Manipulation of host protein expression in primary human cells: a study on HIV infection and replication

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Thesis submitted in fulfillment of the requirements for the degree of Doctor (PhD) in Medical Sciences
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<th>Description</th>
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<tbody>
<tr>
<td>$\Psi$</td>
<td>Encapsidation</td>
</tr>
<tr>
<td>ΔNGFR</td>
<td>Truncated nerve growth factor receptor</td>
</tr>
<tr>
<td>A</td>
<td>Adenine</td>
</tr>
<tr>
<td>AA</td>
<td>Amino acid</td>
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<tr>
<td>AAV</td>
<td>Adeno-associated virus</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>APOBEC</td>
<td>Apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like</td>
</tr>
<tr>
<td>AIDS</td>
<td>Acquired immunodeficiency syndrome</td>
</tr>
<tr>
<td>AKT</td>
<td>Protein kinase B</td>
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<tr>
<td>AP</td>
<td>Activator protein</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
</tr>
<tr>
<td>Arp2/Arp3</td>
<td>Actin related proteins complex</td>
</tr>
<tr>
<td>ARV</td>
<td>Antiretroviral agent</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>ATPase</td>
<td>Adenosine triphosphatase</td>
</tr>
<tr>
<td>BAF</td>
<td>Barrier to autointegration factor</td>
</tr>
<tr>
<td>BM</td>
<td>Bone marrow</td>
</tr>
<tr>
<td>C</td>
<td>Cytosine</td>
</tr>
<tr>
<td>CA</td>
<td>Capsid protein</td>
</tr>
<tr>
<td>CCR</td>
<td>CC chemokine receptor</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>Cdc42</td>
<td>Cell division cycle 42</td>
</tr>
<tr>
<td>cdk9</td>
<td>Cyclin-dependent kinase 9</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>CFSE</td>
<td>Carboxyfluorescein succinimidyl ester</td>
</tr>
<tr>
<td>CLP</td>
<td>Common lymphoid progenitor</td>
</tr>
<tr>
<td>CMEM</td>
<td>Common myeloid–erythroid progenitor</td>
</tr>
<tr>
<td>CMJ</td>
<td>Corticomedullary junction</td>
</tr>
<tr>
<td>CMV</td>
<td>Cytomegalovirus</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>cPPT</td>
<td>Central polypurine tract</td>
</tr>
<tr>
<td>CPSF6</td>
<td>Cleavage and polyadenylation specific factor 6</td>
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<tr>
<td>cSMAC</td>
<td>Central supra molecular activation cluster</td>
</tr>
<tr>
<td>cTEC</td>
<td>Cortical thymic epithelial cells</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic T lymphocytes</td>
</tr>
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<td>CTS</td>
<td>Central termination sequence</td>
</tr>
<tr>
<td>CXCL</td>
<td>CXC chemokine ligand</td>
</tr>
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<td>CXCR</td>
<td>CXC chemokine receptor</td>
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<tr>
<td>CycT</td>
<td>Cyclin T</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>DC-SIGN</td>
<td>Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin</td>
</tr>
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<td>Dicer</td>
<td>RNase III-like enzyme</td>
</tr>
<tr>
<td>DN</td>
<td>Double negative</td>
</tr>
<tr>
<td>DNA</td>
<td>Desoxyribonucleic acid</td>
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<tr>
<td>dNTP</td>
<td>Deoxyribonucleotide triphosphate</td>
</tr>
<tr>
<td>DP</td>
<td>Double positive</td>
</tr>
<tr>
<td>DPC</td>
<td>Distal pole complex</td>
</tr>
<tr>
<td>dSMAC</td>
<td>Distal supra molecular activation cluster</td>
</tr>
<tr>
<td>dsRNA</td>
<td>Double strand RNA</td>
</tr>
<tr>
<td>eGFP</td>
<td>Enhanced green fluorescent protein</td>
</tr>
<tr>
<td>E-MK</td>
<td>Erythroid and megakaryocyte cells</td>
</tr>
<tr>
<td>ENV</td>
<td>Envelope</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
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</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>ERM</td>
<td>Ezrin, radixin, and moesin family of proteins</td>
</tr>
<tr>
<td>ESCRT</td>
<td>Endosomal sorting complex required for transport</td>
</tr>
<tr>
<td>ETP</td>
<td>Earliest thymic progenitor</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence activated cell sorting</td>
</tr>
<tr>
<td>F-actin</td>
<td>Actin filaments</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and drug administration</td>
</tr>
<tr>
<td>FTOC</td>
<td>Fetal thymic organ culture</td>
</tr>
<tr>
<td>G</td>
<td>Guanine</td>
</tr>
<tr>
<td>G-actin</td>
<td>Globular actin</td>
</tr>
<tr>
<td>gag</td>
<td>Group specific antigen gene</td>
</tr>
<tr>
<td>GALT</td>
<td>Gut-associated-lymphoid-tissue</td>
</tr>
<tr>
<td>GAP</td>
<td>GTPase activating protein</td>
</tr>
<tr>
<td>GBD</td>
<td>GTPase binding domain</td>
</tr>
<tr>
<td>GDI</td>
<td>Guanine nucleotide dissociation inhibitor</td>
</tr>
<tr>
<td>GDP</td>
<td>Guanine diphosphate</td>
</tr>
<tr>
<td>GEF</td>
<td>Guanine nucleotides exchange factor</td>
</tr>
<tr>
<td>GOI</td>
<td>Gene of interest</td>
</tr>
<tr>
<td>GT</td>
<td>Gential tract</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanine triphosphate</td>
</tr>
<tr>
<td>GTPase</td>
<td>Guanosine triphosphatase</td>
</tr>
<tr>
<td>HAART</td>
<td>Highly active antiretroviral therapy</td>
</tr>
<tr>
<td>HDAC6</td>
<td>Histone deacetylase 6</td>
</tr>
<tr>
<td>HDCAI</td>
<td>Histone deacetylase inhibitor</td>
</tr>
<tr>
<td>HIV</td>
<td>Human Immunodeficiency Virus</td>
</tr>
<tr>
<td>hnRNP-A1</td>
<td>Heterogeneous nuclear riboprotein A1</td>
</tr>
<tr>
<td>HSC</td>
<td>Hematopoietic stem cell</td>
</tr>
<tr>
<td>ICAM</td>
<td>Intercellular cell-adhesion molecule</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>ILK</td>
<td>Integrin-linked kinase</td>
</tr>
<tr>
<td>Imp</td>
<td>Importin</td>
</tr>
<tr>
<td>IN</td>
<td>Integrase</td>
</tr>
<tr>
<td>IP3</td>
<td>Inositol trisphosphate</td>
</tr>
<tr>
<td>IP3R</td>
<td>Inositol trisphosphate receptor</td>
</tr>
<tr>
<td>IRES</td>
<td>Internal ribosomal entry site</td>
</tr>
<tr>
<td>IS</td>
<td>Immunological synapse</td>
</tr>
<tr>
<td>ISP</td>
<td>Immature signal positive</td>
</tr>
<tr>
<td>ITAM</td>
<td>Immunoreceptor tyrosine-based activation motif</td>
</tr>
<tr>
<td>ITK</td>
<td>Interleukin-2 kinase</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
</tr>
<tr>
<td>kDa</td>
<td>KiloDalton</td>
</tr>
<tr>
<td>LAT</td>
<td>Linker for activation of T cell</td>
</tr>
<tr>
<td>Lck</td>
<td>lymphocyte specific tyrosine-kinase</td>
</tr>
<tr>
<td>LFA</td>
<td>lymphocytes function-associated antigen</td>
</tr>
<tr>
<td>LIMK</td>
<td>LIM domain kinase 1</td>
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<tr>
<td>LT-HSC</td>
<td>Long-Term HSC</td>
</tr>
<tr>
<td>LTR</td>
<td>Long terminal repeat</td>
</tr>
<tr>
<td>MA</td>
<td>Matrix</td>
</tr>
<tr>
<td>MALT</td>
<td>Mucosal-associated lymphoid tissue</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MC</td>
<td>Monocyte</td>
</tr>
<tr>
<td>MFI</td>
<td>Mean fluorescence intensity</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>miRNA</td>
<td>MicroRNA</td>
</tr>
<tr>
<td>MLV</td>
<td>Murine leukemia virus</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>ssRNA</td>
<td>Single stranded RNA</td>
</tr>
<tr>
<td>ST-HSC</td>
<td>Short-term HSCs</td>
</tr>
<tr>
<td>SU</td>
<td>Surface protein</td>
</tr>
<tr>
<td>Tat</td>
<td>Transcriptional activator</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TEC</td>
<td>Thymic epithelial cells</td>
</tr>
<tr>
<td>TGR</td>
<td>Thymocyte generation ratio</td>
</tr>
<tr>
<td>TH</td>
<td>T helper cells</td>
</tr>
<tr>
<td>TM</td>
<td>Transmembrane protein</td>
</tr>
<tr>
<td>TNPO3</td>
<td>Transportin-3</td>
</tr>
<tr>
<td>TPO</td>
<td>Thrombopoietin</td>
</tr>
<tr>
<td>TSP</td>
<td>Thymus seeding progenitors</td>
</tr>
<tr>
<td>Treg</td>
<td>T regulatory cells</td>
</tr>
<tr>
<td>tRNA</td>
<td>Transfer RNA</td>
</tr>
<tr>
<td>U</td>
<td>Uracil</td>
</tr>
<tr>
<td>VAV1</td>
<td>vav 1 guanine nucleotide exchange factor</td>
</tr>
<tr>
<td>VCAM</td>
<td>Vascular cell adhesion molecule</td>
</tr>
<tr>
<td>Vif</td>
<td>Viral infectivity factor</td>
</tr>
<tr>
<td>VLA</td>
<td>Very late antigen</td>
</tr>
<tr>
<td>Vpr</td>
<td>Viral protein R</td>
</tr>
<tr>
<td>Vpu</td>
<td>Viral protein U</td>
</tr>
<tr>
<td>Vpx</td>
<td>Viral protein X</td>
</tr>
<tr>
<td>VS</td>
<td>Virological synapse</td>
</tr>
<tr>
<td>VSG-G</td>
<td>Vescicular stomatitis virus envelope glycoprotein G</td>
</tr>
<tr>
<td>WASP</td>
<td>Wiskott-aldrich syndrome protein</td>
</tr>
<tr>
<td>WH1</td>
<td>WASP homology 1domains</td>
</tr>
<tr>
<td>WPRE</td>
<td>Woodchuck hepatitis virus posttranscriptional regulatory element</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
</tr>
<tr>
<td>ZAP-70</td>
<td>ζ-chain associated protein kinase-70 kDa</td>
</tr>
<tr>
<td>ZFN</td>
<td>Zinc-finger protein</td>
</tr>
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General introduction and objectives

The Acquired Immunodeficiency Syndrome (AIDS) is currently considered as a pandemic. Although a cure is not yet available, the life expectancy of HIV (Human Immunodeficiency Virus) infected individuals has increased over time thanks to the ameliorations achieved by the available treatment, Highly Active Antiretroviral Therapy (HAART). Because of the nature of the infection and the capability of HIV to escape drug pressure by accumulating mutations, new strategies are required to assess methods that would allow us to eradicate the infection. Among them, gene therapy is giving very encouraging results; however, facility requirements and costs to perform such procedures, does not render it as applicable to a wide range of people. Another possibility would be to improve therapy already existing by overcoming one of the major obstacle of the current antiretroviral therapy, which is viral escape. The study presented here could allow identification of cellular proteins, less prone to mutational escape than viral proteins, to be used as alternative target for drug compounds. We therefore focused on the indentification of host cellular proteins that are exploited by HIV-1 to establish a productive infection in the target cells, working on a relevant cellular model, such as primary human CD4+ T lymphocytes or thymocytes.
Chapter 1: Introduction

1 Immune system

The immune system defends the body against attack from external infectious agents. It is composed of two main components: the innate and the adaptive immunity. The innate immunity guarantees a fast response and the activation of the adaptive immunity, whose response is more specific and specialized. The cells that compose the immune system are known as leukocytes. They are comprised of a heterogeneous group of cells that are distributed throughout the body in mucosal tissue and lymphoid organs.

1.1 Innate immunity

Granulocytes, monocytes (MCs), and dendritic cells (DCs) have a myeloid origin and belong to the innate immune system. Also Natural Killers (NK), despite their lymphoid nature, are part of this system. Granulocytes, polymorphonuclear cells, are characterized by the presence of granules containing enzymes in their cytoplasm involved in the defense against pathogens. According to the nature of the granules and their function they are subdivided into neutrophils, active in the defense against fungal and bacterial infection, eosinophils, in the defense against parasites, and basophils involved in allergic responses.

Monocytes are mononuclear cells able to migrate and invade the tissue of interest where they further differentiate into macrophages. They have the ability of responding to bacterial infection by engulfing the pathogens. They activate a stronger immune response by presenting, on their surface, antigens bound to the Major Histocompatibility Complex (MHC) molecules, after processing proteins of the pathogens that are then recognized by the cells of the adaptive response.

Another important cell type specialized in presenting antigens (antigen presenting cell, APC) to the other cells of the immune system is the DCs. They rarely circulate in the bloodstream but reside in the tissue mucosa which acts as the first check point for pathogen invasion. Their main function is to capture and transport the pathogens to lymph nodes where they can initiate adaptive immune responses.
The role of NKs is to surveil for virus-infected cells and tumor cells and kill them by cytolysis. The NKs immune response is determined by the integration of signals downstream of both inhibitory and activating receptors.

1.2 Adaptive immunity

The adaptive immune system is made of T lymphocytes, whose generation mainly takes place in the thymus, and B lymphocyte that instead generate in the Bone Marrow (BM). T lymphocytes are divided into two main cellular populations: αβ or γδ T cells.

The αβ T cells are comprised of T helper (TH1 and TH2) and cytotoxic T lymphocytes (CTLs), and in addition regulatory T cells (TREG) and special cytokine producing T helper cells (IL-17: TH17, IL-9:TH9 and IL-21: TFH (follicular) ). They have different roles in the immune response. The T helper cells are the directors of the adaptive responses. Once activated, they produce cytokines and express, on their surface, molecules able to activate the effector function of the other cells, like B lymphocytes or CTLs. CTLs secrete the content of intracellular granules to kill virally infected cells, while B lymphocytes differentiate in plasma cells that secrete antibodies. T helper cells and CTLs are characterized by the surface expression of two different receptors: CD4 and CD8, respectively. They are able to differentially interact with the Major Histocompatibility Complex (MHC) molecules. The MHC class I molecules (interacts with CD8) are expressed on every nucleated cell of the human body. If the cell is invaded by a pathogen, MCH-I molecules present foreign epitopes recognized by the CTLs. Alternatively, the MHC class II molecules (interacts with CD8) expressed on the APCs allow specific T helper response [1].

TREG have a key role in the modulation of the immune response by contributing to the maintenance of the immune homeostasis, prevention of autoimmunity and moderation of the inflammaroty response induce by external pathogens [2]. TH17 induce expression of antimicrobial peptides in fibroblasts and epitelial cells in fungal and extracellular infections [3]; TH9 are pro-inflammatory and appear to contribute to allergy reactions and autoinflammatory diseases; TFH support B cells in the lymph nodes germinal centers [4].

T lymphocytes express on their surface a receptor, TCR (analogous to BCR for B lymphocytes), able to recognize specific proteic epitopes; the receptor specificity and variability originate during the cellular differentiation by somatic recombination of the genes coding for it, rendering the antigen recognition repertoire highly various and extremely well furnished.
The conventional TCR is made of two variable αβ chains non-covalently associated with CD3 proteins that exist as a series of dimers: γε, δε and ζζ. The complex is associated to CD4 (on T helper cells) or CD8 (on CTL) that mediate the interaction with MHC class II and class I, respectively. Upon antigen recognition, the immunoreceptor tyrosine-based activation motifs (ITAMs) present on the CD3-ζ chains are phosphorylated by the lymphocytes specific tyrosine-kinase (Lck) and recruit ζ-chain associated protein kinase-70 (ZAP-70) which in turn phosphorylates the linker for activation of T cell (LAT). The Src-homology-2 (SH2)-domain – containing leukocyte protein of 76KDa (SLP-76) is then recruited by LAT and both serve as scaffolds for the recruitment and activation of signaling effectors.

Phosphorylated SLP-76 recruits the interleukin-2 kinase (ITK) that has a role, together with LAT and ZAP-70, in the activation of the phospholipase Cγ1 (PLCγ1). PLCγ1 hydrolysates phosphatidylinositol biphosphates in diacylglycerol (DAG) and inositol trisphosphate (IP3). DAG is important for the activation of both Ras and Protein kinase C isoform θ (PKCθ) signaling while IP3 is a soluble messenger that induces calcium release from intracellular stores [5].

Gene expression changes induced by TCR stimulation are mediated by the transcription factors activator protein 1 (AP1, heterodimer constituted of FOS and JUN), nuclear factor of activated T cells (NFAT) ) and nuclear factor-κB (NF-κB). Activation of FOS and JUN occurs downstream of the mitogen-activated protein kinases (MAPK) pathways activated by the protein GTPase Ras. NFAT is activated by the intracellular concentration of Ca\(^{2+}\) regulated by IP3. An increase in the cytoplasmic level of Ca\(^{2+}\) induces NFAT dephosphorylation and nuclear import. NF-κB regulation is dependent on the degradation of its inhibitors mediated by the MAPKs pathway or by PKCθ [6].

The downstream activation of the transcription factors mediates the expression of genes involved in the immune response which can be subdivided into: 1) recognition of the antigen presented in the secondary organ lymphoid by the APC, 2) T cell activation and migration to the site of inflammation and 3) proliferation and differentiation in effectors.

When the pathogen is cleared, most of the T cell effectors undergo apoptosis but some of them revert to a quiescent state and become memory cells that guarantee a faster immune response if the same foreign antigen is encountered again.
Figure 1: Overview of TCR-induced cell signaling [7]

The signaling cascade triggered upon TCR recognition of a cognate antigen involves the phosphorylation of proximal TCR components (blue), signaling by the Ras-Erk pathway (green), activation of the transcription factor NF-κB (orange) by PKC-θ, and Ca²⁺ flux-mediated signaling (yellow). These pathways activate transcription factors that mediate a variety of T cell developmental and effector programs.

2 T cell generation and maturation

2.1 Hematopoiesis

The production of lymphocytes is part of a broader process known as hematopoiesis. In the bone marrow of adults mammals, the hematopoietic stem cells (HSCs) remain as a heterogeneous group of multipotent stem cells that have the ability to self-renew and differentiate into all of the functional blood cell types [8]. HSCs depend on their microenvironment, or niche, that maintains and controls the function of the HSCs by regulating survival, self-renewal ability, and cell fate decision [9, 10]. Their progeny loses pluripotency in a stepwise fashion, giving rise to unipotent progenitors in a process called lineage commitment. The long-term HSC (LT-HSC) is the first cell at the apex of the hierarchy, which maintains the
pluripotent potential to differentiate into every kind of blood cell. The LT-HSC give rise to the short-term HSCs (ST-HSC), from which the multipotent progenitor (MPP) is originated [11].

In the “classical” model of hematopoiesis, the MPP diverges into a common myeloid-erythroid progenitor (CMEP) and a common lymphoid progenitor (CLP), that undergo further commitment steps. The CMEPs give rise to the cells of the myeloid lineage committed to the granulocyte-monocyte or produce erythroid and megakaryocyte fate. On the lymphoid side, CLPs give rise to B cell precursors and the earliest thymic progenitors (ETPs) committed to the T and NK lineage. Dendritic cells arise from both pathways.

2.2 T cell development

The thymus is the primary organ for T cell generation that provides the microenvironment necessary for the development of T lymphocytes [12]. It is an encapsulated organ with a thymic stroma and hematopoietic cells and consists of several lobules containing distinct cortical and medullary areas. It can be divided into four main compartments, each with a distinct function. The first compartment is the subcapsular zone (SCZ), which stromal component is constituted of cortical thymic epithelial cells (cTECs); the second is the cortex, which contains cTECs, fibroblast, and macrophages; the third compartment is the medulla which is made of DCs and medullary TEC (mTECs); the last is the corticomedullary junction (CMJ), which is rich in endothelial cells that allow entry to and exit from the thymus [13].

