Administration of Fludarabine-Loaded Autologous Red Blood Cells in Simian Immunodeficiency Virus-Infected Sooty Mangabeys Depletes pSTAT-1-Expressing Macrophages and Delays the Rebound of Viremia after Suspension of Antiretroviral Therapy


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A major limitation of highly active antiretroviral therapy is that it fails to eradicate human immunodeficiency virus (HIV) infection due to its limited effects on viral reservoirs carrying replication-competent HIV, including monocytes/macrophages (M/M). Therefore, therapeutic approaches aimed at targeting HIV-infected M/M may prove useful in the clinical management of HIV-infected patients. In previous studies, we have shown that administration of fludarabine-loaded red blood cells (RBC) in vitro selectively induces cell death in HIV-infected M/M via a pSTAT1-dependent pathway. To determine the in vivo efficacy of this novel therapeutic strategy, we treated six naturally simian immunodeficiency virus (SIV)-infected sooty mangabeys (SMs) with either 9-[2-(R)-(phosphonomethoxy)propyl]adenine (PMPA) only, fludarabine-loaded RBC only, or PMPA in association with fludarabine-loaded RBC. The rationale of this treatment was to target infected M/M with fludarabine-loaded RBC at a time when PMPA is suppressing viral replication taking place in activated CD4+ T cells. In vivo administration of fludarabine-loaded RBC was well tolerated and did not induce any discernible side effect. Importantly, addition of fludarabine-loaded RBC to PMPA delayed the rebound of viral replication after suspension of therapy, thus suggesting a reduction in the size of SIV reservoirs. While administrations of fludarabine-loaded RBC did not induce any change in the CD4+ or CD8+ T-cell compartments, we observed, in chronically SIV-infected SMs, a selective depletion of M/M expressing pSTAT1. This study suggests that therapeutic strategies based on the administration of fludarabine-loaded RBC may be further explored as interventions aimed at reducing the size of the M/M reservoirs during chronic HIV infection.

The hallmark of human immunodeficiency virus (HIV) infection in humans is a progressive depletion of CD4+ T lymphocytes that is due to either direct killing of infected cells (viral cytopathicity) or activation-induced apoptosis of uninfected bystander cells (9, 30, 46, 47). In the majority of HIV-infected individuals, the introduction of highly active antiretroviral therapy (HAART) induces suppression of viral replication, which is associated with at least partial reconstitution of the CD4+ T-cell pool and ultimately results in a significant decline in mortality/morbidity. Unfortunately, HAART does not eradicate the infection, and in fact, after its suspension, HIV RNA rapidly rebounds to pretherapy levels, indicating the existence of refractory reservoirs (17, 31, 40, 42, 49). Cellular sources of reemerging HIV include latently infected resting CD4+ T cells as well as monocytes/macrophages (M/M) and dendritic cells. Infected M/M persist throughout the course of disease as long-term stable reservoirs able to produce large amounts of virions and to disseminate them in other cells and tissues (6, 22, 24, 33). As such, persistence of active viral replication in M/M despite prolonged antiretroviral treatment represents a major obstacle to HIV eradication (8, 25, 34) It should also be noted that currently available drugs have poor antiviral activity against the M/M compartment (1). Collectively, these considerations define the rationale for designing therapeutic approaches aimed at eliminating, or at least reducing, the levels of active HIV replication in M/M in HIV-infected patients.

