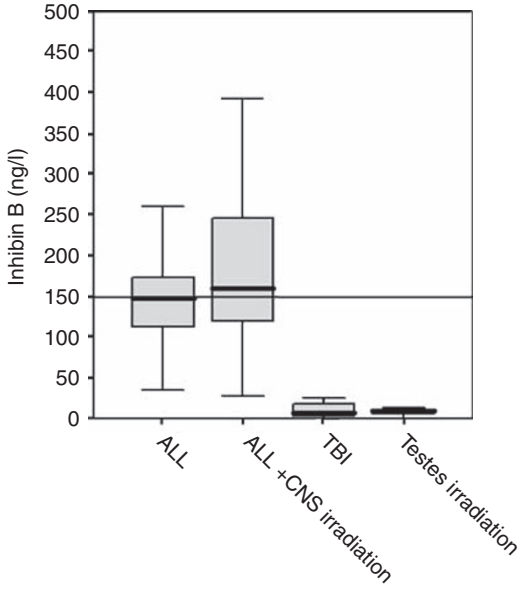

Figure 2 Median Inhibin B values and quartile ranges according to treatment and location of irradiation. One patient in the TBI group and two survivors in the testicular radiated group also received cranial irradiation. The horizontal line represents the lower threshold level for Inhibin B (150 ng/l).

Conflict of interest

The authors declare no conflict of interest.

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Myxoma virus targets primary human leukemic stem and progenitor cells while sparing normal hematopoietic stem and progenitor cells

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Regimens of high-dose chemotherapy followed by autologous blood and marrow transplantation have been used to treat patients with acute myelogenous leukemia (AML), but leukemia relapse is frequent, likely due to marrow contamination with leukemic hematopoietic stem and progenitor cells (HSPCs) and residual disease in protective niches. Purging autologous hematopoietic grafts of contaminating cancer cells before transplant serves as a viable strategy for increasing transplant efficacy but would also require sparing normal HSPCs within the same hematopoietic cell graft. Previous purging strategies using *ex vivo* incubation with cytotoxic agents and cell culture techniques have generally resulted in the loss of normal HSPC numbers or functionality.[1]

Oncolytic poxviruses, such as vaccinia virus and myxoma virus (MYXV), are promising new instruments in targeting human cancer.[2,3] MYXVs natural host tropism is highly restricted to European rabbits (*Oryctolagus cuniculus*), and it is nonpathogenic for all other vertebrate species tested, including humans.[4,5] There have been no reports of MYXV infection in humans and no cases of any clinical complications, such as aplastic anemia, despite widespread MYXV exposure in countries such as Australia. MYXV has a large double-strand

DNA genome permitting insertion of large numbers (at least 25 kb) of therapeutic eukaryotic genes. Despite its narrow host specificity, we have shown in cultured cells and in preclinical animal model studies that MYXV is capable of infecting and killing various established human cancer cell lines *in vitro* and *in vivo*, but its efficacy and safety in targeting primary human cancer cells while sparing normal cells have not been defined.[3] Thus, to determine whether oncolytic viruses can be used as a therapeutic modality for purging leukemia from autologous grafts, we evaluated MYXV in a preclinical model of AML and normal HSPC transplantation. We show that *ex vivo* incubation with MYXV ablates *in vitro* colony-forming potential of *fms*-like tyrosine kinase receptor-3 (FLT3) mutant AML cells and dramatically decreases engraftment levels in mice. High-risk AMLs, such as those with FLT3 internal tandem duplications (ITDs), are notoriously chemotherapy-insensitive and prone to relapse. Moreover, using identical *ex vivo* conditions, MYXV does not affect either *in vitro* colony-forming potential or *in vivo* engraftment of normal HSPCs derived from healthy human bone marrow (BM). These results show safety and efficacy of using oncolytic MYXV to purge AML HSPCs from autologous HSPC grafts intended for hematopoietic rescue.

