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**Host restriction factors against human retrovirus:**  
**the role of CIITA and TRIM22**

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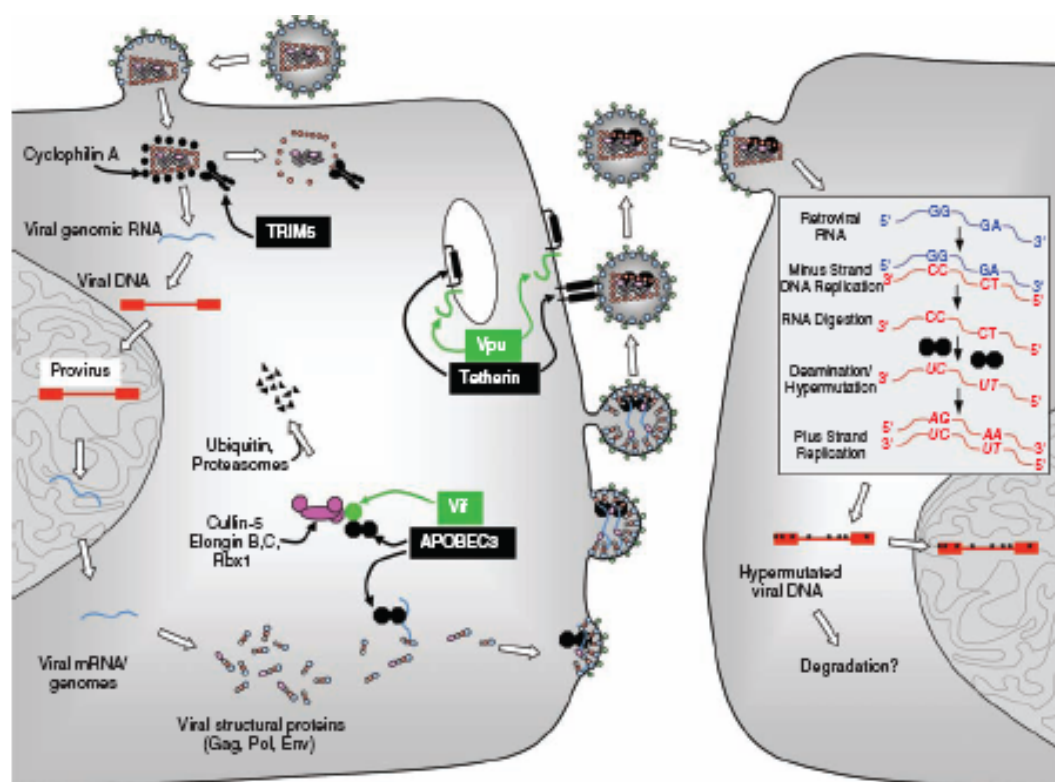
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# 1. Introduction

## 1.1 Host Restriction factors

The innate and acquired immune system represent an effective defence against virus infection, but this response is triggered only by recognition of an already replicating virus (Bieniasz P. et al. 2004 *Nat Immunol*). In addition to the conventional immune response many mammals, including humans, have evolved a complex of cellular proteins, named host restriction factors, that function as inhibitors of retroviral replication. These cellular factors are constitutively expressed or enhanced by interferons (Neil S, Bieniasz P. et al. 2009 *J Interferon Cytokine Res*) and represent an important part of intrinsic immunity against viral infection (Bieniasz P. et al. 2004 *Nat Immunol*). Nevertheless, retroviruses have evolved mechanisms to inactivate or overcome the block of infection through viral proteins that can efficiently counteract the inhibitory effect of some cellular restriction factors (Strebel K. et al. 2009 *BMC Med*; Goff SP. 2004 *Mol Cell*; Rowland-Jones S. et al. 2001 *Cell*). At present, the knowledge of the mechanisms by which restriction factors interfere with retroviral replication, and how their effects are avoided by virus, is sufficiently clear in particular for HIV-1. To date, three major types of antiretroviral restriction factor that are potentially capable of inhibiting HIV-1 replication have been identified (Sheehy AM. et al. 2002 *Nature*; Stremlau M. et al. 2004 *Nature*; Neil SJ. et al. 2008 *Nature*): APOBEC3G, TRIM5 $\alpha$  and tetherin (Scheme 1). Other cellular proteins were shown to interfere with viral infection (Ozato K. et al. 2008 *Nat Rev Immunol*). Here we focused our attention on two cellular factors, namely CIITA, well-studied in our laboratory, and TRIM22, that was shown to have antiviral activity by suppressing HIV-1 transcription (Ozato K. et al. 2008 *Nat Rev Immunol*). Of particular interest, in our laboratory we have demonstrated that the cellular factor CIITA, the master regulator of MHC Class II gene transcription, affected HIV-1 replication by inhibiting the viral

transactivator Tat (Accolla R. et al. 2002 *Eur J Immunol.*). In addition, we have recently described CIITA as a potent inhibitor of HTLV-1 and HTLV-2 replication, affecting their respective viral transactivators Tax. These findings suggest that CIITA acts as a viral restriction factor for human retroviruses, counteracting viral replication and spreading.



**Scheme 1.** The most important host restriction factors capable of inhibiting HIV-1 replication, and their viral antagonists evolved by the virus to avoid these host defenses. TRIM5 recognizes incoming HIV-1 capsids and inactivates them; Cyclophilin A also binds to incoming capsids and can affect recognition by TRIM5. APOBEC3 proteins can be incorporated into assembling HIV-1 particles, and catalyze the deamination of nascent minus strand DNA during the subsequent round of infection. The HIV-1 Vif protein binds to APOBEC3 proteins, and also to a Cullin5-based ubiquitin ligase complex. This results in APOBEC3 protein ubiquitination and degradation in proteasomes. Tetherin is a cell surface protein

that causes entrapment of nascent virions on the infected cell surface. Vpu antagonizes tetherin by sequestering tetherin from virions and by reducing the levels of tetherin on the cell surface. (Neil S, Bieniasz P. 2009 *J Interferon Cytokine Res*)

## APOBEC3 family

Apolipoprotein B mRNA-editing enzyme, catalytic polypeptide-like (APOBEC) family of cytidine deaminases represents the first group of proteins identified as inhibitor of HIV-1 replication (Sheehy AM. et al. 2002 *Nature*). Among them, APOBEC3G is the most studied in the context of HIV-1 (Bishop KN. et al. 2004 *Science*; Zennou V. et al. 2006 *Virology*; Virgen CA. et al. 2007 *J Virol*). Specifically, it can be incorporated into retroviral particles, by binding the packaged RNA (Svarovskaia ES. et al. 2004 *J Biol Chem*; Zennou V. et al. 2004 *J Virol*; Wang FZ. et al. 2007 *Human Herpesviruses*), and carried by the virion to a new target cell. During reverse transcription APOBEC3G deaminates the deoxycytidines of the viral minus-strand DNA, generating minus strand DNA containing many deoxyuracil nucleotides, whose replication results in G to A hypermutations in the complementary plus DNA strand (Chen D. et al. 2003 *Cell*; Lecossier D. et al. 2003 *Science*). However, recent finding suggest that the induced-mutation capacity of APOBEC3 seems to constitute only a part of the mechanism by which antiviral activity is achieved (Newman EN. et al. 2005 *Curr Biol*)

HIVΔ1 Vif protein counteracts APOBEC3G by blocking its incorporation into the viral particles. Vif binds to APOBEC3G and to the Cul5–elongin B–elongin C–Rbx1 ubiquitin ligase complex, causing APOBEC3G polyubiquitination and subsequent degradation by proteasoma (Mariani R. et al. 2003 *Cell*). Of particular interest for this work is APOBEC3A, whose deaminase activity is induced by IFN in monocytes and macrophages (Thielen, B. K. et al. 2010 *J Biol*). In this regard, recent reports

suggested that endogenously produced IFN- contributes to restricted HIV-1 viral replication in U937 promonocytic cells (Mace.K. et al. 1989 *Virology*).

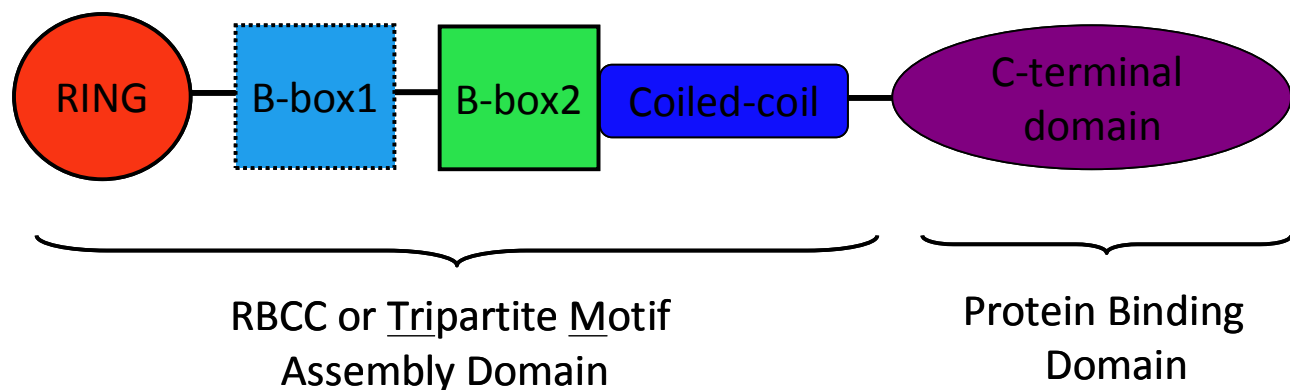
## Tetherin

Tetherin (also known as BST-2, CD317, or HM1.24) is an inhibitor of HIV-1 particles release from the infected cells (Neil SJ. et al. 2008 *Nature.*, 2008; Van Damme N. et al. 2008 *Cell Host Microbe*). It is a membrane protein, with unusual topology, which is essential for its antiviral function (Kupzig S. et al. 2003 *Traffic*). Tetherin seems to induce the formation of protein-based tethers that cause the retention of HIV-1 particles on infected cell surfaces (Neil SJ. et al. 2006 *PLoS Pathog*; Neil SJ. et al. 2008 *Nature*), preventing their spreading to the uninfected cells. Subsequently, tetherin-retained virions can be re-internalized into the infected cell and targeted to late endosomes, where they may be retained or destroyed by lysosomal enzymes (Neil SJ. et al. 2006 *PLoS Pathog*). Tetherin expression is induced by type I interferon, (Blasius AL. et al. 2006 *J Immunol*). In absence of stimulation tetherin was found only in a certain subtype of cells, suggesting that it is part of a specific innate immune defense, that limits the replication of many enveloped viruses (Neil SJ. et al. 2007 *Cell Host Microbe*; Neil SJ. et al. 2008 *Nature*; Jouvenet N. et al. 2009 *J Virol*; Kaletsky RL. et al. 2009 *Proc Natl Acad Sci U S A*; Sakuma R. et al. 2009 *J Biol Chem*). In the case of HIV-1, the viral protein Vpu sequesters tetherin from sites of HIV-1 particle assembly, thereby preventing it for encountering nascent virions (Neil SJ. et al. 2008 *Nature*; Jouvenet N. et al. 2009 *J Virol*). It has been suggested that Vpu may reduce the level of tetherin at the cell surface and trigger its proteasome-dependent degradation (Jouvenet N. et al. 2009 *J Virol*)

## TRIM family

The superfamily of tripartite motif-containing (TRIM) proteins is involved in gene regulation, ubiquitination, DNA recombination and DNA repair (Joazeiro CA. et al. 2000 *Cell*; Reymond A. et al. 2001 *EMBO J* ; Tissot C. et al. 1995 *J Biol Chem*; Tissot C. et al. 1996 *Genomics*). Moreover, mutations

in several TRIM proteins have been linked to human diseases. There are approximately 70 TRIM family members, and they are characterized by the presence of a tripartite motif, which consists of a RING domain, one or two B-box motifs, and a coiled-coil region (Singh R. et al. 2011 *J Virol*) (Scheme 2).



#### **Scheme 2. Schematic representation of TRIM protein family**

TRIMs share a common domain organization, with an N-terminal RING domain, followed by a B-box 2 domain, a coiled-coil motif, and a C-terminal domain. Some TRIMs contain a B-box 1 domain located between the RING and B-box 2 regions.

