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Immune recovery in HIV-infected patients after *Candida* esophagitis is impaired despite long-term antiretroviral therapy

Running Title: Long-term defects after *Candida* esophagitis

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Abstract

Objective. *Candida* esophagitis belongs to the most common AIDS-defining diseases, however, a comprehensive immune pathogenic concept is lacking.

Design. We investigated the immune status of 37 HIV-1-infected patients from the Swiss HIV cohort study at diagnosis of *Candida* esophagitis, 1 year before, 1 year later and after 2 years of suppressed HIV RNA. We compared these patients to 3 groups: 37 HIV-1-infected patients without *Candida* esophagitis but similar CD4 counts as the patients at diagnosis (advanced HIV group), 15 HIV-1-infected patients with CD4 counts >500 cells/µl, CD4 nadir >350 cells/µl and suppressed HIV RNA under combination antiretroviral therapy (cART) (early cART group), and 20 healthy individuals.

Methods. We investigated phenotype, cytokine production and proliferative capacity of different immune cells by flow cytometry and ELISpot.

Results. We found that patients with *Candida* esophagitis had nearly abolished CD4 proliferation in response to *C. albicans*, significantly increased percentages of dysfunctional CD4 cells, significantly decreased cytotoxic NK-cell counts and peripheral innate lymphoid cells and significantly reduced IFN-γ and IL-17 production compared to the early cART group and healthy individuals. Most of these defects remained for more than 2 years despite viral suppression. The advanced HIV group without opportunistic infection showed partly improved immune recovery.

Conclusions. Our data indicate that *Candida* esophagitis in HIV-1-infected patients is caused by an accumulation of multiple, partly *Candida*-specific immunological defects. Long-term immune recovery is impaired, illustrating that specific immunological gaps persist despite cART. These data also support the rationale for early cART initiation to prevent irreversible immune defects.

Keywords. *Candida* esophagitis; HIV ; IL-17 response ; proliferative impairment ; long-term immune recovery ; early cART

Introduction

The risk of opportunistic infections (OI) in patients with human immunodeficiency virus (HIV) infection has markedly declined since 1996 because of the widespread use of combination antiretroviral therapy (cART) [1]. Nevertheless, OIs still remain a leading complication with an incidence of 16% in late presenting patients [2]. Absolute CD4 counts <200 cells/µl and uncontrolled HIV RNA replication are well-described major risk factors for the development of OI, yet they also occur in patients with CD4 counts >200 cells/µl with an incidence of 10.5 per 1000 patient years follow-up, highlighting that apart from the absolute CD4 counts additional risk factors for OI must be present [3]. This is further supported by recent studies documenting that early initiation of cART at CD4 >500 cells/µl is beneficial as it significantly reduces the risk for OI and malignancies [4, 5]. However OIs are not completely eliminated. However, why certain HIV-infected patients are susceptible to specific OIs and how the infection influences long-term immune recovery has only been scarcely investigated.

Candida esophagitis is one of the most common AIDS-defining diseases, occurring in up to 10-15% of HIV-infected patients before introduction of cART [1, 6, 7]. Importantly, *Candida* esophagitis is often the first opportunistic infection and also develops in patients with rather high CD4 counts suggesting that the functionality of immune responses is diminished [7].

Earlier studies considered that susceptibility to *Candida* esophagitis is enhanced by a lack of protective Th1 responses and/or a shift to Th2 responses [8]. However, recent studies show that individuals with impaired IL-17 responses exhibit enhanced susceptibility to chronic mucocutaneous candidiasis [9]. In the context of HIV, progressive infection is accompanied by continuous loss of Th17 cells [10] and a decrease in the ratio of Th17 to Th1 cells in peripheral blood [11]. Recently, it has been demonstrated in a mouse model of oropharyngeal candidiasis

that IL-17 secreting RORγt^+ type 3 innate lymphoid cells (ILC) also contribute to fungal clearance [12]. Moreover, natural killer (NK) cells are increasingly considered as part of the host defense against fungi [13] and their function was shown to be impaired against *Cryptococcus neoformans* in HIV-infected patients [14].

In this study, we took the advantage of prospectively stored patient samples within the Swiss HIV Cohort Study and investigated the numbers and functions of different immune cell subsets in patients with *Candida* esophagitis over a longitudinal follow-up, including samples before disease development and after long-term suppression of HIV RNA and compared them to 3 groups of individuals, including HIV-infected patients with similarly advanced HIV infection without OI, HIV-infected patients that initiated cART at CD4 nadirs >350 cells/µl and were HIV RNA suppressed, and healthy individuals.

Methods

Patients and healthy blood donors

The Swiss HIV Cohort Study (SHCS) is a large prospective observational cohort study with continuous enrolment of adult HIV-infected individuals initiated in 1988 and approved by the local institutional review boards [15]. Basic socio-demographic characteristics, data on clinical course, antiretroviral therapy, immunologic and virologic parameters are collected at enrolment and every 6 months thereafter. Viable PBMC and plasma are stored every 6-12 months. Ethical approval and written informed consent from all patients enrolled in the SHCS have been obtained.