In previous studies, we found that activation of the JAK/STAT (signal transducers and activators of transcription) pathways is associated with survival of HIV-infected M/M (28). STAT proteins comprise a family of transcription factors that are normally located in the cytoplasm as inactive forms and that upon binding of extracellular signaling proteins (i.e., cytokine and growth factors) to specific cell surface receptors enter the nucleus and regulate several cellular events, including activation, differentiation, proliferation, cell survival, and apoptosis. Cytoplasmic STAT proteins are activated by tyrosine phosphorylation, a transient and tightly regulated process that results in dimerization, nuclear translocation, and transcriptional activation of genes that control the cellular response (2, 27, 41). We found that levels of phosphorylated STAT1 (pSTAT1) were three- to fivefold greater in in vitro HIV-infected M/M compared to uninfected M/M (28). Therefore, we proposed that, at least in this in vitro system, the
enhanced expression and phosphorylation of STAT1 may play a role in the development of a persistent state of active HIV replication in M/M. This hypothesis also implies that pSTAT1 may be a useful target in designing treatment strategies to selectively eliminate persistently infected M/M and thereby provides a rationale for the use of 9-(β-D-arabinofuranosyl)-2-fluoroadenine (fludarabine), a potent anti-leukemic nucleoside analog that also acts as a potent inhibitor of cells with a low-growth fraction that overexpresses pSTAT1 (12). To selectively deliver fludarabine to M/M we used red blood cells (RBC) as carriers by taking advantage of the peculiar phagocytic capacity of M/M. Our group has validated the use of RBC as specific carriers for M/M with various compounds and in several different in vitro and in vivo models (13, 15, 28, 29). The fludarabine-loaded RBC technology allows us to overcome the nonspecific toxic effect of fludarabine on actively replicating cells (such as lymphocytes), which are more sensitive than resting cells (such as M/M) to the DNA polymerase inhibition induced by the drug (20, 21). Following its loading in human RBC, fludarabine is converted by the RBC kinases to the corresponding triphosphate derivative, which is the active form of the drug (14). Fludarabine loaded within RBC strongly and selectively contributes, in vitro, to the induction of cell death in HIV-infected M/M, without any cytotoxic effect upon nonphagocytosing cells (28).

In the present study we analyzed for the first time, in an in vivo model of simian immunodeficiency virus (SIV) infection, the safety and efficacy of a new therapeutic protocol based on the weekly administration of autologous RBC loaded with fludarabine, to clear the infected M/M compartment, in combination with daily administration of 9-[2-((phosphonomethoxy)propyl)adenine (PMPA), to suppress viral replication in vivo model of simian immunodeficiency virus (SIV) infection, pathic effect upon nonphagocytosing cells (28).

Drug resistance to antiretrovirals in patients strongly and selectively contributes, in vitro, to the induction of cell death in HIV-infected M/M, without any cytotoxic effect upon nonphagocytosing cells (28).

Lymph node biopsies. For lymph node biopsies, the skin over the inguinal or axillary lymph node was prepared for aseptic surgery. A small skin incision was made over the node, and blunt dissection was used to isolate and remove the node. The subcutis and skin were then closed with absorbable sutures. Either ketamine (10 mg/kg) or Telazol (4 mg/kg) was used for anesthesia. The frequency of administration was determined by the veterinarian performing the procedure so as to maximize animal safety and comfort.

SIV viral load. Quantitative real-time reverse transcription-PCR assay to determine SIV viral load was performed as described previously (44). Encapsulation of fludarabine in SM RBC. SM RBC were loaded with fludarabine by a procedure involving hypotonic dialysis, isotonic resealing, and reannealing, as previously described (28). Some modifications were performed to adapt the procedure for SM RBC. In particular, compared to the loading procedure followed for human RBC, the osmolarity of dialysis buffer was increased and the dialysis time was prolonged. Targeting of fludarabine-loaded RBC to macrophages was achieved by inducing band 3 clustering as described previously (29). To study fludarabine metabolite formation in SM RBC, fludarabine-loaded RBC were incubated at 0.5% hematocrit (Ht) in RPMI 1640 medium containing fetal calf serum. At different times (0, 2 h, 16 h, 2 days, and 5 days) during incubation at 37°C in a 5% CO2 atmosphere and under sterile conditions, 4-mL aliquots were processed to determine the concentrations of fludarabine and its metabolites. Briefly, fludarabine-loaded RBC were extracted with perchloric acid and analyzed by high-performance liquid chromatography as described previously (14), while media were submitted to solid-phase extraction using Isolute C18 columns (International Sorben Technology, Mid-Glamorgan, United Kingdom) according to the manufacturer’s instructions before being analyzed by high-performance liquid chromatography.

