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Effect of macrophage depletion on viral DNA rebound following antiretroviral therapy in a murine model of AIDS (MAIDS)

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ABSTRACT

In the attempt to eradicate HIV-1 infection, a strategy to eliminate macrophages, one of the most important cellular reservoirs in sustaining virus replication during HAART, could be of great benefit in the suppression of viral rebound. Aware of the ability of clodronate to cause macrophage depletion, the effect of the administration of clodronate encapsulated in erythrocytes on disease progression and on viral rebound was evaluated in a murine model of AIDS (MAIDS). One group of LP-BM5 retroviral complex-infected C57BL/6 mice received oral administrations of azidothymidine and dideoxyinosine daily for 12 weeks; two other groups received in addition, either clodronate-loaded erythrocytes or free clodronate at 7–10 day intervals. At the end of the treatment, the three groups maintained parameters characterizing disease progression similar to those of uninfected mice and showed a significantly lower level of BM5d DNA than infected mice in all organs and cells tested. To assess the viral rebound, some animals were left for an additional 4 month period without any treatment. After this time, the BM5d DNA content in blood leukocytes increased in all groups, but the group having received clodronate-loaded erythrocytes, in addition to transcriptase inhibitors, showed a significant delay in viral rebound.

1. Introduction

Human immunodeficiency virus type 1 (HIV-1) eradication is one of the most important aims of novel antiretroviral therapies. In fact, highly active antiretroviral therapy (HAART) is able to suppress plasma viremia to undetectable levels, but it cannot eradicate HIV-1. This is mainly due to the existence of cellular reservoirs. The best characterized cellular reservoirs of HIV-1 are the latently infected resting memory CD4+ T cells, which, containing an integrated copy of HIV-1 genome, become an extremely stable HIV-1 reservoir that can survive in the patient for many years (Chun et al., 1997).

Resting memory CD4+ T cells may archive the wild type and drug-resistant HIV-1 strains, which appear throughout the years of HAART and can produce HIV-1 upon the subsequent activation by the antigen. Apart from latent HIV-1 infection of the resting memory CD4+ T cells providing long-term viral persistence, residual viral replication can also occur in these cells. Such residual replication contributes to viral rebound following cessation of HAART. Unfortunately, the latent reservoir decays slowly, with a half-life of up to 44 months, making it the principal, known obstacle in the eradication of HIV-1 infection (Sedaghat et al., 2008).

Another important cellular reservoir of HIV-1 is represented by the cells of macrophage lineage which were shown to be the first to be infected. Both blood monocytes and tissue macrophages can harbour HIV-1, although monocytes are significantly less susceptible than macrophages. Following infection, these cells are resistant to the cytopathic effects of HIV-1 and hence can persist in the tissues for a long period of time (even in the presence of HAART), support viral replication and contribute to the pathogenesis of disease. Whilst the kinetics of virus release from cells of macrophage lineage are slower in comparison to CD4+ T cells, the lack of HIV-induced cytopathicity enables macrophages to continue to secrete HIV-1 for a longer period of time (Chun and Fauci, 1999; Crowe and Sonza, 2000). Hence, tissue macrophages can act as long-term stable reservoirs for HIV-1 capable of disseminating the virus in other tissues, thus contributing to viral reservoir pools that ultimately lead to disease progression (Shehu-Xhilaga et al., 2005).

Viral compartmentalization has been reported not only within circulating immune cells, but also in various biological fluids and organs such as the brain, the cerebrospinal fluid (CSF), seminal plasma, and lymph nodes, where differences in the concentration of

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HIV inhibitors have been demonstrated (Pomerantz, 2003; Shehu-Xhilaga et al., 2005).

Recent findings have proposed the regulatory Nef protein, expressed by HIV-1 in the early stages of the infection cycle, to counteract the HIV-induced apoptosis in macrophages, thus having a role in the in vivo establishment of the HIV macrophage sanctuary (Olivetta and Federico, 2006). Some researchers have proposed STAT-1 and its activation upon HIV infection in macrophages as a possible survival factor contributing to the role of macrophages as persistent viral reservoirs (Magnani et al., 2003). Other authors have shown that the nerve growth factor (NGF), a neurokine involved in the survival, development and functions of peripheral and central neurons (Levi-Montalcini, 1987), is produced and released by HIV-1-infected macrophages during the first hours/days of infection (Garaci et al., 1999) and is implicated in inhibiting the cytopathic effects normally induced by this virus in other cells.

Besides this well established role in harboring the virus, acting as reservoirs of virions (Gartner et al., 1986), macrophages play an important role in the regulation of T cell apoptosis following HIV-1-infection. In fact, infected activated macrophages are able to trigger the apoptosis of uninfected T cells (Badley et al., 1997; Herbein et al., 1998), and to protect HIV-infected T cells from apoptosis favoring their recruitment and productive infection (Mahlknecht et al., 2000). All these findings clearly demonstrate that new therapeutic approaches targeting macrophages may be relevant in the therapy of HIV infection and, in particular, the transient and organ-specific suppression of their functions may be beneficial, eliminating the so-called cellular sanctuary that plays a role in HIV-1 persistence.

Macrophage depletion by means of liposome encapsulation of the bisphosphonate clodronate is a well documented technique to obtain the transient suppression of macrophage functions (van Rooijen and van Kesteren-Hendrikx, 2002). Clodronate (dichloromethylenebisphosphonate, Clod) is a non-nitrogen containing bisphosphonate widely used in the treatment of metabolic bone diseases such as hypercalcemia in malignant forms, and osteolytic diseases resulting from bone metastases (Fleisch, 1991), Paget's disease (Khan et al., 1996) and osteoporosis (Meunier et al., 1999). Depletion of macrophages through liposome-encapsulated clodronate is shown to prevent corneal graft rejection (Slegers et al., 2003), to increase graft survival after cardiac xenotransplantation in rats, preventing anti-graft antibody production (Koyamada et al., 2005), and to reduce neointimal hyperplasia and restenosis after mechanical arterial injury (Danenberg et al., 2002). Furthermore, it has been studied in several preclinical models of rheumatoid arthritis (van Lent et al., 1996) and neurological disorders (Tran et al., 1998). Moreover, applications of liposomes as drug delivery systems for the treatment of AIDS have already been reported (Lanao et al., 2007).

Despite the wide spectrum of applications and the encouraging evidence, the only application of liposome-encapsulated clodronate that has reached clinical use is in the depletion of synovial macrophages (Barrera et al., 2000).

As an alternative to liposomes, we propose erythrocyte-mediated drug delivery to selectively target clodronate to the macrophage compartment, exploiting the phagocytic capacity of macrophages.