The diagnosis *Candida* esophagitis was based on clinical findings defined according to CDC criteria [16]. From January 2000 until December 2013, 465 HIV-1 infected patients were diagnosed with *Candida* esophagitis. Of these 277 patients had *Candida* esophagitis as first and only AIDS-defining disease. Of these 37 patients with available longitudinal peripheral blood

mononuclear cells (PBMC) were included. We analysed cryopreserved PBMC from three time points: 6-18 months before diagnosis, at diagnosis (+/-6 months) and 6-18 months after diagnosis. For patients with suppressed HIV RNA (<50 copies/ml) over 2 years an additional time point was included. These patients were compared to 3 groups. (i) HIV-1-infected patients with similarly advanced disease but without OI. Patients were matched to *Candida* esophagitis patients according to CD4 counts (+/-25 cells/µl), date of diagnosis of *Candida* esophagitis, use of cART, gender, age and absence of other OI within 6 months prior to sample collection [17]. As for the *Candida* esophagitis patients, four time points were analysed. (ii) 15 SHCS patients with well-controlled HIV-1 infection from outpatients of the HIV clinic at the University Hospital of Basel. Patients had the following criteria: HIV CDC A1 or A2 classification with suppressed HIV RNA (<50 copies/ml) and stable cART therapy for at least 6 months, CD4 counts >500 cells/µl and CD4 nadir >350 cells/µl. (iii) 20 healthy individuals after receipt of informed consent according to the ethic approval from the Ethikkommission Nordwest- und Zentralschweiz (EKBB 242/11). For both latter groups only one time point was analysed. Baseline characteristics of patients and healthy individuals are included in Table 1.

Generation of heat-inactivated *C. albicans*

A mixture of *C. albicans* strain SC5314 yeast and hyphae was cultured and heat-inactivated as previously described [18, 19].

Phenotypic characterization

T-cell activation/exhaustion was analysed by staining with anti-CD3-PerCP, anti-CD4-PacificBlue, anti-CD8-APC, anti-CD25-PE/Cy7 and anti-CD279-PE (PD-1); innate lymphoid cells (ILCs) by staining with anti-Lineage-Cocktail-APC (anti-CD3/CD14/CD16/CD19/CD20/CD56) and anti-CD127-PE; NK-cell subsets by staining with

anti-CD3-PerCP, anti-CD14-FITC, anti-CD19-PE/Cy7, anti-CD56-APC/Cy7, anti-CD16-PacificBlue (all Biolegend), anti-NKG2A-APC (clone #131411) and anti-NKG2C-PE (both R&D Systems) [20]. Samples were acquired on a CyAn ADP Analyzer (Beckman Coulter) and data analyzed with FlowJo software vX.0.7.

ELISPOT assay

Interferon-gamma (IFN-γ) and interleukin (IL)-17 enzyme-linked immunosorbent spot (ELISPOT, Mabtech) were performed according to manufacturer's instructions as previously published [21]. Briefly, 3-5x10^5 cells/well were stimulated in duplicates with *C. albicans* (MOI 0.05), 0.05 µg/ml Cytomegalovirus (CMV) pp65 (JPT Peptide Technologies) or 0.5 µg/ml staphylococcal enterotoxin B (SEB; Sigma-Aldrich) for 72 hours. The number of spot forming counts (SFC) per well was counted by ELISPOT reader (Cellular Technologies Ltd.). Data are shown after subtraction of unstimulated controls.

Proliferation assay

PBMC were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE, Invitrogen) according to manufacturer's instructions and stimulated with *C. albicans* (MOI 0.05) or 0.5 µg/ml SEB for 7 days in RPMI 1640 (Gibco) with 5% pooled human serum. Medium was replenished as needed. Cells were stained with anti-CD3-PerCP, anti-CD4-PacificBlue, anti-CD8-APC and anti-CD56-APC/Cy7 (all Biolegend) and acquired on a CyAn ADP Analyzer (Beckman Coulter) and data analyzed with FlowJo software vX.0.7.

Statistical analysis

Comparisons between 2 groups were performed with the 2-sided Mann-Whitney U test. $P \leq .05$ was considered statistically significant. Statistical analyses were done using GraphPad Prism 6.0f and Stata 13.1 software (StataCorp LP). Shown are median values + interquartile ranges (Tukey plots).

Results

Patients with *Candida* esophagitis have low and dysfunctional CD4 cells and decreased *Candida*-specific cytokine responses and proliferative capacity

We first analysed T-cell phenotype and function of patients diagnosed with *Candida* esophagitis and compared them to HIV-1-infected individuals with early initiation of cART and sustained viral suppression (<50 copies/ml) and healthy individuals (for baseline characteristics of patients and healthy individuals see Table 1).

As expected from earlier studies, absolute CD4 counts were significantly lower in *Candida* esophagitis patients compared to patients with early initiated cART and healthy individuals (Fig. 1a) and the frequencies of exhausted PD-1^+ and activated/regulatory $CD25^+$ CD4 cells were significantly increased compared to healthy individuals (Fig. 1b). The median percentage of $CD25^+$ CD4 cells was 6-fold higher in patients with *Candida* esophagitis compared to healthy individuals. In accordance with the significantly reduced number of functional CD4 cells, the IFN-γ and IL-17 responses of PBMC to the superantigen SEB or *C. albicans* were significantly lower in *Candida* esophagitis patients compared to patients with early initiated cART and healthy individuals. The IFN-γ response to CMV pp65 in CMV seropositive donors was not affected, showing that viral reactivation was still able to trigger functional immune responses independent of the CD4 cell count (Fig. 1c,d).

In line, the proliferative capacity of CD4 and CD8 cells to *C. albicans* was significantly lower in patients with *Candida* esophagitis compared to healthy individuals (Fig. 1e,f). The median percentage of proliferating CD4 cells was 7-fold and of proliferating CD8 cells 12-fold decreased compared to healthy individuals. Interestingly, CD4 proliferation to SEB was comparable to healthy individuals. To examine whether the inability to proliferate was due to a lack of IL-2 production we supplemented some cultures with 50 U/ml recombinant IL-2 on day 2. However, T-cell proliferation was not improved (Supplemental Fig. 1, http://links.lww.com/QAD/A920).

