Gene Expression Pattern and Functional Analysis of $CD8^+$ T Cells from individuals with or without anti HIV/SIV noncytolytic activity.

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I hereby declare that my doctoral thesis entitled "Gene Expression Pattern and Functional Analysis of $CD8^+$ T Cells from individuals with or without anti HIV/SIV noncytolytic activity" has been written independently with no other aids or sources than those quoted.

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Related publications

Within this thesis, the following publication has been submitted.

- Rational design of HIV vaccines and microbicides: report of the EUROPRISE network annual conference 2011. Marie Borggren, Zelda Euler, Fabio Fiorino, KatrijnGrupping, David Hallengärd, **Aneela Javed**, Kevin Mendonca, Charlotte Pollard, David Reinhart, Nicolas Ruffin, Elisa Saba, Enas Sheik-Khalil, Annette Sköld Serena Ziglio, Robin Shattock, Gabriella Scarlatti, Frances Gotch,BrittaWahren. (Submitted, Journal of Translational Medicine).

Parts of this thesis have been presented at international conferences. I want to thank all People who contributed to these studies.

- Oral Presentation "Role of FAM26F in disease course of SIV infection"
  EUROPRISE Network Annual Conference, Prague 14-17th November 2011.

- Oral Presentation"Differential gene expression pattern of $CD8^+$ T cells from individuals with and without anti HIV/SIV Noncytolytic activity"
  "Molecular Medicine" Annual PhD Retreat, September 2011.

- Oral Presentation"Differential gene expression pattern of $CD8^+$ T cells from individuals with and without anti HIV/SIV Noncytolytic activity"
  "Molecular Medicine" Annual PhD Retreat, September 2010.

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ANEELA JAVED

Content

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III List of Abbreviations

Abbreviation	Explanation
°	Degree
Amp	Ampicillin
Bp	Base pair(s)
C	Celsius
CD	Cluster of differentiation
cDNA	Complementary DNA
CNAR	$CD8^+$ T cell noncytolytic antiviral response
CO_2	Carbon dioxide
CXC	Cystein X cystein
d	Day(s)
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DNase I	DNA-hydrolyzing enzyme
dNTP	Deoxynucleotide triphosphate
PBS	phosphate buffered saline
Dpi	Days post infection
dsDNA	Double stranded DNA
E.coli	Escherichia coli
EDTA	Ethylenediaminetetraacetic acid
env	Retroviral envelope protein
FCS	Fetal calf serum
FSC	Forward scatter

FACS	Fluorescence activated cell sorting
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
gDNA	Genomic deoxyribonucleic acid
h	Hour(s)
H_2O	Water
IFN	Interferon
Kb	Kilo base pairs
kDa	Kilo Dalton
LB	Lysogeny broth
LTR	Long terminal repeat
mAb	Monoclonal antibody
min	Minute
mRNA	Messenger RNA
NCBI	National Centre for Biotechnology Information
ORF	Open-reading frame
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
pH	Potentiahydrogenii
PK	Proteinase K
qPCR	Quantitative PCR
RNA	Ribonucleic acid
RNase	RNA-hydrolyzing enzyme
RT	Room temperature (21°C)
RT	Reverse transcriptase
Rpm	Revolutions per minute
ssDNA	Single stranded DNA

TCID50	Tissue culture infectious dose 50%
U	Unit
VSV-G	Vesicular stomatitis virus G protein

ABSTRACT

$CD8^+$ T cell mediated antiviral response (CNAR) is associated with long-term control of HIV-infection and attributed to the secretion of an unknown factor called soluble $CD8^+$ cell antiviral factor (CAF). In order to identify CAF, microarray data from $CD8^+$ cells displaying high CNAR activity and $CD8^+$ cells that lack CNAR were analyzed. Out of more than 50 differentially regulated genes, differential expression of 16 genes was validated by qRT-PCR in CD8 cells from 21 monkeys. FAM26F was identified as a sole candidate that was significantly differentially expressed in samples from SIV-infected as well as non-infected animals. FAM26F expression increased in $CNAR^-$ $CD8^+$ T cells during their co-cultivation with SIV-infected $CD4^+$ T cells in viral inhibition test. FAM26F was found to be expressed on three major blood cell populations ($CD4^+$, $CD8^+$ T cells and B cells). In vitro stimulation studies revealed that FAM26F expression was greatly induced in PBMCs after 6hrs of IFN-γ stimulation, and to some extent by IFN-α. Next, the expression pattern of FAM26F before and after infection was investigated in two independent AIDS vaccine experiments comprising in total 42 monkeys. In both experiments, FAM26F expression along with other innate immune modulators was significantly increased in PBMCs and followed same *in vivo* expression pattern after infection as Mx1, IP-10 and tetherin after SIV-infection. Its expression was also found to be significantly correlated with Mx1, IP-10 and tetherin. In first experiment, preinfection RNA levels of FAM26F were inversely correlated with 2, 12 and 24 wpi viral load while in other experiment, 2wpi expression of FAM26F was positively correlated with plasma viral RNA copies at 12, 24 and 48 wpi. Expression of FAM26F, MX1, IP-10 and tetherin was studied before and after immunization in two groups of animals that were finally boosted with fowlpox virus- or adenovirus-derived vector respectively. Increased level of protection in adenovirus-derived vector group was most likely attributed to significantly elevated expression of IP-10 (24hrs post boosting), FAM26F and tetherin (24 hrs and 48hrs post boosting) indicating more pronounced IFN-γ responses or a unique balance between type I and type II responses. In summary, the results emphasize that FAM26F may be an important regulator of innate or adaptive immune response. FAM26F expression may be an early prognostic marker for SIV/HIV infection. Lower expression of FAM26F before infection may indicate an immune status that is able to limit early viral replication, whereas a strong increase after infection may indicate an early immune dysregulation that is later on associated with higher viral load. Irrespective whether FAM26F is involved directly in regulation of viral replication or indirectly via the immune defense; our study has shown that it is an important molecule that clearly merits further investigation.

Introduction

More than three decades have passed since the first case of an acquired immunodeficiency syndrome (AIDS) [1, 2] and isolation of its causative agent human immunodeficiency virus (HIV) from the lymph node of the infected patient [3]. After its worldwide dissemination, infecting more than 33.4 million people (AIDS Epidemic Update, UNAIDS, WHO, as of December 2007) HIV is one of the most catastrophic examples of emergence, transmission and propagation of microbial genome [4].

HIV belongs to the genus of lentiviridiae of the retroviridae family. Lentiviridiae comprise of five groups each restricted to a single mammalian family namely ovines-caprines (CAEV), bovines (BIV), felines (FIV), equines (EIAV) and primates including SIVs, HIV-1 and HIV-2. Lentiviruses are host-specific enveloped RNA viruses, characterized by a long latency period rendering slow onset of disease [5]. They complicate the development of an effective immune response of the host due to their high mutation rate and variability[6]

1.1- Origin and subtypes

On the basis of genetic differences, geographical distribution and pathogenesis, HIV can be divided into two major types, HIV type 1 (HIV-1) and HIV type 2 (HIV-2). Although the origin of HIV is disputed, evidences based on the identical genomic organization led to the conclusion that HIV originally came from SIV (simian immunodeficiency viruses) infecting non-human primates. HIV-1 is closely related to SIVcpz, simian immuno deficiency virus infecting chimpanzees [7] while HIV-2 originated from SIV in sooty mangabeys (SIVsm) [8]. Furthermore HIV-1 groups M and N are thought to be originated in SIVcpz from Gabon [9] and that group O originated in gorillas [10]. It is important to note that SIVcpzand SIVsm do not cause disease in their natural hosts but induces in other monkeys like Asian macaques, an AIDSlike disease similar to humans [11]. It is considered that most likely the virus has entered the human population through zoonotic or cross-species transmission from non-human primates [12][13]

So far four groups of HIV-1 namely M (major), O (Outlier), N (non-M, non-O) and P (closer to SIVgor) [14] have been identified on the basis of genomic analysis of viral sequences from both env and gag genes [15]. Group 'M' is the cause of 90% of HIV/AIDS cases and is further subdivided into 11 clades (A through K) [16-19]. Simultaneous infection and recombination between different subtypes gives rise to "circulating recombinant forms" orCRFs. These

subtypes have distinct geographic distribution. The most prevalent are subtypes B (found mainly in North America and Europe), A and D (found mainly in Africa), and C (found mainly in Africa and Asia) (***HIV sequence compendium 2008).*** HIV-2 is geographically more restricted [20], less transmissible [21] and less pathogenic [20]. It is most prevalent in Africa and has 8 known HIV-2 groups (A to H) but only group A and B are prevalent.

1.2- Viral phenotypes

HIV can infect the immune cells such as helper T cells (specifically $CD4^+$ T cells) [22] macrophages [23] and dendritic cells. After binding to CD4 (main receptor) on these target cells, HIV uses secondary transmembrane G protein-coupled chemokine receptors, mainly CCR5 and CXCR4 as co-receptors for attachment and subsequent entry into cells [24]. Macrophage tropic (M-tropic) strains of HIV-1 (R5) use β-chemokine receptor CCR5 for entry [25]. They are non-syncytia-inducing strains and replicate *in vitro* slower than the T-tropic or syncytia-inducing (SI) strains which use α-chemokine receptor, CXCR4, for entry [26].

Generally, during acute virus infection, R5 virus emerges as the dominant type. People homozygous with the CCR5-Δ32 deletion are therefore resistant to infection with R5 virus and this homozygosity appears to account for resistance of some multiply-exposed individuals to HIV-1 infection [27]. The reasons for the selective transmission of R5 viruses are still unclear. Over time, in many cases, HIV becomes dual/tropic (that can use both receptors R5/X4) [28] or X4 strain emerge at a later stage causing more rapid progression to AIDS [29].

1.3- HIV-1 virion structure

The viral particle, with a diameter of about 110 nm, has a cone-shaped core composed of almost 2000 copies of p24 viral protein. Inside the capsid are two identical (9.2 kb each) single stranded RNA molecules, found to be closely associated with viral reverse transcriptase (RT) the enzymes integrase and protease and the nucleocapsid proteins P7 and p9 [30]. Viral capsid is surrounded by a layer called the matrix made up of protein p17.Surrounding the matrix is the viral envelope that is composed of two layers of phospholipids taken from the membrane of a human cell when a newly formed virus particle buds from the cell The envelope also contains cellular proteins acquired during virus budding, including ICAM (intracellular adhesion molecule), β 2-microglobulin and the human major histocompatibility

complex (MHC) class I and II molecules [31]. Embedded in the viral envelope are 72 spikes [32]. Each spike consists of three molecules of external surface envelope protein, gp120, (CAP) interacting non-covalently with three molecules of transmembrane protein, gp41, (stem) that crosses the lipid bilayer to anchor the structure into the viral envelope. This glycoprotein complex is essential for attachment and fusion of virus with target cells. Both these surface proteins, especially gp120, have been considered as targets of future treatments or vaccines [33].

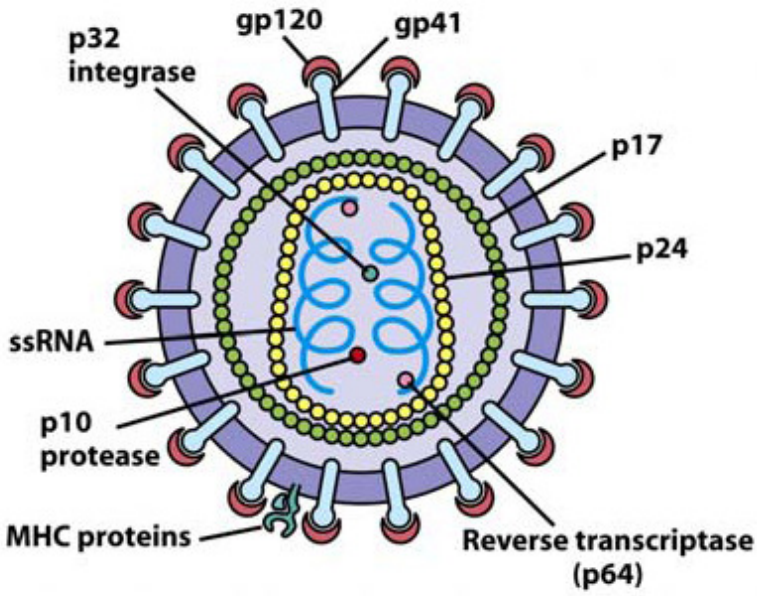


Fig 1.1: The HIV viral structure. HIV virus structure depicting important structural components. p: Protein; gp: Glycoprotein. Adapted from: Thomas K. Kuby."Immunology."

1.4- Genomic organization and gene products

The RNA genome of HIV consists of at least seven structural landmarks (LTR, TAR, RRE, PE, SLIP, CRS, and INS), nine genes (gag, pol, and env, tat, rev, nef, vif, vpr, vpu, (or vpx in the case of HIV-2), sometimes a tenth tev, (a fusion of tat, env and rev) and 19 encoded proteins.The coding regions of each strand of HIV RNA are flanked by long terminal repeat (LTR) composed of in total three elements with regulatory functions, R- and U5 region at the 5´end and U3 and R at the 3´end. The LTRs are duplicated upon intergration of the virus. PE

(Psi element) is involved in viral genome packaging and recognized by Gag and Rev proteins while the SLIP element (TTTTTT) is involved in the frame shift in the Gag-Pol reading frame required to make functional Pol. (*HIV Sequence Compendium 2008 Introduction*)

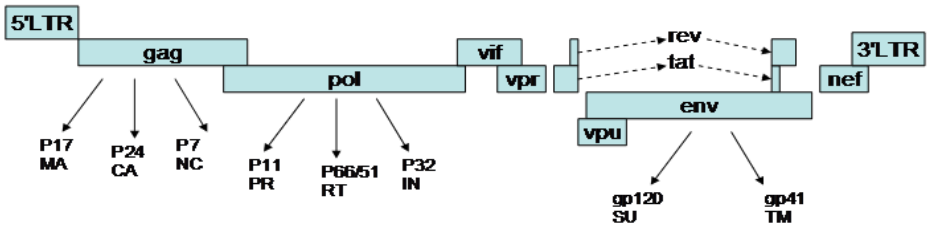


Fig 1.2: Depiction of the ~10 Kb HIV-1 genome showing the organization of its genes. Modified from Costin *Virology Journal* 2007.

1.4.1-Structural proteins

HIV genome contains three important genes to make the structural proteins for new virus particles.

1. The gag gene encodes the precursor p55 (polyprotein) which is further cleaved by the viral protease to the structural proteins; the matrix, the capsid, and the nucleocapsid protein (p24, p17, p7 and p6) [34].

2. The pol gene codes for a precursor protein which, after proteolytic clevage, results in three viral enzymes: p11 protease, p66/51 RT, and p32 integrase. These proteins are vital to virus replication [34]

3. The env gene codes for the precursor gp160 which is later proteolytically cleaved into two envelope proteins gp120 and gp41 that are crucial for the virus to bind and enter a host cell [32]

1.4.2-Accessory proteins

In addition to these genes, the HIV-1 genome encodes accessory proteins with important functions for viral replication and infection.The tat gene composed to two exons encodes Tat protein that acts as transcriptional trans-activator for TAR (Tat responsive region) elements located in the LTR to initiate viral mRNA transcription and promote viral RNA elongation.

The Rev protein (p19 regulator of viral expression) is involved in shuttling the RNAs from the nucleus and the cytoplasm by binding to RRE (Rev responsive element) located in the env gene. This interaction permits un-spliced mRNA to enter the cytoplasm from the nucleus and to give rise to full-length viral proteins needed for progeny production [35]. The Nef protein (p27) is one of the first and most immunogenic HIV proteins to be produced in infected cells and modulates diverse properties to increase the virion infectivity. It down-regulates CD4 [36] as well as the MHC class I and class II molecules [37]. It also induces complex changes in cellular trafficking, antigen presentation, and signal transduction [38]. Furthermore, Nef may also deregulate the communication between T cells and antigen-presenting cells [39]. In conclusion multiple Nef activities cooperate to delay the elimination of HIV-1-infected cells by the immune system and make the cellular environment more conducive for viral spread.

Vif, Vpu, and Vpr, all seem to target antiviral factors for ubiquitin-dependent proteasomal degradation to make the intracellular environment more conducive for viral replication. The Virion infectivity factor (Vif, p23) seems to be important for the cell-cell transmission of virus and prevents the action of viral restriction factor APOBEC3G. It has been reported to be crucial for proviral DNA synthesis and involved in the final stages of the nucleoprotein core packing [40][41]. 14 kDa viral protein R (Vpr) is involved in activation of proviral transcription, cell-cycle arrest in the G2 phase, induction of cell death, and enhancement of reverse transcription [42-44]. HIV-1 viral protein U (Vpu) is a 16 kDa protein with two main functions. On one hand it recruits ligase complex to newly synthesized CD4 in the endoplasmic reticulum for its proteasomal degradation [45, 46]. Thus facilitates the virus release, averts super infection, and enhances the incorporation of functional Env proteins into progeny virions. On other hand it antagonizes the cellular restriction factor tetherin [46].

1.5- Replication cycle

HIV replication cycle starts with the high-affinity attachment of the CD4 binding domains of gp120 (V3 loop)[47] to the CD4 [48] receptor causing conformational changes in the viral protein gp120 and exposure of co receptor (CCR5/CXCR4) binding sites [49]. This more stable two-pronged attachment results in fusion of the membranes and subsequent injection of the viral genome and other enzymes, including reverse transcriptase- (the ribonuclease a subunit of the RT) integrase, and proteaseinto the cell. After entry, viral RNA is reverse

transcribed in cDNA and integrated into the genome of host by the viral integrase. To actively produce the virus, certain cellular transcription factors like NF-κB are needed [50]. When the host cell is activated, cellular RNA polymerase transcribes the viral DNA. Multiply-spliced mRNAs are transcribed and produce the regulatory proteins Tat, Rev and Nef. Transcription and elongation is increased by Tat activity on the TAR in the LTR region while an increase in the level of Rev accelerates the cellular translocation of unspliced viral RNA. Nef makes up to 80% of the early viral transcripts. Expression of late transcripts gives rise to structural proteins Gag and Env. During the post-translation period, the envelope proteins are glycosylated and cleaved by cellular proteases into gp120 and gp41. The envelope proteins, Gag polyproteins, Pol polyproteins and the new viral genomes are assembled into new viral particles at the cell membrane. The virus progeny particles are released by budding through the cell membrane. The maturation of the virus particles is completed during and after budding from the host cell when the viral protease cleaves the Gag and Pol polyproteins into functional proteins.

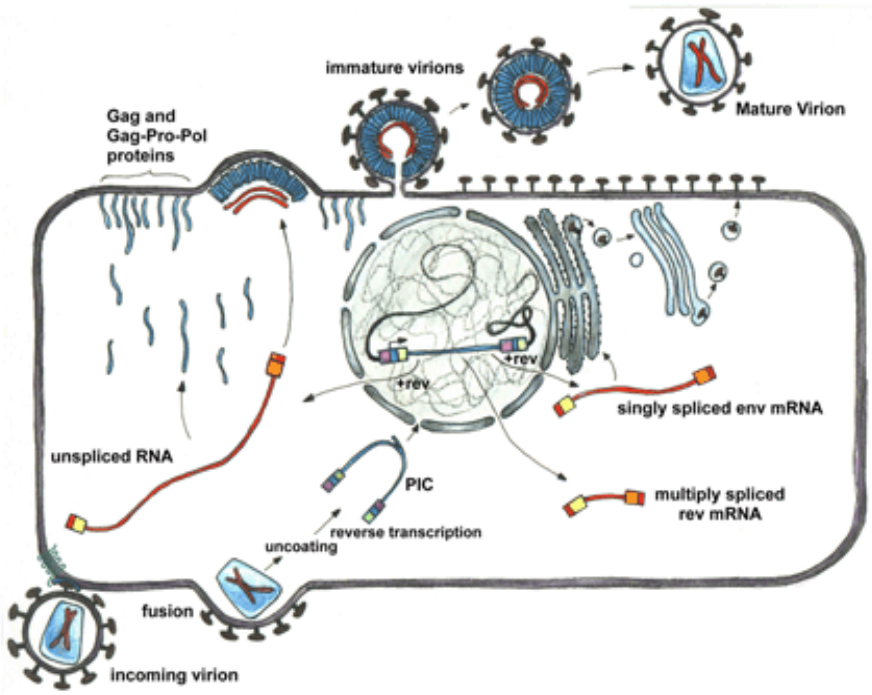


Fig 1.3: Different steps in HIV replication cycle including attachment, entry, reverse transcription, integration, virion formation and budding. (Adapted from Eric M Poeschla. http://mayoresearch.mayo.edu/mayo/research/poeschla/images/hiv_1.gif)

1.6- Infection and disease

HIV infection generally evolves into three phases namely acute infection (primary infection), latency period or chronic infection and finally AIDS leading to death. The acute phase is characterized by rapid viral replication with a burst in plasma viremia allowing systemic dissemination of the virus and may last 1 to 3 months [51][52]. Symptoms include fever, pharyngitis(sore throat), rash, myalgia(muscle pain). Generally, with the onset of humoral and cellular immune response the plasma viremia drops to an individual set point [53]. This stage of HIV infection can vary between two to 20 years. A small percentage of infected individuals are rapid progressors while 80% are "normal" progressors in whom survival time is eight to ten years without antiretroviral treatment [37]. Approximately 5-8% of all HIV-infected people fall into the group of long-term non progressors (LTNPs). These individual remain infected for > 10 years without showing signs of the disease and without therapy [54]. A subgroup called elite controllers are those LTNPs with viral RNA copies <50/ml plasma although they have been infected for 2 to >10 years [55] . When infected individuals progress to AIDS, $CD4^+$ cells number drops below 200 cells/µl leading to the loss of cell-mediated immunity and thus an increase in plasma viremia [37] and opportunistic infections and ultimately, the death of the patient [56]

1.7- The host immune responses against HIV-1 infection

AIDS is essentially an infection of the immune system. An early effective host immune system is crucial to control against HIV infection. Two major defense pathways are described within the host immune system: innate and adaptive immunity.

1.7.1 Innate immunity

Apart from physical barriers (mucus, low pH, and epithelial integrity), a number of secreted cationic peptides and small secreted proteins at the mucosal surfaces can modulate HIV infection. A small cationic peptide SEVI (semen derived enhancer of virus infection) enhances *in vitro* HIV infection through formation of amyloid fibrils that capture and focus virus onto target cells. Small cationic peptides produced by mucosal epithelial cells called defensins, contribute to anti-HIV by several mechanisms including impairment of gp120 binding to CD4 [57], induction of β-chemokines [58], inhibition of the fusion step [59]. They also act as chemo-attractants for T cells, monocytes and dendritic cells (DCs) and regulate

cellular activation and cytokine production [60]. Although local elevations in α-defensin levels during genital tract infections leads to enhanced HIV acquisition [60], it is not clear either pro- or anti-HIV activities of defensins predominate in vivo.

Among the solublecomponents of the innate immune system with anti-HIV activity are the complementsystem and mannose-binding lectins(MBL). These soluble products bind to HIV and either lyse thevirus directly or induce macrophages to phagocytose the virus [61, 62]. TheCC chemokines (RANTES, MIP-1α and MIP-1β may also block HIV access to the CCR5co-receptors [63], and are able to attract the entire immunological repertoire ofcells (T and B cells, DC and macrophage) to the mucosal site.

Two of the 18 human whey acidic proteins family members are documented for their anti HIV activity. Secretory leukocyte protease inhibitor (SLPI) found in saliva elicit its anti HIV activity by binding to annexin II (an enhancer of HIV entry) and thus may contribute to the infrequent oral transmission of HIV[64-66]. Elafin, with unknown underlying mechanism is over-expressed in female genital tract [66]. However another member of same family WFDC1/ps20 (whey acidic protein four-disulphide core domain 1) can enhance HIV infection [67].

A high concentration of stromal-derived factor (SDF1), a ligand of the CXCR4 receptor is expressed in human cervico-vaginal and rectal epithelial cells can preventthe transmission of X4-viruses across mucosal surfaces [68].

1.7.1.1 Cellular HIV restriction factors

Intrinsic retroviral restriction factors such as apolipoprotein B editing complex (APOBEC)3G/F, tripartite motif (TRIM)5α [69]and tetherin [70] are reported to display broad antiviral effects.

APOBEC3G is incorporated into HIV-1 virions and catalyses the deamination of cytidine to uridine during negative-strand DNA synthesis [71, 72]. These hypermutations can lead to the degradation of the viral DNA and/or become fixed as G-to-A changing TGG (W) to TAA/TAG (stop) codons. In both cases the virus is inactivated [72]. To counter the effect of APOBEC3G, virion infectivity factor (Vif) serves as an adaptor molecule to link a cullin 5-based E3 ubiquitin ligase complex to APOBEC3G thus inducing its polyubiquitination and subsequent proteasomal degradation, thereby preventing its packaging into budding

virions.[73] Vif also mediates the degradation of APOBEC3F, which is another potent inhibitor of HIV-1.

TRIM5α (tripartite motif 5-a) represents a host restriction factor that displays species-specific retrovirus restriction. While for instance rhesus TRIM5α can restrict HIV-1 and some SIVsm strains, human TRIM5α cannot restrict HIV-1 but HIV-2. The species specific differences are based on the presence of a target sequence in the viral capsid proteins as well as on species-specific differences in the C-terminal region of TRIM5α. TRIM5a proteins can interact with incoming capsid protein and may induce rapid uncoating by proteasomal degradation [74, 75]

Tetherin is a type II single-pass transmembrane protein that inhibits the release of viral particles [70]. It contains a cytoplasmic N-terminal region, followed by a transmembrane (TM) domain, a coiled-coil extra-cellular domain, and a C-terminal glycophosphatidylinositol (GPI) anchor [76]. After dimerization by three cysteine residues, it directly tethers the nascent virions to the surface of the producer cells with one membrane anchor sticking in the virion and the other in the cell. VPU interacts with TM domain of tetherin and recruits it to Trans Golgi network or early endosomes for its proteasomal and/ or lysosomal degradation [77].

1.7.1.2 Dendritic cells

Dendritic cells (DCs) play a pivotal role in the initiation and regulation of immunity. They are derived from hematopoietic bone marrow progenitor cells. DCs represent a heterogenous cell population depending on their differentiation status and their anatomical location. There are two main types of DCs (1) plasmacytoid dendritic cells (pDC) and (2) conventional dendritic cells (cDC) that differ markedly in their developmental program and function.

Pre-cDC precursors migrate to the lymphoid organs to convert into cDC[78]. cDC express TLR2 and TLR4 and play an essential role in antigen presentation in vivo [79]. In contrast, pDC develop fully in the bone marrow and disseminate through the blood [80]. They express TLR7/8 and TLR9. The main functional feature of pDC is their ability to quickly secrete large amounts of type I IFN (predominantly INF-α) in response to viral products [81], but their antigen-presenting function in vivo remains unknown [82]

pDC recognize HIV ss RNA via TLR7 receptors leading to subsequent upregulation of MHC and costimulatory molecules production of high levels of type 1 IFNs[61].The earliest

systemic cytokine elevations produced by pDCs in acute HIV infections include rapid but transient increase in IFN-a and interleukin (IL)-15 levels in plasma [83]. This is followed by initiation of a rapid but more sustained increase in TNF-α and IP-10 while slightly slower but more sustained increase in IL-18 by cDCs response [84].

A positive correlation betweenblood IPC (interferon producing cells) number, IFN-α production, and clinical state of HIV-infected subjects is well established. High viral load and a progressive disease in HIV individuals have beensuggested to have a close correlation with a decreased number of pDCs in peripheral blood. Furthermore the ability of pDC to produce INF α during acute HIV infection is impaired [85]. While IFN-α has antiviral effects, it should be noted that chronic long term INF-α production could be deleterious as it induces abnormal immune activation potentially leading to HIV pathology [86]. Relatively swift resolution of interferon responses in HIV controllers compared to progressors (as evident from ISG expression pattern in whole genome transcript studies [87] and lack of chronic immune activation in non-pathogenic SIV infections (in sooty mangabeys and African green monkeys) as compared to persistent immune activation in pathogenic SIV infection (e.g. rhesus macaques) [88-90] emphasize that achieving the optimal IFN response by pDC, limiting the IFN dependent immunopathology is the main discriminatory factor between pathogenic and non-pathogenic infections and still to be resolved.

Dendritic cells display another adverse role in HIV-infection as they cancapture and internalize virions via DC-SIGN (lectin dendritic cell-specific, intercellular adhesion molecule-grabbing non-integrin) leading to rapid dissemination of HIV [91][92].

1.7.1.3 NK cells

In addition to pDCs, HIV infection can rapidly activate NK cells predominantly driven by IL-15 and IFN-α secreted by dendritic cells and monocytes. Impairment of NK cell function with persisting viral replication and disease progression is characterized by decrease in CD56high (regulatory) NK cells (in part due to their recruitment to lymph nodes) and accumulation of CD56low (anergic effector) NK cells [93]

NK cells represent a highly heterogenic cell population characterized by differential combination of activating and inhibitory KIRs in conjunction with their HLA class I ligands. These unique combinations not only dictate the functionality of NK cells but also effect their

differential expansion [94]. As an example, co-expression of HLA-Bw480I and KIR3DS1 has been found to be associated with low-level viremia and delayed disease progression in ~~early~~ HIV-infection [95]. The presence of KIR3DL has been associated with decreased NK cell frequency and high viral load in SIV-infected rhesus monkeys [96]. Furthermore, a SNP associated with elevated HLA-C (ligand for receptors of the KIR2DL and KIR2DS family) expression is also associated with lower viral set point and slower disease progression [97].
Nk cells help in maturation of DCs in peripheral tissue (at sites of inflammation) after their recruitment from the blood stream. Resulting mature DCs (mDCs) migrate to secondary lymphoid tissues, where they prime an antigen-specific T cell response while immature DCs are rapidly eliminated from peripheral circulation ensuring that only mature DCs can access to inductive sites (to secondary lymphoid tissues). Thus NK cell act as quality control for DC populations. This function is impaired in chronic HIV-1 infection [98].
Other cellular components of the innate system include γδ T cells, which are involved in early mucosal protection and can lyse HIV-infected target cells [99]. Neutrophils arethe most abundant innate immune cells responding early to infections, and possess virocidaleffects on HIV [100].

1.7.1.4 Interferons

Interferons (IFNs) are proteins interfering with viral replication by triggering the protective defenses of the immune system by activating immune cells, such as natural killer cells and macrophages. About ten distinct IFNs (7 for humans) are divided among three IFN classes: Type I IFN, Type II IFN, and Type III IFN. Type I interferons include IFN-α (family of closely related genes), IFN-β (product of a single gene) and IFN-ω.

HIV-1 stimulates plasmacytoid dendritic cells (pDC) to produce interferons either TLR7/9 dependent or independent mechanisms. Downstream molecules involved in TLR dependent mechanism include, MyD88, (myeloid differentiation primary response protein 88) interferon regulatory factor (IRF)-7 and finally NF-κB activation [101]. On other hand TLR-independent induction of type I IFN by viruses is mediated through cytoplasmic sensors, like retinoic acid-inducible gene I (RIG-I) and the melanoma differentiation-associated gene 5 (MDA5). This leads to IRF3 activation and finally NF-κB activation [102].

Binding of Type I IFNs to their receptors IFNAR (composed IFNAR1 and IFNAR2) stimulates the JAK/STAT signal transduction and ultimately leads to transcription of more

than hundred ISG (interferon stimulated genes) whose products have antiviral, anti-proliferative, apoptotic and immunomodulatory properties (Fig 4). The *in vitro* inhibitory effects of IFNα on HIV-1 replication have been described in macrophages, monocytes [103] and humanized mouse models of HIV-1 infection [104] some important interferon induced proteins are given in table below some of them have already been discussed above in detail.

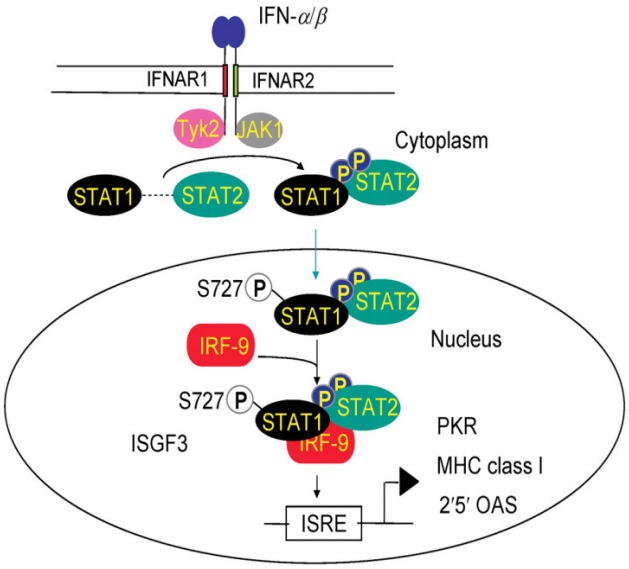


Fig 1.4: Signaling pathway activated by IFN-α/β. The biological activities of IFN-α/β are initiated by binding to the type I IFN leading to activation of the receptor-associated tyrosine kinases JAK1 and Tyk2, which phosphorylate STAT1 on tyrosine 701 and STAT2 on tyrosine 690. Stable STAT1–STAT2 heterodimer is translocated into the nucleus, where it interacts with the DNA-binding protein IRF-9 and a sequence motif (the IFN-stimulated response element or ISRE) in target promoters and brings about transcriptional activation. Adapted from [105].

Interferon-gamma (IFN-γ) is the only member of the type II class of interferons[106] produced by natural killer (NK) cells as part of the innate immune response, and by CD4 (Th1) and CD8 (CTLs) effector T cells once antigen-specific immunity develops [107]. Binding of the ligand to IFNGR leads to the activation of the JAK/STAT pathway that triggers the gamma-activated factor (GAF) in the nucleus that binds to gamma-activated sequences (GAS) in the promoter region of IFN-γ-induced genes.

Some effects of Type I and Type II interferons are

- Type I and II IFN up-regulate cell-surface MHC class I which is important for host response to intracellular pathogens, as it increases the potential for cytotoxic T cell recognition of foreign peptides and thus promotes the induction of cell-mediated immunity[108].
- IFN- γ can efficiently up-regulate the class II antigen presenting pathway and thus promote peptide specific activation of $CD4^+$ T cells[109].
- Release of cytokines such as interferons and interleukins, that co-ordinate the activity of other immune cells.
- Promote T_h1 differentiation by upregulating the transcription factorT-bet, ultimately leading to cellular immunity: cytotoxic $CD8^+$ T-cells and macrophage activity - while suppressing Th2 differentiation which would cause a humoral response.
- Up regulates the expression of adhesion molecules such as ICAM-1 and VCAM-1 to on target surfaces where leukocyte trafficking is required.
- Induce the expression of intrinsic defence factors such as TRIM5alpha, APOBEC, and tetherin

Table 1.1: some important Type I and Type II Interferon induced genes/responses.

