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Characteristics of a Foamy Virus-Derived Vector that allow for safe Autologous Gene Therapy to correct Leukocyte Adhesion Deficiency Type 1

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Characteristics of a Foamy Virus-Derived Vector that allow for safe autologous gene therapy to correct Leukocyte Adhesion Deficiency Type-1

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By

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I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Ryan McNichol ENTITLED Characteristics of a Foamy Virus-Derived Vector that allow for safe Autologous Gene Therapy to Correct Leukocyte Adhesion Deficiency Type 1 BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science

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Abstract

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Characteristics of a Foamy Virus-Derived Vector that allow for safe autologous gene therapy to correct Leukocyte Adhesion Deficiency Type 1.

The hematopoeitic stem cell is a prime target for gene therapy in the attempt to correct a number of single gene inherited genetic defects that affect the immune system. In persons affected by leukocyte adhesion deficiency type 1 (LAD-1) the gene for the Beta-2 subunit of the integrin molecule is mutated. This autosomal recessive gene defect yields a phenotype with little or no beta-2 integrin expression on leukocytes. Beta-2 integrin expression is essential for leukocytes to travel from the blood to the tissues to fight infection. Persons with this disease have lowered leukocyte counts in the tissues and as a result are beset with recurrent infections which often cause death within the first year of life. As an alternative to using sibling bone marrow transplants, a treatment using retroviral vectors in autologous gene therapy is being studied to correct the disease. Problems surrounding retroviral vectors include 1) a dependence on cell division for integration, 2) short term transgene expression, 3) insertional mutagenesis and 4) the potential of becoming replication competent. The latest alternative in the treatment of LAD-1 is the use of vectors derived from human foamy viruses. These viruses show no human pathogenicity. The foamy viruses have displayed promising integration patterns in cells and are able to survive in quiescent cells that will later divide. Furthermore, these viruses can be modified to become replication incompetent. Using the promoter sequence for the integrin subunit CD18 to direct transgene expression will further increase the safety of this vector in treating LAD-1.

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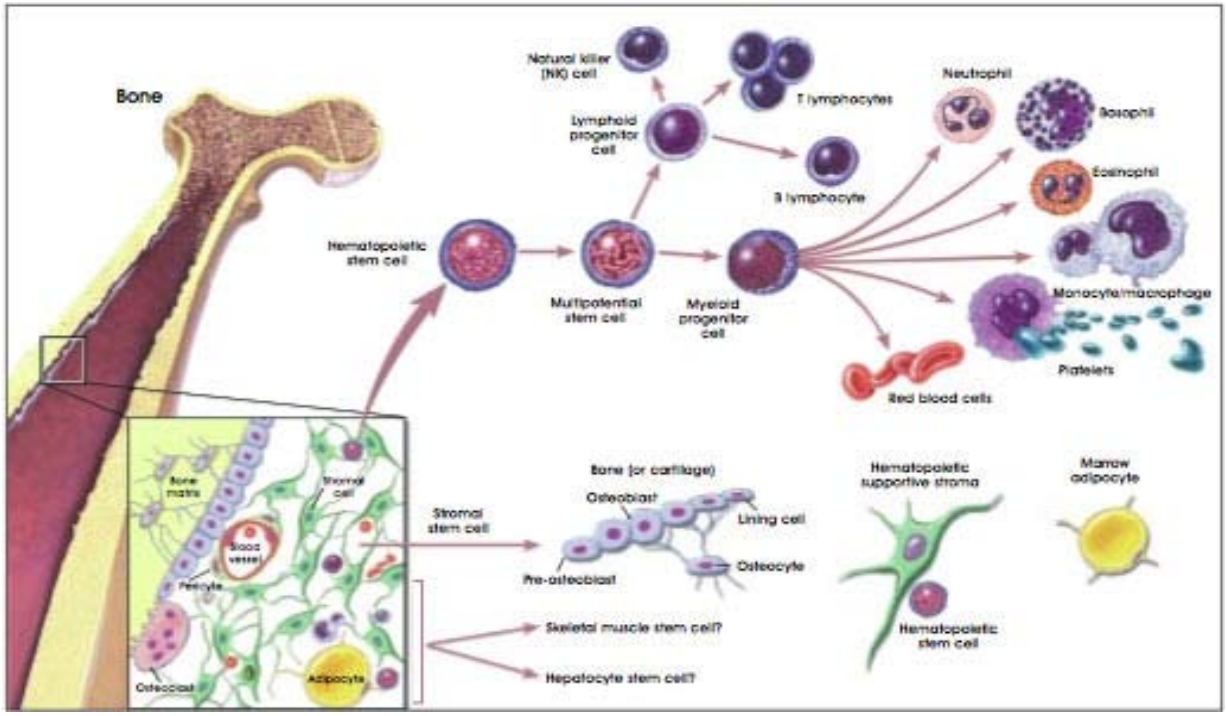
INTRODUCTION

Hematopoeitic stem cells (HSCs) are the self renewing adult stem cell population in the bone marrow which gives rise to all the cell types in the blood. The HSCs undergo asymmetric cell division and eventually give rise to lymphoid and myeloid progenitors. The lymphoid progenitors divide and further differentiate to become the thymocytes in the thymus, the B-cell precursors in the bone marrow, and the natural killer cells. The myeloid progenitors also divide and further differentiate to become mast cells, neutrophils, eosinophils, basophils, erythrocytes, the platelet producing megakaryocytes and the blood monocytes which give rise to tissue macrophages. Dendritic cells arise from both the lymphoid and myeloid progenitors (29, 47).

Figure 1. Origin of the Cells of the Immune System



Adapted from http://stemcells.nih.gov/info/scireport/chapter5.asp

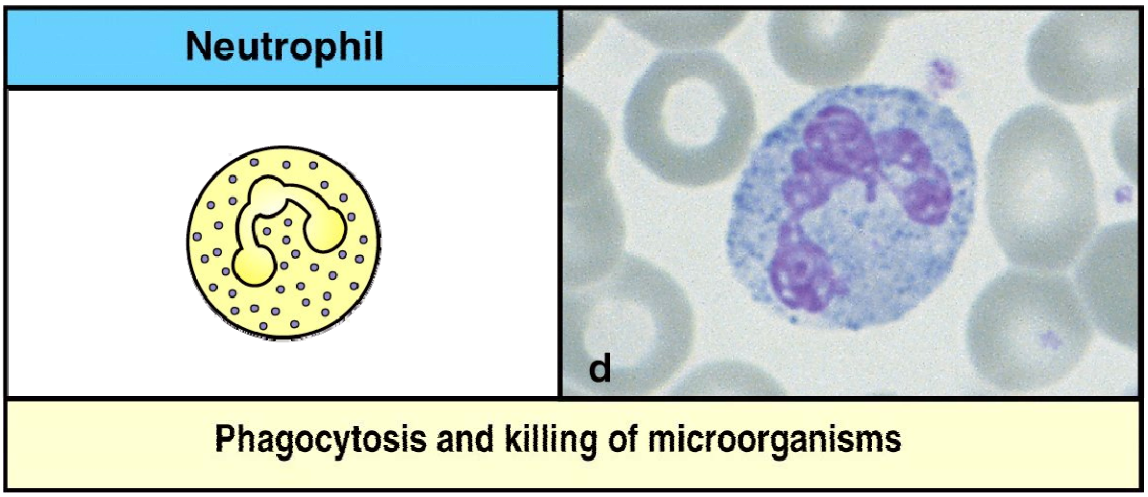
The immune cells, red blood cells and megakaryocytes derived from HSCs will sometimes have malfunctioning proteins or even lack certain cell surface and intracellular signaling proteins. The result is a variety of blood diseases. These problems make the HSC population an attractive target for viral vector-mediated gene therapy to correct a number of genetically inherited diseases. Some of these target diseases include sickle cell anemia (49), beta-thalassemia (44), adenosine deaminase-deficient severe combined immunodeficiency (ADA SCID) (27), X-linked SCID (16), X-linked agammaglobulinemia (67), chronic granulomatous disease (22), Wiskott-Aldrich syndrome (55), and leukocyte adhesion deficiency type 1 (LAD-1) (13), a very rare disorder in which neutrophil migration is impaired.

