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Frequencies of myeloid-derived suppressor cells in relation to the cytotoxic T cell response in HIV-infected patients of the New Era Study

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Zusammenfassung der Arbeit

Die HIV-Infektion ist eine ernstzunehmende Erkrankung, die nahezu 38 Millionen Menschen weltweit betrifft. Dank des Forschungsfortschrittes lässt sich die Infektion heutzutage mit einer kontinuierlichen hochaktiven antiretroviralen Therapie behandeln. Allerdings ist eine Heilung der Erkrankung bislang nicht möglich.
Diese Arbeit untersuchte die Effekte einer Therapieintensivierung auf das Immunsystem von HIV Patienten der New Era Studie (NE). Hierbei wurden 5 antiretrovirale Medikamente über einen Zeitraum von mindestens 5 Jahren verabreicht mit dem Ziel langfristig eine funktionale Heilung der Infektion zu erreichen. Eine Gruppe erhielt das Therapieregime aus 5 Medikamenten während der chronischen Phase der Infektion (CHI), wohingegen eine weitere Gruppe während der akuten Phase begann (PHI). Die Ergebnisse wurden zwischen diesen beiden Gruppen verglichen sowie mit weiteren HIV Gruppen. Darunter befanden sich Patienten mit einer fortgeschrittenen Infektion ("Progressor"), Patienten mit einem konventionellen Therapieansatz und "HIV Controller". Einerseits beschäftigte sich diese Arbeit mit dem Effekt der Behandlung auf die zwei Untergruppen der Myeloid-derived suppressor cells (MDSCs), nämlich den PMN-MDSC und M-MDSC. Andererseits wurde der Effekt von spezifischen CD8-T-Zell Antworten auf zwei HIV Proteine Gag und Nef untersucht. Außerdem wurden mögliche Korrelationen zwischen den immunsuppressiven Zellen und den HIV-spezifischen CD8-T-Zell Antworten überprüft.

Interessanterweise waren die Ergebnisse für die MDSCs und die Immunantworten der NE Kohorte vergleichbar mit den Ergebnissen der Patienten unter konventionellem Therapieansatz. Daher konnte in diesem Zusammenhang kein Vorteil für die Intensivierung der Therapie gezeigt werden. Desweiteren konnte kein klarer Vorteil für die Patienten mit einem frühen Behandlungsbeginn aus den Ergebnissen interpretiert werden. Die antiretrovirale Therapie schien sich stärker auf die PMN-MDSC Untergruppe auszuwirken, da hier nahezu normale Level erreicht wurden. Dagegen war die Frequenz der M-MDSCs im Vergleich zu den gesunden Probanden signifikant erhöht. Obwohl MDSCs für ihre suppressive Funktion auf T-Zellen bekannt sind, konnten keine Korrelationen zwischen den zwei Untergruppen der MDSCs und den HIV-spezifischen CD8-T-Zellen gefunden werden.

Die Level der MDSCs waren vergleichbar zwischen den NE Patienten und den Patienten unter einer konventionellen Therapie mit cART. Allerdings zeigten die NE Patienten überraschend breite CD8-T-Zell Antworten, was auf einen höheren Grad der Erhaltung des Immunsystems dieser Patientengruppe schließen lassen könnte.

Abstract

The HIV infection is a serious health burden that affects almost 38 million people worldwide. Thanks to extensive research, the infection has become manageable for patients under a continuous highly active antiretroviral treatment but a cure has not been found up to now. This thesis investigated the effects of treatment intensification on the immune system of HIV patients of the New Era Study (NE). This involved 5 antiretroviral drugs over a minimal duration of 5 years with the long-term objective of reaching a functional cure of HIV. One subgroup received the 5-drug regimen during the chronic stage (CHI) whereas the other subgroup started during primary infection (PHI). Results were compared within the two NE subgroups and to other HIV groups, including progressors, patients under a conventional cART and natural controllers. On the one hand, this study focused on the effect of treatment on the two subsets of myeloid-derived suppressor cells (MDSCs), namely PMN-MDSC and M-MDSC. On the other hand, the effect on CD8 T cell responses specific to two proteins of HIV, Gag and Nef, was studied. Possible correlations between the immunosuppressive cells and the HIV-specific CD8 T cell responses were also tested.

Intriguingly, the results for MDSCs and immune responses were similar in the NE cohort and patients under cART. Thus, it was not possible to postulate an advantage for the treatment intensification. Furthermore, a clear advantage for the patients with an early treatment start could not be interpreted from the results either. The impact of antiretroviral treatment appeared to be greater on the PMN-MDSC subset as it led to almost normal levels. In contrast, the frequency of M-MDSCs remained significantly higher in comparison to healthy individuals. Although MDSCs are known to exert their suppressive function on T cells, there were no correlations found between the two subsets of MDSCs and the HIV-specific CD8 T cells investigated.

In the NE patients, the level of MDSCs was comparable to those of the conventional cART-treated patients. However, NE patients revealed a surprisingly broad CD8 T cell response which might imply a higher degree of immune preservation in this group of patients.

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List of abbreviations

AIDS	Acquired Immunodeficiency Virus
APC	Allophycocyanin
cART	Combined antiretroviral therapy
CD	Cluster of differentiation
CDC	Centers for Disease Control and Prevention
CHI	Chronic HIV-infected patients
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic T-lymphocyte-associated Protein 4
DNA	Deoxyribonucleic acid
EI	Entry Inhibitors
Elispot	Enzyme-linked immunospot assay
FACS	Fluorescent-activated cell sorting
FITC	Fluorescin Isothiocyanate
FSC	Forward Scatter
HAART	Highly active antiretroviral therapy
HC	Healthy Control
HIV-1, -2	Human Immunodeficiency Virus type 1, -2
HLA	Human Leucocyte Antigen
IL-2	Interleukin-2
INI	Integrase Inhibitors
LAG-3	Lymphocyte Activation Gene 3
M	Monocytic
MDC	Multi-Drug Class
MDSC	Myeloid-derived suppressor cells
MHC1	Major Histocompatibility Complex 1
NE	New Era
NNRTI	Non Nucleoside Reverse Transcriptase Inhibitor
NRTI	Nucleoside Reverse Transcriptase Inhibitor
PBMC	Peripheral Blood Mononuclear Cells
PD-L1	Programmed Death Ligand 1

PE	Phycoerythrin
PerCP	Peridinin Chlorphyll Protein
PHI	Primary HIV-infected patients
PI	Protease Inhibitor
PMN	Polymorphonuclear
PR	Progressor
PrEP	Pre-exposure prophylaxis
RNA	Ribonucleic acid
RPM	Rotations per minute
SIV	Simian Immunodeficiency Virus
SSD	Side Scatter
START	Strategic Timing of Antiretroviral Therapy
TIM-3	T-cell immunoglobin and mucin-domain containing-3
TNF-alpha	Tumor necrosis factor alpha
VISCONTI	Viro-Immunological Sustained Control after Treatment Interruption
VL	Viral load

1 Introduction

1.1 Milestones in the history of HIV

The first isolation of the Human Immunodeficiency Virus (HIV) from a lymph node biopsy dates back to 1983. This finding led to the conclusion that HIV was the causative agent of the Acquired Immunodeficiency Syndrome (AIDS), a severe immune suppression which had previously been described as a new disease by the CDC in 1982. (1) Since its discovery, this virus has infected over 70 million people and over 32 million AIDS-related deaths have been reported worldwide (2). As a global burden for health and economics, HIV has drawn significant attention from the research community and has thereby become the most researched virus amongst all.

In the first years after HIV discovery, an infection inevitably led to death after 8-10 years. Due to intensified research commitment over the past 35 years, it has been possible to develop an effective antiretroviral treatment for patients infected with HIV. Nowadays, HIV patients who consistently follow their treatment regimen can reach a life expectancy comparable to a non-infected individual. One of the latest achievements in the recent years has been the establishment of an effective pre-exposure prophylaxis (PrEP) which reduces the chances for those who are at substantial risk of HIV infection to near-zero. Despite those major breakthroughs in HIV management, neither the attempts to find a cure for HIV infection nor a vaccine to prevent the transmission of HIV in the first place have been successful. It is therefore important to further study the virus in relation to its host in order to identify new approaches in terms of treating and curing HIV, respectively.

1.2 The nature of the Human Immunodeficiency Virus

1.2.1 Transmission routes of HIV

HIV has rapidly evolved into a human pandemic spanning across all continents and different types of people. This was possible due to different routes of transmission including sexual transmission which accounts for the majority of reported HIV cases. However, in some parts of the world, particularly in Eastern and Southern Africa, vertical transmission from a HIV-positive mother to her child is of great concern. (3) Another route of transmission is via blood, for example by sharing needles amongst drug users, and has increased the number of people with HIV particularly throughout Eastern Europe and parts of Asia. (4)

1.2.2 Two Subtypes of HIV: HIV-1 and -2

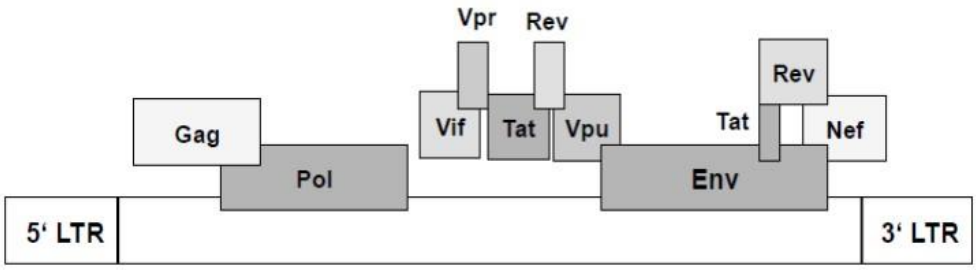
HIV is a lentivirus which is part of the family of retroviruses and can be classified into two major types, HIV-1 and HIV-2. An infection with the former is more common and responsible for the AIDS pandemic. Therefore HIV-1 will be the focus of this study. The latter is mostly found in West Africa. It significantly differs from HIV-1 in terms of its markedly reduced pathogenicity, slower disease progression and its response to the available treatment options. (5)

1.2.3 The HIV Genome

The genome of HIV-1 is comprised of three major structural genes which can also be found in other retroviruses: Gag (*group specific antigen*), Pol (*polymerase*) and Env (*envelope*). In addition, the genome is comprised of 4 accessory (Vif, Vpr, Vpu and Nef) and two regulatory genes (Tat and Rev) which are unique to HIV. The Gag gene codes for a precursor protein that is cleaved to several HIV proteins. Those are involved in HIV assembly and release. (6) Nef (*negative factor*) codes for a regulatory protein that enables the virus to easily replicate particularly in the early stages of infection by downregulating certain parts of the immune system of the host. (7) Due to their high immunogenicity, Gag and Nef are of great interest for this study. (8)

Figure 1: Structure of the HIV-1 genome.

Figure 1 schematically illustrates the organisation of the HIV-1 genome. (9) It is comprised of three structural genes (Gag, Pol and Env) as well as six regulatory and accessory genes (Vif, Vpr, Vpu, Tat, Rev and Nef). The Long Terminal repeat (LTR) regions are found on both ends and drive the expression of the HIV-1 genome.

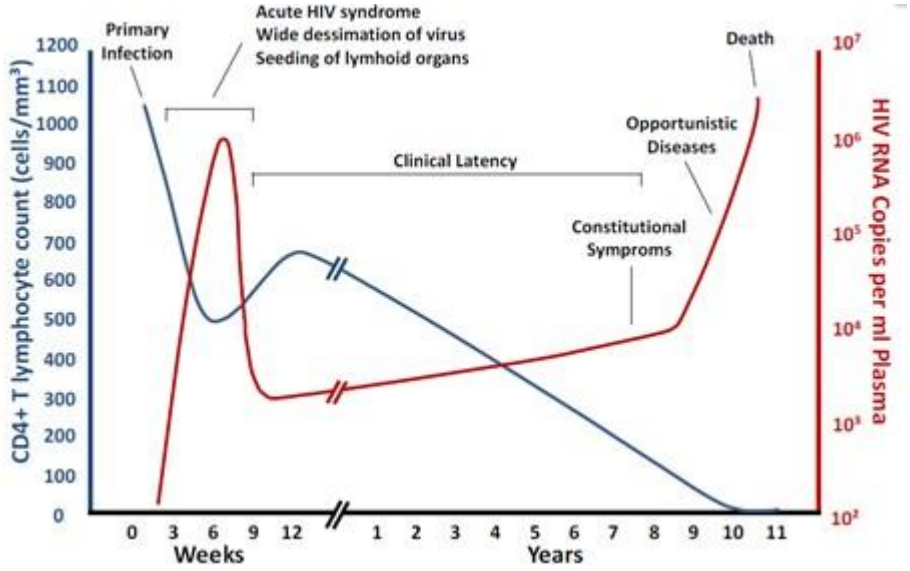


1.2.4 The three stages in HIV infection

The main cellular target of HIV is the CD4 T lymphocyte whose major function is to coordinate the immune response by regulating other immune cells which comprises among others the production of cytokines to the stimulation of B cell maturation and the activation of cytotoxic T cells. When untreated, the HIV infection ultimately leads to the depletion of CD4 T cells. The typical course of an untreated HIV infection is defined by different stages which can be referred to as acute, asymptomatic and AIDS as the final stage.

Figure 2: CD4 cell count and viral load during the course of an HIV infection.

Figure 2 shows a typical course of HIV infection during the acute and chronic stage regarding two clinical parameters, CD4 cell count and viral load. CD4 cell count is given in cells/mm^3 on the left side whereas viral load is given in copies/ml on the right side of the graph. (10)



After an individual has encountered HIV, the virus rapidly multiplies within the human body resulting in extremely high viral loads which lead to a greatly increased risk of transmission. The viral load inversely correlates with the CD4 cell count which drops during the acute phase and even falls below 200/µl. The acute/primary stage of infection takes up to 4 - 6 weeks where the infected person often suffers from non-specific flu-like symptoms. This is followed by an asymptomatic stage in which the CD4 cell count usually recovers at least in part. This results in the control of the viral load at least for a certain period of time before it slowly starts to increase on a long term. This stage varies in length, though most people advance to the final stage within 6 – 8 years in the absence of antiretroviral treatment.

Low CD4 cell counts are characteristic of this stage and show that the immune system has severely been damaged over the course of time. An array of opportunistic infections and cancers can be seen during this final stage which is better known as AIDS.

1.3 Treating HIV: Success and limitations

In 1996 the combination of highly antiretroviral drugs (HAART), nowadays referred to as combined antiretroviral therapy (cART), was introduced and revolutionised the treatment of HIV up to the present day. cART effectively reduces the viral load of the infected patient to an undetectable level of at least ≤ 50 copies/ml. Once considered a fatal illness, HIV has turned into a manageable condition given that treatment is applied for life. (11)

Basically, cART consists of a combination of three drugs which interfere with specific steps in the viral life cycle. An integral part of a typical drug regimen are nucleoside reverse transcriptase inhibitors (NRTIs) and non-nucleoside reverse transcriptase inhibitors (NNRTIs) that stop viral multiplication by blocking 'reverse transcriptase'. Further drugs used in the regimen are protease inhibitors (PIs), integrase inhibitors (INIs) and entry inhibitors (EIs) named with regard to the step they interfere with in the viral life cycle.

