6.

Zufferey, R., D. Nagy, R. J. Mandel, L. Naldini and D. Trono (1997). "Multiply attenuated lentiviral vector achieves efficient gene delivery in vivo." Nat Biotechnol **15**(9): 871-5.

7 APPENDIX

7.1 Abbreviations

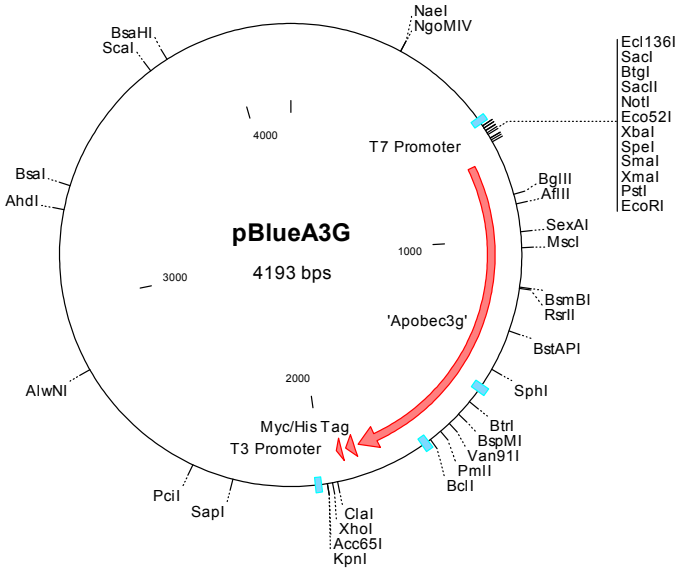
A	Adenine
A3G, APOBEC3G	Apolipoprotein B mRNA-editing enzyme catalytic polypeptide like 3G
AIDS	Acquired Immune Deficiency Syndrome
APS	Ammonium persulfate
ATP	Adenosintriphosphate
c	centi-
C	Celsius
C	Cytosine
CA	Capsid protein
CDC	Center of disease control
Da	Dalton
DCs	Dendritic cells
ddNTP	Dideoxyribonucleotide Triphosphate
DMEM	Dubecco's modified Eagle Medium
DMSO	Dimethyl sulfoxide
dNTP	Deoxyribonucleoside Triphosphate
DNA	deoxyribonucleic acid
def	deficient
dH2O	deionised Water
gp	glycoprotein
dVif	Delta Vif (lacking the Vif protein)
E. coli	Escherichia coli
eGFP	Enhanced green fluorescent protein
Env	Envelope protein
FCS	Fetal calf serum
Flt-3	FMS-like tyrosine kinase 3
g	Gram
G	Guanine
GALT	gut associated lymphoid tissue
GAS	Gamma activated sequence

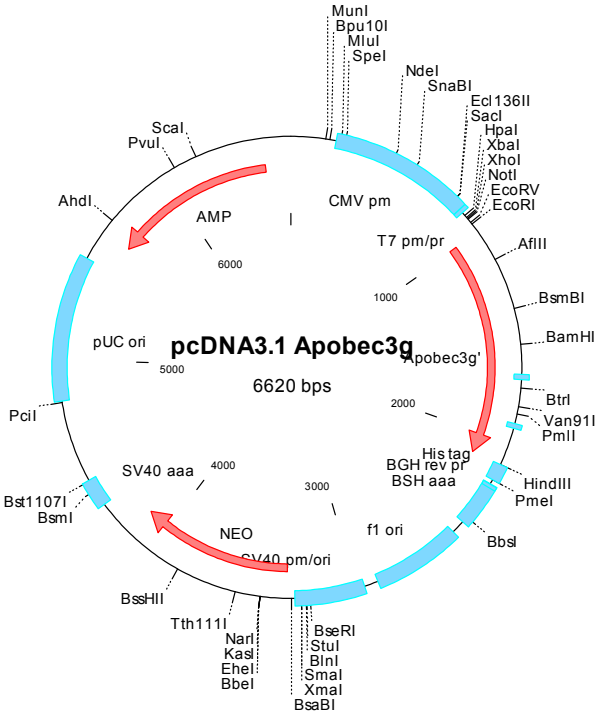
GM-CSF	granulocyte-macrophage colony stimulating factor
h	Hour
H	Hydrogen
HIV	Human immunodeficiency virus
HMM	High molecular mass
IFN	Interferon
IFNAR	Interferon alpha receptor
IMDM	Iscove's Modified Dulbecco's Media
IRF	toll-interleukin 1 receptor (TIR) domain containing adaptor protein
ISRE	Interferon-stimulated response element
ISGF	interferon-stimulated gene factor
k	Kilo-
l	Liter
LB medium	Lysogeny broth medium
iDCs	Immature dendritic cells
IL	Interleukin
IN	integrase
LMM	Low molecular mass
LPS	Lipopolysaccharide
LTR	Long terminal repeats
LVV	Lentiviral vector
m	Meter
m	Milli-
M	Molar
MA	Matrix protein
MAP	mitogen-activated protein
MAPK	mitogen-activated protein kinase
M-CSF	Macrophage colony stimulating factor
MEM	Minimal Essential Medium
min	Minute
mol	Mol
mRNA	messenger Ribonucleic acid
MyD88	Myeloid differentiation primary response gene 88
n	Nano-
NC	Nucleocapsid protein
Nef	Negative factor
OAS1	Oligoadenylate synthetase

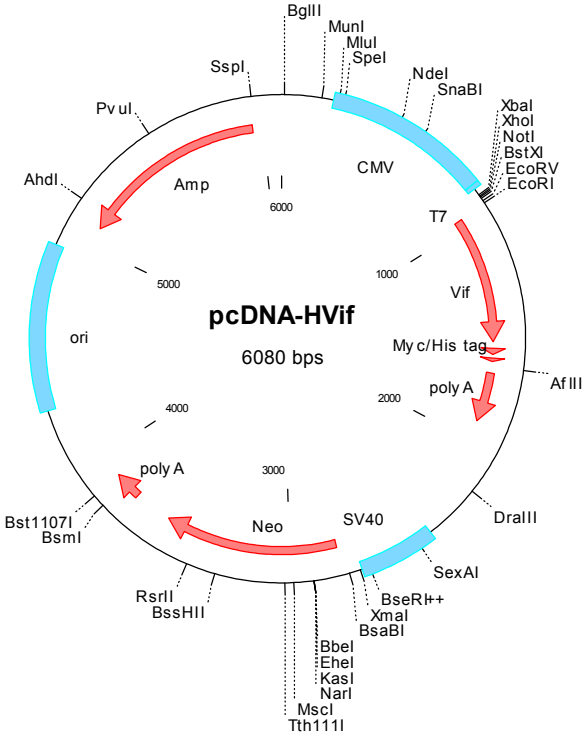
µ	Micro-
PAGE	SDS–polyacrylamide gel electrophoresis
PB region	Primer binding region
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
pDCs	Plasmacytoid dendritic cells
PEG	polyethylene glycol
pH	potentia hydrogenii
PKR	protein kinase R
Pol	polymerase
Rev	Regulator of viral transport and splicing
RNA	Ribonucleic acid
RNAi	RNA interference
Rpm	Rotations per minute
RRE	Rev responsive element
RT	Room temperature
RT	Reverse transcription
SCF	Stem cell factor
SDS	Sodium Dodecyl Sulfate
sec	Seconds
shRNA	Short hairpin RNA
siRNA	Short interfering RNA
ssRNA	Single stranded RNA
SIV	simian immunodeficiency virus
Stat	signal transducer and activator of transcription
T	Thymine
TAR	Trans-activation response element
TAT	Trans-activator of viral transcription
TBE	Tris-Borate-EDTA Buffer
TEMED	N, N, N’, N’-tetramethylethylenediamine
TIRAP	toll-interleukin 1 receptor (TIR) domain containing adaptor protein
TLR	Toll-like receptor
TPO	Thrombopoietin
TRAM	TRIF-related adaptor molecule
TRIF	Toll/IL-1 receptor (TIR) domain-containing adaptor
Tris	tris(hydroxymethyl) methylamine
U	Unit
UV	Ultraviolet

