Chapter 6

Toll-Like Receptors: The Key of Immunotherapy in MSCs

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Abstract

Human mesenchymal stem cells (MSCs) are potential candidates for various applications in the fields of immunotherapy. Their multilineage differentiation capability and immune modulatory features allow their prospective application for the management of different immunological circumstances. However, the local microenvironment, in addition to the source of the MSCs can control diverse biological features of the cells. Indeed, throughout their therapeutic application, MSCs may interact with their microenvironment through their expressed toll-like-receptors (TLRs), producing immune modulating reactions. Stimulation of MSCs before or within the potential treatment procedures with distinct TLR ligands may assist as an effective step controlling the biological function of the MSCs as needed in different therapeutic stages of the disease.

Keywords: TLR, immunotherapy, immunomodulation, mesenchymal stem cells

1. Introduction

Human mesenchymal stem cells (MSCs) are potential candidates for various applications in the fields of immunotherapy [1, 2]. Their multilineage differentiation capability and immune modulatory features allow their prospective application for the management of different immunological circumstances [1, 2]. However, the local microenvironment, in addition to the source of the MSCs can control diverse biological features of the cells [1, 2]. Indeed, throughout their therapeutic application, MSCs may interact with their microenvironment through their expressed toll-like-receptors (TLRs), producing immune modulating reactions of the

cells. Thus, by comprehending these TLR-promoted properties on immune regulating functions of MSCs, potential therapeutic applications of these cells can be optimized [1, 2].

Toll-like receptors (TLRs), major molecules connecting the innate and adaptive immune responses, are germ line-encoded pattern-recognition receptors (PRRs), identifying specific pathogen-associated molecular patterns (PAMPs), thus supporting the activation of immune cells [3, 4]. They work as sensors for different pathogens and play an important role in the pathogenesis of autoimmune, chronic inflammatory and infectious diseases [5]. So far, 10 functional human TLRs have been categorized [2, 6]. Depending on their PAMP ligands and their cellular localization, TLRs are divided into intracellular and extracellular receptors. Extracellular TLRs are expressed on the cell surface and generally identify constituents of microbial membranes as lipids and lipoproteins (TLR1, TLR2, and TLR6), lipopolysaccharide (LPS) (TLR4), and flagellin (TLR5). The intracellular group is expressed inside the cells, where they recognize double-stranded RNA (TLR3), single-stranded viral RNA (TLR7 and TLR8) and unmethylated CpG DNA of viruses and bacteria (TLR9) [7].

Multipotent stromal cells (MSCs) of diverse origin have been presented to express functional TLRs in definite patterns [2, 8], turning them selectively sensitive to specific microbial compounds. When activated by these compounds, TLRs can control MSCs' proliferative, immunomodulatory and differentiation potentials [9]. Differential expression profiles of functional TLRs 1–10 were described on MSCs from various tissues of the human body [10]. Results displayed that the specific profile of expressed TLRs differs according to the tissue origin of the MSCs, which endorses different immunomodulatory and therapeutic potentials of these cells during transplantation in infectious and inflammatory environments *in-vivo* [1, 10].

2. The immune system

Defending the human body against potential threats of invading pathogens depends on a number of natural mediators which are capable of recovering the homeostasis and preserving it [11]. This biological process of protection involves cells and molecules opposing the microorganisms detected by the immune system, originally developed in the human embryo. This mechanism starts with hematopoietic stem cells that differentiate into the key players of the immune reaction of our bodies (granulocytes, monocytes, and lymphocytes). Through the various activities of these major units of immunity, the immune response holds two chief divisions, the innate and the adaptive immune reactions. The innate immunity includes different protective walls of microbiological, as well as chemical and physical nature but also delivers the immune components responsible for abrupt actions against the invading threats. Though this defensive response is fast, it lacks specificity and could damage some normal tissues. On the contrary, the adaptive immune response provides a higher accuracy in its defensive process, nevertheless it takes several days or weeks to develop. This can be clarified by the development of an immunological memory through the adaptive immunity, which permits specific reactions against the pathogens with less harm to the normal tissues than the innate response.

