

# Mesenchymal stem cell-mediated T cell suppression occurs through secreted galectins

MOULDY SIOUD<sup>1</sup>, ANNE MOBERGSLIEN<sup>1</sup>, ABDELLATIF BOUDABOUS<sup>2</sup> and YNGVAR FLØISAND<sup>3</sup>

<sup>1</sup>Department of Immunology, Institute for Cancer Research, Radiumhospitalet-Rikshospitalet University Hospital, Oslo, Norway; <sup>2</sup>Department of Biology, University of Tunis El Manar, Tunis, Tunisia; <sup>3</sup>Department of Haematology, Radiumhospitalet-Rikshospitalet University Hospital, Oslo, Norway

Received August 26, 2010; Accepted October 11, 2010

DOI: 10.3892/ijo.2010.869

**Abstract.** Human galectins are involved in a variety of biological and pathological processes including cell adhesion, apoptosis, differentiation, immune regulation and tumour evasion. Previously, we identified galectin-3 as the first human lectin involved in the modulation of the immunosuppressive potential of mesenchymal stem cells (MSCs). In this study, we report on the expression profiles and potential activities of other galectins expressed in these cells. The data show that MSCs constitutively express galectins-1, -3 and -8 at both the mRNA and protein levels. In contrast to galectin-8, galectins-1 and -3 are secreted and found on the cell surface. MSC-mediated T cell suppression was inhibited by galectin-1-specific siRNAs but not by galectin-8-specific siRNAs. The double knockdown of galectins-1 and -3 almost abolished the immunosuppressive capacity of MSCs. The use of a competitive inhibitor for galectin binding,  $\beta$  lactose, restored alloresponsiveness, implying an extracellular mechanism of action of galectins. Collectively, the data highlight the involvement of secreted galectins-1 and -3 in MSC-mediated T cell suppression. The immunosuppression by MSC-secreted galectins should facilitate the use of recombinant galectin-1 and/or -3 as a novel therapy to alleviate inflammatory reactions such as those seen in graft versus host disease (GvHD) and autoimmune disorders.

## Introduction

Human mesenchymal stem cells (MSCs) represent a relatively rare stromal cell population traditionally found in the bone marrow (BM), but can also be isolated from other tissues including umbilical cord blood, amniotic fluid, adipose tissue

and foetal lungs (1-3). Similar to other stem cells, they have a high capacity for self renewal and retain the potential to differentiate into many different types of tissues, such as bone, cartilage, muscle, cardiomyocyte and adipose tissue. Moreover, MSCs exert a strong inhibitory effect on cells of innate and adaptive immunity, including dendritic, B, T and natural killer cells (4-7). Indeed, they have been used clinically to block graft-versus-host disease after allogeneic hematopoietic stem cell transplantation (8). Although the described mechanisms of MSC-mediated T cell suppression are rather complex, a number of secreted factors have been implicated, including transforming growth factor  $\beta$  (TGF- $\beta$ ), hepatocyte growth factor (HGF), prostaglandin E2 (PGE2), interleukin 10 (IL-10), indoleamine 2,3 dioxygenase (IDO) and interferon  $\gamma$  (9-11).

Certain studies have shown that MSCs and hematopoietic stem cells respond to pathogen-associated molecular patterns through toll-like receptors (TLRs) (12-15). A microarray analysis of human MSCs in response to a TLR-2 ligand, defined a set of genes whose expression was affected by TLR-2 signaling (16). Among the modulated genes are galectins, a family of human lectins that have pleiotropic functions in innate and adaptive immune responses (17). For example, galectins-1, -3 and -9 inhibited a range of T cell processes including T cell development and activation, apoptosis, cytokine secretion and regulatory T cell function (17). A number of studies have also shown the involvement of galectins in various aspects of tumorigenesis, such as cell survival, tumour-host interaction, angiogenesis and tumour metastasis (18-21).