The C-terminal region can contain any of 10 distinct motifs alone or in combination, which results in the designation of 9 families, C-I to C-IX36 (Ozato K. et al. 2008 *Nat Rev Immunol*). Structural analyses of these domains in several family members have greatly increased the understanding of the contribution of each domain to TRIM protein functions. The RING domain is a specialized zinc finger of 40–60 residues that bind two zinc atoms, located in the N-terminal region of almost all TRIM proteins, which can mediate the covalent association of proteins with ubiquitin. Studies have demonstrated that a lot of RING finger proteins could simultaneously bind ubiquitylation enzymes and



the substrates through their RING finger domains and hence act as ligases in the ubiquitylation reaction. This function can contribute to the biological flexibility of TRIM proteins (Ozato K. et al. 2008 *Nat Rev Immunol*). For example, TRIM21 functions as an E3 ligase for IRF-8 (Interferon Interferon regulatory factor 8) 8 (IRF-8) and contributes to the elicitation of innate immunity in macrophages. TRIM25 induces the Lysine 63-linked ubiquitylation of RIG-I (Retinoic Retinoic acid inducible gene I), I (RIG-I), which is crucial for the RIG-I signalling signaling pathway to elicit host antiviral innate immunity (Duan Z .et al. 2008 *Biochem Biophys Res Commun*).

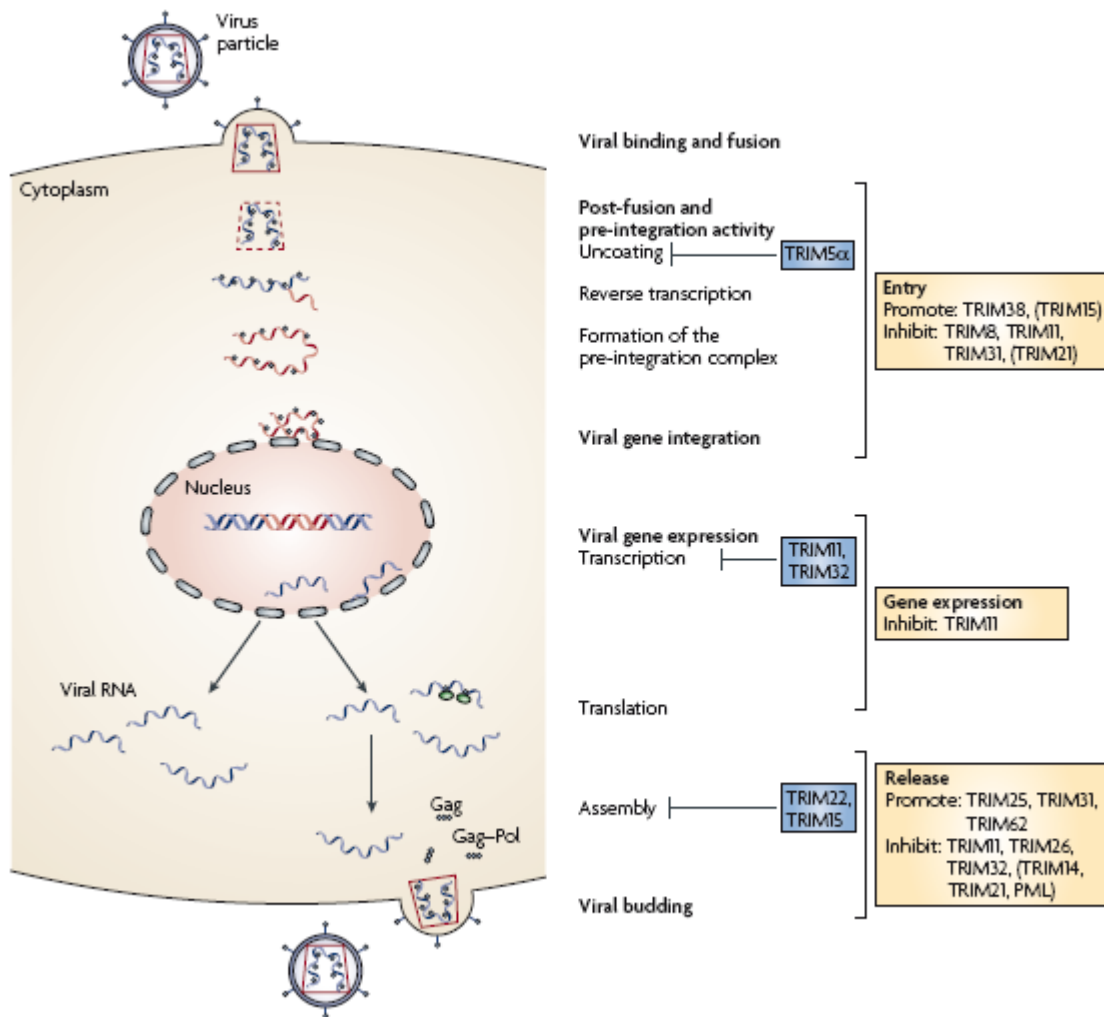
The RING-finger domain of several TRIM proteins, including TRIM5 $\alpha$  and TRIM22 contains a specialized zinc finger (Barr SD. et al. 2008 *PLoS Pathog*), which has been shown to possess an E3 ubiquitin ligase activity that triggers a cascade of ubiquitin transfer reactions to specific proteins leading to the tight control of the concentration or the subcellular localization of the target proteins (Gack MU. Et al 2007 *Nature*; Kallijarvi J. et al. 2005 *Exp Cell Res*; Kong HJ.et al. 2007 *J Immunol*; Kudryashova E. et al. 2005 *J Mol Biol*) (Eldin P. et al. 2009 *J Gen Virol*). The E3 ubiquitin ligase activity of the RING domain is important for the anti-retroviral function of many TRIM proteins. In the case of TRIM22 the conserved cys-15 and cys-18 of its RING domain are especially important for its biological functions (Yu S. et al.2011 *Biophys Res Commun*). Structural studies of several human TRIM B-box 1 and B-box 2 domains have shown they have ternary structures similar to those of RING domains, which suggests that all three domains have evolved from a common ancestral domain (Liu J. et al.2010 *PLoS One*). Indeed the B-box are zinc-binding motifs, and B-box 1 and B-box 2 are characterized by different consensus sequences between members of the TRIM family (Liu J. et al. 2010 *PLoS One*). These domains can mediate protein–protein interactions as well (Borden KL. et al.1998 *Biochem Cell Biol*; Nisole S. et al.2005 *Nat Rev Microbiol*). TRIM5 $\alpha$  and TRIM22 contain only the B-box 2 (Kajaste-Rudnitski A. et al. 2010 *Amino Acids*). In addition, the B-box and coiled-coil domains of several TRIM proteins have also been described to mediate homo- and hetero-

multimerization, promoting the formation of higher order protein complexes (Reymond A. et al. 2001 *EMBO J*). Such complexes are found in different subcellular compartments, thereby determining the specific functions of the various TRIM proteins (Herr AM. et al. 2009 *Immunogenetics*).

Several TRIM proteins also contain a C terminal SPRY domain that has been suggested to promote protein-protein interactions and RNA binding (Barr SD. et al. 2008 *PLoS Pathog*). In particular it was demonstrate that the SPRY domain is required for the nuclear localization of TRIM22, and its deletion would lead to the inability of TRIM22 to activate NF-kB (Yu S. et al. 2011 *Biophys Res Commun*). The C terminus of certain TRIM family members can also contain other different domains, such as, fibronectin type 3 (FN3) domains, characterized by binding sites for DNA and heparin (Ozato K. et al. 2008 *Nat Rev Immunol*). The tissue distribution of TRIM proteins is extremely heterogeneous. The overexpression of enhanced green fluorescent protein (EGFP)-TRIM fusion proteins, have demonstrated that TRIM proteins are localized in specialized sub-cellular compartments both in the nucleus and in the cytoplasm, suggesting that individual TRIM proteins may function in a unique sub-cellular compartments. The coiled-coil, the RING and the B-box domains are responsible for the specific localization of TRIM proteins; indeed mutations in these regions induce a diffuse redistribution of these proteins in the cell (Ozato K. et al. 2008 *Nat Rev Immunol*). The biological functions of TRIM proteins are largely unknown, but the TRIM family includes proteins with different activity such as tumor suppression, control of apoptosis, cell proliferation and senescence (Salomoni P. et al. 2002 *Cell*).

The numerous evidences of the effects of TRIM proteins on viral infections comes from studies of TRIM5 $\alpha$ , which strongly inhibits HIV-1, acting after virus entry into cells. TRIM5 $\alpha$  is responsible for the complete block of HIV-1 replication in Old World monkey cells. This effect is mediated through the interaction of rhesus monkey TRIM5 $\alpha$  trimers with hexameric HIV-1 capsid proteins (Singh R. et al. 2011 *J. Virol*; Stremlau M. et al. 2004 *Nature*). Further studies suggested that, in addition to the

effects of TRIM5 $\alpha$  on HIV via binding to capsid, other mechanisms of viral inhibition are possible. Specifically, in humans the C-terminal domain of the TRIM5 $\alpha$  protein directly recognizes the incoming viral capsids (Sebastian S. et al. 2005 *Retrovirology*) and thereby governs antiretroviral specificity (Perez-Caballero D. et al. 2005 *J Virol*; Stremlau M. et al. 2005 *J Virol*; Perron MJ. et al. 2006 *J Virol*), while a central coiled-coil drives TRIM5 $\alpha$  multimerization, which is essential for inhibition. To antagonized TRIM5 $\alpha$  restriction the virus exploits its capsid sequence ability to bind cyclophilin A (CypA), a very abundant cytoplasmic host protein. It has been suggested that CypA binding can inhibit restriction by TRIM5 $\alpha$  proteins (Zhang F. et al. 2006 *Virology*), perhaps by coating the incoming capsid, or by isomerizing peptidyl–prolyl bonds. In addition, TRIM5 $\alpha$  is responsible for the species-specific post-entry restriction of retroviruses, such as N-tropic murine leukemia virus and HIV-1, in primate cells (Sawyer SL. et al. 2005 *Proc Natl. Acad. Sci. U S A*; Singh R. et al. 2011 *J Virol*). Other TRIM E3 ligases with antiviral activity have been described (Yap MW. et al. 2004 *Proc Natl. Acad. Sci. U S A*). TRIM family proteins affect specific steps in the HIV life cycle. For most TRIM proteins, the exact step of the viral life cycle affected and the precise mechanisms of action are not known (Woods MW. et al. 2011 *Retrovirology*). TRIM proteins appear to mediate their antiviral activities via different mechanisms: interference with the un-coating of the viral pre-integration complex was noted for TRIM5 $\alpha$ , and an inhibition of viral budding has been described for TRIM22 (Ozato K. et al. 2008 *Nat Rev Immunol*). TRIM22 has also been suggested to suppress HIV-1 transcription. TRIM1 has been shown to target the capsid protein at an early stage of infection and TRIM19 and TRIM32 have been demonstrated to affect trafficking of viral proteins (Nisole S. et al. 2005 *Nat Rev Microbiol*). TRIM28 was shown to repress transcription by binding to proviral DNA (Wolf D. et al. 2007 *Cell*) (Scheme 3). Here we describe in detail the features of TRIM22 protein.



### Scheme 3. Effects of human and mouse TRIM proteins on HIV-1 infection

Stages in the life cycle of HIV, from cell binding and fusion to release from the cell are depicted on the left. Here are shown the human TRIM family members that are known to inhibit specific stages of the cycle. TRIM family members that are known to affect viral entry, viral gene expression or viral release, but for which the exact stage affected is not known (shown on the right). Proteins with moderate inhibitory or promoting effects are listed in parentheses. PML, promyelocytic leukaemia (Ozato K. et al. Nat Rev Immunol).

## 1.2 TRIM 22

TRIM22 was identified several years ago as Stimulated Trans-Acting Factor of 50 kDa (Staf50), an interferon-induced cellular gene, which represses the activity of a HIV-1 LTR- luciferase construct in COS-7 cells (Sawyer SL. et al.2007 *Plos Pathog* ; Tissot C. et al. 1995 *Biol Chem*). Beside IFN, TRIM22 is induced by viral proteins like Latent membrane protein 1 (LMP-1) which induces EBV latency and also by latency-associated nuclear antigen (LANA), a product of human herpesvirus 8 (Renne R. et al.2001 *J Virol* ; Zhang J. et al. 2004 *J Biol Chem*). In human leukocytes it is expressed in resting T cells and down-regulated during co-stimulation with CD2 and CD28 (Gongora C. et al. 2000 *J Interferon Cytokine Res*). Recently, TRIM22 has been demonstrated to be a p53 target gene and play an important role in cell proliferation (Obad S. et al.2004 *Oncogene*; Wei CL. Et al. 2006 *Cell*). In addition, TRIM22 is involved in hematopoietic differentiation and T lymphocyte activation (Duan Z .et al. 2008 *Biochem Biophys Res Commun* ). TRIM22 was one of the relatively well-characterized TRIM E3 ligases, which has been shown to be type 1 IFN inducible in vitro, and to possess anti-HIV-1 activity (Meroni G. et al. 2005 *Bioessays*). Recent evidences suggest an in vivo antiviral activity of TRIM22. Indeed, it was demonstrated that the increased expression of type 1 IFNs and TRIM22 is associated with a significantly lower likelihood of HIV-1 acquisition and lower viral loads or higher CD4+ T-cell counts during primary HIV-1 infection. Remarkably, in peripheral blood mononuclear cells (PBMCs) from HIV-1 positive individuals TRIM22 levels strongly correlated with the expression of IFN-a, IFN-b, and MxA, a type 1 IFN-inducible gene, in both HIV-1-negative and -positive PBMCs and were up-regulated in HIV-1-positive subjects. Furthermore, TRIM22 plays a critical role in type 1 IFN-induced anti-HIV-1 activity in tissue cultures.