In order to prevent drug resistances, it is important to use at least two different drug classes at a time. (12) Furthermore, the patient needs to make sure that the prescribed drug regimen is strictly followed without allowing any pauses. Despite the continuous improvement of HIV drug agents, a major factor contributing to poor adherence still are the side effects caused by cART which can lead to a number of different long-term effects including hepatotoxicity, lipodystrophy or osteoporosis. (13)

Since most HIV patients still get diagnosed in their early adulthood, an average HIV patient will need to receive cART for a significant amount of time, approximately 30 to 50 years. (14) The results of the START study (Strategic Timing of Antiretroviral Therapy) published in 2015 further emphasized that the earlier antiretroviral treatment was initiated the better the outcome for the patient. (15) Altogether, this puts an enormous financial burden on health systems worldwide and emphasises the need for new solutions in fighting HIV on a global scale.

1.4 New perspectives on Curing HIV

To date, no cure for HIV has been found yet. In the past it has been difficult to target the virus in a way that allows full eradication from the human body. A major obstacle in the search for HIV eradication is that HIV latently resides in cells which are not detected by the immune system but have the ability of viral reactivation. These cells are memory CD4 T cells, can be found at different tissue sites and are collectively known as the HIV reservoir. (16) Besides a full viral eradication, another concept of curing HIV is referred to as 'functional cure'. This describes a state in which the virus is kept under control by the immune system itself in the absence of antiretroviral drugs.

This phenomenon has already been described in a small group of HIV-infected individuals which make up less than 1% of the HIV population. Those are HIV controllers which are characterised by low viral loads, normal CD4 T cell counts, show no clinical symptoms and do not progress to an advanced stage of infection even in the absence of treatment. On a cellular level, they typically exhibit broader HIV-specific T cell responses, reduced viral reservoirs and protective HLA alleles. (17) Although the determining factors leading to non-progression have not fully been identified, HIV controllers can certainly serve as a model in terms of a 'functional cure'. (18)

Investigating the concept of a 'functional cure' was further encouraged by the French VISCONTI study published in 2013. In this study, researchers identified 14 people who were able to control viremia after treatment interruption. They had received cART within the first weeks after transmission and had followed the drug regimen for approximately 3 years prior to treatment interruption. The results obtained during treatment interruption were surprising: the virus was detectable but significantly less than one would normally expect in untreated HIV patients (<400 copies/ml) and the CD4 cell count did not appear to be affected by the absence of cART. The results were kept stable for a prolonged period of time and there was no need to resume HIV treatment. Similarly surprising were the results from genetic testing performed on those 14 individuals. They revealed that the patients did not come from a favourable genetic background concerning the natural control of HIV, suggesting that other factors must have been responsible for the observed post-treatment control. One of them was the antiretroviral treatment initiated very early in the course of the infection. This might have limited the development of viral diversity and preserved the immune system from chronic immune activation. (19)

1.5 Attempts towards a functional cure for HIV

In the past years, eliminating viral reservoirs in HIV patients has increasingly been of scientific interest as they represent a significant barrier towards a cure for HIV. Although the exact timing is uncertain, viral reservoirs are believed to evolve within the first days to weeks after HIV transmission. Although even an early application of ART cannot block the establishment of the HIV reservoir, early ART appears to result in a reduced reservoir when compared to patients with a later ART start. (20; 21) HIV latently resides in different subsets of resting memory CD4 T cells for a substantial amount of time and is able to escape conventional HIV therapy. There are three main subsets of cells identified which serve as the cellular reservoir even over a prolonged period of ART. Amongst those cells are central, transitional and effector memory CD4 T cells which facilitate viral persistence via different mechanisms. (22) Once antiretroviral treatment is being paused, the cells get reactivated from their resting state and account for the quick and steep increase of viral replicates. Developing a strategy towards targeting those hidden reservoirs, could therefore bring us one step closer to the finding of a ‘functional cure’. (23)

1.5.1 The New Era Study

In 2009 the New Era Study was launched in Germany as an Investigator Initiated Trial (IIT) with the ultimate aim of eradicating HIV amongst study participants. This was performed by initiating a multi-drug class (MDC) regimen during the primary stage of infection. 20 primary infected patients (PHI) had to meet certain inclusion criteria including the presence of less than two bands in the Western blot analysis. In order to compare results from PHI, a second group of 20 chronic HIV patients (CHI) who had already been under cART for at least 36 months was recruited to follow the same MDC cART. Furthermore both groups, PHI and CHI, needed to have a CD4 cell count above 200 cells/ul. The intensified drug regimen included an integrase inhibitor (Raltegravir) and an entry inhibitor (Maraviroc) in addition to a conventional three drug cART which included a protease inhibitor. Based on previously performed calculations, researchers of the New Era study predicted that it would take at least 7 to 8 years of intensified treatment to eliminate latent reservoirs in their patients to possibly reach eradication of the virus. (24)

During the course of the New Era Study, different laboratory parameters were repeatedly taken to monitor the current state of the participants. This included the measurement of cell-associated proviral DNA which is an indicator for existing viral reservoirs.

Those measurements clearly showed the reduction of proviral DNA amongst participants. Nonetheless, post-treatment controllers of the VISONCTI Study showed that low proviral DNA is not a necessary prerequisite for post-treatment control, implying that other predictive factors needed to be taken into account. Following at least 5 years of intensified treatment, an array of additional immunological and viral parameters were investigated in the New Era study to identify markers which could possibly predict a successful post-treatment control. Amongst those were immunosuppressive cells, i.e. myeloid-derived suppressor cells, and CD8 T cell responses which were examined in this thesis.

1.6 Myeloid-derived suppressor cells

1.6.1 Characteristics of Myeloid-derived suppressor cells

Exhaustion of the immune system plays an important role particularly during the advanced stage of HIV infection. It is characterised by a progressive loss of functions of immune cells including CD8 and CD4 T cells. Amongst other consequences, 'immune exhaustion' leads to a loss of CD8 T cells' cytotoxic function disabling them to eliminate HIV. CD4 T cells lose their ability to produce cytokines which in turn is a crucial component in the activation of CD8 T cells. There are several molecules known to be involved in the chronic phase of HIV-1 leading to this phenomenon. Amongst others are PD-1, CTLA-4, LAG-3 and Tim-3. They lead to a downregulation of the process of immune activation. (25) In addition, cell populations that exhibit regulatory effects on immune cells are of great scientific interest. One of those cell populations are myeloid-derived suppressor cells (MDSC). They are a heterogeneous group of immature cells with a myeloid origin which can protect the healthy individual from an overt inflammatory response. Furthermore, they have an impact on tissue repair. Under certain pathological conditions such as infections, cancers and organ transplantations, different factors create an environment that disrupts the maturation of immune cells. In consequence, immature cells rise in numbers and gain certain immunosuppressive functions. This process is facilitated by cytokines such IL-6, IFN-gamma and TNF-alpha during an infection with HIV. (26) Some pathogens and tumours are able to benefit from the regulatory effects of MDSCs and thus manage to escape the immune system response. Although MDSCs have been known and are being studied for more than 20 years, a detailed understanding underlying their involvement in HIV immunopathogenesis is still missing. (27)

Nowadays, MDSCs are not only of great scientific interest in areas such as cancer research but have become increasingly important in the field of infections. Despite extensive research on this specific cell type, study results appear to differ substantially. One factor contributing to this discrepancy in results is certainly the fact that MDSCs represent a vulnerable cell type that has been studied by different research teams with varying approaches. In 2016 *Gruetzner et al* showed that cryopreservation and the time of blood draw are essential when analysing MDSCs and differ between the two existing subsets of MDSCs. (28)

Human MDSCs can be divided into two main subsets according to their distinct morphology and surface phenotype: polymorphonuclear (PMN) and monocytic (M) MDSCs. Their phenotype can be distinguished by flow-cytometry analysis. In the past, different study groups have used different sets of surface molecules to distinguish MDSCs which also contributed to the confusion in study results. The phenotype used for analysis in this study is CD14-, CD11b+, CD66b+/CD15+ for the PMN-MDSC whereas that of M-MDSC is defined as CD11b+, CD14+, HLA-DR-/low, CD15- and corresponds with current recommendations by *Bronte et al.* (29)

1.6.2 Myeloid-derived suppressor cells during HIV infection

Several studies of the past have focused on the investigation of MDSC frequencies at different stages of the HIV infection as well as the effect antiretroviral treatment has particularly on those cells. Despite intensive research in this field, findings of different study groups are inconsistent highlighting the need for further investigations.

In an untreated infection, both subsets of MDSCs, PMN and M-MDSCs, are increased in HIV patients and their frequency correlates with the viral load. Corresponding to their immunosuppressive function, they also appear to correlate inversely with the CD4 cell count during an untreated HIV infection as well as with disease progression. (30; 31; 32) Moreover, PMN-MDSCs were shown to induce CD8 T cell proliferation in vitro whereas M-MDSCs were also shown to inhibit CD8 T cell function. (31; 32) The mechanisms used to suppress the immune system also appear to differ between the two MDSC subsets. (33; 34; 35)

Previous studies have examined the effect of antiretroviral treatment on PMN-MDSC frequencies in HIV patients. In 2012 *Vollbrecht et al* showed that the increased levels of PMN-MDSC in HIV progressors who were not under antiretroviral treatment decreased when cART was initiated. (31) Correspondingly, *Wang et al* showed that PMN-MDSCs were not detected in HIV patients who were under cART with a non-detectable viral load. (36)

Consistent with those results, *Tumino et al* found higher levels of PMN-MDSC in HIV patients when compared to healthy controls. However, they could not find a difference between HIV patients under cART and HIV progressors. (37) Contrastingly, a study by *Zhang et al* was able to find differences in PMN-MDSC frequencies between untreated and treated HIV patients. (38) In a recent study by *Agrati et al*, HIV patients were examined in their primary stage of infection and showed high levels of PMN-MDSC. Interestingly, these high levels of PMN-MDSC were not found to be reduced after antiretroviral treatment had been applied for 48 weeks. (39) Yet another study used SIV-infected macaques to examine the effect of antiretroviral treatment as well as treatment interruption on MDSCs. They found that MDSCs remained elevated over a 7-months course of cART. Upon treatment interruption, they reported a drastic increase in MDSC levels with the major fraction being PMN-MDSCs. (40)

In terms of the dynamics of M-MDSCs during HIV infection, the results are similarly conflicting. In 2013 *Quin et al* demonstrated that M-MDSCs are dramatically increased in the peripheral blood of HIV patients in comparison to healthy controls. They also recorded a decrease in M-MDSC levels in HIV patients under treatment application for 84 weeks. However, the patients did not reach normal M-MDSC levels despite the treatment. (32) Correspondingly, *Wang et al* showed that M-MDSC levels were substantially higher in HIV patients than in healthy controls. This also applied to HIV patients under cART with undetectable levels of viremia. (36) Contrastingly, *Vollbrecht et al* also examined M-MDSCs in HIV patients and found no increase in M-MDSC levels amongst HIV progressors who were not under cART. (31) Overall, this emphasises the need for further research on the function and cellular behaviour of MDSCs and the need to clearly distinguish between the MDSC subsets.

1.7 CD8 T cells

1.7.2 The cytotoxic T cell response

CD8 T lymphocytes, also called cytotoxic T lymphocytes (CTL), are a subpopulation of T cells and part of the adaptive immune system. Besides their clinical relevance in tumour immunology, they are effective in eliminating intracellular pathogens. Thus, CTLs play a crucial role in the control of HIV infection both during the acute and chronic stage. They recognise an infected cell via antigen presentation: An infected cell displays viral peptide fragments on its surface via the Major Histocompatibility Complex-1 (MHC-1), a peptide complex which can be found on every nucleated cell in the body.

CTLs express specific T cell receptors which allow them to bind to only one specific epitope presented in the MHC-1 complex – including HIV-specific epitopes. The interaction between CTLs and the infected cell immediately triggers a cytotoxic response leading to the release of cytokines (TNF-α, IL-2) and the induction of apoptosis. As a result, viral replication is reduced and a decrease in viral load can be noted. A polyfunctional HIV-specific CTL response that is characterised by displaying several effector functions simultaneously might be an important factor for successful control of HIV in HIV controllers. (41; 42) Nonetheless, the CTL response of most HIV patients does not result in a complete suppression of the virus and fails in terms of long-term infection control.

1.7.3 Loss of function in CD8 T cells during HIV infection

Several studies have described a progressive loss of function in CTLs during the course of the HIV infection. CTLs are able to fight HIV by incorporating multiple effector mechanisms. However, the impairment of these mechanisms contributes to the viral immune evasion.

During the later stages of HIV infection, a persistence of HIV antigens will constantly keep CTLs activated. This is known as immune activation and is characterised by the higher expression of immune activation markers such as HLA-DR, CD38 and Ki-67 at the surface of the cell. (43) This constant activation of CTLs then leads to a phenomenon which is termed ‘immune exhaustion’ (see section 1.6.1). The cells gradually lose their ability to respond to HIV in the way they used to during earlier stages and there are multiple factors involved underlying this phenomenon. One study in 2006 compared HIV progressors to non-progressors and found that it is not the mere quantity but rather the quality in functionality of CTLs that matters in terms of disease progression. (44)

There is further evidence that the impaired function of CTLs observed over the course of the infection is also mediated by a change in expression of cellular surface receptors including PD-1, Tim-3 and CD160 which contribute to the exhaustion of the cell. (45) *Buggert et al* showed that transcription factors such as T-bet and Eomes which differentially regulate T cell exhaustion show an inverse expression during HIV infection when compared to the uninfected state and thereby contribute to immune exhaustion. (46)

Interestingly, different studies were able to demonstrate that CTLs' effector functions can at least be restored in part under antiretroviral treatment. (47; 48) *Rehr et al* compared chronic HIV patients during cART initiation and maintenance and found that polyfunctional CD8 T cells rise in numbers when the viral load decreases. (49) Upon treatment interruption this effect is however reversed. (50)

1.7.4 Breadth and magnitude of the CD8 T cell response in HIV patients

Due to their significant role in the control of HIV, CD8 T cells play a central role in curing HIV independent of the pursued strategy, either via a 'functional cure' or HIV eradication. They have also been considered as potential targets in the development of a vaccine. Nevertheless, the factors required for a vaccine of high efficacy are still to be found. Elite controllers are an interesting group of HIV patients to consider in this context as they are able to control viral replication in the absence of antiretroviral treatment. Besides low viremia, they express certain characteristics more commonly such as histocompatibility alleles, i.e. HLA-B57 and HLA-B27, but the underlying mechanisms contributing to the control of HIV are unclear. (51) There are two terms which can be used to describe the HIV-specific CD8 T cell response in relation to HIV epitopes: 'Breadth' is defined by the number of HIV epitopes targeted by the CD8 T cell response whereas 'magnitude' describes how strong the response is towards a certain epitope. In a study by *Addo et al*, two of the most frequently recognised HIV epitopes amongst HIV patients at different stages of infection were 'p24 Gag' and 'Nef'. However, their breadth and magnitude appeared to vary greatly between the study participants. Interestingly, a previously proposed inverse correlation between both, breadth and magnitude, and viral load in plasma could not be confirmed in this study. Moreover, it has been shown that the application of antiretroviral treatment leads to a partial restoration of CD8 T cells even when treatment is initiated at a later stage of the infection. (52) *Addo et al* demonstrated lower and narrower responses in treated patients compared to an untreated cohort. Furthermore, an early initiation of treatment led to low breadth and magnitude. (53; 54) Finally, it is noteworthy that breadth and magnitude are only two possible ways of examining the HIV-specific CD8 T cell response and need to be considered along with other measures.

1.8 Aims of the Study

The New Era study comprised a cohort of HIV patients who had started a 5-drug antiretroviral therapy either during the acute or chronic stage of infection with the ultimate aim of HIV eradication. In order to investigate the impact of an early treatment initiation with a 5-drug cART on parts of the immune system of the HIV-infected patients, the parameters MDSCs and CD8 T cell responses were studied and compared to patients who were treated with a three drug ART, controllers, progressors and healthy controls. Prior to investigation, all New Era study participants had been taking 5-drug cART for at least 36 months.

