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Differential effects of TLR3 and TLR4 activation on MSC-mediated immune regulation

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ABSTRACT

Mesenchymal stromal cells (MSCs) have evolved as an invaluable therapeutic cell type due to their broad therapeutic properties. Bone marrow-derived MSCs are currently being applied in numerous clinical trials, and the initial results have been encouraging. However, heterogeneous responsiveness amongst patients is also being experienced; therefore, the efficacy of MSCs *in vivo* is still debatable. Host microenvironment plays an essential role in determining the fate of MSCs *in vivo*. Recent studies have indicated the role of toll-like receptors (TLR) in modulating the biological properties of MSCs. TLRs are expressed by MSCs, and activation of TLR3 and TLR4 can alter the functionality of MSCs. While MSCs can suppress the effector and memory T cell function by promoting regulatory T cells, the effect of TLR activation on MSC-mediated immune cell induction is still not well understood. This study was performed to understand the TLR licensing of MSCs and its impact on MSC-mediated immunomodulation. We found that TLR3 mediated activation of MSCs (TLR3-MSCs) increased the expression of G-CSF & IL-10 while TLR4-mediated activation of MSCs led to an increase in CXCL-1, CXCL-10, and CXCL-12. To study the immunological aspect, an *in vitro* co-culture model was established-to imitate the brief *in vivo* interaction of MSCs and immune cells. We found that TLR3-MSCs led to increase in CD4 and CD8 naive T (T_{NAI}) cells and vice versa for effector (T_{EFF}) and memory T (T_{MEM}) cells, while TLR4-MSCs did not show any effect.

Moreover, only TLR3-MSCs led to a non-significant increase in the regulatory T cells (T_{REGS}) and Double negative regulatory cells. No change in B cell profile was evident while TLR3-MSCs depicted an increasing trend in regulatory B cells which was not statistically significant. TLR3 MSCs also inhibited the T cell proliferation in our setup. Our data indicate that TLR3 priming may regulate the function of MSCs through immunomodulation.

Understanding the role of TLRs and other microenvironmental factors causing subdued responses of MSCs *in vivo* would allow the uninhibited use of MSCs for many diseased conditions.

1. Introduction

Cell-based therapies specifically using mesenchymal stromal cells (MSCs), have gained the attention of the clinicians and researchers worldwide. Clinically, MSCs have been explored for a plethora of health conditions owing to their immunomodulatory and regenerative capacity [1]. However, the mechanisms underlying the regulatory effect of MSCs are not fully explored.

MSCs possess immunomodulatory properties which can regulate the immune cell responses by hampering immune cell differentiation, maturation, and functional responses [2–5]. MSCs can induce tolerogenic immune responses by activating regulatory cells and suppressing effector and memory immune cell subsets [6–8]. This property makes them suitable for application in solid organ transplantation (SOTx). Numerous studies in preclinical transplant models have successfully demonstrated the ability of MSCs to enhance and improve graft function [9–11]. Moreover, clinical trials conducted in transplant patients till date, have substantiated the safety of MSCs [12,13]. Nevertheless, efficacy of MSCs has not been completely reflected in the clinical trials. MSCs have been shown to affect both humoral and adaptive arms of immunity [3,7]. Our previous studies in kidney transplant (KTx) patients demonstrated the effect of bone marrow-derived MSCs on the interplay of B and T cell subsets. We found MSC infusion lead to an increase in regulatory B (B_{REGS}) [14], and decreased differentiation of

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naive T (T_{NAI}) cell into effector (T_{EFF}) and memory (T_{MEM}) cells [15]. However, MSCs did not produce the same response in all the patients and similar heterogeneity has been reported in other clinical trials [12,16].

Exogenously cultured MSCs after *in vivo* administration interact with the local cytokines or soluble proteins in that specific microenvironment that leads to MSC licensing towards specific functions [17–19].

Host microenvironment can therefore be held responsible for causing anomalies in MSC-responsiveness in heterogenous group of patients where MSC infusion is done. Toll-like receptors (TLRs) form an integral part of the microenvironment, and their importance in MSC-licensing has been highlighted in few studies [19–21]. Following stimulation, TLRs trigger several intracellular signalling cascades [22], and release of paracrine factors.

Out of all TLRs, both TLR3 and TLR4 are known to contribute to MSC licensing. TLR3 and TLR4 activation of MSCs has been shown to enhance immunosuppression by T_{REGS} induction [20,23,24]. TLR3 pre-conditioning of MSCs promoted T_{REG} differentiation in a trinitrobenzene sulfonate (TNBS)-induced mouse model of colitis [25]. Similarly, TLR3-MSCs proved to be therapeutically efficacious in a dextran sulfate sodium (DSS) induced mice model [26]. Tolstova et al. showed that TLR3 priming can enhance the immunosuppressive properties of MSCs [27]. TLR4-MSCs have also resulted in superior therapeutic neo-vascularisation and recovery of cardiac function in acute myocardial infarction model [28]. On the contrary to this, Liotta et al. showed that TLR3 and TLR4 priming inhibited the process of MSC-mediated T-cell immunomodulation [29]. TLR3 activation also failed to enhance the therapeutic effects of MSCs in B6.MRL-Fas(lpr) mice [30]. Another study by Pezzanite et al. demonstrated that TLR3-activated MSC treatment can induce T lymphocytes and suppress innate immune responses in synovium of septic arthritis equine model [31].

