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Source / Izvornik: **Leukemia, 2012, 27, 500 - 503**

Journal article, Published version

Rad u časopisu, Objavljena verzija rada (izdavačev PDF)

<https://doi.org/10.1038/leu.2012.202>

Permanent link / Trajna poveznica: <https://urn.nsk.hr/urn:nbn:hr:184:060561>

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Download date / Datum preuzimanja: **2025-01-03**



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Encapsulated mesenchymal stem cells for *in vivo* immunomodulation

Leukemia (2013) **27**, 500–503; doi:10.1038/leu.2012.202

Mesenchymal stem cells (MSCs) are self-renewable, multipotent progenitor cells able to differentiate into various mesodermal lineages.¹ MSCs are present in basically all tissues, and have a pivotal role in tissue repair and in local control of inflammation.

In vitro studies indicated that MSCs have immunomodulatory activities: they inhibit T and NK cell activation, B-cell terminal differentiation and dendritic cell maturation and functions.² Little is known about the action of MSCs *in vivo* but a recent report showed that MSCs affect dendritic cell homing to lymph nodes, thus impairing T-cell priming.³ Although there is evidence from *in vivo* studies that MSCs are able to reduce inflammatory damage without engraftment,⁴ it is not clear whether their immunomodulatory effects rely on soluble mediators or cell–cell contacts (reviewed in Yagi *et al.*²).

There is a growing interest in using MSCs to treat human inflammatory diseases, including severe (grade III–IV) steroid resistant acute graft versus host disease (aGVHD). Various trials reported non-homogeneous results, with MSCs responses varying from 15 to 55% of treated patients. Although a study using a large cohort of patients provided encouraging results (reviewed in Tolar *et al.*⁵), recent data could not confirm the efficacy of MSCs for the treatment of GVHD.⁶ The reasons for these conflicting results are not clear and, among others, may include differences in the number of MSCs that remain viable in patients overtime, a critical factor that so far is very difficult to control.

Another critical aspect of MSC-based cell therapy is its safety, especially when considering long-term complications: MSCs may cause tumor formation^{7,8} or aberrantly differentiate after ectopic engraftment.⁹ Finally, another risk factor is associated with the

administration route. Indeed, although the number of MSCs required to achieve immunomodulation *in vivo* is basically unknown, injection of large number of cells may be necessary to obtain maximal clinical benefit given the low rate of cell retention and survival. In these conditions, MSCs may form aggregates that could cause pulmonary emboli or infarctions in patients.¹⁰

Considering all these issues, we decided to study the immunomodulatory effects of encapsulated mouse MSCs (E-MSCs). Indeed, this procedure would allow not only to solve all safety problems related to MSCs administration, but also to design precise protocols in which cell viability, and therefore cell dosage, is standardized. Importantly, this approach might provide conclusive data in support of either the endocrine or the paracrine/contact hypothesis for the *in vivo* immunomodulatory activity of MSCs.