NEUTROPHILS

The cells that are primarily responsible for clearing infectious agents from the body are the phagocytes, the macrophages and the neutrophils. The most abundant of these cell types is the neutrophil, which comprises over 50% of the total circulating white blood cells. Due to their short 12-16 day life span neutrophils are produced at a rate of 100 billion cells per day (14, 25, 66). Three billion of these cells daily travel to the mucosal tissues of the nose and throat to protect these areas of high pathogen exposure. This cell type is also known as the polymorphonuclear neutrophil leukocyte and is easily distinguished from eosinophils and basophils by its multilobed nucleus (figure 2) (29). Neutrophils originate in the bone marrow and circulate in the blood until they are summoned to enter infected tissue by complement proteins or inflammatory signals from the site of an infection. Neutrophils move from the blood stream to the tissue by

squeezing between endothelial cells of the blood vessel wall, a process called diapedesis, and follow a chemokine gradient to the site of infection to phagocytose pathogenic invaders. The engulfed pathogens are safely destroyed by reactive oxygen species and microbicidal proteins while causing little or no damage to surrounding tissue (29, 47, 66). After having carried out their function, neutrophils then begin to undergo apoptosis and are ingested by macrophages. The average lifespan of a mature neutrophil is 12-16 days (14, 25, 66).

Figure 2. Diagram and Light Microscope Image of a Neutrophil



Adapted from Parham, The Immune System 2nd ed. 2005

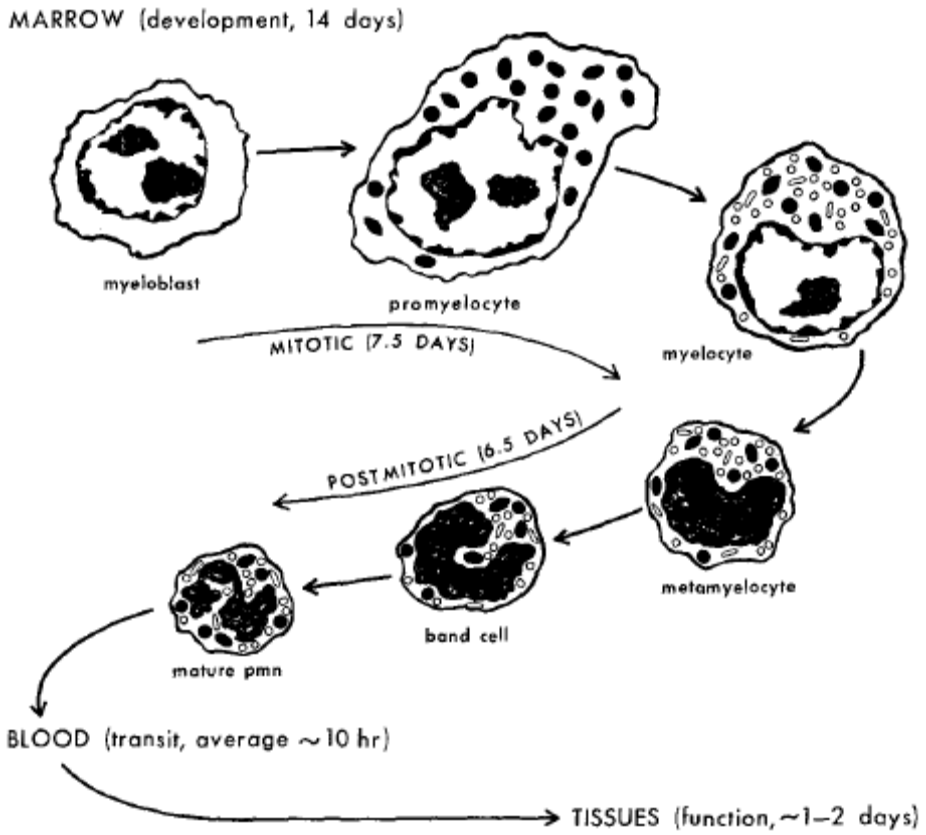
Neutrophil Development

The development of a Neutrophil in the bone marrow from its most immature precursor to a fully functional mature phagocyte includes six stages. The stages are: Myeloblast → Promyelocyte → Myelocyte → Metamyelocyte → Band →Mature Polymorphonuclear Neutrophil (Figure 3) (5). The first three stages of development are

mitotic while the last three are not mitotic. During the promyelocyte, myelocyte, metamyelocyte and band stages of development the azurophilic, specific and gelatinase granules are produced. The azurophilic and specific granules are produced in the promyelocyte and myelocyte stages respectively and contain mainly the potent microbicidal proteins that the neutrophil uses to destroy pathogens. The granules act on the invading pathogen in one of two ways. The granules are delivered to the phagosome or they are exocytosed to destroy foreign invaders in the extracellular matrix. The gelatinase granules are composed primarily of metalloproteases capable of cleaving the constituents of the basement membrane and extracellular matrix and allow the neutrophil to move out of the blood stream and migrate through the tissues (20).

After development, neutrophils can be found in the bone marrow, the blood stream, or in the tissues. In the blood, neutrophils are classified as one of two types based on their relationship to the vascular endothelial wall. The neutrophils which move slowly through circulation making brief, reversible, attachments to the blood vessel wall are called marginal neutrophils while freely circulating neutrophils are referred to as circulating neutrophils (5, 14, 25).

Figure 3. Development of a Neutrophil



Adapted from Bainton et al. 1971

Neutrophil Migration

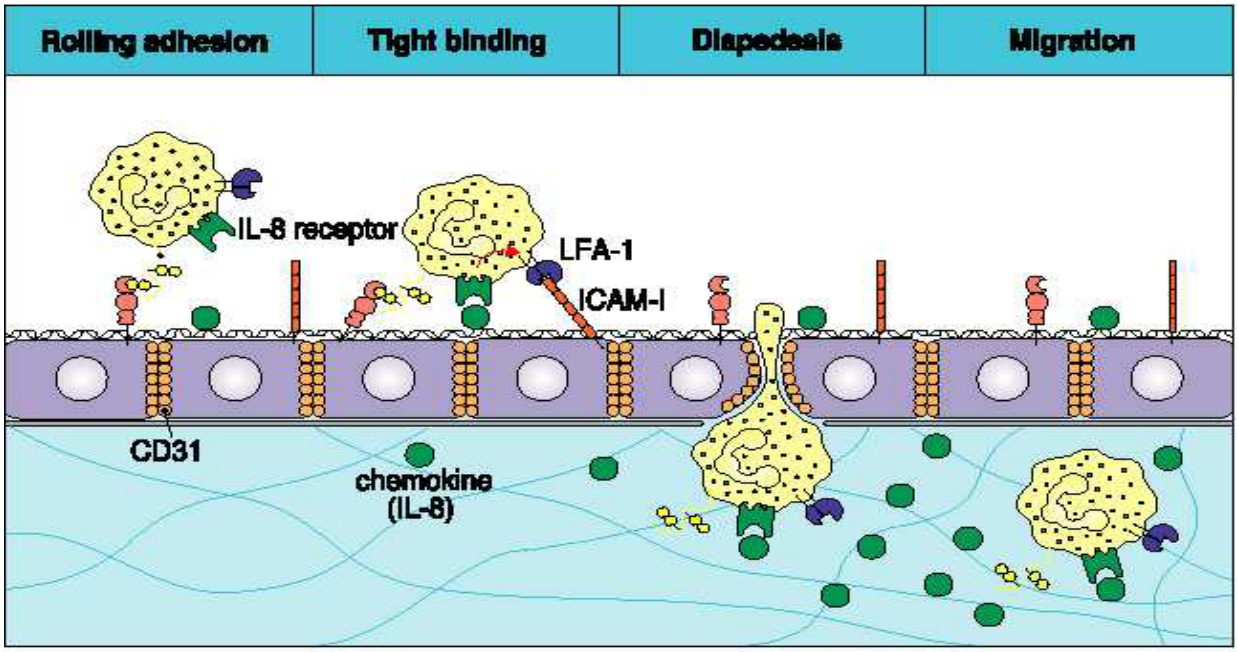
Mature neutrophils in the bone marrow are summoned into the blood by the inflammatory cytokines interleukin-1 (IL-1), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α). Neutrophils in the blood can be converted from circulating neutrophils to marginal neutrophils through the function of selectins. Chemokines and cytokines released by tissue macrophages induce the expression of L-selectin on neutrophils and E- and P-selectins on vascular endothelial cells at the post-capillary venule. Selectins make