Protein induced	**Effect**	**Reference**
PKR	Phosphorylates α-subunit of the protein synthesis initiation factor eIF-2α, leading to inhibition of translation.	[110]
(OAS)2'-5′oligoadenylate synthetases	In the presence of dsRNA, synthesizes 2′5′ oligoadenylates, which activate endogenous cytoplasmic RNase L and ultimately degradation of viral and cellular mRNA	[111]
MxA/Mx1,	Belongs to superfamily of GTPases. Appears to sense nucleocapsid-like structures and trap them into specific subcellular compartments to make them unavailable for the generation of new virus particles.	[112]
Protein IP-10 or Chemokine (CXC motif) ligand 10	IP-10 binds to the CXCR3 receptor and acts as a chemoattractant for monocytes / macrophages, T cells, natural killer cells and promotes their adhesion to endothelial cells thus modulating cellular trafficking.	[113, 114]
Tetherin	Viral restriction factor	[115]

TRIM5α	Viral restriction factor	[69, 116]
APOBEC3G	Viral restriction factor	[117]

1.7.2 Adaptive immune system

Antigen specific adaptive immune system starts late after the initial innate responses against HIV. Adaptive immune system not only recognizes the “non-self” antigen and tailors the responses to eliminate the pathogen infected cells but it also develops the immunological memory. Professional antigen presenting cells (APC) like dendritic cells, B-cells and macrophages (T cells to a lesser extent) process and present the (HIV) antigens on their surface which are recognized by T cells in lymph nodes via T cell receptors (TCR) (Janeway, 1999).

Exogenous antigens displayed on MHC class II molecules activates $CD4^+$ helper T-cells which differentiate into Th1 or Th2 depending on the magnitude and patterns of TCR, co-stimulatory and cytokine signals received. Th1 cells response produce pro-inflammatory cytokines like IFN-γ, IL-2, and lymphotoxin-α (LTα) activating macrophages, natural killer cells (NK), antigen-specific cytotoxicT-lymphocytes thus leading to "cell-mediated immunity". Th2 cells release of IL-4, IL-5, IL-6, IL-10, and IL-13 that activate B-cells to produce neutralizing antibodies leading to"humoral immunity(Janeway, 1999). Intracellular antigens (produced by viruses replicating within a host cell) are bound to MHC class I and activate $CD8^+$ T cells (CTLs). Once activated, CTLs undergo clonal expansion and travel throughout the body to kill infected cells by releasing perforin, granulysin and granzyme. Upon resolution of the infection, most of the effector cells are cleared away by phagocytes while a few will be retained as memory cells. Regulatory T cells (Treg), limits aberrant immune responses to self-antigens (Janeway, 1999). Hence adaptive immune system has two major arms i.e. cellular and humoral immune responses.

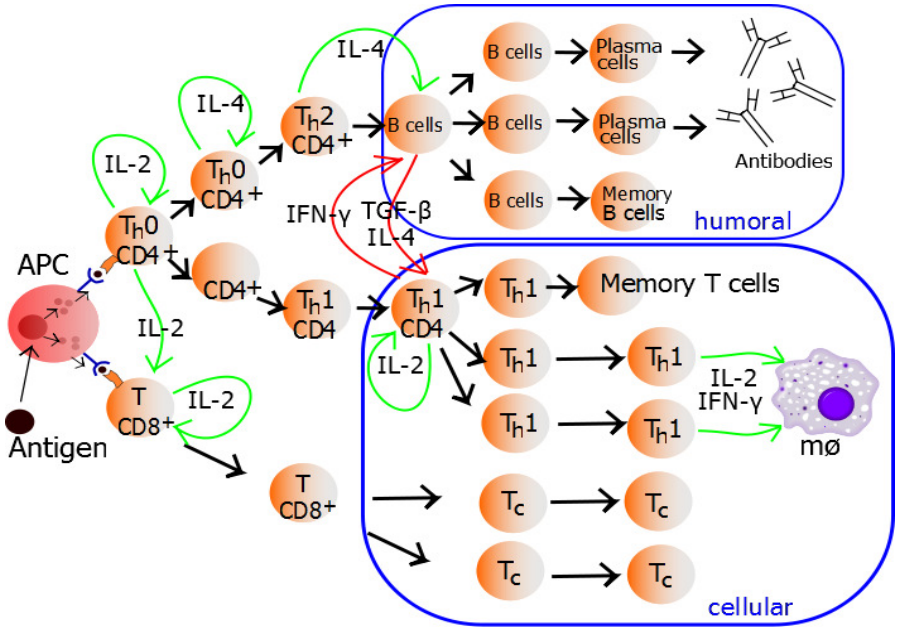


Fig 1.5: T_h1/T_h2 Model for helper T cells and activation of cellular and humoral responses. (Adapted from Rang, H. P. (2003) *Pharmacology* Edinburgh: Churchill Livingstone ISBN: 0-443-07145-4. Page 223)

1.7.2.1 Cellular responses:

The cellular immune system consists of CD8cells, involved in the killing of infected cells, and CD4 Th1 cells that activate different cells of the immune system. (Janeway, 1999). CD8 T cells help to control HIV replication either by MHC class I-restricted antigen-specificmanner to directly kill the HIV infected cells through the production of perforin and granzymes [118] or by the expression of membrane-bound Fas ligand inducing apoptosis in Fas expressing cells[119].Non-cytotoxic effects of $CD8^+$ T cells include IFN-γ production [120][121], CC-chemokine production[122]and non-cytolytic antiviral response.

Substantial evidences like high production of chemokines and strong CD8 cytotoxic antiviral responses delaying HIV and SIV infections [123, 124][125], detection of viral-specific $CD8^+$ T cell inhigh exposed seronegative individual and strong enhancement of the SIV replication after in vivo depletion of $CD8^+$ cells in monkeys (by infusion of anti CD8 monoclonal antibodies) [126] are few examples highlighting the importance of CD8 T cell responses in controlling the viral replication. But it is noted that both cytotoxic as well as noncytotoxic anti-HIVCTL responses of $CD8^+$ often decline with disease progression [127, 128]. Furthermore some LTNP seem to have rather low levels of HIV-specific CTL [129] while

some evidences of increased viral load in rapid progressor despite of strong HIV specific CTL responses are suggestive of HIVs ability to escape antiviral responses. (See viral evasion mechanisms).

1.7.2.2 $CD8^+$ T cell mediated non cytotoxic antiviral response (CNAR)

In addition to their MHC class I-restricted antigen-specific CTL effector function, an important activity of $CD8^+$ cells is the non-cytolytic suppression of HIV. First experimental evidence that $CD8^+$ T cells from HIV-infected individual have an effective anti-HIV immune response without killing the infected cell came in 1986[130]. Substantial reduction in virus replication was observed when $CD8^+$ T cells from HIV-infected LTNP were co-cultured with MHC mismatched CD4 cells acutely infected with HIV [131, 132]. Several groups confirmed the findings [133, 134] and this activity has been referred to in different forms, such as CNAR ($CD8^+$ T cell non-cytotoxic anti-HIV response) or CASA ($CD8^+$ anti-HIV suppressor activity).

CNAR was demonstrated with heterologous effectors and targets, suggesting that it was not restricted by MHC class I antigens [131]. This noncytolytic suppressive activity was also observed when infected CD4 cells were separated by a trans well devices or by adding cell-free culture fluids from $CD8^+$ T cells of HIV-seropositive persons to infected CD4 cells (although maximal effect is observed when cells are in contact) [135]. Both of these observations provided evidence that CNAR is mediated by a soluble secreted antiviral factor that has been termed as the $CD8^+$ T cell antiviral factor (CAF). In contrast, the term CNAR is used to describe one or more antiviral factors secreted by $CD8^+$ T cells.

CAF is predicted as 10–50 kD protein, found to be Serine protease sensitive. It is produced at low levels and resistance to heat (86C, 10 min) and low pH (2.0) [136].Studies show that culture fluids from $CD8^+$ T cells of asymptomatic infected persons can produce more CAF and thus better control the virus replication as compared to cells from AIDS patients . This suggests that CAF production is dependent on the clinical state of the infected individual. Furthermore, an inverse correlation was found between viral load and *in vitro* CAF production i.e. a person with a high $CD8^+$ T cell antiviral response would have a low virus load and vice versa suggesting that, the loss of CNAR or CAF production may contribute to the inability of AIDS patients to control the viral replication[137]. CNAR/CAF is found to be effective before the onset of any humoral response (Ab production) [138] and is

also observed in high risk seronegative subjects, elite controllers [139]. Potent CD8 $^{+}$inhibitory activity or CAF has been noted in the simian immunodeficiency virus (SIV) infected macaques [133] SIVsm-infected sooty mangabey [140] the SIVagm-infected African green monkey model [140, 141] and the HIV-1-infected chimpanzee [142].

Attempts to reveal the suppression mechanism showed that there was no decrease in the number of infected cells in the presence of CAF but a decrease in the expression of viral RNA species indicated that CAF was able to suppress the transcription of HIV-RNA [143]. Further investigation indicated that CAF can control HIV replication at the level of the HIV long terminal repeat (LTR)–driven transcription as a marked decrease in the expression of luciferase reporter gene linked to LTR was observed when CD4 infected cells were treated with CAF containing fluids. In an attempt to clarify the molecular mechanisms CAF was shown to elicit its response by activating the signal transducer and activator of transcription 1 (STAT1) protein leading to IRF-1 induction and inhibition of gene expression regulated by the HIV-1 LTR. [144].

Several cytokines produced by CD8 cells can inhibit HIV replication *in vitro*, however studies have demonstrated that CAF is not related to any known cytokine or chemokine For example, although the β-chemokines can block HIV replication [63], they are distinct from CAF as β-chemokines block replication of only R5 strains while CAF can block the replication of all types of HIV [145]. Furthermore, neutralizing antibodies to RANTES, MIP-1α, and MIP-1 β were unable to substantially block viral suppression [146]. Furthermore, β-chemokines block the entry of HIV into the cell, whereas CAF blocks virus production at the level of transcription. Baier et al [147] attributed the CNAR activity to IL-16 but the fact that relatively high concentrations of recombinant human IL-16 were necessary to inhibit virus production from primary cells doubted these findings.

Others demonstrated that the chemokine stromal-cell derived factor 1 (SDF-1) and/or macrophage derived chemokine (MDC) may be CAF. The role of α- defensins in CD 8-suppression was proposed and then retracted. Although a large number of interleukins, interferons, chemokines, granzymes, growth factors and other cytokines, with anti-HIV activity were initially considered as possible CAF candidates but till date none of them fully attributes to CAF properties. Some of them are listed in table 2.

According to one hypothesis by J.A. Levy, CD8 cells produce a protease (serine protease) and a CAF- precursor protein. The precursor is cleaved by the protease to become active as an antiviral protein most probably at the cell surface. Whether a proteolytic step is needed to activate a CD8 cell product or it affects the infected CD4 cell directly to establish an antiviral state requires further study [148].

In attempts to define the gene(s) mediating CNAR, differential gene expression techniques have been used [149] to examine expression pattern of $CD8^+$ cells from infected subjects with high CNAR and $CD8^+$ cells from uninfected controls that lack this activity. Although many genes involved in different cellular processes were found to be differentially expressed, none of them exclusively identified as CAF and these factors still remained elusive.
Evidence was presented by Tumne and co-workers [150] linking CNAR to exosomes secreted by $CD8^+$ T-cells. They demonstrated that purified exosomes from $CD8^+$ T-cell culture supernatant noncytotoxically suppress both (R5) and (X4) of HIV-1 replication *in vitro* through a protein moiety indicating the existence of an antiviral membrane-bound factor consistent with the hallmarks defining noncytotoxic $CD8^+$ T-cell suppression of HIV-1. However, follow-up studies to identify CNAR have not been published yet.
In a recent study Scott and coworkers tried to identify the phenotype of CD8 cell subsets having strong CNAR activity. They showed that $CD8^+$ T cells from asymptomatic individuals with low-level viremia exhibited the highest HIV-suppressing activity and had elevated frequencies of $CD45RA^-$ $CD27^+$ and $PD\text{-}1^+$ (CD279) cells and maximal CNAR activity was mediated by $CD45RA^-$ $CCR7^-$ $CD27^+$ and $PD\text{-}1^+$ $CD8^+$ T cells. This study suggested that CNAR activity is associated with oligoclonally expanded activated $CD8^+$ cells expressing PD-1 and having a transitional memory cell phenotype [151].

Table 1.2: Proteins that have antiviral effect but lack identity to the $CD8^+$ T cell antiviral factor (CAF).

	Proteins	Reference
1	Interferon-α , and β	[152]
2	Transforming growth factor-b (TGF-β)	[152]
3	Interleukin-8 (IL-8)	[152]
4	IL-10	[152]

5	IL-16	[147]
6	ß chemokines: RANTES, macrophage inflammatory protein-1a (MIP-1α), MIP-1β	[63]
7	Stromal cell-derived factor-1 (SDF-1)	[153]
8	Tumor necrosis factor-a (TNF-α)	[152]
9	Macrophage-derived chemokine (MDC)	[154]
10	Leukemia inhibitory factor (LIF)	[155]
11	Monocyte chemotactic protein-2 (MCP-2)	[156]
12	Lymphotactin	[156]
13	Alpha-defensins 1–3	[157]
14	RNase	[158]
15	Secretory leukocyte protease inhibitor (SLPI)	[159]
16	Alpha-1-antitrypsin	[160]
17	D Lactoalbumin	[161]
18	6 kD protein [42]	[162]
19	Natural killer cell enhancing factors (NKEF) A, B [53]	[163]
20	CD8 cell product modifying anti-thrombin III [50]	[164]

1.7.2.3 Humoral responses

Humoral responses to HIV both against envelope (gp160, gp120, gp41) and core proteins (p55, p24, p17) [165] appear generally within 1 to 3 months after infection. HIV-specific IgM antibodies that appear at the start of seroconversion start to decline within a few weeks and HIV-specific IgG antibodies start to develop [166]. IgG1 is a key player in host defense at all stages of infection, and helps to combat the virus with antibody-dependent cellular cytotoxicity (ADCC) complement-dependent cytotoxicity (CDC), and complement-dependent phagocytosis via Fc mediated effector system [167]. All the other antibody isotypes (IgM, IgA, IgG2, IGG4 and IGD) vary in their levels throughout the course of infection. IgA is reported to neutralize HIV intracellularly and inhibits its epithelial transfer [168][169] but its role in vivo is unclear.

Neutralizing antibodies can inhibit virus binding to the target cell or interfere with post-binding events, including fusion of the virus and cell membranes[170] . High anti-p24 antibody correlates with slower disease progression. However presence of such correlation with gp120 responses has been controversial. Nevertheless the V3 loop [171] as well as V1/V2 region of gp120 has been described to elicit neutralizing antibody responses. [172]. Furthermore, V1V2 antibodies may confer protection against HIV-1 infection [173].

Correlations between humoral responses and disease progression have been established and studies show that slow progressors are able to neutralize primary isolates more frequently than rapid progressors [174, 175]. Some studies show that HIV specific antibodies have limited efficacy during the asymptomatic period, but are unable to clear an infection.

1.8- Immune evasion of HIV

HIV differs from many viruses in that it has very high genetic variability. This diversity is attributed to fast replication cycle with the generation of about 10^{10} virions every day, high mutation rate of approximately 3 x 10^{-5} per nucleotide base per cycle of replication [176, 177], and a high recombination rate [178] that enables the virus to escape from the antibodies or cytotoxic T lymphocytes (CTLs). Conserved functional domains of the Env are masked by variable loops and only transiently exposed during viral entry [179]. The high degree of glycosylation of Env and alterations in the glycan shield of Env during infection also contribute to viral escape. This viral camouflage makes the broadly neutralizing antibodies very rare. Selection pressure from neutralizing antibody can lead to the change of the antibody binding and neutralizing sensitivity [180, 181].

CTL responses also contribute to immune escape. HIV forms ‘escape mutants’ through mutation of viral genes thus altering the antigen processing [182] and loss of epitope binding MHC class I making recognition of virus by $CD8^+$ T cells difficult [183-185].

1.9- Non-human primate models for HIV infection

Non-human primate models for disease pathogenesis are of vital importance for the understanding of human diseases. SIV, first isolated in the early 1980's from monkeys with AIDS-like disease [186][187] is closely related in genetic structure to HIV-2[188]. It was accidently transmitted from juvenile SIV-infected Asian to African macaques when they were

kept together in the same enclosure, remarkably at a similar time point when the first cases of human AIDS were reported. Experimental inoculation of SIV into a number of Asian macaque species, including rhesus, pig-tailed and cynomolgus monkeys, results in pathological manifestations similar to AIDS in humans. [189] Currently, the most commonly used non-human primate model in HIV research is the SIV infected rhesus macaque mainly because rhesus macaques are widely available and numerous laboratory tools for investigating immune responses or macaque genetics have been established) [190]. SIV-infection leads to a decline in $CD4^+$ T cell populations, immunodeficiency and finally to an AIDS-like illness (e.g. Opportunistic infections, neoplastic diseases; hematological and neurological disorders)[190]. Progressive changes in lymph node structure during disease course are very similar to that seen in humans [191]. Rapid and selective depletion of memory T cells in gut-associated lymphoid tissues during SIV infection is also confirmed in HIV infection [192]. Both viruses, replicate not only in activated and proliferating T cells, but also resting T cells and in macrophages [193]. Acute infection in HIV-1 and SIV models resolves with the onset of antigen-specific immune responses [194]. Evasion tactics including modification of glycosylation patterns in viral envelope protein and mutations in neutralization and CTL determinants [195], are also shared by both viruses. In both HIV and SIV infections, plasma viral load after the acute phase ("viral setpoint") is associated with the rapidity of disease progression [196]. Survival time is in macaques as variable as in humans but shorter with an average of 8-10 years for humans vs. 0.5-3 years for macaques infected with the majority of pathogenic SIV strains. However, SIVmac-infected controllers can survive the infection for more than 7 years, and one macaque surviving even more than 13 years with ongoing viral replication has also been observed (Christiane Stahl-Hennig, personal communication). SIV isolates also utilize the CCR5 coreceptor for viral entry [197][198]. However, in contrast to HIV-infection no switch in the utilization of coreceptors occurs [199]. In conclusion common features between HIV-infected humans and SIV infections in macaques define the unique advantage of using this model for the study of HIV pathogenesis.

Material and Methods

2.1 Material

Table 2.1: General laboratory equipments and consumables.

NAME	Manufacturer
Sterile cell culture work bank	ThermoScientific
Real-time pcr system 7500	Applied Biosystems
LSRII (multi-laser flow cytometer)	BD bioscience
Thermal cycler my cycler	Bio-Rad
Centrifuge 5415 r, 5417 r, 5424, concentrator plus	Eppendorf
Pipettes (1-10 µl, 10-100 µl, 2-200 µl, 100-1000 µl)	Eppendorf
Centrifuge 3 s-r	Heraeus
Shaker multi bio 3d, mini rocker mr-1	Kisker
Microbiological safety cabinet	KOJAIR Ltd
-80°c freezer ultralow u57085	Labotect
Incubator series 5400	NAPCO
Microscope ix70 with ccd device and fluorescence imaging unit	Olympus
Spectrophotometer nanodrop	PEQLAB
M48 automated system	Qiagen
-20°c freezer, 4°c fridge	Liebherr
Centrifuge sorvall discovery 90 with rotor tft 80.4	ThermoScientific
Inverted microscope, axiovert 25	Carl Zeiss GmbH
Nitrogen tank	Chronos Messer
Vortexer	Ernst Schütt jr
Microamp optical 96-well reaction plate	Applied Biosystems
Parafilm	Carl Roth
Reaction tube (1.5 ml, 2 ml)	Eppendorf
Pipettor	Hirschmann

Centrifugation tube (15 ml, 50 ml)	Greiner Bio-One
Agar dishes (100 mm)	Sarstedt
Pipette tips rnase/dnase free (10 µl, 100 µl, 200 µl, 1000 µl),	Sarstedt
Reaction tube (0.2 ml, 0.5 ml, 1.5 ml)	Sarstedt
Dishes (60 mm, 353004; 100 mm, 353003) bd falcon	BD Falcon
Multi well plates (12-well, 353043; 24-well, 353047)	BD Falcon
Freezing device "mr. Frosty"	Nalgene
Neubauer counting chamber	Carl Roth
Water bath	GFL
Cell scraper	Kisker
Cryotubes (1.8 ml)	NUNC (ThermoScientific)
Flasks (250 mm 2 , 750 mm 2 , 1750 mm 2)	Sarstedt
Serological pipette (2 ml, 5 ml, 10 ml, 25 ml)	Sarstedt
Cover slips	ThermoScientific
Leukosep tubes	Greiner
Macs multistand	Miltenyi Biotec
Macs separation columns ms and ls	Miltenyi Biotec
Tissue culture treated paltes	BD falcon
Non tissue culture treated plates	BD falcon
Multichannel micropipette	

Table 2.2: Chemicals and Reagents.

Reagent/ chemical	**Source**
Trypsin, trypsin/EDTA 0.25%	PAA laboratories
Penicillin/streptomycin	PAN Biotech
Fetal calf serum,	PAA laboratories
RPMI 1640 cell culture medium	PAA laboratories
Dimethylsulfoxid (DMSO)	Sigma Aldrich

Nuclease free water	Merk
Ethidium bromide	Applichem
Ethanol	Roth
Formaldehyde (37%)	Roth
Concanavalin-A	SERVA
DPBS	Gibco
Milk powder	Roth
B-mercaptoethanol	Roth
Fetal calf serum	PAN Biotech
Interleukin 2 (IL-2)	Pepro Tech
Rhesus Interferon -α	PBL biomedical laboratories
Rhesus Interferon -γ	PBL biomedical laboratories
Rhesus TNF- α	PBL biomedical laboratories
Isopropanol	Roth
Methanol	Roth
QIAshredder columns	Qiagen
dNTPs (10 mM each)	Fermentas
Loading dye (6x)	Bioline
Agrose	Biozyme
FACS flow, FACS rinse , FACS safe	BD biosciences
Lymphocyte separation medium	PAA laboratories
MACS Bovine-Serum-Albumin (BSA)	Miltenyi Biotech
DMEM	PAA laboratories

Table 2.3: Reaction components and commercial kits

Kit/component	Source
Quantitect probe RT-PCR kit	Qiagen
BigDye Cycle Sequencing Kit	Applied biosystem
Immomix	Bioline
CloneJET1.2 PCR Cloning Kit	Fermentas
Superscript III First strand cDNA synthesis kit	Invitrogen
PAXgene blood RNA kit and tubes	PreAnalytix
Endo Free plasmid mini kit	Qiagen
RNA easy plus mini kit	Qiagen
Endo Free plasmid maxi kit	Qiagen
Phusion PCR Master Mix	Thermo scientific
Gel extraction Kit	Qiagen
Bgl 11 enzyme and orange buffer	Fermentas
TransIT transfection reagent kit	MIRUS
Retronectin	Takara/clontech
10x ROX	Bioline
Anti-mouse anti CD3 antibody	Becton Dickinson
Sybr green	Bioline

Table 2.4: List of Antibodies

Name	Clone	Isotype	Fluorochrome
CD4	MT477	Ms IgG1 k	PE
CD8	SK1	Ms IgG1 k	PE
CD11a	HI111	Ms IgG1 k	PE
CD27	MT271	Ms IgG1 k	PE
CD28	28.2	Ms IgG1 k	PE

CD45	TU116	Ms IgG1 k	PE
CD45RA	2H4	Ms IgG1 k	PE
CD62L	SK11	Ms IgG2a k	PE
CD64	10.1	Ms IgG1 k	PE
CD80	L307.4	Ms IgG1 k	PE
CD86	B70/B72	Ms IgG1 k	PE
CD95	DX2	Ms IgG1 k	PE
CD122	Mikb2	Ms IgG1 k	PE
CD127	hIL-7R-M21	Ms IgG1 k	PE
CD153	RM153	Rat IgG2b	PE
CD154	TRAP1	Ms IgG1 k	PE
CD183	1C6/CXCR3	Ms IgG1 k	PE
CD184	12G5	Ms IgG2a k	PE
CD193	61828.111	Rat IgG2a	PE
CD195	3A9	Ms IgG2a k	PE
CD196	11A9	Ms IgG1 k	PE
CD197	3D12	Rat IgG1a k	A647
TCRab	R73	Ms IgG1 k	PE
HLA-DR	L243	Ms IgG2a k	PE
CD271	C40-1457	Ms IgG1, κ	PE
CD3	SP34	Ms IgG_3, λ	PE
CD271	C40-1457	Ms IgG2a k	APC
CD4	L200	MsIgG1k	Per-CP-cy55A
CD8	Sk1	MsIgG1k	Amcyan-A
CD69	TP1.55.3	MsIgG2bk	ECD
HLA-DR	L243	MsIgG2ak	APC-Cy7

2.2 Methods

2. 2.1 Experimental animals

To identify the CNAR/CAF$^+$ animals (whose CD8$^+$ T cells were having the ability to suppress the viral replication), *in vitro* viral inhibition test was performed with CD8$^+$ T cells from 11 SIV-infected and 9 non-infected rhesus macaques (*Macaca mulatta*) of Indian origin. CD4$^+$ T were isolated from MHC-mismatched non-infected rhesus macaques of Indian origin.

At latter stage of study, samples from two vaccine experiments were also included to monitor the changes in expression pattern of selected genes upon immunization and infection. First experiment (Exp-1) included24 animals, while Second group (Exp-2) included6 control (non-vaccinated) and 12 vaccinated animals.

All animals are housed at the German Primate Centre under standard conditions according to the German animal protection law which complies with the European Union guidelines on the use of non-human primates for biomedical research. Animals were handled by the veterinarians and animal keepers of the Department of Infection Models. All samples were processed at biosafety level II.

2.2.2 Preparation of virus stock

To prepare virus stocks, 15 million CEMx174 cells were pelleted and re-suspended in 1ml SIVmac239 virus stock. Suspension was incubated for 1.5hours at room temperature with intermittent swirling every 15 min. Cells were re-suspended in RPMI 1640 complete medium containing 10% (vol/vol) heat-inactivated (56°C, 30 min) foetal bovine serum (FBS) (PAA Laboratories), 1% streptomycin (PAN biotech). After 24 hours incubation at 37°C (CO_2 5%), residual virus was washed out twice and cells were incubated for next three days. On day 3 cultures were replenished with new complete RPMI and returned to incubator till day 6. Cell culture supernatants at day 6 and day 7 were collected. After filter sterilizing with 0, 45µm filter, samples were frozen at -80°C.

2.2.2.2Virus titration

Virus stock was titrated to determine the exact TCID50. For this purpose, 3×10^4 C8166 cells in 50 µl of RPMI complete medium were plated in each well of a 96 well plate. 100 µl of 10 fold serially diluted virus stock was added in 8 replicates. Negative controls contained

medium only. After 4 days of incubation, cell culture medium was replaced by fresh RPMI complete medium. At day 7, virus titer was determined by indirect immunoperoxidase assay.

2.2.2.3 Indirect immunoperoxidase assay

A 96 well plate was equilibrated with 200 µl of RPMI complete media overnight (4°C) or 1 hour (room temperature RT). The plates were coated with 50 µl/well of Concanavalin-A (ConA) at a concentration of 0.5mg/ml in phosphate buffer saline (PBS) and incubated for 1 hour at RT. Plates were either used immediately or frozen at -20°C till needed. Using a multi-channel micropipette, cells from the virus titration assay (2.3) were re-suspended and transferred to respective ConA coated plates. After incubating at 37°Cfor 2 hours, plates were than decanted and cells were immersed in pre-chilled (-20) methanol for 30min or overnight at -20. Plates were carefully washed thrice with cold PBS and blocked with 100 µl/well of 2% milk powder (MP) at RT for 30-60 min. MP was discarded and plates were incubated at 37°C for another 30-60 min with 50µl/well of a 1:3000 diluted (in MP) polyclonal SIV anti-serum from a chronically infected macaque (SIV.S 1604, DPZ). Another washing with PBS was followed by similar incubation with 50 µl/well of 1:1000 diluted anti-human Ig-HRPO conjugate. Plates were washed with PBS and incubated with 50 µl substrate solution (2mg AEC, 300 µl DMF, 25 µl of 30% H_2O_2 and 5ml sodium acetate) for 20-30 min. The reaction was stopped by twice washing in PBS and plates were examined immediately for stained cells under an inverted light microscope. The TCDI50 was scored using the Reed and Muench method as described elsewhere (Fridholm&Everitt, 2005; LaBarre& Lowy, 2001)

2.2.3 Preparation of peripheral blood mononuclear cells (PBMCs)

Peripheral blood mononuclear cells (PBMCs) were isolated from the blood by Ficoll density gradient centrifugation. Ficoll (Lymphocyte separation medium, PAA Laboratories) laid blood was centrifuged at 1045xg for 25 min in 50 ml Leukosep-Tubes (Greiner). The buffy layer containing lymphocytes was collected and washed twice with 10ml PBS by centrifugation for 10min at 300xg. Cell were counted during second wash step using trypan blue exclusion method allowing counting of only live cells on haemocytometer (Neuberger, Germany)

2.2.4 Enrichment of specific cell types

Specific cell types ($CD4^+$ T cells, $CD8^+$ T cells, B cells, $NGFR^+$ T cells) were isolated by magnetically labelled antibodies cell separation technique (MACS) (Miltenyi Biotech). Lymphocytes were suspended in MACS buffer (1:20 dilution MACS BSA Stock Solution with auto MACS Rinsing Solution Miltenyi Biotec) and incubated with respective antibodies. (CD8-PE 10 µl, NGFR-PE 40 µl, CD4-beads 20 µl, CD20-beads 20 µl) for 10-20 min as specified in the respective protocol. The CD4 and CD20 (for B-cells) antibodies were directly conjugated to magnetic beads (CD4 and B cells) whereas the CD8 and anti-NGFR were coupled to Phycoerythrin. Cells labelled with PE-coupled antibodies needed additional 15 min incubation step with 20 µl of anti-PE magnetic beads. Applying the labelled cell suspension on MS columns (Miltenyi Biotec) allowed the unbound cells to pass through and retained the positive cells on column that were eluted in the subsequent wash step with MACS buffer.

2.2.5 *Invitro* viral inhibition assay

Viral inhibition assays were performed to estimate the inhibitory capacity of $CD8^+$ T cells on *in vitro* replication of SIV. In principal, $CD4^+$ T cells from SIV-uninfected, MHC-mismatched donor animals and $CD8^+$ T cells from experimental animals were isolated by MACS as described (section 2.4).Purity of isolated cells was checked with FACS (section 2.12). These cells were then activated with Concanavalin-A (ConA) (10ng/ml) for 24 hours at 2×10^6 cells/ml of RPMI complete medium. Cells were washed and $CD4^+$ T were infected with SIVmac239 at a multiplicity of infection (MOI) 0,001 $TCID_{50}$for 2 hours with intermittent swirling every 15 min. After washing twice with RPMI complete medium, cells were re-suspended at a concentration of 2×10^6 cells/ml in the RPMI 1640 complete medium supplemented with 100 U/ml recombinant human IL-2 (Pepro Tech). *In vitro* infected $CD4^+$ T cells were either cultured alone (controls) or co-cultured with ConA activated $CD8^+$ cells from experimental animals in duplicate at a 2:1 ($CD8^+$T cell: $CD4^+$T cell) cell input ratio.5×10^5 $CD4^+$ T cells and 1×10^6 $CD8^+$ T cells were co-cultured in each well of a 24 well plate. Cells were incubated in sterile conditions at 37°C in a 5% CO_2 humidified chamber. Old culture medium was replaced with fresh RPMI complete medium at day 3. Supernatants were collected at day 5 and 7 for viral RNA isolation. $CD8^+$ T cells were re-isolated from the co-cultures at D7 and were subjected to Microarrays analysis to find the genes differentially expressed in the $CD8^+$T cell of $CNAR^+$ and $CNAR^-$ animals.

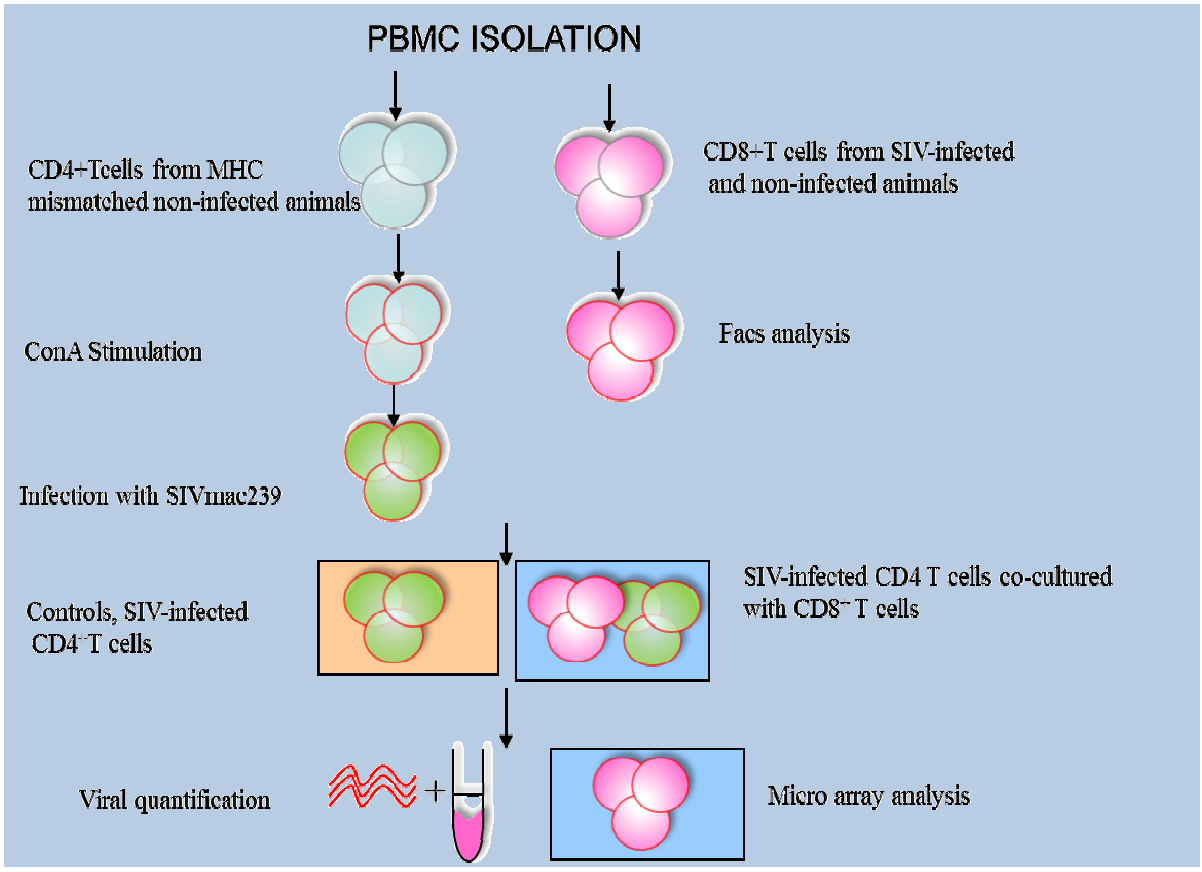


Fig 2.1: A schematic representation of *in vitro* viral inhibition /CAF test.

2.2.6 Viral RNA extraction and quantification

Viral RNA from 200 µl of culture supernatant at day 5 and day 7 post infection were isolated by using the BioRobot® M48 workstation and theMagAttract Virus Mini M48 protocol (Qiagen, Hilden, Germany). This automated RNA isolation procedure involves initial lysis in protease and Al buffer. After lysis, viral RNA is bound to MagAttract suspension beads. Successive steps include automated washing with buffer AW1, AW2 and ethanol followed by elution of viral RNA in 75 µl of buffer AVE. Viral RNA was quantified using TaqMan probe-based one-step RT qPCR on an ABI-Prism 7500 sequence detection system (Applied Biosystems) and in the presence of standard RNA.