Distinct T cell lineages are generated in thymus: TCRαβ T cells (T helper, and CTL), NK, regulatory T cells, and also TCRγδ T cells. The thymic seeding progenitors (TSPs) migrate from the BM and seed the thymus at the level of the CMJ [14]. They express the CD34 marker on their surface and they are able to differentiate into T cells but they are not yet committed to the T cell lineage [15, 16]. They have not recombined the variable (V), diversity (D), and joining (J) segments of the TCR loci and they lack the expression of the recombination-activating gene 1 (RAG-1), CD3ε, and CD1a [17]. After encounter with the thymic epithelium, they become more restricted in their potential, progressing to the early thymic progenitors (ETPs) stage of T cell fate specification with a limited capability of self-renewal [18].

The ETPs undergo several distinct passages that can be discriminated on the basis of cellular surface markers. The transition of CD34+CD1A− cells to a CD34+CD1A+ stage is associated with T cell commitment, because CD34+CD1A− cells have a strong T cell, but little NK-cell nor DC or plasmacytoid dendritic cells (pDC) precursor activity. At a later stage the CD34+CD1A+, thymocytes express CD4 but not CD8 and subsequently differentiate in CD4+CD3+ immature single positive cell (ISP4). The rearrangement of the TCR-β, TCR γ, TCR-δ loci requires
the expression of RAG-1 and RAG-2. The precursors with a productive rearrangement of TCRβ locus pass the β-selection check point: the TCRβ gene product associates with the pre-TCR α-chain to form a precursor of the TCR that transduces signals for cell survival and proliferation. After this passage the receptor CD8 is expressed. The CD4+CD8α-β+ CD3- T cell precursor completes the rearrangement of the TCR α chain to become CD3+ double positive (DP) cells [17]. Notch signaling supports T-cell development by inducing T-cell commitment and by inhibiting macrophages, DCs, or NK differentiation in intrathymic progenitors [19, 20].

DPs undergo a selection process for TCRs with suitable reactivity to exert their function in the host environment. At the level of the cortex, the DP cells die from neglect during the positive selection if they express a TCR that poorly interacts with MHC-presented self-ligand. In the medulla, the strength of the signal generated after engagement of the TCRs with self-peptide ligands induces rapid apoptosis if above a certain threshold, during a phase called negative selection. Cells surviving these two phases will eventually differentiate into single positive cells (SP) and start migration to peripheral lymphoid tissue as recent thymic emigrants that will supply the naïve T cell pool [21].

The most essential cytokines produced by the stromal thymic epithelial cells (TECs) are interleukin-7 (IL-7) and CXC chemokine ligand 12 (CXCL12 (also known as SDF-1α)). The former promotes proliferation and maturation depending on the different stage of differentiation [22, 23] while the latter promotes migration of immature thymocytes [24]. The receptor for CXCL12 is CXCR4 which is expressed during all stages of differentiation [25-28].

In mice, other chemokines beside CXCL12 have been show to have a role in the migration of both the TSPs towards the thymus and of developing thymocytes throughout the thymus. CCL25 (ligand CCR9), CCL19, and CCL21 (ligand of CCR7) are among them [24]. CXCR4 and CCR7 have also been shown to be important in the exit of thymocytes from the neonatal thymus [29, 30].

Thymic output of naïve T cells decreases with age due to an age-dependent thymic involution [31]. However, normal functioning thymic tissue remains in adults, although with a high heterogeneity between individuals [32-36]. The contribution of the thymus to T cell homeostasis makes this organ an important element for the replenishment of the T cell compartment after chemotherapy, bone marrow transplantation, and highly active retroviral therapy (HAART) in HIV infection [21, 37-39].
Figure 2: Early stage of T cell development in the thymus [17]

CD34+ precursors populate the thymus and pass through distinct stages of development that can be discriminated on the basis of cell-surface antigen expression and the status of T-cell receptor (TCR) rearrangements.

It is probable that the CD34+CD1a- cell population contains a mixture of different precursor cells and can also give rise to T/NK (T cell and natural killer cell) precursors and myeloid precursors.

IL-7 produced by TECs and Notch signaling are very important for the T cell commitment of the ETP [19, 20].
Part II

3  Cytoskeleton in T cell development and T cell functions

T lymphocytes are highly dynamic cells. In order to fulfill their functions in the immune defense, they undergo several differentiation steps and functional changes that are strictly regulated. The cellular cytoskeleton is a dynamic structure composed of actin filaments, microtubules, and intermediate filaments that play an important role in this context. In particular, the actin cytoskeleton machinery takes part in virtually all aspects of the T cell’s life cycle and functions. [40].

In this chapter we will describe the cellular actin cytoskeleton and its role during T cell migration, activation, and generation.

3.1  Actin cytoskeleton

Actin filaments (F-actin) are double helical polymers made of globular subunits called globular actin (G-actin). Adenosine triphosphate (ATP) binds to the monomer in a cleft, where it is hydrolyzed to adenosine diphosphate (ADP) after polymerization. Filaments have polarity: a plus end (barbed end) where the G-actin is added during polymerization and a minus end (pointed end) where G-actin units are removed [41]. F-actin forms a complex meshwork underneath the plasma membrane. The filaments are interconnected through cross-linking proteins like filamin that organizes actin filaments in a loose network [42]. Besides its roles in defining cellular shape and maintenance of cell plasticity, actin has a pivotal role in migration [43]. The two structures in which actin filaments are reorganized to promote cell movement are lamellipodia and filopodia. The first are sheet-like actin projections filled with a branched network of filaments; the latter are finger like protrusions organized in tight parallel bundles. The elongation of these filaments pushes the leading edge forward, promoting migration [44].

Master regulators of actin dynamics are the proteins of the Rho GTPase family. The three archetypal members of the family are RhoA, Rac, and Cdc42. Their activity is regulated by the guanine nucleotides exchange factors (GEFs), the GTPase activating proteins (GAPs), and the guanine nucleotide dissociation inhibitors (GDIs). When bound to GTP, the Rho GTPases are in their active form. GAPs increase their intrinsic ability of hydrolyzing the guanine triphosphate...
(GTP) to guanine diphosphate (GDP) and inorganic phosphate (Pi) while GEFs catalyze the release of GDP allowing GTP to bind. The GDIs inhibit Rho GTPase not only by inhibiting the GDP to GTP switch but also by sequestering the protein away from the binding to the membrane. Indeed, the precise membrane localization of Rho GTPase is important not only for their efficient activation but also to spatially organize the activation of effectors [45]. Activation of Rho leads to the formation of stress fibers, Rac induces formation of lamellipodia, while Cdc42 mediates actin reorganization of filopodia [46].

![Diagram of the Rho GTPase cycle](image)

**Figure 3: The Rho GTPase cycle [45]**

The Rho GTPases cycle between an inactive (GDP-bound) and an active (GTP-bound) conformation. The cycle is regulated by three classes of proteins: guanine nucleotides exchange factors (GEFs), the GTPase activating proteins (GAPs), and the guanine nucleotide dissociation inhibitors (GDIs). The Rho GTPases interact with their effectors when they are in the active form.

In mammalian cells, actin nucleation factors favor the process of creation of new filaments. Spontaneous assembly of actin monomers is an unfavorable process due to the instability of actin dimers [41]. However, after a slow nucleation phase in which two or three monomers assemble, elongation is much faster, keeping the balance towards polymerization when required. Actin filaments can bind G-actin and F-actin at the same time, promoting elongation and branching. Two well characterized groups of factors are the formin-related family and the actin-related proteins (Arp2/Arp3) complex. Formin binds actin in a dimeric state, mediating the formation of unbranched filaments by binding the monomers at the barbed end and
preventing the binding of capping proteins that would interrupt the elongation. The new filament grows thanks to the available pool of G-actin bound to a specific protein, profilin, that makes monomers available for elongation while new branches cannot grow rapidly.[47]. The Arp2/Arp3 complex is composed of seven polypeptides (ARPC1-5, Arp2 and Arp3) and serves as a nucleation site that mimics an actin dimer to initiate a new actin filament or to start a branch at 70° on the side of an existing one [41]. The complex is activated by the actin nucleation promoting factors of the Wiskott–Aldrich Syndrome Protein (WASP) family that include N-WASP, WASP, and WAVE/SCAR. At the C-terminus, the family is characterized by the presence of a VCA domain that binds G-actin and interacts with the Arp2/Arp3 complex, and a proline rich domain, which acts as a binding site for SH3 domain-containing proteins, or profilin. WASP and N-WASP contain, at the N-terminus, a WH1 domain and a GTPase binding domain (GBD). Under resting conditions these two proteins are in a state of autoinhibition, and the GBD masks the VCA domain. The binding of the Rho GTPase Cdc42 to the GBD induces a conformational change that renders the VCA domain available to interact with the Arp2/Arp3 complex. Activation of WASP can also be induced via phosphatidylinositol 4-5 bisphosphate (PIP2(a,5)) that mediates its recruitment to the plasma membrane. WAVE-2 exists in a protein complex made of five different proteins. It binds the Rho GTPase Rac indirectly [48].

Actin dynamics are regulated by both capping proteins and actin depolymerization factors. Capping proteins bind the barbed ends and control the length and the direction of growth of the filament [49]. The actin depolymerization factor cofilin induces actin turnover by severing and depolymerizing actin filaments. The actin-binding capacity of cofilin is regulated by the LIM domain kinase 1 (LIMK), which phosphorylates and inactivates cofilin, and by protein phosphatase slingshot homolog 1 (SSH1), which performs the opposite function. LIMK can be activated in turn by the p21-activated kinases (PAKs) or directly by the Rho-associated protein kinases (ROCK) [50].
**Figure 4: Rho GTPases and actin dynamics regulation [51].**

Figure 4 shows the mechanisms that are involved in actin polymerization and actin polymer stability and contraction, by black, grey, and dashed arrow, respectively. Rac and Cdc42 regulate actin nucleation through the activation of the Arp2/Arp3 complex via Wave and WASp, respectively. Capping proteins, like CAPZ and gelsolin, limit the growth of the actin filament at the barbed end. The formin protein DIA1 and Ena/VASP promote actin polymerization by incorporating actin monomers provided by profilin (PFY). Thymosin (Thyβ4) sequesters actin monomers limiting the actin polymerization process. LIMK, activated by either ROCK (activated by RhoA) or PAK (activated by Cdc42 and Rac) inactivates cofilin by phosphorylation. RhoA induces contraction by direct phosphorylation of myosin light chain (MLC) and by inactivation of MLC phosphates via ROCK. PAK induces relaxation by phosphorylation of the MLC kinase and MLC.
3.1.1 Actin cytoskeleton in T cells

To increase the probability of encountering the antigen and guarantee an appropriate immune-surveillance for the body, naïve T cells circulate permanently between the bloodstream, secondary lymphoid organs, and lymphatic vessels [52]. The development of the immune response depends on the ability of T lymphocytes to migrate into the site of inflammation and pass from the bloodstream to the tissue during extravasation. This process involves rolling along the blood vessel, firm adhesion, and crossing through the endothelial barrier during the diapedesis [51].

3.1.2 Adhesion

Low-affinity interaction between adhesion molecules expressed on the endothelium (P and E-selectins) and the T cells (P-selectin glycoprotein, also known as PSGP1, and L-selectins) supports rolling [53]. These molecules are redistributed on microvilli [54], which are small protrusions filled with parallel bundles of actin. The cytoplasmic tail of PSGP1 is anchored to the ezrin, radixin, and moesin (ERM) family of proteins, which connect cytoskeleton proteins to the plasma membrane. This interaction results in transcriptional activation of genes necessary for extravasation [55, 56].

The arrest and firm adhesion of the T cells on the endothelium [57] is mediated by the interaction between the endothelial vascular cell-adhesion molecule 1 (VCAM) and intercellular adhesion molecule 1 (ICAM-1) that bind α4β1 integrins (very late antigen 4, VLA4) and αLβ2 integrins (lymphocytes function-associated antigen-1, LFA-1), respectively. Integrins and actin are interconnected through different membrane adaptors that mediate the activation of the Rho GTPases and therefore of the actin dynamics needed for a stable adhesion of the lymphocytes onto the blood vessels [52]. Integrins are a very important group of adhesion molecules that guarantee the efficient recruitment of T cells into the organs. Their activity is regulated by intracellular signals (inside-out) or by extracellular cues (outside-in). Rac1 and Rac2 have a redundant role in favoring a stable adhesion of lymphocytes [58]. Rac1 is directly linked to the increase in affinity of integrins for their ligand upon CXCL12 stimulation [59].
3.1.3 Migration

During diapedesis the T cells assume a polarized structure with a leading edge and a uropod at the rear. The leading edge is enriched in chemokine receptors and integrins at the front [60], while the uropod is believed to represent a storage zone in the cells for molecules not needed in the migration but readily mobilized if required [51, 61, 62].

Actin polymerization and filament cross-linking at the front edge generate a sufficient force to push out lamellipodia and filopodia from the membrane. Contraction of myosin II, a motor protein associated with actin filament, generates the condition for cytoplasm propulsion [63] in the direction of the migration. The uropod is responsible for retraction and detachment, regulated by RhoA. It is characterized by the segregation of other cytoskeletal structures like microtubules and intermediary filaments and of molecules like ICAMs, CD43, and CD44 mediated by the ERM proteins [64].

Rac1 induces the formation of lamellipodia structures via WAVE-mediated actin polymerization. The roles of Rac1 and Rac2 have been confirmed in primary murine T lymphocytes where the depletion of both GTPases induced a block in the migration upon CCR7 or CXCR4 stimulation [58]. Activation of Cdc42 leads, via WASP, to the formation of filopodia that are pivotal in the migration towards chemoattractants [65]. The formin mDIA1, which is an effector of RhoA have been shown to be important in knock down mice for migration induced response to CXCL12 [66].

3.1.4 Polarization

Once migrated into the tissue the T cell first establishes contact with the APC through integrins, such as LFA1 and ICAM1 and ICAM3 [57]. The cytosolic portion of the LFA1 induces actin reorganization via the protein talin that acts as scaffolding for the formation of PIP2(4,5), which activates WASP [67]. It is also able to recruit integrin linked kinase (ILK) that activates Rac and Cdc42 GEFs for the formation of membrane protrusions that allow a closer contact between T cell and APC [67]. Once TCR-MHC interaction is established, the signaling cascade triggered upon TCR engagement promotes a firm interaction, rearrangements of surface receptors and signaling complexes mediated by actin remodeling [68], and the formation of an actin-rich structure, the distal pole complex (DPC), at the opposite side of the cell [40]. At the site of contact between the two cells a supramolecular structure, called the immunological synapse (IS), is formed. This site of intense interaction between cells is organized in functionally distinct structures composed of different molecules: the central, peripheral, and
distal supramolecular activation cluster (cSMAC, pSMAC and dSMAC respectively) organized in a centripetal fashion. TCRs-MHCs, together with co-stimulatory proteins are found in the first, while the second is characterized by the presence of integrins and cytoskeleton proteins; the third is constituted by inhibitory phosphatases, such as CD45 [68]. After T cell activation, the redirection of molecules and organelles necessary for exocytosis of chemokines or cytotoxins to the site of cell-cell contact, is mediated by the microtubules organization center (MTOC) [69, 70].

### 3.1.5 T cell activation

The engagement of the TCR with MHC molecules triggers a signal cascade and leads to T cell activation that is supported by supplemental signaling events coming from stimulatory proteins [5].

The actin cytoskeleton supports T cell signaling by maintaining the integrity of the multi-protein signaling complex [68]. Depletion of cytoskeletal regulators impairs the IS formation and T cell activation [71-74]. RNA interference (RNAi) studies to silence the expression of actin regulators highlighted the central role of the Arp2/Arp3 complex for actin polymerization at the IS [75]. WASP deficient IS showed impaired signaling transduction, resulting in lower IL-2 production [76]. Multiple GEFs have been shown to participate in TCR signaling upon formation of the IS [52]. VAV1 is a GEF recruited at the level of the TCR by LAT-SLP-76 and the ITK [77-79]. It is able to activate Rac, Cdc42, and RhoH [80, 81], which is a very important role in actin rearrangements. VAV1 deficient cells proliferate less upon TCR engagement, have a reduction of calcium flux, and have impaired activation of downstream transcription factors like NF-kB and NFAT [82-85]. These effects are a consequence of an upstream failure in the activation of the PLCγ1 and PI3K [84, 86]. Together with Rac1 activation, VAV1 has a role in the dephosphorylation and inactivation of the ERM proteins; this promotes a transient deformability of the plasma membrane and favors conjugate formation [87]. It is also required for the signaling transduction induced by the LFA-1 [88]. VAV1 is also believed to have an additional role independent of its GEF activity. Studies in which the GEF activity was eliminated showed that this activity is not important for the activation of the MAPK/ERK pathway or for co-localization in microclusters with SLP-76 [45, 86]; overexpression of a GEF-inactive form of VAV1 induces an increase in NFAT mediated transcription [45, 77].
3.1.6 T cell generation and maturation

HSCs homing and migration depend on Rac GTPases. In mice, Rac1 is necessary for the HSCs engraftment in the BM [89], and for the control of the fetal HSCs trafficking. Rac1 deficient embryos show near complete absence of liver hematopoiesis and a decreased migration in response to CXCL12 [90]. CXCL12 induced chemoattraction is mediated by both Rac1 and Rac2 [91] and negatively regulated by RhoH. Deletion of RhoH in HSCs leads to increased CXCL12 induced chemotaxis and it is associated with Rac1 increased activity [92].

The roles of RhoA, B, and C were studied in transgenic mice by the expression of the C3 transferase from C. botulinum which selectively ADP-ribosylates Rho within its effector domain and thereby abolishes its biological function. The inactivation of the three Rho GTPases showed inhibition in thymopoiesis because of a decrease in the number of double negative (DN) 2 and DN3 thymocytes for apoptosis [93]. In the thymocytes expressing the constitutively active mutant RhoA V14 there is a more efficient selection for SP CD8+ T cells [94].

In RAC1 deficient mice, thymopoiesis is arrested in DN stage due to the lack of the pre-TCR formation. The expression of the constitutively active mutant Rac1 L61 can partially overcome this block with the appearance of some DP thymocytes [95]. Alternatively, transgenic mice expressing for a constitutively active mutant of Rac2 show an increased apoptosis in DP and SP thymocytes [96]. Mice lacking both GTPases demonstrate functional redundancy [97]. They suffer from hampered thymopoiesis at the pre-TCR check-point, a defect in positive selection and an aberrant survival of thymocytes lacking TCRβ expression, showing hallmarks of hyperactive Notch signaling [98]. Moreover, Rac2 T17 dominant negative transgenic mice showed the involvement of Rac2 in the generation of TH1-specific signaling [99].

Murine thymocytes expressing constitutively active Cdc42 (Q61L) showed increased apoptosis and a blockage of T cell development at the level of CD4 CD8 [100].

Mice deficient for vav expression have a block in T cell development at the level of the pre-TCR check-point and a defect in DP thymocytes for TCR induced calcium flux and ERK, protein kinase D, and PI3K activation [101-103]. These defects can be rescued by expression of Rac1 suggesting a role for VAV1 in the activation of this Rho GTPase upon TCR signaling induction [95]. Mutations in the vav gene affect both negative and positive selection, presumably for a defective TCR signaling [102].
Part III

4 Acquired Immunodeficiency Syndrome (AIDS)

The Acquired Immunodeficiency Syndrome (AIDS) was first recognized as a disease in 1981, when the Centers for Disease Control and Prevention described an increasing number of opportunistic infections and rare malignancies occurring in young homosexual men and drug users [104]. The etiological agent of the disease was identified as a new retrovirus two years later by two independent laboratories [105, 106]. In 1986 the virus was defined as the Human Immunodeficiency Virus (HIV) [107]. Since its first identification, three decades ago, HIV-1 has infected over 60 million people and caused more than 25 million deaths; most of the infected people live in Sub-Saharan Africa [108].