The E3 ligase activity of TRIM22 is directly correlated to its ubiquitylation capacity. In vitro and vivo studies have demonstrated that TRIM22 could be self-ubiquitylated in a RING finger-dependent manner (Duan Z. et al. 2008 *Biochem Biophys Res Commun*). Till now, the substrates of TRIM22 other than itself remain not fully understood. So far, only the Gag protein of HIV was reported to interact with TRIM22. However, the interaction between the Gag and TRIM22 did not affect the stability of Gag protein level (Barr SD. et al. 2008 *PLoS Pathog*).

Recent studies in human macrophage cell line U937 have demonstrated that TRIM22 may act as a positive regulator of NF- $\kappa$ B-mediated transcription and the activation of NF- $\kappa$ B could significantly induce the secretion of pro-inflammatory cytokines. Both N terminal RING domain and C terminal SPRY domain were crucial for the TRIM22-mediated activation of NF- $\kappa$ B. In particular the deletion of the RING domain or the mutation of cys15 into alanine abolish TRIM22 ability to activate NF- $\kappa$ B. (Yu S. et al. 2011 *Biochem Biophys Res Commun*).

There have been conflicting reports regarding the localization of ectopically expressed TRIM22. The shorter form of TRIM22 (442 aa) translated from a 1329 nt coding sequence appears to localize as cytoplasmic aggregates with some nuclear localization. In contrast, a c-myc/flag-tagged protein product of the 1497 nt TRIM22 coding sequence amplified from human peripheral blood mononuclear cells localized exclusively in the nucleus. It should be noted that ectopically expressed TRIM22 localized exclusively as a nuclear protein in cells expressing low to or medium amount of protein (Sivaramakrishnan G. et al. 2009 *Epub*).

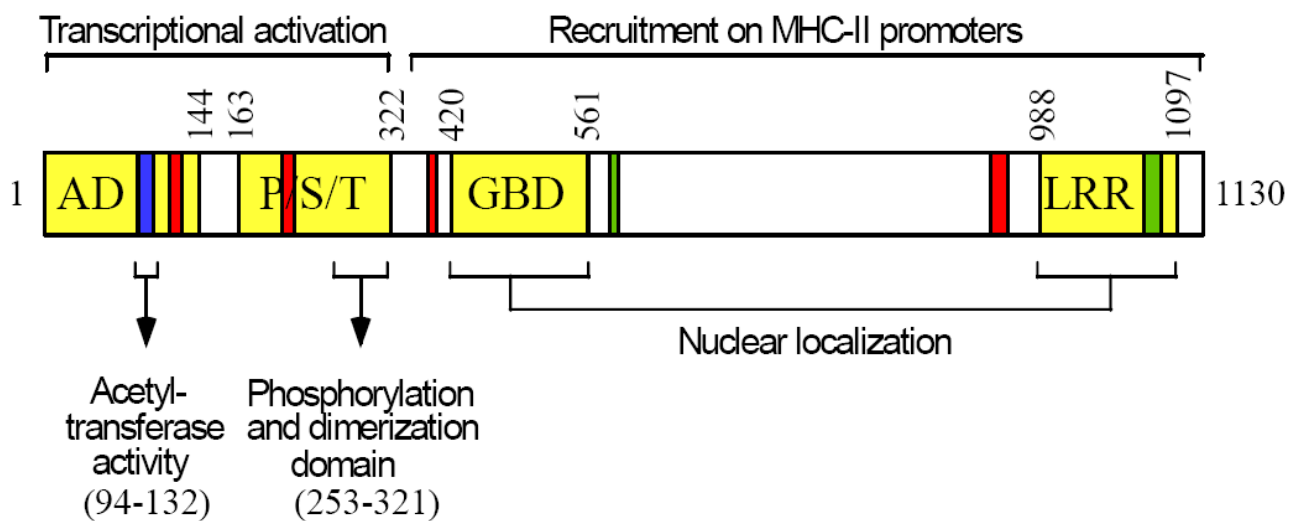
In particular, endogenous TRIM22 forms distinct Nuclear Bodies (NB) that are regulated by signaling molecules including progesterone and IFN, and during the cell cycle. NB are sub-nuclear domains that contain an assortment of components involved in diverse nuclear functions including ribosome biogenesis, pre-mRNA processing, splicing, apoptosis and transport (Matera AG. et al. 1999 *Trends Cell Biol*) . In ABC28 cells, endogenous TRIM22 localized as a nucleoplasmic protein and as distinct NB.

Both nuclear forms of the protein were evidently increased by progesterone treatment. Moreover, TRIM22 NB are adjacent to Cajal Bodies (CB), interact with p80-coilin and undergo dynamic assembly and disassembly during cell cycle progression. It has been suggested that TRIM22 NB are dynamic structures that can form and disintegrate in response to cellular signals (Sivaramakrishnan G. et al. 2009 *Epub*).

### **1.3 Class II transactivator (CIITA)**

The existence and the function of the AIR-1 locus-encoded (Accolla et al., 1986 *J. Exp Med.*) class II transactivator (CIITA), were initially identified in our laboratory by a somatic cell genetic approach (Accolla, 1983 *J. Exp. Med.*; Accolla et al., 1985 *Proc. Natl. Aca. Sci*; Accolla et al., 1985 *J. exp. Med*; Accolla et al., 1986, *J. Exp Med*). Several years later the CIITA cDNA was cloned by a complementation strategy (Steimle et al., 1993 *Cell*) by using a HLA-II negative B cell line, RJ.2.2.5, obtained in our laboratory by mutagenesis and immunological-selection against HLA-II DR expression (Accolla et al., 1983 *J. Exp. Med*).

CIITA is a complex molecule of 1130 aminoacids, composed of four essential functional domains (Scheme 4). Its N-terminal acid region forms the transcriptional activation domain that binds components of general transcriptional machinery and other co-factors to direct the initiation and elongation of MHC-II genes transcription (Kretsovali et al., 1998 *Mol Cell Biol.*; Fontes et al., 1999 *Mol Cell Biol.*; Kanazawa et al.; 2000 *Immunity*). The N-terminus of CIITA also contains the acetyltransferase activity (Raval et al.; 2001 *Mol Cell*). The proline-, serine- and threonine-rich region (P/S/T) is essential for the activity of CIITA (Chin et al., 1997 *Proc Natl Acad Sci*) and contains the phosphorylation/dimerization domain (Tosi, G et al., 2002 *EMBO J*). The GTP-binding domain (GBD) and the C-terminal leucine-rich motif (LRR) are critical for the sub-cellular localization of CIITA (Harton et al., 1999 *Science*; Hake et al., 2000 *Mol Cell Biol.*).



#### Scheme 4. Functional domains of CIITA protein

CIITA is a protein of 1130 amino acids with distinct functional domains indicated in the scheme. The red bars represent the sequences NLS (nuclear localization signal), the green bars indicate sequences NES (nuclear export signal); AD, activation domain, P / S / T, region rich in proline / serine / threonine, GBD, GTP binding domain, LRR, repeated sequences rich in leucine.

The function of CIITA can be modulated by post-translational modifications. For example, phosphorylation of certain residues of CIITA can increase its dimerization, its interaction with other key factors and its ability to trans-activate MHC class II promoters (Sisk, T. et al., 2003 *Int. Immunol*; Tosi, G. et al., 2002 *EMBO J*). In addition, ubiquitination of CIITA increases its ability to transactivate MHC class II genes (Greer, S. F. et al., 2003 *Nature Immunol*). CIITA transcription is driven by a large regulatory region, which contains four distinct promoters, named pI, pII, pIII and pIV. The function of



pII is unknown. Promoters pI, pIII and pIV precede alternative first exons that are spliced to the shared downstream exons, and this gives rise to three types of CIITA mRNA, which differ at their 5' ends. pI promoter is used mainly by cells of myeloid origin, such as dendritic cells (DC) and by IFN- $\gamma$ -activated macrophages; pIII is a lymphoid-cell-specific promoter essential for CIITA expression in B cells and activated T cells; pIV drives the expression of CIITA in thymic epithelial cells (TEC) and mediates induction by IFN- $\gamma$  in cells of non haematopoietic origin, such as fibroblasts, astrocytes, endothelial and epithelial cells (Muhlethaler-Mottet, A. et al.; 1998 *Immunity*), (Muhlethaler-Mottet, A. et al.; 1997 *EMBO J*)

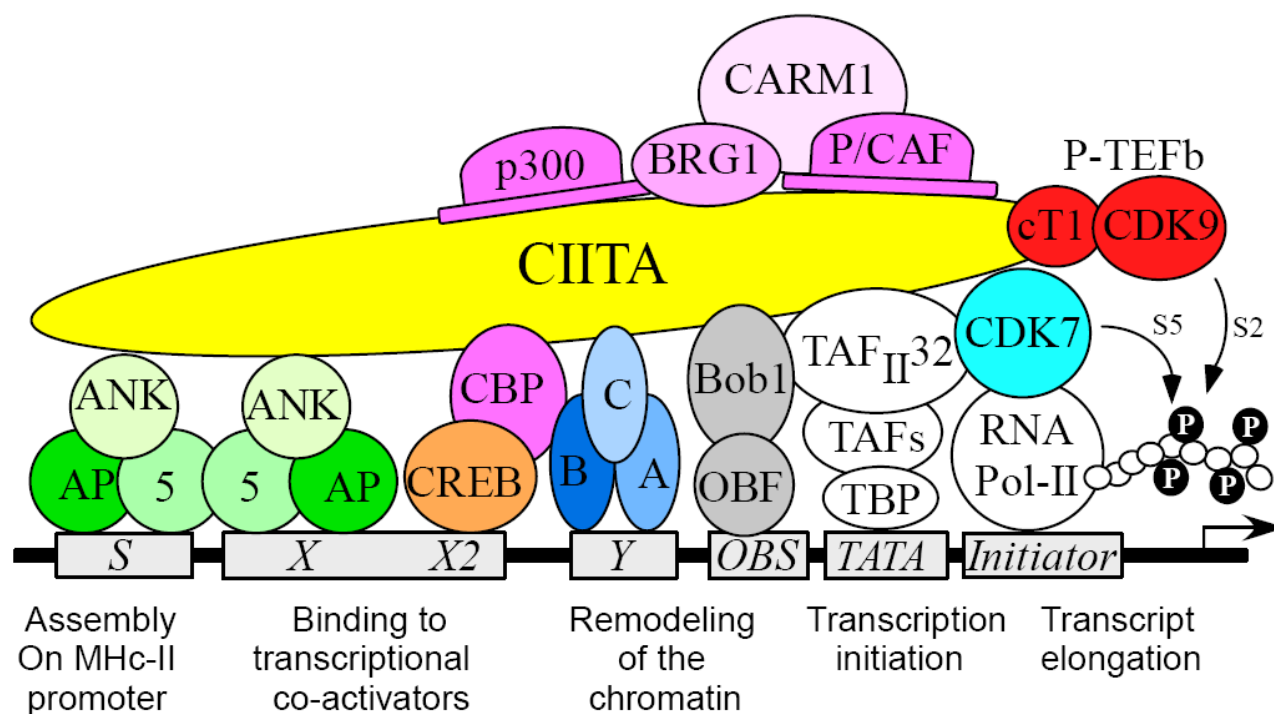
#### **1.4 Dual role of CIITA as modulator of adaptive immunity and restriction factor against human retroviruses.**

