TOLL-LIKE RECEPTOR EXPRESSION PROFILE OF GINGIVAL MESENCHYMAL STEM CELLS IN INFLAMED AND NON-INFLAMED CONDITIONS

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To my dear mother and father
1 Introduction and review of literature

Human gingival tissues cover the tooth-carrying alveolar bone and its inserting teeth. One of its special features is its great regenerative and wound healing ability with little if any scarring evidence [118]. The multiple functions of connective tissue fibroblasts of the human gingiva, their wide spectrum in responsiveness to different growth/differentiation factors as well as in the capacity to form an array of characteristic extracellular matrix proteins throughout the healing processes presents their heterogeneous nature [81, 180, 182, 200, 204] and proposes the presence of a population of stem/progenitor cells, providing this heterogeneous culture of cells. Former studies presented isolation techniques of oral cavity' soft tissue derived stem/progenitor cells [62, 138, 149, 219, 223, 245]. Studies showed favourable characteristics of gingival stem/progenitor cells, including its immunomodulatory properties [256], besides their biocompatibility with scaffolds of alginate hydrogel microbeads [151]. Recently, stem/progenitor cells, originating from the free gingival margin (G-MSCs) showed significant abilities of generation in-vivo [57].

Toll-like receptors (TLRs) are important players connecting innate and acquired immune reactions. They are germ line-encoded pattern-recognition receptors (PRRs) that identify specific pathogen-associated molecular patterns (PAMPs) and induce activation [72, 107]. They can recognize invading microbes and show an association in chronic diseases' pathogenesis, autoimmune disorders and different infections [36]. To date, investigations were able to characterize ten functional human TLRs. [129]. Depending on their localization in the cells and their specific PAMP ligands, TLRs are classified into extracellular and intracellular TLRs. Extracellular TLRs are expressed on cell surfaces and mostly identify constituents of the pathogens' cell membranes including lipids and lipoproteins (TLR1, TLR2 and TLR6), lipopolysaccharide (LPS) (TLR4), and flagellin (TLR5). The second category of TLRs is formed intracellularly and mostly identify double-stranded RNA (TLR3), single-stranded viral RNA (TLR7 and TLR8) and unmethylated CpG DNA of viruses and bacteria (TLR9) [100].

MSCs from various sources within the body have been shown to express functional TLRs in certain patterns, which allow them to become selectively sensitive to
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pathogenic and microbial compounds. When stimulated by their ligands, TLRs can modulate immunosuppressive, proliferative, migratory and differentiation abilities of MSCs [31, 129, 224, 231]. Studies have shown differential expressions of TLRs 1, 2, 3, 4, 5, 6 on human and mural adipose and bone marrow derived MSCs (BM-MSCs), human Wharton Jelly MSCs (WJ-MSCs), on human umbilical cord blood MSCs (UCB-MSCs) and on human MSCs from the dental follicle and the dental pulp [187, 225, 231]. These investigations showed that the specific pattern of TLRs expression differs according to the tissue of origin of the MSCs, which could have an effect on the MSCs’ therapeutic potential during treatment procedures utilizing these cells for transplantation in inflammatory milieus in-vivo [42].

G-MSCs are currently in experimental employment in therapeutic modalities for inflammatory conditions as the treatment of periodontitis [57] and colitis [256]. To date, no expression profile exists for the TLRs of G-MSCs, although gingiva has been proven as a good source and reservoir harbouring MSCs [255]

1.1 The immune system

Protecting the human body against possible threats of invading pathogens relies upon a series of natural mediators that are able to recover the homeostasis and preserve it [143]. This biological mechanism of protection consists of cells and molecules opposing the microbes discovered by the immune system, initially developed in the human embryo. This process begins with hematopoietic stem cells which differentiate into the main players of the immune response in our bodies (granulocytes, monocytes, and lymphocytes). Through the different functions and activities of these main units of immunity the immune response contains two major divisions, the innate and the adaptive responses (Fig.1).

The innate immunity incorporates different barriers of microbiological, chemical and physical nature but also provides the components of our immunity responsible for immediate action against the invading pathogens. Although this defensive action is rapid, it lacks specificity and might cause some damage to normal tissues. On the contrary, the adaptive immune response shows a much higher precision in its defensive mechanism, but takes several weeks or days to develop. This can be
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explained by the development of an immunological memory by the adaptive immunity, which allows specific responses against the pathogens and much less damage to the normal tissues than the innate response.

1.1.1 The innate immune system

The innate immunity refers to semi-specific mechanisms of defence starting immediately or within a short time of a pathogen's appearance in the body [43, 124, 133]. In this immune response the germline-encoded receptors' genetic memory facilitates the recognition of certain molecular motifs of common microbes [21, 43]. It is accountable for primary forms of protection against invading threats. Simple physical and chemical barriers such as epithelial layers of cells and mucous secretions lining multiple tracts, such as the gastrointestinal tract or the oral mucosa, contribute to this first line of defence [26, 35, 84]. In addition, bioactive molecules and soluble proteins present in biological fluids of the body as complement proteins or cellular secretions of cytokines are able to debilitate a wide range of invading pathogens [26]. Furthermore,
cellular components of the innate response include dendritic cells, macrophages and natural killer cells [26, 35, 84]. In order to ensure the restoration of homeostasis and clearance of invading microbes the innate response has to fulfil the fundamental process of early pathogen recognition. This procedure principally takes place by a group of receptors termed the Pattern Recognition Receptors (PRR). These receptors have the ability to recognize conserved microbial patterns known as Pathogen-Associated Molecular Patterns (PAMPs) [121]. The human PRRs include Toll-like Receptors (TLRs) recognizing bacteria, fungi and viruses; Nod-like receptors (NLRs) detecting bacteria and the retinoic acid inducible gene-I (RIG-I)-like receptors (RLRs) that distinguish viruses [37, 114]. Following PAMP-PRR recognition a reaction cascade is initiated by cells of the innate immunity generating antimicrobial agents as reactive oxygen. In conjunction with that, generated chemokines and cytokines facilitate immune cell recruitment favouring the clearance of pathogenic organisms. Moreover, the stimulation of PRR leads to the synthesis of acute phase proteins, such as C-Reactive Protein and complement factors, with antimicrobial functions. The activated innate response of the immune system is fundamental for the onset of the adaptive response as both branches of the immunity do not operate separately, but rely on their interdependent functions [63, 101].

1.1.2 The adaptive immune system

Successive to the innate immune response the second arm of immunity commences. This adaptive (acquired) response is unlike the innate reaction characterized by being highly specific against certain organisms. This is promoted by a special ability of the cells of the adaptive arm of immunity to implement a recombination of their antigen receptors. This provides these cells with specific receptors for invading pathogens and generates the immunological memory of the body, by which pathogens are identified distinctively [35]. As this process may require 3 to 5 days the innate immunity has to coordinate and fulfil its functions in order to protect the body as the first line of defence [143] The adaptive response is composed of a group of specialized cells originating during haematopoiesis from lymphoid cell lineage. Among these are CD4+ and CD8+ T-cells as well as B-Lymphocytes that are responsible for antibody production [102]. The antibodies provide the humoral immunity which play different roles in the defence
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against pathogenic invasion. As B-Lymphocytes produce large numbers of antibodies, pathogens and their products can be identified and cleared. After pathogen recognition the antibodies bind to the pathogens, allowing their neutralization and preventing the access of pathogenic organisms into the host cells. Other functions of antibodies perform an incitement of phagocytic immune cells as macrophages and neutrophils as well as natural killer cells. They also form the first step of complement cascade activation through antigen-antibody complexes to enable the phagocytosis of unrecognized bacteria. This is facilitated through the antibodies’ opsonisation mechanism to microbial organisms. On the other hand killing infected cells is operated by T cells, as the second cellular component of the acquired immune response [35].

1.2 Toll-like receptors

1.2.1 Definition and discovery

The Toll Receptor family was the earliest group of PRRs to be discovered. In 1985 Toll protein was identified and classified as being significant for embryonic growth of the fruit fly, Drosophila melanogaster [9]. Another function described in further studies is mediating host responses to fungal Aspergillus niger infection and inducing the release of antimicrobial proteins [123]. Furthermore, a human toll like homologue was reported in 1997 [145]. This protein was termed as the Toll-like receptor (TLR) and showed an important role in intercommunication between innate and acquired immunity [144]. Toll-like receptors are intracellular and extracellular proteins that distinguish classes of different molecules. This enables the innate immune response to utilize these receptors for sensing microbial pathogenic invasions [216]. By recognizing specific microbial products by the TLRs the early immune reaction can be initiated [115]. Among PAMPs stimulating TLRs are, lipoproteins, peptidoglycan, lipopolysaccharide, bacterial DNA and double-stranded RNA [115]. As a result of this TLR-PAMP complex, expressions of pro-inflammatory and defensive genes are induced. Conjointly signalling pathways are started activating NF-kB and MAPK, along with promoting cytokine (e.g. IL-6 and IL-10) and co-stimulatory molecule production, leading to the commencement of the adaptive immune response [4].
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1.2.2 Identification of TLRs (1-11)

The earliest mammalian TLR to be reported was TLR4 [141, 144]. The PRR-PAMP identification mapped to TLR4 showed a critical role in recognizing the bacterial component LPS [184]. Subsequent studies identified a family of 13 mammalian TLRs, [95], with TLRs 1 to 10 functional in human cells [129], while non mammalian species have shown a broader TLR spectrum [172]. TLRs display a correspondence to IL-1 receptor family in their cytoplasmic portion. Due to this resemblance the intracellular domains of TLRs are termed Toll/IL-1 receptors (TIRs). On the other hand, extracellularly the TLRs show leucine-rich repetitions while IL-1 receptors present immunoglobulin-like areas [70, 145]. Regarding the TLR family, most consistent studies have been aiming attention to TLRs 1-10 in humans and other mammalian species.

