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Utrecht University

Anouk Schuren
3384748

AN ANTIVIRAL IMMUNE RESPONSE TURNING AGAINST THE HOST IN PATHOGENIC HIV INFECTION

HIV infection leads to development of AIDS, a deadly immune deficiency in humans. This is in marked contrast with sooty mangabey monkeys, which have been the natural hosts of the simian immunodeficiency virus (SIV) for millions of years. Although high viral loads are present in these primates, they do not progress to AIDS. Sooty mangabeys do not show immune activation, and therefore antiviral immune activation itself, in combination with other factors, has been suggested as a cause of pathogenic infection. The insights from sooty mangabeys downregulating their immune responses against SIV may potentially be used to render HIV infection nonpathogenic in humans.

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INTRODUCTION

Worldwide an estimated 33.3 million people are infected with HIV (Human Immunodeficiency Virus), the cause of the AIDS epidemic[1]. The viral outbreak first came to light in the Western World in 1981, when five cases of the extremely rare *Pneumocystis carinii* pneumonia were reported among homosexual men in Los Angeles[2]. The disease then spread via San Francisco and New York to the rest of the US. This was however not the initial infection in humans. It is widely known that HIV originated in Africa and is derived from a primate virus (SIV; Simian Immunodeficiency Virus). Transmission to the human population has likely been caused by exposure to infected primate blood during hunting or consumption of contaminated meat[3, 4], and is estimated to have occurred around 1931[5].

There are two types of the virus causing AIDS in humans: HIV-1 and -2, the first of which being the most common. This subtype can be further divided in three groups: M (main), N (non-main/non-outlier) and O (outlier), each of which consists of multiple clades (fig 1). Although these lineages occurred as a result of variability of the virus, HIV-1 and -2 are in fact different viruses which respectively arose from chimpanzees (except the gorilla-derived O-group[6]) and sooty mangabeys[3, 4].

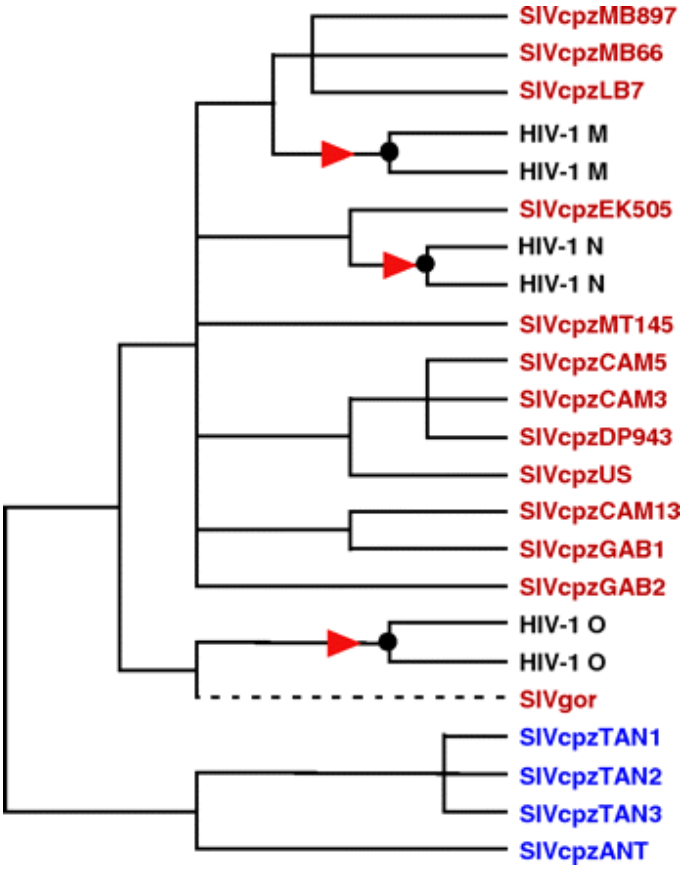

FIGURE 1: EVOLUTION OF HIV-1 FROM SIV VARIANTS.
M and N groups are derived from chimpanzee SIV (SIVcpz), whereas the O group originated from gorilla SIV (SIVgor). HIV-2 is not shown, since it is only distantly related to the SIV variants which led to HIV-1.
Wain, LV, Mol Biol Evol (2007) 24:1853-1860

The natural SIV hosts (sooty mangabeys, chimpanzees, African green monkeys and more) do not suffer from a pathogenic infection, meaning they do not progress to AIDS. SIV is only called an immunodeficiency virus due to its genetic similarity to AIDS-causing viruses, not only in humans but also in rhesus macaques (SIV_{RM}), which are often used as model organisms for disease progression.

AIDS is characterized by the occurrence of rare opportunistic infections and malignancies as a consequence of the collapsing immune system. The virus mainly infects $CD4^+$ T cells (T_{helper} cells), which are depleted almost entirely once the patient progresses to AIDS. However, even during progression to

AIDS, only an estimated 1 in 10,000 to 1 in 100,000 $CD4^+$ T cells are actively infected[7]. Cell death of infected T cells may thus contribute to, but is not sufficient to cause depletion of the entire CD4+ T cell compartment.

Although it may seem paradoxical, immune activation itself has been suggested as a cause of T cell depletion[8, 9]. Like in any viral infection, the immune system is activated when it encounters HIV. It is however unable to clear the infection, so the trigger for immune activation remains and results in chronic general immune activation. Interestingly, not only HIV-specific immune cells are activated in the process, other $CD4^+$ and $CD8^+$ cells are as well. To control itself, the immune system has a built-in negative feedback: activation-induced cell death. In chronic immune activation there will thus be a high number of activated as well as dying T cells. Depletion of T cell reservoirs would then initiate AIDS.

Although there are numerous suggestions in support of the above mechanism, the exact immunologic mechanism leading to this immune deficiency is yet to be elucidated. If immune activation is in fact the main cause of AIDS – as a result of HIV infection – the solution to this problem may lie in the natural SIV hosts which do not progress to AIDS. Sooty mangabeys are intensively studied, since their viral loads are similarly high to humans and despite this, they do not show high antiviral immune responses. Furthermore, both HIV-2 and SIV_{RM} are derived from this SIV variant (SIV_{SM}). It seems the natural SIV hosts can control their immune activation and thereby spare their immunological reservoirs. Once these control mechanisms have been revealed, the hope is that these insights from natural nonprogressors can ultimately be used to control pathogenic HIV infection and prevent progression to AIDS in progressing host species.

CHARACTERISTICS OF DISEASE PROGRESSION

HIV infection is characterized by an acute and a chronic phase, ultimately leading to AIDS in most cases. In the acute phase, spanning weeks to months, rapid HIV replication leads to a peak in viral load. This induces a strong immune response, characterized by aspecific flu-like symptoms. The adaptive immune response, as well as the low number of available uninfected target cells reduces the level of HIV down to a setpoint: the lowest viral load after infection.

From this setpoint on, the viral load will remain relatively stable over the whole chronic phase of infection, whereas $CD4^+$ T cell levels gradually decline. The chronic phase is clinically latent, indicating there are no symptoms despite constant viral replication and rapid T cell turnover. Eventually most nonnatural hosts progress to the symptomatic phase if no therapeutic intervention is applied. This phase is characterized by increased viral load and rapidly decreasing $CD4^+$ and also $CD8^+$ T cell levels. Patients suffer from opportunistic infections, the most frequent being Candidiasis, reactivation of tuberculosis and *Pneumocystis carinii* pneumonia. In further progression, the brain can become affected, leading to AIDS-related dementia, and damaged intestinal integrity can lead to severe wasting[10]. When the disease has progressed this far, it is always lethal.

LESSONS TO BE LEARNT FROM NATURAL HOSTS

Currently, HIV patients are treated with a combination of antiviral drugs, a therapy called HAART (highly active antiretroviral therapy). These drugs have many side effects, have to be taken in combination, and once or more times a day. Therefore, the recommended course of treatment is sometimes not followed well, increasing the risk of drug resistance. Especially because HIV is a highly variable virus – each replication round leads to one point mutation on average – drug resistance occurs relatively frequently[11].

Not all HIV-infected people have to take antiviral drugs to prevent developing AIDS. There are also naturally long-term non-progressing individuals among the nonnatural hosts (2-5% of infected individuals)[12]. These individuals despite often having been infected with HIV for over a decade still do not display any AIDS-related symptoms. It has been suggested that these long-term non-progressors (LTNPs) show a more efficient immune response against HIV. Specific single nucleotide polymorphisms are present in the HLA-I alleles are more often expressed by these individuals[13] and are suggested to present conserved Gag peptides. Most LTNPs show significant and lasting CTL responses against HIV, whereas the majority of patients are unable to do so[12, 14, 15]. In progressors the viral load remains high and continues to induce immune activation, whereas in LTNPs viral load may be controlled beneath the threshold of extensive immune activation. Continuous immune activation in progressors likely leads to exhaustion and reduction of blood T cell levels, including CTLs. It must be noted that in progressing

individuals, it is unknown whether the lack of CTL responses is a cause or a consequence of symptomatic HIV infection.

Natural hosts too control SIV infection, and they can be divided in two classes, based on their immune responses against SIV. The chimpanzee immune response is relatively similar to that in most LTNPs: their viral loads are maintained at low levels, likely as a consequence of their efficient immune response.

The immune response by LTNPs or chimpanzees is related to their HLA alleles. Since these alleles are genetically determined and cannot be altered, it is more promising to focus on the other group of non-progressing hosts: sooty mangabeys and African green monkeys. These primates do not develop a symptomatic phase when they are infected with SIV. Around a decade ago, it was found that these primates, unlike most human LTNPs, maintain limited immune activation despite high viral loads[8, 9] (fig 2). It is thus of