MHC class II molecules are cell-surface glycoproteins that are critical to the adaptive immune system because they present peptides derived mainly from extracellular proteins to the antigen receptor of CD4<sup>+</sup> T cells. MHC-class-II-mediated peptide presentation is essential for the positive and negative selection processes that shape the specificity of the T-cell-receptor repertoire of the CD4<sup>+</sup> T-cell population during its development in the thymus, for the homeostasis of the mature CD4<sup>+</sup> T-cell population in the periphery and for the initiation, amplification and regulation of protective immune responses to pathogens and tumors. To ensure tight control of these functions, MHC class II genes are themselves regulated in a precise cell-type-specific manner. Their expression is largely restricted to thymic epithelial cells (TECs) and to antigen-presenting cells (APCs), that include B cells, cells of monocytes-macrophage lineage and DC, specialized in the capture and presentation of extracellular antigens. MHC class II expression is mainly controlled at transcriptional level. All MHC-II promoters have conserved upstream sequences (CUS), 150–300 base pairs upstream of the transcription-initiation site. CUS contain few boxes present in a tightly constrained order, orientation and spacing that are called (from 5' to 3') S/W, X, X2 and Y boxes. The multiprotein complex that is assembled at the SXY

module, which is known as the MHC class II enhanceosome, is a platform to which CIITA is recruited by multiple synergistic protein–protein interactions. The enhanceosome and CIITA then cooperate to activate transcription of MHC class II genes (De Thé G. et al. 1996 *J Acquir Immune Defic Syndr Hum Retrovirol* ; Hoffman PM. Et al. 1992 *Proc Natl. Acad. Sci. USA*) (Scheme 5). CIITA is a non-DNA binding transcriptional integrator recruited to MHC-II promoters via multiple interactions with transcription factors bound to DNA, including the RFX and the NF-Y complexes (Carette G. et al. 2000 *Mol Biol*; Jabrane-Ferrat N. et al. 2002 *Mol Cell Biol*). It interacts with CBP, p300, PCAF as well as the cyclin T1 subunit of the positive transcription elongation factor b (P-TEFb) to enhance MHC-II gene transcription (Kanazawa S. et al. 2000 *Immunity* ; Kretsovali A. et al 1998 *Mol Cell Biol* ). From one side, CIITA triggers the molecular events leading to transcription of MHC class II genes. In so doing, CIITA governs the CD4+ T cell triggering, leading to optimal activation of immune effector mechanisms, particularly specific antibody production by B cells. Antibody binding is a crucial event for neutralization of extracellular viruses, which cannot infect host cells and are driven to degradation. CIITA has evolved as a general defense mechanism of the host against retroviruses not only because it activates the adaptive immune response against the infectious agents but also because of its intrinsic capacity to act as an endogenous viral restriction factor (Accolla et al, 2002 *Eur J Immunol*, Tosi et al, 2006 *PNAS*, Tosi G. et al. 2011. *J Virol*). For example, CIITA targets the viral transactivator Tat to inhibit the replication of the HIV-1 (Accolla et al, 2002 *Eur J Immunol*), by competing with the binding of P-TEFb in the elongation of HIV-1 viral transcripts (Wei P. et al. 1998 *Cell*). Moreover, CIITA inhibits Tax-1-directed transactivation of the viral LTR and the consequent viral replication. Interestingly, CIITA and Tax-1 interact *in vivo*, and overexpression of CREB, ATF1 and PCAF restore the Tax-1 mediated HTLV-1 LTR transactivation inhibited by CIITA thus suggesting the existence of distinct mechanism of CIITA-mediated inhibition of Tax-1 activity. (Tosi G. et al. 2011. *J Virol*).

Similarly, CIITA inhibits HTLV-2 Tax transactivation and consequently HTLV-2 replication (Casoli et al 2004 Blood, Tosi et al. 2006 PNAS, Orlandi et al. 2011 J Transl Med). Preliminary studies suggested that CIITA could inhibit Tax-2 function via interaction with the common binding factor NF-Y (Tosi et al, 2006 PNAS). CIITA-NF-YB interaction *in vivo* is stabilized and/or favored by the presence of Tax-2. Thus concomitant interaction of Tax-2 with CIITA and NF-YB, most likely in the CIITA-NF-YB molecular complex, is at the basis of the functional inactivation of Tax-2 leading to the inhibition of HTLV-2 retrovirus replication. (Orlandi C. et al. 2011 *J Transl Med*).

Taken together, these results definitely establish that CIITA, in addition to increasing the antigen-presenting function for pathogen antigens, behaves as a viral restriction factor with an intrinsic defensive role against infection of human retroviruses.



**Scheme 5. CIITA is a transcriptional integrator.**

CIITA is recruited to the promoter of MHC-II genes via multiple interactions with proteins bound to DNA. All promoters upstream of the MHC-II genes present in the direction 5'-3' in the form of sequences highly conserved SX-X2-Y. Each of these sequences is the binding site for specific proteins. The heterotrimer RFX (Regulatory Factor X) binds X and S regions, the region X2 CREB/AP-1 binds the complex and NFY (nuclear factor Y), formed by three subunits NF-YA,-YB and-YC, binds the sequence Y. CIITA, once recruited to the promoter, functions as a transcriptional integrator by coordinating the various stages of the transcriptional process. CIITA promotes the recruitment of co-activators that alter the accessibility of chromatin inducing histone acetylation (CBP, p300, P / CAF) or methylation (CARM1) and chromatin remodeling factor, BRG-1, facilitates the 'initiation of transcription factors binding general transcription (TFIIB and TFIID) and supports the elongation of transcription by recruiting the RNA polymerase II into the complex P-TEFb (Positive transcription elongation factor b), formed by the cyclin T1 and the cyclin-dependent kinase CDK9.

## **2. Aim of the study**

The aim of this study was to obtain deeper insight into the cellular and molecular mechanisms implicated in the CIITA-mediated inhibition of retrovirus replication, particularly in the HIV system. As CIITA and TRIM22 share overall actions as inhibitors of HIV-1 replication we began to investigate possible functional and/or molecular interactions between the two factors.

In the first part of this study we have analyzed the anti-viral function of CIITA in a monocyte-macrophage model of HIV-1 infection, composed of two isogenic lines, U937 *Minus* and U937 *Plus* characterized by efficient or inefficient capacity to support HIV-1 replication, respectively (Franzoso G. et al. 1994 *J. Exp. Med* ). We demonstrate that the different expression in MHC class II molecules between the two cell lines correlates with the expression of CIITA (*Minus* is positive for CIITA and MHC class II molecules, *Plus* is almost negative for CIITA and for MHC class II). This correlates with

the capacity of *Minus* cells to strongly inhibit Tat-mediated HIV-1 LTR transactivation. Interestingly the *Minus* and the *Plus* phenotype correlate also with a positive and a negative TRIM22 expression, respectively and recently it was demonstrated that TRIM22 impairs HIV-1 replication by interfering with basal activation of viral LTR promoter (Kajaste-Rudnitski A. et al. 2001 *J Virol*). Thus the two factors have similar functions in the isogenic U937 system. To further investigate molecular correlates of these similarities we transfected 293T cells we demonstrate that CIITA co-immunoprecipitated with TRIM22, suggesting a possible cooperation between the two factors. This hypothesis was reinforced by localization studies of the two overexpressed proteins. When CIITA is co-expressed with TRIM22, most of nuclear CIITA is recruited in the specific TRIM22 nuclear bodies.

In the second part of the study, to further detail the antiviral potential of TRIM22 and CIITA and to understand whether the two factors can functionally cooperate for the inhibition of HIV-1 replication in U937 cells, or whether their restriction activity is completely uncoupled, we decided to develop monoclonal antibodies against TRIM22. Whereas several specific reagents to detect and localize the CIITA protein are available, only a polyvalent rabbit antiserum for TRIM22 is present on the market, but upon direct assessment this reagent has been proved to be insufficient for serious studies of protein expression, intracellular localization and biochemical characterization. Moreover TRIM22 fusion proteins with tags such as GFP have been used but, of course, these proteins cannot be used satisfactorily for sophisticated studies of biochemical and biological properties of endogenously made proteins.

Since our specific aim is to dissect the expression and localization of TRIM22 and to directly correlate its function with the function of CIITA as viral restriction factors, we decided to generate a protein expression system capable to provide us with sufficient amounts of TRIM22 protein and then use this protein to generate monoclonal antibodies against TRIM22.

### **3. Materials and Methods**

### **3.1 Cell lines and animals**

Raji B cells and U937 promonocytic cells were maintained in RPMI-1640 (Lonza) supplemented with 10% heat-inactivated fetal calf serum (FCS, Lonza) and 5mM L-glutamine, at 37° in a humidified atmosphere of 5% CO<sub>2</sub>.

Human embryonic kidney 293T cells, HeLa Hep-2 cells were maintained in DMEM (Lonza) supplemented with 10% FCS and 5mM L-glutamine at 37° in a humidified atmosphere of 5% CO<sub>2</sub>.

Murine plasmacytoma PX-63 Ag8 cells were grown in RPMI-1640 supplemented with 10% FCS, 5mM L-glutamine and  $1 \times 10^{-4}$  M thioguanine.

8-10- week-old female BALB/c mice purchased from Charles River were used for immunization. All animals used in this study were treated in accordance with the guidelines of the University of Insubria Ethical Committee.

### **3.2 Plasmids**

Plasmids expressing myc-tagged CIITA and Flag-tagged CIITA were described in our previous study (Tosi, 2011). Plasmids expressing Flag-tagged TRIM22 was a gift from Prof Guido Poli.

The reporter plasmid pHIV-1 LTRLuc, pTat, pTYB1 vector were obtained from New England Biolabs.

### **3.3 Transient Transfection, Luciferase Assay and Immunoprecipitation**

For Luciferase gene reporter assays 293T cells were seeded on 60-mm-diameter plates and transfected with 150ng of reporter plasmid HIV-1 LTR-Luc, 10ng of Tat expressing vector and increasing amounts of plasmids DNA coding for flag-tagged CIITA or flag-tagged TRIM22 (0.8µg -1.6µg), by using lipofectamine (Invitrogen). All the transfections were carried out in the presence of 5ng of pRL-CMV

expressing the Renilla luciferase. Empty pcDNA3 vector was used as a stuffer DNA to maintain constant the total amount of transfected DNA. The lipofectamine-DNA mixture (3ul/ 1µg DNA) was incubated for 40 min at room temperature (RT) and then added to the cells in the presence of Opti-MEM medium (Gibco). After 5 hours, the DNA-lipofectamine complexes were removed and complete DMEM medium was added to the cells. 24hrs post-transfection the cells were collected and lysed with Passive lysis buffer (PLB, Promega) by three cycles of freezing and thawing. Cell lysates were assayed for luciferase activity by using the Dual Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. Mean luciferase values, normalized to Renilla values, of at least three independent experiments performed in duplicate are expressed as percentages of Tat-dependent luciferase activity set to 100%.

Cell lysates were analysed for the expression of CIITA and TRIM22 by SDS-PAGE on 8% polyacrylamide gels and western blotting with anti Flag M2 (Sigma Aldrich) and HRP-conjugated anti-mouse IgG secondary antibody (Amersham Pharmacia). Blots were developed by chemiluminescence assay (ECL, Biorad).

U937 cells were seeded on 6-multi-well plates ( $2 \times 10^6$  cells/plate) and transfected with 600ng of reporter plasmid HIV-1 LTR-Luc, 150ng of pRL-CMV and increasing amounts of pTat (10ng -20ng) by using FugeneHD (Promega) at 6:1, FugeneHD: DNA ratio. Empty pcDNA3 vector was used as a stuffer DNA. The FugeneHD-DNA mixture was incubated for 10 min at RT and then added to the cells in the presence of Opti-MEM medium (Gibco). After 16 hrs, complete DMEM medium was added to the cells. After 24hrs the cells were collected and lysed with the PLB, as above.

For protein binding assays, 293T cells were seeded in 100-mm-diameter plates and transfected with 3µg of the expression vector of each interacting protein. Empty pcDNA3 vector was used as a stuffer DNA. 24h post-transfection cells were lysed on ice for 50 min with IP lysis buffer containing 1% NP-40, 10mM Tris-HCl pH7.4, 150mM NaCl, 2mM EDTA, 1µM phenylmethylsulfonyl fluoride (PMSF)

and protease inhibitors cocktail (sigma). Cell lysates were centrifuged 14.000 rpm for 10 min and the supernatants were pre-cleared with protein A Sepharose 4 fast flow (GE Healthcare) for 30 min at 4°C by rotation. An aliquot corresponding to 5% of the total cell extract was kept for proteins expression detection (input)

For myc-CIITA immunoprecipitation cell extracts were incubated with anti-CIITA antibody (anti-CIITA7-1H, Santa Cruz Biotechnology) for 1 hour on ice and then reacted with 30µl of Protein A sepharose 4 fast flow, overnight at 4°C by rotation. To precipitate flag-tagged TRIM22 we used the anti-flag M2 agarose beads (Sigma, 30µl/ sample) overnight at 4°C by rotation. The immunoprecipitates were washed 5 times with IP lysis buffer containing 1% NP-40, 10mM Tris-HCl pH7.4, 150mM NaCl, 2mM EDTA, 0.1% protease inhibitors, and 1 time with the same lysis buffer containing 500mM NaCl. Co-immunoprecipated proteins were analysed by western blots with the anti-CIITA monoclonal antibody, or the anti-Flag M2 monoclonal antibody and HRP-conjugated anti-mouse secondary antibodies.

