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The double face of cutaneous MSCs: a target to hit or a therapeutic tool in psoriasis

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ABBREVIATIONS

AD: atopic dermatitis

ALP: alkaline phosphatase

AMPs: antimicrobial peptides

APC: antigen presenting cells

AT-MSCs: MSCs isolated from adipose tissue

BDCA: blood DC antigens

BM-MSCs: MSCs isolated from bone marrow

BSA: Body Surface Area

CEU: clonal ectodermal unit

DCs: dendritic cells

DLQI: Dermatology Life Quality Index

DT: doubling time

EGF: epidermal growth factor

EpiSCs: epidermal stem cells

EpiSCU: EpiSCs unit

EPU: epidermal proliferation unit

ESCs: embryonic stem cells

FGF: fibroblast growth factor

HGF: hepatocyte growth factor

H-MSCs: MSCs isolated from skin of healthy people

IDO: indoleamine-2,3-dioxygenase

iNOS: inducible nitric oxide synthase

iPS: induced pluripotent stem cells

KCs: keratinocytes

LIF: leukemia inhibitory factor

mDCs: myeloid dendritic cells

MMP: matrix metalloproteinase

MSCs: mesenchymal stem cells

NK cells: natural killer cells

NO: nitric oxide

PASI: Psoriasis Area Severity Index

pDCs: plasmacytoid dendritic cells

PDGF: platelet-derived growth factor

PD-L1: programmed death-ligand 1

PGA: Physician's Global Assessment

PGE2: prostaglandin E2

PsA: psoriatic arthritis

PSO-MSCs: MSCs isolated from skin of psoriatic patients

ROS: reactive oxygen species

SOD: superoxide dismutase

solTNF: soluble TNF

TA cells: transit amplifying cells

TLR: Toll-like receptor

tmTNF: TNF-α transmembrane protein

TNFR: receptors of TNF

UC-MSCs: MSCs isolated from umbilical cord

VEGF: vascular endothelial growth factor

ABSTRACT

Psoriasis is a chronic inflammatory skin disease; the triggering events are not yet known but it is generally accepted that genetic and/or environmental factors are involved. Psoriasis manifests with erythema, thickening and squamae, and it is associated with various metabolic and non-metabolic comorbidities, that reduce the life quality of patients. Histologically, psoriatic skin is characterized by an epidermal hyperplasia, associated with angiogenesis and an inflammatory infiltrate. The pathology can be considered a Th1/Th17 model of disease, since there are dysregulated levels of cytokines of these pathways, that play a crucial role in psoriasis onset and progression. Mesenchymal stem cells (MSCs) are self-renewal multipotent stem cells. They can be isolated from different tissues, included the skin. MSCs show immunomodulatory peculiarities both at the level of innate and adaptive responses and exhibit tissue protective and repair-promoting properties. It was demonstrated MSCs are involved in cutaneous wound healing by their interaction with the microenvironment in a paracrine way and the ability to differentiate. However, MSCs of psoriatic patients reveal the same Th1/Th17 imbalance observed in differentiated skin cells and altered expression of VEGF, iNOS and production of NO. These evidences enforce the hypothesis that MSCs are primary involved in psoriasis pathogenesis. To date, several treatments of psoriasis are available and some biological therapies are also been developed. In particular, inhibitors of TNF-α are increasingly applied, but their influence on MSCs has not yet been clarified. In this study, MSCs were isolated from skin of patients affected by psoriasis (PSO-MSCs) before (T0) and after 12 weeks treatment (T12) with the TNF-α inhibitor adalimumab or etanercept, and from healthy people (H-MSCs). Cells were characterized following the minimal criteria of Dominici. Then, the expression of 22 cytokines belonging to Th1, Th2 and Th17 pathways were assessed before and after treatment by Real-Time PCR and ELISA array. PSO-MSCs T0 showed a dysregulated immunological profile, confirming the data of literature. A 12 weeks treatment reduced the levels of up-regulated cytokines and increased the expression of the down-regulated molecules. No difference was observed between MSCs treated with adalimumab or etanercept. In addition, the influence of H-MSCs on PSO-MSCs was investigated to evaluate if healthy cells are able to restore the pathological state of PSO-MSCs. H-MSCs and PSO-

