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Immune suppressive cells in chronic HIV-1 infection

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1. List of abbreviations

AIDS Acquired Immune Deficiency Syndrome

ART antiretroviral therapy

3ART patients treated with a conventional 3-drug ART

Bregs regulatory B cells

CD cluster of differentiation

CHI chronic HIV infection

CNS central nervous system

CO controllers

CTLA-4 cytotoxic T lymphocyte antigen-4

DNA Deoxyribonucleic acid

EC elite controllers

HC HIV-uninfected controls/ healthy controls

HIV Human Immunodeficiency Virus

IFN- γ Interferon γ

IIT investigator initiated trial

IL-2 Interleukin-2

LAG-3 lymphocyte-activation gene 3

M-MDSCs monocytic myeloid-derived suppressor cells

MIP-1β macrophage inflammatory protein-1β

NE New Era

OI opportunistic infection

ON overnight

PBMCs peripheral blood mononuclear cells

PD-1 programmed death-1

PHI primary HIV infection

PMN-MDSCs polymorphonuclear myeloid-derived suppressor cells

PR progressors

RNA ribonucleic acid

SIV Simian Immunodeficiency Virus

TIM-3 T cell immunoglobulin and mucin domain containing protein-3

TNF-α tumor necrosis factor α

Tregs regulatory T cells

ÜN über Nacht

Visconti Viro-Immunological Sustained CONtrol after Treatment Interruption

2. List of publications

2.1. Publications

Grützner E, Stirner R, Arenz L, Athanasoulia AP, Schrödl K, Berking C, Bogner JR, Draenert, R. Kinetics of human myeloid-derived suppressor cells after blood draw. J Transl Med (2016) 14:2.

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3. Introduction

3.1. Human Immunodeficiency Virus (HIV) infection

3.1.1. Epidemiology of HIV infection

In Germany, approximately 88.400 people were living with HIV at the end of 2016. 86% of diagnosed patients are being treated with antiretroviral therapy (ART). Approximately 460 patients died because of their HIV infections in 2016 (1, 2).

Globally, the numbers are even more alarming: 36.7 million people were living with HIV worldwide in 2016 and only 53% (20.9 million people) had access to antiretroviral therapy (ART) in June 2017 (3). And yet, the latest number of treated patients signifies a 1.2-fold increase compared to 2015 and even 2.7-fold compared to 2010. This raise contributes to an encouraging 48% decline of AIDS related deaths from 1.9 million in 2005 to one million in 2016 (the lowest number since 2000) (4).

In the last decades, intense research efforts resulted in an ART with a low pill burden and few side effects. Today, ART is highly efficacious in terms of suppressing viral load and decreasing mortality. Therefore, life expectancy of young treated HIV patients in Europe and North America increased massively in the last two decades and is now comparable to healthy adults (5). Nevertheless, due to highly efficient resistance mechanisms of the HI virus and 47% HIV-infected patients who do not have access to treatment, there is an urgent need for alternative treatment strategies towards a sterilizing cure (eradication of virus) or at least a functional cure (sustained immune control of HIV-1 viremia).

3.1.2. Pathogenesis of HIV infection

For entry and spreading within the human immune system, the HI virus uses the CD4 T cell marker along with chemokine coreceptors (CCR5 and CXCR4 respectively). With CD4 T lymphocytes, dendritic cells, macrophages, central nervous system (CNS) microglia cells expressing these receptors, HIV is able to infect these cells and, thus, to replicate in blood cells, lymphatic organs and CNS. After infection, viral load peaks with often several million copies per ml blood. Over the course of the disease, cytotoxic CD8 T cells of the adaptive

immune system among others are activated to eradicate HIV-infected cells resulting in the decrease of the viral load and the plateau at a certain individual level which predicts the rate of disease progression as the so-called “viral setpoint” (approximately 2 log_{10} copies/ml less than the peak) (6, 7). In the following years, HIV disease persists in a clinically asymptomatic stage. The duration varies due to the age of the patient, the pathogenicity of the virus, or environmental factors. The latent phase lasts for 8 to 10 years but increasingly for a shorter period of time (8, 9). The HIV-specific immune response is not able to prevent the virus completely from replicating. Without antiviral treatment of the patient, the immune system loses the control over viral replication and the patient reaches the stage of AIDS. The CD4 T cell count falls below 200/µl, pathogens can spread and, therefore, opportunistic infections arise (10, 11).

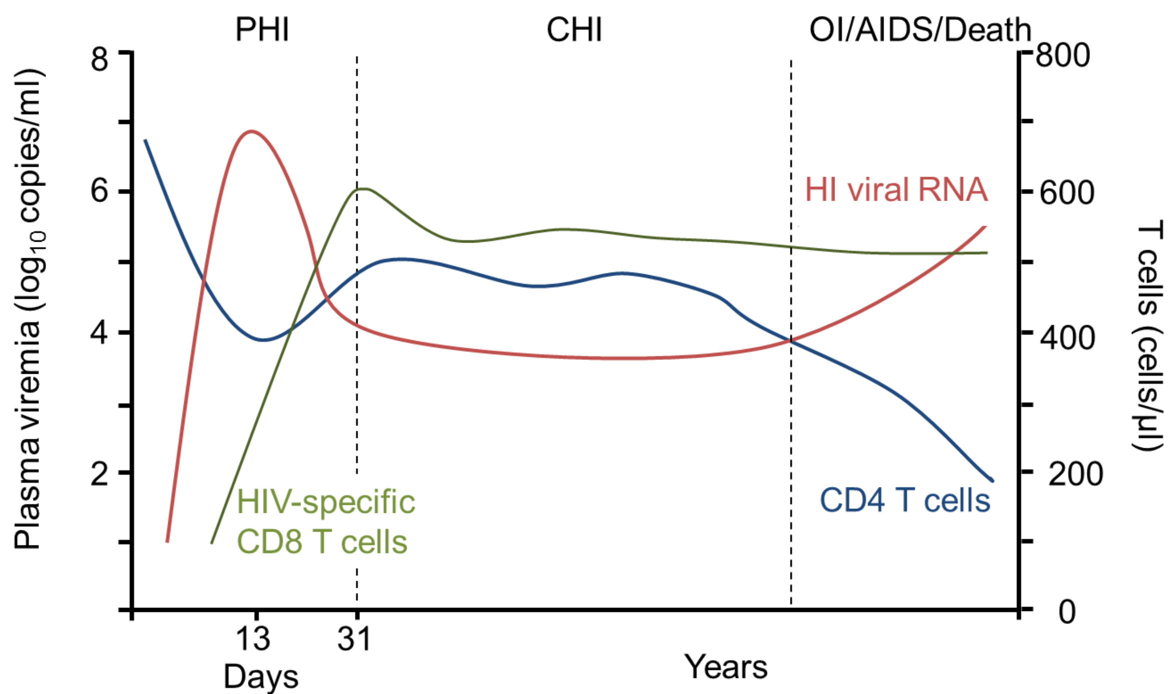

Figure 1 The natural course of HIV infection (diagram adapted to (7, 11)). In the first weeks after viral infection, the viral load increases tremendously (more than 6 log_{10} copies/ml, median 6.7 log_{10} copies/ml (7)) and the number of CD4 T lymphocytes decreases. After CD8 T cell activation, the viral set point is reached (approximately minus 2 logs compared to peak (7)), the viral RNA decreases and plateaus for about 8 to 10 years (latent phase of infection). Without ART AIDS develops. PHI = primary HIV infection, CHI = chronic HIV infection, OI = opportunistic infection, RNA = ribonucleic acid.

However, even in the absence of ART, a certain group of patients – the so-called controllers – can control viremia below 2000 copies/ml spontaneously. Less than 1% of untreated infected patients (“elite controllers”) even suppress

viral loads below 50 copies/ml. The reasons for this singular ability of controllers are not completely clarified, however, there is evidence that immunological CTL defense and restrictions in HLA allele may play a crucial role (6, 12-15).

3.1.3. Antiretroviral therapy (ART)

National and international treatment guidelines recommend a therapy regimen containing three compounds (two different classes of antiretroviral drugs) to interfere substantially with the viral replication cycle (16-18). With conventional 3-drug ART (3ART) a durable suppression of the HI virus below detection limit (state of the art: <20 copies/ml) is possible but ART cannot wipe out latent virus in resting T cells. Proviral HIV DNA is harbored in quiescent CD4 T cells (central memory and transitional memory) building the latent reservoir. Thus, viral eradication using the currently available antiretroviral drugs cannot be achieved (19-22).

After treatment interruption, the virus restarts to replicate and viral load increases (23-25). However, a reduction of reservoir has been shown if ART was early started in primary HIV infection (PHI) (26, 27). Therefore, treatment strategies were contrived to analyze the impact of early treatment in acute infection as well as with treatment intensification. One of them, the Visconti (Viro-Immunological Sustained CONtrol after Treatment Interruption) study, analyzed the influence of ART in 14 individuals who started very early in PHI and controlled viremia for several years after the interruption of the treatment. The authors concluded a beneficial effect of an early prolonged ART e.g. because of low viral reservoir as well as a low proportion of long-lived CD4 T cells (28-30). Furthermore, the New Era study, a German investigator initiated trial (IIT) of treatment intensification with the aim of virus eradication, looked at the impact of an intensified, 5-drug treatment strategy on residual viremia. Two supplementary drug classes (the integrase inhibitor raltegravir and the CCR5 inhibitor maraviroc) were added to a PI based regimen. Patients started intensified treatment in PHI and chronic HIV infection (CHI). The study comprised 40 patients with a CD4 nadir above 200 cells/µl and without history of AIDS or protease inhibitor (PI) resistance. PHI patients (n = 20) had detectable HIV RNA and an early stage of acute infection with less than two bands in Western blot analysis. Before starting the 5-drug regimen, CHI patients

(n = 20) were required to be successfully treated on a stable PI based regimen for at least 36 months. All study participants have been on treatment intensification for more than 5 years. After 24 months of treatment intensification, significantly lower levels of median proviral DNA were observed in PHI versus CHI patients (31-33). With more than 5 years of NE regimen, the immunological status was to be evaluated before an eventual treatment interruption. This was the project for the second manuscript of this dissertation. Similar studies to compare treatment intensification and conventional 3-drug ART have been performed earlier. However, no differences in viral suppression and immune activation have been observed and immune suppressive cells were not evaluated (34, 35).

3.1.4. CD8 T cell response

Back in the late 1980s, the suppression of viral replication by CD8 T cells was first discovered in vitro, closely followed by detecting HIV-specific CD8 T cells in HIV-infected patients (36-38). Already in acute HIV infection, these HIV-specific CD8 T cells are activated and their levels increase tremendously, resulting in a decrease of viral load after the initial peak. In primary HIV infection, the T cell response is very strong and effective, forcing the HI virus to develop escape mutations for further replication (39-41). This temporal association of increasing viral load and activated CD8 T cell response suggests the crucial role of CD8 T lymphocytes in recognizing and killing HIV-infected cells (42-50) which was additionally confirmed by depletion studies in SIV-infected rhesus macaques (51-53). The latter studies showed an increase of viral load in acute and chronic SIV infection and even in antiretroviral treated animals after the complete depletion of CD8 T cells by a monoclonal antibody. Therefore, HIV-specific CD8 T cells were identified as crucial for the control of viremia in acute as well as chronic infection.

3.1.5. Immune exhaustion

After clearing a pathogen during the phase of acute infection, the downregulation of the activated immune system is required to prevent unspecific autoimmune reactions. The physiological role of inhibitory immune signaling is dedicated to reduce the effectivity of T cell lymphocytes e.g. by T

cell proliferation, production of cytokines and chemokines. The infection with HIV is a chronic disease without clearance by the immune system resulting in a chronic, continuous immune activation which is a hallmark of HIV infection. This immune activation strongly predicts disease progression, independently of other markers of disease progression like CD4 decline or viral load (14, 54-60).

During the natural course of untreated HIV infection, the CD8 T cell response loses effectivity – by decreased proliferation and a decline in the production of cytokines and chemokines and, thus, results in disease progression. In contrast to untreated patients with progressive HIV infection (progressors), controllers and elite controllers maintain CD8 T cells which are still polyfunctional, i.e. these cells still have the ability to mobilize CD107a (degranulation), to produce the cytokines IL-2, IFN-γ and TNF-α as well as the chemokine MIP-1β (15, 61, 62).

