Human mesenchymal stromal cells (MSC) possess immunosuppressive and antimicrobial effects that are partly mediated by the tryptophan-catabolizing enzyme indoleamine-2,3-dioxygenase (IDO). Therefore MSC represent a promising novel cellular immunosuppressant which has the potential to control steroid-refractory acute graft versus host disease (GvHD). In addition, MSC are capable of reducing the risk of infection in patients after haematopoietic stem cell transplantation (HST). Recent data indicate that signals from the microenvironment including those from microbes may modulate MSC effector functions. As Cytomegalovirus (CMV) represents a prominent pathogen in immunocompromised hosts, especially in patients following HST, we investigated the impact of CMV infection on MSC-mediated effects on the immune system. We demonstrate that CMV-infected MSC lose their cytokine-induced immunosuppressive capacity and are no longer able to restrict microbial growth. IDO expression is substantially impaired following CMV infection of MSC and this interaction critically depends on intact virus and the number of MSC as well as the viral load. Since overt CMV infection may undermine the clinical efficacy of MSC in the treatment of GvHD in transplant patients, we recommend that patients scheduled for MSC therapy should undergo thorough evaluation for an active CMV infection and receive CMV-directed antiviral therapy prior to the administration of MSC.

1. Introduction

Human multipotent mesenchymal stromal cells (MSC), known for their multilineage differentiation potential, possess pleiotropic immunosuppressive functions that are partly mediated by expression of the tryptophan-catabolizing enzyme indoleamine-2,3-dioxygenase (IDO) [1–4]. Upon stimulation with inflammatory cytokines, MSC exhibit broad-spectrum antimicrobial effector functions directed against various clinically relevant pathogens, and these effects are dependent on IDO and/or the antimicrobial peptide LL-37 [5, 6]. These dual immunosuppressive and antimicrobial
properties render MSC a promising novel cellular immunosuppressant which is currently under intensive clinical investigation for various auto- and alloimmune diseases such as steroid-refractory graft versus host disease (GVHD) after allogeneic hematopoietic stem cell transplantation (HSCT), Crohn’s disease, and multiple sclerosis [7–10]. Emerging data indicate that signals from the microenvironment including those induced by hypoxia [11, 12] or derived from microbes may critically affect IDO and consequently MSC effector functions [13–15]. As the Cytomegalovirus (CMV) represents a prominent pathogen in immunocompromised hosts in particular in patients suffering from GvHD after HSCT, we initiated studies investigating the impact of CMV infection on MSC-mediated effects. During coevolution with its specific host, human CMV has developed several immune evasion strategies [16–18]. For example, CMV has been reported to inhibit the upregulation of MHC class II antigens. Furthermore, it was found that CMV generally inhibits signalling via the IFN-γ receptor and that this is mediated via a reduced phosphorylation of STAT1 and an enhanced degradation of Jak1 [19–21].

Mesenchymal stromal cells and embryonic stem cells are able to inhibit T-cell responses and several mechanisms including the production of prostaglandins, immunosuppressive cytokines [3], of arginase I [22], or of adenosine including the production of prostaglandins, of immunosuppressive and antimicrobial properties, thereby implicating that active CMV infections may undermine the clinical efficacy of MSC treatment.

2. Materials and Methods

2.1. Primary Cells. Human bone marrow-derived MSC were prepared, propagated, and characterized as previously described [5]. Bone marrow aspirates for the generation of MSC were obtained from healthy volunteer donors who had provided written informed consent; the study was conducted according to the Declaration of Helsinki principles and approved by the ethics committee of the Medical Faculty of the Heinrich-Heine-University, Düsseldorf, Germany.

2.2. Cell Lines and Reagents. OKT3 producing hybridoma cells were obtained from the American Type Culture Collection (Rockville, USA). Recombinant human IFN-γ was purchased from R&D Systems (Wiesbaden, Germany). L-Tryptophan, L-kynurenine, 1-L-methyl-tryptophan (1-MT), and Ehrlich’s reagent were ordered from Sigma-Aldrich (Deisenhofen, Germany).

2.3. HumanCytomegalovirus. CMV strains AD169 and TB40E were kindly provided by C. Sinzger (Institute for Medical Virology, Ulm, Germany) and A. Zimmermann (Institute for Virology, Düsseldorf, Germany). Before infection of MSC, the virus-containing solution was thawed and diluted in tryptophan-free RPMI 1640 medium to reach a multiplicity of infection (MOI) of 0.1–10. In some experiments UV-inactivated CMV preparations were used. Viral replication was analysed using real-time PCR as described [5].