MSCs T0 were indirectly co-cultured for 72 hours. Then, conditioned medium was collected and analysed for the expression of 12 cytokines related to inflammation by ELISA array while cells were counted. Non co-cultured PSO-MSCs were used as control. It is known that PSO-MSCs have a proliferation rate higher than normal cells; however, co-culture conditions could reduce psoriatic cells number of about 30%. The ELISA array revealed that H-MSCs also rebalanced the altered levels of cytokines. Moreover, H-MSCs and anti-TNF-α treatments gave comparable results: all the cytokines of the Th1 and Th17 pathways, over-expressed by PSO-MSCs at baseline, resulted down-regulated; also levels of IL-2, IL-13, TGF-β1, dysregulated at baseline, were restored. These data demonstrate that H-MSCs represent a promising therapeutic tool for the treatment of an inflammatory disease such as psoriasis. While on one hand healthy MSCs are able to improve the inflammatory state of PSO-MSCs, few information is available about the influence of PSO-MSCs on keratinocytes (KCs). Therefore, based on the only paper, to the best of our knowledge, that evaluated the influence of MSCs isolated from psoriatic patients on KCs, it was made an indirect co-culture of H-MSCs or PSO-MSCs with a KCs cell line, the HaCaT. After 72 hours of co-culture, HaCaT were counted and the secretome was analysed by ELISA array for the expression of inflammatory cytokines. Co-culture conditions did not induce a variation in cell proliferation; even more the altered levels of secreted cytokines after co-culture with PSO-MSCs were comparable to those after co-culture with H-MSCs. These data can not clarify whether MSCs are able to reach the most superficial skin layers and move KCs towards psoriasis.

In conclusion, our research demonstrates that biological treatments currently in use for psoriasis are effective also at the staminal level of the skin and can modify the cytokines milieu of MSCs. Furthermore, also healthy MSCs seem able to invert the psoriatic MSCs profile towards a more physiological one. With regards to that, further studies are necessary to clarify the H-MSCs action on psoriatic cells, and evaluate their possible use as therapeutic tool.

1. INTRODUCTION

Psoriasis is a chronic inflammatory skin disease. Its pathogenesis is complex and the exact mechanisms are not clear. However, it is thought to have a multifactorial etiology, related to genetic and non-genetic causes, including environmental factors and lifestyles (infections, drugs, smoking, diet, stress). Psoriasis is associated with several metabolic and non-metabolic comorbidities, with high impact on both physical and emotional health-related quality of life. Cutaneous mesenchymal stem cells (MSCs) are early involved in psoriasis onset.

1.1. Epidemiology of psoriasis

Onset of psoriasis varies depending on geography, age, gender and ethnicity. Also if several epidemiological studies have been made, there is not an exhaustive evaluation of people affected by psoriasis worldwide. It is estimated that psoriasis affects 2-4% of population in western countries but it is less frequent in countries closer to the equator (Egypt, Tanzania, Sri Lanka, Taiwan). This difference can be explained by the fact that psoriasis is a complex disease in which genetic but also environmental factors occur to its pathogenesis (Parisi R *et al*, 2013). In Italy, based on a 5-year observational study from a national primary care database during the years 2001-2005, incidence of psoriasis is 2,30-3,21 cases per 1,000 person/years in people more than 18th years old (Vena GA *et al*, 2010). Psoriasis can appear at any time of life, nevertheless Michalek and colleagues in a systematic review evaluated that worldwide in adult psoriasis ranges from 0,51% to 11,43% and in children it is from 0% up to 1,37% (Michalek IM *et al*, 2017). More specifically, the prevalence in children is up to 0,71% in Europe (Augustin M *et al*, 2010), with a peak of 2,1% in Italy, based on a study of 13 and 14 years old children (Naldi L *et al*, 2009), while it is almost absent in Asia (Yang YC *et al*, 2007). To further support, the most common form of psoriasis, psoriasis vulgaris, arises at a mean age of 33 years, and 75% of cases happen before 46 years of age (Nevitt GJ and Hutchinson PE, 1996). About gender, there are conflicting reports in literature. In fact, while some studies described no difference on developing psoriasis based on sex, others registered high prevalence of psoriasis in female subjects in Germany, United States,

Norway. Other researches highlighted that psoriasis is more frequent in men than in woman in Denmark and Australia (Parisi R *et al*, 2013).