Quantitect probe RT-PCR Kit (Qiagen) was used for this purpose. Use of 2x QuantiTect Probe RT-PCR Master Mix (HotStarTaq DNA Polymerase, QuantiTect Probe RT-PCR Buffer, and ROX passive reference dye) and QuantiTect RT Mix (Omniscript and Sensiscript RT blend), together with SIV primers SIV gag forward: 5'-ACCCAGTACAACAAATAGGTGGTAACT-3'. SIV gag reverse: 5'-

TCAATTTTACCCAGGCATTTAATGT-3' and probe (FAM 5'-TGTCCACCTGCCATTAAGCCCGAG-3'-TAMRA) (M33262) allowed both reverse transcription of RNA (100ng) and PCR to take place in a single tube. Quantity of each component is given in Table 2.5.

Amplification conditions were as follows: reverse transcription: 50°C (30 min); activation: 95°C (15 min); amplification: 45 cycles of denaturation at 94°C (15s); extension/annealing 60°C (60s). Amplified viral RNA was expressed as SIV-RNA copies /ml supernatant.

Component	Volume	Final Concentration
TaqMan Probe	0,375 µl	0,2 µM
SIV gag forward primer	0,625 µl	0,4 µM
SIV gag reverse primer	0,625 µl	0,4 µM
RNasefree water	5,625 µl	-
2x QuantiTect RT-PCR master mix	12,5 µl	1x
QuantiTect RT-mix	0,25 µl	-
RNA	5 µl	
Total	25 µl	

Table 2.5: Reaction components and their respective concentration for cDNA synthesis.

2.2.7 Cellular RNA extraction

Total $CD8^+$ T cellular RNA was isolated with RNeasyPlus Mini Kit according to the manufacture´s instruction (Qiagen). Briefly, proteins were denatured by suspending the cells in RLT buffer (guanidine isothiocyanate with 1% β-mercaptoethanol) and centrifugation (Table-top centrifuge 5417 R, Eppendorf) in a QIAshredder column (Qiagen). Genomic DNA (gDNA) was removed by passing the homogenized lysate through gDNA eliminator spin column, allowing only unbound cellular RNA to pass through. After precipitation with 70% ethanol, RNA was bound to membrane of RNeasy spin columns and subsequently washed with RW1 and RPE Buffers. Finally RNA was eluted with 20 µl of RNase-free water. Quantity of isolated cellular RNA was determined by spectrophotometer (Nano Drop,PeqLab) using RNAse free water as reference. Quality of RNA was checked by Agilent 2100

Bioanalyzer (Agilent Technologies). Average RIN values (RNA Integrity Number) for most samples was greater than 8.5. RNA was either stored at -80°C or immediately used for cDNA synthesis.

2.2.8 RNA isolation from blood

From whole blood collected in a PAXgene Blood RNA Tube (PreAnalytix), total cellular RNA was manually isolated by PAX gene Blood RNA Kit according to the instruction of the manufacturer (Qiagen). PAXgene Blood RNA Tubes contain a special RNA stabilization reagent that protects RNA molecules from degradation by RNases thus minimizing the *ex vivo* gene expression changes. Previously frozen blood was first incubated at room temperature for 2 hours in order to achieve complete lysis. To begin with purification, blood was centrifuged at 3000×g for 10 min to pellet nucleic acids. After washing with RNase free water, the pellet was incubated in optimized buffers together with proteinase K (55°C, 10 min) for protein digestion. Centrifugation at maximum speed (3 min) through the PAXgene Shredder spin column allowed homogenization of the cell lysate and removal of residual cell debris. 100% ethanol was added to supernatant of the flow-through and applied to a PAXgene RNA spin column. During 1 min centrifugation at 17000 rcf, RNA was selectively bound to the PAXgene silica membrane and contaminants pass through. Remaining contaminants were removed in several wash steps. Between the first and second wash steps, the membrane is treated with DNase I (at room temperature for 15 min) to remove trace amounts of bound genomic DNA. After the final wash steps, RNA was eluted in 40 µl elution buffer and heat-denatured (65°C, 5 min).

2.2.9 Primer design and optimization

The cDNA sequences used for the primer design were taken from Genbank of National Centre of Bio-Informatics (NCBI) and EMBL (European Molecular Biology Laboratory). Primers for target genes were designed by NCBI primer3online software (http://www.ncbi.nlm.nih.gov). Special attention and precautions were taken to avoid secondary structures or dimers. The lengths of the amplicons were kept between 80bp to 150bp.Specificity was determined by agarose gel electrophoresis of PCR fragments and by checking the dissociation curve. Efficiency was tested by amplifying serial dilutions of cDNA with various primer concentrations.

Only reaction conditions with efficiency of amplifying the target sequence more than 95% were used for further experiments. App Table 1 summarize the primer sequences used for amplification of selected genes along with amplicon lengths. The sequences for the GAPDH and MxA primers were already described by [200] and [201]respectively.

2.2.10 cDNA synthesis

For initial experiments (candidate gene expression in $CD8^+$ T cells) 500ng RNA was reverse transcribed using SuperScript III First-Strand Synthesis System (Invitrogen) according to the manufacturer´s protocol. For initial denaturation each 10 µl reaction mixture containing 500 ng RNA, 50 ngOligodT primers and 10 mMdNTP was incubated at 65°C for 1 min. 10 µl master mix containing, 10 x RT buffer, 25 mM $MgCl_2$, 0.1 M DTT, 40 U RNase Out and 200 U of SuperScript III was added to each reaction mix. No-reverse transcriptase reaction (NRT) that lacked the reverse transcriptase was included to assure there is no gDNA contamination. The reaction condition for cDNA synthesis were as follows

Hybridization: 10 min 25°C

Elongation : 50 min 50°C

Stopping : 5 min 85°C

cDNA was directly stored at -20°C and was used later on for RT-PCR.

RNA isolated from whole blood was reverse transcribed by QuantiTect Reverse Transcription Kit (Qiagen). Reaction comprises two main steps: elimination of genomic DNA and reverse transcription. In the first step gDNA was removed by incubating 100ng RNA with gDNA wipeout buffer for 2 min at 42°C. In the second step a master mix prepared from Quantiscript Reverse Transcriptase (1 µl), Quantiscript 5x RT Buffer5x (4 µl) and RT Primer Mix (1 µl) was added to the reaction mixture. Reaction was started at 42°C for 15 min and then was then terminated at 95°C for 5 min. cDNA samples were stored at -20°C for subsequent use in RT-PCR.

2.2.11 Real Time PCR quantification of cellular genes

Expression of cellular genes was quantified by Real Time PCR in an ABI Prism 7500 cycler. Reactions were performed in 96 well MicroAmp Optical Reaction Plates (ABI). Each 25 µl reaction mixture contained 12.5 µl Immomix (Bioline), 6.6 µl 10xRox (Bioline), 0.5 µl Sybr green (Bioline), 2 µM of each primer, and 2 µl of 1:5 diluted cDNA products. The reactions were run as one cycle at 95°C (7 min) followed by 40 cycles at 95°C (15 s) and 60°C (33 s).

Additional melting temperature detection step was included to ensure the amplification of right product. No-reverse transcriptase (NRT) and Non-template controls (NTC) were run simultaneously. RNA levels of the housekeeping gene Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were determined as reference for calculating the relative expression levels of the target genes. All samples were run at least in duplicates. The results were analysed by Sequence Detection Software (ABI). The relative expression level was calculated as the difference between the mean Ct values (calculated from the duplicates) of each target and reference gene GAPDH (Mean Δ Ct = Ct (target gene) - mean Ct (GAPDH).

2.2.12 Flow cytometry

For qualitative as well as quantitative analysis of desired cell surface molecules, flowcytometry analysis was performed using LSR II Flow cytometer (BD). Approximately 5 × 10^5 cells were washed with FACS-PBS (PBS supplemented with 0.1% BSA, 0.03% sodiumazide, pH 7.2) and fluorescently labelled antibodies for 30 min at 4°C. Cells were than washed with FACS-PBS and fixed in 1.5 ml of formaldehyde (7% in PBS) in dark at room temperature. After washing, the cell pellet was suspended in 50 µl of PBS and stored at 4°C in the dark. Samples were measured by BD FACSDiva software (BD Biosciences) and later data was analysed with FlowJo version 6.4.7 differentiating positive, double positive and negative cells. Antibodies used are given in table 2.4.

2.2.13. Cloning and sequence analysis of FAM26F

2.2.13.1 PCR-amplification of FAM26F

FAM26F gene from previously characterized CAF^+ and CAF^- group of animals was cloned and sequenced. For this purpose *FAM26F*cDNA was amplified using Phusion High-Fidelity PCR Master Mix (Thermo Scientific). The components and their respective concentrations used per reaction are given in table 2.6 Primers (forward 5'-GGACGAGGCTCATGGAGAAG-3'; reverse, 5'-AAGGTCATAACCCAGGAGTGC-3) used in this reaction were designed as described (section2.9). The primers amplify the cDNA sequence of *FAM26F* from position 155 to 1117 (963bp product) encompassing the whole protein coding region. PCR conditions were as follows: initial denaturation at 98°C for 3min, 30 cycles of denaturation 98°C (30s); extension/annealing 58°C (30s) with incrementsof4sat each cycle and 72°C (10min). 5 µl of PCR product was run on 1% agarose gel to confirm the expected 971bp *FAM26F* product.

Components	Volume
Water	32,5 µl
5x Phusion HF Puffer	10 µl
Forward Primer 10 µM	2,5 µl
Reverse Primer 10 µM	2,5 µl
dNTP 10 mM	1 µl
Phusion DNA Polymerase (2 U/ µl)	0,5 µl
cDNA	1 µl

Table 2.6: Reaction components and their concentrations for PCR.

2.13.2 Extraction and purification of DNA from agarose gel

DNA band from the agarose gel was excised with a clean, sharp scalpel and weighed. DNA was extracted using QIAEXII Extraction kit (Qiagen) according to the instructions of the supplier. Addition of Buffer QX1 in 1:3 (by weight) solubilised the gel by disrupting the hydrogen bonds between sugars. Mixture was incubated with QIAEX II at 50°C for 10 min and vortexed after every 2 min. This allowed the DNA to quantitatively adsorb on the QIAEX II silica-gel particles. After centrifugation for 30s, supernatant was removed and the pellet was washed twice with 500 µl of Buffer QX1. Pellet was air dried. DNA was eluted by adding 20 µl of 10 mMTris-Cl (pH 8.5) to the pellet and centrifuging at 17000 rcf for 30 s. The supernatant containing purified DNA was carefully pipetted into a clean tube. Product was later confirmed by running 1 µl of the extracted sample on 1% agarose gel and quantified using NanoDrop (PeqLab) spectrophotometer.

2.13.3 Cloning

CloneJET™ PCR Cloning Kit (Fermentas, now Thermo Scientific) was used for cloning of bluntend DNA fragments. Briefly stating, 10 µl of 2X Reaction Buffer, 0.05 pmol of pJET1.2/blunt Cloning Vector, 1 µl of T4 DNA Ligase and 0.15 pmol of purified DNA fragment were mixed and scaled up to 20 µl by adding nuclease free water. Mixture was incubated for 30min at room temperature.

This ligation mixture was then used to transform TOP10 E.coli strain by using heat schok transformation method. Ligation mixture was added to the competent cells.Cells were given a heat shock by placing first at 42°C for 1min and then immediately transferring them to ice. After adding 1ml LB media, transformed cells were agitated at 200 rpm for 1hour at 37°C and then centrifuged for 1min at 300xg. 900 µl of the supernatant was discarded and 150 µl of cells were streaked on pre-warmed ampicillin selective agar plates. Plates were incubated overnight at 37°C and subsequently stored at 4°C.

2.13.4 Purification of plasmid

For sequence analysis *FAM26F* small scale plasmid production was done. Positive clones from agar plates were picked and a starter culture of 2–5 ml LB medium containing ampicillin (100µg/ml) was inoculated. These bacterial cultures were incubated for approximately 8 hours at 37°C with vigorous shaking. For cell culture applications, large scale endotoxin free plasmid preparation was required. For this purpose, a starter culture of 5ml LB-ampicillin medium was set up (as for small scale production) and this culture was used further to inoculate 100ml of LB-ampicillin medium. Cultures were incubated for approximately 14-16 hours at 37°C with vigorous shaking. Cells were harvested by centrifugation at 6000 x g for 15 min at 4°C.

Plasmids were purified from bacterial cells using plasmid isolation Mini (for small scale production) or Maxi (large scale production) kits (Qiagen). Briefly, bacterial cell pellet was first re-suspended in Buffer P1 and then incubated with P2 at room temperature for 5 min to precipitate genomic DNA and cell debris. Chilled buffer P3 was added and after vigorously inverting the tubes 4–6 time, mixture was incubated on ice for 5 min. After centrifugation at maximum speed in which centrifuge or g numbers for 10 min supernatant containing plasmid DNA was promptly applied to pre-equilibrated (buffer QBT) QIAGEN-tip 20. After washing the column twice with buffer QC, plasmid DNA was eluted with buffer QF. Isopropanol precipitation and centrifugation was followed by washing with 70% ethanol. Resulting pellet was air dried, dissolved in a TE buffer (pH 8.0) and concentration was determined by NanoDrop (PeqLab) spectrophotometer.

2.13.5 BglII restriction digestion

BglII restriction digestion was performed to identify the positive colonies carrying *FAM26F*cDNA fragment. For this purpose 1µg of plasmid (propagated and isolated from

selected colonies) was incubated overnight at 37°C along with 1U of Bgl II enzyme (Fermentas) and orange buffer (Fermentas). Product was run on 1% agarose gel and positive clones were further subjected to sequencing.

2.13.6 DNA sequence analysis

Sequencing was performed using Big Dye Terminator sequencing kit (ABI). Beside vector specific Forward 5'- CGACTCACTATAGGGAGAGCGGC -3' or reverse primer 5'-AAGAACATCGATTTTCCATGGCAG -3' (provided with CloneJET™ PCR Cloning Kit), two internal primers were also used for complete sequencing. Internal Forward 5'-CTATTTGGAACAGGAGCAGC-3' Internal reverse 5'-CAGTTG TGGTCGCGGTCGAG-3'. 20 µl sequencing reaction contained 3.3 pmol Primer, 200 – 300 ng plasmid, 1.5 µl 5x sequencing buffer and 1 µl Big Dye. Sequencing PCR amplification conditions were as follows 25 cycles of 96°C (30 sec), 50°C (15 sec), 60°C (4 min). Ethanol precipitation of PCR product was done by adding 250 µl 100 % ethanol and 10 µl 3M sodiam acetate and centrifugation at 13.000 rpm (15 min). Product was afterward washed with 250 µl of 70% ethanol, air dried in a SpeedVac andsequenced by ABI PRISM 3130xL Genetic Analyzer (Applied Biosystems).

2.14 *In Vitro* stimulation of PBMC with interferons

In vitro stimulation experiments were performed to study the differential expression pattern of desired genes upon activation by Interferon alpha (INF-α), interferon gamma (INF-γ) and Tumour necrosis factor alpha (TNF-α). For this purpose PBMCs were isolated from blood (section 2.3) and activated by concanavalin-A (10 ng/ml) for 8 hours. After washing twice, cells were re-suspended in complete RPMI media supplemented with 100 U/ml recombinant human IL-2 (Pepro Tech). Cells were plated in duplicate at a density of 5×10^6 cells/2 ml/well and INF- α(10 ng/ml), or INF-γ(100ng/ml)or TNF-α(10, 50 or 100ng/ml) were added in respective experiments. In parallel, unstimulated PBMCs were used as controls. Cells were harvested by centrifugation at different time points and frozen in RNA later (Qiagen).

2.15 T Cell line development

The ability of human telomerase reverse transcriptase (hTERT) to stabilize telomere length and extend cellular proliferative capacity is well documented. Infecting cultures of antigen

stimulated PBMCs with MuLV-based vectors, carrying hTERT gene results in selective immortalisation of primary T cells. To produce the viral vectors, GP2-293 cells were transfected with constructs carrying viral envelope (RD114) and human telomerase gene (hTERT) genes. Inclusion of a surface marker gene ΔLNGFR (C-terminally truncated human low-affinity nerve growth factor receptor) allowed the efficient identification and purification of hTERT transduced cells. Fig 2.2 represents different steps of immortalisation procedure.

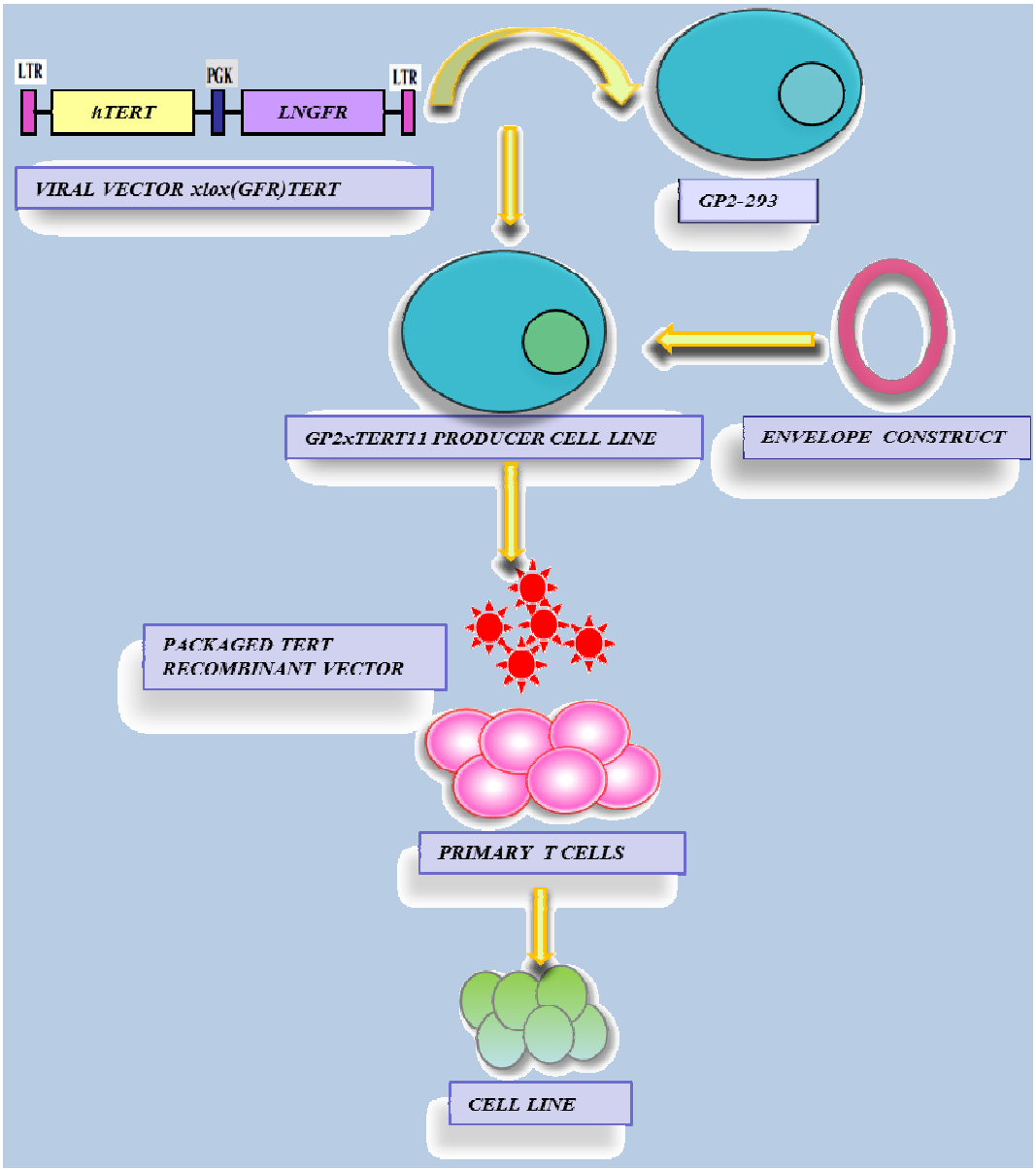


Fig 2.2: Schematic representation of the steps leading to conversion of primary cells into cell line

2.15.1 Culturing of Packaging Cell Line GP2XTERT11

The cell line GP2xTERT11 (kindly provided by Eugene Barsov) were maintained in DMEM complete growth medium (DMEM supplemented with 10% of FBS, penicillin, and streptomycin) and incubated at 37 °C (5% CO2). Cells were split every 3–4 days as per routine cell culture practices. For maximal transfection, cells that have been either longer than 1 month or less than 1 week in culture were avoided. Reselection of packaging function was done at intervals by growing the cells for 5 days in the growth medium supplemented with aminopterin (0.1 μM), then in HAT medium (DMEM + 10% FBS + HAT Supplement) for next 5 days and for last 5 days in the HT medium (DMEM + 10% FBS + HT Supplement). After that, the cells were maintained in the regular growth medium.

2.15.2 Viral Vector production

To produce the viral vector, one day before transfection (D0) 6 million GP2xTERT11 cells were plated on 10cm tissue-culture treated polystyrene plates (BD Falcon). On the day of transfection (D1), cells were transfected with a plasmid construct carrying Feline endogenous retrovirus RD114 envelope gene (kindly provided by Eugene Barsov [202]. 25 µl of TransIt-293 transfection reagent (Mirus Bio Corporation) was first incubated with 300 µl RPMI 1640 (FCS and antibiotic free) medium and incubated for 20–30 min at room temperature. Mixture was then incubated with 10 µg of RD114 envelope carrying plasmid for 20 min and added to the cells. Plated were rocked gently and overnight incubated at 37°C (5% CO2). 24hours post transfection (D2), old growth medium was replaced with new DMEM complete medium and cells were incubated for another 24 hours. 48hours post transfection (D3) the cell culture supernatant was harvested and the equal volume of fresh DMEM complete medium was added for a second harvest. Supernatants containing the retroviral vectors were centrifuged at 3,000–4,000 × g (4°C) to remove residual cells debris and aliquoted to store at -80.

2.15.3 Activation of T Cells

$CD4^+$ or $CD8^+$ cells were isolated from 30 ml blood as described in section 2.3. 50µgof anti-mouse anti CD3 antibody (Becton Dickinson) was dissolved in 1ml PBS. Each well of a 12-well tissue culture plate was coated with 500 µl of this solution. Plates were incubated at 37°C for 3 hours and afterwards washed once with PBS. T Cells were re-suspended in complete RPMI medium (100U IL-2) at density of 2×10^5cells/ml and plated on antibody coated plates. Cells were cultured at 37°Cin a 5% CO_2 humidified chamber for 48 hours.

2.15.4 Transduction

12-well non-tissue culture treated plates were coated with 25µg of Retronectin (TaKaRa/Clontech) solution in PBS. After blocking the non-specific binding by 2% bovine serum albumin (BSA in PBS) for 30 min, wells were washed twice with PBS. Retroviral vector stocks (2-5ml/well) were then added to the wells and plates were centrifuged (1950×g) at 32°C for 2 hours. 3-4 million activated T cells in the complete RPMI medium (supplemented with 100U IL-2) were added to the wells. In one well, 2×10^6 C8166 cells (in complete RPMI medium) were added as a positive control of transduction. After centrifugation of cells at (215 × g) at 32°C for 30 min, plates were incubated at 37°C (5% CO_2). Fresh RPMI complete media was added after 24 hours and plates were incubated for another 24 hours.48 hours post-transduction, cells were stained with APC labelled anti-NGFR antibody (Miltenyi) and analysed by FACS (section 2.12) for the presence of $NGFR^+$ cells. Non-transduced cells were used as negative controls for FACS analysis. On the basis of % positive cells, the viral titers were calculated as follows:

Number of viral vector particles = Number of cells initially plated × % of $NGFR^+$ cells.

2.15.5 Preparation of feeder cells

For preparation of feeder cells, PBMCs were isolated from buffy coats kindly provided by Transfusion Centre University Clinic Göttingen, UMG. PBMCs were isolated by ficoll density gradient centrifugation as described in section 2.3. Cells were then resuspended in 15ml of the complete RPMI media and irradiated with 60 G using γ-irradiator situated at the Department of Radiotherapy and Radiation Oncology UMG. After irradiation, cells were washed twice with complete RPMI medium and cryo preserved in freezing media containing 20% FCS and 10% DMSO till further use.

2.15.6 Post transduction cell culturing and sorting

Once the transduction of xlox (NGFR) TERT in T cells was verified by FACS analysis, the transduced $NGFR^+$ cells were purified by magnetically labelled antibodies cell separation technique (MACS) (section 2.4) using anti-NGFR (CD271) antibody (Becton Dickinson). 40 µl of antibody was used to label 1×10^7 cells and LS columns (Miltenyi) were used for sorting of positive cells. 0.5-1 million $NGFR^+$ sorted cells were cultured in T25 tissue culture flasks with 4-5 million feeder cells in RPMI medium (20% FCS) supplemented with 30–50 ng/ml anti-CD3 antibody. Flasks were initially placed in incubator in upright position for 48 hours.

Cells were replenished with fresh RPMI complete medium and flasks were placed in bottom position. Cells were counted and split every second day to keep the cell density at 2×10^6 cells/ml. After 1 week feeders were died leaving the cell line in culture. Cells were re-analysed for NGFR expression and resorted if necessary. Cell lines were propagated further for at least 3 months in culture and sufficient aliquots were stored at different passages. .

Statistical Analysis

All data analyses and respective graphs were drawn by graph pad prism 5. In all cases data with $p<0.05$ was taken as significant.

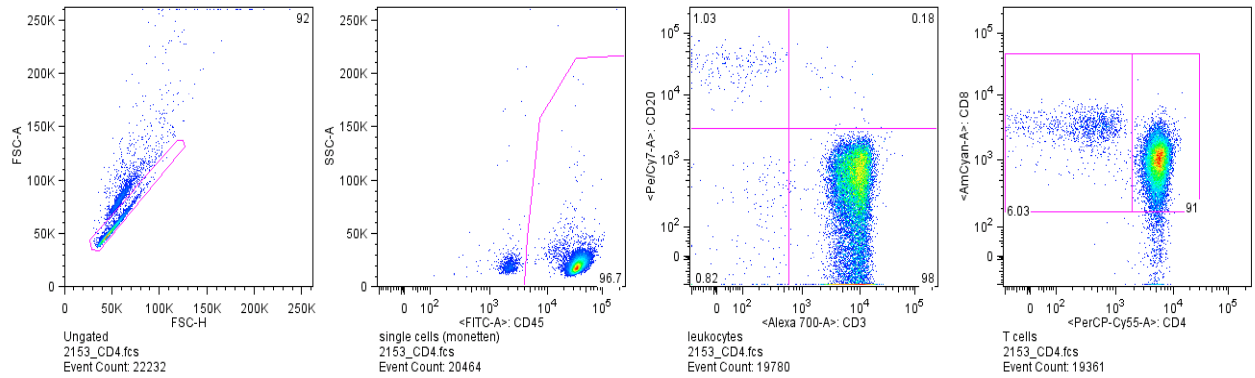
Results

Identification of the genes and factors potentially involved in $CD8^+$ T cell mediated non-cytolytic antiviral response (CNAR) is still unsolved. To this end, the aim of our study was to focus on the discovery of novel genes and on the elucidation of their biological functions contributing to CNAR activity by using SIV-infected macaques as animal model.

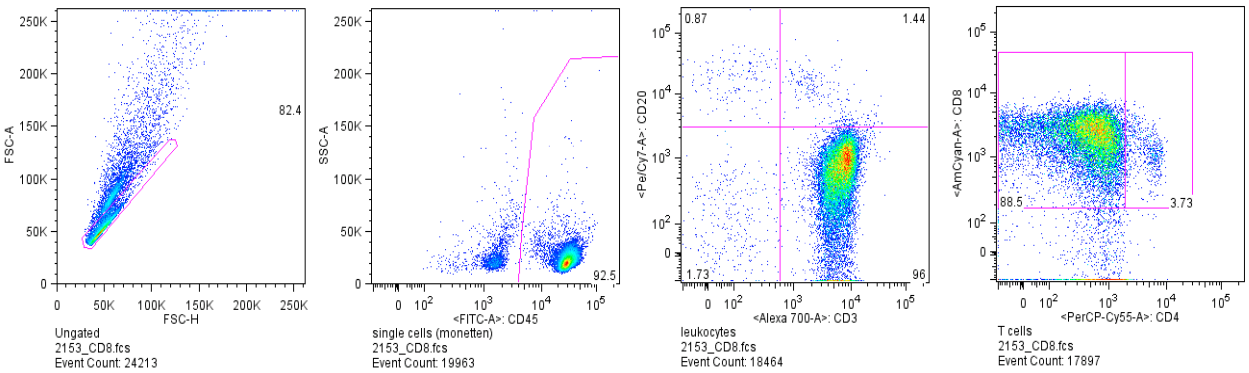
3.1 Characterization of $CNAR^+$ and $CNAR^-$ animals

11 SIV-infected and 10 non-infected rhesus macaques (*Macaca mulatta*) of Indian origin were selected for the study. In order to identify the $CNAR^+$ and $CNAR^-$animals we performed *in vitro* viral inhibition test at initiation of the study. All SIV-infected animals were long term non progressors (LTNP) who were positive for the presence of virus for more than three years but were showing no symptoms of AIDS. We expected that most of these LTNP will appear to be $CNAR^+$ as it is well known CNAR activity may contribute to long term non progression. Another advantage of using LTNP was the fact that LTNP in terms of gene expression pattern are similar to non-infected individual as compared to progressors thus making the study more accurate.

In vitro viral inhibition assay was performed with the $CD8^+$ T cells isolated from both SIV infected and non-infected animals. $CD4^+$ and $CD8^+$ T cells were isolated by MACS (section 2.4) and their purity was checked by FACS analysis prior to performing the viral inhibition test.For qualitative as well as quantitative analysis of desired cell surface molecules, flowcytometry analysis was performed. A flow cytometer works on the principle of hydrodynamic focusing and exposure of the cells to one or more laser beams. Scattered light is measured by a number of detectors mainly the Forward Scatter (FSC, in line with the light beam), Side Scatter (SSC, perpendicular light beam) and several fluorescent detectors. LSR II Flow cytometer (BD) was used to check the purity of MACS separated $CD4^+$ and $CD8^+$ T cells.More than 90% of the single cells were positive for the respective $CD4^+$ or $CD8^+$ population. Fig 3.1 represents one of the representative experiments.



(A)



(B)

Fig 3.1: Flow cytometric dot plot to determine the purity of a MACS-separated cell population A) degree of purity of $CD8^+$ T cells; B) $CD4^+$ T cells from PBMCs.

$CD4^+$ T cells, isolated from the uninfected and largely MHC unmatched donor animals were activated with ConA to exclude effects due to classical cytolytic activity. After 24 hours cells were infected with SIVmac239. They were either cultured alone as controls (control wells) or with ConA activated $CD8^+$ T cells from experimental animals (experimental wells). Supernatants were collected at day 5 and 7 for viral RNA quantification. All $CD8^+$ T cells were tested with $CD4^+$ T cells from a reference monkey (2163) to obtain some level of standardisation since the susceptibility to the CNAR activity might differ between monkeys. At day 5 and 7, viral RNA was isolated from the culture supernatants and quantified by RT qPCR. The extent of viral replication inhibition was calculated as the ratio of the viral copies/ml in culture supernatants from control wells to the viral copies/ml from the experimental wells. $CD8^+$ T cells from each animal were tested with $CD4^+$ cells from at least 3 different donor animals. If the $CD8^+$ T cells inhibited SIV-replication more than 50fold in at

least three independent assays, the animal was categorised as $CNAR^+$ and vice versa (Table 3.1).

Table 3.1: Animal Grouping on the Basis Of Viral Inhibition Test*

SIV-infected (LTNP) N=11		**Non infected N=10**	
$CNAR^+$	**$CNAR^-$**	**$CNAR^+$**	**$CNAR^-$**
N=6	**N=5**	**N=4**	**N=6**
2151*	12535	2247	2163
2155*	12543	2338*	2272
2153*	9794	2328*	2324*
2139*	2219*	2290*	2251*
2172*	12672		2249
12671			2278

* Monkeys marked with an asterisk are related (sibs or half-sibs)

$CNAR^+$ animals were able to supress the viral replication between 50 to 3000fold in at least three independent experiments as compared to $CNAR^-$ monkeys where suppression was less than 50fold (Fig 3.2). Most of the animals that were found to be $CNAR^+$ were descendants of same parental lineage (siblings) and were therefore over represented among the LTNPs. Thus it appears as if the CNAR activity is partially an inherited trait. Furthermore it is observed that $CNAR^+$ animals were able to suppress the viral replication in vivo and had very low viral load as compared to $CNAR^-$ animals Fig 3.3. Two animals (12672, 12536) were found to be $CNAR^+$ at the start of study but later on (after a year) became $CNAR^-$. This decrease in their CNAR activity preceded an increase in viral load. Therefore it is difficult to say if the emergence of virus is solely due to loss in CNAR activity or vice versa. Other animals (listed as $CNAR^+$) were quite stable towards this activity even over a period of 2 years.

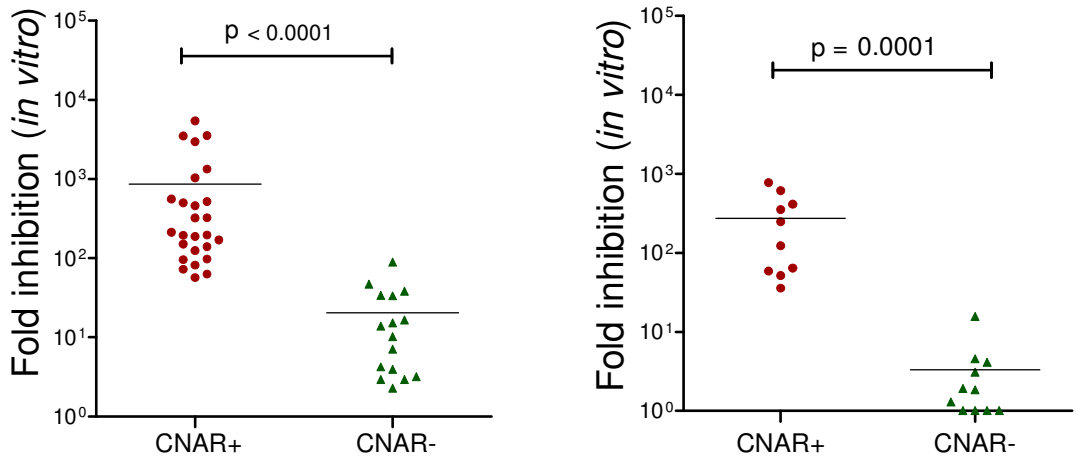


Fig 3.2: Fold suppression of SIV replication by $CD8^+$ cells from $CNAR^+$ and $CNAR^-$ animals from SIV-infected and non-infected monkeys. Each dot represents an independent viral inhibition test.