As to erythrocytes (RBC) as drug carriers, these are readily available in large quantities, biocompatible (when autologous RBC are used), and completely biodegradable. They have a large capacity, so that a significant amount of drug can be encapsulated. Furthermore, it is possible to achieve a selective targeting of drugs to macrophages without affecting the non-phagocytic cells. To specifically target the drug-containing RBC to the phagocytic cells, in particular to the monocyte-derived macrophages, it is possible to artificially induce senescent signals on the RBC membrane, thus promoting the macrophages to exploit their physiologic role (Magnani et al., 1992).

In our previous experiences, we demonstrated that the administration of Clod-loaded RBC both in vitro and in vivo was able to eliminate macrophage cells (Rossi et al., 2005). Prompted by these considerations, we propose to take advantage of this strategy to obtain the elimination of viral macrophage reservoirs in a murine model of immunodeficiency. Murine AIDS (MAIDS) is a severe immunodeficiency syndrome induced by a complex of retroviruses, called LP-BM5 murine leukemia virus (MuLV), in susceptible strains of mice (Morse et al., 1992). LP-BM5 retroviral isolates consist of pathogenic defective murine retrovirus (BM5def) that requires replication-competent ecotropic helper viruses (e.g. BM5eco) for its entry into cells and proliferation in vivo. The LP-MB5 infection of C57BL/6 mice is considered an efficient mouse model for human AIDS due to the similarity of symptoms (Morse et al., 1992). Some of these similarities include development of profound immunodeficiency characterized by deficits in B- and T-lymphocyte function as well as deficiencies in macrophage functions. The early stage of the disease is characterized by early-onset hypergammaglobulinemia and polyclonal activation of lymphocytes and proliferation associated with progressive lymphoadenopathy and splenomegaly (Chattopadhyay et al., 1991). In advanced stages of the disease, infected mice become increasingly immunodeficient resulting in increased susceptibility to opportunistic infections (Doherty et al., 1995) and development of secondary neoplasm, especially B-cell lymphomas (Buller et al., 1987); in fact, both CD4 T cells and B cells are required for disease induction and progression. It has been reported that the main targets of initial LP-BM5 retrovirus infection are B cells, and to some extent, macrophages and T cells.

In this study, we have evaluated the effects of macrophage depletion induced by the periodical administration of Clod-loaded RBC in immunodeficient LP-BM5-infected C57BL/6 mice treated with two antiretroviral drugs (azidothymidine, AZT and dideoxyinosine, DDI) to suppress viral replication taking place in activating/proliferating CD4+ T cells. To this end, infected mice were treated with AZT plus DDI in drinking water and received weekly administrations of Clod by means of RBC or given free. Comparative studies were performed with mice treated with the two antiretrovirals alone or with only Clod. After 12 weeks of treatment, drug administrations were interrupted and the mice, left without drugs for a further four months, were used to assess some signs of the disease and the viral load. In fact, the primary aim of this study was to evaluate if the depletion of the macrophage population could have some effect on disease progression and, in particular, on viral rebound in mice treated with the combination of AZT + DDI.

2. Materials and methods

2.1. Virus and animals

The LP-BM5 viral mixture was kindly provided by Robert Yetter (Veterans Administration Hospital, Baltimore, MD, USA) and was maintained in a persistently infected SC-1 cell line as previously described (Mosier et al., 1987). Five-week-old female C57BL/6 mice (Harlan, Italy) were inoculated with 0.250 ml of the virus stock containing 0.33 Units (IU) of reverse transcriptase by means of two consecutive intraperitoneal injections at 24-h interval. Mice were housed in specified conditions at $22 \pm 1\,^{\circ}C$ with a 12-h light/dark cycle, $60 \pm 5\%$ humidity, and 12-h air changes/hour. The use and care of the animals used in this study were approved by the Ethical Committee of the University of Urbino "Carlo Bo", Italy.

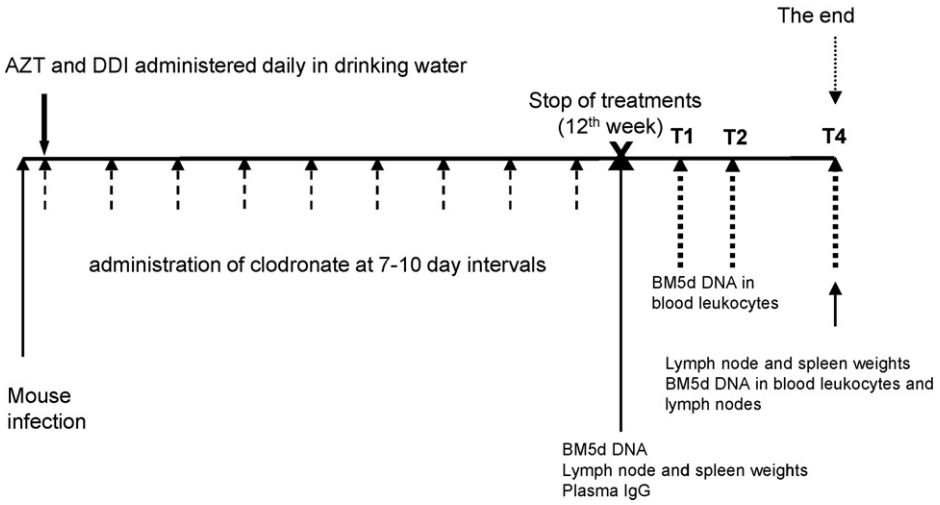

Fig. 1. Treatment schedule. T1, T2 and T4 = one, two and four months after stopping therapy.

2.2. Drugs and animal treatments

Azidothymidine (AZT) and dideoxyinosine (DDI) were obtained from Sigma (Milan, Italy); clodronate (Clod) was a gift from Roche Diagnostics GmbH (Mannheim, Germany).

AZT and DDI were administered ad libitum in drinking water at the concentrations of 250 and 134 mg/L, respectively, for the duration of the entire experiment (12 weeks) beginning 24 h after the first virus inoculation.

Clodronate was encapsulated into murine RBC by a procedure of hypotonic dialysis and isotonic resealing to a final concentration of 10 μmol/ml RBC (mM) as previously described (Rossi et al., 2005). Clod-loaded RBC were further processed to increase macrophage phagocytosis. In particular, Clod-loaded RBC suspension (10% Ht) was incubated with 1.0 mM $ZnCl_2$ and treated with 1.0 mM bis(sulfosuccinimidyl)suberate (BS3) for 15 min at room temperature under gentle mixing, then washed once in washing buffer containing 10 mM ethanolamine (pH 7.4), once in washing buffer containing 1% (w/v) bovine serum albumin (BSA) and finally in washing buffer (Rossi et al., 2005).