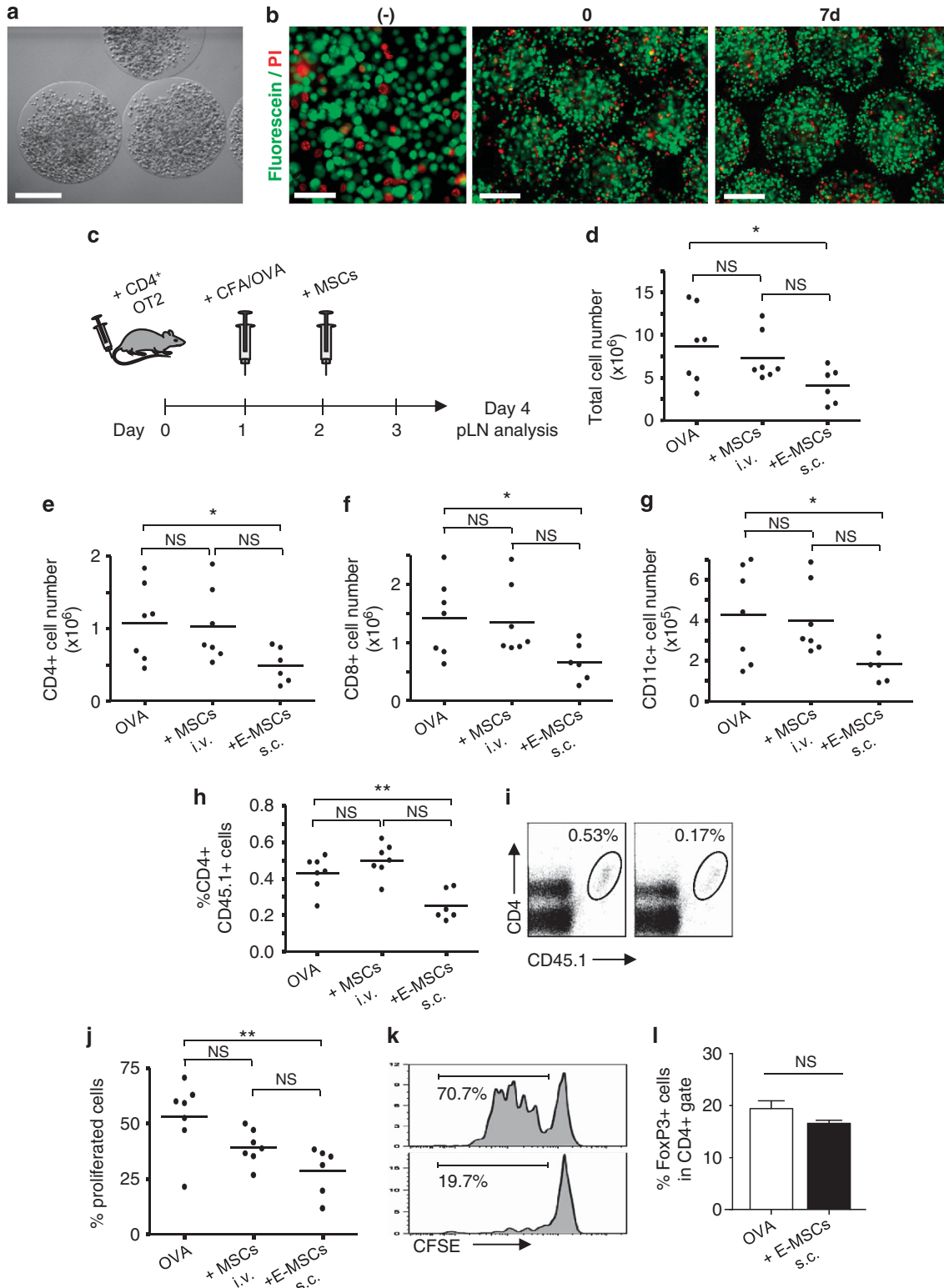
Alginate is the most commonly employed polymer for cell microencapsulation because of its excellent biocompatibility and *in vivo* stability.¹¹ MSCs derived from B6 mice were used for the preparation of alginate microcapsules that, when analyzed by optical microscopy, showed uniform and spherical morphology (Figure 1a), with an average diameter of 600–700 μm. Staining with fluorescein diacetate and ethidium bromide showed that the microencapsulation procedure did not affect MSCs viability, even after 7 days of *in vitro* incubation (Figure 1b). In order to demonstrate that E-MSCs do not lose their multipotentiality, we compared the osteogenic and adipogenic differentiation of MSCs extracted from 7-day-old microcapsules with that of control MSCs. Both populations displayed analogous positivity for Alizarin Red staining after 15 days of conditioning with osteogenic stimuli (Supplementary Figure 1a). Similarly, the staining with Oil Red after adipogenic stimulation showed comparable differentiation