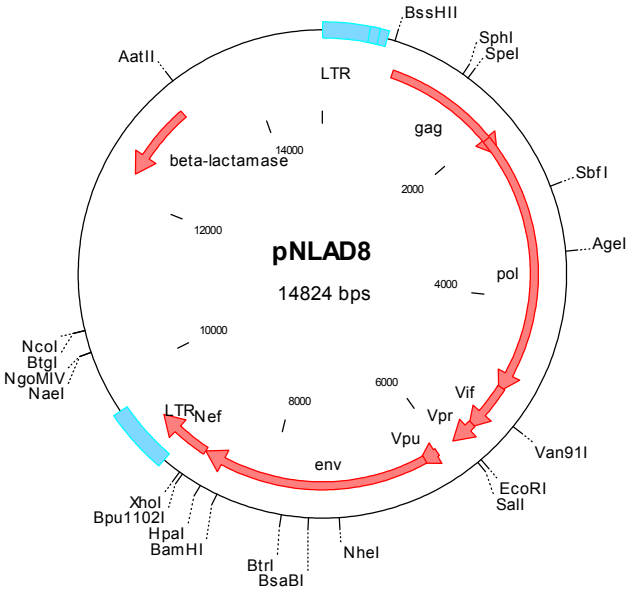
Vif	Viral infectivity factor
Vpr	Viral protein rapid
Vpu	Viral protein out
VSV-G	Vesicular stomatitis virus G-protein
WPRE	woodchuck postregulatory regulatory element
wt	Wild type