Free clodronate was dissolved in distilled water in 500 mM stock solution, adjusted to pH 7.4 with 6.0N sodium hydroxide and filter sterilized prior to use; finally, this solution was diluted in Hepes saline solution to a final concentration of 1 mM just before use.

Clodronate administration was performed as follows: infected mice receiving clodronate by means of RBC (I + AZT + DDI + Clod-RBC and I + Clod-RBC groups) were intraperitoneally (ip) injected with 250 μl of Clod-loaded RBC at 6% Ht corresponding to 56 μg clodronate/mouse, of which 2.8 μg of drug is free (5%), as calculated previously (Rossi et al., 2005); infected mice, receiving free clodronate (I + AZT + DDI + free Clod and I + free Clod groups), were injected intraperitoneally with 156 μl of 1 mM clodronate (56 μg Clod/mouse). Both Clod-loaded RBC and free clodronate were administered 9 times in all at 7–10 day intervals starting from the day after the first virus inoculation.

The groups of mice studied were seven and were marked as: control mice (uninfected and untreated, $n = 10$, CTR), infected mice (infected and untreated, $n = 20$, I), infected mice treated with AZT + DDI alone ($n = 20$, I + AZT + DDI) or with AZT + DDI plus Clod-loaded RBC ($n = 20$, I + AZT + DDI + Clod-RBC) or with AZT + DDI plus free clodronate ($n = 20$, I + AZT + DDI + free Clod), and infected mice treated with Clod-loaded RBC ($n = 20$, I + Clod-RBC) or with free Clod ($n = 20$, I + free Clod).

After 12 weeks of infection, the mice of each experimental group (except for 6 mice of groups I + AZT + DDI, I + AZT + DDI + Clod-RBC and I + AZT + DDI + free Clod) were euthanized by cervical dislocation after CO_2 anesthesia, and several parameters characterizing the progression of the disease (splenomegaly, lymphoadenopathy, hypergammaglobulinemia, and levels of BM5d DNA in lymph nodes, spleens, whole blood leukocytes, peritoneal and bone marrow macrophages) were evaluated. Lymphoadenopathy was assessed by investigating the mediastinal, brachial, lumbar, mesenteric and inguinal nodes.

At the first, the second and the fourth month after stopping therapy, 6 mice of the I + AZT + DDI, I + AZT + DDI + Clod-RBC and I + AZT + DDI + free Clod groups were re-tested for BM5d DNA content in whole blood leukocytes. Moreover, at the fourth month these mice were sacrificed and the analysis of the BM5d DNA content in lymph nodes, as well as the evaluation of lymph node and spleen weights, were performed. The complete experimental schedule is reported in Fig. 1.

Another group of control mice ($n = 6$) were ip injected with the same amount of Clod-loaded RBC and the hematological parameters were recorded (CTR + Clod-RBC group).

2.3. Plasma immunoglobulin G determination

At 12 weeks post-infection, blood was drawn from the retro-orbital sinus for determining plasma IgG levels via an enzyme-linked immunosorbent assay (ELISA) technique. Briefly, polystyrene microtiter plates (Dynex Technologies, Inc., Chantilly, VA, USA) were coated with goat anti-mouse IgG (Sigma BioSciences, St. Louis, MO, USA) diluted 1:100 in 0.135 M NaCl and incubated for 24 h at 37 °C. The plates were washed four times with 0.1% Tween 20 in 10 mM NaH_2PO_4, 154 mM NaCl, pH 7.0 (TPBS) and blocked with 5% bovine serum albumin (BSA) in PBS for 1 h at 37 °C. After four washings in TPBS, serial dilutions of murine plasma in 50 mM sodiumborate, pH 8.5, were added and incubated for 1 h at 37 °C. After four washings in TPBS, 100 μl of goat anti-mouse IgG-horseradish peroxidase (HRP) conjugate (Bio-Rad, Richmond, CA, USA), diluted 1:1000 in PBS, were added. After incubation for 1 h at 37 °C, plasma IgG levels were determined using a color development solution containing 2.2 mM *o*-phenylenediamine. Absorbance was measured at 492 nm on a Model 2550 enzyme immunoassay (EIA) reader (Bio-Rad). Absolute plasma IgG concentrations were obtained using known concentrations of standard mouse IgG.

2.4. Nucleic acid purification and real-time PCR

Total cellular DNA was isolated from frozen lymph nodes, spleens, whole blood leukocytes, peritoneal and bone marrow macrophages of uninfected, infected and infected/treated mice. For the peritoneal and bone marrow macrophages, the same protocol reported in our previous paper (Fraternale et al., 2002a) was used. The isolation of DNA from frozen tissues required an initial homogenization step in lysis buffer, while the white blood cells of the peripheral blood required an initial washing step with 0.1% NP-40 in saline solution. The subsequent steps of DNA extraction protocols were already reported elsewhere (Casabianca et al., 2004). Cellular DNAs were resuspended in TE buffer (1 mM Tris HCl, 0.1 mM EDTA, pH 8.0), digested for 30 min at 37 °C with DNase-free RNasi and quantified by a NanoDrop ND-1000 Spectrophotometer following the Manufacturer's instructions (NanoDrop Technologies, Wilmington, DE, USA). All samples were diluted in TE buffer to a final concentration of 20 ng/μl.

A real-time PCR assay was used to analyze BM5d DNA content (Casabianca et al., 2004). The PCR was performed starting from 100 ng (lymph nodes and peripheral blood) or 500 ng (spleens) of genomic DNA or from 5 μl of cellular lysate (macrophages). For spleens, lymph nodes and whole blood leukocytes, the PCR results were normalized to 500/100 ng of genomic DNA. The 18S rRNA housekeeping gene was used to generate a standard curve of dilutions of a known number of cells and the results were normalized to 10^6 macrophages.

A sample having a BM5d DNA level below the limit of quantification of the assay (2 copies) was defined as "undetectable" (UN) and for the statistical analysis we attributed a zero value.

2.5. Preparation of murine peritoneal macrophages

Murine peritoneal macrophages were collected from the peritoneum of C57BL/6 mice at the end of the 12 weeks of treatment. Cold phosphate-buffered saline (5 ml) containing 100 IU/ml penicillin, 100 mg/ml streptomycin and 5 IU/ml heparin was injected into the abdominal cavity, the peritoneum was massaged, and the peritoneal fluid containing the cell suspension was collected by a sterile syringe and transferred into sterile centrifuge tubes. After centrifugation, cells were suspended in RPMI 1640 medium supplemented with 20% FCS and placed in culture plates (PBI International, Italy) and incubated at 37 °C in a 5% CO_2 atmosphere for 18 h, which is a long enough time to permit macrophage adhesion. Non-adherent cells were removed, adherent cells were gently washed with PBS several times, then scraped, collected, and used for the evaluation of BM5d DNA.