transient attachments to their carbohydrate ligands on an opposing surface to mediate "neutrophil rolling" where neutrophils roll along the blood vessel wall prior to migration into tissues. E- and P-selectins bind to the carbodrate molecules sialyl-Lewis X and P-selectin Glycoprotein Ligand-1 (PSGL-1) of neutrophils respectively. L-selectins bind to sulfated sialyl-Lewis X of the endothelial cell surface. Rolling is a prerequisite for neutrophils that migrate from the blood to the site of infected or inflamed tissue (14, 25, 29, 47, 66).

The next step in migration involves the interaction of integrin molecules on the neutrophil with intercellular adhesion molecules (ICAMs) on the endothelial cell. This interaction mediates the events of arrest and migration across the blood vessel wall at the site of infection or inflammation. The integrins CD11a/CD18 (leukocyte functional antigen-1 (LFA-1)), CD11b/CD18 (Mac-1 or complement receptor 3 (CR3)) and CD11c/CD18 (p150,95) are composed of an alpha protein (CD11) and a beta-2 protein (CD18). These are the three main integrins involved in arresting the neutrophil. The beta-2 protein of this pair of integrin molecules assumes an active form when neutrophils are exposed to inflammatory chemoattractants such as C5a, and IL-8. These chemoattractants not only increase the integrin affinity for ICAMs but they also induce the neutrophil to discard L-selectin molecules. On the endothelial cell, the ICAM-1 is upregulated mainly by the inflammatory cytokines interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α). The activated integrin molecule attached to the ICAM then brings the rolling neutrophil to rest and sets the stage for transmigration across the endothelial wall (14, 25, 29, 47, 66).

At the stage of arrest, the neutrophil crosses the endothelial wall through the interaction of platelet endothelial cell adhesion molecule-1 (PECAM-1) (also known as CD31). PECAM-1 is expressed both on neutrophils and at the junction between endothelial cells. At this intercellular junction, CD31 on neutrophils binds to CD31 on the endothelial cell to perform transmigration (25, 29, 47, 66).

Figure 4. Extravasation of a Neutrophil From the Blood to the Tissues



Adapted from Parham, 2001

At this point the only remaining barrier between the neutrophil and the intended tissue is the basement membrane of the post capillary venule wall. The activated neutrophil releases enzymes from its gelatinase granules known as collagenolytic metalloproteases which are responsible for cleaving the basement membrane of the blood vessel wall (20).

Following the neutrophil entry into the tissues it migrates to the foreign invader that elicited its migration. The neutrophil travels to the pathogen along a chemical gradient by an amoeboid movement. Some of the chemoattractants for which the neutrophil possesses receptors are C5a (a product of the complement cascade), C5a des Arg (a processed form of C5a), interleukin-8 (IL-8) (also known as the chemokine CXCL8) and proteins containing N-formylmethionine (an amino acid found only on bacterial proteins) (66).

Phagocytosis

A neutrophil expresses a variety of cell surface molecules by which it recognizes foreign invaders and discriminates against human tissue. Infectious bacteria possess pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharide (LPS) on their surface. The neutrophil will bind to LPS by its CD14 and CR4 receptors to initiate phagocytosis. The neutrophil can also bind to opsonins such as complement protein and antibody which coat the surface of the pathogen. Complement receptor 3 (also known as CD11b/CD18) binds to the abundant complement protein C3b while the Fc receptor binds to the Fc region of pathogen bound antibody (14, 25, 29, 47, 66).

The process of engulfment (endocytosis) follows pathogen binding and psuedopods of the neutrophil project out around the bound pathogen and fuse together to form a phagosome, a membrane bound chamber within the neutrophil. It is in the phagosome that killing and degradation take place. Many mechanisms are responsible for dismantling the engulfed pathogen, but they can be simplified into two main categories: those processes that involve microbicidal proteins released during the

formation of the phagolysosome and those that involve the generation of reactive oxygen species (ROS). Generation of ROS is initiated when the neutrophil's integrin molecule binds to an ICAM (66).

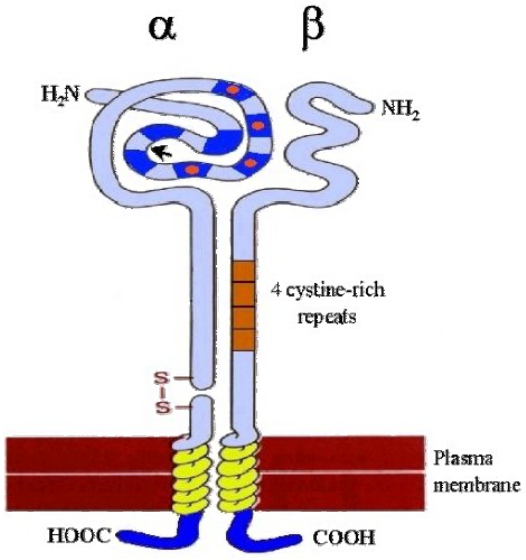
Apoptosis and Clearance

The neutrophil's lifespan ends with programmed cell death. During this process the phospholipid, phosphatidyl-serine (PS) is exposed to the outer leaflet of the neutrophil membrane. The PS receptors of the macrophage bind to the exposed PS and engulf the dead neutrophil (14).

LEUKOCYTE ADHESION DEFICIENCY TYPE 1

Leukocyte adhesion deficiency type 1 (LAD-1) is a defect in which the expression of the three main leukocyte integrin molecules LFA-1, Mac-1 and p150,95 is decreased, completely absent, or expressed but nonfunctional. These molecules are responsible for allowing leukocytes to firmly attach to blood vessel walls and transmigrate into the tissues. The integrin molecules allow leukocytes to bind to each other and also to intercellular adhesion molecule-1 (ICAM-1) on activated endothelial cells of the post capillary wall. These three integrin molecules consist of different alpha-1 subunits (CD11a in LFA-1, CD11b in Mac-1, CD11c in p150,95) and a common beta-2 subunit (CD18) which pairs with the alpha subunit in the assembly of each of these molecules. The alpha and beta subunits are separately translated, undergo carbohydrate additions, and paired with each other for transport to the cell surface (53).