A study by Cassatella et al. suggested that TLR3 or TLR4 primed MSCs might trigger inflammatory disorders [32]. Interestingly, a study by Waterman et al. showed that TLR3 priming of MSCs could induce an anti-inflammatory phenotype (MSC2) while TLR4 priming induced a pro-inflammatory phenotype (MSC1) [19]. Supporting this view, a few studies have shown that TLR4 inhibition in MSCs can improve their therapeutic potential [33] and survival [34].

Keeping these findings in mind, the current *in vitro* study was designed to study the effect of TLR3/4 primed MSCs on the immune cell responsiveness. Ours is the first study to report the impact of TLR primed MSCs on the T and B cell profile of the responder cells.

Our study shows that TLR3 primed MSCs induce immunomodulation and anti-inflammatory microenvironment.

2. Materials and methods

2.1. Isolation and characterisation of mesenchymal stromal cells

Mesenchymal stromal cells (MSCs) were isolated from the bone marrow (BM) aspirate of a healthy kidney donor. This donor was recruited for another study to elucidate the effect of MSC infusion on immune cells of kidney transplant patients (KTx) in an allogeneic setting (NCT- NCT02409940). After the infusion of specific numbers in originally recruited KTx patients, the remaining MSCs were used for the current experimental setting. All protocols were approved by the Institutional Committee for Stem Cell Research of PGIMER (PGI-IC-SCRT-39-2013/1471), Chandigarh. Briefly, BM aspirate was diluted with 1X PBS (1:1) and was subjected to density gradient centrifugation at 550*g* for 30 min. The mononuclear cells were then separated and resuspended in α-minimal essential medium (α-MEM) (Sigma-Aldrich, USA) containing 7–10 % pooled human platelet lysate (pHPL), 5 IU/mL heparin (Caprin®, India), 1 % penicillin-streptomycin (Sigma-Aldrich, USA) and 2 mM L-glutamine (Sigma-Aldrich, USA). Cells were maintained under standard culture conditions (5 % CO_2 and 37 °C). After 5–7 days non-adherent cells were washed off. MSCs were subsequently trypsinised at 70–80 % confluency and sub-cultured (10,000 cells per cm^2) till passage-3.

2.2. Characterisation of mesenchymal stromal cells

Phenotypic and functional characterisation of MSCs was performed as described in the International Society for Cellular Therapy guidelines [35].

Phenotypic characterisation of MSCs was carried out by multi-parametric flow cytometry (BD FACS Aria II; BD Biosciences, USA) after staining with PE-conjugated negative cocktail antibody (anti-CD11b, anti-CD19, anti-CD34, anti-CD45 and anti-HLA-DR), APC conjugated anti-CD73, FITC conjugated anti-CD90, and PerCP Cy5.5 conjugated anti-CD105. Compensation was performed using single colour controls, and the unstained sample was used as a negative control. Data were analysed with FlowJo software (Tree Star, Inc, USA).

MSCs were functionally assessed by their potential to differentiate into different lineages as described in our previous study [36]. Briefly, MSCs at passage-4 were kept in the adipogenic, osteogenic and chondrogenic medium. After 21 days, cells were fixed, stained and observed microscopically for the presence of lipid droplets (adipocytes), calcium deposits (osteocytes) and aggrecans (chondrocytes).

Before using MSCs for the experiments, karyotyping was performed [37,38] to confirm chromosomal stability.

2.3. Priming of MSCs

The concentration and timings of TLR agonist and antagonist were decided upon by standards mention in the literature.

2.3.1. TLR-3 priming

MSCs (passage-3) at 70 % confluency were incubated with a TLR3 agonist (ago) - polyinosinic-polycytidylic acid (poly I:C) (100 μg/mL) [39,40], for 1 h in fresh complete α-MEM. MSCs treated with bafilomycin A1 (BafA1) (100 μM) for 1 h and then with poly I:C (100 μg/mL) for 1 h were used as control. TLR3-primed MSCs (TLR3-MSCs) were then washed and used for further assays.

2.3.2. TLR-4 priming

MSCs (passage-3) at 70 % confluency were incubated with a TLR4 agonist-lipopolysaccharide (LPS) (250 ng/mL), for 24 h in fresh complete α-MEM. MSCs treated with a polymyxin B (poly-B) (10 μg/mL) for 1 h and then with LPS (250 ng/mL) for 24 h were used as control. TLR4-primed MSCs (TLR4-MSCs) were then washed and used for further assays.

2.4. Flow cytometric analysis for TLR priming

Flow cytometry was performed for the primed MSCs to confirm the efficacy of the priming protocols. Primed MSCs were stained with PE-conjugated anti-TLR3, or APC conjugated anti-TLR-4 and were acquired on BD FACS Aria II (BD Biosciences, USA) and analysed by FlowJo software (Tree Star, Inc, USA). Unprimed-MSCs were used as a negative control, and gating was performed using unstained MSCs.

2.5. Human cytokine, chemokine array and analysis

The expression of different cytokines, chemokines and acute-phase proteins in the culture supernatant of primed-MSCs was determined using the Proteome Profiler Human Cytokine Array Kit (ARBY005B; R&D Systems) per the manufacturer instructions. Primed MSCs (TLR3/TLR4) were serum starved for 24h in α-MEM. MSC culture supernatant was then collected and incubated with the array membrane. Manufacturer's instructions were followed without any deviation. The change in expression of the cytokines was determined by volumetric analysis using ChemiDoc™ XRS+ (Bio-Rad, USA) and is expressed as fold change

relative to the control sample (unprimed-MSCs).

