

Treatment-dependent Loss of Polyfunctional CD8+ T-cell Responses in HIV-infected Kidney Transplant Recipients Is Associated with Herpesvirus Reactivation

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Antiretroviral-therapy has dramatically changed the course of HIV infection and HIV-infected (HIV(+)) individuals are becoming more frequently eligible for solid-organ transplantation. However, only scarce data are available on how immunosuppressive (IS) strategies relate to transplantation outcome and immune function. We determined the impact of transplantation and immune-depleting treatment on CD4+ T-cell counts, HIV-, EBV-, and Cytomegalovirus (CMV)-viral loads and virus-specific T-cell immunity in a 1-year prospective cohort of 27 HIV(+) kidney transplant recipients. While the results show an increasing breadth and magnitude of the herpesvirus-specific cytotoxic T-cell (CTL) response over-time, they also revealed a significant depletion of polyfunctional virus-specific CTL in individuals receiving thymoglobulin as a lymphocyte-depleting treatment. The disappearance of polyfunctional CTL was accompanied by virologic EBV-reactivation events, directly linking the absence of specific polyfunctional CTL to viral reactivation. The data provide first insights into the immune-reserve in HIV+ infected transplant recipients and highlight new immunological effects of thymoglobulin treatment. Long-term studies will be needed to assess the clinical risk associated with thymoglobulin treatment, in particular with regards to EBV-associated lymphoproliferative diseases.

Key words: HIV, immunological monitoring, kidney transplantation, T cell

Introduction

The availability of antiretroviral therapy (ART) has changed the long-term clinical management of HIV infection and chronic renal failure is now increasingly prevalent in the HIV(+) population. In fact, in HIV(+) individuals treated with ART, chronic kidney, liver and heart diseases have replaced opportunistic malignancies and infections as the leading cause of mortality (1–3). Chronic renal failure in HIV(+) individuals can be due to direct effects of HIV infection (HIV-associated nephropathy/HIVAN), or result from immune-complex glomerulonephritis, ART drug toxicity or co-morbid diseases such as hypertension and diabetes (4–6). Kidney transplantation is considered the most effective treatment modality for end-stage renal disease (ESRD) and has been successfully attempted in the HIV(+) setting, notwithstanding a somewhat elevated occurrence of acute rejection episodes (7–14). Accordingly, the use of appropriate immunosuppressive (IS) regimens in this setting is a matter of debate more so than the generally well tolerated ART. In particular, IS regimens including thymoglobulin (ATG), have been associated with an accelerated long-term CD4+ T-cell depletion and increased risk of non-opportunistic infections and may thus hamper overall clinical outcome in this patient group (15).

Among opportunistic complications, Epstein-Barr virus (EBV)- and Cytomegalovirus (CMV)-associated diseases are among the most dreaded ones in solid-organ transplant recipients (16–21). Although effective immune control of these pathogens has been demonstrated in healthy subjects and by adoptive T-cell transfer approaches, the knowledge on HIV-, EBV- and CMV-specific immunity in the context of solid-organ transplantation is limited and the understanding of the virus-specific immunological reserve of HIV(+) kidney transplant recipients subjected to iatrogenic IS being especially poor. However, a better understanding of factors associated with loss of immune control over viral infections may be needed to ensure best possible clinical outcome of organ transplantation and to make optimal use of available organs. By longitudinally enumerating CD4+

T-cells, quantifying HIV, EBV and CMV viral loads, and assessing the breadth, magnitude and functionality of virus-specific CD8+ T-cell responses in HIV(+) kidney transplant recipients we sought to gain insight into the complex relation between iatrogenic IS, viral pathogens and host immunity.

Material and Methods

Study participants

Twenty-seven HIV(+) transplant recipients were included in this study. Inclusion criteria included undetectable plasma HIV RNA for six months before transplantation (measured using either the Amplicor Monitor Ultrasensitive PCR assay (Roche Diagnostics) or the bDNA Versant version 3.0 assay) (Siemens Healthcare Diagnostics), CD4+ T-cell-counts >200 cells/ μ L and no use of IL-2 or GM-CSF in the six months prior to transplantation. Exclusion criteria were pregnancy and significant wasting or weight loss. Of the 27 subjects, none had any other organ transplantation before enrolling into the study. Twenty-six, 18 and six out of the 27 transplant recipients received prophylactic antiviral medication (Ganciclovir/Acyclovir) during the first three, the second three and the last six months of the observation period, respectively. Ganciclovir was used prominently, with only two and one patients receiving Acyclovir and both Ganciclovir and Acyclovir, respectively.

The Institutional Review Boards of the participating hospitals approved the study and all subjects provided written informed consent before recruitment.

Viral loads

Plasma CMV DNA was quantified as previously described (22). The lower limit of detection for CMV DNA was 1000 copies/mL (23). CMV reactivation was defined here as any single or repeated newly detectable plasma CMV viral load in latently infected individuals.

Cellular EBV viral loads were also determined using previously methods (24,25). The lower limit of sensitivity of this assay is 1 copy EBV DNA per 10^5 PBMCs. Consistent with recent reports, reactivation of latent EBV-infection was defined as either single or repeated occurrence of detectable viral load (>100 copies/ 10^5 cells) in previously aviremic patients or as a significant increase in patients that continuously had detectable EBV viral loads; an increase being considered significant if the peak value exceeded threefold standard deviation of the individuals mean viral load (calculated without the values of the obvious viral load peak) (26).