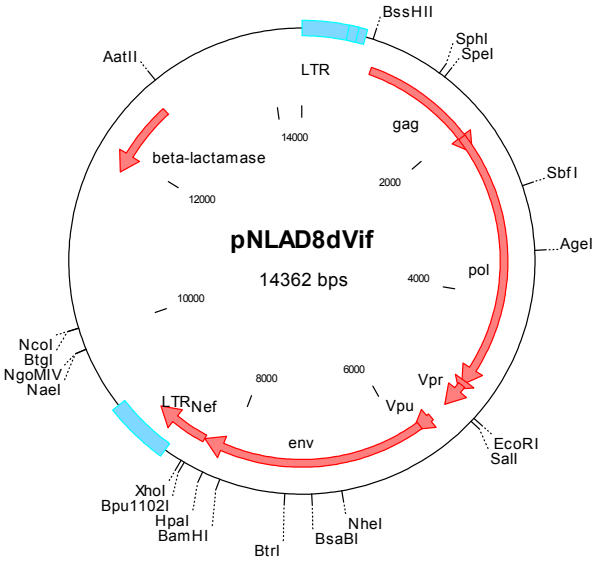
7.2 Plasmid Maps

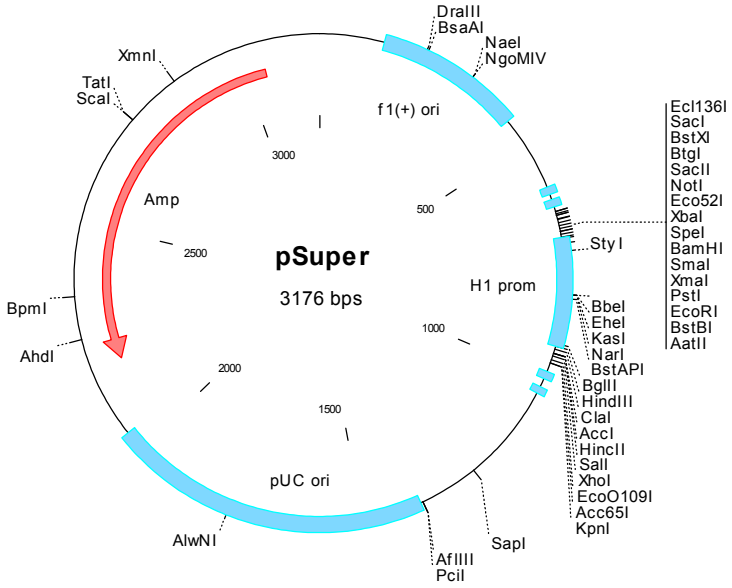


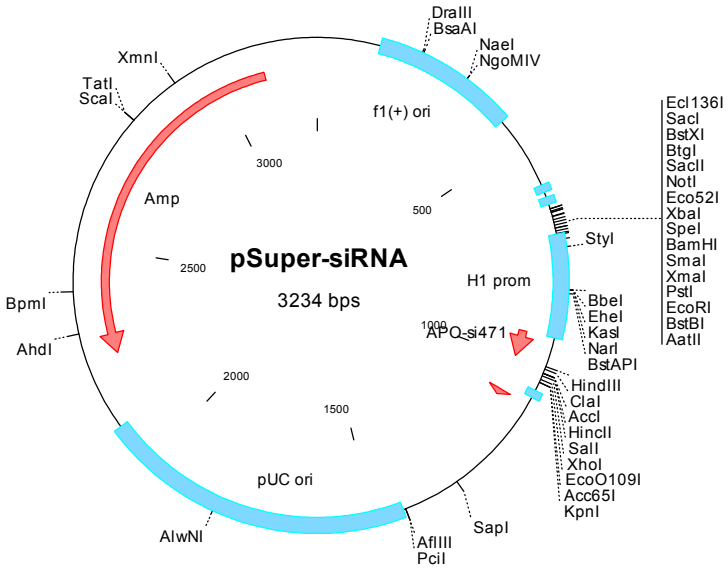


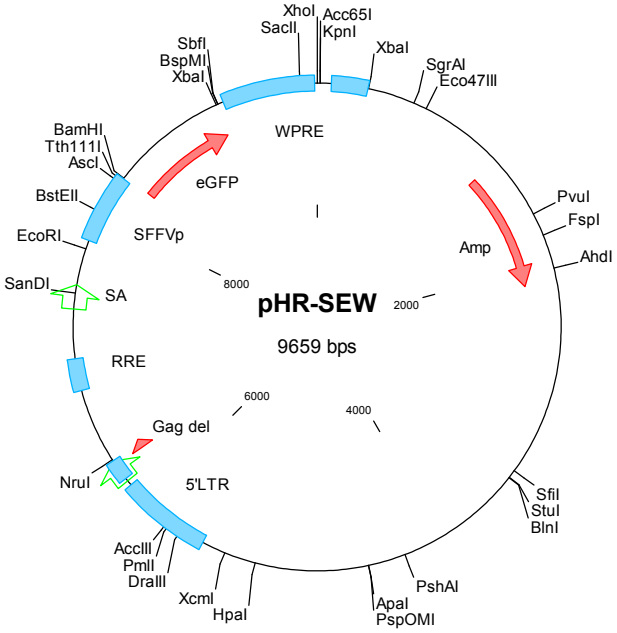

pcDNA-HVif
6080 bps
BglII
MunI
MluI
SpeI
NdeI
SnaBI
XbaI
XhoI
NotI
BstXI
EcoRV
EcoRI
AflII
DraIII
SexAI
BseRI++
XmaI
BsaBI
BbeI
EheI
KasI
NarI
MscI
Tth111I
RsrII
BssHII
Bst1107I
BsmI
AhdI
PvuI
SspI
CMV
T7
Vif
Myc/His tag
poly A
SV40
Neo
poly A
Amp
ori
1000
2000
3000
4000
5000
6000

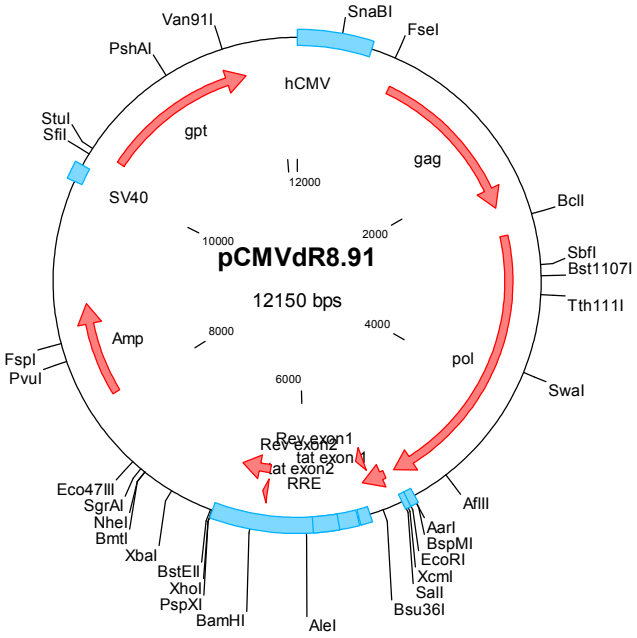

pNLAD8
14824 bps
BssHII
SphI
SpeI
SbfI
AgeI
Van91I
EcoRI
SalI
NheI
BtrI
BsaBI
XhoI
Bpu1102I
HpaI
BamHI
NcoI
BtgI
NgoMIV
NaeI
AatII
LTR
gag
pol
Vif
Vpr
Vpu
env
Nef
LTR
beta-lactamase
2000
4000
6000
8000
10000
12000
14000

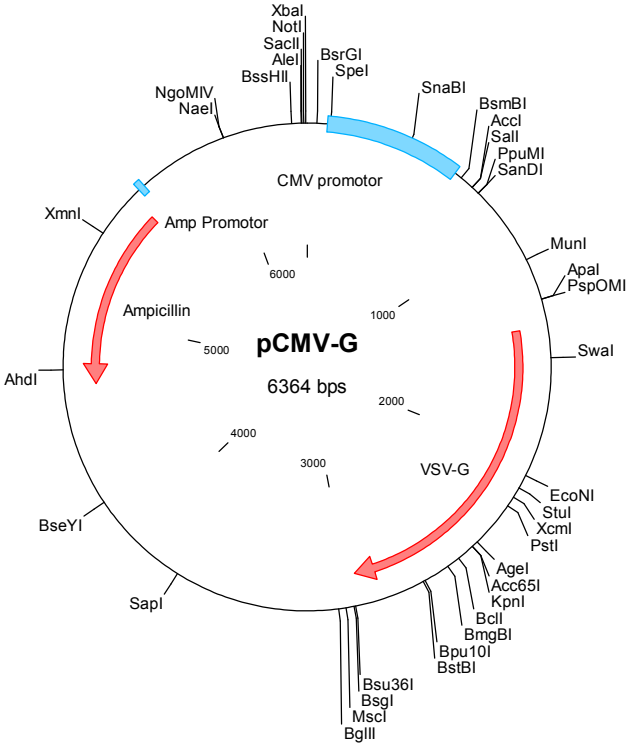

pNLAD8dVif
14362 bps
BssHII
SphI
SpeI
SbfI
AgeI
AatII
LTR
gag
pol
Vpr
Vpu
env
Nef
LTR
beta-lactamase
14000
2000
4000
6000
8000
10000
12000
NcoI
BtgI
NgoMIV
NaeI
EcoRI
SalI
XhoI
Bpu1102I
HpaI
BamHI
BtrI
BsaBI
NheI

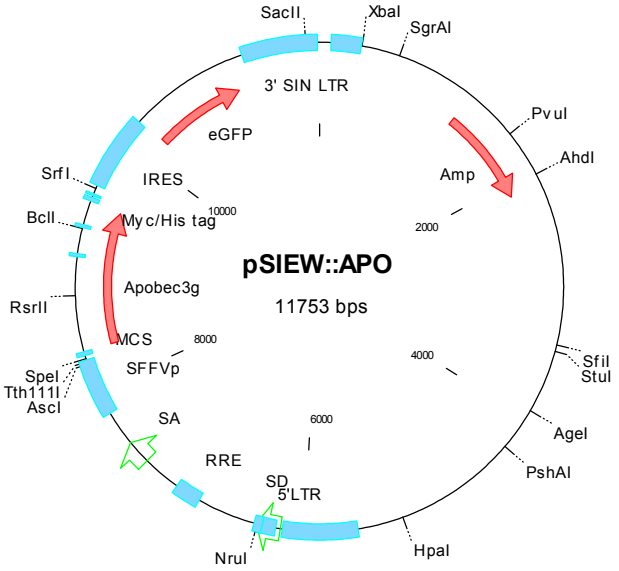

pSuper
3176 bps
DraIII
BsaAI
NaeI
NgoMIV
f1(+) ori
H1 prom
pUC ori
Amp
XmnI
TatI
ScaI
BpmI
AhdI
AlwNI
AflIII
PciI
SapI
StyI
Ecl136I
SacI
BstXI
BtgI
SacII
NotI
Eco52I
XbaI
SpeI
BamHI
SmaI
XmaI
PstI
EcoRI
BstBI
AatII
BbeI
EheI
KasI
NarI
BstAPI
BglII
HindIII
ClaI
AccI
HincII
SalI
XhoI
EcoO109I
Acc65I
KpnI
3000
500
2500
1000
2000
1500