2.1. The innate immune system

The innate branch of the immune system refers to none- or partially-specific defensive mechanisms starting directly or after a short interval of a pathogen's invasion of the body [12–14]. In this immune reaction, the genetic memory of the germline-encoded receptors enables the detection of certain molecular patterns of common pathogens [12, 15]. It is responsible for primary steps of protection against microbial threats. Simple chemical and physical barriers as epithelial layers and mucous secretions lining numerous tracts, such as the oral mucosa or the gastrointestinal tract, contribute to this defensive first line [16–18]. Furthermore, soluble proteins and bioactive molecules within biological body fluids as cellular secretions of cytokines or complement proteins are able to weaken a varied spectrum of invading pathogens [16]. Cellular constituents of the innate reaction consist of dendritic cells, macrophages and natural killer cells [16–18]. In order to confirm its role restoring the homeostasis and clearing the invading microorganisms the innate immune response has to accomplish the fundamental mechanism of early pathogen recognition. This mechanism primarily is initiated by a group of receptors termed the Pattern Recognition Receptors (PRR). These receptors are able to detect conserved microbial patterns known as Pathogen-Associated Molecular Patterns (PAMPs) [19]. One of the most important PRRs are TLRs, as they are able to recognize bacteria, fungi and viruses [20, 21]. Following PAMP-PRR detection, a reaction cascade is introduced by cells of the innate immunity creating antimicrobial mediators as reactive oxygen. In conjunction with that reaction, produced chemokines and cytokines enable recruitment of immune cells favoring the clearance of pathogens. Likewise, the ligation of PRR promotes the synthesis of antimicrobial acute phase proteins, such as complement factors. The initiated innate immune response is essential for the inception of the adaptive response as both divisions of the immunity do not function separately, but depend on their inter-reliant activities [7, 22].

2.2. The adaptive immune system

Consecutive to the innate immune reaction the second branch of immunity begins. This adaptive or acquired reaction is unalike the innate response considered highly specific against certain microbes. This is endorsed by a special capability of the cells of this arm of immunity to perform a recombination of their antigen receptors, creating the immunological memory, by which pathogens can be identified specifically [17]. As this mechanism may need 3–5 days, the innate response has to coordinate to fulfill its functions, creating the first line of defense in the body [23]. The adaptive immune response is composed of a number of specialized cells that originate during hematopoiesis from lymphoid cell lineage. Among these cells are $CD4^+$ and $CD8^+$ T-cells as well as B-lymphocytes, which are accountable for antibody production [24]. The antibodies deliver the humoral immunity, a main defense against pathogenic invasion. After pathogen detection, the antibodies bind to the microbes, triggering their neutralization and averting the pathogenic access into the host cells. Other functions of antibodies implement an incitement of phagocytic immune cells including macrophages and neutrophils, as well as natural killer cells. Through forming the first step of complement cascade activation by antigen-antibody complexes, they also allow the phagocytosis of unrecognized bacteria. This is enabled through the opsonisation mechanism to microbial pathogens.

Additionally, killing infected cells is operated by T cells, a second cellular constituent of the adaptive immune response [17].

3. Toll-like receptors

3.1. Discovery and description

The TLRs family is considered the first PRR group of to be discovered. The Toll protein was identified in 1985 and categorized as being substantial for embryonic growth of the fruit fly, *Drosophila melanogaster* [25]. An alternative function described in auxiliary studies is facilitating host responses to fungal infections and encouraging the release of antimicrobial mediators [26]. In 1997 a human toll like homolog was described [27]. This protein was named the TLR and displayed an important function in interplay between innate and acquired immunity [28]. TLRs are extracellular and intracellular proteins, which differentiate classes of various molecules. This allows the innate immune reaction to utilize the TLRs for detecting microbial pathogens [29]. By recognizing definite microbial products or patterns by the TLRs the early immune response can be commenced [30]. Among PAMPs activating TLRs are, peptidoglycan, lipoproteins, lipopolysaccharide, bacterial DNA, as well as double-stranded RNA [30]. Resulting from this TLR-PAMP complex, expressions of defensive or pro-inflammatory genes are induced within the cells. Simultaneously, signaling pathways are initiated promoting NF-κβ and MAPK pathways, along with supporting cytokine production, leading to the instigation of the adaptive immune response [31].

3.2. Identification of TLRs

The first reported mammalian TLR was TLR4 [28, 32]. The PRR-PAMP complex associated with TLR4 presented a critical function in identifying the bacterial element LPS [33]. Further studies reported a family of 13 TLRs in mammalian species [34], with functional TLRs 1–10 in human cells [6]. TLRs show a resemblance to IL-1 receptor family in their cytoplasmic fragment. Due to this correspondence, the intracellular areas of TLRs were called Toll/IL-1 receptors (TIRs). Extracellularly the TLRs display leucine-rich replications, while IL-1 receptors show immunoglobulin-like domains [27, 35]. Due to their functionality, most reliable investigations have been pointing attention to TLRs 1–10 in humans, as wells as other mammalian species.

