Human mesenchymal stem cells (hMSCs) can be genetically modified with viral vectors and hold promise as a cell source for regenerative medicine, yet how hMSCs respond to viral vector transduction remains poorly understood, leaving the safety concerns unaddressed. Here, we explored the responses of hMSCs against an emerging DNA viral vector, baculovirus (BV), and discovered that BV transduction perturbed the transcription of 816 genes associated with five signaling pathways. Surprisingly, Toll-like receptor-3 (TLR3), a receptor that generally recognizes double-stranded RNA, was apparently upregulated by BV transduction, as confirmed by microarray, PCR array, flow cytometry, and confocal microscopy. Cytokine array data showed that BV transduction triggered robust secretion of interleukin-6 (IL-6) and IL-8 but not of other inflammatory cytokines and beta interferon (IFN-β). BV transduction activated the signaling molecules (e.g., Toll/interleukin-1 receptor domain-containing adaptor-inducing IFN-β, NF-κB, and IFN regulatory factor 3) downstream of TLR3, while silencing the TLR3 gene with small interfering RNA considerably abolished cytokine expression and promoted cell migration. These data demonstrate, for the first time, that a DNA viral vector can activate the TLR3 pathway in hMSCs and lead to a cytokine expression profile distinct from that in immune cells. These findings underscore the importance of evaluating whether the TLR3 signaling cascade plays roles in the immune response provoked by other DNA vectors (e.g., adenovirus). Nonetheless, BV transduction barely disturbed surface marker expression and induced only transient and mild cytokine responses, thereby easing the safety concerns of using BV for hMSCs engineering.
Microarray Scanner (Agilent Technologies), and the fluorescence intensities of gene probes for transcription expression profiling and 1,082 experimental control probes. Array data sets were deposited in the NCBI Gene Omnibus Express database under the accession number GSE15810.

RESULTS

Expression of hMSC surface markers. To examine whether BV transduction altered surface characteristics, hMSCs were mock transduced or transduced with a BV carrying no mammalian gene cassette at an MOI of 100, followed by immuno-
fluorescence labeling and flow cytometry analyses at 24 h hpt. In agreement with the surface marker profiles in normal hMSCs (18, 31), the mock-transduced hMSCs expressed CD29, CD44, CD73, CD90, CD105, and HLA-I but were negative for CD14, CD19, CD34, CD45, and HLA-II (Fig. 1). BV transduction did not apparently alter the surface expression profile, except that CD73 expression was slightly diminished while HLA-I expression was elevated.

**BV transduction-upregulated genes associated with the TLR signaling pathway.** To explore the global responses of hMSCs to BV transduction, hMSCs were treated as described in the legend of Fig. 1 and subjected to microarray analysis at 24 hpt. Of the 30,968 human genes on the microarray, we identified 548 upregulated (≥2-fold) and 268 downregulated (<0.5-fold) known genes after BV transduction compared with the mock transduction control (see Tables S1 and S2 in the supplemental material). Pathway analysis using the Pathway-Express tool demonstrated five signaling pathways that were profoundly disturbed: cell adhesion molecules, TLR, Jak-STAT, apoptosis, and antigen processing and presentation (see Tables S3 to S8 in the supplemental material). Since the activation of the TLR pathway is essential for initiating innate immunity and can trigger the other four pathways, we focused on the TLR pathway in subsequent experiments and analyses.

The microarray data revealed significant upregulation of TLR1, TLR2, and TLR3 but not of other TLR genes (Table 1). Certain genes encoding the TLR signaling molecules (e.g., MyD88 and IRAK2), downstream cytokines (e.g., IL-6 and IL-8), and other genes downstream of the TLR3 pathway (e.g., RSAD2, INDO, and PTGS2) were also significantly upregulated. To confirm the data, transcription was also quantified by using the RT² Profiler PCR Array, which detects 84 genes involved in the TLR pathway (including TLR1 to TLR10). In accord with the microarray data, the PCR array revealed the upregulation of such genes as TLR3, MyD88, IRAK2, IL-6, IL-8, and PTGS2 (Table 1). In contrast, the PCR array detected upregulation of neither TLR1 nor TLR2 but revealed the upregulation of other genes involved in the TLR pathway (e.g., NFkBIA, TRIF, and TRAF6). The discrepancy between the microarray and PCR array data sets probably arose from the relatively weak stimulation of these genes by BV transduction.

**BV transduction of hMSCs triggered IL-6 and IL-8 production.** To screen the BV-induced cytokines at the protein level, the conditioned medium collected at 24 hpt was analyzed by a multiplex cytokine array which simultaneously detects 11 cytokines (Fig. 2A and B). Compared with the mock transduction control, BV transduction did not significantly (P > 0.05) elicit
Despite the secretion of IFN-γ, tumor necrosis factor alpha (TNF-α), TNF-β, IL-1β, IL-2, IL-4, IL-5, IL-10, and IL-12 but provoked high-level secretion of IL-6 (∼3,722 pg/ml) and IL-8 (∼1,334 pg/ml). Such induction was dose dependent as IL-6 and IL-8 expression increased with elevating MOIs (Fig. 2C and D). To confirm the result and examine the kinetics, the protein expression of both IL-6 and IL-8 peaked at 24 hpt and fell to background levels at 96 hpt, indicating a transient cytokine response. It is also noteworthy that BV transduction did not provoke the cytokine response.