1.2.3 TLR activating PAMPs and their signalling pathways

Studies have displayed various molecular components of pathogenic microbes, including combinations of nucleic acids, proteins, lipids and carbohydrates as ligands (PAMPs) activating the TLRs. Bacterial lipopolysaccharides, lipoproteins and flagellin, besides viral RNA are considered amongst the most important members of these PAMPs [90, 93, 115]. By activating TLRs by their specific ligands, intracellular signalling pathways are induced, promoting factors as MyD88 and NF-κB. MyD88, a molecule structurally related to the IL-1R family, is considered one of the main proteins utilized by almost all TLRs to start the signalling pathway, promoting the production of the transcription factor NF-κB [3]. This nuclear factor can cause both pro- and anti-inflammatory responses and increases the expression of various genes, including chemokines, cytokines, and adhesion molecules [119]. Through these intracellular reactions the innate immune response is finally initiated and a signalling cascade is formed to resist the invading pathogen (Fig.2). This critical step is the first protective mechanism against the microbes, which later also commences the adaptive immunity to fight against the pathogens by specific means [4]. Corresponding to their activating PAMPs, TLRs can be classified into different subfamilies. For instance, studies have demonstrated the recognition of lipids by (TLRs 1, 2 and 6), nucleic acid by (TLRs 7, 8 and 9) and various ligands by TLR4 [4, 115]. Regarding their site of cellular expression,
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TLRs 1, 2, 4, 5, 6 and 11 are expressed on the cell surface, while the rest are expressed intracellularly [115]
The stimulation of the TLR signalling pathway initiates from the cytoplasmic Toll/IL-1 receptor (TIR) domain which associates with a TIR domain-containing adaptor, MyD88. After activation with PAMPs, MyD88 recruits IL-1 receptor-associated kinase-4 (IRAK-4) to TLRs by communicating the death domains of the two molecules. IRAK-1 is triggered by phosphorylation and together with TRAF6 it activates the IKK complex and leading to stimulate of MAP kinases (JNK, p38 MAPK) and NF-κB. Tollip and IRAK-M interact with IRAK-1 and negatively regulate the TLR-mediated signalling cascades. Other modes of control regarding these pathways include TRIF-dependent initiation of TRAF6 signalling by RIP1 besides the negative regulation of TIRAP-mediated downstream signalling by ST2L, TRIAD3A, and SOCS1. Activation of MyD88-independent pathways occurs via TRIF and TRAF3, directing to the recruitment of IKKε/IKKB1, phosphorylation of IRF3, and production of interferon-β. TIR domain containing adaptors such as TIRAP, TRIF, and TRAM regulate TLR-mediated signalling reactions by offering specificity for individual TLR signalling pathways.

Source: http://www.cellsignal.com/pathways/nk-kappab-signaling.jsp

Figure 2: Explanation of Toll-like receptors’ activation and their signalling pathways
1.2.4 TLR subfamilies

1.2.4.1 TLR1, TLR2, TLR6 and TLR10

TLR2 has displayed a broad spectrum of recognized PAMPs. These involve pathogenic lipoproteins, gram-positive bacterial peptidoglycans and lipoteichoic acid, mycobacterial lipoarabinomannan, *Porphyromonas gingivalis* fimbriae and fungal zymosan [115, 217]. LPS originating from bacteria as *Porphyromonas gingivalis*, *Capnocytophaga ochracea* and *Bacteroides fragilis* is also a TLR2 identified ligand [115].

Two mechanisms have been proposed considering the TLR2 recognition of various pathogenic components. In the first one, TLR2 creates heterophilic dimers with other members of the TLR family that show structural resemblance to it, as TLR1, TLR6 and TLR10. Consequently, TLR1, TLR6 and TLR10 are considered associated in their function with TLR2 and able to recognize similar or correlated types of PAMPs as triacyl and diacyl lipopeptides [114, 216]. The second model suggests the identification of fungal proteins by TLR2. This attribute explains why TLR2 functionally associates with dectin-1, a receptor recognizing β-glucan, a fungal cell wall constituent [67]. Regarding this functional coordination with various types of related or unrelated proteins, TLR2 gains its ability to identify various pathogenic molecules at an early stage, activating the immune responses.

1.2.4.2 TLR3

TLR3 mainly identifies dsRNA that is produced in the replication period of most viruses. It has the function of activating NF-κB and type I Interferon production [6]. It has been also reported that TLR3 can homodimerize with TLR4 and TLR9 [168, 240].

1.2.4.3 TLR4

TLR4 is an important receptor recognizing PAMPs as LPS from several bacterial species [115]. This type of LPS shows structural differences from the LPS identified by TLR2 in the number of acyl chains, which widens the spectrum of pathogen
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identification by TLRs [164]. In addition other ligands of endogenous nature including heat shock proteins (HSP60 and HSP70) can stimulate TLR4 in higher concentrations [68].

1.2.4.4 TLR5

TLR5 can identify flagellin through a mechanism of physical interaction [68, 115]. Expression of TLR5 has been reported on epithelial cells of mucosal surfaces of the intestine [73] and the lung [85], as well as on mesenchymal cells of adipose, bone marrow and umbilical cord origin [120], which presents the important function of detecting microbes at these surfaces.

1.2.4.5 TLR7 and TLR8

TLR7 and TLR8 both show the ability to recognize similar ligands in certain cases. It has been reported that the two are activated by organic molecules as Imidazoquinoline [115] and by viral ssRNA [45, 87, 132], while host ssRNA is not identified by them, as stated by other studies [216]. This recognition starts by viral internalization and replication releasing the viral RNA into endosomes. The interaction between the viral ssRNA and TLR7/8 activates the recruitment of the adapter molecule MyD88 leading to production of NF-κB and other factors as well as proinflammatory cytokines and chemokines [105].

1.2.4.6 TLR9

TLR9 has the ability to distinguish bacterial DNA [115]. This contains unmethylated CpG generating its immunostimulatory effect in contrast to the vertebrate DNA that contains methylated CpG only [142]. By activation of TLR9 through the bacterial CpG DNA the production of inflammatory cytokines such as IL-12, IFN-α and TNF-α is potentiated [89]. The competence of TLR9 to induce IFN-α production besides its
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competence identifying unmethylated CpG motifs indicates that it may also be important in mechanisms of viral pathogen recognition [113].

1.3 Human Mesenchymal Stem Cells

1.3.1 Definition and history

Human mesenchymal stem cells were initially defined in 1970 by Friedenstein et al. [64]. They were described as bone marrow isolated, non-hematopoietic and plastic-adherent cells, which hold the capabilities of multipotent differentiation and self-renewal \textit{in vitro} [16, 25, 183]. These undifferentiated cells originate from different tissues of the human body [104]

The multilineage potential and the ability of self-renewal both define the major characteristics of MSCs [60]. Self-renewal is the technique by which stem cells expand their number during development. This occurs asymmetrically or symmetrically where daughter stem cells have a capacity of development similar to their mother cells that become committed progenitor cells [86]. This ability is essential for MSCs to expand within the tissues and could therefore be very important in stem cell related therapies [86].

On the other hand this self-renewal depends on the life span of the cells. In humans MSCs are restricted to a maximum of 44 weeks [15] or 55 population doublings [91] in culture. In addition cell density during culture also influences “stemness” characteristics of the MSCs [202].

Multipotency, or the multilineage potential of MSCs forms the unique ability of the cells for differentiation into cells of the mesodermal lineage like osteocytes, chondrocytes and adipocytes (Fig.3). In addition MSCs can also differentiate into cells of other embryonic lineages [228].

These special characteristics of MSCs as well as their interaction with specific signals of the human body show great therapeutic potential and may become basic treatment options for severe diseases in the future [176, 237].
1.3.2 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 amongst 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 [48]. 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 [48, 116, 177].

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 [25, 48, 174]. 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 [54, 174].

1.3.3 Adult sources of mesenchymal stem cells

Risks and morbidity of stem cell based therapies have become one of the most discussed topics in recent years. This led to wide investigations regarding the tumorigenicity of embryonic stem cells [13, 19, 214]. Concurrently, ethical discussions about the application of these cells have raised a lot of controversies throughout the society, leading a large number of researchers to explore possible sources for adult (somatic) stem cells. Despite the fact, that bone marrow has been settled to become the primary source of adult mesenchymal stem cells [29, 156], various efforts are being done to establish new sources that could provide large numbers of MSCs with less donor site morbidity (Fig.4). Among these sources, umbilical cord blood (UCB) [178, 257], placental tissue [59, 258], wharton jelly [8] and adipose tissue [12, 247] have been reported to be possible niches of MSCs. Furthermore, MSCs could be isolated from tissues related to the dental field as alveolar bone proper [56], gingiva [54, 103], periodontal ligament [80], dental pulp [209] and dental follicle [238]. In spite of the phenotypic similarity of MSCs derived from various sources, discrepancies in activities and functions of these cells have been observed, underlining the individuality of MSCs from every source [186, 197]
1.3.4 Gingival mesenchymal stem cells (G-MSCs)

Gingival tissues have gained the interest of different researchers as a unique reservoir for MSCs [103, 248, 255]. While skin, oral mucosa and gingiva show a number of analogies in their functions and histological appearance [212], specifically the gingiva has displayed particular biological properties suggesting its unique distinction compared to other oral mucosal tissues [218]. For instance gingival tissues are continuously under influence of factors like oral bacteria, friction or food rests. Furthermore, inflammatory responses, drug induced overgrowth and gingival fibrosis...
also differ gingival tissues from other oral mucosa [69, 154]. Among the studies presenting gingival tissues as MSC-harbouring reservoirs, some results proposed the presence of specific markers related to pluripotent cell characteristics, as Stro-1, SSEA-4 and Oct-4 in cells originating from the gingiva [54, 218, 254], in addition to the positive staining of p75, a marker normally present in neural stem cells [138]. Besides the ability of G-MSCs for self-renewal and multipotent differentiation [218, 222, 239], human G-MSCs express CD29, CD44, CD73, and CD90 (> 80%) and CD105, CD146 and Stro-1, while they show no expression of CD34 and CD45 [255]. This surface expression profile of G-MSCs confirms their “stemness” concomitant to the minimal criteria for human MSCs as it was proposed in 2006 [48].

1.3.5  Immunobiology of MSCs

1.3.5.1  MSCs mediated immunomodulation

Among the properties of MSCs demonstrated by recent studies, the ability to modulate immune responses and inflammatory reactions within the body has been underlined [134]. This interaction between MSCs and immunological factors displays an important role played by these cells in repairing damaged tissues or protecting them during inflammation [206].

Tissue injury promotes the activation of inflammatory cells, including CD4⁺ T cells and CD8⁺ T cells besides the macrophages and neutrophils, releasing specific mediators, as IL-1β and TNF-α [134]. These inflammatory changes lead to an organization and differentiation of MSCs to repair the damaged tissue. By entering an inflammatory milieu released cytokines as IL-1, TNF-α and IFN-γ, in addition to the tissue hypoxia activate MSCs to produce growth factors like epidermal growth factor (EGF), fibroblast growth factor (FGF), platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF). These factors in turn play an effective role in regeneration and repair of affected tissues [97, 135, 207]. Even in cases like myocardial infarction some studies reported a possible recovery by factors released by MSCs [221]. Moreover, MSCs produce a number of molecules as stem cell factor (SCF), macrophage colony-stimulating factor (M-CSF), and Ang-1, that promote the repair process intrinsically [158, 207, 232].
Supplementary to the described tissue repairing ability, immunomodulatory functions of the MSCs were further demonstrated and explained [134]. Recently, the immunosuppressive ability of MSCs has been observed in combination with a milieu containing IFN-γ and proinflammatory cytokines as TNF-α, IL1α or IL1β [134]. Such inflammatory environment could lead to higher expressions of adhesion molecules and chemokines, bringing the immune cells in close vicinity to the MSCs and augmenting their potency of immunosuppression [191, 192]. Nevertheless, other evidences concerning MSCs mediated immunomodulation confirm the ability of MSCs to elevate the immune responses in media of low inflammatory levels [126]. This indicates the flexibility of MSCs mediated immunomodulation, depending on the amount of inflammation affecting the cells.