ELISpot assays

ELISpot assays were carried out as previously described (27,28). Briefly, frozen PBMC-samples were thawed and plated directly, without prior *in vitro* expansion, into 96-well ELISpot plates with 100 000 cells per well in the presence of 10 μ g/mL of peptide(s). Plates were then incubated for 16 h and developed for the secretion of IFN- γ . Spots were counted using an AID ELISpot Reader Unit (Autoimmun Diagnostika GmbH, Strassberg, Germany). Results were expressed as spot forming cells per 1×10^6 input cells. Thresholds for positive responses were determined as at least 5 spots per well and spot counts exceeding the mean plus 3 standard deviations of negative control wells.

In order to identify specificities for single peptides, we carried out a 2-step ELISpot procedure. In the first step, we tested the cells for responses to a matrix of peptide pools where each peptide was present in two peptide

pools. Each pool was tested in a different well on the plate and the patterns of reactive pools allowed us to predict the individual targeted peptide(s) present in these pools. To confirm the identity of the targeted epitope, a second ELISpot assay was performed, now testing the suspected reactive peptides individually. This method represents a time- and sample-saving way to test large libraries of peptides, without having to test each peptide separately.

Antigenic peptides

The peptide sets used consisted of previously described HLA class I restricted CD8+ T-cell epitopes for HIV, CMV and EBV. For HIV, all 184 optimally defined HIV-derived CD8+ T-cell epitopes listed in the 2001 edition of the Los Alamos National Laboratory HIV Immunology Database CD8+ T-cell epitope list were included (29). The set of used EBV-derived CD8+ T-cell epitopes consisted of 92 HLA class I restricted CD8+ T-cell epitopes as recently described (27). A set of 38 CMV-derived CD8+ T-cell epitopes was included to assess CMV-specific T-cell reactivity (27). Given the wide promiscuity of CD8+ T-cell epitopes, all individuals were tested with the entire sets of virus-specific peptides, regardless of their HLA type and the peptides described HLA restriction, (30) using a matrix system that allowed us to get a complete screen with two rounds of ELISpot assays and minimal amounts of cells.

Multi-parameter flow cytometry

Thawed cells were washed twice and re-suspended in R10 at 10^6 /mL and one million cells were typically used per stain. Anti-CD28 and anti-CD49d (BD Biosciences, CA) were added at a final concentration of 1 μ g/mL along with selected (using ELISpot results) HIV-, EBV- or CMV-derived single peptides (final concentration 2 μ g/mL) and incubated with the cells at 37°C for 1 h. Selection of individual peptides was based on positive responses observed in the screening ELISpot data. Then, Brefeldin A (Sigma) was added (final concentration 10 μ g/mL) and the cells cultured for six more hours at 37°C. A UV viability dye (LIVE/DEAD[®] Fixable Blue Dead Cell Stain Kit, Invitrogen, CA) was then used according to the manufacturers protocol to be able to discriminate between viable and apoptotic cells before cells were washed twice in fluorescence activated cell sorting (FACS) buffer (PBS/1% FCS) and stained with all surface antibodies (i.e. anti-CD3-Pacific Blue and anti-CD8-Pacific Orange, both from BD Biosciences) for 1 h at 4°C. The cells were again washed twice in FACS Buffer and permeabilized using the Fix/Perm Kit from BD Biosciences following the manufacturers instructions. After washing, cells were incubated with 250 μ L BD Fix/Perm solution for 15 min at 4°C, washed with BD PermWash solution and subsequently incubated for 1 h at 4°C with all intracellular antibodies (i.e. anti-IFN- γ -PE-Cy7, anti-TNF- α -Alexa 700, both from BD Biosciences and anti-IL2-APC from Biolegend [San Diego, CA]). After two washing steps in BD PermWash solution and an additional washing step in FACS Buffer, cells were resuspended in a small volume of FACS Buffer and placed at 4°C until use.

Statistical analysis

The breadth and magnitude of virus-specific CD8+T-cell responses as well as the CD4+ T-cell counts in study participants were compared between time points using non-parametric unpaired Kruskal-Wallis tests and non-parametric paired Wilcoxon Signed Rank tests, whereas the frequencies of individual CD8+ T-cell responses, stratified by functionality, were analyzed by Mann-Whitney U-tests. All reported p-values are two-sided, and p-values <0.05 were considered statistically significant. Where data were missing, the number of missing observations is indicated and no assumptions about missing data were made. GraphPad Prism (Version 5.0; GraphPad Software Inc., La Jolla, CA) and StatXact (Version 6.0; Cytel Inc., Cambridge, MA) software were used for statistical analyses.

Results

Study population, immunosuppressive regimens, graft function and other clinical outcomes

Demographic, immunological and clinical characteristics of the study population are summarized in Table 1. A total of 27 HIV (+) kidney transplant recipients were included. At the time of transplantation, all study participants were infected with EBV and CMV infection was prevalent (25/27 [92.6%]). Allo-sensitization (defined by peak panel-reactive antibodies [PRA] >10%) was present in seven of 27 (25.9%) HIV(+) transplant recipients. ART consisted of nucleoside/nucleotide reverse transcriptase inhibitors (RTI) and/or non-nucleoside RTI and/or protease inhibitors, mostly combined as a three-class therapy.