3.3. TLR activating PAMPs and their signaling pathways

Investigations have presented different molecular components and patterns of pathogenic microorganisms, comprising combinations of nucleic acids, lipids, proteins, and carbohydrates functioning as ligands (PAMPs) stimulating the TLRs. Among the these PAMPs, bacterial lipoproteins, lipopolysaccharides, flagellin and viral RNA are considered significant components detected by TLRs [30, 36, 37]. By triggering TLRs through their specific ligands, signaling pathways are initiated, promoting elements as MyD88 and NF-κβ within the cells.

MyD88, a structurally related molecule to the IL-1R family, was reported as one of the major factors employed to initiate the signaling pathway of most TLRs, producing the transcription factor NF-κβ [38]. This nuclear factor can induce both pro- and anti-inflammatory reactions and promotes the expression of different genes, as cytokines, chemokines and adhesion molecules [39]. Through these intracellular responses, the innate immune reaction is commenced and a signaling cascade is provided to resist the pathogenic invasion. This important step is the first defensive tool of the cells against the pathogens, leading to the adaptive response as a second stage defense to defend the cells by specific means [31]. Corresponding to their specific PAMPs, TLRs can be categorized into subfamilies. Studies have displayed the detection of lipids by (TLRs 1, 2 and 6), nucleic acids by (TLRs 7, 8 and 9) and different ligands by TLR4 [30, 31]. TLRs can also be classified regarding their cellular expression, as TLRs 1, 2, 4, 5, 6 and 11 are existent on the cell surface, while the rest are expressed inside the cells [30].

3.4. TLR subgroups

3.4.1. TLR1, TLR2, TLR6 and TLR10

TLR2 has the ability to recognize a wide range of PAMPs. These include pathogenic lipoproteins, gram-positive bacterial lipoteichoic acid and peptidoglycans, *Porphyromonas gingivalis* fimbriae and fungal zymosan, as well as mycobacterial lipoarabinomannan [30, 40]. Additionally, bacterial LPS originating from *Porphyromonas gingivalis*, *Capnocytophaga ochracea* and *Bacteroides fragilis* can also be identified by TLR2 [30]. Two possible processes have been suggested considering the TLR2 identification of different pathogenic components. In the first mechanism, TLR2 produces heterophilic dimers with other TLRs that show structural similarity to it, as TLR1, TLR6 and TLR10. Therefore, TLR1, TLR6 and TLR10 are considered associative in their function with TLR2, being able to identify correlated types of PAMPs as diacyl and triacyl lipopeptides [20, 29]. The second model proposes the TLR2 mediated recognition of fungal proteins. This feature explains why TLR2 associates with dectin-1, a fungal cell wall constituent [41]. Through this functional coordination with different types of proteins, TLR2 gains its aptitude to detect various pathogenic invasions at early stages, activating the immune reactions.

3.4.2. TLR3

TLR3 primarily identifies dsRNA formed in the replication phase of most viruses. It activates the formation of NF-κβ and type I Interferon [42]. TLR3 can also homodimerize with TLR4 and TLR9 creating an intercommunicative response against invading pathogens [43, 44].

3.4.3. TLR4

TLR4 is a significant receptor identifying PAMPs as LPS from different bacterial species [30]. This LPS pattern shows structural variances from the LPS detected by TLR2, seen in the number of acyl chains of the bacterial protein [45]. Other molecules of endogenous nature, as heat shock proteins (HSP60 and HSP70), also showed activation of TLR4 in higher concentrations [46].

3.4.4. TLR5

TLR5 can detect flagellin through a process of physical interaction with the pathogens [30, 46]. Its expression has mainly been reported on epithelial cells of mucosal surfaces of the lung [47] and the intestine [48], promoting the detection of microbes at these surfaces.

3.4.5. TLR7 and TLR8

TLR7 and TLR8 both show the capability to distinguish similar ligands in certain conditions. Studies have reported that the two are stimulated by organic materials as Imidazoquinoline [30] and viral ssRNA [49–51], whereas host ssRNA is not identified by them [29]. The recognition process initiates by internalization and replication of the virus releasing its viral RNA into the cellular endosomes. The interaction mechanism between the viral ssRNA and TLR7/8 triggers the recruitment of MyD88 and production of NF-κβ, as well as proinflammatory cytokines [52].