The loss of effectivity of CD8 T cells during HIV infection was termed immune exhaustion and the reasons for this are not completely understood so far. With increasing immune activation, immune inhibitory signals (also called “immune checkpoints”) are expressed more and more on CD8 T cells, e.g. programmed death-1 (PD-1) (63-66), CTLA-4 (67), LAG-3 (68), and TIM3 (69) (all reviewed in (70)). However, just recently, immune inhibitory cells have been described to play a decisive role in immune exhaustion. Studying the role of these cells in chronic HIV infection is the core of this dissertation. The three main groups are: myeloid derived suppressor cells (MDSCs), regulatory T cells (Tregs) and regulatory B cells (Bregs).

3.2. Immune suppressive cells

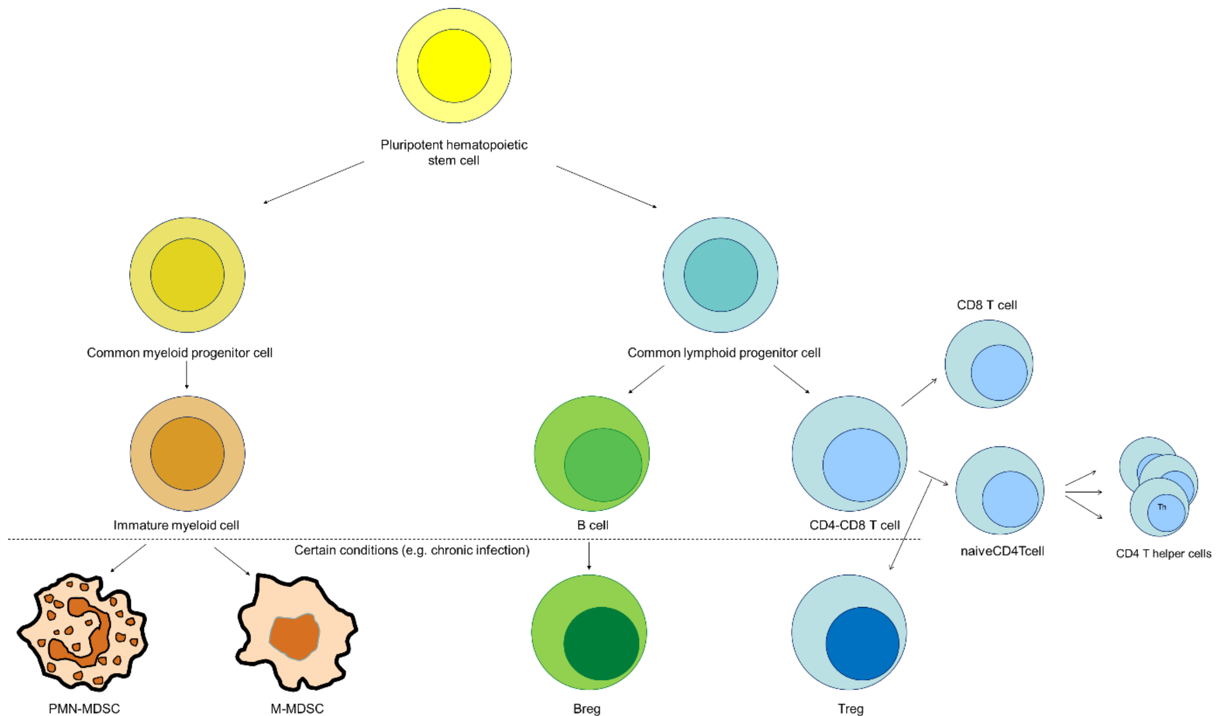

Figure 2 Development of immune suppressive cells (simplified scheme adapted to (71-75)). Regulatory T cells (Tregs) and regulatory B cells (Bregs) develop of lymphatic progenitor cells in thyme and bone marrow respectively. Myeloid-derived suppressor cells (MDSCs) originate from myeloid cells and, therefore, belong to the innate immune system. All three cell types accumulate under certain conditions e.g. in chronic infections, suppress CD8 T lymphocytes and are said to be involved in T cell exhaustion. PMN-MDSC = polymorphonuclear MDSC, M-MDSC = monocytic MDSC.

3.2.1. Myeloid-derived suppressor cells (MDSCs)

MDSCs comprise a heterogeneous population of immature myeloid cells that inhibit the functions of T lymphocytes as well as functions of cells of the innate immune system (72). Following published evidence from oncology studies the suppressive role of MDSCs in infectious diseases, e.g. HIV, has been investigated and established (76-78). In natural untreated HIV infection, polymorphonuclear (PMN)-MDSCs directly correlate to markers of disease progression namely decreasing CD4 cell count and increasing viral load. ART suppressed viral replication leads to a substantial decrease in PMN-MDSC levels. PMN-MDSC levels in patients who spontaneously control HIV infection were comparable to those of treated patients (76). However, data in SIV-infected monkeys report higher levels of PMN-MDSC during ART than prior to infection (79).

Recently, MDSC classification as well as an agreement for minimal criteria of the phenotyping were introduced in a consensus paper. Thus, the two major subsets are termed and defined PMN-MDSCs with a $CD14^-$, $CD11b^+$, $CD66b^+/CD15^+$ phenotype, and monocytic (M) MDSCs with a $CD11b^+$, $CD14^+$, $HLA\text{-}DR^{-/low}$, CD 15^- phenotype (80). The same definitions were used for our studies.

The growing scientific interest in these cells and the fact that MDSCs are a vulnerable cell type, resulted in the aim to harmonize methodological approaches in MDSC research. We identified MDSC phenotyping and the time frame between blood draw and cell processing as well as the recovery after freezing as critical in terms of comparing studies in the field. Vollbrecht et al., Tumino et al. and Qin et al. found different elevated MDSC subsets in chronic HIV infected patients compared to healthy controls. In contrast to elevated PMN-MDSC frequencies in chronic HIV infection (Vollbrecht et al. and Tumino et al. respectively), Qin et al. found the frequencies of these cells comparable to healthy controls (76, 77, 81). One probable explanation for discrepancies in study results is the difference in the handling of the cells after blood draw. In order to suggest the best time point to analyze frequencies of MDSCs, we conducted the first study of this dissertation. Besides the kinetics of cells after blood draw, we analyzed the sensitivity of MDSCs to freezing procedures.

Finding a sterilizing or at least a functional cure for HIV infection is an ultimate research goal. Treatment intensification and start of treatment during acute infection are strategies with the aim of reaching this goal that is the rationale of the second study of this dissertation. We were provided with the unique opportunity to evaluate the immunosuppressive cells in the treatment strategy of New Era (see chapter 3.1.3) before an intended treatment interruption: It was reported that 3ART consisting of three antiretroviral drugs influences the levels of immunosuppressive cells (76, 82-85). Thus, PMN-MDSC levels in patients with 3ART regimen are decreased (76). Our research objective was, whether an ART regimen consisting of 5 drugs intensifies this effect on PMN-MDSCs and other immunological markers.

3.2.2. Regulatory T cells (Tregs)

Tregs develop of progenitor T cells in the thymus after the CD8 T cell population has differentiated. Phenotypically, Tregs are described to be $CD4^+$, $CD25^+$, and $FoxP3^+$. Expressing the CD4 surface marker, Tregs are a target of HIV.

Ample evidence for the immunosuppression by Tregs in HIV infection can be found in the literature (73-75, 82, 86-90). In fact, these cells are the most well-known immunosuppressive cell type in HIV disease. Tregs act immunosuppressively by inhibiting T cell responses resulting in viral persistence. However, there are still open questions about their ambivalent role in the immune system.

Tregs are known to reduce immune activation and excessive immune reactions (tissue damage) during acute infection (73-75, 86, 88, 90). Given that immune activation leads to fast progression in chronic HIV infection (56, 59, 60), this would be a beneficial effect of Tregs in HIV infection. However, Cao et al. found high Treg levels in parallel to a high proportion of activated T lymphocytes in advanced HIV disease which stands for a lack of suppression of immune activation (90). During ART, Tregs were found to have the ability to control residual immune activation but 12 months after treatment interruption, the cells did not control immune activation anymore (82).

In the course of untreated HIV infection, the absolute number of Tregs decreases because of the reduction of CD4 cell counts while the relative frequencies increase (73, 74, 82, 86, 88). Successful antiretroviral treatment (ART) decreases proportionally Treg frequencies (75, 86, 89) whereas treatment interruption results again in increased Treg levels with suppressive potential (82). Although Tregs are well-described immune suppressive cells in HIV infection, to our knowledge, there is no data of the impact of an intensified treatment itself and its start in acute infection on the Treg levels. To fill this gap, we analyzed relative and absolute Treg levels in patients of the New Era study and compared them to control groups.

3.2.3. Regulatory B cells (Bregs)

Bregs were identified only recently as cells with immune suppressive capabilities in HIV infection (reviewed in (73)). For evolvement of Bregs from progenitor B cells toll-like receptor (TLR) stimulation is an important promoter (71). TLR agonists are products of well-known microbial translocation described in HIV infection (83, 91, 92). Phenotypically Bregs are defined as $CD19^{+}$, $CD24^{hi}$, $CD38^{hi}$ and produce IL-10 which has been shown to inhibit T cell function (71, 93). Depletion experiments confirmed the immunosuppressive function of Bregs. Breg-depleted peripheral blood mononuclear cells (PBMCs) showed augmented proliferation and levels of cytotoxic HIV-specific CD8 T cells followed by diminished HIV-infected CD4 T cells. Increased Breg levels have been related with a higher viral load, immune activation, and CD8 T cell exhaustion (84). Looking at $IL10^{+}$-Breg frequencies in various patient groups and disease stages compared to uninfected subjects, the cells were already substantially increased in untreated early infection as well as in chronic HIV-infected patients without ART and even in patients with ART and in elite controller (85). For a more comprehensive evaluation of the immune status of the New Era population, we thought it indispensable to include Bregs in our studies. At the same time we were able to gain data and a better understanding of these cells as relatively newly defined participants in immunosuppressive processes in HIV infection.

4. Summary

4.1. Abstract

The loss of efficacy of the CD8 T cell response is a crucial process for the progression of HIV infection. Despite intense research efforts in the last years, the reasons for the so-called immune exhaustion remain elusive. Forefront among supposed causes are inhibitory T cell receptors and immunosuppressive cells. The aim of this dissertation was to specify the impact of immune suppressive cells in chronic HIV infection.

In our first publication (94), two subsets of MDSCs were analyzed in 42 patients with chronic viral infection ($n = 25$) or with solid tumors ($n = 17$) to determine the best time point (2, 4, 6 hours and overnight rest (ON) respectively) for further processing after blood draw as well as freezing properties. Our results show that the frequencies of PMN-MDSCs were comparable within 6 hours after blood draw ($p > 0.5$). Whereas frequencies of M-MDSCs were reduced 6 hours after blood draw and after ON respectively (2 hours vs. 6 hours after blood draw and ON respectively: $p < 0.0051$). In both subsets frequencies were significantly decreased after freezing (PMN-MDSCs: $p = 0.0001$; M-MDSCs: $p = 0.04$) and we, therefore, recommend to analyze MDSCs in fresh PBMCs only within 6 hours (PMN-MDSCs) and 4 hours (M-MDSCs) respectively.

In the second publication (95), we analyzed the frequencies of immune suppressive cells (two subsets of MDSCs, Tregs and Bregs) as well as the HIV-specific CD8 T cell response in the special cohort of patients of the New Era study compared to different control groups. The treatment intensification had no influence on frequencies of MDSCs and Bregs compared to those of conventional 3-drug ART treated patients ($p > 0.21$). In contrast, the levels of Tregs in NE were significantly lower compared to 3ART patients ($p < 0.0001$) as well as to other control groups with HIV-infected patients ($p < 0.0033$). The CD8 T cell response in NE was broader ($p = 0.0134$) with a higher magnitude ($p = 0.026$) compared to 3ART patients. This second project was done in cooperation with a medical student who worked on part of the project.

