Rapid Communication

Loss of HIV-specific memory B-cells as a potential mechanism for the dysfunction of the humoral immune response against HIV

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ABSTRACT

A central, yet unresolved issue in the pathogenesis of HIV disease is the mechanism of antibody perturbation. In this study, HIV-specific memory B-cells were quantified in groups of infected subjects and compared with memory responses to other antigens and antibody titers. HIV-specific memory B-cell responses were vigorous in individuals with CD4 T-cell counts >350/μl and weak or undetectable in subjects with CD4 T-cell numbers <200/μl. Memory B-cell loss was permanent, because antiretroviral therapy failed to restore HIV-specific memory responses while influenza- and tetanus toxoid-specific memory B-cells remained unaffected or recovered. Antibody titers to Gag strongly correlated with memory B-cell frequencies. In contrast, Env-specific antibodies were maintained in advanced disease despite low or undetectable levels of memory B-cells. These results provide a potential mechanism by which destruction of HIV-specific CD4 T-cells affects the humoral immune response against HIV and compromises the ability to maintain an effective antibody response.

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Introduction

Infection with the human immunodeficiency virus (HIV) leads to B-lymphocyte dysfunction that is already detectable in the primary phase of the disease (Titanji et al., 2005). Infected individuals show signs of polyclonal B-cell hyperactivity with hypergammaglobulinemia (Lane et al., 1983; Terpstra et al., 1989) and increased frequencies of activated and terminally differentiated B-cells (Fournier et al., 2002; Moir et al., 2001; Shirai et al., 1992). Moreover, levels of memory B-cells in the blood decline (De Millo et al., 2001; Nagase et al., 2001) and as the disease progresses to AIDS, the B-cell population becomes poorly responsive to mitogens and common recall antigens (Hart et al., 2007; Janoff et al., 1991; Jiang et al., 2008; Kroon et al., 2000; Lane et al., 1983; Malaspina et al., 2005, 2008; Moir et al., 2008a). Initiation of antiretroviral therapy (ART) reduces B-cell hyperactivity, normalizes total B-cell numbers and partially restores the proportion of B-cell subpopulations in the blood indicating that ongoing viral replication and low CD4 T-cell numbers are the major pathogenic factors (Moir et al., 2008b; Morris et al., 1998). Dysfunction specifically affects the HIV-specific antibody response leading to a decline of anti-Gag antibody titers in the blood that is not or only weakly responsive to therapy. It is thought that the decline is a surrogate marker for the loss of T-cell help, but the mechanism that leads to the fall of antibodies is incompletely understood. In contrast, Env-specific antibodies are maintained and may only decline as frank AIDS develops but the underlying process is likewise unclear (Binley et al., 1997; Brown et al., 1995; Cheingsong- Popov et al., 1991; Strathdee et al., 1995; Zwart et al., 1994).

Viral infections usually induce the generation of memory B-cells in a process called the germinal center (GC) reaction. Initiation of the GC reaction requires the help of antigen-specific CD4 T helper cells (Gourley et al., 2004). It has been reported that HIV preferentially infects and progressively depletes HIV-specific CD4 T helper cells (Douek et al., 2002; Pitcher et al., 1999; Rosenberg et al., 1997) suggesting that the GC reaction and specifically the development of HIV-specific memory B-cells might be affected. However, there is limited knowledge about HIV-specific memory B-cells. While previous studies provide evidence that HIV infection induces virus-specific memory B-cells (Bonsignori et al., 2009; Fondere et al., 2003; Guan et al., 2009; Scheid et al., 2009),
the effect of HIV disease progression on these cells has not yet been investigated. One reason for that is that analyses of HIV-specific memory B-cells are considered inherently difficult because of the immune dysfunction associated with HIV pathogenesis (Guan et al., 2009).

In the present study, we examined the possibilities of permanent versus transient or reversible loss of HIV-specific memory B-cell responses in groups of subjects with different clinical characteristics including individuals with CD4+ T-cell counts above 350/μl, below 200/μl, and under antiretroviral therapy using a highly sensitive memory B-cell ELISpot protocol. We compared the results with memory B-cell responses against influenza virus and tetanus toxoid and with antibody titers. Our findings provide evidence of selective depletion of HIV-specific memory B-cells during disease progression that is not responsive to ART. Moreover, the results demonstrate a correlation of Gag- but not Env-specific memory B-cell and antibody responses.