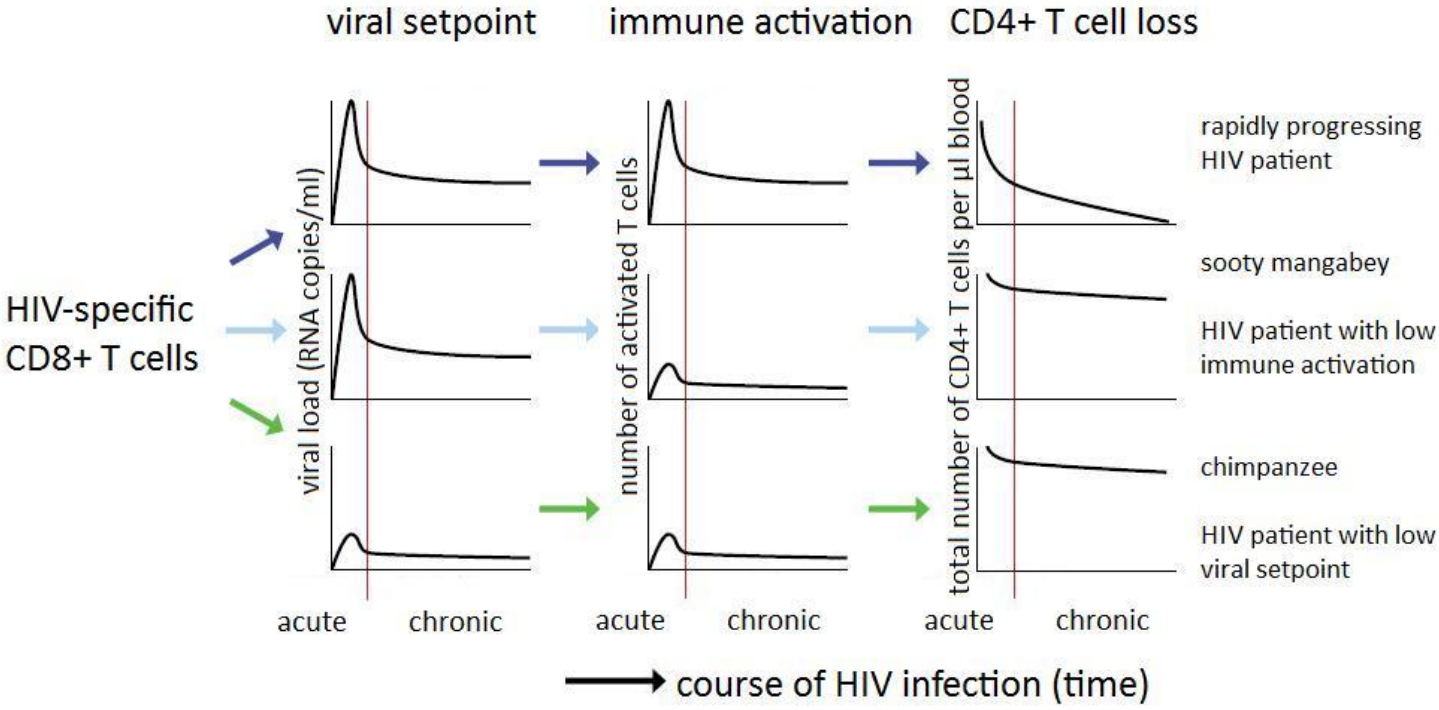

FIGURE 2: VIRAL LOAD, IMMUNE ACTIVATION AND BLOOD CD4⁺ T CELL LEVELS OVER THE COURSE OF PATHOGENIC (TOP) AND NONPATHOGENIC (MIDDLE AND BOTTOM) HIV OR SIV INFECTION.

The viral load (on the left) initially peaks during acute infection in either host, likely because high numbers of mucosal CCR5⁺ T cells are packed tightly at the site of entry into the body. In contrast with either progressing HIV patients or sooty mangabeys, chimpanzees suppress viral load during acute as well as chronic infection. At the transition of acute to chronic infection (indicated by the vertical red line), the setpoint is reached, which is the lowest viral load after infection.

Neither sooty mangabeys nor chimpanzees have high T cell activation. Chimpanzees likely would have similar immune activation as do nonnatural hosts, if their viral load had been sufficient. Sooty mangabeys on the other hand, retain low immune activation despite similar viral loads to those seen in progressing HIV patients. These progressing patients respond strongly against HIV infection by T cell-mediated immune activation (top middle). However, via an immunological negative feedback mechanism, overstimulation of T cells elicits an apoptotic pathway in these cells. It has been suggested that this enhanced T cell activation is the key to CD4+ T cell depletion seen in progressing nonnatural hosts (on the right). This hypothesis is based in part on the natural hosts which retain normal T cell levels and do not progress to AIDS, likely due to the lack of immune activation.

Van Baarle, D, Tijdschr Infect (2006) 1:93-99

large interest to find the key to immune downregulation as a potential therapeutic application. If indeed the detrimental effect of chronic immune activation is the main cause of T cell depletion and subsequent progression to AIDS, downregulating this response may render HIV infection less pathogenic.

THE T CELL AS A TARGET OF HIV INFECTION

The most striking characteristic of HIV infection is the constant decline in $CD4^+$ T cell levels, not only in the blood, but also in lymphoid and mucosal tissues. These T lymphocytes are potential host cells for HIV replication, because the virus utilizes CD4 and a chemokine co-receptor (CCR5 or CXCR4) for cell entry. CCR5 is the predominant co-receptor, since only HIV types utilizing this receptor (R5 tropic) can be transmitted to other individuals. Within 50% of HIV-infected patients, the virus switches tropism (to X4), but the R5 variant in that case remains present as well[16].

Although somewhat contradictory results have been obtained as to whether or not SIV-infected sooty mangabeys maintain normal blood $CD4^+$ T cell levels[8, 17], even when T cells were severely decreased in this species, no AIDS was observed for over a decade[17]. Thus, AIDS does not seem to be directly linked to the number of infected T cells, nor to the total levels of peripheral blood $CD4^+$ T lymphocytes, but rather to the interaction between the host immune system and the virus.

Regardless of whether T cell depletion is a direct consequence of HIV infection, T CD4 T cell depletion occurs mainly in patiens progressing to AIDS.There are many subsets of $CD4^+$ T cells, generally divided in naïve, effector and memory cells, the latter two subclasses expressing CCR5[18]. Effector and memory $CD4^+$ T cells (and also macrophages, but these will not be discussed) are thus primarily infected with HIV. The switch of tropism towards X4 is associated with poor prognosis, since naïve T cells express CXCR4 and the reservoir for effector and memory cells is then also infected. The unconventional observation of severe T cell decreases in sooty mangabeys could also be explained by this principle: a multitropic (R5/X4/R8) SIV strain had developed within the infected hosts, which would deplete naïve T cells[19].

T cell depletion seems an obvious consequence of cytopathic infection by HIV. However, since only limited numbers of blood T cells are infected, cytopathic HIV infection of T cells itself cannot account for the extensive T cell depletion seen in HIV-infected individuals. Even more striking, apoptosis of non-infected $CD4^+$ and even $CD8^+$ T cells occur as well. Most T cells dying during HIV infection were found not to be productively infected[20]. The viral replication cycle is aborted in these cells during reverse transcription and the accumulation of reverse transcripts would induce apoptosis, rather than cytopathic infection itself[21]. For T cell apoptosis, direct killing by HIV as a result of infection thus seems inferior to the indirect effect of the virus: apoptosis of non (productively) infected cells. The exact cause of cell death in noninfected CD4 and CD8 T cells is currently unknown, although there are a number of suggestions, which will be explained below.

T CELL HOMEOSTASIS

T cell levels are the result of an equilibrium between production and loss of cells. Like all blood cells, T lymphocytes are produced in the bone marrow. They leave this organ in an immature state and travel to the thymus, where selection and maturation take place. A shift in the equilibrium causing depletion could thus result either from decreased T cell production, from thymic output or peripheral proliferation, or by increased loss of T cells. Indeed, in pathogenic situations including HIV infection, decreased thymic function has been observed. Intrathymic lymphocytes may become infected in later stages of infection and therefore not leave the thymus but die instead[22]. Thymic output however decreases with age and may thus not be of great importance in HIV-infected adults.

Where the thymus leads to increases in T cell levels, these are declined because of peripheral apoptosis. This process often occurs after activation of the T cell. Activation of a naïve T helper cell is facilitated by an antigen-presenting cell (APC), usually a dendritic cell (DC). APCs continuously sample their environment by endocytosis and subsequent presentation of processed internalized peptides on MHC class II. $CD4^+$ T cells recognize MHC II with their TCR and CD4, which is the first signal for activation. Co-stimulation is required to complete the activation process, and consists of an interaction between B7 from the DC and CD28 from the T_H cell. The latter interaction induces T cell proliferation, as well as expression of both IL-2 and the IL-2 receptor α-chain. Autocrine signaling of IL-2 further enhances proliferation of the activated lymphocyte[23].

Rapid clonal expansion is important in clearing an infection. However, strong activation of the immune system is potentially dangerous and therefore tightly regulated. One of the built-in negative feedback mechanisms of the immune system is activation-induced cell death (AICD). This process is not only important to regulate the number of activated T cells at any timepoint, but also to diminish the expanded population of antigen-specific T cells after clearance of the infection. It has been suggested that immune activation followed by this negative feedback mechanism may be involved in the depletion of T cells seen in progressive HIV or SIV infection.

Although immune activation involves both CD4 and CD8 T cells, only $CD4^+$ cells are depleted in HIV infection, whereas $CD8^+$ T cell levels remain constant. This suggests that the two types of T cells respond differentially in HIV infection. HIV viral load is a stimulating factor in proliferation of either T cell type, and in the case of $CD4^+$ cells, so are the reduced cell counts. The inflammatory environment caused by HIV is correlated with strongly enhanced CD4 T cell activation and proliferation. It has been suggested to rank the proliferating CD4 and CD8 T cells in a slowly and a rapidly proliferating pool. In this model, both CD4 and CD8 T cells would proliferate in the slow pool to maintain normal lymphocyte levels in healthy conditions. HIV infection induces additional inflammatory forces and compensatory mechanisms for $CD4^+$ T cell depletion. Upon these stimuli CD4 (but not CD8) T cells are recruited to the rapidly proliferating pool,

where overstimulation drives activation-induced cell death. The rapidly dividing $CD4^+$ T cells expressed STAT5, a protein in the lymphoproliferative IL-7 signaling pathway. The slowly proliferating pool would not induce cell death and, however slowly, leads to expansion of cell populations, whereas the rapid pool eventually leads to cell death and further depletion[24].

The rapid T cell turnover seen in humans thus clearly has adverse effects and no additional proliferation or associated immune activation is observed in CD4-depleted sooty mangabeys[19]. Since rapid proliferation of lymphocytes is associated with the suicidal potential of activation-induced cell death, preventing this compensatory mechanism may actually preserve immunologic function and avoid progression to AIDS.

Although the above compensatory mechanism in nonnatural hosts describes CD4 depletion as a result of enhanced peripheral division in response to previous reductions, it does not explain the initial reduction in CD4 T cells. This 'chicken or the egg' causality could only be solved if the initial CD4 T cell decline caused directly by cytopathic HIV infection is sufficient to induce general CD4 T cell division and entry into the pool of rapidly dividing and dying cells. Generally, when adaptive immunity is unable to solve an infection, ongoing innate stimuli further drive DC and subsequent T cell activation, resulting in a detrimental vicious circle.

IMMUNE ACTIVATION: THE TLR HYPOTHESIS

The inflammatory environment involved in T cell activation and apoptosis during HIV infection is generated at least in part by signaling of Toll-like receptors (TLRs; fig 3), which are expressed by cells of the innate immune system. Dendritic cells play an important role in innate immunity and are at the interface between the innate and adaptive branch of the immune system. These cells can be divided in plasmacytoid DCs (pDCs) and myeloid or mDCs. Myeloid dendritic cells are the the classic antigen-presenting and T cell-activating cells, whereas the main function of pDCs is the production of IFN-α[25-27].

Plasmacytoid dendritic cells express TLR7 and 9, whereas TLR1 to 6 and 8 are expressed on mDCs. Molecular patterns from HIV are recognized via TLR7, 8 or 9 signaling. TLR7 and 9 recognize viral GU-rich single stranded RNA sequences[28] and unmethylated CpG DNA, respectively[29]. Both these TLRs are located at the limiting membrane of the endosome. Although pDCs express CD4, CCR5 and CXCR4, and thus are potential HIV target cells[26], whether or not they are infected, remains controversial. These HIV entry receptors can however be used by HIV to bind the cell. Binding does not necessarily result in infection: the virus can be endocytosed instead, allowing for TLR activation. For activation of the pDC via TLR7 signaling, fusion of the viral envelope subsequent to endocytosis should be prevented[30].