Despite tremendous efforts, neither a sterilizing nor a functional cure in HIV infection was achieved in more than 30 years of HIV research. For the

persistence of the HI virus, an involvement of immune exhaustion of T cell responses is obvious among other factors. For this phenomenon, immune suppressive cells are a decisive part of the puzzle. The aim of this dissertation was to further elucidate the role of immune suppressive cells in HIV infection with the final goal of therapeutical use. We found that MDSCs should be analyzed in fresh PBMCs and that Tregs were the only immunosuppressive cell type that was significantly reduced by intensified treatment independent of its start in acute or chronic infection. However, more research will be needed. Recently, we gained evidence that MDSCs lose effector functions in advanced HIV disease as already shown for CD8 T cells (96).

4.2. Deutsche Zusammenfassung

Ein entscheidender Prozess in der fortschreitenden HIV-Infektion ist der Verlust der Effektivität der CD8 T-Zellantwort. Trotz intensiver Forschung in den letzten Jahren, sind die Ursachen dieser sogenannten Immunerschöpfung noch nicht vollständig geklärt. Dazu gehören u. a. inhibitorische T-Zellrezeptoren und immunsuppressive Zellen. Ziel dieser Arbeit war es, die Bedeutung von immunsuppressiven Zellen in der chronischen HIV-Infektion näher zu bestimmen.

In unserer ersten Veröffentlichung (94), wurden zwei Subtypen von MDSCs bei 42 Patienten mit chronischer viraler Infektion (n = 25) oder soliden Tumoren (n = 17) hinsichtlich ihrer zeitlichen Weiterverarbeitung 2, 4 und 6 Stunden nach Blutentnahme bzw. Inkubation über Nacht (ÜN) sowie der Einfrierbarkeit untersucht. Unsere Ergebnisse zeigten, dass die Frequenzen von PMN-MDSCs innerhalb von 6 Stunden ($p > 0,5$) nach Blutentnahme vergleichbar waren. Bei M-MDSCs dagegen waren die Frequenzen nach 6 Stunden bzw. ÜN signifikant erniedrigt (2 Stunden vs. 6 Stunden nach Blutentnahme bzw. ÜN: $p < 0,0051$). Bei beiden Zelltypen erniedrigten sich die Frequenzen der Zellen nach dem Einfrieren signifikant (PMN-MDSCs: $p = 0,0001$; M-MDSCs: $p = 0,04$). Deshalb empfehlen wir, MDSCs nur aus frischen PBMCs innerhalb von 4 bzw. 6 Stunden nach Blutentnahme zu analysieren.

In der zweiten Veröffentlichung (95), untersuchten wir die Frequenzen von immunsuppressiven Zellen (zwei Subtypen MDSCs, Tregs und Bregs) sowie

HIV-spezifische CD8 T-Zellantworten bei dem besonderen Patientenkollektiv der New Era Studie im Vergleich zu verschiedenen Kontrollgruppen. Die Therapieintensivierung hatte keinen Einfluss auf die Frequenzen von MDSCs und Bregs im Vergleich zu denen bei Patienten mit konventioneller 3fach-ART ($p > 0,21$). Dagegen waren Tregs bei den NE Patienten signifikant erniedrigt sowohl im Vergleich zu 3ART Patienten ($p < 0,0001$) als auch zu den weiteren HIV-infizierten Kontrollgruppen ($p < 0,0033$). Die CD8 T-Zellantwort der Patienten mit Therapieintensivierung war breiter ($p = 0,0134$) und stärker ($p = 0,026$) als bei 3ART Patienten. Dieser zweite Teil der Dissertation wurde in Zusammenarbeit mit einer medizinischen Doktorandin durchgeführt, die einen Teil der Arbeiten übernahm.

Trotz 30 Jahre intensiver HIV-Forschung wurde weder eine sterilisierende noch eine funktionelle Heilung in der HIV-Infektion erreicht. Zur Persistenz des HI Virus trägt, neben anderen Faktoren, die Immunerschöpfung entscheidend bei. Bei diesem Phänomen sind immunsuppressive Zellen ein Stück des Puzzles. Das Ziel dieser Dissertation war es, die Rolle der immunsuppressiven Zellen in der HIV-Infektion weiter aufzuklären mit dem letztendlichen Ziel einer therapeutischen Anwendung dieser Zellen. Wir zeigten, dass MDSCs aus frischem PBMCs analysiert werden sollen und dass Tregs die einzige immunsuppressive Zellsorte war, die durch eine intensivierte Therapie signifikant reduziert wurde unabhängig vom Therapiestart in der akuten oder chronischen Infektion. Jedoch sind weitere Studien zu diesen Zellen nötig, um deren Einsatz therapeutisch zu nutzen. So konnten wir kürzlich nachweisen, dass PMN-MDSCs ebenso wie CD8 T Zellen mit Fortschreiten der HIV-Infektion Effektorfunktionen verlieren (96).

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6. Published articles

6.1. Kinetics of human myeloid-derived suppressor cells after blood draw

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METHODOLOGY Open Access

Kinetics of human myeloid-derived suppressor cells after blood draw

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Abstract

Background: Human myeloid-derived suppressor cells (MDSC) have been described as a group of immature myeloid cells which exert immunosuppressive action by inhibiting function of T lymphocytes. While there is a huge scientific interest to study these cells in multiple human diseases, the methodological approach varies substantially between published studies. This is problematic as human MDSC seem to be a sensible cell type concerning not only cryopreservation but also time point after blood draw. To date data on delayed blood processing influencing cell numbers and phenotype is missing. We therefore evaluated the kinetics of granulocytic MDSC (gMDSC) and monocytic MDSC (mMDSC) frequencies after blood draw in order to determine the best time point for analysis of this recently defined cell type.

Methods: In this study, we isolated peripheral blood mononuclear cells (PBMC) of patients with HIV infection or solid tumors directly after blood draw. We then analyzed the frequencies of gMDSC and mMDSC 2, 4 and 6 h after blood draw and after an overnight rest by FACS analysis using the standard phenotypic markers. In addition, part of the cells was frozen directly after PBMC preparation and was measured after thawing.

Results: gMDSC levels showed no significant difference using fresh PBMC over time with a limitation for the overnight sample. However they were massively diminished after freezing ($p = 0.0001$ for all subjects). In contrast, frequencies of fresh mMDSC varied over time with no difference between time point 2 and 4 h but a significantly reduction after 6 h and overnight rest ($p = 0.0005$ and $p = 0.005$ respectively). Freezing of PBMC decreased the yield of mMDSC reaching statistical significance ($p = 0.04$). For both MDSC subgroups, FACS analysis became more difficult over time due to less sharp divisions between populations.

Conclusions: According to our data human MDSC need to be studied on fresh PBMC. gMDSC can be studied with delay, mMDSC however should be studied no later than 4 h after blood draw. These results are crucial as an increasing number of clinical trials aim at analyzing MDSC nowadays and the logistics of blood processing implies delayed sample processing in some cases.

Keywords: Myeloid-derived suppressor cells (MDSC), MDSC subsets, MDSC, Kinetics, Blood processing

Background

In the last decades newly described suppressors of the cellular immune system have become increasingly important: myeloid derived suppressor cells (MDSC). These cells are part of a group of immature cells with myeloid origin which act immunosuppressively by inhibiting function of T lymphocytes among others [1]. Firstly discovered in mice, MDSC have been shown in various studies in patients with solid tumors [2] like hepatocellular carcinoma (HCC) [3], non-small cell lung cancer (NSCLC) [4], head and neck cancer [4], gastrointestinal cancer [5] or melanoma [6, 7]. Recently MDSC were also found in peripheral blood of infectious diseases namely HIV [8] or tuberculosis [9]. All these studies on human MDSC vary substantially in their methodological

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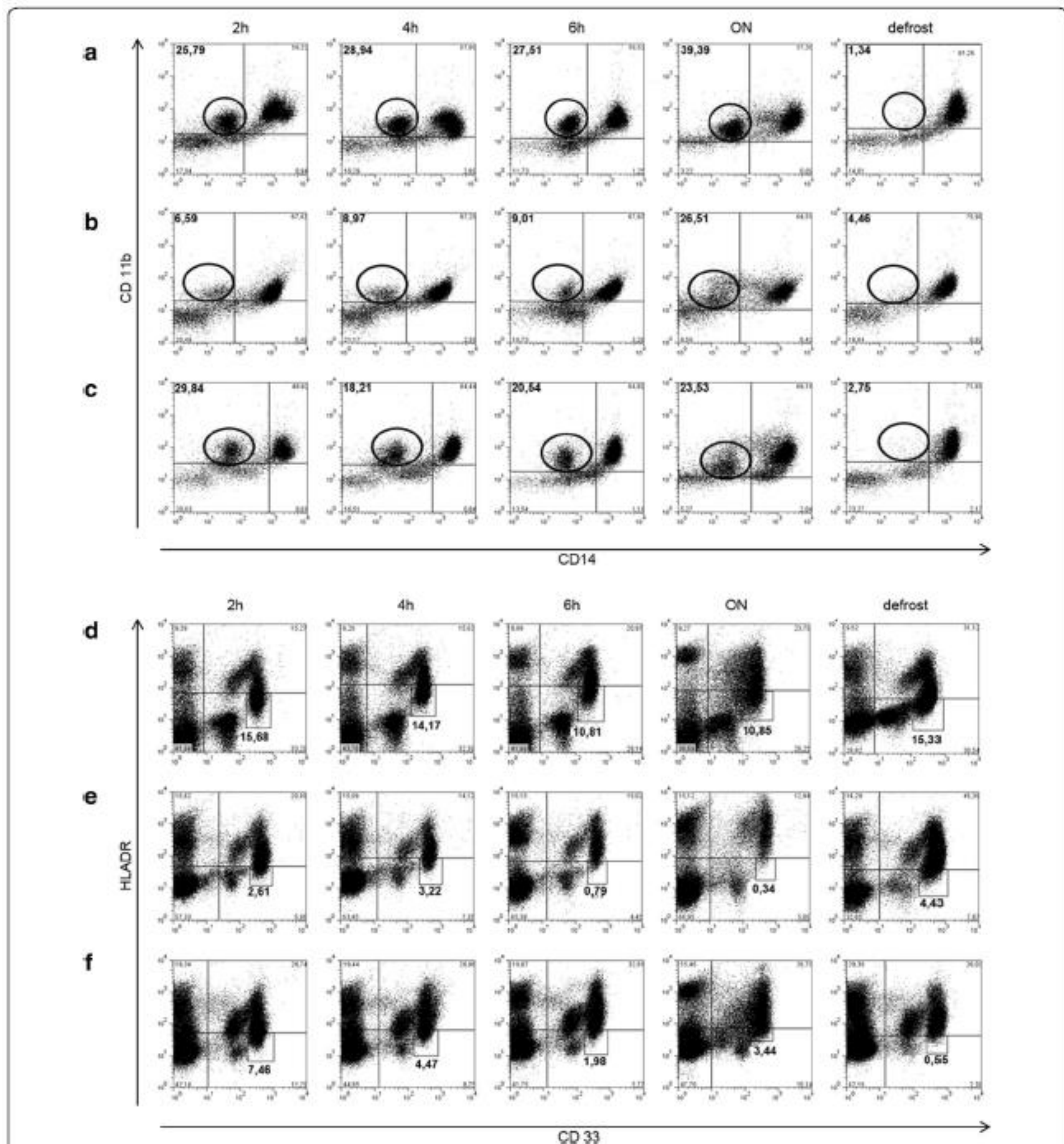

Fig. 2 Dot blot kinetics of MDSC. Representative dot plots of gMDSC (**a–c**) and mMDSC (**d–f**) after 2, 4, 6 h, overnight rest and freezing/thawing procedure of three HIV patients (gMDSC) and three patients with solid tumors (mMDSC). PBMC were stored in RPMI media after the ficoll separation gMDSC gated on CD11b$^+$ and CD14$^-$ population (*oval* gate); for CD15$^+$/CD66b$^+$ gating see Additional file 2: Figure S2; mMDSC gated on CD33$^+$ and HLA-DR$^{low/-}$ (*rectangle* gate); CD14$^+$ gating not shown. The majority of dot blots (**a**) of the CD11b$^+$/CD14$^-$ population remained similar after 2, 4 and 6 h. However, it was not clearly definable after ON and almost disappeared after the freezing/thawing procedure. Divergent minor presentations were seen and are presented in the remaining panels: increasing gMDSC frequencies after overnight rest and relatively high gMDSC levels after freezing/thawing (**b**) as well as reduction of gMDSC numbers at 4, 6 h and overnight rest compared to the 2 h time point (**c**). Looking at mMDSC (CD33$^+$ and HLADR$^{-/low}$ population), again the majority of dot blots presented as shown in 2d: similar numbers of mMDSC after 2 and 4 h and a clear reduction in mMDSC frequencies after 6 h. Populations are not well discriminable after overnight rest and after freezing/thawing. Minor divergent presentations are shown in the remaining panels: very high mMDSC frequencies after freezing/thawing (**e**) as well as increased frequencies after overnight rest and loss of mMDSC after freezing/thawing (**f**)

the bloodstream until they differentiate e.g. to macrophages in inflamed tissue. Therefore monocytes have to adhere and migrate through the vascular endothelium [26, 27]. In vitro the ability of monocytes to adhere to glass surfaces was first described in 1966 [28]. This adhesion was shown to be dependent on the surface material [29–32]. Interestingly, mMDSC frequencies were lowest at the 6 h time point and again higher after overnight rest which was not statistically significant. We cannot explain this phenomenon at the moment. We can only speculate that the monocytic cells release their attachment to the vial surface after some time.