Given the immune regulatory function of galectins, we anticipated that this family of human lectins could be used by MSCs to elicit immunosuppressive effects. Along this line, galectin-3-specific siRNA treatment reduced the MSC immunosuppressive effect on T cells, but did not totally eliminate the immunosuppressive capacity of MSCs, thus indicating the involvement of other factors (16). Galectin-3 is the Mac-2 antigen that is expressed constitutively on the surface of macrophages (22). It is a monomeric protein, but it can form pentamers, creating lattice structure on the cell surface. A recent study has shown that galectin-3 is secreted by umbilical cord blood-derived MSCs (23). Although we have previously shown that galectin-3 is involved in MSC function, little is known about the expression profiles and the

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*Correspondence to:* Professor Mouldy Sioud, Department of Immunology, Institute for Cancer Research, Radiumhospitalet-Rikshospitalet University Hospital, Montebello, N-310 Oslo, Norway  
E-mail: mosioud@medisin.uio.no

**Key words:** mesenchymal stem cells, galectins, immunosuppression, tumour evasion, RNA interference

function of other galectins in these cells. Accordingly, herein we report on the expression profiles of all known human galectins and the potential effects that they could have on MSC functions. The results indicate that secreted galectins-1 and -3 are essential for MSC-mediated T cell suppression.

## Materials and methods

**Antibodies and reagents.** PE-conjugated anti-CD3 and anti-CD14 were purchased from DakoCytomation (Copenhagen, Denmark). PE-conjugated anti-CD105 was purchased from BD Biosciences (Franklin Lakes, NJ, USA). Biotin-conjugated anti-galectin-1, -3, -8 and -9, and PE-conjugated streptavidin were purchased from R&D Systems (Abingdon, UK). The total RNA isolation kit, the cDNA synthesis kit and MSC culture medium were purchased from Invitrogen (San Diego, CA, USA).  $\beta$  lactose, amphotericin B and trypsin/EDTA, were purchased from Sigma-Aldrich.

**Isolation of MSCs from BM.** MSCs were isolated and expanded from BM taken from the iliac crest of adult volunteers who had given informed consent. Heparinised BM was mixed in a 2/3 volume of Dulbecco's Modified Eagle's medium/F12 (DMEM/F12) and mononuclear cells were prepared by gradient centrifugation (Lymphoprep, Nycomed Pharm, Oslo, Norway). Subsequently, the mononuclear cells were washed and then cultured in T175 flasks at a concentration of  $50 \times 10^6$  cells per 30 ml medium supplemented with 10% heat-inactivated FBS, 1% amphotericin B and antibiotics. The cultures were then incubated at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. After 3 days in cultures, non-adherent cells were removed and adherent cells were washed 3 times with DMEM. Following 12-15 days in culture, adherent cells that constitute the MSC population were detached by treatment with trypsin/EDTA. These cells were frozen down and/or re-plated at a density of  $10^6$  cells/75 cm<sup>2</sup> flask. Amphotericin B was omitted after the first passage.

**Flow cytometry.** For surface staining, MSCs ( $5 \times 10^5$  cells/well/100  $\mu$ l) were incubated for 30 min at 4°C with biotinylated antibodies against human galectins or isotype control antibodies diluted in PBS containing 1% foetal calf serum (FCS) and 0.1% sodium azide (FACS buffer). After washing, the cells were incubated with PE-streptavidin for 30 min. The cells were then washed in FACS buffer before being analysed using a BD FACS Canto II flow cytometer equipped with an argon laser.

**Mixed lymphocyte reactions (MLRs).** Human peripheral blood mononuclear cells were prepared by density gradient centrifugation (Lymphoprep, Nycomed Pharm) from buffy coats obtained from healthy adult donors. Cells were washed and then re-suspended in RPMI-1640 medium containing 10% heat-inactivated FCS and antibiotics. The cells were then cultured in RPMI-1640 supplemented with 10% heat-inactivated FCS and antibiotics. In order to study the effect of MSCs on T cell activation, MLR assays were performed in the presence of irradiated allogeneic MSCs. The cells were co-cultured in 96-well U-bottom microtiter plates. After 4 days of culture, the cells were pulsed with 1  $\mu$ Ci per ml of

[<sup>3</sup>H]-thymidine (20  $\mu$ l) 18 h before harvesting. All experiments were run in triplicate.