3.4.6. TLR9

TLR9 is capable of distinguishing bacterial DNA [30]. This DNA contains unmethylated CpG promoting immunostimulation dissimilar to the vertebrate DNA that contains methylated CpG only [53]. By triggering of TLR9 by bacterial DNA the production of cytokines as IL-12, IFN-α and TNF-α is potentiated [54]. The proficiency of TLR9 to induce IFN-α production and to identify unmethylated CpG designates that it may also play a role in processes of viral pathogen identification [55].

4. Human mesenchymal stem cells

4.1. History and description

Human mesenchymal stem cells were defined originally by Friedenstein et al. [56] and designated as bone marrow isolated, non-hematopoietic and plastic-adherent cells, holding the abilities of self-renewal and multipotent differentiation *in vitro* [57–59]. These undifferentiated cells arise from different niches of the human body [60].

The multilinenage ability and the potential of self-renewal both describe the main characteristics of MSCs [61]. Self-renewal is the mechanism through which stem cells can expand their number throughout development. This capacity is essential for MSCs to allow their expansion within the tissues and plays a very important role in stem cell related therapies [62]. Investigations on this characteristic displayed its dependence on the life span of the cells. Most human MSCs are limited to a maximum of 44 weeks [63] or 55 population doublings *in vitro* [64].

Multilineage potential, or multipotency of MSCs forms the exceptional capacity of the cells for differentiating into other mesodermal lineage cells, as osteocytes, chondrocytes and adipocytes. Nevertheless, they can correspondingly differentiate forming cells of other embryonic lineages [65].

These exceptional features of MSCs as well as their communication with specific signals and mediators of the human body display great therapeutic prospectives and may develop into possible treatments for different diseases in the future [66, 67].

4.2. Identification of mesenchymal stem cells

The identification of MSC populations and the verification of their "stemness" have been confronting researchers in recent years. Without an ability to recognize MSCs among mixed cell populations' cultures of MSCs of higher purity would be very effortful to achieve.

Considering this, numerous studies investigated different characteristics of MSCs identification. In 2006 the plastic adherence of MSCs maintained under basic culture conditions was defined [68]. In addition, the multilineage differentiation potential of MSCs *in-vivo* or *in-vitro* after stimulation by specialized media was postulated by a number of studies [68–70].

Another widely reported method for MSCs' recognition is the analysis of the expression of specific surface markers of the cells by flow cytometry. Markers as CD29, CD44, CD71, CD73, CD90, CD105, CD106, CD120, CD124, CD166 and Stro-1 show positive expressions on the cell surface, while markers as CD11, CD14, CD18, CD31, CD34, CD40, CD45, CD56, CD80 and CD86 are missing or weakly expressed [59, 68, 71]. Colony forming units (CFUs), which are cellular colonies formed by the MSCs after isolation, were also reported as a method of MSC recognition by CFU assays [71, 72].

4.3. Sources of adult mesenchymal stem cells

Hazards and morbidity risks of stem cell based therapies have turned into one of the most debated subjects in the latest years. These discussions led to multiple studies regarding the carcinogenic potential of embryonic stem cells [73–75]. Simultaneously, ethical discussions about the use of these cells have raised many disagreements within the scientific society, promoting a large number of investigators to discover potential sources for safer adult (somatic) stem cells. Although bone marrow has been established to be the primary source of adult mesenchymal stem cells [76, 77], several efforts and investigations are being prepared to establish new stem cell bases that could deliver large quantities of MSCs with less risks and donor site morbidity. Among these niches, umbilical cord blood (UCB) [78, 79], placental tissue (PT) [80, 81], adipose tissue (AD) [82, 83] and Wharton jelly (WJ) [84] have been described as possible sources of MSCs. Additionally, MSCs can be extracted from oral tissues as gingiva [72, 85–87], alveolar bone proper [88, 89], periodontal ligament [90], dental follicle [91] and dental pulp [92]. Despite the phenotypic resemblance of MSCs isolated from various niches of the body, differences in their actions and functions of have been reported, emphasizing the individuality of MSCs derived from every source [10, 93].

4.4. Immunobiology of MSCs

4.4.1. MSCs mediated immunomodulation

Among the MSCs characteristics presented recently, their therapeutic ability to modulate immune inflammatory reactions by various means has been noticeably highlighted [94]. This

communication between active MSCs and different immunological aspects in the human body presents a significant role played by them for restoring damaged tissues, as well as protecting them during inflammatory conditions [95].