Activation of TLR7 induces two different activation pathways: expression of the pro-inflammatory cytokine TNF-α, via expression of NFκB, or the antiviral cytokines IFN-α and -β (via MyD88 and the interferon regulatory factor-7 [IRF-7]). As a consequence of

IFN-α/β, an antiviral state is induced in responding cells by autocrine and paracrine signaling. Although pDC depletion is seen in HIV infection, this too may be explained by activation of these cells, via expression of the maturation marker CCR7. This chemokine receptor may induce migration out of the blood and into secondary lymphoid organs, where its ligand, CCL19, is expressed[31]. No upregulation of the CCR7 chemokine receptor was observed in either DC type in sooty mangabeys[32], suggesting that their dendritic cells may be less active.

The interferon-α produced in part by active pDCs has the primary function of inducing an antiviral state in virus-infected macrophages or potentially infected neighbouring cells. This state is reached by upregulation of MHC I, and activation of the protective proteins protein kinase R (PKR) and 2'-5' oligo-A synthetase (OAS). Both proteins are activated upon contact with viral RNA, a common viral pattern. PKR inhibits translation initiation, thereby preventing potential viral replication. 2'-5' OAS exerts its function via synthesis of a 2'-5' linked adenosine oligonucleotide. This oligo-A nucleotide is recognized by RNAse L, which subsequently degrades all mRNAs present in the cytosol. Either pathway may lead to apoptosis of the infected or activated cell[33].

Beside this beneficial antiviral state, interferons may also have adverse effects in the context of HIV or SIV infection. These cytokines not only induce an antiviral state in infected or neighbouring cells, they are also implicated in the activation of several cell types, including T lymphocytes and myeloid dendritic cells. Overstimulation of these T lymphocytes by elevated levels of interferons 22wzzultimately leads to activation-induced cell death. Individuals repeatedly exposed to HIV without becoming infected (exposed seronegative or ESN individuals) show a higher overall cytokine production, but with a striking reduction in IFN-β[34]. Heightened production of IFN-β, and likely also the closely related IFN-α, could thus play a role in HIV pathogenesis. Sooty mangabeys, and also African green monkeys express lower levels of IFN-α upon immune activation by SIV[35], suggesting that this cytokine may indeed play a role in the detrimental immune response contributing to progression to AIDS. The exact mechanism of IFN-α production and signaling will be discussed below, but first it is important to realize that TLR7 signaling in response to HIV is not the only stimulus for IFN-α production.

IMMUNE ACTIVATION: THE LEAKY GUT HYPOTHESIS

Alternatively, not HIV itself, but immune activation primed by intestinal microbes may be implicated in inducing the interferon response (fig 3). This hypothesis is based on damage to the intestinal integrity. When transmitted sexually, HIV usually enters the body via the intestinal tract, where a broad microbial flora is present. Although these bacteria are harmless, they are immunogenic and will thus elicit an immune response when they cross the intestinal border as a result of damaged integrity. This process is called microbial translocation and leads to general immune activation. Indeed, elevated levels of immunogenic bacterial molecules have been observed in HIV^+ individuals and SIV-infected rhesus macaques[36, 37]. One of these is lipopolysaccharide (LPS), a highly

immunogenic compound of Gram-negative bacterial cell walls Not only LPS, but also other bacterial proteins are translocated. Because of its high immunogenicity and the availability of LPS assays, LPS is most often measured and hereafter the focus will be on this immunogenic bacterial molecule.

To explain the effect of LPS on T cell levels, it is important to start with some hallmarks of HIV infection first. In the acute phase of HIV infection, blood T cell levels are only slightly affected, in marked contrast with the intestinal mucosa, where extensive cell death takes place. The mucosa-associated lymphoid tissue (MALT) contains numerous memory and effector memory T_H17 cells, which act rapidly upon microbial infections derived from the gut lumen. However, these cells express CCR5 and are thus potential target cells for HIV in early stages of infection.

Before HIV or SIV can encounter these mucosal T cells, the virus first has to cross the tight epithelial border lining the entire intestinal tract. T lymphocytes are located in between the enterocytes or beneath this epithelial layer, but either way direct contact of these T cells with the intestinal lumen is prevented by numerous tight junctions between the enterocytes.

The intestinal epithelium is severely damaged during acute HIV infection. In this enteropathy, villous atrophy as a result of apoptosis is an important characteristic. 15-30 times higher apoptotic rates have been observed in enterocytes as early as two to four weeks after vaginal transmission of SIV_{RM}. The epithelial apoptosis was likely caused by infection of enterocytes. Since these cells do not express CD4, it was suggested that cell entry was facilitated by GPR15/Bob, an alternative SIV_{RM} and HIV-2 receptor. This protein is transcytosed to the apical cell surface prior to enteropathy[38]. In the case of HIV-1, disruption of tight junctions has been suggested as another way to cross the epithelium[39].

Damage to intestinal integrity does not occur in sooty mangabeys during SIV infection and functional integrity of the epithelial border is retained. This was confirmed by tight junction intactness, and the lack of LPS detection in the lamina propria[37] or blood plasma[36]. In some more severe cases, a transient rise in LPS was observed, which correlated with greater localized as well as systemic immune activation, but this was rapidly downregulated during the chronic phase of infection[40]. Loss of epithelial integrity is likely one of the first steps in systemic pathogenic HIV or SIV infection: the virus can now cross this tight barrier and access a Walhalla of T cells: the MALT.

MUCOSAL T CELL DEPLETION

In contrast with the low proportion of infected blood lymphocytes during acute infection, dense packaging of $CCR5^+$ T cells in the mucosa allows for high infection rates during acute HIV infection. Despite the overall intact epithelium in sooty mangabeys, extensive $CD4^+$ T cell apoptosis in the MALT has been observed in both progressing and nonprogressing hosts with decreases of up to 95%[40]. Also, these effector memory CD4

cells are metabolically active cells and thus capable of supporting HIV replication, thereby further increasing the amount of virus. After depletion, these mucosal $CD4^+$ T cell levels remained low, also over the chronic phase of infection.

Mucosal T cells are derived from peripheral blood lymphocytes, a small subset of which expresses the $\alpha_4\beta_7$ integrin. This protein is a so-called homing receptor for Peyer's patches, mesenteric lymph nodes and the lamina propria. HIV-specific $\alpha_4\beta_7^+$ cells are also present in the genital mucosa and within hours to days after sexual transmission of this virus, infected T lymphocytes translocate to the gut (GALT) where extensive infection of mucosal T cells takes place[41, 42]. The MALT covers a number of lymphoid tissues, the GALT being one of these.

The extremely high infection rates at these mucosal locations can be explained not only by the dense packaging of $CCR5^+$ T cells, but also by the presence of MALT-specific additional co-receptors. The $\alpha_4\beta_7$ integrin has been shown to interact with gp120, the HIV spike protein, and to function as co-receptor in a complex with CD4[42, 43]. CCR6 is another homing receptor specifically expressed for lymphocyte migration into Peyer's patches of the ileum. In contrast with $\alpha_4\beta_7$, which serves as a marker for HIV susceptibility only in T_H1 cells, CCR6 expression has been suggested as a predictor of T_H1 as well as T_H17 infection by HIV[44]. These studies however did not include a $CCR5^-$ negative control, and it can therefore not be excluded that these cells, expressing CCR5, are infected via this ordinary co-receptor instead. On the other hand: the interaction between gp120 and CCR5 is CD4-dependent, which is not the case for $\alpha_4\beta_7$. Considering the distance between complexed CD4 and $\alpha_4\beta_7$ (about 1.2 nm) it is much more likely that gp120 interacts with this integrin subsequent or even prior to CD4 encounter[42].

Direct infection and killing by HIV provides a good explanation for the depletion of mucosal T cells. This high infection rate occurs in the entire MALT, with the greatest depletion in the rectal lamina propria. An additional mechanism has been proposed for T cell depletion: T cells in the lamina propria must balance protective immunity against micro-organisms and maintenance of intestinal integrity, which is damaged by extensive immune activation. To prevent overstimulation of immune activation they become more prone to apoptosis upon high exposures to microbial proteins; in this case gp120. This leads to upregulation of FasL (CD95L)[41, 45], which interacts with the already expressed Fas (CD95) on the same or a neighbouring T cell and induces apoptosis of the Fas-bearing cell. A combination of T cell infection and apoptosis upon direct contact with extracellular viral proteins provides the most likely cause of high rate of T cell depletion in the mucosa.

Cell death in the GALT leads to severe impairment of its function. The important role of the GALT is to eliminate microbial infections entering from the intestinal tract. Therefore, the extensive damage to the gut induced by HIV could result in bacteria (or HIV) crossing the mucosal barrier, spreading throughout the body and activate peripheral blood T cells[46]. However, the GALT does not exist solely of T lymphocytes, it also contains numerous macrophages which fulfill a role as antigen presenting cells. Damage to the epithelium would not immediately cause extensive infection in the GALT

and subsequent microbial translocation, since these microorganisms would immediately be phagocytosed and cleared by macrophages. Interestingly, during the late acute phase of HIV infection, an increased number of macrophages is present in the GALT. These seem however unable to efficiently phagocytose HIV, which likely contributes to extensive GALT damage[37].

In contrast with nonnatural hosts, peripheral blood $CD4^+$ lymphocytes in sooty mangabeys are maintained at 50-90% of the pre-infection rate[40]. Somewhere in between the mucosal depletion and systemic infection, a differential response from nonnatural hosts must therefore occur. In nonnatural hosts, high numbers of immunogenic intestinal bacteria stimulate immune activation very strongly and may lead to activation-induced cell death of T lymphocytes. A lack of microbial translocation in sooty mangabeys may be the key to this distinct immune response.

The few remaining $CD4^+$ T cells in the MALT of sooty mangabeys are possibly capable of maintaining intestinal integrity. These hosts have limited a $CD4^+$ $CCR5^+$ proportion of T cells, likely as a result of co-evolution[47]. This supports the hypothesis of a non-$CD4^+$ T cell-mediated immune response. It is unclear how these low $CD4^+$ $CCR5^+$ numbers in natural hosts could lead to the massive CD4 T cell depletion seen in the mucosa, since most of these cells do not express CCR5. In the case of African green monkeys, which share a generally similar response to sooty mangabeys, partial recovery of GALT T cells occurs, which may prevent microbial translocation[48]. Avoiding microbial translocation prevents the occurrence of the highly immunogenic bacterial components in the blood, and the subsequent immune activation seen in nonnatural hosts.

LPS AND ITS ROLE IN A DETRIMENTAL IMMUNE RESPONSE

The bacterial component LPS activates TLR4 on myeloid dendritic cells, which in turn induces an interferon response. Beside the T cell stimulatory effects of interferon, the activated myeloid dendritic cells can now act as antigen-presenting cells, activate these T lymphocytes as well and lead to strong immune activation.

Because of its strong effects, LPS levels are tightly regulated. This bacterial molecule can be bound or neutralized by a number of secreted proteins, including IgA, IgG and IgM antibodies directed against the endotoxin core (EndoCAbs), soluble CD14 from monocytes and macrophages and LPS binding protein (LBP) from mucosal epithelia. sCD14 is a marker for immune activation in response to LPS. It can be hypothesized that with low LPS levels, sCD14 is sufficient to neutralize LPS and prevent excessive immune activation. When LPS levels rise, so will sCD14 levels, but these may no longer be sufficient to control LPS-induced immune activation. High sCD14 levels are associated with mortality in HIV infection[49], suggesting that elevated LPS levels contribute to pathogenesis and mortality.