Use of IS and antiretroviral agents within the study and graft function of all study participants are listed in Table 2. Ten of the 27 [37.0%] transplant recipients received anti-thymocyte globulin (ATG) perioperatively (i.e. immediately prior to transplantation [n = 9], or within the first 12 weeks posttransplantation [n = 1]). In addition, one other patient received ATG as treatment against a suspected rejection 22 weeks after transplantation. Twenty-five of the 27 [92.6%] individuals were initiated on a standard triple IS regimen consisting of steroids (Prednisone), a calcineurin inhibitor (Cyclosporine A [CyA] or Tacrolimus/FK506 [FK]) and a nucleotide/DNA synthesis inhibitor (Mycophenolate Mofetil [MMF] or Azathioprine). At 12 months post-transplantation, graft survival was overall 100% and graft function (glomerular filtration rate [GFR] estimated by the MDRD equation) was 60.7 ± 23.1 mL/min/1.73m².

Table 1: Demographic, immunological and clinical characteristics of HIV(+) renal transplant recipients

Variable	
Patient number	27
Age—median (range)	46 (32–71)
Race:	
-African Americans—no. (%)	18 (66.7)
-Caucasian—no. (%)	9 (33.3)
Male gender—no. (%)	24 (88.8)
Living donor—no. (%)	12 (44.4)
Epstein-Barr virus infected—no. (%)	27 (100)
Cytomegalovirus infected—no. (%)	25 (92.6)
- Donor +/Recipient -	0 (0)
- Donor +/Recipient +	15 (55.5)
- Donor -/Recipient +	10 (37.1)
- Donor -/Recipient -	2 (7.4)
HLA-A/B mismatches—median (range)	3 (0-4)
HLA-DR mismatches—median (range)	2 (0-2)
Panel reactive Ab (50) >10%—no. (%)	7 (25.9)
Cause of end stage renal disease—no. (%)	
Hypertensive renal disease	9 (33.3)
HIV-associated nephropathy	7 (25.9)
Glomerulonephritis	2 (7.4)
Others	9 (33.3)

Table 2: Immunosuppressive regimens, graft function and antiretroviral therapy

Variable	
Immunosuppressive regimen—no. (%)	
Anti-thymocyte globulin (ATG)	10 (37.0)
Simulect® (Basiliximab)	11 (40.7)
At transplantation	
Triple therapy ¹	25 (92.6)
Dual therapy	1 (3.7)
Monotherapy	1 (3.7)
At 6 months	
Triple therapy	23 (85.2)
Dual therapy	3 (11.1)
Monotherapy	1 (3.7)
At 1 year	
Triple therapy	22 (81.5)
Dual therapy	4 (14.8)
Monotherapy	1 (3.7)
Glomerular filtration rate—mL/min/1.73m ² mean ± SD 1 year	60.7 ± 23.1
Biopsy-proven clinical rejection episodes—no. (%)	
<6 months	7 (25.9)
6 months to 1 year	2 (7.4)
Antiretroviral therapy—no. (%)	
Three-class therapy ²	22 (81.5)
Two-class therapy	5 (18.5)

¹Immunosuppressive triple therapy consisted of Cyclosporine A (CyA) or Tacrolimus (FK), Mycophenolate Mofetil (MMF) or Azathioprine, and Prednisone (Pred). In some cases (n = 10), Sirolimus supplemented or replaced either MMF or Pred. Dual therapy consisted of CyA or FK and Pred or MMF, alternatively of MMF or Azathioprine and Sirolimus, and in one case of FK or Azathioprine and Pred. Monotherapy consisted of either Tacrolimus, MMF, Azathioprine or Pred.

²Three-class therapy consisted of nucleoside/nucleotide reverse transcriptase inhibitors (nRTI), non-nucleoside RTI (nnRTI; or in one case fusion inhibitors [FI]) and protease inhibitors (PI). Two-class therapy included nRTI and nnRTI or PI.

Seven early (<6 months posttransplantation) and two late (6–12 months posttransplantation) biopsy-proven clinical rejection episodes were observed over the study time-frame. Finally, none of the patients developed lymphoproliferative diseases during the one-year follow-up period.

Transient reduction in CD4+ T-cell counts is driven by ATG treatment

CD4+ T-cell counts were determined before and at 12, 26 and 52 weeks posttransplantation. The median CD4+ T-cell count at study entry was 483 CD4+ T-cells/mL (range 108–1843). Subsequently, a significant decrease in CD4+ T-cell counts was observed 12 weeks posttransplantation (Figure 1). While statistically significant when compared to pretransplantation time point for all 27 subjects, the initial decline in CD4+ T-cell counts at week 12 was largely driven by the patients treated with ATG (pretransplant vs. week 12 CD4+ T-cell-counts in ATG-treated vs. non-ATG-treated individuals: p = 0.01 [median CD4+ T-cell-counts pre- and

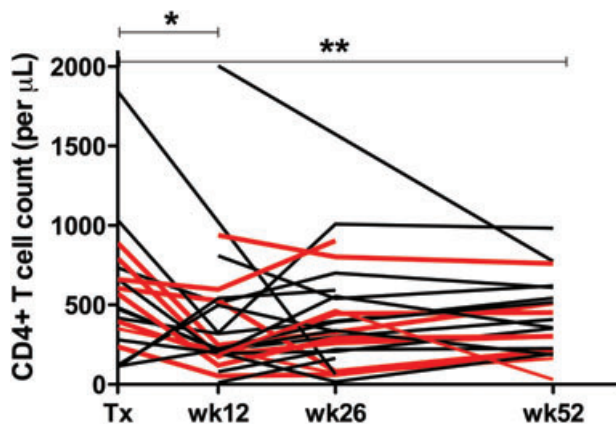


Figure 1: CD4+ T-cell-counts in HIV(+) kidney transplant recipients before and at 12, 26 and 52 weeks posttransplantation. Counts are expressed as CD4+ T-cells per μL of peripheral blood. Each line represents one individual, red lines represent individuals that were treated with ATG. Note the significantly lower CD4+ T-cell counts at week 12 (* $p < 0.05$) and week 52 (** $p < 0.01$, [Wilcoxon signed rank]) compared to pretransplantation levels.

