Baculovirus Transduction of Mesenchymal Stem Cells Triggers the Toll-Like Receptor 3 Pathway†‡

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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.

Toll-like receptors (TLRs) are pattern recognition receptors that recognize a variety of pathogen-associated molecular patterns and are essential for activating innate immunity and potentiating adaptive immunity against pathogens (for a review, see references 2, 15, and 23). To date, 11 TLRs have been identified in humans (2). For example, TLR2 recognizes bacterial lipoproteins and peptidoglycans, TLR3 recognizes virus-derived double-stranded RNA (dsRNA) and a synthetic dsRNA analogue poly(I:C) (polyriboinosinic-polyribocytidylic acid), TLR4 recognizes lipopolysaccharides, and TLR9 recognizes the unmethylated CpG DNA motifs. Upon the engagement of cognate ligands, TLRs are activated and recruit Toll/IL-1 receptor-containing adaptor molecules such as myeloid differentiating factor 88 (MyD88) and Toll/interleukin-1 receptor-domain-containing adaptor-inducing beta interferon (TRIF). Among the TLRs, the TLR3 pathway is unique in that its signaling cascade begins by recruiting TRIF (2, 15, 33). TRIF can signal through interferon regulatory factor 3 (IRF-3) phosphorylation, leading to downstream beta interferon (IFN-β) expression. TRIF also can orchestrate with TRAF6 and RIP1, leading to NF-κB activation and subsequent expression of cytokines and chemokines such as interleukin-1 (IL-1), IL-6, IL-8, IL-12, MCP-1 (CCL2), RANTES (CCL5), and MIP-2 (CXCL2).

The baculovirus (BV) Autographa californica multiple nucleopolyhedrovirus is a DNA virus that infects insects as its natural hosts and that has been developed as a biological insecticide. However, BV also efficiently transduces a broad range of mammalian cells in which BV neither replicates nor is toxic. Also, recombinant virus construction, propagation, and handling can be performed readily in biosafety level 1 facilities. These attributes have inspired the development of BV vectors for in vitro and in vivo genome delivery (6, 28), cartilage tissue engineering (3), development of cell-based assays, delivery of vaccine immunogens, production of viral vectors, and cancer therapy (for a review, see references 14 and 17). Furthermore, BV transduces human mesenchymal stem cells (hMSCs) derived from bone marrow at efficiencies greater than 80% (12) and accelerates osteogenesis of hMSCs in vitro and in vivo when expressing an osteogenic growth factor (4). hMSCs are capable of differentiating into multiple cell types (e.g., chondrocytes, osteoblasts, adipocytes, and myoblasts).
osteoblasts, and endothelial cells) and possess immunosuppressive and immunomodulatory properties (32). Therefore, hMSC-based cell therapy has captured a growing attention in regenerative medicine and has advanced to various phases of clinical trials for the treatment of damaged myocardium, knee injuries, graft-versus-host disease, and Crohn’s disease (22). hMSCs also serve as a gene delivery carrier for the treatment of cancer, osteogenesis imperfecta (13), and various neurological disorders (27). As such, the efficient BV transduction of hMSCs offers a new, attractive option for hMSC engineering.

Despite the potential of hMSCs for cell and gene therapy, whether the genetic modification provokes undesired cellular responses has yet to be explored. The lack of safety evaluation will hamper future clinical applications of genetically modified hMSCs. Therefore, the overriding objective of this study was to assess how hMSCs respond to BV transduction.

**MATERIALS AND METHODS**

**BV and hMSCs.** A recombinant BV harboring no mammalian transgene cassette was used for transduction. The virus was amplified and harvested, and titers were determined by an endpoint dilution assay based on virus infectivity in insect cells (25). Bone marrow-derived hMSCs were obtained from Cambrex Co., selected, enriched, cultured in alpha minimal essential medium (α-MEM) containing 10% fetal bovine serum (HyClone) as described previously (11), and expanded to passage 11 for all experiments.

The virus transduction was performed on six-well plates as described previously (21) with minor modifications. Depending on the multiplicity of infection (MOI), a certain volume of virus supernatant was mixed with NaHCO3-deficient RPMI (104 mJ/cm2) as described previously (21) before transduction. The virus transduction was performed on six-well plates as described previously (21) with minor modifications. Depending on the multiplicity of infection (MOI), a certain volume of virus supernatant was mixed with NaHCO3-deficient RPMI (104 mJ/cm2) as described above. The spent supernatant was discarded, and the cells were incubated with PolyI:C (ODN 2006; Invitrogen). The cytoplasmic and nuclear proteins were separately extracted using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher) and then analyzed for TLR expression in a similar fashion.

**Expression of hMSC surface markers.** hMSCs were nucleofected with 5 µg of a control small interfering RNA (siRNA) plasmid (InvivoGen) or a plasmid expressing the TLR3 siRNA (psiTLR3; InvivoGen) using a Human Plasmid Nucleofector Kit (Amaxa Biosystems). Among the genes tested, the highest knockdown was achieved by using a siRNA targeting TLR3. The knockdown efficacy was assessed using a fluorescence bead immunoassay (Bender MedSystems) that detects 11 cytokines simultaneously. IL-6 and IL-8 were also measured using Module Sets of enzyme-linked immunosorbent assays (ELISAs) (Bender Medsystems).