Flow cytometry for surface and intracellular markers. Cells derived from peripheral blood (PB) and LN were isolated by gradient centrifugation. Four-color flow cytometric analyses were performed according to standard procedures, using a panel of monoclonal antibodies (MAbs) that were originally designed to detect human molecules but that we and others have shown to be cross-reactive with SMs (43, 44). The MAbs used included CD8-phycocerythrin (clone SK1), CD4-phycoerythrin (clone SP34-2), CD4-peridinin chlorophyll protein (clone L200), and Ki67-fluorescein isothiocyanate (FITC) (clone B56) (BD PharMingen, San Diego, CA) and CD14-FITC (clones RMO52 and MY4) (Beckman Coulter, Miami, FL). Samples used for Ki67 were surface stained, fixed and permeabilized using the PharMingen CytoFix/Perm kit, and stained intracellularly with the proper MAb and control. Flow cytometric acquisition and analysis of samples was performed on at least 100,000 events on a FACScalibur flow cytometer driven by the CellQuest software package (Becton Dickinson). Analysis of the acquired data was performed using FlowJo software (Tree Star, Inc., Ashland, OR).

Western blot analysis. Expression levels of STAT1, STAT3, and STAT5 and of their corresponding phosphorylated forms in peripheral blood- and lymph node-derived cells were measured by Western blotting. Briefly, cells were lysed for 20 min on ice with 20 mM HEPES (pH 7.9), 25% glycerol, 0.42 M NaCl, 0.2 mM EDTA, 1.5 mM MgCl2 containing 0.5% NP-40, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 1 mM sodium fluoride, and 1 mM sodium orthovanadate. From the total protein extract, 30 μg was fractionated by 7% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then was electrically transferred to a nitrocellulose membrane. Blots were incubated with anti-STAT1, anti-STAT3, and anti-STAT5 (1:1,000; Santa Cruz Biotechnology Inc., Santa Cruz, CA) and with anti-pSTAT1, anti-pSTAT3, and anti-pSTAT5 (1:1,000; Cell Signaling Technology, Danvers, MA) MAbas overnight at 4°C, followed by incubation with horseradish peroxidase-conjugated secondary antibody. Blots were treated with
enhanced chemiluminescence reagents, and proteins were detected and quanti-
tated with the ChemiDoc system (Bio-Rad, Hercules, CA). Equal protein load-
ing was confirmed by the level of actin protein present in the membrane tested
with antiactin antibody (1:500; Sigma).

**Histology, immunohistochemistry (IHC), and indirect immunofluorescence
analysis.** Sections were cut from paraffin-embedded paraformaldehyde-fixed
lymph node tissues, deparaffinized, and stained with hematoxylin and eosin. M/M
and pSTAT1-positive cells were detected with NCL-Macro (1:100; Novacastra
Laboratories, Newcastle, United Kingdom) and pSTAT1 monoclonal antibodies
(1:100; Zymed Laboratories, San Francisco, CA), respectively. For indirect im-
munofluorescence staining, sections prepared as described above were deparaf-
fined, hydrated, treated for antigen unmasking, and incubated with the first
monoclonal primary antibody, anti-pSTAT1, according to the manufacturer’s in-
teructions. Subsequently, the samples were incubated overnight 4°C with the second
colonal primary antibody, anti p24 (1:100; Diatheva, Fano, Italy). Primary anti-
bodies were detected with FITC- and tetramethyl rhodamine isocyanate-conjugated
goat anti-mouse or -rabbit antibodies (1:200; Sigma, St. Louis, MO). DNA was
stained with 4,6-diamino-2-phenylindole (DAPI) (0.1 g/ml). Images were col-
lected on a Leica DML equipped with a charge-coupled device camera.

**Statistical analysis.** The Mann-Whitney U test was used for comparisons
between groups, while correlations involving different sets of data within the
same group were analyzed using either the standard Pearson correlation coeffi-
cient or Spearman’s rank correlation test. Significance was assessed at \( P \)
values of \(<0.01\) and \(<0.05\) levels. All analyses were performed using SAS software.