week 12 posttransplantation: 588 and 187 cells/ μL , respectively] vs. $p = 0.30$ [median CD4+ T-cell-counts pre- and week 12 posttransplantation: 475 and 320 cells/ μL , respectively]). Although most individuals recovered their CD4+ T-cell counts thereafter, their numbers remained significantly below pretransplant levels at the end of the 1 year follow-up period (pre- vs. week 52 posttransplantation; all HIV(+) study participants: $p < 0.01$, ATG-treated: $p = 0.01$ [median CD4+ T-cell-counts week 52 posttransplantation: 302 cells/ μL], non-ATG-treated: $p = 0.2$ [median CD4+ T-cell-counts week 52 posttransplantation: 517 cells/ μL]).

Stable HIV viral loads and pre- versus posttransplantation HIV-specific CD8+ T-cell responses

HIV viremia was well controlled in all study participants both before (inclusion criterion) and after transplantation (Figure 2, Panel A).

The breadth (i.e. the number of epitopes recognized) and the magnitude (i.e. the frequency of epitope-specific T-cells, expressed as IFN γ spot forming cells per million PBMC [abbreviated here as SFC]) of the immune responses to optimally-defined CD8+ T-cell restricted epitopes, as measured by IFN- γ ELISpot, was stable between pretransplantation time points and 12, 26 and 52 weeks posttransplant (Figure 2, Panels B and C: median of three epitopes recognized). Only minor variations in the magnitude of these responses were observed (pre, 12 weeks, 26 weeks and 52 weeks posttransplantation medians: 540, 370, 780 and 720 SFC; Figure 2, Panel C). No difference in response rates were detected between HIV(+) transplant recipients that were receiving ATG (median 2–3 responses

over time) and those not receiving ATG (median 1.5–3 responses over time).

Posttransplantation cellular host immune responses to EBV are increased compared to baseline values

In accordance with previous reports indicating a high prevalence of EBV DNA in peripheral blood mononuclear cells and saliva of treated HIV(+) individuals, EBV DNA was detected at least once in every subject over the course of follow-up (Figure 2, Panel D) (31,32). Importantly, the rate detection of EBV DNA did not differ between pre- and posttransplantation PBMC samples (5/21 [23.8%] pre- vs. 47/147 [31.3%] available posttransplantation samples with detectable EBV viral load). Using the criteria described in the 'Material and Methods' section, eight out of 10 [80%] HIV(+) individuals treated with ATG reactivated EBV. The remaining two ATG treated individuals also showed ongoing viral replication but could not be classified formally as reactivators since pretransplantation viral load measurements were missing. In contrast, EBV-reeactivation was observed in only 1/15 [6.7%] ATG-naïve transplant recipients ($p < 0.001$, Figure 2, Panel D). EBV viral loads remained statistically significant higher in the ATG-treated versus non-ATG-treated sub-groups of transplant recipients, both at week 12 and week 52 posttransplantation compared to baseline (pretransplantation) values ($p = 0.007$ and $p = 0.01$, respectively, Figure 2, Panel D, red vs. black lines).

Before transplantation, EBV-specific T-cells were detected in 10/13 [76.9%] patients for whom EBV-specific immune responses could be assessed. Comparison of pre- and posttransplantation time points by non-parametric unpaired analysis of variance (ANOVA; Kruskal–Wallis test) revealed no significant difference. However, excluding subjects for whom longitudinal data were unavailable, by means of a paired non-parametric analysis (Wilcoxon), revealed significantly increased breadths and magnitudes of the EBV-specific CD8+ T-cell response over time (week 12 and 52 breadth/magnitude (in SFC): 1/119 and 2/410, respectively; $p = 0.01$ and $p = 0.007$ for both breadth and magnitude, respectively; Figure 2, Panels E and F). ATG-treated individuals did not differ significantly from the ATG untreated group ($p > 0.05$).

Cellular host immune responses to CMV increase over the first year posttransplantation

CMV reactivation was detected in four of the 25 transplant recipients that were CMV infected at the pretransplantation time point (Figure 2, Panel G). These included 3 of the 7 [42.8%] subjects that received ATG and for whom follow-up CMV viral loads were available and one of the nine [11.1%] transplant recipients that did not receive ATG ($p = 0.26$). We then assessed the CMV-specific T-cell reactivity and pretransplantation responses were detected in 23 [92.0%] of the 25 CMV-infected subjects. CMV-specific responses pre- and posttransplantation were again

