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Research article

The immunogenic profile and immunomodulatory function of mesenchymal stromal / stem cells in the presence of Ptychotis verticillata

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A B S T R A C T

Mesenchymal stromal/stem cells (MSCs) are considered to be a promising immunotherapeutic tool due to their easy accessibility, culture expansion possibilities, safety profile, and immunomodulatory properties. Although several studies have demonstrated the therapeutic effects of MSCs, their efficacy needs to be improved while also preserving their safety. It has been suggested that cell homeostasis may be particularly sensitive to plant extracts. The impact of natural compounds on immunity is thus a fascinating and growing field. *Ptychotis verticillata* and its bioactive molecules, carvacrol and thymol, are potential candidates for improving MSC therapeutic effects. They can be used as immunotherapeutic agents to regulate MSC functions and behavior during immunomodulation. Depending on their concentrations and incubation time, these compounds strengthened the immunomodulatory functions of MSCs while maintaining their immune-evasive profile. Incubating MSCs with carvacrol and thymol does not alter their hypoimmunogenicity, as no induction of the allogeneic immune response was observed. MSCs also showed enhanced abilities to reduce the proliferation of activated T cells. Thus, MSCs are immunologically responsive to bioactive molecules derived from PV. The bioactivity may depend on the whole phyto-complex of the oil. These findings may contribute to the development of safe and efficient immunotherapeutic MSCs by using medicinal plant-derived active molecules.

1. Introduction

Mesenchymal stromal/stem cells (MSCs) represent a promising immunotherapeutic tool for different clinical applications. Due to their special immunological features, MSCs have been studied for regenerative medicine and cell therapy purposes [1]. MSCs, as adult cells, represent one of the safest populations of stem cells with nearly no risk of endogenous teratogenic potential characterizing normal pluripotent stem cells such as ESCs and iPS cells [2]. They are present in connective tissues throughout the body, with bone marrow being the most popular and most used source of MSCs. Thus, MSCs can be easily obtained, expanded by in vitro culture, and characterized for different biological properties. MSCs are defined according to a set of in vitro criteria, such as the phenotype and multi-lineage potential. Currently, there are no surface markers exclusively defining MSCs [3]. During standard culture conditions,

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MSCs are fibroblast-like shaped and highly adhere to the plastic. According to the ISCT guidelines, MSCs express CD73, CD90, and CD105 but lack the expression of CD34, CD45, CD14, CD19, and HLA-Dr [4]. The most well-established characteristic of MSCs is their ability to give rise to three important mesodermal cell lineages under controlled in vitro conditions: osteoblasts (bone), adipocytes (adipose), and chondroblasts (cartilage) [5].

Allogeneic MSC therapy is suggested for being safe because it does not generate an immune response in the recipient after transplantation [6]. The growing knowledge of their intrinsic immunological properties has demonstrated that the tissue repair capacity of MSCs is not linked to their long-term engraftment and differentiation [7]. The therapeutic effects of MSCs for damaged and injured tissues may be attributed to alternative pathway modes. MSCs' healing process includes the enhancement of local progenitor cell viability and proliferation and, most importantly, the modulation of local inflammatory and immune responses [8]. Nowadays, it becomes more apparent that the immunomodulatory functions of MSCs play a major role in their therapeutic effects [9]. MSCs modulate the immune response by interacting and inducing multiple immunomodulatory effects on several immune cells, including T-cells, B-cells, DCs, monocytes, Treg cells, and NK cells. These effects are likely involved in tissue repair and regeneration. They can inhibit local inflammation and immune responses by expressing and secreting several regulatory mediators [10]. MSCs regulate the local microenvironment during tissue repair and provide a good "soil" for tissue regeneration. Several data have shown that the therapeutic effect of MSCs is mainly a combination of immunomodulation and local cell "empowerment". The expression of trophic and immunomodulatory factors may underline these therapeutic effects [11]. The intrinsic secretory activity promotes the repair or regeneration of damaged tissues by establishing a regenerative microenvironment at the injury sites.

The immune system plays a key role in stem cell repair-mediated tissue regeneration. As inflammation always fluctuates with the progression of diseases, various inflammatory mediators can influence the immunoregulatory plasticity of MSCs. Depending on their type and intensity, inflammatory stimuli may allow MSCs to present a plasticity regarding their phenotype and functions. MSCs mat thus suppress the immune response in some cases or to enhance it in others [12]. During acute inflammation, the activation of MSC is critical for the production of immunoregulatory factors, in contrast with non-activated MSCs, which do not exhibit a significant production of these molecules. Regarding the mechanisms underlying their therapeutic features, it is reported that Treg cells play a key role in the immunomodulatory functions of MSCs. Indeed, MSCs may increase Treg cell proliferation via TLR2 and TLR3 signaling, thrombospondin, IL-2, and TNF-α through activation of Stat 5 that increases CD39 and CD73 expression, both molecules involved in the adenosine production necessary for Treg cell function. On the other hand, a close relationship has been established between TNFR2 expression and Treg cell induction by MSCs [13]. In parallel, activated MSCs could take up and process antigens and inhibit the proliferation of activated effector T-cells in an antigen-specific manner without overtly increasing the immunogenicity of allogeneic MSCs [14]. Due to their plasticity, MSCs adapt their response to changes in the microenvironment by regulating different immune cells [15]. In this respect, a study recently revealed that the TNFα-TNFR2 signaling controls several aspects of MSC immunomodulatory properties, including their ability to suppress T cells and their conversion towards Foxp3-expressing Tregs [13]. MSCs have been shown to respond to different aspects of tissue injury microenvironment, such as inflammatory cytokines, hypoxia, and acidosis. Their secretome is also altered by modulating the release of regulatory and trophic factors [16].

The results from both pre- and clinical studies indicate the need to improve both the efficiency and safety of MSC therapy [17]. Several strategies have thus been developed and are being explored. Because the inflammatory niche (cytokines and alarmins) plays a key role in triggering reparative and immunomodulatory functions, priming of MSCs with bioactive molecules has been proposed as a way to foster the therapeutic potential of these cells [18]. The priming of MSCs by inflammation favor a proregenerative and tolerogenic phenotype by modulation their secretome and therefore factors and EV content [19,20]. Recently, the immunological profile of equine MSCs was shown to be differentially modulated by the immune cell reaction that depends on the inflammatory priming and human leukocyte antigen (HLA) compatibility [21]. The use of recombinant cytokines and growth factors to modulate some features of stem cells may be hindered by efficiency and safety shortcomings. Thus, the use of natural products may represent a promising alternative strategy to regulate the functions of stem cells with minimum side effects, low toxicity, and high availability and affordability [22]. In terms of future perspectives, we have proposed the use of herbal extracts to enhance the immunological features of MSCs [23]. Cell homeostasis may be particularly sensitive to certain bioactive molecules from plants, nutrients, and food components [24]. They are becoming increasingly important in this context due to their merits in preventing and controlling a wide range of diseases and disorders [25]. Previous studies have reported distinct therapeutic effects of natural compounds against several diseases. These effects were attributed to their multipharmacological properties including but are not limited to antioxidant, antibacterial, and anticaner activities [26]. However, little is known regarding the impact of *Ptychotis* verticillata (PV) on the immunomodulation-immunogenicity balance of MSCs. This balance is relevant for the therapeutic properties of MSCs. As an aromatic plant, PV and its phytochemical constituents (thymol and carvacrol) were reported to be valuable medicinal candidates [5,27,28]. Based on this evidence, we aimed to investigate the impact of PV and its compounds on the immunological profile of MSCs. Our original study highlights for the first time that PV extracts can maintain a hypoimmunogenic profile and, at the same time, strengthen the immunomodulatory function of MSCs. The positive effects of the essential oil (HE) thymol (THY) and carvacrol (CAR) on the immune properties of MSCs will open a new area for using natural compounds in cellular therapy. Such products require more characterization regarding their activities, concentration, and incubation time. Such development would provide a cellular product with high efficiency and safety qualities.