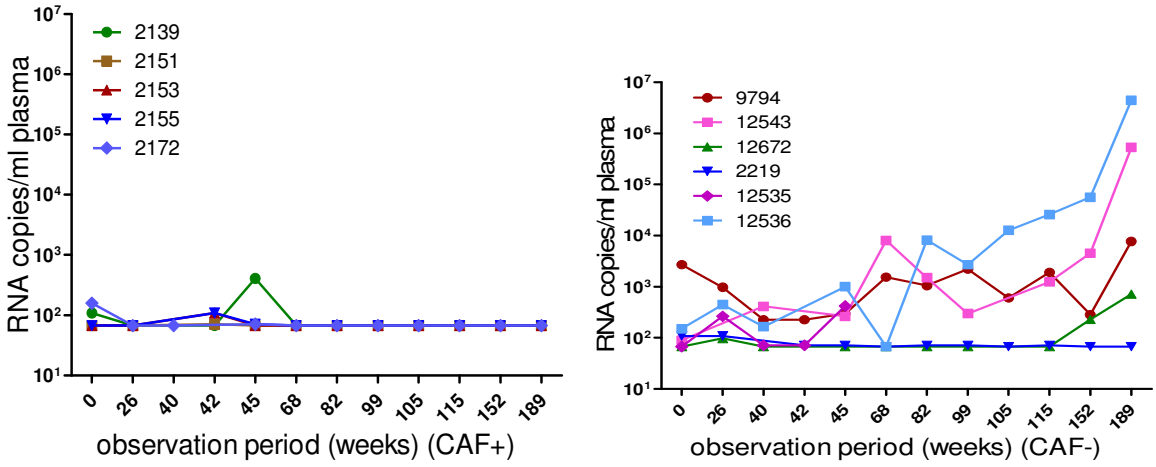
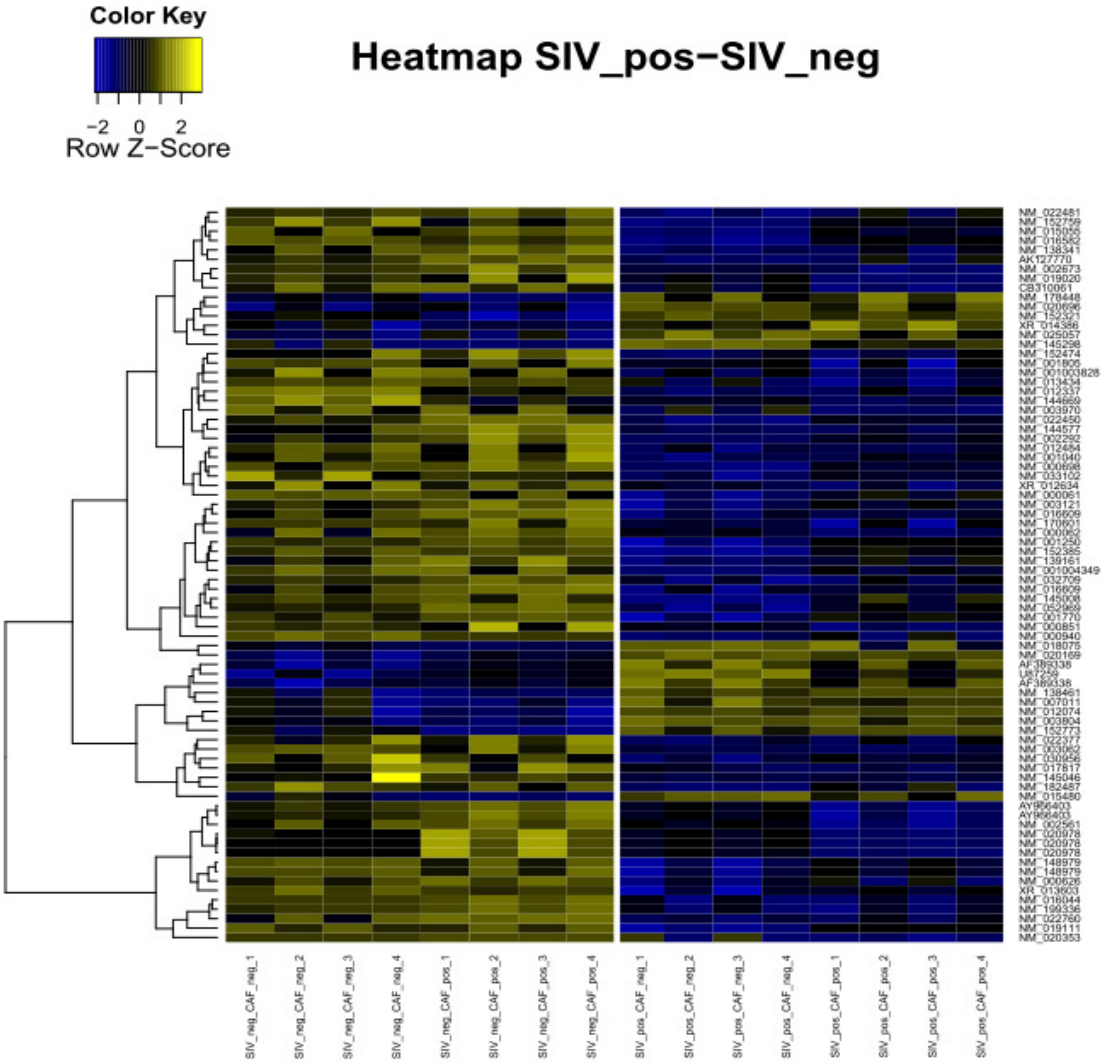


Fig 3.3: Viral load of $CNAR^+$ and $CNAR^-$ long term non progressors over a period of 189wpi.

3.2 Microarray expression analysis

Microarray analysis was performed previously with the $CD8^+$ T cells that were re isolated at D7 of viral inhibition test. This allowed us to directly identify the genes that were differentially expressed in $CD8^+$ T cells of $CNAR^+$ animals as compared to $CNAR^-$ animals. Global gene expression analysis were applied (DNA Microarray and Deep-Sequencing Facility, Göttingen) using the new Macaque 4x44K Design Array (AMADID 015421) from

Agilent Technologies, which was generated in collaboration with the University of Washington (Katze laboratory). The conceptual design of the study not only enabled us to compare CNAR-producing ($CNAR^{+}$) from CNAR-non-producing ($CNAR^{-}$) animals but also allowed us to compare SIV-infected from uninfected animals (Fig 3.4).



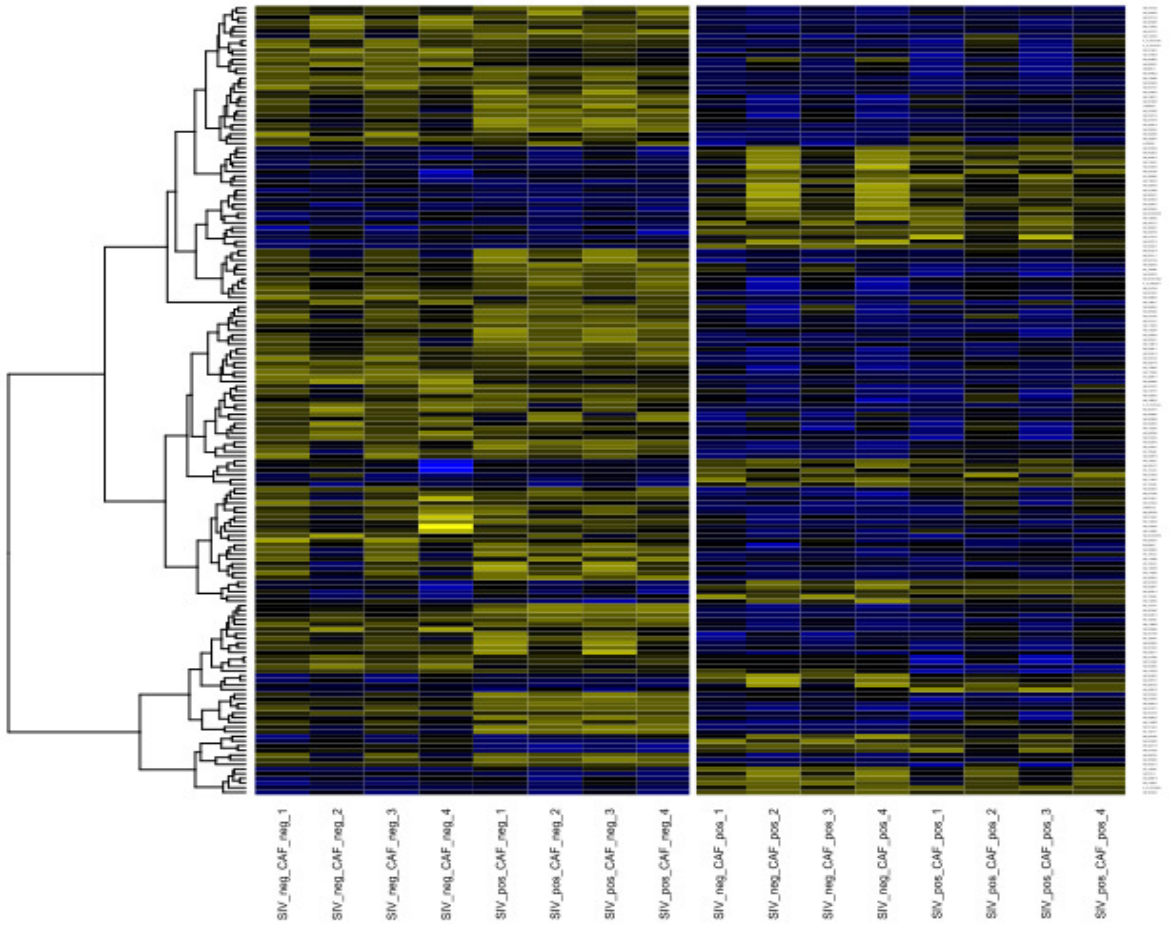


Fig 3.4: Heat Map of the microarrays performed with the $CD8^+$ T cells for the comparison of (A) SIV $^+$ vs. SIV- samples(B). $CNAR^+$ vs. $CNAR^-$ samples

Comparison of expression analysis revealed 50 genes to be at least two fold deregulated in $CD8^+$ T cells from SIV-infected as compared to non-infected animals (Infectivity Markers), while 78 genes were at least twofold deregulated in $CD8^+$ T from of $CNAR^+$ as compared to $CNAR^-$ monkeys (CNAR markers). Most transcriptomic changes were observed to participate in signalling, regulation of transcription, chromatin maintenance, cell cycle, apoptosis, immune response, extracellular matrix, cell adhesion, metabolism and transport (Fig 3.5).

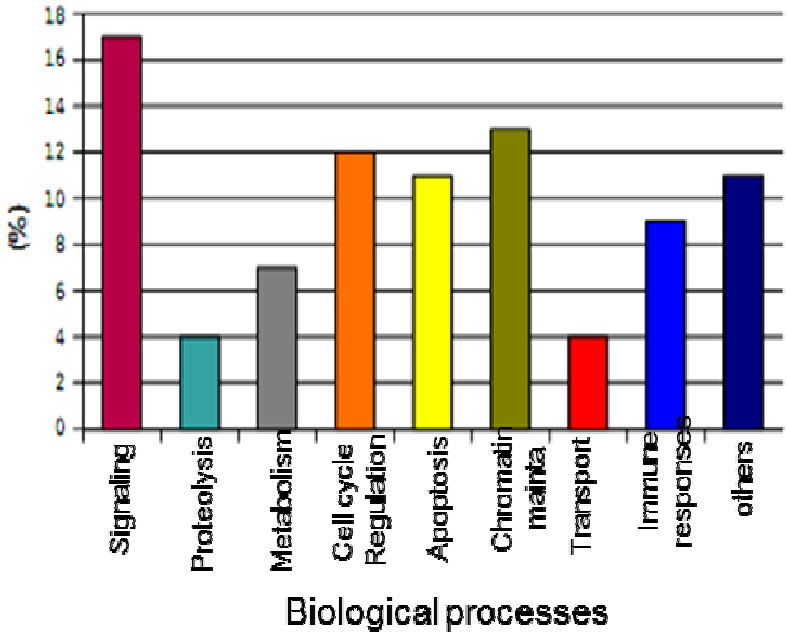


Fig 3.5: Biological processes as defined by the Gene Ontology consortium for significantlychanged genes. Numbers of genes (%) are shown on the y-axis.

3.3. Quantitative PCR analysis of selected differentially expressed genes

16 genes were selected for further validation by real time quantification. (App Table 2 with short description of the validated genes) The selection criterion were

1- Extent of differential expression
2- Immunological importance of the gene
3- Relevance with already defined hallmarks of CNAR (protease activity)

The expression of selected genes was quantified in $CD8^+$ T cells. RNA was isolated from the $CD8^+$ T cells of all animals at three different stages

a) Unstimulated $CD8^+$ T cells right after isolation from PBMCs.
b) ConA stimulated (D1) of *in vitro* viral inhibition tests
c) Co-cultivated and re-isolated at D7 of the *in vitro* viral inhibition tests

All selected genes were quantified by qRT PCR using housekeeping gene GAPDH as reference gene. Primers for the validation assays were designed. Efficiency of the primers was checked with four fold dilutions of the template in duplicate for each primer pair at different temperatures and concentrations. Primers with slope of -3.3 on standard curve of RT-PCR, equivalent to 100% efficiency, were selected.

ΔCt values were calculated as a difference between the mean Ct values (calculated from the duplicates) of each target and reference gene GAPDH

Δ Ct = Mean C_T(target gene) - mean C_T (GAPDH).

(A higher **Δ**Ct values corresponds to lower expression values and vice versa)

For comparison of any of two groups

$\Delta\Delta C_T$ was calculated as

ΔΔC_T= Δ C_T of respective gene in $CNAR^+$ animals - Δ C_T of respective gene in $CNAR^-$ animals.

For the comparison of SIV-infected and non-infected animals

ΔΔC_T= Δ C_T of respective gene in SIV-infected animals - Δ C_T of respective gene in non-infected animals.

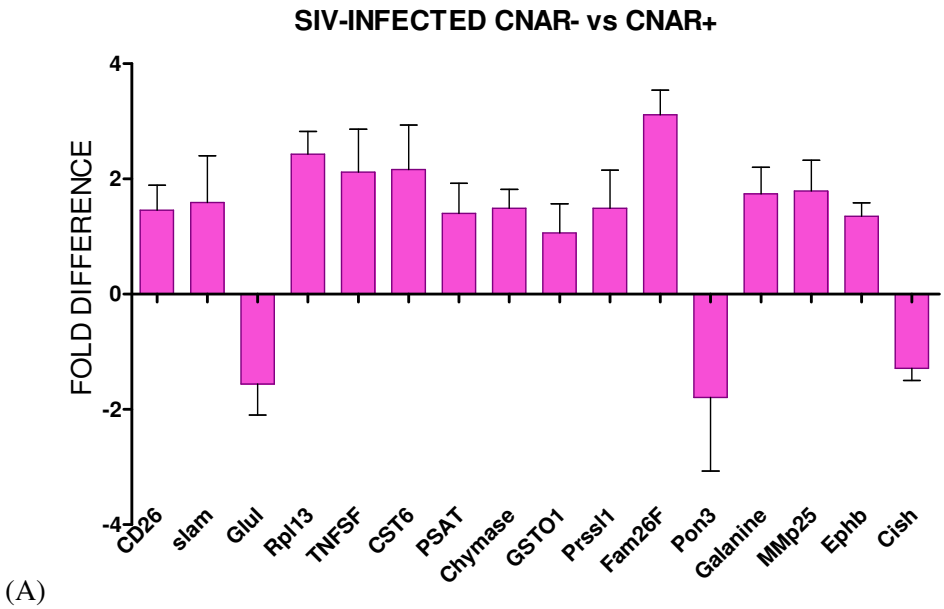
A higher value of **Δ**C_T will indicate that the respective gene is less expressed in respective group and vice versa. Differential expression in all graphs is presented in terms of fold changes calculated as

Fold Difference in expression of a gene = $2^{\Delta\Delta Ct}$

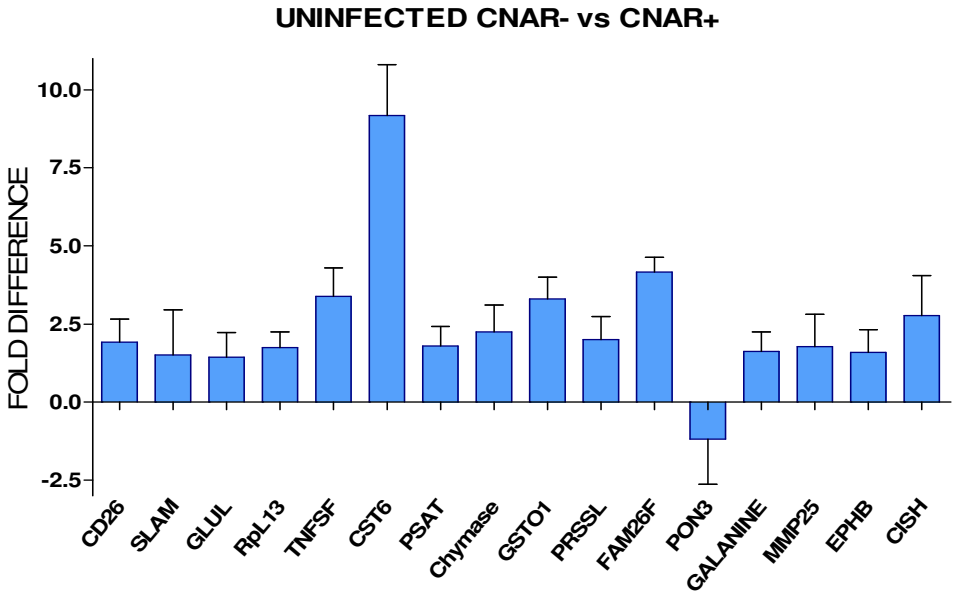
3.4 Comparison of $CNAR^+$ and $CNAR^-$ animals

In our attempts to identify a CNAR-associated gene, relative gene expression was compared in $CNAR^+$ and $CNAR^-$ animals both in SIV-infected and non-infected animals.

Upon 7-day co-culture of $CD8^+$ T cells with SIV-infected $CD4^+$ T cells, 7 out of 16 genes, namely TNFSF-13B, CST6, Chymase, GSTO1, PRSSL1, FAM26F, and CISH, were found to be more than 2 fold up regulated in $CNAR^-$ animals as compared to $CNAR^+$ animals in SIV-infected group of animals (Fig 3.6A). In non-infected group 4 genes namely RPL13, TNFSF-13B, CST6, and FAM26F were found to up-regulated in $CNAR^-$ animals (Fig 3.6B).



(A)



(B)

Fig 3.6: Fold difference in mRNA expression of selected genes in (A) SIV-infected (B) Un-infected $CNAR^-$ animals as compared to $CNAR^+$ animals.

This data showed that only three genes namely TNFSF-13B, CST6, and FAM26F were more than twofold deregulated in both SIV-infected and non-infected group of animals. Only FAM26F was significantly upregulated in $CNAR^-$ animals in comparison to $CNAR^+$ animals, both in SIV-infected and non-infected groups (Fig 3.6C).

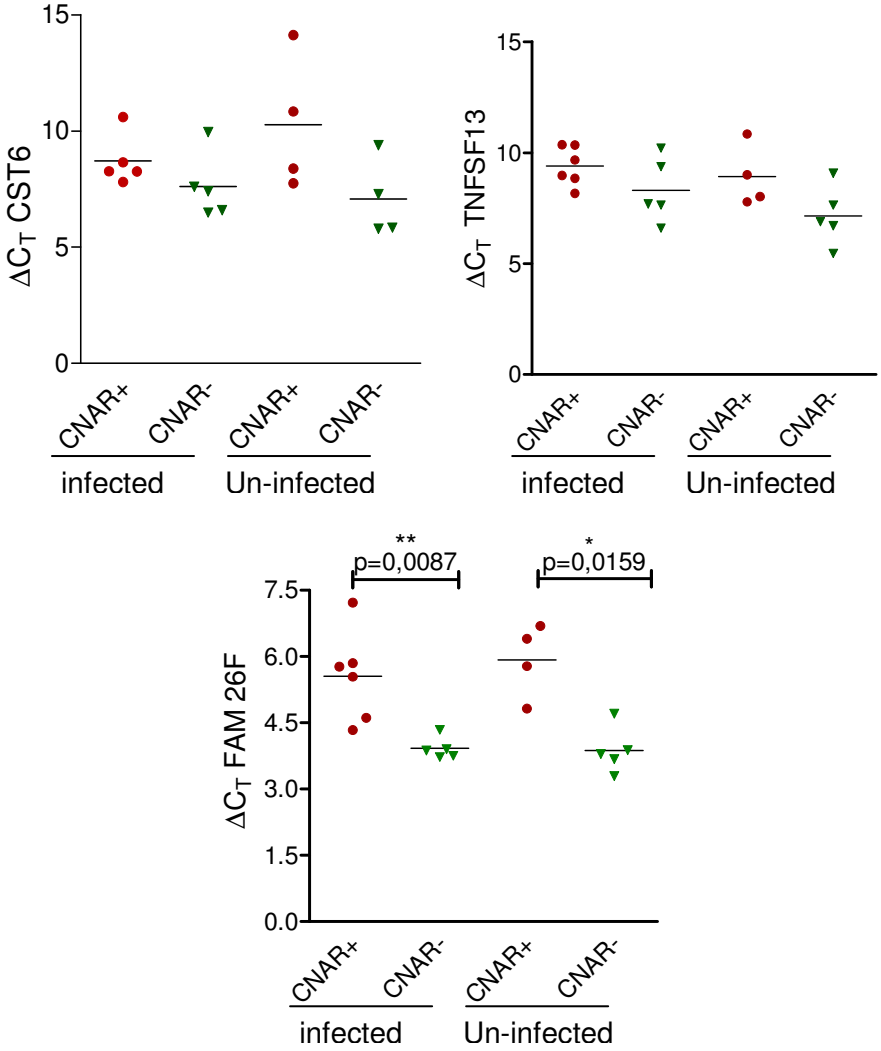


Fig 3.6C: Relative gene expression of FAM26F, CST6 and TNFSF-13B in $CD8^+$ T cells from the SIV-infected and non-infected animals. The difference in expression of FAM26F between $CNAR^+$ and $CNAR^-$ animals is significant in both groups. Δ Ct was calculated as Mean C_T (target gene) - mean C_T (GAPDH) so a higher ΔCt values corresponds to lower expression and vice versa)

To investigate whether the observed difference in the gene expression was due to ConA-stimulation or it was the result of co-cultivation during virus inhibition test, we compared the mRNA levels of the candidate genes in $CD8^+$ T cells directly after ConA-stimulation (D1) between $CNAR^-$ and $CNAR^+$ animals. RNA levels of CST6 and FAM26F were more than two fold deregulated in infected group, while CST6, Chymase, MMP25, PRSSL1 and PON3

differed more than twofold in non-infected animals but none of the differences were significant (Fig 3.7A).

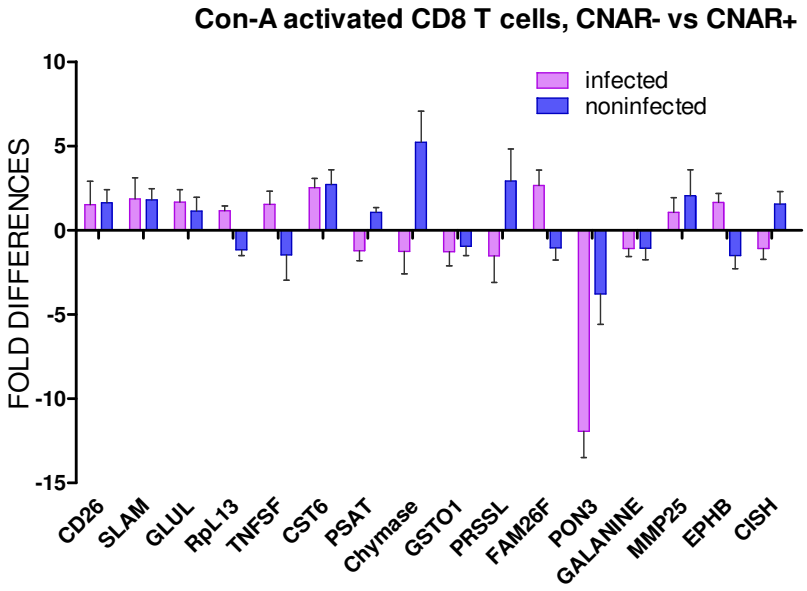


Fig 3.7A: Fold difference in mRNA expression of ConA stimulated $CD8^+$ T cells from $CNAR^-$ animals as compared to $CNAR^+$ animals in both SIV-infected and non-infected groups. None of the gene was found to be significantly differentially regulated in both groups after ConA stimulation.

Next it was investigated whether and how gene expression in $CD8^+$ Tcells changes upon co-culture with SIV-infected $CD4^+$ T cells. Therefore the ΔCT values obtained after co-culture were compared with those obtained after ConA-stimulation, because the CT-value of the reference gene GAPDH values did not change upon co-culture. Although many genes were deregulated upon co-culture only the regulation of FAM26F expression resulted in a significant difference between $CNAR^+$ and $CNAR^-$ monkeys. As clear from Fig 16B, co-cultivation of $CD8^+$ T cells with SIV infected CD4 T cells has led to an increase in FAM26F expression. In case of $CNAR^+$ animals this difference was 2.69 in co-cultured samples as compared to ConA stimulated samples while in case of $CNAR^-$ samples this difference was 6,48fold. This emphasises that differential FAM26F expression in $CNAR^+$ vs $CNAR^-$ animals was a consequence of differential up regulation of FAM26F upon co-cultivation (Fig 3.7B).We further investigated if the selected genes were differentially expressed in $CNAR^+$ and $CNAR^-$ animals intrinsically. For this purpose, mRNA expression levels of selected genes were measured by qRT-PCR directly after isolation $CD8^+$ T cells from PBMCs. None of them

was significantly differentially expressed in CNAR$^+$ vs. CNAR$^-$, in both SIV-infected and non-infected animals Fig 3.8.

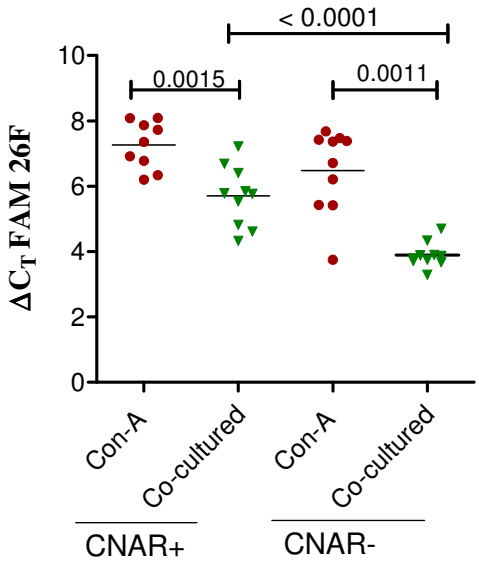


Fig 3.7B: Relative gene expression of FAM26F in CD8$^+$ T cells from CNAR$^+$ and CNAR$^-$ animals before and after co-cultivation with SIV-infected CD4$^+$ T cells. T test was applied to calculate the significance. ΔCt was calculated as Mean C_T (target gene) - mean C_T (GAPDH) so a higher ΔCt values corresponds to lower expression and vice versa)

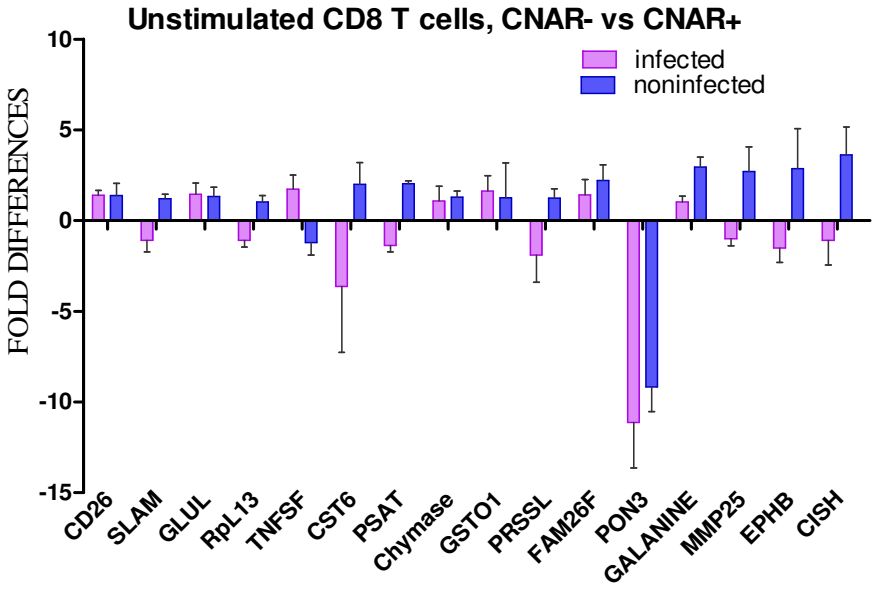


Fig 3.8: Fold difference in the expression of genes in un-stimulated CD8$^+$ T cells from

CNAR$^+$ and CNAR$^-$ animals in SIV-infected and non-infected animals

3.5 Comparison of gene expression in CD8$^+$ T cells between SIV-infected and non-infected animals

In order to find the markers of SIV-infection, gene expression level were also compared between SIV-infected and non-infected animals irrespective of their CNAR activity in CD8$^+$ T cells directly isolated from PBMC (Fig 3.9A)

ΔΔ Ct= ΔCt of respective gene in CNAR$^+$ animals - ΔCt of respective gene in CNAR$^-$ animals.

3 genes SLAMF8, CHYMASE, and PRSSL1 were found to be significantly down-regulated while TNFSF-13B and EPHB were found to significantly up-nregulated in SIV-infected animals as compared to non-infected macaques (Fig 3.9B).

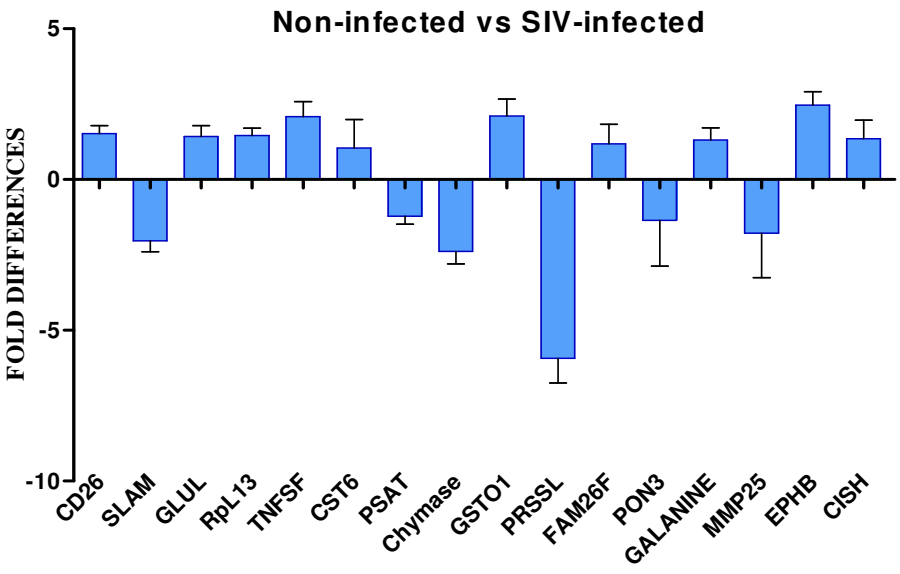


Fig 3.9A: Fold difference in gene expression of non-infected animals as compared to the SIV-infected animals.

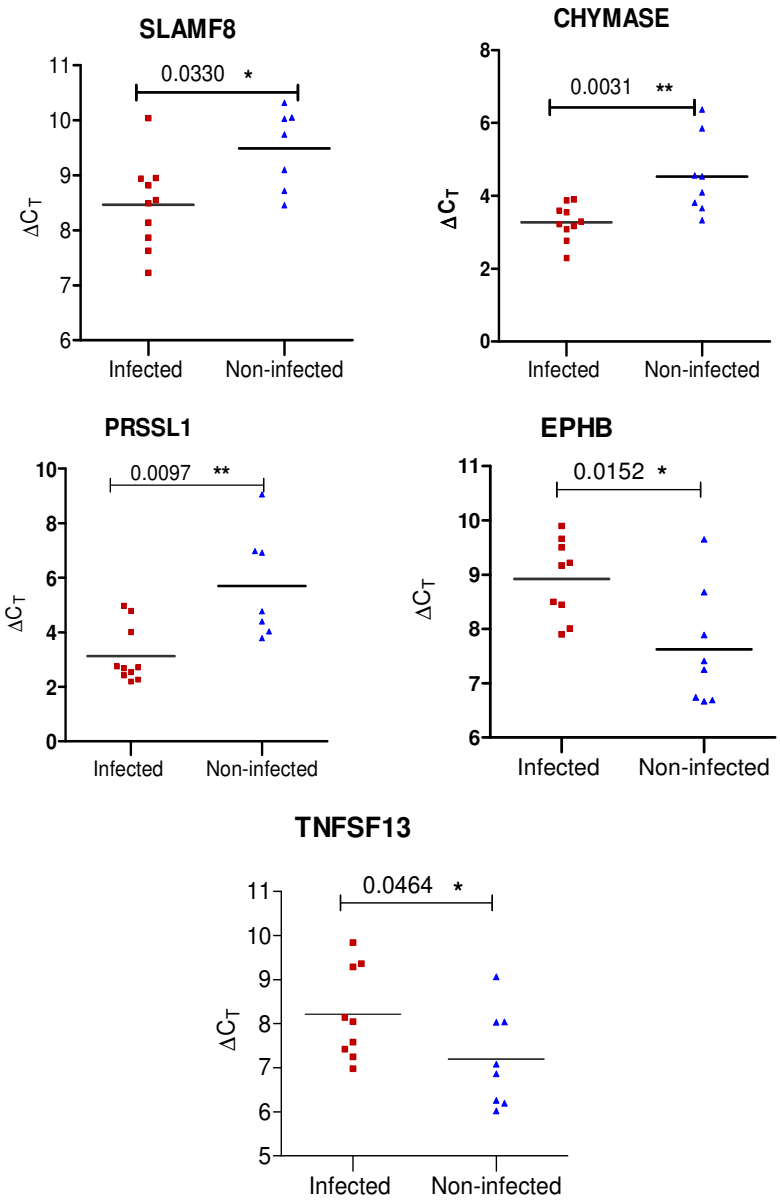


Fig 3.9B: ΔC_T of genes that were found to be significantly differentially expressed (T test applied) in SIV-infected animals as compared to non-infected animals. . Δ Ct was calculated as Mean C_T (target gene) - mean C_T (GAPDH) so a higher ΔCt values corresponds to lower expression and vice versa)

After finding FAM26F as sole differentially expressed between $CNAR^+$ vs $CNAR^-$ for our further investigation we focussed on following aspects

- Genetic studies of FAM26F
- Regulation of FAM26F during course of immunisation and infection in two independent vaccine experiments.
- Correlation of FAM26F with viral load.
- Correlation of FAM26F with other important genes of immune system (Mx1, IP-10, tetherin).
- Establishment of permanent T cell lines to obtain sufficient standardized material for further investigations into non-cytolytic antiviral activity.

3.6 Genetic Studies of *FAM26F*

In order to investigate if CAF activity can be related to genetic differences in *FAM26F* gene, cDNA sequence of *FAM26F* was cloned and sequenced from our previously characterized $CNAR^+$ and $CNAR^-$ group of animals. *FAM26F* cDNA was amplified from each of the four groups (SIV-infected CAF^+ and CAF^-, non-infected CAF^+ and CAF^-) (Fig 3.10) and amplified fragment was then cloned and sequenced as described in section 2.13.