Figure 1. OVA-specific T-cell proliferation is hampered by E-MSCs. **(a)** Phase-contrast microscopy of mouse MSC-loaded microcapsules shows their uniform size and cell distribution (average capsule diameter 0.6 mm; scale bar 200 μm). **(b)** Fluorescence images of fluorescein diacetate viability assay of: MSCs monolayer before the encapsulation procedure (–) (scale bar 30 μm) and after encapsulation at day 0 and 7 in culture (scales bar 200 μm). Red staining indicates dead cells. **(c)** Diagram of the experimental protocol designed to investigate the influence of MSCs upon the activation of OT2 (OVA-specific) T cells: OT2 CD45.1 CFSE-labeled cells (1×10^6) were transferred into wild-type mice. After 24 h mice were immunized with 100 ng OVA_{323–339} peptide in complete Freund's adjuvant by s.c. injection into the footpad. On day 2 a group of animals received 0.4×10^6 MSCs intravenously and another group received 0.4×10^6 encapsulated MSCs injected s.c. in the back. Four days after immunization, the popliteal dLNs were collected, counted **(d)**, stained for CD4, CD8 and CD11c and analyzed by flow cytometry to determine absolute numbers **(e, f, g)**. The same cell suspensions were stained to identify the CD4⁺ CD45.1⁺ population **(h)**, with one representative dot plot shown in **(i)**. CD4⁺ CD45.1⁺ cells were analyzed for CFSE expression and data are expressed as percentage of cells that have divided **(j)**. One representative histogram is shown in **(k)**. The percentage of CD4⁺ FoxP3⁺ cells was determined by i.c. staining with FoxP3 antibodies **(l)**. The results of 1 representative experiment out of 3 are shown ($n = 3$ or 4; * $P < 0.05$; ** $P < 0.005$). CFSE, carboxyfluorescein succinimidyl ester; NS, not significant; PI, propidium iodide.

to adipogenic fate (Supplementary Figure 1b). mRNA expression of differentiation-related genes, such as fatty acid binding protein 4, Osteocalcin and Collagen2a1 did not increase (Supplementary Figure 1c), suggesting that the multipotency of E-MSCs was preserved *in vitro*.

We next determined the biocompatibility and viability of E-MSCs administered subcutaneously (s.c.) *in vivo*. Subcutaneous

administration was chosen because the procedure is barely traumatic, does not elicit innate immune responses and is clinically applicable.¹² E-MSCs injected s.c. in the back of mice did not leave the inoculation site and remained as a clear cluster with no tissue capsule surrounding them (data not shown). A high percentage of MSCs extracted from these capsules 30 days after inoculation were viable cells (Supplementary Figure 1d). This



long-term viability may represent an important achievement for the clinical use of MSCs considering that MSCs injected in the circulation are viable for few days only in the homing tissue.¹³ Furthermore, their multipotency was preserved as shown by the low levels of mRNA expression of differentiation-related genes (Supplementary Figure 1e). These results show that E-MSCs are biocompatible and maintain their viability and stem cell properties *in vivo* over long periods of time.

The ability of E-MSCs to modulate a local, antigen-specific immune response *in vivo* was then tested. B6 mice were adoptively transferred with CD4⁺ CD45.1⁺ Ovalbumin-specific T cells obtained from OT2 mice (TCR transgenic for Ovalbumin peptide 323-339) and were immunized 1 day later with Ovalbumin peptide in complete Freund's adjuvant, by s.c. injection into the footpad. On day 2, animals received 0.4×10^6 MSCs intravenously or 0.4×10^6 E-MSCs s.c. Recipient mice were killed at day 4, the popliteal draining LNs (dLNs) were collected and cells were analyzed by flow cytometry (Figure 1c). Compared with MSC-treated or untreated mice, those treated with E-MSCs showed a decreased cellularity in the dLNs (Figure 1d) and in absolute numbers of CD4⁺, CD8⁺ and CD11c⁺ cells (Figures 1e–g). The percentage and the proliferation of Ovalbumin-specific CD4⁺ T in dLN were greatly reduced in E-MSC-treated mice in comparison with the other groups (Figures 1h–k). However, the CD4⁺FoxP3⁺ population was not affected by E-MSC transplantation (Figure 1l).

The fact that, in our experimental system, MSCs injected intravenously could not inhibit T-cell activation is in contrast with a recent report.³ This difference may be due to the fact that in our study we injected lower numbers of MSCs in a more inflammatory context. At low numbers, E-MSCs may be more efficient than intravenously injected MSCs that get trapped and die in the lungs (described in references^{10–13} and our unpublished observations).