2. Materials and methods

2.1. MSCs

BM was harvested from the sternum or iliac crest of five healthy volunteers (n = 5). The mean age of the donors was 33 ± 2 years (range 18–41 years). Mononuclear cells (MNCs) were isolated from bone marrow aspirates by density-gradient centrifugation (Linfosep; Biomedics) and washed in Hank's buffered salt solution (HBSS; Lonza). MNCs were seeded at a cell density of 2 x 104 cells/cm2 in low-glucose DMEM (DMEM-LG; Lonza) supplemented with 15 % (v/v) heat-inactivated FBS, 2 mM L-glutamine, and 0.5 % (v/v) antibiotic/antimycotic solution (all from Life Technologies). Cells were incubated at 37 °C in a 5 % CO2 humidified atmosphere. After 48 h, nonadherent cells were removed by washing, and the medium (DMEM-LG) was changed twice a week. When subconfluency (80–90 %) was achieved, adherent cells were recovered by adding TryplSelect solution (Lonza) and expanded by replating at a lower density (200 cells/cm2) using DMEM-LG as a culture medium. MSCs are characterized by a plethora of criteria established by the ISCT, including a combination of cell surface markers and differentiation potential [23].

2.2. Plant material

The aerial parts of PV were collected during the flowering season in May 2018 and 2019 (full bloom) from Morocco [29]. The Voucher specimens were deposited in the herbarium of the Faculty of Science at Mohamed 1st University, Oujda, Morocco.

2.3. Essential oil extraction

The essential oil (EO) was extracted by hydrodistillation using a modified Clevenger setup apparatus according to the method recommended in the European Pharmacopoeia [30]. 100 g of the plant's aerial fresh part was introduced into a 5-L flask containing 400 mL of distilled water. After loading the plant material and water, the apparatus was closed and set up properly. This mixture was then positioned within a heating apparatus equipped with a cooling system to facilitate the condensation of essential oils [31]. As both constant and controlled heating conditions are necessary to guarantee efficient and high-quality EO [32]. Thus, the heating source was turned on and sfixed to the optimum temperature 100 °C during a 3-h period for stable and consistent extraction. Upon completion of the distillation process, two distinct phases became apparent: an aqueous phase (aromatic water) and a less dense organic phase (essential oil). The EO was gathered, dehydrated using anhydrous sodium sulfate, and then stored in light-sealed glass vials at an ambient temperature ensuring its preservation for future use.

The yields of essential oil were expressed in g relative to 100 g of dry vegetable matter; it was calculated according to Equation:

$$\text{Yield } (\%) = \frac{\text{Amount of extracted oil (g)}}{\text{Amount of dry vegetal matter mass (g)} \times 100}$$

The EO yields were 2 % (w/w).

2.4. Preparation of chemical reagents

Unless otherwise stated, all chemicals were of the highest quality and were used as supplied. Carvacrol (99.9 %) and thymol (98.5 %) were purchased from Sigma–Aldrich (St. Louis, USA).

2.5. Preparation of PV compounds

2.5.1. Essential oil

Fifty microliters of essential oil was dissolved in 50 µl of dimethylsulfoxide (DMSO, Merck, Germany) and diluted (0.01 %, 0.025 %) with culture medium before the assays.

2.5.2. Carvacrol

One hundred microliters of carvacrol was dissolved in 900 µL of DMSO and diluted (6 µM and 25 µM) with culture medium before the assays.

2.5.3. Thymol

One hundred milligrams of thymol powder was dissolved in 1 mL of DMSO and diluted (3 µg/mL and 6 µg/mL) with culture medium before the assays.

2.6. Treatment of BM-MSCs with PV compounds

Different concentrations and solutions of essential oil, carvacrol, and thymol were added to BM-MSC cultures for 24 h, 48 h, and 72 h of treatment.

2.7. Collection of immune cells

Peripheral blood samples were obtained from 3 healthy adult donors (2 males and 1 female with age range 26–46 years). The samples were collected according to the approval of the institutional Research Ethic Authority and after obtaining donor signed written informed consent. About 50 ml of blood was obtained from each donor using BD Vacutainer tubes containing acid-citrate-dextrose anticoagulant, solution A (ACD-A; BD) from which peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Paque