HIV spread occurs by sexual, percutaneous, or perinatal route, although for 80% of the adults the first route is the most common [109].

The consequence of HIV infection is the slow and progressive deterioration of the integrity and function of the immune system. The infection is usually initiated with a single virus infecting a single target cell from the immune system that expresses the main receptor for the virus, the CD4 receptor [110].

The clinical presentation of HIV-1 associated immune system dysfunction varies from individual to individual. However, without therapeutic intervention, the infection evolves generally through four phases [111]:

I. Eclipse phase: (1-3 weeks) in which the virus replicates freely and spreads from the site of infection to other tissue. Viremia (viral RNA copies present in the blood) is undetectable and immune response is not yet active.

II. Acute (primary) infection: (2-4 weeks) characterized by high level of viremia due to the large fraction of CD4+ T lymphocytes in the blood and in lymph nodes that support viral replication. The viral replication also occurs in the CD4+ T cells residing in the gut-associated lymphoid tissue (GALT). Here, replication and cellular depletion (TH1, T reg and above all TH17), correlates with the onset of epithelial barrier dysfunction[112]. The immune response starts to appear both in the form of
antibodies and CTL response. At the end of this phase, the level of viremia drops together with the number of CD4\(^+\) T lymphocytes.

III. Chronic infection: (1-20 years) characterized by a constant or slowly increase in level of viremia and normal or gradually falling number of CD4\(^+\) T lymphocytes. The incomplete and slow restoration of the GALT is concomitant with a persistent immune activation and a general inflammatory response that cause tissue damage and microbial translocation [113]. Patients are asymptomatic. A large number of CD4\(^+\) T cells become infected and die every day.

IV. Clinically apparent disease (AIDS): typified by the reappearance of high level HIV-1 viremia, a drastic decline in CD4\(^+\) T cells and a general decline in the immune function leading to the occurrence of numerous opportunistic infections.

5 Human Immunodeficiency Virus (HIV)

HIV belongs to the genus of Lentivirus of the family of Retroviridae. Retroviruses are viruses that use their enzyme reverse transcriptase (RT) to retrotranscribe their RNA genome into a double strand DNA molecule in order to integrate their genetic information into the host genome. The term lentivirus is derived from the long incubation period (lenti is the Latin word for slow) that occurs between the viral infection and the development of the disease.

There are two distinct type of HIV: HIV-1 and HIV-2 [114]. Worldwide, the predominant type is HIV-1 while the HIV-2 type is concentrated in West-Africa and is rarely found elsewhere.

5.1 HIV-1

5.1.1 Genome

The HIV-1 genomic organization is depicted in Figure 5. The genome encodes for 9 genes: gag, pol, env, tat, rev, vif, vpr, vpu and nef. The gag (group-specific-antigen) gene encodes for the Pr55gag, a polyprotein that is cleaved by the viral protease in p24 capsid (CA), p17 matrix (MA), p7 nucleocapsid (NC) and p6 protein. The pol (polymerase) gene encodes for viral
enzymes first produced as the polyprotein Pr160, post-translationally cleaved in Pr55gag and 3 enzymes: RT, integrase (IN) and protease (PR). The gene env (envelope) encodes for a precursor protein gp160, cleaved into two proteins gp120 and gp41 by a cellular protease after post-translation modifications. The gene tat and rev encodes for regulatory proteins essential for viral replication: Tat enhances viral transcription while Rev regulates viral RNAs transport from the nucleus to the cytoplasm [115].

The genes vif, vpr, vpu and nef code for proteins that are defined as accessory proteins since they are not essential for viral replication in some cell lines in vitro, but useful for the spread and pathogenesis in vivo. Vif and Vpu suppress the antiviral activity of cellular restriction factors [116]; Vpr supports viral genome transport integration into the nucleus and viral transcription [117]; Nef and Vpu regulate the expression and localization of proteins that influence replication, dissemination, and persistence [118, 119] by down-modulating the expression of CD4 receptor from the plasma membrane. Vpu induces degradation of the cellular protein that inhibit new viral particle release. Nef contributes to partial evasion from adaptive immunity, modulates T cell activation, and has a role in the increase of viral particle infectivity in vitro and viral load in vivo [120-122].

The protein coding regions on the genome are flanked on both sides by the Long terminal Repeat (LTR) regions which are 640 base pairs in length and segmented into the U3, R, and U5 regions. The LTRs have a role in integration of the provirus into the host genome. Once integrated, the LTR on the 5’ end serves as the promoter for the entire retroviral genome, while the LTR at the 3’ end provides for polyadenylation signaling for nascent viral RNA. The HIV-1 LTR is a particularly complex promoter, which operates at several levels of regulation. At first, it interacts with host cellular transcription factors, such as NF-κB, to support LTR regulation and viral gene expression, and then with viral proteins, including Tat and Vpr, that activate the LTR to support higher levels of viral gene expression [123].

5.1.2 Virions composition

The virion has a spherical shape (100 nanometer diameter) surrounded by a lipoproteic bilayer. On the surface, there are proteic complexes of Env, made of transmembrane glycoprotein (TM, gp41) trimers, each non-covalently bound to a surface glycoprotein (SU, gp120). The inner surface of the envelope is surrounded by the MA protein, and the core is made of the CA protein. Here, the two RNA molecules are complexed with the NC protein. Associated with the NC and the RNA there are virion specific enzymes, RT, PR, and IN all
essential for viral replication. Other viral proteins present in the virions are the accessory proteins Nef, Vif, and Vpr [124].

Figure 5: A) HIV-1 genome[125]; B) HIV-1 virion [126]
The two Long Terminal Repeats flanking the HIV-1 genome are made of regulatory sequences that are important for viral genome replication and integration and expression in the host. The genes gag, pol, and env code for structural proteins and enzymes. The gag gene codes for the protein of the matrix (p17), capsid (p24), nucleocapsid (p7), and p6; pol codes for the reverse transcriptase, the integrase, and the protease; env codes for the proteins of the envelope (gp120 and gp41). The gene product of tat mediates the enhancement of viral transcription while rev’s product contributes to the trafficking of viral RNA from the nucleus to the cytoplasm. The products of the accessory genes nef, vpu, vpr, and vif are important for the spreading and pathogenesis of the virus in vivo and replication in vitro.

5.1.3 Life cycle

The first phase of the replication cycle starts with the adhesion of the virus to the host’s cellular surface. The gp120 Env subunit recognizes the CD4 receptor present on the surface of the cells of the immune system such as monocytes, macrophages, dendritic cells, and on T lymphocytes at high density.

After that first binding a conformational change in Env occurs, allowing the interaction with HIV-1 co-receptors CXCR4 and CCR5, which are transmembrane chemokines receptors whose tropism is cellular type dependent. This second interaction mediates conformational changes in the gp41 subunit that ends with the fusion of the viral and cellular membranes, at the cell surface or in an endosome.
After fusion the viral core is released in the cytoplasm, the capsid is partially disassembled and reverse transcription is initiated. The viral RNA genome is retrotranscribed in a full-length double stranded DNA molecule and transported towards the nucleus in the pre-integration complex constituted of IN, MA, RT, and Vpr. Once in the nucleus the proviral DNA is integrated into the host genome thanks to IN activity. The provirus serves as a template for the generation of viral messenger RNAs (mRNAs) synthesized by the cellular RNA polymerase II. These mRNAs are necessary for the production of viral proteins or for RNA genomic molecules that will be incorporated into the newly produced viral particles.

The budding of the new viral particles starts at the plasma membrane after that the new viral components are recruited. Once released, maturation of the new particles is achieved after the cleavage of the viral polyproteins Pr160 and its fragment Pr55gag by the viral protease [127].

![HIV-1 replicative life cycle](image)

**Figure 6: HIV-1 replicative life cycle [128]**

After binding the receptor and coreceptor expressed on the target cell, the virus enters the cytoplasm and uncoats. The RNA molecule that constitutes the viral genome is then retrotranscribed into a double stranded DNA molecule that is transported into the nucleus and integrated into the host’s genome. The viral proteins are expressed by exploiting the cellular machinery for RNA transcription and proteins translation. Viral proteins assemble at the level of the cellular membrane and create new viral particles that bud from the infected cells.
The mucosal tissue plays an important role in viral transmission and replication. Mostly, a HIV-1 virus with CCR5 tropism is transmitted [110] and the primary target for infection would be the memory CD4⁺ T cells expressing this co-receptor [129, 130]. The way the virus reaches these cells depends on the transmission route. In the rectal mucosa, the virus encounters a large amount of memory CD4⁺ T cells already populating the intestine [131]. In the vagina, mucosal intraepithelial DCs, play an important role [132]. DCs capture the virus, migrate towards the lymph nodes, and transmit it to target CD4⁺ T lymphocytes, also in the absence of viral replication, by capturing the virus via the dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN), a C-type lectin, and by sialic acid-binding Ig-like lectin 1 (siglec-1), both present on the cellular surface [133, 134]. The CD4⁺ T cells come into contact with DCs and are contemporarily infected and activated [135]. This early replication is followed by the spreading of the virus to the blood and dissemination to other tissues, like the central nervous system (CNS), genital tract (GT), the GALT and thymus. The lymphoid tissue is where most of the viral replication is occurring because it is densely populated by the viral target cells [136]. As immunodeficiency progresses, the virus can evolve to enter cells using the CXCR4 co-receptor [137] and naïve CD4⁺ T lymphocytes become increasingly infected [138].

HIV-1 can establish a state of reversible non-productive infection, defined as latency, at the single cell level [139]. There are two forms of viral latency: pre-integration and post-integration. The first is the result of a partial or complete block of the viral life cycle at the steps prior to integration of the viral genome into the host genome. The second occurs when the provirus, once integrated, fails to express its genome because of transcriptional silencing. Several factors contribute to this including the site of integration into the host cell genome, absence of transcriptional factors like NF-kB or NFAT, which are excluded from the nuclei in resting cells, the epigenetic control of the LTRs, or a sub-optimal presence of the viral transactivator Tat [140].

The explanation of this phenomenon is based on the normal physiology of the CD4⁺ T cells and their state of activation. Resting CD4⁺ T cells start to proliferate after antigen recognition that in fact renders them able to support viral replication. At the end of the immune response, most effectors die, but others revert to a resting state and become memory cells with a long-term survival capability. If it infects one of these cells, then the virus remains there in a silent form state that will become transcriptionally active once the host cells are reactivated after reencountering the antigen [141]. This mechanism of viral latent reservoirs represents one of the obstacles in the eradication of the HIV-1 from the body [142].
5.1.4 Treatment

Untreated HIV-1 infection is one of the most lethal infectious diseases known, with a rate of mortality near to 95%. Thirty years after the discovery of the virus, a cure, defined as a permanent remission of the disease without requiring any therapy, is not yet available, despite the progress made in the treatment of the infection [143]. A few years after the identification of the disease, the first antiretroviral drug Zidovudine was used; since then five different classes of antiretroviral drugs have been developed, all of which target vulnerable passages in the replication cycle of the virus. Zidovudine belongs to the first class to be approved for use by the U.S. Food and Drug Administration (FDA): the nucleoside/nucleotide reverse transcriptase inhibitors. The other ones are: non-nucleoside reverse transcriptase inhibitors, integrase inhibitors, protease inhibitors, and entry/fusion inhibitors. Presently the treatment of HIV-1 infection consists of the administration of a cocktail of antiretroviral agents (ARVs).

With the advent of the combination therapy (highly active, antiretroviral therapy, HAART) and the introduction of drugs with a increased potency, mortality rates associated with HIV-1 infection decreased [144]. The use of three antiretroviral agents directed to at least two distinct molecular targets, can reduce or suppress viral replication for decades and has turned the infection into a manageable chronic illness. Unfortunately, non-adherence, low drug tolerability, or any event that causes a suboptimal level of the antiretroviral agent increases the probability of viral escape due to adaptive mutations [145], which will lead to viral resistance and failure of the treatment. Indeed, a viral particle produced for residual viral replication during the therapy that gains one or more mutation necessary to escape the drug pressure, will become the dominant pseudospecies and the drug will therefore lose effect. Moreover, because of the nature of the infection, the virus persists in the body by populating sanctuary sites such as the CNS, the GALT which are poorly accessible by ARVs, or by establishing a state of cellular latency where ARVs have no effect since there is no replication happening [142].

Because of the reasons listed above, researchers are now focused on developing alternative strategies to the HAARTs or as adjuvant to the drug regimen.

New protein and nucleic acid-based inhibitors have been tested for their efficacy in antiretroviral activity with good success. Example of these are the dominant negative inhibitor M10 protein, which mimics Rev [146-148], or the use of RNAi against viral transcripts [149]. The disadvantages concerning in vivo use of these systems include the possible induction of the immune response, cellular toxicity, or off-target effects, but also viral escape [150, 151]. An
approach to overcome this is to target cellular proteins interacting with the virus that are less prone to mutational escape [152].

Other strategies aim at the complete eradication of the virus from the body purging the HIV-1 latent reservoirs by reactivating the resting cells, for example by the administration of IL-2 [153, 154], IL-7 (T lymphocytes mitogen) [155] or of histone deacetylase inhibitors (HDCAIs) [156, 157]. The reactivated cells start viral production and the ARVs can be effective again.

Another promising approach is the use of HSCs transplantation and gene therapy, already proposed in 1988 by David Baltimore with the term of “intracellular immunization”[158]. Gene therapy involves genetically manipulated cells (HSCs), to express genes that produce an anti-HIV-1 effect by blocking infection and replication or by potentiating the immune responses.

One strategy would be to reduce the expression of HIV-1 cellular cofactors, like the CCR5 co-receptor. Individuals with a natural occurring homozygous deletion of 32 base pairs in the gene coding for CCR5 (CCR5 Δ32) are less prone to be infected with HIV [159]. Small molecule competitors of the HIV-1 Env binding to these co-receptors have been shown to reduce HIV-1 infection and replication [160, 161]. The famous “Berlin” patient was the first example of an individual apparently being cured from HIV-1 [162] after he received a transplant of HSCs carrying the CCR5 Δ32 deletion from a compatible donor to cure a concomitant case of acute myeloid leukemia [163].

A different approach aims at the ex vivo modification of autologous CD4+ T cells or HSCs to reduce risks and cytotoxicity of bone marrow ablation and transplant. An example is a clinical trial on HIV-1 patients with concomitant cases of lymphoma that was recently performed. Autologous cells were harvested and modified ex vivo before reinfusion to deliver a ribozyme targeting the CCR5 co-receptor, a short-interfering RNA (siRNA) to silence the expression of the HIV-1 proteins Tat and Rev, and a RNA decoy to Tat [164]. The trial demonstrated long-lived engraftment and multilineage hematopoiesis of vector-expressing cells, safety and feasibility, but low engraftment [165]. Another approach is the use of the Zinc-finger proteins (ZFNs), which mediates a error prone DNA repair mechanism that induces mutations in target genes products. ZFNs designed to disrupt CCR5 [166, 167] are used in a clinical trial conducted in people with HIV-1 infection. A CCR5-specific ZFN is delivered to CD4+ T ex vivo with an adenovirus vector, and the genetically modified cells are re-infused into the individual. One of the potential limitation of this strategy is that latently infected cells are not eliminated [165].

Together with the progresses in gene therapy [168], there is evidence that supports ex vivo gene therapy as an appealing strategy to prevent the progression and eventually clear the infection.
5.1.5 Virus-host interplay

To ensure successful infection, the HIV-1 virus interacts with host proteins. If the interaction is beneficial for the infection, then the host proteins, on which the virus depends, are defined as cofactors [169]. However, the interaction with the host can also be detrimental for the infection, with the virus evolving mechanisms to counteract the action of the restriction factors that have the potential to decrease viral infectivity [116].

5.1.5.1 Restriction factors

The factors recognized as bona fide restriction factors interact with at least one viral protein [116].

One example is APOBEC3 DNA deaminase subfamily. These enzymes are able to catalyze the complementary DNA (cDNA) cytosine (C) to uracil (U) deamination that leads to a guanine (G) to adenine (A) mutation in the viral DNA prior to integration [170]. HIV-1 Vif mediates the polyubiquitination and the proteasome-mediated degradation of the four restrictive forms (of the seven in total) belonging to APOBEC3 DNA deaminase subfamily [171]. Vif mediates this degradation (mainly) in the producer cells to impede the incorporation of the enzyme in the nascent virions and its release into the newly infected cells where it would cause hypermutation of the viral genome [172, 173].

A second example is the protein tetherin: HIV-1 Vpu, which acts at the late stage of the infection, induces internalization and then proteasomal degradation of tetherin away from viral budding sites at the surface of the cell. If this protein is on the cellular surface it is able to physically interact with the new nascent particles and tether them to the plasma membrane, hampering release [174, 175].

Another restriction factor is the SAMHD1 dNTPs hydrolase that in DCs, monocytes, macrophages, and resting CD4^+ T cells decreases the intracellular deoxyribonucleotide triphosphates (dNTPs) levels, resulting in inhibition of reverse transcription [176, 177]. Its viral protein counterpart is the protein Vpx, which is coded only by HIV-2. The restriction activity of SAMHD1 requires the cells to maintain a state of inactivation; indeed CD4^+ T lymphocytes, once activated, support HIV-1 replication even though they still express SAMHD1 [178].
5.1.5.2 Co-factors

As obligated intracellular parasites, viruses rely on host cellular machinery during the different phases of their life cycle. The HIV-1 genome codes for a limited number of proteins that evolved to interact with a variety of cellular proteins in order to guarantee virus survival and propagation.

Without the interaction of the host cellular receptor CD4 and co-receptors CCR5 and CXCR4 at the very beginning, the virus would not be able to start the infection process [179-182]. Once in the cell, other host factors like the cellular protein cyclophilin A (CyPA) [183, 184], which promotes uncoating, or the human tRNA(Lys)3 [185, 186], which serves as the primer for reverse transcription, contribute to the progression of the infective cycle. Proteins known as importins (Imp7 [187], Impα1 [188] and Impα3 [189, 190]) or nucleoporins (Nup153 [191] and Nup358 [192]) mediate the transport into the nucleus. Transportin-3 (TNPO3, TRN-SR2) is a karyopherin that mediates nuclear entry of serine rich (SR)-proteins and is required for HIV-1 infectivity through its interaction with CA at a step before entry into the nucleus [193]. Recent studies have shown that this process requires TNPO3 to keep the cleavage and polyadenylation specific factor 6 (CPSF6) in the nucleus, impeding its transfer into the cytoplasm which would inhibit the infection [194]. The integration of the viral genome into the genome of the host cell is assured by BAF (barrier to autointegration Factor [195, 196]) and LEDGF/p75 [197]. Transcription depends on host cellular transcription factors like NF-κB or NFAT [198] enhanced by the interaction of Tat with cyclin T (CycT) and cyclin kinase 9 (cdk9) [199, 200]. Export of viral transcripts is mediated by the interaction of Rev with host RNA transport cofactors like the heterogeneous nuclear riboprotein A1 (hnRNP-A1) [201], involved in mRNA splicing, and the hnRPN-A2, which is responsible for HIV-1 transcripts trafficking [202]. Rab GTP proteases are involved in the trafficking or HIV-1 proteins towards the plasma membrane for assembly [203-206], where the newly produced particles exploit the endosomal sorting complexes (ESCRT) machinery for budding [207-209].

5.1.5.3 Actin cytoskeleton

Both early and late phases of the HIV (and retroviruses in general) life cycle are intimately interconnected to the host cell cytoskeleton. Retroviruses have evolved several strategies to manipulate the host cytoskeleton with a highly tuned regulation depending on the step of the viral life cycle; indeed, the cytoskeleton can represent either a physical barrier to be overcome or a track that facilitates intracellular motility [210]. First, actin-dependent receptor and co-
receptor clustering is induced to facilitate viral entry [211]. This is achieved by the signaling cascade triggered upon binding of the viral gp120 protein with the CD4 receptors and coreceptors on target cells which induce: the activation of actin-cross-linkers, such as filamin A [212] and Ezrin and Moesin (belonging to the ERM proteins family) [213] or the inactivation of the histone deacetylase 6 (HDAC6) to increase microtubule stabilization [214]. Next, the actin cytoskeleton becomes an impediment for the delivery of the viral core into the cytoplasm. The signaling induced by the interaction of Env with the HIV co-receptor triggers a cascade that activates regulators of cytoskeleton reorganization such as Rho GTPases [215]. Targets of this pathway are: coflin [216], which is finely regulated to first stabilize actin filaments [217] and then to enhance actin remodeling [218] and the Arp2/3 complex that is involved in the first step of actin-based motility for the transport of the pre-Integration complex (PIC) to the nucleus. These steps are followed by microtubules mediated motility [219, 220].