**Transfection.** Cells were transfected with siRNAs using lipofectamine according to the manufacturer's instructions (Invitrogen). Cells were plated at  $2 \times 10^5$  per well onto 6-well plates the day before transfection. The knockdown efficacy was monitored by Western blotting 2 days after transfection. The target site sequences of the used siRNAs were the following: Galectin-1, 5'-CCAGAUGGAUACGAAUUCA-3' and 5'-CCAGAUGGAUACGAAUUCA-3'; galectin-3, 5'-GUCUGGGCAUUCUGAUGUU-3', and 5'-GCUCCAUGAUGCGUUAUCU-3'; galectin-8, 5'-GGCCUUUCAUUUCAAUCCU-3', and 5'-GGCAAGAUGCAUUCAAUUU-3'. An arbitrary non-specific chemically made siRNA was used as the control (5'-UUGAUGUGUUUAGUCGCUA-3').

**RT-PCR.** Total RNA was isolated from confluent MSCs using TRIzol reagent (Invitrogen) according to the instructions of the manufacturer. Contaminating DNA was removed using DNase I treatment. Complementary DNA was synthesized from 5  $\mu$ g total RNA using the first strand cDNA synthesis kit and oligo(dT) primer in 30  $\mu$ l volume according to the manufacturer's instructions (GE Healthcare). PCR was conducted in 50  $\mu$ l in a 1/30 volume of cDNA using 2 units of TaqDNA polymerase. The thermal profile for all reactions was as follows: 2 min at 95°C, followed by 30 amplification cycles of 1 min at 95°C, 1 min at 58°C and 1 min at 72°C, with 4 min final extension at 72°C. RT-PCR products were separated on 1.5% agarose gels, were visualised by staining with ethidium bromide and photographed using the Molecular Imager ChemiDoc XRS System (BioRad). We used the following PCR primer pairs (all from Invitrogen): Galectin-1 forward, 5'-ATGGCTTGTTGGTCTGGTC-3' and reverse, 5'-TCAGTCAAAGGCCACACA-3'; galectin-2 forward, 5'-ATGACGGGGAACTTGAG-3' and reverse, 5'-TTATTCCTTTAACTTGAAAGAGGA-3'; galectin-3 forward, 5'-ATGGCAGACAATTTTTTCG-3' and reverse, 5'-TAATATCATGGTATATGAAGCAC-3'; galectin-4 forward, 5'-ATGGCCTATGTCCCCCGCA-3' and reverse, 5'-TTAGATCTGGACATGGACAAGG-3'; galectin-7 forward, 5'-ATGTCCAACGTCCCCAC-3' and reverse, 5'-TCAGAAGATCCTCACGGA-3'; galectin-8 forward, 5'-AGAATGATGTTGTCCTTAAAC-3' and reverse, 5'-CTACCAGCTCCTTACTTCC-3'; galectin-9 forward, 5'-ATGGCCTTCAGCGGTTCC-3' and reverse, 5'-CTATGTCTGCACATGGGTCAG-3'; galectin-10 forward, 5'-ATGTCCTGCTACCCGTG-3' and reverse, 5'-TATCTCTTTAAATAGCTGACAT-3'; galectin-12 forward, 5'-ATGAGTCAGCCCAGTGGG-3' and reverse, 5'-TCAAGGAGTGGACACAGTAGAG-3'; galectin-13 forward, 5'-ATGTCTTCTTTACCCGTG-3' and reverse, 5'-TCAATTGCAGACACACT; galectin-14 forward, 5'-ATGTCCCTGACCCACAG-3' and reverse, 5'-TCAATCGCTGATAAGCACT-3'; and  $\beta$  actin forward, 5'-ATCTGGCACCACACCTTCTAC-3' and reverse, 5'-CGTCATACTCCTGCTTGTGATC-3'.