Our analyses also showed that resting of cells increased the gating difficulties for mMDSC as well. The marker HLA-DR is difficult to gate in general and we used an isotype control to overcome this problem. Short term storage led to an additional blurring of the HLA-DR marker in the dot blots that was even challenging to overcome with the isotype control.

The data on freezing of gMDSC was impressive with an almost complete loss of the gMDSC population after thawing. This seems natural as gMDSC are closely related to granulocytes and granulocytes do not tolerate freezing [33–35]. It is in line with data by Trellakis et al. who showed a reduction of gMDSC frequencies of more than 50 % in all tested samples after freezing [16]. Another study on freezing/thawing of MDSC used a lineage cocktail rendering the comparison of their data with ours difficult. However the population closest to our gMDSC, namely HLA-$DR^{-}/LIN^{-}/CD15^{+}$, also showed a 69 % reduction after freezing [15]. There is even less data on freezing/thawing of mMDSC. In our hands, mMDSC were more resistant to the freezing procedure. However the difference between the 2 h time point and the frozen sample still reached statistical significance. Published data is conflicting in this point: while one study stated similar sensitivity towards freezing comparing mMDSC and gMDSC [16], two studies found no significant difference between the frequency of fresh and frozen cells using the markers HLA-$DR^{-}/CD14^{+}$ for definition of cells. Duffy et al. even showed increased frequencies within frozen PBMC [5]. However, Kotsakis et al. reported abolished function of MDSC after freezing [15]. Consequently, based on our research and the literature, we cannot recommend using frozen samples for gMDSC or mMDSC analyses. If mMDSC have to be analyzed in frozen PBMC, ficoll separation and freezing should be done directly after blood draw as suggested by the results of the kinetics of mMDSC. In view of the reality of translational research with the clinical parts of studies being often conducted at different sites than the laboratory evaluation—sometimes even in different countries—this is unfortunate. However the data clearly shows that freezing of MDSC has to be avoided in order to accurately describe the situation in vivo.

Conclusions

MDSC are a very sensitive type of cells of the immune system in terms of time point of analysis. Their precise role in cancer and infectious diseases still remains an urgent target for future investigations which should be facilitated by harmonizing the analytical process. Our data show differences in sensitivity of gMDSC and mMDSC and are crucial for defining the ideal time point for analysis. For frozen/thawed gMDSC and mMDSC, we support the results from previous studies to analyse fresh PBMC. Of particular importance is our finding determining the first 4 h after blood draw as ideal time point for analyses of mMDSC but allowing processing of gMDSC within the same day.

Additional files

Additional file 1: Figure S1. Gating strategies for gMDSC and mMDSC.

Additional file 2: Figure S2. Gating of $CD15^{+}/CD66b^{+}$ population.

Abbreviations

gMDSC: granulocytic myeloid-derived suppressor cells; HCC: hepatocellular carcinoma; MDSC: myeloid-derived suppressor cells; mMDSC: monocytic myeloid-derived suppressor cells; NSCLC: non small cell lung cancer; PBMC: peripheral blood mononuclear cells.

Authors' contributions

EG acquired data, performed research, analyzed data, performed statistical analysis and drafted the manuscript. RS and LA acquired data and performed research. APA, KS and CB identified eligible patients with solid tumors and provided samples. JRB interpreted data, wrote manuscript. RD designed research, analyzed and interpreted data, performed statistical analysis, wrote manuscript and revised final version of the manuscript. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

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6.2. Treatment Intensification in HIV-Infected Patients Is Associated With Reduced Frequencies of Regulatory T Cells

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Treatment Intensification in HIV-Infected Patients Is Associated With Reduced Frequencies of Regulatory T Cells

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In untreated HIV infection, the efficacy of T cell responses decreases over the disease course, resulting in disease progression. The reasons for this development are not completely understood. However, immunosuppressive cells are supposedly crucially involved. Treatment strategies to avoid the induction of these cells preserve immune functions and are therefore the object of intense research efforts. In this study, we assessed the effect of treatment intensification [=5-drug antiretroviral therapy (ART)] on the development of suppressive cell subsets. The New Era (NE) study recruited patients with primary HIV infection (PHI) or chronically HIV-infected patients with conventional ART (CHI) and applied an intensified 5-drug regimen containing maraviroc and raltegravir for several years. We compared the frequencies of the immune suppressive cells, namely, the myeloid-derived suppressor cells (MDSCs), regulatory B cells (Bregs), and regulatory T cells (Tregs), of the treatment intensification patients to the control groups, especially to the patients with conventional 3-drug ART, and analyzed the Gag/Nef-specific CD8 T cell responses. There were no differences between PHI and CHI in the NE population ($p > 0.11$) for any of the studied cell types. Polymorphonuclear myeloid-derived suppressor cell (PMN-MDSC), monocytic myeloid-derived suppressor cell (M-MDSC), and the Breg frequencies were comparable to those of patients with a 3-drug ART. However, the Treg levels were significantly lower in the NE patients than those in 3ART-treated individuals and other control groups ($p \leq 0.0033$). The Gag/Nef-specific CD8 T cell response was broader ($p = 0.0134$) with a higher magnitude ($p = 0.026$) in the NE population than that in the patients with conventional ART. However, we did not find a correlation between the frequency of the immune suppressive cells and the interferon-gamma+ CD8 T cell response. In the treatment intensification subjects, the frequencies of the immune suppressive cells were comparable or lower than those of the conventional ART-treated subjects, with surprisingly broad HIV-specific CD8 T cell responses, suggesting a preservation of immune function with the applied treatment regimen. Interestingly, these effects were seen in both treatment intensification subpopulations and were not attributed to the start of treatment in primary infection.

Keywords: HIV-1, treatment intensification, immune suppressive cells, myeloid-derived suppressor cells, regulatory T cells, regulatory B cells, CD8 T cell response, New Era study

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INTRODUCTION

Since 2010, the number of HIV-infected people with access to highly active antiretroviral therapy (ART) increased from 7.5 to 18.2 million in 2016. However, with 36 million people living globally with HIV, only half of them are treated despite intense efforts toward access to ART (1). Thus, there is still an urgent need for alternative treatment strategies with the aim of either eradicating the virus or achieving the so-called "functional cure," meaning a state of complete suppression of the virus in the absence of medication and a preservation of the immune response.

Conventional 3-drug ART suppresses viral replication and results in an undetectable viral load (VL) in plasma, but an eradication of the virus has not been achieved so far. Within the New Era (NE) study, treatment intensification by adding raltegravir and maraviroc to a 3-drug regimen has been investigated with the goal of eradication or at least a functional cure. The study recruited patients with primary HIV infection (PHI) or patients with chronic HIV infection (CHI). PHI was defined by less than two bands in a Western blot analysis. CHI patients were already treated successfully for at least 36 months with a protease inhibitor (PI) based conventional ART. All the patients had a CD4 nadir above 200 cells/μl and no evidence of resistance to the PI-based regimen. The aim of the study was to stop residual viremia, as well as to reduce and limit the viral reservoir measured by proviral DNA (2–4). Before a possible treatment interruption, correlates of immune control were assessed in all the patients. In earlier studies, a comparable 5-drug ART (also called treatment intensification) was tested with the result that there was no difference with regard to virus suppression or immune activation between the treatment intensification and conventional ART groups (5, 6). However, none of the previous studies evaluated the immune suppressive cells in their cohorts. Therefore, in this study, we hypothesized that an intensified treatment will have a beneficial effect on the development of immune suppressive cells in comparison to conventional 3-drug ART.

In early untreated HIV infection, a strong cytotoxic CD8 T lymphocyte response (CTL)—among others—effectively decreases VL (7–11). However, only <1% of the infected population, namely, elite controllers (EC), reaches complete viral suppression without ART (12–15). One reason for the viral persistence and eventual disease progression is the loss of CTL function over the disease course (e.g., production of cytokines/chemokines, degranulation) (16, 17). This phenomenon is termed immune exhaustion. In addition to immune checkpoints, e.g., PD-1 and CTLA-4, immune suppressive cells are said to be responsible for immune exhaustion. In HIV infection, well-known immune suppressive cells are regulatory T cells (Tregs). More recently, myeloid-derived suppressor cells (MDSCs) and regulatory B cells (Bregs) have been described to inhibit T lymphocyte function as well, leading to the loss of immune control in chronic viral infections.

In HIV infection, the role of Tregs is ambiguous and has not been evaluated conclusively (18–25). On the one hand, Tregs were found to reduce immune activation, which is a positive effect in uncontrolled HIV infection. On the other hand, these cells inhibit T cell responses, leading to viral persistence (18, 20, 23).

MDSCs are immature cells with a myeloid origin, which act immunosuppressively by inhibiting the functions of T lymphocytes, among others. MDSCs are divided into two major subsets, including polymorphonuclear (PMN) and monocytic (M) MDSCs (26, 27). In natural HIV infection, PMN-MDSC as well as M-MDSC frequencies correlate with the VL and are inversely related to the CD4 cell count. ART decreases PMN-MDSC levels within 6 weeks (28, 29). However, data on SIV-infected monkeys report a minor percentage of M-MDSCs within the complete MDSC population and higher levels of PMN-MDSCs during ART than that before infection (30).

Recently, studies by Siewe et al. described a direct correlation of Breg frequencies and CD8 T cell exhaustion in HIV-infected patients (31). Interleukin (IL)-10^{+} Breg frequencies are significantly elevated in viremic patients compared with HIV-uninfected individuals (32, 33). However, even ART-treated patients and EC still have elevated Breg levels when compared with uninfected controls (33).

With the NE study, we had the opportunity to evaluate the impact of treatment intensification on the described immunosuppressive cells in correlation with HIV-specific CD8 T cell responses. We hypothesized that these patients, especially the PHI group, have preserved immune functions and, therefore, low levels of Tregs, MDSCs, and Bregs in comparison to patients treated with conventional 3-drug ART.

MATERIALS AND METHODS

Study Subjects

A total of 116 individuals participated in the study after signing an informed consent form.

New Era: 24 patients within the scope of an amendment of the NE study [EudraCT Number 2008-002070-35, approved by the Bayerische Landesärztekammer (BLAEK) and the German federal institute for drugs and medical devices (BfARM)] and 92 individuals within 4 control groups (study approval by the Institutional Review Board of the Ludwig-Maximilians-Universität, Munich, Germany). The treatment intensification within the NE study consisted of two nucleoside reverse transcriptase inhibitors, a PI, and maraviroc and raltegravir in all cases. Patients with a virus with CXCR4 tropism were excluded from the study.

Primary HIV infection (=subgroup of NE patients with PHI, which was defined by less than two bands in a Western blot analysis; four patients were recruited in Fiebig stage 3 and six patients in Fiebig stage 4; $n = 10$).

Chronic HIV infection (=subgroup of NE patients with CHI; the patients were successfully treated with PI-based ART for at least 36 months; $n = 14$).

Abbreviations: 3ART, patients treated with a conventional 3-drug ART; Bregs, regulatory B cells; CHI, chronic HIV infection; CO, controllers; EC, elite controllers; HC, HIV-uninfected controls; M-MDSCs, monocytic myeloid-derived suppressor cells; NE, New Era; PHI, primary HIV infection; PMN-MDSCs, polymorphonuclear myeloid-derived suppressor cells; PR, progressors; Tregs, regulatory T cells.