2.6. Preparation of conditioned medium

1X10 [6] MSCs (unprimed, TLR3-primed and TLR4-primed) were cultured in α-MEM for 24 h (without supplements) in a CO_2 incubator at 37 °C. The culture supernatant was then collected and concentrated 20 times by using centrifugal filters (cut-off 3 kDa MW) (Millipore, Germany) at 4000g for 20–25 min. The concentrated supernatant or conditioned medium (CM-MSC) was then snap-freezed and stored at −80 °C till further use.

2.7. Isolation of peripheral blood mononuclear cells

Peripheral blood was collected from healthy donors (n = 6). Peripheral blood mononuclear cells (PBMCs) were then separated using Lymphoprep (Stem cell technologies, Canada) by density gradient centrifugation [41]. The samples collected from healthy donors were randomly assigned to two different groups, namely, responder cells (n = 3) and stimulator cells (n = 3).

2.8. Labelling of responder cells and inactivation of stimulator cells

Responder cells were labelled with 5 μM carboxyfluorescein succinimidyl ester (CFSE) [42]. Untreated CFSE-labelled responder cells were used as controls. Stimulator cells were inactivated using mitomycin-c at a concentration of 30 μg/mL for 2 h.

2.9. Mixed lymphocyte reaction

CFSE labelled responder cells were co cultured with inactivated stimulator cells (MLR) at a number of 0.3×10^6 for each to induce lymphocyte proliferation. Either TLR3/TLR4 primed or unprimed MSCs or CM-MSC were added to the MLR at a 1:10 ratio; MSC:PBMC ratio. Co-cultures were incubated for four days at 37 °C in a CO_2 incubator, before analysing the immune cell population & proliferation in the cultures.

2.10. Immune cell profiling of responder cells

On the 5th day, immune cell subsets were determined for responder cells. For this, CFSE labelled responder cells were assessed for T cell (CD3, CD4, CD8, CD45RA, CD45RO, CD62L, CD25, CD127 and FoxP3), B cell (CD19, CD5, CD1d, CD24, CD27 and CD38) and cell viability marker (7-amino actinomycin D (7-AAD)). Lymphocyte subsets (Table S1) were analysed on a flow cytometer using fluorochrome-conjugated monoclonal antibodies according to previously published protocols [36]. Cells were acquired on a flow cytometer and analysed on FlowJo software. Gating strategy for the subsets is provided in Supplementary Figs. S2–S5.

2.11. Proliferation of responder cells

T and B cell proliferation was measured by CFSE dilution assay. For this, CFSE labelled responder cells were stained for T cell markers (CD3, CD4, CD8), B cell (CD19) and cell viability marker (7-amino actinomycin D (7-AAD)). Cells were acquired on a flow cytometer and analysed on FlowJo software. Gating strategy for the subsets is provided in Supplementary Figure S5 (A-E) and S6.

2.12. Statistical analysis

All experiments were performed in duplicates and at least twice. The analysis was performed using GraphPad Prism software (GraphPad, USA). Statistical analysis for multiple group comparison was performed using one-way analysis of variance (ANOVA). Comparison between two groups was performed by two-tailed Student's t-test or Mann-Whitney test and $p < 0.05$ was considered statistically significant. All data are expressed as mean ± SD.

3. Results

3.1. Phenotypic and functional characterisation of mesenchymal stromal cells

MSCs stained with fluorochrome labelled antibodies showed >95 % positivity for CD73, CD90 and CD105 and < 2 % positivity for negative markers (CD11b, CD19, CD34, CD45 and HLA-DR) (Supplementary Fig. S7). MSCs differentiated into adipocytes, osteocytes and chondrocytes following standard protocols (Supplementary Fig. S8). Moreover, cultured MSCs demonstrated genetic stability as analysed by karyotyping (Supplementary Fig. S9).

3.2. Patterns of TLR3/4 expression on mesenchymal stromal cells

Flow cytometric analysis revealed that MSCs in their native form express both TLR3 (intracellular) and TLR4 (on the cell surface) (Fig. 1A). Upon exposure to poly I:C (TLR3 agonist), there was an increase in the percentage of TLR3 expressing MSCs (TLR3-MSCs) in comparison to the unprimed MSCs (un-MSCs) (20 ± 5.9 % Vs 6.08 ± 1.66 %; p = 0.01; Fig. 1A). Similarly, MSCs exposed to LPS (TLR-4 agonist) showed higher percentage of TLR4 expressing MSCs (TLR4-MSCs) than un-MSCs (27.07 ± 5.57 % Vs 7.06 ± 3.91 %; p = 0.007; Fig. 1A).

3.3. Paracrine factor secretion patterns of TLR3/4 primed mesenchymal stromal cells

Supernatant from TLR3-MSCs and TLR4-MSCs was tested for expression of 36 chemokines, cytokines and acute phase proteins (C5a, CD40L, G-CSF, GM-CSF, CXCL1, CCL1, ICAM-1, IFN-γ, IL-1 α, IL-1β, IL-1RA, IL-2, IL-4 IL-5, IL-6, IL-8, IL-10, IL-12 p70, IL-13, IL-16, IL-17, IL-17E, IL-18, IL-21, IL-27, IL-32α, CXCL-10, CXCL-11, CCL-2, MIF, MIP-1, CCL-5, CXCL12, Serpin E1, TNF-alpha, TREM-1). Out of 36, only 11 factors were detectable in the MSC samples. We found that TLR4-MSCs showed an increased expression of CXCL-1(1.1 ± 0.07 *vs* 0.8 ± 0.06; p = 0.001; Fig. 1B), CXCL-10(2 ± 0.2 *vs* 0.9 ± 0.04; p = 0.0007; Fig. 1B), CXCL-12 (1 ± 0.1 *vs* 0.8 ± 0.1; p = 0.02; Fig. 1B) while TLR3-MSCs showed an increase in expression of G-CSF (1.7 ± 0.1 *vs* 0.9 ± 0.08; p = 0.001; Fig. 1B).