Thus, development of *Candida* esophagitis was associated with reduced and dysfunctional CD4 cells that showed significant impairments in cytokine production and proliferation to specific antigens.

Patients with *Candida* esophagitis have decreased peripheral NK cells and innate lymphoid cells (ILC)

NK cells and ILC are increasingly considered as part of the host defense against fungi. We therefore investigated whether these cells and their functionality are also impaired in patients with *Candida* esophagitis.

Absolute NK-cell counts ($CD3^-CD56^+$) and especially the cytotoxic $CD16^+$ NK-cell subset were significantly lower in patients with *Candida* esophagitis compared to patients with early initiated cART and healthy individuals (Fig. 2a). The absolute number of $CD16^+$ NK cells was with a median of 42 cells/µl nearly 5-fold lower than in healthy controls (201 cells/µl). The percentages of NK cells expressing the inhibitory receptor NKG2A was higher in cases with *Candida* esophagitis than in the other groups, while the percentage of cells expressing the activating receptor NKG2C was higher in *Candida* esophagitis cases and in HIV-1-infected virologically suppressed patients than in healthy individuals (Fig. 2b). The proliferative capacity of NK cells to *C. albicans* was not significantly affected (Fig. 2c).

Similar to NK cells, also peripheral ILC counts (lineage$^-$CD127$^+$) were significantly reduced in patients with *Candida* esophagitis compared to patients with early initiated cART and healthy individuals (Fig. 2d).

In conclusion, additionally to defective CD4 cell responses, patients with *Candida* esophagitis had significantly reduced numbers of NK cells and ILC.

Despite higher CD4 counts at diagnosis, the proliferative responses to *C. albicans* and NK-cell counts and function are impaired

Next, we examined whether higher CD4 counts at diagnosis of *Candida* esophagitis are associated with better functionality of the different cell subsets (Fig. 3).

Patients with CD4 counts >350 cells/µl (18 of 37 patients) showed decreased percentages of dysfunctional PD-1$^+$ and CD25$^+$ CD4 cells and increased cytokine responses to SEB and *C. albicans* compared to patients with CD4 counts <200 cells/µl (12 of 37 patients; Fig. 3a-c; supplemental Table 1). By contrast, CMV-specific responses were comparable regardless of the absolute CD4 counts. The proliferative response to *C. albicans* was reduced even in patients with CD4 counts >350 cells/µl (Fig. 3d).

Although NK-cell counts and the percentage of cytotoxic CD16$^+$ NK cells increased with higher CD4 counts, the absolute numbers of cytotoxic NK cells in patients with CD4 counts >350 cells/µl still remained more than 4-fold reduced compared to healthy individuals (Fig. 3e). The number of ILC in peripheral blood also significantly increased with higher CD4 counts (Fig. 3f).

These data highlight, that CD4 phenotype and cytokine production and ILC counts normalized with increasing CD4 counts whereas the proliferative capacity of CD4 cells specifically to *C. albicans* and the number of cytotoxic NK cells were strongly impaired in all patients with *Candida* esophagitis irrespective of the CD4 count.

Patients with *Candida* esophagitis show a significant drop in CD4 counts and nearly abolished T-cell proliferation to *C. albicans* at diagnosis and retain immunological impairments even after disease resolution and successful cART

We further analysed the immune status of patients with *Candida* esophagitis prior to disease development to identify immunological changes associated with disease development and after disease resolution and successful cART to identify possible long-term defects. We therefore additionally examined PBMC 6-18 months before diagnosis, after disease resolution (6-18 months after diagnosis) and after successful cART with stably suppressed HIV RNA (<50 copies/ml) for more than 2 years (Fig. 4).

Interestingly, patients with *Candida* esophagitis showed a significant drop in absolute CD4 counts at diagnosis and nearly abolished proliferation of CD4 and CD8 cells in response to stimulation with *C. albicans*. Proliferation to SEB was not significantly affected. Also NK-cell proliferation in response to *C. albicans* dropped during development of *Candida* esophagitis.

In line with previous studies, *Candida* esophagitis patients with low CD4 nadirs (<320 cells/µl, median 87 cells/µl) restored CD4 counts after viral suppression to a lower level compared to patients with early cART and higher CD4 nadirs (>350 cells/µl, median 397 cells/µl) (Fig. 4a). The percentage of $CD25^+$ CD4 cells and the IFN-γ responses to SEB and *C. albicans* normalized after suppression of viral replication, whereas the IL-17 response remained impaired despite long-term viral suppression under cART (Fig. 4b-d). The proliferative capacity of CD4 cells to *C. albicans* after suppression of HIV replication recovered but remained in median lower than in patients with early initiated cART and healthy individuals (Fig. 4e,f).

Median NK-cell counts remained below 200 cells/µl despite viral suppression. Although the percentage of $CD16^+$ cells increased, the absolute number of cytotoxic $CD16^+$ NK cells remained more than 3.5-fold lower compared to healthy individuals (Fig. 4g). By contrast, the percentages of $NKG2A^+$ and $NKG2C^+$ NK cells and the proliferative responses were

comparable to healthy controls (Fig. 4h,i). Similarly to NK cells, also ILC counts remained 2.5-fold reduced for at least 2 years after viral suppression (Fig. 4j). NK-cell and ILC reconstitution seemed to correlate directly with CD4 reconstitution (Supplemental Fig. 2, http://links.lww.com/QAD/A920).