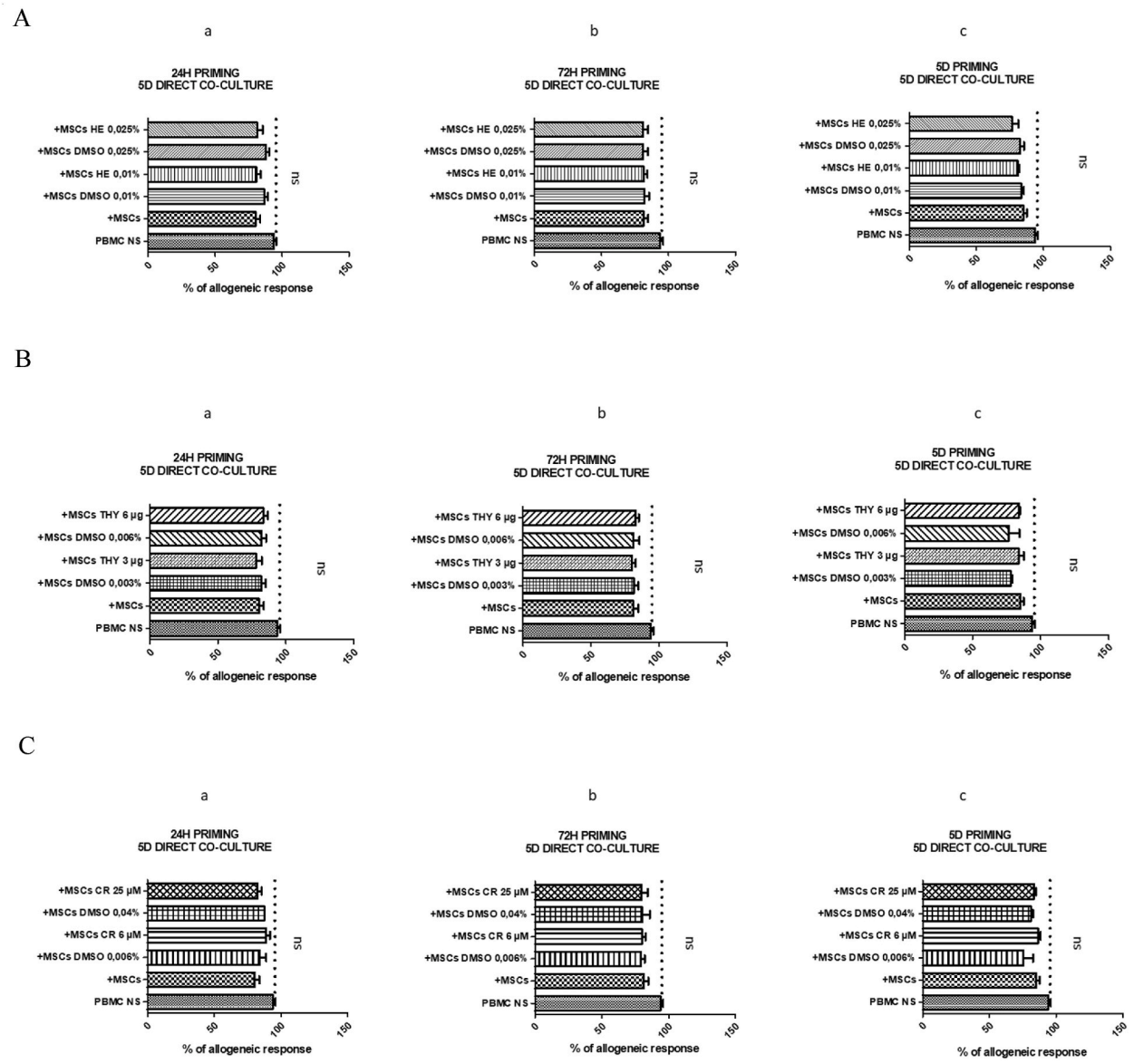

Fig. 1A. MSCs do not induce PBMC allogeneic response. MSCs were or were not pretreated at different times with various concentrations of PV compounds Essential Oil (HE) for 24h (a), 72h (b), and 5 days (c). Allogeneic PBMC were incubated in the presence or absence of MSCs and cultured with direct contact for 05 days. The proliferation of PBMC was assessed by flow cytometry measurement of the CFSE fluorescence. The data are expressed as the mean ± SEM of the % of the allogeneic PBMC response.

Fig. 1B. MSCs do not induce PBMC allogeneic response. MSCs were or were not pretreated at different times with various concentrations of PV compounds Thymol (THY) for 24h (a), 72h (b), and 5 days (c). Allogeneic PBMC were incubated in the presence or absence of MSCs and cultured with direct contact for 05 days. The proliferation of PBMC was assessed by flow cytometry measurement of the CFSE fluorescence. The data are expressed as the mean ± SEM of the % of the allogeneic PBMC response.

Fig. 1C. MSCs do not induce PBMC allogeneic response. MSCs were or were not pretreated at different times with various concentrations of PV compounds Carvacrol (CR) for 24h (a), 72h (b), and 5 days (c). Allogeneic PBMC were incubated in the presence or absence of MSCs and cultured with direct contact for 05 days. The proliferation of PBMC was assessed by flow cytometry measurement of the CFSE fluorescence. The data are expressed as the mean ± SEM of the % of the allogeneic PBMC response.

gradient centrifugation [33].

T cells were purified from PBMCs by positive selection using the MACS system (Miltenyi Biotec). The purity of the selected cells as evaluated by flow cytometry was always above 95 %. T-cell activation was performed by using a cocktail of 5 μg/m^{2}L phytohemagglutinin (PHA, Remel Europe, Kent, UK) and 20 U/mL interleukin 2 (IL2, Biotest AG, Dreieich, Germany).

2.8. Coculture of MSCs and immune cells

2.8.1. Immunogenicity assay

To assess the immunogenic potential of MSCs, they must be evaluated for their inability to induce a cell-mediated immune response [34]. Before starting the coculture, MSCs were plated in a flat-bottomed 24-well plate for an overnight period of adherence. This step is important to obtain adherent MSCs and to remove those that were not attached to the plate. Then, allogeneic PBMCs were incubated with MSCs for five days of coculture (1/2 cell ratio) in RPMI 1640 medium supplemented with 10 % FBS.

2.8.2. Immunomodulation assay

To assess their immunomodulatory potential, MSCs must be evaluated for their ability to modulate the activated lymphocyte response [35]. Before starting the coculture, MSCs were plated in a flat-bottomed 24-well plate for an overnight period of adherence. This step is important to obtain adherent MSCs and to remove those that were not attached to the plate. Then, allogeneic activated T cells were incubated with MSCs for five days of coculture (1/2 cell ratio) in RPMI 1640 medium supplemented with 10 % FBS. To assess the role of cellular interactions during coculture, a Transwell® system (Transwell Permeable Supports, Life Sciences, Acton, MA, USA) was used in some assays to avoid any direct contact between cells and thus assess the role of soluble mediators (through several pores