Endogenous CIITA was precipitated from U937 cells and Raji B cells ( $30 \times 10^6$  or  $15 \times 10^6$  cells, respectively). After pre-clearing, the cleared lysates were incubated with 1µg of anti-CIITA antibody (anti-CIITA7-1H, Santa Cruz Biotechnology) 1 hour on ice and then reacted with 50µl of Protein A beads overnight at 4°C by rotation. The immunoprecipitated proteins were analysed by western blots using anti-CIITA monoclonal antibody.

### **3.4 SDS/PAGE and Western blotting**

U937 cells ( $8 \times 10^6$ ) were collected and lysed with lysis buffer containing 1% NP-40, 10mM Tris-HCl pH 7.4, 150mM NaCl, 2mM EDTA, 1µM PMSF and protease inhibitors cocktail. Cell debris were removed by centrifugation at 14.000 rpm for 10 min at 4°C and the supernatants were collected. Cells lysates were analysed for the expression of TRIM22 proteins in denaturing and in not-denaturing gel electrophoresis, followed by western blotting. For the denaturing gel electrophoresis the lysates were



diluted with 6x SDS-sample buffer (0.3M Tris-HCL pH6.8, 0.6 M DTT, 10% SDS, 0.6% bromophenol blue, 60% glycerol) and boiled for 3 min before loading on the gel. For the not-denaturing gel electrophoresis, the lysates were diluted with 6x SDS-sample buffer lacking SDS, without boiling. Proteins were then separated by electrophoresis on 8%-polyacrylamide gel containing 0.1% SDS, and then transferred into a nitrocellulose membrane (Hybond-C, GE Healthcare) by electroblotting (360mA, for 1hour) at 4°C. After blocking with 5% non-fat dry milk in PBS for 1 hour at RT, the membrane was incubated with the primary antibodies (Supernatants of hybridoma cell lines, see below) diluted 1 to 2 with 5% non-fat dry milk in PBS, overnight at 4°C under agitation. The membrane was then washed three times for 10 min with PBS then incubated with the secondary antibody solution (HRP-conjugated goat anti-mouse secondary antibodies) for 2 hrs at RT, under agitation. After five washes (10min each) with PBS and one wash with PBS containing 0,05% Tween 20, blots were developed by ECL.

### **3.5 FACS analysis**

U937 Plus and Minus cells ( $1 \times 10^5$ ) were collected and washed with PBS. Cells were then incubated with the anti HLA-II DR (D1.12) monoclonal antibody, for 30 min at 4°C. After washing, cells were incubated with fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG secondary antibody for 30 min at 4°C. As a control, cells were incubated only with the secondary antibody. Expression of HLA molecules was analysed by flow cytometer (EPICS XL flow cytometer) and EXPO32 analysis software (Beckman Coulter S.p.A., Milan, Italy).

### **3.6 Real time RT-PCR**

Total RNA was extracted using TRIzol reagent (Life Technologies). cDNA was synthesized from 2 µl total RNA using oligo(dT) primers and M-MLV reverse transcriptase (Promega). Quantitative real-time

PCR (QRT-PCR) was performed using IQSYBR Green PCR reagent on an ABI Prism 7000 Sequence Detection System (Applied Biosystems) according to the manufacture's protocol. The HLA-II DR, CIITA, TRIM22 and RPS7 (Ribosomal protein S7, used as control to normalize for cDNA quantity) transcripts were amplified using specific primers (Table 1). Changes in fluorescence of the SYBR Green I dye in each cycle were monitored, and the cycle threshold (Ct), which is defined as the cycle number at which the amount of amplified target reaches a fixed threshold, was obtained for each gene. The relative amount of PCR products, generated from each primer set was determined on the basis of the Ct value. The expression difference for each gene (gene x) between control and samples was calculated by normalizing Ct values with RPS7 gene expression according to the formula:

Relative mRNA expression =  $2^{[Ct(\text{control}) \text{ gene x} - Ct(\text{sample}) \text{ gene x}] - [Ct(\text{control}) \text{ RPS7} - Ct(\text{sample}) \text{ RPS7}]}$ .

**Table 1 – Forward and reverse primers used in QRT-PCR**

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
hCIITA	CCTGCTGTTCGGGACCTAAA	GGATCCGCACCAGTTTGG
HLA-DR	CTCTTCTCAAGCACTGGGAGTTT	ATGAAGATGGTCCCAATAATGATG
hTRIM22	CACTCTTCTCCCCTGATTCAA	TCACAAACTCCTGCAGTGCT
hRPS7	TGGAGATGAACTCGGACCTC	CGACCACCACCAACTTCAA

### 3.7 Immunofluorescence analysis

Hep-2 cells were transfected with plasmids expressing myc-tagged CIITA (0.5µg) and flag-tagged TRIM22 (0.5µg). At 24 hrs post-transfection, cells on coverslips were fixed in methanol for 6 min at -20°C and blocked with 0.5% BSA in PBS for 1 hour at RT. Cells were then incubated with anti-myc polyclonal antibody (anti-mycA14, 1:200 dilution 0.1% BSA in PBS, Santa Cruz Biotechnology) and

with anti-FITC conjugated Flag monoclonal antibody (1:200 dilution 0.1% BSA in PBS, Sigma) overnight at 4 °C in the dark, followed incubation with Alexa-Fluor 546 goat anti rabbit secondary antibody (1:500 dilution in 0.1% BSA in PBS, Life technology) for 1 hr at RT, in the dark. Cells were then examined under a confocal laser-scanning microscope, Leica TCS SP5 (objective lenses: HCX PL APO, 63X original magnification, numerical aperture: 1.25) and imported into LAS AF software.

### **3.8 Immunization and monoclonal antibody production**

TRIM22-intein recombinant protein was used as antigen for the generation of TRIM22 monoclonal antibodies and was injected (about 5µg) intraperitoneally into BALB/c mice three times, specifically at day 1, 14 and 28. The first immunization was performed in presence of Complete Freund's Adjuvant (CFA), whereas the second and third immunizations were performed in presence of Incomplete Freund's Adjuvant (ICFA). Three to five days after the last immunization, the immunized animals were killed and the splenocytes used for fusion. Hybrid cell lines were obtained by fusing antibody-producing B cells with HGPRT<sup>neg</sup> myeloma cells. The fusion was accomplished using polyethylene glycol (PEG). Fused cells were cultured in four 24-multi-well plates on monolayer of peritoneal macrophages of mice BALB/c in presence of HAT (Hypoxanthin Aminopterin Thymidine) medium. Aminopterin blocks the pathway that allows for nucleotide synthesis. Hence, unfused myeloma cells die, as they cannot produce nucleotides by the *de novo* or salvage pathways because they lack HGPRT. Removal of the un-fused myeloma cells is necessary because they have the potential to outgrow other cells. Unfused B cells die as they have a short life span. In this way, only the B cell-myeloma hybrids survive, since the HGPRT gene coming from the B cells is functional. After about 14 days, hybridoma culture supernatants were collected and we identified and selected only those hybridomas that produce antibodies of appropriate specificity as assessed by western blot (see above).

## 4. Results

### 4.1 CIITA is expressed only in U937 *Minus* cells

Previous results demonstrated that the MHC-II transactivator CIITA inhibits HIV-1 replication in human T cells by competing with the viral transactivator Tat for the binding to the Cyclin T1 subunit of the PTEF-b transcriptional elongating complex (Accolla R. S. et al. 2002 *Eur. J. Immunol*).

In order to assess whether the anti-viral function of CIITA is present in mononuclear phagocytes, which represent another important target of HIV-1 infection, we studied two phenotypically and functionally distinct isogenic cell clones of U937 characterized by efficient or inefficient capacity to support HIV-1 replication, named *Plus* and *Minus*, respectively (Franzoso G. et al. 1994, *J Exp Med*).

First, we analyzed the cell surface expression of MHC-II molecules in U937 *Plus* and *Minus* clones by FACS analysis. Interestingly, only U937 *Minus* cells express HLA-II DR whereas U937 *Plus* cells do not (Fig.1A). This correlates with HLA-II DR mRNA expression in U937 *Minus* cells and in U937 *Plus* cells, as assessed by QRT-PCR. Raji B cells were used as a positive control for HLA-II DR mRNA expression (Fig.1B).

To verify whether this different phenotype correlates with the expression of functional CIITA protein restricted to *Minus* clones, CIITA mRNA expression in both U937 *Minus* and *Plus* cells was assessed by real-time PCR. U937 *Minus* cells clearly express CIITA mRNA, whereas *Plus* cells do not (Fig.2A). Raji B cells were used as a positive control for CIITA mRNA expression. We further assessed whether the observed differences in CIITA mRNA expression correlated with the protein expression. Immunoprecipitation following immunoblotting (IP:  $\alpha$ CIITA, WB:  $\alpha$ CIITA) with an anti-CIITA

antibody detected CIITA only in *Minus* cells, confirming the expression of CIITA only in these cells (IP:  $\alpha$ CIITA, WB:  $\alpha$ CIITA Fig. 2B).

In conclusion, the phenotype of U937 *Minus* and *Plus* clones shows the additional correlation of expressing or not expressing MHC class II molecules as a function of expression or non expression of CIITA, respectively.

#### **4.2 Both CIITA and TRIM22 inhibit Tat-dependent HIV-1 LTR transactivation**

Previous data demonstrate that CIITA inhibit HIV-1 transcription, by blocking Tat activity in Jurkat T cells (Accolla RS. et al.2002 *Eur J Immunol*). In addition, it has been reported that TRIM22 inhibits basal HIV-1 transcription in U937 cells without affecting HIV-1 Tat transactivation (Kajaste-Rudnitski A. et al. 2011 *J. Virol*). Interestingly, TRIM22 is specifically expressed in U937 *Minus* cells but not in *Plus* cells as reported by Kajaste-Rudnitski A. et al. (*J. Virol* 2011), and further confirmed in by our studies (Fig.2C).

To investigate the possible relationship between CIITA and TRIM22 in the inhibition HIV-1 transcription, we first examined the effect of expression of the two factors on the transcriptional activity of HIV-1 Tat, by reporter gene assay. We co-transfected human 293T cells with the HIV-1 LTR luciferase reporter construct and the expression vector for Tat, alone or together with increasing amounts of a plasmid coding for Flag-tagged CIITA, or for Flag-tagged TRIM22 (0.8 $\mu$ g – 1.6 $\mu$ g). We found that the overexpression of CIITA or, surprisingly, TRIM22 could inhibit Tat activity in dose-dependent manner, (Fig.3A, B).

The observed inhibition of Tat transactivation by CIITA and TRIM22, prompted us to investigate whether this suppressive effect was confirmed also in the isogenic U937 cell system. To this aim U937 *Minus* and *Plus* cells were co-transfected with fixed amounts of the reporter construct HTLV-1 LTR-luciferase and increasing amounts of Tat expression vector (10ng - 20ng). Interestingly, we found that

Tat-dependent LTR transactivation is strongly reduced in *Minus* cells with respect to *Plus* (Fig.3C), indicating a possible cooperation between CIITA and TRIM22 in the inhibition of Tat-mediated HIV-1 transcription.

### **4.3 CIITA interacts with TRIM22 in vivo**

To further investigate the molecular and functional relationship between CIITA and TRIM22 we assessed whether the two proteins physically interact each other. To test this hypothesis, myc-tagged CIITA and Flag-tagged TRIM22 were co-expressed in 293T cells. Cell lysates were immunoprecipitated with anti-flag antibody and TRIM22-bound proteins were assessed for the presence of CIITA by Western blotting. As shown in Figure 4, CIITA co-precipitated with TRIM22 (IP:  $\alpha$  flag, WB:  $\alpha$  CIITA, lane 4). The interaction between the two factors was not so evident when the immunoprecipitation step was first performed with anti-CIITA antibodies, instead of anti-FlagTRIM22 antibody (Fig.4, IP:  $\alpha$  CIITA WB:  $\alpha$  flag, lane 4), possibly suggesting that the epitope recognized by anti-CIITA antibody spatially overlaps the site of interaction between CIITA and TRIM22.