Results

Study subjects

While a better understanding of the HIV-specific B-cell-mediated immune response may provide new opportunities to raise the level of immune protection, current knowledge about the virus-specific memory B-cells is limited. To address the question to what extent HIV-specific memory B-cell responses are affected during disease progression, we examined the HIV-specific memory B-cells in groups of subjects with CD4+ T-cell counts above 350/μl and patients with a fall of CD4+ T-cell counts to below 200/μl. Subjects with CD4+ T-cell counts above 350/μl were arranged in groups of individuals with viral loads smaller (hereafter: controllers, 10 subjects) and greater than (hereafter: early progressors, 10 subjects) 1,000 RNA copies/ml plasma (Fig. 1A). Individuals with CD4+ T-cell counts below 200/μl were grouped in ART untreated (hereafter: late progressors, 8 subjects) and treated (hereafter: treated late progressors, 10 subjects) individuals. A group of 10 healthy uninfected individuals was added as control. None of the subjects outside the treated group received ART.

Treated late progressors received ART for more than 18 months and had undetectable viral loads or levels below 100 RNA copies/ml in the three measurements preceding the examination. The median CD4+ T-cell count in this group was 413 cells/μl. CD4+ T-cell numbers were lower in the untreated late progressor group (Fig. 1B). In addition, total numbers of memory B-cells were significantly lower and plasma cell frequencies higher than in the groups of uninfected individuals, controllers, and ART-treated subjects. In contrast, memory B-cell frequencies and plasma cell numbers in the ART-treated group were comparable to those in uninfected healthy individuals (Figs. 1C, D). Together the data show that in the study population HIV progression led to perturbations of the total memory and plasma cell frequencies in the blood. ART restored plasma and memory B-cell

![Fig. 1. Viral load and percentage of CD4+ T-cells and B-cell populations. (A) Viral load in the plasma of viral controllers, subjects with early disease and with late disease progression without and with antiretroviral therapy. Symbols represent values from individual subjects. Viral load values were significantly different between the groups; (B) CD4+ T-cell counts; (C) CD19+ CD20+ CD27+ memory B-lymphocytes and (D) CD19+ CD20− CD38hi plasma cells in groups of infected individuals and in healthy uninfected subjects. Statistical p-values were calculated using the Kruskal–Wallis (values in boxes) and Mann–Whitney test. Bars indicate median values.]
frequencies together with a reduction of the viral load and an increase of total helper T-cell numbers.

**Loss of Gag- and Env-specific memory B-cells upon disease progression**

We next examined Gag/p24- and Env/gp120-specific memory B-cell responses in the HIV-infected subjects of the study groups. Vigorous Gag-specific responses were observed in the group of controllers with a median frequency of 0.5%. In the cohort of subjects with early progressive disease, the median memory B-cell frequency was 0.135%. The difference between the two groups was not significant ($p = 0.198$). In contrast, the median frequencies in these groups were significantly higher than in the group of subjects with late disease progression (median frequency 0.0065%) indicating a loss of the Gag-specific memory B-cell responses in individuals with advanced disease. Particularly notable is that in the treated late progressor group, Gag-specific memory B-cell frequencies were as low as in the untreated group (median frequency 0.019%) indicating that antiretroviral therapy does not reverse the loss of specific memory B-cells (Fig. 2A).

In addition to Gag-specific memory B-cells, robust Env/gp120-specific memory B-cell responses were present in the controller group (median: 0.111 %), and in the group with early progressive infection (median: 0.068%) indicating that HIV normally induces vigorous memory B-cell responses. Similar to the Gag-specific responses, the median Env-specific memory B-cell frequencies in these groups were significantly higher than those in the late progressor group (median frequency: 0.002%) and ART-treated group (median frequency: 0.005%) (Fig. 2B) indicating progressive and irreversible Env-specific memory B-cell loss.

**Maintenance of influenza- and tetanus-specific memory B-cells in progressive HIV disease**

To investigate whether these data would be reflected in other parts of the B-cell response or whether memory B-cell depletion was limited to HIV-1-specific cells, we compared HIV-specific responses with those to other common recall antigens such as influenza nucleoprotein (NP, Fig. 3A) and tetanus toxoid (Ttx, Fig. 3B). While it is likely that all individuals had repeatedly been naturally exposed to influenza virus, several individuals had also been immunized against influenza and all study subjects had previously been vaccinated against tetanus. Memory B-cell responses against influenza and Ttx were not statistically different in the study groups, although there was a trend towards lower frequencies in the late progressor group. Notably, the memory B-cell responses against influenza and Ttx were normal in the group of ART-treated subjects.