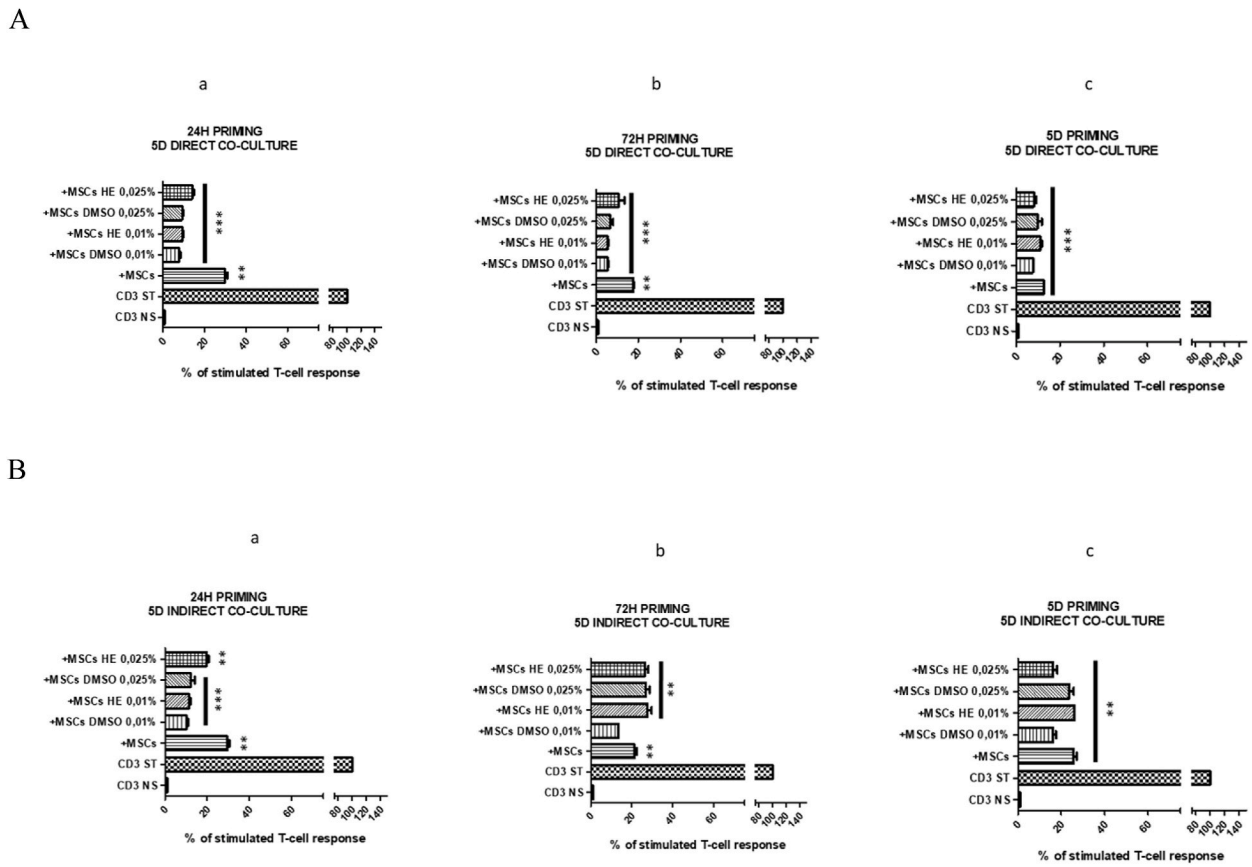

Fig. 2A. Pretreated MSCs differentially modulate activated T-cell response. MSCs were or were not pretreated at different times with various concentrations of PV compounds Essential Oil (HE) for 24h (a), 72h (b), and 5 days (c). Allogeneic T-cells were activated and incubated in the presence or absence of MSCs and cultured with direct contact for 05 days. The proliferation of activated lymphocytes was assessed by flow cytometry measurement of the CFSE fluorescence. The data are expressed as the mean ± SEM of the percentage of stimulated T-cell response.
Fig. 2B. Pretreated MSCs differentially modulate activated T-cell response. MSCs were or were not pretreated at different times with various concentrations of PV compounds Essential Oil (HE) for 24h (a), 72h (b), and 5 days (c). Allogeneic T-cells were activated and incubated in the presence or absence of MSCs and cultured without direct contact for 05 days. The proliferation of activated lymphocytes was assessed by flow cytometry measurement of the CFSE fluorescence. The data are expressed as the mean ± SEM of the percentage of stimulated T-cell response.

0.4 μm in size).

2.9. Proliferation assay

The proliferation of PBMCs and T cells was assessed using the CellTraceTM CFSE (carboxyfluorescein succinimidyl ester) Cell proliferation kit (Invitrogen, Molecular Probes, USA). After cell labeling, CFSE fluorescence was analyzed on a MacsQuant analyzer (Miltenyi Biotec). Immune cells were gated according to their forward and side scatter features to exclude dead cells and cell debris. A total of 5000–10000 gated events were usually acquired [36].

2.10. Statistical analysis

The results are presented as the mean ± standard error of the mean (SEM). For statistical comparison, we have performed a one-way ANOVA with Bonferroni's correction. A p-value less than 0.05 is considered statistically significant (Prism v5.0d, Graph-Pad Software, La Jolla, CA).

3. Results

3.1. MSCs do not induce an allogeneic response in PBMCs

As immunogenicity is an important challenge for cell therapy, we addressed the immunogenic potential of MSCs regarding PBMCs. Thus, we evaluated the ability of MSCs to modulate the allogeneic PBMC response. MSCs and PBMCs were cocultured for 5 days. MSCs were pretreated with various concentrations of essential oil (Fig. 1a), thymol (Fig. 1b), or carvacrol (Fig. 1c) for different incubation times. MSCs were not able to activate and increase the proliferation of PBMCs. All the changes observed were very weak and most importantly no significant. A slight decline in the allogeneic response of PBMCs was induced by MSCs. Moreover, the presence of PV extracts resulted in a decrease in PBMC immunogenicity regardless of the compound concentration and incubation time. During direct coculture with PBMCs, the presence of MSCs did not increase the proliferation of PBMCs compared to that in the corresponding control

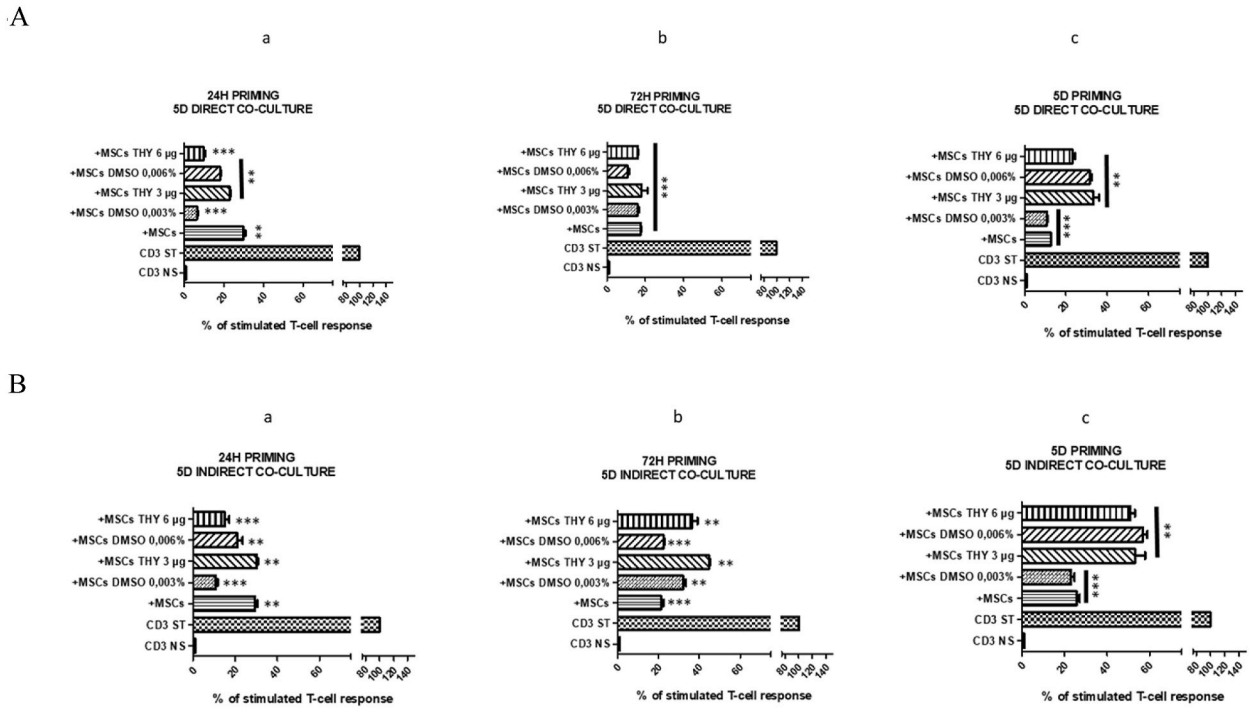

Fig. 3A. Pretreated MSCs differentially modulate activated T-cell response. MSCs were or were not pretreated at different times with various concentrations of PV compounds Thymol (THY) for 24h (a), 72h (b), and 5 days (c). Allogeneic T-cells were activated and incubated in the presence or absence of MSCs and cultured with direct contact for 05 days. The proliferation of activated lymphocytes was assessed by flow cytometry measurement of the CFSE fluorescence. The data are expressed as the mean ± SEM of the percentage of stimulated T-cell response.
Fig. 3B. Pretreated MSCs differentially modulate activated T-cell response. MSCs were or were not pretreated at different times with various concentrations of PV compounds Thymol (THY) for 24h (a), 72h (b), and 5 days (c). Allogeneic T-cells were activated and incubated in the presence or absence of MSCs and cultured without direct contact for 05 days. The proliferation of activated lymphocytes was assessed by flow cytometry measurement of the CFSE fluorescence. The data are expressed as the mean ± SEM of the percentage of stimulated T-cell response.

(100 %). Independent of the pretreatment incubation times (1, 3, and 5 days), the essential oil (0.01 % and 0.025 %), thymol (6 μg and 3 μg), and carvacrol (6 μM and 25 μM) did not increase the percentage of allogeneic response. In contrast, a slight decrease in the proliferation of PBMCs was observed in all the conditions.