**FIG. 1.** Stat1 activation in SIV-infected sooty mangabeys. (a) Expression of total and phosphorylated Stat1 in peripheral blood- and lymph
node-derived cells from three SIV-infected (SIV+ ) and three uninfected (SIV− ) SMs was measured by Western blot analysis. Histograms show
the means ± standard deviations of band intensity, expressed as number of pixels. (b) The expression of Stat1 in lymph node tissues isolated from
three SIV-infected and three uninfected SMs was determined by IHC. The results are reported as number of pStat1-positive cells per mm² of tissue.
In panels a and b statistical analysis were performed between SIV-infected (black bars) and uninfected (gray bars) animals, and significant values
are marked by asterisks.
RESULTS

Chronic SIV infection in SMs is associated with increased STAT1 activation in peripheral blood- and lymph node-derived cells. Our previous in vitro experiments indicated that HIV infection of M/M results in a marked increase of pSTAT1 expression in these cells, a finding that suggests that these cells may be specifically targeted by fludarabine in vivo. To determine whether increased STAT-1 expression is also present during in vivo SIV infection, we analyzed by Western blotting the intracellular concentrations of STAT1 and pSTAT1 in PB- and LN-derived cells isolated from three SIV-infected and three uninfected SMs. All infected animals, whose characteristics are listed in Table 1, were studied during the chronic stage of infection. SIV-infected SMs express significant higher level of pSTAT1 than uninfected ani-

FIG. 2. Treatment protocol and longitudinal analysis of viral load in SIV-infected sooty mangabey. (a) SMs were assigned to three groups treated with different protocols. Animals were studied for 64 days. At various time points through the study, plasma, peripheral blood, and lymph node were collected. Flu, fludarabine. (b) Temporal trend of viral load at various time points during the study in SIV-infected SMs treated with fludarabine (gray dotted line), PMPA (dark gray dashed line), and fludarabine plus PMPA (black). Viral load is presented as mean ± standard deviation of SIV RNA copies/milliliter of plasma of the two animals included in each group. Numbers in square bracket at day 36 and day 43 represent the fold increase in viral load compared to day 28 (end of treatment) found in group 2 and group 3.
mals, in both PB (P < 0.01)- and LN (P < 0.05)-derived cells (Fig. 1a). In the PB of SIV-infected SMs we also found a significant increase (P < 0.05) in the level of pSTAT1. As shown in Fig. 1b, the number of pSTAT1-positive cells per mm² of LN tissue (measured by IHC) was increased in SIV-infected SMs compared to uninfected animals. Importantly, the majority of these cells show morphological features that are typical of tissue macrophages. It is of note that no significant differences in the levels of either total or phosphorylated STAT3 and STAT5 were found between SIV-infected and uninfected SMs in either PB- or LN-derived cells (data not shown). In all, these results indicate that, similarly to what was observed in vivo HIV-infected M/M, PB- and LN-derived mononuclear cells of chronically SIV-infected SMs manifested increased pSTAT1 expression.

Study design. Six naturally SIV-infected SMs were assigned to three groups and treated with the following protocols (Fig. 2a): two with four weekly administrations of fludarabine-loaded autologous RBC (group 1), two with PMPA at a dose of 30 mg/kg per day for 28 consecutive days (group 2), and two with 30 mg/kg PMPA per day for 28 consecutive days and four weekly administrations of fludarabine-loaded autologous RBC (group 3). One uninfected SM was treated with fludarabine-loaded autologous RBC as a control. Animals were studied for 64 days, during which time we collected plasma, PB, and LN at different time points (Fig. 2a). Fludarabine was encapsulated in SM RBC at a final concentration of 1.84 ± 1.23 μmol/ml RBC. The study of fludarabine metabolites shows that in SM RBC, fludarabine is phosphorylated to the same active forms (fludarabine diphosphate and fludarabine triphosphate) that were found in human RBC (14). At the time of loading, both the fludarabine diphosphate and fludarabine triphosphate forms are present, and at 18 h, fludarabine triphosphate represents 23% of all metabolites (data not shown). Three SIV-infected and three uninfected SMs were left untreated and used for ex vivo analysis (Table 1).

Administration of fludarabine-loaded RBC in SMs does not induce any significant toxic side effect. We carefully examined the possibility of toxic side effects associated with the above-described protocol of administration of fludarabine-loaded autologous RBC alone or in association with PMPA. In particular, we evaluated several basic hematological parameters (white blood cells, RBC, hemoglobin, Ht, mean cell volume, mean cell hemoglobin, and lymphocyte number) at different time points (days 7, 14, 21, 30, 36, 43, and 64) in all treated SMs, and we found no significant difference from the pretreatment levels. Table 2 summarizes the hematological values before (day 0) and 30 days after the initiation of treatment, i.e., after four fludarabine administrations and 28 days of PMPA treatment. Similarly, no changes were observed in terms of either body weight variation or animal behavior, and all treated SMs are alive and in good health 2 years after treatment suspension. These results indicate that in vivo administration of fludarabine-loaded autologous RBC alone or in combination with PMPA is a safe intervention in this nonhuman primate model of SIV infection.