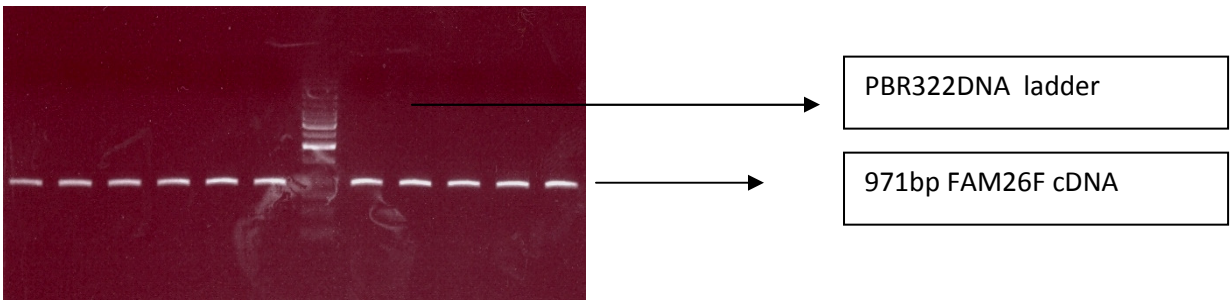


Fig 3.10: Electropherogram of ethidium bromide stained 1% agarose gel showing PCR amplified *FAM26F* cDNA products in 12 selected animals.971bp FAM26F fragment along with DNA ladder.

Chromatograms were analyzed by Bioedit software. The sequences were aligned and compared with *Macaca mulatta FAM26F* sequence (Genbank accession number XM_001111520) (Fig 3.11). We were able to identify two alleles on the basis of nucleotide

difference at position c.208 and c.805 of *FAM26F* gene (Table 3.2). Nucleotide change at position 208 does not lead to a change in amino acid sequence whereas nucleotide change at position 805 is indicating a change of Lys to Glu amino acid.

Majority of animals (10) were heterozygotes for these two alleles. Only two were homozygotes for allele B and both were $CNAR^{+}$. Tendency for over-representation of allele B in CAF^{+} animals was regarded as a reason for further investigations. It may be possible that polymorphism have an influence on survival time or viral load at set-point in SIV-infected macaques. Therefore, polymorphism typing was initiated in a larger sample of about 170 SIV-infected macaques using Taqman-based genotyping assay. Primers and probes were designed by TIB Molbiol synthesis-lab but the assays were not able to discriminate the polymorphisms probably of high GC regions of *FAM26F*.

	Position c.208	Position c.805
Reference allele	C	A
Allele A	T	A
Allele B	T	G

Table 3.2: Alleles identified after sequencing of *FAM26F* cDNA clones from selected $CNAR^{+}$ and $CNAR^{-}$ animals.

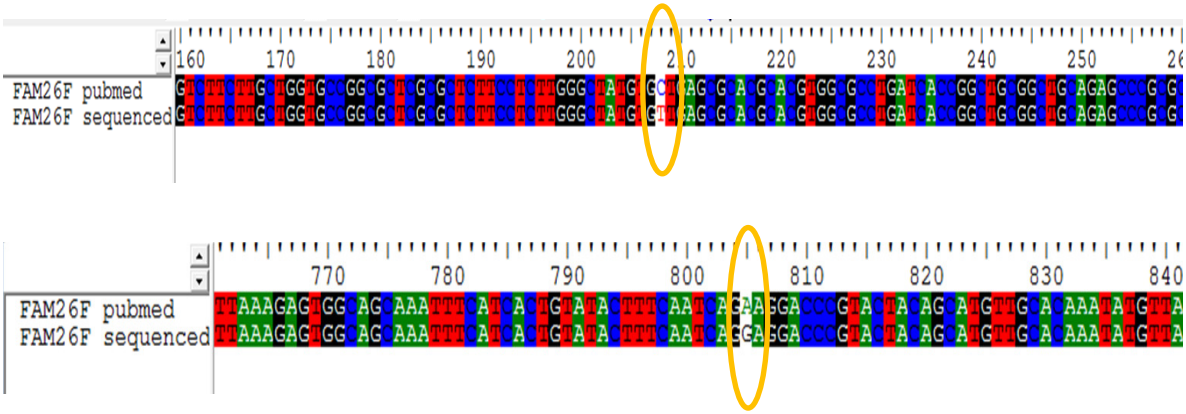


Fig 3.11: *FAM26F*sequence alignment with the Genbank *FAM26F* sequence (XM_001111520).

3.7 FAM26F regulation during course of infection

In order to investigate a possible connection of FAM26F with viral load and other immune components, we undertook a cross-sectional study. Total RNA isolated from whole blood (section 2.8) of SIV-infected rhesus macaque, 24 weeks after infection, was quantified using housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Genbank accession number CO774281) as reference. All samples were run at least in duplicates. The results were analysed by Sequence Detection Software (ABI) and ΔCt of FAM26F were plotted against respective viral loads (Fig 3.12).

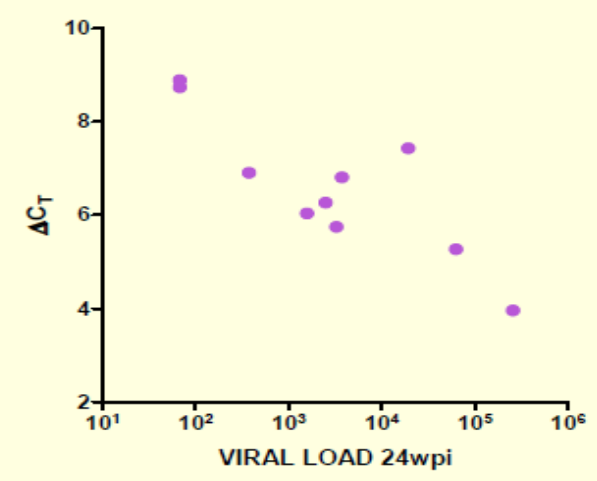


Fig 3.12: ΔC_T of FAM26F relative to GAPDH plotted against log viral load. Data shows a direct correlation of FAM26F expression with viral load in selected animals (p= 0.0202).

A significant (p=0.0202) correlation was found when viral load was plotted against ΔCT and data was analysed by two-tailed spearman correlation. In other words FAM26F expression seems to be associated with viral load and can be further investigated as a potential marker of infection. This investigation was extended to more animals in longitudinal studies. To find out expression variation of FAM26F upon infection and immunisation and its correlation with some other well-known genes of immune components, samples from two independent vaccine experiments were investigated.

The first experiment (Exp-1) included 6 control (non-vaccinated) and 12 vaccinated animals that were studied before and after SIV infection. The monkeys have been infected by a so-called repeated low dose challenge that is supposed to depict the "natural" infection more closely than a single high dose challenge. Macaques from experiment 1 were challenged weekly intrarectally with escalating doses of SIVmac251 (30 TCID50 up to 7 times and 60 TCID50 up to 8 times) until they became productively infected (viral RNA copies >50/ml plasma). Blood for RNA quantification was drawn at the day of the third, fourth and fifth challenge, therefore these data may not represent true pre-infection values. After infection blood samples were also collected at indicated time points Fig 3.13.

Second group (Exp-2) included 24 animals and both immunization and infection studies were performed. Animals were challenged intra rectally with 120 TCID50 of SIVmac251 up to 8 times until they became productively infected (viral RNA copies >50/ml plasma). Blood for RNA quantification was drawn at the day before the first inoculation of SIV. After infection blood additionally was drawn at the indicated time points (Fig.13).

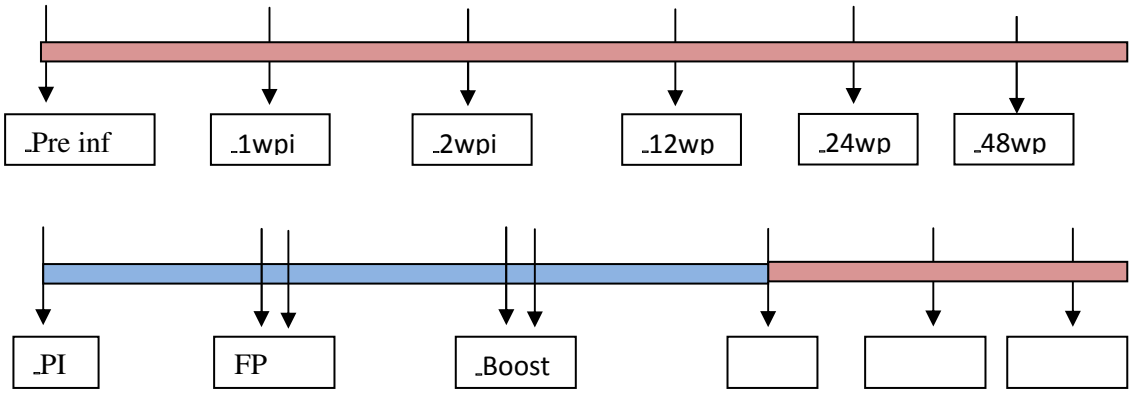


Fig 3.13 : Time lines for the (A) Experiment 1 (B) Experiment 2 indicating the time points when blood samples were taken for quantification of specified genes. Pre inf stand for pre infection, PI for pre immunization, FP for 24hrs and 48hrs after a final priming, Boost 24hrs and 48hrs after boosting (final vaccination), DOI for Day of first challenge and wpi for weeks post infection.

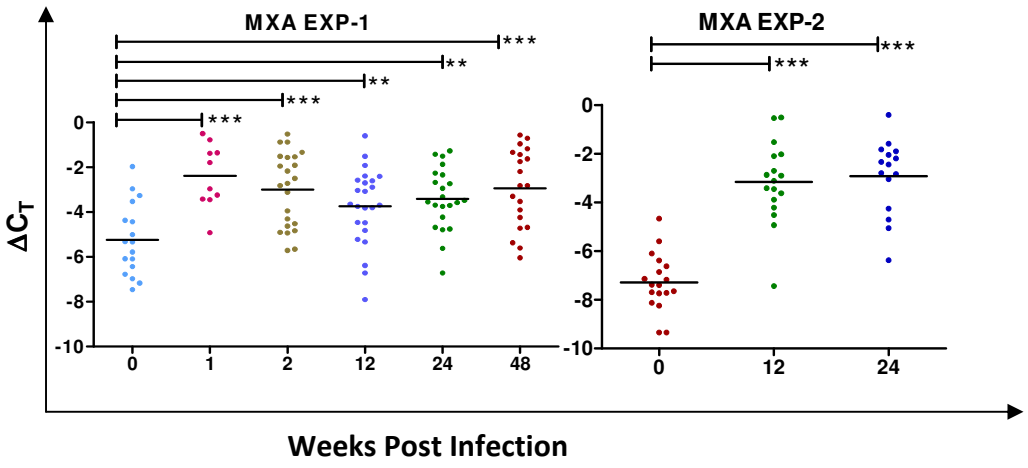
To simplify the interpretation of data, Δ CT was calculated as

$$\Delta Ct = \text{mean } C_T \text{ (GAPDH)} - \text{Mean } C_T \text{ (target gene)}$$

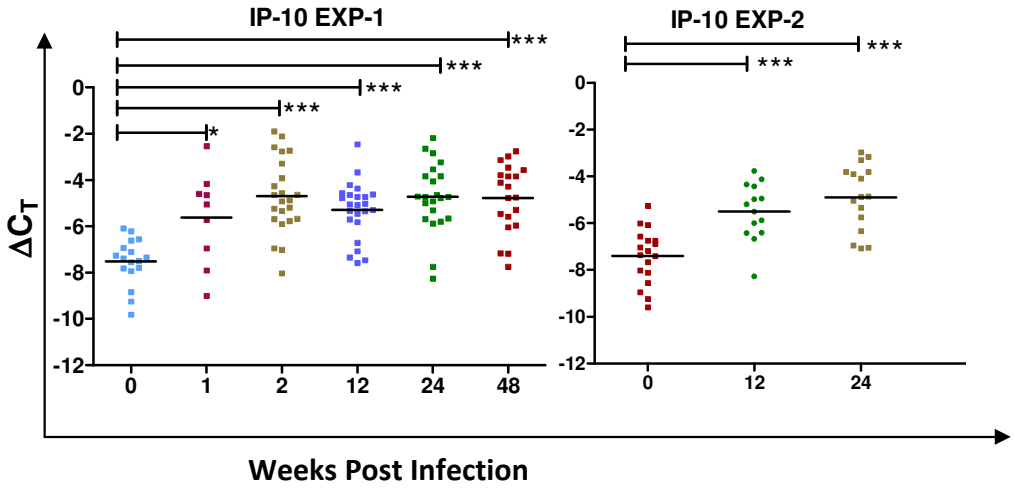
This implies that a higher value of Δ Ct represents the higher expression and vice versa (unlike the Δ Ct calculations made in data sets mentioned earlier)

3.7.1 Gene expression variations after infection:

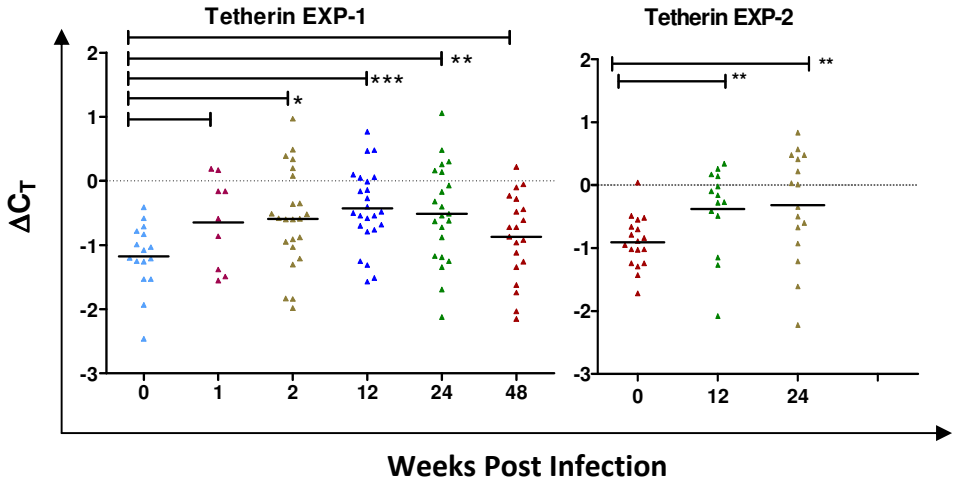
In both experiments FAM26F expression was quantified at the specified time points (Fig 3.13) along with Mx1, IP-10 and tetherin. These genes are well documented for their importance in innate immunity with respect to viral load or/and immune activation or SIV/HIV restriction. All genes under investigation follow almost same expression pattern with only minor fluctuations. All of them were significantly upregulated after infection as compared to their pre infection expression values. Mx1 showed highest expression 1wpi compared to IP-10, FAM26F and tetherin. 12 wpi Mx1 expression increased on average 3fold in Exp1 and 16fold in Exp2. For IP-10, tetherin and FAM26F this induction was 4.7, 1.6 and 2.2folds respectively. (Fig: 3.14). This increased expression of genes upon infection is maintained after 48 weeks post infection in experiment 1 for all genes except for tetherin, where difference was significant till 24 weeks post infection. The importance of Mx1, IP-10 and tetherin in innate immunity is well known (references). Our data suggests that expression pattern of FAM26F after infection is similar to above mentioned genes so these findings may suggest a role of FAM26F in innate immunity. It should also be noted that the expression of the investigated genes at pre-infection levels were different among animals in both vaccine groups. Furthermore, there were large interindividiudal differences in expression before infection (e.g. for Mx1 16fold,). However, except for FAM26F these differences did not correlate with viral load after infection (see below).



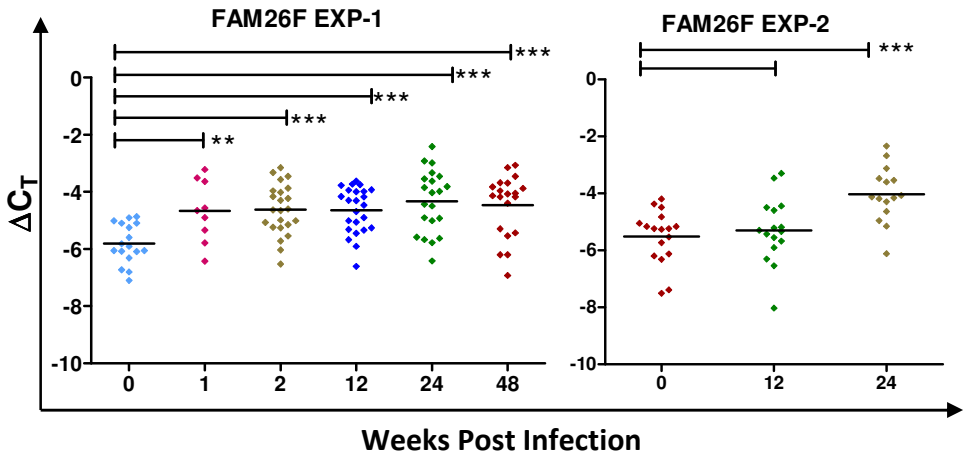
(A)


IP-10 EXP-1
IP-10 EXP-2
ΔCT
0
-2
-4
-6
-8
-10
-12
0
1
2
12
24
48
*
***
Weeks Post Infection

(B)


Tetherin EXP-1
Tetherin EXP-2
ΔCT
2
1
0
-1
-2
-3
0
1
2
12
24
48
*
**
***
Weeks Post Infection

(C)



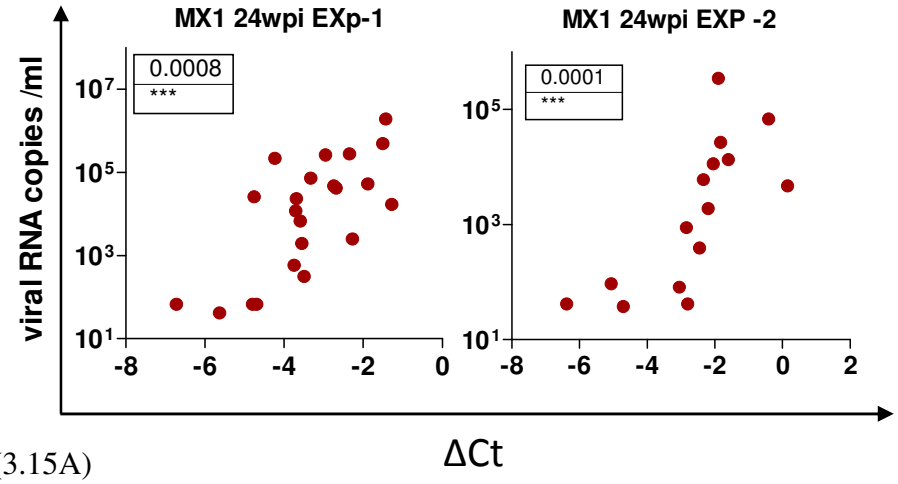
(D)

Fig 3.14: Expression variations of (A) Mx1 (B) IP-10 (C) Tetherin (D) FAM26F during course of SIV infection in two independent experiments.A higher Ct values correspond to higher gene expression values.

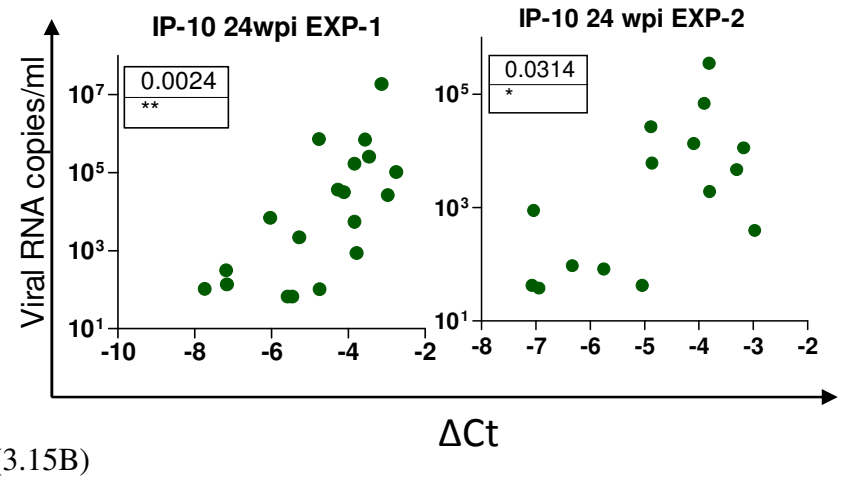
3.7.2 Correlation of gene expression with viral load

We were interested to know whether the expression of the genes under investigation is correlated to the viral load at respective time point. In both experiments Mx1, IP-10 expression were significantly positively correlated with the viral load (Fig 3.15 A, B) (For simplicity only 24wpi data is shown) as described by others [203].

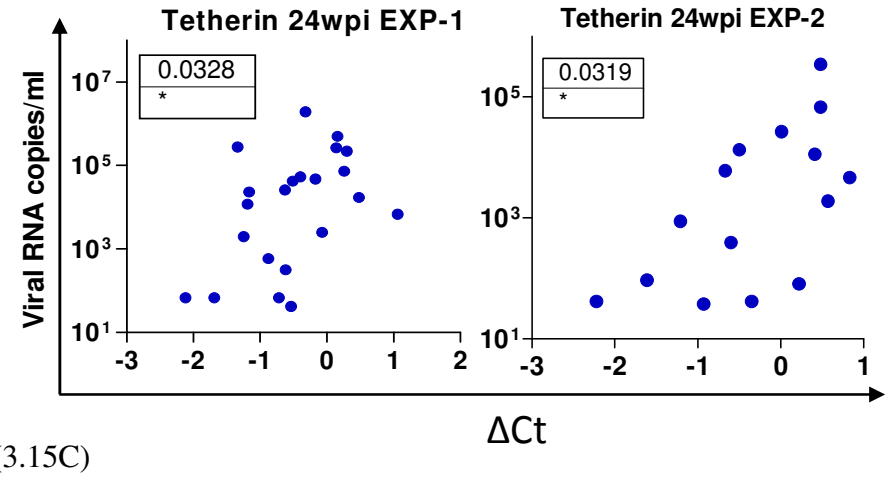
For the first time a significant correlation between tetherin expression and respective viral load was found (Fig 3.15C). In contrast, FAM26F was not found to be correlated with viral load (Fig 3.15D).



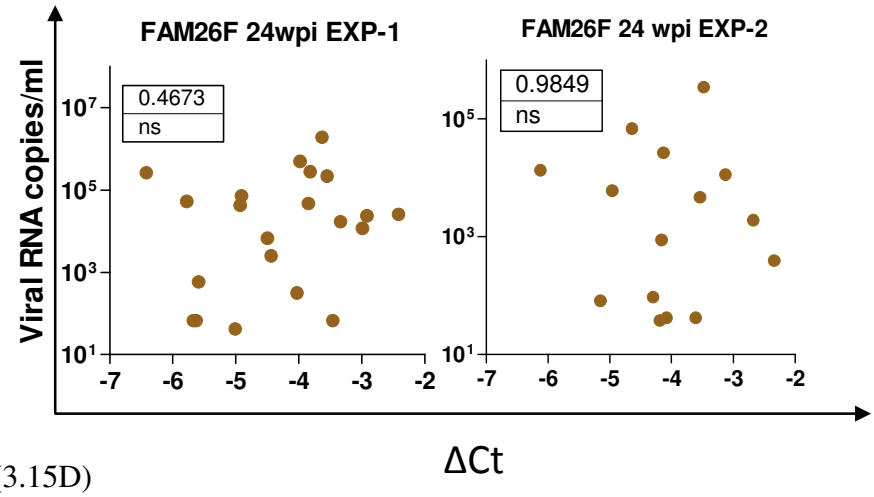
(3.15A)



(3.15B)



(3.15C)



(3.15D)

Fig 3.15: Correlation of (A) Mx1 (B) IP-10 (C) tetherin (D) FAM26F gene expression levels (ΔCT) with viral load at week 24 post infection. P values are indicated. ΔC_T was calculated as mean C_T (GAPDH) - Mean C_T (target gene) so that higher Ct values correspond to higher expression values

3.7.3 FAM26F – an early predictor of viral load

In both vaccine experiments we also studied whether expression of FAM26F may be an early predictor/indicator of viral load during the chronic phase of infection. In experiment 1, FAM26F expression 2 weeks post infection was found to be positively correlated with plasma viral RNA copies at 12, 24 and 48 wpi (Fig 3.16). Unfortunately this could not be validated with data from exp. 2 because from this experiment, 2 wpi samples were not available.

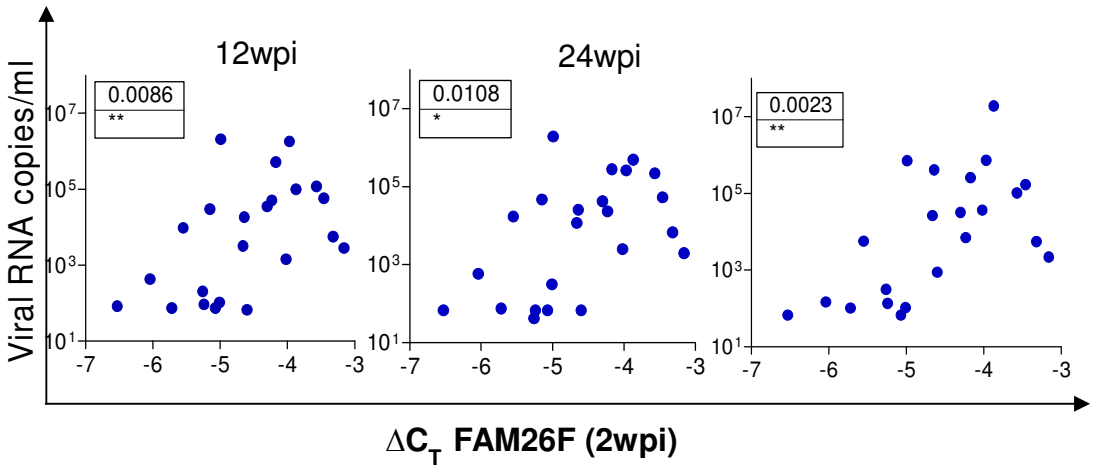
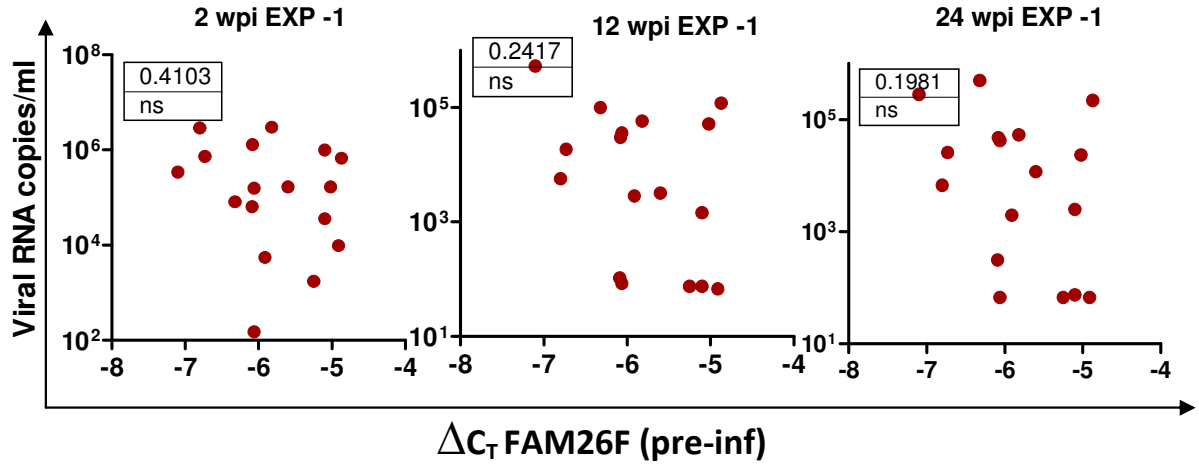


Fig 3.16: Significant correlation of 2wpi FAM26F expression with (A) viral load 2wpi (B) viral load 12wpi (C) viral load 24wpi. P values are indicated in boxes. ΔC_T was calculated mean C_T (GAPDH) - Mean C_T (target gene) so that higher Ct values correspond to higher expression values

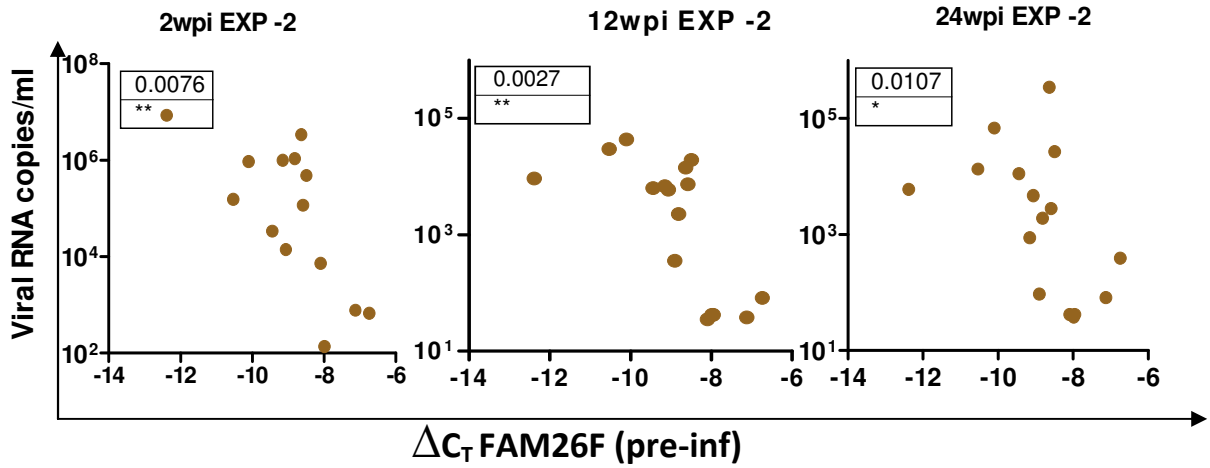
Next it was calculated whether the pre-infection levels of any of the studied genes are correlated to set point viral load. In both experiments, pre infection values of Mx1, IP-10 and tetherin were not correlated with set point viral load. Interestingly, in experiment- 2, pre-

infection values of FAM26F were inversely correlated with viral load 2, 12 and 24 wpi (Fig 3.17 A). This suggests FAM26F as an indicator of chronic phase viral load. However this was not the case for Exp. 1 (Fig: 3.17 B) possibly because of two outliers. Furthermore, one has to keep in mind that the blood samples for Exp. 1 were drawn after the animals had been inoculated with virus.

Interestingly, the pre infection values were positively correlated with the viral load at set point, while the post infection values (wpi 2, Exp. 2) were inversely correlated with viral load at set point like Mx1 and IP-10.



(3.17 A)



(3.17 B)

Fig 3.17: Correlation of pre infection expression of FAM26F with viral load 2, 12 and 24wpi in (A) experiment1 (B) experiment 2. P values are shown in boxes. ΔC_T was calculated mean C_T (GAPDH) - Mean C_T (target gene) so that higher Ct values correspond to higher expression values

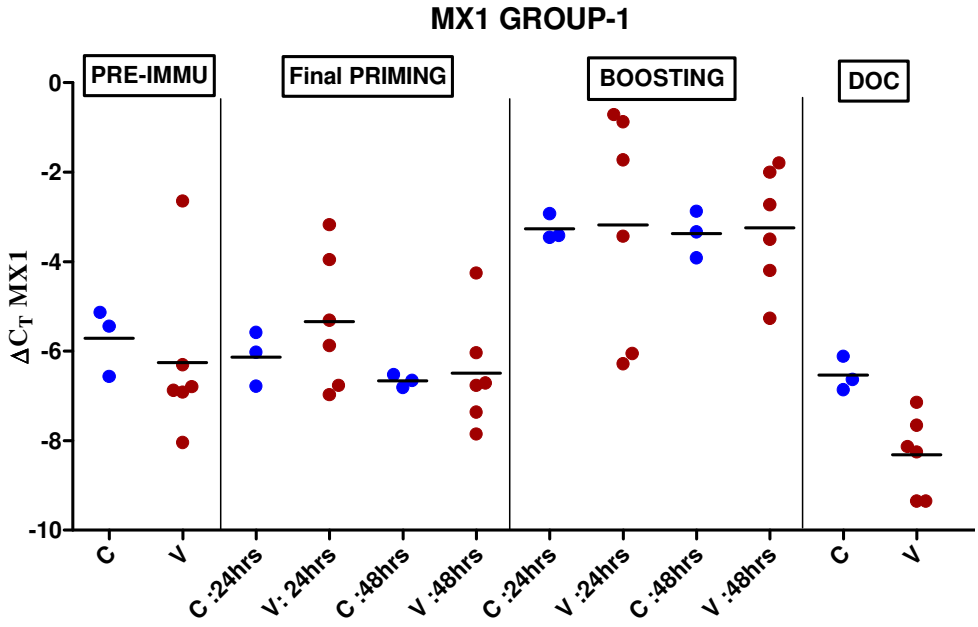
3.8 FAM26F regulation during course of immunization

Exp-1 it was investigated whether the expression levels of genes belonging to the innate immunity (Mx1, IP-10), a viral restriction factor (tetherin) and FAM26F varied during immunisation, and whether this variation may be related to vaccine efficacy. Briefly, the immunisation procedure followed a prime-boost strategy and included at first two injections of single-cycle immunodeficiency viruses within a four-week interval [204].Thereafter the monkeys were split in two groups of 6 animals. Group A received two times an adenovirus-derived vector expressing SIV genes via the tonsils [205] followed by an intramuscular immunisation with a fowlpox virus-derived vector expressing SIV genes [206]. Group B received two times a fowlpox virus-derived vector expressing SIV-genes via the tonsils followed by an intramuscular immunisation with the adenovirus-derived vector expressing SIV-genes. Thus group A was primed with an adenovirus-derived vector followed by a boost with a fowlpox virus-derived vector, whereas the procedure for group B was vice versa. For each vaccine group, 3 “control” animals were “immunised” with so-called empty vectors. These controls are important as the virus-derived vectors may also stimulate innate immunity and may confer some level of protection.

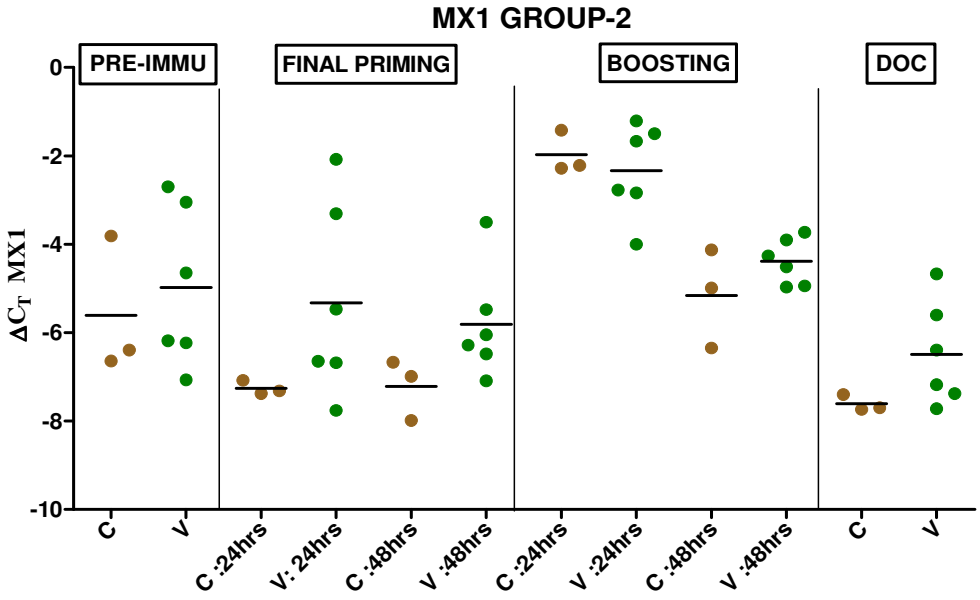
Blood samples for RNA quantification were drawn before initiation of the immunisation procedure, 24 and 48 hours after immunisation for the final prime (fowlpox virus vector constructs via the tonsil or adenovirus vector constructs via the tonsils), the boost (adenovirus vector or fowlpox virus vector intramuscular) and at the day of first challenge with SIV (see above).