3.2. *Pretreated MSCs differentially modulate the activated T-cell response*

As immunomodulation (either suppression or stimulation of the immune response) is an essential feature of MSCs, we examined the capacity of MSCs to modulate the response of activated lymphocytes (% of stimulated T-cell response). MSCs and T cells were cocultured for 5 days in the presence or absence of cellular contact. MSCs were pretreated with various concentrations of essential oil (Fig. 2), thymol (Fig. 3), or carvacrol (Fig. 4) for different incubation times.

The activation of CD3 T cells allowed a major increase in lymphocyte proliferation compared to nonactivated CD3 T cells. In the presence of untreated MSCs, the proliferation of activated CD3 T cells is significantly decreased by more than half. The level of inhibition during such direct coculture is substantially low when no contact (indirect coculture) between MSCs and MSCs is established.

Fig. 2A reports the profile of the activated CD3 T-cell response in the presence of MSCs pretreated for 1, 3, and 5 days with different concentrations of the essential oil (0,01 % and 0,025 %) during direct coculture. After 1 day, MSCs pretreated with essential oil, regardless of their concentration, showed significant inhibition of the T-cell response compared to untreated MSCs. Under such conditions, the percentage of activated T-cell response was considerably reduced to reach less than 20 % proliferation when direct coculture was conducted with essential oil-pretreated MSCs. After 3 and 5 days, MSCs pretreated with essential oil showed a similar profile of T-cell inhibition to that of 1 day. Indeed, activated T cells in the direct presence of essential oil-pretreated MSCs presented a significant reduction in their proliferation rate with an approximately 15 % response compared to the respective control.

In contrast, (Fig. 2B), the absence of direct coculture between pretreated MSCs and activated T cells showed a distinct profile for lymphocyte response inhibition. Independent of the concentration of the essential oil (0,01 % and 0,025 %) and the incubation times

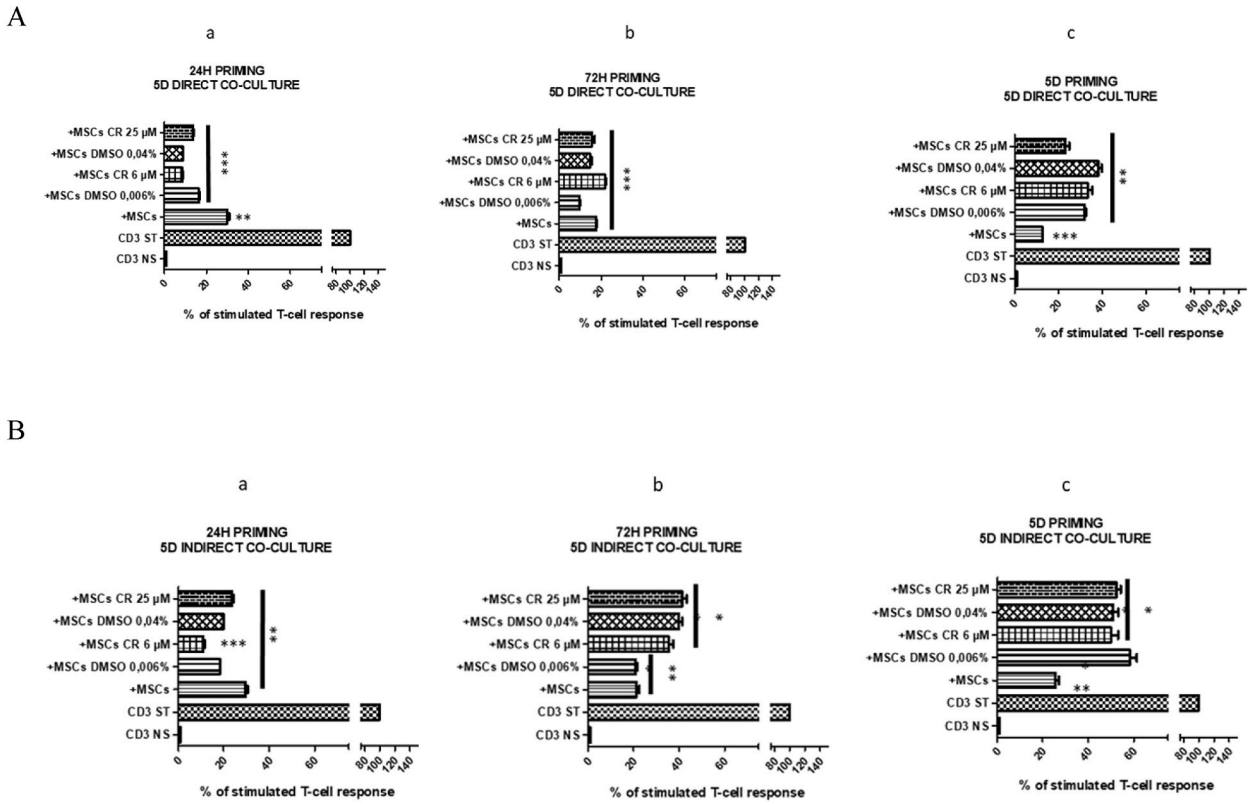

Fig. 4A. Pretreated MSCs differentially modulate activated T-cell response. MSCs were or were not pretreated at different times with various concentrations of PV compounds Carvacrol (CR) for 24h (a), 72h (b), and 5 days (c). Allogeneic T-cells were activated and incubated in the presence or absence of MSCs and cultured with direct contact for 05 days. The proliferation of activated lymphocytes was assessed by flow cytometry measurement of the CFSE fluorescence. The data are expressed as the mean ± SEM of the percentage of stimulated T-cell response.

Fig. 4B. Pretreated MSCs differentially modulate activated T-cell response. MSCs were or were not pretreated at different times with various concentrations of PV compounds Carvacrol (CR) for 24h (a), 72h (b), and 5 days (c). Allogeneic T-cells were activated and incubated in the presence or absence of MSCs and cultured without direct contact for 05 days. The proliferation of activated lymphocytes was assessed by flow cytometry measurement of the CFSE fluorescence. The data are expressed as the mean ± SEM of the percentage of stimulated T-cell response.

(1, 3, and 5 days) used for pretreating MSCs, the inhibition of activated T cells was effectively lower than that observed during direct coculture. Indeed, the proliferation of activated T cells reached only 20–30 %.

Fig. 3A illustrates the activated CD3 T-cell response profile in the presence of MSCs pretreated for 1, 3, and 5 days with different concentrations of thymol (3 μg/mL and 6 μg/mL) during direct coculture. In comparison to essential oil, MSCs pretreated with thymol presented a distinct profile regarding the modulation of the T-cell response. After 1 day, MSCs pretreated with thymol showed significant inhibition of activated T cells during direct coculture. Interestingly, such inhibition depended on the concentration of thymol used for pretreating MSCs. Indeed, MSCs pretreated with 6 μg/mL thymol were more efficient in reducing the activated T-cell response than MSCs pretreated with 3 μg/mL. The lymphocyte proliferation reached 10 % and 20 % in the presence of MSCs pretreated with 6 μg/mL and 3 μg/mL, respectively. After 3 days, MSCs pretreated with thymol, regardless of its concentration, seem to present a decreased capacity to inhibit activated T cells, as less reduction in lymphocyte proliferation is shown during direct coculture. After 5 days, MSCs pretreated with thymol, in a dose-dependent manner, maintained their inhibitory effect on the activated T-cell response, although with less importance. Specifically, MSCs pretreated with 6 μg/mL thymol displayed a more pronounced inhibitory effect than those pretreated with 3 μg/mL thymol. The lymphocyte proliferation reached 20 % and 40 % in the presence of MSCs pretreated with 6 μg/mL and 3 μg/mL, respectively.