Changes in viral replication induced by administration of fludarabine-loaded autologous RBC in SIV-infected SMs. We next sought to evaluate the effects of the three therapeutic approaches used (i.e., PMPA only, fludarabine-loaded RBC only, or the combination of the two) on the level of virus replication. To this end, we longitudinally measured plasma viral load in the six SIV-infected SMs at different time points before and after treatment. As shown in Fig. 2b, we found that daily PMPA administration was followed, in all four treated SMs, by a significant decline in viral load to levels below the limit of detection of our assay between day 14 and day 28 of treatment. As expected, the two SMs treated with fludarabine-loaded RBC alone maintained viral replication at levels similar to the baseline, thus confirming that the administration of fludarabine within RBC does not act on actively replicating CD4+ T cells, which are known to be a major source of virus production in SIV-infected SMs (G. Silvestri, unpublished observations). Importantly, we observed that after interruption of PMPA treatment, the two SIV-infected SMs treated only with PMPA showed a faster rebound of viral replication than the animals that received PMPA-plus-fludarabine-loaded RBC. In particular, the plasma viral load was significantly higher in the SMs treated with PMPA only at days 36 (P = 0.05) and 43 (P = 0.06) of follow-up. This faster rebound can be better perceived by calculating the ratio of viral loads between days 36 and 43 and the end of treatment (day 28). At days 36 and 43 animals treated with only PMPA increased their viral load, compared to that at day 28, by 7.75- and 1.482-fold, respectively. In contrast, in SMs treated with PMPA-plus-fludarabine-loaded RBC, these variations were 1.81- and 215-fold. It is of note that one animal (FMr) treated with PMPA-plus-fludarabine-loaded RBC maintained a lower level of viremia until day 64, i.e., the end of the study (data not shown). This delayed rebound in viral replication found after PMPA suspension in fludarabine-treated SMs suggests that this combined therapeutic approach
may have effectively reduced the number of viral reservoirs in naturally SIV-infected SMs.

**Treatment with fludarabine-loaded RBC does not induce any change in the levels of T cells and their main subsets.** The delayed viral replication rebound found in SIV-infected SMs treated with fludarabine-loaded RBC and PMPA after PMPA suspension is compatible with two possible mechanisms: a reduction in the levels of total and actively proliferating CD4 T cells and/or a depletion in the number of long-lasting SIV reservoirs (i.e., M/M). To discriminate between these mechanisms, we performed a detailed four-color flow cytometric analysis of PB- and LN-derived cells isolated from the six SIV-infected SMs treated with the three different protocols. First, we measured the levels of CD4 and CD8 T cells in PB and LN and found that fludarabine treatment did not significantly change either the absolute number or the percentage of these T-cell subsets compared to those in PMPA-treated animals (Fig. 3). We next measured the levels of proliferating, i.e., Ki-67-positive, T cells and found that treatments with PMPA alone or PMPA-plus-fludarabine-loaded RBC had comparable effects on the levels of proliferating CD4+ T cells in PB and LN (Fig. 3). In particular, the percentage of circulating CD4+ Ki67+ T cells decreased slightly during the 28 days of PMPA administration, as expected given the suppression of viral replication. After suspension of therapy, SIV-infected SMs treated with PMPA alone experienced an increase in the levels of proliferating CD4+ T cells that temporally correlated with the rebound in viral replication. Interestingly, SMs treated with fludarabine-loaded RBC and PMPA also exhibited an increase in the levels of CD4+ Ki67+ T cells after PMPA suspension (Fig. 3), despite the delay in viral rebound (Fig. 2b). These data suggest that the different kinetics of viral replication rebound in SMs treated with PMPA alone and those treated with PMPA-plus-fludarabine-loaded RBC are unlikely to be due to direct effects of fludarabine on the actively proliferating CD4+ T-cell compartment. In addition, no significant differences were found in the proliferation levels of PB- or LN-derived CD8+ T lymphocytes between SIV-infected SMs that were treated with PMPA alone or with PMPA-plus-fludarabine-loaded RBC (Fig. 3). Surprisingly, SMs treated with fludarabine experienced a significant increase only in the levels of proliferating CD4+ and CD8+ T cells, which was maintained until the end of the study (day 64). The increment in the pool of Ki-67+ T cells in this group may be related to immune system activation induced by the administration of RBC in the context of active viral replication (no PMPA treatment). In the other two groups, this phenomenon may be counterbalanced by the decreased levels of T-cell proliferation that follow the PMPA-dependent control of viral replication.