1.2. Etiology of psoriasis

The triggering factors of psoriasis are not well known. It is believed that a component of inheritance is present; in fact, individuals with onset of disease prior to 40 years old have a family history pf psoriasis. Even more, population-based studies and twin studies indicated that psoriasis is a polygenic hereditable disease with variable penetrance (Elder JT *et al*, 1994). Genome analyses identified 9 chromosomal loci that are statistically significant for psoriasis onset (called PSORS1-9). PSORS1 accounts for about 35-50% of the heritability of the disease (Trembath RC *et al*, 1997). Nevertheless, other genetic and/or environmental factors must be considered. Increasing data focused on epigenetic modifications in driving psoriasis. Hypomethylated level of 26 regions of the human genome and abnormal DNA methylation of CpG in genes such as p15 and p21 were found in psoriasis patients (Han J *et al*, 2012; Zhang K *et al*, 2009). Histone modifications have been also identified (Zhang P *et al*, 2011). In addition, psoriatic skin lesions present aberrant levels of many microRNAs, among which miR-31, miR-203, miR-99a, miR-125b (Wang MJ *et al*, 2017). Furthermore, infections, particularly strep throat, smoking, alcohol consumptions, obesity, some medications have been linked to psoriasis onset. Psoriasis can also occur after a skin trauma, in the site of a recent sunburn or excoriation or on periwound skin (Young M *et al*, 2017; Villaseñor-Park J *et al*, 2012). Emotional stress, such as financial difficult, loss of employment, family loss, can exacerbate the disease (Heller MM *et al*, 2011). In woman, hormone change levels are involved in psoriasis, with worsening of disease during puberty, pregnancy, postpartum and menopause (Ceovic R *et al*, 2013).

1.3. Clinical aspects of psoriasis

Psoriasis can affect different area of the body, with prevalence of the scalp, the face, the hands and the skin fold (Fig. 1). Facial psoriasis is present mostly in the eyebrows, the upper lip, the upper forehead and the hairline. There are various skin manifestations of psoriasis that can be present simultaneously in the patients. All the forms share common features:

erythema, thickening and squamae. The size of the lesions can vary up to 20 cm of diameter, and have usually round, oval or polycyclic borders.

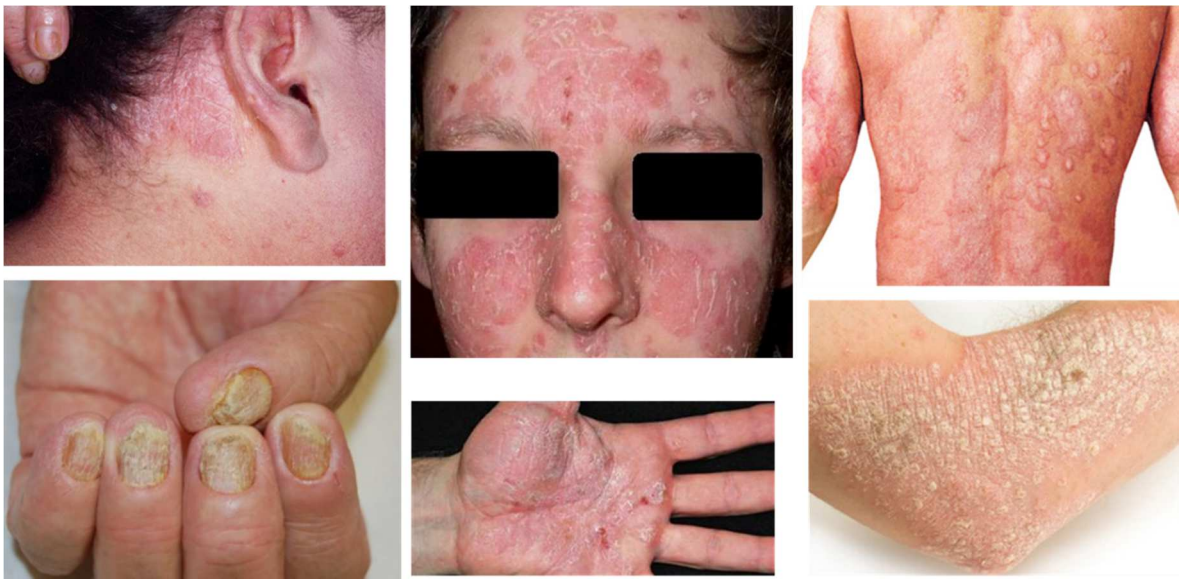

Figure 1. Different body areas affected by psoriasis.