At time of immunological analysis, the median duration of intensified treatment (=5 drugs) of all the NE patients was 5.5 years. Thus, CHI had a median ART duration of 11/9.5–16.5 years (median/range).

Control groups are defined as follows:

Progressors (PR) (untreated patients in CHI; $n = 27$): CD4 cell count below 400/μl, VL above 10,000 copies/ml.
Controllers (CO) (patients who control HIV infection spontaneously in the absence of ART for at least 3 years; $n = 21$): CD4 cell count above 500/μl and VL below 2,000 copies/ml (including EC: patients with VL below 40 copies/ml; $n = 8$).
3ART (patients treated with a conventional 3-drug ART for at least 4 years; $n = 21$): any CD4 cell count and VL below 50 copies/ml. The median duration of treatment: 10.5/4–18.5 years (median/range). One individual in this group was treated with maraviroc, but no one was treated with raltegravir. No threshold was set for the CD4 count, because this was not done in any of the treated groups.

HIV-uninfected controls (HC) ($n = 23$).

We included two exceptions of those definitions: PR 22 had a low CD4 cell count of 80 cells/μl and, therefore, we accepted a low-level viremia of 2,100 copies/ml. Whereas CO 13 was an EC (infected for 29 years) with a VL below 40 copies/ml and a CD4 count of 467 cells/μl in the absence of ART.

None of the ART-treated patients experienced treatment failure.

To further interpret our data, we stratified the cohort in the following subgroups:

All the patients on any treatment regimen were summarized in the subgroup with ART (w ART) and in analogous patients untreated in the subgroup without ART (w/o ART).

The clinical characteristics of the study subjects are shown in **Table 1**.

Peripheral Blood Mononuclear Cell (PBMC) Isolation

Peripheral blood mononuclear cells were isolated from freshly obtained EDTA blood by Ficoll density gradient centrifugation (Biocoll Separation Solution, Biochrom, Germany). The frequencies of the PMN-MDSCs and M-MDSCs (including isotype control) were determined in fresh PBMCs directly after blood draw (≤2 h after blood draw). For this reason, only individuals living in Munich were included in the MDSC studies. The frequencies of the Bregs and Tregs as well as the IL-10 production by the Bregs and the T cell immune responses by interferon-gamma enzyme-linked immunospot (Elispot) assay were determined using frozen PBMCs from all the study subjects.

Flow Cytometric Analysis

The extracellular staining was performed with fluorescent antibodies using fresh (PMN-MDSCs/M-MDSCs) or frozen (Tregs/Bregs) PBMCs as previously described (28). The following

TABLE 1 | Characteristics of the study subjects.

	NE ($n = 24$)	PHI ($n = 10$)	CHI ($n = 14$)	3ART ($n = 21$)	PR ($n = 27$)	CO ($n = 21$)	HC ($n = 23$)
Gender	m = 20/f = 4	m = 9/f = 1	m = 11/f = 3	m = 18/f = 3	m = 21/f = 6	m = 13/f = 8	m = 9/f = 14
Age at blood draw (years)	48 (24–62)	47.5 (24–56)	49.5 (36–62)	49 (30–71)	40 (20–60)	39 (23–66)	45 (25–62)
CD4 pre-ART (abs., %)	434 (212–751) 16.5% (7–37)	467 (339–751) 23.5% (7–37)	247 (212–489) 13.5% (8–23)	294 (12–492) 19% (1–35)	n.a.	n.a.	n.a.
CD4 at blood draw (abs., %)	713 (551–1,342) 37% (19–57)	892 (557–1,342) 42.5% (37–57)	676 (551–1,052) 31% (19–50)	604 (104–1,243) 31% (9–56)	136 (25–357) 11.5% (1–26)	706 (467–1,442) 35% (16–48)	n.a.
CD4 nadir (abs.)	360 (212–751)	467 (339–751)	237 (212–530)	235 (6–472)	143 (12–357)	534 (270–1,259)	n.a.
VL pre-ART (cp/ml)	299,230 (6,769–39,791,860)	1,410,751 (33,873–39,791,860)	173,642 (6,769–501,187)	40,749 (2,583–758,578)	n.a.	n.a.	n.a.
VL at blood draw (cp/ml)	<40 (<40–150)	<40 (<40–150)	<40 (<40)	<40 (<40)	123,818 (2,100–2,970,000)	103 (<40–1,214)	n.a.
Treatment length (years)	5.5 (4.5–6.5) (=only intensified ART)	6 (5–6.5)	5.5 (4.5–6.5) Total years of ART: 11 (9.5–16.5)	10.5 (4–18.5)	n.a.	n.a.	n.a.

NE, patients in the New Era study; PHI, subgroup of the NE patients with primary HIV infection (4 patients of Fiebig stage 3; 6 patients of Fiebig stage 4); CHI, subgroup of the NE patients with chronic HIV infection; HC, HIV-uninfected controls; CO, controllers; PR, progressors; 3ART, patients treated with a conventional 3-drug ART; m, male; f, female; n.a., not applicable; abs., absolute; ART, antiretroviral therapy.
The data are presented as the median and range.
For the subgroup PHI: the CD4 count pre-ART and CD4 nadir are the same, since there was only one blood draw after the diagnosis of HIV infection and before the start of ART.
For viral load: the limit of detection was 40 cp/ml in this study.
The CD4 counts between the NE and 3ART patients at blood draw were significantly different ($p = 0.044$; Mann–Whitney U test). However, there was no difference in the CD4 count at blood draw between the CHI and 3ART patients ($p = 0.288$, Mann–Whitney U test). Therefore, the difference was driven by the PHI patients who had higher CD4 counts.

antibodies were used: PMN-MDSCs: CD11b-FITC, CD14-APC, CD15-PerCP, and CD66b-PE; M-MDSCs: CD11b-FITC, CD14-APC, CD33-PE, HLA-DR-PerCP, and PerCP isotype control; Bregs: CD19-PerCP, CD24-APC, and CD38-FITC; and Tregs: CD4-APC and CD25-FITC (all BioLegend, USA). IL-10 production in the Bregs was determined with an intracellular cytokine staining protocol for IL-10-PE (BioLegend). The Tregs were stained intracellularly with anti-FoxP3-PE (BioLegend) using the FoxP3 staining buffer set (eBiosciences, USA) [as described in Ref. (21)]. The cells were analyzed on a FACSCalibur (BD, Germany), and the data analysis was done using FlowJo software 7.2.1 (TreeStar, Inc., Ashland, OR, USA). The gating strategies were according to Vollbrecht et al. (28) and Rieber et al. (34, 35) for PMN-MDSCs. For the gating of the M-MDSCs, we used a PerCP isotype control to gate for HLA-DR and our gating strategy was according to Dumitru et al. (36). Bregs were gated as $CD19^+$, $CD24^{hi}$, and $CD38^{hi}$ (31, 33), and the Tregs were $CD4^+$, $C25^+$, and $FoxP3^+$. For the gating strategies of all the cell types, see Figure S1 in Supplementary Material. For the statistics, we indicated the MDSCs, Bregs, and Tregs as the percentage of PBMCs in all the subgroups because the number of monocytes vary substantially in the subjects with chronic viral infections. Each patient was tested once for flow cytometric analysis.

Functional Analysis of the Bregs

The Bregs show an immunosuppressive function by the production of IL-10. For measuring IL-10 production, we stimulated the thawed PBMCs of five PR and five HC in two ways [according to Siewe et al. (31, 33)]. First, the cells (10^6 PBMCs/ml) were incubated for 48 h with 10 µg/ml CPG-B (oligodeoxynucleotide-2006), 2 µg/ml PAM (palmitoyl-3-cysteine-serine-lysine-4), and 2 µg/ml CD40L (all InvivoGen, San Diego, CA, USA) at 37°C and in 5.0% CO_2, and during the last 5 h, the cells were supplemented with 1 µg/ml ionomycin, 50 ng/ml phorbol-12-myristate-13-acetate (PMA), 50 µg/ml brefeldin A (all Sigma), and 1 µl/ml monensin (BD Golgi Stop™ BD Biosciences). Second, we only stimulated the cells with ionomycin/PMA. After 2 h of incubation at 37°C/5.0% CO_2, brefeldin A and monensin were added, and the cells were incubated for another 5 h. After the incubation, the cells were washed and stained intracellularly for IL-10. The background range was <0.02% for IL-10 production.

Peptides

Overlapping synthetic peptides corresponding to the HIV proteins Gag and Nef were used for screening (15–20 amino acids long, overlap of 5–10 amino acids; Gag: HIV-1 SF-2, Nef: HIV-1 Bru, NIBSC, England). The peptides had a purity of ≥70%.

Interferon-Gamma Elispot

HIV-specific CD8 T cell responses were quantified by an interferon-gamma Elispot assay using frozen PBMCs ($0.5–1 \times 10^5$/well) and peptides (final concentration: 12.5 µg/ml) as described previously (37, 38). Interferon-gamma producing cells were counted by direct visualization on an AID Elispot Reader (Autoimmun Diagnostika GmbH, Strassberg, Germany) and are expressed as spot-forming cells (SFC)/10^6 PBMCs. According to Addo et al., the wells were counted as positive if they had at least the total number of the three control values and were >50 SFC/10^6 PBMCs (39). The mean of the control values was subtracted of SFC/10^5. As the upper cutoff limit, 2,000 SFC/10^6 PBMCs was chosen. The breadth of the CD8 T cell responses was also determined according to Addo et al. (39): responses to two neighboring overlapping peptides were counted as one response toward one epitopic region, since some T cell epitopes are located in the overlap of the two peptides. In addition, for the total magnitude of the CD8 T cell response, we only considered the higher response of the two neighboring overlapping peptides. This conservative approach may potentially underestimate the real number or the real magnitude of the CD8 T cell responses.

Statistical Analysis

The statistical analyses were performed using GraphPad Prism version 5.0. In most cases, our data were not normally distributed. Therefore, we only used non-parametric tests. Comparisons between two groups were done with the Mann–Whitney *U* test, and comparisons between more than two groups were first tested with the Kruskal–Wallis test. If this was significant ($p < 0.05$), we also did pairwise Mann–Whitney *U* tests with Bonferroni Correction for multiple testing. Four pairwise comparisons were considered relevant and were tested for each experiment as follows: NE vs. 3ART; NE vs. HC; NE vs. PR; and NE vs. CO. The corrected level of significance, therefore, was $p < 0.0125$ when HC was included, and it was $p < 0.0167$ in the experiments without HC. Spearman rank test was used for correlation analyses and Wilcoxon signed rank test for paired comparisons (level of significance $p < 0.05$). Only tests with significant results are indicated in the figures.

RESULTS

Comparable PMN-MDSC Frequencies in NE (=Treatment Intensification) and 3ART Patients

To evaluate the impact of the intensified ART regimen in NE patients on the frequencies of PMN-MDSCs, the levels were compared with 3ART patients and to the HC, CO, and PR patients. We observed significantly lower PMN-MDSC frequencies in NE vs. PR patients ($p = 0.002$). However, there was no difference to the 3ART group ($p = 0.65$) (**Figure 1A**). The treatment intensification subgroups PHI and CHI had comparable PMN-MDSC frequencies ($p = 0.97$) (**Figure 1B**). We further stratified all the patients with any ART regimen (w ART: NE and 3ART) and patients without therapy (w/o ART). Both groups had significantly higher percentages of PMN-MDSCs vs. the HC group (w ART vs. HC: $p = 0.048$; w/o ART vs. HC: $p = 0.01$) (**Figure 1C**).