### **4.4 Nuclear CIITA is mainly recruited into TRIM22 nuclear bodies**

To assess where CIITA-TRIM22 physically interact at the sub-cellular level, we performed the confocal microscopy analysis on Hep-2 cells transiently transfected with myc-tagged CIITA and flag-tagged TRIM22. As we expected, CIITA is homogeneously diffused in the nucleus and in the cytoplasm of Hep-2 cells (Fig. 5A, panel a), whereas TRIM22 localizes as a nucleoplasmic protein in a spot-like distribution reminiscent of nuclear body structures (Fig.5A, panel b). Interestingly, in the presence of TRIM22, nuclear CIITA is mainly recruited into TRIM22 nuclear bodies, further suggesting a functional relationship between the two factors (Fig.5 B, panel a'', overlay).

Future work in this area should include an investigation into the association between TRIM22's nuclear localization, CIITA recruitment into TRIM22 nuclear bodies and their antiviral properties in U937 *Minus* cells.

Unfortunately, no specific reagents, such as monoclonal antibodies, are available for the detection and the localization of endogenous TRIM22 protein. Thus we decide to generate mouse monoclonal antibodies against recombinant TRIM22 protein.

## 4.5 TRIM22 Cloning

The first step for the generation of monoclonal antibodies was to produce sufficient amount of antigen to immunize mice. This was accomplished by the following strategy. TRIM22 c-DNA was cloned into an *E.coli* expression vectors with specific expression tags or fusion proteins to express them in a basic *E.coli* strain. The second step was the purification of the protein used as antigen for the production of the monoclonal antibodies in mice.

First, the TRIM22 coding sequence was amplified by polymerase chain reaction (PCR) from TRIM22-pcDNA3.1, using the following primers:

The forward (FW) 5'-GGCCCATATGACCATGGATTTCTCAGTA-3', which introduced an NdeI restriction site (blue) into the N-terminus of TRIM22 coding sequence (red) and the reverse (RW) 5'-CCCACCGAGCTCCCTCGAGTATCTA-3', which introduced an XhoI restriction site (blue), immediately before the first TGA stop codon of TRIM22 sequence (red). Each primer contains extra bases (black) near the restriction site, in order to allow a complete digestion by the corresponding enzyme. The amplified sequence was sub-cloned into a pGEMT easy vector (Promega) and easily digested using NdeI and XhoI restriction enzymes.

The digested fragments containing the TRIM22 gene were cloned into pTYB1 (NEB) in frame with the Intein gene, to generate TRIM22-Intein fusion protein expression in *E.coli* (Fig.6, pTYB1-TRIM22). The inserted cDNA and the flanking regions were sequenced to control the integrity of the sequence.

#### **4.6 Expression and purification of TRIM22-intein recombinant protein**

The great advantage of pTYB vectors is the possibility to obtain a TRIM22-untagged protein. This system utilizes the inducible self-cleavage activity of the Intein, to separate the target protein from the tag. The Intein contains also a chitin-binding domain necessary for the purification of the fusion protein on chitin beads. After beads purification, 1,4-Dithiothreitol (DTT) induces cleavage, resulting in the elution of the untagged TRIM22.

A protein expression competent *E.coli* strain (ER2566) was transformed with pTYB1-TRIM22 vectors. Four colonies from the overnight LB-Agar plate culture were picked to select one for the best expression. Colonies were amplified in 200ml of LB media of at 37°C, overnight under agitation to reach the desired concentration ( $OD_{600nm}=0.6$ ) TRIM22-intein protein expression was then induced by adding 0.5 mM isopropyl-1-thio- $\beta$ -D-galactopyranoside (IPTG) and culturing for 3.5 hrs at 30 °C. Bacteria were finally centrifuged and corresponding pellet was lysed. We tried different lysis buffers and lysis conditions, as the TRIM22-intein recombinant protein was found to be rather insoluble. The final selected procedure was the following. Bacterial pellet was collected by centrifugation at 4500 rpm, at 4°C for 15 min, re-suspended in Lysis Buffer (1 ml) containing urea 8M in PBS and protease inhibitors, lysed by sonication and then solubilized overnight at 4°C, by rocking. After centrifugation at 14000 rpm, at 4°C for 15 min, both the soluble fraction (SN) and the pellet fraction (PT) were analyzed by SDS-PAGE and Comassie blue staining. As shown in figure 7, a band corresponding to TRIM-Intein recombinant protein (100 KDa) was detected on IPTG-induced bacterial cultures (Fig.7, compare



lane 3 to lane 2). We were able to solubilize at least 50% of the protein using 8M urea lysis buffer (fig. 7, compare lane 8 to lane 4).

The TRIM22-intein recombinant protein was purified using chitin beads (NEB). Washed beads (40 ul) were added to the soluble protein extracts (SN) and incubated at 4°C for 3 hrs. After washes, the Intein-self cleavage was induced by adding 40 mM DTT (200 ul) to the beads and further incubation at 4°C for 2 hrs in the dark. The eluted proteins were detected by SDS-PAGE, and Comassie blue staining. Unfortunately, we were not able to obtain purified TRIM22 protein, possibly for the partial denaturation of the intein protein, during the urea treatment (Fig.7, lane 5). Thus, we decide to use TRIM22-intein fusion protein as antigen for the production of monoclonal antibodies in mice.

#### **4.7 Generation, screening and characterization of TRIM22 monoclonal antibodies**

In order to generate monoclonal antibodies (mAb) specific for TRIM22, BALB/c mice were injected intraperitoneally three times with 3µg TRIM22-intein every two weeks; first injection was carried out in CFA followed by the second injection in ICFA, and by the third in PBS. Spleen cells from immunized mice were used to prepare somatic cell hybrids with the plasmocytoma PX-63 Ag8. mAb containing supernatants were checked for anti-TRIM22 specificity by western blots in denaturing and non-denaturing condition, using protein extracts obtained from U937 Plus and Minus cells.

Cumulative results of the screening are summarized in the Table1. Figure 8 shows some of the most representative results of the screening. Similar results were obtained in denaturing and not-denaturing gels. Interestingly, the hybridoma supernatant L91 detects a band at 55 kDa only in U937 *Minus* cells, probably corresponding to TRIM22 (Fig.8A, lane 3). We also found another hybridoma supernatant, the L92, with the same specificity (not shown in the figure). Interestingly, the hybridoma supernatant L85 detects a band of 100 kDa only in U937 *Minus* cells, which may be related to an ubiquitinated form of TRIM22 (Duan Z .et al. 2008 *Biochem Biophys Res Commun*) (Fig.8 lane 3). Figure 8 also

shows two negative results related to the mAb L40, which could not detect any band (Fig.8A, lane 1), and the mAb L95, which detects a band at 72 kDa in both *Plus* and *Minus* cells (Fig. 8A, lane 2).

To further characterized the L91 and L92 mAbs, which detect a band at 55 kDa in *Minus* cells possibly corresponding to TRIM22, we looked for the antibody subtype by western blot using HRP-conjugated goat anti-mouse IgG or goat anti mouse IgM secondary antibodies. The anti-HLA-I antibody B9.12.1 (IgG subtype) was used as control.

As shown in figure 8 B, the goat anti-mouse IgG could detect the light and heavy chains (50 kDa and 25 kDa) of B9.12.1 antibody, whereas the goat anti mouse IgM antibody could only detect the light and heavy chains (50 kDa and 25 kDa) of L91and L92 antibodies. This result clearly indicates that the two monoclonal antibodies L91 and L92 against TRIM22 are of IgM subclass.

Further experiments are planned to definitively assess the specificity of these antibodies for TRIM22.

## 5. Discussion

Characterization of host factors restricting HIV-1 infection may provide important information on the mechanisms used by the virus to efficiently infect and replicate in different target cells. Recent studies have demonstrated that the restriction factor TRIM22 inhibited HIV-1 LTR-driven transcription and virus replication in a model represented by isogenic U937 promonocytic cell lines characterized by efficient (*Plus*) or inefficient (*Minus*), capacity to support productive HIV-1 replication respectively (Kajaste-Rudnitski A. et al. 2011 *J Virol*)

In this work we have investigated the inhibitory effect of the MHC-II transactivator CIITA on HIV-1 transcription in U937 *Plus* and *Minus* promonocytic cell lines. In addition to TRIM22, also CIITA was differentially expressed in U937 *Plus* and *Minus* cells, with *Plus* cells lacking detectable mRNA and protein expression. Thus, the phenotypic analysis of these cells shows that, in contrast to *Minus* cells, *Plus* cells do not express HLA-II molecules because of a defect in MHC-II gene transcription due to

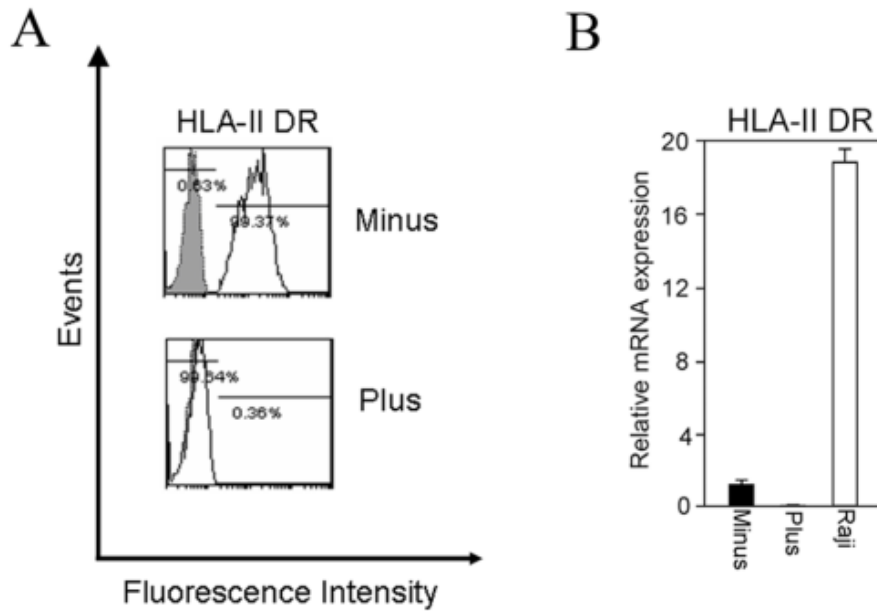
the absence of the MHC-II transactivator CIITA. In searching for the molecular mechanisms at the basis of CIITA and TRIM22-mediated inhibition of HIV-1 transcription, we demonstrated that CIITA and TRIM22 inhibit HIV-1 replication by targeting the LTR-dependent transcriptional activating function of the viral transactivator Tat, in 293T cells transfected with increasing amounts of CIITA or TRIM22 expressing plasmid. In contrast to our results, Kajaste-Rudnitski A. et al. observed that TRIM22 did not interfere with Tat-mediated HIV-1 replication when overexpressed in 293T cells. To date, we cannot explain these different data but since this inhibitory effect was observed when Tat was transfected into U937 *Minus* cells expressing endogenous CIITA and TRIM22, we conclude that physiologic amounts of the two proteins might contribute to the “nonpermissive” phenotype of U937 *Minus* cells for HIV-1 infection. At present, the relative contribution of CIITA or TRIM22 to the inhibition of viral replication in *Minus* cells cannot be precisely assessed. Within this frame, it will be important to investigate the Tat activity in U937 *Minus* cells in which CIITA or TRIM22 were silenced. That CIITA and TRIM22 may cooperatively act for the resistant phenotype to HIV-1 replication observed in *Minus* cells is also strongly suggested by our additional findings on the physical interaction and subcellular colocalization of the two restriction factors. Indeed we found, for the first time, that CIITA, a restriction factor also for HTLV infection (Tosi, 2011, Orlandi 2011), interacts *in vivo* with TRIM22. We also analyzed the sub-cellular localization of CIITA and TRIM22 and we observed that the nuclear CIITA is mainly recruited into TRIM22 nuclear bodies, in Hep-2 cells over-expressing the two proteins. Interestingly, TRIM22 nuclear bodies are also frequently observed next to and within nucleoli. It is likely therefore that TRIM22 is involved in one or more nucleolar functions, which include rDNA transcription, pre-rRNA processing, ribosome assembly and formation of the telomerase ribonuclear protein complex, and more importantly the nucleolus has been suggested as a gateway for viral replication (Sivaramakrishnan G et al. 2009 Exp Cell Research). Viral proteins are known to interact with nucleolar proteins such as B23 and fibrillarin and use the nucleolus as a site of

replication. In addition Tat was reported to be present primarily in the nucleolus of transfected and infected cells (Cullen et al., 1988, Luznik et al., 1995, Stauber and Pavlakis 1998). In contrast, CIITA has never been found in the nucleoli. The findings of CIITA recruitment into TRIM22 nuclear bodies certainly warrants future work to possible correlate the association between TRIM22's nucleolar localization, CIITA and their antiviral properties.