**Western blot analysis.** Equal amounts of proteins were separated by 10% SDS-PAGE and transferred onto nitrocellulose membranes (GE Healthcare). After overnight

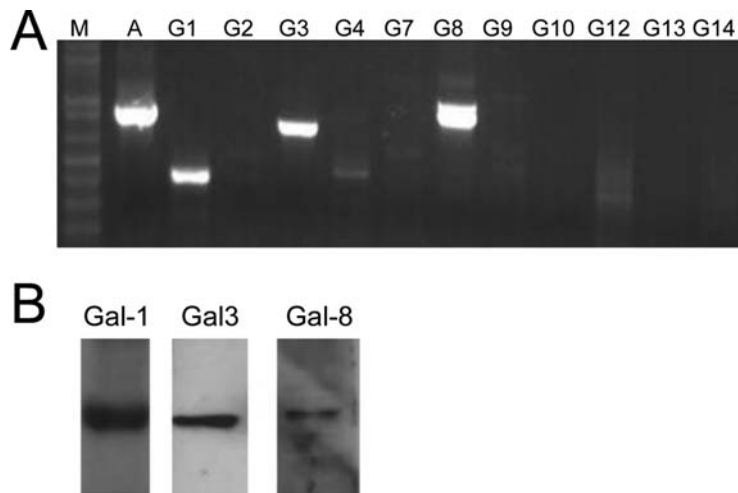


Figure 1. Galectin expression profiles in human MSCs by RT-PCR and Western blot analysis. (A) An agarose gel electrophoresis showing the PCR products. Total RNA was purified from MSCs and subjected to galectin expression by RT-PCR. Results are from 1 donor and are representative of 4 donors. M, 1 Kb marker; A,  $\beta$  actin, G, galectin. (B) Western blot analysis. Protein extracts from MSCs were analysed by Western blot analysis using anti-galectin specific antibodies.

blocking with 5% dry non-fat milk in phosphate-buffered saline containing 0.1% Tween-20, the membranes were incubated with biotin-conjugated anti-galectin antibodies for 1 h at room temperature followed by HRP-conjugated streptavidin. The reactive proteins were visualised with enhanced chemiluminescence plus reagent (GE Healthcare). In certain cases, the blots were stripped and re-probed with anti- $\beta$  actin polyclonal antibodies (Santa Cruz Biotechnology).

**Statistical analysis.** Statistical significance was determined using a two-tailed unpaired Student's t-test. P-values of <0.05 were considered to indicate statistical significance.

## Results

**Expression pattern of galectins in human BM MSCs.** In a search for the potential functions of galectins in MSCs, we first examined the expression of all known human galectins by RT-PCR. As shown in Fig. 1A, MSCs mainly expressed galectins-1, -3 and -8. In order to confirm the expressions at the protein level, Western blots were performed (Fig. 1B). In accordance with the PCR data, galectin-1 showed a strong band at the expected molecular weight of 14 kDa. Galectin-3 also displayed a prominent band at 30 kDa, and galectin-8 a faint band at 33 kDa.

**Cell surface expression.** Although galectins lack the signal sequence required for protein secretion via the usual secretory pathway, some are found on the cell surface and extracellular space (17). Therefore, we investigated the cellular localisation of galectins in MSCs. Cell surface staining experiments indicated that galectins-1 and -3 are on the cell surface (Fig. 2A). In contrast, only a very small fraction of MSCs showed staining for galectin-8. Given the cell surface staining of galectins-1 and -3, we questioned whether these proteins could be secreted by MSCs. Both galectins were detected in culture supernatants from MSCs (Fig. 2B).