2.4. Kynurenine Assay. The enzymatic activity of IDO directly correlates with the concentration of kynurenine in supernatants of tissue culture cells and therefore, the measurement of kynurenine can be used to determine IDO activity [25]. MSC (2 × 10^4 per well) were plated in 96-well flat-bottomed microtiter plates in IMDM containing 5% FCS and 0.6 mM L-tryptophan. The cultures were stimulated with IFN-γ at concentrations indicated in the respective experiments. The plates were incubated at 37°C and after 72 h 160 µL were removed from each well and transferred to a 96-well V-bottomed plate. After the addition of 10 µL 30% trichloroacetic acid to each well, the plates were incubated at 50°C for 30 minutes to hydrolyze N-formyl-kynurenine to kynurenine. After centrifugation for 10 min at 600 g, 100 µL supernatant was transferred to 96-well flat-bottomed plates and 100 µL 1.2% (w/v) 4-(dimethylamino) benzaldehyde (Ehrlich’s reagent) in glacial acetic acid was added. After 10 minutes at room temperature, the extinction was determined at 492 nm with a microplate reader (Tecan, Crailsheim, Germany). Data are given as mean kynurenine content of triplicate cultures. In some experiments IDO was induced by coculturing MSC with OKT3-stimulated peripheral blood lymphocytes for three days. As a control 1-L-MT (1.5 mM) or a neutralizing anti-IFN-γ antibody (10 ng/mL) was added at the time point of MSC stimulation. In addition, IDO protein was detected in stimulated MSC using Western blot analysis as described [5].

2.5. T-Cell Proliferation Assay. 1 × 10^5 peripheral blood lymphocytes (PBL), obtained from heparinised blood of healthy donors after Ficoll purification, were stimulated with a monoclonal anti-CD3 antibody (OKT3, American Type Culture Collection, Rockville, USA) in the presence of different amounts of MSC as described [26]. In some experiments MSC (0.5–2 × 10^4 per well) were infected with CMV and/or stimulated with IFN-γ at the start of the culture. After three days the cultures were pulsed with 0.2 µCi [3H] thymidine for 24 hours. T-cell proliferation was measured by [3H] thymidine incorporation using liquid scintillation spectrometry (1205 Betaplate, PerkinElmer, Jugelsheim, Germany).

2.6. Staphylococcus aureus. MSC were infected with 10–100 cfu/well. Bacterial growth was monitored after further incubation of 16 h by measuring optical density at 620 nm [27].

2.7. Statistical Analysis. All data are given as mean ± SEM of at least 3 independent experiments and each experiment was performed in triplicate. Data of representative experiments, also performed in triplicate, were given as mean ± SD. For
various numbers of CMV-infected MSC. We found that formed a series of 15 subsequent experiments employing could be abrogated by the addition of CMV or of 1-MT. MSC inhibited T-cell responses and this inhibitory effect L-methyl-tryptophan (1MT). As shown in Figure 2(b) human MSC [4]. To prove this finding we used the IDO inhibitor 1-L-methyl-tryptophan (1MT). As shown in Figure 2(b) human MSCs inhibited T-cell responses and this inhibitory effect could be abrogated by the addition of CMV or of 1-MT.

To substantiate these experimental findings, we performed a series of 15 subsequent experiments employing various numbers of CMV-infected MSC. We found that 2 × 10⁴ MSC provided substantial suppression of T-cell proliferation and the addition of CMV consistently antagonizes this T-cell inhibitory effect. In all 15 experiments performed an inhibitory effect of CMV on MSC-mediated T-cell suppression was observed; however, the magnitude of this effect varied (Figure 3(a)). In contrast, 5 × 10³ MSC were unable to restrict T-cell proliferation and, under these conditions, the same amount of CMV that was employed in the experiments shown in Figure 3(b) did not have any impact on T cells, thus ruling out an unspecific effect of CMV. Taken together, this data demonstrates that CMV infection of human MSC substantially impedes their T-cell inhibitory effector function.

In previous studies we and others have identified a significant role of the IFN-γ-inducible tryptophan-catabolizing enzyme indoleamine-2,3-dioxygenase (IDO) in MSC-mediated T-cell inhibition [4, 28]. In additional studies, the role of IDO in tolerance induction was demonstrated in in vivo studies using IDO deficient animals [29]. Based on these findings we went on to assess the impact of CMV infection on cytokine-induced IDO activity of human MCS. As shown in Figure 4(a), we observed a substantial IDO-mediated kynurenine production when MSC were cultured in the presence of OKT3-activated T cells. As expected, IDO activity induced in MSC by activated T cells could be blocked by neutralising antibodies directed against IFN-γ as well as by the IDO-specific inhibitor 1-L-methyl-tryptophan. However, it is of particular interest that CMV-infected MSC were unable to express IDO activity in the presence of activated T cells, while UV-inactivated CMV preparations had no impact on IDO activity. Thus, an infection with replication-competent CMV substantially impairs IFN-γ-induced IDO expression in human MSC.

In addition to their immunosuppressive capacity, MSC have recently been shown to possess cell-autonomous antimicrobial effects directed against various clinically relevant pathogens [5, 6, 15]. We therefore proceeded to analyse the potential impact of CMV on MSC-mediated antibacterial effects induced by cytokines released from OKT3-stimulated T cells. As shown in Figure 4(b), MSC cocultured with activated T cells are able to restrict bacterial growth and CMV infection of MSC abrogated their antimicrobial effect against Staphylococcus aureus. The functional relevance of IDO-mediated antibacterial effects was confirmed by demonstrating that addition of excess amounts of tryptophan completely abolished the antimicrobial effector function of MSC (Figure 4(b)).