1.3.5.2 MSCs and TLRs

One of the most important factors directing immunomodulatory functions of MCSs into pro- or anti-inflammatory responses are TLRs. In the light of these results TLRs expression profiles and their effects on MSCs mediated immunomodulation have become an important field of scientific research in recent years to comprehend possible encounters of TLR ligands with MSCs and their influence in inflammatory sites. Several studies have been performed on TLR expression profiles of human MSCs. The reported results presented different expressions of TLRs corresponding to the tissue origin of these cells. While bone marrow-derived MSCs showed an expression of TLRs 1, 2, 3, 4, 5, 6, 8, 9 and 10 [125, 186, 188], MSCs originating from the umbilical cord blood and Wharton jelly displayed the same results with an exception of TLR8, TLR10 [108, 230] and TLR4 [146, 186]. Investigations on oral tissue related MSCs, demonstrated an expression of all TLRs except TLR7 in periodontal ligament MSCs [125], besides TLRs 2, 3 and 4 in MSCs derived from dental follicle [28, 226] and dental pulp [226, 249]. Furthermore, the evidence has shown the possible modulation of this expression pattern by microenvironmental factors surrounding the MSCs. Inflammatory conditions have been suggested to upregulate the expression of TLR2 [188, 193], TLR4 [188, 205] and TLR7 [193]. On the other hand, TLR6 was downregulated under the same conditions [188]. Similarly, in human BM-MSCs viral infections [30, 32] and hypoxia [32] induced an increased expression of TLRs 1, 2 and
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3 and TLRs 1, 2, 5, 9 and 10 respectively. Furthermore, fatty acids also showed a suppressive effect on TLR2 expression in mouse AD-MSCs [96].

In many cases the consequence of TLRs' activation on MSCs and the local immune reaction appears to be related to the origin of cells and the type of TLR activated. In recent studies, results have shown no significant change by TLR stimulation on human AD-MSCs [130], BM-MSCs [186], UCB-MSCs [230], and wharton jelly MSCs' [186] immunosuppressive effect. Nevertheless, other scientific groups confirmed the support of human BM-MSCs mediated immunosuppression by TLR ligands in different mechanisms. In case of TLR3 and TLR4, some groups observed the increased immunosuppressive influence after TLR activation without correlation with IDO activity or PGE2 levels [40]. Others had dissimilar results presenting the indirect induction of IDO1 production as the main mechanism leading to the same effect by TLRs on BM-MSCs [170]. In another investigation TLR3 and TLR4 ligands were reported to have reducing effects on human BM-MSCs mediated suppression of T-cell proliferation [129], whereas other investigations reported the opposite result through stimulated TLR3 and TLR4 with the same type of MSCs [170]. Moreover, TLR3 activation enhanced the suppressive role of DF-MSCs and DP-MSCs to the local immune response, while activated TLR4 promoted the immunosuppression in DF-MSCs and decreased it in DP-MSCs [226].

In addition, TLRs of MSCs have shown the ability to trigger the production of pro- or anti-inflammatory cytokines regulating the immune response [244]. In consonance with this, the kinetics of TLR activation, besides the ligand concentration and timing, have been proposed as important factors controlling the cytokine release [244]. This function also seems to depend on the type of TLR and the origin of MSCs. Triggering of TLR4 promoted the expression of pro-inflammatory molecules as IL-6 or IL-8. TLR3 activation on the other hand enhanced anti-inflammatory functions by molecules as IL-4, or IDO. These pro-inflammatory and anti-inflammatory mediators act in concert together, controlling the immune response against the invading pathogens. While pro-inflammatory response increases the production and activation of immune cells and mediators, this process is counter-regulated by the anti-inflammatory cytokines on cellular and humoral levels [253].
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Even on lymphocyte proliferation level and its relation to TLR3 and TLR4 activation, the same pro- and anti-inflammatory influence was observed [244]. In regard to the tissue origin of TLR stimulated, MSCs, BM-MSCs and UCB-MSCs showed no changes in their cytokine production, while PL-MSCs showed a weaker immunosuppressive ability [24, 58].

MSC induced cytokine secretion by TLR activation has also an effect on neutrophils as another part of their immunomodulation. Investigations displayed that TLRs of BM-MSCs delayed neutrophil apoptosis through cytokines as IL-6 and IFN-γ and increased their respiratory burst. This effect was confirmed in the same study by MSCs originating from adipose tissue, thymus and spleen [23].

Studies also reported the possible impact of activated TLRs on the differentiation potential of MSCs. Adipogenic differentiation showed no changes after UCB-MSCs and AD-MSCs’ TLR3 and TLR4 stimulation [98, 108, 130]. Alternatively, osteogenic differentiation of BM-MSCs, AD-MSCs and UCB-MSCs was intensified with TLRs 2, 3 and 4 activation [98, 108, 130] and inhibited with TLR9 ligands [129, 130, 165]. Interestingly, chondrogenic differentiation showed only an enhancement with TLR2 stimulation [108], while TLRs 3, 4 and 7 agonists had no obvious effect [129].

Considering the proliferation rate and migration of MSCs, inhibition of proliferation was observed with concurrent TLR9 activation [98]. In mouse BM-MSCs TLR2 and TLR4 ligands increased the proliferative rate of the MSCs [179, 243]. Other studies performed on the migration of MSCs to injury sites after TLR stimulation showed no amplification of the MSCs’ movement [122, 179], except with stimulated TLR3 of human BM-MSCs [224].

Concerning the TLR activation in MSCs and its relation to therapeutic benefit in vivo, different results have been published so far. Several studies reported therapeutic benefits of using LPS stimulated MSCs treating induced lung injury in animal models [74, 161, 162]. Other investigations about the survival and engraftment of MSCs for cardiac protection and its modulation by TLRs showed varying results. First, positive effects of TLR4 modulation of MSCs used for the treatment of acute myocardial infarctions were demonstrated in rats [252]. On the other hand a contrasting result was revealed by a different investigation [242]. In this regard it can be concluded, that various modulations affected by TLR stimulation on MSCs of different origins need
further investigations in the future to clarify the importance of these factors and their role in possible therapeutic administrations of MSCs.

1.4 Periodontium and periodontitis

1.4.1 The healthy periodontium

The periodontium forms the supporting structures surrounding every tooth. It consists of alveolar bone proper, cementum, periodontal ligaments and the gingival tissues, formed of connective tissue and epithelium. Besides supporting the teeth these structures also play a fundamental role of protection against different pathogens and in sensation of touch and pressure to the tooth [82, 155].

The word incorporates two Greek terms peri- and -odonts, meaning literally taken “around the tooth”. Various studies have investigated the structure of the periodontal apparatus in healthy conditions. Compassing a dimension of approximately 1mm the gingival epithelium is attached to the tooth 0.67-1mm apical to the cementoenamel junction (CEJ). The connective tissue of the gingiva forms the base for the epithelium and connects to the tooth a further millimetre apically. It mainly holds collagen, fibroblasts and ground substance. In an apical direction to the gingiva the alveolar bone proper continues the attachment to tooth through the periodontal ligament connecting between the alveolar bone proper and the cementum of the tooth root. Among the people, minor differences regarding the dimensions of these structures might occur [155]. The dimension of soft tissues attached to the tooth surface in coronal direction to the alveolar bone is called the “biologic width” [166]. Although the “biologic width” does not have a constant dimension and may vary between teeth and their aspects, it was reported that a dimension of 3 mm is enough to provide the needed functions and protection to the tooth and stay in a healthy condition for at least 6 months. In healthy conditions measurements of “biologic width” compose of approximately one millimetre each for the sulcus depth, epithelial attachment and connective tissue attachment [166].

The functionalities of the periodontium vary between its different structures. The main role of the epithelium is to create a barrier against bacterial invasion into the soft and hard tissues of the periodontium. [155]. Moreover, the alveolar bone is the main
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support of the tooth against the strong masticatory forces. These forces are additionally distributed through the periodontal ligaments, which also stabilize the tooth within its socket and supply it with nutrition and somatosensation [160].

1.4.2 The diseased periodontium

Periodontal diseases include several inflammatory disorders affecting the periodontium. These conditions are cumulative and influenced by different factors that play major roles in defining the host response towards the disease [171]. Periodontitis is always preceded by gingival inflammation initiated by bacterial pathogens. Further development of the disease is then provided by host factors of every patient [110]. This interaction between acquired, environmental and genetic factors has been reported to be the main process affecting the host susceptibility to the periodontal inflammation and destruction [171].

Gingivitis starts by colonization of the gingival sulcus and surrounding surfaces by the bacterial pathogens. These form a structure called bacterial biofilm, sticking to the tissues and generating bacterial products as LPS which are recognized by Toll-like receptors of the gingival cells [136, 216]. This process activates an inflammatory response of the gingiva leading to the gingival disease. At early stages of the disease, mechanical removal of the biofilm and the tooth-tissue interface is the first step to treat the inflammation and prevent any progression of the disease [50]. If bacterial deposits are left to accumulate they start invading deeper structures of the periodontium and by the same process the destruction of the attachment apparatus is continued. As the junctional epithelium of the periodontium is destroyed, it detaches from the tooth surface and can start an apical migration, exposing the root surface of the tooth to more inflammatory and bacterial insults and creating a periodontal pocket or recession [215].

Periodontitis can be classified into one of two main types, chronic and aggressive. In chronic periodontitis the tissue destruction is not continuous but shows periods of acute exacerbation causing the loss of periodontal attachment. This can be correlated to increased proportions of so called periodontal pathogens including Porphyromonas gingivalis, Prevotella intermedia, Bacteroides forsythus, Aggregatibacter (formerly
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Actinobacillus) *actinomycetemcomitans* and *Treponema denticola* [10]. On the other hand, aggressive periodontitis is characterized by rapid damage to the periodontal ligaments and the supporting bone. It may also display raised amounts of bacterial deposits of *Aggregatibacter actinomycetemcomitans* and/or *Porphyromonas gingivalis* and secreted inflammatory molecules such as prostaglandin E2 (PGE2) and interleukin-1β [10, 61]. Although both types of periodontitis share a number of clinical features, aspects as the age of onset, clinical signs of correlated gingivitis, patterns of destruction and plaque and calculus formation are considered to differentiate between them [159]. While chronic periodontitis shows generalized or localized pattern of inflammation, usually aggressive periodontal inflammations affect a large number of teeth. In addition to that, younger ages of aggressive periodontitis patients and their fewer clinical signs of gingivitis are also points of contrast between the two types.

1.4.3 Etiology and risk factors of periodontal disease

Various reports provide scientific evidence that periodontal disease etiology can be associated with a large number of risk factors. These can be classified into modifiable factors as smoking, Diabetes and stress and non-modifiable factors as osteoporosis and blood disorders [7] Nevertheless, the main and most important etiology of periodontitis are the periodontal pathogens, including different species of bacteria coexisting together in mixed microbial infections of the periodontium [18, 185, 194].