(1) The first aim of my thesis was to measure levels of MDSC in the New Era cohort from peripheral blood using flow cytometry. We hypothesised that reduced levels in both, PHI and CHI, could be seen due to the intensified treatment. We furthermore hypothesised that PHI had benefited from early treatment by resulting in lower levels of MDSC when compared to CHI. Since it is not clear to what extent the two subpopulations of MDSCs react to antiretroviral treatment, PMN-MDSCs and M-MDSCs were both measured in each study participant.

(2) The second aim was to measure CD8 T cell responses from patients of the New Era cohort by the means of an interferon-gamma-based enzyme-linked immunospot assay (Elispot) in relation to the MDSC frequency. The two viral proteins of interest in this thesis were Gag and Nef as they had previously been recognized to be major targets of CD8 T cells.

(3) The third aim was to compare the results of the New Era cohort with healthy controls as well as with other groups of HIV-infected individuals. Amongst those were HIV controllers, HIV patients under cART for at least 4 years and HIV progressors who were not under any antiretroviral treatment.

2 Materials and Methods

2.1 Materials

2.1.1 Consumables and technical devices

Cell counter, CASY®1 Model TT	Schärfe System, Reutlingen
Cell Culture Flask, 6- und 24-well	PAA, Cölbe
Cell Culture Flask, Filter, 25 und 75 cm²	TPP, Ibbenbüren
Centrifuge 5810R	Eppendorf AG, Hamburg
Centrifuge Tubes	TPP, Ibbenbüren
Centrifuge, Sorvall Super T21	Thermo Scientific, Schwerte
EDTA Monovette, KE/ 9ml	Sarstedt, Nümbrecht
Elispot-Reader, ELR04	AID, Strassberg
FACS Tubes	Becton Dickinson, Heidelberg
Flow cytometer, FACSCalibur	BD, Heidelberg
Freezing Container Mr. Frosty	Nalgene, Wiesbaden
Heated Bath, Model 1083	GFL, Burgwedel
Incubator, Heracell®	Heraeus, Hanau
Microscope Dialux 20 EB	Leitz, Wetzlar
Microscope Slides Kova	Hycor Biomedical, Kassel
Model Labgard 437 Class II, Type A2	Plymouth, USA
Multiscreen IP – 96 Well Elispot plate	Millipore, Schwalbach
Nunc Cryo-Tubes 1,8 ml	Thermo Scientific, Langenselbold
Safe-Lock Tubes 1,5 ml	Eppendorf AG, Hamburg
Serological Disposable Pipettes, 5 and 10 ml	TTP, Ibbenbüren
Sterile Workbench,	NuAire Biological Safety Cabinets,
Sterile Workbench, Model Gelaire BSB 4A	Gruppo Flow s.p.a. OPERA, Italy
Vortex Mixer, Vortex-Genie® 2	IKA® Werke, Staufen

2.1.2 Chemicals and reagents

Biocoll (Ficoll®)	Biochrom, Berlin
DMSO (Dimethylsulfoxid)	Merck KGaA, Darmstadt
Dulbecco's PBS (1x)	PAA, Cölbe
FCS (fetal calf serum) Gold	PAA, Cölbe
Fixation Medium – Medium A	Invitrogen, Karlsruhe
Hank's BSS (1x)	PAA, Cölbe
HEPES Buffer Solution (1M)	PAA, Cölbe
L-Glutamin 200mM (100x)	PAA, Cölbe
Penicillin/ Streptomycin (100x)	PAA, Cölbe
Permeabilization Medium – Medium B	Invitrogen, Karlsruhe
PHA (Phytohaemagglutinin)	Sigma Aldrich, Taufkirchen
RPMI1640, without L-Glutamin	PAA, Cölbe
Tween-Solution	Sigma Aldrich, Taufkirchen

2.1.3 Solutions

Hanks:

Hank's Balanced Salt Solution (HBSS) containing no glutamine was modified by adding 5 ml L-Glutamin, 5 ml Penicillin/Streptomycin and 5 ml HEPES buffer.

R10:

RPMI1640 medium containing no glutamine and was modified by adding 5 ml L-Glutamin, 5 ml Penicillin/Streptomycin, 5 ml HEPES buffer and 50 ml FCS (inactivated via heat at 56°C for 1 hr).

R20:

RPMI1640 medium containing no glutamine was modified by adding 5 ml L-Glutamin, 5 ml Penicillin/Streptomycin, 5 ml HEPES buffer and 100 ml FCS (inactivated via heat at 56°C for 1 hr).

2.1.4 Antibodies

Table 1: Antibodies used to detect myeloid cell surface antigen.

Antigen	Fluorochrome	Species	Supplier	per 10^6 cells
CD11b	FITC	Mouse	BioLegend, USA	3 µl
CD14	APC	Mouse	BioLegend, USA	3 µl
CD15	PerCP	Mouse	BioLegend, USA	3 µl
CD66	PE	Mouse	BioLegend, USA	3 µl
CD33	PE	Mouse	BioLegend, USA	3 µl
HLA-DR	HLA-DR-PerCP	Mouse	BioLegend, USA	1 µl
HLA-DR	PerCP isotype control	Mouse	BioLegend, USA	2 µl

2.1.5 Peptides

Synthetic peptides corresponding to the HIV proteins Gag and Nef were used for screening (Gag: HIV-1 SF-2, Nef: HIV-1 Bru, NIBSC, England) with a purity of ≥ 70%. (55)

Table 2: Peptides corresponding to HIV protein Gag.

Peptide	Sequence	Peptide	Sequence
1	MGARASVLSGGELDK	2	GELDKWEKIRLRPGG
3	LRPGGKKKYKLKHIV	4	LKHIVWASRELERFA
5	LERFAVNPGLLETSE	6	LETSEGCRQILGQLQ
7	LGQLQPSLQTGSEEL	8	GSEELRSLYNTVATL
9	TVATLYCVHQRIDVK	10	RIDVKDTKEALEKIE
11	LEKIEEEQNKSKKKA	12	SKKKAQQAAAAAGTG
13	AAGTGNSSQVSQNY	14	PIVQNLQGQMVHQAIISPRTL
15	VHQAISPRTLNAWVKVVEEK	16	NAWVKVVEEKAFSPEVIPMF
17	AFSPEVIPMFSALSEGATPQ	18	SALSEGATPQDLNTMLNTVG
19	DLNTMLNTVGGHQAAMQMLK	20	GHQAAMQMLKETINEEAAEW
21	ETINEEAAEWDRVHPVHAGP	22	DRVHPVHAGPIAPGQMREPR
23	IAPGQMREPRGSDIAGTTST	24	GSDIAGTTSTLQEQIGWMTN
25	LQEQIGWMTNNPPIPVGEIY	26	NPPIPVGEIYKRWIILGLNK
27	KRWIILGLNKIVRMYSPTSI	28	IVRMYSPTSILDIRQGPKEP

Peptide	Sequence	Peptide	Sequence
29	LDIRQGPKEPFRDYVDRFYK	30	FRDYVDRFYKTLRAEQASQD
31	TLRAEQASQDVKNWMTETLL	32	VKNWMTETLLVQNANPDCKT
33	VQNANPDCKTILKALGPAAT	34	ILKALGPAATLEEMMTACQG
35	LEEMMTACQGVGGPGHKARV	36	AEAMSQVTNPANIMM
37	ANIMMQRGNFRNQRK	38	RNQRKTVKCFNCGKE
39	NCGKEGHIAKNCRAP	40	NCRAPRKKGCWRCGR
41	WRCGREGHQMKDCTE	42	KDCTERQANFLGKIW
43	LGKIWPSYKGRPGNF	44	RPGNFLQSRPEPTAPPE
45	EPTAPPEESFRFGEE	46	RFGEEKTTPPQKQEPI
47	QKQEPIDKELYPLTS	48	YPLTSLRSLFGNDPSSQ

Peptide	Position	Sequence
SL9	Gag77-85	SLYNTVATL

Table 3: Peptides corresponding to HIV protein Nef.

Peptide	Sequence	Peptide	Sequence
49	GGKWSKSSVVGWPTVRERMR	50	GGKWSKSSVVGWPTVRERMR
51	RAEPAADGVGAASRDLEKHG	52	AASRDLEKHGAITSSNTAAT
53	AITSSNTAATNAACAWLEAQ	54	NAACAWLEAQEEEEVGFPVT
55	EEEEVGFPVTPQVPLRPMTY	56	PQVPLRPMTYKAAVDLSHFL
57	KAAVDLSHFLKEKGGLEGLI	58	KEKGGLEGLIHSQRRQDILD
59	HSQRRQDILDLWIYHTQGYF	60	LWIYHTQGYFPDWQNYTPGP
61	PDWQNYTPGPGVRYPLTFGW	62	GVRYPLTFGWCYKLVPVEPD
63	CYKLVPVEPDKVEEANKGEN	64	KVEEANKGENTSLLHPVSLH
65	TSLLHPVSLHGMDDPEREVL	66	GMDDPEREVLEWRFDSRLAF
67	EWRFDSRLAFHHVARELHPE	68	HHVARELHPEYFKNC

2.1.6 Software

CellQuest	BD, Heidelberg
EliSpot Reader Version 5.0	AID, Strassberg
FACSDiva Version 6.0	BD, Heidelberg
FlowJo Version 7.2.1	Tree Star Inc., Ashland, USA
GraphPad Prism Version 5.0	GraphPad Software, La Jolla, USA

2.2 Methods

2.2.1 Study Subjects

43 individuals participated in the study after signing informed consent: 19 patients (PHI, n = 8, CHI, n = 11) within the scope of an amendment of the New Era study (EudraCT Number 2008-002070-35, approved by the Bayerische Landesärztekammer (BLAEK) and the German federal institute for drugs and medical devices (BfARM)) and 23 individuals within 4 control groups (study approval by the Institutional Review Board of the Ludwig-Maximilians-Universität, Munich, Germany). (55)

The control groups were defined as follows:

- PR (Progressor) were defined as untreated patients during chronic HIV infection at the time of blood draw. We considered 6 PR with a CD4 cell count below 400 cells/µl and viral load (VL) above 10000 copies/ml
- CO (Controller) were defined as patients who were able to control HIV infection spontaneously in the absence of cART. We considered 7 individuals with a CD4 cell count above 500/µl and a VL below 2000 copies/ml. This also included 3 Elite Controllers who were defined as patients with a viral load below 50 copies/ml.
- 3ART were defined as patients who had received a conventional cART for at least 48 months. We considered 5 patients for the 3 ART control group with any CD4 cell count but an undetectable VL below 40 copies/ml.
- HC (Healthy controls) were 5 individuals who were not infected with HIV.

A summary of study participants and their clinical characteristics, CD4 cell counts and VL, can be found in Table 4 below.

Table 4: Study subjects and their clinical characteristics.

Clinical characteristics of study participants at the time of blood draw are shown below. Clinical data is shown in median values of each group as well as its range. CD4 cell count is reported in cells/µl and viral load (VL) is reported in copies/ml. The detection limit for VL was 40 copies/ml in this study. Age is given in years and treatment length is given in months. Treatment in PHI and CHI refers to the length on intake of the intensified treatment. For CHI the length of conventional treatment (cART) prior to the intensified regimen is also shown. Treatment for 3ART refers to a conventional antiretroviral treatment (cART). Not applicable is abbreviated as 'n.a.', female as 'f' and male as 'm'.

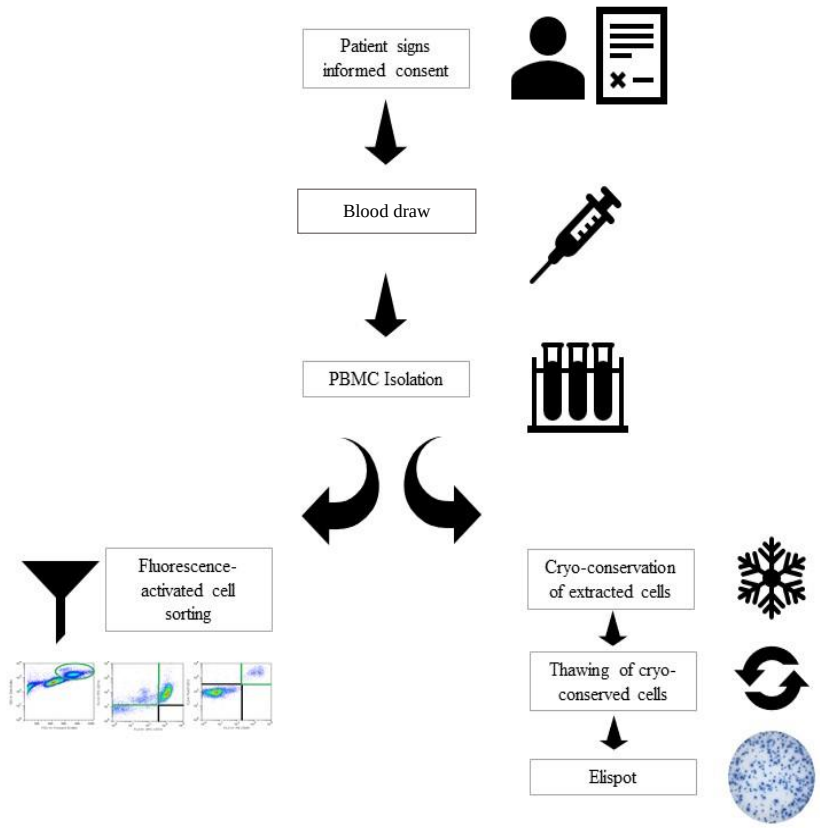
	HC (n=5)	PHI (n=8)	CHI (n=11)	3 ART (n=5)	CO (n=7)	PR (n=6)
Gender	2m/3f	7m/1f	9m/2f	5m/0f	5m/2f	5m/1f
Age	48.6	47.5	49.5	45.8	45.3	38.6
Range	42 - 55	42 - 56	36 - 62	30 – 70	30 - 66	20 - 56
CD4 cell count	n.a.	790.5	662	591	578	81
Range	n.a.	557 - 1342	551 - 1133	487 - 687	467 - 1442	49 - 205
VL	n.a.	<40	<40	<40	51	177453
Range	n.a.	n.a.	n.a.	n.a.	<40 - 702	2100 - 540539
Treatment length	n.a.	72.5	65 cART: 77.6	94	n.a.	n.a.
Range	n.a.	68 - 77	55 - 71 cART: 49 - 126	51 - 207	n.a.	n.a.

2.2.2 Procedures

A schematic outline of the sequence of procedures that was carried out in this study can be seen in Figure 3. The individual steps are described in detail in the following sections.

Figure 3: Outline of the sequence of procedures.

Figure 3 briefly outlines the sequence of procedures carried out in this study. After signing the informed consent, 6-12 large EDTA tubes of blood were drawn from the patient. PBMC were isolated from the whole blood within a time frame of 4 hrs to be studied by FACS on the same day. The Elispot technique did not need to be carried out on the same day so that all extracted cells were first cryo-conserved and thawing could be performed on another day independent of the time of blood collection.



2.2.3 Blood sample collection procedure

Peripheral venipuncture was carried out to obtain 6 to 12 EDTA tubes, each containing a volume of 30 to 50 ml of whole blood. After obtaining the patient's whole blood, the viral load was measured by the means of Real-time-polymerase chain reaction (RT-PCR) whereas the CD4 cell count was measured by flow cytometry (BD Tritest).