Expression of all the genes under investigation showed non-uniform fluctuations upon final priming as compared to pre immunization values (Fig 3.18). These changes were partially independent of SIV-vaccines as the empty vector control groups also showed such fluctuations. However, the final boosting resulted in marked differences between the

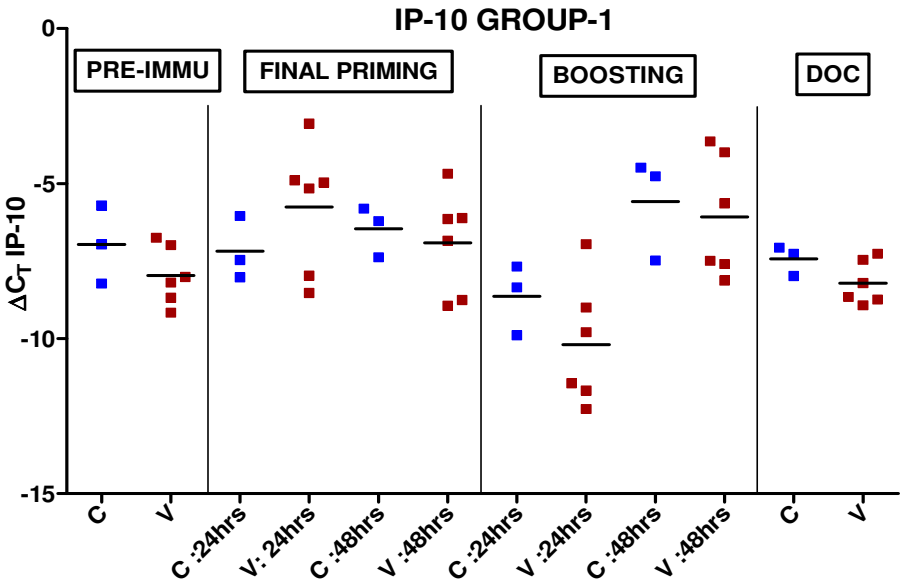
twogroups. Notably boosting resulted in an increase in expression of Mx1 in both groups. This enhanced expression was persistent till 48 hrs only in group 1 (Fig 3.18 A) whereas it dropped rapidly in group 2. Although the difference in expression of Mx1 between both vaccinegroups was not significant 48 h after boosting, it was significant at the day of 1st challenge (pre infection) Fig 3.19

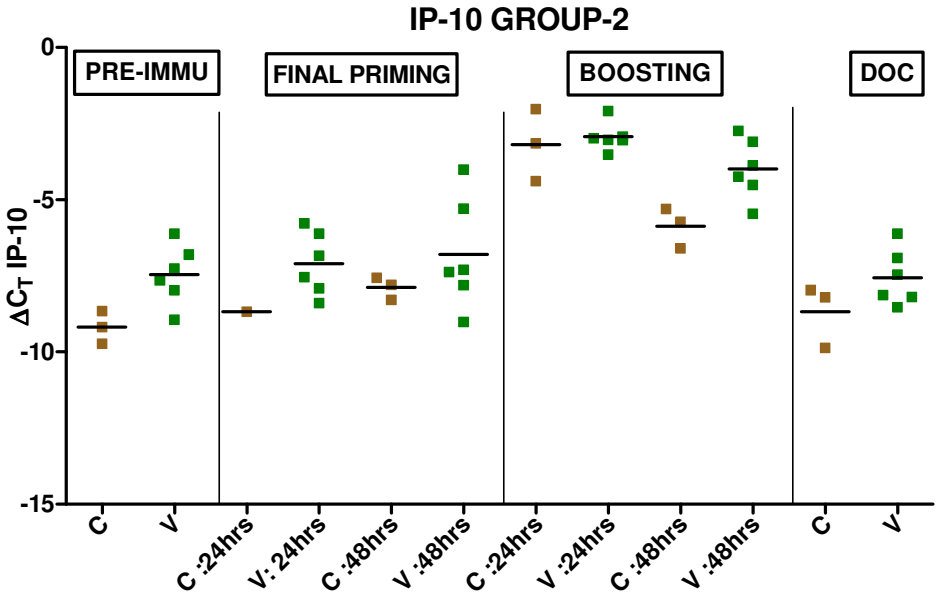


(A).

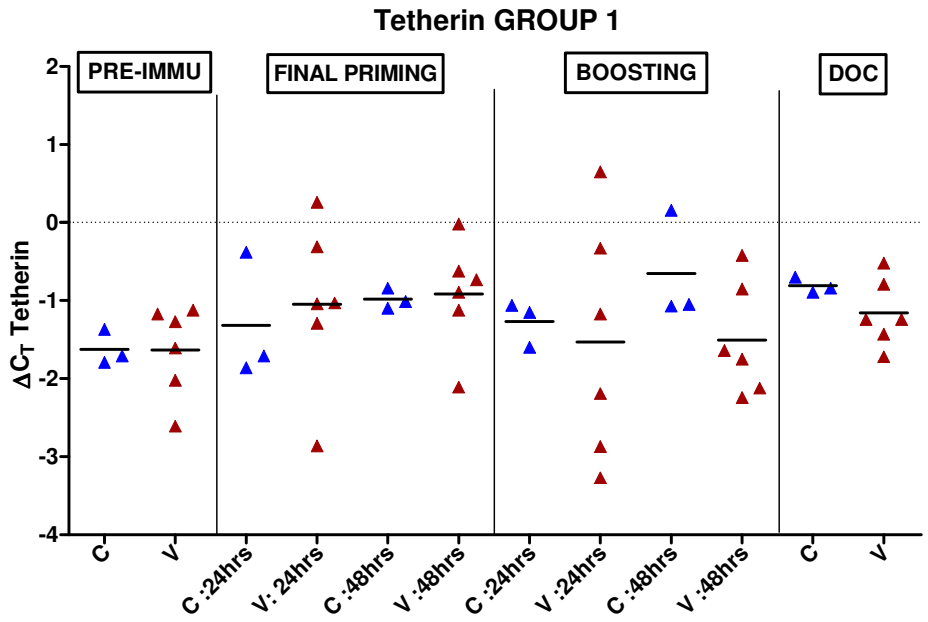


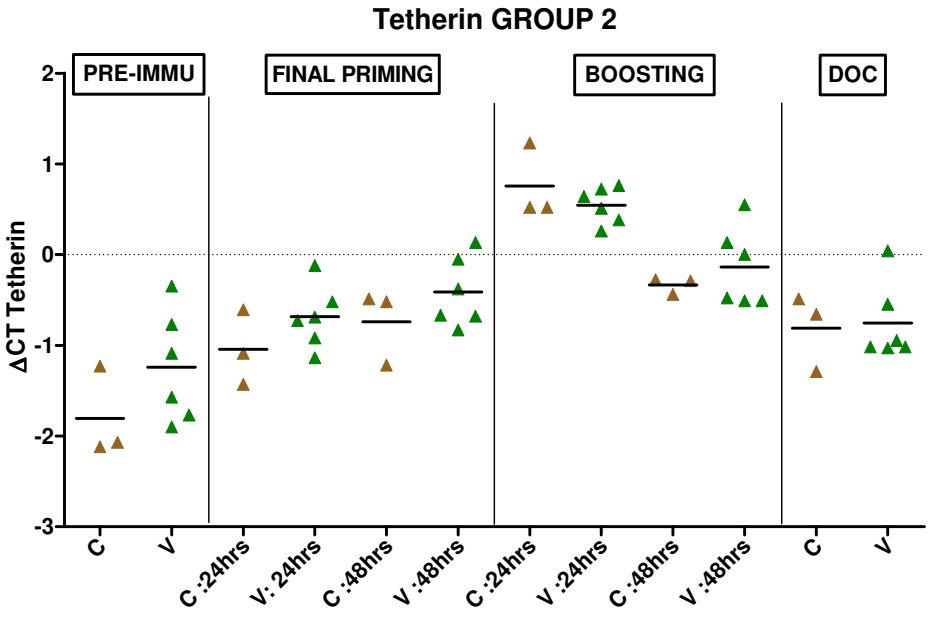
(3.18A)


IP-10 GROUP-1
PRE-IMMU
FINAL PRIMING
BOOSTING
DOC
0
-5
-10
-15
ΔCT IP-10
C
V
C :24hrs
V: 24hrs
C :48hrs
V :48hrs
C :24hrs
V :24hrs
C :48hrs
V :48hrs
C
V

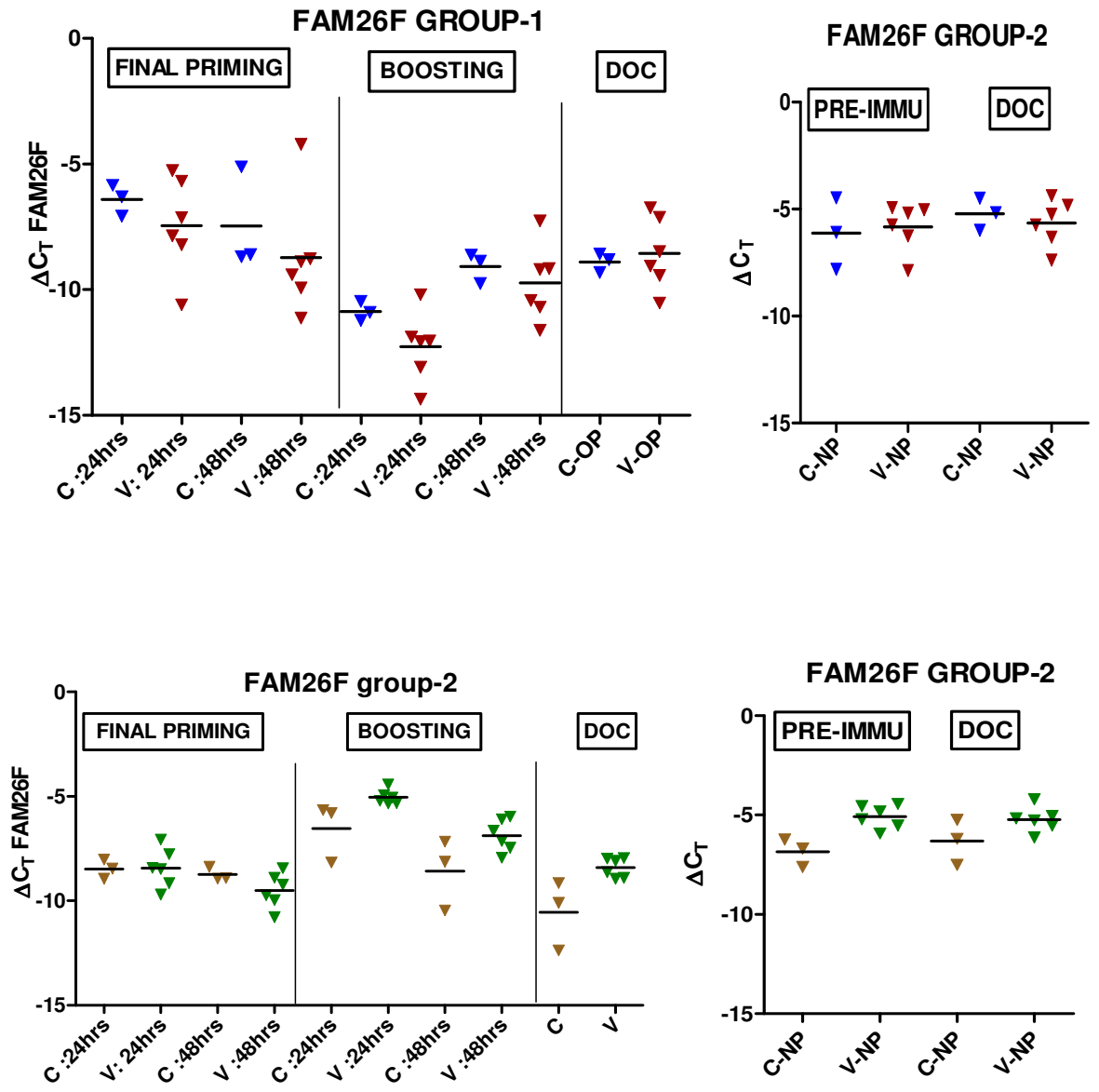

IP-10 GROUP-2
PRE-IMMU
FINAL PRIMING
BOOSTING
DOC
0
-5
-10
-15
ΔCT IP-10
C
V
C :24hrs
V: 24hrs
C :48hrs
V :48hrs
C :24hrs
V :24hrs
C :48hrs
V :48hrs
C
V

(3.18B)


Tetherin GROUP 1
PRE-IMMU
FINAL PRIMING
BOOSTING
DOC
ΔCT Tetherin
2
1
0
-1
-2
-3
-4
C
V
C :24hrs
V: 24hrs
C :48hrs
V :48hrs
C :24hrs
V :24hrs
C :48hrs
V :48hrs
C
V


Tetherin GROUP 2
PRE-IMMU
FINAL PRIMING
BOOSTING
DOC
ΔCT Tetherin
2
1
0
-1
-2
-3
C
V
C :24hrs
V: 24hrs
C :48hrs
V :48hrs
C :24hrs
V :24hrs
C :48hrs
V :48hrs
C
V

(3.18C)

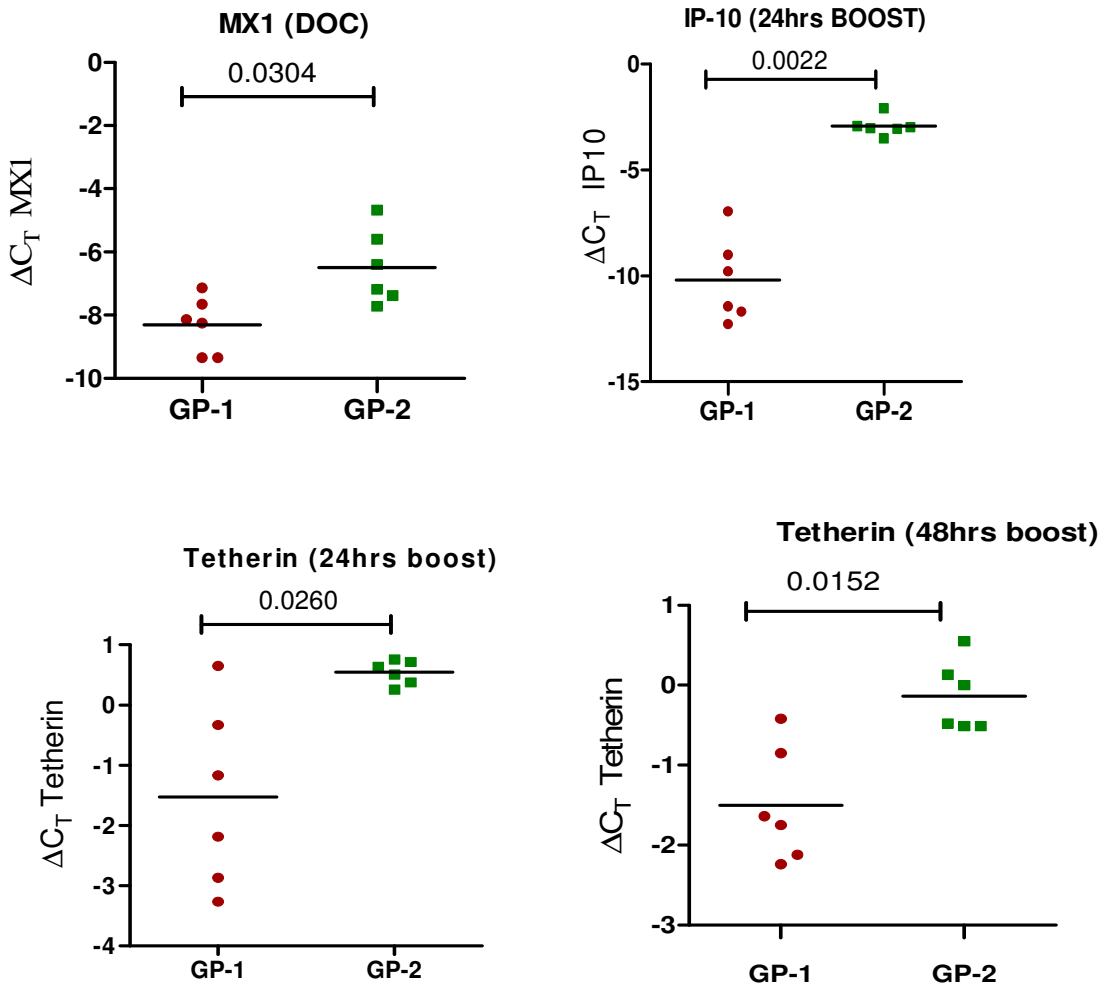


(3.18D)

Fig 3.18: Expression variations of (A) Mx1 (B) IP-10 (C) tetherin (D) FAM26F before (PRE-IMMU), during course of immunization in two vaccine experiments, and at the day of challenge (DOC)(C: controls and V: vaccinees). For comparison of FAM26F pre-immunization and day of challenge values a new batch of primers (NP) had to be usedΔC_T was calculated mean C_T (GAPDH) - Mean C_T (target gene)so that higher Ct values correspond to higher expression values.

In contrast, IP-10, tetherin and FAM26F upregulation was delayed and more variable in group 1 compared to group 2 where the expression of these genes peaked 24 hrs post immunisation. The difference of IP-10, FAM26F and tetherin expression after 24hrs of boosting is significantly different between both vaccine groups Fig 3.19 (B, C, D and E). Furthermore, tetherin and FAM26F expression were even significantly enhanced 48 hrs post immunisation in group 2 compared to group 1 (Fig. 3.19).

Notably, the results show that FAM26F is as fast deregulated as the other components of the innate immune system (Mx1, IP-10), this also emphasises our observation of infection study suggesting FAM26F as an important player of innate immunity.



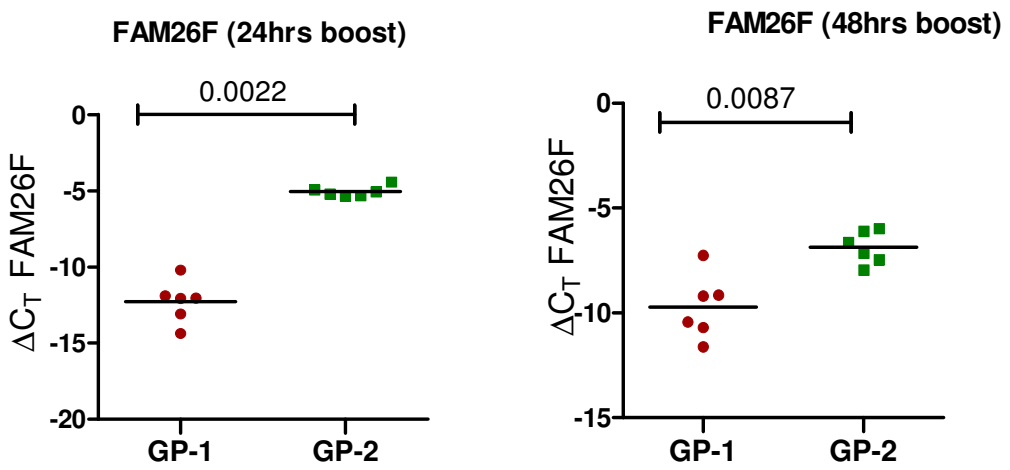


Fig 3.19: Significant differences of gene expression between group 1 (GP1) and group 2 (GP2) after immunization that was attributed to protection and vaccine efficacy in group 2.ΔCT was calculated mean C_T (GAPDH) - Mean C_T (target gene) so that higher Ct values correspond to higher expression values.

In the light of above mentioned analysis, we can say that different immunisation procedure had certainly different effects (statistically significant) on the innate immune response/activation. Interestingly this relates with the difference in set point viral load after infection of animals. In group 2 animals had lower viral load and greater protection against infection as compared to group 1 (Fig 3.20A). Moreover, numbers of challenges to infect the animals in group 2 were more than to infect the animals in group 1 (Fig 3.20B). It seems as if a different pattern in activation of components of the innate immune system (e.g. increased expression of IP-10, FAM26F and tetherin after boosting) has resulted in observed lower viral load and increased level of protection in group 2. It is also important to note that all the significant differences were only in vaccinees and not in control groups.

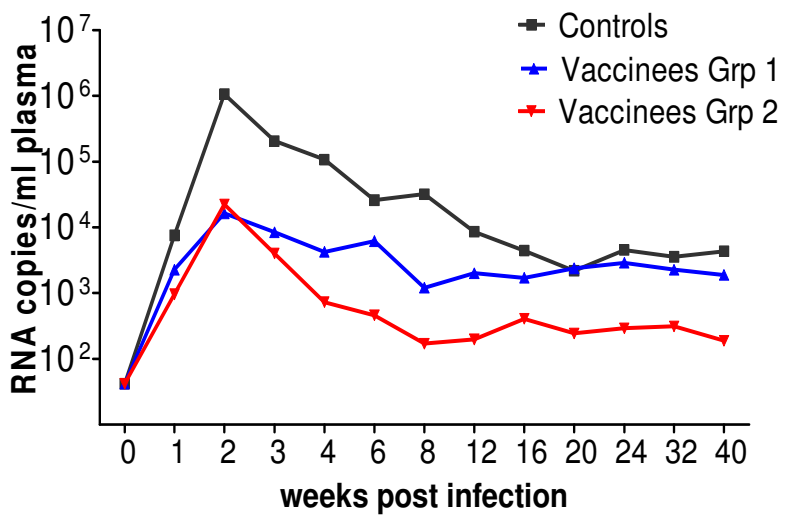


Fig 3.20A: Viral load of three infected groups over time of 41wpi.

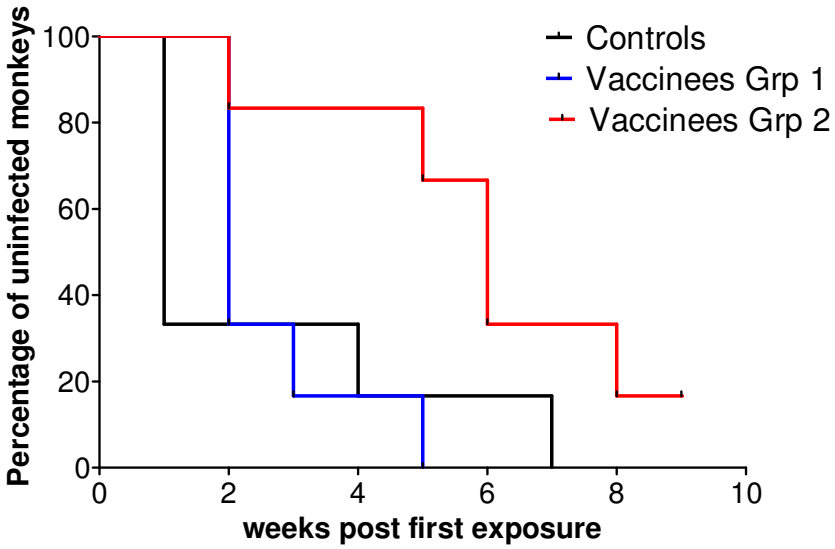


Fig 3.20B: Fraction of uninfected animals in controls and both vaccine groups during the course of challenge.

3.9 FAM26F correlation with other immune components

Furthermore the expression of FAM26F and its correlation with the expression of other genes (Mx1, IP-10, tetherin) was also investigated. FAM26F was significantly correlated with all

three genes under investigation Mx1, IP- and tetherin in both independent experiments (Fig 3.21). However its strongest correlation was found with IP-10. These observations were further confirmed by *in vitro* experiments (see below).

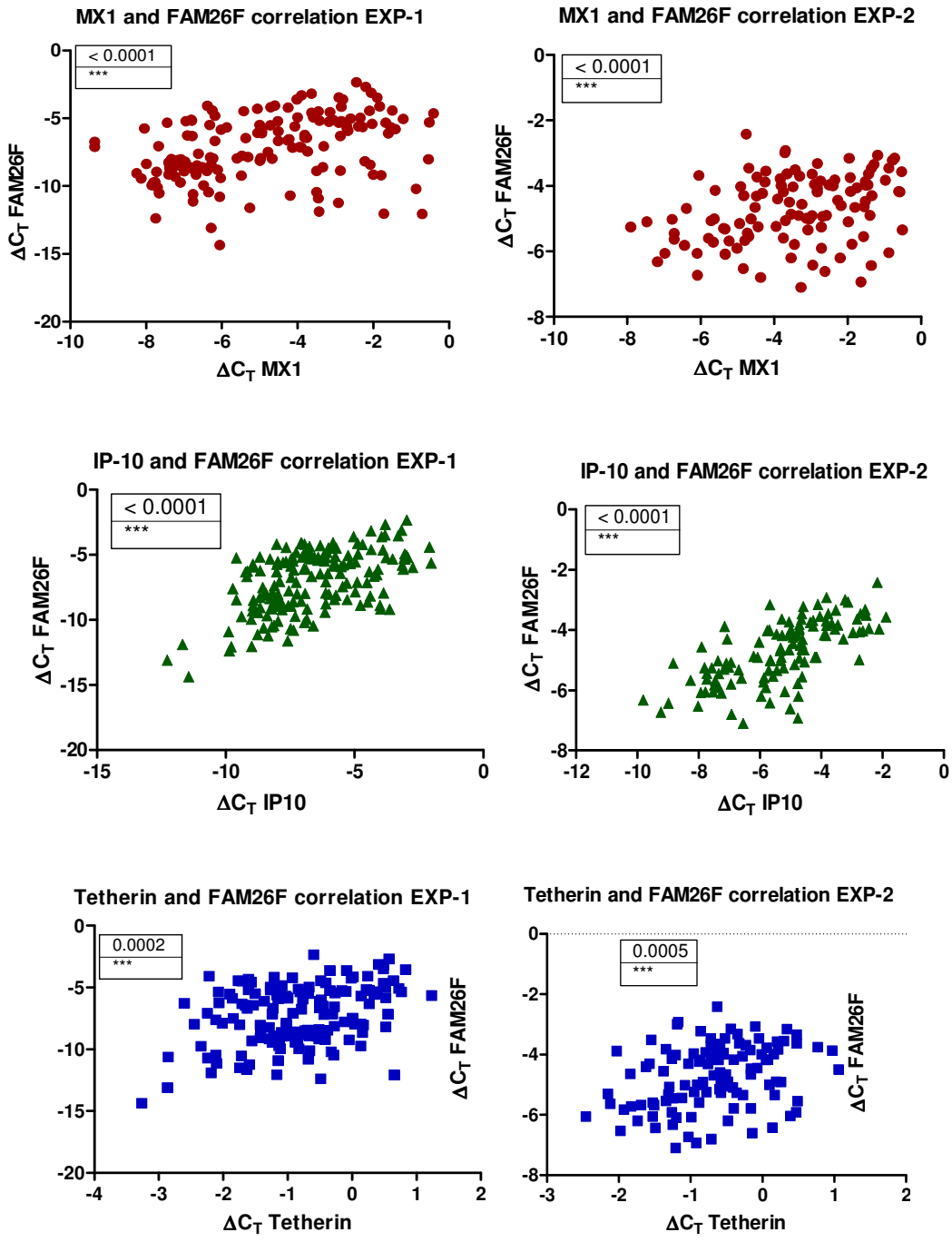


Fig 3.21: Correlation of FAM26F with (A) Mx1 (B) IP-10 and (C) tetherin RNA levels in PBMCs from two independent vaccine experiments.

3.10 Expression of FAM26F in different lymphocyte populations:

FAM26F expression was quantified by qRT-PCR in different cell populations including $CD8^+$, $CD4^+$ and $CD20^+$ (B cells) along with Mx1, tetherin, and IP-10 in 4 animals. Results are expressed as copies per 100 copies GAPDH using following formula(Pfaffl, 2001)

Copies per 100 copies of GAPDH (rE) = $1/(2\ \Delta Ct) * 100$

Maximum expression of FAM26F was observed in CD4 cells. Mx1 and IP10 were highly expressed in CD20 while highest levels of tetherin were found in CD8 + T cells (Fig3.22).

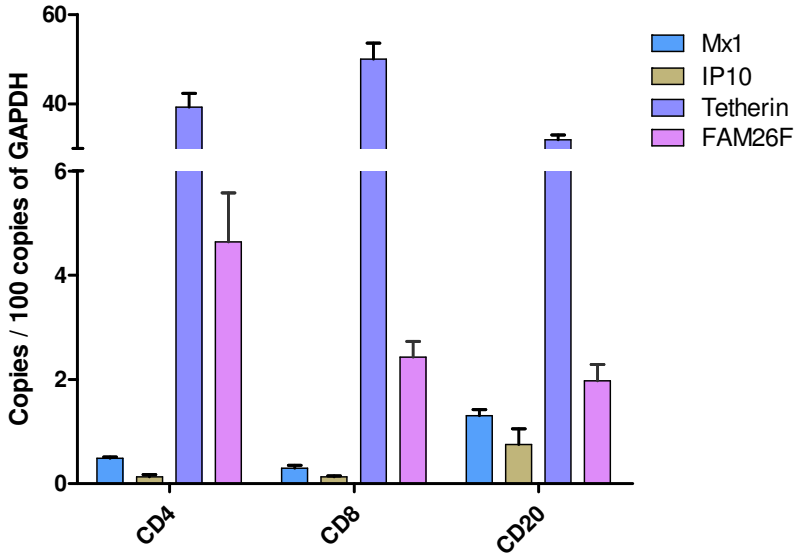


Fig 3.22: Expression of FAM26F in different cell types in four independent experiments.

3.11 *In vitro* activation studies of FAM26F

In order to investigate the effect of Interferon alpha (INF-alpha), Interferon gamma (INF-gamma) and Tumor necrosis factor alpha (TNF-alpha) stimulation on the expression of FAM26F, *in vitro* PBMC stimulation experiments were performed as described in 2.14. RNA expression was measured after 6, 12, 24 and 48 hours after stimulation. Δ Ct was calculated as follows

$\mathbf{\Delta\ C_T}$= Mean $\mathbf{C_T}$ (target gene) - mean $\mathbf{C_T}$ (GAPDH).

$\Delta\Delta\mathbf{C_T}$ was calculated as

$\Delta\Delta C_T = \Delta C_T$ of candidate gene in interferon stimulated samples - ΔC_T of respective gene in control samples.

Differential expression in all graphs is presented in terms of Fold changes calculated as

Fold change = $2^{\Delta\Delta CT}$

Interferon Alpha stimulation resulted in 100fold increase in Mx1 expression, as expected since Mx1 is an interferon alpha regulated gene. IP-10 was 13fold while tetherin and FAM26F were increased 4fold and 12 fold respectively after interferon alpha stimulation. Expressions of all genes under investigation increased 6 hours post stimulation. Tetherin and FAM26F levels continued to increase and reached their maximum levels 12 hours post stimulation. IP-10 levels started to decline 6 hrs post stimulation while Mx1 levels declined after 12 hours (**Fig 3.23A**).

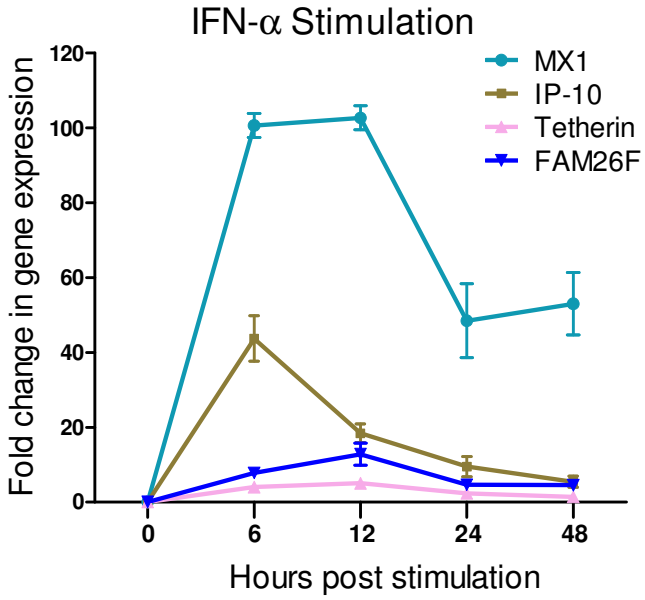


Fig 3.23A: Changes in the expression pattern of selected genes after 100ng of Interferon-alpha (INF-α) stimulation in three independent experiments.

Interferon Gamma stimulation resulted in maximum increase in FAM26F expression as compared to Mx1, IP-10 and tetherin. Expression of all the genes under investigation increased 6 hours post stimulation and returned to baseline 48 hours post stimulation (Fig 3.23B).

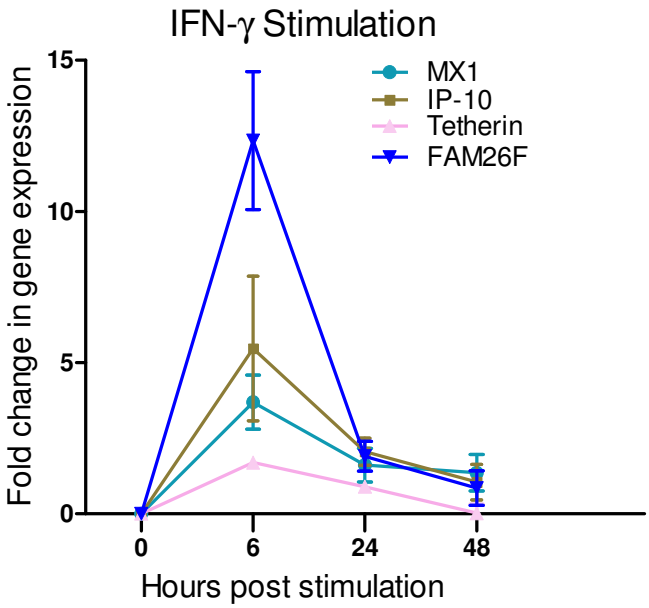


Fig 3.23B: Changes in the expression pattern of selected genes after 10ng of Interferon-gamma (INF-γ) stimulation in three independent experiments.

PBMCs were stimulated with 10, 50 and 100ng of **tumor necrosis factor alpha** but no significant effect in any gene of interest was observed after 6 hours of stimulation (3.23C). Further investigations were therefore dismissed.