These data show that development of *Candida* esophagitis is associated with a drop in absolute CD4 counts and proliferative capacity of *Candida*-specific T cells and that despite successful cART, patients with previous *Candida* esophagitis have prolonged immune defects of CD4 cells, NK cells and ILC.

Patients with similarly advanced HIV infection but without an opportunistic infection show overall better immune recovery after successful cART

The last comparator group consisted of patients with similarly advanced HIV infection but without OI. These patients were matched to *Candida* esophagitis patients according to CD4 counts, use of cART, gender, age and absence of other OI within 6 months prior to sample collection. Also the viral load was comparable between these two patient groups. Notably, patients with similarly advanced HIV infection but without OI showed overall very similar immune cell impairments and reconstitution as patients with *Candida* esophagitis (Supplemental Fig. 3, http://links.lww.com/QAD/A920). However, they differed by a two-fold lower percentage of $CD25^+$ CD4 cells prior to disease development and consistently higher CD4 proliferation to *C. albicans*. Additionally, long-term recovery of the IL-17 response, total NK cells, $CD16^+$ cytotoxic NK cells and ILCs was better in these patients without *Candida* esophagitis.

Discussion

The detailed pathogenesis of OIs is still unknown for many pathogens. *Candida* esophagitis is one of the most frequent opportunistic diseases in untreated HIV-infected individuals but also occurs in patients with other underlying conditions.

In this study investigating in depth 37 HIV-1-infected *Candida* esophagitis patients compared to advanced HIV-1-infected patients without OI, HIV-1-infected patients with early initiation of cART and healthy individuals we found that patients with *Candida* esophagitis showed i) a significant drop in CD4 counts at diagnosis, ii) a nearly abolished proliferative capacity to *C. albicans*, iii) an impaired IFN-γ and IL-17 production to *C. albicans* and iv) a dysfunction of CD4 cells with increased percentages of $CD25^+$ and $PD\text{-}1^+$ cells. Additionally, these patients had significantly decreased peripheral ILCs and cytotoxic NK-cell counts. Recovery of the proliferative capacity of CD4 cells and IL-17 production to *C. albicans* and of ILCs and cytotoxic NK cells was impaired for years despite effective cART.

HIV infection is commonly associated with an inability to proliferate and produce IL-2. Even patients with normal IFN-γ production often have a proliferative defect to different antigens, especially patients with previously low CD4 counts and persistent HIV replication [22-24]. In line with these data, we observed an overall decreased proliferative capacity to *Candida* in patients with *Candida* esophagitis at diagnosis and even after recovery of the IFN-γ response. These findings suggest that the proliferative defect could contribute to disease development and that these patients might remain vulnerable despite effective cART.

CD4 T cells from chronically infected HIV patients show diminished IFN-γ and IL-17 production [25], but data on cytokine production in response to different fungi in HIV-infected patients is scarce. This study demonstrates that all patients with advanced HIV infection including patients with and without *Candida* esophagitis had an overall impaired IFN-γ and IL-17 production to *C. albicans*, which probably is partly due to the significantly reduced CD4

count. However, HIV-infected patients with early initiation of cART had comparable or even higher *Candida*-specific cytokine responses compared to healthy individuals despite significantly reduced CD4 counts, showing that low CD4 counts do not necessarily lead to reduced antigen-specific responses. Similarly, CMV pp65-specific IFN-γ production by CMV-seropositive patients was comparable in all groups, independent of CD4 counts. It is however possible, that patients with acute *Candida* esophagitis have additional impairments in antigen-presenting cells leading to reduced T-cell responses or that *Candida*-specific T cells in these patients are recruited to sites of infection and therefore disappear from the blood. Interestingly, the recovery of IL-17-producing cells was slower in patients with *Candida* esophagitis compared to patients with advanced HIV infection without OI. This data fortify previous findings in chronic mucocutaneous candidiasis in humans and oropharyngeal candidiasis in mice [9-11, 25, 26].

We further found that patients with *Candida* esophagitis had a significantly higher percentage of $CD25^+$ CD4 cells before disease development compared to healthy individuals. Patients with advanced HIV without *Candida* esophagitis showed a two-fold lower percentage compared to patients with *Candida* esophagitis. We cannot state if these cells were activated or regulatory T cells, as we did not include additional markers. However, previous studies showed that a higher percentage of regulatory T cells was associated with lower HIV- and *Candida*-specific responses [27]. Furthermore, in vitro depletion of the T_{reg}-containing $CD25^+$ T-cell population greatly enhanced the response to HIV and CMV antigens [28-30]. Moreover, $PD\text{-}1^+$ CD4 cells significantly increased at diagnosis of *Candida* esophagitis, which further supports the assumption that a dysfunction of CD4 cells might be one factor leading to susceptibility to *Candida* esophagitis.

Healthy individuals also show proliferation of CD8 cells in response to *C. albicans*. Previous work in mouse models has shown that in the absence of CD4 cells, CD8 cells were able to confer protection to the fungal pathogens *Blastomyces dermatitidis* and *Histoplasma*

capsulatum [31]. Interestingly, in our study we did not observe compensatory proliferation of CD8 cells in patients with low CD4 cell responses after stimulation with *C. albicans*. It is possible, that the CD8 cells proliferating to *C. albicans* in healthy individuals are mucosal-associated invariant T (MAIT) cells that respond to *C. albicans* and are depleted in the course of HIV infection [32-34].