LBP is another LPS-neutralizing protein and it has been suggested that T_H17 cells produce this protein in response to elevated LPS levels. This would link the increased blood LPS levels directly to mucosal T_H17 depletion.

T_H17 CELLS

Elevated LPS levels are observed after about four weeks of SIV_{RM} infection, when most mucosal T_H17 cells have already been depleted[37]. It could thus be hypothesized that the mucosal T cells somehow control LPS levels. Indeed, T_H17 cells have been suggested to play a crucial role in controlling LPS levels because IL-22, one of the predominant cytokines produced by this cell type, induces the production of LPS binding protein[50]. This could thus at least in part explain the rising LPS levels after T_H17 apoptosis.

The presence of T_H17 cells may, via induction of LBP, limit LPS levels and thereby control pathogenic SIV infection. In humans, a significant decline in T_H17 cells is observed in the MALT, whereas sooty mangabeys retain normal T_H17 cell levels in both the MALT and the blood. T_H1 cell levels remain relatively unaffected. T cell depletion in the MALT is thus not simply the result of infection with HIV. Upon T_H17 depletion by HIV, naïve blood T helper cells in the blood are skewed away from differentiation into T_H17 and mainly differentiate into T_H1 cells[51]. T_H17 cells had similar proliferative capacity and susceptibility to activation-induced cell death when compared with T_H1 lymphocytes, confirming that their depletion is caused by HIV infection. Within the T_H1 cell population of nonnatural hosts, the proportion of cells expressing CCR5 was similar to those in uninfected individuals. Even though $CCR5^+$ T_H1 cells were present, these did not seem to be affected by HIV infection. Normally, both a $CCR5^+$ and a $CCR5^-$ pool of T_H17 are present in the mucosa, but the remaining T_H17 cells in HIV infection were mainly $CCR5^-$. Since the $CCR5^+$ T_H17 pool was absent in HIV-infected subjects, a preferential infection of this cell type is expected in the mucosa[51].

T_H17 depletion is seen only in nonnatural hosts and therefore seems to be an important characteristic of pathogenic HIV or SIV infection. In sooty mangabeys, despite overall $CD4^+$ depletion in the gut, no preference for T_H17 depletion was observed[51]. T_H17 lymphocytes themselves are not directed against viruses entering at mucosal surfaces, but they do respond against bacteria and fungi and therefore may prevent microbial translocation as is observed in pathogenic HIV infection[37]. Maintenance of T_H17 cells in sooty mangabeys seems contradictive with the 95% decrease[3, 40] in their mucosal T cells, and it is unclear how these results are associated.

The important role for T_H17 in controlling HIV pathogenicity could be appointed to polyfunctionality of these lymphocytes. Apart from IL-17 and IL-22, these cells produce antimicrobial defensins as well as neutrophil-attracting chemokines, and play a role in enterocyte homeostasis[51].

Preserving the T_H17 subset in the intestinal mucosa, for example by shifting infection preference more towards T_H1 as seen in sooty mangabeys, could possibly provide a less

pathogenic infection in nonnatural hosts. It would be interesting to define in more detail the role of T_H17 cells in preventing microbial translocation and controlling blood LPS levels via the production of LBP.

REGULATORY T CELLS

Loss of intestinal integrity only occurs in nonnatural hosts, and is associated with microbial translocation and immune activation. In sooty mangabeys, intestinal integrity is not affected by SIV infection, nor microbial translocation is observed. Despite the intact intestinal epithelium in these primates, their mucosal $CD4^+$ T cells are infected with SIV. Although it is unclear how these T cells can be infected while the intestine remains intact, a more striking question is why no immune activation occurs, even though MALT T cells are depleted.

This lack of immune activation is the most striking difference between natural and nonnatural hosts, and seems associated with asymptomatic infection. Sooty mangabeys, displaying high viral loads but controlling their immune response, suggestively have strong regulatory T cell (T_{reg}) activity. These cells counteract an ongoing T cell response by secreting anti-inflammatory cytokines (TGF-β and IL-10) or by direct contact with an activated T lymphocyte[23]. The clonal expansion of antigen-specific T cells has to be turned back once an infection has been cleared and this function is exerted by T_{regs}. Since the cause of pathogenic SIV or HIV infection has been suggested not to be the viral infection itself, but rather the lack of downregulation of the immune response against the virus, HIV pathogenicity may be due to impaired regulatory T cell function.

Indeed, loss of functional T_{regs} has been observed in the chronic phase of pathogenic SIV infection. Sooty mangabeys on the other hand, retain functional regulatory T cells. In rhesus macaques, the number of T_{regs} is negatively correlated with viral load. The absence of T_{regs} is correlated with elevated viral load and the associated immune response, whereas higher T_{reg} levels are found in nonprogressing animals. This supports the potential role for regulatory T cells in suppressing SIV-induced immune activation.

The loss of regulatory T cells can be explained by direct infection and killing by HIV or SIV. The absolute number of regulatory T cells, expressing CD4 and CD25 (IL-2 receptor), is decreased in chronic pathogenic HIV or SIV infection, thereby contributing to the overall CD4 T cell depletion. However, also in the remaining regulatory T cells the activity is lowered. Normally, T_{regs} respond to IL-2 secreted by activated T cells. It has been suggested that lower IL-2 levels are produced by activated T effector cells during the chronic phase of HIV infection and that T_{regs} thus may not sense overactive T cell responses[52].

The respective depletion or preservation of regulatory T cells in chronic infection likely originates during the acute phase of infection. Rhesus macaques show a strong regulatory T cell response after two weeks of infection, in contrast with sooty mangabeys. During these first two weeks, $CD4^+$ T effector cells were extensively diminished[53] in rhesus macaques, while this is the time they are most needed to

eliminate the early infection[54]. Sooty mangabeys did not induce this acute T_{reg} response, suggesting they do not downregulate the immune response against SIV during acute infection.

By contrast, sooty mangabeys do retain T_{regs} during the chronic phase, whereas T_{reg} function is impaired in chronic SIV infection of rhesus macaques. Targeting the virus this early by a non-downregulated immune response might help control immune activation at later timepoints and lead to nonpathogenic infection. It can therefore be hypothesized that in nonnatural hosts, blunting the acute immune response by T_{reg}-mediated downregulation enables the virus to establish a persistent infection[54].

The strong acute immune response exhibited by natural hosts is rapidly downregulated, preventing immune activation during chronic infection. This downregulation was shown not to be facilitated by regulatory T cells, but by PD-1 expression[55], a protein further discussed below. How regulatory T cells are induced to control immune activation during chronic SIV infection of a natural host remains elusive, but may provide a potential target for immune downregulation in nonnatural hosts.

THE PROS & CONS OF INTERFERON

Apart from T_{regs}, interferon-α, as already mentioned, likely plays an important role in immune activation during pathogenic HIV or SIV infection. Interferon-α is primarily an antiviral cytokine, suggesting a positive effect of this cytokine during viral infections. On the contrary, the adverse effects of an overwhelming interferon response become clear in HIV infection. Continuous activation of T cells, via mDCs or pDCs, may drive activation-induced cell death of these lymphocytes, while an antigen-specific antiviral response by the adaptive immune system is mostly inefficient.

Interferon is produced in response to triggers of the innate immune system, including LPS and viral RNA. Once neutralization of LPS no longer occurs during the chronic phase of progressive HIV or SIV infection, its levels in the blood will rise and trigger TLR4 signaling on myeloid dendritic cells. This does however not rule out the hypothesis of HIV signaling directly via TLR7 and 9 on plasmacytoid DCs, which do not express TLR4. In fact, pDC and mDC activation in response to HIV or LPS seem to be linked. One of the bystander effects of IFN-α secretion by plasmacytoid DCs is the activation of mDCs[31]. These myeloid cells, responding to LPS with their TLR4, are the professional T cell activating DCs. It has to be noted that pDCs, although not the professional T cell activators, are also capable of this function[31].

The main function of plasmacytoid dendritic cells is the production of IFN-α, and these cells therefore play an important role in the suggested detrimental IFN-α response. Interferon-α from plasmacytoid dendritic cells is strongly correlated with T cell activation and activation-induced cell death in nonnatural SIV hosts[56]. Since natural hosts retain higher T cell levels and maintain low immune activation, it would be expected that these primates do not express interferon-α during SIV infection. However, in sooty mangabeys a similarly strong INF-α response is induced as in nonnatural hosts,

even though no or less microbial translocation or elevated LPS are observed as triggers for this cytokine.

Despite similar levels of viral replication and IFN-α, the levels of $CD4^+$ and $CD8^+$ T cell activation and apoptosis remain much higher in rhesus macaques than sooty mangabeys[57]. In sooty mangabeys, this interferon response is rapidly downregulated during the transition from acute to chronic SIV infection. This is in marked contrast with rhesus macaques, where the IFN-α response continues throughout the entire chronic and symptomatic phase of infection and keeps inducing immune activation[58, 59]. This differential interferon response in the context of similar viral replication thus suggests a host-specific regulatory mechanism of IFN-α expression. One possibility could be that sooty mangabey T cells become increasingly resistant against IFN-α. This suggestion was falsified because *in vitro,* immune responses their T cells could repeatedly be activated using IFN-α. It is more likely that suppression of IFN-α production itself occurs *in vivo*[59]. The IFN-α response was induced, at least in large part, by plasmacytoid dendritic cells in either primate species[58].

Like the transient IFN-α response in sooty mangabeys, pDC depletion from the blood takes place only transiently in African green monkeys[35]. Since in rhesus macaques this depletion may be caused by migration of DCs into lymphoid tissues as a result of activation, less activation may occur in African green monkeys.

Positive correlation between the absolute $CD4^+$ T cell and pDC number has been observed during chronic SIV infection in AGMs. If pDCs in peripheral blood are inactive – in contrast with the activated cells in lymph nodes – increased $CD4^+$ T cell numbers in the presence of blood pDCs could be explained by the lack of IFN-α production. Another explanation for this correlation could be an intrinsic difference between natural and nonnatural hosts. The latter may result in pDCs with a beneficial effect on T cell survival, in contrast with the AICD-inducing DCs from rhesus macaques and humans.

Interestingly, pDCs from African green monkeys have been shown to produce far less IFN-α than do nonnatural hosts[35]. Therefore, the low level of IFN-α may have limited influence on T lymphocytes, or induce a normal immune response. Either may contribute to decreased immune activation and associated SIV pathogenicity in this primate species. In macaques and humans however, the far higher IFN-α levels induce T cells much stronger and possibly drive them into a rapidly proliferating pool of cells[24], predisposing them to activation-induced cell death.

The intrinsic difference in IFN-α levels per host possibly lies in the regulation of IFN-α by its target genes. In rhesus macaques, IRF-8 (interferon regulatory factor-8) is expressed in response to SIV-induced IFN-α[59]. This transcription factor can, in a positive feedback mechanism, further enhance IFN-α production[60]. African green monkeys on the other hand, mainly produce negative regulators of IFN-α[59]. Rhesus macaques thus likely fail to produce an efficient negative feedback mechanism, possibly because immune activation is too overwhelming. This loss of control would likely not occur in natural hosts.