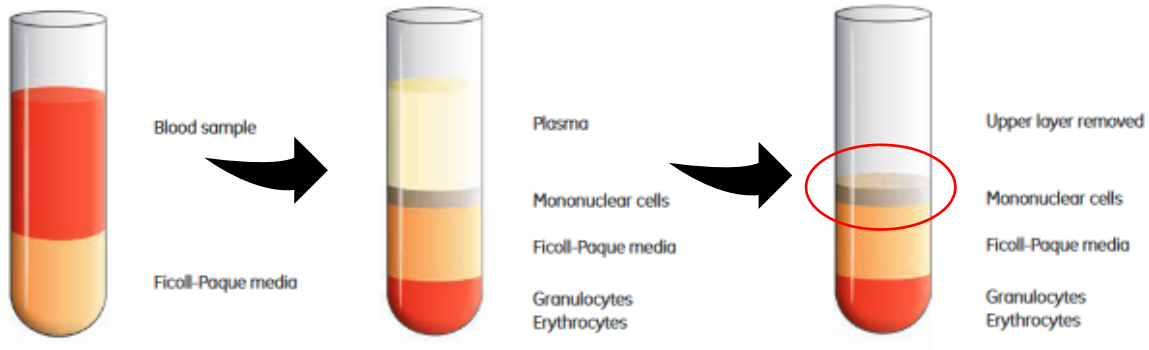
The blood draw was performed by the nurses whereas the laboratory methods were carried out by the medical technical assistants. Following the collection of clinical data relevant in monitoring HIV progression, isolation of the cell population of interest was performed.

2.2.4 PBMC Isolation from whole blood

Cell population of interest are lymphocytes which belong to the group of peripheral blood mononuclear cells (PBMC). They were separated from fresh whole blood via density gradient centrifugation using Ficoll. The Ficoll media is a neutral hydrophilic polysaccharide with a density of 1.077 g/ml. The blood sample was carefully layered with the Ficoll media in order to counteract mixing. Individual cell populations of blood were centrifuged at a high speed of 1500 U/min for 30 min at 18 to 20°C. The centrifuge brake was turned off during centrifugation resulting in a separation of different cells divided into three layers: the upper layer contains light cells (thrombocytes) whereas lymphocytes as well as monocytes can be found in the central layer just above the Ficoll media. Red blood cells and granulocytes gather at the bottom of the centrifuge tube (see the red circle in Figure 4).

Figure 4: PBMC isolation from whole blood.

Figure 4 shows the different layers of cells which form after Ficoll media has been added to a blood sample with the aim of isolating mononuclear cells (red circle). (56)



Lymphocytes and monocytes were carefully obtained with a sterile pipette and transferred to a sterile centrifuge tube of 50 ml. The cell suspension was washed three times (10 min, at room temperature, 1500 U/min). Hanks medium was used for the first two washings whereas R10 was used for the third wash. The supernatant was removed and the remaining cell pellet containing mononuclear cell was resuspended in 10 ml of R10.

2.2.5 Cell counting

Isolated PBMC were counted with an electric field multi-channel count system called CASY®. 50 µl of the previously obtained cell pellet were suspended in CASY®Ton, an isotonic salt solution with a physiological pH. To analyse viability of counted cells, 10 µl of cells were mixed with an equal amount of trypan blue and inserted into the KOVA® cell chamber system. The used dye only stains non-viable cells whereas viable cells are spared. Thus, determining the count of viable cells was based on an exclusion method. The ratio of viable vs. non-viable cells from the cell chamber multiplied by the result of CASY® cell counter led to the number of viable cells of the cell pellet.

2.2.6 Cryo-conservation of extracted cells

Cryo-conservation of cells enables the researcher to preserve cells for a significant amount of time in liquid nitrogen. The cell suspension was first centrifuged at 1500 U/min for 10 min at 4°C. Supernatant was removed and the remaining cell pellet was resuspended in a 1 ml solution consisting of two cryo-protective agents composed of 90% pure FCS and 10% DMSO. Then an additional amount of the cryo-protective solution was quickly added and the total volume was equally transferred into cryovials. Each cryovial received a total volume of 1.8 ml with a cell count of 10^6 cells/ml. The cryotubes were immediately transported to the Thermo Scientific Mr. Frosty freezing container filled with isopropanol. Frozen cells were stored overnight at -80°C. On the consecutive day they were transferred to a liquid nitrogen tank (-196°C) for long-term storage.

2.2.7 Thawing of frozen cells

Frozen cells which were stored in a liquid nitrogen tank were quickly thawed in a heated bath and transferred into cold centrifuge tubes to reduce cell damage. After 10 ml of R10 had been added, two washings of the cell suspension were carried out for 10 min with a speed of 1500 U/min. The first washing was performed at 4°C whereas the second was performed at room temperature. Following each centrifugation step, supernatant was removed and cells were resuspended in 2 ml of R10. 10 µl of the cell suspension was used to count the cells and determine their viability as outlined in 2.2.5. Afterwards cells were transferred into cell culture flasks with a volume of 25 cm^3. Each flask contained 1.5 x 10^6 cells per 1 ml of R10. For optimal cell storage, they were kept in an incubator at 37°C and 5% CO_2 overnight.

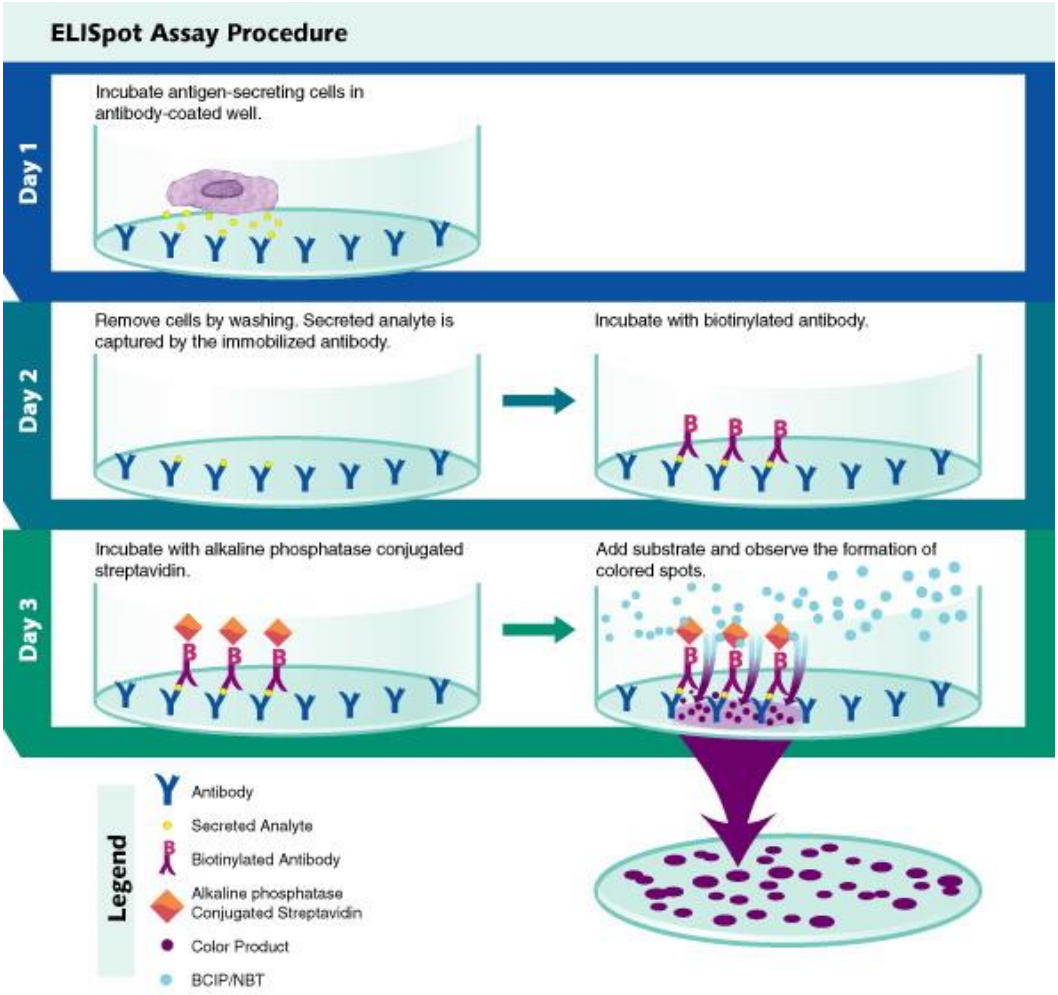
2.2.8 Interferon-γ Elispot

2.2.8.1 Procedure of the Elispot assay

The enzyme-linked immunospot assay is a sensitive and accurate method that identifies cell-based immune responses by measuring secretory products of immune cells in response to a certain stimulus (see Figure 5).

Figure 5: Principle of the Elispot assay.

Figure 5 briefly outlines the principles of the Elispot assay which lasts 3 days and employs an antibody 'sandwich' technique. The first day focuses on coating the polyvinylidene diflouride (PVDF) microtiter plate with a primary antibody specific to the analyte of interest. Cells that are capable of secreting this analyte are given into the wells of the 96-well plate and are incubated overnight to allow binding of the secreted analyte and antibody. The second day is used to wash off any cells not bound to the primary antibody. This is followed by the addition of a secondary antibody specific to the analyte of interest. It is also biotinylated to allow binding to the Alkaline phosphatase conjugated streptavidin on the following day. A second incubation period is carried out. On the third day, a substrate solution (BCIP/NBT) is added so that streptavidin catalyzes a reaction that leads to the precipitation of blue spots at the site of cytokine secretion on the plate. (57)



In this study the secretion of interferon-gamma by CD8 T lymphocytes was measured. At first, a 96-well PVDF-membrane microtiter plate was coated with 100 µl of a solution made up of 11 µl of the primary antibody, mAb 1-D1K (1 mg/ml), and 11 ml of sterile PBS. The 96-well microtiter plate were kept in the fridge overnight at 4°C.

On the consecutive day, each well of the microtiter plate was repeatedly washed with 200 µl of PBS and 1% of FCS for 6 times. After the final wash, 50 µl of R10 was given to each of the 96 wells. Then, 10 µl of a certain peptide (200 µg/ml) depending on the position of the well on the plate was added (see 2.1.5). Finally, an equal amount of PBMC cell suspension (100000 cells per well) were given into each well. Negative controls contained PBMC and R10 only whereas positive controls contained PBMC with either PHA or FEC, a mixture of as optimally defined CD8 T cell epitopes of Influenza virus, Epstein-Barr-Virus and Cytomegalovirus. Both types of controls were run as triplicates. The plate was placed in the incubator overnight at 37°C and 5% CO_2 to allow sufficient time for cytokine production.

On the third day of the procedure, the plate was washed 6 times with PBS only. 5.5 µl of the secondary antibody, mAb 7B6-1-biotin, was resuspended in 11 ml of PBS and the solution was used to coat the 96-well microtiter plate, each well receiving 100 µl. The plate was placed in the dark for an incubation period of 1 hr and was once again washed 6 times with PBS. Following the washings, streptavidin which is a strong binding agent to the previously added biotin was applied. For this purpose, 5.5 µl of streptavidin had been suspended in 11 ml of sterile PBS and a 100 µl had been given into each well. The plate was again kept in the dark for an incubation time of 45 min. Before applying a colouring solution, the plate underwent a series of 6 washings using PBS. The colouring solution contained the following reagents: 12 ml of distilled water, 500 µl of Tris-HCl-Buffer and two colouring reagents, AP colour reagents A and B, of 125 µl each. Due to colour development, blue spots became clearly visible in positive wells. Then, each well of the microtiter plate was sterilised with 200 µl of 0.05% Tween solution which had previously been suspended in PBS. Following a 10 min rest, plates were generously cleaned with water three separate times and were left to dry.

2.2.8.2 Analysis of the Elispot assay

Elispot plates were read out by an automated Elispot Reader. The number of spot-forming cells (SFC) per well was counted by the reading device and calculated per 10^6 PBMCs. In case of minor colour development in the negative controls (≤ 5 SFC/10^6 PBMCs), spots were counted individually for each well and were considered positive when at least 50 SFC/10^6 PBMCs was reached. If the median in the negative controls exceeded 5 SFC/well, a well was only considered positive if it exceeded the total number of SFC/10^6 PBMCs in the negative controls at least 3 times. Since the Elispot reader reaches its limit to discriminate all the spots formed at 2000 SFC/10^6 PBMCs, this was chosen as the upper cut-off limit. To determine the number of CD8 T cell responses, the following formula was used:

$$Number\ of\ spotforming\ cells - \ mean\ of\ triplicate\ control\ values = actual\ number\ of\ CD8\ T\ cell\ responses$$

Since neighbouring synthetic peptides overlapped each other for 5-10 amino acids at their ends (Table 5), it is possible to overestimate the breadth and magnitude of CD8 T cell responses. To avoid overestimation, we referred to the method used in *Addo et al* from 2003: In terms of breadth, responses to neighbouring peptides were only counted once. In terms of magnitude, the higher response according to the number of SFC/10^6 PBMCs was chosen. (53)

Table 5: Overlapping synthetic peptides in Gag p17 and Gag p24.

In order to demonstrate the overlap of sequences in the synthetic peptides used in this study, two HIV-specific sequences from Gag p 17 and Gag p24 were chosen as examples. The sequences are found at the beginning and end of the sequence and vary in length from 5 to 10 amino acids. They are underlined in blue.

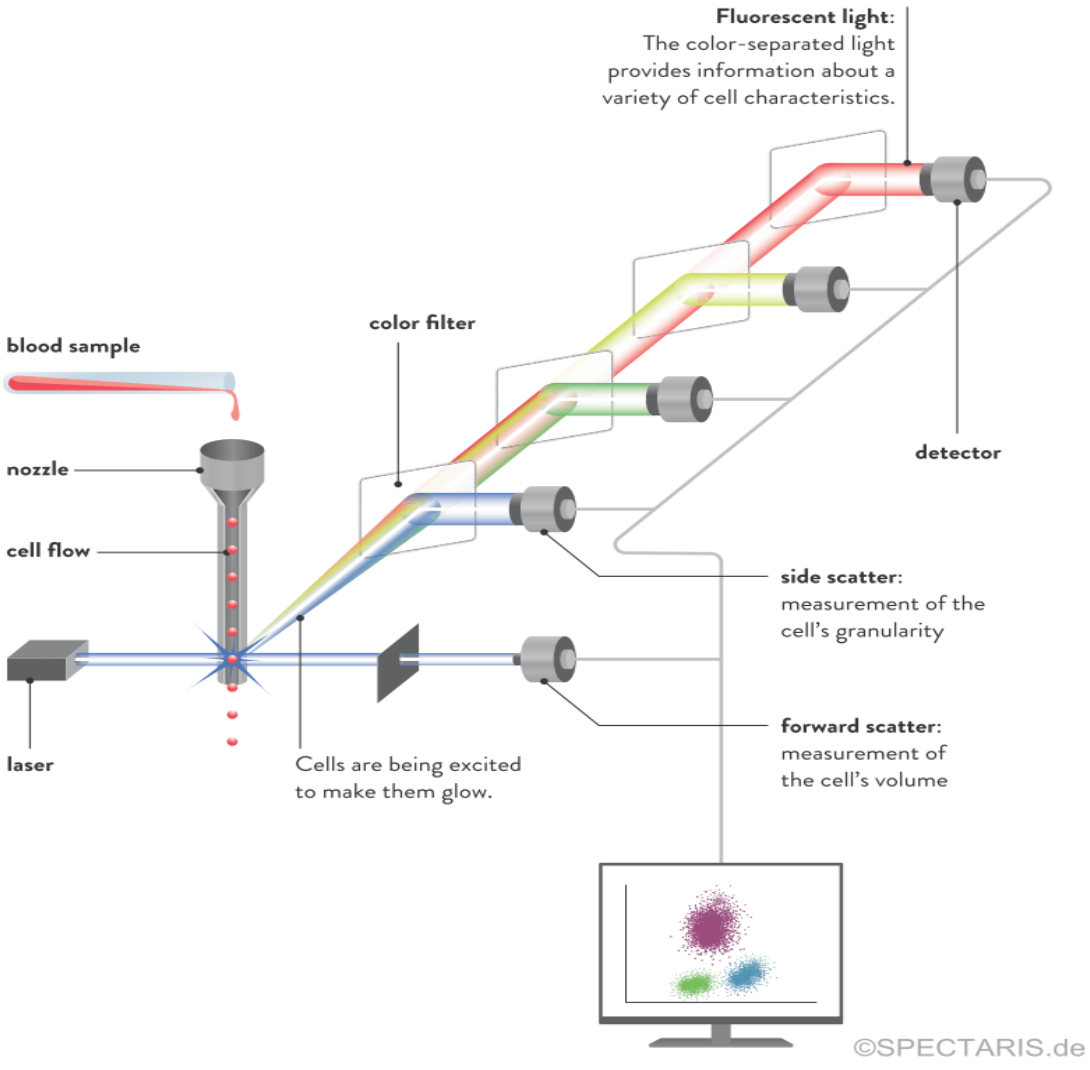
Peptide Gag	***Peptide No.***	***Sequence***	***No. of overlapping amino acids***
p17	1	MGARASVLSGGELDK	5
p17	2	GELDKWEKIRLRPGG	
p24	26	NPPIPVGEIYKRWIILGLNK	10
p24	27	KRWIILGLNKIVRMYSPTSI	

2.2.9 Flow cytometric analysis

Flow cytometry is a laser-based technology which assesses physical as well as chemical properties of thousands of particles. It allows rapid separation of cell types from a heterogeneous mixture of different cells. In this study, expression of phenotypic markers of two cell populations of interest, PMN and M-MDSC, was detected with the help of several fluorescent antibodies (see Table 1). Figure 6 briefly outlines the principle of fluorescence-activated cell sorting (FACS).