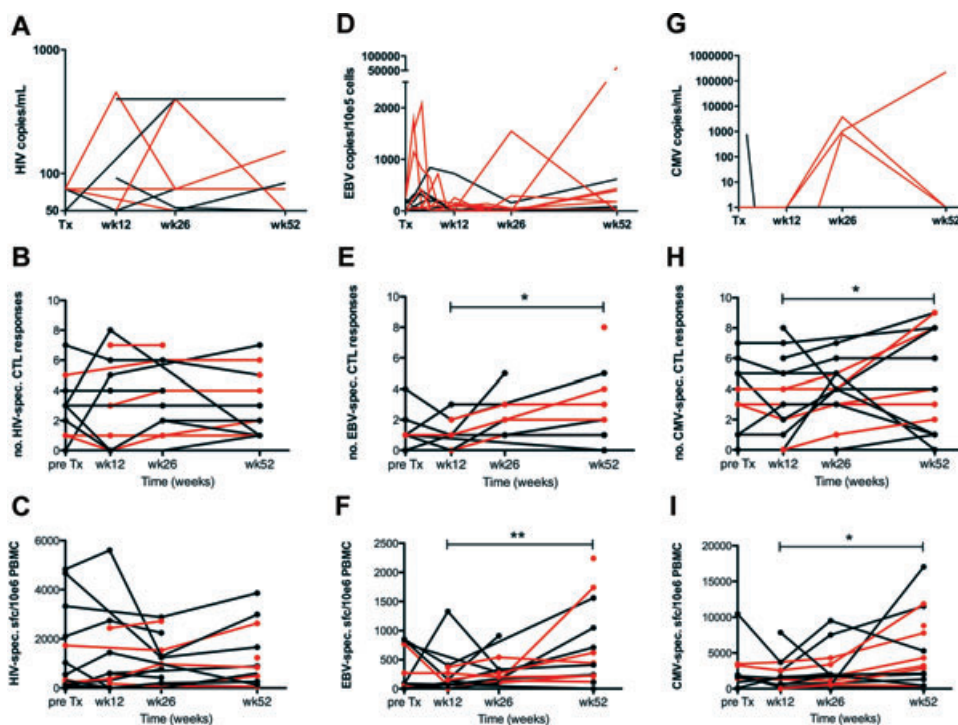


Figure 2: Kinetics of viral loads, and breadth and magnitude of HIV-, EBV- and CMV-specific CD8+ T-cell responses in HIV(+) kidney transplant recipients. (Panel A) shows HIV viral loads before and after transplantation. HIV viral loads are expressed as copies per mL plasma/serum. Note that most measurements showed viremia below the detection limit of the assay (<50 copies/mL) and thus fall on the x-axis. (Panel B and C) show the *ex vivo* assessment of the breadth and magnitude of HIV-specific CD8+ T-cell responses before and after transplantation as assessed by ELISpot assays. Similarly, (Panels D-F) show longitudinal measurements of EBV viral loads (in copies/10⁵ cells; median of 7 measurements per patient) and breadth and magnitude of the EBV-specific CD8+ T-cell response, respectively. Note the significant increase in both breadth (Panel E, **p* < 0.05) and magnitude (Panel F, ***p* < 0.01) of EBV-specific T-cell responses between weeks 12 and 52. Finally, (Panels G-I) show CMV viral loads (expressed as copies per mL plasma; median of 7 measurements per person), and CMV-specific CD8+ T-cell responses pre- and posttransplantation, respectively. Again, note the significant increase in both breadth (Panel H, **p* < 0.05) and magnitude (Panel I, **p* < 0.05) of CMV-specific CD8+ T-cell responses between weeks 12 and 52. Each line or symbol represents a single individual; red lines and symbols identify individuals that were treated with ATG. Bold horizontal lines indicate medians.

undistinguishable both in terms of breadth and magnitude by ANOVA analyses (Figure 2, Panels H and I). Nevertheless, and analogous to the EBV-specific CD8+ T-cell response, paired analyses (Wilcoxon) on longitudinal data points from the same subjects revealed statistically significant broader and stronger responses at week 52 (breadth/magnitude: 3/2850) compared to week 12 (3/690; *p* = 0.02 and *p* = 0.01 for breadth and magnitude, respectively). ATG treatment did not impact the breadth or magnitude of the CMV-specific CD8+ T-cell response.

Reduction in effector functions in virus-specific T-cell responses posttransplantation are driven by ATG and are associated with EBV reactivation episodes

To investigate the reason(s) why ATG-treated and ATG-untreated patients show such differences in control of EBV-reactivation, notwithstanding very comparable breadths and magnitudes of their EBV-specific CD8+ T-cell responses, the effector functionality of HIV-, EBV- and CMV-derived peptide specific CD8+ T-cell responses were

assessed longitudinally. Although a potential association between functional competence of specific CTL populations and viral control could only be expected, if at all, in the case of EBV, for which prophylactic or therapeutic effect of antiviral agents is less well established as for HIV and CMV, T-cell responses to HIV and CMV (which were under efficient pharmacological control) were analyzed as well to determine the effect of ATG on antiviral immune responses across different viruses (33).

The functional analyses were based on the ability of CD8+ T-cells to react to antigen stimulation with the secretion of the cytokines IFN- γ , TNF- α and/or IL-2. First, each response was assigned one of the seven possible combinations of effector functions as listed in Figure 3 and the relative (%) contribution of each combination to the total virus-specific response across all subjects (from each group; i.e. ATG-treated and ATG-naïve transplant recipients) at any given time point was recorded. As shown in Figure 3, ATG treatment appears to induce a more vigorous and