Additional data shown in Fig. 3A and B demonstrated that both BV transduction and poly(I:C) treatment stimulated a gradual increase in TRIF expression for 4 h and accumulation of phosphorylated IRF-3 in the nuclei. The nuclear trafficking of IRF-3 and NF-κB was further confirmed by confocal microscopy (Fig. 5B), which illustrated the absence of IRF-3 and NF-κB in the nuclei of untreated hMSCs and the presence of IRF-3 and NF-κB in the nuclei after BV transduction and poly(I:C) treatment.

**DISCUSSION**

The present study primarily aimed to explore the hMSC response to BV transduction and to decipher the molecular pathway. We determined that most hMSC surface markers remained undisturbed after BV transduction (Fig. 1), suggesting that hMSC characteristics are retained. This response contrasted markedly with the evident BV-induced upregulation of surface molecules (e.g., HLA-II) in dendritic cells (29) but was in line with the negligible perturbation of hMSC marker expressions.
expression (e.g., CD34 and CD105) after poly(I:C) treatment (18). BV transduction only slightly upregulated HLA-I, which is desirable since HLA-I is responsible for presenting endogenously synthesized proteins to CD8+ T cells. BV transduction also marginally downregulated CD73, but the physiological significance of this is unknown.

We identified 816 known genes that were significantly perturbed by BV transduction. Among all TLR genes, TLR3 expression showed the most pronounced upregulation. Concurrent with the TLR3 pathway (see introduction), BV transduction upregulated not only TLR3 but its downstream genes such as TRIF, TRAF6, NFKB1A (encoding IκB), IL-6, IL-8, IL12A, CCL2, CCL5, and CXCL2 (Table 1; see also Tables S1 and S2 in the supplemental material). At the protein level, BV elicited transient IL-6 and IL-8 production in a dose-dependent manner (Fig. 2 and 3), which concurred with the activation of TLR3 (Fig. 4) and its signaling molecules like TRIF, IRF-3, and NF-κB (Fig. 5). Critically, silencing TLR3 expression considerably abolished BV-induced cytokine secretion and augmented hMSC migration (Fig. 6). These data

**FIG. 2.** Cytokine production by BV-transduced hMSCs. (A and B) Cytokine production at an MOI of 100. (C and D) IL-6 and IL-8 production at different MOIs. (E and F) IL-6 and IL-8 production at different times. The mock-transduced hMSCs served as the controls. Cytokine production was measured using a fluorescence bead immunoassay that detects 11 cytokines (A to D) or ELISA kits (E and F).

**FIG. 3.** Cytokine production required infectious BV. (A and B) IL-6 and IL-8 production by the hMSCs transduced with virus (MOI of 100) that was untreated (−) or treated (+) with UV light. (C and D) IL-6 and IL-8 production by the hMSCs transduced with virus (MOI of 100) that was pretreated with 2 μl of RNase A (+RNase) or 2 μl of TNM-FH (−RNase) for 1 h at 37°C. In parallel, cells were mock transduced and served as controls. All spent media were collected at 24 hpt for ELISAs.

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collectively confirmed the activation of the TLR3 signaling pathway by BV.

However, BV transduction provoked no secretion of IL-1β, IFN-γ, IL-12, and TNF-α (Fig. 2A). These proteins were highly expressed by BV-transduced dendritic cells (1) but were not robustly secreted by the poly(I:C)-treated hMSCs (18, 31). Nor did we detect IFN-β secretion from 0.25 to 24 h after BV transduction or poly(I:C) treatment (see Table S9 in the supplemental material). IFN-β is the signature IFN induced after TLR3 activation in murine cells (2, 15), but its expression was not reported in studies that treated hMSCs with poly(I:C) (18, 31). In contrast, Opitz et al. recently showed that poly(I:C) treatment of hMSCs induced detectable IFN-β secretion and a subsequent signaling loop (24). One key difference was the poly(I:C) dose (50 μg/ml) these investigators used, which was markedly higher than amounts used in this (10 μg/ml) and other studies. As such, it appears that in hMSCs TLR3 ligation could elicit IFN-β secretion but at a fairly low magnitude. This suggests that in hMSCs certain pathways downstream of IRF-3 might be lacking or blocked unless potently stimulated. In this study, the virus dose (MOI of 100) used is sufficient to transduce 80 to 90% of hMSCs (12) and induce ectopic bone formation in vivo when hMSCs express an osteogenic factor (4). Given that these IFNs and cytokines are pivotal in establishing the antiviral state and immune responses, the undetectable induction of these proteins at an MOI of 100 is instrumental for the safe use of BV-transduced hMSCs for tissue regeneration.