Recently, it has been shown that not only Th17 cells, but also other cells such as ILCs can be a source of IL-17 and are involved in the host defense against fungal infections [12]. We found that the ILC counts in peripheral blood of *Candida* esophagitis patients were unable to recover even with suppressed viral replication. Remarkably, patients with CD4 counts >350 cells/µl had significantly higher ILC counts in the peripheral blood compared to patients with CD4 counts <200 cells/µl, suggesting that the loss of ILCs occurs in parallel with the loss of CD4 counts over time and may additionally increase the risk of developing *Candida* esophagitis. Furthermore, recovery of ILCs after suppressed viral replication seems to be correlated with CD4 recovery. Nevertheless, these data should be interpreted with caution, as the number of patients is low and we did not investigate the different subsets of ILCs and their involvement in the mucosa.

Also NK cells are increasingly considered as part of the host defense against fungi [13, 14, 35-37]. Impaired NK-cell activity was observed in patients with chronic mucocutaneous candidiasis [38]. Also HIV-infected individuals show quantitative und functional NK-cell impairments that continue during disease progression, such as a decrease of the $CD3^{-}CD56^{+}$ cell subset, a decreased cytotoxic capacity and aberrant expression of several surface receptors [39-42]. In fact, we found significantly lower NK-cell counts and a significantly lower percentage of $CD16^{+}$ cytotoxic NK cells in patients with *Candida* esophagitis compared to healthy individuals and patients with early initiated cART. They did not recover under stable, virologically successful cART. Furthermore, similar to ILCs, NK-cell recovery seemed to correlate with CD4 recovery.

Recently, evidence has accumulated that early initiation of cART is beneficial for virological as well as immunological parameters. Early treatment decreases cell-associated HIV RNA and DNA and limits the HIV reservoir [43-47], maintains numbers and function of the CD4 compartment [4, 48-50] and reduces the risk for disease transmission and the development of opportunistic viral and fungal infections and malignancies [4, 51, 52]. However, it was not clear, how early or late treatment affects T-cell responses to opportunistic pathogens and how other immune cell subsets such as NK cells or ILC are affected. In this study we could confirm improved overall and *Candida*-specific CD4 recovery in patients with early cART. We could further show that ILC and NK-cell reconstitution, immune cells with a likely role in antifungal defense, correlated with CD4 recovery and was therefore superior in HIV-1-infected patients with early treatment, further arguing for early initiation of cART in HIV-1-infected individuals.

The strength of this study is the comprehensive longitudinal analysis of quantitative and qualitative immune responses in a large number of HIV-infected patients with *Candida* esophagitis. This allowed identifying significant immunological impairments compared to healthy individuals and HIV-infected patients with early initiation of cART. However, due to the limited availability of PBMC we did not further characterize different cell subsets such as T_{reg} or T_H17 cells and functional analysis could not be performed in every sample.

In conclusion, this study demonstrates that HIV-1-infected patients with *Candida* esophagitis not only have deficient T-cell responses, but an accumulation of multiple, partly *Candida*-specific immunological defects. This may explain the fact that despite the high frequency of *Candida* esophagitis only a part of AIDS patients develop this OI. These defects are only partially reversible under cART and long-term immune impairments remain. This is particularly apparent in patients with low CD4 cell counts at initiation of cART showing greater general and *Candida*-specific immune impairments initially and under stable cART. Nevertheless, certain

individuals even experienced *Candida* esophagitis at higher CD4 counts. These patients showed similar immune defects as patients with low CD4 counts highlighting that the presence of specific immunological gaps is relevant. We hypothesize that specific gaps due to underlying genetic and/or immunological predisposition may explain why certain individuals also develop OI at higher CD4 counts. In line with other current studies, our study similarly supports the rationale for early initiation of cART.

Notes

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Authors' contributions: The study was conceived and designed by N.K., C.S., M.B. and C.B. Data acquisition and analysis was performed by C.B., and C.S. Sample recruitment and acquisition of clinical data was performed by C.B., M.S., S.Z., H.F. and H.F.G. Statistical

analysis was done by L.E. The paper was written by N.K., C.S., M.B., and S.L. and reviewed by all co-authors.

Supplemental Digital Content 1.doc

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Figures

Fig. 1. Patients with *Candida* esophagitis have low and dysfunctional CD4 counts with decreased *Candida*-specific cytokine responses and proliferative capacity. (a) Absolute $CD4^+$ T-cell counts, (b) percentage of $PD1^+$ and $CD25^+$ $CD4^+$ T cells, (c) IFN-γ response of PBMC to SEB or heat-inactivated *C. albicans*, (d) IL-17 response of PBMC to SEB or heat-inactivated *C. albicans*, (e) percentage of proliferating ($CFSE^{dim}$) cells in the $CD4^+$ T-cell population after 7 days stimulation with SEB or heat-inactivated *C. albicans* and (f) percentage of proliferating ($CFSE^{dim}$) cells in the $CD8^+$ T-cell population after 7 days stimulation with SEB or heat-inactivated *C. albicans* of patients at diagnosis of *Candida* esophagitis (ESO), patients with early initiated cART and a viral load (VL) <50 c/ml (VL<50) and healthy donors (HD). Shown are median values + interquartile ranges (Tukey plot). Data (c)-(f) are shown after subtraction of unstimulated controls. Number of ESO/VL<50/HD were n=37/15/20 (a), n=33/15/20 (b), n=18/11/19 (c), n=12/14/20 (d) and n=4/8/14 (e,f). * $P \leq .05$, ** $P \leq .01$, **** $P \leq .0001$ (Mann-Whitney test).