Tissue injuries endorse the stimulation of inflammatory cells, as $CD4^+$ T cells and $CD8^+$ T cells, the macrophages and neutrophils, promoting the release of specific factors, as IL-1β and TNF-α [94]. These inflammatory alterations lead to a differentiation and organization of MSCs to repair the damaged tissue. In an inflammatory environment produced mediators as IL-1, TNF-α and IFN-γ, besides the tissue hypoxia trigger MSCs to release growth factors like epidermal growth factor (EGF), fibroblast growth factor (FGF), platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEG), playing an important role in regeneration and repair of damaged tissues [96–98]. In some studies, even myocardial infarction was reported to be recovered by MSCs related factors [99]. Furthermore, MSCs can release a number of other molecules as stem cell factor (SCF), macrophage colony-stimulating factor (M-CSF), and angiopoietin-1 (Ang-1), promoting the repair mechanism intrinsically [96, 100, 101].

Supplementary to the tissue repairing ability, immunomodulatory effects of the MSCs were further demonstrated [94]. Recently, the immunosuppressive capacity of MSCs has been reported in combination with an environment containing IFN-γ and inflammatory cytokines as TNF-α, IL-1α or IL-1β [94]. In such inflammatory higher expressions of adhesion molecules and chemokines are promoted, bringing the immune cells closer to the MSCs and enhancing their effectiveness of immunosuppression [102, 103]. Nevertheless, in other reports, MSCs showed the ability to raise the immune reactions and support the pro-inflammatory milieu [104]. This designates the immunomodulatory flexibility of MSCs, depending on many factors, as the source of the MSCs, or the level of inflammation surrounding them [1, 2].

4.4.2. MSCs and expression of TLRs

TLRs are considered one of the most significant factors directing the immunomodulatory role of MCSs into pro- or anti-inflammatory reactions. Observing these results, the profiles of TLRs expression and their effects on immunomodulation have become a central field of scientific investigations to understand possible interplay of TLR ligands with MSCs in inflammatory and non-inflammatory sites. Multiple studies have been implemented on TLR expression profiles in human MSCs. The reported outcomes displayed different expressions of TLRs depending on the tissue origin of these cells. Although bone marrow-derived MSCs displayed an expression of TLRs 1, 2, 3, 4, 5, 6, 8, 9 and 10 [10, 105, 106], MSCs isolated from the umbilical cord blood and Wharton jelly presented the same outcomes with an exclusion of TLR8, TLR10 [107, 108] and TLR4 [10, 109]. Studies on oral tissue related MSCs, showed an expression of all TLRs except TLR7 in periodontal ligament MSCs [106], in addition to TLRs 2, 3 and 4 in MSCs derived from dental follicle [110, 111] and dental pulp [110, 112]. On the other hand, MSCs isolated from the free gingiva showed an expression of all TLRs 1–10 [2]. Moreover, the evidence has revealed the potential modulation of this pattern of expression by micro-environmental factors surrounding the MSCs. Inflammatory conditions have been suggested to upregulate the expression of TLR2 [105, 113], TLR4 [105, 114] and TLR7 [113]. On the other

hand, TLR6 was downregulated under the same conditions [2, 105] . Likewise, in human bone marrow MSCs (BM-MSCs) viral infections [115, 116] and hypoxia [115] encouraged an augmented expression of TLRs 1, 2 and 3 and TLRs 1, 2, 5, 9 and 10 respectively.

In many circumstances the results of TLRs' activation on MSCs and the delivered immune response appears to be linked to the origin of cells, as well as the type of TLR triggered. Recent studies have shown no significant alteration by TLR activation on human adipose tissue MSCs (AD-MSCs) [117], BM-MSCs [10], UCB-MSCs [107], as well as Wharton jelly MSCs' [10] immunosuppressive effect. However, other scientific results confirmed the BM-MSCs mediated immunosuppression by TLR ligands explained by different mechanisms. Regarding TLR3 and TLR4, some groups detected the increased immunosuppressive effect after TLR activation without association with IDO activity or PGE2 levels [9]. Others presented different results showing the indirect induction of IDO1 production leading to a similar effect by TLRs on BM-MSCs [118]. In another study TLR3 and TLR4 ligands were reported to have reducing effects on human BM-MSCs facilitated suppression of T-cell proliferation [6], while other examinations reported the opposite result by stimulated TLR3 and TLR4 in the same type of MSCs [118]. Furthermore, TLR3 activation enhanced the suppressive role of DF-MSCs and DP-MSCs and G-MSCs to the local immune response, while activated TLR4 promoted the immunosuppression in DF-MSCs and decreased it in DP-MSCs and G-MSCs [1, 2, 110].