While the oral cavity includes over 700 bacterial species, 400 among these are found in subgingival plaque [14, 175]. Further investigations specified only certain types to play major roles in the etiology of periodontitis [259]. Especially gram negative anaerobic rods and spirochetes are dominating species in deep periodontal pockets [78, 150]. Furthermore *Porphyromonas gingivalis* [112] and *Aggregatibacter actinomycetemcomitans* [46] have been associated with periodontal pathology. Moreover, *Prevotella intermedia* [131], *Bacteroides forsythus* [233] and *Fusobacterium nucleatum* [196] present important etiological factors of the disease progression. *Capnocytophaga species* [34], *Peptostreptococcus micros* [5, 190], as well as herpes viruses [39] were also reported in to have associations with periodontal disease.
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Many studies have investigated the different factors characterizing these pathogens and their virulence towards the periodontium. In particular, *Porphyromonas gingivalis* [152], *Aggregatibacter actinomycetemcomitans* [2], *Treponema denticola* [211], *Tannerella forsythia* [157] and were intensively investigated by different research groups. These microorganisms are considered important pathogenic factors for periodontal diseases through a number of possible virulent mechanisms [94]. While agents as adhesins, invasins and bacteriocins promote colonization and persistence of the bacteria, other factors as, chemotactic inhibitors, leukotoxins, Fc-binding proteins and immunosuppressive proteins interfere with the host’s defences. Furthermore, cytotoxins, bone resorption agents, stimulators of inflammatory mediators and collagenase are responsible for the tissue destruction affecting the periodontium. In addition, host tissue repair is inhibited by inhibitors of bone formation and inhibitors of fibroblast proliferation. Concerning these factors of virulence, the TLR signalling pathway is considered an element of great importance defining the pathogenicity of bacteria to periodontal tissues and correspondent responses of the body on local and systemic levels [11, 79]

1.5 Aim of the thesis

This study aims to describe for the first time the distinctive TLRs’ expression profile of G-MSCs in inflamed and uninflamed environments. As the oral cavity is habitat to a large number of bacterial species under physiological conditions and even clinically healthy marginal tissues of the periodontium can show histological signs of inflammation [27, 83], the regeneration of destroyed periodontal tissues has to be performed under the influence of inflammatory stimuli. Changes occurring in the expression of TLRs responding to such stimuli could impact the therapeutic potential, regenerative capacity and immunomodulatory effects of G-MSCs in inflamed tissues in-vivo.
2 Materials and Methods

2.1 Isolation and culture of G-MSCs

2.1.1 Ethical committee approval and informed consents

The current study was approved by the ethical committee of the Christian-Albrecht’s University of Kiel (IRB- Approval number D 444/10). Before excising the gingival samples needed for the study, an informed consent was obtained from every patient individually.

2.1.2 Sample isolation

G-MSCs isolation was done as previously described [51]. Free gingival collars from five individuals (n=5) were surgically excised at the department of periodontology of the Christian-Albrecht’s-University-Kiel, Germany. Instantly after surgery, every sample was placed in a 50 ml sterile polypropylene tube containing Minimum Essential Medium Eagle Alpha Modification (α-MEM; Sigma-Aldrich GmbH, Hamburg, Germany) supplemented with antibiotics (100 U/ml penicillin, 100 µg/ml streptomycin) and 1% amphotericin (all from Biochrom AG, Berlin, Germany). The free gingival tissue collars were detached, de-epithelised and cut into small pieces (2 mm-3 mm) under the laminar flow hood. They were rinsed several times with Minimum Essential Medium Eagle Alpha Modification (α-MEM; Sigma-Aldrich GmbH, Hamburg, Germany) supplemented with antibiotics (100 U/ml penicillin, 100 µg/ml streptomycin) and 1% amphotericin (all from Biochrom AG, Berlin, Germany) and placed into dry 75 ml culture flasks (Sarstedt AG, Nümbrecht, Germany) for 30 minutes to adhere to their bottoms. Subsequently, the basic medium consisting of α-MEM, supplemented with 15% fetal calf serum (FCS; HyClone, Logan, UT, USA), 400 mmol/ml L-glutamine (Biochrom), 100 U/ml penicillin, 100 µg/ml streptomycin and 1% amphotericin was gently added. The flasks were incubated in 5% carbon dioxide at 37°C for 1 week without changing the medium and cells were left to grow out.
2.1.3 Cell culturing and isolation

2.1.3.1 Gingival mixed cell culture

Following the sample isolation procedure, the culture flasks were intermittently checked by phase contrast inverted microscopy to observe any outgrowing cells. The change of basic medium added to the flasks started 7 days after the placement of the samples into the flasks and was performed three times per week. This was continued until the outgrowing mixed cell culture reached 80-85% confluence.

2.1.3.2 Cell passage

After the cells reached a confluence of 80-85%, cells in the flasks were washed twice with 10 ml phosphate buffered saline (PBS) (Biochrom). Afterwards 5 ml of accutase (EMD Millipore) was added to each of the flasks which were then incubated in a 5% CO₂ incubator at 37°C for 10 min to allow the cells to detach from the flasks’ bottom. Subsequently the cell suspensions were transferred to sterile Falcon tubes (Sarstedt) and centrifuged at 1700 rpm for 10 min. The supernatant was then removed and cells were resuspended in 2 ml of Minimum Essential Medium Eagle Alpha Modification, supplemented with 15% FCS, 4 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 1% amphotericin. Finally, the cells were counted and tested for viability with Trypan blue (Sigma-Aldrich) and seeded at a density of 30 cells/cm² in 75 ml culture flasks. 10 ml of basic culture medium was added to each and the flasks were incubated in 5% CO₂ at 37°C. Medium change occurred 3 times per week.

2.1.3.3 Magnetic activated cell sorting (MACS)

After the cells’ first passage reached 80-85% confluence, magnetic activated cell sorting (MACS) was done to isolate the G-MSCs from the mixed gingival cell culture. At first the medium within the flasks was aspirated and cells in every flask were washed twice with 5 ml of PBS. 5 ml of accutase were then added to each culture flask and the flasks were incubated in 5% CO₂ incubator at 37°C for 5 min to detach the cells from
Materials and Methods

The cells were then transferred to new falcon tubes and centrifuged for 5 min at 1500 rpm. This allowed the supernatant to be easily discarded and the cell pellet was resuspended in 10 ml of fresh basic medium. Furthermore, a cell count was performed and the cell suspension was again centrifuged for 5 min at 1500 rpm to discard the supernatant and subject the cells to the MACS technique for MSCs’ isolation.

2.1.3.3.1.1.1 MACS Separator and MACS column preparation

First of all, the magnetic field was disinfected with 70% ethanol and the column attached to the magnetic field of the MACS Separator according to manufacturer’s instructions (Miltenyi Biotec, Bergisch-Gladbach, Germany). The column was prepared by rinsing it with 500 µl MACS-buffer consisting of 0.5% bovine serum albumin (BSA) and 2 mM EDTA in PBS (all from Biochrom). Two tubes were labelled and prepared to receive the cells below the column; one for MACS positive and one for MACS negative cell populations.

2.1.3.3.1.1.2 Magnetic labelling

After incubating the cells with r FcR- blocking reagent (Miltenyi Biotec) for 5 min, anti STRO-1 (BioLegend, San Diego, CA) and anti IgM Micro Bead antibodies (Miltenyi Biotec) were added to the cells afterwards according to manufacturer’s instructions for 10 min at 4 ºC. Ten times MACS-buffer volume was added to the cell suspension and the suspension centrifuged for 10 min at 1700 rpm at 4 ºC. The supernatant was discarded and the resultant cell pellet resuspended in 500 µl MACS-buffer (cells< 10^8) or 1000 µl MACS-buffer (cells>10^8) (Fig. 5).
Materials and Methods

2.1.3.3.1.2 Magnetic separation

The magnetic labelled and resuspended cells were transferred to the MACS Column to start the separation procedure (Fig. 6). Cells that passed through the column were collected by the MACS negative labelled tube. 500 µl MACS-buffer was then added three times to the column to perform the washing step and new buffer was only added when the column reservoir was empty. Following that the column was removed from the separator and positioned on the MACS positive collection tube. 1 ml of MACS buffer was pipetted into the column and the buffer with the cells within the column were eluted by a provided plunger into the MACS positive labelled tube. The obtained MACS positive cells were filled with MACS buffer to a volume of 5 ml. The MACS positive cell count was performed and their cell suspensions were centrifuged for 10 min at 1700 rpm at 4 ºC to seed the MACS positive cells out to form colony forming units (CFUs).

Figure 5: Schematic image of MSC surface after magnetic labelling with Microbeads

Materials and Methods

2.2 Colony-forming units (CFUs)

To assess the cells' efficiency to form colonies, MACS positive (G-MSCs) were cultured in basic medium at a density of 1.63 cells/cm². Aggregates of 50 or more cells were scored as colonies. On day 12 a representative sample of the cultures were fixed with 4% formalin, stained with 0.1% crystal violet. From the remainder of the CFUs forming G-MSCs single colonies were then detached by cell scrapers [71, 117] and seeded in new 75 ml flasks in basic medium to grow.

2.3 Flow cytometric analysis predefined MSCs’ surface markers)

After reaching 80%-85% confluence, a sample of the G-MSCs were characterized by flow cytometry for the predefined MSCs’ surface marker constellation [47]; namely CD14, CD34, CD45, CD73, CD90 and CD105 (all from Becton Dickinson). Primary antibodies’ reaction with the corresponding isotype controls was performed according to standard protocols using FcR Blocking Reagent (Miltenyi Biotec) and finally
Materials and Methods

evaluated with FACSCalibur E6370 and FACSComp 5.1.1 software (Becton Dickinson).

2.4 Multilineage differentiation potential

Cells obtained by the isolation procedures were tested independently for their multilineage differentiation ability into osteogenic, adipogenic and chondrogenic lineages. Cells at their third passage were cultured on 6-well culture plates (Sarstedt, Germany) for osteogenic and adipogenic differentiations and in 96-well plates (Sarstedt, Germany) for chondrogenic differentiations with specific inductive media.

2.4.1 Osteogenic differentiation

To investigate the osteogenic differentiation potential, third passage $2 \times 10^4$ G-MSCs were cultured on 6-well culture plates in osteogenic differentiation inductive medium (Promo Cell, Heidelberg, Germany). As control cells, G-MSCs were cultured in basic medium. At day 14, cell cultures were stained with Alizarin Red (Sigma-Aldrich) [47], to mark calcified deposits and evaluate the staining qualitatively.

2.4.2 Adipogenic differentiation

To test the adipogenic differentiation potential, $3 \times 10^5$ G-MSCs of the third passage were cultured on 6-well culture plates in adipogenic differentiation inductive medium (Promo Cell). As a control G-MSCs were cultured in basic medium. The existence of lipid drops was evaluated by staining with Oil-Red-O (Sigma-Aldrich) qualitatively [47].