2.6. Peripheral blood parameters

After 12 weeks of treatment, blood was withdrawn from mice of CTR, I, I + AZT + DDI + Clod-RBC, I + AZT + DDI + free Clod, I + Clod-RBC, I + free Clod and CTR + Clod-RBC groups to evaluate the hematological parameters by ABX Micros "OT" (ABX, Parc Euromedicine, Montpellier, France).

2.7. Statistical analysis

Statistical analysis of data was performed with the following nonparametric tests: the Mann–Whitney U test for comparison between continuous variables in two groups, the Kruskal–Wallis test for comparison among continuous variables in more than two groups, and the Wilcoxon signed rank test for paired analysis within the same group. Statistical significance was accepted for p values below 0.05.

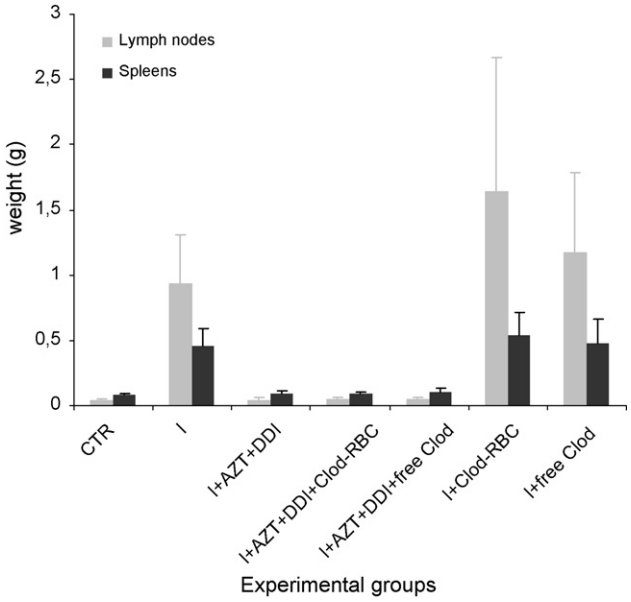

Fig. 2. Effect of drug treatment on inhibition of viral-induced lymphoadenopathy and splenomegaly in LP-BM5-infected mice at 12 weeks post-infection. LP-BM5-infected animals were treated daily for 12 weeks with AZT + DDI in drinking water ad libitum alone or in combination with free Clod or Clod-loaded RBC intraperitoneally injected as described in Section 2. Infected mice receiving free Clod and Clod-loaded RBC were used as controls. After 12 weeks of drug treatment, animals were sacrificed and their lymph nodes (including the mediastinal, brachial, lumbar, mesenteric and inguinal nodes) and spleens were weighed. Values represent the mean ± standard deviation of at least 10 animals per group. CTR = control uninfected mice. I = infected mice. I + AZT + DDI = infected mice treated with AZT + DDI. I + AZT + DDI + Clod-RBC = infected mice treated with AZT + DDI + Clod-RBC. I + AZT + DDI + free Clod = infected mice treated with AZT + DDI + free Clod. I + Clod-RBC = infected mice treated with Clod-RBC. I + free Clod = infected mice treated with free Clod.

3. Results

3.1. Inhibition of lymphoproliferation and splenomegaly after 12 weeks of treatment

Progression of murine AIDS is associated with extensive lymphoproliferation and is characterized in part by the development of splenomegaly (Morse et al., 1992). In this study, all infected animals developed peripheral lymphoadenopathy and splenomegaly. Spleen and lymph node weights from each experimental group are shown in Fig. 2. By the end of the 12 weeks of treatments, lymph nodes and spleens of infected mice weighed 22 and 6-fold higher than CTR mice, respectively (Mann–Whitney test, $p = 10^{-4}$).

Mice of the I + AZT + DDI group did not develop splenomegaly or lymphoadenopathy, since the spleen and lymph node weights were equivalent to those of control mice ($p > 0.05$), while the administration of clodronate alone (both free and by means RBC) caused an increase both in lymphoadenopathy and in splenomegaly ($p \leq 10^{-4}$). When the antiretroviral therapy was administered together with encapsulated clodronate or free drug, a significant inhibition of lymphoadenopathy and splenomegaly in the I + AZT + DDI + Clod-RBC and I + AZT + DDI + free Clod groups was observed (Fig. 2).

3.2. Inhibition of development of hypergammaglobulinemia

Early progression of MAIDS is also associated with polyclonal activation of B-cells resulting in increased levels of plasma immunoglobulins (IgG) (Klinman and Morse, 1989). Mean plasma IgG concentrations for each experimental group are reported in

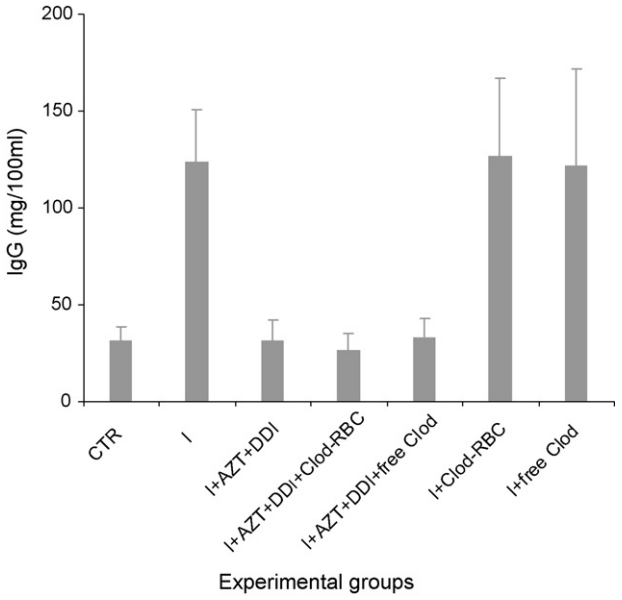

Fig. 3. Effect of drug treatment on inhibition of viral-induced hypergammaglobulinemia in LP-BM5-infected mice at 12 weeks post-infection. LP-BM5-infected animals were treated daily for 12 weeks with AZT + DDI in drinking water ad libitum alone or in combination with free Clod or Clod-loaded RBC intraperitoneally injected as described in Section 2. Infected mice receiving free Clod and Clod-loaded RBC were used as controls. After 12 weeks of drug treatment, animals were sacrificed and their plasma was collected. IgG plasma levels were measured by an enzyme-linked immunosorbent assay (ELISA) technique as described in Section 2. CTR = control uninfected mice. I = infected mice. I + AZT + DDI = infected mice treated with AZT + DDI. I + AZT + DDI + Clod-RBC = infected mice treated with AZT + DDI + Clod-RBC. I + AZT + DDI + free Clod = infected mice treated with AZT + DDI + free Clod. I + Clod-RBC = infected mice treated with Clod-RBC. I + free Clod = infected mice treated with free Clod.