It is important to realize that IFN-α does not solely lead to harmful effects. Not expressing IFN-α, like the partial downregulation in natural SIV hosts, would have

adverse effects to the host during viral infections in general, because of the antiviral function of IFN. Interestingly, sooty mangabeys did produce similar amounts of IFN-α as did rhesus macaques, but only when they were stimulated by a virus signaling through TLRs other than TLR7 or 9[61]. This suggests an intrinsic negative feedback mechanism in pDCs, tightly regulating IFN-α production by these cells.

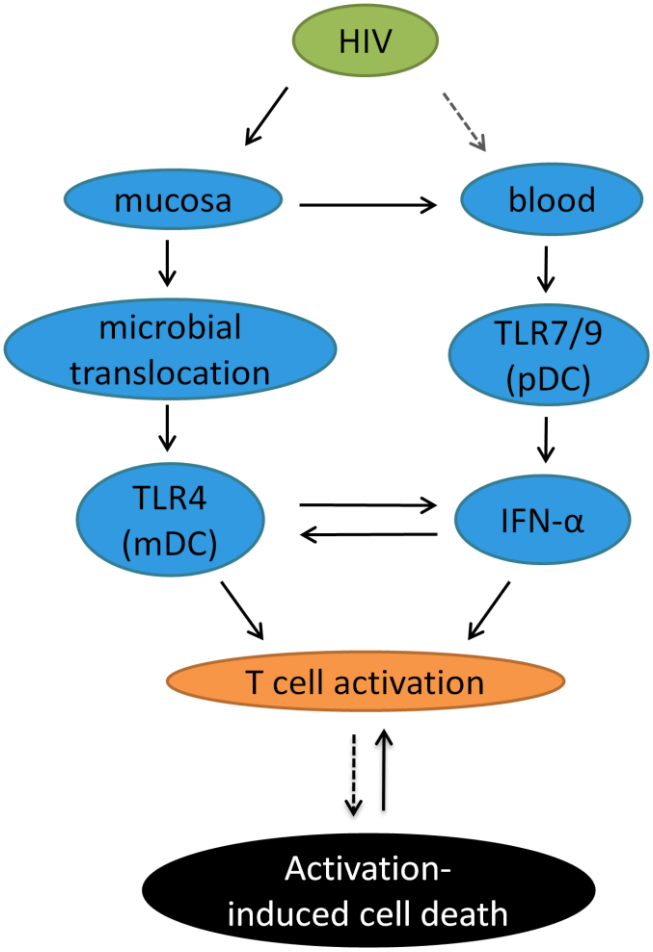

FIGURE 3: SCHEMATIC OVERVIEW OF THE SUGGESTED FACTORS INVOLVED IN HIV PATHOGENESIS. HIV enters the body via mucosal surfaces (in the case of sexual transmission) or directly via blood-blood contact (intravenous drug users or transfusion). In the mucosa, high numbers of T cells in the mucosa-associated lymphoid tissue (MALT) are infected, leading to damaged integrity of the gut. The natural function of the MALT, preventing intestinal bacteria from entering the body and cause an infection, is impaired as a result of damaged intestinal integrity. Bacteria can now spread throughout the body (microbial translocation), such that LPS levels in the blood rise. LPS activates TLR4 on myeloid dendritic cells, and these antigen-presenting cells in turn can activate T cells. TLR4 also leads to IFN-α production by the mDCs. This cytokine further stimulates T cell activation and in high concentrations can lead to activation-induced cell death.
From the mucosa, not only bacteria, but also HIV can enter the blood. A different explanation for T cell activation is based on HIV directly activating TLR7 and 9 on plasmacytoid dendritic cells. These DCs are the main IFN-α producers. This cytokine can act directly on T cells, but also activate myeloid dendritic cells and stimulate their activation and TLR4 expression, thereby enhancing their T cell activation capacity. Overwhelming activation of T cells induces a negative feedback mechanism: activation-induced cell death, which in a feedback mechanism stimulates T cell activation.

Plasmacytoid dendritic cells use both TLR7 and 9 in response to HIV. The downstream signaling pathways of these TLRs bifurcate: the path leading to IFN-α production is dependent on the transcription factor IRF-7, whereas TNF-α, the other path, requires NFκB. Although IFN-α production is much lower in sooty mangabeys compared with rhesus macaques or humans, similar amounts of TNF-α were produced in response to TLR7 or 9 signaling. The partial lack of IFN-α expression in SIV infection is thus not the result of TLR defects but rather originates from a distinct downstream signaling pathway. Interestingly, a number of sooty mangabey-specific genetic polymorphisms have been identified in the transactivation domain of IRF-7[32]. This transcription factor induces expression of the IFN-α gene when driven by TLR7 signaling. Polymorphisms in this transcription factor may influence its activity, and

thereby the expression of IFN-α. In addition, two SNPs have been found in human IRF-7, which correlated with reduced IFN-α production upon *ex vivo* infection with HIV-1[62]. These studies suggest that IFR-7, being an IFN-α inductory transcription factor, plays a crucial role in the level of IFN-α production during HIV or SIV infection.

ACTIVATION-INDUCED CELL DEATH

A detrimental effect of high IFN-α expression is that it drives activation-induced cell death subsequent to overactivation of T cells. Activation-induced cell death driven by interferon-α mainly occurs through signaling of PD-1 (programmed cell death-1), TRAIL, and possibly also by upregulation of Fas and soluble FasL by activated T cells[63]. These latter two proteins are involved in AICD during negative selection of T cells in the thymus.

TRAIL, a TNF-related gene involved in $CD4^+$ T cell apoptosis, is expressed in SIV-infected rhesus macaques but not African green monkeys[59] or sooty mangabeys[57]. These TRAIL levels positively correlated with $CD4^+$ T cell apoptosis in pathogenic SIV infection. In the *in vivo* situation, African green monkeys did produce IFN-α – albeit lower than nonnatural hosts – but did not express TRAIL in response to this cytokine. By contrast, *in vitro* IFN-α-stimulated PBMCs from African green monkeys do produce TRAIL, suggesting that AGMs suppress expression of this gene *in vivo* in a yet to be elucidated mechanism. Since IFN-α is involved in TRAIL induction[64], and IFN-α production is likely suppressed by IRF-7 or other factors *in vivo* in natural hosts, the lower IFN-α levels and the lack of TRAIL expression in these hosts may be related.

PROGRAMMED CELL DEATH-1

PD-1 is suggested to be the most significant protein facilitating activation-induced cell death in the context of IFN-α and HIV or SIV infection. This protein is nearly absent in naïve T cells, but rapidly induced upon lymphocyte activation. PD-1 is a CD28-related protein which, like CTLA-4, can competitively inhibit co-stimulatory signals during T cell activation and induce anergy. In order to do so, PD-1 contains an immunoreceptor tyrosine-based inhibitory motif (ITIM), counteracting ITAM (activation motif)-bearing receptors, such as CD28[65].

It has been suggested that PD-1 counteracts T cell activation by interfering in the PKB pathway[65]. Interference in this important signaling pathway may render the T cell more susceptible to apoptosis. In CTLA-4 signaling, the anti-apoptotic protein Bcl-xL was upregulated to prevent cell death, whereas in PD-1 signaling it was not[65]. In addition to the induction of anergy, PD-1 signaling may thus render lymphocytes more prone to apoptosis and contribute to their exhausted phenotype.

PD-1 expression is driven by TCR-mediated T cell activation, but also by interferons. The PD-1 promoter region contains an IFN-stimulated regulatory element (ISRE) which is responsible for PD-1 expression synergistically with TCR-mediated T cell activation. This regulatory element is a binding motif for IFN-stimulated gene factor-3 (ISGF3), a transcriptional activator produced in response to IFN-α. In mice, IFN-α has been shown to enhance PD-1 expression and to likely contribute to activation-induced cell death[56]. Even reverse transcription-deficient HIV leads to increased IFN-α and PD-1 expression in human macrophages and $CCR5^+$ T cells[66]. PD-1 expression is thus not induced upon viral replication, but rather by IFN-α, produced purely by the presence of HIV, likely signaling through TLR7 or 9.

INTERFERON-ALPHA AND CCR5 EXPRESSION

The $CCR5^+$ macrophages and T cells producing increased levels of PD-1 were found to highly express IFNAR2, the IFN-α specific subunit of the IFN-α/β receptor, irrespective of HIV infection[66]. Expressing this receptor, these cells were more susceptible to IFN-α, even in the absence of HIV. During infection, production of IFN-α is enhanced, signaling particularly to these cells with IFNAR2 upregulation, and inducing PD-1-mediated cell death. This type of cell death thus seems an indirect consequence of HIV infection through elevated IFN-α levels, despite coinciding HIV susceptibility of the affected cell populations.

Strikingly, this $CD4^+$ $CCR5^+$ T cell population in general is extremely scarce in SIV-infected sooty mangabeys, African green monkeys, chimpanzees and other natural hosts[47]. Paucity of these T lymphocytes in the blood, lymphoid and mucosal tissues extensively limits the availability of SIV target cells[67]. The massive depletion of T cells in the sooty mangabey mucosa seems unlikely in this context, and it must be noted that a definitive conclusion on T cell depletion in this SIV host remains elusive.

It could be hypothesized that PD-1 expression is involved in the general downregulation of CCR5. In sooty mangabeys, a rapid and much stronger upregulation of PD-1 is observed during acute SIV infection than that seen in rhesus macaques[55]. This peak in PD-1 expression precedes the downmodulation of their immune response. If sooty mangabeys, like humans, highly express the IFN-α receptor on $CCR5^+$ T cells[66], it could be hypothesized that they too might upregulate PD-1 in these cells. The high peak in PD-1 expression may then result from an interferon response stronger than that seen in nonnatural hosts. Subsequently, these CCR5-expressing lymphocytes would die of PD-1-induced cell death during acute SIV infection. This would also explain the T cell depletion seen in sooty mangabeys during acute SIV infection[40]. Once the $CCR5^+$ T cell population has been depleted, this low expression profile might be maintained. Alternatively, it has been suggested that CCR5 expression becomes restricted to activated T cells, which are predisposed to activation-induced cell death already[67]. This would help maintaining T cell levels because most cells cannot be infected.

There may be even more disadvantages of the interferon response: interferon-α is implicated in enhanced CCR5 expression on thymocytes, thereby expanding HIV tropism to immature T lymphocytes[22]. This would lead to enhanced cell death, as a consequence of infection or potential IFNAR2 expression. Because these cells are immature T cell precursors this will also abrogate the production of new cells and thus partly explain T cell depletion in R5-tropic HIV infection. Normally only a small percentage of T cells normally expresses CCR5. It is unclear whether this CCR5 upregulation contributes to a similar PD-1 expression as seen in macrophages and mature T lymphocytes, which were also $CCR5^+$.

Upregulation of CCR5 in thymocytes has a detrimental effect to these cells. Intrathymic T-cell progenitors (ITTPs) represent the thymocytes already devoted to CD4 expression, but still present in the thymus. These cells are $CXCR4^+$ $CCR5^-$ and can therefore directly be infected in X4-tropic HIV infection, possibly explaining the more severe pathogenesis of this HIV type. Since most infections occur with R5-tropic virus, induction of CCR5 expression would have a similar effect. Infected $CCR5^+$ macrophages migrate into the thymus, where they phagocytose apoptotic thymocytes and can transmit the virus once thymocytes express CCR5, likely in response to IFN-α. Many cytokines have been implicated in regulation of CCR5 expression, of which IFN-α was found to induce the strongest upregulating effect. Infection of ITTPs with R5-tropic HIV could be prevented by IFN-α-neutralizing antibodies[22].