2.4.3 Chondrogenic differentiation

To test the differentiation potential of the cells in a chondrogenic direction, micro-masses of third passage $3 \times 10^4$ G-MSCs were incubated with chondrogenic differentiation inductive medium (Promo Cell) in 96-well culture plates with rounded bottoms (Sarstedt AG, Germany). As a control, G-MSCs were cultured in basic
Materials and Methods

medium. Chondrogenic differentiation was assessed qualitatively at day 21 by staining of glycosaminoglycans with Alcian Blue (Sigma-Aldrich) [47, 127]. All media were renewed three times per week.

2.5 Inflammatory medium

To test the result of the inflammatory environment on the G-MSCs, TLR expression profile, a combination of 25 ng/ml IL-1β, 10³ U/ml IFN-γ, 50 ng/ml TNF-α, and 3×10³ U/ml IFN-α (inflammatory medium, all from PeproTech, Hamburg, Germany) [189] was used. G-MSCs were incubated for 18 hours in the inflammatory medium (G-MSCs-i) as well as basic medium (G-MSCs).

2.6 Flow cytometric determination of TLR expression

G-MSCs and G-MSCs-i were characterized by flow cytometry for the presence of the different TLRs 1-10 at protein level. The following antibodies were used: anti-TLR1, anti-TLR3 and anti-TLR9 (all from eBioscience), anti-TLR2, anti-TLR4 and anti-TLR8 (all from Enzo Life Sciences, Lörrach Germany), anti-TLR5 (R&D Systems, Hessen, Germany), anti-TLR6 (BioLegend), anti-TLR7 (Perbio Science, Bonn, Germany) and anti-TLR10 (Acris Antibodies, Herford, Germany). The antibodies and their isotype controls were each conjugated with PE, FITC or Alexa Flour 488 fluorescent dyes. For intracellular TLRs staining, cells were first fixed and permeabilized with Fix & Perm cell permeabilization kit (BD Biosciences) before adding the antibodies and their isotype controls to the cells. The addition of the primary antibodies and the corresponding isotype controls was performed according to standard protocols by first adding FcR Blocking Reagent (Miltenyi Biotec) to each sample tube, followed by the specific antibodies or isotype controls according to the protocol. Finally the samples were evaluated with FACSCalibur E6370 and FACSComp 5.1.1 software (Becton Dickinson).

The following sample protocol (13 FACS tubes for each cell line) was used for TLR-antibody FACS preparation:
Materials and Methods

- **Extracellular TLRs:**
  1) Anti TLR1 PE (5 μl = 1 μg) + anti TLR2 FITC (10 μl = 1 μg)
  2) PE Mouse IgG1 (5 μl = 1 μg) + FITC Mouse IgG2a (1 μl = 1 μg)
  3) Anti TLR4 FITC (2 μl = 2 μg) + anti TLR10 PE (2 μl = 1 μg)
  4) FITC Mouse IgG2a (2 μl = 2 μg) + PE Mouse IgG2 (0.1 μl = 1 μg)
  5) Anti TLR5 Alexa Fluor 488 (5 μl = 0.5 μg) + anti TLR6 PE (5 μl = 1 μg)
  6) Alexa Fluor 488 isotype ctrl (5 μl = 0.5 μg) + PE Mouse IgG1K isotype ctrl (5 μl = 1 μg)

- **Intracellular TLRs:**
  7) Rat IgG1k PE isotype ctrl (0.5 μl = 0.1 μg)
  8) Rat IgG1k PE isotype ctrl (5 μl = 1 μg)
  9) Anti TLR3 PE (5 μl = 1 μg)
  10) Anti TLR7 PE (2 μl = 0.1 μg)
  11) Anti TLR8 PE (2 μl = 1 μg)
  12) Mouse IgG2ak PE isotype ctrl (20 μl = 2 μg)
  13) Anti TLR9 PE (10 μl = 2 μg)

### 2.7 TLR gene expression profile

#### 2.7.1 m-RNA Extraction and cDNA Synthesis

m-RNA extraction was completed for G-MSCs and G-MSCs-i using the RNeasy kit (Qiagen, Hilden, Germany). At first the medium was aspirated off the culture wells and each well was washed twice with 5ml of PBS. After that the cells were scraped in PBS and 300 μl of buffer RLT with β-mercaptoethanol was added to each well after scraping leading to cell lysis. Afterwards 300 μl of 70% ethanol was supplemented to the lysate in each well and mixed well by proper pipetting. Each sample was then pipetted onto
an RNeasy mini column rested in collection tube of 2 ml volume. The tube was closed and centrifuged for 30 seconds at 10000 rpm. The flow through within the collection tube was discarded after the centrifugation and the tube was replaced by a new one. Moreover 700 μl Buffer RW1 was pipetted onto each RNeasy column and again centrifuged for 30 seconds at 10000 rpm. After discarding the flow through and replacing the collection tube 500 μl Buffer RPE was applied into each RNeasy column and centrifuged for 30 seconds at 10000 rpm. Finally, the flow through was removed, the columns relocated to new 1.5 ml collection tubes and 30–50 μl RNase-free water directly applied onto the RNeasy silica-gel membrane. This was followed by the last centrifugation step for 1 minute at 10000 rpm to collect the RNA in the 1.5 ml collection tubes. The collected RNA was purified afterwards utilizing RNase-free-DNase (Promega, Mannheim, Germany), and photometrically quantified.

2.7.2 Reverse transcription and real time polymerase chain reaction (rt-PCR)

Complementary cDNA was produced from 1-13 μl of RNA (1 μg/μl) by reverse transcription (RT) utilizing QuantiTect reverse transcription kit (Qiagen) according to the manufacturer’s instructions (Mastercycler gradient; Eppendorf) in a volume of 20 μl reaction mixture containing 4 pmol of each primer, 10 μl of the LightCycler Probes Master mixture (Roche Diagnostics) and 5 μl specimen cDNA. To diminish the risk of RNA degradation all reactions were set up on ice. The reverse transcription reaction consisted of the following components according to table 1.
Materials and Methods

Table 1: Reverse-transcription reaction components

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume/reaction</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reverse-transcription master mix</td>
<td></td>
<td></td>
</tr>
<tr>
<td>• Quantiscript Reverse Transcriptase</td>
<td>1 µl</td>
<td>1 x</td>
</tr>
<tr>
<td>• Quantiscript RT Buffer, 5x (includes Mg²⁺ and dNTPs and RNase inhibitor)</td>
<td>4 µl</td>
<td></td>
</tr>
<tr>
<td>• RT Primer Mix (includes Mg²⁺ and dNTPs)</td>
<td>1 µl</td>
<td></td>
</tr>
<tr>
<td>Template RNA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>• Entire genomic DNA elimination reaction</td>
<td>14 µl</td>
<td></td>
</tr>
<tr>
<td>Total volume</td>
<td>20 µl</td>
<td></td>
</tr>
</tbody>
</table>

Before adding the aliquots of every completed reverse-transcription reaction to the real-time PCR mix, tubes containing reverse-transcription master mix and template RNA were incubated for 15 min at 42°C, then for 3 min at 95°C to disable Quantiscript Reverse Transcriptase.

Real time polymerase chain reaction (rt-PCR; LightCycler 96 Real-Time PCR System, Roche Molecular Biochemicals, Indianapolis, Indiana, USA) was completed according to the manufacturer’s instructions. Cycling conditions were as follows: 95°C for 10 minutes to achieve initial denaturation and activation of Taq polymerase (pre-incubation). Then the cycling continues by 45 thermal cycles of 95°C for 10 seconds, 60°C for 30 seconds, and 72°C for 1 second, with a ramping rate of 4.4°C/second, 2.2°C/second, and 4.4°C/second, respectively (amplification). Following the amplification process the plates were cooled to 40°C for 30 seconds. Fluorescence was observed continuously throughout the melting procedure.

Relative quantities of each transcript were normalized according to the expression of PGK1. Primers for TLRs 1 to 10 and the reference gene PGK1 were supplied by Roche and tested on G-MSCs and G-MSCs-i (Table 2).
2.8 Statistical analysis

To test normal distribution of the data the Shapiro-Wilk-Test was used. Differences in TLRs’ expression on m-RNA and Protein levels in G-MSCs and G-MSCs-i were evaluated using the nonparametric Wilcoxon Signed Rank test using SPSS software (SPSS version 11.5, SPSS, Chicago, IL). The level of significance was set at p=0.05 as the expression of each TLR in the non-inflammatory condition was compared to its expression under inflammatory stimuli.
3 Results

3.1 Phase contrast inverted microscopy and colony forming units

Subsequent to the adherence of the tissues, cells grew out of the fragments of gingival tissues, forming fibroblast-like clusters adherent to the surface (Fig. 7A). The expanding cell cultures were regularly examined every 24 hours by the phase contrast inverted microscopy. Twelve days after seeding of the MACS positive cell population of each cell line, G-MSCs displayed CFUs (Fig. 7B).

Figure 7: Microscopic display and colony-formation of G-MSCs. (A) Phase contrast microscopic display of the adherent tissue fragment with new outgrowing cells (2nd week). (B) Colony-forming units of G-MSCs (crystal violet).
Results

3.2 Flow cytometric analysis (predefined MSCs’ surface markers)

With regard to the flow cytometric analysis following the magnetic cell sorting to inspect characteristic markers of MSCs, cells derived from the G-MSCs isolation protocol showed negative results for CD14, CD34 and CD45, while positive expressions were presented for CD73, CD90 and CD105 (Fig. 8).

![Flow cytometric analysis](image)

Figure 8: Flow cytometric analysis of characteristic surface marker expression profile of G-MSCs

3.3 Multilineage differentiation potential

Osteogenic differentiation of G-MSCs was shown by the formation of sediments of calcification marked with Alizarin Red dissimilar to their controls (Fig. 9). Adipogenic differentiation of G-MSCs resulted in the development of lipid droplets that could be positively stained with Oil-Red-O dissimilar to their controls (Fig. 10). Chondrogenic differentiation of G-MSCs resulted in the generation of glycosaminoglycans that could be positively stained with Alcian Blue dissimilar to their controls (Fig. 11).
Results

3.3.1 Osteogenic differentiation potential

G-MSCs were cultured on 6-well culture plates in an osteogenic differentiation inductive medium. After 2 weeks, the formation of calcified sediments was observed, by Alizarin Red staining. This calcium formation displayed the osteogenic differentiation of the isolated G-MSCs. In contrast, the control cells lacked any osteogenic differentiation and did not show any formation of Alizarin Red-positive deposits (Fig.9).

![Figure 9: Alizarin Red staining of the osteogenically stimulated MACS+ G-MSCs and their controls](image)

3.3.2 Adipogenic differentiation potential

G-MSCs were cultured on 6-well culture plates in an adipogenic differentiation inductive medium. After 3 weeks, the development of lipid droplets was seen, by Oil Red O staining. This lipid formation displayed the adipogenic differentiation of the isolated G-MSCs. In contrast, the control cells presented very weak or lacked any adipogenic differentiation and Oil Red O stained droplets (Fig.10).
Results

3.3.3 Chondrogenic differentiation potential

G-MSCs were cultured on 96-well plates with rounded bottoms in a chondrogenic inductive medium. After 3 weeks, the presence glycosaminoglycans was demonstrated by Alcian Blue staining. This glycosaminoglycans formation displayed the chondrogenic differentiation of the isolated G-MSCs. In contrast, the control cells displayed very weak or lacked any chondrogenic differentiation and Alcian Blue stained particles (Fig. 11).