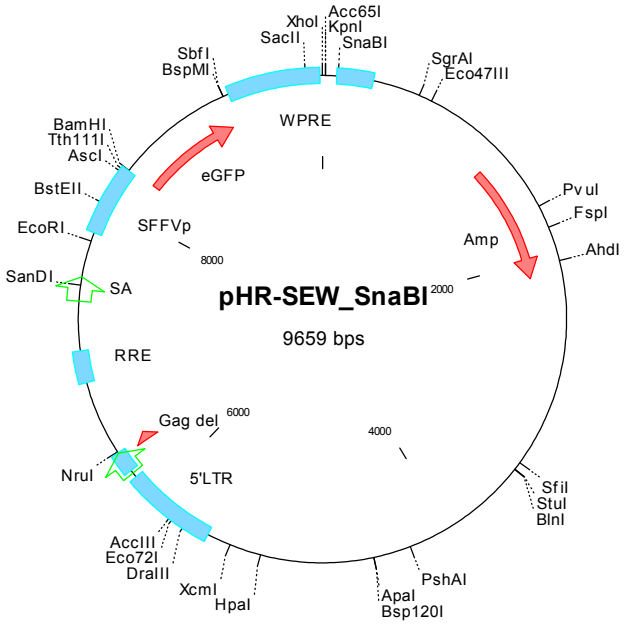

pSuper-siRNA
3234 bps
f1(+) ori
Amp
H1 prom
APO-si471
pUC ori
DraIII
BsaAI
NaeI
NgoMIV
XmnI
TatI
ScaI
Ecl136I
SacI
BstXI
BtgI
SacII
NotI
Eco52I
XbaI
SpeI
BamHI
SmaI
XmaI
PstI
EcoRI
BstBI
AatII
StyI
BbeI
EheI
KasI
NarI
BstAPI
HindIII
ClaI
AccI
HincII
SalI
XhoI
EcoO109I
Acc65I
KpnI
SapI
AflIII
PciI
AlwNI
AhdI
BpmI
3000
500
1000
1500
2000
2500


pHR-SEW
9659 bps
WPRE
eGFP
SFFVp
Amp
RRE
Gag del
5'LTR
XhoI
SacII
Acc65I
KpnI
XbaI
SgrAI
Eco47III
SbfI
BspMI
XbaI
BamHI
Tth111I
AscI
BstEII
EcoRI
SanDI
SA
NruI
PvuI
FspI
AhdI
SfiI
StuI
BlnI
PshAI
ApaI
PspOMI
HpaI
XcmI
AccIII
PmlI
DraIII
2000
4000
6000
8000

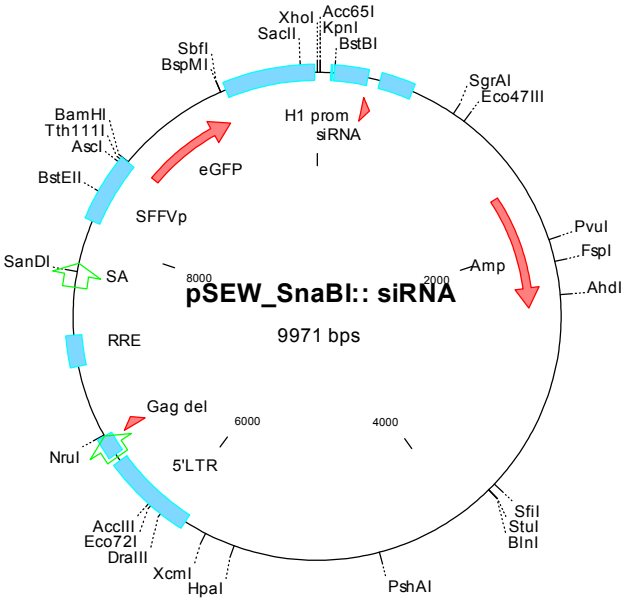

pCMVdR8.91
12150 bps
hCMV
gpt
gag
pol
Amp
SV40
Rev exon1
Rev exon2
tat exon1
tat exon2
RRE
2000
4000
6000
8000
10000
12000
Van91I
PshAI
StuI
SfiI
SnaBI
FseI
BclI
SbfI
Bst1107I
Tth111I
SwaI
AflII
AarI
BspMI
EcoRI
XcmI
SalI
Bsu36I
AleI
BamHI
PspXI
XhoI
BstEII
XbaI
NheI
BmtI
SgrAI
Eco47III
FspI
PvuI


pCMV-G
6364 bps
CMV promotor
Amp Promotor
Ampicillin
VSV-G
1000
2000
3000
4000
5000
6000
XbaI
NotI
SacII
AleI
BssHII
BsrGI
SpeI
SnaBI
BsmBI
AccI
SalI
PpuMI
SanDI
NgoMIV
NaeI
XmnI
AhdI
BseYI
SapI
MunI
ApaI
PspOMI
SwaI
EcoNI
StuI
XcmI
PstI
AgeI
Acc65I
KpnI
BclI
BmgBI
Bpu10I
BstBI
Bsu36I
BsgI
MscI
BglII





The vector pSEW_SnaBI, was derived from the pSEW vector, but contains a SnaBI digestion site in the 3'LTR. The site can be used to introduce a shRNA promoter with a shRNA via blunt end ligation. Oligonucleotides for shRNAs against APOBEC3G were ordered and introduced into the pSuper vector containing an H1 promoter. The complete HI promoter and the shRNA sequence were then cloned into the pSEW_SnaBI vector using the SnaBI digestion site. The vector should express the shRNA in addition to the eGFP protein as a marker for cells that were successfully transduced.


XhoI
Acc65I
KpnI
SacII
BstBI
SbfI
BspMI
SgrAI
Eco47III
H1 prom
siRNA
BamHI
Tth111I
AscI
eGFP
BstEII
SFFVp
PvuI
FspI
SanDI
SA
8000
2000
Amp
AhdI
pSEW_SnaBI:: siRNA
9971 bps
RRE
Gag del
6000
4000
NruI
5'LTR
SfiI
StuI
BlnI
AccIII
Eco72I
DraIII
XcmI
HpaI
PshAI