FIG. 4. BV transduction of hMSCs triggered TLR3 activation. (A) hMSCs were mock transduced or transduced with BV. At 0.5 hpt the cells were subjected to fixation, permeabilization, immunostaining with anti-TLR antibodies, and flow cytometry analyses. The green and pink lines indicate the mock-transduced and transduced cells, respectively. As controls, cells were treated with TLR ligands for 30 min and subjected to the same analyses. The peak shift as indicated by the arrows demonstrated TLR3 activation after BV transduction and poly(I:C) treatment. (B) Cells were treated as described in panel A, labeled with anti-TLR3 antibody, counterstained with DAPI, and examined by confocal microscopy (Nikon TE2000 equipped with a confocal upgrade laser kit). Magnification, ×1,000. LPS, lipopolysaccharide.
Among the cytokines investigated, we detected only IL-6 and IL-8. This is conceivable as they are constitutively expressed by hMSCs (19) and are potently stimulated after poly(I:C) treatment (Fig. 6) (18, 31). IL-6 dictates the regulation of both inflammatory responses and hematopoiesis (26), and its overproduction relates to the pathology of autoimmune diseases and tissue remodeling. Conversely, IL-8 is present in the inflammatory milieu during tissue repair (34). The robust secretion of IL-6 and IL-8 thus suggests that BV transduction might impact hMSC differentiation and potentiate the inflammatory response after transplantation. To date, the consequences of TLR3 activation and IL-6/8 expression on hMSCs remain elusive. It was reported that TLR3 activation in hMSCs promotes migration (31) and hampers immunosuppressive properties but interferes with neither the phenotype nor the differentiation potential (18). However, it was also shown that TLR3 activation enhances the immunosuppressive properties of hMSCs (24). The discrepancy likely stems from the differences in experimental procedures, poly(I:C) dose, and duration of ligand treatment (24). For example, hMSCs have been incubated with poly(I:C) for 5 days (18) or 24 h (24) prior to evaluation of the immunosuppressive properties. In our hands, BV transduction of hMSCs did not impair long-term proliferation (11), differentiation (12), and immunosuppressive properties (5). The disparity in the immunosuppressive properties could arise from the differences in the protocols because the cells were exposed to BV for only 4 h, after which the virus was withdrawn. Also the BV-induced cytokine response was transient, precipitously dropping after 24 hpt and vanishing at 96 hpt (Fig. 2E and F). As a result, unlike results of previous studies that continuously stimulated hMSCs with poly(I:C), BV transduction only transiently activated the TLR3-mediated responses, which accounts for the intangible adverse effects. Our data, however, suggest that hMSCs be transplanted after cytokine responses wane in order to circumvent the disturbance of hMSC functions and elicitation of immune responses in vivo.

Our findings also raised an intriguing question: how did BV, a DNA virus, trigger the TLR3 pathway that is generally regarded as a sensor of dsRNA? Since BV genes (e.g., immediate-early gene ie1) can be expressed at low levels in mamma-
larian cells (20), the most likely explanation is that some BV genes were transcribed in hMSCs and that the RNA intermediates were recognized by TLR3. However, the underlying mechanism(s) awaits further investigation. Another intriguing is that BV DNA activated the TLR9 pathway in mouse immune cells (1), yet only TLR3 activation was detected in hMSCs. Because hMSCs express high levels of TLR3 and TLR4 but low levels of TLR1, TLR2, TLR5, and TLR6 and negligible levels of TLR7 to TLR10 (18), the undetectable activation of TLR7 to TLR9 may be explained by the lack of viral DNA-sensing (TLR9) and single-stranded RNA-sensing (TLR7) receptors.

In summary, hMSCs can be genetically engineered with various viral vectors (8) and serve as a promising cell and gene therapy vehicle, yet little is known about how hMSCs respond to viral vector transduction. This study, for the first time, systematically explored the cellular responses of hMSCs to virus transduction at the molecular level. We revealed that BV transduction of hMSCs barely perturbed surface marker expression even while altering the expression of genes implicated in several pathways. We also provided the first evidence that a DNA viral vector can activate the TLR3 pathway in hMSCs, leading to a cytokine expression profile distinct from that in immune cells. Although TLR3 has been implicated in controlling the infection of two DNA viruses (vaccinia virus [10] and mouse cytomegalovirus [30]), there was no direct evidence confirming the induction of the TLR3 pathway by a DNA virus until the recent discovery that Kaposi’s sarcoma-associated herpesvirus triggers the TLR3 pathway in human monocytes (35). Since DNA vectors including adenovirus, herpes simplex virus, and adeno-associated virus have been employed for gene therapy (35). 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