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Naoya Uchida
National Institutes of Health

Aylin Bonifacino
National Institutes of Health

Allen E. Krouse
National Institutes of Health

Mark E. Metzger
National Institutes of Health

Gyorgy Csako
National Institutes of Health

See next page for additional authors

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Accelerated lymphocyte reconstitution and long-term recovery after transplantation of lentiviral-transduced rhesus CD34$^+$ cells mobilized by G-CSF and plerixafor

Naoya Uchida[a], Aylin Bonifacino[b], Allen E. Krouse[b], Mark E. Metzger[b], Gyorgy Csako[c], Agnes Lee-Stroka[d], Ross M. Fasano[d], Susan F. Leitman[d], Joseph J. Mattapallil[e], Matthew M. Hsieh[a], John F. Tisdale[a], and Robert E. Donahue[b]

[a]*Molecular and Clinical Hematology Branch, National Heart Lung and Blood Institute/National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Md., USA;* [b]*Hematology Branch, National Heart Lung and Blood Institute, National Institutes of Health, Rockville, Md., USA;* [c]*Department of Laboratory Medicine, Clinical Center, National Institutes of Health, Bethesda, Md., USA;* [d]*Department of Transfusion Medicine, Clinical Center, National Institutes of Health, Bethesda, Md., USA;* [e]*Department of Microbiology and Immunology, Uniformed Services University of Health Sciences, Bethesda, Md., USA*

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Objective. **Granulocyte colony-stimulating factor (G-CSF) in combination with plerixafor produces significant mobilization of CD34$^+$ cells in rhesus macaques. We sought to evaluate whether these CD34$^+$ cells can stably reconstitute blood cells with lentiviral gene marking.**

Materials and Methods. **We performed hematopoietic stem cell transplantation using G-CSF and plerixafor-mobilized rhesus CD34$^+$ cells transduced with a lentiviral vector, and these data were compared with those of G-CSF and stem cell factor mobilization.**

Results. **G-CSF and plerixafor mobilization resulted in CD34$^+$ cell yields that were twofold higher than yields with G-CSF and stem cell factor. CD123 (interleukin-3 receptor) expression was greater in G-CSF and plerixafor-mobilized CD34$^+$ cells when compared to G-CSF alone. Animals transplanted with G-CSF and plerixafor-mobilized cells showed engraftment of all lineages, similar to animals who received G-CSF and stem cell factor−mobilized grafts. Lymphocyte engraftment was accelerated in animals receiving the G-CSF and plerixafor-mobilized CD34$^+$ cells. One animal in the G-CSF and plerixafor group developed cold agglutinin-associated skin rash during the first 3 months of rapid lymphocyte recovery. One year after transplantation, all animals had 2% to 10% transgene expression in all blood cell lineages.**

Conclusions. **G-CSF and plerixafor-mobilized CD34$^+$ cells accelerate lymphocyte engraftment and contain hematopoietic stem cell capable of reconstituting multilineage blood cells. These findings indicate important differences to consider in plerixafor-based hematopoietic stem cell mobilization protocols in rhesus macaques.**

Hematopoietic stem cell (HSC) transplantation is a therapeutic strategy for hematologic malignancies, immunodeficiency states, nonmalignant hereditary and acquired hematologic diseases, and inherited metabolic disorders. HSC transplantation is an ideal platform for delivering stem cell therapies, such as ex vivo cell manipulation for gene therapy applications [1,2]. HSCs can be mobilized into peripheral blood and collected by apheresis. This method of peripheral blood stem cell collection is frequently used clinically for obtaining HSCs for allogeneic and autologous HSC transplantation. Currently, injection of granulocyte colony-stimulating factor (G-CSF) is the clinical standard for mobilization of human HSCs [1]. Sufficient numbers of HSCs for transplantation, however, are not obtained from all donors by G-CSF mobilization. Plerixafor (also known as AMD3100 or Mozobil) represents a new alternative agent for mobilization of HSCs. This low molecular weight, highly charged compound ($C_{28}H_{54}N_8$) inhibits chemokine stromal cell−derived

Offprint requests to: Robert E. Donahue, V.M.D., Hematology Branch, 5 Research Court, Building 5RC, 1B06, MSC-3315, Rockville, MD 20892; E-mail: donahuer@nhlbi.nih.gov

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factor-1α binding to CXC chemokine receptor 4 (CXCR4) to interrupt adherence of HSCs to the stem cell niche [3].

Rhesus macaques are Old World monkeys frequently used in preclinical studies evaluating HSC transplantation [4–6]. In the rhesus transplantation model, the combination of G-CSF and stem cell factor (SCF) is a standard strategy to mobilize $CD34^+$ cells in which $CD34^+$ cell numbers are approximately twofold greater than those achieved with use of G-CSF alone [5]. Recently, we found that G-CSF and plerixafor mobilization in rhesus macaques increased $CD34^+$ cell yields approximately three- to fivefold more than mobilization with G-CSF alone, plerixafor alone, or G-CSF and SCF combined together [7]. These $CD34^+$ cells demonstrated different gene expression profiles in each mobilization strategy, suggesting that the composition of mobilized $CD34^+$ cells is dependent on the mobilization protocol [7].

Based on these differences, we hypothesized that G-CSF and plerixafor-mobilized $CD34^+$ cells might include different hematopoietic progenitor cells when compared to previous mobilization strategies. In this study, we performed HSC transplantation using transduced rhesus $CD34^+$ cells that were mobilized by G-CSF and plerixafor to evaluate whether these $CD34^+$ cells would reconstitute long-term hematopoiesis differently and to determine what kind of progenitor cells were contained in the mobilized population of $CD34^+$ cells.

Materials and methods

Rhesus HSC transplantation

Rhesus HSCs were obtained after five daily injections of 10 μg/kg recombinant human G-CSF (Amgen, Inc., Thousand Oaks, CA, USA), followed by a single injection of 1 mg/kg plerixafor (Genzyme Corporation, Cambridge, MA, USA), or five-daily injections of both 10 μg/kg G-CSF and 200 μg/kg SCF (Amgen, Inc.). Two to four hours after the last dose of mobilizing agent, the $CD34^+$ cells were harvested by leukapheresis and immunoselection was performed as described previously [4,5]. The rhesus $CD34^+$ cells were cultured in serum-free X-VIVO10 media (Lonza, Allendale, NJ, USA) containing SCF, FMS-like tyrosine kinase 3 ligand, and thrombopoietin (all at 100 ng/mL; R&D Systems, Minneapolis, MN, USA) on fibronectin CH-296–coated (RetroNectin, TaKaRa, Otsu, Shiga, Japan) cell culture flasks for 1 day. These cells were then transduced with enhanced green fluorescent protein (EGFP)–expressing lentiviral vector at multiplicity of infection 50 in the same media and cytokines for 1 day [8,9]. The rhesus macaques to be transplanted received a total of 10 Gy total body irradiation, delivered as 5 Gy daily on 2 consecutive days. The transduced $CD34^+$ cells were infused into these irradiated rhesus macaques. Complete blood cell counts, cell surface markers of differentiation, and EGFP expression rates in circulating blood cells were assessed periodically for a minimum of 1 year.