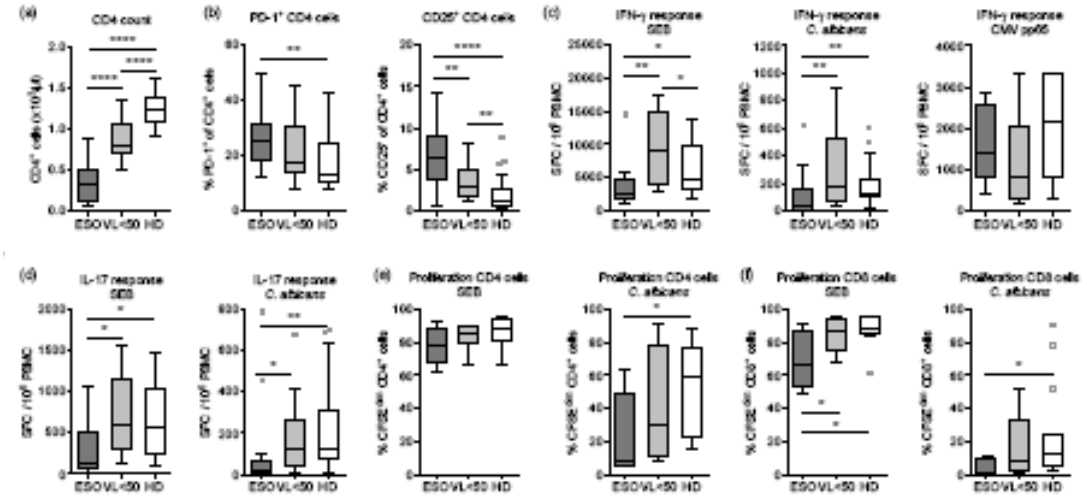

Fig. 2. Patients with *Candida* esophagitis have decreased peripheral NK cells and innate lymphoid cells (ILC). (a) Absolute $CD3^{-}CD56^{+}$ NK-cell counts, percentage of $CD16^{+}$ NK cells and absolute $CD16^{+}CD3^{-}CD56^{+}$ NK-cell counts, (b) percentages of $NKG2A^{+}$ and $NKG2C^{+}$ NK cells, (c) percentage of proliferating ($CFSE^{dim}$) cells in the NK-cell population after 7 days stimulation with SEB or heat-inactivated *C. albicans* and (d) absolute $lineage^{-}CD127^{+}$ ILC-count of patients at diagnosis of *Candida* esophagitis (ESO), patients with early initiated cART and a viral load (VL) <50 c/ml (VL<50) and healthy donors (HD). Shown are median values + interquartile ranges (Tukey plot). Data in (b) are shown after subtraction of unstimulated controls. Number of ESO/VL<50/HD were n=31/15/20 (a,b,d) and n=4/8/14 (c). ** $P \leq .01$, *** $P \leq .001$ **** $P \leq .0001$ (Mann-Whitney test).

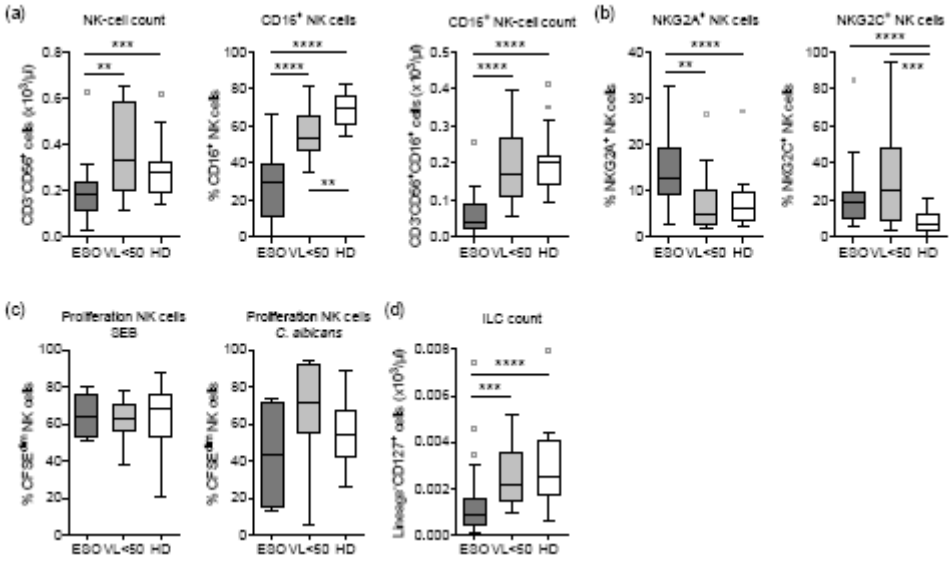

Fig. 3. Proliferative response to *C. albicans* and NK-cell counts and function are impaired despite higher CD4 counts at diagnosis. (a) Percentage of $PD1^+$ and $CD25^+$ $CD4^+$ T cells, (b) IFN-γ response of PBMC to SEB or heat-inactivated *C. albicans*, (c) IL-17 response of PBMC to SEB or heat-inactivated *C. albicans*, (d) percentage of proliferating ($CFSE^{dim}$) cells in the $CD4^+$ T-cell population after 7 days stimulation with SEB or heat-inactivated *C. albicans*, (e) absolute $CD3^-CD56^+$ NK-cell count, percentage of $CD16^+$ NK cells, absolute $CD16^+CD3^-CD56^+$ NK-cell counts, percentages of $NKG2A^+$ and $NKG2C^+$ NK cells and (f) absolute $lineage^-$ $CD127^+$ ILC-count in patients with *Candida* ESO and $CD4^+$ T-cell counts <200 cells/µl or >350 cells/µl. Shown are median values + interquartile ranges (Tukey plot). Broken and dotted lines represent medians of healthy individuals (HD) and HIV-1-infected patients with early initiated cART and a viral load (VL) <50 c/ml (VL<50), respectively. Data (b)-(d) are shown after subtraction of unstimulated controls. Number of patients <200 / >350 CD4 were n=10/16 (a), n=8/7 (b), n=5/5 (c), n=1/3 (d), n=9/13 (e) and n=11/13 (f). * $P \leq .05$, ** $P \leq .01$ (Mann-Whitney test).