*Galectin-1, like galectin-3, modulates MSC immunosuppressive effects on T cells.* Having demonstrated that MSCs express a large amount of galectin-1 and given the role played by this lectin in inhibiting T cell proliferation (24-26), we thus investigated its involvement in MSC immunomodulatory function. For this purpose, we knocked down the expression of galectins-1, -3 and -8 in MSCs using RNA interference and then investigated the immunomodulatory function of the cells. Immunoblot analysis showed that the expression of galectins-1, -3 and -8 was effectively inhibited in MSCs by siRNAs (Fig. 3A). As expected, the addition of MSCs to MLR cultures inhibited T cell proliferation (Fig. 3B). Gene silencing of galectin-1 ( $P < 0.01$ ), but not galectin-8, in MSCs restores T cell proliferation (Fig. 3B). These results indicate that galectin-1 expression is also important for the immunosuppressive capacity of MSCs. The double knockdown of galectins-1 and -3 almost abrogated the MSC inhibitory effect on T cells ( $P < 0.001$ ). Moreover, the addition of  $\beta$  lactose, a competitor inhibitor of galectin binding, restored the proliferation of T cells (Fig. 3B). The data with  $\beta$  lactose imply that secreted galectins are essential for MSC-mediated T cell suppression. When culture supernatants from MSCs were added to the T cells, a significant inhibition was observed. In contrast, supernatants from galectin-1- and -3-knocked-down MSCs, were extremely less immunosuppressive (data not shown).

## Discussion

Previous studies have shown that protein-glycan interactions play an essential role in controlling the responsiveness and tolerance of immune cells (17,27). In this study, we show that like galectin-3, galectin-1 is expressed on the cell surface of MSC and that it is secreted in culture supernatants where it can function as an extracellular protein to activate cells and mediate cell-cell and cell-ECM interactions. The knockdown of galectin-1 in MSCs inhibited the immunosuppressive

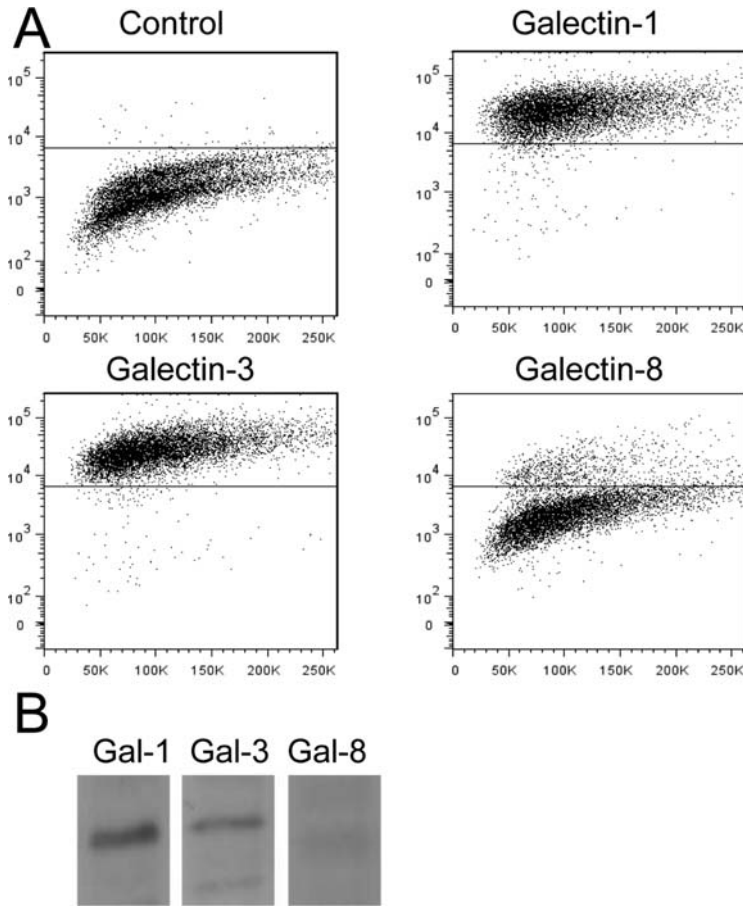


Figure 2. Galectins-1 and -3 are expressed on the cell surface of MSCs. (A) Surface-staining. A representative example of flow cytometry analysis of cultured MSCs with antibodies against galectins-1, -3 and -8. (B) MSCs secreted galectins-1 and -3 in culture supernatants. Proteins in 0.5 ml culture medium were precipitated with trichloroacetic acid, separated by SDS-PAGE and then immunoblotted with anti-galectin antibodies.