Figure 10: Oil-Red-O staining of the adipogenically stimulated MACS+ G-MSCs and their controls

Figure 11: Alcian Blue staining of the chondrogenically stimulated MACS+ G-MSCs and their controls
Results

3.4 Flow cytometric TLR expression

Evaluation of the prepared samples for TLR expression on protein level was performed with FACSCalibur E6370 and FACSComp 5.1.1 software (Becton Dickinson). MSCs incubated in basic medium expressed TLRs 1, 2, 3, 4, 5, 6, 7 and 10 (Table 3 and Fig.12). The inflammatory medium significantly (Wilcoxon Signed Ranks Test) upregulated the expression of TLRs 1, 2, 4, 5, 7, and 10, while TLR6 was no longer expressed on G-MSCs-i (Table 3 and Fig.13). No difference was noted for the expression of TLR3 in both media. Both G-MSCs and G-MSCs-i did not express TLRs 8 and 9.

<table>
<thead>
<tr>
<th>TLRs expression</th>
<th>G-MSCs Median Fluorescence Intensity, Q25/Q75</th>
<th>Significantly upregulated G-MSCs-i Median Fluorescence Intensity, Q25/Q75 and p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8.12, 3.91/11.33</td>
<td>25.82, 18.37/37.92 p=0.043</td>
</tr>
<tr>
<td>2</td>
<td>26.33, -2.21/41.72</td>
<td>280.51, 213/354.90 p=0.043</td>
</tr>
<tr>
<td>3</td>
<td>5.79, 0.23/15.90</td>
<td>no significant changes</td>
</tr>
<tr>
<td>4</td>
<td>13.89, 12.92/22.63</td>
<td>105.19, 91.46/120.83 p=0.043</td>
</tr>
<tr>
<td>5</td>
<td>7.16, 5.43/15.67</td>
<td>242.84, 216.80/287.38 p=0.043</td>
</tr>
<tr>
<td>6</td>
<td>3.25, 0.53/11.73</td>
<td>no expression</td>
</tr>
<tr>
<td>7</td>
<td>7.83, 5.48/9.74</td>
<td>24.41, 17.08/38.39 p=0.043</td>
</tr>
<tr>
<td>8</td>
<td>no expression</td>
<td>no expression</td>
</tr>
<tr>
<td>9</td>
<td>no expression</td>
<td>no expression</td>
</tr>
<tr>
<td>10</td>
<td>1.16, -6.70/14.96</td>
<td>55.83, 51.32/96.44 p=0.043</td>
</tr>
</tbody>
</table>
Results

Figure 12: Median Fluorescence Intensity (MFI) of expressed TLR1 to TLR10 of G-MSCs (green curve) and of their isotype controls (red curve) after incubation in basic medium. Differences in fluorescence intensity of TLR1 to TLR10 (n=5; box- and whisker plots with medians and quartiles).
Results

Figure 13: Median Fluorescence Intensity (MFI) of expressed TLR1 to TLR10 of G-MSCs (green curve) and of their isotype controls (red curve) after incubation in inflammatory medium. Differences in fluorescence intensity of TLR1 to TLR10 (n=5; box- and whisker plots with medians and quartiles). The green coloured boxes show significantly changed MFI after stimulation by the inflammatory medium.

3.5 TLRs’ m-RNA expression
Results

TLR expression on m-RNA level inspected by rt-PCR presented the following: G-MSCs incubated in basic medium expressed TLRs 1, 2, 3, 4, 5, 6, 7, 8 and 10 (Table 4 and Fig. 14A). G-MSCs-i showed a significantly higher expression (Wilcoxon Signed Ranks Test) of TLRs 1, 3 and 7, in addition to downregulated TLRs 6 and 10 (Table 4 and Fig.14B).

Table 4: Median gene copies/PGK2copies, Q25/Q75 and p-values of expressed TLRs on m-RNA level in G-MSCs and G-MSCs-i

<table>
<thead>
<tr>
<th>TLRs m-RNA expression</th>
<th>G-MSCs Median gene copies/PGK2copies,Q25/Q75</th>
<th>Significantly upregulated G-MSCs-i Median gene copies/PGK2copies,Q25/Q75 and p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.0037, 0.0005/0.0171</td>
<td>0.0172, 0.0094/0.0611, p=0.043</td>
</tr>
<tr>
<td>2</td>
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Figure 14: Expression of TLR m-RNA after incubation in basic medium (A) and inflammatory medium (B). Relative quantities of TLR m-RNA expression normalized according to the expression of PGK1. The green coloured boxes show significantly changed m-RNA expression after stimulation by the inflammatory medium.
4 Discussion

The development of inflammatory responses follows most tissue injuries and is considered a major part of the healing process in its early stages. Periodontal tissues show histologically pre-inflammatory circumstances, even under clinically healthy conditions or after a successful anti-inflammatory periodontal treatment [155]. Regarding this biological evidence in the field of tissue engineering and during possible therapeutic approaches, G-MSCs’ transplantation may be employed directly into inflamed milieus [57, 256], exhibiting the cells to inflammatory incitements and resulting conclusively in a direct communication between the G-MSCs and the PAMPs through their TLRs. Subsequently, the inflammatory stimulus and the TLR-PAMP interface affects the MSCs mediated immunomodulation as well as its reparative functions [134]. The aim of the present study was to characterize for the first time the distinctive TLRs’ expression profile of G-MSCs in inflamed as well as in uninflamed conditions as a first stage of exploring this possible interaction.

4.1 Methods

4.1.1 Tissue and G-MSCs’ isolation and identification

For many years, most of the procedures used to extract and isolate MSCs from donor tissues have been associated with major surgical operations under general anaesthesia, a high ratio of donor site morbidity, painful harvesting or in some cases infection and loss of teeth [147, 153]. Regarding these risks and burdens on the patient, El-Sayed et al. established and patented a minimally invasive technique to isolate MSCs from gingival tissues and alveolar bone proper [52, 56]. In further animal experiments the periodontal regenerative potential of these cells was reported to be successful [54, 55]. Building upon these results the same technique was used in the present study to isolate G-MSCs from the collected gingival tissues. As proposed in the same studies by El-Sayed et al, the isolated gingival tissues were not digested enzymatically afterwards by collagenase in comparison to other investigations [44],
Discussion

but relied on the tissue adherence to the culture dishes to avoid immense mechanical manipulation to the tissues and the outgrowing cultured cells.

To determine the criteria needed for the identification of MSCs derived from the cultured tissues and to differentiate them from other cells outgrowing from the samples as fibroblasts, the study design was based on the standards of MSC identification as described by Dominici et al. in their position paper in 2006 [48] and confirmed by different research groups in their investigations [55, 255]. Following these criteria, the G-MSCs displayed in the present study were only accepted being plastic adherent under normal culture conditions, expressing the surface markers CD73, CD90, and CD105, with minimal or no expressions of CD45, CD34 and CD14, in addition to demonstrating the ability to form CFUs.

Moreover, the G-MSCs had to be able to differentiate into the osteoblastic, chondroblastic and adipocytic lineages after stimulation by specialized differentiating media.

For isolating potential G-MSCs from the tissue outgrowing gingival cells, anti-STRO-1 antibodies and anti-IgM Micro Bead antibodies were applied during the magnetic activated cell sorting technique (MACS), similar to the procedures utilised in other investigations reporting the isolation of MSCs from alveolar bone proper [56], gingiva [54] and bone marrow aspirate [77]. The MACS separator, the device used to perform this type of cell sorting, depends on magnetic columns of cell separation which are needed for the attachment of marked cells in a magnetic field created by an external magnet that can be attached to the device. These columns contain a matrix of ferromagnetic substance that has the function to direct and focus the lines of the magnetic field and create potent gradients that are able to attract magnetized cells and bind them to the ferromagnetic matrix. In the present study, this process of cell isolation depends on the physical fact that anti STRO-1 antibodies bind to the required MCSs expressing the STRO-1 receptor conjointly with anti-IgM Micro Bead antibodies and therefore can successively be attracted by the magnetic field of the MACS separator and attached to the matrix within the separating column. Following that, the marked cells are eluted outside the magnetic field, where the ferromagnetic matrix of the MACS column is not able to hold the cells. Although this technique shows great ability
Discussion

to isolate STRO-1 expressing cells in a simple and rapid procedure it is still not able to provide a MSCs’ culture of complete purity when the cells are isolated from mixed cell populations.

4.1.2 Multilineage differentiation and colony forming units (CFU) assay

Two other major criteria used in this investigation to identify the isolated MSCs were the ability of the cells to differentiate into the osteoblastic, chondrogenic and adipogenic directions and to form colony forming units (CFUs), distinguishing the MSCs from morphologically analogous fibroblasts [48, 56].

The multilineage differentiation potential was tested by adding specialized inductive media to initiate the cell differentiation process into the three lineages. The composition of these media was decided according to standard investigations in the literature and the protocol of differential staining, as published by the International Society for Cellular Therapy, was used as a proof for positive differentiation of the cells [48, 56]. Calcified deposits were stained with Alizarin red, lipid droplets with Oil Red O and glycosaminoglycans with Alcian Blue to demonstrate the successful differentiation into the three mentioned lineages.

Furthermore, to test the G-MSCs' forming ability of CFUs the cells had to be able to form multiple single clusters of fibroblast-like cells in comparison with fibroblasts lacking this characteristic under the same culture conditions [52]. By the rise of these colonies from each of the MSCs the capabilities of these cells for self-replication and generation of clonogenic daughter cells can be clearly demonstrated.

4.1.3 Analysis by flow cytometry

The laser based technology of flow cytometry is a technique employed in cell sorting and counting, in addition to detecting biomarkers and microscopic particles, such as cell surface receptors. It allows a synchronized analysis of thousands of particles within seconds and an evaluation of their physical and chemical characteristics according to multiple parameters. The cellular investigations are based on the concept of coating the analysed cells with fluorescent labelled antibodies and suspending them
Discussion

in a fluid. During the “real time” data acquisition the narrow liquid stream containing the cells is passed through the device rapidly. This torrent of cells is broken down into individual droplets by a vibrating mechanism, decreasing the probability of having more than one cell in every single droplet. When the stream passes through an optical detection apparatus, a laser beam is directed onto the cellular units inside the running fluid. This allows every suspended particle between 0.2 and 150 μm in size to scatter the aimed ray, while fluorescent molecules within or attached to the cells become excited and emit their fluorescence subsequently. Several detectors aimed at the liquid stream passing channel and surrounding it are then able to detect the combination of scattered and fluorescent light and analyse it. According to the fluctuations of light brightness picked up by every detector it is possible afterwards to develop the data needed about physical and chemical properties of the examined particles.