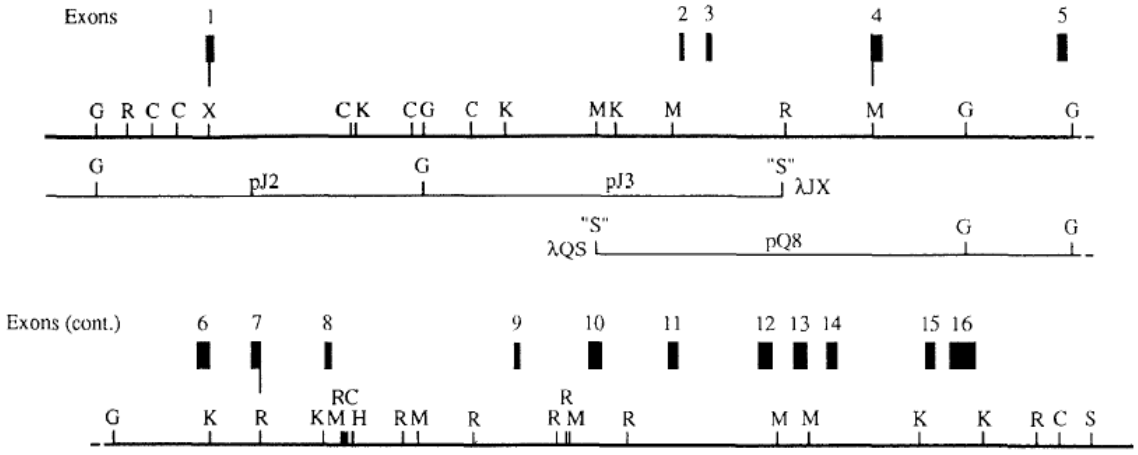
Figure 5. Diagram of a Beta-2 Integrin Molecule with Alpha and Beta Subunits



Adapted from www.medicine.ox.ac.uk/ndog/mardon/integrin.htm

The gene for the beta-2 subunit contains 16 exons and stretches about 40 kilobases. Along this region of DNA a variety of mutations may occur. Three main types of mutations give rise to the disease phenotype. The first type of mutation alters the region of the beta-2 subunit known as the I-domain which is encoded by exons 5-9 of the gene. Such a mutation may decrease the ability of the beta-2 subunit to pair with one of its three alpha partners, but more importantly will decrease the ability of the beta-2 integrin to bind to its ICAM ligands (6). The second type of mutation affects the 13th exon of the beta-2 subunit gene. This region of the gene encodes for a cysteine rich region that gives stability to the protein through multiple disulfide bonds within the beta-2 subunit (51). The third type of mutation affects the ability of the beta-2 mRNA to splice. This mutation, which can occur at any exon/intron border within the gene, alters splice acceptor or donor sites and gives a resulting larger-than-usual mRNA molecule which in turn is not translated into the beta-2 subunit (51).

Figure 6. Gene organization of the CD18 subunit (exons represented by black bars)



Adapted from Weitzman et al., 1991

As stated above, in LAD-1 patients, a mutation in the gene for the common beta-2 subunit results in little or absent expression on the leukocyte cell membrane (53). The cell types that are most affected by this mutation are the macrophages and neutrophils. As neutrophils are clearly the most abundant white blood cell type, their levels in blood and tissue are used to measure disease severity. B and T lymphocytes have other adhesion molecules which can compensate for the loss CD11/CD18 heterodimers and are thus able to perform adhesion in LAD-1 patients (3).

The percentage of CD18 protein expression corresponds with the level of disease severity. Those patients that express less than 1% of normal levels of functional integrins are said to have the severe form of the disease, whereas patients whose levels are in the 1% to 10% range have the mild form of the disease. Those born with severe LAD-1 often die by the age of 1 and those with milder forms of the disease rarely live beyond their 40s (41, 42). Persons who are heterozygous for a LAD-1 mutation have about 50% of blood neutrophils expressing functional integrins and exhibit no disease phenotype (41).

Patients with LAD-1 usually have chronic bacterial infections with *Staphylococcus aureus* and *Escherichia coli*, and fungal infections like *Candida* and *Aspergillus* (42). Infections of this nature necessitate regular antibiotic/antifungal treatment and frequent transfusions with normal granulocytes. Another treatment option is bone marrow transplantation, but as with granulocyte transfusion, there always exists the risk of graft rejection through alloantibody formation (3).

GENE THERAPY HISTORY OF LAD-1

Before gene therapy could be considered to treat LAD-1, researchers needed to uncover the molecular basis of the disease and the gene or genes affected. The molecular basis was discovered when a hybrid experiment was performed with T cells from a patient with LAD-1 and normal mouse T cells. Before fusion of the cells, the T cells from the LAD-1 patient expressed neither the alpha nor the beta subunit of the integrin LFA-1 on their surface. After fusion, it was apparent that the human alpha protein coupled with the mouse beta protein to form a hybrid LFA-1 integrin molecule that could be expressed at the cell surface of the hybrid cell. None of the resulting hybrids in this study expressed the human derived beta-2 subunit. This finding allowed researchers to trace the genetic defect of LAD-1 to the gene encoding the beta-2 subunit of the integrin molecule (43). The gene for this common beta-2 subunit was later discovered to be on chromosome 21 (64). The gene spans about 40 kilobases and contains 16 exons, but its coding sequence alone was determined to be a mere 2.3 kilobases (64). The largest transgene sequence that can be accommodated by a retroviral vector is 9.2 kilobases (58); therefore, LAD-1 is an ideal candidate for retroviral-mediated gene therapy (4).

Based on the above findings, some laboratories began to use various viral vectors to transfect and transduce the CD18 gene into cells from LAD-1 patients. These early studies relied on EBV-, RSV-, and Moloney MLV-based vector systems and EBV transformed cell lines derived from LAD-1 subjects (4, 11, 26, 65). The next step toward correction of this disease began with a clinical trial where the Moloney MLV-based vector system was used to target the CD34+ cells from two patients with the severe form of LAD-1. The CD34+ cells were obtained and purified from the peripheral blood

through G-CSF-induced mobilization followed by apheresis and CD34+ HSC purification. Researchers attempted to transduce the hematopoeitic stem cells of these LAD-1 patients *ex vivo* and then reintroduce these cells into these patients, with the hope that their own gene-corrected stem cells would give rise to gene corrected progeny *in vivo*. These patients both exhibited a temporary increase of more than double the percentage CD18+ neutrophils in the blood and a temporary improvement of disease phenotype. These researchers concluded that the procedure with this vector system required further enhancement and modification (personal communication with Dennis Hickstein and Thomas Bauer, unpublished results from 1999). Later, this lab concluded that the temporary cure seen in this patient was due to the fact that the oncoretroviral vector requires cell division to integrate its target cell (10). Therefore, there are two possible explanations for this failed gene therapy trial. 1) The cell that these vectors transduced was not the HSC but the transient myeloid progenitor. 2) It is possible that this vector did transduce the HSC but that the forced *ex vivo* cell division by multiple growth factors caused both of this cell's daughters to become committed myeloid progenitors (23).

FURTHER STEPS IN GENE THERAPY TREATMENT OF LAD-1

At present, dogs with Canine Leukocyte Adhesion Deficiency type 1 (CLAD-1) are being used to test autologous HSC therapy in hope that the therapy could be successfully used in humans. In the treatment of CLAD-1, bone marrow transplantation has been used to successfully correct CLAD-1. While bone transplantation has a history of failure with human LAD-1 patients (57), this large animal bone marrow

transplantation revealed that only a small increase in the number of CD18 expressing neutrophils is sufficient to reverse or improve the disease phenotype (8). Persons who are heterozygous for a LAD-1 mutation will exhibit no disease phenotype and these persons express at most 50% of the normal amounts of CD18 on their neutrophils (41), while those with the most moderate phenotype of the disease can express up to 31% of normal levels on their neutrophils (2). In view of these points, it is clear that this disease only requires partial restoration of normal numbers of CD18 expression on neutrophils in order for the disease phenotype to be reversed.