Lentiviral vector preparation

We developed a chimeric HIV1-based lentiviral vector system (χHIV vector), which included simian immunodeficiency virus–capsid instead of HIV1-capsid, for efficient transduction of rhesus $CD34^+$ cells [8]. The vesicular stomatitis virus glycoprotein pseudotyped χHIV vector, including the EGFP-expressing cassette under the control of a murine stem cell virus–long term repeat promoter, was prepared and its titer quantitated as described previously [10,11]. The basic HIV1 vector plasmid was kindly provided by Dr. Arthur Nienhuis (St. Jude Children's Research Hospital, Memphis, TN, USA) [12,13].

Flow cytometry analysis

Transduced rhesus $CD34^+$ cells were cultured for an additional 3 to 4 days in vitro and EGFP expression rates were evaluated by FACSCalibur (BD Biosciences, Franklin Lakes, NJ, USA). After transplantation, we analyzed EGFP expression and cell surface markers in rhesus peripheral blood cells and bone marrow cells using phycoerythrin (PE) or allophycocyanin (APC)-conjugated antibodies (CD3-PE, clone 10D12; CD4-PE, clone M-T466; CD8-PE, clone BW135/80; CD20-PE, clone LT20; CD33-PE, clone AC104.3E3; CD34-PE, clone 563; CD41a-PE, clone HIP8; CD56-PE, clone AF12-7H3; CD71-APC, clone L01.1; RBC, clone T3G6; mouse IgG1-PE, clone A85-1; BD Pharmingen). After immunoselection, $CD34^+$ cells were assessed for purity using a mouse anti-human CD34-PE (clone 563; BD Pharmingen), for interleukin (IL)-3 receptor expression (CD123) using a mouse anti-human CD123-APC (clone 7G3; BD Pharmingen), and for CD45RA using an anti-human CD45RA-FITC (clone 5H9; BD Pharmingen).

EGFP expression on lymphocytes was determined in peripheral blood, lymph nodes, and rectal mucosa collected post-transplantation using multicolor flow cytometry. Cells were isolated from these tissues as described previously [14–16]. Freshly isolated cells were labeled with CD3-Cy7-APC, CD4-PB, CD8-Alexa700, CD45RA-ECD, CD28-Cy5PE, and/or CD95-APC for phenotypic analysis of T cells. To determine the frequency of EGFP-expressing B-cell subsets, cells were labeled with CD20-PE, CD3-Cy7APC, and IgM-Cy5PE. Labeled cells were fixed in 0.5% paraformaldehyde and analyzed on an LSR-II flow cytometer. Approximately 1 million events were collected for analysis.

Red blood cell serologic testing

Direct antiglobulin testing was performed by standard tube technique using heavy chain specific human anti-IgG and murine monoclonal anti-C3d (Ortho Clinical Diagnostics, Raritan, NJ, USA). Cold agglutinin titers were performed using serial 10-fold dilutions of serum in phosphate-buffered saline, incubated with normal human adult group O or normal rhesus macaque red blood cells (RBCs) for 24 hours at 4°C. Rhesus RBCs were tested for polyagglutination using the following lectins: Glycine soja, Arachis hypogea, Salvia sclarea and Salvia horminum (Gamma Lectin System; Immucor/Gamma, Norcross, GA, USA).

Antibody screening of animal sera was performed by standard tube technique against a panel of human adult group O, umbilical cord group O, and rhesus macaque control RBCs at immediate spin (22°C) and antiglobulin (37°C) phases of testing, using a low ionic strength solution potentiator. Sera were treated with 0.01 M dithiothreitol to differentiate IgG from IgM reactivity. To determine if a drug-related RBC antibody was present, a 1 mg/mL solution of fluconazole in phosphate-buffered saline was incubated at 37°C for 2 hours with animal sera plus native

or papain-treated group O RBCs, with or without a fresh source of complement. After a serial washing step, anti-IgG was added to all tubes.

Determination of serum immunoglobulins

Serum IgG, IgA, and IgM levels were quantified by immunonephelometry on a Dimension Vista 1500 automated analyzer using the manufacturer's methods for human subjects (Siemens Healthcare Diagnostics, Deerfield, IL, USA).

Serum protein and immunofixation electrophoresis

For analysis of various protein fractions, all sera were electrophoresed in agarose gel by a semi-automated electrophoretic system developed for human subjects (Hydragel 7 or 15 Protein(e) gels, Hydrasys system; Sebia, Norcross, GA, USA). Gels were stained with Amidoblack. Select specimens were further analyzed with immunofixation electrophoresis in agarose gel (Hydragel 2F or 4F; Sebia) using the same semi-automated system as for protein electrophoresis (Hydrasys) and the manufacturer's monospecific anti-human immunoglobulin antibodies. The immunofixation electrophoresis method uses a more sensitive protein stain (acid violet) for improved detection of possible paraproteins.

Statistical analysis

Statistical analyses were performed using JMP 8 software (SAS Institute Inc., Cary, NC, USA). Comparison between G-CSF and plerixafor vs. G-CSF and SCF mobilization was evaluated by Student's *t*-test. A p value <0.05 or 0.01 was considered significant. Standard errors of the mean are shown as error bars in all figures. For G-CSF and SCF-mobilized $CD34^+$ cells, we applied the linear regression *t*-test to %EGFP data between in vitro bulk $CD34^+$ cells and lymphocytes at 1, 3, and 6 months post-transplantation. The 90% confidence intervals are also included.

Results

To evaluate whether G-CSF and plerixafor-mobilized $CD34^+$ cells could stably reconstitute blood cells, we performed HSC transplantation using rhesus macaque $CD34^+$ cells transduced with an EGFP-expressing χHIV vector, which was developed for efficient transduction of rhesus $CD34^+$ cells [8]. First, we evaluated the number and %EGFP of immunoselected $CD34^+$ cells, which were transplanted into rhesus macaques. As shown in Figure 1, G-CSF and plerixafor mobilization (n = 3) yielded twofold greater $CD34^+$ cell numbers compared to yields observed with the G-CSF and SCF combination (n = 5) ($p < 0.01$). Transduction rates with the χHIV vector, however, were fourfold lower in G-CSF and plerixafor-mobilized $CD34^+$ cells when compared with G-CSF and SCF-mobilized harvests ($p < 0.01$). G-CSF and plerixafor mobilization tended to result in a lower overall number of $EGFP^+CD34^+$ cells compared to G-CSF and SCF mobilization ($9.6 \pm 0.9 \times 10^6$ vs. $2.1 \pm 0.5 \times 10^7$, respectively; not significant).