Based on the morphologic evaluation, psoriasis can be classified in:

- ❖ Non-pustular psoriasis, including
    - Psoriasis vulgaris
    - Guttate psoriasis
    - Erythrodermic psoriasis
    - Palmoplantar psoriasis
    - Psoriatic arthritis (PsA)
    - Inverse psoriasis
- ❖ Pustular psoriasis, comprising
    - Generalized pustular psoriasis (von Zumbusch type)
    - Impetigo herpetiformis
    - Localized pustular psoriasis (palmoplantar pustular psoriasis-Barber type and acrodermatitis continua of Hallopeau).

1.3.1. Non-pustular psoriasis

Psoriasis vulgaris

Psoriasis vulgaris is the most frequent form of psoriasis, affecting more than 80% of psoriatic patients (Menter A *et al*, 2011). It is clinically characterized by well-defined erythematous plaques covered with pearlescent squamae. Lesions are symmetrically distributed principally on knees, elbows, scalp, and sacral region. If the surface of a psoriatic plaque is scraped with a blunt scalpel, squamae fall off as layers of coherent white lamellae, similar to candle wax (wax spot phenomenon). It is a sign of hyperkeratosis with presence of nucleated cells (parakeratotic hyperkeratosis) (Paragraph 1.4.). A further scraping evidences a wet layer, that is the last layer of the dermal papillae of the epidermis (last membrane phenomenon). If psoriatic plaque is scraped again erythema and bleeding foci with small red pinpoints, the so-called Auspitz sign, due to papillomatosis on tips of dermal papillae, are showed. Around the healed plaques, a hypo-pigmented non well yet clarified macular ring, called Woronoff ring, is visible (Sarac G *et al*, 2016).

Guttate psoriasis

Guttate psoriasis more frequently occurs in children and young adults under 30 years of age. It is often triggered by Streptococcus infections (Griffiths CE and Barker JN, 2007), but it is self-limiting and usually resolves within 3-4 months of onset. Lesions are distributed in the trunk and extremities. Guttate psoriasis is characterized by small, oval, pink, scattered lesions with silvery scaling. Only one third of guttate psoriatic patients will develop plaque psoriasis (Martin BA *et al*, 1996).

Erythrodermic psoriasis

Erythrodermic psoriasis is characterized by exfoliative dermatitis distributed in the large percentage of body's surface. Generally, there are erythematous lesions and the typical psoriasis plaques lose their typical features. Hypothermia can be present due to widespread vasodilatation, and patients can be affected by fever and fatigue. Additionally, erythrodermic psoriasis is usually associated with hair loss and nail dystrophy. Protein loss and associated systemic problems, such as edema of the lower extremities, may occur as a

result of desquamation; cardiac, hepatic and renal failure can happen (Sarac G *et al*, 2016). The concrete risk of cardiovascular or septic shock is the reason why patients have to be closely followed; in some cases, it is necessary hospitalization (Egeberg A *et al*, 2016).

Palmoplantar psoriasis

Palmoplantar psoriasis affects palms of the hands and soles of the feet. It is characterized by squamae and, when erythema is visible, by pinkish-yellow lesion (Sarac G *et al*, 2016).

Psoriatic arthritis (PsA)

Psoriatic arthritis (PsA) affects 0,1-1,0% of population, but its prevalence increases up to 30-40% among psoriatic patients with severe skin involvement, that develop PsA approximately within 5-10 years the onset of disease (Maese PJ and Armostrong AW, 2014). Different clinical forms of PsA are recognizable. Following Moll and Wright criteria (Moll JMH and Wright V, 1973), PsA can be classified in five subgroups:

- classical psoriatic arthritis: it affects distal interphalangeal joints of the hands and feet, with usually nail involvement;
- asymmetric oligoarticular arthritis: it is the most characteristic form of joint involvement. It can lead to dactyly;
- symmetric poliarticular form: it is similar to rheumatoid arthritis. It frequently involves distal interphalangeal joints and can provoke bone ankylosis;
- arthritis mutilans: it is characterized by progressive phalangeal and metacarpal bones osteolysis, frequently associated with sacroiliitis;
- spondylitic form: it is generally associated with peripheral arthritis. This form resembles ankylosing spondylitis but has a better prognosis.