3.4. TLR-3 primed MSCs modulate the proliferation of T lymphocytes

TLR3-MSC or TLR4-MSC treatment led to no change in frequency of CD3, CD4 or CD8 responder cells in a MLR reaction in comparison to un-MSCs (Fig. 2A).

Proliferated responder cells were identified by CFSE dilution [42]. The proliferation index of CD4 T cells was significantly reduced in TLR3 group when compared to TLR4 or unprimed group (Fig. 2B).

3.5. TLR-3 primed MSCs favour the survival naïve T cells

T_{NAI} cells are metabolically inactive until they encounter a cognate antigen, which results in their activation and differentiation into T_{EFF}/T_{REG} and T_{MEM} cells. Effect of TLR-primed MSCs was assessed to identify alterations in the T-cell profile of responder cells. We compared the ratios of $T_{NAI,}$ T_{EFF} and T_{MEM} cells for both helper and cytotoxic T cells.

An increase in the responder CD4 T_{NAI}:T_{EFF} cells was observed for TLR3-MSC group in comparison to the un-MSC group (0.4 ± 0.1 Vs 0.2 ± 0.06; p = 0.01; Fig. 3A). Responder CD4 T_{NAI}:T_{MEM} cells in TLR3-MSC group showed slight increase in comparison to un-MSC, though statistically not significant (0.4 ± 0.1 Vs 0.2 ± 0.06; p = 0.06; Fig. 3A). Further, the responder CD4 T_{NAI} cells were compared against the T_{MEM}

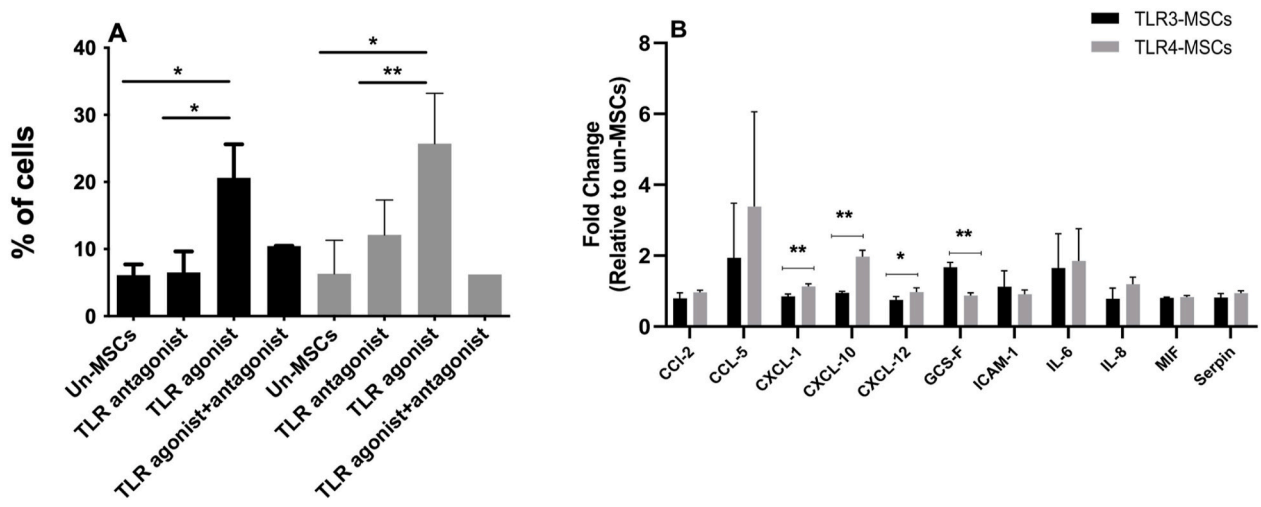

Fig. 1. Comparison of TLR expression on MSCs and their secretory cytokine/chemokine under different culture conditions. Flow cytometric analysis plots indicating the percentage of MSCs expressing **(A)** TLR3 and TLR4. Volumetric analysis indicating the fold change in expression of **(B)** CCl-2, CCl-5, CXCL-1, CXCL-10, CXCL-12, GCS-F, ICAM-1, IL-6, IL-8, MIF and Serpin. Data are represented as mean ± SD. * represents the statistical difference between the respective groups (*p < 00.05; **p < 00.005). TLR3 antagonist- BafA1 (bafilomycin A1); TLR3 agonist-poly I:C (polyinosinic-polycytidylic acid); TLR4 antagonist - poly-B (polymyxin B); TLR4 agonist - LPS (lipopolysaccharide).