Additionally, we evaluated the composition of the $CD34^+$ cell population in three different mobilizations from a single animal using cell surface analysis. Time between mobilizations was 1 month or longer. $CD123^+$ (IL-3 receptor) expression has been found to vary on $CD34^+$ cell subpopulations [17]. $CD34^+CD123^+$ populations were higher in $CD34^+$ cells mobilized by G-CSF and plerixafor or plerixafor alone when compared with that of G-CSF alone (Fig. 2).

To determine repopulating ability, G-CSF and plerixafor-mobilized $CD34^+$ cells were transduced with EGFP-expressing lentiviral vector and transplanted into 5 Gy × 2 days (total dose 10 Gy) irradiated rhesus macaques

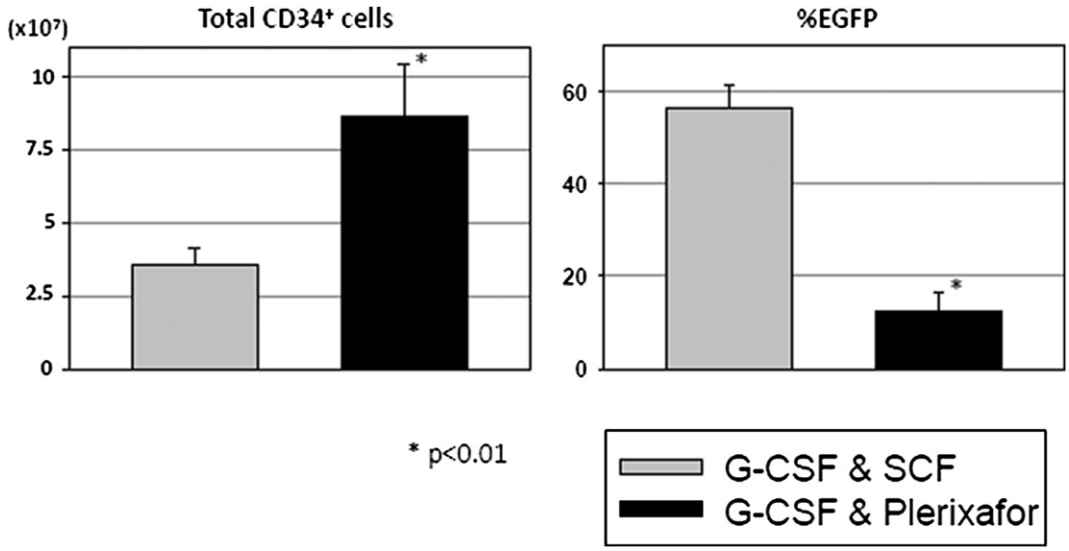

Figure 1. G-CSF and plerixafor mobilization increased $CD34^+$ cell apheresis yields. G-CSF and plerixafor mobilization (n = 3) yielded a twofold higher number of $CD34^+$ cells, compared to that observed with G-CSF and SCF mobilization (n = 5) ($p < 0.01$). Transduction rates with χHIV vector, however, were fourfold lower in G-CSF and plerixafor-mobilized $CD34^+$ cells, compared to that observed for G-CSF and SCF-mobilized cells ($p < 0.01$).

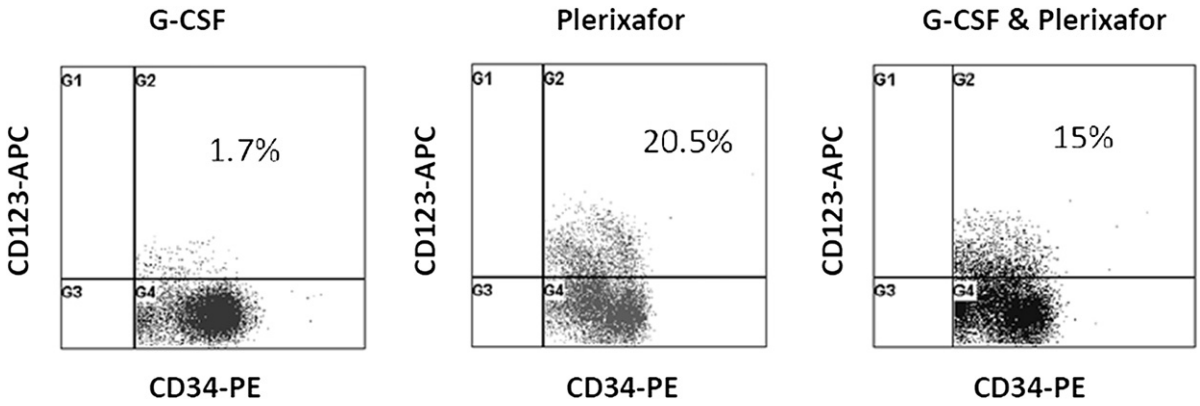

Figure 2. Cell surface analysis of G-CSF and plerixafor-mobilized CD34$^+$ cells. CD34$^+$ cells were immunoselected from a single animal (RQ6712) and immunophenotyped with murine anti-human CD34-PE and CD123 (IL-3 receptor)-APC antibodies known to be cross-reactive with rhesus macaques. Mobilizations using the designated regimens and leukapheresis procedures were separated by at least 1 month. The percentage of CD34$^+$CD123$^+$ expression for each mobilization regimen is shown.

(n = 3, Fig. 3A). Whole cultures of mobilized and immunoselected CD34$^+$ cells were transduced and transplanted without sorting. Blood cell counts and transgene expression levels were followed during 1 year, and these data were compared to those of G-CSF and SCF-mobilized animals (n = 5) transplanted previously [8]. As shown in Figure 3B, animals transplanted with G-CSF and plerixafor-mobilized cells had similar recovery of myelocytes (2900 ± 700/μL vs. 3500 ± 700/μL on day 30, not significant) and earlier recovery of lymphocytes (3300 ± 900/μL vs. 1200 ± 300/μL on day 30; $p < 0.05$) compared to animals who received G-CSF and SCF-mobilized grafts. Although hemoglobin concentration and platelet counts showed a tendency toward earlier recovery in animals receiving transduced CD34$^+$ cells obtained after G-CSF and plerixafor mobilization, there was no significant difference between the two groups (hemoglobin: 10.5 ± 0.3 g/dL vs. 9.9 ± 0.6 g/dl; not significant; platelet: 1.9 ± 0.2 × 10^5/μL vs. 1.2 ± 0.3 × 10^5/μL on day 30; not significant). These data suggested that G-CSF and plerixafor-mobilized CD34$^+$ cells contained increased numbers of early lymphoid progenitor cells.