7.3 P24 amounts during Infection

7.3.1 CD34+ cells

7.3.1.1 Donor #1

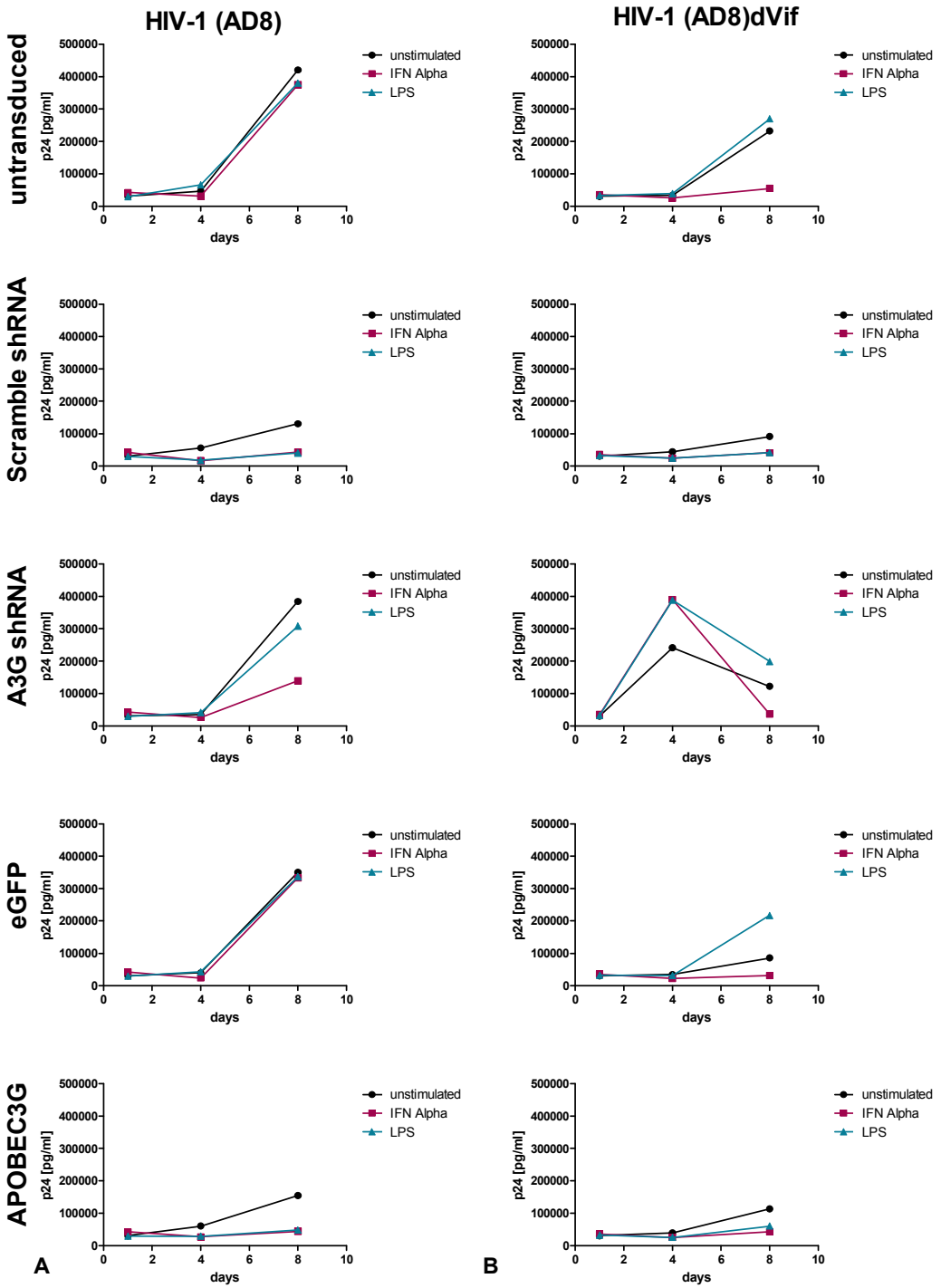


Figure 40. Infection of CD34+ cells from donor #1. The p24 expression in transfected CD34+ cells from donor #1 infected with either (A) HIV-1 (AD8) or (B) HIV-1 (AD8) dVif virus.

7.3.1.2 Donor #2

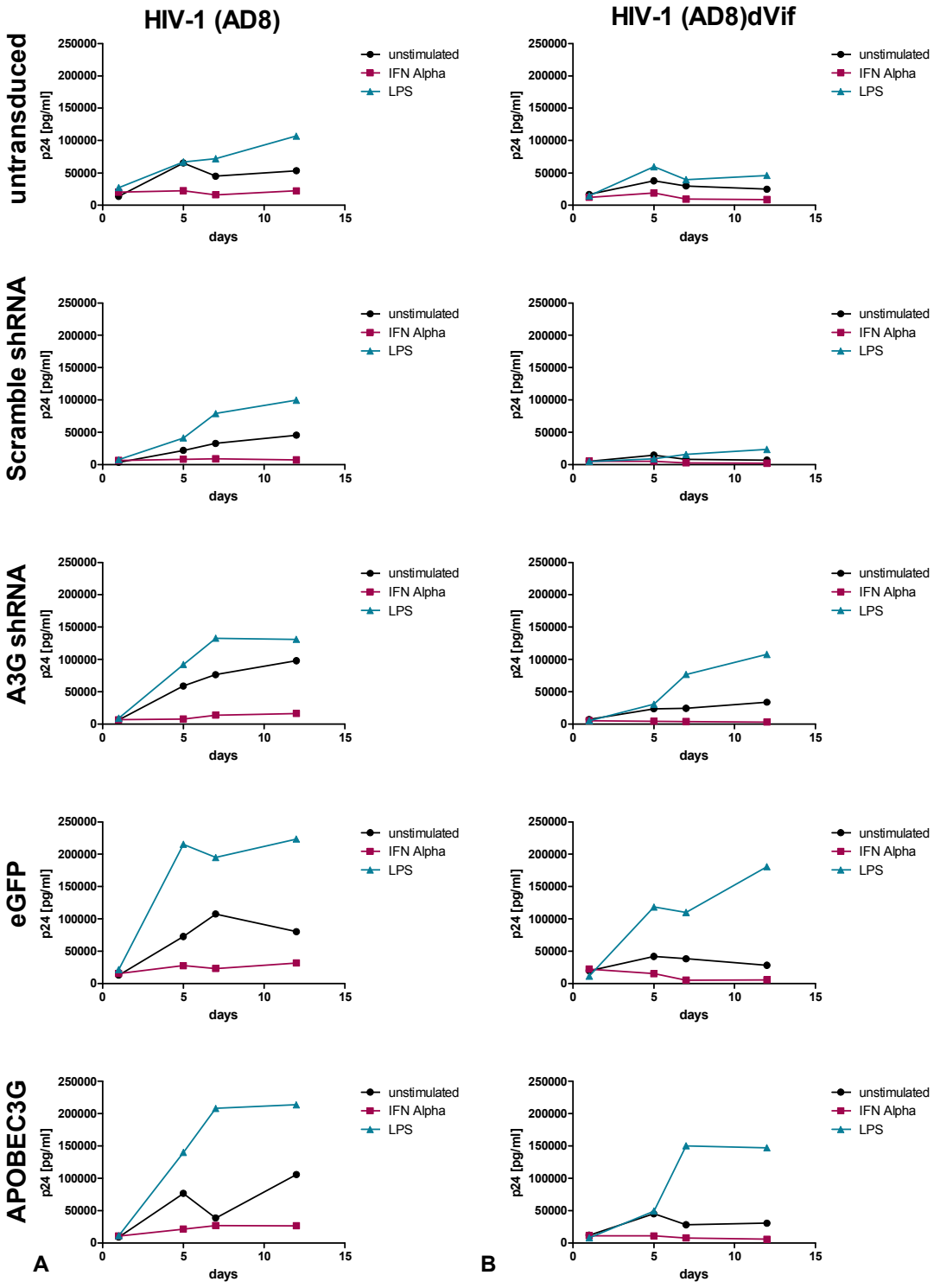


Figure 41. Infection of CD34+ cells from donor #2. The p24 expression in transfected CD34+ cells from donor #2 infected with either (A) HIV-1 (AD8) or (B) HIV-1 (AD8) dVif virus.