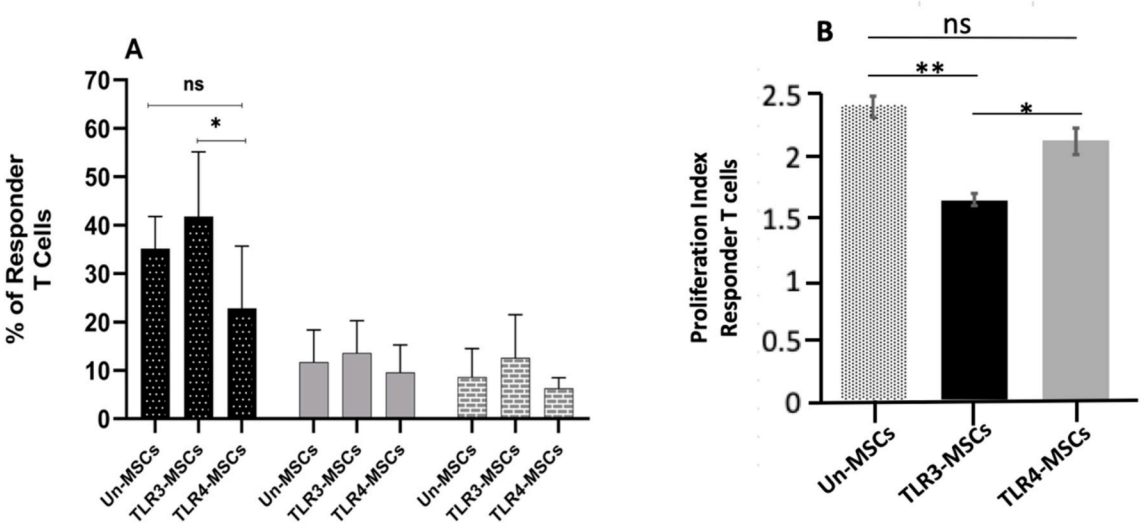

Fig. 2. Comparison of responder T lymphocyte subsets and their proliferation in response to primed/unprimed MSCs. Flow cytometric analysis plots indicating the percentage of **(A)** Responder T cells and **(B)** Proliferation index of Responder T cells, flow cytometry was performed on Day-5 post-co-culture. Data are represented as mean ± SD. * represents the statistical difference between the respective groups (*p < 00.05; **p < 00.005, ns = not significant).

cell subsets, i.e. $T_{MEM\text{-}EM}$ and $T_{MEM\text{-}CM}$. An increase in responder CD4 $T_{NAI:}$ $T_{MEM\text{-}EM}$ cells was evident in TLR3-MSC group in comparison to un-MSC (5 ± 6.2 Vs 2.3 ± 2.1; p = 0.02; Fig. 3A) while no change was observed for CD4 $T_{NAI:}$ $T_{MEM\text{-}CM}$ cells (Fig. 3A).

Further, the analysis of CD8 subset ratios was performed which revealed a slightly higher responder CD8 T_{NAI}:T_{EFF} and CD8 T_{NAI}:T_{MEM} cells for TLR3-MSC group in comparison to the un-MSC group, however, this increase statistically insignificant (Fig. 3B). Subsequent analysis of CD8 T_{NAI} cells against memory cell subsets showed no difference in CD8 T_{NAI}:$T_{MEM\text{-}CM}$ for TLR3-MSC or TLR4-MSC group in comparison to un-MSC group (Fig. 3B).

T_{REGS} have been identified as important mediators of immune tolerance [43,44]. A small but slightly significant increase in T_{REGS} was observed for responder T_{REGS} of TLR3-MSC group in comparison to the un-MSC group (1 ± 0.8 % Vs 0.4 ± 0.2 %; p = 0.06; Fig. 3C).

3.6. TLR-primed mesenchymal stromal cell pre-treatment does not modulate the responder B lymphocytes

Similar to T cells, B cells are also considered imperative mediators of the immune system. We found that treatment of responder cells with TLR-3,4 or unprimed MSCs in a MLR setup did not result in any change in the frequency (Fig. 4A) or proliferation (Fig. 4B) of B cells.

We analysed all relevant regulatory B cell subsets including B_{regs}, B_{IM} and B_{10} cells as well, which are known to contribute to immunotolerance.

A slight increase in B_{regs}, B_{10} and B_{IM} cells (Fig. 4C) was evident for responder cells in TLR3-MSC group. However, these changes were statistically insignificant. A minor increase in the percentage of responder B_{regs} was also observed in TLR4-MSC group.