Although there have been no case reports of human BM failure despite widespread MYXV exposure, to document MYXV safety in the context of normal human HSPC function, we incubated BM-derived HSPCs from healthy donors ($n = 3$) with a GFP-expressing MYXV construct (vMYX-GFP) *in vitro* over a 3-day period at a high multiplicity of infection (MOI) of 10 per cell. After exposure to vMYX-GFP, BM-derived mononuclear cells (MNCs) were analyzed by flow cytometry to determine the extent of infectivity. Over $99.6 \pm 0.2\%$ of BM-derived MNCs were free of vMYX-GFP infection (Figure 1a). A small population of MNCs accounting for $0.5 \pm 0.2\%$ of the total population was GFP-positive by this treatment, which was essentially indistinguishable from mock (vehicle only)-treated cells and is typical of a nonpermissive infection by MYXV. No GFP expression was

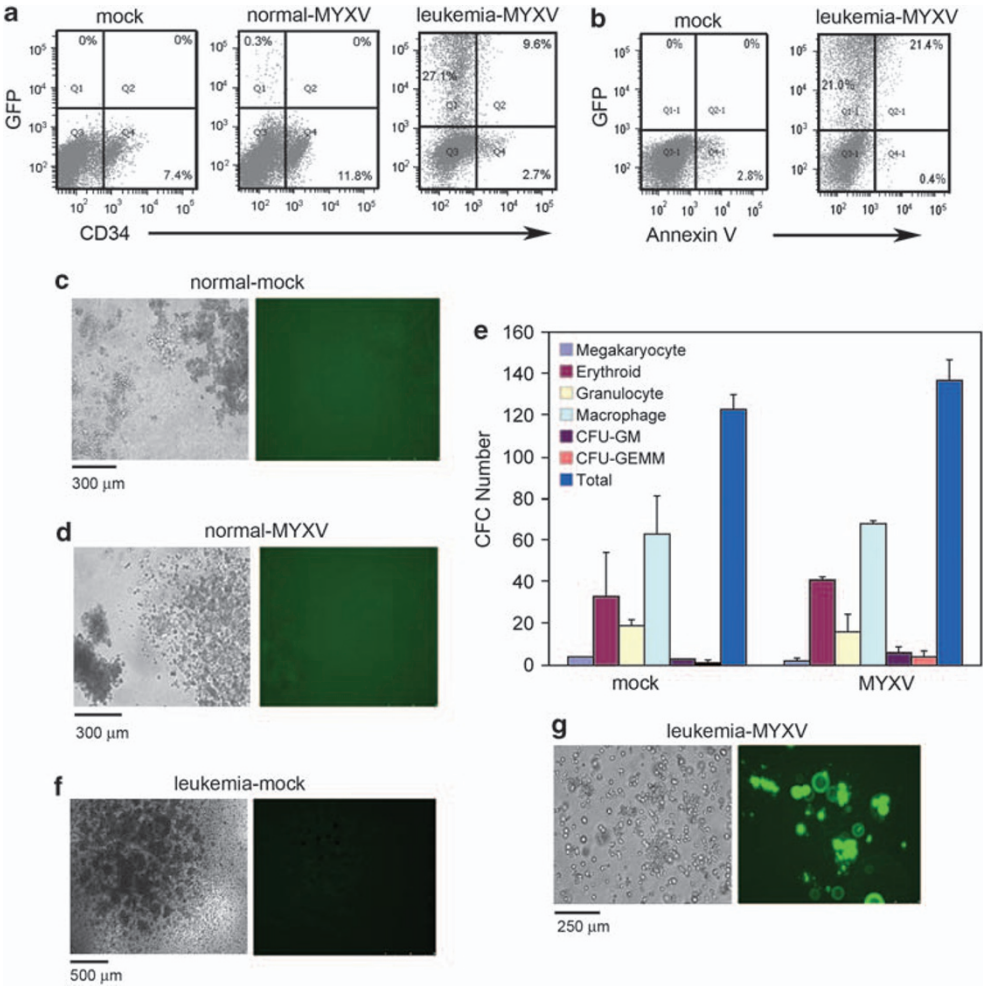

Figure 1 Myxoma virus (MYXV) treatment of normal and leukemic hematopoietic stem and progenitor cells (HSPCs) *in vitro*. (**a**) Normal and leukemic cells (*fms*-like tyrosine kinase receptor-3 internal tandem duplication, FLT3 ITD$^+$) were incubated with MYXV expressing GFP. Flow cytometric analysis showed a small population of normal mononuclear cells (MNCs) infected, whereas a significant proportion of acute myelogenous leukemia (AML) cells were susceptible to MYXV infection. (**b**) Apoptosis was mainly observed in the GFP$^+$ MYXV-infected cell population as shown by Annexin V staining. (**c**, **d**) Normal bone marrow (BM) cells were incubated with MYXV expressing GFP and assessed for colony-forming potential. Various types of colony-forming cell (CFC) colonies were formed and showed no GFP expression in either MYXV-treated (**d**) or mock-treated samples (**c**). (**e**) The frequency of each colony type was enumerated after MYXV or mock treatment. Similar frequencies were observed between both cohorts. Values mean ± s.d. (**f**) Mock-treated AML cells formed AML-CFU colonies. (**g**) MYXV-treated AML cells showed signs of infection (GFP$^+$) and did not form colonies.

observed in normal CD34$^+$ HSPC cells. On a lineage subset analysis, CD15$^+$ myeloid cells and CD19$^+$ B lymphocytes also showed no signs of infection (data not shown).