Fig. 3 and confirm the results obtained regarding splenomegaly and lymphoadenopathy. By the end of the 12 weeks of drug treatment, hypergammaglobulinemia was evident in infected mice, which showed a four-fold increase in IgG levels versus control animals ($p < 10^{-4}$). All treatments including AZT + DDI were effective in significantly lowering the level of IgG to control values ($p > 0.05$), while in infected mice treated only with Clod-RBC or free Clod, hypergammaglobulinemia showed high levels similar to those of infected mice ($p > 0.05$). Again, the injection of Clod-loaded RBC in mice receiving AZT + DDI did not modify the IgG levels, which resembled CTR mice ($p > 0.05$).

3.3. Inhibition of BM5d DNA in target organs and cells after 12 week treatment

BM5d is the pathogenic virus in the LP-BM5 retroviral complex and its quantification with the previously developed real-time PCR assay is used as a marker for disease progression (Casabianca et al., 1998, 2004; Morse et al., 1992). In this study, after 12 weeks of drug treatment, the BM5d DNA levels in lymph nodes, spleens, peripheral blood leukocytes and in peritoneal and bone marrow macrophages of all infected groups were evaluated. All tested organs and cells of infected and treated mice belonging to I + AZT + DDI, I + AZT + DDI + Clod-RBC and I + AZT + DDI + free Clod groups displayed significantly lower levels of BM5d DNA copy number than those of infected mice (I group), while the decrease in BM5d DNA copy number in bone marrow macrophages of I + Clod-RBC and I + free Clod groups was not significant. In lymph nodes, spleen, blood leukocytes and peritoneal macrophages of I + Clod-RBC and I + free Clod groups, the BM5d DNA copy number was higher than in the infected group, reaching a significant value in peritoneal macrophages of the I + Clod-RBC group (Fig. 4). Because of the very low level of BM5d DNA in I + AZT + DDI, I + AZT + DDI + Clod-RBC and I + AZT + DDI + free Clod groups, the majority of spleen samples in these groups were classified as undetectable, thus it was necessary to test a greater amount of DNA (500 ng). The results showed that 57% and 28% of samples from I + AZT + DDI and I + AZT + DDI + Clod-RBC groups, respectively, were still undetectable, while all samples from I + AZT + DDI + free Clod group contained quantifiable levels of BM5d copy number. Similarly, the majority of peritoneal and bone marrow macrophage samples, showed undetectable BM5d DNA levels. Among the peritoneal macrophage samples, 75%, 88% and 78% from I + AZT + DDI, I + AZT + DDI + Clod-RBC and I + AZT + DDI + free Clod groups, respectively, were undetectable, whereas in bone marrow the percentages were 50, 44 and 56 for the same groups, respectively. The drug combinations I + AZT + DDI, I + AZT + DDI + Clod-RBC and I + AZT + DDI + free Clod appeared to be equally efficient in decreasing proviral load in all organs and cells analyzed (Kruskal–Wallis test, $p < 0.05$) except in the spleen (I + AZT + DDI vs. I + AZT + DDI + Clod-RBC, $p > 0.05$; I + AZT + DDI vs. I + AZT + DDI + free Clod, $p < 0.01$; I + AZT + DDI + Clod-RBC vs. I + AZT + DDI + free Clod, $p > 0.05$).

3.4. Inhibition of lymphoproliferation and splenomegaly 4 months after treatment interruption

Four months after therapy was interrupted, only lymph node weights of I + AZT + DDI + Clod-RBC group remained similar to those of the control group (Kruskall–Wallis test, $p > 0.05$), while weights of I + AZT + DDI and I + AZT + DDI + free Clod groups showed significant differences from control mice (Fig. 5). As regards spleen weights, those of mice belonging to I + AZT + DDI and I + AZT + DDI + Clod-RBC groups remained similar to those of control mice ($p > 0.05$), while in the I + AZT + DDI + free Clod group, spleen weights increased becoming significantly different. However, no significant differences in weight values were observed for lymph nodes and spleens when comparing the three groups.

3.5. Inhibition of BM5d DNA in target organs and cells 1, 2 and 4 months after treatment interruption

BM5d DNA content in blood leukocytes was analyzed at the first (T1), the second (T2) and the fourth month (T4) after stopping therapy in six mice of each I + AZT + DDI, I + AZT + DDI + Clod-RBC and I + AZT + DDI + free Clod group to determine whether a delay in the reappearance of the virus in circulation would occur (Fig. 6). After 4 months, the mice were sacrificed and the BM5d DNA in lymph nodes was analyzed. At T1, the BM5d DNA copy numbers in blood leukocytes of I + AZT + DDI, I + AZT + DDI + Clod-RBC and I + AZT + DDI + free Clod groups were similar (median value: 10, 11, 10 copies/100 ng of DNA, respectively), while at T2 a five-fold increase in BM5d DNA and a slight increase in the median value were found in I + AZT + DDI and I + AZT + DDI + free Clod groups (median value: 52, 17 copies/100 ng of DNA, respectively). In the I + AZT + DDI + Clod-RBC group, the median value was similar to that of T1 (median value: 12 copies/100 ng of DNA). At T4 the median values of I + AZT + DDI, I + AZT + DDI + Clod-RBC and I + AZT + DDI + free Clod groups were 414, 273, and 388, respectively. The viral rebound of I + AZT + DDI and I + AZT + DDI + free Clod groups observed at T4 was statistically significant when compared with T1 (Wilcoxon test, $p = 0.03$; $p = 0.03$) whereas no significant increase in BM5d DNA levels was observed for the I + AZT + DDI + Clod-RBC group ($p = 0.13$) (Fig. 6). Moreover, no significant differences in BM5d DNA copy number were observed when the three groups were compared at T4 (Kruskal–Wallis test,