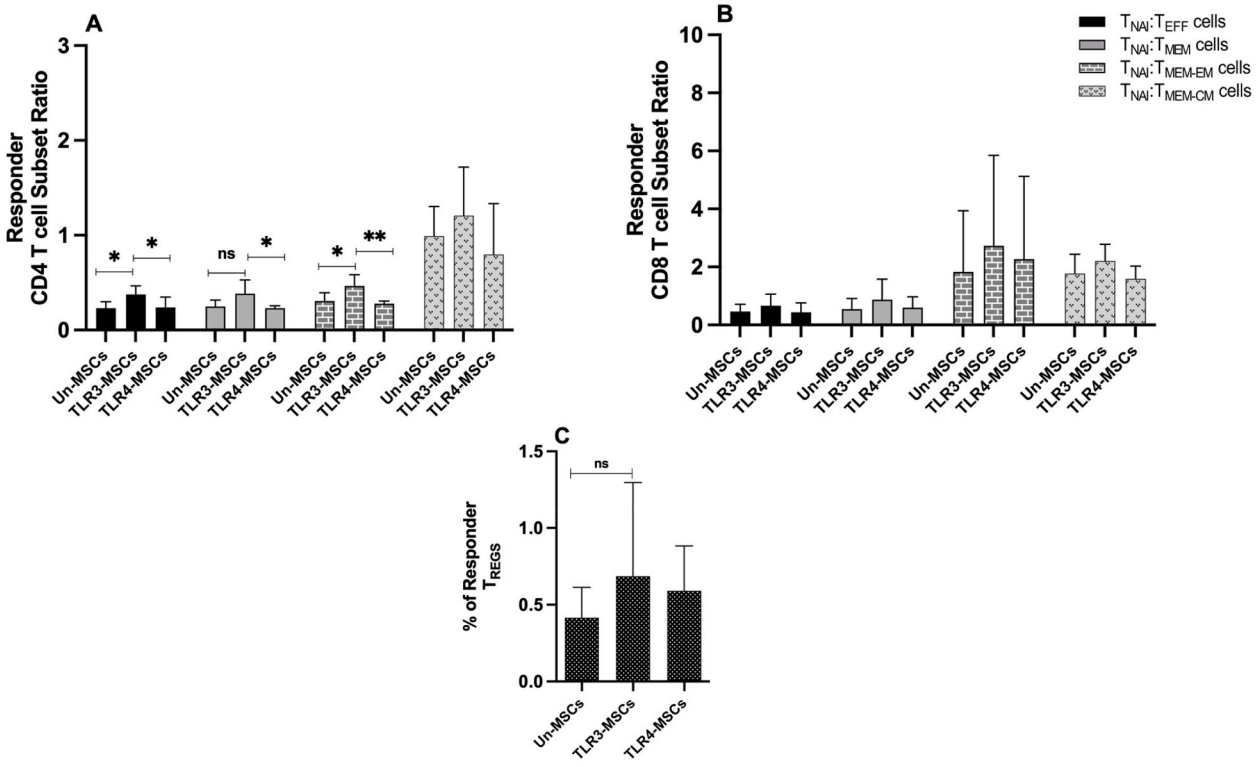

Fig. 3. Comparative analysis of responder T lymphocyte distribution under different culture conditions. Flow cytometric analysis plots indicating **(A)** Responder CD4 T cell subset ratio (CD4 T_{NAI}: T_{EFF} cells, CD4 T_{NAI}: T_{MEM} cells, CD4 T_{NAI}: T_{MEM-EM} cells and CD4 T_{NAI}: T_{MEM-CM} cells) and **(B)** Responder CD8 T cell subset ratio (CD8 T_{NAI}: T_{EFF} cells, CD8 T_{NAI}: T_{MEM} cells, CD8 T_{NAI}: T_{MEM-EM} cells and CD8 T_{NAI}: T_{MEM-CM} cells) and percentage of **(C)** T_{REGS}. Responder cells in a MLR setup were cultured with TLR3 primed MSCs or TLR4 primed MSCs, or unprimed MSCs. Flow cytometry was performed on Day-5 post-co-culture. Data is represented as mean ± SD. * represents the statistical difference between the respective groups (*p < 00.05; **<0.005, ns = not significant).

3.7. Conditioned medium from TLR-primed mesenchymal stromal cells failed to modulate the T and B lymphocytes

Conditioned medium derived from MSCs has been suggested as alternate cell therapy in many studies. However, we found CM to be ineffective in modulating the response of T and B cells in our setup (Figs. S10–12).

4. Discussion

MSCs display unique immunomodulatory properties both *in vitro* and *in vivo*, which makes them desirable as a therapy for organ transplantation [45,46]. Paracrine factors secreted by MSCs [47,48] that aid in tolerance induction makes their use even more appealing. However, the mechanisms involved in the MSC immunomodulation *in vivo* are still not clear, thus limiting their use.

TLR3 and TLR4, have been reported to influence the biological properties of MSCs [49], including their immunomodulation [19,29]. This study was designed to understand the effect of TLR3 and TLR4 primed MSCs on the immune profile of responder cells upon stimulation. Taken together, the results from our study indicate three significant findings which contribute to understanding the role of TLRs in modulating properties of MSC. First, TLR3 primed MSCs increase expression of GCS-F while TLR4 primed MSCs showed an increase in the expression of CXCL-1, CXCL-10 and CXCL-12. Second, TLR3-MSC treatment had an impact on the ratio of CD4 T_{NAI} cells into $T_{EFF,}$ T_{MEM} or T_{MEM-EM} cells and increased T_{REGS} marginally. Third, TLR3 MSCs were able to alter the proliferation profile of T cells. Fourth, CM failed to produce any relevant results in comparison to the equivalent number of MSCs used.

In our study, the pre-treatment of MSCs with TLR3 agonist (poly I:C) led to an increase in the anti-inflammatory protein and cytokine GCS-F and IL-10 while TLR4 agonist (LPS) led to an increase in the proinflammatory cytokines CXCL-1, CXCL-10 and CXCL-12. MSCs are known to cause direct immunomodulation of T_{EFF} cells which are attracted towards them by the secretion CXCL-1, CXCL-10, CXCL-12 [47,50].

Studies have shown that both TLR3-MSCs and TLR4-MSCs when directly co-cultured with T cells, act by suppressing the proliferation of T cells [20,23]. However, our findings from our co-culture assay revealed that only TLR3-MSCs had an effect on proliferative ability of T cells.