IL-7 COUNTERACTS IFN-ALPHA INDUCED CELL DEATH

Interferon-α as an antiviral cytokine is commonly used to treat hepatitis C virus (HCV) infection. Lymphopenia is an adverse side effect to this treatment, also in HCV, and it could be hypothesized that this is the result of IFN-α induced AICD. Around 16% of the HIV-infected individuals are co-infected with HCV[68]. These patients already suffer from lymphopenia because of HIV infection, which would be worsened upon administration of IFN-α. HIV-infected patients by themselves express elevated levels of IFN-α and the potential detrimental effects with respect to activation-induced cell death would be strongly enhanced if IFN-α treatment were applied. To prevent further lymphopenia, this IFN-α treatment is not used in HIV/HCV co-infected patients.

Refraining patients from obtaining IFN-α treatment may save these people from worsened lymphopenia, but it also gives a free hand to the hepatitis C infection which is left untreated. An interferon-α treatment, but without the lymphopenia would be a solution to treat HCV in these patients.

IL-7 may counteract the IFN-α-mediated lymphopenia, being a cytokine involved in hematopoiesis and peripheral B- and T-cell homeostasis[69]. Signaling of IL-7 is decreased in T cells from HIV-infected patients, which may explain the detrimental effects of IFN-α[70]. Furthermore, this cytokine has been shown to enhance the increase in T cell levels in HAART-treated HIV patients[71]. Rhesus macaques co-infected with SIV and HCV showed two-fold decreases of circulating T cells when administered with IFN-α. By contrast,

when co-administered with IFN-α and IL-7, around five-fold increases were seen for naïve CD4 and CD8 cell populations. The numbers of proliferating cells were maintained, and also the anti-apoptotic Bcl2 was upregulated, suggesting that both proliferation and survival contribute to better maintenance of lymphocyte numbers. Furthermore, young lymphocytes recently having left the thymus, matured more rapidly into naïve T cells and also contributed to T cell numbers. Administration of IL-7 also led to decreased PD-1 expression on $CD8^+$ T cells, and a higher number of SIV-specific CTLs, suggesting a better antiviral response[72, 73]. IL-7 could thus potentially be used to counteract IFN-α-induced lymphopenia. However, infusion of exogenous cytokines comes with the risk of unforeseen immune activation and must be applied only with great carefulness.

Immune activation is strongly correlated with disease progression in HIV infection and this activation is strikingly absent in natural hosts such as sooty mangabeys[74]. It is important to focus on the potential causes of the natural host lack of immune activation, since these differences may be the key to render SIV or HIV infection nonpathogenic in nonnatural hosts. A number of differences between the immune responses of natural and nonnatural hosts have been identified so far.

Although massive $CD4^+$ T cell depletion in the MALT occurs in both sooty mangabeys and rhesus macaques, this depletion does not lead to loss of intestinal integrity in natural hosts. Immune activation in the gut is an unlikely cause of intestinal damage and subsequent microbial translocation, because sooty mangabeys, retaining intestinal integrity, show strong immune activation during acute infection. During this phase, sooty mangabeys express high levels of interferon-α and do not activate regulatory T cells to suppress the acute immune response. However, during chronic infection, their regulatory T cells are functional and likely control their immune activation. In nonnatural hosts, T_{regs} are strongly activated during acute infection and therefore have been suggested to suppress a crucial early immune response against SIV[54]. During chronic infection T_{reg} function is impaired, allowing for the detrimental vicious circle of T cell depletion.

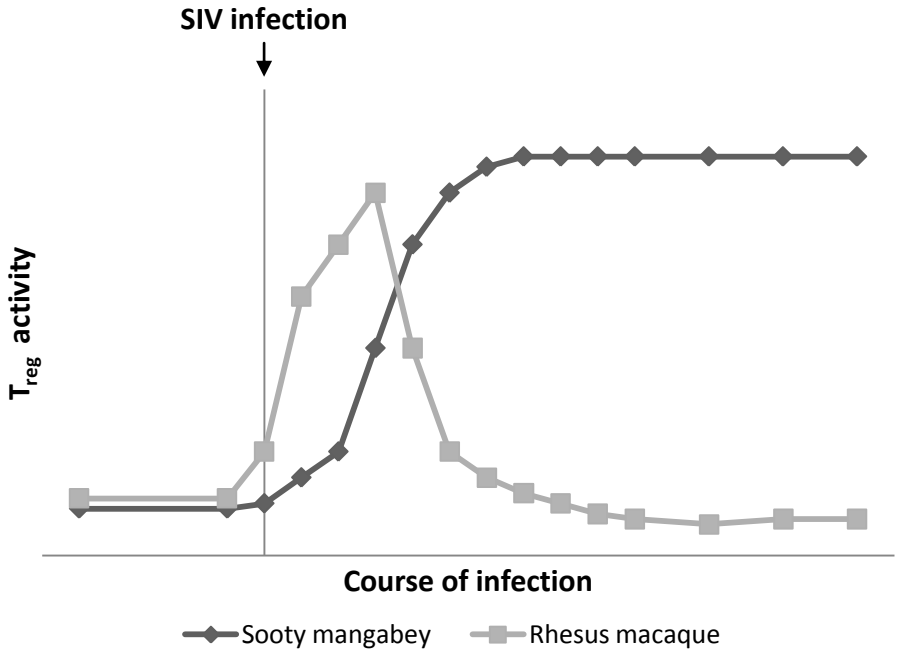

FIGURE 4: ACTIVITY OF REGULATORY T CELLS IN SIV INFECTION OF SOOTY MANGABEYS OR RHESUS MACAQUES. Sooty mangabeys are able to control chronic immune activation by T_{reg} activity. By contrast, these primates do not control their immune response during acute SIV infection. In rhesus macaques this is the other way around. Limiting the acute immune response may allow the virus to establish an effective infection, whereas the detrimental immune response during chronic infection cannot be downregulated. This graph is purely to illustrate the general activity of T_{regs} and both the activation rate, as well as the time course of infection are in arbitrary units.

Apart from regulatory T cells, interferon-α has been suggested as an important factor in immune activation leading to HIV pathogenesis. HIV and SIV molecular patterns are recognized by TLR7 and 9, being expressed on plasmacytoid dendritic cells. In response to TLR7 signaling, the IRF-7 transcription factor is activated, stimulating expression of IFN-α. IFN-α then induces T cell activation, either directly or via activation of myeloid dendritic cells. This leads to activation-induced cell death when overactivation of T cells occurs. Strikingly, natural host pDCs produce intrinsically lower levels of IFN-α as a consequence of polymorphisms in IRF-7[32].

Activation-induced cell death via IFN-α-mediated T cell activation is caused by expression of the apoptosis-inducing proteins PD-1 or TRAIL. Their expression patterns are lower in natural hosts, but this may simply be a consequence of lower IFN-α levels. PD-1 may contribute to associated T cell depletion seen during early infection in nonnatural hosts. In sooty mangabeys however, this protein has a beneficial effect when downregulating the acute immune response against SIV[55].

	Nonnatural host	**Sooty mangabey**
Development of AIDS	Yes	No
$CD4^+$ T cell depletion	Yes	No
Viral load	High	High
Immune activation	High	Low
Microbial translocation	Yes	No
Interferon-α level	High	Low
T_{reg} activity (acute/chronic)	High/Low	Low/High

TABLE 1: OVERVIEW OF THE GENERAL AND IMMUNOLOGICAL DIFFERENCES BETWEEN SIV INFECTION OF SOOTY MANGABEYS AND NONNATURAL HOSTS.

For over 20 years, the development of a vaccine against HIV has been in progress, with no effective result so far. However, if HIV pathogenicity is actually caused by immune activation, a classic vaccine, activating an immune response, would deteriorate the disease rather than prevent it.

The STEP vaccine trial showed an example of a failing vaccine. In this trial, three HIV genes (*gag, pol* and *nef)* were expressed in a replication-defective Adenovirus-5 vector. However, injection with the vaccine led to an increased risk of HIV infection, compared with the placebo. In retrospect, this too could be correlated to immune activation. When individuals with a history of adenovirus infection were injected with this vector (closely resembling the common cold-causing adenovirus), their immune response was boosted[75]. Classic vaccines, including the STEP trial, have always aimed for eliciting a humoral immune response. As a consequence of HIV variability, antibodies however do

not seem effective against this virus because their optimization takes days and they are always one step behind at the rapidly changing virus.

T-cell mediated immunity is thought to be more efficient against HIV infection. However, an effective T cell-mediated immune response against lentiviral infections does not occur in sooty mangabeys, in which the virus has been present for millions of years[76]. Evolution has not selected for sooty mangabeys actively suppressing viral load, but rather induce downmodulation of their immune response in the presence of high viral loads[67]. Chimpanzees and LTNPs are able to elicit and effective T cell response, but these responses are highly dependent on MHC I alleles, which cannot be altered in other individuals. All attempts in creating an HIV-vaccine have failed so far, and nature has not selected for an active immune response against SIV for over a million years. Therefore, it may be time for a different approach.

One such approach could be to mimic the low sooty mangabey immune response. Two main distinct features in nonnatural hosts are the early regulatory T cell activation (with impaired function during chronic infection) and higher levels of interferon-α, which has been suggested to drive activation-induced cell death when its levels are too high.

Controlling T_{reg} activity during chronic infection would induce a stronger immune response against HIV, but increases the risk of immune overactivation or autoimmunity already occurring in nonnatural hosts. The opposite, activating T_{regs} during chronic infection using IL-2, has been suggested. IL-2 is secreted by activated T cells and induces activation and proliferation of T cells, including T_{regs}. Administration of IL-2 therefore would cause general T cell activation, which can be extremely dangerous. This treatment did not cause a protective effect in combination with antiretroviral therapy and is therefore no longer extensively tested in the context of HIV infection[77].

Instead of trying to activate T_{regs} themselves, their activity can be mimicked. A subset of regulatory T cells secretes the anti-inflammatory cytokines IL-10 and TGF-β and administration of these cytokines would dampen the activity of the immune system in general. This could however induce an immune deficiency and would thus potentially induce AIDS-like symptoms. All in all, targeting the immune system as a whole does not seem the best solution against HIV infection.

It would be better to focus on the branches of the system going out of control in HIV infection. High levels of IFN-α have been suggested as a cause of T cell depletion and a different cytokine, IL-7, is being tested with more promising results than IL-2 in the context of HIV pathogenesis. IL-7 is involved in lymphopoiesis and homeostasis of peripheral T lymphocytes and counteracts IFN-α-mediated cell death. However, administration of cytokines comes with the risk of robust immune activation and therefore is potentially dangerous. Alternatively, IFN-α signaling can also be inhibited by neutralizing antibodies against this cytokine. IFN-α, being an antiviral cytokine, is however important in the context of viral infections other than HIV. In sooty mangabeys, low IFN-α levels are produced upon SIV infection, whereas this cytokine seemed crucial in controlling influenza infection[32, 61].