**HIV-specific antibody titers**

Memory B-cells may periodically differentiate into antibody-secreting long-lived plasma cells to maintain long-term antibody production (Gourley et al., 2004). We determined the magnitude of the antibody responses to Gag and Env to examine the association with memory B-cell frequencies. Vigorous Gag- and Env-specific antibody titers were present in the controller and early progressor groups. However, whereas Env-specific antibodies remained high in the late progressor group, there was a trend towards lower levels of Gag-specific antibodies in this group compared with the group of disease controllers ($p = 0.091$). ART significantly reduced Env-specific antibodies but did not further reduce Gag-specific antibody titers (Figs. 4A, B).

Gag-specific antibody titers correlated highly significantly with the memory B-cell frequencies indicating an association of the plasma cell activity with the memory B-cell response. In contrast, memory B-cell frequencies and antibody responses to Env were divergent. Robust antibody titers were observed in individuals with advanced disease despite low or undetectable memory B-cells indicating that at least some of the Env-specific antibody response is memory B-cell independent (Figs. 4C, D).

**Discussion**

While it is well known that in HIV infection the B-cell population becomes poorly responsive to common recall antigens indicating global memory B-cell dysfunction (Janoff et al., 1991; Kroon et al., 2000; Lane et al., 1983; Malaspina et al., 2005), there is limited knowledge about HIV-specific memory B-cells in early and advanced disease. The results of this study show that HIV induces vigorous virus-specific memory B-cell responses in early stages of the infection (CD4+ T-cell count above 350/μl) and preferential elimination of HIV-specific memory B-cells later in the disease (CD4+ T-cell count below 200/μl). In particular, compared with subjects with CD4+ T-cell counts above 350/μl, individuals with helper T-cell counts below 200/μl, as a group, had significantly lower numbers of HIV-specific memory B-cells in the peripheral blood. Moreover, ART for more than 18 months failed to normalize HIV-specific memory B-cell responses suggesting that the cells were depleted permanently.

Consistent with previous studies that showed an increase of total memory B-cell numbers after initiation of ART (Moir et al., 2008b;
Morris et al., 1998) we observed normal total memory B-cell numbers in the group of late disease progressors under therapy. Similarly, in agreement with previous findings that demonstrated a correlation of influenza-specific memory B-cell responses and CD4+ T-cell counts after booster vaccination with an influenza vaccine (Malaspina et al., 2005), we observed that memory B-cell responses to influenza and Ttx were normal in ART-treated individuals with partially restored CD4+ T-cell counts. In striking contrast, HIV-specific memory B-cell responses were low to absent in the group of ART-treated subjects despite significant increases in CD4+ T-cells. As the generation of memory B-cells depends on T-cell help, the preferential loss of HIV-specific memory B-cells supports the concept of preferential infection and depletion of HIV-specific CD4+ T-cells described previously (Douek et al., 2002; Pitcher et al., 1999; Rosenberg et al., 1997).

While previous studies indicated that total memory B-cell numbers were not completely restored after initiation of ART (Cagini et al., 2008; De Milito et al., 2001; Moir et al., 2008b), the group of ART-treated subjects in this study had normal memory B-cell frequencies. This was possibly due to longer and more sustained viral suppression because the proportion of memory B-cells depends on the degree of viral suppression (De Milito et al., 2001). Whereas it has previously been reported that common recall antigens are frequently reduced in advanced HIV disease (Janoff et al., 1991; Kroon et al., 2000; Lane et al., 1983; Malaspina et al., 2005), memory B-cell responses to influenza and Ttx were normal in the group of individuals with late progressive disease in this study. This was not surprising as we tolerated variability in natural exposure to influenza virus, influenza and tetanus vaccine coverage and time point of booster immunizations. This approach may have obscured potential differences in influenza- and Ttx-specific immune responses between the groups. However, it highlights the significance of our findings with respect to the HIV-specific memory B-cells because despite maximal recovery of the total memory B-cells under ART and minimal damage to the influenza and Ttx-specific memory B-cells, HIV-specific memory B-cell responses were greatly reduced.