One year after transplantation, animals transplanted with G-CSF and plerixafor-mobilized cells showed engraftment of EGFP$^+$ cells in all lineages. Transgene expression levels in peripheral blood cells were 2% to 5% in the first animal (RQ7424), 2% to 5% in the second animal (RQ7375), and 5% to 10% in the third animal (RQ7277) in all lineage cells (Fig. 4A). Multilineage marking was observed by cell surface analysis in peripheral blood cells and bone marrow cells (Fig. 4B). These data indicated that G-CSF and plerixafor-mobilized CD34$^+$ cells could stably reconstitute peripheral blood in the rhesus macaque. Additionally, both naïve and memory CD4 and CD8 T cells stably expressed EGFP in peripheral and mucosal lymphoid compartments (Fig. 5A, B, and D). Likewise, both CD3$^-$CD20$^+$IgM$^+$ and CD3$^-$CD20$^+$IgM$^-$ (includes IgG and IgA) cells expressed EGFP (Fig. 5C, E), indicating long-term reconstitution of multiple phenotypes in both primary and secondary lymphoid tissues of rhesus macaques.

Because lymphocyte recovery was more rapid in the G-CSF and plerixafor group, we compared transgene expression levels (%EGFP) among the CD34$^+$ cells present in the component (graft, in vitro) and among peripheral blood lymphocytes (repopulating cells, in vivo) at 1, 3, and 6 months post-transplantation. In G-CSF and SCF-mobilized animals, there was a significant correlation of %EGFP between the CD34$^+$ cells in the component (graft) and the circulating lymphocytes at all three time points ($p < 0.01$) (Fig. 6, line of regression drawn for data from G-CSF and SCF-mobilized products, n = 5). Data from G-CSF and plerixafor mobilizations showed a lower ratio of %EGFP labeling of CD34$^+$ cells in the component to %EGFP labeling in circulating lymphocytes at 1 and 3 months after transplantation when compared to the G-CSF and SCF-mobilized transplants. Six months after transplantation, however, the ratios were similar to those obtained in animals receiving G-CSF and SCF-mobilized CD34$^+$ cells, with all points being inside 90% confidence intervals of the regression line for the G-CSF and SCF mobilized products (Fig. 6). These results suggest that in vitro transduction rates predict in vivo EGFP expression in peripheral blood lymphocytes, and also that G-CSF and plerixafor-mobilized CD34$^+$ cells might include a larger proportion of early lymphoid progenitor cells when compared to G-CSF and SCF-mobilized cells.

Thirty days after transplantation, a transient skin rash developed in one of the three animals transplanted with G-CSF and plerixafor-mobilized cells (Fig. 7A). The rash resolved 79 days after transplantation (Fig. 7B). This animal (RQ7375) had the most rapid lymphocyte recovery of the three animals transplanted (Fig. 3B). Skin biopsy (day 49) showed an acanthotic epidermis with superficial dermal edema and perivascular lymphocytic infiltrates