Actin, myosin, and actin-binding proteins have been identified in HIV-1 viral particles [221] suggesting a role as a structural component. Moreover, the cytoskeleton might be involved in the transport of virion components to the plasma membrane, since association of Gag with actin [222, 223] or with the microtubule protein KIF4 [224] was already shown. The use of actin and microtubules disrupting drugs highlighted the necessity of an intact and functional cytoskeleton for correct virion assembly but resulted in little inhibition of HIV-1 production [225]. HIV-1 could use the ESCRT cellular machinery to destabilize actin at the plasma membrane [226, 227] during budding.

The cytoskeleton is also involved in HIV-1 cell-to-cell transmission. The virus can spread through cell-to-cell transfer [228, 229] which has been proven to be more effective via the formation of the virological synapse (VS), a multimolecular structure that forms physical contacts between a HIV-1 infected cell and uninfected target [230, 231]. In the infected cells, the polarization at the VS involves the reorientation of the MTOC that can contribute to the delivery of viral proteins to the site of cell contact [231, 232]. At the site of assembly on the plasma membrane, Env and Gag co-localize together on the infected cell in lipid rafts [233-235]. In the target cell, polarization and accumulation of viral receptors and co-receptors occurs at the site of cell-cell contact in an Env dependent process [236] that can trigger downstream CD4 or CXCR4 to induce cytoskeleton rearrangements to facilitate entry [237].
6 Manipulation of cells for studying gene function and gene therapy

Genetic manipulation of primary human cells has become a powerful tool for basic research, such as functional gene study, but it also has an application in translational medicine for gene therapy purposes [238]. In both cases, an exogenous gene is transferred into the target cell to alter the expression of a gene of interest (GOI). In our study we induced overexpression or down-modulation of the GOI for functional study. Efficient transgene delivery and expression is guaranteed by the use of viral vectors. The knock-down of the gene of interest is accomplished by virtue of the endogenous RNA interference (RNAi) pathway that mediates the degradation of target mRNA.

6.1 Gene transfer

Although the plasma membrane is a major barrier for the uptake of nucleic acids, exogenous delivery can be achieved by several methods. The net negative charge of the DNA needs to be neutralized to interact with the negatively charged plasma membrane and allow the transfer. Physical methods generate forces to directly inject the DNA, such as “gene gun”, microinjection, or electroporation. These techniques are reliable and easy to use but in some applications they can cause tissue damage. Chemical carriers such as cationic lipids, liposomes, synthetic and natural polymers are non-immunogenic and have the advantage of low toxicity [239]. However, the only system that guarantees highly efficient transfer and expression with lasting stability is viral gene transfer (transduction) based on the natural ability of viruses to deliver genetic material to the target cell [240]. Among those, retroviral vectors offer the most attractive properties as gene-delivery vehicles. Throughout the years, several changes have been made: the virus was turned from a pathogen into a transgene-carrying vehicle. The viruses they originated from were engineered to be replication deficient. After transfer and integration of the transgene into the target cell genome, no replication can occur and the exogenous gene is expressed. To optimize safety without hampering transduction, sequences necessary for production of the vector particles are provided in trans to reduce the risk of recombination once the vector DNA is inserted into the host genome. The producers, called packaging cells, are provided with all the plasmids necessary for the production of vector
particles. The particles accumulate in the medium, are harvested, and then are used on the cells of interest.

The use of retroviral vectors offers several advantages: sustained gene delivery through stable integration into the host genome; broad tissue tropism; easy system for vector manipulation and production [238].

<table>
<thead>
<tr>
<th>Genome</th>
<th>Packaging capacity</th>
<th>advantages</th>
<th>disadvantages</th>
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<tbody>
<tr>
<td>Retroviruses</td>
<td>Oncoretroviruses</td>
<td>ssRNA ~8 kb</td>
<td>integration host genome stable expression</td>
</tr>
<tr>
<td></td>
<td>Lentiviruses</td>
<td>ssRNA ~8 kb</td>
<td>integration host genome stable expression transfer in non-dividing cells</td>
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<tr>
<td></td>
<td>Adenovirus</td>
<td>dsDNA ~30 kb</td>
<td>large insert capacity</td>
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<tr>
<td></td>
<td>Adeno-associated</td>
<td>ssDNA ~5 kb</td>
<td>long lasting expression high stability and safety</td>
</tr>
<tr>
<td></td>
<td>Herpes simplex virus</td>
<td>dsDNA ~160 kb</td>
<td>large insert capacity</td>
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Table 1: overview and comparison of viral vectors based on [239] and [241]

Retroviruses are RNA viruses. The genome is constituted of *gag*, *pol*, and *env* genes that code for structural proteins, enzymes, and surface proteins, respectively. The genome is flanked by two important sequences for replication and gene expression, called Long Terminal Repeat (LTRs). The genomic RNA is retrotranscribed into a double strand DNA molecule that is inserted into the genome of the host cell for stable expression. The viruses belonging to this family used as viral vectors are oncoretroviruses, such as Moloney murine leukemia virus (MoMLV), and lentiviruses like HIV [242].
6.1.1 Oncoretroviral gene transfer

Oncoretroviranae are a subfamily of Retroviruses. The most common onconretroviral vector is derived from the MoMLV.

From the original genome, the genes \textit{gag}, \textit{pol}, and \textit{env} are removed leaving only the \textit{cis}-acting sequences required for reverse transcription primer binding site (PBS) and polypurine tract (PPT), transcription and integration (LTRs including \textit{att} sequences at the end) and encapsidation (\psi) [243]. The viral particles are e.g. produced in Phoenix-Ampho packaging cells, a second-generation producer cell line, that originated from 293T cells stably transfected with two packaging constructs. One of the two constructs provides the \textit{gag} and \textit{pol} genes; the second one encodes the \textit{env} gene that, in order to spread the tropism of the viral particles to mammalian cells, is replaced with the amphotropic envelope protein that recognizes the Pit-2 protein expressed on a large variety of mammalian cells [244].

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure7.png}
\caption{MoMLV genome adapted from [125]
As with every other retrovirus, the MoMLV virus genome has the three main genes \textit{gag}, \textit{pol}, and \textit{env} that code for structural proteins and enzymes necessary for viral replication. The LTRs are regulatory sequences essential for viral genome replication, integration into the host’s genome, and expression.}
\end{figure}

In the system we used, the transgene is cloned into the retroviral vector together with a marker gene, the two are separated by an Internal Ribosomal Entry Sequence (IRES), allowing for the evaluation of the transfer efficiency. We used two reporters: the enhanced green fluorescent protein (eGFP), expressed intracellularly, in the LZRS-GOI-I-E, or the nerve grow factor receptor (NGFR) in the LZRS-GOI-I-N, expressed at cell surface, that carries a mutation in the cytoplasmic tail so that no signal transduction is induced.

The disadvantage of using retroviral vectors is that they only operate efficiently in cycling cells [241], rendering its use very efficient in cell lines but limited in primary cells.
Figure 8: Oncoretroviral vectors generation and transfer (adapted from [245]).

The shuttle (or expression) plasmid that contains the GOI is transfected into the packaging cell line, the amphotropic Phoenix cells. These packaging cells already express the structural proteins and enzymes necessary for assembly of the viral vector. The viral genome will include the GOI and the viral sequences for integration into the host’s genome and expression of the GOI driven by the viral promoter.

Transducing particles are collected in the medium that will be purified and then used to manipulate the target cells. Transfer efficiency is easily evaluated by quantification of marker expression.
6.1.2 Lentiviral gene transfer

Lentivirinae, as already mentioned in the description of HIV-1 biology, also belongs to the family of Retroviruses. Different from oncoretroviruses, lentiviruses are actively transported to the nucleus of target cells, which is advantageous because it has the capability of transducing both dividing and non-dividing cells. This makes them suitable for a more widespread use in cells very difficult to manipulate such as primary or stem cells [241].

Most lentiviral vectors currently in use are based on the HIV-1 genome. The viral particles are produced in 293T packaging cells by simultaneous transfection of plasmids encoding for packaging of a vector genome together with the transgene encoding shuttle vector plasmid. In order to increase the safety of the system, the vector particles are produced with a second generation transfection system, constituted by three plasmids that provide in trans the sequences necessary for the production of the particles. All the accessory proteins, not necessary for viral production, are removed [246]. The Env protein, pseudotyped to the Vesicular Stomatitis Virus glycoprotein (VSG-G) [247], is encoded by a different plasmid than Gag and Pol; the gene of interest is located on the third plasmid and its expression is under the control of a mammalian promoter so that the Tat protein is not required for its expression [248]. In a third generation transfection system, to further increase safety for use in vivo, the Rev protein is also provided on another plasmid [249]. The LTR sequences necessary for integration in the viral genome contain a mutation/deletion in the 3’-U3 LTR [250, 251]. This Self Inactivating (SIN) approach reduces the likelihood of the propagation of spontaneously produced replication-competent recombinant HIV viruses, insertional activation of cellular oncogenes by residual activity of the integrated LTRs, and transcriptional interference of suppression by the latter [238]. Moreover, as a further optimization of the system, the shuttle vector is provided with a central polypurine tract (cPPT) and the central termination sequence (CTS) that increases nuclear transport [252], and the Woodchuck hepatitis virus post-transcriptional element (WPRE) sequence that increases transgene expression [253]. As a control of transduction in our system, the transgene is expressed with a marker gene such as eGFP or the antibiotic resistance gene for puromycin selection of transduced cells.

Lentiviral technologies are now in use for stable transgene overexpression, gene silencing, immunization, induction of pluripotent cells, stem cells modification, and site-directed gene editing [238].
Figure 9: Lentiviral vectors generation and transfer (adapted from [245]).
Producer cells, 293HEK, are simultaneously transfected with the shuttle plasmid (or expression plasmid) and the helper plasmids that provide, in trans, proteins necessary for the production of transducing particles. The lentiviral particles newly produced are collected in the medium, sometimes purified, and then used to manipulate target cells. The GOI is under the control of a cellular promotor. The quantification of the expression of the marker is a measure of the efficiency of the transfer.
6.1.2.1 Gene silencing by lentiviral vector

RNA interference (RNAi) is a post-transcriptional mechanism of gene silencing occurring in a large variety of eukaryotic organisms [254, 255]. Both plants and mammals produce double strand RNA (dsRNA) as precursor molecules that mediate sequence specific knock-down in gene expression. Three types of small RNA have been described: short interfering RNA (siRNA), repeat-associated short interfering RNA (rasiRNA), and microRNA (miRNA). In nature, dsRNA can be created during RNA viral replication or by hybridization of overlapping transcripts produced by repetitive sequences of transposons. Finally, endogenous dsRNA are produced as precursors, and processed into miRNAs for gene regulation involved in several mechanisms of cell differentiation and development [256].

The natural miRNA pathways use the RNA polymerase II or III (Pol II or Pol III) to produce long primary miRNA transcripts (pri-miRNA) in the nucleus. They are processed by the RNase III-like enzyme Drosha into 70 nucleotides (nt) stem-loop structures know as miRNA precursors (pre-miRNA) [257]. The dsRNA-binding protein exportin 5 transfers the pre-miRNA to the cytoplasm [258, 259]. Here, the cytoplasmic RNase III-like enzyme Dicer processes the pre-miRNA and loads the +/- 20 nt mature miRNA into the RNA-induced silencing complex (RISC) [260]. RISC unwinds the miRNA and keeps one strand loaded onto the complex, the guide strand, while the other one is degraded [261]. The cleavage of the mRNA complementary to the guide strands takes place in the cytoplasm and it is mediated by cellular exonucleases [262].

Most applications of the RNAi are to exploit the mechanism for targeted gene silencing by transfer of artificial siRNA of 20-30 nt into the cells. The chemically synthesized siRNA sequence can be introduced by direct transfection into the cytoplasm where it becomes a substrate for Dicer. However, there are many disadvantages in the use of siRNA that include low efficiency in delivery, short duration silencing, and innate immune response induction. Indeed, studies showed that the delivered siRNA complexed with cation lipids or polymers is endocytosed by the cells [263, 264] and processed in the endocytic pathway towards lysosomal degradation. Therefore, the siRNA must escape the endosomes to reach the cytosol [265, 266]. Moreover, other studies showed that the RNAi induced by siRNA is only effective for a relatively short time due to the successive dilutions resulting from cellular division [267, 268]. Finally, it has been shown that siRNA delivery induces up-regulation of interferons [269, 270]. To overcome most of these problems, a short hairpin RNA molecule (shRNA) can be used.
to induce knock-down of the gene of interest instead of siRNA. A RNA sense sequence is combined to an antisense sequence separated by a loop under the control of a Pol III promotor such as U6 or H1 [271]; the transcript generated assumes a hairpin-like structured RNA molecule that undergoes the same enzymatic processing described for miRNAs. The shRNA sequence can be delivered and integrated into the genome of the target cells via lentiviral transduction.

However, there are risks associated with the use of RNAi: one is the “off-target” effect [272], which mediates the degradation of unintended targets due to partial aspecificity of the RNA sequence; another is the saturation of the pathway that could also lead to aspecific degradation of mRNAs [273]. This can be easily checked by monitoring the gene and protein level expression, and performing functional rescue studies while improving the shRNA design [274].

Lentiviral delivery of shRNAs has become essential for gene functional studies in normal physiology or diseases; the feasibility of using such a technology increased over the years thanks to the many commercially available shRNA lentiviral vectors [238]. Sigma Aldrich is the licensed multinational company for assistance and distribution of the largest shRNA construct libraries (MISSION® shRNA) designed and developed by the Broad Institute of MIT and Harvard University, Boston [275]. Up to five shRNAs per gene are individually cloned in the pLKO.1 lentiviral vector. The constructs are generated by using a specific algorithm with a cross comparison with the Basic Local Alignment Search Tool (BLAST) database to reduce the possibility of an off-target effect. The vector has all the features for efficient delivery and expression of the transgene combined with a puromycin resistance gene to allow selection of the transduced cells.
Figure 10: shRNA mediated gene silencing by RNA interference pathway.
The shRNA coding sequence is integrated into the host’s genome after the delivery into the target cells by lentiviral transduction. The short hairpin RNA molecule formed after Polymerase III-mediated transcription is transported to the cytoplasm. There, the enzyme Dicer processes the RNA molecule that is then loaded onto the RISC complex. The mRNA complementary to the guide strand loaded onto the complex will be degraded by cellular exonucleases and the expression of the corresponding protein silenced.

Much progress has been made in the field of gene transfer for manipulation of cellular expression. This is due to the advances made by gene therapy applications. However, to widen the study of a specific phenomenon from cell line to primary cells is necessary to optimize in vitro manipulation.
Part V

7 Research goals

Cellular genetic manipulation has become an important tool in basic molecular research. The induction of change in specific gene expression gives pivotal hints on defining the function of a protein. The transfer of the gene of interest into the target cell can be achieved by several chemical and physical methods. Among all transfer types, gene transfer based on the use of a retroviral vector is, in most cases, the method of choice because of its high efficiency in transgene transfer and stability in expression. More specifically, the use of lentiviral based vectors makes it possible to transfer alien genes into non-dividing cells, a fact that broadens the applicability of primary cells. The importance of performing such studies in primary cells is corroborated by the fact that often studies performed in different cell lines give discordant outcomes.

One of the goals of this thesis was to optimize a protocol to transfer genes by single or combined retroviral and lentiviral transduction into CD4\(^+\) T lymphocytes \textit{in vitro}. This allows us to broaden the range of studies in primary CD4\(^+\) T lymphocytes, making it possible to investigate the role of endogenous gene products in cells that express a transgene.

Another goal was to use genetic manipulation of primary cells to study gene functions in T cell development and Human Immunodeficiency Virus (HIV) infection of CD4\(^+\) T lymphocytes. T cell development in the thymus is the mechanism that results in the generation of naive T lymphocytes. Its perturbation during HIV-1 infection can contribute to the progression towards the Acquired Immunodeficiency Syndrome (AIDS). We induced overexpression of Rho GTPases (wild-type and dominant negative mutants) to investigate their role during T cell development in humans to gain more insights into the mechanism that generates cells of the immune system.

The Human Immunodeficiency Virus (HIV)-1 virus, as an obligate parasite takes advantage of host cellular proteins for its survival and propagation. We applied and implemented our protocol, referred to above, in the study of proteins related to T cell activation and the cytoskeleton to identify host factors that interact with the virus. By using RNA interference we silenced specific genes in primary CD4\(^+\) T lymphocytes and evaluated the effect on HIV-1 infection/replication. The aim was to offer new interfaces for the development of alternative therapeutic strategies aiming at inhibiting host-virus interactions, thus replication.
Chapter 2: Rho GTPase Cdc42 is essential for human T-cell development.

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RHO GTPASE CDC42 IS ESSENTIAL FOR HUMAN T-CELL DEVELOPMENT

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Key words: Lymphopoiesis, Rho GTP-Binding Proteins, Rac GTP-Binding Proteins, hematopoietic stem cells, T lymphocytes
Abstract

Background

Rho GTPases are involved in the regulation of many cell functions, among which a number are related to the actin cytoskeleton. Different Rho GTPases were shown to be important for T-cell development in mice. However, their role in human T-cell development has not been explored so far.

Design and methods

We examined the expression and activation of Rho GTPases along different stages of T-cell development in the human thymus. Early stages of human thymocytes were transduced with constitutively active and dominant negative mutants of different Rho GTPases to explore their role in human T cell development, as analyzed in fetal thymus organ culture. The use of these mutants as well as Rho GTPase-specific inhibitors allowed us to explore their role in thymocyte migration.

Results

We show that several Rho GTPases are differently regulated in expression during subsequent stages of T-cell development in man, suggesting a specific role in human thymopoiesis. In chimeric fetal thymus organ culture, T-cell development was not or only mildly affected by expression of dominant negative Rac1 and Rac2 but was severely impaired in the presence of dominant negative Cdc42, associated with enhanced apoptosis and reduced proliferation. Kinetic analysis revealed that in human T-cell development, Cdc42 is necessary both before and after expression of the pre-T cell receptor. Using inhibitors and retrovirally transferred mutants of the aforementioned Rho GTPases, we showed that only Rac1 is necessary for migration towards SDF-1α of different thymocyte subsets, including the early CD34+ fraction. Constitutively active mutants of Rac1, Rac2 and Cdc42 all impaired migration towards SDF-1α and T-cell development in different degrees.

Conclusion

This is the first report on Rho GTPases in human T-cell development, showing the essential role of Cdc42. Our data suggest that enhanced apoptotic death and reduced proliferation rather than disturbed migration explains reduced thymopoiesis induced by dominant negative Cdc42.
Introduction

The family of Rho GTPases comprises a major subgroup of the Ras superfamily of small GTPases. RhoA, Rac1 and Cdc42 are the best characterized members of this family of at least 23 genes. Most Rho GTPases cycle between an active GTP-bound and an inactive GDP-bound state. In the GTP-bound conformation, Rho GTPases are able to interact with downstream effectors, thereby initiating diverse signaling cascades. Because each Rho GTPase can recognize multiple effectors, and some effectors are recognized by more than one Rho GTPase, these interactions generate a complex network.1-4

Cycling of Rho GTPases is tightly regulated by different types of regulatory molecules, including Rho guanosine nucleotide exchange factors (GEF), Rho GTPase activating proteins (GAP) and Rho GDP dissociation inhibitors (GDI).