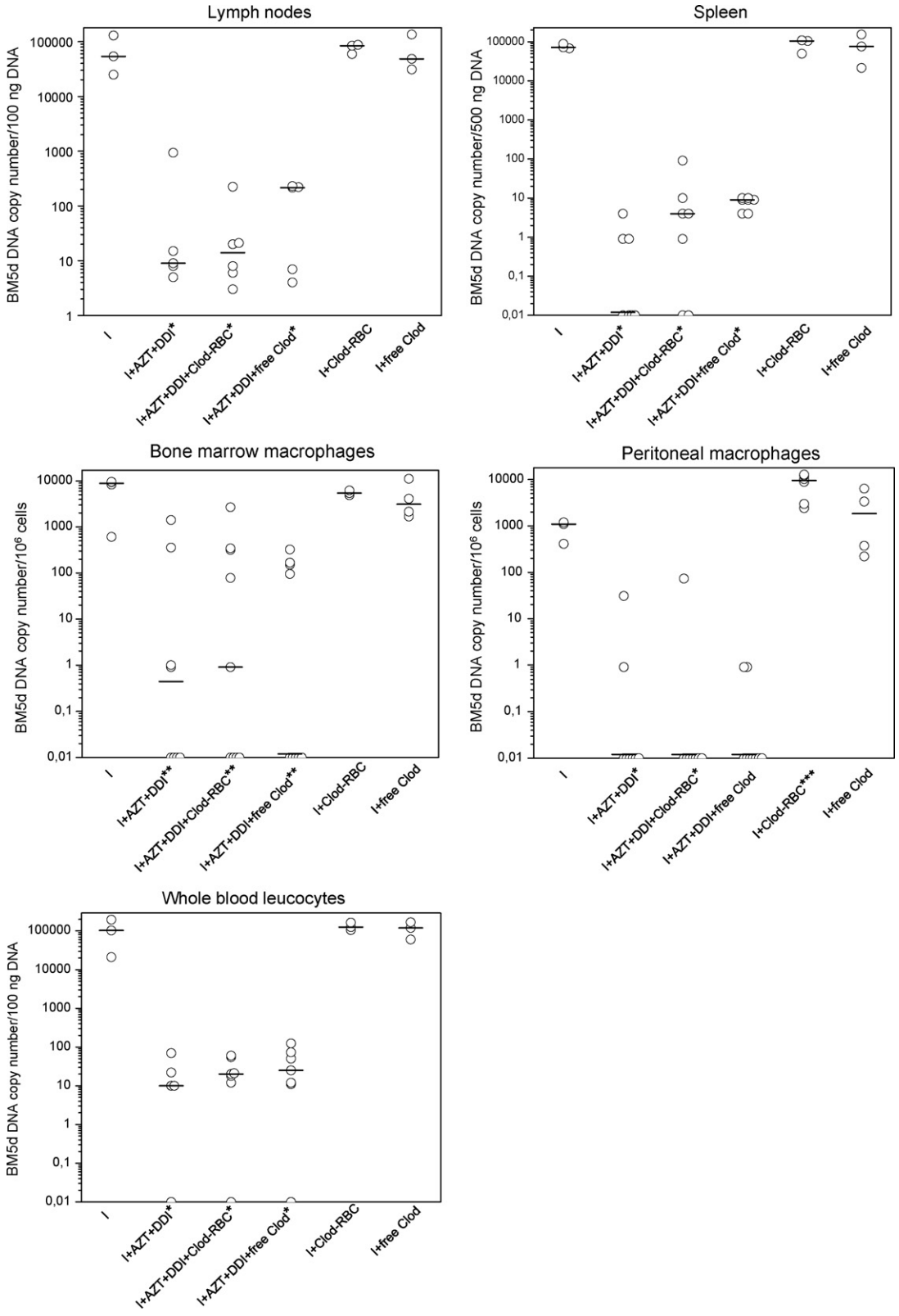

Fig. 4. Effect of different drug treatments on BM5d DNA content in target organs and cells of LP-BM5-infected mice at 12 weeks post-infection. In the logarithmic graphics, BM5d DNA copy number values are reported; they are calculated by quantitative real-time PCR as described in Section 2. The data were analyzed statistically using the Mann–Whitney test. Bars show the median for each group. The real-time PCR performed in uninfected and untreated mice gave negative results. The undetectable samples appear on the *x*-axis. The number of analyzed samples was in the range of 3–9. $^{*}p < 5 \times 10^{-2}$ vs. infected mice. $^{**}p < 1 \times 10^{-2}$ vs. infected mice. $^{***}p < 1 \times 10^{-4}$ vs. infected mice.

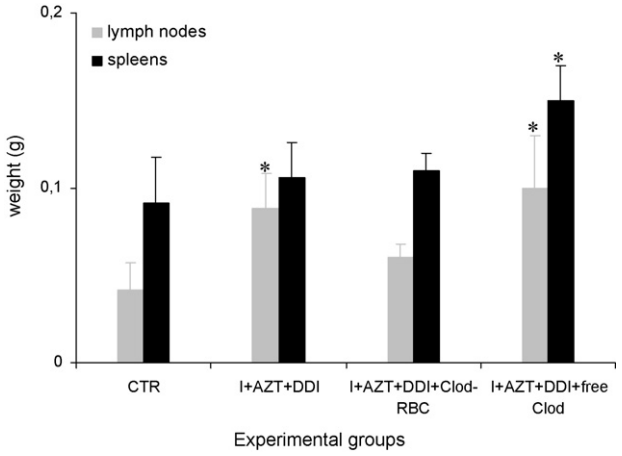

Fig. 5. Effect of drug treatment on inhibition of viral-induced lymphadenopathy and splenomegaly in LP-BM5-infected mice 4 months after stopping therapy. LP-BM5-infected animals were treated daily for 12 weeks with AZT + DDI in drinking water ad libitum alone or in combination with free Clod or Clod-loaded RBC intraperitoneally injected as described in Section 2. After 12 weeks, the drug treatment was stopped and 6 mice in each group treated with AZT + DDI (I + AZT + DDI, I + AZT + DDI + Clod-RBC and I + AZT + DDI + free Clod) were observed for 4 successive months. After this period, they were sacrificed and their lymph nodes (including the mediastinal, brachial, lumbar, mesenteric and inguinal nodes) and spleens were weighed. Values represent the mean ± standard deviation of 5 animals per group. CTR = control uninfected mice. CTR = control uninfected mice. I + AZT + DDI = infected mice treated with AZT + DDI. I + AZT + DDI + Clod-RBC = infected mice treated with AZT + DDI + Clod-RBC. I + AZT + DDI + free Clod = infected mice treated with AZT + DDI + free Clod. $^{*}p < 0.05$ vs. CTR group.

$p = 0.24$). The results obtained for lymph nodes at T4 after interrupting therapy revealed that in the I + AZT + DDI group ($n = 6$), one mouse had an undetectable number of BM5d DNA copies and one mouse a number of >5 000 copies (median value: 15; range: 0–5 495). In the I + AZT + DDI + free Clod group ($n = 6$), one mouse revealed a number of copies >20 000, and the median value was 11 (range: 10–22 240). For the I + AZT + DDI + Clod-RBC group ($n = 6$), the median copy number of BM5d DNA was 9 (range: 8–18). Statistical analysis however showed no significant differences among the three groups (Kruskal–Wallis test, $p = 0.57$).