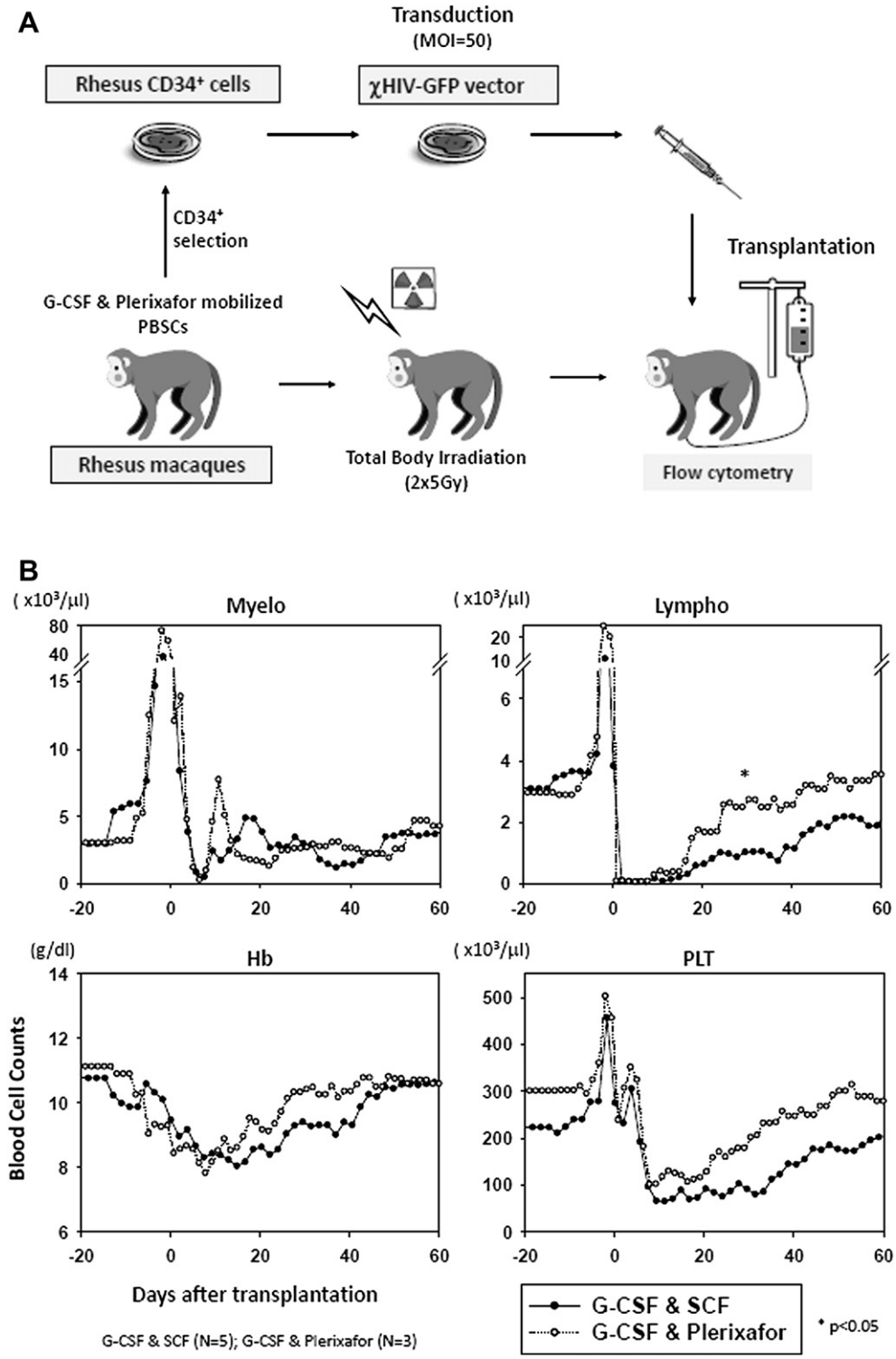

Figure 3. G-CSF and plerixafor-mobilized cells demonstrated early lymphocyte recovery. (**A**) G-CSF and plerixafor-mobilized $CD34^+$ cells were transduced with EGFP-expressing chimeric HIV1-based lentiviral vector, including the simian immunodeficiency virus−capsid (χHIV vector), and these cells were transplanted into lethally irradiated rhesus macaques. Blood cell counts and transgene expression levels in each lineage of peripheral blood cells were assayed for at least 1 year. (**B**) After transplantation of transduced $CD34^+$ cells, recipients of G-CSF and plerixafor-mobilized autologous grafts (n = 3) showed engraftment of all lineage cells and accelerated recovery of lymphocytes (lymphocytes: 3300 ± 900/μL vs. 1200 ± 300/μL on day 30, $p < 0.05$) compared to recipients of G-CSF and SCF-mobilized grafts (n = 5). There was also a trend for an accelerated recovery of hemoglobin concentration (Hb) and platelet count (PLT), but this did not reach statistical significance.

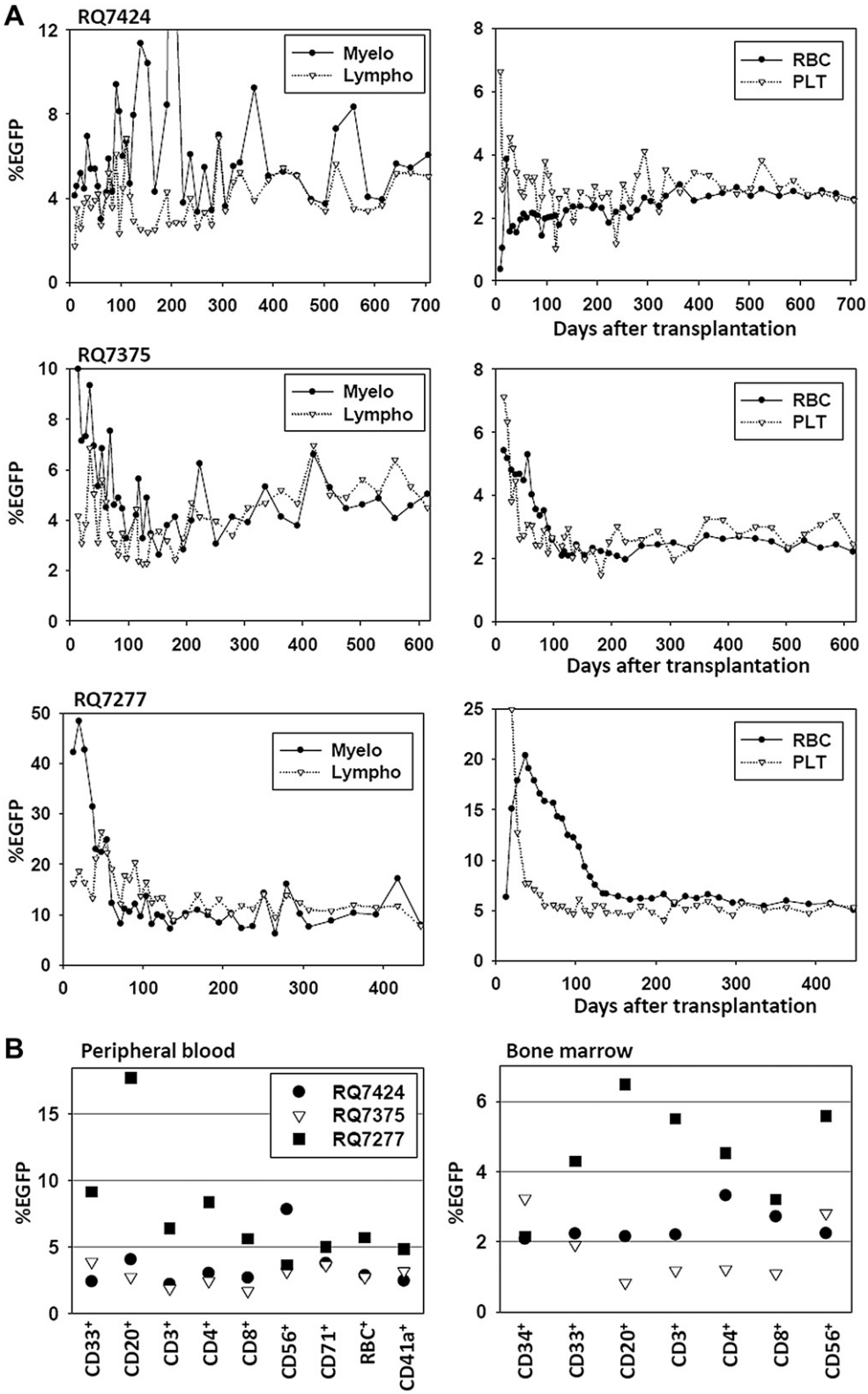

Figure 4. G-CSF and plerixafor-mobilized cells demonstrated long-term reconstitution of peripheral blood in rhesus macaques. (**A**) After infusion of G-CSF and plerixafor-mobilized $CD34^+$ cells transduced with EGFP-expressing lentiviral vector, EGFP expression rates in peripheral blood were evaluated for at least 1 year in three rhesus macaques. EGFP expression rates were 2% to 4% in all lineage cells in the first animal (RQ7424) at 2 years, 2% to 5% in the second animal (RQ7375) at 1½ years, and 5% to 10% in the third animal (RQ7277) at 1 year after transplantation. (**B**) In lineage marker analysis, all lineage cells demonstrated EGFP expression in peripheral blood cells (RQ7424 on day 503, RQ7375 on day 559, and RQ7277 on day 391) and bone marrow cells (RQ7424 on day 503, RQ7375 on day 700, and RQ7277 on day 532).