7.3.1.3 Donor #3

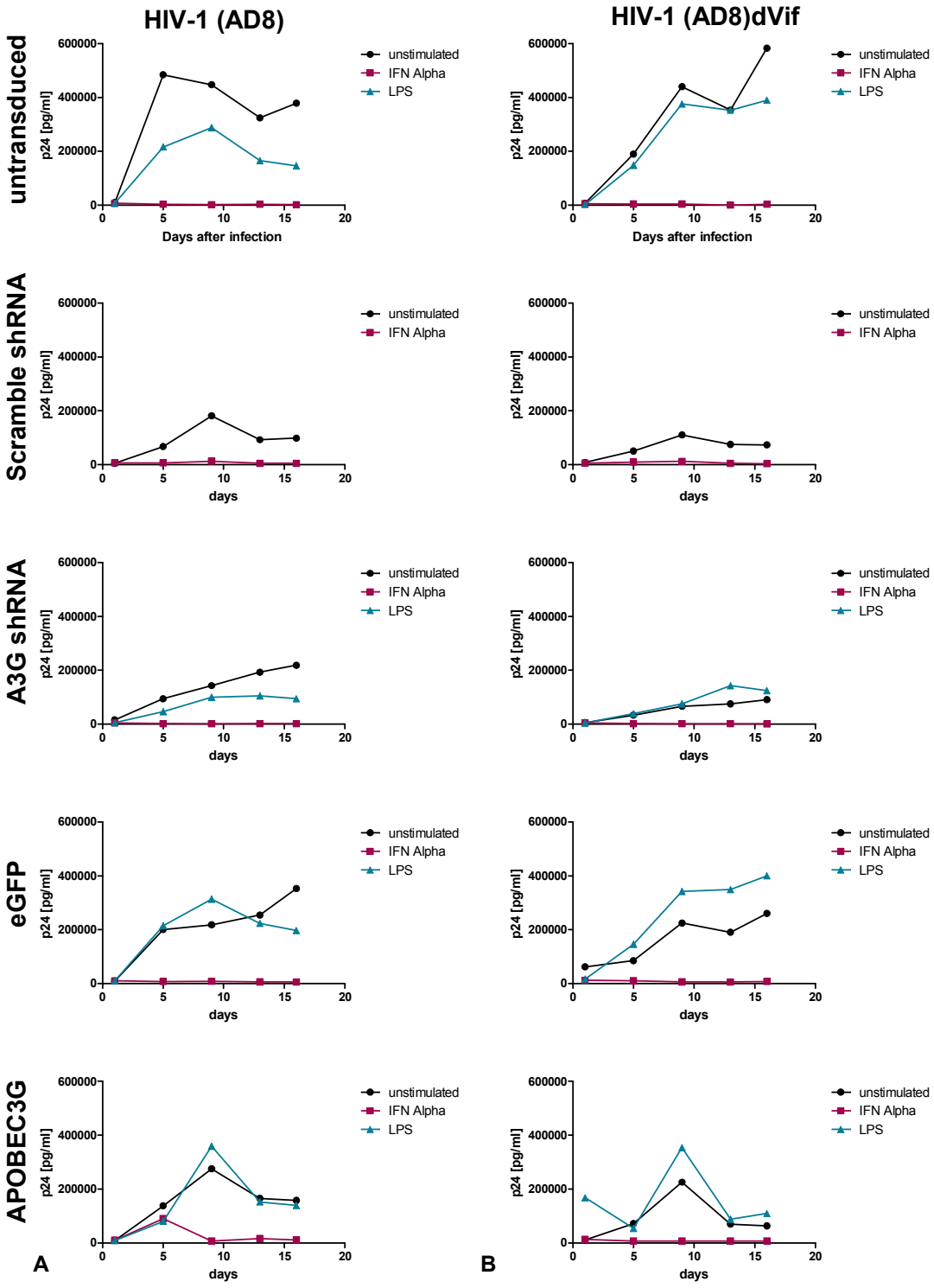


Figure 42. <u>Infection of CD34+ cells from donor #3.</u> The p24 expression in transfected CD34+ cells from donor #3 infected with either (A) HIV-1 (AD8) or (B) HIV-1 (AD8) dVif virus.