Rho GTPases are involved in the regulation of many cell functions, sometimes cell type specific, among which a number are related to the actin cytoskeleton: gene transcription, cell cycle progression, survival, adhesion, migration, cell polarity, enzymatic activities, and axon guidance.1,3,4 These functions can be both overlapping and unique to different Rho GTPases, what complicates interpretation of loss-of-function studies.

Rac1, Cdc42 and RhoA are ubiquitously expressed, while Rac2 is restricted to the hematopoietic system.5 In the past 10 years, efforts were made to elucidate the specific roles of Rho GTPases in murine T-cell development using gain and loss of function strategies. Thymi lacking functional Rho are drastically reduced in size and cellularity.6 Thymocyte specific expression of C. Botulinum C3 exoenzyme, an inhibitor of RhoA, B and C, results in a survival defect of early thymocyte progenitors and CD4⁺CD8⁺ double positive (DP) cells.7,8 Thymocyte development is not perturbed in Rac2⁻/⁻ mice and the effect on T cell development is limited in conditional Rac1⁻/⁻ mice.9-11 However, the generation of conditional Rac1⁻/⁻Rac2⁻/⁻ double knockout mice showed that proliferation, apoptosis, adhesion and migration of thymocytes were disturbed, revealing a crucial but redundant role of Rac1 and Rac2.10 This was confirmed by a recent study that used an alternative approach to create conditional double knockout mice, which in addition links these results to altered Notch signalling.12 A dominant negative mutant of Rac1 was used to demonstrate that Rac1 is required for generation of CD4 SP cells from a murine DP cell line by preventing apoptosis.13 Cdc42 loss of function causes embryonic lethality. For this Rho GTPase, conditional knockout mice were recently generated and used to study hematopoietic stem cells but so far, the effect on T-cell development has not been studied.14,15
Expression of constitutively active RhoA promotes positive selection and generates hyperresponsive mature T cells.\textsuperscript{16} Transgenic mice expressing constitutively active Rac1 in the thymus, have revealed a role for Rac1 as positive regulator of $\beta$ selection.\textsuperscript{17} However, development into single positive (SP) cells is not possible due to exacerbated negative selection.\textsuperscript{18} Thymocyte specific expression of constitutively active Rac2 and Cdc42 results in severely reduced thymic cellularity due to deletion of DP cells, which could be explained by induction of apoptosis.\textsuperscript{19,20} Although the importance of Rho GTPases in T-cell development is well established, the mode of action is not clear. In case of Rho, it was suggested that the inability of thymocytes lacking Rho function to migrate correctly could explain why Rho is necessary for T-cell development.\textsuperscript{21} It has been shown that directed migration is essential for T-cell development. During their journey through the thymus, developing thymocytes encounter specific microenvironments that provide the appropriate signals for a particular stage in T-cell development, such as cell surface molecules, secreted proteins and extracellular matrix components.\textsuperscript{22} Therefore it is plausible that factors disturbing migration may also disturb development as cells fail to make the necessary cell-cell contacts. Besides migration, (pre-) T cell receptor (TCR) signaling that affects thymic selection by balancing survival over apoptosis and that induces proliferation, might explain the essential role of Rho GTPases.

We researched the importance of different Rho GTPases in human T-cell differentiation. We show that Cdc42 but not Rac1 or Rac2, is absolutely required for normal development. There is no correlation between the ability of Rho GTPase mutants to block migration towards SDF1-\(\alpha\) and to disturb thymopoiesis. Dominant negative Cdc42 blocks early T-cell development also beyond the point of pre-TCR expression, most probably by a migration independent mechanism affecting survival and proliferation.
Design and Methods

Monoclonal antibodies and reagents

Mouse anti-human monoclonal antibodies used were CD4-allophycocyanin (APC) or phycoerythrin (PE) (SK3), CD34-APC (8G12), HLA-DR-APC (L243), CD3-PE or fluorescein isothiocyanate (FITC) (SK7), Ki-67-PE (B56) and CD8-FITC (SK1), all from Becton Dickinson Immunocytometry Systems (BDIS, Erembodegem, Belgium); CD8β-PE (2ST8.5H7) from Coulter (Miami, FL); CD1-biotin (OKT 6), unlabeled CD3 (OKT 3) and CD8 (OKT 8) from American Type Culture Collection (ATCC, Rockville, MD); anti-glycophorin-A was a kind gift from Dr. L. Lanier (University of California, San Francisco, CA). Stem cell factor (SCF) and interleukin (IL)-7 were from R&D Systems (Abingdon, United Kingdom), recombinant human stromal cell-derived factor-1 (SDF-1α/CXCL12) was from Peprotech (London, UK). Rho GTPase inhibitors used were Rac Inhibitor NSC23766 (Calbiochem, Nottingham, UK) and Secramine A.23

Cell purification

Child thymus tissue, removed during cardiac surgery, was obtained and used following the guidelines of the Medical Ethical Commission of Ghent University Hospital. Informed consent was provided according to the Declaration of Helsinki. For purification of immature single positive (ISP4) cells, immunomagnetic depletion was performed on total thymocytes using antibodies against glycophorin-A, CD3 and CD8 and sheep anti-mouse Dynabeads (Dynal Biotech, Hamburg, Germany) according to the manufacturer’s instructions. The enriched population was labelled with CD4-PE, CD34-APC, CD8-, CD3- and HLA-DR-FITC and the CD34 CD4+FITC- fraction was sorted on a FACS Vantage (BDIS) using CellQuest software (BDIS). DP cells were sorted from total thymocytes after labeling with CD3-FITC, CD4-APC and CD8β-PE. For isolation of mature CD3+ SP cells and CD34+ cells, thymus mononuclear cells were isolated over a Lymphoprep density gradient (Axis-Shield PoC AS, Oslo, Norway). For SP cells, this was followed by depletion of CD1+ cells using CD1-biotin and Streptavidin MicroBeads (MACS; Miltenyi Biotec, Bergisch Gladbach, Germany). Cells were subsequently labelled with CD3-FITC, CD4-APC and CD8β-PE, and sorted for either CD3+CD8+ or CD3+CD4+ cell subsets. Purity of all these thymocyte subsets was at least 98.4%. CD34+ thymus cells were enriched by positive selection using either CD34 MACS (Miltenyi Biotec) or EasySep (StemCell Technologies SARL, Grenoble, France). Purity was on average 95.3 ± 3.3 %.
Constructs, viral production and transduction

All plasmid constructs were made into the retroviral vector LZRS-IRES-EGFP as described before. Dominant negative mutants Rac1N17, Rac2N17 and Cdc42N17 (placenta isoform), and constitutively active mutants Rac1V12 and Rac2V12 were obtained from the UMR cDNA Resource Center (www.cdna.org) and EcoRI-XhoI transferred from pcDNA3.1+ to LZRS-IRES-EGFP. Cdc42V12 (placenta isoform) was BamHl transferred from pEBG-Cdc42V12/GST to LZRS-IRES-EGFP. Direct sequencing (ABI, Foster City, CA), Western blot or a pulldown based Rho GTPase activity assay (G-LISA™, Cytoskeleton, Denver, CO), were used as instructed by supplier and using standard protocols to confirm the integrity of all constructs and to measure RhoA, Rac1, total Rac (Rac1+Rac2+Rac3) and Cdc42 activity in thymocyte subsets.

For the production of retroviral supernatant, the Phoenix-Amphotropic packaging cell line was transfected with the LZRS-IRES-EGFP (control) and the LZRS-(insert)-IRES-EGFP plasmids using calcium-phosphate precipitation. Viral supernatants contained between 3.0 and 15.5 x 10^6 transducing units per mL titrated on 293T cells (ATCC). For chemotaxis and fetal thymus organ culture (FTOC), CD34⁺ thymus cells were cultured in Iscove modified Dulbecco medium (IMDM) supplemented with penicillin (100 IU/mL), streptomycin (100 µg/mL) and 10% heat-inactivated fetal calf serum (complete IMDM, Invitrogen) and in the presence of SCF (5 ng/mL) and IL-7 (10 ng/mL) for 24h prior to transduction. After transduction, performed as previously described, cells were cultured at same cytokine concentrations for an additional period of 1 day prior to FTOC or 2 days prior to chemotaxis.

Real-time PCR

After sorting of thymocyte subsets, cells were resuspended in TRlzol (Invitrogen) and stored at -70°C until use. Total RNA was extracted from thymocytes as instructed by supplier (TRlzol), DNase-treated (DNase I, Invitrogen) and reverse transcribed (Reverse Transcription Core Kit, Eurogentec, Seraing, Belgium). Genes assayed included RhoA (Sybr Green I detection, Eurogentec), Rac1, Rac2 and Cdc42 (TaqMan detection chemistry, Eurogentec). For normalisation, YWHAZ mRNA was chosen from 10 housekeeping genes based on GENORM (Sybr Green I detection, Eurogentec). Primers (Invitrogen) and Taqman probes (Eurogentec) were described previously or were designed with Primer
Express 2.0 software (Applied Biosystems, Foster City, CA) for RhoA (forward CGGAATGATGAGCACACAAGG; reverse ATGTACCCAAAAGCGCCAATC). Primer specificity was confirmed with plasmids expressing one of the Rho GTPases. Quantitative real-time PCR was performed on ABI Prism 7300 Sequence Detection System (Applied Biosystems) as described before.\textsuperscript{29} Comparative quantification of the target gene expression was performed based on the standard curve method. To compare different donors, donor subsets were normalized to own total mean relative expression.

\textbf{FTOC}

Chimeric FTOC and subsequent flow cytometric analysis at day 21 were performed as described previously.\textsuperscript{30,31} The total progeny of $10^4$ pre-cultured CD34$^+$ or ISP4 cells, transduced 24h earlier, was used for each lobe in hanging drop. The excess of transduced progenitor cells, which were not used for hanging drop, were kept in culture to determine transduction efficiency after 2 or 4 days. Mean transduction efficiency of different viruses varied between 8 ± 3\% and 26 ± 4\%. After 14 days, half of the medium of the FTOC was replaced. Mean human cell number per thymic lobe harvested after 21 days of culture and starting from control transduced cells is $408 \times 10^3$ cells ranging from $190 \times 10^3$ to $880 \times 10^3$ cells (n=10). The large variation can be attributed to differences between human donors and murine thymic lobes. However, the fraction of transduced cells was previously shown to be very reproducible, what allows quantification of thymic development using the Thymocyte Generation Ratio (TGR); i.e., the ratio of the fraction of EGFP$^+$ thymocytes harvested to the fraction of EGFP$^+$ progenitors that were put in FTOC.\textsuperscript{31} The TGR does not take into account the total number of cells that is generated in each lobe, but compares development of transduced cells compared to non-transduced cells, the latter serving as an internal control since they are derived from the same donor and cultured in the same thymic lobe.\textsuperscript{31}

\textbf{Chemotaxis}

Chemotaxis assays were performed in duplicate using 5 µm pore filters (Transwell, 24 well cell cluster, Corning Costar, Cambridge, MA). Migration medium (600 µl IMDM supplemented with penicillin (100 IU/mL), streptomycin (100 µg/mL) and 0.5% bovine serum albumin (Sigma-Aldrich, Bornem, Belgium)) containing 50 ng/mL SDF-1α was added to the lower compartment; 100 µl of cell
suspension in migration medium without SDF-1α (between $1.2 \times 10^6$ and $5.0 \times 10^6$ cells/mL for total thymus single cell suspension and between $2.0 \times 10^5$ and $1.7 \times 10^6$ cells/mL for sorted CD34⁺ cells) was placed in the upper well. In experiments with inhibitors for Rho GTPases, total thymus or CD34⁺ thymus cells were cultured overnight in complete IMDM with SCF (5 ng/mL) and IL-7 (10 ng/mL) and NSC23766 (200 µM or 100 µM for total thymocytes or CD34⁺ cells respectively) or Secramine A (2 µM). These inhibitors were added at the same concentration to the upper well during migration. Transwells were incubated for 3 hrs at 37 °C, 7% (v/v) CO₂. Upper wells were removed and cells migrated into the lower compartment were harvested after addition of a fixed amount of Flow-Count Fluospheres (Beckman Coulter, Fullerton, CA). In experiments starting from total thymocytes, migrated cells were stained with CD4-APC, CD8-PE and CD3-FITC. Flow cytometry was done on a FACS® Calibur (BDIS) to determine the absolute number of input and migrated cells as well as the relative frequency of specific subsets of thymocytes in initial and migrated population. Fraction of migrating cells (e.g. DP thymocytes) was calculated as follows: (% DP cells in migrated population x total amount of migrated cells) / (% DP cells in initial population x total amount of input cells). For each subset, migration was evaluated relative to migration of the same population in the absence of an inhibitor. When thymus CD34⁺ cells were transduced with mutants of Rho GTPases, mean transduction efficiency was 17 %, giving us the opportunity to use the nontransduced EGFP⁻ cells, present in each well, as an internal control for migration. In this case, relative migration was calculated as follows: fraction of migrating EGFP⁺ cells / fraction of migrating EGFP⁻ cells, where fraction of migrating cells is calculated as indicated above. Percentage of migration typically fluctuates around 25% for both total and CD34⁺ cells.

**Statistical analysis**

FTOC and chemotaxis data were analysed with the paired sample t-test (SPSS, version 12; SPSS, Chicago, IL); p<0.05 was considered significant.
Results

Expression of Rho GTPases in the thymus

Whereas Rac1, Cdc42 and RhoA are ubiquitously expressed in mammalian tissue, Rac2 expression is restricted to the hematopoietic system. We examined the expression pattern of Rho GTPases in different developmental thymocyte stages, to have a clue on their potential role in human T-cell development. Rac1 and RhoA are broadly expressed with the highest levels in the early stages of T-cell development (CD34+, ISP4) (Figure 1). Rac2 expression is upregulated during development towards SP cells and Cdc42 is expressed in very early (CD34+) as well as in late (SP) stages of development. Because activity of Rho GTPases is the result of post-transcriptional regulation by GEFs, GAPs and GDIs, we used a pulldown based Rho GTPase activity assay (G-LISA™, Cytoskeleton; data not shown) to examine activity in different thymocyte subsets. We found activities found RhoA and Rac1 activity in most fractions (CD34+, ISP4, DP and CD3+) comparable to that of unseparated thymocytes +/- 50%. The Cdc42 G-LISA was not sensitive enough to reproducibly measure Cdc42 activity. Different expression and activation patterns reflect modulation during T-cell development, suggesting a role for Rho GTPases in human thymocyte development.

Human T-cell development is disturbed by deregulating activity of Rho GTPases

To investigate the role of Rho GTPases in human T-cell development, we used in vitro chimeric FTOC, the best validated in vitro model for human T cell development at the moment. This FTOC has previously shown to support development of retrovirally transduced CD34+ and immature CD3−CD4+CD8− single-positive ISP4 cells. A flowchart to explain the FTOC experiment is presented in Figure 2. The percentage of EGFP+ cells after FTOC corrected for initial transduction efficiency (Thymocyte Generation Ratio, TGR) is a parameter for T-cell development. It allows comparison of the effect of the transgene to untransduced cells, and is not blurred by individual differences between the donors or between murine thymic lobes. A large set of data has shown that the median TGR of control transduced cells is well above 1. This indicates that transduced cells have a slight advantage over non-transduced cells in this assay. Because retroviral vectors are used for the gene transfer, this suggests that cycling cells (after 24h of culture in the presence of appropriate cytokines) within the CD34+ fraction are more potent T cell precursors. As lentiviral vectors expressing shRNA against Rho GTPase family members described before expressed only transiently in FTOC, human
CD34⁺ thymus cells were retrovirally transduced with dominant negative or constitutively active mutants of Rho GTPases, and assayed for T-cell development. Dominant negative Cdc42 severely impairs T-cell development, as measured by the TGR (Figure 3A). The few T cells that are generated while expressing dominant negative Cdc42 display a normal phenotype (Figure 3C), the relative frequency of DP and SP cells is comparable to control transduced cells (Figure 3C and data not shown). Absence of Rac1 or Rac2 activity results in only very small albeit statistically significant effects on thymopoiesis.

All constitutively active mutants disturb T-cell development (Figure 3B). Although the relative number of transgene-expressing cells is reduced, the percentage of DP cells or CD3 positive cells is comparable between EGFP⁺ and EGFP⁻ cells (Figure 3D). In the CD3 positive population, no difference in the fraction expressing TCRαβ or TCRγδ was observed between EGFP⁺ and EGFP⁻ cells (data not shown). However, we observed a positive correlation between transgene expression level (measured by EGFP) and CD3 expression levels for cells transduced with constitutively active Rac1, Rac2 or Cdc42 but not with control (Figure 3D).

**Cdc42 is essential from early stages of human T-cell development**

Among all Rho GTPases tested, manipulation of Cdc42, whether this is mediated by expression of constitutively active or dominant negative mutants, resulted in the most profound effect on T-cell development. As Cdc42 has been shown to be important in different processes that could be relevant in the context of T-cell development, like proliferation, apoptosis and migration, we examined whether any of these processes could be linked to its mechanism of action in T cell development. Both thymus CD34⁺ and ISP4 cells transduced with control vector or mutant Cdc42, were cultured in medium supplemented with cytokines (SCF+IL-7) or used to initiate T-cell development in FTOC. Cell culture, initiated with transduced CD34⁺ cells, for more than one week resulted in comparable amounts of Cdc42N17 expressing cells and control transduced cells (% of EGFP⁺ cells after 8 days of culture / % of EGFP⁺ cells after 4 days of culture is 0.92 ± 0.08 and 1.01 ± 0.14 for Cdc42N17 and control transduced cells respectively, n=3). By contrast, T-cell generation from these progenitors was greatly reduced in FTOC already after 4 days (Figure 4). This was observed both with CD34⁺ progenitors and with ISP4 thymocytes, the latter known to be in part TCR-β selected.³³ Cells transduced with the constitutively active mutant of Cdc42 hamper T-cell development only after longer culture periods (Figure 4, beyond d8), suggesting that the effect of Cdc42 overactivity on T-cell generation arises only later in development.
To explore whether apoptosis was induced and proliferation was hampered in the absence of functional Cdc42, we performed additional FTOC experiments.

First, we analyzed the fraction of Annexin V and 7-AAD positive cells in FTOC initiated with Cdc42N17 and control transduced cells. Double positive Annexin V+ 7-AAD+ dead and Annexin V+ 7-AAD− apoptotic cell fractions are increased in the presence of Cdc42N17 (Figure 4D).

Second, the proliferation marker Ki-67 was stained in these cultures. As shown in Figure 5, both at day 4 and day 21 of FTOC, Cdc42N17 transduced thymocytes showed a reduced fraction of Ki-67 positive cells (less than 10%) compared to control transduced cells (up to 20%). The observation that transduced cell numbers are not reduced after cell culture of CD34+ thymocytes, but are drastically reduced in FTOC over the same period, indicate a thymus-specific effect of dominant negative Cdc42 on CD34+ cell proliferation. Cdc42 is necessary early in T-cell development as well as past the point of pre-TCR expression, as both CD34+ and ISP4 progenitor cells expressing dominant negative Cdc42 both show reduced T-cell generation.