Figure 6: Principle of FACS.

Figure 6 illustrates the principle of FACS with a flow sorter. Cells have been stained with a fluorescent dye specific to a certain extracellular marker. The cell flow allows the analysis of one cell at a time. The sheat fluid within the cell flow carries a cell 'single file' through a lightbeam with multiple lasers of different wavelengths and fluorescence detectors. The detector converts forward and side scattered light as well as fluorescence signals into digital signals which are visualised as digital graphs. (28)



2.2.9.1 FACS procedure

For the detection of MDSCs in previously isolated PBMCs, it was crucial to use fresh PBMCs. According to *Grützner et al*, it is important to study particularly M-MDSCs within 4 hrs of blood draw to avoid a significant loss of these cells. This finding does not apply to PMN-MDSCs to the same extent. However, a significant delay in FACS analysis will also limit proper analysis of FACS data. (28)

Fresh PBMCs with a concentration of 1 x 10^6 cells/ml were washed in a solution of 2 ml of PBS and 20 µl of FCS. This was followed by a centrifugation of 10 min at 4°C with a speed of 1500 rpm. Supernatant was removed and extracellular staining was carried out in the dark. First, 50 µl of PBS were added to resuspend the cell pellet. Then, antibodies were added and immediately stored in the fridge for a 30 min incubation period. Afterwards they were again resuspended with PBS and centrifuged at the previously used setting. Following the removal of supernatant, 100 µl of a fixation reagent, Fix Perm Solution A, was added and left in the dark for incubation for 15 min at room temperature. Another wash with 2 ml of PBS and a final centrifugation were carried out. At last, stained cells were resuspended in 200 µl of PBS. They were analysed on FACSCalibur and 100000 cells were counted for each sample. The obtained data were further analysed using the FlowJo software.

2.2.9.2 Gating strategies

Gating strategies were according to *Vollbrecht et al* and *Rieber et al* for PMN-MDSCs. (31; 58) To identify M-MDSCs, gating strategies were used according to *Dumitru et al.* (59) A detailed description of the strategy can be found in Figure 7.

Figure 7: Representative dot blots and gating strategy for PMN-MDSCs.

Figure 7 describes the gating strategy for determining the frequency of PMN-MDSC within a heterogeneous cell mixtures of fresh PBMCs. Since PMN-MDSCs are defined as CD11b+/CD14- and CD66b+/CD15+, the following strategy was applied to identify cells with these characteristics. First, the monocytic fraction in FSC and SSC is found and gated (green gate in the first panel). MDSCs are mainly found in the smaller fraction located just above the monocytic 'bulk'. The green squared gate in the second panel then includes a CD11b+/CD14- cell population. In the third panel, those cells are further gated into CD66b+/CD15+ (green gate).

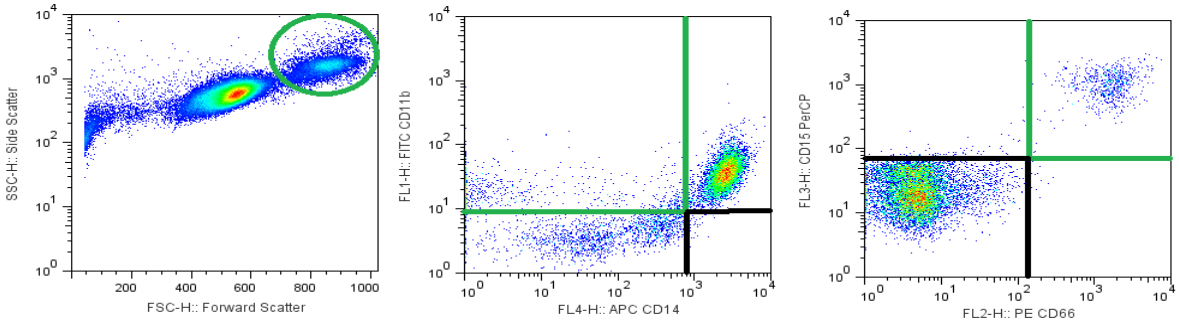
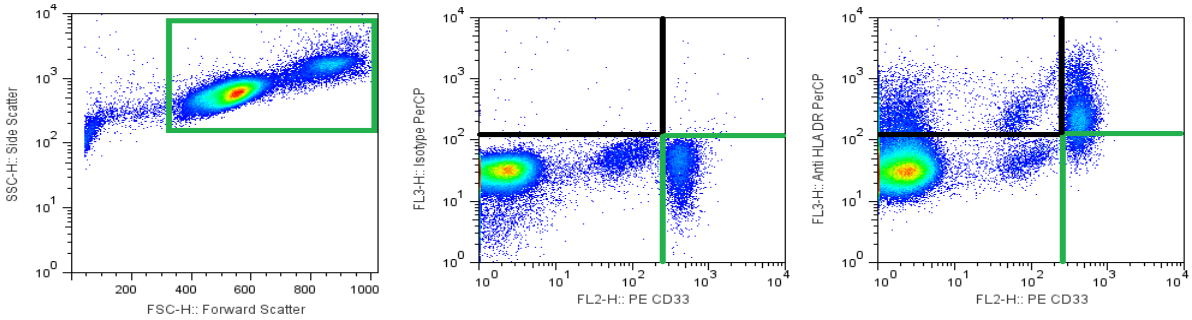


Figure 8: Representative dot blot and gating strategy for M-MDSCs.

Figure 8 describes the gating strategy used to identify M-MDSCs from a fresh PBMC mixture. They are defined as CD14+/CD11b+/HLA-DR- and CD33+. The first panel serves to identify the fraction of lymphocytes and monocytes in FSC and SSC which is located in the green squared gate. Once this fraction is identified, the second panel uses a PerCP isotpye control to identify the HLA-DR negative population. In the third panel, cells that are HLA-DR- and CD33+ are identified in the green gate.



2.2.10 Statistical analysis

Statistical analysis was performed using GraphPad Prism version 5.0. Obtained data was found to be non-parametric and was therefore analysed by non-parametric tests. Comparisons done within one study group were analysed using the Mann-Whitney-U-test. Regarding the comparison of different groups of HIV patients, the type of analysis was chosen according to the number of groups considered. When comparing two groups, Mann-Whitney-U-test was again applied whereas Kruskal-Wallis testing was performed when more than two groups were included in the comparison. A p-value of < 0.05 was considered to be statistically significant. For multiple comparisons, Bonferroni correction was applied when p was significant in Kruskal-Wallis testing. p-value significance changed to < 0.0167 for 4 groups and < 0.0175 for 5 groups, respectively. For correlation studies, the Spearman rank test was used.

3 Results

3.1 Low frequencies of PMN-MDSC amongst NE, 3ART and CO

Frequencies of PMN-MDSC of New Era patients (NE) were compared to other groups of HIV patients of this study as well as to healthy control. NE patients showed comparably low frequencies of PMN-MDSC to 3ART who had also received treatment for HIV. Furthermore, both groups receiving HIV treatment, NE and 3ART, showed similarly low frequencies of PMN-MDSC when compared to HC. Furthermore, CO patients also showed comparably low levels of PMN-MDSC with respect to HC and HIV patients with treatment, NE and 3ART. The only group of patients showing a significantly higher level of PMN-MDSC to NE patients was the PR group who had not received any treatment for their HIV infection ($p = 0.0008$).

These results suggest that despite differing drug regimens, HIV treatment must have had a similar impact on NE and 3ART regarding PMN-MDSC frequencies. The significant difference between the NE and PR patients further underlines the effect of treatment on PMN-MDSC frequency in HIV patients. It is also noteworthy that CO patients' ability to naturally control HIV infection appeared to lead to similarly low frequencies of PMN-MDSC as seen in HC.

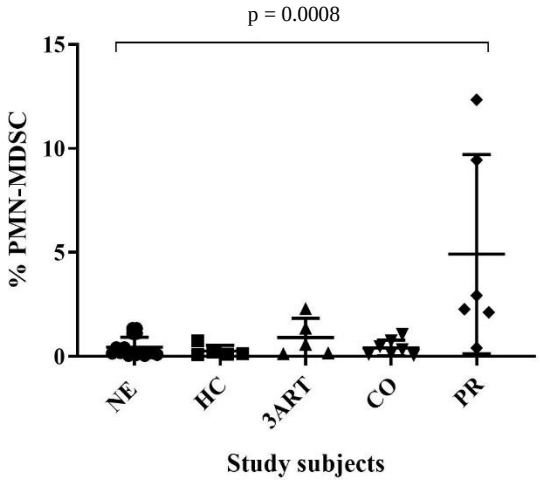


Figure 9: Frequencies of PMN-MDSC in NE patients and control groups.

3.2 No difference between frequencies of PMN-MDSC in NE subgroups

When analysing NE subgroups individually, frequencies of PMN-MDSC in both, PHI and CHI, were low. Comparison to the control groups of this study showed similar results to the analysis which considered NE patients collectively (Figure 10).

Thus, both subgroups showed similar levels of PMN-MDSC frequency as seen in HC, 3ART and CO whereas significantly lower frequencies were observed when compared to PR ($p = 0.0047$; $p = 0.0019$). In a comparison between PHI and CHI, no significant difference between their PMN-MDSC frequencies was seen ($p = 0.999$). This suggests that the starting point of the 5-drug regimen did not have a major impact on frequencies of PMN-MDSC in NE patients.

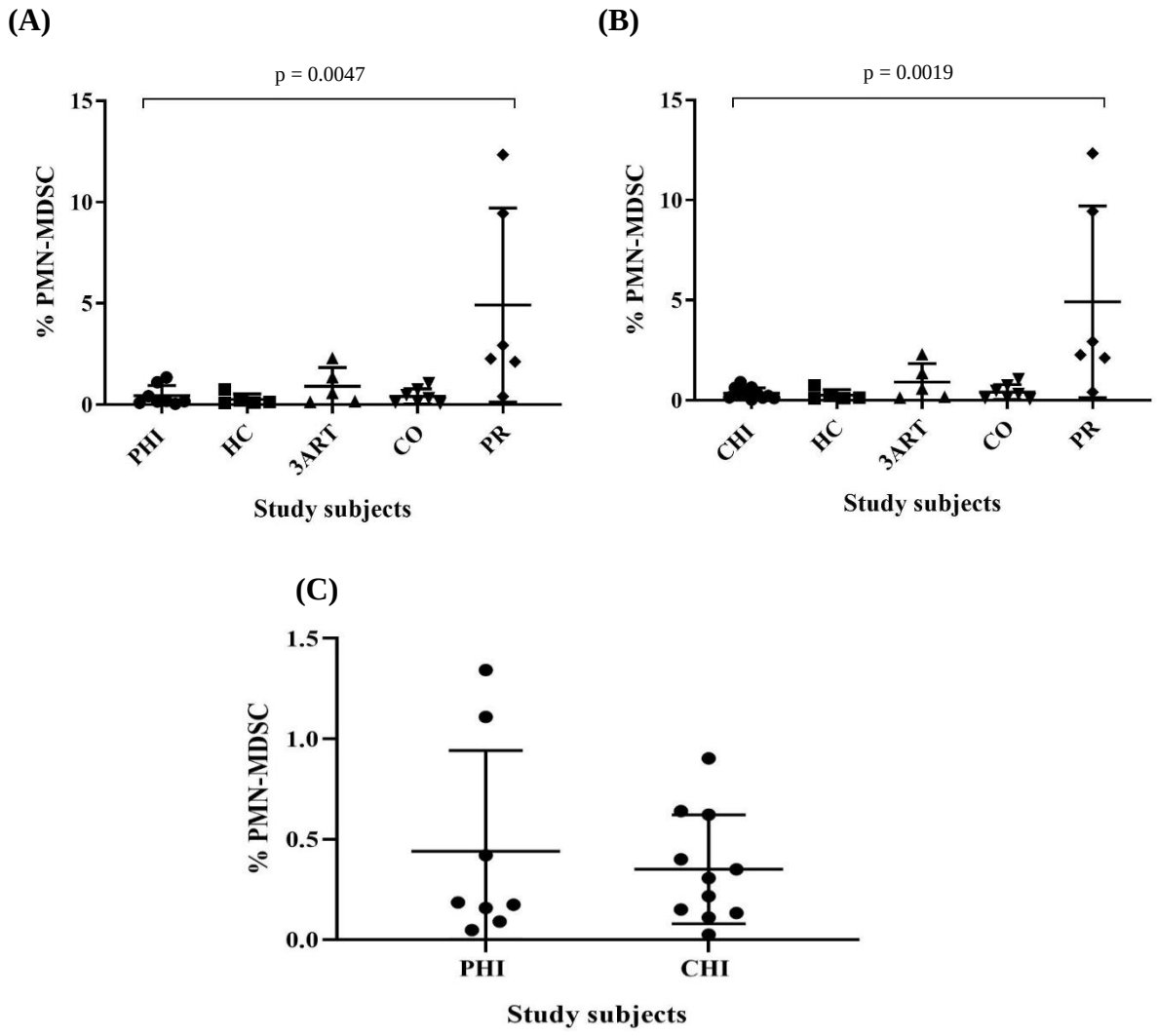


Figure 10: Frequencies of PMN-MDSC in NE subgroups. **A** Comparison between PHI and control groups (Kruskal-Wallis test). **B** Comparison between CHI and control groups (Kruskal-Wallis test). **C** Comparison between PHI and CHI (Mann-Whitney-U-Test).