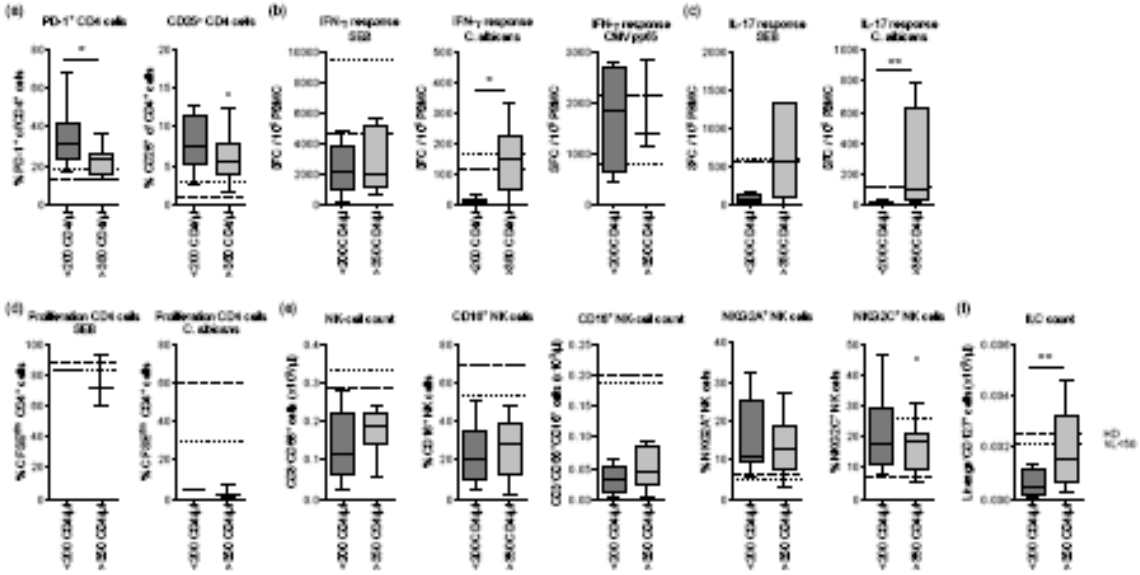

Fig. 4. Patients with *Candida* esophagitis retain immunological impairments after disease resolution and successful cART. Immune cell numbers and function of patients with *Candida* esophagitis 6-18 months before diagnosis (T-1), at the time of diagnosis (ESO), 6-18 months after disease resolution (T1) and after suppression of HIV-1 RNA for at least 2 years (T2). (a) Absolute $CD4^+$ T-cell counts, (b) percentage of $PD1^+$ and $CD25^+$ $CD4^+$ T cells, (c) IFN-γ response of PBMC to SEB or heat-inactivated *C. albicans*, (d) IL-17 response of PBMC to SEB or heat-inactivated *C. albicans*, (e) percentage of proliferating ($CFSE^{dim}$) cells in the $CD4^+$ T-cell population after 7 days stimulation with SEB or heat-inactivated *C. albicans*, (f) percentage of proliferating ($CFSE^{dim}$) cells in the $CD8^+$ T-cell population after 7 days stimulation with SEB or heat-inactivated *C. albicans*, (g) absolute $CD3^-CD56^+$ NK-cell counts, percentage of $CD16^+$ NK cells, absolute $CD16^+CD3^-CD56^+$ NK-cell counts, (h) percentages of $NKG2A^+$ and $NKG2C^+$ NK cells, (i) percentage of proliferating ($CFSE^{dim}$) cells in the NK-cell population after 7 days stimulation with SEB or heat-inactivated *C. albicans* and (j) absolute $lineage^-$ $CD127^+$ ILC-count. Shown are median values + interquartile ranges. Broken and dotted lines represent medians of healthy individuals (HD) and HIV-1-infected patients with early initiated cART and a viral load (VL) <50 c/ml (VL<50), respectively. Data (c)-(f) and (i) are shown after subtraction of unstimulated controls. Number of patients T-1/ESO/T1/T2 were n=12/37/37/8 (a), n=10/33/34/8 (b), n=3/18/27/6 (c), n=1/12/22/6 (d), n=3/4/6/6 (e,f), n=10/31/34/8 (g,h), n=3/4/6/5 (i) and n=10/31/36/8 (j).