7.3.2 CD34 derived macrophages

7.3.2.1 Donor#1

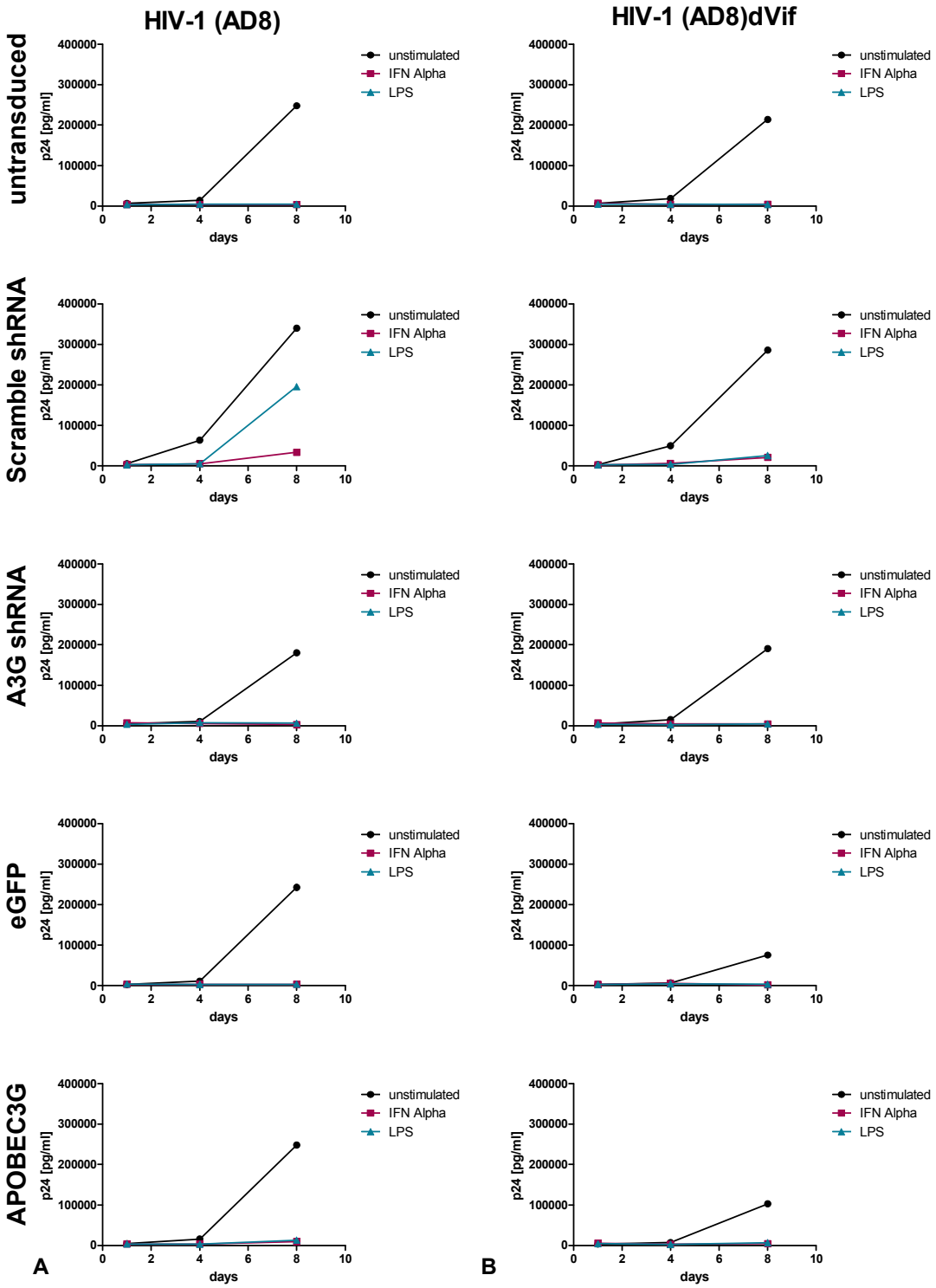


Figure 43. Infection of CD34+ derived macrophages from donor #1. The p24 expression in transfected CD34+ cells from donor #1 infected with either (A) HIV-1 (AD8) or (B) HIV-1 (AD8) dVif virus.

7.3.2.2 Donor #2

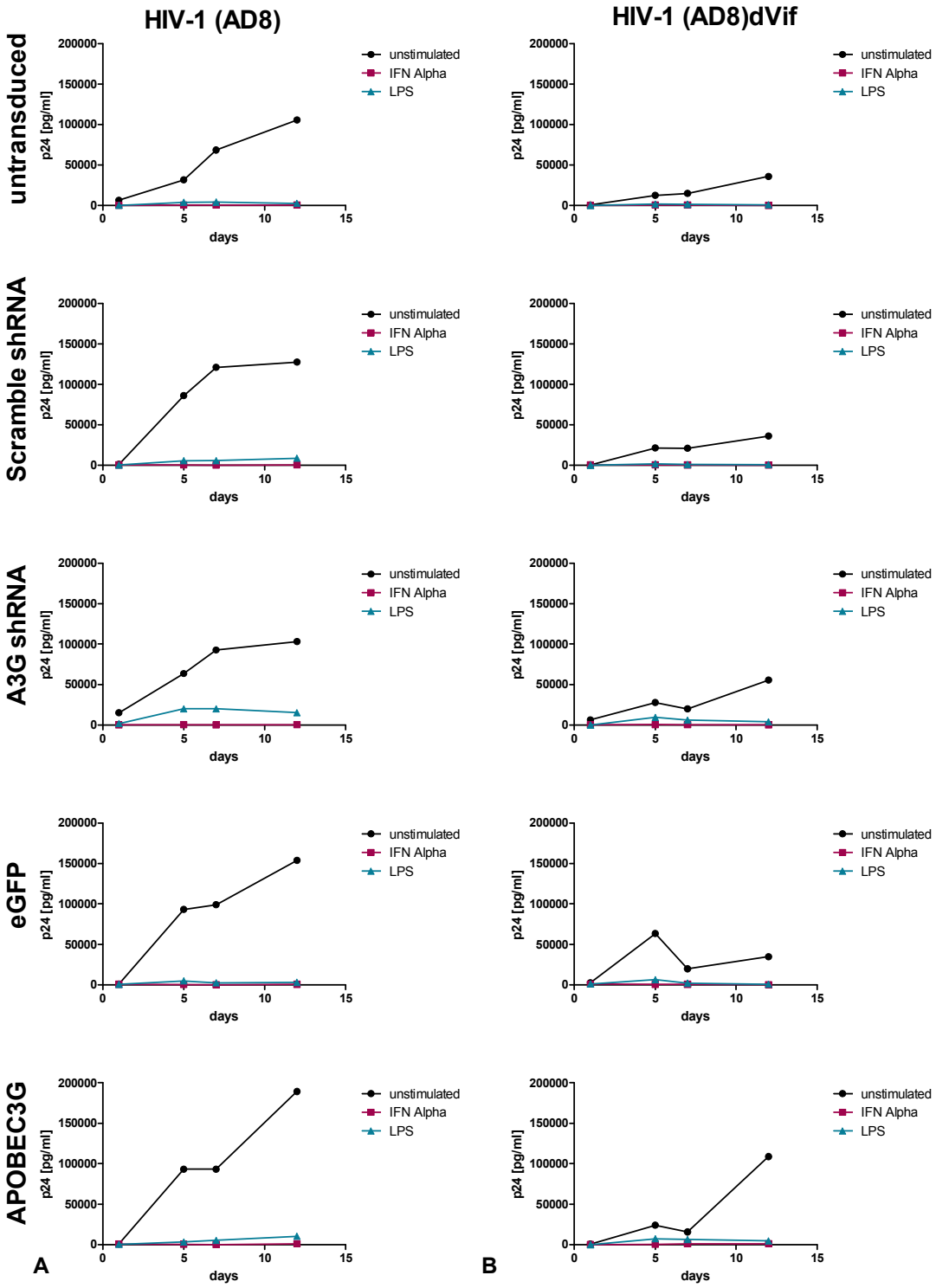


Figure 44. <u>Infection of CD34+ derived macrophages from donor #2.</u> The p24 expression in transfected CD34+ cells from donor #2 infected with either (A) HIV-1 (AD8) or (B) HIV-1 (AD8) dVif virus.