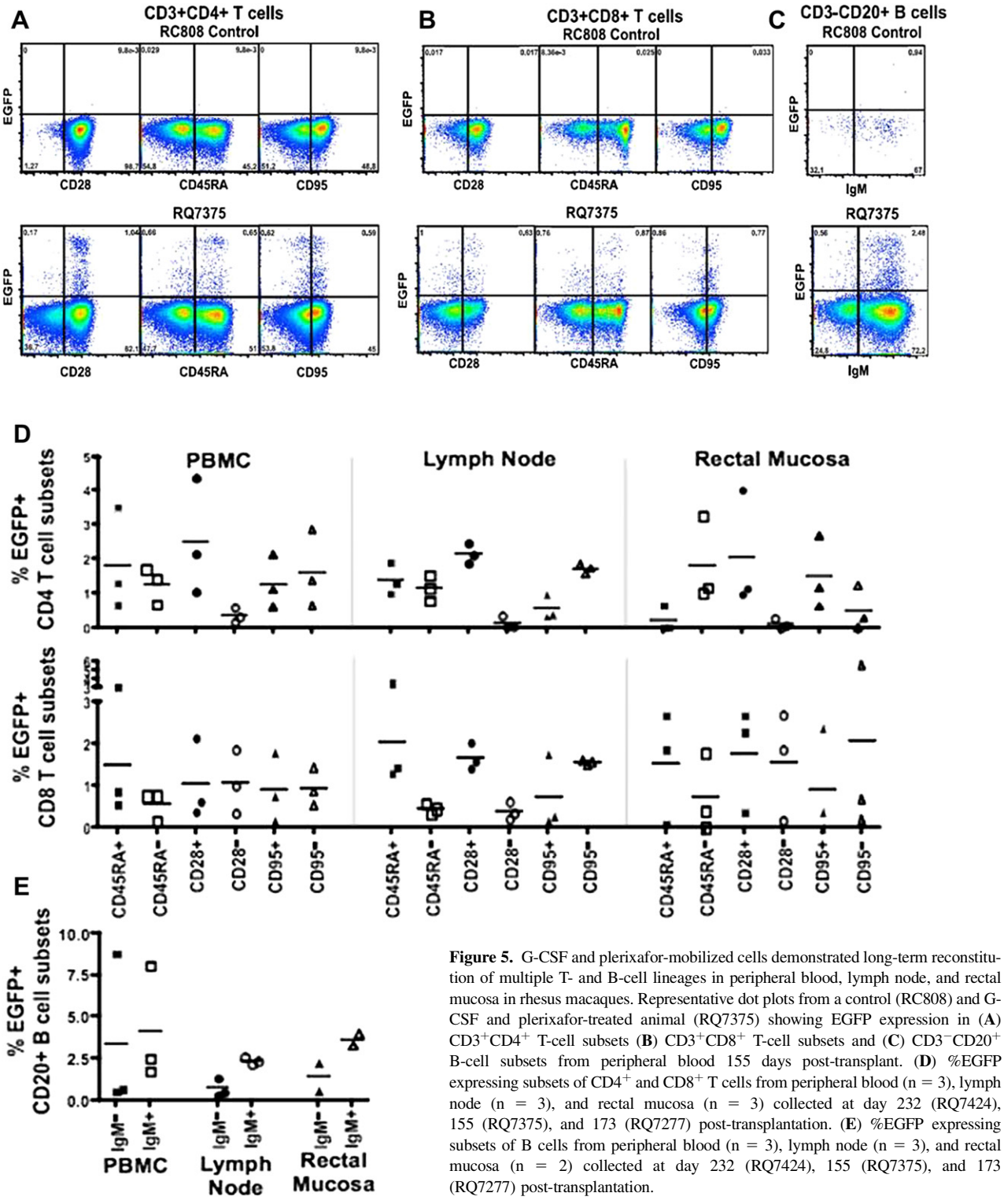

Figure 5. G-CSF and plerixafor-mobilized cells demonstrated long-term reconstitution of multiple T- and B-cell lineages in peripheral blood, lymph node, and rectal mucosa in rhesus macaques. Representative dot plots from a control (RC808) and G-CSF and plerixafor-treated animal (RQ7375) showing EGFP expression in (**A**) $CD3^+CD4^+$ T-cell subsets (**B**) $CD3^+CD8^+$ T-cell subsets and (**C**) $CD3^-CD20^+$ B-cell subsets from peripheral blood 155 days post-transplant. (**D**) %EGFP expressing subsets of $CD4^+$ and $CD8^+$ T cells from peripheral blood (n = 3), lymph node (n = 3), and rectal mucosa (n = 3) collected at day 232 (RQ7424), 155 (RQ7375), and 173 (RQ7277) post-transplantation. (**E**) %EGFP expressing subsets of B cells from peripheral blood (n = 3), lymph node (n = 3), and rectal mucosa (n = 2) collected at day 232 (RQ7424), 155 (RQ7375), and 173 (RQ7277) post-transplantation.

(Fig. 7C). Fifty-five days post-transplantation, clumping of RBCs was observed in vitro at room temperature, but not at 37°C. Direct antiglobulin testing revealed that the animal's RBCs were coated with complement (C3d) but not immunoglobulin G (IgG). Serological evaluation revealed an IgM cold agglutinin with a titer of 1:640 when tested against rhesus macaque control RBCs. Antibody screening revealed that the serum was reactive at immediate spin