On the other hand (Fig. 3B), the absence of direct coculture between pretreated MSCs and activated T cells provided a different inhibitory profile of lymphocyte response. Compared to direct coculture, inhibition by MSCs was maintained but to a lesser extent. Both concentrations and incubation times with thymol greatly influenced such a profile. After 1 day, MSCs pretreated with 6 μg/mL thymol exhibited significantly more lymphocyte proliferation inhibition than MSCs pretreated with 3 μg/mL thymol. After 3 days, the effect of MSCs pretreated with thymol was likely less pronounced, with a reduced capacity to inhibit lymphocyte proliferation. Compared to untreated MSCs that showed a lymphocyte proliferation rate of 20 %, thymol-pretreated MSCs, regardless of their concentration (3 μg/mL and 6 μg/mL), allowed a lymphocyte proliferation rate of 40 %. After 5 days, the decreased capacity of pretreated MSCs to inhibit activated T cells in the absence of contact is accentuated. Independent of thymol concentrations, pretreated MSCs maintained a rate of lymphocyte proliferation between 50 % and 55 % compared to untreated MSCs, which showed a rate of lymphocyte proliferation of 20 %.

Fig. 4A demonstrates the profile of the activated CD3 T-cell response in the presence of MSCs pretreated for 1, 3, and 5 days with different concentrations of carvacrol (6 μM and 25 μM) during direct coculture. MSCs pretreated with carvacrol presented a similar effect to those with thymol regarding the modulation of the T-cell response but were distinct from those with essential oil. After 1 or 3 days, MSCs pretreated with carvacrol, independent of its concentration, showed significant inhibition of activated T-cell response cells during direct coculture. Compared to that of untreated MSCs, the percentage of lymphocyte proliferation did not exceed 20 %. After 5 days, the inhibition of activated T cells by carvacrol-pretreated MSCs was likely different than that observed with essential oil and thymol. Although a direct coculture of carvacrol-pretreated MSCs with activated T cells induced a decrease in lymphocyte proliferation, this decrease was reduced compared to untreated MSCs. The reduced lymphocyte proliferation is inverted with significantly less inhibition of the activated T-cell response.

In the absence of direct coculture (Fig. 4B), the profile of T-cell inhibition after 1 day of carvacrol pretreatment of MSCs was quite similar to that observed during direct coculture. Despite a slight decrease, the inhibition of lymphocyte proliferation is still observed. The profile of T-cell inhibition after both 3 and 5 days of carvacrol pretreatment of MSCs in the absence of direct coculture is entirely different from that with direct coculture. A substantial decrease in the T-cell inhibition level was observed. Compared to untreated MSCs, 3- and 5-day carvacrol-pretreated MSCs were less capable of inhibiting activated T cells. The percentage of activated T-cell response reached 40 % and 50 % after 3 and 5 days of carvacrol treatment, respectively.

4. Discussion

With cell therapy progress, considerable efforts are being directed to achieve repair and regeneration effects following tissue injury and damage. The immune tolerance of certain types of stem cells has led to the emergence of using allogeneic human stem cells. Thus, mesenchymal stromal cells (MSCs) may be a promising cellular immunotherapeutic tool for different diseases [10]. As reviewed by García-Bernal et al. [37], the therapeutic properties of MSCs depend on the crosstalk of these cells with the host tissues as suggested by the mechanisms which control their immunomodulatory properties and the status of pre-sensitization of the host. These findings suggest that MSCs are sensitive to local microenvironmental cues that may significantly modulate their activation and immunomodulatory functions to facilitate tissue repair and regeneration [38]. However, poor engraftment, the low survival rate of transplanted MSCs, and impaired donor-MSC potency under host age/disease result in unsatisfactory therapeutic outcomes. According to the literature, there is a need to improve the therapeutic efficiency of MSCs with persevering their safety [28]. Enhancement strategies, including genetic manipulation, pre-activation, and modification of culture method, have been investigated to generate highly functional MSCs [39]. Developing approaches to enhance the immunomodulatory capacity of MSCs while maintaining their hypo immunogenicity state is of importance [40]. This could be achieved by using natural medicinal compounds to preserve such a balance [41]. In addition, plant extracts induce few reported side effects compared to those of immunomodulatory pharmaceutical drugs.

During tissue injury, several immune cells proliferate and migrate to sites of damage where they exert their inflammatory functions [42]. MSCs are mobilized by pro-inflammatory factors produced by both innate and adaptive immune cells [43]. MSC-mediated tissue repair implies that they closely interact with lymphocytes and modulate their fate accordingly. The effect of transplantation itself and delivery to a hostile environment can trigger increased immunogenicity of MSCs. Thus, the behavior of MSCs during an immune response, as well as their immunomodulatory effects, need to be improved. Indeed, the ultimate goal of cell therapy is to produce “off-the-shelf” cells with specific immune tolerance features capable of repairing and regenerating damaged or injured tissues [44].

The capacity of a cell population to stimulate an active immune response determine their immunogenicity state [45]. Thus, the response that will be generated following BM-MSC and immune cell interactions is of utmost importance. The proliferation of PBMC indicates immune cell recognition and subsequent activation which may lead to the death and clearance of the transplanted MSCs. Both the loss of protection against cytotoxic lysis and the induction of complement-activating antibodies will likely impact the use of allogeneic MSCs for therapeutic applications [46]. Research on exploring the immunogenic potential of plant-derived natural products has increased owing to the growing awareness about immune system modulation and the desire to achieve positive effects on disease prevention [47]. They can interact with the immune system that may lead to undesired immunological responses, including immunogenicity, and others. The immunogenicity can impact the safety, efficacy, and pharmacokinetic profile of a product, ultimately leading to unwanted side effects. Strategies for the assessment and prediction of immunogenicity should be implemented early in drug development [48]. In this context, we demonstrated for the first time that MSCs maintained their immune-evasive profile in the presence of PV extracts. Indeed, MSCs pretreated with natural medicinal compounds did not alter their hypo immunogenicity profile, as no induction of allogeneic immune response was observed. Independently of the pretreatment concentration and incubation times, the essential oil, Thymol, and Carvacrol have not increased the proliferation of allogeneic PBMC. In contrast, a slight but not significant decrease of PMBC proliferation was observed in some conditions. Indeed, plant-based products are considered as less immunogenic and safer, as compared to the animal proteins. Furthermore, they possess low molecular weight and are therefore more water-soluble [49]. Derived from plants, EOs are highly concentrated natural oils containing several aromatic, volatile and secondary metabolites presenting different bioactivity profiles. Such components of the EO may display synergistic, additive or antagonistic effects that may impact the bioactivity of EO [50,51]. Several factors including environmental conditions (temperature, light, location), physiology of the plant (plant age and plant parts), and genetic aspects are reported to influence the chemical composition of the EO and therefore their bioactivity [52].