Furthermore, TLRs of MSCs have presented the aptitude to elicit the production of pro- and anti-inflammatory cytokines modulating the immune response [119]. The kinetics of TLR stimulation, besides the concentration and timing of the active ligand, have been reported as the main factors controlling this cytokine and mediator release [119]. This function also appears to be contingent on the TLR type and the MSC niche. TLR4 activation endorsed the production of pro-inflammatory mediators as IL-6 or IL-8. TLR3 activation on the contrary enhanced anti-inflammatory responses by triggering molecules as IL-4, IDO, or PGE2. These cytokines act in concert together, directing the immune reaction against the invading microorganisms. While pro-inflammatory immune modulating responses increase the production and stimulation of immune cells and cytokines, this mechanism is counter-regulated at the same time by the anti-inflammatory mediators on cellular and humoral levels [120]. Correspondingly, a pro- and anti- inflammatory influence was reported in relation to MSC TLR3 and TLR4 activation on the level of lymphocyte proliferation [119]. MSC induced secretion of mediators by TLR activation has also shown a modulating effect on neutrophils as another mechanism of their immune regulating function. TLRs of BM-MSCs delayed neutrophil apoptosis by triggering the production of cytokines as IL-6 and IFN-γ. This outcome was reported to be similar in MSCs originating from adipose tissue, thymus and spleen [121].

Studies also presented the possible effect of active TLRs on the differentiation potential of MSCs. Adipogenic differentiation presented no changes following UCB-MSCs and AD-MSCs'TLR3 and TLR4 activation [108, 117, 122]. Otherwise, osteogenic differentiation potential of BM-MSCs, AD-MSCs and UCB-MSCs was strengthened after activation of TLRs 2, 3 and 4 [108, 117, 122] and repressed with TLR9 ligands [6, 117, 123]. Chondrogenic differentiation displayed only an improvement with TLR2 stimulation [108], while TLRs 3, 4 and 7 activation had no obvious effect [6].

MSC proliferation rate and migration was reported to be influenced by TLRs, as inhibition of proliferation was detected with TLR9 activation [122]. Studies implemented on MSC migration to injury sites after TLR activation displayed no amplification of the MSCs' movement [124, 125], except for TLR3 activated human BM-MSCs [126]

Regarding the TLR triggering in MSCs and its potential therapeutic benefits *in vivo* diverse results have been issued so far. Many studies described therapeutic benefits of LPS triggered MSCs for the treatment of induced lung injuries in animal models [127–129]. Other surveys about MSCs engraftment for cardiac protection and its inflection by TLRs exhibited varying results. Positive effects of TLR4 triggered MSCs in the treatment of acute myocardial infarctions were reported in rats [130]. A contrasting outcome was shown by a different study [131]. This concludes that different modulations promoted by TLR stimulation on MSCs originating from various niches of the body need further investigations to explain the prominence of these factors and their possible administrations in MSC related therapies.

4.4.3. MSC immunomodulation through TLR activation

One of the special abilities of MSCs is presented in sensing the microenvironment surrounding the cells and accordingly adjusting the biologic functions of various immune cells and responses [132]. Therefore, MSCs can display immune interactions performing their immunomodulating effects. Triggering of BM-MSC TLRs initiate pathways of downstream signaling particularly for TLR3. Accordingly, this activation promotes the production of cytokines mainly active in cell migration mechanisms [126]. Indeed, migration of MSCs was endorsed by exposure with TLR3 ligand as a primary mediator of MSC stress migration responses compared to TLR2 and TLR9. TLR3 (Poly I:C) and TLR4 (LPS) activation have consequently transformed BM-MSCs into special chemotactic cells proficient of improving the inflammatory immune cell recruitment by promoting the production of IL-6, IL-1β, IL-8, IP10, monocyte chemotactic protein (MCP)-1 and CCL5 (RANTES) by NF-κβ signaling activation [113]. Analogous outcomes have been attained in AD-MSC, as TLR ligands for TLR2 and for TLR4 promoted mRNA synthesis of MCP-1 and -2, IL-1β, granulocyte chemotactic protein-2 (GCP-2) and macrophage inflammatory protein-3α (MIP-3α) [115]. Human turbinated MSC (hTMSC) were reported expressing high percentages of TLR3 and TLR4. Nevertheless, hTMSCs were only responsive to TLR4 as displayed by the significant changes in their cytokine profiles [133]. Macrophage-activating ligand-2 (MALP-2), an agonist of TLR6, as well as its heterodimer partner TLR2, initiated the activation of NF-κβ pathway leading AMC to obtain a pro-inflammatory profile by highly secreting cytokines as IL-4, IL-8 and IL-6 [134].