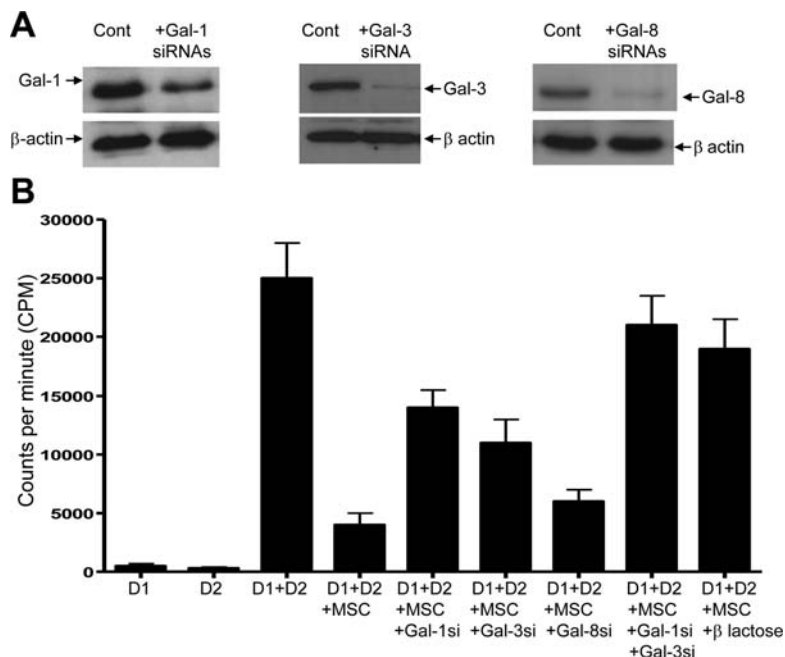


Figure 3. The knockdown of galectin-1, but not galectin-8, reduced the immunosuppressive effect of MSCs on T cell proliferation. (A) Knockdown of galectins-1, -3 and -8 in MSCs. The cells were transfected with the indicated siRNAs for 48 h. For each target, 2 different siRNA sequences were used. Protein extracts from control and treated cells were prepared and analysed by Western blot analysis. Control cells received an irrelevant siRNA. (B) Effects of galectin gene silencing on MSC immunosuppressive function. MSCs were transfected with galectin siRNAs for 24 h and then the cells were washed and cultured with peripheral blood mononuclear cells from 2 donors (D1 and D2). After 4 days incubation, alloantigen-driven proliferation was measured by [<sup>3</sup>H]-thymidine incorporation. Data are expressed as the mean counts per minute (cpm) ± standard deviation. Results are from 1 experiment performed in triplicate and are representative of 2 other independent experiments.