**Fludarabine-loaded RBC induce a significant reduction in both the percentage and absolute number of M/M in SIV-infected SMs.** To test the hypothesis that the delayed rebound in viral replication observed in SIV-infected SMs treated with PMPA-plus-fludarabine-loaded RBC is related...
to a decreased M/M reservoir, we measured the percentage and absolute number of M/M in PB of treated SMs by multiparametric flow cytometric analysis. In particular, we calculated the absolute number and percentage of cells that, based on forward scatter/side scatter, were gated as monocytes and also expressed CD14. We found that SIV-infected SMs treated with fludarabine-loaded RBC manifested a significant reduction in both the percentage and number of monocytes independent of PMPA administration (Fig. 4). In contrast, SMs treated with PMPA alone did not experience any significant decline in the levels of monocytes (Fig. 4). Importantly, SMs treated with fludarabine-loaded RBC plus PMPA showed significantly lower numbers of monocytes during days 28 to 42 after initiation of therapy, i.e., when the different rebound in viral replication was found (Fig. 2b), than did animals treated with PMPA alone. It is of note that treatment with fludarabine-loaded RBC did not modify levels of monocytes in the uninfected SM (data not shown), suggesting that only M/M from SIV-infected animals became a target for fludarabine.

**Administration of fludarabine-loaded RBC in SMs depletes SIV-infected M/M via a pSTAT1-dependent pathway.** To further test the hypothesis that fludarabine-loaded RBC specifically deplete M/M that overexpress pSTAT1 as a result of their infection with SIV, we measured the fraction of macrophages expressing pSTAT1 in LN tissue by IHC. We analyzed LN samples collected from the six SIV-infected SMs at three different time points, i.e., before (day 0) and at day 14 and day 31 after initiation of therapy. pSTAT1-positive cells were identified by staining with a specific monoclonal antibody, whereas...
M/M were identified by morphological features. When treated with fludarabine-loaded RBC, and independently of PMPA administration, SIV-infected SMs showed a significant reduction \( (P < 0.01) \) in the amount of M/M expressing pSTAT1 (Fig. 5A). Interestingly, no effects were found in SIV-infected animals treated with PMPA alone (Fig. 5A) or in the uninfected SM treated with fludarabine-loaded RBC (data not shown). Finally, to confirm that macrophages that overexpressed pSTAT1 and that are selectively depleted by fludarabine treatment are SIV infected, we performed, by fluorescence microscopy, p24 and pSTAT1 double staining in LN tissue isolated from infected animals. The results confirm that the vast majority of pSTAT1-positive cells are SIV-infected M/M, as indicated by the morphology of these cells and the expression of p24 viral protein (Fig. 5B to F). In all, these findings are compatible with the hypothesis that treatment with fludarabine-loaded autologous RBC is able to selectively deplete SIV-infected macrophages via a pSTAT1-dependent pathway in SMs.