The effects of Rho GTPases on migration and T-cell generation do not correlate

Migration is crucial for T-cell development. Rho GTPases have been shown to be important for migration of many cell types, including hematopoietic stem cells or thymocytes in the mouse. We therefore examined the in vitro migration capacity of different thymocyte subsets after manipulation of Rho GTPase activation by treatment with different inhibitors. NSC23766 is a rationally designed Rac specific inhibitor that interferes with binding to specific GEFs. The recently described small molecule inhibitor Secramine A blocks Cdc42 activation in a RhoGDI1 dependent manner. In the absence of inhibitors, the migration response to SDF-1α differs between thymocyte subsets. The immature CD4−CD8− cells are most responsive towards SDF-1α (43.5 ± 19.1 %) compared to total thymocytes (29.5 ± 15.7 %, mean percentage ± standard deviation, n=7, p=0.062), which is in agreement with previously reported data on murine thymocytes. We analyzed migration of T cell subsets in the presence of an inhibitor relative to the migration of this subset in the absence of this inhibitor. The Rac specific inhibitor NSC23766 reduces migration to SDF-1α of total thymocytes and of each subset (Figure 6A). Secramine A does not interfere with chemotaxis. As CD34+ progenitor cells migrate in response to SDF-1α and these precursors are used to initiate T-cell development in our experiments, we specifically analyzed migration of sorted child thymus CD34+ cells using chemical inhibitors and gene transfer of constitutively active and dominant negative
mutants of different Rho GTPases. The influence of Rho GTPase inhibitors on the migration capacity of child thymus CD34⁺ cells was comparable to that on the other thymocyte subsets (Figure 6A). All constitutively active mutants impaired migration of CD34⁺ cells to SDF-1α but only Rac1 seems essential as all other dominant negative mutants show normal migration (Figure 6B-C). By gating on intermediate and strong EGFP⁺ cells, reflecting intermediate and strong transgene expression, a dose response is evident for the mutants that affect migration. These results confirm the data derived from experiments with inhibitors and suggest that the inhibitory effect of NSC23766 on migration of CD34⁺ cells, is mediated by Rac1 but not Rac2. Collectively, these data suggest that Rac1, but not Rac2 or Cdc42, is essential for thymocyte migration towards SDF-1α. The essential role of Cdc42 for human T-cell development we show in this report is therefore unlikely to be mediated by a role in thymocyte migration.
Discussion

With this study, we show that Cdc42 is necessary for T-cell development. Cdc42 has been shown to be important for hematopoietic stem cell (HSC) quiescence and retention in the bone marrow in conditional knockout mice, but no data are available on the effect of loss-of-function of Cdc42 in T-cell development. However, Wiskott-Aldrich syndrome patients mutated in the activated-Cdc42 binding protein WASP show reduced T-cell development and function. Progenitor cells transduced with dominant negative Cdc42 sustain in vitro culture with cytokines, but not in FTOC for the same period, showing that Cdc42 is specifically necessary for T-cell development.

As inhibition of migration was considered a possible mechanism for mutant Rho GTPases to disturb migration, we investigated this option. However, migration towards SDF-1α is not impaired after treatment of thymocytes with Cdc42 inhibitor Secramine A, or after transduction of the dominant negative mutant of Cdc42. We also ruled out the possibility that Cdc42N17 transduced cells are unable to enter the thymic lobe during hanging drop, as transduced cells were not enriched in the fraction not entering the lobe (data not shown). Also from the ISP4 population, expressing in part a pre-TCR, thymocyte generation was greatly reduced due to dominant negative Cdc42 expression. Pre-TCR signaling therefore cannot overcome the effect of dominant negative Cdc42. Although we could not reliably measure Cdc42 activity in thymocyte fractions CD34+ nor in ISP4, these results show that the mutant protein competed in sufficient amount for Cdc42 specific GEFs in ISP4 cells.

Another process that could be affected is proliferation. Our observation that T-cell development is hampered overall, with no obvious block at a specific developmental stage and unaltered subset ratios is in agreement with the hypothesis that proliferation is hampered when Cdc42 cannot be activated, possibly related to β-selection. Indeed, thymocytes generated are present in normal DP over SP ratio’s, but are greatly reduced in number. Moreover, Ki-67 staining showed that thymocytes expressing dominant negative Cdc42 had a reduced proliferating fraction. The signal transduction pathways downstream of the pre-TCR that lead to induction of proliferation have not been fully elucidated yet, but recently a role for cyclin D3 was demonstrated in mice and humans. Cdc42 has a role in G1 cell cycle progression through stimulation of cyclin expression and translation. A role for Cdc42 in proliferation seems to be highly cell type specific, as conditional Cdc42-/- mouse HSCs display increased proliferation, while cell cycle progression of embryonic stem cells and embryonic fibroblasts is respectively unaffected and reduced in the absence of Cdc42. However, it has been shown that Cdc42 is activated upon TCR engagement of murine T cells, and expression of dominant negative Cdc42 results in reduced proliferation upon full T-cell activation. It is therefore...
tempting to speculate that disturbance of proliferation (downstream of the pre-TCR) is at least in part responsible for the observed defects in T-cell development in our model. In line with this, apoptotic death, the default pathway for thymocytes not triggered to proliferate and mature, was enhanced in thymocytes expressing dominant negative Cdc42 compared to control transduced cells. Together, these experiments reveal an important role for Cdc42 in proliferation and cell survival during normal T cell development.

Transgenic mice expressing a constitutively active mutant of Cdc42 have reduced thymocyte numbers due to increased apoptosis in the thymus. We show that expression of a constitutively active mutant in human CD34+ progenitor cells also results in reduced thymocyte generation. Thus, Cdc42 activity has to be kept in check to allow T-cell development.

The discrepancy between the inhibition of T-cell development mediated by expression of constitutively active Rac2, and the significant but minor effect of the dominant negative mutant of Rac2, is similar to studies of transgenic and knockout mice. One explanation that should be considered is that Rac2 is not abundantly active in the early stages of T-cell development, which is supported by expression analysis. The enforced expression and activation might cause aberrant effects, not related to its true function. However, another possibility is that Rac1 activation partially compensates for loss of active Rac2 and vice versa. Double transduction experiments could resolve this question, but due to the low frequency of double transduced cells, this was technically not possible. However, this hypothesis is supported by mice studies, by comparison of T cell development in Rac1−/−/Rac2−/− double knockout mice and single knockout mice. Expression of dominant negative Rac1 did not result in a lower, but even a higher thymocyte generation ratio, as compared to control transduced cells, while migration was severely impaired. This suggests that Rac1 dependent migration to SDF-1α is not necessary for T-cell development in this model, or that residual migration is enough to provide the necessary signals to developing thymocytes. Among the dominant negative mutants, only Rac1N17 disturbs migration, while all constitutively active mutants impair migration to a certain extent. We conclude that in our experiments, there is no correlation between thymocyte development and migration to SDF-1α after manipulation of Rho GTPase activity.

All constitutively active mutants disturb T-cell development and we noticed that a high transgene expression level correlates with higher CD3 expression and higher TCR expression. It is unclear whether this skewing of CD3 is the result of a direct effect of Rho GTPases on CD3 expression, or an
indirect developmental effect. Apart from this, we found no phenotypical differences between cells expressing these constitutively active mutants or EGFP only, most CD3 positive cells being CD4⁺CD8⁻.

Dominant negative Rho GTPase mutants operate by sequestering GEFs away from the endogenous counterparts. The first implication is that these mutants need to be overexpressed in large excess in order to effectively inhibit activation of endogenous protein. Rac1N17 disturbs migration while Cdc42N17 hampers T-cell development. Hence, these functional effects combined with the performed controls (material and methods section) convincingly demonstrate that the constructs are functional. Rac2N17 does not result in major alterations of cell behavior. We demonstrated that expression of the transgene effectively led to generation of the protein, detected by Western blot (data not shown). However, we cannot exclude that the expression level is too low to result in effective block of endogenous Rac2 activation.

A second implication of the use of dominant negative mutants is the concern about specificity, especially with regard to Rho GTPases from the same subgroup, like Rac1 and Rac2, that share GEFs.⁴⁹,⁵⁰ To account for this, we applied two different methods to inhibit the function of Rho GTPases in chemotaxis, i.e. administration of inhibitors and gene transfer of dominant negative mutants. Due to the duration of the FTOC and the short half-life of the inhibitors, inhibitors could not be used in FTOC. Moreover, inhibitors could also affect thymic stroma besides the thymocytes. However, the differential outcome of both chemotaxis assays and FTOC upon expression of Rac1N17 and Rac2N17 argues for biological activity of these mutants. For example, we found that Rac1N17 blocks migration while Rac2N17 doesn’t. If Rac2N17 would reduce Rac1 activity, it should also block migration. Yet, we cannot exclude that Rac1N17 has a broader effect than competing only with GEFs specific for Rac1. Despite their very high overall amino acid sequence similarity, Rac1 and Rac2 can be targeted to different subcellular locations via the hypervariable C-terminus.⁵¹ This can explain their specific functions, and it could also explain the rather specific action of the mutants although they can be activated by the same GEFs and activate mutual effectors.

**Conclusion**

We demonstrate that Cdc42 is essential for T-cell development but not for migration of thymocytes towards SDF-1α. Increased apoptosis and reduced proliferation were found in thymocytes expressing dominant negative Cdc42. The role of Cdc42 in pathological development of human T cells and a possible Cdc42 deregulation in their malignant counterparts merits further research.
Authorship and disclosures

KS and BV designed the study, co-ordinated research and wrote the paper. KS, VI, VS, PVH, EN, PM, KA, MB and BV collected data and participated in statistical analysis and interpretation. JP interpreted data. All authors revised and approved the final manuscript. The authors reported no potential conflicts of interest.
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Expression pattern of Rho GTPases in human thymus.

Real time PCR analysis of sorted thymocyte subsets. Bars indicate mRNA expression level of the indicated gene relative to the housekeeping gene YWHAZ for the corresponding subset. For comparison of the 3 donors, levels of individual subsets were normalized to mean donor expression overall subsets, designated zero. Error bars indicate standard deviation.
Figure 2

Flowchart of fetal thymus organ culture (FTOC). TGR: Thymocyte generation ratio.
Figure 3

A

B

C

D
Influence of expression of mutant Rho GTPases on human T-cell development.

(A-B) Overview of T-cell development as evaluated in FTOC. Cells transduced with dominant negative mutants (A) and constitutively active mutants (B) of Rho GTPases. TGR was calculated as indicated in ‘Material and Methods’. Within each graph, each symbol represents data derived from the same thymus donor. Mean is represented by the thick horizontal line. Asterisk indicates statistically significant difference between mutant Rho GTPase and control (* p<0.05; ** p<0.01).

(C-D) Representative flow cytometric analysis of control, dominant negative mutant (C) and constitutively active mutant (D) Rho GTPase transduced cells analyzed after 21 days of FTOC. Upper panels are gated on EGFP+ fraction, lower panels (EGFP versus CD3) are gated on total human cells. Figures indicate fraction of DP cells (upper dot plots) and mean fluorescence intensity of CD3 for EGFP− (left) and EGFP+ (right) cells (lower dot plots). Quadrants are set according to isotype controls.
Figure 4

Kinetic analysis of T-cell development of progenitor cells expressing Cdc42 mutants.

(A-B) FTOC started from transduced CD34\(^+\) cells (A) or ISP4 cells (B), analyzed at different time points. Bars represent TGR and results shown are representative of at least 2 independent experiments.

(C-D) Representative flow cytometric analysis after 4 (C; upper panel) and 21 (C; lower panel) or 14 (D) days of FTOC started from CD34\(^+\) cells. Gates are set on human EGFP\(^+\) cells. Figure indicates the percentage of cells in the corresponding quadrant. Quadrants are set according to isotype controls. Results shown are representative of at least 2 independent experiments.
Impaired proliferation in thymocytes expressing dominant negative Cdc42N17 mutant.

Representative flow cytometric analysis after 4 (upper panels) and 21 (lower panels) days of FTOC started from CD34+ cells. Plots show thymocytes stained intracellularly with anti-Ki-67-PE. Figure indicates the percentage of cells in the corresponding quadrant. Quadrants are set according to isotype controls. Results shown are representative of at least 3 independent experiments.
Chemotaxis of thymocyte subsets in response to SDF-1α.

(A) The percentage of migrated cells in the presence of Rho GTPase inhibitors is calculated for each thymocyte subset. Relative migration is displayed, i.e. migration of a thymocyte subset in the presence of inhibitor normalized to migration of the same donor thymocyte subset in the absence of inhibitors. For total thymocytes (TOTAL), CD4⁺CD8⁻ (SP4), CD4⁺CD8⁺ (DP), CD4⁺CD8⁻ (DN) and CD4⁻CD8⁺ (SP8) cell fractions, thymocytes migrated ‘in bulk’ and subsets were determined by labeling with monoclonal antibodies and subsequent flow cytometry. For CD34⁺ cells (CD34), cells were sorted and analyzed in separate transwells. Asterisk indicates statistically significant difference between inhibition by NSC 23766 and control (* p<0.05; ** p<0.01).

(B) Representative flow cytometric analysis of EGFP expression of CD34⁺ cells transduced for chemotaxis assays. Gates E⁻ to E++ indicate expression intervals used for the calculation of migration of transduced cells as shown in (C).

(C) Migration of CD34⁺ thymocytes expressing mutant Rho GTPases, normalized to non-transduced EGFP⁻ cells in the same transwell. Asterisk indicates statistically significant difference between EFGP⁺ or EGFP⁺⁺ cells and EGFP⁻ (* p<0.05; ** p<0.01).

(A,C) Bars indicate mean of at least 3 independent experiments and error bars indicate standard deviation.
Chapter 3: Gene and transgene silencing in human primary CD4$^+$ T lymphocytes

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Title: Gene and transgene silencing in human primary CD4+ T lymphocytes

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Short title: Gene and transgene silencing in CD4+ T lymphocytes
Abstract

In biomedical research, translational relevance is increased by experiments in \textit{ex vivo} cultured primary cells such as human peripheral blood \( \text{CD}4^+ \) T lymphocytes (CD4\(^+\) T cells). However, manipulation of gene expression levels in CD4\(^+\) T cells \textit{in vitro}, without affecting the viability of the cells, remains a challenge. We present here an optimized lentiviral transduction method to knock-down gene expression by short-hairpin RNA (shRNA) transfer. Sustained downregulation of endogenously expressed cell surface receptor CD4 or cytoplasmic protein ADRBK1 can be achieved without needing to select transduced cells. In addition, consecutive transduction rounds were possible without compromising cellular survival. Expression of an eGFP transgene introduced by retroviral or lentiviral gene transfer was amenable to knock-down via a sequential transduction with a shRNA expressing lentivirus. Our approach allows us to study the function of host proteins in wild-type or transgenic CD4\(^+\) T cells.
Introduction

To study cellular function in health and disease, *in vitro* cell culture models are frequently a method of choice for practical and ethical reasons. Especially when manipulation of gene expression is required, *ex vivo* cell culture is frequently not an option given the sensitivity of primary cells to cellular stress and lack of survival signals [1]. Therefore, researchers often have to settle for the use of transformed cell lines, which may behave differently from primary cells [2,3]. For the study of human cells, peripheral blood is a readily available source of different cell types, such as lymphocytes which can be cultured for at least several weeks *in vitro*. Although methods for retroviral or lentiviral transduction of CD4⁺ T cells are available for over a decade [4-6], consecutive transductions remain a challenge. Furthermore, if manipulated cells must remain metabolically uncompromised, a straightforward method to achieve this in freshly isolated CD4⁺ T cells is still lacking. As a consequence, e.g. genome-wide siRNA screens to identify host proteins involved in HIV infection and replication were all done in cell lines [7], and hardly any of the identified partners were validated in primary cells so far. A major challenge in manipulation and culture of CD4⁺ T cells is to achieve sufficient survival of the cells. Experience in silencing "difficult-to-silence" genes, such as *SAMHD1* in post-activation resting CD4⁺ T cells for evaluating their role in HIV infection [8,9] indicated that shRNA transduction is less cytotoxic than repeated siRNA electroporation (Dr. O. Fackler and Dr. O. Keppler, personal communication). Moreover, unless chemically modified, transfected siRNA is lost over time in active primary T cells [10]. We therefore chose to use shRNA encoding lentiviral vectors to silence gene expression. We could show knock-down of endogenously expressed genes such as cell surface receptor CD4 and the cytoplasmic enzyme adrenergic, beta, receptor kinase 1 (ADRBK1) with high efficiency. Although selection of transduced cells could be omitted, a more sustained knock-down was achieved by including a single round of puromycin resistance selection. Furthermore, sequential transduction allowed the knock-down of transgenic eGFP expression.

We present a method that allows reaching sustained shRNA expression without affecting survival in CD4⁺ T cells. In addition, the ability to sequentially transduce CD4⁺ T cells with lentiviral and retroviral vectors opens the possibility to investigate the role of endogenous gene products in the function of a transgene.
Materials and Methods

Ethics statement
Peripheral blood from healthy donors was obtained from the Red Cross (Ghent, Belgium) after written informed consent as approved by the Ghent University Hospital Ethical Committee.

CD4 positive T lymphocytes isolation
Peripheral blood CD4+ T lymphocytes (CD4+ T cells) were isolated by negative selection using paramagnetic beads (MACS, Miltenyi Biotec, Bergish Gladbach, Germany) from buffy coat peripheral blood mononuclear cells obtained on Lymphoprep (Axis-Shield PoC, Oslo, Norway). Purity of the CD4+ T cell population was measured by flow cytometry (MACSquant® Analyzer using MACSQuantify v2.4 software, Miltenyi Biotec, Leiden, The Netherlands), showing a fraction of at least 95% CD4+CD3+ double positive cells. Antibodies used for staining were CD3-PE (clone SK7, Becton Dickinson Biosciences, Erembodegem, Belgium) and CD4-APC (clone MT4 66, Miltenyi Biotech). CD4+ T cells were cultured in Gibco® RPMI medium 1640 (Life Technologies, Ghent, Belgium) supplemented with 2 mM L-glutamin, 10% heat-inactivated fetal calf serum (FCS, Hyclone), 100 U/mL penicillin, and 100 µg/mL streptomycin (Life Technologies), supplemented with 20 ng/mL interleukin-2 (IL-2; specific activity 10 U/ng, Peprotech, London, United Kingdom), and stimulated with 1 µg/mL phytohemagglutinin (PHA) mitogen (Thermo Fisher Scientific, Waltham, USA) for the first 2 or 3 days of culture after isolation. Subsequently, cells were cultured in IL-2 supplemented medium without PHA, refreshed for half of the volume every 2 days. Cell survival was measured by flow cytometric absolute cell number counting (MACSQuant, Miltenyi Biotec) using propidium-iodide exclusion to gate out dead cells.

Vector production
All shRNAs encoding lentiviral vectors (TCR.1 LKO.1-puro vectors) that also encode a puromycin resistance gene were purchased from the Sigma MISSION® product line (Sigma-Aldrich, Bornem, Belgium, complete list is available in Table 1). The self-inactivating replication incompetent lentiviral particles were produced in HEK293T (DZSM, Braunschweig, Germany) in a 96-well production format by co-transfection of the shRNA encoding lentiviral vector plasmids with the MISSION Lentiviral Packaging Mix (Sigma Aldrich, VSV envelop) using FuGENE® HD (Promega, Leiden, The Netherlands) as instructed by the suppliers. The medium was refreshed after 1 day, and the supernatant was harvested two days after transfection and stored in aliquots at -80 °C until use. Additional shRNA vectors were the LKO.1 scrambled shRNA puromycin resistance encoding vector (SHC002, encoding a shRNA not targeting any known human expressed sequence nor eGFP), the LKO.1-sheGFP-puro
lentiviral vector encoding a shRNA sequence directed against the transcript of the eGFP protein (SHC005), and the LKO.1-eGFP vector obtained by replacing the puromycin resistance gene sequence in the Non-Mammalian scrambled shRNA control vector (SHC002) by eGFP [11]. To express eGFP as a transgene, the pTRIP-ires eGFP lentiviral vector (VSV envelop) or the Moloney Murine Leukemia Virus-derived retroviral vector LZRS-IRES-eGFP retroviral vector (amphotropic envelop), produced as described before [12], were used.

**Lentiviral transduction**

After isolation, CD4⁺ T cells were resuspended at a density of 5.4 x 10⁶ cells/ml in medium supplemented with IL-2, PHA and polybrene (Sigma Aldrich) to facilitate transduction. To perform the transduction, 300,000 cells (55 µL) were plated per well in flat bottom 96-well plates and 45 µL of unconcentrated lentiviral vector supernatant (between 700-3,000 mU RT/mL, based on measurement of reverse transcriptase activity [11]) was added to each well to obtain a final volume of 100 µL. Final concentration of polybrene at the moment of transduction was 8 µg/mL. Cells were immediately spinoculated for 30 minutes, 950 g at 32 °C and then cultured at 37 °C, 5% CO₂ (v/v).