To test whether primary AML cells permitted intracellular MYXV replication and oncolysis *in vitro*, leukemia cells were obtained from a 71-year-old white male presenting with *de novo* AML (normal male karyotype, FLT3 ITD$^+$ mutation, NPM1c$^+$ mutation). The myeloblasts were collected by peripheral blood leukapheresis and made up >98% of the collected product. These AML cells were incubated with vMYX-GFP at 10 MOI for 3 days, and analyzed for infectivity and apoptotic events by Annexin-V staining. Experiments were carried out in triplicate. In these samples, 40.3 ± 5.0% of AML cells were infected with the virus, which was significantly higher in comparison with normal BM-derived MNCs ($P<0.05$; Figure 1a). Of the total AML CD34$^+$ population, we found that 65 ± 6% were infected with MYXV, which was significantly higher in comparison with normal BM CD34$^+$ cells ($P<0.05$). Cellular infection correlated with higher levels of apoptotic cell death, as assessed by Annexin V staining, and was restricted to the GFP$^+$ cell population (Figure 1b). Kinetic analysis of cells incubated with vMYX-GFP at either 1.5 or 3.5 days after infection showed that there was a time-dependent increase in the number of HSPCs infected with vMYX-GFP (data not shown). These data indicate that MYXV efficiently infects AML HSPCs *in vitro* and induces apoptotic cell death through a classic permissive oncolytic poxvirus infection.

To further examine MYXV safety for HSPCs, experiments were carried out to determine whether MYXV alters normal HSPC differentiation function *in vitro*. MNCs derived from normal BM ($n=3$) were incubated with vMYX-GFP at 10 MOI for 3 h at 37 °C, after which cells were assayed for normal HSPC differentiation using an *in vitro* methylcellulose-based colony-forming cell (CFC) assay. We observed differentiated colonies forming normally after 14 days using either mock (vehicle only)- or vMYX-GFP-treated cells, indicating that the CFC potential of HSPCs was not adversely affected by MYXV (Figures 1c and d). Resulting colonies were analyzed for viral infection using fluorescent microscopy and showed a uniform lack of GFP$^+$ colonies, further demonstrating that vMYX-GFP did not infect or perturb the viability of normal CFCs *in vitro*.