These data suggest that MSCs even in the presence of PV extracts retain their immuno-privileged state, can escape immune recognition upon allogeneic transplantation, or at least possess hypoimmunogenic properties [53]. The lack of proliferative responses following the culture of MSCs with PBMC demonstrates the absence of activation and differentiation of donor lymphocytes [54]. MSCs show low levels of human leukocyte antigen (HLA) class I, and they do not express HLA-DR, which is necessary to escape immune surveillance [6]. The lack of HAL-II and co-stimulatory molecule expression sustains their hypo-immunogenicity as they are unable to activate an immune response. The presence of HLA class I at low levels is important to protect cells from cell-mediated recognition and lysis by natural killer cells [33]. On the contrary, cells that do not express HLA class-I are targeted and destroyed easily [55]. According to the literature, MSCs can be considered non-immunogenic as they are not capable to initiate an immune response due to the lack of effective T-cell activation [56]. They are capable of immune escape and fail to induce an immune response by the transplanted host. One of the major hurdles for developing biopharmaceutical agents is the generation of unwanted immune responses. Due to the absence of immunogenicity effect of PV compounds, they can be explored for their immunomodulatory effect on MSCs.

The combination of MSCs and PV extracts are likely immune-privileged due to the absence of immunogenicity and need to preserve their immunosuppressive effects. MSCs may modulate different immune cell populations mainly by through their secretome [57]. Understanding the immunological interplay between T-cells and MSCs pretreated with PV extracts may be helpful for the development of improved immunotherapy strategy. In this study, MSCs were able to inhibit the proliferation of activated T cells in a contact-dependent manner. In the presence of contact, MSCs efficiently inhibited lymphocyte proliferation compared to the absence of direct contact. Moreover, independently of the compound's concentrations and incubation time, we demonstrated that MSCs are immunologically responsive to PV extracts by demonstrating enhanced abilities to inhibit activated T-cell proliferation. MSCs in the presence of PV extracts were thus more suppressive than normal MSCs as they induced more inhibition of lymphocyte proliferation. The essential oil (0,01 % and 0,025 %) pretreated MSCs showed significant inhibition of T-cell response compared to untreated MSCs during the direct co-culture. After 3 and 5 days, the inhibition profile is maintained and in some cases enhanced indicating the capacity of the essential to modulate the immunomodulatory effect of MSCs. As expected, the absence of direct co-culture between pretreated MSCs and activated T-cells showed lower inhibition capacity in particular after 3 and 5 days. Essential oils (EOs) are reported to display various pharmacological effects such as antiallergic, anticancer, anti-inflammatory, and immunomodulatory effects. The immunomodulatory activity of EO can be linked to the secretion of a panel of cytokines probably through the regulation of NF-κB, and MAPK signaling pathways. According to the review of Sandner et al. [58], several cytokines and regulatory factors such as IL-1α, IL-1β, IL-3, IL-4, IL-5, IL-6, IL-8, IL-10, IL-13, TNFα, NO, and IFNγ are significantly reduced in monocytes and activated macrophages. In parallel, an increase of IL-5, IL-13 and HO-1 that promote the switch toward a M2 macrophage profile is reported. The current literature strengthens the potential of using EOs as a suitable immunomodulatory tool for the alternative disease management.

In comparison to essential oil, MSCs pretreated with Thymol presented a distinct profile regarding the modulation of T-cell response. In the presence of Thymol (3 μg/mL and 6 μg/mL), MSCs displayed an increased capacity to inhibit T-cell response during direct co-culture. The high concentration of Thymol was more efficient in reducing activated T-cell response by MSCs. The incubation time has also influenced the inhibitory profile of MSCs pretreated with Thymol as a small reduction is observed after 5 days. The absence of direct co-culture between pretreated MSCs and activated T-cells resulted in a different inhibitory profile. T-cell inhibition was significantly altered during the time with a pronounced decreased capacity after 5 days. Accordingly, these compounds are thus capable of modulating the regulatory functions of MSCs.

Exploring the immunomodulatory profile of BM-MSCs after treatment by PV compounds is required to ensure their functional immunotherapeutic effect. Several studies suggested that these compounds (EO, carvacrol and thymol) are strong candidates for the development of new therapeutic products for wound healing and tissue repair [59]. Thus, thymol and carvacrol were showed to modulate different phases of the wound healing process. In the first phase, they modulate the expression and function of inflammatory, oxidative stress and antimicrobial mediators. In the second phase, they promote tissue re-epithelialization, angiogenesis and

development of granulation. In the third phase, they improve the deposition of collagen and modulate the growth of fibroblasts and keratinocytes. Mechanistically, carvacrol and thymol may decrease the inflammatory response by modulating the JNK, STAT-3, AP-1, and NFATs pathways [60]. Orally administered carvacrol essential oils may suppress the immune and inflammatory responses in broilers challenged by lipopolysaccharide (LPS) by affecting the TLRs/NF-κB pathway and inhibiting the secretion of inflammatory cytokines [61].

Moreover, Carvacrol and Thymol have been shown to alter the maturation and function of dendritic cells (DCs) as well as T cell responses and activation. Thus, the expression of co-stimulatory CD40 and CD86 was reduced, the secretion of cytokines IFN-γ and IL-4 was decreased, and T-cell proliferation, either induced by mitogenic or in mixed lymphocyte culture (MLR), was inhibited [62]. Thymol may act as an anti-inflammatory agent by inhibiting the release of human neutrophil elastase [63], cytokine responses by macrophages [64], and inflammatory edema and leukocyte migration [65]. Carvacrol and Thymol may also suppress the antigen-specific immune response by reducing TH-1 cell-related cytokines\specific transcription factors.

Indeed, Carvacrol and Thymol through modulation of the JNK, STAT-3, AP-1 and NFATs pathways may reduce the inflammatory response. It is likely that these compounds decrease the expression of the interleukin (IL)-2, interferon (IFN)-γ, IL-4 and IL-17A, T-box 21 (T-bet) GATA binding protein 3 (GATA-3) and RORγc [66]. In parallel, they increase the levels of IL-10 and transforming growth factor (TGF)-β [67].

Moreover, different plant-derived components have been shown to promote MSC migration and homing to damaged sites to enhance tissue repair and healing. The activation of phosphatidylinositol 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) signaling pathways, the enhanced expression of the CXCL12/CXCR4 axis as well as matrix metalloproteinases (MMPs) may stimulate extracellular matrix remodeling, and consequently, promote cell migration of MSCs [68]. The recruitment of transplanted MSCs into damaged tissues is required to display their therapeutic effect.