As a preliminary step to further refine the subcellular localization and the intimate function of endogenous TRIM22, we are exploring the possibility to produce monoclonal reagent against TRIM22. Out of two somatic cell fusions we have obtained a series of mAbs that are presently under accurate scrutiny. The antibodies L91 and L92 which detected a band at around 50–60 kDa in U937 *Minus* cells are good candidates for being TRIM22-specific. Additionally, the monoclonal antibody L85 detected a band at around 100 kDa. We have hypothesized that L85 mAb should react against an ubiquitinated form of TRIM22 (Duan Z. et al. 2008 Biochem and Biophysical Res). To confirm our hypothesis it will be necessary to sequence the bands recognized by the two antibodies by using Mass-spectrometry analysis.

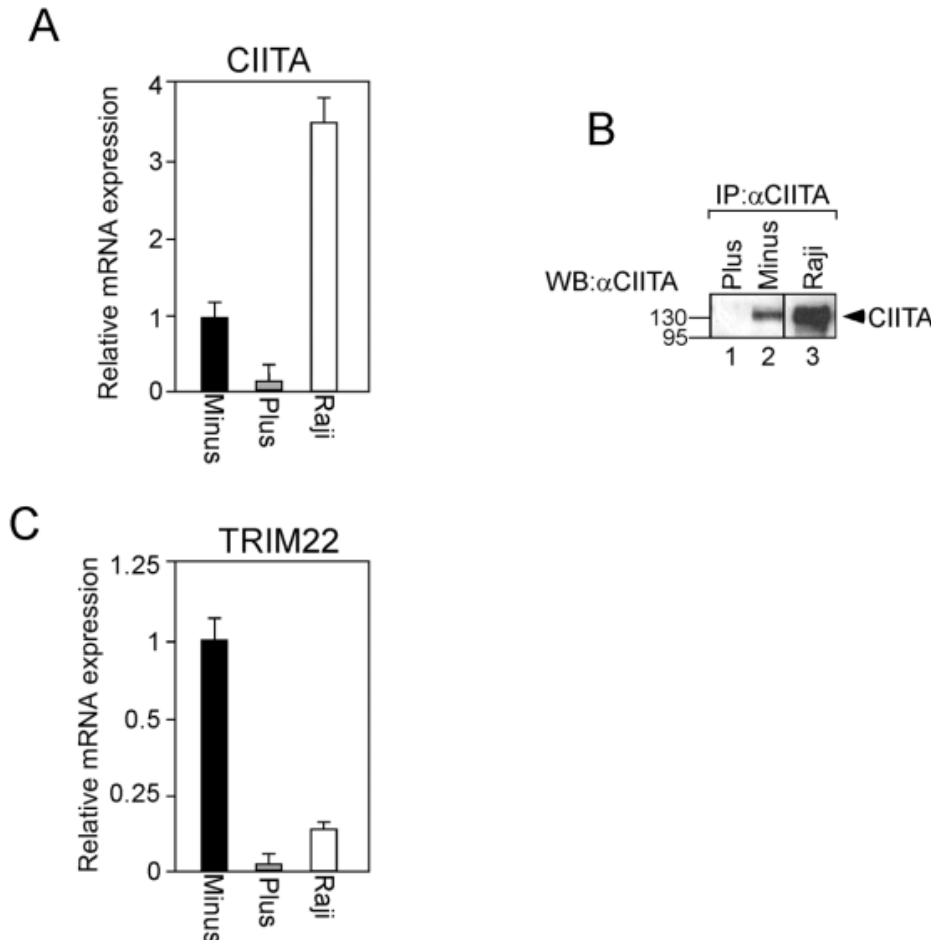
In conclusion these results confirm the important role of CIITA and TRIM22 in the pathogenesis of HIV-1 infection and indicate that different host factors may contribute to inhibit HIV-1 expression in monocytes-macrophages target cells. We believe that CIITA acts as a new versatile antiviral factor, which may be useful to develop innovative preventive and therapeutic approaches to counteract HIV-1 spreading in the host.

## 6.Figures



**Fig.1 U937 *Minus* cells expresses MHC-II molecules**

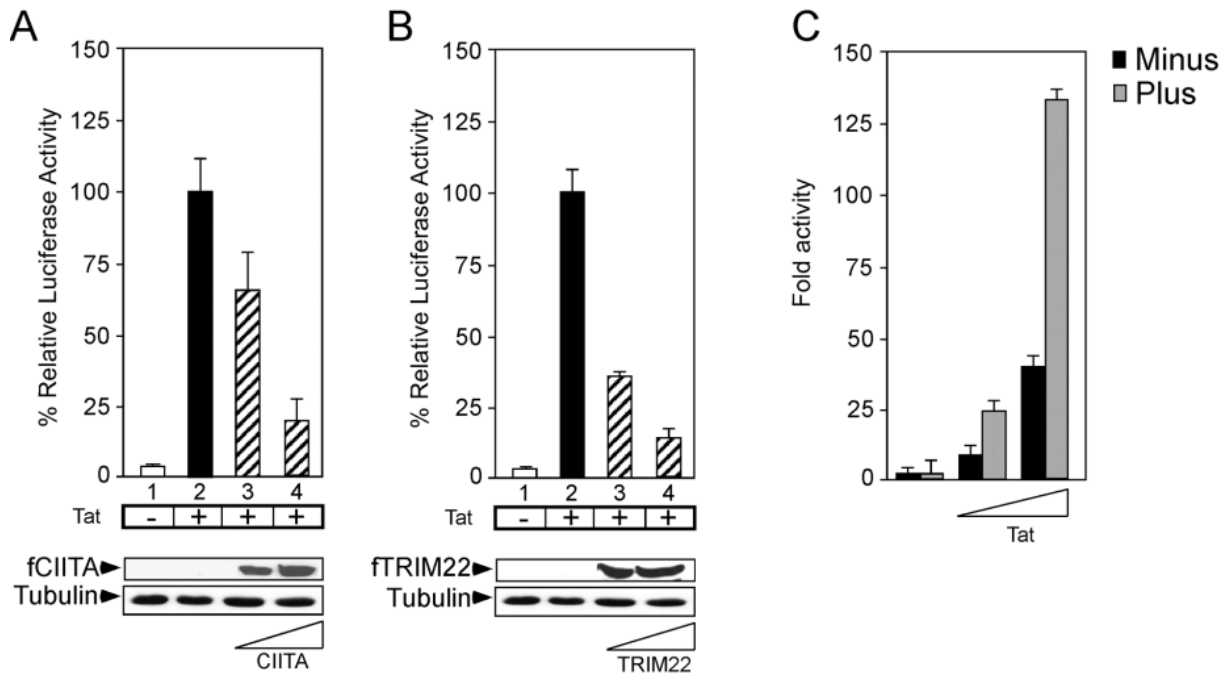
(A) The cell surface expression of MHC-II molecules was assessed by FACS analysis in U937 *Plus* and *Minus* clones. Only U937 *Minus* cells express HLA-II DR (white histogram in upper panel) whereas U937 *Plus* cells do not. Gray histogram represent negative control. (B) HLA-II DR mRNA expression in U937 *Minus* cells and in U937 *Plus* cells was assessed by QRT-PCR. HLA-II DR mRNA expression in *Minus* cells was normalized to 1. All mRNA levels are relative to RPS7 mRNA expression. Raji B cells were used as a positive control for HLA-II DR mRNA expression



**Fig.2 U937 *Minus* cells constitutively express endogenous CIITA**

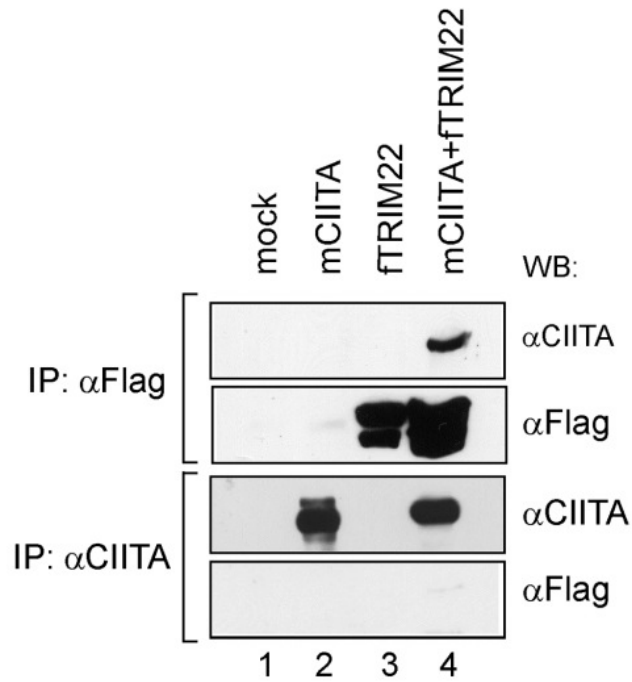
(A) CIITA mRNA expression in both U937 *Minus* and *Plus* clones, was assessed by real-time PCR.

U937 *Minus* cells clearly express CIITA mRNA, whereas *Plus* cells do not. Raji B cells were used as a positive control for CIITA mRNA expression. (B) Total cell lysates were immunoprecipitated with an anti-CIITA antibody and then analyzed for the expression of CIITA by Western Blotting (IP:  $\alpha$ CIITA, WB:  $\alpha$ CIITA). CIITA protein is exclusively expressed in U937 *Minus* cells. (C) TRIM22 mRNA levels were analyzed by QRT-PCR. TRIM22 mRNA is detected only in *Minus* cells.



**Fig.3 CIITA and TRIM22 inhibit Tat-mediated HIV-1 LTR transactivation in a cotransfection system and in U937 *Minus* cells.**

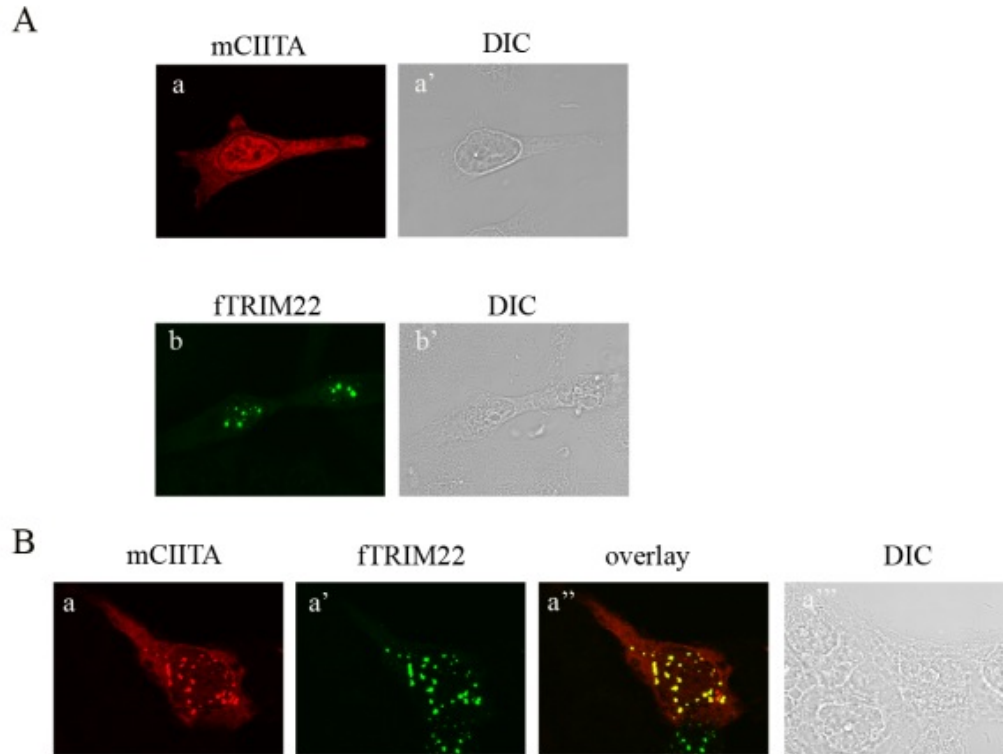
293T cells were co-transfected with fixed amounts of pHIV-1 LTR-Luc, phRL-CMV, pTat (10 ng) and with increasing amounts (0.8-1.6 $\mu$ g) of plasmid coding for flag-tagged CIITA (A) or flag-tagged TRIM22 (B) (hatched columns 3-4). Mean luciferase activities, normalized to Renilla activity, are presented in the ordinate as percentages relative to activation by Tat set to 100% (black, column 2). Column 1 represents the activity of pcDNA3 vector in the absence of Tat. Expression of recombinant flag-CIITA (fCIITA) and flag-TRIM22 (fTRIM22) proteins was assessed in cell extracts by Western blotting with anti-flag antibody. (C) U937 *Plus* (gray columns) and *Minus* (black columns) cells were co-transfected pHIV-1 LTR-Luc, phRL-CMV, and increasing amounts of a Tat expression vector (10, 20 ng). Mean luciferase activities, normalized to Renilla activity are presented in the ordinate as fold of Tat activity. Columns 1 represent basal reporter activity in the absence of Tat.