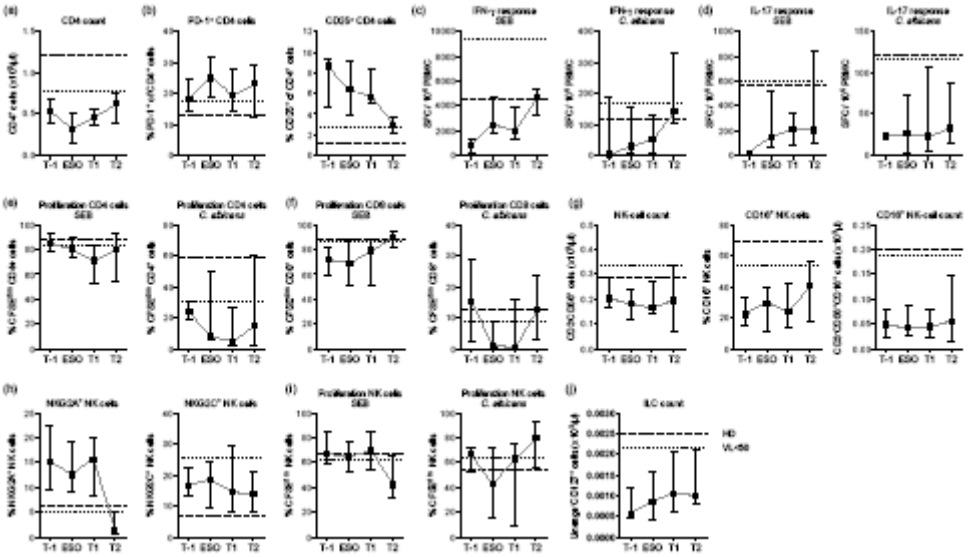

Table 1. Baseline characteristics.

	Time point T-1 6-18 months before diagnosis		Time point T0 Diagnosis of *Candida* esophagitis						Time point T1 6-18 months after diagnosis		Time point T2 After suppressed HIV RNA for > 2 years			
	ESO (n=12)	No OI (n=8)		ESO (n=37)			No OI (n=37)		ESO (n=37)	No OI (n=37)	ESO (n=8)	No OI (n=8)	Healthy (n=20)	HIV patients with early cART (n=15)
			<200 CD4 (n=12)	200-350 CD4 (n=7)	>350 CD4 (n=18)	<200 CD4 (n=12)	200-350 CD4 (n=7)	>350 CD4 (n=18)						
Median age	42	43		45			44		46	45	47	47	39	47
(IQR)	(36-55)	(36-54)		(38-54)			(38-53)		(39-55)	(39-54)	(41-57)	(41-58)	(36-50)	(36-52)
			49	37	45	48	38	43						
			(41-55)	(33-40)	(39-55)	(40-54)	(31-41)	(37-56)						
Sex, male	6	5		22			22		22	22	3	3	10	14
(%)	(50)	(62.5)		(59.5)			(59.5)		(59.5)	(59.5)	(37.5)	(37.5)	(50)	(93.3)
			8	4	10	8	4	10						
			(66.7)	(57.1)	(55.6)	(66.7)	(57.1)	(55.6)						
Median CD4$^+$	540	374		326			321		463	418	624	428	1225	785
cells/ul (IQR)	(382-692)	(212-627)		(136-489)			(146-471)		(360-563)	(259-494)	(400-764)	(233-601)	(1088-1378)	(701-1067)
			104	260	503	95	265	472						
			(77-136)	(224-320)	(414-678)	(58-140)	(226-305)	(401-668)						
Median CD8$^+$	1032	1217		922			781		1004	870	936	882	555	680
cells/ul (IQR)	(795-1271)	(972-1343)		(630-1533)			(457-1265)		(660-1580)	(613-1280)	(607-1254)	(519-957)	(463-638)	(610-1290)
			895	463	1063	420	671	1173						
			(457-1536)	(439-922)	(837-1588)	(327-749)	(561-1191)	(842-1768)						
Median	2261	2164		1863			1457		2013	1765	2268	1881	-	2002
lymphocytes	(1584-2522)	(1736-2888)		(1232-2420)			(1146-2000)		(1528-2773)	(1323-2274)	(1446-2999)	(1229-2383)		(1588-3030)

cells/ul (IQR)			1232 (728-2000)	1446 (1060-1848)	2056 (1782-2922)	876 (640-1201)	1307 (1148-1890)	2365 (1690-2881)						
HIV RNA log10 copies/ml (IQR)	2.3 (1.2-4.3)	3.1 (1.2-4.5)	5.3 (3.2-5.6)	3.6 (2.7-5.6) 5.1 (3.3-6.9)	3.0 (2.2-3.7)	3.9 (2.6-5.2)	4.0 (2.7-4.4) 4.5 (4.0-4.8)	3.6 (2.3-4.3)	2.5 (1.6-4.3)	3.4 (2.3-4.2)	<50 copies/ml	<50 copies/ml	-	<50 copies/ml
cART (%)	11 (91.7)	7 (87.5)	6 (50)	26 (70.3) 6 (85.7)	14 (77.8)	8 (66.7)	23 (62.2) 5 (71.4)	10 (55.6)	36 (97.3)	31 (83.8)	8 (100)	8 (100)	-	15 (100)
CMV-positive (%)	11 (91.7)	7 (87.5)	11 (91.7)	31 (83.8) 4 (57.1)	16 (88.9)	9 (75)	31 (83.8) 5 (71.4)	17 (94.4)	31 (83.8)	31 (83.8)	6 (75)	2 (25)	-	13 (86.7)
HCV-positive (%)	5 (41.7)	2 (25)	4 (33.3)	10 (27) 1 (14.3)	5 (27.8)	3 (25)	8 (21.6) 1 (14.3)	4 (22.2)	10 (27)	8 (21.6)	4 (50)	2 (25%)	-	1 (6.7)
Sample dates (year-year)	2001-2010	2001-2007		2001-2011			2000-2012		2002-2012	2001-2013	2008-2014	2008-2014	2014	2014

ESO, patients with *Candida* esophagitis; No OI, patients with advanced HIV infection without opportunistic infection; cART, combined antiretroviral therapy; IQR, interquartile range; CMV, cytomegalovirus serology; HCV, hepatitis C serology.