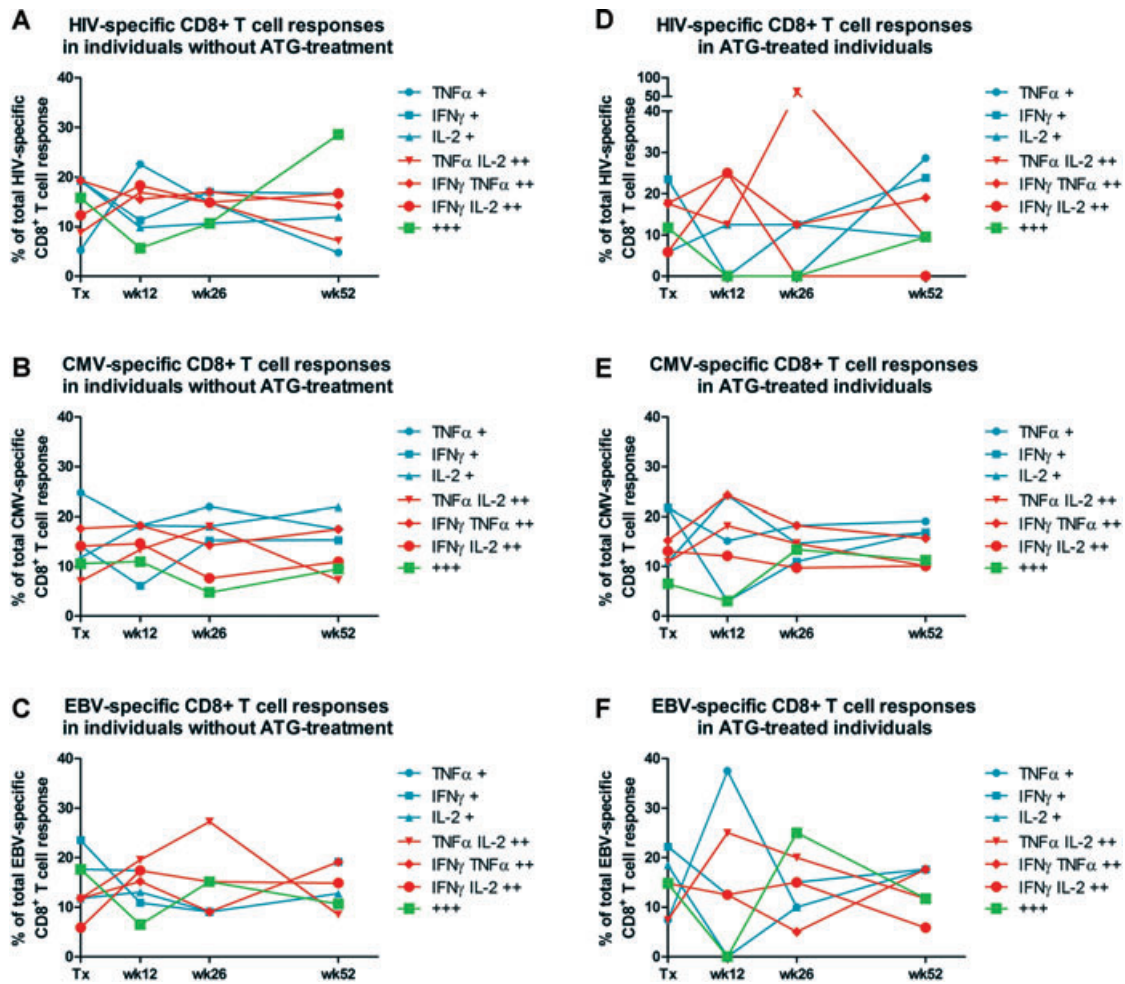


Figure 3: Impact of ATG treatment on the functional composition of virus-specific CD8+ T-cell responses in HIV(+) kidney transplant recipients. (Panels A and D) represent the functional composition, pre- and posttransplantation, of HIV-reactive CD8+ T-cells of ATG-treated and ATG-untreated HIV(+) kidney transplant recipients, respectively. The composition is expressed as percentage of total HIV specific CD8+ T-cells, i.e. encompassing all ATG-treated and -untreated recipients, respectively. Likewise, (Panels B and E and C and F) represent the functional composition of CMV- and EBV-responsive CD8+ T-cells in patients that did or did not receive ATG treatment, respectively.

transiently complete, depletion of tri-functional antiviral CD8+ T-cell responses than ATG-free IS regimen, although this comparison did not reach statistical significance. ATG-treated individuals experienced a similar drop in tri-functional cells specific for CMV at week 12.

Since this analysis was designed to encompass all individual immune responses within each group and to provide an overall assessment of the effector function patterns, responses of smallest or greatest magnitude were attributed the same weight. We thus potentially overestimated the contribution of weak responses to the total virus-specific immunity. To compensate for this potential bias, we then compared all individual responses based on their effector function profile and their frequency in the CD8+ T-cell pool of each patient, for each virus separately and between pre-

and posttransplantation time points (Figure 4). Responses were recorded either as mono-, bi- or tri-functional and their frequency compared between ATG-treated and ATG-untreated individuals. As shown in Figure 4, panel A, the frequencies of HIV, EBV and CMV-specific CD8+ T-cells that responded with only one detectable effector function were largely unaffected by IS regimens, as for all viruses the ATG treated and ATG untreated individuals showed comparable magnitudes of responses pre- versus post-transplantation. In contrast, we observed an almost complete absence of bi- and tri-functional responses in ATG-treated subjects compared to ATG-untreated individuals at posttransplantation time points (i.e. panels B and C: EBV-specific CD8+ T-cell immune responses [$p < 0.001$] as well as bi- and tri-functional HIV-specific CD8+ T-cell responses [both $p < 0.05$]). Importantly, ATG-treated and