The goal of autologous gene therapy experiments is that the virally transduced autologous cell (the HSC) will persist in the patients and permanently give rise to gene corrected immune cell types to improve or reverse the disease phenotype. The only viral vectors which are capable of this kind of cure are the retroviruses because only this family of viruses is capable of integration into the target cell chromatin (32). This goal has been reached in Irish Setters with CLAD on two separate occasions where two different retroviral vectors were employed. In the first of these two studies the foamy virus-derived vector was used to completely reverse the disease phenotype in four out of five dogs. All four of these dogs are completely disease free and have not required antibiotics at two years post- treatment (Personal Communication with Dennis Hickstein, unpublished data from 2005). In another experiment, six out of 11 treated dogs had the disease phenotype reversed or improved using an MLV-derived vector and reinfusing *ex vivo* corrected HSCs. Slight increases in CD18+ neutrophil levels were maintained up to 21 months following treatment (9). HIV-1-derived vectors have also been used in attempt to correct other blood diseases (44, 49) in small animal models, but never in

LAD-1 studies. The lentiviruses will be discussed as they have a significant advantage over the oncoretroviruses for delivering gene to the HSC.

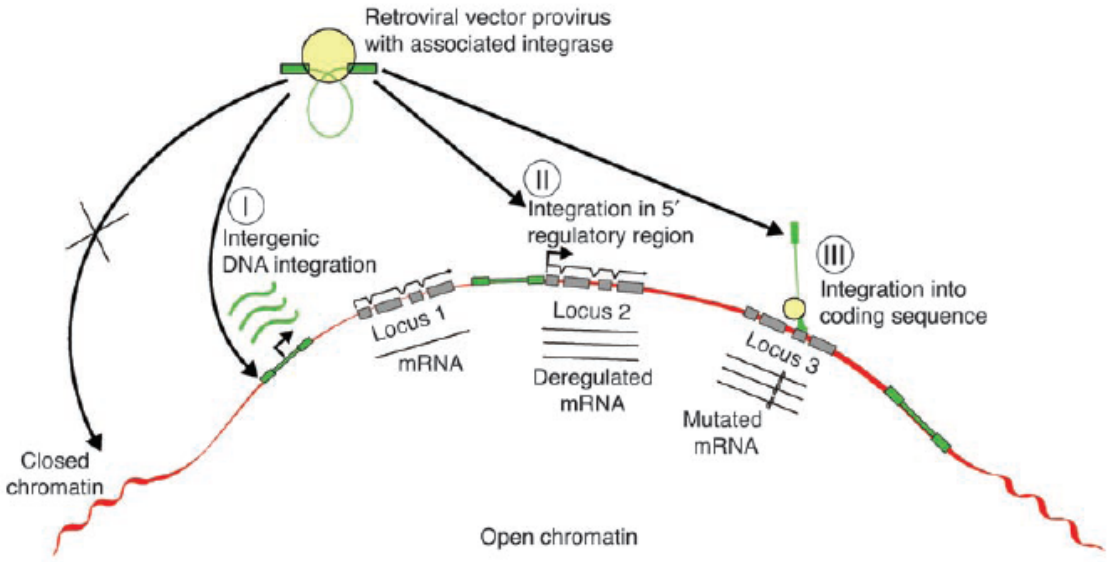
The Problems with Retroviral Vectors

The preintegration complex of an MLV-derived vector is too large to pass through the nuclear envelope of a non-dividing cell (i.e. HSCs) (59). Therefore, in order for integration to take place using these popular vectors, HSCs must be forced to divide. Forcing a stem cell to divide can be detrimental to the goal of achieving a permanent cure because if the cell is exposed to growth factors for a long enough period of time the cell may differentiate (21). Although integration will be accomplished, the daughter cells of the HSC might be committed to myeloid or lymphoid progenitors which have a limited lifespan *in vivo*. This commitment to a progenitor was the explanation for the failure of an early LAD-1 clinical trial (10).

Retroviral vectors still remain as attractive tools for gene therapy because they are capable of integrating genetic information into target cells. If the vector is derived from a lentivirus, it can integrate into non-dividing cells. However, the retroviruses exhibit preferential integration (figure 7). Preference for integration into active genes has been seen in Beta-globin gene transduction with HIV-1-derived vectors (28, 62). Preference for integration close to promoter sequences has also been observed in γ chain gene transduction with MLV-derived vectors (24, 62) The dangers of the MLV integration preference have been demonstrated in an X-linked SCID trial where 2 of 11 young patients after having received successful HSC correction (16) later developed leukemia. The retroviral integration in both of these leukemia patients was found near the promoter

for the LMO-2 gene, a proto-oncogene that is normally only expressed in progenitor cells (24).

Figure 7. Areas of Common Integration by Oncoretroviruses and Lentiviruses



Adapted from von Kalle et al. 2004

Additionally, replication competent retrovirus (RCR) may form via recombination during vector production. While HIV-1-derived vectors have been generated that are claimed to be self-inactivating, these viruses can still rebuild their promoter region even after significant deletion (19, 68). The process involved in producing such vectors involves much risk as a single genetic recombination event can mean the generation of one replication competent retrovirus. Furthermore, HIV-1 and MLV do not naturally infect HSCs and for this reason these viruses need to be psuedotyped with an envelope protein that allows entry into this cell type. If a recombination event occurred that produced a broad host tropic HIV-1 virus, the result could be the production of a more

harmful virus than the pre-existing wild-type HIV-1 (18). However, such an event is very unlikely as the genes that are used to produce these vectors are located on three or four separate plasmids within producer cells. Nevertheless, the risks involved in manipulating this deadly pathogen have not been completely eliminated (18).

Taken together, retroviral vectors show three major problems. 1) The oncoretroviruses cannot infect non-dividing cells. These viruses may not survive in a quiescent cell during the G_0 and G_1 phases of the cell cycle since they lack sufficient nucleotide pools required for reverse transcription prior to nuclear entry (59). These nucleotide pools are also required for HIV-1 as seen by the fact that they transduce dividing cells more efficiently than quiescent cells (59). 2) The oncoretroviruses and lentiviruses have dangerous integration preferences putting stem cells at risk for insertional mutagenesis (62). 3) Genetic recombination puts labs at risk for producing highly pathogenic replication competent retroviruses (RCR) (18).