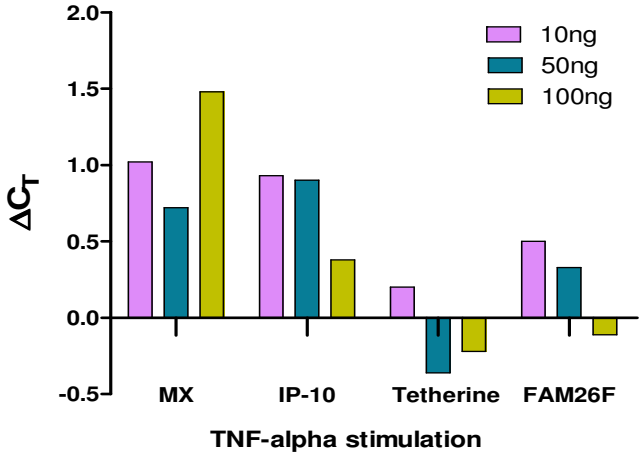


Fig 3.23C: Changes in the expression pattern of selected genes after 10, 50 and 100ng of Tumor necrosis factor-alpha (TNF-α) stimulationafter 6 hours of stimulation

3.12 Establishment of permanent monkey T cell lines.

After confirming the differential expression of FAM26F by Real time quantification, we planned to continue further investigations of this membrane bound protein with FACS analysis or Western Blot. But unfortunately till date no antibody against FAM26F for rhesus macaques (*Macaca mulatta*) is available. Shortage and lack of standardized material is a key drawback when working with primary cells, especially from monkeys. A study stating that noncytotoxic suppression of human immunodeficiency virus type1 transcription is possibly mediated by exosomes secreted from $CD8^+$T cells[150].We wanted to investigate the differential protein expression in exosomes secreted by $CD8^+$T cells of our $CNAR^+$ and CAF^- groups. For such type of studies there was a demand to establish immortal $CD8^+$ T cell lines, as material from primary cells is not enough for such type of study. So we decided to establish these $CD8^+$ T cell lines from our already characterized $CNAR^+$ and $CNAR^-$ group of animals.The insertion of oncogenes to immortalize various mammalian cell types [207] had resulted in altered cellular signalling pathways and a loss of normal cell-cycle functions [208]. Telomerase expression vectors, used to immortalize normal human fibroblast and epithelial cells have been proven a better option in this regard [209]. The ability of human telomerase reverse transcriptase (hTERT) to extend cellular proliferative capacity by stabilizing the telomere length is well documented. Many cell types immortalized by hTERT did not indicate cancer-associated changes and show normal cellular responses to DNA damage signals [210]. We undertook this strategy to establish permanent cell lines for our study. Murine leukaemia virus (MuLV)-based retroviral vectors can transduce dividing cells. Infecting cultures of antigen-stimulated PBMCs with MuLV-based vectors carrying immortalizing genes, results in the selective immortalization of T cells.

3.12.1 Principle

The retroviral vectors are widely used to stably integrate the gene of interest in target cells**.** Vector xlox (NGFR) TERT co-expresses the human telomerase gene (hTERT) and a surface marker, C-terminally truncated human low-affinity nerve growth factor receptor (NGFR). To facilitate the production of high viral vector titers, we used a producer cell line GP2xTERT11 carrying stably integrated xlox (ΔNGFR) TERT vector. Thus to produce the retroviral vector, GP2xTERT11 cells are transfected with envelope construct (e.g. RD114). Anti-CD3 antibody-stimulated T cells are infected with these replication defective retroviral vectors.

The transduced cells co-express hTERT and a surface marker, ΔLNGFR, and thus can be easily purified by immunomagnetic cell sorting. TERT gene continuously produces telomerase resulting in immortalization of transduced cells while preserving the properties of primary cells.

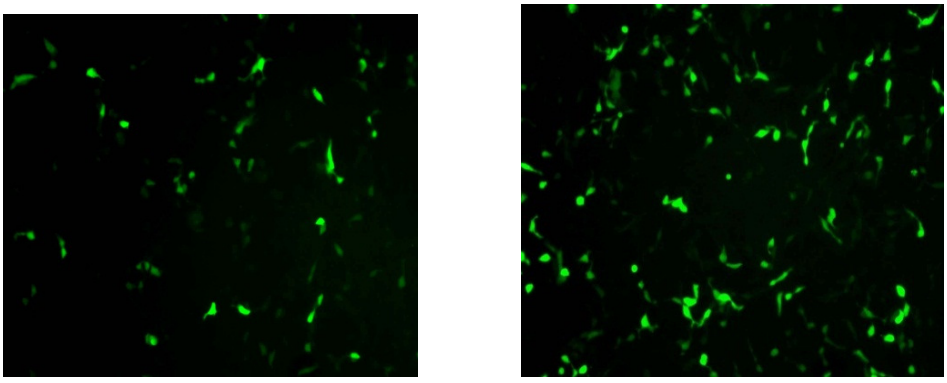
3.12.2 Vector stock preparation and titer determination

Before starting with the viral vector production, initial optimization of the transfection efficiency was done using different concentrations of GFP (green fluorescent protein) construct and transfection reagent at various incubation times were tested with GP2xTERT11. Optimal transfection efficiency (30%) was obtained with 10µg of plasmid using 22µl of TransIt-293 transfection reagent (Fig: 3.24). To produce the viral vectors carrying hTERT gene, GP2xTERT11 cells were transfected with RD114 envelope construct and collecting the supernatant as described in section 2,15,2.

Before transducing the target primary T cells, exact titers of viral vector stocks were determined by retronectin mediated transduction of C8166 cells.48 hrs post-transduction, percentage of $NGFR^+$ cells was determined by FACS analysis as described in section 2,15,4. Non-transduced C8166 cells were used as negative controls for FACS analysis. On the basis of % positive cells, the viral titers were calculated as follows:

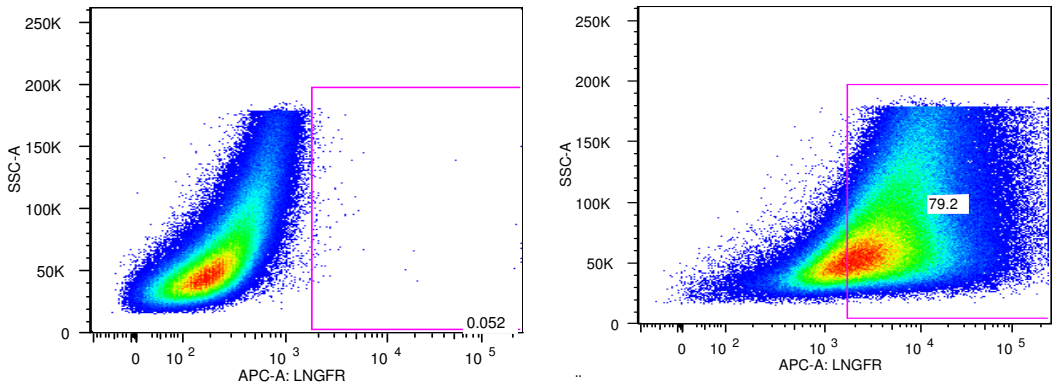
Number of viral vector particles = Number of cells initially plated × % of $LNGFR^+$ cells.

For most of viral vector preparations titers were in range of $5\text{-}9 \times 10^5$ viruses/ml



(3.24A) (3.24B)

Fig 3.24: GFP transfected GP2xTERT11 producer cell line (A) 24 hours post transfection (B) 36 hours post transfection.

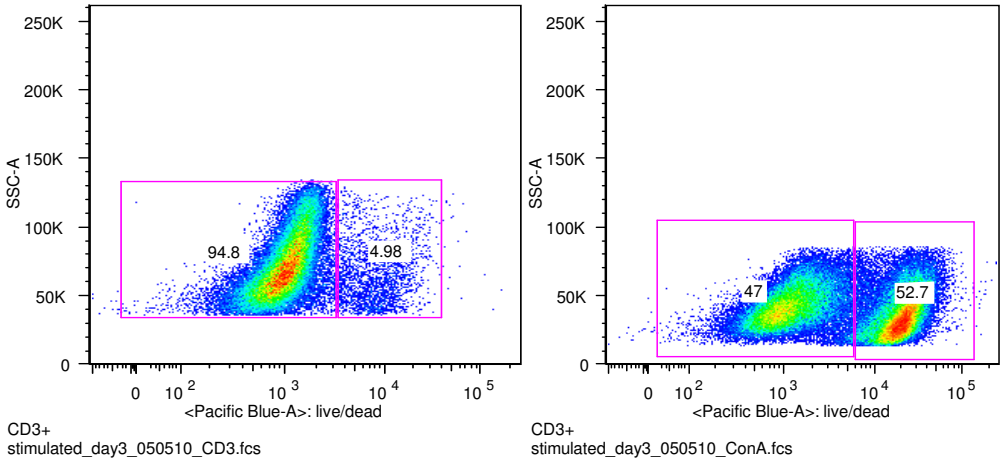


(3.25A) (3.25B)

Fig 3.25: FACS analysis of C8166 cells (A) non-transduced C8166 cells as negative control (2B) C8166 cells transduced with undiluted viral vector stock. 79.2% cells were LNGFR$^+$ which is equivalent to 7.9×10^5 viral vector particles/ml.

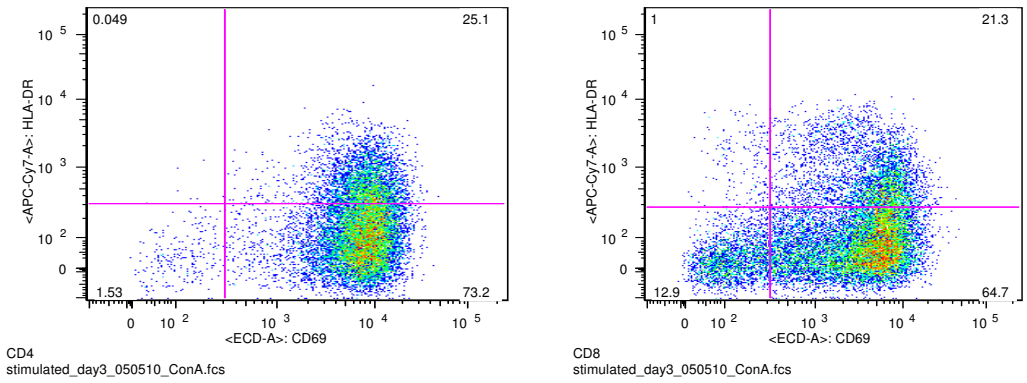
3.12.3 Activation of target primary T cells:

In our initial experiments Concanavalin-A (ConA) was used to activate the target primary T-Cells. But we were not able to get the desired transduction efficiency with ConA as a T cell stimulant. It was found that ConA activation leads to increased cell death (52.7% dead cells) as compared to plate bound anti CD3 antibody (4.98 % dead cells) (Fig 3,26A, B). This gave us an explanation of lower than the expected transduction efficiency. Latter on use of plate bound anti CD3 antibody in subsequent experiments lead us to improved transduction rate. With ConA stimulation, 25.1% and 21.3% CD69$^+$-HLA-DR$^+$ double positive cells were found on CD4$^+$ and CD8$^+$ cells populations respectively (Fig 3.26C, D). While 10.7% and 17.5% double positive cells were found on CD4$^+$ and CD8$^+$ cells respectively with anti CD3 activation (Fig 3.26E, 3.26F), indicating a slight difference in the activation markers CD69 and HLA-DR in both activations. This difference is most likely not affecting the CAF activity as anti CD3 antibody stimulation is used by others for viral inhibition assays [6].



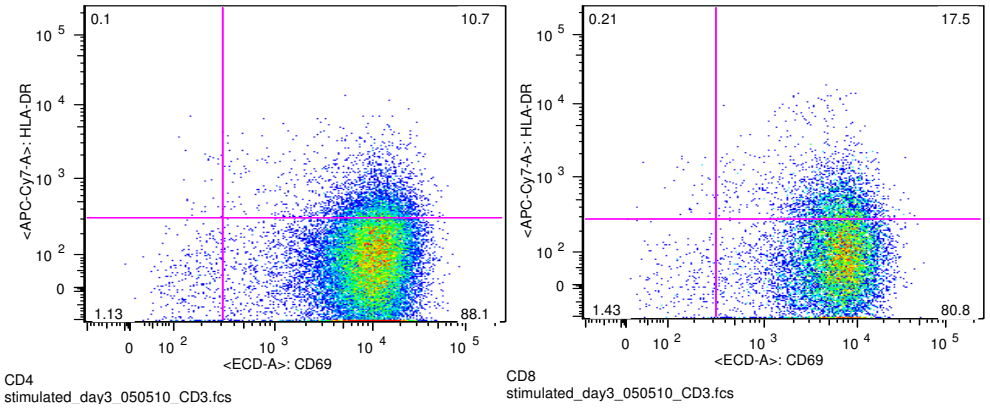
(3.26A) (3.26B)

Fig 3.26: Percentage of live/dead cells, after 48 hours activation of PBMC activation with (A) plate bound anti CD3 antibody (B) Concanavalin-A.



(3.26C) (3.26D)

Fig 3.26: Percentage ofCD69-HLADR double positive cells on $CD4^+$ cells (C) $CD8^+$ cells (D) after Concanavalin-A stimulation.

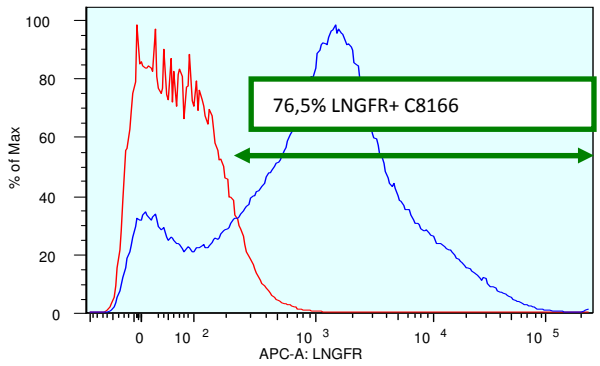


(3.26E) (3.26F)

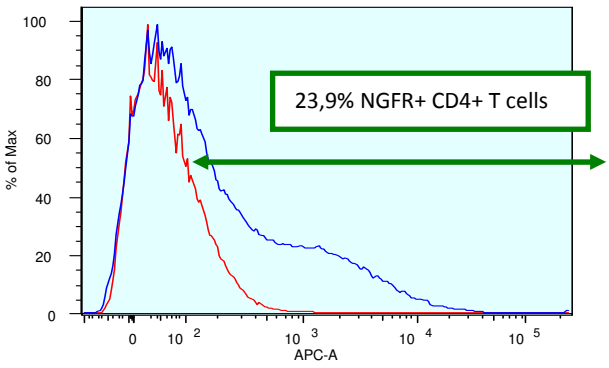
Fig 3.26: Percentage ofCD69-HLA-DR double positive cells on $CD4^+$ cells (E) $CD8^+$ cells (F) after plate bound anti CD3 antibody stimulation.

3.12.4 Transduction of target primary T-cells

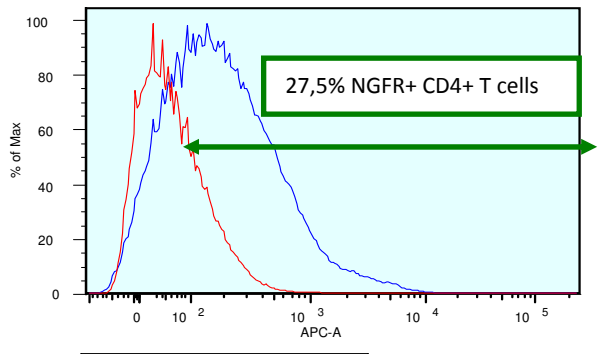
Blood was drawn from the target animal and $CD4^+$ T cells or $CD8^+$ T cells were isolated from the PBMC (section 2.2 and 2.3). Cells were activated by plate bound anti CD3 antibody (section 2.15.3) and transduced with the retroviral vector stocks whose titers were above 5×10^5 as described in section 2.15.4. Transduction efficiency of target primary T cells varied from 6-27%. We are able to get maximum of 27.5% transduction efficiency for CD4 cells and 23.9% for CD8 cells (Fig: 7B, 7C). $LNGFR^+$ cells were sorted by (MACS) and were cultured along with feeders as described in section 2,15,5.



(3.27A)



(3.27B)



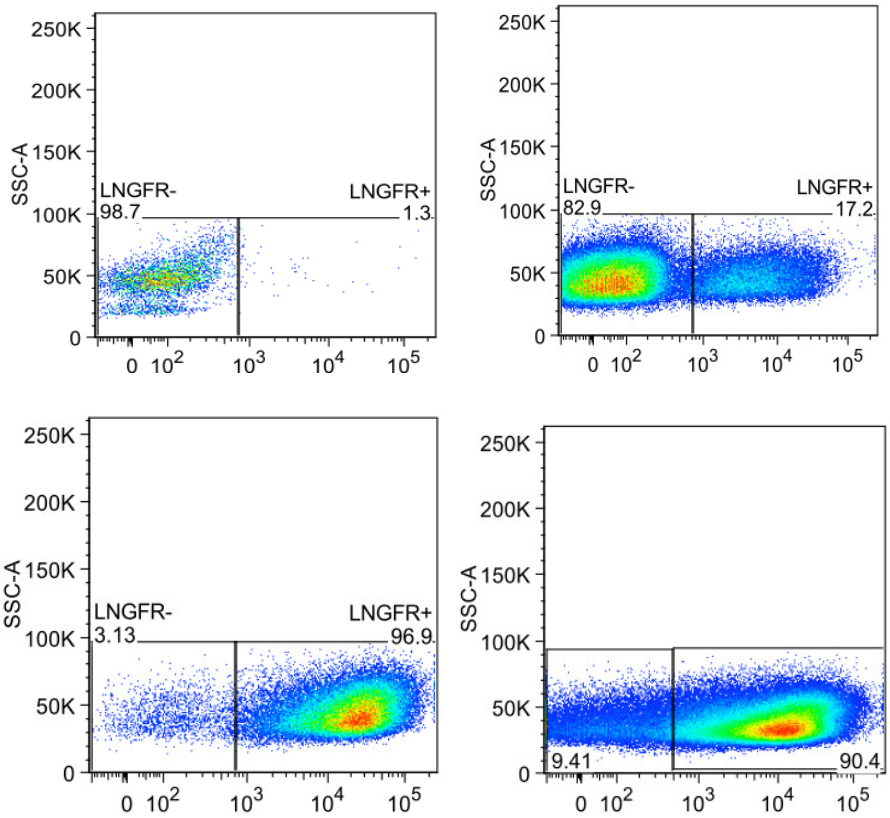
(3.27C)

Fig 3.27: FACS histograms of transduced $NGFR^+$ (A) C8166 positive control of transduction (B) Target primary $CD4^+$T cells (C) Target primary $CD8^+$ T cells. Red-lined histograms

indicate the non-transduced negative controls while blue-lined histograms are representatives of transduced cells.

3.12.5 Post transduction culturing

Once the transduced NGFR$^+$ cells were purified by (MACS), they were co-cultured with feeder cells. Cells were counted and split every second day. Cells were re-analysed for NGFR expression after every 3 days and In several experiments, the number of NGFR$^+$ cells increased (Fig: 3.28 representative of 1 experiment). We were successfully able to establish a CD4$^+$ T cell line that was propagated further for at least 3 months in culture and sufficient aliquots were stored at different passages. This cell line is in culture for more than 1 year now. Expression of different surface markers was analysed by FACS (fig 3.29). Although we were able to efficiently transduce the CD8$^+$ T cells and LNGFR expression was more than 90% at d9 (fig 3.28) these cells showed a problem with the post culturing and were not able to survive in culture for more than 3 weeks.



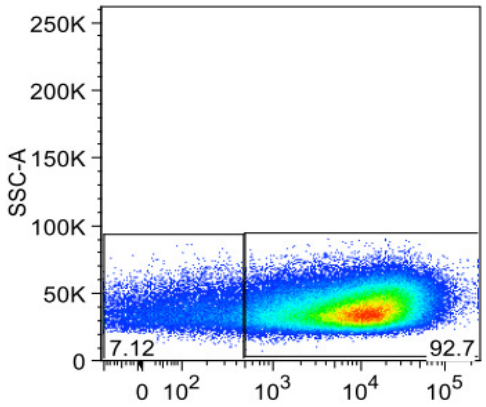
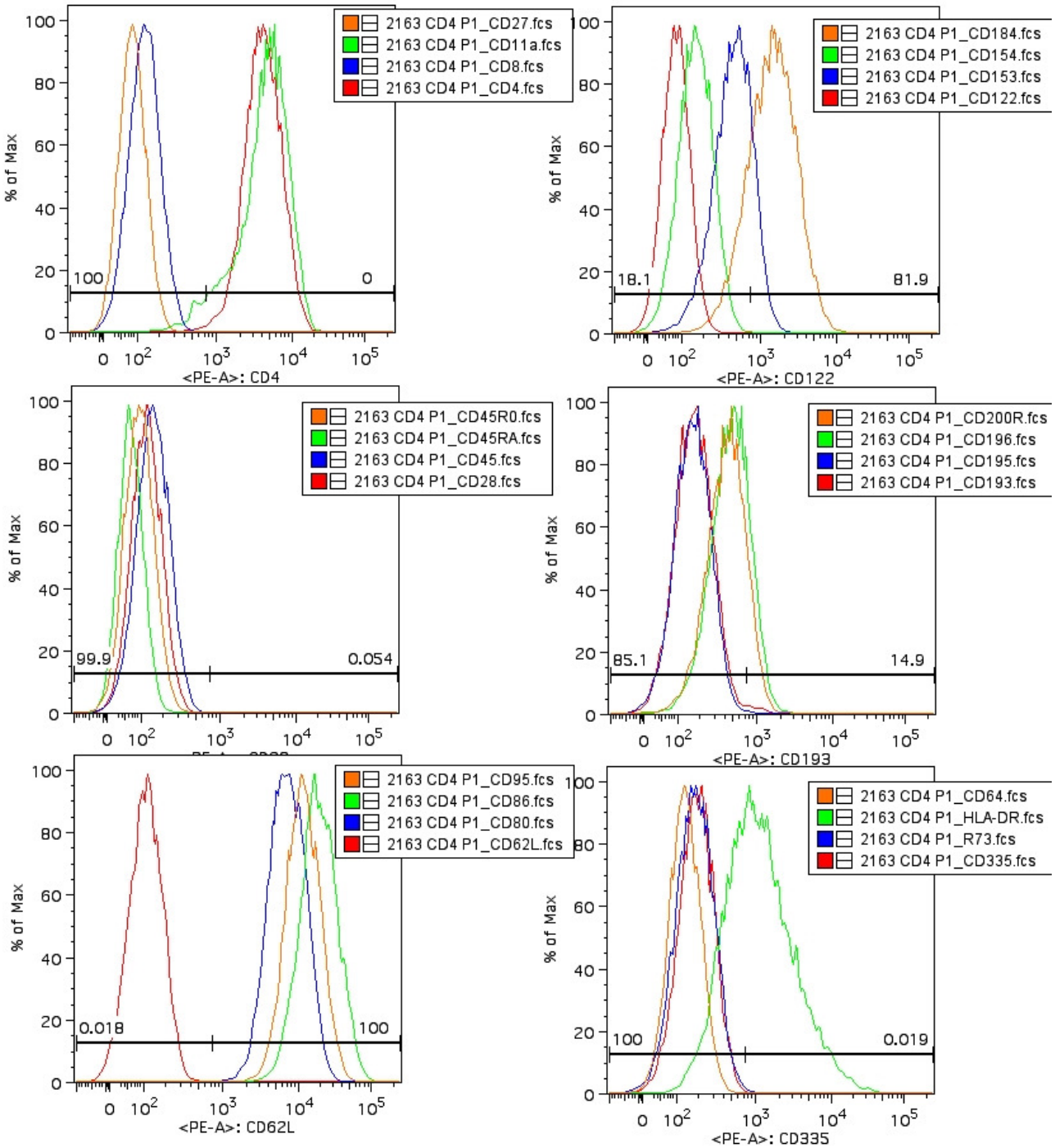


Fig 3.28:FACS dot plots of $CD8^+$ T cells showing percentages of $LNGFR^+$ cells in (A) controls (B) Transduced d1 (C) d3 of culture (D) d6 of culture (E) d9 of culture.

3.12.6 FACS analysis of CD4 cell line



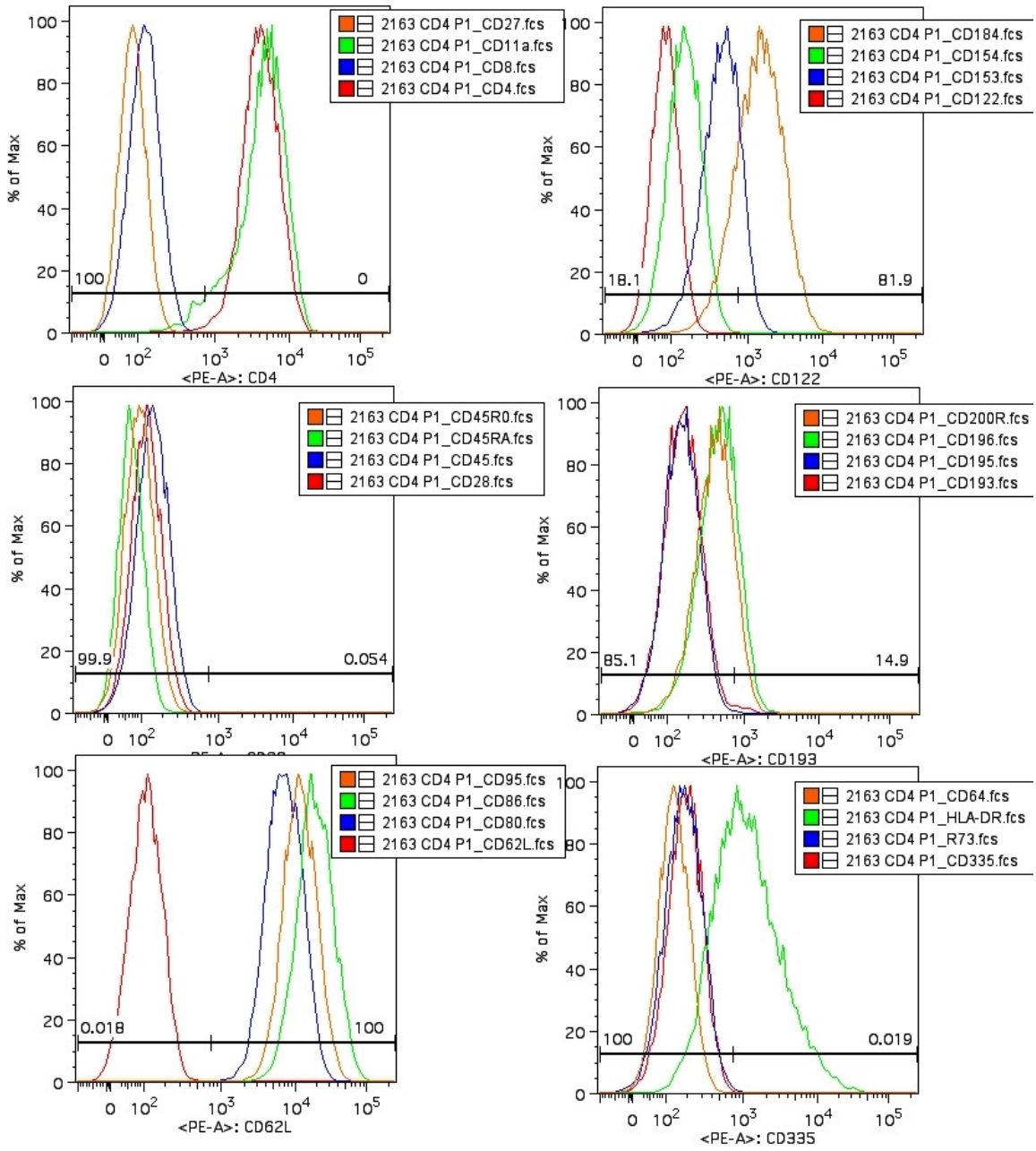


Fig 3.29:FACS chromatograms of $CD4^+$ T cell line showing expression of different cell surface markers.

Discussion

HIV infection usually results in a progressive immunodeficiency disease culminating in the development of AIDS however a small subset of infected individuals, known as elite controllers has been described to escape disease progression for prolonged periods. Strong immune responses and low virus loads in these individuals [211] raise the possibility that specific and/or particularly effective antiviral immune responses may confer long-lasting protection.

In addition to their MHC class I-restricted antigen-specific anti-HIV-1 CTL effector function, noncytolytic suppression of HIV-1 via secretion of soluble factors (CNAR) has also been described for more than 2 decades. It is now apparent from the results of many laboratories that $CD8^+$ T cells from asymptomatic infected individuals can potently inhibit HIV-1 replication through noncytolytic mechanisms. However, the nature of this inhibitory activity has not been discovered yet and its role as a component of protective immunity has remained controversial.

To this end our study focused on the identification of these factors responsible for noncytolytic viral suppression of virus. For this purpose SIV-infected rhesus macaques were used as animal models. At first *in vitro* viral inhibition tests were performed to identify $CNAR^+$ and $CNAR^-$ animals. Notably, CNAR activity of the $CD8^+$ T cells was tested not only from SIV-infected long term non- progressors (LTNPs) but also from the non-infected animals. So for the first time it was shown that $CD8^+$ T cells of the non-infected animals can display CNAR activity. However, the extent of inhibition is lower in non-infected than in SIV-infected monkeys.

We also observed that $CNAR^+$ LTNPs had on average a lower in vivo viral load as compared to $CNAR^-$ LTNPs. This observation is in line with the observation made in HIV-infected humans where an inverse relation between the extent of this CD8 non cytolytic antiviral response and level of plasma viraemia was documented [138].

It is well documented that a decrease in CNAR activity is found to be associated with increase viral load and disease progression in HIV-infected humans [128]. Similar results were found in our study where the loss of CNAR activity preceded the increase in viral load of two animals (12672, 12536). It is difficult to conclude if the emergence of virus is solely due to loss in CNAR activity. Presence of genetically similar animals (half-siblings) in $CNAR^+$

group of LTNPs and noninfected macaques indicated that CNAR activity can partially be an inherited trait.

In order to identify the genes mediating CNAR, differential gene expression between $CD8^+$ T cells from $CNAR^+$ and $CNAR^-$ monkeys was evaluated from infected as well as non-infected groups. This was two pronged strategy that not only allowed us to identify the differentially expressed genes among $CNAR^+$ and $CNAR^-$ animals but also helped us to find the differential gene expression among infected and non-infected $CD8^+$ T cells. Furthermore, this allowed double screening for the candidate genes. As few genes were found to be differentially regulated in infected groups but only those genes were considered that showed a significant differential expression in both infected and non-infected group on animals. Almost 78 genes were found to be differentially expressed among $CNAR^+$ and $CNAR^-$ animals. They are involved in a wide variety of cellular processes including immunity, signaling, metabolism and cell cycle etc. 16 genes were selected for further validation by real time qPCR. The selection was based not only on the extent of differential expression (>2 fold) but also on the immunological importance of the genes and their relevance to the already defined characteristics of CAF. For example CAF is reported to have a protease activity as the antiviral effect is diminished by protease inhibitors. In our selected genes three members (Chymase, PRSSL-1 and MMP25) were proteases in nature and were more than two fold differentially regulated in the initial microarray study.

Evaluating the differential expression of $CD8^+$ T cells at three different levels i.e. un-stimulated, ConA activated and co-cultivated with infected CD4, helped us to clearly evaluate if observed differential expression was intrinsically present in $CD8^+$ T cells of $CNAR^+$ and $CNAR^-$ animals or if it had emerged during co-cultivation of the cells. The results showed that none of the selected genes was differentially expressed after isolation from blood or after Con-stimulation.However, after co-culture with SIV-infected cells one gene, namely FAM26F, was found to be significantly overexpressed in $CNAR^-$ animals as compared to $CNAR^+$ animals in both, infected and non-infected group of animals (Fig 3.6C). Since this differential expression was not significant in un-stimulated and ConA activated $CD8^+$ T cells, FAM26F was up-regulated in $CNAR^-$ samples to larger extent than in $CNAR^+$ samples as a result of their co-cultivation with $CD4^+$ T cells during viral inhibition test (Fig 3.7B).

4.1 Difference between $CD8^+$ T cells from infected and noninfected animals

As mentioned earlier our microarray study enabled us to identify the genes differentially expressed between $CD8^+$ T cells of infected and non-infected animals. 3 genes, namely SLAMF8, (signalling lymphocytic activation molecule), the proteinases CHYMASE, and PRSSL1 were found to be significantly up regulated while TNFSF13B, and EPHB1 were found to significantly down regulated in SIV-infected animals as compared to non-infected macaques. SLAM-related receptors (in presence of SAP adaptors are reported to be involved in stimulatory signalling for B cell signalling while in absence of adaptors mediate inhibitory signals. Chymase and PRSSL1 are serine proteases and reported to be involved in various physiological functions. TNFSF13B is reported to be involved in B cell activation and its reduced level has been associated with prolonged time to AIDS in HIV-infected viremic patients [212]. Furthermore its expression distinguished pathogenic (rhesus macaque model) from non-pathogenic (sooty mangabey model) SIV-infection [212]. EPHB1 is reported to be implicated in development of nervous system. Although up-regulation of these genes in $CD8^+$T cells from SIV-infected animal seems relevant in this context, further investigations will help to clearly define their exact function.

4.2 FAM26F expression after interferon stimulation

FAM26F was found to be expressed on $CD4^+$ T, $CD8^+$ T and B cells. *In vitro* stimulation with IFN- α induced slight increase in the expression of FAM26F after 6 hrs that diminished 48 hrs post stimulation. This increase was much less than the well-known IFN-α stimulated gene Mx1 (100fold vs. 12fold respectively). Stimulation with IFN-γ induced FAM26F expression greater than all genes under investigation i.e. Mx1, IP-10 and tetherin. IFN-γ mediated FAM26F induction was even greater than IP-10 which is a well-known IFN-γ responsive gene (12fold vs. 5fold respectively), indicating that FAM26F is an IFN-γ responsive gene. IFN-γ is a pro inflammatory cytokine involved in modulation of diverse immune responses. Similar to FAM26F it is produced by NK cells, activated CD4 helper cells, $CD8^+$ T and B cells. IFN-γ induced responses are very diverse including up regulation of MHC-1, MHC-II, PKR, ADAR1, GBPI and 2. IFN-γ elicits also apoptotic functions by upregulating proteins like IRF1, caspases, cathepsins, Fas and TNF alpha receptors. Antimicrobial mechanisms involve induction of ROS (reactive oxygen species) and many other genes [108]. Most important to note are the genes that are involved not in the cytolytic effector function but involved in the immunomodulation, Th development and leukocyte trafficking. These include IL-12,

CXCL10 (IP-10), CXCL9 (MIG), CCL3, CCL4 (MIP1 alpha and beta, RANTES, ICAM-1 VCAM-1. Most of the above mention members have been previously attributed to be secreted by $CD8^+$ T cells or represent membrane proteins and were studied specially in relationship to general viral inhibition. This may indicate that FAM26F could play an important role in diverse IFN-γ responses, both in innate and adaptive arms of immunity. We have investigated FAM26F expression in whole PBMCs; however further studies focusing on the IFN- γ mediated FAM26F induction in different subpopulations (e.g. CD4, CD8. NK and B cells) will be a step forward to fit it in diverse range of INF - γ functions.

Identification of FAM26F as a protein that distinguishes $CNAR^+$ and $CNAR^-$ animals in the *in vitro* inhibition test, its regulation by IFN-gamma, and expression on major immune cells suggest that it plays a role in immunity. These findings lead us to investigate its expression pattern during infection and immunization.

4.3 FAM26F expression during infection

After finding FAM26F correlation with viral load in a small cross sectional study, the expression pattern of FAM26F before and after infection was investigated in two independent experiments. The monkeys have been infected with SIVmac239 by a so-called repeated low dose challenge that is supposed to depict the "natural" infection more closely than a single high dose challenge[213].