7.3.2.3 Donor #3

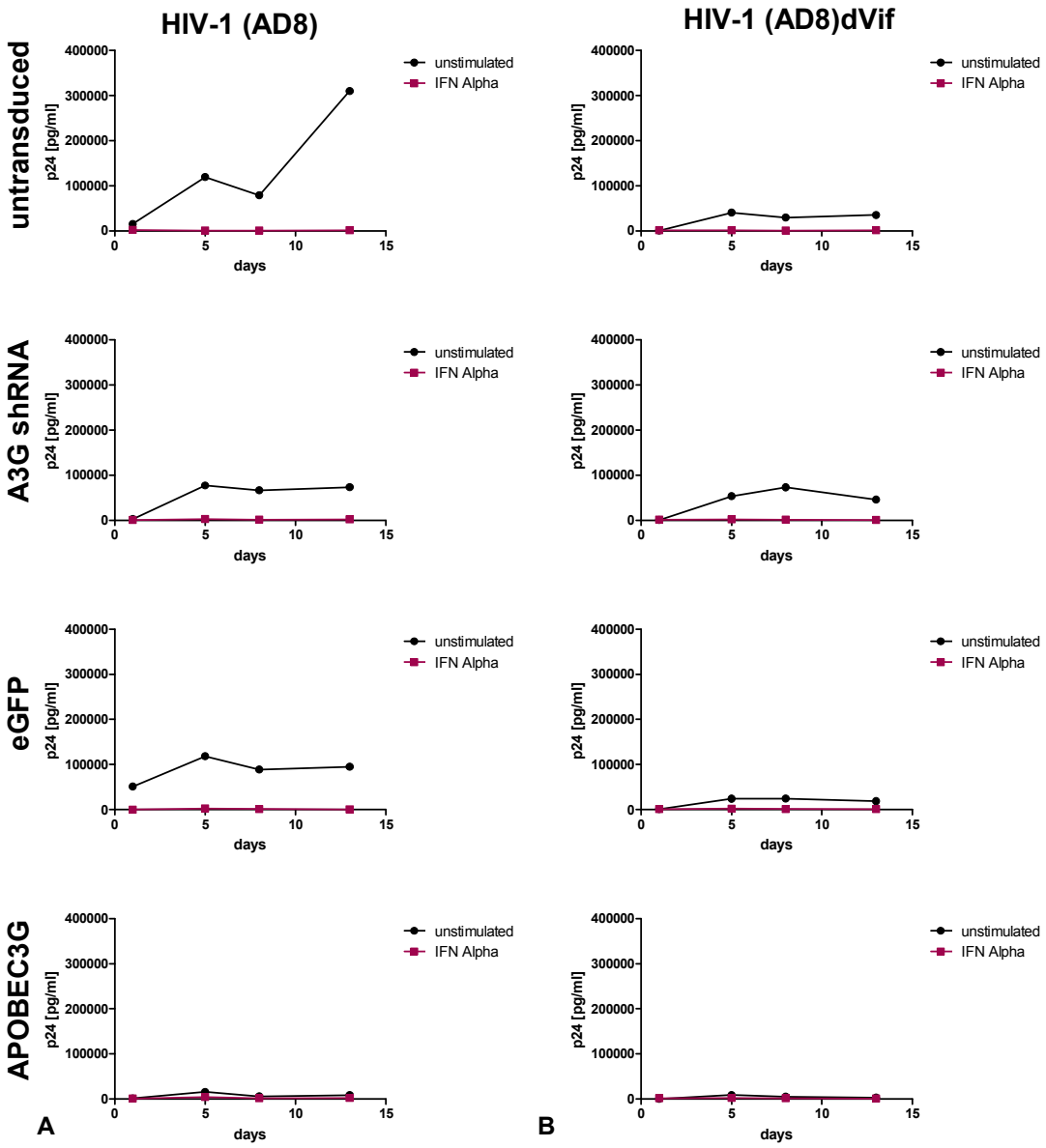


Figure 45. Infection of CD34+ derived macrophages from donor #3. The p24 expression in transfected CD34+ cells from donor #3 infected with either (A) HIV-1 (AD8) or (B) HIV-1 (AD8) dVif virus.

LEBENSLAUF

Name und Anschrift	Anna Heinzelmann Fuchstanzstraße 61 60489 Frankfurt am Main Tel.: 069/26012233 Heinzel@gmail.com
Persönliche Daten	
Geburtsdatum und -ort	28.05.1978, Altoona, Pennsylvania (USA)
Familienstand	verheiratet
Staatsangehörigkeit	USA
Schulbildung	
1993 -1996	Altoona Area High School, Altoona, Pennsylvania (USA) Abschluss: High School Diploma, Note 92.1/100
Studium	
1/2008 – 7/2008	Doktorarbeit: Fraunhofer-Institut für Biomedizinische Technik Weiterführung der Doktorarbeit unter der Betreuung von PD Dr. Hagen von Briesen und Dr. Ursula Dietrich.
10/2004 – 1/2008	Georg-Speyer-Haus Doktorarbeit zum Thema „HIV neutralisierende Mechanismen bei der Infektion von Makrophagen“ an der biochemischen Fakultät der Johann Wolfgang Goethe-Universität Frankfurt. Betreuung durch PD Dr. Hagen von Briesen und Dr. Ursula Dietrich.
10/2000 – 09/2004	Philipps-Universität Marburg Schwerpunktfächer: Virologie, Mikrobiologie und Genetik Studiendauer: 8 Fachsemester Diplomarbeit: Die Rolle von APOBEC3G in HIV-1-infizierten Monocyten/Macrophagen Note 1,8
10/1998 – 07/1999	Austauschjahr: Philipps-Universität Marburg Schwerpunkte Biologie und Deutsch
08/1996 – 05/2000	Juniata College Huntingdon, Pennsylvania (USA) Schwerpunktfächer: Biologie und Deutsch Abschluss: Bachelor of Science (BS) Note 3.02/4.00
Praktische Erfahrungen	
06/2001 - 08/2001	Praktikum, Max-Planck-Institut für terrestrische Mikrobiologie, Marburg
05/2000 - 10/2000	Kelly Scientific Resources: Quality Assurance Analyst at Wyeth-Ayerst Labs, Marietta, Pennsylvania (USA)

01/1997 - 05/1997 und 07/1999 - 05/2000	Studentische Hilfskraft, Juniata College Biology Department Forschung in der Zellbiologie bei Dr. Jeffery Demarest Thema: Untersuchung der chlor-ionenkanal Hemmer in der *Acornus calamus* Wurzel und die Entwicklung eines Protokolls, um den Einfluss von Chemikalien an den Pflanzen Stomata zu untersuchen. Ergebnisse bei der National Conference for Undergraduate Research in Montana, USA vorgestellt.
Posterpräsentationen	Heinzelmann, A., Meyerhans, A., Dietrich, U., von Briesen, H. (2007) APOBEC3G in infected myeloid cells as an antiviral target. Third European Congress of Virology (Jahrestagung GfV), Nürnberg, Germany. Heinzelmann, A., Hüther, A., Dietz, J., Königs, C., Dervillez, X., Humbert, M., Dietrich, U. (2007) Selection of peptides for HIV-1 inhibition by the phage display technology. Targeting Replication and Integration of HIV (TRIoH) General Assembly, Leuven, Belgium. Heinzelmann, A., Crnomut, D., Meyerhans, A., v. Briesen, H., Dietrich, U. (2006) APOBEC3G in infected myeloid cells as an antiviral target. Targeting Replication and Integration of HIV (TRIoH) General Assembly, Barcelona, Spain. Heinzelmann, A., Bracharz, S., Landersz, M., Bouazzaoui, A., Meyerhans, A., Dietrich, U., v. Briesen, H. 2006 Upregulation of APOBEC3G in Macrophages with Restricted HIV-1 Replication. Jahrestagung der Gesellschaft für Virologie, München, Germany. 2006
Publikationen	Bouazzaoui A, Kreutz M, Eisert V, Dinauer N, Heinzelmann A, Hallenberger S, Strayle J, Walker R, Rübsamen-Waigmann H, Andreesen R, von Briesen H. Stimulated trans-acting factor of 50 kDa (Staf50) inhibits HIV-1 replication in human monocyte-derived macrophages. Virology. 2006 Dec 5-20;356(1-2):79-94. Epub 2006 Aug 22.
Kenntnisse	
Labor Methoden	Allgemeine Zellkultur Methoden Prämare Zellen Isolierung und Kultivierung FACS Analyse allgemeine virologische Arbeit Arbeiten mit lentiretroviralen Vektoren allgemein Protein biochemische Methoden Molekulare Biologie/Klonierungen
EDV-Anwendung	Microsoft Word, Excel, PowerPoint, Endnote, Clone Manager, DNAstar
Sprachen	Englisch und Deutsch fließend