**DISCUSSION**

The introduction of HAART has resulted in a major reduction of virus load and in a significant decline in mortality and morbidity among HIV-infected individuals (17, 18, 32, 49). Unfortunately, HAART is unable to eradicate HIV infection due to its limited effects on viral reservoirs carrying replication-competent HIV (5, 7, 10, 35). The existence of HIV reservoirs represents a major obstacle to eradication of HIV with current antiretroviral regimens and underlines the importance of exploring novel therapeutic approaches that specifically target
these reservoirs. In this perspective, an ideal anti HIV therapy should include drugs that protect new cells from infection (i.e., HAART) as well as agents able to reduce the reservoir of infected cells. The first reservoir that has been identified consists of resting CD4+ memory T lymphocytes, and most efforts were aimed at designing treatment strategies able to reduce them (11, 26). Other reservoirs, however, and in particular M/M, are also involved in maintaining HIV infection in chronically treated patients and must be considered when protocols aimed at eradicating HIV infection are designed. Several lines of evidence indicate the importance of M/M in virus persistence: (i) macrophage and microglia in the central nervous systems of patients with AIDS dementia complex were identified as one of the first nonlymphocyte cell lineages that support viral replication (23, 48); (ii) in rhesus monkeys infected with a highly pathogenic virus such as SHIVpDL12, tissue M/M sustain high virus loads for several months after the depletion of CD4+ T cells (22); (iii) in HIV-infected persons the number of infected M/M, compared to infected CD4+ T lymphocytes, increases substantially over time (33); and (iv) M/M become exposed to virus very early in the infection, are relatively resistant to cytotoxic effects of HIV, and are involved in antigen presentation to CD4+ T cells (24, 50).

The results of our previous in vitro study indicate that survival of human primary M/M during HIV infection is associated with activation of STAT1 pathway and that the HIV-infected M/M can be depleted with fludarabine, a nucleoside analog able to act on low-growth-fraction cells overexpressing pSTAT1 (12, 28). In the present study, we describe the results of an experiment aimed at testing the feasibility, safety, and efficacy of an antiviral therapeutic protocol based on the administration of PMPA in association with fludarabine-loaded RBC. We hypothesized that this approach may be of interest since it conceivably targets viral replication in both recently infected proliferating CD4+ T cells (by PMPA) and persistently HIV-1-infected M/M (by fludarabine). To the best of our knowledge, this is the first time that this type of study has been conducted in vivo in a primate model of HIV infection.

Although the current study was designed as an exploratory pilot trial involving a limited number of animals, several interesting indications emerged from the analysis of the results obtained. First, we found that, similarly to what was observed in pathological HIV infection in humans, natural, nonpathogenic SIV infection of SMs resulted in a significant activation of STAT1 signaling in M/M, thus providing a rationale for the use of fludarabine as a “reservoir-reducing” agent in this nonhuman primate model. Second, we found that administration of fludarabine-loaded autologous RBC is a feasible therapeutic approach that is not associated with any relevant toxicity in nonhuman primates. Third, we determined that SIV-infected SMs treated with four fludarabine-loaded RBC administrations plus PMPA showed a delayed rebound in SIV replication after suspension of treatment compared to animals treated with PMPA alone. It is worth noting that in both animals treated with fludarabine-loaded RBC plus PMPA, viremia remained at lower levels until day 43, i.e., 22 days after the last fludarabine administration, with one animal maintaining the lower level of SIV replication until the end of the study (day 64). Fourth, we found that the two SIV-infected SMs treated with fludarabine-loaded RBC only (i.e., without PMPA) did not show any change in viral replication, confirming that this intervention selectively targets M/M and is unlikely to have any effect on nonphagocytosing cells, such as the actively replicating CD4+ T cells that are the major source of SIV production. Consistently, the absence of any measurable effects of fludarabine-loaded RBC plus PMPA on the level of activated/proliferating CD4+ T cells suggests that the delayed rebound in viral replication is not due to direct effects of the drug on CD4+ T cells. Importantly, the potential efficacy of the presently described therapeutic protocol was confirmed by the observed changes in the M/M compartment. In SMs, treatment with fludarabine-loaded RBC induced a significant reduction in the absolute number of SIV-infected M/M, which remained significantly lower than that in animals treated only with PMPA at the time when the different kinetics of viral rebound were observed. It is of note that treatment with fludarabine-loaded RBC selectively depleted macrophages in chronically SIV-infected SMs but not in the uninfected control. This specificity may be due to the fact that M/M overexpressed pSTAT1 following SIV infection, as demonstrated by IHC and indirect fluorescence analysis of LN tissues.

In conclusion, this study suggests that anti-HIV therapeutic strategies based on the combination of antiretroviral (PMPA) and cytotoxic (fludarabine) drugs targeted to M/M may be well tolerated and effective in both suppressing the level of ongoing viral replication and reducing the reservoirs of infected cells.

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