3.3 Frequencies of M-MDSC in NE patients are significantly higher than in healthy controls

In contrast to PMN-MDSC frequencies, NE patients showed a significantly higher level of M-MDSC frequencies when compared to HC ($p = 0.0014$). No significant difference between the HIV groups, NE and 3ART, receiving HIV treatment could be detected. Although not statistically significant, M-MDSC frequencies of HIV patients were higher when compared to HC, even in HIV patients receiving continuous treatment.

Moreover, NE patients did not show a significant difference of their M-MDSC frequencies when compared to the HIV patients, CO and PR, who did not receive any treatment ($p = 0.91$; $p = 0.78$). However, the difference between NE patients and PR patients would probably become statistically significant if the groups were bigger.

The results suggest that HIV treatment had a similar effect on M-MDSC frequencies independent from the type of drug regimen applied. Although not statistically significant, PR patients showed considerably higher M-MDSC frequencies when compared to the other groups in this study. This underlines the effect of HIV treatment on those cells. Nonetheless, frequencies of M-MDSC in any HIV group did not reach the level observed in HC. Thus, HIV treatment does not appear to have the same effect on M-MDSC frequencies as it does on PMN-frequencies.

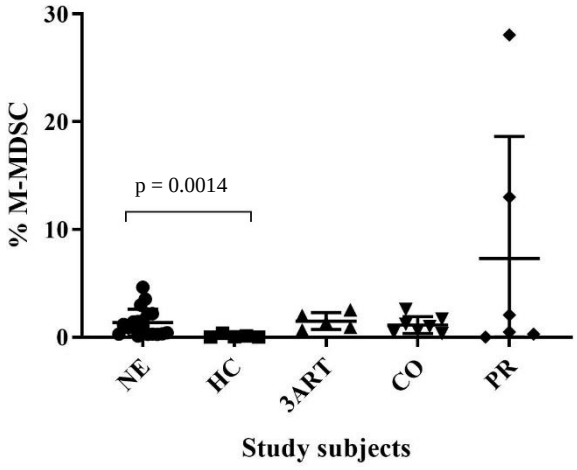


Figure 11: Frequencies of M-MDSC in NE patients and control groups (Mann-Whitney-U-test).

3.4 M-MDSC frequencies differ between NE subgroups

In contrast to PMN-MDSC frequencies, NE subgroups showed significant differences in their M-MDSC frequencies. PHI showed a trend towards higher levels of M-MDSC frequencies when compared to HC ($p = 0.0186$) whereas this trend was not observed in CHI ($p = 0.038$). Besides, CHI also appeared to have significantly lower M-MDSC frequencies than 3ART who had also received HIV treatment ($p = 0.0009$). In fact, their level of M-MDSC frequency was comparable to the level seen in HC. Furthermore, CHI showed significantly lower M-MDSC frequencies than CO who are able to naturally control HIV infection and did not receive any treatment ($p = 0.0114$).

Comparing NE subgroups, no significant difference could be detected ($p = 0.21$). Nonetheless, Figure 12 C illustrates that M-MDSC frequencies of PHI were generally higher and showed a wider distribution than CHI.

Although there was no significant difference observed in the comparison of M-MDSC frequencies between the NE subgroups, the results suggest that PHI drive the significantly higher frequencies of M-MDSC in NE patients when compared to HC (Figure 12). Despite their late onset of HIV treatment, CHI had been treated with a cART regimen for at least 36 months prior to the start of the 5-drug regimen. Thus, they were expected to be in an adequate immunological status at the start of the 5-drug regimen which might not have been the case for the PHI patients.

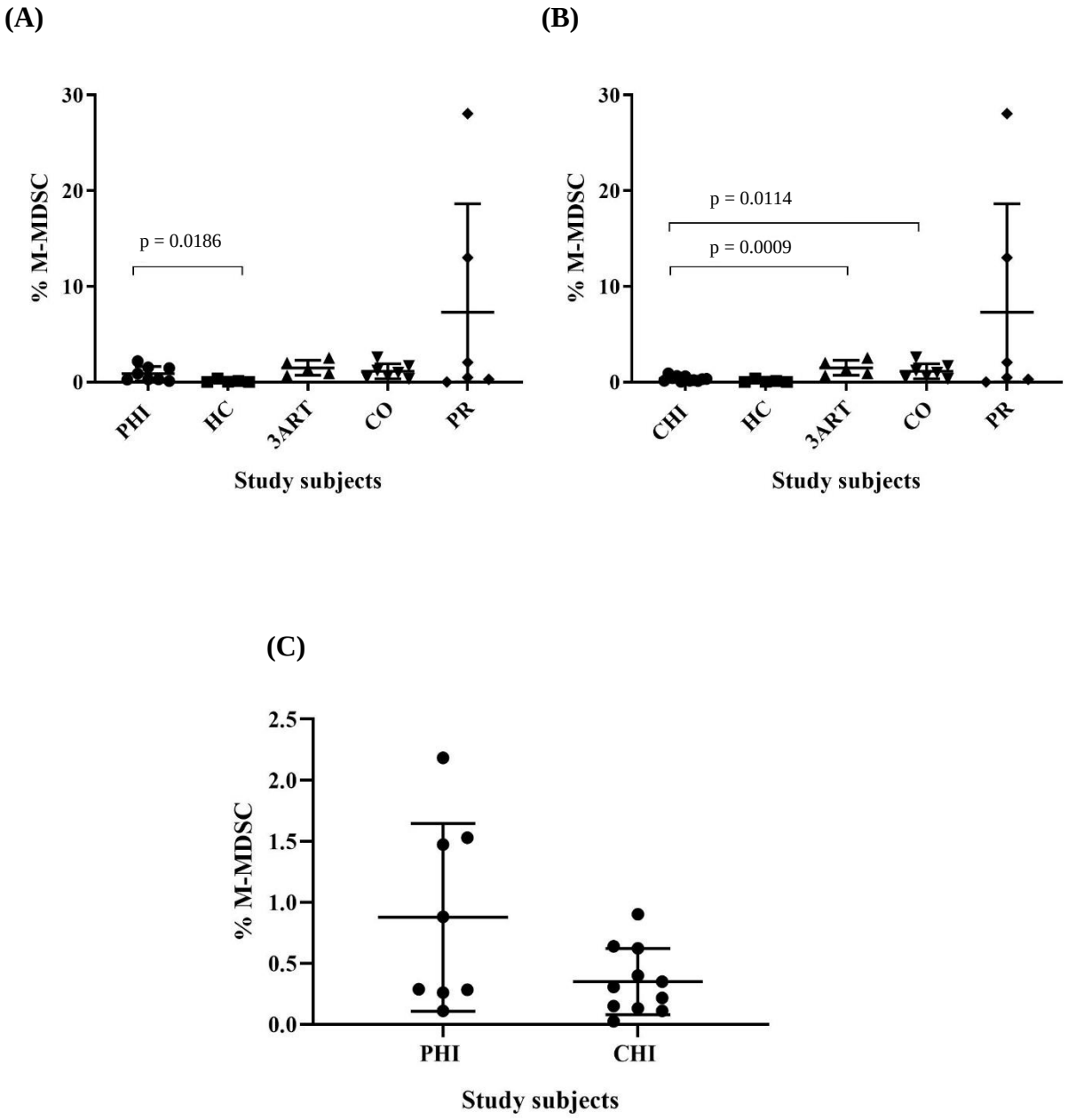


Figure 12: Frequencies of M-MDSC in NE subgroups. **A** Comparison between PHI and control groups (Kruskal-Wallis test). **B** Comparison between CHI and control groups (Kruskal-Wallis test). **C** Comparison between PHI and CHI (Mann-Whitney-U-test).

3.5 CHI tend to have broader HIV-specific immune responses than PHI

The HIV-specific CD8 T cell response was examined in the four groups of HIV patients of this study and breadth and magnitude were analysed. Despite an intensified HIV treatment, the breadth of HIV-specific CD8 T cell responses did not significantly differ between the NE cohort and the other groups of HIV patients (p = 0.3458).

Comparing the two subgroups within the NE cohort with regard to their individual level of breadth, it is worth noticing that CHI tended to show a broader CD8 T cell response specific to HIV than PHI (p = 0.062). Moreover, the mere number of responses towards Gag and Nef varied considerably between patients of the CHI subset (range: 0 to 16). Consequently, CHI appeared to be the dominant subset of patients within the NE cohort that drives the level of breadth observed for immune responses in NE patients.

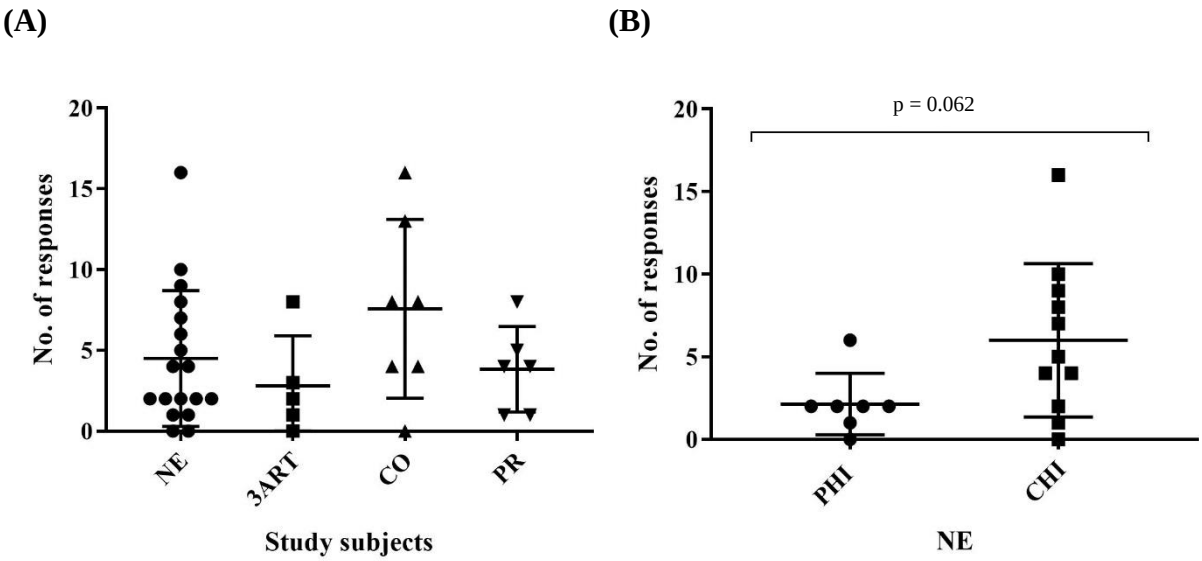


Figure 13: Breadth of CD8 T cell responses towards Gag and Nef. **A** Comparison between NE and control groups (Kruskal-Wallis test). **B** Comparison between PHI and CHI (Mann-Whitney-U-test).

3.6 NE patients have slightly stronger immune responses than 3ART

The HIV groups of this study were analysed according to their individual levels of magnitude of the CD8 T cell response. In contrast to the results obtained for the level of breadth, the NE cohort showed slightly stronger CD8 T cell responses to HIV proteins when compared to the 3ART patients (p = 0.083). When considering the level of magnitude for the two subsets of NE patients, no significant difference between PHI and CHI was detected (p = 0.2185). Thus, CHI tended to have a broader response of HIV-specific CD8 T cells than PHI, but their magnitude of immune responses was comparable.

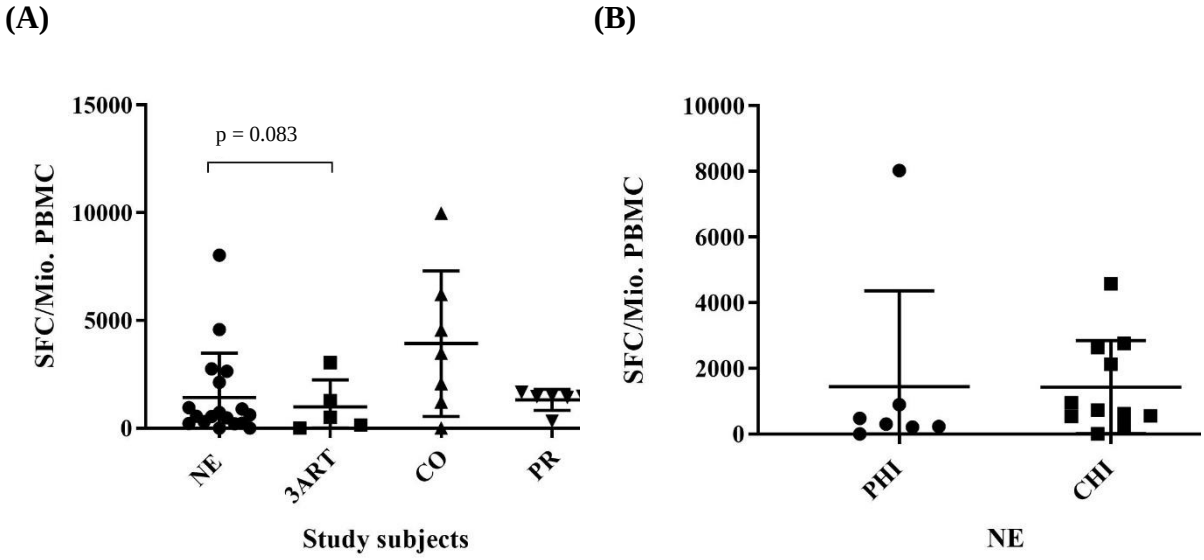


Figure 14: Magnitude of CD8 T cell responses towards Gag and Nef. **A** Comparison between NE and control groups (Kruskal-Wallis test). **B** Comparison between PHI and CHI (Mann-Whitney-U-test).

3.7 CHI patients target all studied HIV-proteins – similar to CO patients

In order to investigate CD8 T cell responses which are specific to HIV, 4 of the most commonly recognized HIV-1 proteins, namely Gag p17, Gag p24, Gag p15 and Nef were assessed in the experiments. The average number of CD8 T cell responses was determined for the viral proteins investigated within every group of HIV patients of this study.

As shown above, CHI patients had generally broader responses to Gag and Nef, especially when compared to 3ART (p = 0.0286). Interestingly, CD8 T cells in CHI targeted all HIV proteins studied when compared to PHI patients (p = 0.0857). The CO patients who did not receive any treatment over their course of infection showed broad CD8 T cell responses for all 4 proteins.

In this respect, CHI patients were similar to CO patients. 3ART patients who received a standard antiretroviral therapy showed generally narrow responses of CD8 T cells to all 4 HIV-1 proteins. This represents an interesting finding, also in comparison to the 3ART patients as duration of HIV treatment was similar for both groups. It emphasizes that this subgroup of the NE cohort was a special group of patients selected.

The PR group which had already reached an advanced stage of HIV infection also showed broad responses in Nef and Gag p24 whereas responses to Gag p17 and Gag p15 were relatively narrow. The broadest responses in NE patients were observed for Nef followed by Gag p24 as earlier studies have also identified Nef as a highly immunogenic protein. Correspondingly, these two proteins were also found to have the broadest responses among all other HIV study groups.