We next examined whether MYXV could alter the efficiency of normal HSPC differentiation by calculating the frequency of different colonies derived from either mock- or vMYX-GFP treated cells. Following infection, cells were plated in the CFC assay ($n=3$) and the number and type of colonies determined after 16–18 days. The frequency of colonies formed was similar between mock- and vMYX-GFP-treated groups (Figure 1e). As the number and type of hematopoietic colonies generated were similar between groups, it suggested that vMYX-GFP did not alter CFC viability or frequency in normal HSPC specimens. The data showed that MYXV does not adversely affect normal HSPC developmental potential after *in vitro* incubation.

To determine whether MYXV infects AML cells and perturbs their growth and differentiation *in vitro*, primary AML cells were incubated with vMYX-GFP at MOI of 10 for 3 h, and then grown in methylcellulose-based medium ($n=3$). Mock-treated AML cells formed pleomorphic colonies consistent with AML colony-forming units (CFUs) (Figure 1f). When exposed to vMYX-GFP, AML cells did not form recognizable AML-CFU colonies and instead remained heterodispersed (Figure 1g). Before plating in methylcellulose-based medium, AML infection was confirmed using flow cytometric analysis. Together, these data indicated that MYXV effectively infects AML *in vitro* and impairs clonal proliferation.

On the basis of the *in vitro* safety results showing normal colony-forming potential from MYXV-treated normal HSPCs, we next tested safety by *in vivo* repopulation assays. Normal BM-derived MNCs ($n=3$) or CD34$^+$-selected cells ($n=6$) were exposed to 10 MOI MYXV for 3 h *ex vivo* and then xenotransplanted into sublethally irradiated (325 cGy) NOG mice. After 6–8 weeks, BM were harvested from the mice and analyzed by flow cytometry for human cell engraftment using antibodies to human CD45$^+$ and HLA-abc. Mock-treated BM MNCs ($n=4$) and CD34$^+$ cells ($n=6$) were also transplanted into NOG mice and served as controls. Typical human engraftment with CD45$^+$/HLA-abc$^+$ cells was observed in both treatment groups (Figures 2a). Quantitatively, similar numbers of mice engrafted with human hematopoietic cells in mock- and MYXV-treated cohorts (70 vs 78%; $P=0.72$; Figure 2b). The percentage of engraftment varied within individual mice as determined by double staining for CD45$^+$ and HLA-abc, but overall levels of engraftment were indistinguishable between mice receiving mock- or MYXV-treated normal HSPCs (1 vs 2%, $P=0.41$; Figure 2b). Interestingly, some MYXV-treated mice generated higher engraftment levels in comparison with mock-treated mice, but this trend was not statistically significant. Histological analysis of various tissues from mice transplanted with MYXV-treated cells showed normal tissue morphology (data not shown). None of the immunocompromised NOG mice developed any evidence of viral replication in their skin or in any internal tissues, indicating that even severely immunodeficient hosts are nonpermissive for MYXV infection. The data indicate that *ex vivo* treatment with MYXV does not reduce *in vivo* engraftment potential of normal BM-derived MNCs or CD34$^+$ HSPCs. This critical safety data support the notion that MYXV would be a safe candidate for purging leukemic cells from autologous hematopoietic grafts, even in immunocompromised recipients.

Finally, we set out to test MYXV oncolytic effects on primary AML HSPCs *in vivo*. An AML specimen harboring a FLT3 ITD mutation was used in this study because this high-risk AML represents a hematological malignancy that is notoriously chemotherapy-insensitive and can be tracked at the molecular level using PCR. Primary AML FLT3 ITD$^+$ MNCs were treated with MYXV at 10 MOI for 3 h and transplanted into sublethally irradiated NOG mice. Experiments were carried out in duplicate and mouse numbers maximized due to the rarity of obtaining AML cells. At 6–8 weeks after transplantation, mouse BM cells were harvested and examined for AML engraftment. By flow cytometric analysis, mock-treated cells resulted in CD45$^+$/HLA-abc$^+$ human engraftment in 100% of transplanted mice ($n=7$; Figures 2c and e). Conversely, MYXV-treated AML cells were engrafted only in 10% of mice, whereas the remaining 90% of the recipients were phenotypically free of any detectable AML cells ($n=10$; Figures 2c and e). In both cohorts, PCR analysis for human AML FLT3 ITD$^+$ cells in mouse BM was used to confirm leukemic engraftment (Figure 2d). Comparing results, MYXV treatment resulted in significantly lower mean levels of engraftment in comparison with mock-treated controls (4.5 vs 24%, $P<0.05$; Figure 2e). In the primary, high-risk AML specimen tested, normal HSPC levels were extremely low, thus normal HSPC engraftment in immunocompromised mice did not occur, as expected. Taken together, the data indicate that MYXV incubation *ex vivo* significantly inhibited subsequent AML HSPC engraftment *in vivo*, resulting in phenotypic and molecular remissions of FLT3 ITD AML.