Considered a proven technique for studying cellular populations with high precision [181], flow cytometry was utilised in this study to investigate two different aspects of the examined cell population. First of all, it was applied to characterize positive or negative expressions of MSC characteristic surface markers of the MSCs isolated from the collected gingival samples. As stated before, choosing these selected markers including CD14, CD34, CD45, CD73, CD90 and CD105 constructs on the position paper of Dominici et al. and other recent publications [48, 52, 55], presenting them as one of the important minimal criteria defining mesenchymal stem cells. Corresponding to this identification criteria, MSCs should display positive expressions of CD73, CD90 and CD105, whereas lacking the receptors of CD14, CD34 and CD45 or expressing them very scarcely.

To clarify the negative expression of the latter three markers on MSCs, investigations postulated CD14 to be normally expressed on cells of the innate immunity as monocytes, macrophages and neutrophil granulocytes [250], while CD34 is mainly expressed on early [173] and CD45 on differentiated cells of the hematopoiesis [22]. Moreover, the flow cytometric analysis was performed to explore the TLRs expression profile of G-MSCs on protein level in inflamed and non-inflamed environments. Similarly, this was the technique of choice in multiple studies inspecting TLRs of MSCs and other cell types [128, 193, 213, 224]. Nevertheless, exploring the TLRs of G-MSCs by flow cytometry provided only the evidence needed on protein level without an
Discussion

assessment of the TLRs’ m-RNA condition within the cells, leading consequently to
the introduction of the polymerase chain reaction (PCR) analysis to the study protocol
to enable the assessment of TLRs’ m-RNA levels in inflamed and non-inflamed
milieus.

4.1.4 Analysis by real time polymerase chain reaction (rt-PCR)

The polymerase chain reaction is a technique of molecular biology, generating an
enzymatic process of DNA amplification, which allows to some extent the mimicking
of in vivo DNA replications through a series of cycles of the reaction. This technique,
considered as one of the revolutionary scientific ideas of the 20th century [234], solved
an essential problem faced by biologists for a long time, a method to achieve a de
novo synthesis of DNA in vitro, hence providing the ability to amplify any required DNA
target sequence, which normally has a concentration too scarce for downstream
analysis.

In optimal reaction conditions for the PCR, the quantity of the target DNA fragment of
the inspected sample can be doubled in every cycle of the process, promoting an
exponential accumulation of the reaction product. When the newly synthesized
molecules have been produced sufficiently, fluorescent dyes are utilised to visualize
the created amplicons. In fact, the sensitivity of the PCR technique is so high, that only
one molecule could be detected in a complex sample of DNA.

Among the PCR technology, the technique of real-time PCR provided another step of
advance in the area of DNA analysis. Compared to the conventional PCR technique,
this advanced version allows a stronger quantitative and qualitative analysis, as well
as highly sensitive amplification reactions of the sample DNA. As the process of DNA
amplification occurs this advanced technique gives biologists the ability to observe it
as it happens. This key feature of the rt-PCR is possible, as the enzymatic reaction
and detection process of the PCR amplicons both take place in the same PCR reaction
vessel.
Discussion

For monitoring the rt-PCR process a special equipment is needed, the Light Cycler, which performs an online data collection of PCR products from each cycle of the reaction. This measurement is enabled by means of fluorescent dyes which fluoresce on binding to DNA. The dye-DNA contact may be achieved directly, using intercalating dyes that attach to the double stranded DNA molecule, as SYBR Green I, as well as indirectly through fluorescence-labeled oligonucleotides. These fluorescent oligonucleotide probes can identify and bind to a specific site on one strand of the DNA molecule by a base pairing process. Following that, the measurement of the fluorescence of the tested samples at every cycle of the PCR provides the possibility to display the accumulation of the reaction product on a plotted curve. As the amount of fluorescence measured by the device is proportional to the quantity of the PCR product in the Light Cycler, the time and number of cycles needed to provide detectable signals on the plotted curve increase when the initial concentration of the product is low in the inspected PCR sample. Subsequently, the data of cycle numbers can be easily used for qualitative and quantitative analysis of the explored gene.

In the present investigation, the LightCycler 96 Real-Time PCR System from Roche Molecular Biochemicals was applied to inspect the expression of TLRs’ m-RNA in G-MSCs, with or without inflammatory stimuli. The real time reaction was performed by using fluorescent probes and the fluorescence was monitored throughout the rt-PCR process. In several other investigations on MSCs, PCR analysis was employed to explore the m-RNA expression of different TLRs [40, 179, 193]. Hereafter, it seemed important in this study to examine the TLR expression profile on m-RNA, as well as on protein levels. This might even be necessary, as m-RNA and protein expression levels of the same gene do not always correlate or reflect each other, but could actually present conflicting results in some cases [1, 137, 236]. Accordingly, some investigators explained, that the link between both levels of gene expression is subjected to a number of technical and biological factors, leading to such discrepancy [137, 236]. Looking upon the possibility of the preceding postulations regarding the TLR genes, this study combined both expression levels of TLRs in G-MSCs to cover the whole spectrum of G-MSCs’ TLRs and provide a detailed and evident picture of their expression in inflamed and non-inflamed milieus.
4.2 Results

As seen in previous investigations [51, 57], the characteristic stem cell marker, STRO-1, was capable of isolating and purifying BM-MSCs [76] and alveolar bone proper-derived stem/progenitor cells [53] using an immunomagnetic technique for cell selection. This technique has been exploited to isolate the G-MSCs. The characterized G-MSCs showed all classical features defined for MSCs [47, 65], being positive for CD105, CD73, CD90, while negative for CD45, CD14 and CD34 as well as demonstrating a remarkable CFUs ability, plastic adherence and a multilineage differentiation ability into lineages of osteogenic, adipogenic and chondrogenic nature.

In previous studies MSCs showed sensitivity to inflammation [38, 88, 187, 189]. In the current study, G-MSCs were cultured in an inflammatory medium supplemented with IL-1β, IFN-γ, TNF-α, and IFN-α, the cytokines mostly active at sites of inflammation [187], as well as in basic medium. This change of milieu surrounding the G-MSCs showed influential outcomes affecting the cells and their expression profile of TLRs. On protein level, the G-MSCs in uninflamed condition distinctively expressed a TLR profile of TLRs 1, 2, 3, 4, 5, 6, 7 and 10 in different amounts without TLR8 and TLR9 expression. According to their median expression values, TLR2 was highest expressed followed by TLRs 4, 1, 7, 5, 3, 6 and finally 10. The inflammatory medium significantly promoted an up-regulation of the expression of TLRs 1, 2, 4, 5, 7, and 10, while it diminished TLR6 expression in G-MSC-i. Besides of that it partially changed the quantitative order of expression leaving TLR2 as the highest expressed TLR followed by TLRs 5, 4, 10, 1, 7 and finally 3. The m-RNA level of most G-MSCs’ TLRs in inflammatory and non-inflammatory conditions corresponded with the protein expression, showing a statistically significant upregulation for TLRs 1, 3 and 7 as well as a downregulation in TLR6.

Different TLRs expression profiles have been described on human MSCs seized from various tissues. TLRs 1, 2, 3, 4, 5, 6 and 9 showed expressions in UCB-MSCs [108, 230]. BM-MSCs displayed wider expression motifs with added TLRs 8 and 10 expressions [125, 186, 188]. WJ-MSCs exhibited a comparable pattern with marginal or deficient expression of TLR4 [146, 186]. Investigations on MSCs derived from dental tissues recorded the expression of TLRs 2, 3 and 4 in dental follicle MSCs [28, 226] and dental pulp MSCs [226, 249]. TLRs 1, 2, 3, 4, 5, 6, 8, 9 and 10 were expressed in
Discussion

periodontal ligament MSCs [125]. Resembling the outcome of our investigations inflammation tended to upregulate the expression of TLR2 [188, 193], TLR4 [188, 205] and TLR7 [193] as well as to downregulate the expression of TLR6 [188] in BM-MSCs. On the other hand, oral cavity derived fibroblasts showed TLRs’ m-RNA expression profiles dissimilar to G-MSCs, as gingival fibroblasts expressed TLRs 1,2,3,4,5,6,7,8 and 9 [229] and periodontal fibroblasts expressed TLRs 1,2,4,6 and 7 [199] under normal culture conditions. In inflammatory conditions periodontal fibroblasts presented an upregulation of TLRs 1, 4 and 7 m-RNA, while gingival fibroblasts displayed no change of TLR expression [199].

The presently defined TLR expression profile, especially under inflammatory conditions, may affect the therapeutic potential of G-MSCs in-vivo. An upregulation of the LPS recognizing TLRs 2 [75, 251] and 4 [75, 106] could raise the G-MSCs’ capacity to identify gram-negative periodontal pathogens, including Aggregatibacter actinomycetemcomitans, Prevotella intermedia, Porphyromonas gingivalis and Tannerella forsythia. The inflammatory upregulation of TLR5 expression could also favor the recognition of bacterial flagellin of periodontal pathogens [106, 198] as Treponema denticola [203, 208], Campylobacter rectus [99, 208] and Eubacterium species [208, 210]. Upregulation of TLR1 and TLR2 could augment lipoproteins’ identification [208], while an upregulated TLR7 and TLR9 will promote the ability to sense viral pathogens [113, 246]. This pronounced biological process of TLRs’ pathogen recognition in inflamed tissue might have positive as well as negative effects regarding the tissue regeneration. Understanding the basis of consequences following TLR activation and its contribution to the inflammatory response is the first step to comprehend this dual role of TLRs. First of all, NFkB activation triggered by TLR signaling pathways allows for transcription of genes of different cytokines and chemokines responsible for immunomodulatory effects. This in turn leads to a higher recruitment of host defensive cells and an overall increased antimicrobial response of the host immune system. While this reaction of the host immunity is important to clear infections and pathogens, the TLR activated response may have harmful effects to the host tissues due to tissue damage and even septic shocks [140]. Furthermore, TLRs show close involvement in the phagocytosis of pathogens. This process occurs through MyD88, IRAK4, and p38 genes increasing scavenger receptors [49], besides
Discussion

a TLRs mediated internalization of pathogens and maturation of host phagosomes [17].

TLR activation is also capable of inducing the release of non-specific antimicrobial peptides by triggering of direct antipathogenic pathways, while these released molecules may trigger more TLR activation [92, 241]. In addition, TLR mediated response is considered a phase of development and preparation for the adaptive immunity by increasing expressed co-stimulatory molecules on dendritic cells allowing a more effective T cell activation, as CD80 and CD86 [227]. TLR triggered immunomodulation has also been reported to play an important role in influencing the adaptive immunity through releasing specific cytokines and soluble factors by the MSCs, directing the differentiation of T cells into T-helper 1 or T-helper 2 subsets with either cytotoxic T cell responses or B cells and antibody production [220]. Some studies also reported an apoptotic effect through TLR stimulation. LPS has been presented to trigger cellular apoptosis through TLR4 [33].