During optimization, polybrene and/or spinoculation were omitted to test the effect on transduction efficiency. Twenty-four hours after transduction, the medium was removed and 200 µl of fresh medium containing IL-2 and PHA was added to the cells. In those experiments where lentiviral transduction was only performed 2 days after isolation, freshly isolated CD4⁺ T cells were first cultured for 2 days at a density of 7.5 X 10⁵ cells/ml in RPMI medium supplemented with IL-2 and PHA before transduction as described above, and further cultured in IL-2 supplemented medium after transduction. In the case of puromycin resistance gene encoding vectors, selection of transduced cells was performed with puromycin (1 µg/ml, Sigma Aldrich) supplemented medium, where indicated. For easy monitoring of the transduction efficiency, cells were transduced with a control LKO.1 vector expressing eGFP in some experiments [11] and the percentage of eGFP expressing cells was evaluated by flow cytometry (MACSquant® Analyzer, Miltenyi Biotec) 3 days later. Transduction efficiency varied between 60-80% over donors used.

**Sequential lentiviral transduction**

For sequential transduction of CD4⁺ T lymphocytes with lentiviral vectors, cells were transduced with the pTRIP-IRES-eGFP on the day of isolation as described above, to express eGFP. Two days later, a second lentiviral transduction with LKO.1-sheGFP-puro or the LKO.1 scrambled shRNA (SHC002) vectors was performed. Three days after the second transduction, the medium was replaced with fresh medium supplemented with IL-2 and, where indicated, with puromycin (1 µg/ml) to select the transduced cells for an additional three days.
Sequential lentiviral - retroviral transduction

Lentiviral LKO.1-sHEGFP-puro transduction was performed on the day of isolation as described above. Two days later, cells were transferred to a 96-well non-tissue culture plates (BD Falcon™, Erembodegem, Belgium) coated with retronectin (24 µg/ml; Takara, Saint-Germain-en-Laye, France) in 100 µl of fresh medium supplemented with IL-2 and were transduced with the LZRS-IRES-eGFP retroviral vector encoding eGFP [12]. After addition of 100 µl of retroviral particles containing supernatant to the cells, the plate was centrifuged for 90 min, 950 g at 32 °C and then cultured at 37 °C, 5 % CO₂ (v/v). After overnight incubation, the medium was replaced by 200 µl of fresh RPMI medium containing IL-2 and puromycin.

Quantitative real-time PCR

After transduction with shRNA encoding lentivirus and puromycin selection, the expression level of the target genes were measured with quantitative real-time PCR (qPCR). Reverse transcription was performed with SuperScript® III reverse transcriptase (Life Technologies), starting from 1 µg of total RNA extracted from the transduced cells (RNeasy kit, QIAGEN, Venlo, The Netherlands). Gene-specific primers were designed using Primer Blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) and the oligo analyzer 3.1 tool available online on Integrated DNA technologies website (http://eu.idtdna.com). Primer sequences are listed in Table 2. All qPCR reactions were performed using the LightCycler® 480 SYBR Green I Master mix (Roche Applied Science, Vilvoorde, Belgium) in a final volume of 10 µl on the LightCycler® 480 Real-Time PCR system. The thermal cycling conditions were composed of a first step for activation of FastStart Taq DNA polymerase and denaturation of the DNA at 95 °C for 10 min, followed by 45 amplification cycles at 95 °C for 10 s, 60 °C for 30 s and 72 °C for 30 s. The relative expression levels were calculated using the qBasePLUS software 2.0 (Biogazelle, Zwijnaarde, Belgium) with UBC (ubiquitin C) and YWHAZ (tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein) as reference genes. The stability of UBC and YWHAZ expression in manipulated CD4+ T cells was confirmed by the geNorm algorithm included in the qBasePLUS software.

Western Blot

CD4+ T cells transduced with the different ADRBK1 shRNA sequences were first selected with puromycin as described above and subsequently lysed in Laemmli sample buffer (0.125 M Tris [pH 6.8], 25 % glycerol, 2.3 % sodium dodecyl sulphate). Protein concentrations were determined by RC DC Protein Assay (BioRad, Nazareth, Belgium). Equal amounts of protein (10 µg) were run on a NuPAGE 4-12% Bis-Tris polyacrylamide gel (Invitrogen) and blotted onto PVDF membranes.
(Invitrogen) in reducing conditions. Antibodies used were: anti-ADRBK1 rabbit monoclonal antibody (1:5000 dilution, clone Y137, Abcam, Cambridge, U.K.); secondary antibody was donkey anti-rabbit IgG ECL antibody, HRP conjugated (1:5000 dilution, GE Healthcare, Diegem, Belgium). β-actin was stained with anti-β-actin antibody Beta Actin Loading Control Antibody (1/5000 dilution, BA3R, Thermo scientific) and secondary antibody sheep anti-mouse whole ECL antibody, HRP conjugated (1:5000 dilution, GE Healthcare). The signal generated using the Super Signal West Femto Maximum Sensitivity Substrate (Thermo Scientific) was detected by chemoluminescence with the VersaDoc Gel Imaging System model 3000 (Bio-Rad, Nazareth Eke, Belgium) and analyzed by Quantity One software version 4.4.1.

Statistics
Analyses were performed using the GraphPad Prism version 5.00 statistical software (GraphPad Software Inc., La Jolla, CA, USA), non-parametric tests used were Mann-Whitney U test and Kruskal-Wallis with Dunn’s correction for multiple testing.
Results

Optimization of lentiviral transduction of human peripheral blood lymphocytes (CD4$^+$ T cells) ex-vivo.

We chose to use commercially available shRNA encoding lentiviral LKO.1 vectors of the Mission consortium (Sigma-Aldrich, St. Louis, MO) to silence gene expression [13]. These vectors encode a U6 promoter to drive expression of the shRNA, as well as a puromycin resistance selection marker driven by a human phosphoglycerol kinase (PGK) promoter. We found the PGK promoter to drive expression very efficiently in CD4$^+$ T cells, in contrast to the widely used CMV promoter in other lentiviral shuttle vectors (data not shown). CD4$^+$ T cells were transduced either immediately after isolation at the start of culture or after 48 h of culture. Selection of the transduced cells with puromycin was started 3 days post-transduction. Cultures of selected cells showed equal survival rates compared to control non-transduced non-selected cultures from the same donor, indicating efficient transfer of puromycin resistance (Fig. 1A). There was no significant difference in survival rates in cultures of cells transduced directly upon isolation or after 48 h of culture and subsequently selected with puromycin. By contrast, non-transduced cells died in puromycin supplemented medium, as expected (Fig 1A). To measure transduction efficiencies most accurately, we replaced the puromycin resistance gene with eGFP [11]. Based on the fraction of eGFP expression, percentage of transduction was comparably efficient (60 to 80%) over donors tested, either transduced immediately or two days after the start of culture (Fig. 1B). This suggests that freshly isolated CD4$^+$ T cells which are effectively transduced, sustain to reach similar transduction efficiencies as in cells cultured for two days before transduction. In these experiments we used spinoculation after addition of the cationic polymer polybrene, the latter more important and described before to enhance transduction efficiency [14] (Fig. 1C).

Independent experiments with puromycin resistance encoding vectors showed that full puromycin resistance is only reached after 2 to 3 days post-shRNA transduction, and that a single selection cycle of 3 days is sufficient to select the effectively transduced cells (data not shown). Therefore, to keep culture periods as short as possible our standard protocol was to transduce CD4$^+$ T cells with the shRNA-encoding lentiviral vectors upon isolation, followed by 3 days of culture before selection with puromycin supplemented medium.

Transfer of shRNA by lentiviral vectors efficiently downregulates expression of endogenous genes.

Once the efficient transduction protocol was established, we determined whether the encoded shRNA was expressed sufficiently to downregulate the expression of an endogenous gene. We chose cell surface protein CD4 as the first target, and used four lentiviral vectors each encoding a different shRNA sequence targeting the CD4 transcript (Table 1, not validated by the supplier to effectively...
downregulate CD4 expression). As a control, a LKO.1 vector encoding a scrambled shRNA, not targeting any known human transcript, was used. Transduced CD4⁺ T cells were analyzed up to 13 days after transduction to determine mRNA and protein expression levels. Only one of the four vectors could reduce both mRNA expression levels (Fig. 2A) and cell surface expression (Fig. 2B, representative staining profile Fig. 2C) significantly, another clone reduced only cell surface expression modestly. When parallel cultures, not selected with puromycin, were assayed by flow cytometry, reduction of cell surface expression was similar early after transduction (Fig. 3). However, an increase in CD4 expression was evident 13 days after transduction in non-selected CD4⁺ T cells, but not in selected ones. This indicates that a selection round is especially useful to obtain long lasting knock-down. We also chose to downregulate expression of a cytoplasmic protein, adrenergic, beta, receptor kinase 1 (ADRBK1). Corresponding shRNA encoding lentiviral vector clones from the Mission consortium were not validated to effectively knock-down target transcript expression, similar to the shRNAs targeting the CD4 transcript (Table 1). One vector reduced mRNA levels significantly (Fig. 4A). The same clone reduced protein levels as measured by Western blot (Fig. 4B and C). These results show that vectors that transduce at similar efficiency, can differ significantly in their effect on the target gene expression. Most probably, the encoded shRNA sequences differ greatly in their efficiency to target the transcripts in CD4⁺ T cells.
Sequential transduction allows downregulation of transgene expression

To determine the feasibility of sequential transduction, we transduced eGFP expressing CD4⁺ T cells with a LKO.1 vector that encodes a shRNA targeting eGFP transcript. The first approach was to transduce CD4⁺ T cells with lentiviral vector encoding shRNA as described above, followed by retroviral transduction to introduce eGFP 2 days later. Retroviral transduction efficiency was on average 30%, as measured by eGFP expression in scrambled shRNA expressing cells. Cells expressing eGFP shRNA showed significantly reduced eGFP expression levels (70 to 80% reduction, Fig 5A and B). We also tested whether retroviral transduction could be delayed, if endogenous gene products with long protein half-life need to be knocked-down first. Efficient retroviral transduction at later time points required restimulation of CD4⁺ T cells with PHA after puromycin selection, but was similar to transduction levels in non-lentiviral transduced cells (data not shown). The second approach to knock-down transgene expression was sequential lentiviral transduction. Different from the experiments above, we here transduced CD4⁺ T cells upon isolation with a lentiviral vector encoding eGFP, and subsequently with the eGFP shRNA encoding vector 2 days later. Based on measurement of eGFP mean fluorescence intensity, a knock-down of over 80% was reached this way (Fig. 6C and D). These results show that irrespective of the order, successive transductions of CD4⁺ T cells allows to knock-down a transgene, encoded either by a lentiviral or a retroviral vector, using lentiviral vector encoded shRNA. In this setting, eGFP levels remained significantly reduced for at least 11 days of culture (Fig. 6). However, similar to downregulation of an endogenous gene expression (Fig. 3), selection of transduced cells could prevent a decrease in downregulation of transgene expression over the observation period.
Discussion

In this report, we present optimized cell culture and transduction conditions for silencing endogenously and transgenically expressed gene products, using lentiviral-mediated transfer of shRNA in CD4⁺ T cells. It is increasingly recognized that the use of cell lines in in vitro models is inherently flawed by the transformed phenotype, thus reducing relevance of the experimental data obtained for (patho)physiological processes [2,3]. The use of primary cells of human or animal origin is however not always feasible because of ethical reasons, or because of difficulties to culture the cell types studied. The great advantage of hematopoietic cells is that they can be harvested from the study objects with minimal invasive techniques without putting the health of the individuals at risk. Peripheral blood lymphocytes are attractive study material given their relative resistance to apoptosis in cell culture [15]. However, manipulation of gene expression puts additional stress on the cells in culture especially if physical methods such as transfection or electroporation are used [2]. Several papers report successful transduction of CD4⁺ T cells, including effective transfer of shRNA in post-stimulation resting cells [8,9]. However, an efficient method that allowed sustained knock-down and consecutive transduction without compromising cellular survival was lacking to date. During optimization, we observed that the timing of IL-2 addition and appropriate cell density is important in the different phases of culture. By refreshing half of the medium volume every two days, cytokines and other factors produced by the cells are not depleted while IL-2 and serum factor levels are maintained. Moreover, we chose not to work with concentrated virus preparations, as this is laborious in screening efforts and in our hands some preparations are toxic to the cell culture (data not shown). Rather, we produced and applied vector supernatants in a standardized way while keeping IL-2 concentrations constant at all time. In this way, we did not observe any effect on growth in transduced compared to not-transduced cultures.

To reach efficient knock-down 3 to 4 days after transduction, selection of transduced cells was not essential, leaving researchers the option not to expose cells to puromycin. However, a single round of selection in puromycin supplemented medium was sufficient to obtain sustained knock-down up to almost two weeks after transduction. CD4⁺ T cells can be cultured for extended periods in vitro with intermittent restimulation, although this selects for clonal expansion in the population [16]. We therefore did not culture for more than 2 weeks, but knock-down will most likely last longer. This period is sufficient to answer a diverse range of questions, e.g. we currently aim to combine the silencing protocol with HIV infection in order to study host protein involvement in infection and replication in CD4⁺ T cells (Iannucci et al., manuscript in preparation).
The ability to perform consecutive transductions opens the opportunity to silence either a transgene, or host genes in transgenic CD4^+ T cells. This allows to investigate the functional role of host proteins in the (patho)physiology of dominant active mutants like receptors (e.g. Notch mutations [17]), signaling proteins (e.g. constitutive active GTPases [18]), cytoskeleton proteins (e.g. ezrin [19]), transcription factors, and others. For gene therapy purposes, T cells are modified by transduction to either express or downmodulate the expression of a protein [20,21]. With the methods presented here, the function of such transgenes can be studied. Another application is the study of the function of microbial proteins such as viral proteins when expressed in CD4^+ T cells [12]. The methods presented in this paper will thus be of interest for application in a wide range of bio-medical research fields.
Acknowledgement

We thank Drs. Van Steendam and Deforce for assistance in Western blot quantification and the BCCM/LMBP Plasmid Collection of the Department of Biomedical Molecular Biology, Ghent University for distributing the pLKO.1 shRNA encoding lentiviral vector plasmids.

Author contribution

V.I., A.L., J.V., A.V.N., W.W. and A.B. performed experiments on transduction of primary cells, V.I. and A.L. performed optimization of sequential transduction, E.V., H.V. and K.V.L. produced virus and constructs, M.B. and B.V. initiated and supervised the study, V.I., M.B. and B.V. analyzed data, V.I. and B.V. wrote the manuscript.
References


Table 1: List of shRNA sequences used for knock-down of host proteins.

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Table shows gene symbol and full name description according to the HUGO Gene Nomenclature Committee database (www.genenames.org); gene ID and RefSeq code according to the Reference sequence database of National Center for Biotechnology Information (www.ncbi.nlm.nih.gov); TRC number from the RNAi consortium clones distributed by Sigma-Aldrich (www.sigmaaldrich.com), corresponding region in the transcripts targeted (UTR untranslated region, CDS: coding DNA sequence). Validation data of transcript level knock-down with at least 70% in one or more cell lines was not available for either of the clones with the supplier on July 4th 2013.
Table 2: List of primer sequences used to detect transcripts of host proteins.

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Table shows gene symbol according to the HUGO Gene Nomenclature Committee database (www.genenames.org); RefSeq code, transcript variant type of known protein coding transcripts according to the Reference sequence database of National Center for Biotechnology Information (www.ncbi.nlm.nih.gov), orientation and sequence of primers used to amplify the transcripts, amplification region in corresponding transcript and PCR product length.
Optimization of shRNA encoding lentiviral vector transduction of CD4⁺ T cells.

A. Percentage of surviving cells in cultures treated as indicated, measured by flow cytometric cell counting, of CD4⁺ T cells 6 days post-transduction (including 3 days of puromycin selection for selected cultures) with LKO.1 scrambled shRNA–puro vector (SHC002). Survival of CD4⁺ T cells transduced immediately upon isolation (Day 0) or after 2 days of culture (Day 2) is shown, N=3 donors. Each column represents average, no significant difference between “not transduced, not selected” and “transduced, selected” cultures, error bars indicate standard deviation (SD). B. Percentage of transduced cells measured by eGFP expression, 3 days post-transduction with LKO.1 scrambled shRNA and eGFP encoding vector. Measurements in CD4⁺ T cells of different donors transduced immediately after isolation (Day 0) or after 2 days of culture (Day 2) are shown, N=6 data points per donor connected, no significant difference (p>0.05). C. Percentage of transduced cells measured by eGFP expression 3 days post-transduction with LKO.1 scrambled shRNA and eGFP encoding vectors. The effect of polybrene and application of spinoculation on transduction efficiency is shown. Each column represents average, error bars indicate SD (N=3 donors), ** p<0.005, Mann-Whitney U test.
Figure 2

A. mRNA levels of CD4 transcript in CD4⁺ T cells expressing CD4 specific shRNA sequences (clones are numbered consecutively in the order of Table 1), relative to cells expressing scrambled shRNA, at different points in time after transduction. Each column represents average, error bars indicate SD (N=2 donors), ** p<0.005, Kruskal-Wallis test.

B. Mean fluorescence intensity of CD4⁺ T cells expressing CD4 specific shRNA sequences (clones are numbered consecutively as in A.) or scrambled shRNA, stained for CD4 surface expression and analysed by flow cytometry at different points in time after transduction. Each column represents average, error bars indicate SD (N=2 donors), * p<0.05, *** p<0.001, Kruskal-Wallis test.

C. Representative CD4 staining profile of CD4⁺ T cells, either expressing scrambled shRNA or CD4 shRNA (selected or not selected). Profile of not stained cells is shown as a reference.

Specific shRNA sequences expressed in CD4⁺ T cells reduce CD4 expression.

A. mRNA levels of CD4 transcript in CD4⁺ T cells expressing CD4 specific shRNA sequences (clones are numbered consecutively in the order of Table 1), relative to cells expressing scrambled shRNA, at different points in time after transduction. Each column represents average, error bars indicate SD (N=2 donors), ** p<0.005, Kruskal-Wallis test. B. Mean fluorescence intensity of CD4⁺ T cells expressing CD4 specific shRNA sequences (clones are numbered consecutively as in A.) or scrambled shRNA, stained for CD4 surface expression and analysed by flow cytometry at different points in time after transduction. Each column represents average, error bars indicate SD (N=2 donors), * p<0.05, *** p<0.001, Kruskal-Wallis test. C. Representative CD4 staining profile of CD4⁺ T cells, either expressing scrambled shRNA or CD4 shRNA (selected or not selected). Profile of not stained cells is shown as a reference.
Sustained reduction of cell surface CD4 expression in CD4$^+$ T cells expressing a functional shRNA sequence.

Mean fluorescence intensity of CD4$^+$ T cells expressing scrambled or CD4 specific shRNA sequence 3 (clone is numbered as in Fig. 3) either selected for transduction with puromycin or not, stained for CD4 surface expression and analyzed by flow cytometry at different points in time after transduction. Each point represents average of at least 2 donors, error bars indicate SD.
Figure 4

A

mRNA relative expression

0.0 0.5 1.0

1 2 3 4 5

ADRBK1 shRNAs

B

1 2 3 4 5

ADRBK1

β-actin

C

Protein relative expression

0.0 0.5 1.0

1 2 3 4 5

ADRBK1 shRNAs
Specific shRNA sequences expressed in CD4+ T cells reduce ADRBK1 expression.

A. mRNA levels of ADRBK1 gene expression in CD4+ T cells expressing ADRBK1 specific shRNA sequences (clones are numbered consecutively in the order of Table 1), relative to cells expressing scrambled shRNA, 3 days after puromycin selection started. Each column represents average, error bars indicate SD (N=3 donors), * p<0.05, Kruskal-Wallis test. B Western blot of protein lysates of CD4+ T cells expressing ADRBK1 transcript specific shRNA sequences (clones are numbered consecutively as in A), scrambled shRNA (both 3 days after puromycin selection started) or not-transduced as indicated. Blots were stained for ADRBK1 protein and β-actin as indicated. Bands are present at the expected corresponding molecular weight, marker with indication of molecular weight (in kDa) is shown. C. Protein expression levels of target proteins quantified on Western blot in CD4+ T cells expressing target ADRBK1 transcript specific shRNA encoding clones (clones are numbered consecutively as in A.), relative to scrambled shRNA expressing CD4+ T cells, first normalized on β-actin. Each column represents average, error bars indicate SD (N=3 donors), * p<0.05, Kruskal-Wallis test.
Reduction of eGFP transgene expression upon transduction with shRNA encoding lentiviral vector.