Activation-induced cell death is not induced by IFN-α itself, but by its downstream signaling target PD-1. Therefore, downregulating PD-1 would protect T cells from dying, but also comes with a risk of autoimmunity. Expression of PD-1 on cytotoxic T cells is elevated in progressive HIV infection but not in nonprogressors. PD-1 therefore is a potential therapeutic target and competitive inhibition of the interaction between PD-1 and PD-L1 has been suggested. A PD-1 blocking antibody is being tested in monkeys[74]. However, PD-1 is expressed broadly on many T cell subsets and inhibiting this immune repressing protein may have unwanted side effects, similar to the induction of T_{reg} or exogenous cytokine signaling.

Sooty mangabeys do not show an all-or-nothing response when it comes to interferon-α production and the associated responses. When induced by TLR7 and IRF-7, IFN-α levels are markedly low, as opposed to the production induced by non-pDC-expressed TLRs[32]. This more subtle difference in IFN-α may lie in genetic polymorphisms present in the transactivation domain of IRF-7. Similar polymorphisms have been correlated to low IFN-α production in humans[62], and have been suggested to result in an LTNP phenotype when infection with HIV occurs.

A more gentle control of IFN-α thus seems the best solution against pathogenic HIV infection, if indeed pathogenicity is the result of an overactive IFN-α response. The induction of a genetic polymorphism in the human population is obviously not feasible but testing this polymorphism could give great insight in the significance of IFN-α levels in detrimental immune activation. Of course there are ethical objects against introducing the primate IRF-7 polymorphism in a human. However, if the human IRF-7 gene would be introduced in a natural SIV host, it is expected that this primate would progress to AIDS when infected with SIV, and it can be defined whether IRF-7 is a crucial factor in pathogenesis of the human and simian immunodeficiency viruses. This experiment would give rather similar information to creating a human not progressing.

With gene therapy being still in its infancy and many dangerous potential side effects, the genetic ‘correction’ of IRF-7 polymorphisms in the human race is far from being applied in the clinic. If the human variant of the IRF-7 gene could induce AIDS in a sooty mangabey however, the importance of interferon-α in the pathogenesis of HIV is confirmed. IFN-α and associated proteins may then be utilized as potential therapeutic targets in HIV therapy.

MINI INTERNSHIP

During maturation of T and B lymphocytes, their antigen-specific T cell receptor (TCR) or B cell receptor (Ig) is formed. Concerning the enormous number of potential epitopes, high variability among the antigen receptors has to be reached. This is achieved by V(D)J recombination, the process in which multiple gene segments are joined together to form one receptor chain. Most TCRs consist of an α and a β chain, which develop in V-J and V-D-J recombination respectively.

In humans, there are 54 V and 61 J segments for the α chain, and 67 V, 2 D and 14 J segments for the β chain[23]. Any Vα segment can be joined to any Jα segment, and any Vβ segment to any Dβ segment, which in turn can be joined to any Jβ. Apart from this, the rearranged VDJ segments from the β chain can be joined to two different constant regions. When both the α and β chain have been completed, they are combined, which further enhances TCR variation. In total, this leads to a variation in the order of 10^7 combinations for the human αβ TCR.

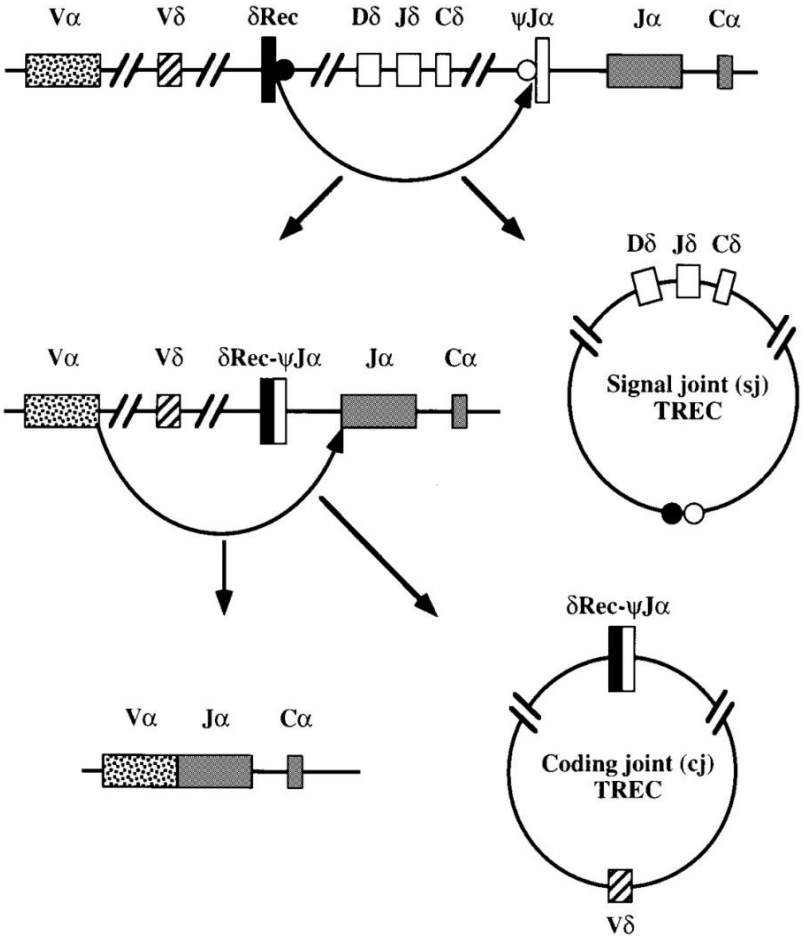

FIGURE 1: ORIGIN OF T CELL RECEPTOR (TCR) EXCISION CIRCLES (TRECS).
In V-J recombination of the TCR α-chain, two excision events take place, each resulting in a TREC. These TRECs can subsequently be used to measure the proportion of T cells recently having left the thymus, and thereby determine thymic output.
Haynes, BF, *Annu Rev Immunol* (2000) 18:529-560

The alternative for an αβ TCR is a receptor consisting of γδ chains. α and δ V, (D) and J segments are located at the same region on chromosome 14. Vδ and Dδ segments are located in between the Vα and Jα segments. Therefore, during V-J joining of the α chain, these δ segments have to excised from the genomic DNA. These DNA fragments circularize, rendering them stable, and they are called T cell receptor excision circles (TRECs)[23]. These so-called signal joint TRECs are the most commonly used to determine T cell dynamics.

As shown (fig 1), two TRECs are produced per cell from single TCRα development. TCR formation occurs just prior to, or inside the thymus. Since these excision circles are not replicated during the S

phase of cell division, they are diluted rapidly when a mature T cell divides. Naïve T cells containing two TRECs thus have recently left the thymus and the average number of TRECs per T cell can be used to measure T cell dynamics, including thymic output, cell death and peripheral division[78].

With ageing, both the size of the thymus as well as its output decrease. Therefore, TREC numbers too decrease with age. In healthy individuals, a negative correlation can be found between TREC numbers and age, although the rate of this decline varies per individual. In HIV infection, where enormous rates of T cell activation occur as a compensatory mechanism for $CD4^+$ T cell depletion, TREC numbers decline much faster.

In the current study, TREC numbers are compared between HIV-free individuals of different ages to verify the common notion of decreasing TREC numbers with increasing age.

MATERIALS AND METHODS

Blood samples

Blood samples were obtained from donors involved in the Mini Donordienst at the University Medical Center Utrecht. Dates of birth were included.

Isolation of T cell subsets

Blood samples were diluted 1:1 in 5% FCS and mononuclear leukocytes were separated using a Ficoll-Paque gradient and isolating the buffy coat. From these PBMCs, $CD4^+$ and $CD8^+$ T cells were isolated using MACS. PBMCs, $CD4^+$ and $CD8^+$ T cells were tested for the proportion of naïve and memory cells, and purity after MACS using flow cytometry with BD FACS Diva software. Antibodies against CD3, CD4, CD8, CD14, CD16, CD27, CD31 and CD45RO were used. The monocyte marker CD14 was included as a negative control for lymphocytes and *vice versa*.

DNA isolation

DNA was isolated using a NucleoSpin Blood QuickPure DNA extraction kit and frozen after step 1 of the protocol. After continuing the protocol, elution was performed with demi water instead of elution buffer, with twice 50 µl/10^6 cells, the resulting volume being 100 µl water per 10^6 cells, or 70 µl for every sample containing less than 10^6 cells.

TREC analysis

The number of TRECS was determined using quantitative Taqman PCR of the PBMC, $CD4^+$ and $CD8^+$ samples. Signal joint TREC numbers were quantified by comparison with Cα DNA. These TREC numbers (per µg DNA) were divided by 150.000 to obtain the mean TREC number per T cell. Also, proportions of naïve T cells were calculated, to correlate TREC numbers with these cells.

Proportion of naïve T cell subsets ranges between blood donors

TRECs are almost exclusively present in naïve T cells, because of their rapid dilution once a T cell divides and becomes an activated effector cell. Therefore, to determine TREC numbers, naïve T cells have to be isolated. After isolating the buffy coats from mini donordienst donors, PBMCs were obtained. From these PBMCs, a CD4 and a CD8 T cell population were isolated using MACS. The absolute number of total PBMCs differed widely between the donors, ranging from $5{,}74 \cdot 10^6$ (donor 2000071) to $21{,}9 \cdot 10^6$ (donor 98071).

Using forward scatter (FSC) and side scatter (SSC), the expected lymphocyte population was gated. From this population, CD3-positive cells were divided in CD4 and CD8. Of these cells, roughly two-third was $CD4^+$ and one-third $CD8^+$, which are normal ratios for T cells.