3.6. Peripheral blood parameters

Hematological parameters of all groups of mice (I, I + AZT + DDI + Clod-RBC, I + AZT + DDI + free Clod, I + Clod-RBC, I + free Clod and CTR + Clod-RBC groups) are not significantly different ($p > 0.05$, Kruskal–Wallis test) compared to control mice thus showing no signs of toxicity (Table 1). In particular, following the repeated ip administrations of Clod-RBC (nine consecutive in all), mice of the CTR + Clod-RBC group showed only a slight increase in RBC counts, hematocrit value and hemoglobin content compared to CTR mice; furthermore, an increase in WBC counts was evidenced, as observed by other authors (Giuliani et al., 2007) following 4 weekly clodronate-liposome administrations. On the contrary, LP-BM5 infection causes a slight decrease in all hematological parameters compared to control mice, except in MCH and MCHC values; the prolonged injections of Clod-RBC (I + Clod-RBC group) did not modify RBC parameter values, while only WBC cell count tends toward control values.

4. Discussion

Although treatment of HIV-1 infected individuals with HAART has effectively decreased viral load to undetectable levels, efforts to eliminate HIV-1 from these individuals have been unsuccessful due to the presence of stable, latent viral reservoirs, represented by CD4+ T lymphocytes and macrophages (Crowe et al., 2003; Kulkosky and Bray, 2006). In particular, it is known that these viral reservoirs can be crucial in sustaining virus replication during HAART therapy, thus their elimination could have a role in delaying the viral rebound after the suspension of therapy. Previously, we have proven that the selective administration of clodronate by means of RBC to macrophages causes transient macrophage depletion (Rossi et al., 2005). In this paper, we show that the depletion of macrophages through injections of clodronate-loaded RBC in a murine model of immunodeficiency (C57BL/6 mice infected with LP-BM5 virus) is able to delay the viral rebound once antiretroviral

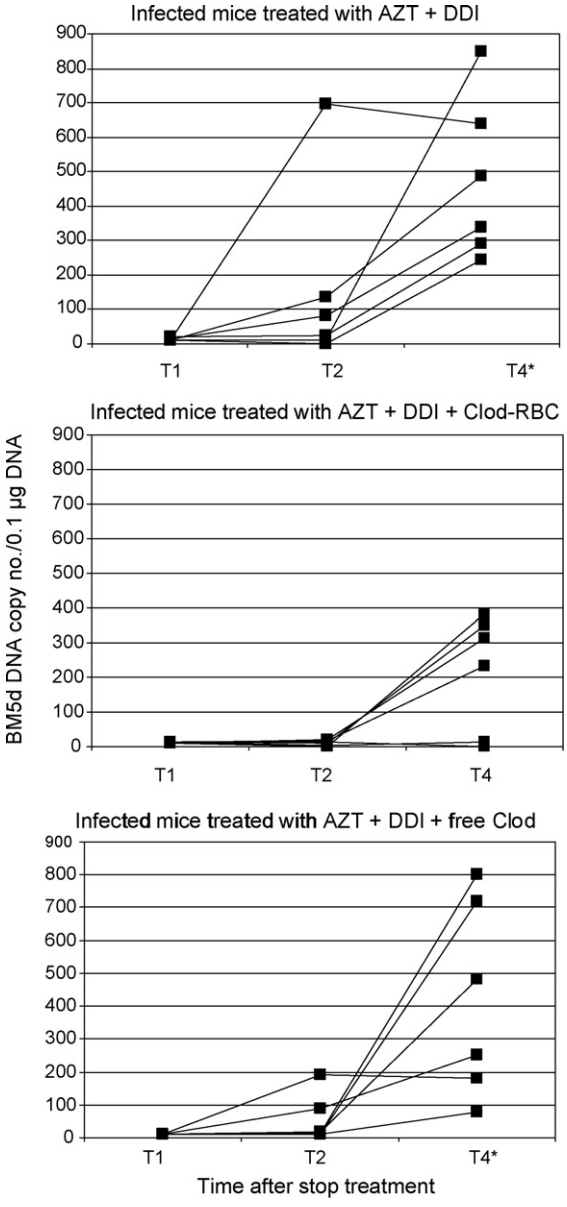

Fig. 6. BM5d DNA copy number in the blood of I + AZT + DDI, I + AZT + DDI + Clod-RBC and I + AZT + DDI + free Clod groups one (T1), two (T2) and four (T4) months after stopping treatments (12 weeks). At each time, 100 ng of genomic DNA extracted from blood leukocytes was subjected to quantitative real-time PCR as reported in Section 2. For each group, five mice were analyzed. $^{*}p = 0.03$ vs. T1 (Wilcoxon test). The viral rebound between T1 and T4 in the I + AZT + DDI + Clod-RBC group is not statistically significant (Wilcoxon test). The Kruskal–Wallis test shows no significant differences in BM5d DNA copy number among the I + AZT + DDI, I + AZT + DDI + Clod-RBC and I + AZT + DDI + free Clod groups at the fourth (T4) month.

Table 1
Hematological parameters in C57BL/6 at 12 weeks post-infection with LP-BM5 virus.

Drug treatments	RBC (cells × 10^6/μl)	Ht (%)	Hb (g/dL)	MCV (fl)	MCH (pg/cell)	MCHC (g/dL)	WBC (cells × 10^3/μl)
CTR	10.4 ± 1.3	44.4 ± 6.3	15.3 ± 2.0	43 ± 1	14.7 ± 0.4	34.4 ± 1.4	4.4 ± 2.3
Infected (I)	9.9 ± 0.7	42.2 ± 3.7	14.9 ± 0.9	43 ± 2	15.1 ± 0.9	35.6 ± 3.0	3.1 ± 1.8
I + AZT + DDI + Clod-RBC	8.6 ± 1.1	40.2 ± 3.8	13.1 ± 1.0	47 ± 1	15.3 ± 0.7	32.6 ± 0.5	4.0 ± 2.3
I + AZT + DDI + free Clod	7.85 ± 0.7	37.5 ± 3.4	12.3 ± 0.8	47 ± 2	15.3 ± 0.8	32.9 ± 0.5	2.5 ± 0.8
I + Clod-RBC	9.5 ± 1.6	40.2 ± 5.5	13.9 ± 2.0	42 ± 1	14.8 ± 1.0	34.7 ± 2.2	4.3 ± 2.1
I + free Clod	9.9 ± 1.3	42.9 ± 5.3	14.7 ± 1.8	42 ± 2	14.7 ± 0.4	34.2 ± 1.5	5.3 ± 5.7
CTR + Clod-RBC	11 4 ± 0.6	47.7 ± 4.1	16.4 ± 0.7	42 ± 1	14.4 ± 0.5	34.6 ± 2.4	6.3 ± 2.6

All values are means ± standard deviations of 6 animals. RBC, Red blood cells; Ht, hematocrit; Hb, hemoglobin; MCH, mean cellular hemoglobin; MCHC, mean cellular hemoglobin concentration; WBC, white blood cells.

therapy (AZT + DDI) has been stopped. AZT and DDI combination therapy was chosen since it efficiently reduced AIDS signs in mice (mainly lymphoadenopathy, splenomegaly and hypergammaglobulinemia) (Fraternale et al., 2002a,b). Moreover, the addition of drug-loaded RBC to nucleoside analogue therapy (AZT + DDI) to protect both macrophages and lymphocytes had already proven to give additive and/or synergistic effects in delaying the progression of MAIDS and to inhibit BM5d DNA content in LP-BM5-infected organs (Fraternale et al., 1996a, 1999; Magnani et al., 1998).