Gag and Env-specific antibodies were significantly lower in the ART-treated group indicating that short-lived plasma cells are maintaining antibody titers in viremic individuals. It is well known that Gag-specific antibodies decline during HIV disease progression (Binley et al., 1997; Brown et al., 1995; Cheingsong-Popov et al., 1991; Strathdee et al., 1995; Zwart et al., 1994) and the results of our study are consistent with this finding. A potential mechanism for the decline of Gag-specific antibodies in these studies that emerges from our memory B-cell findings is that viral replication early in infection induces vigorous memory B-cell and plasma cell activity. Subsequent destruction of HIV-specific helper T-cells causes depletion of the HIV-specific memory B-cells. As memory B-cells contribute to sustain long-term antibody production through periodic differentiation into antibody-secreting long-lived plasma cells (Crotty and Ahmed, 2004), depletion of Gag-specific memory B-cells may reduce the ability to renew the pool of HIV-specific plasma cells leading to a decline of antibodies to levels maintained by long-lived plasma cells primed early in infection.

As pointed out previously, Env-specific antibodies do not decline spontaneously during disease progression indicating that Gag- and Env-specific antibody responses are differentially regulated (Binley et al., 1997; Bonsignori et al., 2009). The drop of Env-specific antibodies in ART-treated individuals to basal levels indicates that Env-specific antibodies are partially secreted by short-lived plasma cells. This is consistent with recent observations that indicate maintenance of
Env-specific antibodies by short-lived plasma cells based on the short half-life of the Env-specific antibody titer in ART-treated individuals (Bonsignori et al., 2009).

A possible explanation how Env antigen-dependent antibody titers are maintained in this situation is that Env activates B-cells in a helper T-cell independent fashion. This has been considered previously (He et al., 2006) but the fact that Env does not have the characteristics of a typical T-cell independent antigen and that T-cell independent antibody responses are generally weak and IgM-mediated argue against this hypothesis (Binley et al., 1997). Alternatively, antibodies are generated in a helper T-cell dependent fashion. Possibly, the high variability of Env in contrast to Gag causes occasional recruitment of new Env-specific helper T-cells resulting in sufficient Env-specific T-cell help to support the generation of memory B-cells at quantities below the level of detection, and short-lived plasma cells.

The preferential loss of HIV-specific memory B-cells may be relevant to HIV disease progression. Although the study did not specifically address memory B-cells for neutralizing antibodies, the observation that gp120-specific memory B-cells were lost suggests that the depletion includes memory B-cells and affects the plasma cell pools relevant for viral inhibition. This facilitates genetic reversion of the virus to variants that were previously encountered and likely controlled by the immune system. This scenario has practical implications because it favors the concept of early antiviral intervention to preserve the HIV-specific memory B-cells (Gourley et al., 2004).

Materials and methods

Study participants and antigens

HIV-infected individuals with CD4+ T-cell counts above 350/μl, patients with progressive disease and CD4+ T-cell counts below 200/μl, and infected persons having experienced a fall in CD4+ T-cell counts to below 200/μl or an AIDS-defining illness that led to the initiation of ART were enrolled in this study. CD4+ T-cell counts were determined by flow cytometry. Uninfected healthy individuals were examined as controls. The study was approved by the Ethics Committee of the Faculty of Medicine at the University of Leipzig and informed consent was obtained from the study subjects.

Influenza-specific immunity was examined with recombinant influenza H3N2 nucleoprotein as maltose binding protein (MBP) fusion product. Tetanus toxoid (Novartis-Behring, Marburg, Germany) was used to determine tetanus-specific responses. Immune responses to HIV were tested with HIV-1_W136D gp120 expressed in Chinese hamster ovary cells (GlaxoSmithKline, Rixensart, Belgium) and HIV-1_EK22 Gag/p24 expressed in Escherichia coli as MBP fusion proteins.
protein (Reiche et al., 2009). MBP and bovine serum albumin served as control antigens.