Regarding these mechanisms of TLR- mediated immune response, TLR expression at sites of the host–pathogen interface is considered a necessary player for pathogen recognition, immune response initiation and adaptive response preparation [195].

On the other hand, various studies have shown negative effects of an increased TLR response in different organs resulting in more aggressive inflammations and damaging of the host tissues. For instance, high expressions of TLR1 and TLR2 have been linked to dermatological diseases as Leprosy, Psoriasis and Acne vulgaris [140]. Tissue destruction in several immune disorders has also been described in a number of studies to be induced by TLR-mediated pathogen recognition. Among these, TLR4 was involved in the development of chronic enterocolitis [111] and atherosclerosis [148], while TLR9 activated pathway induced tissue damage in SLE and rheumatoid arthritis [20, 235]. In his study, O’Neill describes the dual role of TLR2 and TLR4 signaling in the lung promoting both, tissue inflammatory destruction and tissue repair simultaneously [167]. Nevertheless, TLRs are not only involved in the initiation of the inflammatory responses towards the pathogens, but even control the secondary induced anti-inflammatory reactions [163]. In the light of this, TLR-mediated immunosuppression could create great dangers to the tissues if activated too early before total clearance of pathogens in severe infections as investigated with TLR2,
Discussion

TLR4 and TLR9 in [163]. Due to these wide and discrepant repair and damage induction roles of TLRs, even TLR targeting procedures have been investigated using agonists and antagonists to test possible therapeutic effectiveness of TLR activation or blocking during disease [169].

Interestingly, one of the biggest challenges facing scientists in therapeutic applications of MSCs up till now remains the unpredictability of the results, as well as the unstandardized manipulation techniques of MSCs [109, 139]. Several explanations are possible for these findings. For example, the source of the MSCs seems to play a role defining the function and results gained following clinical treatment trials using the cells [66]. More importantly, MSCs’ potential clinical applications encompass a wide variety of conditions, ranging from inflammatory and degenerative disorders to autoimmune diseases and allograft rejections [40]. Throughout such an extensive spectrum of possible therapeutic approaches biological properties of MSCs can be restrained, modified or expanded by diverse factors, depending on the microenvironment or the stimuli they face at the sites of application [40, 201]. One of the factors shown recently to direct the mode of action and biological performance of the MSCs in response to the microenvironment are the TLRs [40, 41], which in turn also differ according to their site of origin [186]. Based on these outcomes, it can be evidently suggested, that knowledge of the source of clinically applied MSCs and their TLR expression profile in fields of cell transplantation could show major improvements directing the cells to the biological functions needed and avoiding undesirable or unexpected fallouts.

Therefore, although it might be difficult to predict the effects of an enhanced pathogen recognition response in vivo, or other cellular properties promoted by an increased expression of TLRs in G-MSCs-I (G-MSCs surrounded by inflammatory factors), this study took us one step closer to a better understanding of G-MSCs’ potential application in sites of inflammation. However, this subject might need further investigations in the future, due to the contrasting possible functions of TLRs in detecting infection, regulating the immune response and even in inducing disease.
5 Summary and conclusion

Gingival margin-derived stem/progenitor cells (G-MSCs) show a significant potential of periodontal regeneration in vivo. During the process of regeneration, G-MSCs may interact with their inflammatory milieu via toll-like-receptors (TLRs). The present study aimed to depict the G-MSCs TLRs expression profile.

Cells were isolated from free gingival margins, then STRO-1-immunomagnetically sorted and seeded to obtain single colony forming units (CFUs). G-MSCs were characterized for the expression of CD14, CD34, CD45, CD73, CD90, CD105, CD146 and STRO-1 and for multilineage differentiation potential. Following the incubation of the cells in basic or inflammatory medium (IL-1β, IFN-γ, IFN-α, TNF-α) a TLR expression profile was created.

G-MSCs showed all stem/progenitor cells characteristic features. In basic medium G-MSCs expressed TLRs 1, 2, 3, 4, 5, 6, 7, and 10. The inflammatory medium significantly up-regulated TLRs 1, 2, 4, 5, 7 and 10 and diminished TLR 6 (p≤0.05, Wilcoxon-Signed-Ranks-Test). The current study portrays for the first time the distinctive TLRs expression profile of G-MSCs under uninflamed and inflamed conditions.

To conclude, the current study describes for the first time the distinctive TLRs’ expression profile of G-MSCs in inflamed and uninflamed conditions, which could impact its therapeutic potential in inflammatory environments in-vivo [42]. In light of the present results, inflammation tends to upregulate most TLRs’ expression, promoting the ability of G-MSCs to recognize important periodontal PAMP in-vivo.
6 References

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References


References


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Derived from Human Umbilical Cord Blood.

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7.3 Abbreviations

AD-MSCs          adipose-derived mesenchymal stem cells
BM-MSCs           bone marrow-derived mesenchymal stem cells
CEJ               cemento-enamel junction
CFUs              colony forming units
DF-MSCs           dental follicle-derived mesenchymal stem cells
DNA               deoxyribonucleic acid
dNTP              nucleoside triphosphate containing deoxyribose
DP-MSCs           dental papilla-derived mesenchymal stem cells
dsRNA             double stranded RNA
EDTA              ethylenediamintetraacetic acid
FACS              fluorescence activated cell sorting
FCS               fetal calf serum
Appendix

FITC  fluorescein isothiocyanate

G-MSCs  gingiva-derived mesenchymal stem cells

G-MSCs-i  G-MSCs in inflammatory medium

H-MSCs  human mesenchymal stem cells

IDO  indoleamine-pyrrole 2, 3-dioxygenase

IFN  interferon

IL  interleukin

LPS  lipopolysaccharide

MACS  magnetic activated cell sorting

MAPK  mitogen-activated protein kinases

MFI  mean fluorescence intensity

m-RNA  messenger ribonucleic acid

NF-κB  nuclear factor kappa beta

NLRs  nod-like receptors

PAMPs  pathogen associated molecular patterns

PBS  phosphate buffered saline

PE  phycoerythrin
<table>
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<th>Abbreviation</th>
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<tr>
<td>PGE2</td>
<td>prostaglandin E2</td>
</tr>
<tr>
<td>PGK-1</td>
<td>phosphoglycerate kinase 1</td>
</tr>
<tr>
<td>PL-MSCs</td>
<td>placenta-derived mesenchymal stem cells</td>
</tr>
<tr>
<td>PRRs</td>
<td>pattern-recognition receptors</td>
</tr>
<tr>
<td>RLRs</td>
<td>(RIG-I)-like receptors</td>
</tr>
<tr>
<td>rt-PCR</td>
<td>real time polymerase chain reaction</td>
</tr>
<tr>
<td>ssRNA</td>
<td>single stranded ribonucleic acid</td>
</tr>
<tr>
<td>TIRs</td>
<td>toll/interleukin-1 receptors</td>
</tr>
<tr>
<td>TLRs</td>
<td>toll-like receptors</td>
</tr>
<tr>
<td>TNF</td>
<td>tumour necrosis factor</td>
</tr>
<tr>
<td>UCB-MSCs</td>
<td>umbilical cord blood-derived mesenchymal stem cells</td>
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<tr>
<td>WJ-MSCs</td>
<td>wharton jelly-derived mesenchymal stem cells</td>
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Acknowledgement

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"This is from the favor of my Lord to test me whether I will be grateful or ungrateful. And whoever is grateful - his gratitude is only for [the benefit of] himself. And whoever is ungrateful - then indeed, my Lord is Free of need and Generous." (Quran 27:40)
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**Mai 2012 - Juli 2012**
Assistenzahnarzt an dem Dar Al Fouad Krankenhaus in Kairo, Ägypten

**September 2012**
Landesgraduiertenstipendium der CAU

**Seit September 2012**
Doktorand und Gastwissenschaftler an der Klinik für Zahnerhaltung und Parodontologie der CAU in Kiel

**Juni 2014**
Teilnahme an der IADR (Forschungstagung) in Südafrika mit einer Poster Präsentation

**Seit Oktober 2014**
Masterstudiengang (I-Masder) an der Zahnklinik der CAU in Kiel

**November 2014**
Stipendium der Fazit Stiftung

**März 2015**
Teilnahme an der IADR (Forschungstagung) in Boston, USA mit einer Poster Präsentation

**November 2015**
Einreichung der Dissertationsarbeit

**Februar 2016**
Einreichung der Masterarbeit

**Juni 2016**
Teilnahme an der IADR (Forschungstagung) in Seoul, Südkorea mit einer Poster Präsentation

**Seit August 2016**
Zahnarzt und Wissenschaftler an der Klinik für Zahnerhaltungskunde und Parodontologie des Universitätsklinikums Schleswig-Holstein zu Kiel.

**September 2016**
Ertteilung der deutschen zahnärztlichen Approbation

**Oktober 2016**
Auszeichnung mit dem wissenschaftlichen Jahrespreis der Schleswig-Holsteinischen Gesellschaft für Zahn-, Mund- und Kieferheilkunde

**November 2016**
Öffentliche Disputation und Abschluss der Promotion (Magna cum laude)
Curriculum vitae

**Zahnärztliche Mitgliedschaften:**
International Association for Dental Research (USA)
Deutsche Gesellschaft für Parodontologie (Deutschland)
Deutsche Gesellschaft für Zahn-, Mund- und Kieferheilkunde (Deutschland)
Zahnärztekammer Schleswig-Holstein (Deutschland)
Ägyptische Zahnärztekammer (Ägypten)

**Sprachen:**
Arabisch (Muttersprache)
Deutsch (fließend bzw. Muttersprache in Sprache und Schrift)
Englisch (fließend in Sprache und Schrift)

**Aktivitäten:**
2008-2009
Mitglied des Alumni-Vereins der Deutschen Evangelischen Oberschule in Kairo

2007-2010
Teilnahme an mehreren Veranstaltungen und Workshops der “Bridges Foundation” in Kairo (eine Stiftung für interreligiösen Dialog)

2007-2008
Teilnahme an mehreren Veranstaltungen der “Resala Charity Organization” in Kairo

2012-2014
Leiter der medizinischen Gruppe im Rahmen des DocsBuildBridges Programmes für die Unterstützung ausländischer Doktoranden der Medizinischen Fakultät der CAU in Kiel und für interkulturelle Trainingskurse

2013-2015
Mitglied des Migrantenforums der Stadt Kiel.

Seit 2016
Interkultureller Trainer und Sprachlehrer an dem Bildungsinstitut Kolenda in Kiel
Eigene Publikationen

Originalarbeiten

  
  http://www.nature.com/ijos/journal/vaop/ncurrent/full/ijos201441a.html

  

- Dr. Karim M. Fawzy El-Sayed, Mohamed Mekhemar, Prof. Dr. Sabine Adam-Klages, Prof. Dr. Dietrich Kabelitz, Prof. Dr. Christof E. Dörfer: *TLR Expression Profile of Human Gingival Margin-Derived Stem/progenitor Cells*. Med Oral Patol Oral Cir Bucal 2015(accepted)
Eigene Publikationen

Zitierfähige Abstracts