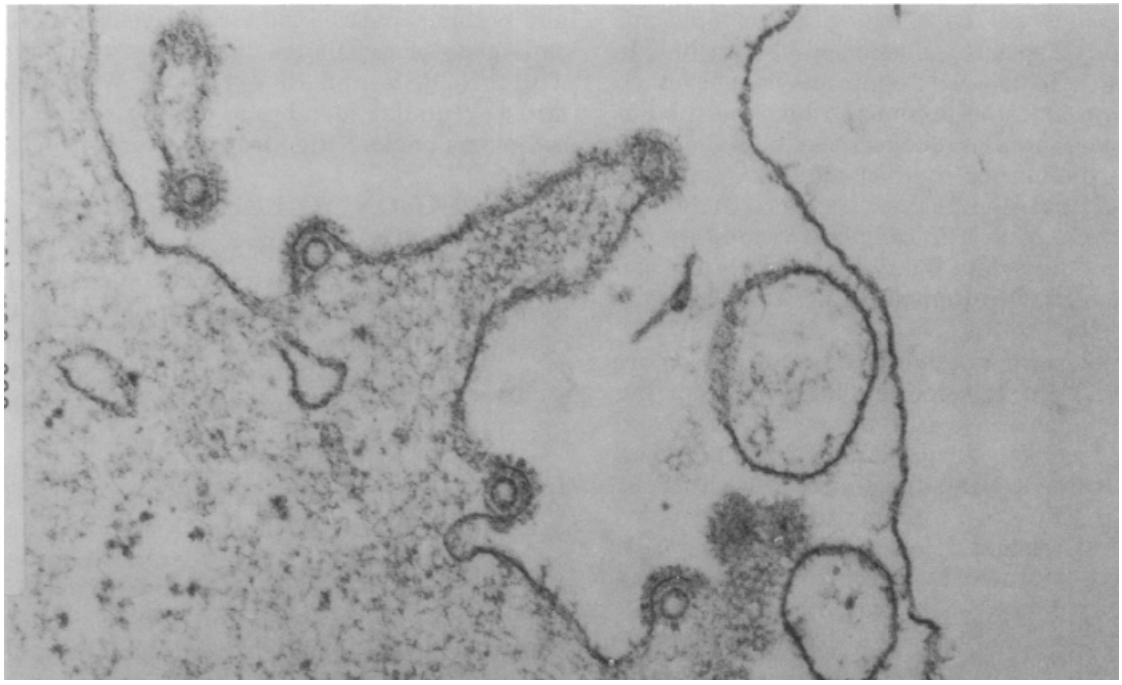
Table 1. Problems With Current Retroviral Vectors

	Cell Cycle Requirement	Integration Preference	RCR?
Oncoretroviruses	**Mitosis**	**Upstream of promoters**	**Risk not completely eliminated**
Lentiviruses	**None**	**Within active genes**	**Risk not completely eliminated**

FOAMY VIRUSES

The foamy viruses, named for the characteristic foamy appearance that they produce in culture, have not been heavily researched until recently. These viruses, of the spumavirus subfamily, are endogenous in animals and they have also been isolated from humans. In 1971 the first human foamy virus (HFV) was isolated from the nasopharynx of a Kenyan man. The closest characterized relative of the "HFV" is the SFVcpz isolated from a chimpanzee species which is native to Kenya (1). It is still debated as to whether or not there is an actual human variant of this virus. Most researchers claim that the virus isolated from a human specimen in 1971 is possibly a primate variant that the specimen received by animal to human transmission. Therefore, while the use of the letters HFV is common, it should probably be called SFVcpz(hu) (38).

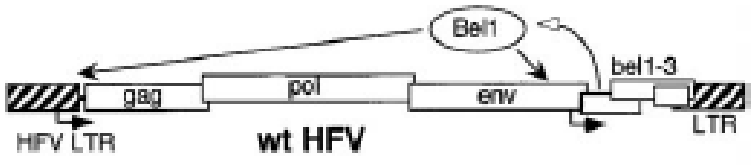
Figure 8. Electron Micrograph of Budding Foamy Viruses



Adapted from Hooks and Gibbs. 1975

While having the typical *gag*, *pol* and *env* genes that are characteristic of all retroviruses, these viruses are unique in that they seem to be dsDNA viruses. The reason for this classification lies in a distinct feature of their life cycle where reverse transcription happens just prior to budding (50, 52). Another unique feature of the foamy virus life cycle is their gene regulation. The foamy viruses have two promoters, the LTR and an internal promoter (IP) near the end of the *env* gene. Both promoters are driven by the non-structural *tas* (transactivator of spumavirus) protein (50). This protein is also known as the bel-1 gene product (figure 8).

Figure 9. The Human Foamy Virus Genome

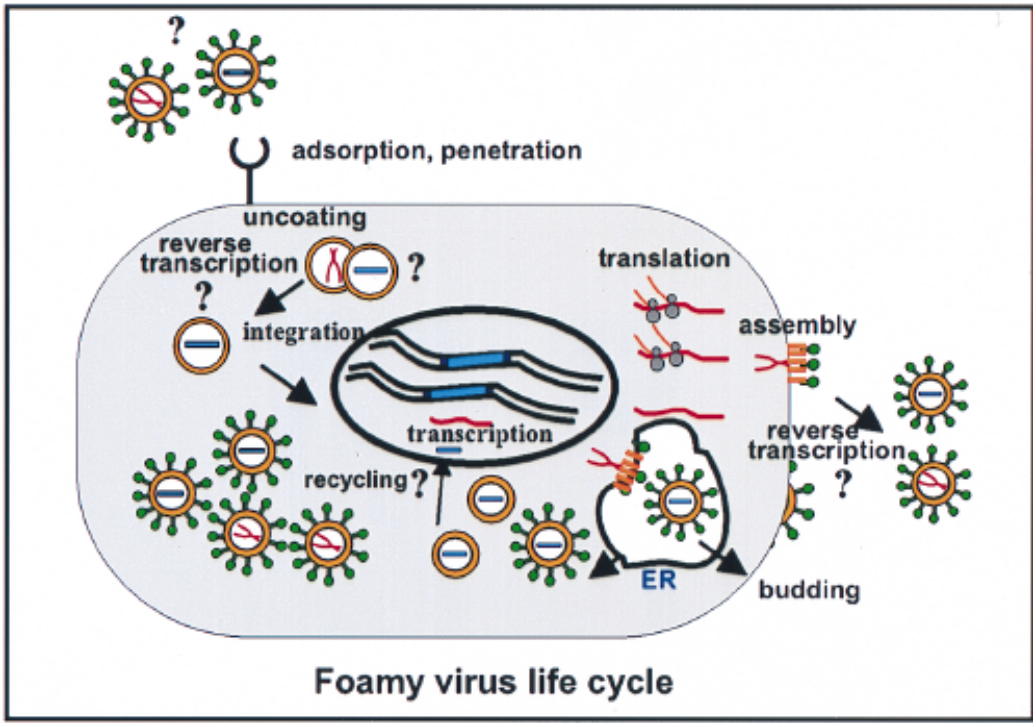


Adapted from Vassilopoulos et al. 2001

While the IP has a basal level of activity the LTR is transcriptionally inactive without *tas* binding (40). The fact that the LTRs are transcriptionally inactive without *tas* is an excellent safety feature in light of this virus being a gene delivery tool. Foamy vectors constructed without the *tas* protein are unable to induce expression of nearby genes (7). Foamy viruses further deviate from the other retroviruses in that many virions never become extracellular viruses but remain associated with the plasma membrane; their foamy appearance in culture results from syncytial formation (39). Another unique

feature of the foamy virus when compared to other retroviruses is their co-requirement of *gag* and *env* for budding and infectivity (37). The *env* protein is processed into three subunits after translation: a leader peptide (LP) at the N-terminus, a surface subunit (SU) and a transmembrane subunit (TM). The *env* protein is processed by cellular furin-like cellular proteases and must be cleaved at least into SU and TM subunits to be capable of producing infection. The LP of *env* and the N-terminus of *gag* interact with each other. This interaction is required in order for HFV capsids to be incorporated into the HFV envelope (37).

Figure 10. The Life Cycle of the Human Foamy Virus



Adapted from Linial 1999

Foamy Virus Vectors: Advantages

Foamy viruses have been reported to persist in humans for up to 20 years without any pathogenic activity. A number of comprehensive studies, each of which used several assays to confirm the presence of the virus (Table 2), have failed to attribute any disease condition to the virus (45). Such a quality makes this virus an excellent choice as a gene therapy vector.