B-cell isolation and flow cytometric characterization

Peripheral blood mononuclear cells (PBMCs) were prepared from heparinized blood by density gradient centrifugation using a ficoll cushion (Histopaque, Sigma-Aldrich, Inc.). B-cells were isolated with immunomagnetic anti-CD19 beads (Dynal® Pan CD19 kit, Invitrogen, Inc.). Cells were resuspended in RPMI-1640 medium (Invitrogen, Inc.) supplemented with 10% fetal calf serum (Biochrom AG), 1% non essential amino acids, 1% sodium pyruvate, 1% l-glutamin (Invitrogen, Inc.), penicillin (100 IU/l, Jenapharm GmbH & Co. KG) and streptomycin sulfate (100 mg/l Pharma Wernigerode GmbH). CD19-mediated selection of the cells had no negative effect on subsequent measurements (data not shown). B-cell purity and the distribution of B-cell subpopulations in PBMCs were analyzed by flow cytometry using anti-CD19, anti-CD20, anti-CD27 and anti-CD38 mAbs (BD Bioscience). Cell preparations contained on the average 97.2% B-lymphocytes.

Memory B-cell assay

PBMCs depleted of B-cells were treated with mitomycin C (50 mg/l, Merck KgA) and used as feeder cells. B-cells (2 × 10^5) and feeder cells (1.8 × 10^6) were cultured for 6 days at 37 °C with a mixture of mitogenic agents including pokeweed mitogen (PWM, Sigma-Aldrich, Inc.), Staphylococcus aureus lysate (Sigma-Aldrich, Inc.), interleukin (IL)-2 (Proleukin, Novartis AG), IL-10 (Hiss Diagnostics GmbH) and phosphorothioated CpG ODN-2006 (Metabion GmbH) to induce differentiation to plasma cells.

Antibody secretion was examined by ELISPOT using 96-well Multiscreen HA plates (Millipore, Inc.). Total numbers of antibody secreting cells were determined with plates coated with goat anti-human IgG recognizing IgG F(ab')2 (Dianova GmbH). Antibody-specific responses were examined with plates coated with Env/gp120 (2 μg/well), Gag/p24 (2 μg/well), influenza virus NP (NP, 2 μg/well) or Ttx (1 μg/well) in quadruplicates. Wells coated with BSA (1 μg/well) or MBP (1 μg/well) were used as controls. One hundred (lg-coated) wells or 50,000 (antigen-coated wells) cultured cells were added and the plates incubated for 20 h at 37 °C. Plates were washed and alkaline phosphatase-conjugated goat anti-human IgG recognizing IgG, A and M (Dianova GmbH) was added for two hours. The plates were developed using the AP Conjugate Substrate Kit (Bio-Rad Laboratories, Inc.). Spots were counted using an AID ELISPOT 04 plate reader (Autoimmun Diagnostika GmbH). Spots in wells with control proteins were subtracted from the number of spots in wells with antigen.

The frequency of antigen-specific cells was calculated as the percentage of total antibody-secreting cells according to the formula:

\[
\text{% Ag specific spots} = \frac{100 \times \text{Ag spots} - \text{control spots}}{50,000} \times 100
\]

No antibody secreting cells were detected with unstimulated B-cells after culture for 6 days.

Antibody ELISA

96-well microtiter plates (Greiner Bio-One GmbH) were coated overnight with recombinant protein (0.05–0.1 μg/well) in coating buffer (0.2 M NaHCO₃, pH 9.0). The plates were washed with phosphate-buffered saline/0.05%Tween-20 (PBST) and blocked with 3% BSA in PBST. Sera were serially threefold diluted with PBST/BSA, added to the wells and incubated for 2 h at 37 °C. HRP-conjugated rabbit anti-human IgG (Dako GmbH, diluted 1:6,000) was added for 1.5 h. Plates were incubated for 30 min at room temperature with developing solution. The reaction was stopped with H₂SO₄ and the OD at 450 nm was determined. Midpoint titers were calculated. Midpoint titers were defined as the reciprocal value of the serum dilution at half maximal OD after background subtraction (Binley et al., 1997). Pooled serum from HIV-infected individuals was used as reference and tested in each series of experiments to adjust the half maximal OD values. A fictitious antibody titer of 10 was taken for those sera that did not reach half-maximum OD at a dilution of 1:30.

Statistical analysis

Statistical analyses were performed using SPSS (version 12.0) software. We used the Kruskall–Wallis test to determine significance of differences when comparing more than two study groups at a time. If differences were significant, pairwise comparisons were performed with the Mann–Whitney test. A non-parametric Spearman test was used for regression analysis to calculate correlations of memory B-cell frequencies and antibody titers. The minimum level of significance was 0.05.

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