Thus, our analysis shows low PMN-MDSC frequencies in the treatment intensification-treated individuals, which were, however, comparable to the 3ART patients. In addition, all the

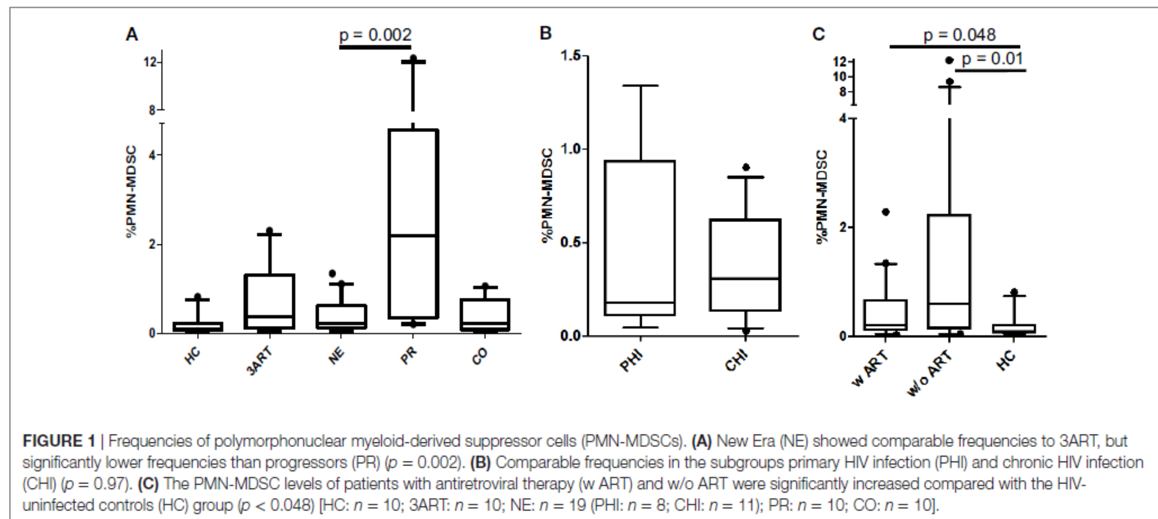

FIGURE 1 | Frequencies of polymorphonuclear myeloid-derived suppressor cells (PMN-MDSCs). **(A)** New Era (NE) showed comparable frequencies to 3ART, but significantly lower frequencies than progressors (PR) ($p = 0.002$). **(B)** Comparable frequencies in the subgroups primary HIV infection (PHI) and chronic HIV infection (CHI) ($p = 0.97$). **(C)** The PMN-MDSC levels of patients with antiretroviral therapy (w ART) and w/o ART were significantly increased compared with the HIV-uninfected controls (HC) group ($p < 0.048$) [HC: $n = 10$; 3ART: $n = 10$; NE: $n = 19$ (PHI: $n = 8$; CHI: $n = 11$); PR: $n = 10$; CO: $n = 10$].

ART-treated subjects had PMN-MDSC levels that did not reach the level of the HIV-uninfected controls.

Comparable M-MDSC Frequencies in All the HIV-Infected Groups

In HIV infection, M-MDSCs are suggested to play a role in T lymphocyte suppression (29, 40). Interestingly, in the NE patients, the frequencies of the M-MDSCs were significantly higher than the PMN-MDSCs ($p = 0.008$) (**Figure 2A**), whereas there was no significant difference between these cells in the PR patients ($p = 0.65$) (data not shown). In contrast to the PMN-MDSCs, the percentages of M-MDSCs in our cohort were significantly higher in the treatment intensification patients than those in the HC patients ($p < 0.0001$) but were comparable to those in the 3ART patients ($p = 0.21$) (**Figure 2B**). Again, within the NE groups, the PHI and CHI subgroups showed comparable values ($p = 0.2$) (**Figure 2C**). In accordance with these data, the analyses in patients with or without ART showed comparable frequencies, which were significantly higher than those in the HC group ($p < 0.002$) (**Figure 2D**).

Taken together, in our study, the M-MDSC levels in treatment intensification patients were not different than the 3ART group and were significantly higher than the PMN-MDSCs.

Low Breg Frequencies in the Treatment Intensification and the 3ART Groups

Since Bregs are a group of immunosuppressive cells only recently described in HIV infection, we evaluated them in our cohort (31–33). According to Siewe et al., we defined the Bregs as $CD19^{+}$, $CD24^{hi}$, and $CD38^{hi}$ and showed IL-10 production in these cells for a subset of the PR and CO patients. We found significantly increased IL-10 production in stimulated vs. unstimulated Bregs in all 10 samples tested independent of the stimulation type [for ionomycin $p = 0.002$, for toll-like receptor (TLR) agonists $p = 0.002$; Figure S2 in Supplementary Material]. Overall these results showed that, in our assays, phenotypically defined Bregs are capable of IL-10 production, and therefore, they are Bregs. As previously described, we observed a positive correlation of Breg frequencies with VL in viremic HIV-infected patients (rho = 0.46, $p = 0.009$) (**Figure 3D**).

The NE patients had comparable levels of Bregs compared with those of the 3ART patients and the HC group (**Figure 3A**). Their Breg frequencies were significantly lower than those of the untreated group of PR patients ($p = 0.0004$) (**Figure 3A**). The PHI and CHI subgroups showed no difference in Breg frequencies among each other ($p = 0.46$) (**Figure 3B**). Stratifying the patients in the w ART and w/o ART groups revealed that the w ART group showed significantly lower frequencies than those of the w/o ART group ($p = 0.002$), and these frequencies were comparable to those of the HC group ($p = 0.61$) (**Figure 3C**).

In conclusion, Breg levels in the NE patients were not different than those of the 3ART patients.

Significantly Lower Relative Treg Frequencies in the NE Patients Compared With 3ART Subjects

As a subset of CD4 T cells, Treg frequencies vary during the course of HIV infection, when CD4 T cells diminish with disease progression. Thus, the differentiation between the relative frequencies and the absolute numbers of Tregs is important to compare our results to those of other studies. In the NE group, the relative Treg levels were highly significantly lower compared with those of the 3ART patients ($p < 0.0001$) (**Figure 4A**). They were also lower than those in the PR and CO groups ($p < 0.0001$ and $p = 0.0033$, respectively), whereas the difference compared with

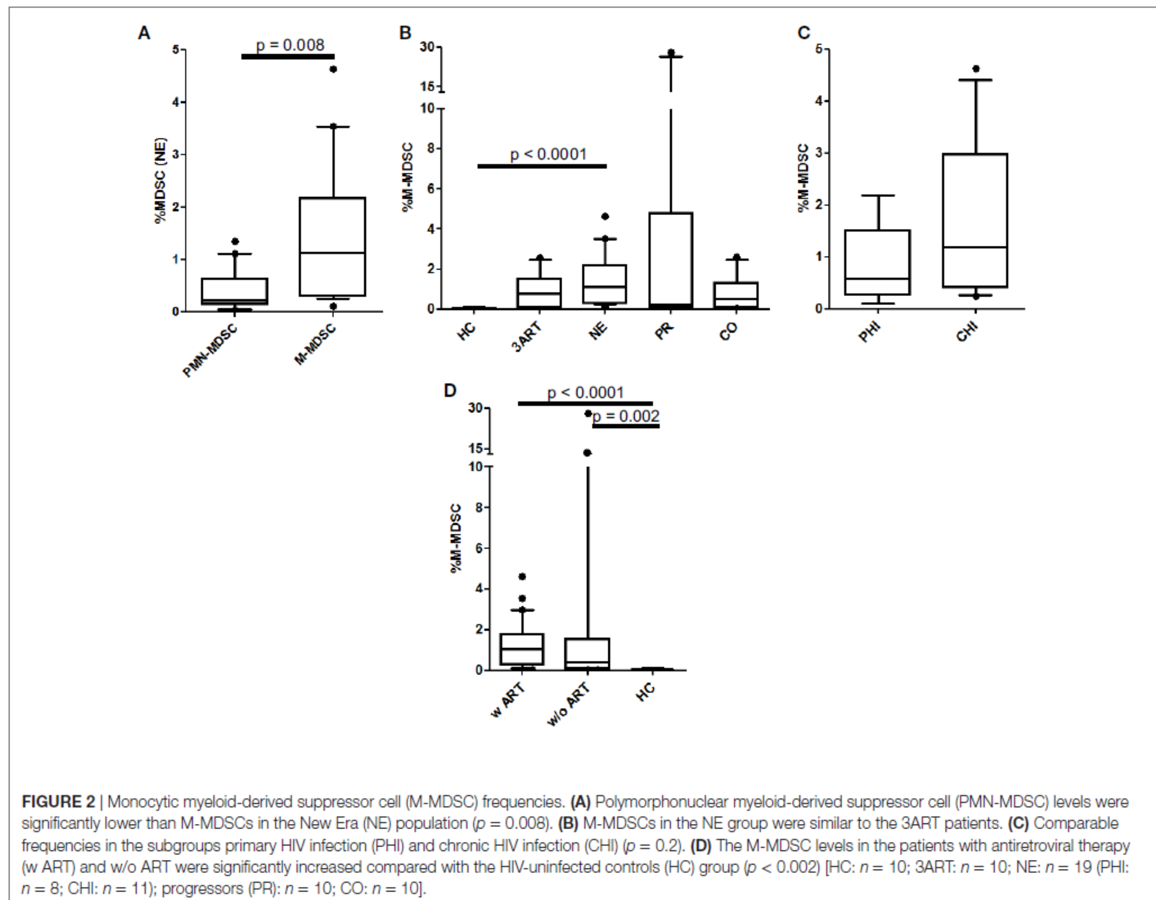

FIGURE 2 | Monocytic myeloid-derived suppressor cell (M-MDSC) frequencies. **(A)** Polymorphonuclear myeloid-derived suppressor cell (PMN-MDSC) levels were significantly lower than M-MDSCs in the New Era (NE) population ($p = 0.008$). **(B)** M-MDSCs in the NE group were similar to the 3ART patients. **(C)** Comparable frequencies in the subgroups primary HIV infection (PHI) and chronic HIV infection (CHI) ($p = 0.2$). **(D)** The M-MDSC levels in the patients with antiretroviral therapy (w ART) and w/o ART were significantly increased compared with the HIV-uninfected controls (HC) group ($p < 0.002$) [HC: $n = 10$; 3ART: $n = 10$; NE: $n = 19$ (PHI: $n = 8$; CHI: $n = 11$); progressors (PR): $n = 10$; CO: $n = 10$].

the HC group failed to reach statistical significance (**Figure 4A**). The PHI and CHI subgroups were again similar ($p = 0.11$) (**Figure 4B**). The entity of patients with ART had lower frequencies of relative Tregs than those of the control groups without ART ($p = 0.002$) (**Figure 4C**).

In absolute cell numbers, the NE subgroups and the total NE population had markedly higher Tregs than those of the PR group ($p = 0.0004$, PHI; $p = 0.02$, CHI; $p = 0.0006$, NE) (data not shown). A higher median CD4 cell count in the PHI subgroup than that in CHI (median = 875 cells/μl and median = 676 cells/μl, respectively, however, not statistically significant $p = 0.107$) along with the abovementioned comparable Treg frequencies resulted in higher absolute Treg cell counts in the PHI groups compared with those in the CHI group, which just missed statistical significance ($p = 0.08$) (**Figure 4D**). In addition, the NE patients showed a significantly strong correlation in the absolute numbers of Tregs with the CD4 cell counts (rho = 0.68, $p = 0.0003$) (**Figure 4E**).

Taken together, the relative Treg levels were significantly lower in the NE patients than those in the 3ART patients. For this cell type, treatment intensification seems to be advantageous compared with conventional ART.

No Correlation Between the Levels of Immune Suppressive Cells and the Gag/Nef-Specific CD8 T Cell Response in Treatment Intensification

Immune suppressive cells inhibit CD8 T cell responses. Therefore, we evaluated if there was a correlation between the HIV-specific CD8 T cell responses and the levels of immune suppressive cells studied here for the patients treated with a 5-drug ART. However, we were not able to demonstrate any correlation between the breadth or magnitude of interferon-gamma^{+} CD8 T cells toward Gag and Nef and the inhibitory cells, namely, the PMN-MDSCs, M-MDSCs, Bregs, and Tregs (data not shown).

A direct association between the levels of inhibitory immune cells and the interferon-gamma positive CD8 T cell response was not found in the treatment intensification.