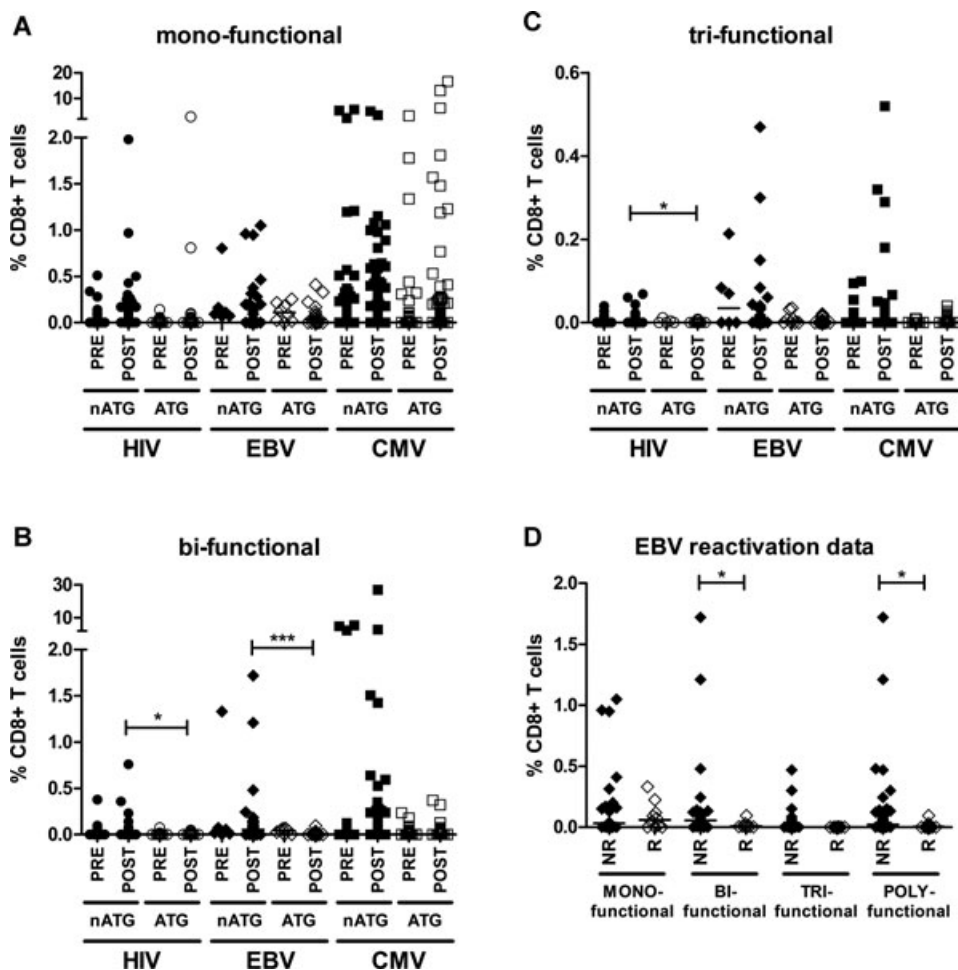


Figure 4: Impact of ATG treatment on the individual frequency of mono-, bi- and tri-functional HIV-, EBV- and CMV-specific CD8+ T-cells pre- and posttransplantation and the occurrence of EBV-reactivation posttransplantation in HIV(+) kidney transplant patients. (Panel A) shows individual frequencies of mono-functional) HIV-, EBV- and CMV-specific CD8+ T-cells over time in transplant recipients that were treated with or without ATG (labeled 'ATG' and 'non-ATG', respectively). Similarly, (Panels B and C) represent individual frequencies of bi- and tri-functional virus-specific CD8+ T-cells in the same groups. (Panel D) shows individual frequencies of EBV-specific CD8+ T-cells with the indicated level of polyfunctionality that where measured at the time of an EBV-reactivation event or conversely, at time points that were not followed by any reactivation event. Horizontal lines indicate medians. * $p < 0.05$; *** $p < 0.001$. Closed symbols represent non-ATG-treated individuals, while open symbols represent ATG-treated individuals. PRE = pretransplantation; POST = posttransplantation; nATG = non-ATG-treated individuals; ATG = ATG-treated individuals; NR = individuals for whom no EBV-reactivation was observed; R = individuals that reactivated EBV-replication.

ATG-untreated individuals had comparable pretransplantation frequencies of mono-, bi- and tri-functional CD8+ T-cells specific for HIV, EBV and CMV. To test for a possible link between the relative absence of bi- and tri-functional CD8+ T-cells and viral control, the effector function profiles of CTL specific for EBV were compared to the occurrence of reactivation of EBV-replication (Figure 4 panel D). Individual EBV-specific CD8+ T-cells were recorded based on their number of detectable effector functions and their frequency and compared between time points with signs of viral reactivation and time points that were not followed by a reactivation event. As shown, the relative magnitude of

mono-functional EBV-specific CD8+ T-cell responses did not again significantly differ between those patients that subsequently reactivated EBV-replication versus those that did not. However, bi-functional EBV-specific immune responses of greater magnitude were detected in patients that maintained viral control versus individuals that reactivated virus ($p = 0.016$). Overall, 'poly'-functional immune responses (defined as bi- or tri-functional responses) were detected less often prior to EBV-reactivation events, and if detected, had significantly lower frequencies than responses seen at time points that were not followed by viral reactivation ($p = 0.024$).

Discussion

Despite good outcomes after solid-organ transplantation in HIV(+) recipients, accepting HIV(+) individuals on transplantation waiting-lists continues to be a matter of debate (34). In this prospective study in HIV(+) kidney transplant recipients we sought to shed light on issues related to immune competence that may influence organ allocation for transplantation in HIV(+) patients. In particular, we investigated how posttransplant immunosuppression (IS), and in particular ATG treatment, impacts antiherpesviral immunity. The advent of ART has largely contributed to the decreased occurrence of herpesvirus-related diseases; however, these remain a significant concern for HIV-infected individuals undergoing solid-organ transplantation as pharmacological IS might diminish the antiherpesviral immunity even further (35,36). The present study demonstrates that HIV viremia remained well controlled and HIV-specific immune responses were stable throughout the observation period. However, the individuals who were treated with lymphocyte-depleting regimen (ATG) show a particularly profound absence of polyfunctional virus-specific T-cells and experience the majority of viral-reactivation episodes, indicating that ATG treatment in this setting may put these individuals at a considerable risk for the development of herpesvirus-associated diseases. The short-term follow-up of our study did unfortunately not allow us to substantiate this finding clinically.