Various autograft purging strategies have been attempted; however, their major limitations are a lack of specificity for AML HSPCs vs normal HSPCs and inadequate elimination of cancer

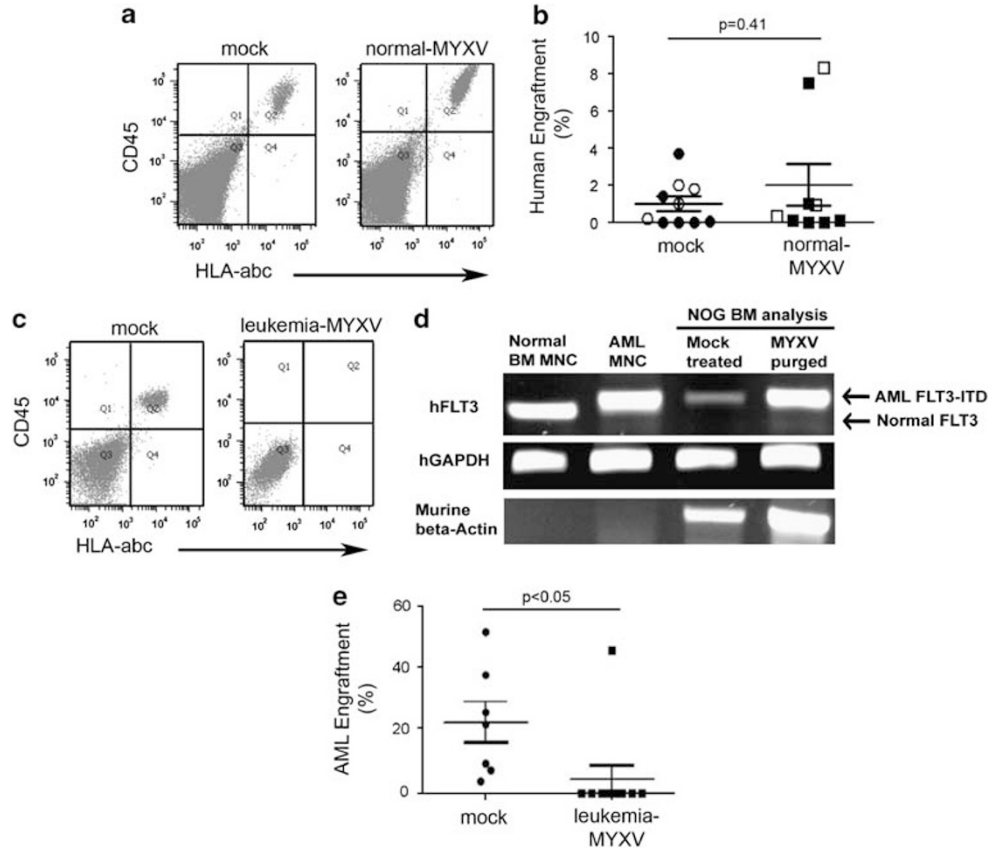

Figure 2 Normal and leukemic human hematopoietic stem and progenitor cell (HSPC) engraftment *in vivo* after myxoma virus (MYXV) treatment. (**a**) Normal bone marrow (BM)-derived mononuclear cells (MNCs) or $CD34^+$-selected cells were exposed to MYXV and transplanted into irradiated NOG mice. The analysis of mouse BM after 6–8 weeks showed normal human cell engraftment with $CD45^+$/HLA-abc^+ cells observed in both MYXV-treated and mock-treated groups. (**b**) Similar engraftment frequencies were observed between mock- and MYXV-treated cells. As expected, the percentage of engraftment values varied between mice; however, the overall levels of engraftment were similar between mice receiving mock- or MYXV-treated cells (circle: mock-treated, square: MYXV purged). (**c**) Acute myelogenous leukemia (AML) cells were mock-treated or exposed to MYXV and subsequently transplanted into irradiated NOG mice. Mock-treated AML cells engrafted in all mice. In contrast, MYXV-treated AML cells engrafted in only 10%. (**d**) Leukemic engraftment was confirmed using PCR for the FLT3 ITD mutation. All human cells in AML-transplanted mice exhibited the FLT3 ITD genotype. Only engrafted samples are shown: one representative of seven from mock-treated and the single FACS-positive engrafted sample from the 10 MYXV-purged mice. Normal BM cells and AML MNCs were used as PCR controls. (**e**) MYXV treatment resulted in significantly lower percentage engraftment levels in comparison with mock-treated controls. Values represent mean ± s.e.m. Gating was established using appropriate isotype controls.

cells upon purging. In this report, we show, for the first time, that MYXV specifically targets and eliminates primary AML HSPCs using a novel *ex vivo* purging technique. We also show that MYXV does not affect normal HSPCs, as assessed by *in vitro* colony-forming potential and *in vivo* engraftment in immunodeficient mice. Interestingly, we show susceptibility of a primary AML specimen harboring an activating mutation in FLT3, which represents an aggressive malignancy well known for insensitivity to conventional chemotherapy. Efficacy against this leukemia signifies the effectiveness of this strategy and an opportunity for disease eradication in an otherwise grim clinical setting. Altogether, these results show that oncolytic virotherapy using MYXV represents a promising therapeutic approach to eradicate malignant hematopoietic cells, including residual cancer stem cells, in autografts from patients treated with myeloablative immunosuppression and that require hematopoietic cell rescue.