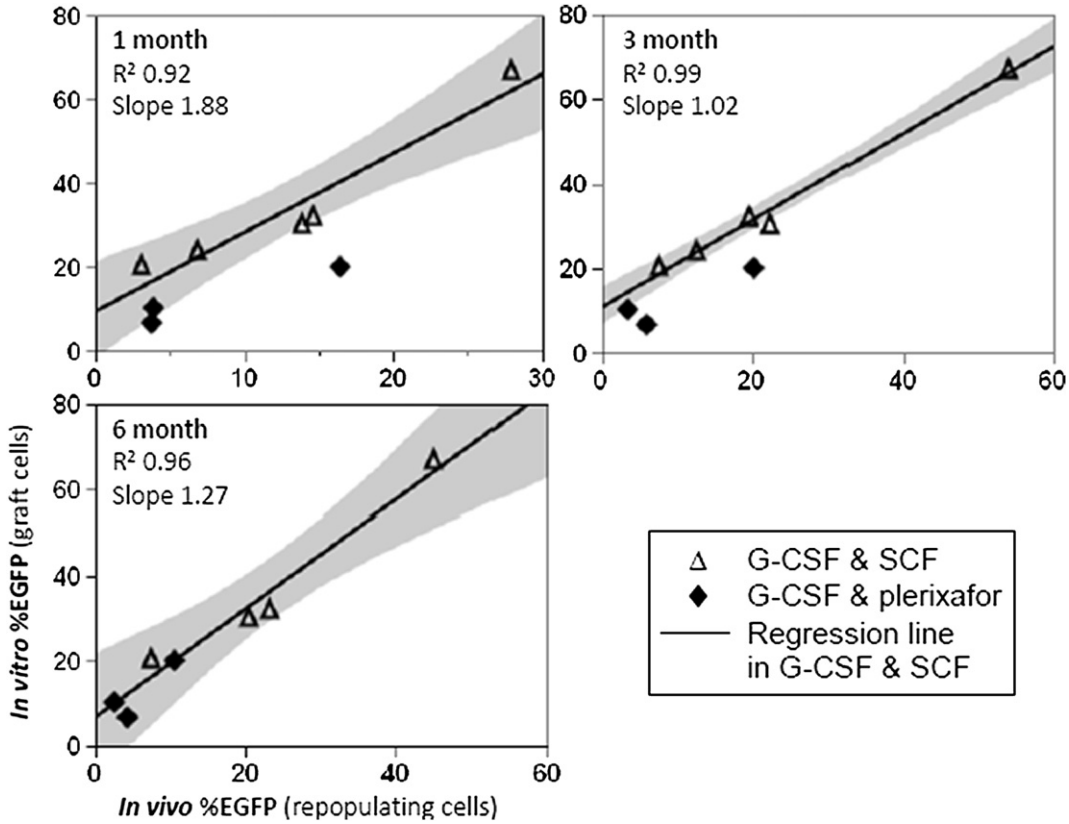

Figure 6. Correlation between in vitro and in vivo transduction rates. We evaluated the ratio of %EGFP in the immunoselected CD34$^+$ cells of the graft to %EGFP of circulating lymphocytes at 1 month, 3 months, and 6 months post-transplantation. In G-CSF and SCF-mobilized animals (n = 5), there was a significant linear correlation of %EGFP among in vitro CD34$^+$ cells in the graft and in vivo circulating lymphocytes at all three time points ($p < 0.01$). (The regression line and 90% confidence intervals (shaded areas) are shown for the G-CSF and SCF components.) In G-CSF and plerixafor-mobilized animals (n = 3), a lower ratio at 1 and 3 months after transplantation was seen, compared to the ratios in the G-CSF and SCF-mobilized transplants. In contrast, the ratio was similar in both types of mobilizations at 6 months after transplantation.

(22°C) and antiglobulin (37°C) phases of testing with a panel of human adult (I-positive) group O RBCs, but was nonreactive at immediate spin and only weakly reactive, microscopically, at antiglobulin phase when tested against human adult I-negative and umbilical cord RBCs. IgG cold-reactive RBC autoantibodies were not present. Further serological testing did not detect evidence of polyagglutination or drug-induced agglutination or hemolysis using medications administered to the monkey in the peritransplant period. Serum immunoglobulin measurements (Fig. 7D), serum protein electrophoresis (Fig. 7D), and immunofixation electrophoresis (Fig. 7E) revealed elevations in serum IgG and IgM with transient appearance of corresponding paraproteins and transient disappearance of IgA levels at the time when the cold agglutinin and skin rash were apparent. By day 79, both the skin rash and cold agglutinin had resolved. This animal had no evidence of infection when the skin rash developed or upon skin biopsy, suggesting that an autoimmune reaction initiated by cells in the graft might be occurring. Aside from the transient skin rash, cold agglutinin disorder, and serum paraproteins in one of the three transplanted animals, no other complications were observed.

Discussion

The CD34 antigen is the most frequently used antigen for identifying human HSCs [10,18,19], and cells expressing this antigen contain both HSCs and committed progenitors. In this work, we found that G-CSF and plerixafor mobilized higher numbers of CD34$^+$ cells, and this increase, compared with that of G-CSF and SCF mobilization in rhesus macaques [7], likely represented lymphoid progenitors, as evidenced by higher IL-3 receptor (CD123) expression and faster peripheral blood lymphocyte recovery after transplantation. This is consistent with our previous in vitro microarray data, which showed CD34$^+$ cells isolated with either plerixafor alone or G-CSF and plerixafor had gene expression profiles predominantly associated with lymphocyte development [7].

When we followed peripheral blood reconstitution after transplantation of these two types of mobilized