A number of previous studies on renal transplantation in the HIV(+) host have yielded promising results but were often based on highly selected individuals (7,8,10,37). By contrast, the HIV(+) population studied here was—with regards to ethnicity, level of presensitization and renal pathologies—representative of HIV(+) US residents currently or in the near future, awaiting renal replacement therapy (38). The data demonstrate that the breadth and magnitude of the HIV specific T-cell response remained stable between pre- and up to one-year posttransplantation while the herpesvirus-specific response gained in both, breadth and total magnitude. While the stable HIV-specific response may have been predicted given the well-controlled HIV viremia, the increased herpesvirus-specific immunity posttransplantation was more surprising. While this expansion may be a consequence of antigen driven activation of EBV- and CMV-specific T-cells, it is also important to note that such expansion was not limited to subjects with documented viral reactivation. Further analyses of plasma and cellular viral replication, excluding possible artifacts such as non-productively infected, apoptotic cells will be needed to better understand the balance between viral reactivation and immune reactivity. However, the present data suggest that an immunologically relevant viral replication is ongoing as comparative analyses in HIV-negative kidney transplant recipients show a slow decay in herpesvirus-specific immune responses and an absence of viral reactivation (Gasser et al. manuscript in preparation)

Naturally, the observed increase in immune reactivity does not necessarily imply improved immune control, as antigen driven T-cell responses to infection such as HIV, KSHV and other pathogens have been described (28,39,40). To address whether the expanded cells were functional and whether the ATG treatment, shown to cause a more pronounced CD4+ T-cell depletion, was associated with higher frequencies of functionally impaired T-cells, we conducted flow-cytometric analyses on T-cells specific for HIV, EBV and CMV (15,41–43). Guided by earlier studies on T-cell function in these infections, we measured three effector functions widely used to characterize CTL-functionality, that is IFN- γ , TNF- α and IL-2 (44–46). Overall, the posttransplantation time points revealed T-cell populations with fewer detectable effector functions, affecting T-cells specific for all three viruses tested and suggesting that IS and/or ATG treatment may severely cripple these preexisting immune responses. A subsequent stratification of tested subjects into ATG-treated versus non-ATG-treated transplant recipients demonstrated that the observed effects were strongly driven by ATG treatment as ATG-treated patients consistently lacked polyfunctional antiviral CTL responses of high frequency. Thus, while we could not detect any reduction in T-cell effector functions associated with 'routine' (i.e. ATG-free) IS treatment, the data indicate that ATG treatment has rather devastating effects on T-cell functionality. Importantly, these effects were not limited to weak and borderline responses, as ATG treatment was associated with a wide depletion of polyfunctional response of high magnitude. Linking the initial observation that EBV-reactivation was almost exclusively restricted to ATG-treated patients to the subsequent functional immune-analyses of antiviral CTL responses, we found a direct association between the absence of polyfunctional EBV-specific CTL responses and EBV-reactivation (Figure 4, panel D). Importantly, we have not observed the development of any lymphoproliferative disease, which represented a big concern in the beginning of the study. A longer follow-up period is however needed to properly assess the long-term consequences of EBV reactivation episodes. Although HIV- as well as CMV-specific CD8+ T-cell responses were affected by similar functional impairments, the virological consequences were very likely silenced by antiviral treatments. Intriguingly, three out of the four patients that reactivated CMV-replication were treated with ATG, an observation that will need to be expanded to more subjects and combined with refined detection approaches to assess a potential association between ATG treatment and subclinical viral reactivation in the setting of HIV+ organ transplantation (47,48). Further studies will also need to include properly matched HIV-negative patients to address the role of HIV-infection in the ATG-driven loss of immune-functionality. Preliminary observations in an independently designed cohort of HIV-negative kidney recipients indicate that ATG drives, expectedly, a similar drop in total CD4+T-cell counts and might adversely affect breadth and magnitude of herpesvirus-specific CTL as well. If confirmed and extended to functional analyses,

these data would suggest that the ATG-mediated effects described here for HIV-infected individuals would likewise influence other groups of transplant recipients. However, differences between transplant centers in their inclination to ATG treatment, the readiness to include HIV-infected individuals on transplantation waiting lists and impact of the local donor/recipients ethnicities will require substantial efforts in the design and execution of well-controlled analyses of immune reactivity and outcome of transplantation in HIV+ versus HIV- organ recipients.

In summary, the present study is the first report on immunological competence in HIV-infected individuals undergoing solid-organ transplantation. The data demonstrate a profound effect of ATG on T-cell function, reminiscent of the functional impairment observed in patients with persistent HIV viral load, (49) and reveal an association between T-cell functionality and viral reactivation, at least for EBV. As the loss of effector function was not limited to EBV specific T-cells, the immune suppressive treatment using ATG will likely need to be sustained by prophylactic treatment of ATG-treated HIV(+) patients for other (viral) co-pathogen and might ultimately argue in favor of non-depleting IS regimen to be used in HIV(+) transplant recipients.

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