In the present study, at 12 weeks post-infection, none of the groups treated with the antiretroviral therapy (AZT plus DDI, with or without clodronate) developed lymphoadenopathy, or splenomegaly, or hypergammaglobulinemia, since they exhibited lymph node and spleen weights (Fig. 2), and IgG values (Fig. 3) similar to controls. In detail, our results show that in infected mice treated with the antiretroviral therapy, the additional treatment with Clod (both free, I + AZT + DDI + free Clod, and by means of RBC, I + AZT + DDI + Clod-RBC) did not modify the positive AZT and DDI effects. This is noteworthy since the injection of clodronate alone (free or encapsulated) in infected mice caused a worsening of the signs of the disease, especially with regard to lymphoadenopathy. We can assume that this worsening may be due to an enhanced viral replication. This can be explained as a consequence of a substantial depletion of immune cells (macrophages) followed by the stimulation of other cells having a role in the immune response (e.g. CD4 + T lymphocytes), in which viral production is higher than in macrophages.

Obviously, no improvement in the disease could be expected following macrophage depletion in infected mice receiving no drugs able to protect lymphocytes from infection. Furthermore, at the end of the 12th week, upon treatment with the antiretroviral drugs (I + AZT + DDI, I + AZT + DDI + Clod-RBC and I + AZT + DDI + free Clod), a very high reduction in the proviral DNA content in all target organs (lymph nodes, spleens) and cells (peritoneal and bone marrow macrophages and whole blood leukocytes) was achieved, as shown by the significantly lower levels of BM5d DNA copy numbers than those of infected mice obtained for the three groups (Fig. 4). On the contrary, the groups treated only with Clod-RBC (I + Clod-RBC group) or free Clod (I + free Clod group) showed levels of proviral DNA similar to those of infected mice. Consequently, the percentages of inhibition of BM5d DNA content in all organs and cells analyzed, reached more than 99% in I + AZT + DDI, I + AZT + DDI + Clod-RBC and I + AZT + DDI + free Clod groups, while, no reduction was found in organs, blood cells and peritoneal macrophages examined in I + Clod-RBC and I + free Clod groups. The bone marrow macrophages of these latter groups exhibited a reduction of 39% and 65% as compared to infected mice (I group), respectively. Based on these results, it is clear that the AZT plus DDI combination alone was able to completely inhibit MAIDS progression during the 12-week treatment, making it impossible to appreciate the additive effect of macrophage depletion. Probably, the fact that mice were infected with 0.33 Units of LP-BM5 reverse transcriptase instead of the one Unit used in previous studies justifies the complete MAIDS inhibition reached by AZT plus DDI (Fraternale et al., 1996a, 2002b).

Furthermore, some mice belonging to the three groups treated with the antiretroviral therapy have been kept under observation to monitor the development of MAIDS until four months after the suspension of the treatment. Even though no significant differences in lymph node and spleen weights were reported among the three groups at the fourth month, it is noteworthy that only in the group treated with Clod-RBC (I + AZT + DDI + Clod-RBC group), both lymph node and spleen weights were not significantly different from the CTR group, supporting the hypothesis of a slow rebound of the disease in those mice in which macrophages had been depleted by Clod-RBC.

During the four months after suspension of the treatments, BM5d DNA content in whole blood leukocytes varied among the AZT + DDI treated groups. In fact, although the BM5d DNA copy numbers were similar in the three groups one month after stopping therapy and viral rebound was evident in all three groups after the fourth month, the increase in DNA copy number from month 1 to month 4 was statistically significant only in I + AZT + DDI and I + AZT + DDI + free Clod groups ($p = 0.03$), while in the I + AZT + DDI + Clod-RBC group it was not ($p = 0.16$). This difference may be attributed to the targeted treatment based on Clod-loaded RBC, which, although not selectively eliminating the macrophage population, reduced the number of important viral reservoirs necessary for viral replication and storage. When Clod was injected as a free drug, a higher viral rebound was observed, confirming the reduced efficacy of free Clod in depleting macrophages as compared to Clod-loaded RBC delivery, as already reported (Rossi et al., 2005).

Thus, AZT + DDI therapy combined with RBC-encapsulated clodronate was able to slow viral rebound in peripheral blood more efficiently when compared to the treatment with AZT and DDI alone. These data are encouraging despite the non-selective macrophage depletion obtained using clodronate. In fact, by this approach both infected and non-infected macrophages are eliminated, as demonstrated by the administration of clodronate-loaded RBC to human macrophages, where both in HIV-infected and non-infected macrophages, the same percentage of depletion was obtained (unpublished results). We previously demonstrated that the lympholytic drug fludarabine, when administered by means of RBC, was able to selectively deplete only infected macrophages, sparing those which were not infected via a pSTAT-1 dependent pathway both in an in vitro study (Magnani et al., 2003) and in SIV-infected Mangabeys (Cervasi et al., 2006). This approach is valid for the delivery of fludarabine to human macrophages since human RBC possess the enzymatic apparatus necessary to transform fludarabine into its active triphosphate derivative (Fraternale et al., 1996b). Unfortunately, mouse RBC enzymes are not able to phosphorylate fludarabine to its active triphosphate form, but rather de-phosphorylate the drug, which in turn, is released from the cells. As a consequence of this different metabolism, encapsulating the

lympholytic drug in RBC and targeting them to macrophages of LP-BM5 mice is not advantageous. Instead of using fludarabine to reach macrophage depletion, although having different mechanisms of action (Frank et al., 1999; Lehenkari et al., 2002), we chose to encapsulate the bisphosphonate drug clodronate into mouse RBC, being aware of its ability to cause macrophage depletion.

In conclusion, the results reported in this paper confirm the efficacy of macrophage depletion in delaying viral rebound and reveal the possibility of using a simple animal model of retroviral infection, such as MAIDS, to collect important data supporting the role of engineered RBC as a drug delivery system to reach viral reservoirs.

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