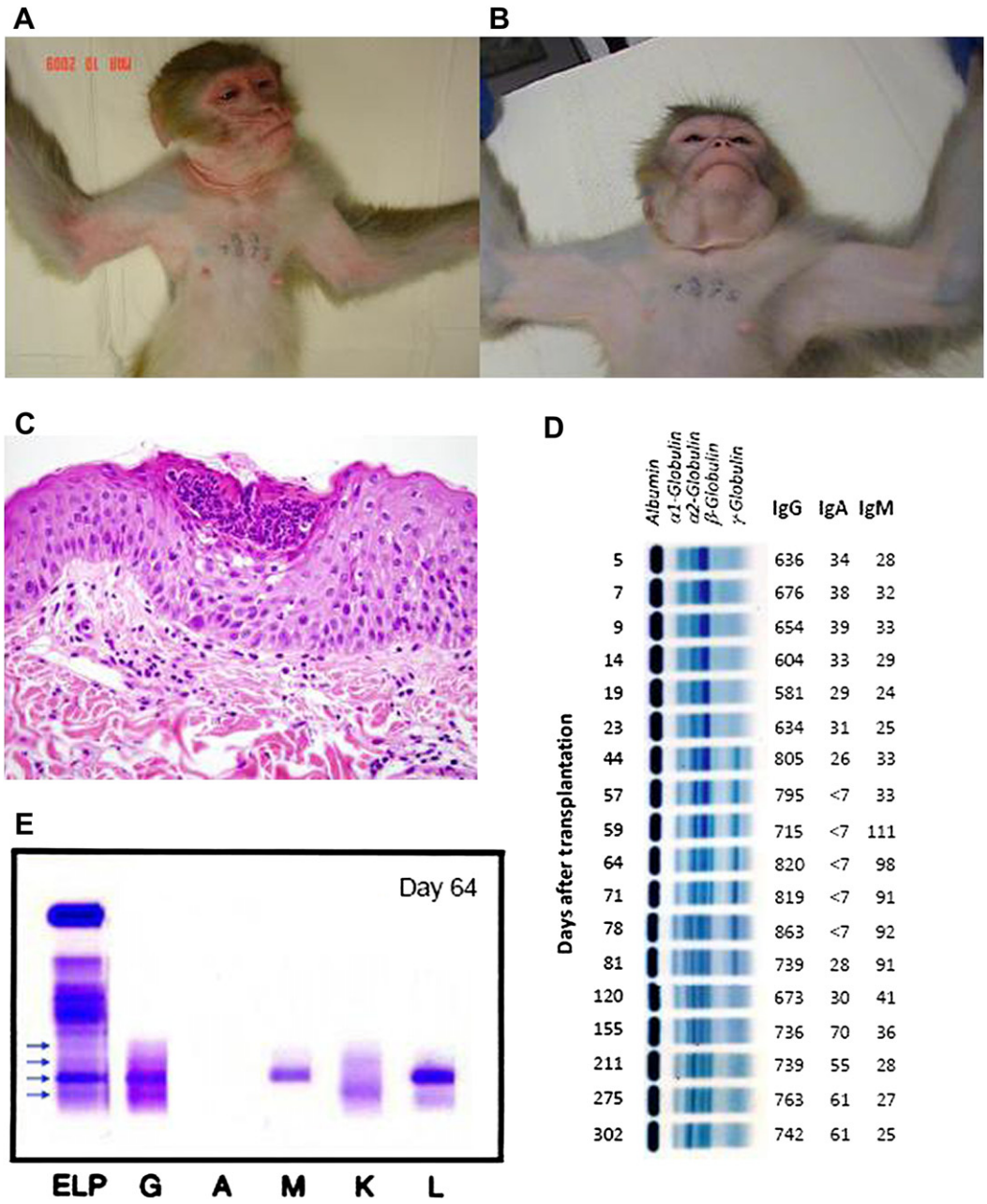

Figure 7. Complication of cold agglutinin disease in one animal after G-CSF and plerixafor-mobilized cell transplantation. In the animal with the most accelerated lymphocyte recovery, RQ7375, an erythematous skin rash developed on the face, neck, and axilla 30 days after transplantation (**A**: day 49). This rash was transient and resolved by day 79 (**B**). A skin biopsy taken on day 49 revealed an acanthotic epidermis with superficial dermal edema and perivascular lymphocytic infiltrates (**C**). On day 55, clumping of RBCs at room temperature was observed. Serological evaluation revealed an IgM-mediated cold agglutinin with a titer of 640 against healthy monkey red cells. Serum immunoglobulin measurements (**D**) and serum protein electrophoresis (**D**) and IFE (**E**) revealed transient primarily oligoclonal elevations in IgG and IgM levels, and non-measurable levels of IgA (all in mg/dL). These levels returned to near pretransplantation levels after resolution of the rash and cold agglutinin disorder. In inset (**E**): anode at the top, short horizontal arrows point at oligoclonal bands in the γ-globulin region of the acid-fixed lane (ELP), and lanes G, A, M, K, and L refer to immunofixation with monospecific antibodies against human γ-, α-, and μ-immunoglobulin heavy chains and human κ and λ immunoglobulin light chains, respectively.

$CD34^+$ cells, we found that lymphoid recovery after transplantation with G-CSF and plerixafor-mobilized $CD34^+$ cells was accelerated. Although the exact lymphoid subpopulations involved within this more rapid recovery were not identified, this novel observation may explain the autoimmune reaction seen in rhesus macaque RQ7375. This animal had the fastest lymphocyte recovery and developed a transient skin rash and RBC cold agglutinin associated with oligoclonal elevation in IgG and IgM immunoglobulin levels and loss of measurable IgA.

Although the pattern of anti-I reactivity found in the animal's serum during the reaction is consistent with that typically seen in human idiopathic cold agglutinin disease, this disease in nonhuman primates has not been reported before, and this animal did not have the hemolytic anemia that is usually seen in humans. The rash and cold agglutinin were transient and disappeared when IgM and IgG levels decreased to baseline and IgA reappeared. The relationship of this transient autoimmune dysregulated immunoglobulin expression to the use of G-CSF and plerixafor-mobilized $CD34^+$ cells remains speculative.

We also provided clear evidence for the first time that G-CSF and plerixafor-mobilized $CD34^+$ cells contain an HSC population capable of reconstituting multilineage hematopoietic cells by lentiviral gene marking. These results corroborate and expand upon our earlier studies using plerixafor alone to mobilize and transplant HSCs in rhesus macaques [20]. In the current study, we used an EGFP-expressing χHIV vector optimized for transduction of rhesus $CD34^+$ cells [8] to mark and follow the reconstitution of G-CSF and plerixafor-mobilized $CD34^+$ cells. In rhesus gene transfer preclinical studies as well as human gene therapy trials, transgene expression rates among peripheral blood cells generally fluctuate for the first few months after transplantation, then plateau at low levels approximately 6 months after transplantation [8,21,22]. One explanation for this observation is that committed progenitor cells contribute to the reconstitution of circulating blood cells in the first few months and stem cells contribute significantly later. In this study, three rhesus macaques showed long-term (1−2 years) reconstitution of multiple lineage blood cells that contained $EGFP^+$ cells originating from the G-CSF and plerixafor-mobilized $CD34^+$ cells and confirmed that HSCs are included in this $CD34^+$ cell population. The proportion of $CD34^+$ progenitors to HSCs, however, appears to be higher within the G-CSF and plerixafor-mobilized than with other mobilization regimens based on the reconstitution data.

We were initially surprised that G-CSF and plerixafor-mobilized $CD34^+$ cells had reduced in vitro transduction efficiency (Fig. 1B) and lower in vivo %EGFP (Fig. 6). This observation occurred even when we used a chimeric vector that was constructed specifically for rhesus $CD34^+$ cells [8]. This reduced transgene expression may have resulted from higher number of lymphocytes and lymphoid progenitors in the mobilized graft because the specific conditions required for efficient gene transfer to lymphocytes (e.g., CD3 antibody and IL-2) were not present [23]. Other possible explanations include an alteration in the $CD34^+$ progenitor to stem cell ratio, which may have increased signaling via cascades that block efficient transduction; or a higher number of plasmacytoid dendritic cell precursors may have been mobilized, which were resistant to transduction [24].

In summary, G-CSF and plerixafor mobilization increased $CD34^+$ cell yields by apheresis. This population of $CD34^+$ cells appears to contain more lymphoid progenitors and a similar number of HSCs capable of long-term reconstitution and stable multilineage transgene expression in vivo. The lymphoid progenitors within the $CD34^+$ cell population accelerated lymphoid recovery. These findings should be helpful in developing plerixafor-based mobilization protocols in HSC transplantation studies.

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Conflict of interest disclosure

No financial interest/relationships with financial interest relating to the topic of this article have been declared.

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