INTRODUCTION
Our study investigated the ability of muscle-derived stem cells (MDSCs)-based ex vivo gene transfer to ameliorate the efficiency of non-viral transduction of myofibers. We hypothesized that the use of a plasmid vector that encodes for a given transgene under the control of a viral promoter may trigger an immune response because the transduced cells can act as antigen presenting cells (APCs)[1]. We used the ex vivo gene transfer approach based on MDSCs transduced with two plasmid vectors: mini-dystrophin gene under the muscle creatine kinase (MCK) promoter; and mini-dystrophin gene under the cytomegalovirus (CMV) promoter to determine whether the promoter is a major determinant in the persistence of transgene expression in skeletal muscle.

MATERIALS AND METHODS
Animals and plasmids: All animal experiments were performed in accordance with the guidelines established by the Institutional Animal Research and Care Committee (ARCC) at Children’s Hospital of Pittsburgh (Protocol # 04/03). Plasmids carrying the mini-dystrophin reporter gene under the CMV or the MCK promoter were constructed in pcDNA 3.1(+) plasmid DNA backbone. Mini-dystrophin A3990 gene fragment [2] was obtained by restriction enzyme digestion from plasmid DNA called PCR-dys-3990-X2 [2].

Ex vivo approach using MDSCs: Primary MDSCs were isolated from C57BL/10ScSn-DMD™ mice (4–8 weeks of age) by using the preplate technique, as described previously [3, 4]. MDSCs were transduced with either CMV-dys or MCK-dys. After 24 h transduction, 1x10^5 cells were injected percutaneously into the gastrocnemius muscles of mdx mice (six mice per time point). Mice were sacrificed 10, 60, or 120 days after injection.

Immunocytochemistry: In cases involving a secondary anti-mouse antibody, the mouse on mouse (MOM) kit (Vector Laboratories) was used. Muscle sections were blocked with 10% goat serum for 30 min. The sections then were incubated overnight at 4°C with the following primary antibodies: purified rat anti-mouse CD4 (1:100), purified rat anti-mouse CD8a (1:100, all from BD Biosciences). Dystrophin staining: sections were incubated percutaneously into the gastrocnemius muscles of mdx mice (six mice per time point). Mice were sacrificed 10, 60, or 120 days after injection.

RESULTS
Ex vivo gene transfer mediated by plasmid transduced MDSCs. Examination of the muscle sections at early time points (10 days after injection) revealed similar levels of dystrophin expression by both populations of plasmid transduced cells (Figure 1a, b). However, at time points of 60 days after injection, the dystrophin expression decreased in the muscle injected with CMV-dys-transduced MDSCs (data not shown). Dystrophin expression was more persistent in the muscle injected with MCK-dys-transduced MDSCs (data not shown). We detected more dystrophin expression in the MDSC-MCK group 120 days after injection than in the MDSC-CMV group (Figure 1c, d). We quantified the dystrophin expressing myofibers in the different groups at different time points. Result showed that MCK-dys-transfected MDSCs displayed significant higher number of dystrophin positive myofibers at 60 and 120 days post-injection (10 days post-injection) (Figure 2).

Determination of Immune response after cell transplantation. Immuno-staining of muscles that were injected with plasmid transduced cells at all time points after injection revealed that the injection of the CMV-dys-transfected MDSCs triggered greater CD4 lymphocyte infiltration than did the injection of MCK-dys-transduced MDSCs (data not shown). Similar results were found for CD8-positive cell infiltration (data not shown).

DISCUSSION
In this project, we demonstrated that the use of different promoters (CMV or MCK) within a given plasmid construct affects the efficiency and the persistence of dystrophin expression after plasmid-mediated gene transfer into the skeletal muscles of mdx mice. Ex vivo gene transfer of MCK-dys triggered a lower immune response that led to more efficient and more persistent dystrophin expression when compared to the ex vivo gene transfer of CMV-dys. We believe that MDSCs transfected with CMV-dys and delivered via ex vivo gene transfer acted as APCs after implantation in skeletal muscle as we have described previously[1]. Our results suggest that the CMV-dys-transfected MDSCs expressed dystrophin, and therefore rapidly initiated an immune response, while the MCK-dys-transfected MDSCs expressed the dystrophin only after differentiation into myotubes and myofibers, ultimately leading to longer persistence of the transgene in the injected skeletal muscle. These results demonstrate that the use of a muscle-specific promoter to restrict the transgene expression to skeletal muscle can reduce the host immune response and prevent MDSCs from acting as APCs, and thereby improve both the efficiency and long-term benefits of gene transfer in skeletal muscle.

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