Table 2. Disease Association with Foamy Viruses

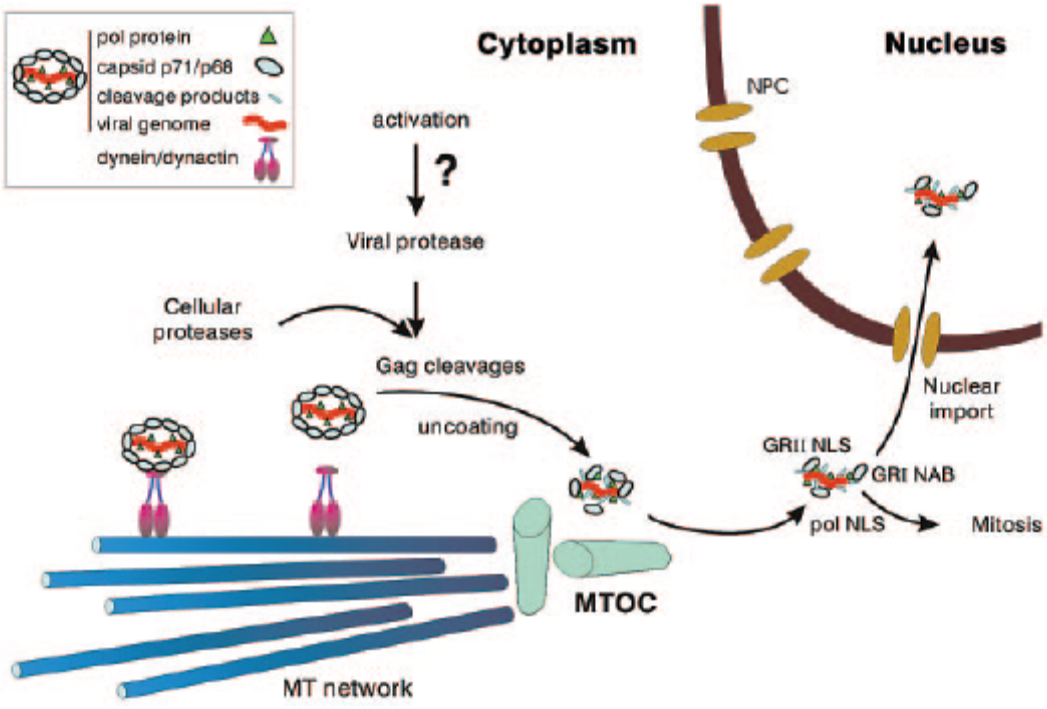
Disease	No. of positive samples/total	Methods[a]	Disease association[b]	Reference(s)
Thyroiditis de Quervain	1/1	CC	FS	Stancek et al. (106)
	5/8	CC, IFA, NAB	FS	Stancek et al. (107)
	±	IFA, EM	Yes	Werner et al. (119)
	0/19	IFA, RIPA, WB, ELISA	No	Debons-Guillemin et al. (26)
	0/58	IFA, RIPA, WB, PCR	No	Schweizer et al. (103)
Graves' disease	7/7	IFA	FS	Wick et al. (122, 123)
	19/29	SB, PCR	Yes	Lagaye et al. (61)
	0/41	SB, PCR	No	Schweizer et al. (104)
	4/4[c]			
	5/99	PCR	No	Yanagawa et al. (128)
	0/45	ELISA, WB	No	Mahnke et al. (71)
	0/28	IFA, RIPA, WB, PCR	No	Schweizer et al. (103)
	13/24	PCR	Yes	Lee et al. (62)
Multiple sclerosis	33/85	ELISA	FS	Westarp et al. (120, 121)
	0/11	CC, PCR	No	Svenningsson et al. (111)
	0/48	ELISA, WB	No	Mahnke et al. (71)
	2/60	ELISA	No	Lycke et al. (70)
	0/34	IFA	No	Rosener et al. (90)
Myasthenia gravis	1/1	WB	Yes	Saib et al. (92)
	4/4	PCR, NAB	FS	Liu et al. (64)
Chronic fatigue syndrome	0/41	ELISA, WB	No	Mahnke et al. (71)
	0/30	WB, IFA	No	Gow et al. (40)
Familial Mediterranean fever	3/3	PCR, SB	Yes	Tamura et al. (113)
Sensorineural hearing loss	4/30	IFA	FS	Pyykko et al. (84)
Dialysis encephalopathy	1/1	CC	FS	Cameron et al. (22)

Adapted from Meiering and Linial 2001

Foamy virus vectors have cell cycle requirements that are lower than the MLV-derived vectors, which require cell division, yet higher than the HIV-1-derived vectors, which do not require cell division. Another suitable quality of foamy viruses is their ability to persist at the microtubule organizing center (MTOC) of non-dividing cell types

that will divide later (36, 59, 61). This ability to survive in the G_0 and G_1 cell cycle environment is due to fact that these viruses have already reverse transcribed and do not require any cellular nucleotide pools before entry into the nucleus (59). In order to integrate genetic information, they only require cell cycle progression into the G_1/S phase (48, 59). The events taking place that allow the preintegration complex to migrate through the Ran GTPase-controlled nuclear pore complex at G_1/S phase are still a mystery (56). However, it is known that prior to nuclear entry, FV *gag* processing and viral capsid disassembly occur and this *gag* activity is totally dependent on some unknown cellular factor which is probably linked with cell cycle progression into G_1/S at the MTOC (figure 11) (35).

Figure 11. Foamy Virus Capsid Disassembly and Nuclear Import



Adapted from Lehmann-Che et al. 2005

An additional feature of the foamy virus vector that sets it apart from the lentiviruses and oncoretroviruses is its integration patterns in infected cell types. In contrast to the lentiviruses and oncoretroviruses, the foamy viruses do not show a preference for integrating within active genes nor do they show a preference for transcriptional start sites (46). Furthermore, the HFV vector integration with respect to the LMO-2 proto-oncogene was about 700 kb from the gene's start site. Although these data appear promising, this study does not report on the effects that a constitutive promoter would have on nearby genes (60).

The foamy viruses seem to have unique characteristics that serve as solutions to the above three fundamental problems found in the other retroviral vectors. These vectors 1) are non-pathogenic in humans (45), 2) they are able to persist in non-dividing cell types in culture which will later be stimulated to divide (48, 59), 3) they appear to have significantly reduced risk of insertional mutagenesis compared with the other retroviral vectors (46, 60) and it is possible to generate a safe, self-inactivating vector using these viruses (7). Based on a recent success in the treatment of CLAD in Irish setters, the NIH is considering a clinical trial with humans who have LAD-1 and are using a newer and presumably safer HFV-derived vector (personal communication w/ Hickstein and Bauer, unpublished data). These vectors are potentially the next great tool to correct LAD-1 as well as a number of other genetic immunodeficiencies.

Disadvantages of the Foamy Virus Vectors

While the foamy viruses appear to be a more suitable vector than the other retroviruses the amount of virus that can be generated by packaging cells is quite low. A small percentage of the virons generated within a producing cell will actually bud and become extracellular particles. The reason for the low production of extracellular virus is that the leader peptide of the envelope protein in foamy viruses is often ubiquitinated during assembly and ushered to the proteasome for destruction. This leader peptide is absolutely required for the foamy virus to bud from the cell (54). The LP is so often ubiquitinated and destroyed, that the foamy virus is left with an incomplete *env* protein that is incapable of allowing the virus to bud (54). The result is that there are many cell membrane-associated virions and few viruses which are able to transfer gene to a target cell. The target cell of the foamy virus vector in an autologous gene therapy experiment to correct LAD-1 is the patient's HSC. This cell type has a low amount of foamy virus receptor (63). The low amount of Foamy receptor coupled with the low number of extracellular viruses produced by a packaging cell line leaves the foamy virus with a lower possibility of delivering the gene for the CD18 subunit into the HSC. However, such disadvantages pale in comparison to this virus's biosafety features and stability within the quiescent cell.