**Immunofluorescence labeling/confocal microscopy.** The cells were fixed and permeabilized as described above, followed by extensive washing and primary antibody staining (1:150 dilution) for 1 h at 4°C in the dark. The primary antibodies were specific for human TLR3, IRF-3 (ab30772, Abcam), or phospho-epitope NF-κB (Cell Signaling Technology). Following the washing step, the cells were incubated with Alexa Fluor 488-conjugated, goat anti-mouse IgG (Invitrogen) or a mixture of Alexa Fluor IgGs previously described in our laboratory. The images were acquired using a Zeiss LSM 710 confocal microscope and were analyzed using ImageJ software.

**Statistical analysis.** All data represent the mean ± standard deviation or mean values of at least three independent culture experiments. The data were statistically analyzed by one-way analysis of variance. A P value of <0.05 was considered significant.

**Microarray data accession number.** 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...
TABLE 1. Significantly changed genes associated with the TLR signaling pathway by BV transduction

<table>
<thead>
<tr>
<th>Gene group and name</th>
<th>Relative change in expression (n-fold) as determined by:</th>
<th>Microarray</th>
<th>PCR array</th>
</tr>
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<tr>
<td><strong>TLR genes</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TLR1</td>
<td>2.6</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>TLR2</td>
<td>6.6</td>
<td>ND</td>
<td></td>
</tr>
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<td>TLR3</td>
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<td></td>
</tr>
<tr>
<td><strong>TLR signaling-associated molecules</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MYD88</td>
<td>8.9</td>
<td>5.8</td>
<td></td>
</tr>
<tr>
<td>IRAK2</td>
<td>5.7</td>
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</tr>
<tr>
<td>NFKBIA</td>
<td>ND</td>
<td>9.4</td>
<td></td>
</tr>
<tr>
<td>TRIF</td>
<td>ND</td>
<td>3.4</td>
<td></td>
</tr>
<tr>
<td>TRAF6</td>
<td>ND</td>
<td>1.8</td>
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</tr>
<tr>
<td><strong>Cytokines and chemokines</strong></td>
<td></td>
<td></td>
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<td>CCL10</td>
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<td><strong>Other genes associated with TLR3</strong></td>
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<tr>
<td>RSAD2</td>
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</tr>
<tr>
<td>PEL11</td>
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*The data represent the average values from three to five independent culture experiments. ND, not detectable; NA, not available in the PCR array.

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 concentrations were measured again by ELISA at 24, 48, and 96 hpt. The results shown in Fig. 2E and F demonstrate that the 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 secretion of antiviral IFN-α (5) and IFN-β (see Table S9 in the supplemental material).

Whether cytokine induction required infectious BV was explored by inactivating the BV with UV prior to transduction. The ELISA data (Fig. 3A and B) showed that UV inactivation significantly (P < 0.05) abolished the BV-induced IL-6 and IL-8 secretion, indicating the essential role of the live virus. Since IL-6 and IL-8 can be elicited by dsRNA as a result of TLR3 activation (18, 31), the virus solutions were treated with RNase or TNM-FH medium prior to transduction. The results shown in Fig. 3C and D show that RNase treatment retarded secretion of neither IL-6 nor IL-8 after BV transduction, thus ruling out a role for RNA. These data collectively confirmed that BV itself provoked the cytokine response.

**BV transduction of hMSCs triggered the TLR3 pathway.** To examine the induction of TLR3 and other TLRs at the protein level, hMSCs were transduced with BV or treated with different TLR agonists and were subjected to immunofluorescence labeling/flow cytometry analyses (Fig. 4A). Compared with the mock transduction control, BV transduction led to the emergence of a peak when cells were labeled with the TLR3 antibody. Such a peak shift was due to receptor activation, internalization, and degradation (31) and was similarly observed for the sample treated with the TLR3 ligand, poly(I:C). However, BV transduction did not apparently provoke TLR2, TLR4, or TLR9.

**TLR3 activation was further visualized by confocal microscopy** (Fig. 4B). TLR3 expression was diffuse in the cytoplasm of the untreated hMSCs but was more focused along the edge of the BV-transduced cells, which was likewise observed in the poly(I:C)-treated hMSCs (Fig. 4B) (31). The results shown in Fig. 4, in conjunction with the microarray and PCR array data, concretely attested to TLR3 activation by BV transduction.

In immune cells, TLR3 activation induces TRIF expression and results in the nuclear translocation of phosphorylated IRF-3 and NF-κB (16). Western blot analyses of hMSCs (Fig. 5A) 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 and NF-κB in the nucleus. 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.

**TLR3 knockdown diminished BV-induced cytokine secretion and promoted migration.** To correlate TLR3 activation and cytokine secretion, cells were nucleofected with a plasmid expressing the control siRNA or psiTLR3. After 48 h of culture, the cells were mock transduced, transduced, or treated with poly(I:C). As depicted in Fig. 6A and B, psiTLR3 treatment of hMSCs considerably abrogated poly(I:C)-induced IL-6 and IL-8 secretion, confirming the TLR3 knockdown by psiTLR3. Accordingly, TLR3 silencing by psiTLR3 treatment significantly (P < 0.05) attenuated BV-induced IL-6 and IL-8 secretion.

Additionally, we examined the effect of TLR3 knockdown on BV-induced migration by the transwell migration assay. The results shown in Fig. 6C indicate that the migration of cells treated with the control siRNA was remarkably impeded by both poly(I:C) treatment and BV transduction, but psiTLR3 treatment significantly (P < 0.05) ameliorated the migration of poly(I:C)-treated and BV-transduced hMSCs.

**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 ex-
pression (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 IxB), 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-kB (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.
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. 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 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-
BACULOVIRUS TRIGGERS TLR3 PATHWAY IN hMSCs

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