Interestingly, TLR3-MSC pre-treatment increased the percentage of T_{NAI} cells while decreasing T_{EFF} and T_{MEM} subset proportions. T_{MEM} cells indicate heightened T cell responsiveness, and they are known to interfere with the graft survival in the transplant patients by directly stimulating the T_{EFF} cells [50,51]. Therefore, expansion of T_{NAI} cell population with low T_{EFF}/T_{MEM} cell differentiation post TLR3/4 primed MSC treatment is of immense clinical importance.

The immunomodulatory capacity of MSCs is often evaluated by their ability to induce regulatory cells. We found that TLR3-MSC treatment led to slight expansion of T_{REGS} . B_{REGs} cells however showed a slight increase that was statistically insignificant in the current *in vitro* set-up.

Many reports have been published in favour of cell-free therapy [52,53]. Few studies have shown that conditioned medium derived from MSCs (CM-MSCs) can produce the same effects as the live MSCs [54,55]. However, we found that CM prepared from an equivalent number of cells failed to produce any relevant change in immune cell subsets. A comparative study (MSC Vs CM-MSCs) in mice model of acute kidney injury also demonstrated the incompetence of CM-MSCs in kidney repair [56]. Another study in a preclinical model of ventilation-induced lung injury indicated the ineffectiveness of CM-MSCs in tissue repair and restoration [57].

This study is first of its kind which has used an *in vitro* co-culture based assay to imitate an *in vivo* transplantation setting, to elucidate the effect of MSC priming on the immune cell profile. The current report

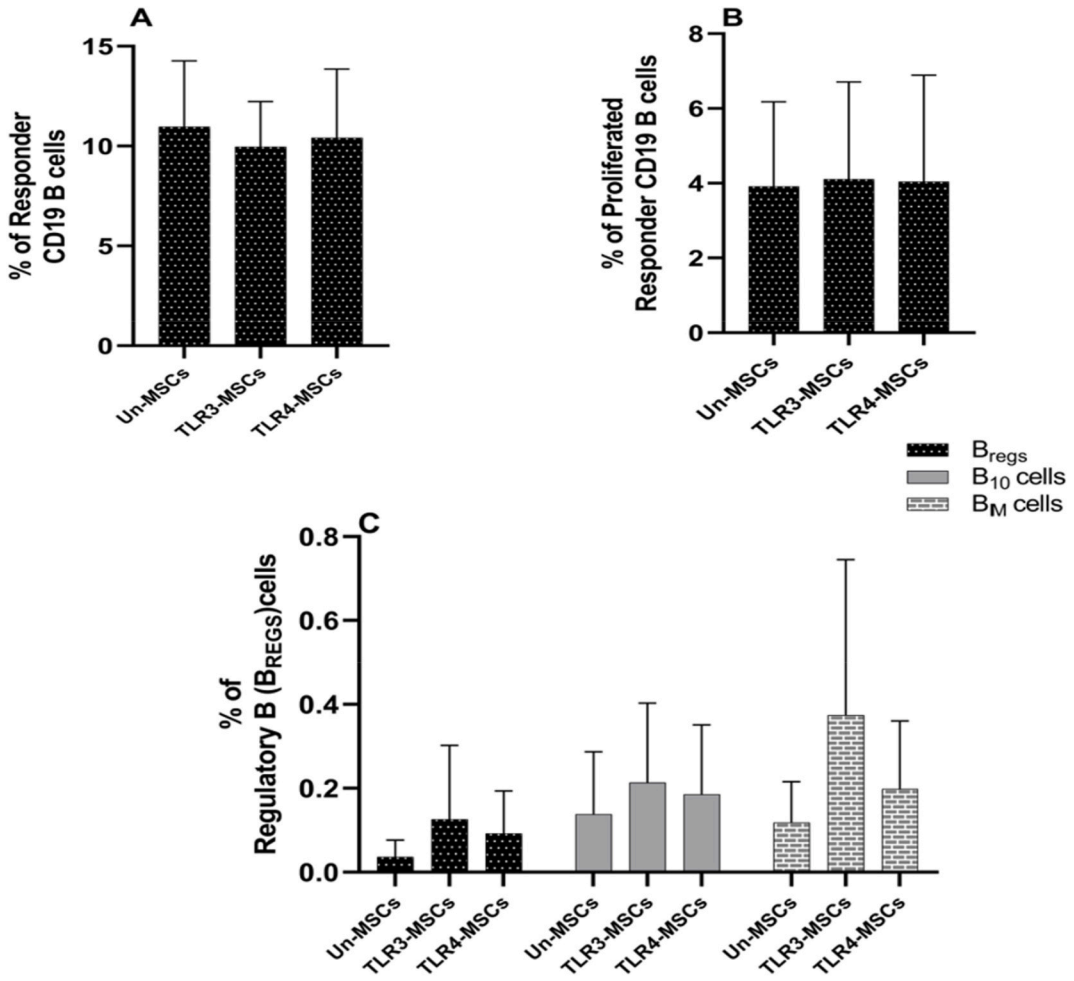

Fig. 4. Comparison of responder B cell distribution under different culture conditions. Flow cytometric analysis plots indicating the percentage of responder **(A)** CD19 B cells, **(B)** proliferated CD19 B cells and **(C)** B_{REGS} (B_{regs}, B_{10} cells and B_{IM} cells). Responder cells in a MLR setup were cultured with TLR3 primed MSCs or TLR4 primed MSCs, or unprimed MSCs were co-cultured with inactivated stimulator cells. Flow cytometry was performed on Day-5 post-co-culture. Data are represented as mean ± SD. * represents the statistical difference between the respective groups (*p < 00.05; **p < 00.005).