Previous studies indicate that an increased expression of innate immune modulators in blood and other tissues (e.g. LN and lungs) during acute and chronic SIV infection is correlated with viral load. Increased Mx1 mRNA levels in lymphoid tissues [214] and IP-10 (CXCL10) in lymph nodes as well as peripheral blood of chronically infected macaques are associated with higher viral RNA levels and more rapid disease progression [203]. Similar results were observed in our study where expression of innate modulators (e.g. Mx1, IP-10) as well as FAM26F was significantly increased after infection. This elevated expression was maintained till the end of investigation period in both experiments (48 wpi for Exp1 and 24 wpi for Exp2). Furthermore, FAM26F followed same *in vivo* expression pattern after infection as Mx1, IP-10 and tetherin. FAM26F was also found to be significantly correlated with Mx1, IP-10 and tetherin. Correlation of FAM26F and IP-10 expression was expected because both are IFN-γ-regulated/induced genes. All these observations emphasize that FAM26F could also be a possible candidate player in immune system, most probably of innate immunity.

Results of our study also showed that in both experiments Mx1, and IP-10 were significantly correlated with viral load. These results are in agreement with afore mentioned studies for Mx1 and IP-10. For the first time a correlation between tetherin and viral load was found in our study.

In contrast, pre infection values of only FAM26F in experiment 2 were inversely correlated with 2, 12 and 24 wpi viral load. However this correlation was not significant (although there was a trend) in experiment 1 possibly because of two outliers. Furthermore, one has to keep in mind that the blood samples for Exp1 were drawn after the animals had been inoculated with virus, but did not become infected. In experiment 1, 2 weeks post infection expression of FAM26F was found to be positively correlated with plasma viral RNA copies at 12, 24 and 48 wpi (Fig 16). None of the other genes under investigation showed this correlation. Unfortunately this could not be validated with data from experiment 2 because of non-availability of the samples. Nevertheless, both of these observations indicate that FAM26F may be an early predictor/indicator of viral load at set-point during the chronic phase of infection and can be considered as early prognostic marker for SIV/HIV infection. Further investigations using larger number of animals are required to validate these findings as well as samples from HIV-infected humans.

It should be noted that initially FAM26F expression was found to be correlated with viral load in a small cross sectional study. However; the results were not reproduced later on. This could be explained by the fact that former study was conducted in a small group of Chinese macaques, which have a slower disease course as compared to macaques of Indian origin. This sub-species difference may have led to contrasting results.

4.4 FAM26F expression upon immunization

As explained earlier in section 3.8, immunization study included two groups. Group 1 was primed (final priming) with an adenovirus-derived vector followed by a boost with a fowlpox virus-derived vector, whereas the procedure was vice versa for group 2. Control animals for each group were “immunized” with so-called empty vectors.

Expression of already defined surrogate innate immune markers Mx1 and IP-10, a viral restriction factor tetherin and new possible candidate of innate immunity, FAM26F were studied before immunization, 24 and 48 h after final priming, after the boost and at the day of 1st challenge. In both groups, expression of all the genes under investigation showed non-uniform fluctuations upon final priming as compared to pre immunization values (fig. 18).

The empty vector control groups also showed such fluctuations indicating that virus-derived vectors may also stimulate innate immunity. However, the final boosting resulted in marked differences between the two groups.

For example, expression of Mx1 in group 1 boosted with fowlpox virus-derived vector was more variable and more persistent as compared to group 2 boosted with Adenovirus-derived vector. In contrast group 2 Mx1 expression was more uniform, peaked 24hrs post boosting and declined earlier in comparison to group 1. Thus fowlpox virus-vector induced a stronger Mx1/IFN-α response in some animals as compared to boosting with Adenovirus-derived vector. In contrast, up-regulation of IP-10, tetherin and FAM26F was more pronounced and less divergent in group2 boosted with Adenovirus-derived vector and peaked 24hrs post immunisation unlike group 1 boosted with fowlpox-derived vector where it peaked 48hrs post immunization. There was a significant difference in elevated expression of IP-10 (24hrs post boosting), FAM26F and tetherin (24 hrs and 48hrs post boosting) in vaccinees of group 2 (Adenovirus-derived vector group) as compared to group1 (fowlpox-derived vector group). This difference is notable in the context that group 2 had lower viral load and increased level of protection as compared to group1 (Fig 3.19).

As the expression of Mx1 and tetherin is driven by IFN-α while those of IP-10 and FAM26F is induced by IFN-γ, we can extrapolate that both groups differed in terms of level/extent of type I and type II responses. The increased level of protection in group 2 can be attributed to more pronounced IFN-γ responses or a unique balance between type I and type II responses, which may have caused a differential activation of the innate cells and finally to differential adaptive immune responses.

FAM26F expressions pattern during immunization and after SIV-infection similar to Mx1, IP-10 and tetherin. These observations emphasize the importance of FAM26F in innate immune system. FAM26F (family with sequence similarity 26, member F) is member of FAM26 gene family. It has been also named as IRF-3–dependent NK-activating molecule (INAM) by Tskashi and coworkers [215] . Humans and mouse have 6 members in FAM26 family. In human, 3 members of the family FAM26A/CALHM3, FAM26B/CALHM2, and FAM26C/CALHM1 are located on chromosome 10 (19 in mouse), while 3 members FAM26D, FAM26E, and FAM26F/INAM are on chromosome 6 (10 in mouse). In *Macaca mulatta* only 3 members FAM26D, FAM26E, and FAM26F are present on chromosome 4.[215]

Human FAM26F is a 315 amino acid 34 kD (without phosporylation) protein (in mouse: 40–55-kD protein with one N-glycosylation site) and possesses four trans-membrane motifs aspredicted by the TMHMM Server (version 2.0) (Fig: 4.1) similar to the cell adhesion tetraspanins, which may support cell–cell contact (Levy and Shoham, 2005). Alignment of the predicted *Macaca mulatta* and mouse FAM26F amino acid sequence with that of the human FAM26F revealed that both shared 88.57 % and 71.7% homology respectively. (Fig: 4.2). FAM26F does not have a normal signal peptide as predicted by CBS SignalIP 4.0 prediction Server (http://www.cbs.dtu.dk/services/SignalP/).

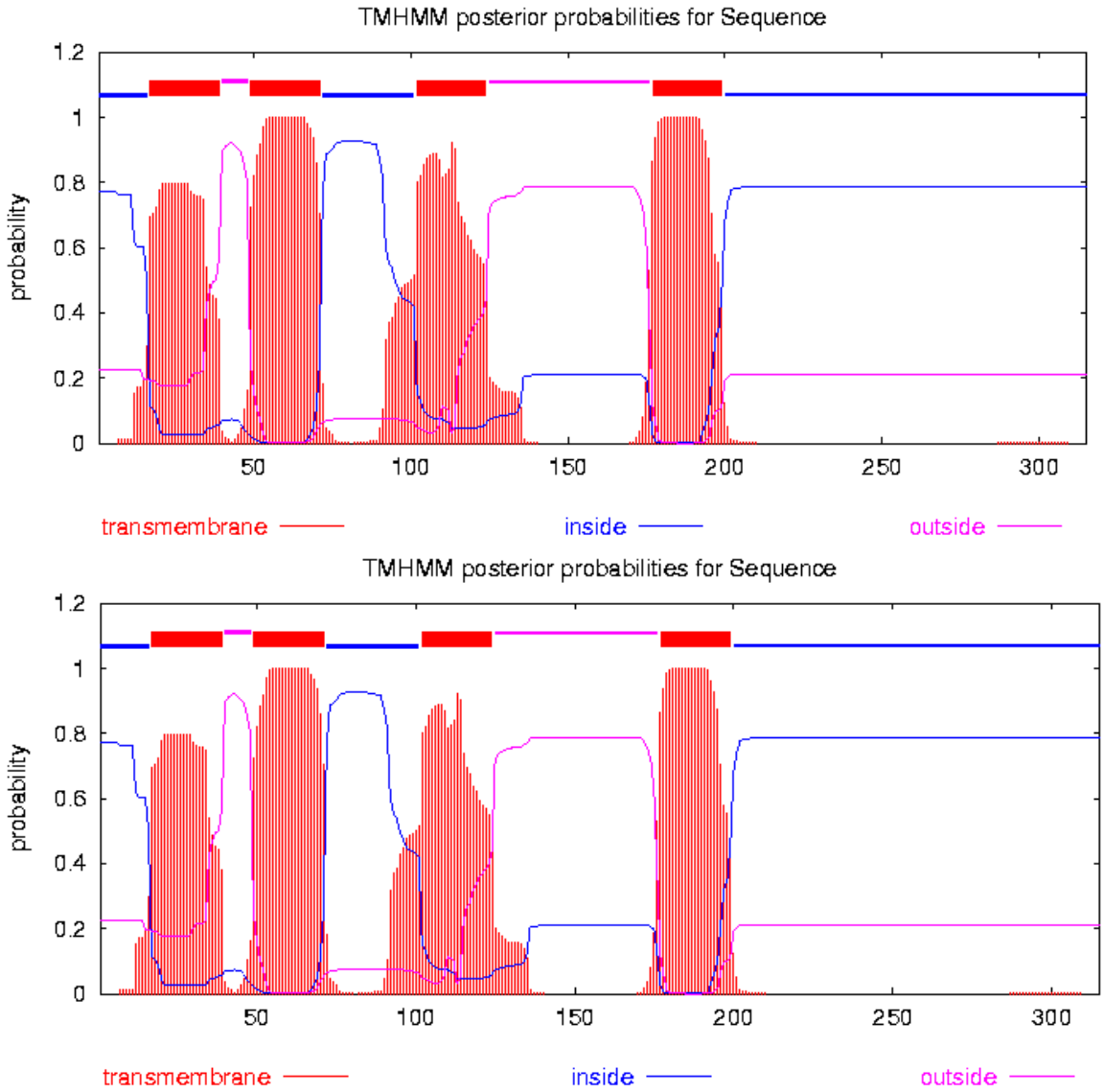


Fig 4.1: Graphic representation of probability of FAM26F transmembrane sequence motifs in (A) *Homo sapiens* (B) *Macaca mulatta* as predicted by TMHMM Server (version 2.0) .

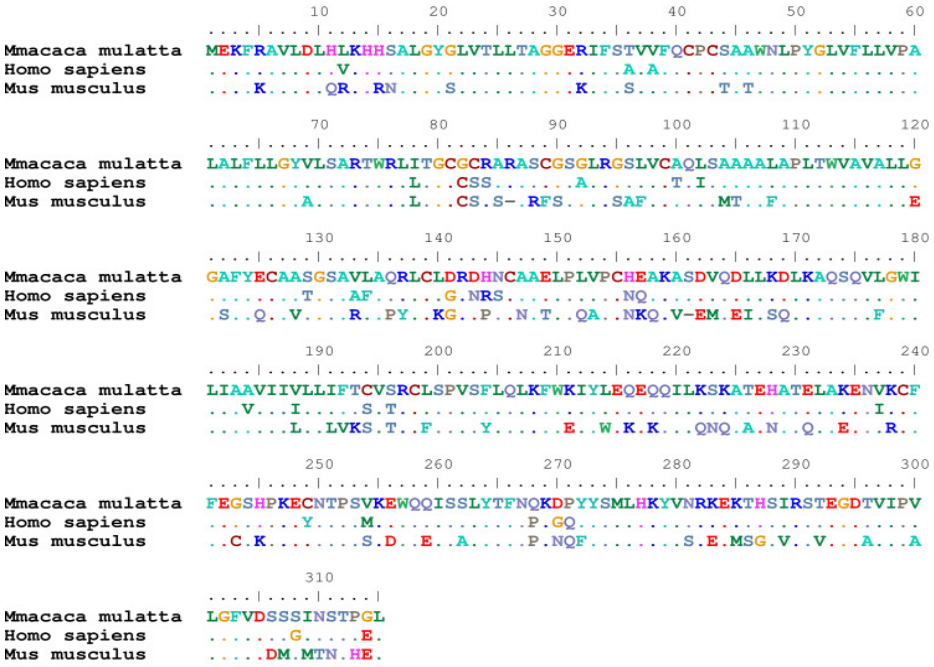


Fig 4.2: Sequence alignment of human, monkey and mouse FAM26F.

Thus it is potentially transported differently to the cell membrane as compared to classical endoplasmic reticulum-Golgi route. One suggestive pathway for the proteins that lack signal peptide FAM26F (e.g. IL-1β) is via intracellular vesicles. Thus it is possible that FAM26F may also be secreted via similar pathways.

Although exact function and its modulatory pathways are not known for FAM26F, there are a lot of studies documenting a role of FAM26F. For example, in an attempt to identify global protein responses of IFN-γ and LPS-treated primary human macrophages, FAM26F along with several other proteins was identified as activation-specific fingerprint that distinguishes primed macrophages from basal (resting) or LPS-activated macrophages [216]. Villitis of unknown etiology (VUE) is a destructive inflammatory lesion of villous placenta characterized by co-presence of histoincompatible (placental macrophages) and maternal T cells leading to placental inflammation. In an attempt to investigate the placental transcriptome of VUE, FAM26F was found to one of the most significant up-regulated along with several chemokines, MHC class I and class II molecules [217].

Another study focusing to identify gene signatures associated with early liver graft failure documented FAM26F among top classifier genes along with other genes functionally associated with oxidative stress and inflammation [218]. Genome-wide transcriptional profiles from the whole blood of patients with septicemic melioidosis (infectious disease caused by

the Gramnegative bacillus Burkholderia pseudomallei) identified FAM26F also as a differentially expressed gene [219]. In addition, FAM26F was also found to be induced by staphylococcal superantigens (SAgs) treatment of PBMCs. [220]. The Atlas database for gene expression of unknown genes (Atlas Data or G2SBC database) reports that FAM26F has been identified to be differentially expressed in cancer micro-array studies of various organs of the human body, including the breast, cervix, mammary gland, uterus etc. [221, 222][221, 223]. Song and co-workers associated FAM26F with potential cell growth, proliferation, and differentiation during synthetic estrogen analog diethylstilbestrol (DES) induced oviduct development in chickens [224]. All these aforementioned studies suggest an important role of FAM26F during infection, immunity or cell differentiation.

So far there is only one publication reporting some insight into the function of FAM26F in mice. Takashi and co-workers identified FAM26F as a TLR signal-derived membrane molecule that modulates mDC–NK contact-mediated NK activation[215]. They named it INAM (IRF-3dependent NK-activating molecule) and showed that it was initially minimally expressed on myeloid dendritic (mDC) and NK cells but co-culture of mDCpretreated with the TLR3 ligand polyI:C and natural killer (NK) cells resulted in an increased expression of FAM26F on both cell types. FAM26F induction was dependent on TICAM-1 and IRF-3 activation, as TICAM-$1^{-/-}$or $IRF3^{-/-}$knockout mDC failed to induce full NK cytotoxicity. They further showed that FAM26F contributed to mDC–NK reciprocal activation via its cytoplasmic tail, which was crucial for the activation signal in NK cellsbut not for the maturation of mDC. The authors suggest furthermore that FAM26F is an IFN type I-inducible gene. However, activation of FAM26F results in production of IFN-γ by NK cells. These observations do not contradict our study where we showed that *invitro* IFN-γ and IFN-α stimulation of PBMCs resulted in a strong and moderate FAM26F induction, respectively. Potentially,these interferons can induce the expression of FAM26F in auto or paracrine manner. Thus FAM26F may not only induce IFN-γ production but its expression can be also further enhanced by an auto feedback mechanism. Nevertheless, identification of the exact signal mechanism will be an issue for further analysis.

Further studies unfolding the functional aspects of FAM26F will shed more light on the relevance of this protein in the whole scenario of immune responses.

Although it is clear that FAM26F doesn't fit to the criterion to be a candidate for CAF as its properties are different than the CAF (e.g. size and protease activity) and it is higher expressed in $CNAR^{-}$ than in $CNAR^{+}$ animals, it is nevertheless possible that FAM26F acts

somewhere intermediate in the mechanism of CAF inhibition. It is possible that virus-infected CD4 cells induce the expression of FAM26F expression either directly or indirectly via interferons. Our microarray validation by qRT PCR showed that an increased expression of FAM26F on $CD8^+$ T cells from $CNAR^-$ animals might have resulted in strong viral replication. Increased/enhanced levels of FAM26F induced by virus can block the production of CAF either at transcription, translational or post translational stage. This inhibition can be achieved either directly, via trafficking of other proteins or activating other pathways that in turn can inhibit CAF production. In contrast, $CD8^+$ T cells from $CNAR^+$ animals may be able to suppress the FAM26F expression by certain unknown mechanisms so that CAF^- inactivation is suppressed. However these mechanisms are too much speculation and our hypothesis needs to be confirmed by further experiments. Unfortunately lack of availability of antibody against FAM26F has halted further research in this direction. Further studies to find the interacting partners of FAM26F will help to understand the function of this protein and its relevance in immune system. In summary, our *in vitro* stimulation as well as infection and immunization studies emphasize that FAM26F may be an important regulator of innate or adaptive immune response which may indicate an activation state of the immune system. FAM26F expression may be an early indicator/marker of the immune status that is especially important during primary infection. This could mean that stronger expression of FAM26F before infection may indicate an immune status that is able to limit early viral replication, whereas a strong increase after infection may indicate already early immune dysregulation that is later on associated with higher viral load. In this case FAM26F could be at least regarded an early diagnostic marker.

Irrespective whether FAM26F is involved directly in regulation of viral replication or indirectly via the immune defense, our study has shown that it is an important molecule that merits further investigation.

4.5 Phenotypic characterization of $CD4^+$ T cells

Expression of different markers on the surface of CD4 cell line was analyzed by FACS. Cells were $CD4^+$, $CD8^-$ and highly expressed CD11a, an antigen found on all leukocytes. Expression of the leukocyte common antigen CD45 was very low as was the expression of the T-cell markers CD3, TCRab, CD27 (none), CD28 and CD45RA. These moleculesare normally present on the naïve T cells and their expression is downmodulated afteractivation.

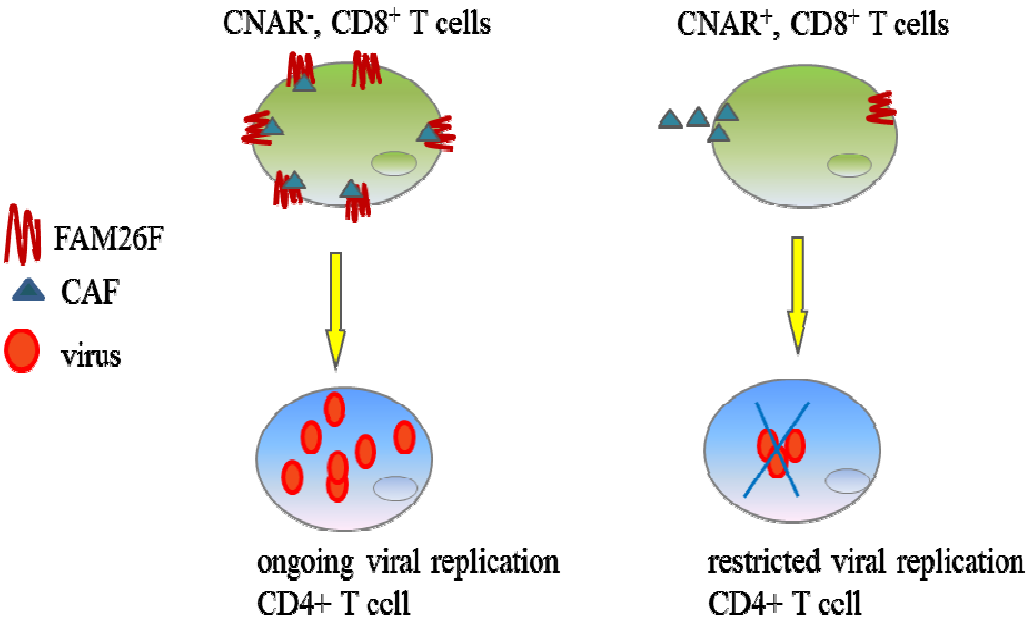


Fig 4.3: A hypothetical model for the mode of action of FAM26F. Increased expression of FAM26F induced by interferons results in the capture of virus inhibitory molecules/CAF in CNAR$^-$ cells. CD8$^+$ T cells from CNAR$^+$ animals are able to down regulate the expression of FAM26F and are able to release their viral inhibitory protein/s.

High levels of the co-stimulatory molecules, CD80 and CD86 that are usually only found on APC were found on the cells; however their expression is also reported in activated T cells. CD95 (TNFRSF6) adeath receptor, was also expressed at higher levels than the naïve cells. There was substantial expression of the chemokine receptors CD183 (CXCR3), CD184(CXCR4),CD196 (CXCR6),CD197 (CXCR7), whereas CD193 (CCR3), and CD195 (CCR5), wereonly weakly expressed. The immunoregulatory molecules CD153, CD154, CD200R, CD69 as well as HLA-DR were expressed at higher levels than on primary T-cells as were the cytokine receptors CD122 and CD127. There was negligible staining for the homing receptors CD49d, CD62, CD103 and integrin ß7. The NK-cell marker (not in rhesus) CD56 is not expressed.The expression pattern of these surface markers was homogenous indicating that the cell line is either monoclonal or the transduction process lead to the same phenotype. Expression of all above mentioned markers suggest that cells somehow represent an unusual activated phenotype. Furthermore the cell line grows well in absence of IL-2 and thus has the characteristics of a transformed CD4$^+$ Tcell line. This is the only rhesus macaque

T cell line available to date. It will be of high value for primate research, as it can replace the existing human T cell lines, e.g. used for propagation of SIV. Preliminary results indicate that this cell line is better infected with SIV strains (e.g. SIVagm) that otherwise poorly replicate on human T cell lines.

Summary and conclusion

A small subset of HIV/SIV infected individuals can control viral replication, do not exhibit immunosuppression or disease for prolonged periods and are termed as long term non progressors (LTNP). One element of the protective responseto HIV-1 infection is the $CD8^+$ T cell mediated noncytolytic antiviral response or CNAR. CNAR is associated with resistance to HIV infection among exposed seronegative individuals, long-term control of HIV-infection and reduced transmission of HIV. A lack ofmajor histocompatibility complex (MHC) restrictionand suppressionof HIV-1 replication in heterologous$CD4^+$ cell targets distinguish this antiviral mechanismfrom the classical cytotoxic activity of $CD8^+$ T cells. CNAR is reported to be mediated at least in part by production of a soluble $CD8^+$ cell antiviral factor (CAF). To date no $CD8^+$ cell-secreted cytokine, chemokine,or inflammatory molecule has been shown to identify with thehallmarks defining the HIV-1 transcription-suppressing activityof $CD8^+$ T cells. To this end, our study focused on the discovery of novel genes and elucidation of their biological functions contributing to CNAR activity by using SIV-infected macaques as animal models.

In order to identify the $CNAR^+$ and $CNAR^-$ animals, *in vitro* viral inhibition tests were performed on both infected and non-infected animals. For the first time it was shown that $CD8^+$ T cells of the non-infected animals (though to a lower extent than infected $CNAR^+$) can display CNAR activity. In line with the previous studies animals that lacked CNAR activity had a higher viral load, and furthermore, loss of CNAR activity preceded the increase in viral load in two of the investigated animals.

The gene expression profile of $CD8^+$ cells with high CNAR activity and $CD8^+$ cells that lack this antiviral activity were evaluated using microarray technology. Out of more than 50 differentially regulated genes, expression of 16 genes was validated by qRT-PCR. FAM26F was identified as a sole candidate that was significantly differentially expressed in both infected as well as non-infected group of animals. Furthermore this increased FAM26F expression in $CNAR^-$ $CD8^+$ T cells samples evolved during their co-cultivation with SIV-infected $CD4^+$ T cells during viral inhibition test (Fig 3.7B). FAM26F was found to be expressed on all three major cell populations (CD4, $CD8^+$ T cells and B cells). *In vitro* stimulation studies revealed that FAM26F expression was greatly induced in PBMCs after 6hrs of IFN-γ gamma stimulation, indicating that FAM26F could play an important role in diverse IFN-γ responses, both in innate and adaptive arms of immunity.

After finding FAM26F correlation with viral load in a small cross sectional study, the expression pattern of FAM26F before and after infection was investigated in two larger independent AIDS vaccine experiments. In both experiments, FAM26F expression increased after SIV-infection and followed same *ex vivo* expression pattern as Mx1, IP-10 and tetherin. FAM26F RNA levels were also found to be significantly correlated with Mx1, IP-10 and tetherin. All these observations emphasize that FAM26F could also be a possible candidate player in immune system, most probably of innate immunity. FAM26F in experiment 2 was inversely correlated with 2, 12 and 24 wpi viral load while in experiment- 1, 2wpi expression of FAM26F was found to be positively correlated with plasma viral RNA copies at 12, 24 and 48 wpi. Both of these observations indicate that FAM26F may be an early predictor/indicator of viral load at set-point during the chronic phase of infection and can be considered as early prognostic marker for SIV/HIV infection. Further investigations using larger number of animals are required to validate these findings as well as samples from HIV-infected humans.

Expression of FAM26F along with already defined surrogate innate immune markers (Mx1 and IP-10) and a viral restriction factor (tetherin) was studied before immunization, 24 and 48 h after final priming, after the boost and at the day of 1st challenge in two groups of animals. Group 1 was boosted with a fowlpox virus-derived vector whereas group 2 was boosted with adenovirus-derived vector. Expression levels of IP-10 (24hrs post boosting), FAM26F and tetherin (24 hrs and 48hrs post boosting) were significantly elevated in vaccinees of group 2 as compared to group1.This difference was notable in the context that group 2 had lower viral load and increased level of protection as compared to group1.

As the expression of Mx1 and tetherin is driven by IFN-α while those of IP-10 and FAM26F is induced by IFN-γ, we can extrapolate that both vectors elicited different immune responses that effected the viral outcome. The increased level of protection in group 2 can be attributed to more pronounced IFN-γ responses or a unique balance between type I and type II responses, which may have caused a differential activation of the innate cells and finally to differential adaptive immune responses.

Although it is clear that FAM26F doesn't fit to the criterion to be a candidate for CAF as its properties are different than the CAF (e.g. size and protease activity) and it is higher expressed in CNAR^{-} than in CNAR^{+} animals, it is nevertheless possible that FAM26F acts somewhere intermediate in the mechanism of CAF inhibition. In summary, our *in vitro* stimulation as well as infection and immunization studies emphasize that FAM26F may be an

important regulator of innate or adaptive immune response. FAM26F expression may be an early indicator/marker of the immune status that is especially important during primary infection. This could mean that lower expression of FAM26F before infection may indicate an immune status that is able to limit early viral replication, whereas a strong increase after infection may indicate already early immune dysregulation that is later on associated with higher viral load. Thus FAM26F expression can be regarded an early diagnostic marker. Irrespective whether FAM26F is involved directly in regulation of viral replication or indirectly via the immune defense; our study has shown that it is an important molecule that clearly merits further investigation.

Further result of the study was the establishment of a permanent CD4 T cell line. Further genetic and functional characterization of this cell line will be of great importance. This cell line will be a valuable tool for in primate basic as well as biomedical research.

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Appendix

APP Table 1: Table briefly summarizing the possible function/s of genes selected for validation.

Gene	Brief summary of Functions
CD26(DPP4)	CD26 is an ecto-enzyme with help of its extracellular domain´s dipeptidyl-peptidase IV (DPP-IV) activity cleaves amino terminal dipeptides from polypeptides. On human T cells appears late in thymic differentiation and can deliver a potent co-stimulatory T-cell activation signal. Elicit signal transduction/ immunoregulatory mechanism via interaction with CD45 and adenosine deaminase (ADA), each of which is capable of functioning in a signal transduction pathway. It may be involved in cell migration and the HIV-1-associated loss of $CD4^+$ cells through the process of programmed cell death.
SLAMF8	It is member of the CD2 family of signaling lymphocytic activation molecule (SLAM), expressed in lymphoid tissues and characterized by Ig domains. SLAM-related receptors physically associate with SAP-related adaptors and mediate stimulatory signals that promote immune cell activation or differentiation. In the absence of SAP-family adaptors, though, the SLAM family undergoes a "switch-of-function," to mediate inhibitory signals that suppress immune cell functions.
GLUL	The protein encoded by this gene belongs to the glutamine synthetase family. It catalyzes the synthesis of glutamine from glutamate and ammonia. Glutamine is a main source of energy and is involved in cell proliferation, inhibition of apoptosis, and cell signaling. This gene is expressed during early fetal stages, and plays an important role in controlling body pH by removing ammonia from circulation
RPL13	The protein belongs to the L13E family of ribosomal proteins. It is located in the cytoplasm. This gene is expressed at significantly higher levels in benign breast lesions than in breast carcinomas.
TNFSF 13B	The protein encoded by this gene is a cytokine that belongs to the tumor necrosis factor (TNF) ligand family. It is a ligand for receptors

	TNFRSF13B/TACI, TNFRSF17/BCMA, and TNFRSF13C/BAFFR. This cytokine is expressed in B cell lineage cells, and acts as a potent B cell activator. It has been also shown to play an important role in the proliferation and differentiation of B cells
CST6	CST6 is typical secretory protease inhibitors. A candidate tumor suppressor gene for cancers of the breast, prostate, brain, lung, cervix and melanocytes.
PSAT	Encodes a phosphoserine aminotransferase which is involved in serine biosynthesis in the chloroplast which operates via the phosphorylated pathway.
CHYMASE-1	This gene product is a chymotryptic serine proteinase that belongs to the peptidase family S1. It is involved in the degradation of the extracellular matrix, the regulation of submucosal gland secretion, and the generation of vasoactive peptides
GSTO-1	The protein encoded by this gene is an omega class glutathione S-transferase (GST) with glutathione dependent thiol transferase and dehydroascorbate reductase activities.
PRSSL-1	Also called (kallikrein-related peptidase 10KLK10) in human. Kallikreins are a subgroup of serine proteases having diverse physiological functions. Growing evidence suggests that many kallikreins are implicated in carcinogenesis and some have potential as novel cancer and other disease biomarkers
FAM26F	Member F of the family with sequence homology 26 . It is involved in dendritic cell mediated activation of NK cells.
PON-3	This gene is a member of the paraoxonase family and lies in a cluster on chromosome 7 with the other two family members. The encoded protein is secreted into the bloodstream and associates with high-density lipoprotein (HDL). The protein also rapidly hydrolyzes lactones and can inhibit the oxidation of low-density lipoprotein (LDL),
LOCUS(Galanin)	Galanin is small neuropeptide that functions as a cellular messenger within the central and peripheral nervous systems, modulating diverse physiologic functions

MMP25	Proteins of the matrix metalloproteinase (MMP) family are involved in the breakdown of extracellular matrix in normal physiological processes, such as embryonic development, reproduction, and tissue remodeling, as well as in disease processes, such as arthritis and metastasis
EPHB	This gene belongs to the ephrin receptor subfamily of the protein-tyrosine kinase family. implicated in mediating developmental events, particularly in the nervous system This gene is expressed in some human cancer cell lines and has been implicated in carcinogenesis
CISH	The protein thus belongs to the cytokine-induced STAT inhibitor (CIS). CIS family members are known to be cytokine-inducible negative regulators of cytokine signaling. The expression of this gene can be induced by IL2, IL3, GM-CSF and EPO in hematopoietic cells

APP Table 2: Table depicting accession number of the genes validated by qRT PCR along with primer sequences used for amplification on along with amplicon lengths.

Gene	Accession Number	Primer Sequence (5` to 3`)		Product Size (bp)
GAPDH	CO774281	F	CCT GCA CCA CCA ACT GCT TA	74
		R	CAT GAG TCC TTC CAC GAT ACC A	
Mx1	EF101561	F	AGG AGT TGC CCT TCC CAG A	76
		R	TCG TTC ACA AGT TTC TTC AGT TTC A	
IP10,CXCL10	AY044446	F	GAT TTG CTG CCT TGT CTT TCT GA	74
		R	CAG GTA CAG CGT ACA GTT CTT GAG A	
CD26(DPP4)	DQ324530	F	CAA ATT GAA GCA GCC AGA CA	108
		R	TCC CAG GAC CAT TGA GGT TA	
SLAMF8	XM_001117299.2	F	GCA GAT CCA CAC TGC TCA AA	110
		R	ATT GGT CTC ACG GAA GCA CT	
GLUL	XM_001114930.2	F	CCG GAT TAG AAA CCA AGC AT	107
		R	GCA GAA ACC CAG AAG TGG TC	
RPL13	XM_002802597.1	F	CAA AGC CTT CGC TAG TCT CC	98
		R	TTT CAA CAT CCT GTT CTG CG	
TNFSF 13B	XM_001082247.2	F	GCG ATA AGTG GAG TCA GAG TTT C	105
		R	GCA AAA GGC AAT GAA GGT TT	
CST6	XM_001111664.2	F	TAC AAC ATG GGC AGC AAC AG	100
		R	CCA TCT CCA TCG TCA GGA A	
PSAT	XM_001101767.2	F	CCT GCT TAT TTT GCC TTT GC	108
		R	TGT GTT CCC ATG ACT CCA GA	
CHYMASE-1	AANU01225520.**1**	F	CAC AGA ATG CAA GCC ACA CT	104
		R	TGT CAG CAC AAA GTT CCG TC	
GSTO-1	XM_001099994.2	F	GGC TGG AAG CAA TGA GGT TA	95
		R	GGC TGA GAC TGT GGG ATC TT	
PRSSL-1	XM_001117143.2	F	CTG ACT TTG AGG ATC TGC CG	104
		R	CAT CGT ATG GCT CAG GTG G	

FAM26F	XM_001111520.2	F	TGT TGG GCT GGA TCT TGA TAG	98
		R	CTG CTG CTT CCT GTT CCA A	
PON-3	NM_000940.2	F	ACT TCC TAG TGG GCT GGC TT	107
		R	GTT ACT TCA GAT CCA TCA AG	
LOCUS(Galanin)	XR_091573.1	F	CCA TGC CTG AGA ACA ATA TCA	130
		R	GAC CGC TCC ATG TCT TCT	
MMP25	XM_001091146.2	F	CGG ACC TGT TTG CCG TGG CT	117
		R	TGT CAG GGT TGC CCA CCG GA	
EPHB1	XM_001115263.2	F	CGG CGA GAG CGC GAA AGG AT	100
		R	GAG CCC GAG CTG AGG CAG CA	
CISH	XM_001097824.2	F	GGA TGT GGT CAG CCT TGT	110
		R	CAG GCA GTG CTG GAT CAT TA	

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- Oral Presentation"Differential gene expression pattern of $CD8^+$ T cells from individuals with and without anti HIV/SIV Noncytolytic activity"
  "Molecular Medicine" Annual PhD Retreat, September 2011.

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- Workshop "Patenting issues in science". Organized by Molecular Medicine program, Göttingen.

- Workshop "scientific integrity and responsible conduct for research". Organized by German Primate Center, Göttingen.

- Workshop “Scientific writing skills”. Organized by Molecular Medicine program, Göttingen.

- Workshop “Project management in scientific research”. Organized by Molecular Medicine program, Göttingen.

- Workshop “Sex and gender aspects in biomedical science”. Organized by Molecular Medicine program, Göttingen.

- Workshop “Team work and leadership qualities”. Organized by Molecular Medicine program, Göttingen.

- Workshop “Career management”. Organized by German Primate Center, Göttingen.

List of publications:

- Rational design of HIV vaccines and microbicides: report of the EUROPRISE network annual conference 2011.

  Marie Borggren, Zelda Euler, Fabio Fiorino, Katrijn Grupping, David Hallengärd, **Aneela Javed**, Kevin Mendonca, Charlotte Pollard, David Reinhart, Nicolas Ruffin, ElisaSaba, Enas Sheik-Khalil, Annette Sköld Serena Ziglio, Robin Shattock, Gabriella Scarlatti, Frances Gotch,Britta Wahren. (submitted Journal of Translational Medicine).