Dissimilar to TLR3, ligation of TLR4 significantly encouraged expression of cytokines as IL-6, IL-12, IL-8, RANTES (CCL5), IP-10 (CXCL10), TNF-α and GM-CSF. Furthermore, it was reported that TLR3 activation by Poly(I:C) a Janus kinase (JAK) 2/signal transducer and activator of transcription (STAT) 1 pathway is triggered with an increased simultaneous expression of suppressor of cytokine signaling (SOCS) proteins [135]. These outcomes further showed that SOCS1 and SOCS3 can perform a distinct function in modulating TLR3, JAK/STAT, and CXCR4/CXCR7 signaling pathways in BM-MSCs. These results propose that as negative regulation mediators, SOCS proteins can influence the way MSCs react to signals *in vivo*, thus manipulating TLR signaling pathways to elevate the distribution of infused MSCs at injury sites [135].

Immune cell binding and migration to MSCs surrounding milieus has been presented to be a main stage for inaugurating immunomodulation [136]. Under TLR3 activation, tonsillar mesenchymal (T-MSCs) obtain a chemoattractant character permitting the migration of immune cells into the environment surrounding the MSCs. This is achieved by an augmented secretion of CXCL5, CXCL1, CXCL6, CXCL10 and CXCL8 active chemokines [137]. Regarding the leukocyte binding ability of MSCs after TLR activation, TLR3 triggering of BM-MSCs elevated the leukocyte number binding to MSCs, through hyaluronic acid structures while TLR4 activation raised VCAM-1 and ICAM-1 promoted binding of leukocytes to MSCs [138].

B cell activating factor (BAFF), known for its prominent stimulating action on B cells was also investigated in human BM-MSCs and displayed a higher expression after TLR4 activation by LPS, while other TLR agonists had no significant outcome. This proposed that TLR4 in human MSCs could play an important role in the regulation of B lymphocyte-associated immune responses [139].

Once the MSCs are in the area of injury or inflammation, surrounded by immune cells and different regulatory mechanisms, multiple factors can play a role in the process of immunomodulation. To date, results of TLR activation and immune modulatory responses by MSCs are discrepantly reported in different studies.

The secretion and differential expression of immune regulatory mediators was described to be controlled mainly by two elements; specifically the tissue origin of the MSCs and the TLR triggered [140]. TLR stimulation in MSCs has been presented to start the intracellular pathways of MAPK, AKT and NF-κβ [6, 118, 126] and to influence other biologic functions of MSCs promoting the secretion of pro-inflammatory, or/and anti-inflammatory mediators [9, 141, 142]. In one investigation, a new pattern for MSC immunomodulation was explained, as MSCs could be polarized by different TLR agonists into pro-or anti-inflammatory phenotypes. TLR4-activated BM-MSCs (MSC1 phenotype), mostly produced pro-inflammatory mediators and were able to trigger T-lymphocyte stimulation, whereas TLR3-activated BM-MSCs (MSC2 phenotype), mainly expressed immunosuppressive factors as IDO (indoleamine-2,3-dioxygenase) and (prostaglandin E2) leading to T-cell inhibition [119]. In another study on G-MSCs outcomes were in accordance to the same paradigm, as a distinct proinflammatory phenotype of G-MSCs (G-MSC1) was triggered by all TLR agonists except TLR3, which promoted the immunosuppressive phenotype of G-MSCs (G-MSC2) [1]. This was also in confirmed by different studies, presenting an immunosuppressive character of MSCs created by TLR3 triggering in MSCs originating from human umbilical cord [143, 144], human bone marrow [113, 118], human dental pulp and dental follicle [145], as TLR3 agonist Poly (I:C) significantly raised the expression of anti-inflammatory cytokine IDO in these investigations [1].