**Fig.4 CIITA and TRIM22 interact *in vivo***

293T cells were transfected with myc-CIITA (mCIITA) and flag-TRIM22 (fTRIM22) expression vectors in different combinations, as indicated. pCDNA3 was used as stuffer DNA (mock). Anti-flag agarose beads (*IP:a flag*) or anti-CIITA (*IP:a CIITA*) precipitated proteins were analyzed for the presence of TRIM22 and CIITA by immunoblotting with anti-CIITA (*WB:a CIITA*) or with anti-flag (*WB:a flag*), respectively.

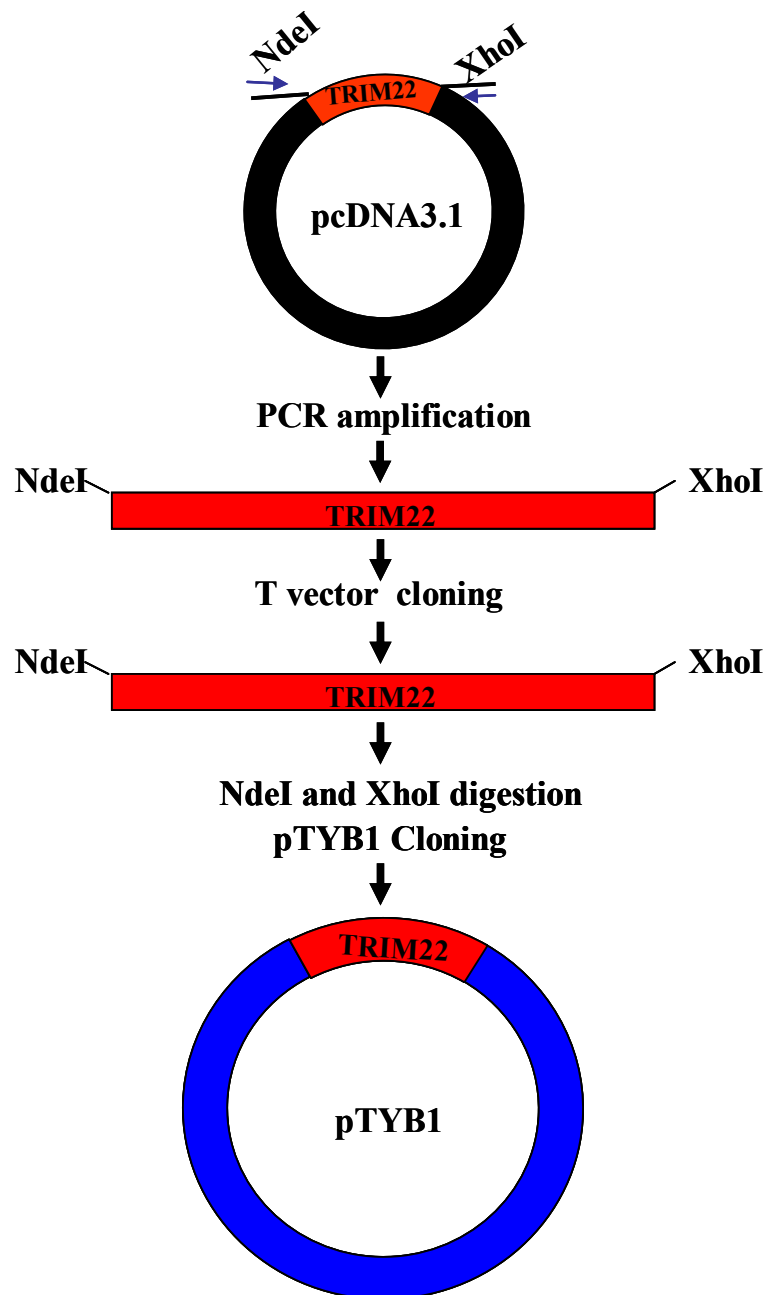




**Fig.5 *CIITA and TRIM22 co-localizes in vivo***

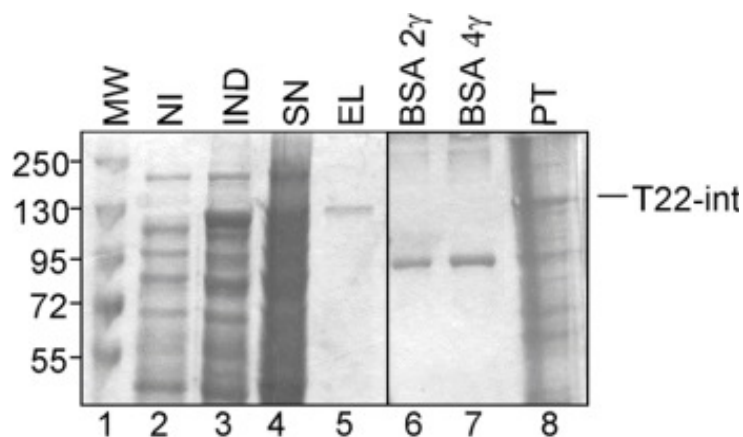
Expression of TRIM22 proteins leads to the recruitment of nuclear CIITA in TRIM22 nuclear bodies.

(A) Hep2 cells were transfected with the myc-CIITA (mCIITA) or flag-TRIM22 (fTRIM22) expression vectors. The cells were fixed and stained with anti-myc rabbit polyclonal antibody for the detection of CIITA proteins (A, a) or with FITC-conjugated anti-FLAG monoclonal antibody for the detection of TRIM22 proteins (A, b). (B) Hep2 cells were co-transfected with the vectors expressing mCIITA and fTRIM22. The cells were fixed and stained by double immunofluorescence staining with anti-CIITA rabbit polyclonal antibody (B, a) and FITC-conjugated anti-FLAG monoclonal antibody directed against the flag epitope to detect TRIM22 proteins (B, a'). The the overlay images are depicted in a''. Differential interference contrast (DIC) images are also shown.



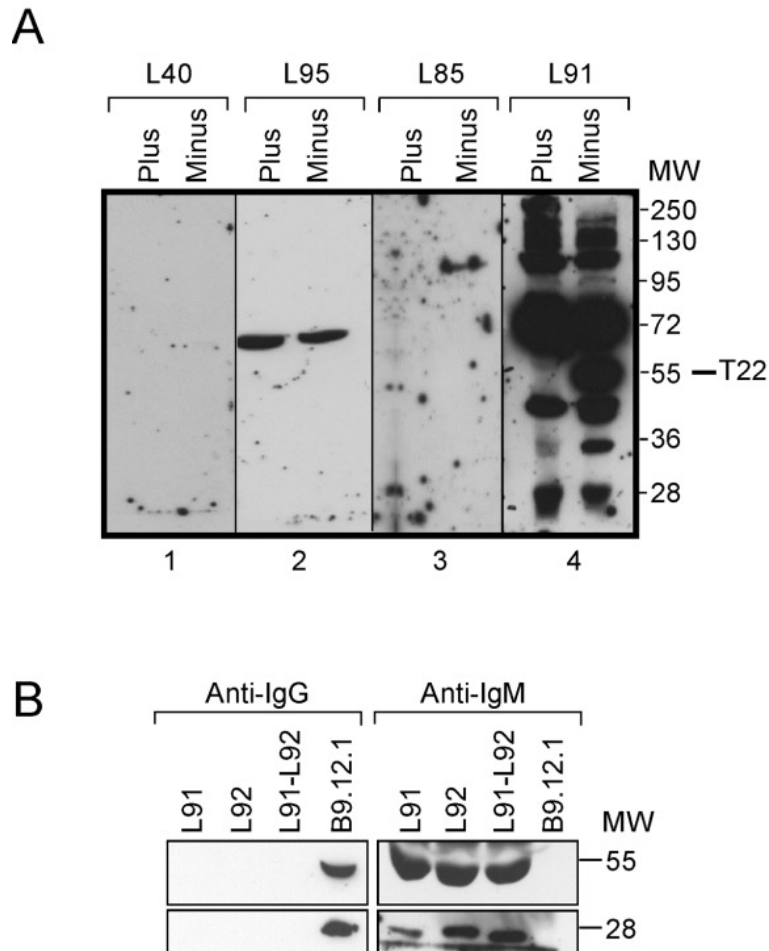
**Fig.6 TRIM22 Cloning**

TRIM22 coding sequence was amplified by polymerase chain reaction (PCR) from pcDNA3.1-TRIM22. using primer partly homologous to the consensus TRIM22 sequence. The amplified sequence was sub-cloned into a pGEMT easy vector and was digested using NdeI and XhoI restriction enzymes. The digested fragment containing the TRIM22 gene was cloned into pTYB1 into nNdeI and XhoI sites.



**Fig.7 Expression and purification of TRIM22-intein recombinant protein**

Lysates from bacteria transformed with TRIM22-intein plasmid construct. Samples were analyzed by SDS-PAGE and Comassie blue staining. Protein molecular weight marker (MW lane 1); the total lysate containing all bacterial proteins before induction by IPTG (NI, lane 2), and after IPTG induction (IND, lane 3). The Urea supernatants (SN, lane 4) and the corresponding protein pellet (PT, lane 8) were analysed. TRIM22-intein eluted protein (EL) was shown in lane 5. Lanes 6 and 7 are fixed amounts of BSA.



**Fig.8 Generation, screening and characterization of TRIM22 monoclonal antibodies.**

(A) Western blot of the selected TRIM22 hybridoma supernatants (L40, L95, L85, L91) on U937 *Plus* and *Minus* cell lysates. (B) The sub-class of L91 and L92 monoclonal antibodies use in different combination (as indicated) was identified by western blot. The HRP-conjugated goat anti-mouse IgG or goat anti mouse IgM secondary antibodies were used. The anti-HLA-I antibody B9.12.1 (IgG subtype) was used as control.

Hybridoma Supernatants	U937 Cells			Hybridoma Supernatants	U937 Cells	
	Plus	Minus			Plus	Minus
L1	ND	ND		L49	ND	ND
L2	ND	ND		L50	ND	ND
L3	ND	ND		L51	Bands	Bands
L4	ND	ND		L52	ND	ND
L5	ND	ND		L53	ND	ND
L6	28 KDA	28 KDA		L54	ND	ND
L7	ND	ND		L55	200KDA	200KDA
L8	ND	ND		L56	ND	ND
L9	ND	ND		L57	ND	ND
L10	ND	ND		L58	ND	ND
L11	ND	ND		L59	72KDA	72KDA
L12	ND	ND		L60	ND	ND
L13	ND	ND		L61	ND	ND
L14	ND	ND		L62	ND	ND
L15	ND	ND		L63	ND	ND
L16	72 KDA	72 KDA		L64	ND	ND
L17	28KDA	28KDA		L65	ND	ND
L18	Bands	Bands		L66	ND	ND
L19	28KDA	28KDA		L67	46KDA	46KDA
L20	28KDA	28KDA		L68	50KDA	50KDA
L21	Bands	Bands		L69	ND	ND
L22	Bands	Bands		L70	ND	ND
L23	28KDA	28KDA		L71	250KDA	250KDA
L24	28KDA	28KDA		L72	ND	ND
L25	ND	ND		L73	36KDA	36KDA
L26	ND	ND		L74	ND	ND
L27	ND	ND		L75	ND	ND
L28	ND	ND		L76	ND	ND
L29	ND	ND		L77	ND	ND
L30	ND	ND		L78	ND	ND
L31	ND	ND		L79	ND	ND
L32	ND	ND		L80	ND	ND
L33	ND	ND		L81	ND	ND
L34	45KDA	45KDA		L82	ND	100KDA
L35	ND	ND		L83	ND	ND
L36	ND	ND		L84	72KDA	72KDA
L37	ND	ND		L85	ND	100KDA
L38	50KDA	50KDA		L86	ND	ND
L39	ND	ND		L87	Bands	Bands
L40	ND	ND		L88	ND	ND
L41	ND	ND		L89	46KDA	46KDA
L42	ND	ND		L90	ND	ND
L43	ND	ND		L91	Bands	55KDA
L44	250KDA	250KDA		L92	Bands	55KDA
L45	ND	ND		L93	72KDA	72KDA
L46	ND	ND		L94	72KDA	72KDA
L47	ND	ND		L95	72KDA	72KDA
L48	ND	ND		L96	ND	ND

### **Table1: Summary of the analysis of the hybridoma supernatants**

Hybridoma supernatants (column 1) were analysed by western blot using U937 *Plus* and *Minus* cell lysates (column 2). In this table were indicated molecular weights of specific bands detected; ND: Not Detected; Bands: more than one band detected.

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