The capacity of E-MSCs to modulate acute GVHD was then tested. Previous studies on mouse models for GVHD have provided conflicting data about the efficacy of MSCs. Whereas in some models repeated administrations of MSCs were required to inhibit moderate GVHD,^{5,14,15} in others MSCs were found to be ineffective in preventing or treating the disease (reviewed in Tolar *et al.*⁵).

Irradiated BALB/c mice were transplanted with either syngeneic (control mice) or allogeneic (GVHD mice) cells (Figure 2a), and the effects of E-MSCs were evaluated. Although E-MSCs had no effect on the increase of the serum levels of CCL8 and soluble tumor necrosis factor receptor 1 (Supplementary Figures 2a and b), examination of the liver revealed a trend in the decrease of the cumulative histologic score, inflammatory cell infiltration and tissue damage (Supplementary Figure 2c). The morphological observation (Supplementary Figures 2d and e) and the immunohistochemical detection of CD3 (Supplementary Figure 2f) revealed numerous foci of lymphoid cells infiltrating hepatic lobules in GVHD livers that were reduced upon E-MSCs treatment.

However, the most striking effect was on the survival of transplanted mice. Although GVHD mice that did not receive MSCs died between day 6 and 13 after transplantation, the treatment with E-MSCs s.c., significantly increased survival rate and clinical score of GVHD mice at day 8 (Figure 2b and c, respectively). Notably, in the same experiments, the intravenous injection of MSCs did not modify disease onset, in agreement with previous reports.⁵ Increased survival was also obtained with free MSCs injected s.c. (Supplementary Figure 3), demonstrating that the encapsulation procedure does not modify the immunoregulatory properties of the cells.

In conclusion, the use of encapsulated cells unequivocally demonstrates that MSCs do not require homing to specific organs or cell–cell contacts to control inflammation and that, in this context; their immunosuppressive action is based on the release of soluble factors that may act systemically. Although the

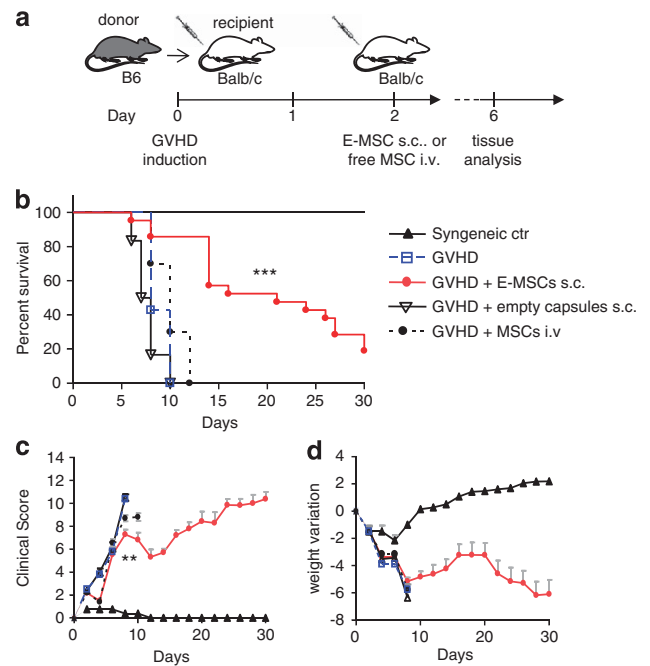


Figure 2. E-MSCs improve the survival of GVHD mice. **(a)** Diagram of experimental protocol. BALB/c mice irradiated with 700 rad were inoculated intravenously with 10^7 bone marrow cells (BMC) and 15×10^6 spleen cells derived from C57 donor mice for GVHD mice or from BALB/c donors for syngeneic controls. On day 2 mice received 400,000 E-MSCs s.c., the same amount of empty capsules or 400,000 MSCs intravenously. Mice were checked daily for weight, survival and they were given a score for a series of clinical parameters indicative of GVHD symptoms. **(b)** Survival curves of the five experimental groups. Data from three experiments were pooled (syngeneic control: $n=4$; GVHD: $n=14$; E-MSCs treated: $n=21$; empty capsules treated: $n=6$; MSCs intravenously treated: $n=10$). The results are presented as Kaplan–Meier survival curves. Statistical difference was demonstrated between GVHD-positive controls and mice treated with E-MSCs ($***P<0.0001$) while no dose–response effect was observed with higher doses of E-MSCs (1×10^6 or 2×10^6) (data not shown). Clinical GVHD signs **(c)** and weight **(d)** were monitored for 1 month after transplantation ($**P<0.005$).

molecular identification of such factors will require further substantial effort, possible candidates that have demonstrated to have a role *in vivo* and that can act at a distance are PGE2 and TSG-6.¹⁶ Both are upregulated by TNF- α , suggesting a level of control based on the inflammatory microenvironment.