While different investigations described no significant outcome of TLR triggering on BM-MSC, AD-MSC and T-MSC-mediated immunosuppressive responses [117, 133], other studies reported decreased responses. TLR3 and TLR4 activated MSCs originating from human nasal mucosa (nmMSCs) preserved their capability of leukocyte suppression, partially mediated by prostaglandin secretion. Nevertheless, another study described an impairment of leukocyte suppression after TLR3 and TLR4 activation in BM-MSCs [6]. These mechanisms were associated mainly with jagged-1 down-regulation initiated by TLR3 or TLR4 activation.

Contrasting to these outcomes, TLR3 and TLR4 triggering promoted the immunosuppressive ability of BM-MSCs presented by the increase of regulatory molecules of kynurenines by the enzyme IDO1 [118]. In a comparative investigation, activation of TLR3 by Poly(I:C) and TLR4 by LPS differentially influenced the suppressive ability of BM-MSCs, as well as WJ- and AD-MSCs [10]. While BM-MSC displayed decreased inhibition of lymphocyte activation, the immunosuppressive function of WJ- and AD-MSC was scarcely changed.

Furthermore, alterations in the amounts of HGF and PGE2 secreted after TLR triggering in MSCs have been also postulated to emphasize these immunomodulatory changes. One of the studies reported that, TLR treated CB-MSCs significantly increased their abilities of immunosuppression only after TLR3 triggering by Poly(I:C). This was explained by an increased expression of cyclooxygenase-2 (COX-2) [143]. Later outcomes showed that miR-143 regulates the influence of Poly(I:C) on the immunosuppressive function of MSCs by targeting COX-2 gene.

Investigations have previously underlined that MSC-facilitated T-cell suppression arises through the discharge of galectins. After TLR2 triggering, galectin-3, a main modulator of T-cell biology, was elevated at both protein and mRNA levels in BM-MSCs, but showed no change in immunomodulation [146]. Moreover, galectin-9 expression was differentially induced by TLR activated BM-MSCs [147]. While TLR2, TLR3 and TLR4 triggering promoted the expression of galectin-9, activated TLR5 and TLR7/8 did not present significant changes on galectin-9 expression. Consequently, in the occurrence of particular infectious incitements through TLR activation, BM-MSCs can preserve or enhance their immunosuppressive ability by increased galectin-9 expression.

Another immunomodulatory effect of MSCs can be directed toward the cellular component of the innate immune response. TLR3- and TLR4-activated MSCs were presented to differently prolong the function and survival rate of neutrophils (PMN) [121]. TLR3 triggered BM-MSCs had higher anti-apoptotic effects on PMN than TLR4 activated ones. Both TLR ligands could in addition augment the respiratory burst ability and CD11b expression by PMN. These biological functions exerted on PMN by TLR3 triggered BM-MSCs were mediated by the action of secreted mediators as IL-6, IFN-β, and GM-CSF, while TLR4-triggered BM-MSCs depended on GM-CSF in their PMN regulating mechanism. In addition, MSCs and NK cells were reported to interact in complex mechanisms with bidirectional regulation. This was described as TLR3 and TLR4-activated MSC enhanced their suppressive functions against NK cell proliferation and cytotoxicity, which may provide a potential stroma-targeted therapy of tumors [148].

5. Conclusion

MSCs are exceptional applicants for use in cellular treatments which can possibly transform the current field of immunotherapy. Although MSCs display pronounced potentials in the therapy of many immune conditions, the wide inconsistency in the quality of cells isolated from various donors and tissue sources, varying protocols, fluctuating measures and changing patterns of transfusion may decrease their therapeutic advantage. Therefore, a watchful assessments of suitable cell sources and tissues, more consistent scientific results, as well as better understanding of immunomodulation mechanisms of MSCs are required. Factors as, standardized cell culture protocols for cell expansion, differentiation and cryopreservation

need to be applied to allow better controlled therapeutic results. Another factor as comprehending the influence of TLR triggering on the immunobiology of MSCs plays a major role to allow correct and efficient therapeutic application of the cells. Despite the great amount of information obtained about that subject, there are many conflicts of the outcomes among the investigations. These may be related to the variety of experimental situations used to investigate the influence of TLR triggering on MSCs. Especially, the effect of specific culture conditions or the MSC source, as well as the TLR triggered seem to be the most influential factors among the studies. Therefore, this topic has to be studied in a more critical manner in standardized and well-designed investigations. Stimulation of MSCs before or within the potential treatment procedures with distinct TLR ligands may assist as an effective step controlling the biological function of the MSCs as needed in different therapeutic stages of the disease.

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