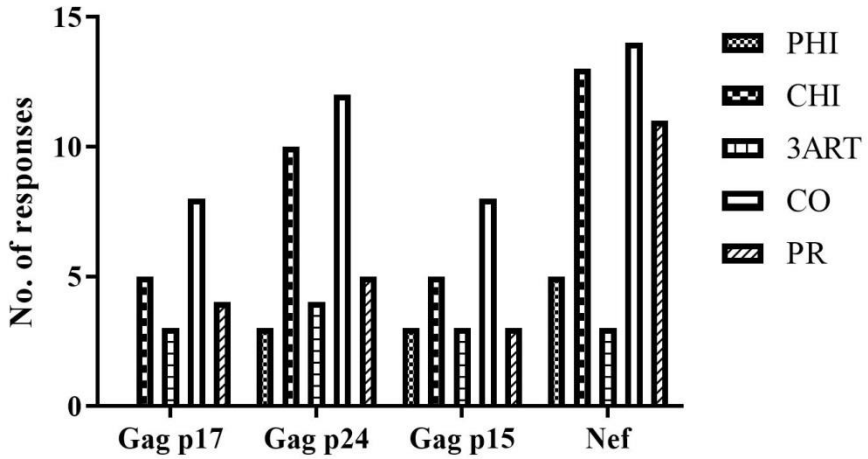


Figure 15: Breadth of CD8 T cell responses to Gag and Nef (Kruskal-Wallis test).

3.8 PHI and CHI patients show similar levels of magnitude within the different HIV proteins studied

Whilst CHI showed broader HIV-specific immune responses than PHI, no major difference in magnitude between PHI and CHI was seen ($p = 0.2927$) (see Figure 14 B). Immune responses in 3ART appeared to be rather similar to the NE cohort except for Gag p17 and Nef. Similarly to the results regarding the levels of breadth, Nef also triggered strong responses in most of the HIV-infected study groups with the exception of the 3ART patients.

CO patients had the highest results in magnitude amongst all study groups. PR patients only showed strong responses to Nef but considerably low levels of magnitude for the other 3 HIV proteins investigated.

In summary, Nef did not only elicit the most responses but also the strongest. In contrast to breadth, magnitude in the NE subgroups, PHI and CHI, was comparable and did not differ to 3ART patients. However, CO patients who had no antiretroviral therapy showed stronger responses. This suggests that HIV treatment has a reducing effect on the magnitude but differing drug regimens do not lead to significantly different levels of magnitude. Most likely the important variable is viral load – which is reduced in all regimens. (55) Moreover, the low level of magnitude observed in the PR patients again underlines the potential effect of immune exhaustion on effector cells.

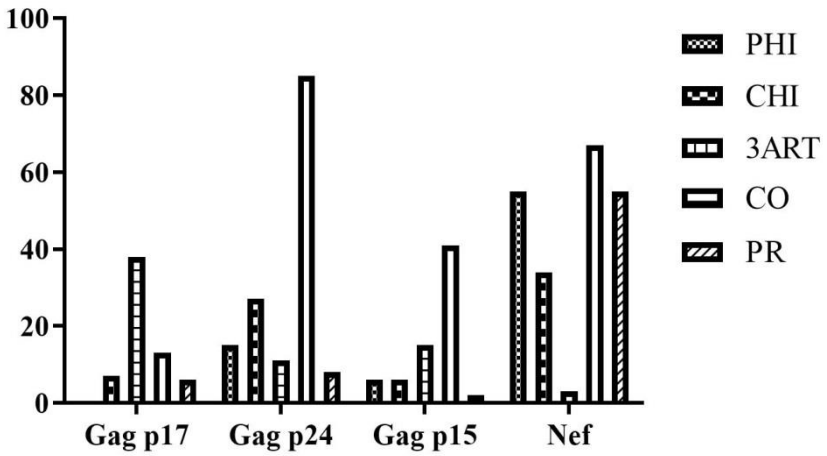


Figure 16: Magnitude of CD8 T cell responses to Gag and Nef (Kruskal-Wallis test).

3.9 No correlation between frequencies of immune suppressive cells and the level of Gag/Nef-specific immune responses in the NE cohort

Immune suppressive cells are known for their inhibitory effect on CD8 T cell responses. Therefore, this analysis further focused on the correlation between MDSCs and HIV-specific CD8 T cells in the NE patients. However, there were no correlations found between the different subsets of MDSCs including PMN- and M-MDSC and breadth or magnitude of HIV-specific CD8 T cell responses, respectively (see Figure 17). This implies that both subsets of immune suppressive cells had no obvious effect on the CD8 T cell responses in NE patients.

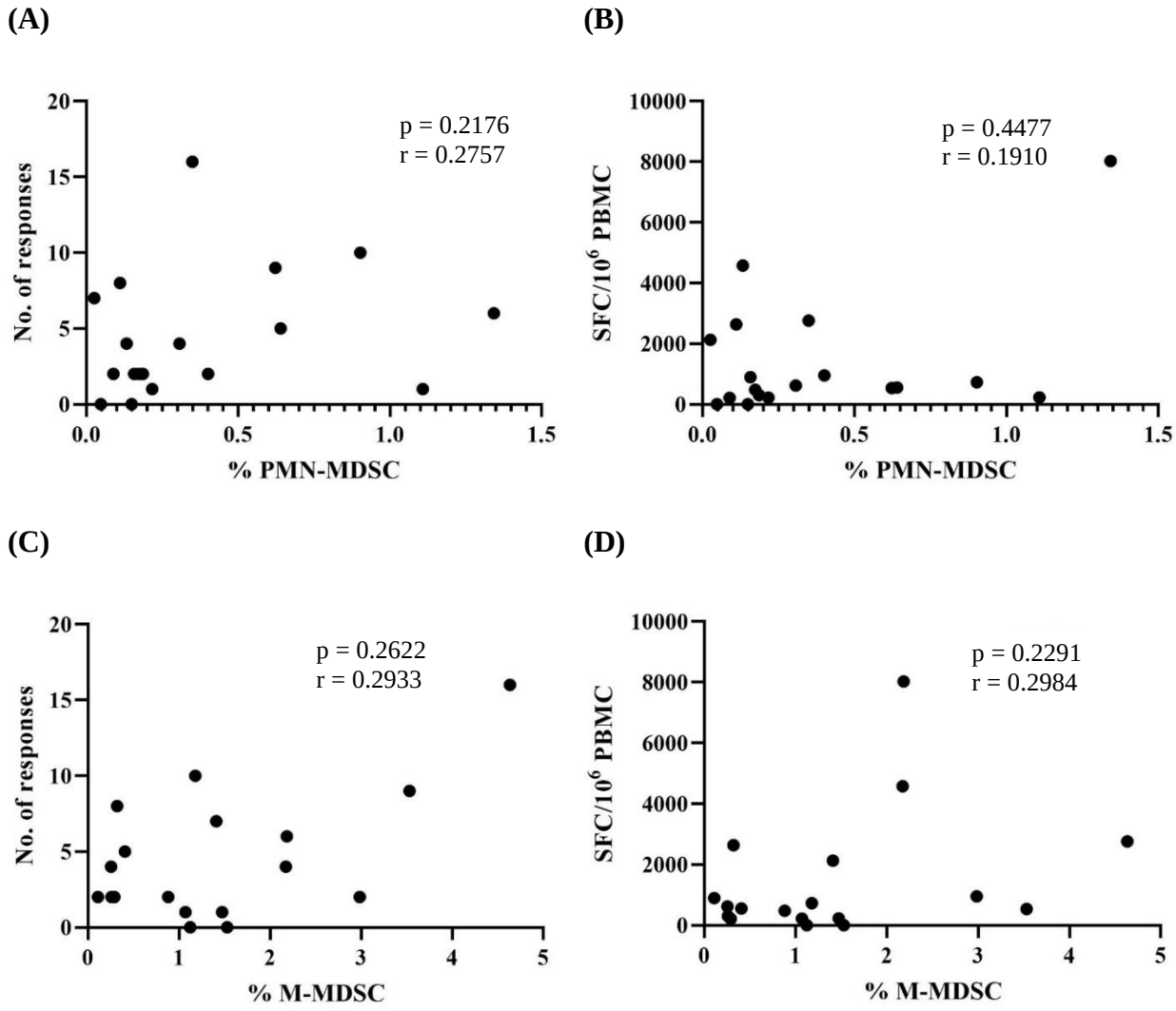


Figure 17: Frequencies of MDSC plotted against HIV-specific immune responses of the NE cohort. (Spearman rank test). **A** Correlation between breadth and PMN-MDSC frequency. **B** Correlation between magnitude and PMN-MDSC frequency. **C** Correlation between breadth and M-MDSC frequency**. D** Correlation between magnitude and M-MDSC frequency.

4 Discussion

This study investigated the effect of an intensified HIV treatment on MDSCs and the breadth and magnitude of CD8 T cell responses in correlation to these in the NE cohort and other groups of HIV-infected individuals as controls. Of particular interest was the impact of different starting points of the intensified treatment on the two NE subsets. As the NE study aimed to eradicate HIV-1 on a long term, these findings were acquired previously to a possible interruption of HIV treatment. The results of this study showed that antiretroviral treatment leads to a reduced level of MDSC frequencies in HIV patients whereas the absence of treatment resulted in higher frequencies as has been shown before. This particularly applied to the levels of PMN-MDSC in treated individuals since this subset of cells showed almost normal levels as seen in HC. There was no advantage for NE patients under an intensified drug regimen in comparison to the HIV patients with a conventional drug regimen in terms of MDSCs and immune responses. Interestingly, NE patients showed surprisingly broad immune responses. Furthermore, the early start of therapy in the PHI subgroup did not result in lower MDSC frequencies when compared to the CHI patients. Finally, there were no correlations identified between MDSC frequencies and breadth or magnitude of CD8 T cell responses, respectively.

4.1 Myeloid-derived suppressor cells

4.1.1 Antiretroviral treatment reduces PMN-MDSCs close to normal levels

Within the NE cohort and 3ART patients, PMN-MDSC levels were low and close to normal when compared to the healthy individuals of this study. These findings are consistent with other studies which show the decline of MDSCs after initiation of HIV treatment implying that HIV treatment is effective in reducing MDSC levels. (31) In our analysis, we were not able to find differences between a conventional ART consisting of three drugs and the intensified treatment regimen used in the New Era study. Nevertheless, a few HIV patients under treatment showed higher PMN-MDSC levels than what one would expect to find. Thus, antiretroviral treatment does not necessarily lead to normal levels in every HIV patient and apparently also applies to the intensified HIV treatment of this study. As a limitation of this study, there are no data available for MDSCs prior to HIV infection and thus it is not possible to compare individual levels of MDSC before and after infection for the HIV patients. However, the kinetics of MDSC frequencies before and during infection were also investigated in the longitudinal study by *Dross et al*. Healthy rhesus macaques were infected with SIV and several blood draws were performed during the following weeks.

In week 6 of the infection, the macaques started to receive an antiretroviral treatment which is comparable to a standard drug regimen in humans. Results showed low MDSC frequencies in the macaques prior to infection whereas the acute stage of the infection led to a slight yet significant elevation. Despite effective treatment starting at week 6 this elevation remained over the full course of treatment which lasted 31 weeks. (40)

4.1.2 Antiretroviral therapy has a greater effect on PMN-MDSCs than on M-MDSCs

M-MDSC levels are generally higher than PMN-MDSC levels within the NE cohort as well as the 3ART patients and thus do rarely reach normal levels. Therefore, it can be concluded that M-MDSC levels appear to be higher no matter which treatment regimen was chosen. Overall, it suggests that the intensified treatment had a lesser effect on the reduction of M-MDSC frequencies than it had on PMN-MDSC levels.

4.1.3 Early intensified treatment does not lead to an observable advantage

Surprisingly, no statistical differences were detected between PHI and CHI in regard to MDSC frequencies. Although the highest percentages regarding MDSCs were found in CHI patients, it is not possible to deduce a beneficial effect for the patients with an early treatment start from this data, at least not in terms of the investigated suppressive cells of this thesis. However, it needs to be taken into account that the number of HIV patients in every group was relatively small in order to reveal statistical significances.

4.1.4 CHI patients represent a special group within the NE cohort

A difference in M-MDSC levels can be found, though not statistically relevant, when considering the two subgroups within the NE cohort individually. Interestingly, patients who had started their intake of the 5-drug regimen during their acute phase of infection tended to have M-MDSC frequencies which were higher than in healthy controls. In contrast, M-MDSC frequencies of CHI were comparable to HC. This underlines that CHI represent a special group of HIV patients as they had to fulfil certain criteria to be included in the NE study in the first place. One of those had been a continuous HIV treatment regimen with a protease inhibitor for at least 36 months prior to the start of the intensified treatment. Another one was the requirement that patients of the CHI groups were not allowed to have had a viral blip in the years before inclusion into the study. This most likely led to a specific immunological state in the CHI patients.

4.1.5 The lack of antiretroviral treatment correlates with high frequencies of MDSC

In untreated HIV progressors, levels of MDSC were considerably elevated when compared to uninfected individuals for both MDSC subsets, especially when compared to healthy controls. This confirms results from past studies (31) and emphasises the effect the virus has on the human immune system. As MDSCs are known to be capable of suppressing T cell functions, they might directly affect the immune system's functions and may contribute to the exhaustion which can be seen during an advanced stage of the infection. (see 4.2.4)

4.2 Cytotoxic T cell response

This study evaluated the breadth and magnitude of CD8 T cell responses on an epitope level. From an array of possible epitopes, Gag and Nef were chosen for study purposes as they are known to be two of the most recognised HIV-1 proteins. Therefore, results only reflect immune responses to those proteins. It also needs to be noted that continuous HIV treatment has been shown to result in a reduction of both, breadth and magnitude of CD8 T cells responses. (53) Amongst the study participants under antiretroviral treatment, the NE cohort had continuously taken the 5-drug regimen for at least 7 years and the 3ART patients were under conventional treatment for at least 4 years. Furthermore, results of CD8 T cell responses were considered for detection of possible correlations between the immunosuppressive cells investigated in this study.

4.2.1 No difference of the CD8 T cell response between the intensified treatment group (NE) compared to the conventional cART group

NE patients showed broad responses towards the epitopes of this study. The number of responses is comparable to the other HIV groups, in particular to the patients receiving cART and implies that there is no difference between the two treatment strategies.

In contrast, NE patients` immune responses appeared to be slightly stronger than the responses patients showed under a conventional HIV treatment, but this difference was only a trend and did not reach statistical significance. However, it might imply that preservation of immune responses is better when using an intensified treatment strategy.

4.2.2 An early HIV treatment results in a narrower immune response

The NE cohort showed immune responses which were similarly broad to those of the other HIV groups. Nevertheless, differences in breadth were found between PHI and CHI patients. Interestingly, the CHI subgroup showed broader responses to all epitopes than the PHI patients.

This shows that early treatment intervention can lead to a narrower immune response; most likely due to fact that early treatment intervention prevents CD8 T cells to be exposed to the HIV antigen for a long period and allows less time to react to the antigen.

This is also consistent with the findings of a study conducted by *Streeck et al* who showed that a broad CD8 T cell response was associated with a delayed initiation of cART. (60) The overall breadth and magnitude can, however, not be taken as predictors for HIV-1 progression. (53) The specific response to certain HIV epitopes appears to be rather important in disease progression. *Streeck et al* also showed that disease outcome is better when Gag is getting recognised well during primary infection. Besides Gag, the response to the HIV epitope Nef has been linked to slower disease progression. (8) Furthermore, *Radebe et al* showed that the point of time when the HIV epitope is recognised is essential: it appears that responses to Nef are important in the very early course of infection whereas responses to Gag seem to be crucial in the maintenance of HIV at a later stage. (61)

4.2.3 Recognition of the epitopes Gag and Nef amongst HIV patients

In this study, Gag p24 and Nef were recognised the most by the HIV groups of this study whereas Gag p15 and p17 were less recognised. This is coherent with findings of other groups and is likely due to the fact that both epitopes, Gag p24 and Nef, are known to be highly immunogenic because of their long and mostly conserved amino acid sequences while Gag p15 and p17 are shorter and more variable in sequence. (62; 63) The varying responses between the single epitopes underline the importance of evaluating the specificity of the CD8 T cell response. Clear differences between individual HIV proteins have also been described in a study by *Kiepiela et al* who looked at the viral load of untreated HIV patients and found that it correlated inversely with the breadth of T cell responses towards Gag. (64) In contrast, they found a direct correlation of the viral load with the breadth of Env-specific T cell responses. An interesting study by *Mothe et al* considered the entire proteome of HIV in 950 patients. Based on cohort-level associations with the viral load, they identified several epitopes to be protective or non-protective. Among others, protective epitopes appeared in regions of Gag whereas Nef was associated with increased viral loads and was thereby described as non-protective. Moreover, they could link the protective epitopes with a higher degree of conservation whereas non-protective epitopes were less conserved in sequence. The highly conserved sequences also appeared to be more stable towards possible viral escape mutations. This is likely to be a determining factor for the protectiveness of an epitope (65).