In additional experiments we analysed CMV-mediated inhibition of IFN-γ induced IDO activity in human MSC in more detail. We found that CMV infection impairs cytokine-induced IDO activity of human MSC in a dose-dependent manner with significant inhibition observed at CMV doses as low as a MOI of 0.6 and with a maximum effect at a MOI of 5 (Figure 5(a)). Furthermore, Western blot analysis, depicted in Figure 5(b), showed that CMV infection of MSC results in a reduced expression of IDO protein.

In our present work we describe for the first time that a CMV infection critically impairs the immunosuppressive
Figure 2: Influence of MSC and CMV on T-cell proliferation. (a) PBL were cultured in the absence or presence of CMV (corresponding to MOI 5) for three days and were stimulated with OKT3 or not. Data are given as mean cpm $[^{3}H]$ thymidine incorporation of four independent experiments with cells from different donors. (b) PBL were cultured in the presence of MSC ($3 \times 10^4$/well) and in the absence or presence of 1-MT (1.5 mM) or CMV (MOI 5). T-cell proliferation was determined as described above. Data are given as % of positive control (OKT3-activated PBL without MSC). The significant inhibition of T-cell proliferation by MSC is marked with one asterisk ($P < 0.05$); the significant antagonistic effect on T-cell inhibition by CMV or 1-MT is marked with two asterisks ($P < 0.05$).

Figure 3: Dose dependency of MSC-mediated effects on T-cell proliferation. Different amounts of MSC ((a) $2 \times 10^4$/well and (b) $2 \times 10^4$ or $5 \times 10^3$/well), either CMV infected (MOI 5) or not, were cocultured with OKT3-activated PBL. Thereafter, T-cell proliferation was determined as described above. Data are given as % of positive control, that is, OKT3-activated PBL without MSC. Each dot represents a single data point from a total of 15 individual experiments, each performed in triplicate. A significant inhibition of T-cell proliferation by MSC is marked with asterisks.
and antimicrobial effector functions of human MSC possibly via an interaction with the IFN-γ-induced IDO pathway. We demonstrate that this interaction between CMV and IDO-mediated effector functions of stromal cells critically depends on intact virus as well as the number of host cells and virus employed.

We have recently reported that human fibroblasts lose their immunosuppressive capacity after a CMV infection and that this finding might be relevant for host-versus-graft reaction disease triggered by CMV infection/reactivation after solid organ transplantation [26]. Here we show that a similar CMV-mediated effect on MSC might influence
the clinical effectivity of these cells as immunosuppres-
sant.

We are aware of the fact that our observations are
derived from in vitro cell culture experiments. In the in vivo situation an immunosuppressive therapy, necessary in
transplant patients, might result in an inhibition of IFN-
y production and therefore inhibit IDO induction [30].
Immunosuppressive substances such as glucocorticoids at
high concentrations are able to enhance IDO activity [31, 32].
Furthermore the broad organ tropism, different cell types,
and differences in cell tropism might have an impact on
CMV-mediated inhibition of IDO activation [33]. However,
based on the species specificity of CMV infection as well
as IDO expression [5, 34], murine in vivo experiments
including humanized xenograft models will not be
informative with regard to the in vivo impact of a
CMV infection on MSC-mediated immunosuppressive and
antimicrobial effects.

We put a particular focus on MSC in our study as
these cells represent a promising novel therapeutic approach
for a variety of clinical applications ranging from tissue
engineering and regenerative medicine to the treatment of
auto- and alloimmune diseases refractory to conventional
pharmacologic immunosuppression [35, 36]. One might
speculate that the inhibitory interaction of CMV with IDO-
mediated immunosuppressive effects may explain the consis-
tent clinical observation that CMV infection frequently trig-
grs GvHD following hematopoietic stem cell transplantation
[37].

The findings presented here may have implications for
transplantation medicine and the future clinical use of MSC.
We found that MSC and fibroblasts are permissive for CMV
infection and are able to inhibit viral growth due to IDO-
dependent mechanisms [5]. Both cell types are also sensitive
to CMV-mediated immune escape mechanisms [26]. We
suggest that the balance between IFN-γ dose-dependent
IDO induction and CMV dose-dependent IDO inhibition
might influence the clinical outcome of organ or stem cell
transplantation in CMV-infected patients. We are aware that
the magnitude of virus load and of IFN-γ concentrations
in virus plaques within infected human tissues is unknown.
However we recommend that patients scheduled for MSC
therapy should undergo thorough evaluation for an active
CMV infection and receive CMV-directed antiviral therapy
prior to administration of MSC, if appropriate, since overt
CMV infection of MSC recipients might underlie the clinical
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Conflict of Interests

The authors declare that there is no conflict of interests
regarding the publication of this paper.

Authors’ Contribution

Roland Meisel and Kathrin Heseler share first authorship.

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