From a translational point of view, subcutaneous transplantation of E-MSCs might represent the optimal strategy for therapeutic intervention in inflammatory diseases because it eliminates all potential problems related to stem cell transplantation, such as tumor growth, ectopic engraftment and uncontrolled differentiation. It also circumvents potential risks linked to the stimulation of donor graft rejection in non-myeloablative settings when using allogeneic MSCs (reviewed in Tolar *et al.*⁵). Moreover, removable microcapsules would allow a precise dosage over time, an important aspect of MSC-based therapy that at the moment is very difficult to evaluate. Notably, cell encapsulation using alginate is already being tested in clinical trials for Alzheimer's disease and type 1 diabetes (NCT01163825; NCT01379729, www.clinicaltrials.gov), indicating that the translation of our results into the clinic could be rapidly achieved.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

We thank Marta Lezama for assistance and Alberto Mantovani for helpful discussions and critical reading of the paper. This work has been supported by grants from Associazione Italiana Ricerca sul Cancro (AIRC, 'Lombardia Molecular Imaging'), Ministero della Salute (Bando cellule staminali, Bando Giovani Ricercatori) and European Community 7th Framework Programme (TIMER project). We would like to thank 'Fondazione Matilde Tettamanti', 'Comitato Maria Letizia Verga', 'Comitato Stefano Verri', 'Fondazione MBBM' and CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, Brazil) for their support. AS was supported by Inserm, France and Fondazione Cassa di Risparmio delle Province Lombarde (grant 5808/2007). MC was supported by a fellowship from European Community 6th Framework Programme INNOCHEM project.

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Supplementary Information accompanies the paper on the Leukemia website (<http://www.nature.com/leu>)

Biclonal and biallelic deletions occur in 20% of B-ALL cases with IKZF1 mutations

Leukemia (2013) **27**, 503–507; doi:10.1038/leu.2012.204

The IKZF1 gene encodes the Ikaros transcription factor, a key regulator of lymphocyte differentiation.¹ IKZF1 is mutated in 20–30% of B-cell acute lymphoblastic leukemia (B-ALL) mostly by genomic deletions.^{2–4} Several reports have shown that IKZF1 deletions are associated with an adverse prognosis, especially in pediatric patients.^{3–10} Ikaros mutations fall mainly into three types: (a) deletions of exons 4–7 ($\Delta 4-7$), which lead to the synthesis of the Ikaros-6 (Ik6) dominant-negative isoform; (b) deletions of exons 2–7 ($\Delta 2-7$), which delete the initiation codon and lead to haploinsufficiency; and (c) larger deletions of various sizes, which affect the coding exons (referred to below as 'complete' deletions). Thus, IKZF1 mutations have until now been separated

into dominant-negative and haploinsufficient groups. Here we report that about 20% of B-ALL patients with IKZF1 mutations present two distinct deletions. These deletions are biallelic, leading to a complete loss of Ikaros function, or biclonal, marking distinct clones within the leukemia. These results highlight a more complex picture of IKZF1 loss of function in B-ALL than thought previously.

IKZF1 status was analyzed in bone marrow samples from 139 B-ALL patients (46 adult and 93 pediatric), followed at the Onco-Hematology Departments of the Strasbourg and Mulhouse Hospitals from 2000 (Supplementary Table S1). To identify $\Delta 2-7$ or complete deletions, we quantified exon 2 and 8 DNA levels by quantitative PCR (qPCR) (Figure 1a). The $\Delta 4-7$ deletions were identified by PCR analysis with primers on each side of the conserved breakpoint³ (Supplementary Figure S1). The copy number of all IKZF1 exons was also assessed by multiplex ligation