A. Histogram shows fluorescence intensity of eGFP expressing CD4+ T cells (eGFP encoded by a retroviral vector used to transduce CD4+ T cells after 2 days of culture), transduced on the day of isolation with a scrambled shRNA (grey line) or shRNA targeting eGFP (black line) encoding lentiviral vector. Cells were selected 3 days after lentiviral transduction with puromycin for 3 days and cultured for another 2 days (CD4+ T cells culture 8 days in total at the moment of measurement). For comparison, the profile of CD4+ T cells not transduced to express eGFP is shown (dotted grey line).

B. Mean fluorescence intensity of CD4+ T cells transduced as in A., and cultured several days post shRNA encoding lentivirus transduction, as indicated. Each column represents average, error bars indicate standard deviation (SD, N=3 donors), ** p<0.003 for each time point, Mann-Withney U test.

C. Histogram shows fluorescence intensity of eGFP expressing CD4+ T cells (eGFP is encoded by a lentiviral vector used to transduce CD4+ T cells at the moment of isolation), transduced 2 days after isolation with a vector encoding scrambled shRNA (grey line) or shRNA targeting eGFP (black line). Cells were selected 3 days later with puromycin for 3 days and cultured for another 5 days (PBL culture 13 days in total at the moment of measurement). For comparison, the profile of CD4+ T cells not transduced to express eGFP is shown (dotted grey line).

D. Mean fluorescence intensity of CD4+ T cells...
cells transduced as in C, and cultured several days after transduction with shRNA encoding lentivirus, as indicated. Each column represents average, error bars indicate SD (N=3 donors), * p<0.05 for each time point, Mann-Whitney U test.

**Figure 6**

![Stability of knock-down of lentiviral encoded eGFP transgene](image)

**Stability of knock-down of lentiviral encoded eGFP transgene.**

Mean fluorescence intensity of eGFP in CD4⁺ T cells (eGFP encoded by a lentiviral vector used to transduce freshly isolated CD4⁺ T cells), transduced 2 days after isolation with a vector encoding scrambled shRNA (●) or shRNA targeting eGFP, either puromycin selected for 3 days (single round) (■) or not selected (♦), and cultured for up to an additional 11 days. D: day after shRNA lentiviral transduction, error bars indicated SD.
Discussion and future perspectives

Three decades after its first identification, the complete eradication of the HIV-1 virus from the body of infected individuals remains most challenging. Despite the many progresses in the field of the treatment, the infection is still virtually incurable and will, without treatment, give rise to AIDS in the overwhelming majority of patients. The goal of this study was to identify host cellular factors that play a role in the viral life cycle to gain a better understanding of the molecular mechanisms that regulate it. The final aim was to propose alternative substrates for the design of a therapeutic strategy that would be less prone to viral escape.

In order to study the function of specific proteins in T cell development and HIV-1 infection, we either induced overexpression or down-modulation of specific genes then analyzed consequential functional effects. Several methods were available for transfer of genetic material into a cell. However, chemical and physical methods, like transfection and electroporation, despite lower costs and less safety issues, are not suitable for all human primary cells and only allow for transient transgene expression [239]. To obtain higher transgene transfer efficiency and stability of expression over time while preserving viability, we preferred the use of viral vector-mediated transfer of the gene of interest.

Although the use of cell lines has some advantages over primary cells in costs and ease of manipulation in vitro, as we show in Chapter 4, they may lack reliability. Indeed, these transformed cells have undergone mutations to acquire the ability to divide infinitely [276]. Therefore, when it comes to performing studies based on protein expression profiles, they might behave differently from primary cells and be deceptive. For this reason, we decided to use primary human cells in all of our investigations.

It is true that methods for retroviral transduction of primary cells are already available [277, 278], but in Chapter 2, we show an implemented method that allows gene transfer by single or combined retroviral and lentiviral transduction at high efficiency. In our method, transgene expression and/or gene knock-down is stable for up to two weeks in culture and not toxic. Our approach results in a targeting of more than one gene simultaneously achieved by consecutive transductions. This allows the expression of the two transgenes to be temporally separated which is of pivotal importance to distinguish between primary and secondary effects in a complex phenotype. For instance, it could be used to induce a
sequential knock-down of redundant proteins, instead of a single transducing vector with multiple cassettes of expression [279], or by co-transduction with multiple vectors. Stable knock-down of more than one protein leads to a reduction in viral escape, which is useful for RNA interference-based therapy in chronic viral infection [280]. It would be of particular interest to construct a vector carrying multiple transgenes under the control of inducible promoters that would allow us to achieve a controlled regulation of the transgene expression [281, 282]. Even though inducible promoters have already been used, [283, 284] such an inducible system might not be ideal since the use of an inducer could result in lower expression efficiencies and/or interfere with cellular survival [285, 286].

The thymus plays an important role in the regeneration of a functional immune system during HAART treatment in HIV-1 infection. One of our goals was to gain more insights into the cellular factors that regulate T cell development for a better understanding of the mechanisms that guarantee intact thymic function, namely the output of naïve T cells, which is impaired during HIV-1 infection of the thymus [36].

Thymocytes at different stages of development are susceptible to HIV-1 infection and the generation of T cells can be impaired by the unique expression of the viral protein Nef [287]. This protein has the ability to alter the expression of several surface receptors such as CD4, MHC class I and II, CD28, CD8αβ but its effect on thymic function is related to the protein domains necessary for interference with T cell signaling pathways [288]. More specifically, the disruption of interaction with the PAK2 protein and its upstream activator VAV1 restores T cell generation in the presence of Nef. VAV1 is a GEF that is able to activate the members of the Rho GTPases family Rac1, Rac2, and Cdc42 that in turn activate PAK2 [289]. Nef-PAK2 interaction induces both cytoskeletal rearrangements [290] and enhancement of T cell activation [291]. We therefore focused on the study of Rho GTPases function during T-cell development in humans FTOC, since their role was already demonstrated in mice studies [45].

The expression of Rho GTPases varies during T cell development, implying a specific role at different stages of thymopoiesis. Expression of all constitutively active mutants affected thymopoiesis, with a stronger effect in the case of Rac2. These constitutively active mutants also impaired thymocyte migration.

Expression of Rac1 and Rac2 dominant negative mutants in the CD34+ progenitors or ISP4 thymocytes resulted in only minor differences in T cell development. Only when dominant
negative Cdc42 was expressed, reduced thymopoiesis from transduced progenitors cultured in FTOC was observed. However, on thymocyte migration, studies with dominant negative mutants and specific drug inhibitors showed only a role for Rac1. Therefore, the effect of the mutants on thymopoiesis and on thymocyte migration does not correlate.

The fact that progenitors expressing dominant negative mutants can sustain in cell culture in the presence of cytokines, suggests that the role for Cdc42 is specific for thymopoiesis, and not due to toxicity. However, the perturbation of T cell development could be the result of a reduced capability of proliferation and/or increased apoptosis during development. T cell differentiation, although greatly reduced in numbers, could still occur in Cdc42 dominant negative expressing thymocytes. Cdc42 might be involved in cell cycle progression by inducing the expression of cyclin D1 [292] or p21 degradation after integrin-mediated adhesion signals [293]. Furthermore, Cdc42 was found to play a role in the control of mitosis in the attachment of the spindle microtubules to the kinetochore or in cytokinesis [294].

In order to better define the specificity of the role of Cdc42 in thymopoiesis, we could silence the expression of this protein by shRNA under the control of an inducible promoter in FTOC [295]. This would allow us to target specifically the expression of the protein at different stages of thymopoiesis to gain a better understanding of which stage requires Cdc42 to function properly. Once the critical step is identified, it would be of particular interest to compare gene expression profiles in the presence or absence of Cdc42 to analyze differences, for example in genes necessary for cell cycle progression such as cyclin D1 or apoptosis [296]. Cell cycle progression could also be analyzed in cells at different expression levels of the protein, by the use of carboxyfluorescein succinimidyl ester (CFSE), a molecule that is taken up by the cells and diluted upon cellular division, which also allows us to discriminate different lymphocytes subset by concomitant surface labeling [297].

We have shown that Cdc42 is necessary for T cell development at early stages. It was shown before that HIV-1 is able to infect CD34⁺ multipotent progenitors [298] and immature thymocytes [36]. Since the output of mature CD4⁺ and CD8⁺ thymocytes originating from Nef transduced precursors is reduced [299], this implies a possible contribution of deregulation in Cdc42 activity by HIV-1 Nef in the infected thymus. A first step could be the analysis of the gene expression profiles of thymocytes, expressing Nef or not, and compare them with the expression profiles of thymocytes expressing a Cdc42 mutant, especially for genes known to be potential Cdc42 effector targets [300]. The determination of Cdc42 activity in the presence
of Nef could give more information on a direct or functional interaction between the two proteins. Unfortunately, the most common assay to measure Cdc42 activity (G-LISA, Cytoskeleton Inc.), which is based on the quantification of GTP-bound active form of the GTPase, was not sensitive enough to measure protein activity in thymocytes.

In order to identify cellular factors involved in HIV-1 infection and replication, we developed a method that allowed the screening of gene products in primary CD4⁺ T lymphocytes, one of the most relevant targets of HIV in vivo. By the lentiviral transfer of a shRNA sequence to CD4⁺ T lymphocytes, we achieved stable knock-down of the protein of interest. In addition, subsequent HIV-1 infection and assessment of the effect of knock-down on the spread of the infection remains possible. The use of a HIV-1 reporter virus, that expresses the eGFP protein [301] allows easy measurement of infection by flow cytometry, ideal for high-throughput experiments. The high rate of infection achieved with spinoculation allows us to identify with more sensitivity shRNA’s affecting HIV infection. However, others reported that spinoculation can interfere with cellular cytoskeleton organization [302]. The requirement of cytoskeletal deregulation by Nef to overcome cellular restriction in the first phases of the HIV-1 life cycle is known [303]. Therefore, before starting the study presented here, to rule out the possibility that spinoculation could mask the virus’ effect on the cytoskeleton, we compared the ratio of infection rates of WT Nef over that of the less infective ∆Nef HIV-1 [304] in cells infected by spinoculation vs. no spinoculation. If spinoculation alters cytoskeleton reorganization in favor of the virus, then this would result in a loss of dependency on Nef for infectivity. However, this was not the case, supporting the validity of our method. Indeed, in the attempt to validate proteins identified as potential HIV-1 cofactors by RNAi screening in cell lines, we were able to confirm TNPO3 [305] as a true HIV-1 cofactor in primary CD4⁺ T lymphocytes using our experimental setup.

We identified six gene products that appeared to support HIV-1 infection and replication, by studying shRNA transduced CD4⁺ T lymphocytes of different donors. These proteins are ZAP-70, SLP-76/LCP2, JNK2/MAPK9, MAP2K4/MKK4, WASL/N-WASP and VAV1. All six identified gene products have a role as an effector or regulator in cytoskeleton pathways, and/or in the signaling cascade triggered upon engagement of the TCR, integrin, or chemokine receptors [306]. Although some of these proteins play a role in several pivotal cellular functions, we did not observe any cellular toxicity associated with the use of some of the
shRNA sequences used for gene silencing. This could be explained by insufficient knock down, by the expression of other proteins with a redundant function or by the adaptation of the cells using compensatory pathways.

Even though the biological effect associated with a given shRNA sequence is reproducible over different donors, the evaluation of expression knock-down both at mRNA and at a protein level is mandatory next to rule out any chance of off-target effects [307].

While our study highlights the importance of these proteins in HIV-1 infection and replication, it does not give any information on how and at which stage of the life cycle they are involved. A first step to research this is to clarify whether the shRNA induced knock-down of the proteins alters the activation status of the cells. We could show that surface expression patterns of activation markers such as CD69 or CD25 on CD4+ T lymphocytes, transduced with vectors encoding scrambled shRNA, do not differ from those of untransduced cells, suggesting that the introduction of the shRNA per se does not alter the activation state of the cells. However, the gene products mentioned above could support HIV infection and replication, as mediators of cellular activation pathways [308]. Beside surface markers, expression or activity could be determined for other molecules that are regulated by the proteins of interest mentioned. For example, specific activation of transcription factors such as NF-kB, AP-1, or NFAT; measurement of Ca2+ flux, or the total phosphorylation state of MAPKs [309] could be tested in the presence or absence of the expression of the proteins of interest. Indeed, the comparison between profiles of activation would allow us to identify which pathways are altered in the presence of the shRNA that in our experiments are necessary to support infection and replication of the virus.

To determine the mechanism of how the identified proteins play a role, different assays could be performed. First, shRNA expressing CD4+ T lymphocytes could be infected with an HIV-1 reporter virus in the presence of a viral protease inhibitor to analyze the effect of the shRNA on infection. In this way, we would inhibit the formation of new viral particles so that we could focus on the analysis of the shRNA effect in a single round infection, measuring only in the first phases of the viral life cycle, from entry to integration of the genome into the host. Second, the quantification of viral particles in the supernatant collected at different time points after infection would be a measure of viral replication; moreover, by using such a viral supernatant to infect untransduced cells of the same donors, we would be able to detect if the shRNA affects infectivity of the virions. Finally, to analyze viral transmission, we could use
a quantitative flow cytometry based assay [310] that selectively monitors cell-to-cell viral transfer. CD4⁺ T lymphocytes transduced with pLKO.1 vectors encoding the shRNA sequence of interest or a scrambled shRNA will be infected with the HIV-1 reporter virus. Next, these cells would be co-cultured with CD4⁺ T lymphocytes from the same donor, either transduced with a vector encoding the same shRNA or scrambled shRNA. This can be done in direct contact or separated by a virus permeable membrane in a Transwell® system. Prior to the co-culture, the infected cells will be stained with CFSE. The measure of cell-to-cell viral transmission is a quantification of CFSE negative cells expressing the HIV-1 marker in direct co-culture conditions corrected by the percentage of CFSE negative cells HIV-1 infected in the Transwell® system. The comparison between the percentages obtained in the different conditions would allow us to discriminate if the protein of interest is necessary in the donor or in the recipient cells for cell-to-cell transmission of the virus.

Among the genes identified, VAV1 has a central role in the TCR signal transduction to regulate cytoskeletal rearrangement by activation of the JNK, ERK, Ras, NF-kB, and NFAT pathways [311]. In a first attempt to define the role of this protein during HIV-1 infection, we expressed a constitutively active and dominant negative mutant of the Rho GTPases Rac1, Rac2, and Cdc42, and a VAV1 protein mutated in its GEF domain, in CD4⁺ T lymphocytes. We did not observe any significant difference in the rate of infection in the cells expressing the protein mutants compared to the controls. Perhaps the role of VAV1 during HIV-1 infection is independent from its GEF activity [312]. The next steps will be to use VAV1 mutants in different protein domains to identify which one is necessary to support HIV-1 infection/replication.

Although preliminary, our results show the validity of our method in the identification of several proteins involved in cytoskeletal organization and T cell activation that are important for supporting HIV-1 infection in primary CD4⁺ T lymphocytes.

In conclusion, with this work, we show that in vitro manipulation of gene expression in primary cells is a feasible method to research host factors involved in HIV infection. First, we established a protocol that advances the ability to knock-down genes and transgene in primary CD4⁺ T lymphocytes. Next, we showed cells manipulated by this approach can be infected with HIV to identify new cellular factors or pathways that support HIV-1 infection and replication.
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Summary

The aim of this work was to study proteins related to T cell activation and the cytoskeleton in primary cells, in particular during T cell development and infection with Human Immunodeficiency Virus (HIV), by using genetic manipulation to study gene function.

In order to do so we first developed a protocol for an efficient viral gene transfer in primary CD4\(^+\) T lymphocytes isolated from the blood of healthy donors. Due to the nature of these cells, manipulation in vitro requires careful handling and their relatively short life span obliges fast and sometimes limited procedures. We ideated a system that allows researchers to use primary cells for combined procedures of retro-lentiviral gene transfer that can be used in a broad variety of applications. We showed the possibility of achieving high transfer efficiency and stable transgene expression without toxicity or hampering the survival of the cells.

Second, we used gene manipulation of Rho GTPases to investigate their role in T cell development. We expressed both constitutively active and dominant negative mutants of three members of the Rho GTPases family, Rac1, Rac2, and Cdc42 in CD34\(^+\) T cells progenitors and analyzed T cell development in fetal thymus organ culture. We identified a very important role for Cdc42 in replication and/or apoptosis. Intriguingly this protein was previously shown to be part of a proteic complex constituted of other cellular proteins such as PAK2, VAV1, and Nef, a HIV-1 cellular protein that alone is able to perturb T cell development.

Finally, we used the RNA interference–mediated gene silencing to down modulate the expression of proteins related to the cellular cytoskeleton and T cell activation that could play a role in HIV infection of CD4\(^+\) T lymphocytes. In our approach, we combined specific silencing of host cellular proteins by short-hairpin RNA and subsequent infection with a fully replicative HIV-1 virus, to study the consequences of the missing proteins on the outcome of the infection. The reliability of the system was shown by confirming the role of a protein already identified as important for HIV infection in cell lines but not yet in CD4\(^+\) T lymphocytes, transportin 3 (TNPO3).

After screening 36 genes, we identified six proteins whose knock-down caused inhibition of the HIV-1 infection and replication in CD4\(^+\) T lymphocytes of five different donors. The products of these genes are: ZAP-70, SLP-76/LCP2, JNK2/ MAPK9, MAP2K4/MKK4, N-WASP.
and VAV1. Although some of the factors have been already shown to have a role in HIV infection, we give hints for new possible roles and implications regarding their functions.
Curriculum Vitae

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Publications, Presentations and Abstracts

Peer reviewed publications:


  *equally contributed

Oral Presentations at international conferences:

- **RNA interference in primary CD4+ T lymphocytes allows to validate relevance of host proteins for HIV infection/replication**

- **Identification of HIV host cellular cofactors by RNAi in primary T lymphocytes**
  HIV workshop XI, Rome, Italy, September 21-22, 2012

- **RNA interference to discover pathways involved in HIV infection: from deceptive cell lines towards primary cells**
  HIV workshop X, Rome, Italy, October 7-8, 2011
- **Transduction of RNA interference to discover pathways involved in HIV infection and replication**
  Interuniversity Attraction Poles 3rd meeting, Amsterdam, The Netherlands, January 21-22 2010

- **Transduction of RNA interference to discover pathways involved in HIV infection and replication**
  HIV workshop VIII, Rome, Italy, October 15-17 2009

**Abstracts and Posters:**

- **Use of RNA interference to discover pathways involved in HIV infection and replication: cell lines tell many stories, primary cells might tell the truth.**
  Retrovirology. 2011; 8(Suppl 2): P35
  Frontiers of Retrovirology, Amsterdam, The Netherlands October 3-5 2011
  [Iannucci V.], Landi A., Vermeire J., Naessens E., Vanderstraeten H., Meuwissen P., Eekels J., Berkhout B., Verhasselt B.

- **Evaluation of pathways and new host proteins involved in CD4 down-modulation during HIV-1 infection**
  Frontiers of Retrovirology, Amsterdam, The Netherlands 3-5 October 2011
  Landi A., Bentahir M., [Iannucci V.], Vermeire J., Vanderstraeten H., Verhasselt B.

- **RNAi strategies to silence HIV**
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  Eekels J., [Iannucci V.], Jeeninga R., Verhasselt B., Berkhout B.

- **Transduction of RNA interference to discover pathways involved in HIV infection and replication**
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  [Iannucci V.], Landi A., Vermeire J., Naessens E., Vanderstraeten H., Meuwissen P., Eekels J., Berkhout B., Verhasselt B.
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