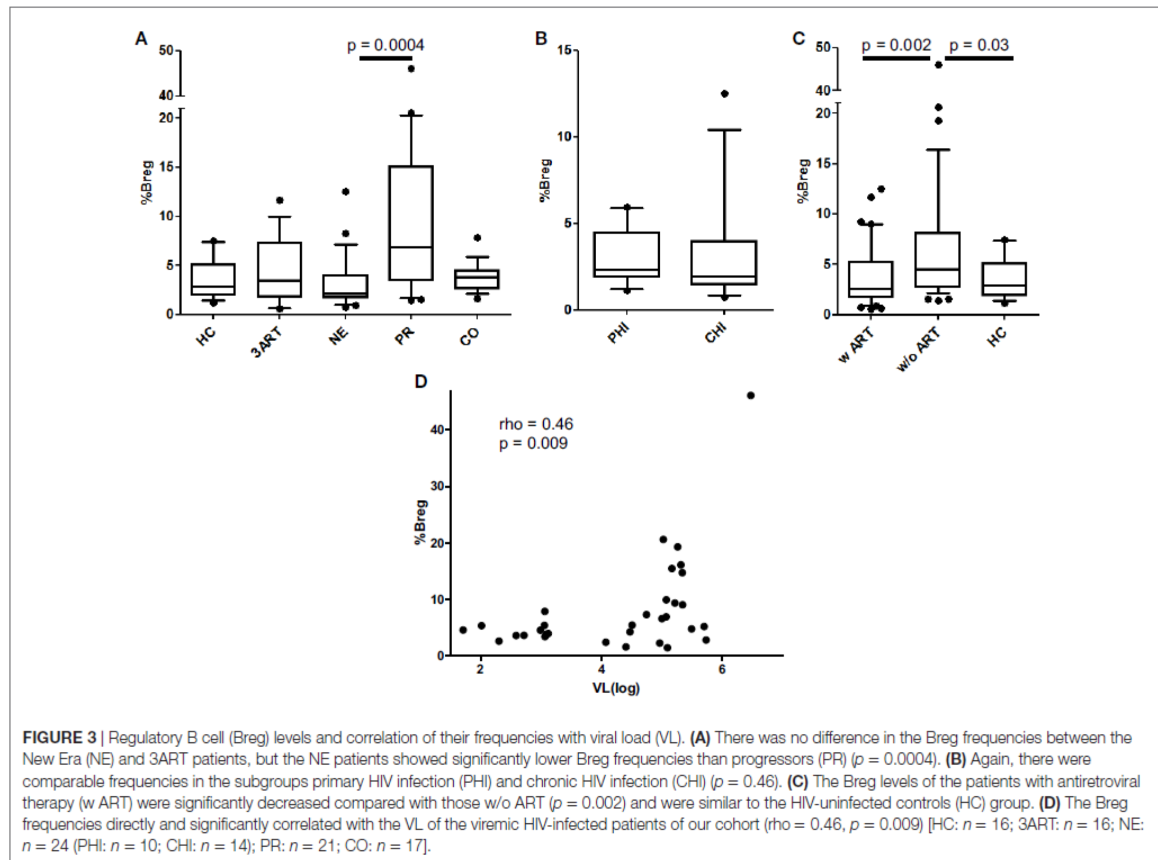

FIGURE 3 | Regulatory B cell (Breg) levels and correlation of their frequencies with viral load (VL). **(A)** There was no difference in the Breg frequencies between the New Era (NE) and 3ART patients, but the NE patients showed significantly lower Breg frequencies than progressors (PR) ($p = 0.0004$). **(B)** Again, there were comparable frequencies in the subgroups primary HIV infection (PHI) and chronic HIV infection (CHI) ($p = 0.46$). **(C)** The Breg levels of the patients with antiretroviral therapy (w ART) were significantly decreased compared with those w/o ART ($p = 0.002$) and were similar to the HIV-uninfected controls (HC) group. **(D)** The Breg frequencies directly and significantly correlated with the VL of the viremic HIV-infected patients of our cohort (rho = 0.46, $p = 0.009$) [HC: $n = 16$; 3ART: $n = 16$; NE: $n = 24$ (PHI: $n = 10$; CHI: $n = 14$); PR: $n = 21$; CO: $n = 17$].

Stronger Immune Responses Toward Gag p17 and Gag p15 in the NE Patients Compared With the 3ART Patients

Interestingly, the NE patients revealed a higher number of CD8 T cell responses in Gag and Nef than that of the 3ART patients ($p = 0.0134$) (**Figure 5A**). In contrast to the comparable frequencies of immunosuppressive cells between the PHI and CHI groups, the breadth of the CD8 T cell responses in the PHI subgroup (median 2, range 1–6) was significantly lower than that in the CHI subgroup (median 7, range 0–17; $p = 0.029$) (**Figure 5B**), showing that the difference between the NE and 3ART groups was driven by the CHI subgroup. This difference was especially interesting, as both groups—CHI and 3ART—were chronically HIV infected and had comparable times of treatment. In addition, the CD4 counts at blood draw as well as the CD4 counts before the start of ART were comparable in both groups ($p = 0.288$ and $p = 0.97$, respectively). This suggested that it was a very special group that was selected for the CHI group in the NE study.

The total magnitude of the immune responses toward Gag and Nef in the NE patients ranged from 0 to 8,030 SFC/10^6 PBMCs (median 700) and was therefore significantly higher compared with that of the 3ART patients ($p = 0.026$) (**Figure 5C**). In contrast to the results in the breadth of the responses, the NE subgroups PHI and CHI showed a comparable magnitude of CD8 T cell responses ($p = 0.28$) (**Figure 5D**). Thus, the fewer responses in the PHI groups generated stronger amplitudes.

Looking at the distribution of the CD8 T cell responses within the viral proteins, Gag p17 and Gag p15 were more often recognized by the CO group and, interestingly, also by the NE group than by the other groups. Studying this finding in detail showed that the CHI group recognized the Gag subunits p17 and p15, and the PHI group did not. Therefore, the CHI patients were similar to the CO patients in this respect. Gag p24 and Nef were the most frequently recognized protein subunit within all the groups as was to be expected (**Figure 5E**).

Overall, the CHI subgroup had a higher breadth of CD8 T cell responses compared with that of the 3ART patients and was comparable to the CO patients but did not lead to a higher magnitude of responses than that in the PHI group. There were no significant correlations between the CD8 T cell responses and frequencies of the immune suppressive cells.

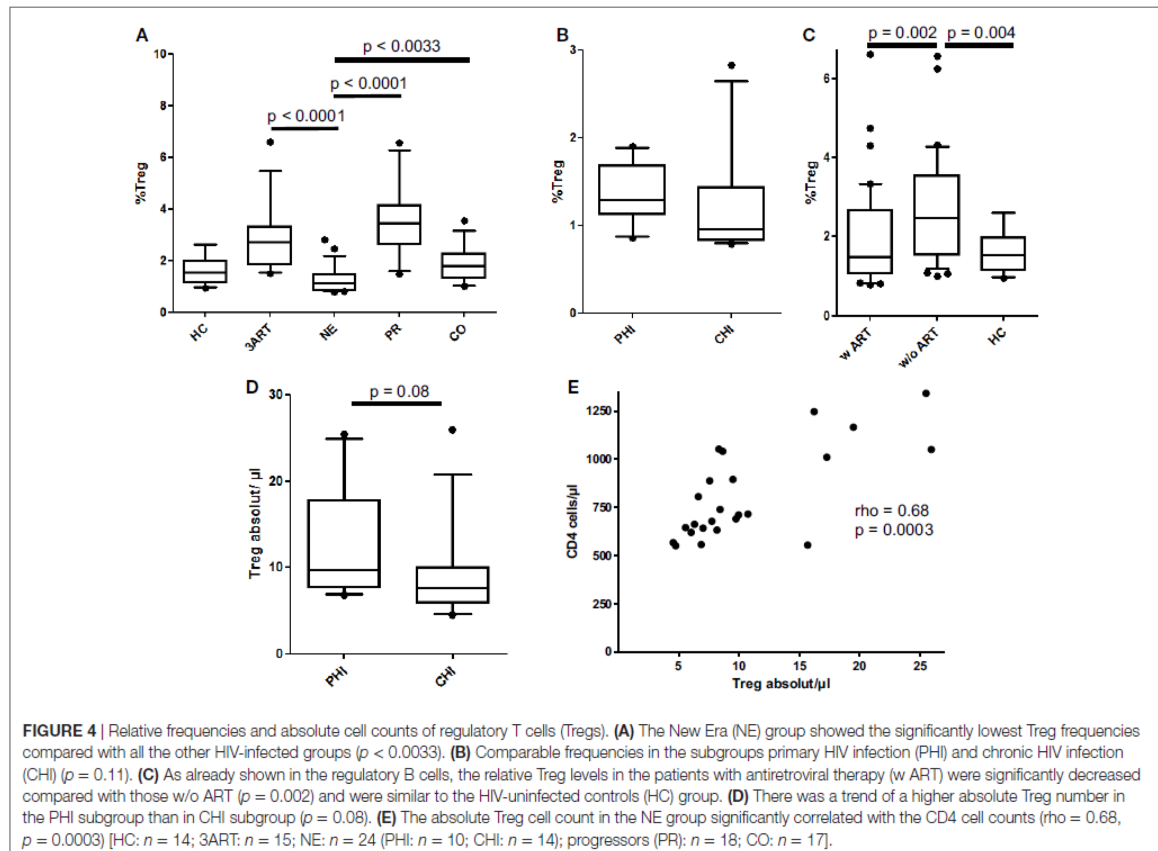

FIGURE 4 | Relative frequencies and absolute cell counts of regulatory T cells (Tregs). **(A)** The New Era (NE) group showed the significantly lowest Treg frequencies compared with all the other HIV-infected groups ($p < 0.0033$). **(B)** Comparable frequencies in the subgroups primary HIV infection (PHI) and chronic HIV infection (CHI) ($p = 0.11$). **(C)** As already shown in the regulatory B cells, the relative Treg levels in the patients with antiretroviral therapy (w ART) were significantly decreased compared with those w/o ART ($p = 0.002$) and were similar to the HIV-uninfected controls (HC) group. **(D)** There was a trend of a higher absolute Treg number in the PHI subgroup than in CHI subgroup ($p = 0.08$). **(E)** The absolute Treg cell count in the NE group significantly correlated with the CD4 cell counts (rho = 0.68, $p = 0.0003$) [HC: $n = 14$; 3ART: $n = 15$; NE: $n = 24$ (PHI: $n = 10$; CHI: $n = 14$); progressors (PR): $n = 18$; CO: $n = 17$].

DISCUSSION

The NE study evaluated an intensified antiretroviral treatment strategy with the primary goal of the eradication of HIV-1. We assessed the impact of this 5-drug treatment on several subsets of immune suppressive cells in the NE patients before a possible treatment interruption. Here, we showed that the levels of PMN-MDSCs, M-MDSCs, and Bregs were comparable to those of patients treated with a conventional 3-drug ART. However, the frequencies of the Tregs were significantly lower than those in the 3ART patients, indicating an advantage of the 5-drug regimen.

Interestingly, this effect was seen in both the NE subpopulations (PHI and CHI) and was not attributed to an early start of ART in primary infection. In addition, the NE population showed broader and stronger HIV-specific CD8 T cell responses, mainly driven by the CHI subgroup.

Myeloid-derived suppressor cell levels are significantly elevated in chronic progressive HIV infection. This association is shown for both subsets of MDSCs, namely, PMN-MDSCs (28, 41) and M-MDSCs (29, 42). In SIV infection, PMN-MDSCs increase substantially in acute infection, remain higher than before infection during antiretroviral treatment and rebound after treatment interruption (30). In addition, in human disease, MDSC frequencies decline after the start of ART (28, 29). MDSCs inhibit CD8 and CD4 T cell functions (proliferation, interferon-gamma production) and induce Treg levels (28, 29, 42), thereby contributing to the immune exhaustion in late stage disease. In our cohort, the intensified treatment regimen led to a near-to-normal level of PMN-MDSCs compared with that of the HC subjects, while it was significantly lower than in the PR patients, and there was no difference compared with that of the patients who were treated with a 3-drug ART. As a natural limitation, we do not have PMN-MDSC levels of the time before HIV infection and cannot therefore comment on this comparison. However, within the NE cohort, there are a few patients with elevated PMN-MDSCs to a level we usually do not find in healthy individuals. This might hint to the fact that, also in human disease, PMN-MDSC frequencies do not return to pre-infection levels, as shown in macaques (30)—not even with the start of an intensified treatment regimen in the very early phase of the infection.