Promoters used to Drive expression of the Beta Subunit Gene

The promoters that are most often used in retroviral vectors to direct the expression of the gene of interest are the Cytomeglovirus (CMV) promoter, the MLV

promoter and the phosphoglycerine kinase (PGK) promoter. A potential problem with any retroviral vector is that using of one of these constitutive promoters has the potential cause inappropriate expression of nearby genes. The key to solving this problem will be the integrin promoter for the CD18 subunit gene. This promoter is myeloid tissue specific and has been used in previous MLV-derived vector systems albeit with little success (12).

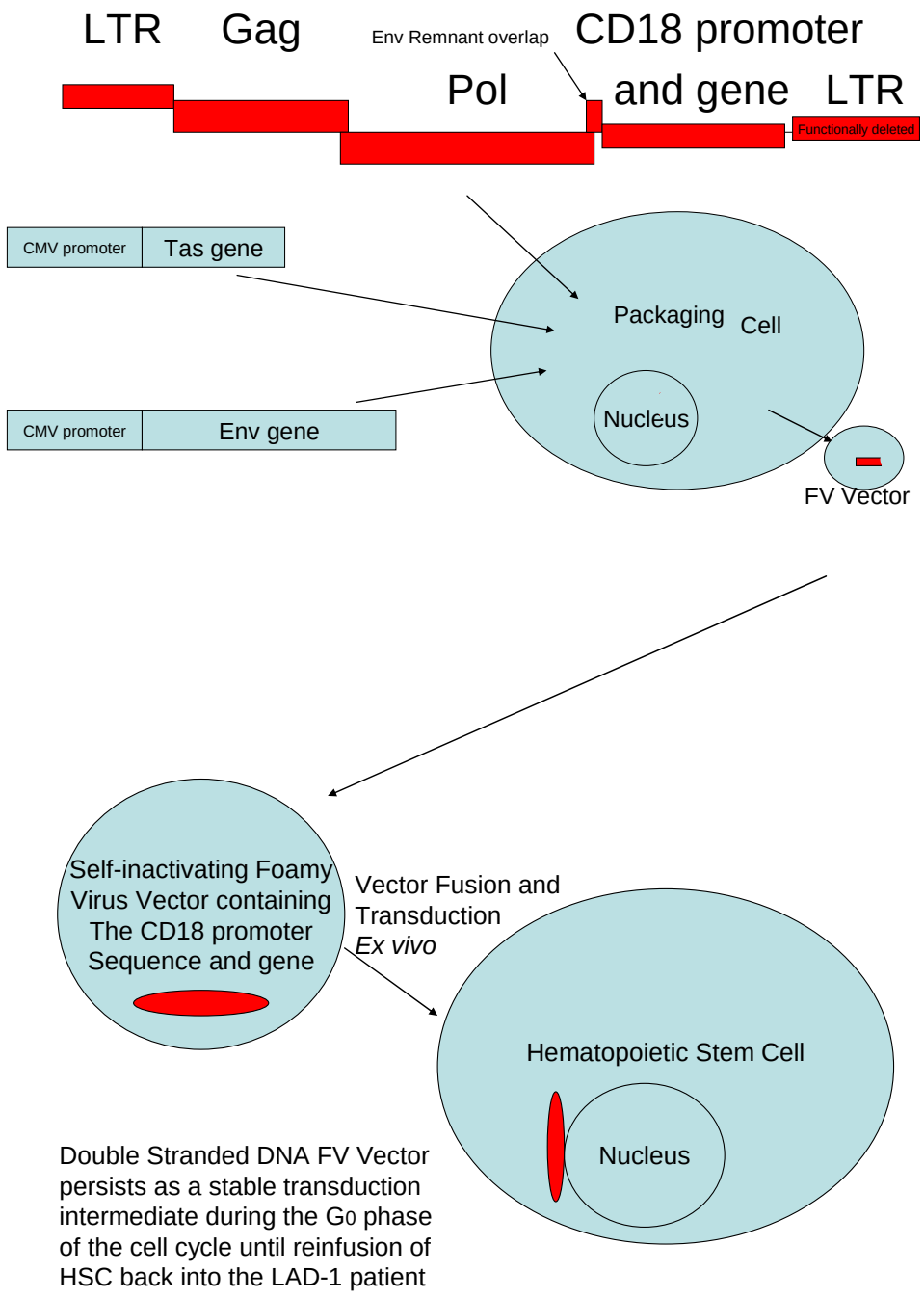
HEMATOPOIETIC STEM CELLS: The Target Cell of the Autologous Gene Therapy Experiment to Correct LAD-1

The CD34+ Hematopoietic stem cell is the target cell that needs to be harvested, transfected by the retrovirus-derived vector, and reintroduced back into LAD-1 patients (9). Therefore, it is important to understand how researchers are attempting to preserve the "stemness" of this cell population during an *ex vivo* autologous gene therapy experiment. As stated earlier, this cell population may differentiate in the presence of growth factors *ex vivo*. One method that is being practiced to attempt to ensure that the trandsduced HSCs retain their "stemness" is a shorter transduction protocol. The duration of these protocols when using retrovirus-derived vectors have been reported to be as short as 24 hours (9) with MLV vectors, 18 (33) and 10 hours (30, 31) when using FV vectors, and even 1hour when using HIV vectors (34). The results of all these studies were that gene corrected progeny could be observed long after the stem cell transduction and reinfusion. However, just the fact that these cells are exposed to growth factors could diminish their repopulating capacity even if these cells remain in the G_0 phase of the cell cycle (23). Since the FV vectors do not need circulating nucleotide pools (59) and are able to survive as a stable transduction intermediate at the microtubule organizing

center (MTOC) for at least 30 days in quiescent cells (36) it is not necessary to even expose HSCs to growth factors when using this vector. While the HSCs normally divide on average once every 30 days in the normal bone marrow microenvironment (15, 17), reinfusion of gene corrected HSCs could prompt them to cycle earlier, allowing the FV vector to enter the nucleus (36). Therefore, the use of a FV vector eliminates the need to place the HSCs in conditions that will jeopardize their long-term repopulating capacity.

Possible Vector Design and Experimental Diagram

Figure 12. Foamy Virus Vector Production and Transduction



CONCLUSIONS

In the near future LAD-1 patients should benefit from the use of Foamy Virus-derived vectors in autologous gene therapy. This retrovirus is non-pathogenic and able to integrate genetic information into the chromosome for long term gene expression. Also, this virus has a unique cell cycle requirement that will allow hematopoietic stem cells to maintain their "stemness". Forced division of the stem cell in vitro may cause it to become a progenitor with a limited life span, leading to a mere temporary disease correction. Furthermore, some single gene blood diseases require a complete correction to reverse the phenotype of the disease. LAD-1 is an autosomal recessive genetic defect and will require at most 50% of normal levels of CD18 expression on neutrophils in order for the disease phenotype to be reversed. In addressing the matter of insertional mutagenesis, the FV LTRs are inactive in the absence of the *tas* protein. These vectors will be unable to cause an inappropriate overexpression of nearby genes. In addition, foamy virus vectors that have a tissue specific CD18 integrin promoter will be exceptionally safer than those possessing constitutive promoters used in previous studies.

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