The results of this study show the state of HIV-specific CD8 T cell responses several years after 5-drug cART had been initiated in NE patients. Therefore, it is difficult to compare these to results from study groups who had drawn blood from untreated patients during their early phase of infection. Although a paper by *Poizot-Martin et al* showed that the long-term application does not affect CD8 T cell counts, the question remains whether the responses specifically to a certain HIV epitope remain correspondingly. (66) *Smith et al* compared primed responses of naïve CD8 T cells with responses from memory CD8 T cells of HIV patients which had been exposed to cART for a substantial amount of time. (67) They showed that those cells were less potent in responding to HIV epitopes than their primed naïve counterparts suggesting that CD8 T cells can lose their ability to respond to HIV epitopes over the course of an antiretroviral treatment. Whether or not this loss occurs towards the whole range of HIV epitopes or rather to a specific subset of epitopes continues to be subject of research.

4.2.4 Immune exhaustion during the advanced stage of an HIV infection

In chronic progressors (PR) the CD8 T cell responses are neither strong nor broad when compared to other HIV groups of this study. Conversely, MDSC frequencies are significantly elevated. MDSCs are known to be capable of inhibiting functions of CD8 T cells and the lack of HIV treatment over a long time has likely led to a certain degree of immune exhaustion in the PR group. In contrast to CD4 T cells which decrease early in an untreated HIV infection, CD8 T cell frequencies typically remain high until they deplete during later stages of the disease. In the absence of cART, CD8 T cells of PR also lose their effector functions and their ability to respond to HIV adequately over time. (68) Overall, this emphasises the importance of antiretroviral treatment in HIV patients.

4.3 Limitations of the study

4.3.1 Different approaches on investigating MDSCs

MDSCs are a group of immune cells which have been subject to intense research in recent years. However, the approach to investigate this distinct cell group differed among research groups. It particularly accounts for the used staining techniques to target surface antigens as well as intracellular targets. This can primarily be observed in cancer research and needs to be considered in comparative analyses of MDSCs. Besides phenotyping, differing gating strategies can have a great influence on the results obtained from flow cytometry. Therefore, attempts have been made to establish a common gating strategy as a reference for future studies which is according to *Bronte et al*. (29)

When comparing our study results to results of papers of the past, it must be noted that the methods used to isolate PBMC varied amongst research groups. Several groups first froze isolated cells and thawed them prior analysis. *Grützner et al* showed that the kinetics particularly in regard to M-MDSCs needs to be carefully taken into account. When isolating PBMCs from peripheral blood, M-MDSCs need to be analysed in a fresh state and isolation should not be performed later than 4 hrs after blood draw. Otherwise, frequencies will decrease significantly. (28) However, this was observed in this study.

Finally, this thesis has only focused on human MDSCs and the HIV-specific CD8 T cell response in peripheral blood. Although most research has been carried out on PBMCs, HIV replication mostly occurs in other tissues such as the lymphoid or mucosal tissue. MDSCs are usually maintained in the bone marrow under normal conditions and migrate into a number of other compartments in response to an infection, i.e. an infection with HIV. Amongst those compartments are immunological tissue sites such as the spleen and lymph nodes. Within the tumour microenvironment of mouse models, it was demonstrated that localization impacts fate as well as function of MDSC. (69) Furthermore, mechanisms of activation as well as their own mechanisms of immunosuppression can vary depending on the tissue site and the conditions the tissue is facing in that particular moment. Altogether, these results highlight the need for future investigations of MDSCs in tissues other than the peripheral blood.

4.3.2 Effects of age and gender on HIV progression

In medicine, it is a well-established concept that age, gender and race of an individual can play a role in acquiring a disease and in its progression. Regarding age, multiple studies have shown that HIV acts differently in different age groups. In elderly HIV patients, disease progress is faster and their recovery under cART is slower than in younger adults. This is most likely explained by the aging of the immune system itself which leads to a change in distribution and competence of immune cells. (70) This ultimately results in a dysfunctional immune system in the elderly. In relation to the frequency of MDSCs, *Verschoor et al* first showed that MDSC frequency is increased with age. Interestingly, this was the case particularly for the PMN-MDSC subset. (71)

Similarly, the gender of an individual can change susceptibility, progression, drug response and disease outcome. More recent studies found clear differences in clinical characteristics between the two genders: Women tend to have significantly lower viral loads than their male counterparts when first diagnosed with HIV. Despite their low viral loads, they reach the final stages of the HIV infection in about the same time as men. (72) As far as we know there is no data on gender and its effect on immunosuppressive cells in HIV patients. Nevertheless, it needs to be considered as a potential factor in determining frequencies and functions of immunosuppressive cells.

In the New Era Study, the patients‘ age ranged from 32 to 62 years at the time of blood draw. This range was even bigger in the control groups with a range from 20 to 70 years and might have impacted the results in terms of immunological aging. In terms of gender, the majority of NE patients were male (n= 16) and the 3ART group only consisted of male patients. Contrastingly, there was a higher number of women (n= 6) included in the remaining HIV groups and in particular in the healthy controls. This needs to be considered when comparing a male-based NE cohort to healthy controls with a higher proportion of females.

4.3.3 An additional control group: Primary infected HIV patients

To understand disease progress on a cellular and molecular level, it is necessary to know the typical conditions at the very beginning of an HIV infection. In a study by *Zhang et al* released in 2017, it was found that primary infected individuals showed high frequencies of PMN-MDSC which were functionally related to the suppression of CD8 T cells via the ligand PD-L1. (38) Thus, it would have been very interesting to compare the obtained results of immunosuppressive cells with HIV patients during primary infection. Nonetheless, this study included a unique set of HIV patients and gives first insights into immunosuppression at a cellular level during an intensified drug regimen.

4.4 Debate on Treatment interruption in the New Era cohort

This study was performed to monitor the status of immunosuppressive cells in the NE cohort with the aim to identify markers that could potentially help in the process of decision making on whether treatment should be interrupted or not in the NE patients. Nonetheless, this topic is being debated amongst clinicians, researchers and other HIV experts. On the one hand discontinuation of cART is known to be associated with a poor outcome for the patients. This, however, applies mostly to long-term discontinuation or repeated cycles of treatment interruption. On the other hand, a recent study by *Clarridge et al* found that a short interval of cART interruption did not exert adverse effects on the HIV cohort studied. (73) This suggests that a pause from the antiretroviral treatment for a limited amount of time and simultaneously frequent clinical controls in short intervals might be ethically acceptable and could give more insights into a functional cure for HIV.

Resources

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Statutory Declaration

Eidesstattliche Versicherung

Hoffmann, Tanja

Name, Vorname

Ich erkläre hiermit an Eides statt, dass ich die vorliegende Dissertation mit dem Titel:

Frequencies of myeloid-derived suppressor cells in relation to the cytotoxic T cell response in HIV-infected patients of the New Era Study

selbständig verfasst, mich außer der angegebenen keiner weiteren Hilfsmittel bedient und alle Erkenntnisse, die aus dem Schrifttum ganz oder annähernd übernommen sind, als solche kenntlich gemacht und nach ihrer Herkunft unter Bezeichnung der Fundstelle einzeln nachgewiesen habe.

Ich erkläre des Weiteren, dass die hier vorgelegte Dissertation nicht in gleicher oder in ähnlicher Form bei einer anderen Stelle zur Erlangung eines akademischen Grades eingereicht wurde.

Werder, den 03.11.2021

Ort, Datum

Tanja Hoffmann

Unterschrift Doktorandin bzw. Doktorand

Publications

Conference presentation

13. Kongress für Infektionskrankheiten und Tropenmedizin (2016), Poster

LMU KLINIKUM DER UNIVERSITÄT MÜNCHEN

MEDIZINISCHE KLINIK UND POLIKLINIK IV
SEKTION KLINISCHE INFEKTIOLOGIE

DIREKTOR: PROF. DR. MED. M. REINCKE

Niedrige Level von granulozytären myeloid-derived suppressor cells bei HIV-Infizierten mit 5-fach HAART (New Era-Studie)

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Hintergrund

Die New Era-Studie untersucht den Einfluss einer 5-fach HAART auf das virale Reservoir bei Patienten, die in der akuten HIV-Infektion mit der Therapie begonnen haben (= PHI), sowie bei Patienten, die nach ≥ 36 Monaten erfolgreicher 3-fach HAART auf eine 5-fach Therapie intensiviert wurden (= CHI).
In den letzten Jahren wurde klar, dass es durch zunehmende inhibitorische immunologische Faktoren zur Immunerschöpfung in der chronischen HIV-Infektion kommt. Zu diesen suppressiven Faktoren zählen in der HIV-Infektion auch die MDSC.
Wir stellten die Hypothese auf, dass durch eine frühe HAART in der PHI die Ausbildung von MDSC verhindert wird. Hierfür untersuchten wir die Frequenz von granulozytären (gMDSC) und monozytären MDSC (mMDSC) in PBMC von Patienten der New Era-Studie (= NE; PHI und CHI), Patienten mit fortgeschrittener, unbehandelter HIV-Infektion (Progressoren= PR), mit 3-fach HAART behandelten Patienten (= 3ART) und gesunden Kontrollen (= HC) per FACS-Analyse (Tabelle 1). Zudem wurde im Interferon-gamma Elispot nach HIV-spezifischen CD8 T-Zellantworten auf die viralen Proteine Gag und Nef gesucht.

Tabelle 1: Study Subjects. Klinische Daten der Patienten (CD4-Zellzahl in Zellen/µl; Viruslast in Kopien/ml)

	Median CD4 Zellzahl	Range	Median Viruslast	Range
HC (n=5)	-	-	-	-
PHI (n=8)	790,5	557 - 1342	<40	-
CHI (n=11)	662	551 - 1133	<40	-
3ART (n=5)	591	487 - 687	<40	-
CO (n=7)	578	467 - 1442	51	<40 - 702
PR (n=6)	81	49 - 205	177.453	2.100 - 540.539

Myeloid-derived suppressor cells

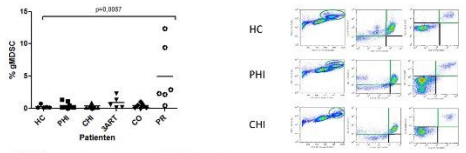

Abbildung 1: Frequenz von gMDSC in PBMC
Die NE-Kohorte (PHI und CHI) wies ähnlich niedrige Frequenzen von gMDSC zu den HC auf (p= 0,66; 0,97). Kein Unterschied bestand zwischen PHI und CHI (p= 0,97). Allein die Progressors (PR) unterschieden sich signifikant von den HC (p= 0,0087).

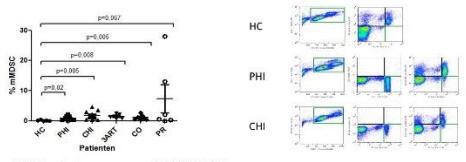

Abbildung 2: Frequenz von mMDSC in PBMC
Die NE-Kohorte (PHI und CHI) zeigte einen signifikanten Unterschied in mMDSC Frequenz zu den HC (p= 0,02; 0,005). Kein Unterschied konnte zwischen den PHI und CHI vermerkt werden (p= 0,2). Nur die PR wiesen keinen signifikanten Unterschied zu den HC auf, jedoch eine tendenz zur höheren mMDSC Frequenz (p= 0,067).

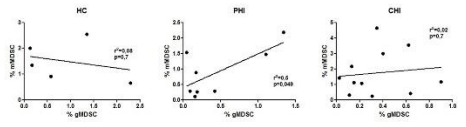

Abbildung 3: Korrelation zwischen der Frequenz von g-und mMDSC
Mit Ausnahme der PHI, bei denen sich ein Trend zur Korrelation zwischen g-und mMDSC Frequenz zeigte (r^2= 0,5; p= 0,049), konnte bei keiner Patientengruppen (HC, CHI, 3ART, CO und PR) eine signifikante Korrelation festgestellt werden. Exemplarisch werden die Graphen der Gesunden und NE-Kohorte abgebildet.

HIV-spezifische CD8 T-Zellantworten

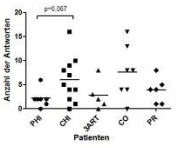

Abbildung 4:
Breite der CD8 T-Zellantwort
Innerhalb der NE-Kohorte wiesen die PHI tendenziell weniger Elispot Antworten auf als die CHI (p= 0,067). Allerdings konnten keine signifikanten Unterschiede zwischen den Patientengruppen gefunden werden.

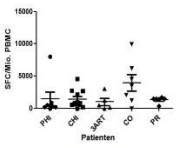

Abbildung 5:
Stärke der CD8 T-Zellantwort
Die Stärke der Antworten innerhalb der NE-Kohorte (PHI und CHI) ähnelte sich (p= 0,2). Weiterhin konnten keine signifikanten Unterschiede zwischen den restlichen Patientengruppen vermerkt werden (p< 0,11).

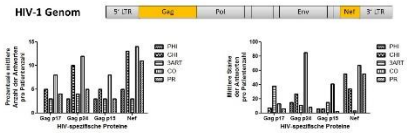

Abbildung 6: Verteilung der Breite der Antworten im HIV-1 Genom
PHI zeigten wie die 3ART Gruppe bei allen 4 Proteinen vergleichsweise wenig Antworten. Die CHI zeigten deutlich mehr Antworten bei Gag p24 und Nef. Controllers (CO) wiesen bei allen Proteinen die höchste Anzahl an Antworten auf.

Abbildung 7: Verteilung der Stärke der Antworten im HIV-1 Genom
Die Stärke der Antworten von der NE-Kohorte (PHI und CHI) war wie bei den CO und PR bei Nef mit Abstand am höchsten, gefolgt von Gag p24. Im Vergleich zeigte die 3ART Gruppe überwiegend schwache Antworten mit Ausnahme von Gag p17.

Fazit

Die NE-Kohorte zeigt eine niedrige Frequenz an gMDSC, die vergleichbar mit gesunden Probanden ist, wohingegen sich die Frequenz der mMDSC signifikant von den gesunden Probanden unterscheidet. Zwischen PHI und CHI konnten keine signifikanten Unterschiede bei der MDSC Frequenz festgestellt werden. Insgesamt zeigt dies, dass unter 5-fach HAART die immuninhibitorische Komponente MDSC auf Normalniveau eingedämmt ist, wenn auch kein Vorteil für die PHI bestand. Die HIV-spezifische CD8 T-Zellantwort der NE-Kohorte unterschied sich weder in der Stärke der Antworten signifikant von den restlichen Gruppen. Erwartungsgemäß zeigten die PHI im Vergleich zu den CHI eine tendenziell niedrigere Breite. Die meisten und stärksten HIV-spezifischen CD8 T-Zellantworten der NE-Kohorte konnten auf dem Protein Nef vermerkt werden, gefolgt von Gag p24 – vergleichbar zu anderen, bereits publizierten Kohorten.

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