There are several results in the literature underlying the effects of Thymol and carvacrol. Through a drug screening, Thymol was identified to inhibit the expression of interleukin 4 induced 1 (IL4I1) reported to have immunoinhibitory and tumor-promoting effects in several cancers. Thymol treatment restored thus the antitumor immune response and suppresses the progression of lung adenocarcinoma (LUAD) in an orthotopic mouse model [69]. In the presence of Carvacrol, MSCs pretreated showed significant inhibition of activated T-cell response cells during the first days of the direct co-culture. After 5 days, there is however a less ability of MSCs to reduce the proliferation of lymphocytes. In the absence of direct co-culture, the profile of T-cell inhibition is different with a significantly decreased capacity of MSCs to reduce the proliferation of lymphocytes. The lower inhibition was very pronounced after 5 days of co-culture. New results demonstrated that carvacrol alleviated vascular endothelial cell injury. carvacrol reduced the expression levels of inflammatory cytokines and the activation of the TLR4/NF-κB signaling pathway in vivo and in vitro. These findings demonstrated that carvacrol could alleviate endothelial dysfunction and vascular inflammation in Type 2 diabetes mellitus (T2DM) [70]. Carvacrol attenuated inflammatory responses by inhibiting the LPS-induced production of inflammatory cytokine interleukin-6 (IL-6) in vivo and in vitro. Mechanistically, carvacrol inhibited IL-6 production mainly through the ERK1/2 signalling pathway in macrophages in the pathogenesis of LPS-induced sepsis [71]. In parallel, results have showed that carvacrol can prevent intestinal inflammation and damage as well as mitigate gut dysbiosis in rabbits following LPS challenge [72]. Further findings disclosed that carvacrol reduced the protein levels of NLRP3, apoptosis-associated speck-like protein (ASC), caspase 1, interleukin (IL)-18, IL-1β, and the pyroptosis-indicative protein, gasdermin-D (GSDMD) in the sepsis-induced myocardial dysfunction model [73]. Carvacrol protected hepatocytes from the toxic effects of Diclofenac by enhancing the activity of antioxidant enzymes such as catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione (GSH) as well by diminishing the expression of tumor necrosis factor (TNF)-α [74]. Importantly, these compounds were found to be non-cytotoxic to Vero cells and did not cause hemolysis in erythrocytes at concentrations that effectively inhibited bacterial growth [75].

Both compounds have shown positive effects on MSCs by preserving their morphology, sustaining their viability, promoting their proliferation, and protecting them from cytotoxicity and oxidative stress [29]. Thus, Carvacrol and Thymol may improve the healing and repair capacities of MSCs in injured tissue by promoting their immunomodulatory potential. Because lymphocyte proliferation is a key event leading to the initiation and development of inflammation, increasing the inhibitory capacity of MSCs by using Carvacrol and Thymol is, therefore, a valuable strategy.

5. Conclusion

As natural, nontoxic immunomodulators, these bioactive molecules may improve the immune-therapeutic functions of MSCs. Following incubation with these compounds, MSCs displayed enhanced immunomodulatory capacities while preserving their immune-evasive profile. Collectively, carvacrol and thymol derived from PV are biologically active and capable of influencing the features of MSCs. Further investigations regarding the preparation and effects of these compounds will significantly improve their biological impact. Owing to diverse chemical complexes, plant-based natural products could provide appropriate combinations useful in immune modulation. In addition, understanding the mechanism of action of these functionally enhanced MSCs will allow for the identification of the main regulatory factors involved in their effects. It is increasingly recognized that the modulation of the immunological fate of MSCs is relevant to therapeutic approaches. The development of these features should enhance the value of MSCs by providing a therapeutic product with high immunological quality, safety, and efficiency.

6. Limitations and perspectives

Following these findings, it is important to underline some limitations and in parallel to highlight some perspective regarding the

development of safe and efficient immunotherapeutic MSCs by using medicinal plant-derived active molecules.

There are some limitations for the use of essential oil and natural compounds that may have an impact on the final results. The nature, composition and formulation, purity and concentration of the bioactive constituents present in herbal formulations as well as the technical issues linked to their isolation stand out as the main challenges for future use [76]. Using different solvents and methods to prepare these coumpounds may also influence the quality and the quantity of bioactive molecules, which may lead to different outcomes and undesirable side effects. These products may also induce an unwanted immune response, which can be influenced by various factors, including patient-/disease-related factors and product-related factors. It is essential to adopt an appropriate strategy for the development of adequate screening and confirmatory assays to measure an immune response against a therapeutic protein. In addition, their use could also be limited by the concentrations that they can achieve at the site of action. Further studies are necessary to obtain optimal ratios and dosing for higher therapeutic efficacy and to decrease toxicological profiles. Correlations studies of in vitro and in vivo toxicity data are necessary in order to develop models that allow a prediction of systemic toxicity in vivo from cell culture data [77]. As these compounds are extracted under different conditions or by using different parts of the plant, it is necessary to evaluate and compare accurately the effects by using standardized approaches. Accordingly, standardized protocols to ameliorate the manufacturing and characterization will help in improving several features of plant-derived products such as quality, efficacy, safety, dose, delivery, patient compliance and reproducibility of the therapeutic effects. Compared to classical vesicles and synthetic carriers, the development of plant nanovesicles as a next-generation drug platform to deliver EO is in progress to avoid the volatility, low water solubility and stability of the EO that are known to impair their therapeutic clinical translation [78]. Indeed, plant nanovesicles are reported to be safer, more stable, non-toxic and low immunogenic. They also increase the bioavailability, improve chemical stability, reduce volatility and toxicity and allow to deliver EO to target tissues. These plant nanovesicles can be produced from various plant parts at a large scale which may boost the local economy and valorize natural product. MSCs are considered as a valuable therapeutic agent because of their promising preclinical features and good safety profile. The main therapeutic effects of MSCs are now attributed to the stimulation of several endogenous repair processes in injured tissues as well as the modulation of inflammatory and immune responses, which translates into a positive outcome of MSC-based therapies [2]. However, several reports indicate that the quality of the human MSC product depends on the isolation and culture methods as well as the age, genetic traits, and medical history of the donor. Another important aspect is the cellular heterogeneity of MSCs, which makes results frequently variable and depending on the different MSC origin as well as harvesting and culture procedures. Factors contributing to the failure of MSC clinical development include but not limited to the poor-quality control and inconsistent characteristics of MSCs in terms of immunocompatibility, stability, heterogeneity, differentiation, and migratory capacity [79]. A previous study demonstrated size-dependent differentiation potency and secretome profile of MSC subpopulations, and provided an effective and practical technology to isolate the respective subpopulations, which may be used for more targeted tissue repair and regeneration [80]. In an interesting review, the authors dissected several aspects of pharmacokinetics (PK) - pharmacodynamics (PD) of MSCs as key aspects to overcome unsatisfactory clinical benefits of MSC application. They suggest a new model that underscores the importance of PK and PD for the success of MSC-based therapies that should consider possible bioactive substances and biomarkers to improve the prediction of a clinical dosing regimen with higher efficacy [81]. Further studies both in vitro (3D model injury model) as well as invivo model are also needed to evaluate the beneficial/deleterious effects of such combination of MSCs and plant derived compounds. Defining the therapeutic mechanisms by which such combination may regulate the trophic, inflammatory and immune response by using AI and Deep Learning may ultimately lead to successful personalized therapeutic options.

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Institutional review board statement

The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the local ethics committee.

Informed consent statement

Clear and Informed written consent was obtained from all donors involved in this study.

Data availability statement

The data presented in this study might be available depending on the type of demand and use and are linked to authorities’ authorization. A request must be sent to the corresponding author with the permission of all authors.

CRediT authorship contribution statement

Mehdi Najar: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources,

Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Fatima Bouhtit:** Writing – review & editing, Writing – original draft, Investigation, Formal analysis, Data curation, Conceptualization. **Saida Rahmani:** Software, Resources, Methodology, Investigation, Formal analysis. **Abderrahim Bouali:** Writing – original draft, Software, Methodology, Formal analysis. **Rahma Melki:** Software, Resources, Methodology, Investigation, Formal analysis. **Mustapha Najimi:** Supervision, Resources, Funding acquisition, Formal analysis. **Philippe Lewalle:** Writing – original draft, Supervision, Resources, Project administration, Funding acquisition. **Makram Merimi:** Writing – review & editing, Writing – original draft, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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