Subsequent studies will test additional leukemic and myelodysplastic specimens to determine the breadth of applicability of this novel approach, and will focus on understanding the mechanisms involved in the successful purging of AML using MYXV. In addition, more specific studies examining the effects of MYXV on cancer stem cells are ongoing. Given that 65% of AML $CD34^+$ HSPCs showed evidence of MYXV infection after incubation and leukemic engraftment was prevented in 90% of recipients, it is possible that leukemic stem cells may be infected and marked by MYXV incubation. Further investigation of this finding is warranted. At present, the precise method of MYXV specificity for human cancer cells is only partially understood. It is known that poxviruses such as MYXV can bind and initiate entry into most mammalian cells, but then discriminates permissive vs nonpermissive cells by virtue of the cell signaling circuitry of the infected cell.[4] For example, we have previously shown that hyperactive AKT signaling, either as constitutive phosphorylation or as induced by the virus infection, dictates MYXV permissiveness in a wide variety of solid tumor cell lines.[6] However, considering the complexity of cancer cell populations, this may not be the only leukemia-specific signaling pathway by which MYXV discriminates normal vs cancerous HSPCs. For example, when normal human macrophages are infected with MYXV, the cells rapidly co-induce two anti-viral cytokines (tumor necrosis factor and type I interferon)

by a RIG-I-dependent signaling mechanism, which then aborts MYXV infection in normal human somatic cells in a paracrine-like manner.[7] Thus, another model would be that normal HSPCs are competent for this synergy, whereas cancerous HSPCs, such as AML cells, would be defective in some aspect of the tumor necrosis factor/interferon synergistic pathway. The aforementioned studies will provide insights into leukemia tropism and the antineoplastic mechanisms of MYXV-mediated oncolytic purging.

Conflict of interest

The authors declare no conflict of interest.

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