The results for the M-MDSCs are not as straightforward. In our study, there was no difference for these cells in any of the HIV-infected groups (including PR), and all the HIV-infected patients had higher M-MDSC levels than those of the HC group. This is

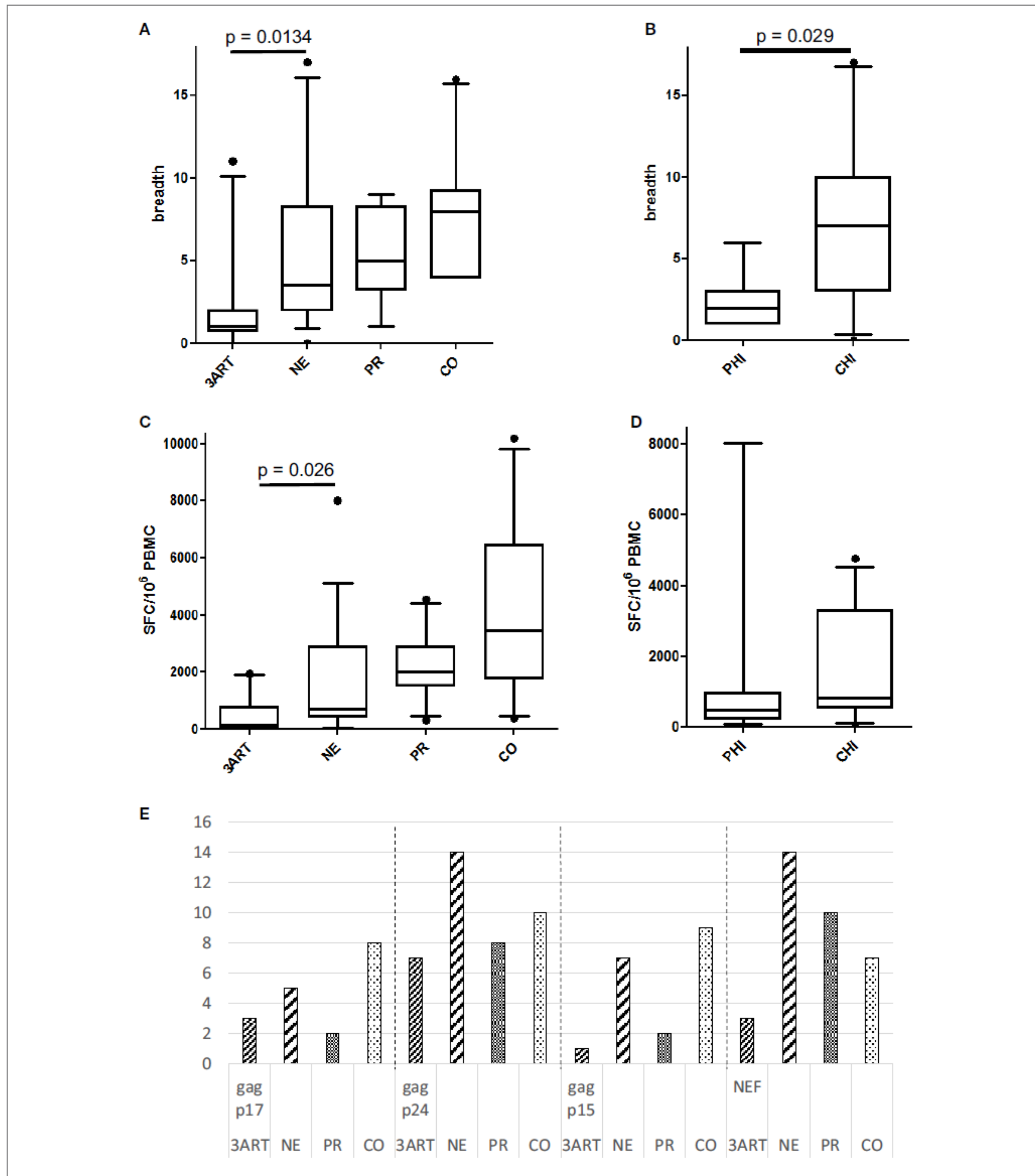

FIGURE 5 | (A) Breadth of immune responses in the New Era (NE) and control groups. The NE patients had significantly more CD8 T cell responses in Gag and Nef than the 3ART patients ($p = 0.0134$). **(B)** The chronic HIV infection (CHI) subgroup showed a significantly higher breadth of immune responses than the primary HIV infection (PHI) subgroup ($p = 0.029$). **(C)** The magnitude of the NE group was significantly higher than in the 3ART patients ($p = 0.026$). **(D)** There was no difference between the PHI and CHI subgroups, with regard to the magnitude of the CD8 T cell response ($p = 0.28$). **(E)** Gag p17 and Gag p15 were more often recognized by the NE (especially CHI) and CO groups than by the progressor (PR) and 3ART groups. Spot-forming cells (SFC)/10^6 peripheral blood mononuclear cells (PBMCs). For the magnitude of the CD8 T cell response, all the individual CD8 T cell responses within Gag and Nef were added for each patient [3ART: $n = 10$; NE: $n = 18$ (PHI: $n = 7$; CHI: $n = 11$); PR: $n = 10$; CO: $n = 10$].

in contrast to data published by others (29, 42). One cause for this difference might be environmental or genetic reasons, as these other studies were conducted in dissimilar circumstances. With regard to the data from this study, we cannot postulate a beneficial effect of the 5-drug regimen on M-MDSC levels.

Regulatory B cells were defined just recently as an immune inhibitory cell type in HIV infection and were therefore included in our analysis. They are best characterized by their capability to produce IL-10. Similar to a study by Siewe et al., we defined Bregs by phenotype ($CD19^{+}CD24^{hi}CD38^{hi}$) (31, 33). However, we studied IL-10 production in a subset of individuals to show that we were in fact dealing with Bregs. Liu et al. had a different approach and evaluated Breg frequencies solely based on IL-10 production (32). In chronic, untreated HIV infection, Breg levels are significantly higher than those in HC, correlate directly with VL and are inversely related with the CD4 count (31, 32, 43). In addition, Bregs inhibit T cell function (31, 32). Our data are consistent with these findings, as we found a significant correlation between the relative Bregs and VL in our viremic patients. The treatment intensification patients had significantly lower Breg frequencies than the CO and PR patients but levels similar to those of the HC group and 3ART-treated subjects. Breg frequencies were already elevated in early HIV infection and may contribute to establishing a viral set point, as well as to disease progression (32). Thus, with an early start of ART, keeping Breg frequencies in a normal range, especially for patients with acute infection, might be a benefit from this treatment strategy. However, since Breg levels between the NE and 3ART patients were comparable, it is debatable if treatment intensification represents an advantage over conventional ART.

In HIV infection, Tregs are the most described and well-known cells with immunosuppressive capabilities. The ambiguous role of Tregs is discussed in several publications (18, 21–23, 44). Cao et al. argued, in reliance to their results, that Tregs have a role rather in disease progression than in hindering immune activation. They described elevated relative frequencies with disease progression (44). Indeed, we observed similar results. The relative Treg frequencies in the HC and CO groups were comparable ($p = 0.34$) and were significantly lower than those in PR and 3ART patients ($p < 0.009$). Surprisingly, the lowest relative Treg frequencies were found in the NE patients with comparable levels in PHI and CHI. The difference between the NE patients and 3ART patients was especially remarkable ($p < 0.0001$), as the levels for those two groups were rather similar for all other cell types. Therefore, treatment intensification seems to have a beneficial effect for this specific cell type. Another reason for this finding could be the inclusion of maraviroc to intensify the treatment in the NE patients. Two studies analyzed the influence of maraviroc on Treg frequencies with contrary results (45, 46). While one study found a decrease in the relative Treg levels in maraviroc-treated individuals compared with maraviroc-free regimens (45), the other trial concluded that the reduction in viremia was the only reason for the Treg decline (46). While all the NE patients received maraviroc as part of their ART, there was only one individual in the 3ART group in our study with a maraviroc containing regimen. This patient had indeed a Treg frequency that was lower than the 3ART median and among the lowest values measured in this group. A third reason for the low Treg levels might be the intensified treatment itself, with lower levels of proviral DNA (especially in PHI) than usually measured in ART-treated patients. In the NE patients, we not only found low Treg frequencies but the absolute Treg numbers correlated well with the CD4 cell counts. This also reflected the healthy Treg compartment in this subgroup.

There was not a significant difference in any of the studied cell types between the PHI and CHI subgroups of the treatment intensification patients, which was surprising to us. We might have found a difference if our study groups had been larger as the highest values were always found in the group of CHI (with the exception of PMN-MDSCs). However, this finding, as it stands, questions the necessity for treatment start in acute HIV infection with regard to immune suppressive cells. On the other hand, the CHI patients in the NE study were chosen very selectively. The patients had to be virologically suppressed by a PI-based ART for at least 3 years and without a history of virological failure or blip (2, 3). It is possible that our findings emphasize this special selection, which is also reflected in the CD8 T cell response, and those patients would have had a benign disease course no matter which treatment.

In our study, we evaluated the CD8 T cell responses on an epitope level to assess possible correlations between the suppressive cells and the breadth of these responses. A limiting factor for this analysis is that the NE patients were treated for several years, and it is known that CD8 T cell responses decline both in breadth and magnitude after the start of ART with the disappearance of viremia (47). We restricted our analyses toward Gag and Nef, which are two of the most recognized HIV-1 proteins by CD8 T cells (39, 48, 49). Therefore, the results are limited to this part of the HIV genome. In our analyses, we did not find any correlations between the levels of the immune inhibitory cells and the CD8 T cell responses. A broad and strong immune response does not necessarily result in control of viral replication (39). In fact, EC control the HI virus below the detection limit but show less breadth and magnitude of immune response than PR (50) and preferentially target the HIV protein Gag (41, 51). In contrast to the similar frequencies of the analyzed cell subsets in the PHI and CHI subgroups, we found a significant difference between these groups in the breadth of the immune response. CHI had a broader CD8 T cell response despite the fact that PHI had higher CD4 counts than CHI. This outcome is in agreement with data from Altfeld et al. (52) and Addo et al. (39), showing that treatment in early infection results in an immune response that is narrower because the CD8 T cells were exposed to an HIV antigen for a shorter period of time. Consistently, all the HIV-1 proteins analyzed were less recognized in the PHI subgroup than in the CHI subgroup.

Gag p17 and p15 contain more variable regions and, therefore, are often less recognized in HIV-infected individuals (39, 49, 53). The CO group had more responses toward these protein subunits than the other groups. Most interestingly, we also found strong responses toward Gag p17 and p15 in the CHI group; these responses were higher than those in the PR and 3ART groups. Assuming that the CO subjects benefit from responses toward Gag p17 and p15, this again stresses the specially selected patients of the CHI group.

With this study, we comprehensively described several immunosuppressive cell subsets in a unique patient population. However, there were some limitations. First, the correlations might not have reached significance because of the small group sizes. The NE study was a multi-center study with a necessity for overnight shipment of samples for some centers. Because of the fragile nature of MDSCs (54–56), we were only able to include patients from Munich in the MDSC analyses. This did not apply to the same degree for the Bregs and Tregs. Therefore, the groups for those assays were slightly larger. Second, we determined the immunosuppressive cells in the peripheral blood. Tregs are found to be underrepresented in this compartment (21), because the cells traffic to the tissues and lymph nodes. To the best of our knowledge, there are no data on humans for the other cells, at present, about other compartments than blood. Thus, future studies might provide insights if these cells act directly on CD8 T cells in the lymphoid tissue.

Among the investigators of the study, it was highly discussed whether to stop treatment in this study population or not. In addition, if the treatment was stopped, should this be done with an additional intervention (e.g., vaccine or drug) or without. We think that it would be particularly interesting to stop treatment in the CHI group because, according to our results, the patients of this group seem very special as described earlier.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the Institutional Review Board of the Ludwig-Maximilians-Universität, Munich, Germany with written informed consent from all subjects. Samples of New Era patients were obtained 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)] with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the Institutional Review Board of the Ludwig-Maximilians-Universität, Munich, Germany.

AUTHOR CONTRIBUTIONS

EMG designed the research, acquired the data, performed the experiments, analyzed the data, performed the statistical analysis, and drafted the manuscript. TH acquired the data, performed the experiments, analyzed the data, and performed the statistical analysis. RS and AN acquired the data and performed the experiments. RP, AU, and JB provided samples and edited the manuscript. EW, EG, and HJ provided samples, contributed to the interpretation of the results, and edited the manuscript. JRB provided samples, interpreted the data, and wrote and edited the manuscript. RD designed and oversaw the research, analyzed and interpreted the data, performed the statistical analysis, wrote the manuscript, and revised the final version of the manuscript. All the authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fimmu.2018.00811/full#supplementary-material.

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