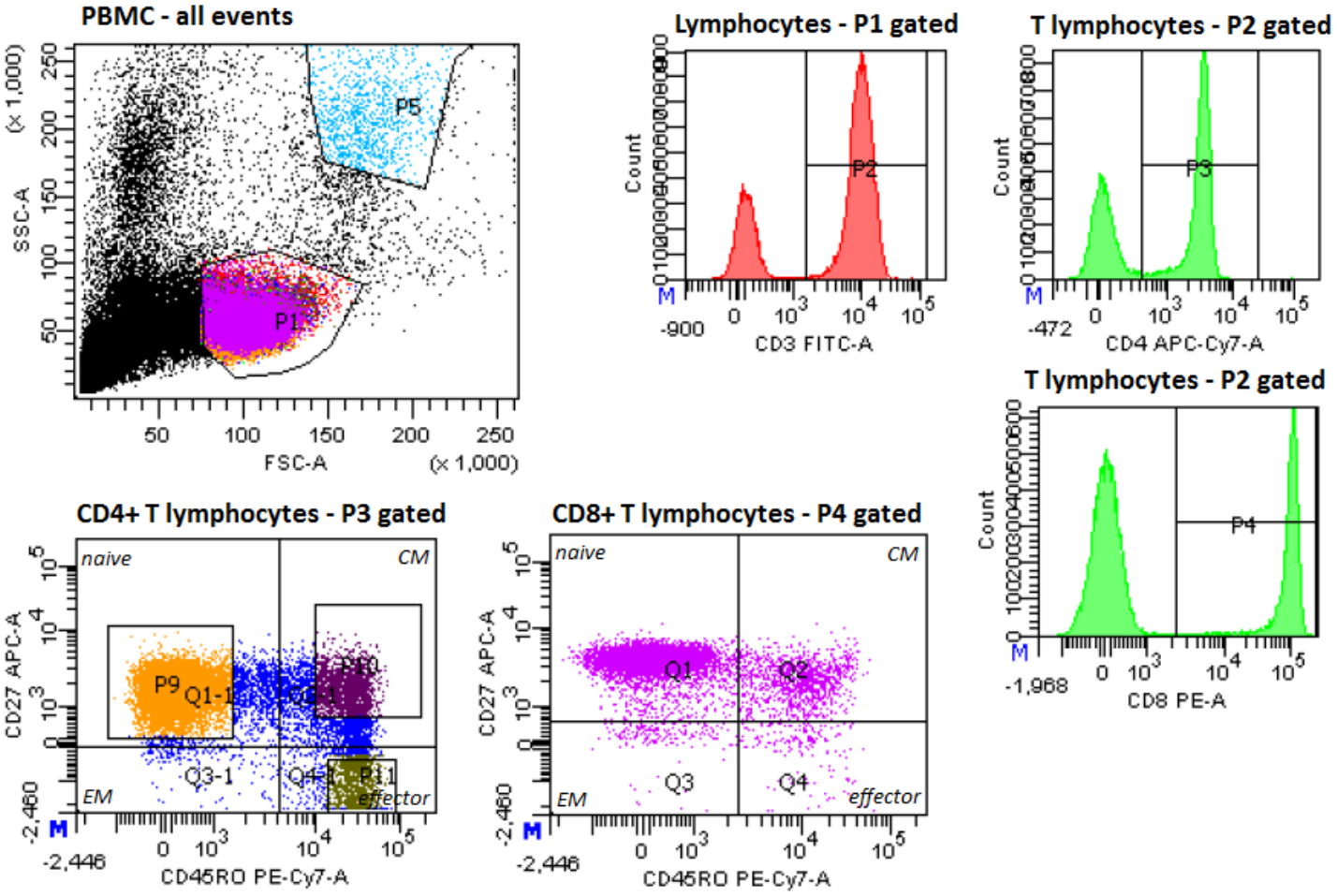

FIGURE 2: FLOW CYTOMETRIC RESULTS OF DONOR 2009032 PBMCS. Lymphocytes were selected on basis of FSC and SSC, then T lymphocytes were identified by CD3 expression, which were further divided on basis of CD4 and CD8 expression. Out of either T cell subset, the proportions of naïve (Q1-1 and Q1) naïve cells were determined.

PBMCs, and also MACS-purified $CD4^+$ and $CD8^+$ T cells were then divided in four populations using CD27 and CD45RO expression. Naïve T cells are indicated as $CD27^+$ $CD45RO^-$, central memory (CM) as $CD27^+$ $CD45RO^+$, effector memory (EM) as $CD27^-$ $CD45RO^+$ and effector T cells as $CD27^-$ $CD45RO^-$. Effector memory $CD4^+$ T cells should not be present in healthy individuals. The percentages of naïve T cells in PBMCs and $CD4^+$ and $CD8^+$ purified populations are shown in table 1. In the CD4 subset, a higher percentage of naïve T cells was observed as compared with CD8. However, total CD8 numbers were relatively low and may thus not be reliable, especially for donor 5 at 21-06, where only one naïve cell was found in the CD8 purified population.

		% naïve cells, TREC no./µg DNA and TREC no./T cell		
Donor no.	Age (years)	Total PBMC	CD4 T cells	CD8 T cells
2009032	24,2	n/a 3817 $2{,}54 \cdot 10^{-2}$	65,8% 3527 $2{,}35 \cdot 10^{-2}$	82,2% 6814 $4{,}54 \cdot 10^{-2}$
2009020	26,5	n/a 2928 $1{,}95 \cdot 10^{-2}$	71,7% 4725 $3{,}15 \cdot 10^{-2}$	76,3% 4143 $2{,}76 \cdot 10^{-2}$
2004063	37,1	n/a 2646 $1{,}76 \cdot 10^{-2}$	56,7% 2505 $1{,}67 \cdot 10^{-2}$	44,1% 4373 $2{,}92 \cdot 10^{-2}$
98071	42,1	n/a 1435 $9{,}57 \cdot 10^{-3}$	54,1% 1891 $1{,}26 \cdot 10^{-2}$	35,0% 1617 $1{,}08 \cdot 10^{-2}$
2000092	50,3	n/a 552 $3{,}68 \cdot 10^{-3}$	24,4% ND ND	5,8% 440 $2{,}93 \cdot 10^{-3}$
95047	52,0	n/a ND ND	63,5% 770 $5{,}13 \cdot 10^{-3}$	41,6% ND ND

TABLE 1: NAÏVE T CELL PERCENTAGES AS OBTAINED BY FLOW CYTOMETRY AND CORRESPONDING TREC NUMBERS. TREC numbers were determined as described in the materials and methods, both per µg DNA and per T cell. Naïve T cell proportions were determined to correlate TREC numbers, because TRECs are only present in naïve cells. These naïve cells are indicated as $CD27^+$ $CD45RO^-$.

Determination of TREC numbers

TREC numbers were determined using qPCR. One µg of DNA was present for each PCR reaction, thus TREC numbers per µg DNA could directly be determined. On average, 1 T cell contains 1\150.000 µg DNA, thus the TREC number per T cell could be determined by dividing this amount by 150.000.

As expected from literature, TREC numbers declined with age (fig 3). Apart from the TREC number per T cell, the proportions of naïve CD4 and CD8 T cells were determined (fig 4), because only these cells are expected to contain TRECs. These proportions too

declined by age, except from donor 95047 who had extremely high numbers of naïve T cells. The decline of naïve T cells supports the decreasing TREC number in older people.

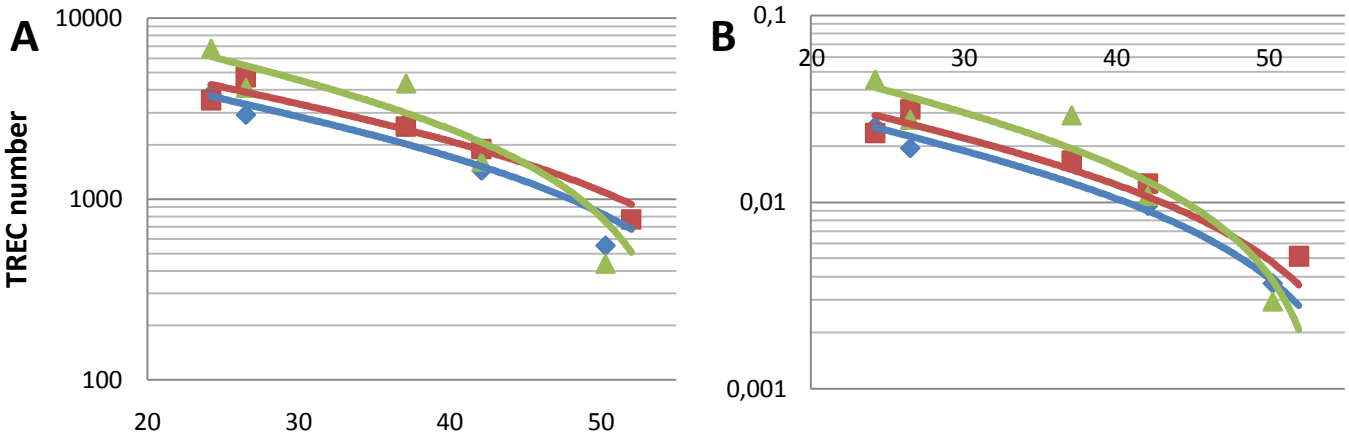

FIGURE 3: TREC NUMBERS PER µl DNA (A) OR PER T CELL (B), CORRELATED WITH AGE.

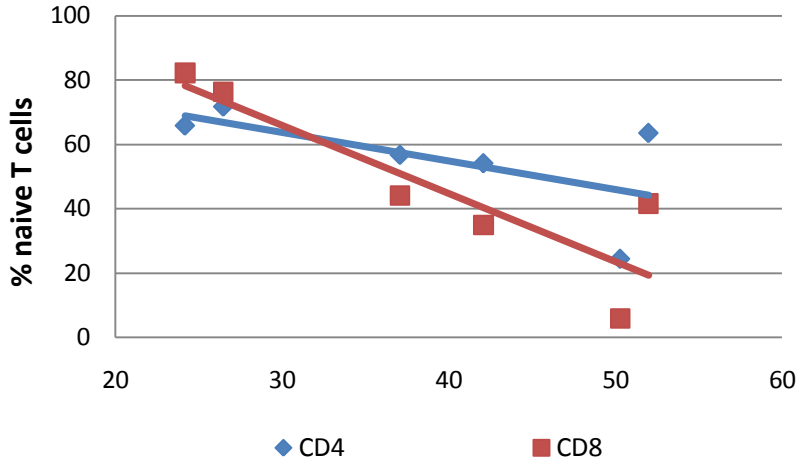

FIGURE 4: PERCENTAGES OF CD4 AND CD8 T CELLS CORRELATED WITH AGE.

DISCUSSION

In this experiment, the phenomenon of decreasing TREC numbers was investigated. This process has been described in the literature, and TREC numbers are utilized to determine peripheral T cell division, cell death and thymic output. Under healthy conditions, TREC numbers decline with age, but in HIV infection, these declines are much stronger.

As expected, TREC numbers were found to negatively correlate with age. It must be noted that due to short time and the low number of donors, these result are not significant, but they suggest a correlation in agreement with literature.

In these results, donor 95047 seemed an outlier when the percentages of naïve T cells were determined. If this donor is removed from the data, a much stronger correlation is

found between decreasing naïve T cells with increasing age (R^2 = 0,8388 without donor 95047 vs. R^2 = 0,3852 including donor 95047 for CD4, and R^2 = 0,9949 vs. R^2 = 0,7765 for CD8). However, due to the low number of donors, it is unclear whether this donor really is an outlier and the data are therefore not removed.

Even when these prospective outlier data are taken into account, a clear reduction in the percentage of naïve T cells is observed, which is correlated indeed with lower TREC numbers. This supports the current notion on declining TREC numbers and suggests that TREC numbers are a useful tool for the determination of various aspects in T cell dynamics.

ABBREVIATIONS

AICD	activation-induced cell death
AIDS	acquired immune deficiency syndrome
APC	antigen presenting cell
DC	dendritic cell
GALT	gut-associated lymphoid tissue
HIV	human immunodeficiency virus
IEL	intra-epithelial lymphocyte
IFN	interferon
ITTP	intra-thymic T cell precursor
LTNP	long-term non-progressor
LTR	long terminal repeat
MALT	mucosa-associated lymphoid tissue
mDC	myeloid dendritic cell
PBMC	peripheral blood mononuclear cell
PD-1	programmed cell death-1
pDC	plasmacytoid dendritic cell
SHP	Src homology phosphatase
SIV	simian immunodeficiency virus
SIV_{RM}	SIV variant in rhesus macaques
SIV_{SM}	SIV variant in sooty mangabeys
TCR	T cell receptor
TLR	Toll-like receptor
TNF	tumor necrosis factor
TRAIL	TNF-related apoptosis-inducing ligand
TREC	T cell receptor excision circle

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