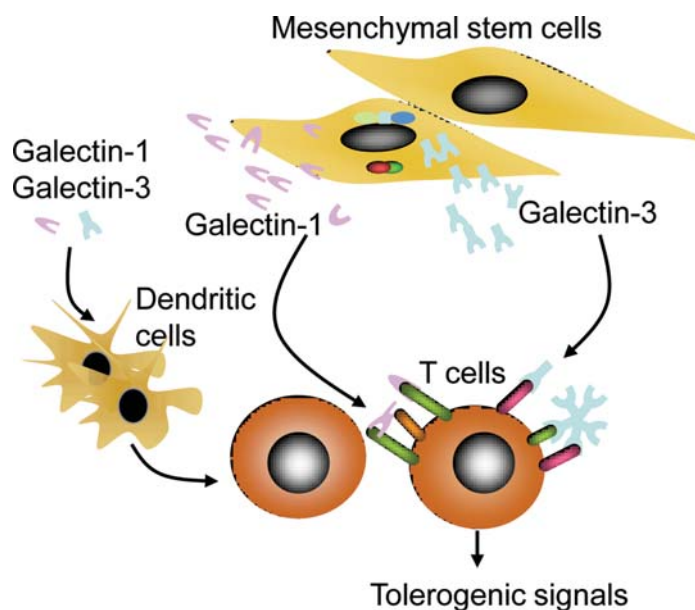


Figure 4. Galectins could be a core mechanism by which MSCs suppress T cell proliferation. The binding of secreted galectins-1 and -3 to T cells can induce tolerogenic signals in T cells. Alternatively, secreted galectins-1 and -3 could impair the function of DC, which can in turn inhibit T cell function.

effects of MSCs on allogeneic T cells, in comparison to the galectin-8 knockdown. The simultaneous knockdown of galectin-1 and -3 expression nearly abrogated MSC immunosuppressive potential.

Although several studies have reported on MSC immunosuppressive functions, the mechanisms through which MSCs suppress alloresponses are not fully understood. The inhibition could involve the factors secreted by MSCs, such as TGF- $\beta$ , HGF, IL-10, IDO and PGE2 (6,28). However, in certain experimental settings, adding IDO inhibitor or neutralizing antibodies against IL-10 or TGF- $\beta$  does not affect T cell suppression, suggesting the involvement of other factors with predominant effects (29,30). The data from our previous as well as our current study, link the expression of galectins, particularly galectins-1 and -3, to MSC-mediated immune suppression. Indeed, the double knockdown of galectins-1 and -3 almost abolished the immunosuppressive potential of MSCs, suggesting the involvement of both proteins. Of note, it has been reported that galectins-1 and -3 interact with similar T cell ligands (31). The finding that  $\beta$  lactose, a galectin antagonist, inhibited MSC inhibitory effects on T cell proliferation, suggests that the carbohydrate recognition domain of MSC-derived galectins is responsible for the immunosuppression of T cells and supports an extracellular mechanism of action, as is illustrated in Fig. 4. The immunomodulatory function of MSC-secreted galectins-1 and -3 as reported here should facilitate the use of these galectins to treat GvHD and autoimmune disorders in humans.

In addition to immune cells, galectins also play a number of key roles in cancer immune escape. In this respect, Peng *et al* demonstrated that tumour-associated galectin-3 impaired T cell response (18). Similarly, galectin-1 contributed to tumour immune escape by inhibiting tumour-reactive T cells (19). Comparable to galectin-3, galectin-1 inhibited the proliferation of mitogen-activated T cells and reduced the clonal expansion of antigen-primed CD8<sup>+</sup> T cells and human

leukemic T cells (24). With regard to the mechanisms of T cell inhibition, a certain study demonstrated that the effects of galectin-1 could result from the altered expressions of IL-10 and IFN- $\gamma$  in T cells as opposed to directly altering T cell viability (31). The study also showed that galectin-3, but not galectin-1, induces both phosphatidylserine exposure and apoptosis in primary activated human T cells and that both proteins recognised T cell surface ligands with similar affinity. Therefore, it would be of interest to analyse the tolerogenic signals delivered by MSCs to T cells through galectins-1 and -3 and whether there is a functional difference between the various forms of galectins-1 and -3 (e.g., monomers vs. dimers or pentamers). It would also be of interest to study the interaction between galectins-1 and -3 in MSC.

#### Acknowledgements

This study was supported in part by grants from the Gene Therapy Program at the Norwegian Radium Hospital and the Norwegian Cancer Society to M.S. We thank Dr Anne Dybwad for reading the manuscript.

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