is based on the results of an *in vitro* setup and additional studies using an *in vivo* model are required. TLRs have been known to influence the biology of MSCs, which in return affects their therapeutic potential. Our data showed that MSCs after priming with TLR-3 agonist have a higher capacity to induce a state of immunotolerance. Our findings, however, primarily rely on cell proliferation and phenotypic markers to characterize T and B cell populations that have differentiated in response to TLR3/4 primed MSCs. While immune cell proliferation and differentiation can serve as a surrogate for function, incorporating intracellular cytokine staining to confirm cell activation could be beneficial. Furthermore, employing multiomic techniques like single-cell sequencing could provide a more comprehensive evaluation of reduced heterogeneity in immune cell populations following stimulation with TLR-primed MSCs.

Further, the effect of microenvironment on MSCs has been reported; however, the underlying decision of MSCs to bind to a specific TLR with higher affinity still needs to be investigated. In this context, it would be interesting to explore the possibility of induction of a similar or better level of immunotolerance after engagement of other TLRs or pro or anti-inflammatory cytokines. Besides this, such studies would form a basis for a better understanding of the *in vivo* mechanism of MSC immunomodulation. The graphical in Fig. 5 depicts our experimental setup with changes in analysed parameters..

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CRediT authorship contribution statement

Urvashi Kaundal: Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation. **Aruna Rakha:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Aruna Rakha reports financial support was provided by Department of Scince and Technology (DST) under the Indian Ministry of Science and Technology.

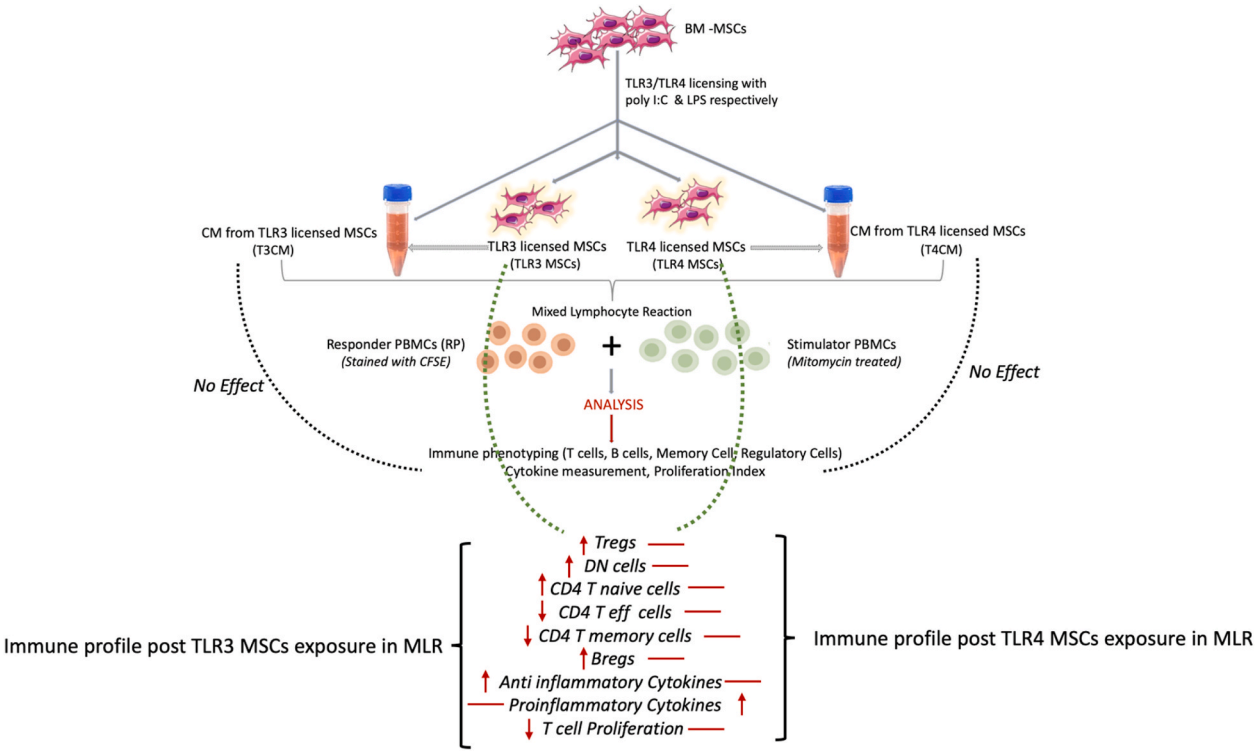

Fig. 5. Schematic of the study depicting study design, parameters analysed and changes observed by various priming protocols. Dotted black lines depict no effects in our experimental setup while green dotted lines depict significant changes in parameters analysed.

Data availability

Data will be made available on request.

Abbreviations

B_{REGS}	regulatory B cells (all subsets)
B_{reg}	regulatory B cell subset
B_{10}	transitional B cells
B_{IM}	immature transitional B cells
CM-MSCs	conditioned medium derived from mesenchymal stromal cells
MSC	mesenchymal stromal cells
T_{EFF}	effector T cells
T_{MEM}	memory T cells
T_{MEM-EM}	effector memory T cells
T_{MEM-CM}	central memory T cells
T_{NAI}	naive T cells
T_{REGS}	regulatory T cells
TLR	toll-like receptors

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrep.2024.101809.

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