Inverse psoriasis

This form of psoriasis is localized in skinfolds, such as armpits, under the breast, or in abdominal folds. It is more frequent in obese patients. Lesions appear as symmetric, shiny, red plaques with defined contours. Inverse psoriasis is usually more resistant to classical treatments (Griffith CE and Barker JN, 2007).

1.3.2. Pustular psoriasis

Generalized pustular psoriasis (von Zumbusch type)

Generalized pustular psoriasis is a rare form of psoriasis more frequent in young individuals. It onsets independently or as a complications of psoriasis vulgaris. Patients affected by generalized pustular psoriasis present erythema, fever, lassitude and polyarthralgia, sometimes associated with leukocytosis, lymphopenia and negative nitrogen balance. Peripustular erythema disseminates, so this form of psoriasis must be promptly treated to avoid it develops in an acute and fatal way.

Impetigo herpetiformis

Also called generalized pustular psoriasis of pregnancy, impetigo herpetiformis can appear in the last trimester of pregnancy or in the puerperal period. This disorder is characterized by erythematous plaques and grouped pustules at their margins. In some cases, it affect mucous membranes and induce onycholysis secondary to subungual pustules. Lesions are usually associated with itch, burning sensation and foul odor; in addition, fever, shivering, nausea and vomiting may be present. Hypocalcemia is a condition commonly present in these patients.

Localized pustular psoriasis

Localized pustular psoriasis includes two forms: palmoplantar pustular psoriasis-Barber type and acrodermatitis continua of Hallopeau. The first one is more common in woman; it is characterized by pustules of 2-4 mm diameter localized on palmoplantar region. The etiology is not known; nevertheless, there is an underlying contact sensitivity and smoking, tonsillitis, humidity and high temperature are associated with disease onset. On the contrary, the acrodermatitis continua of Hallopeau affects fingers and toes with loss of nails and distal phalanges in the most serious cases. Vesicles appear small, polycyclic, purulent and fluid-filled (Sarac G *et al*, 2016).

1.4. Histological features of psoriasis

The specific features identifiable in the psoriatic lesion depend by the disease stage. In general, three main histological features are present in psoriasis: an epidermal hyperplasia, dilated and prominent blood vessels in the dermis, and lastly, an inflammatory infiltrate of leukocytes, in prevalence into the dermis (Fig. 2).

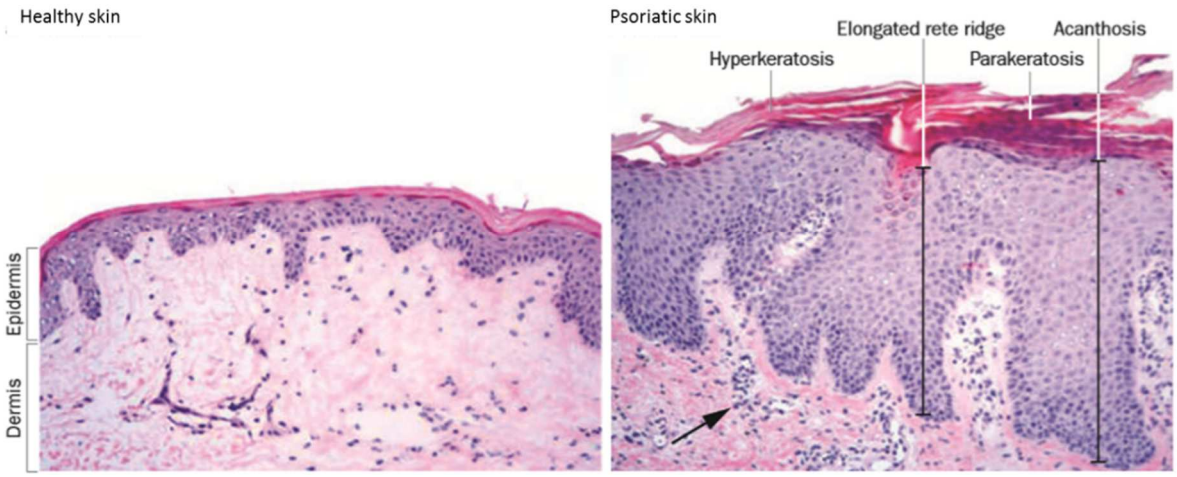

Figure 2. Principal histological features of healthy (left) and psoriatic skin (right; 20x magnification). In detail, in the psoriatic skin acanthosis (epidermal hyperplasia) is evident, associated with hyperkeratosis (thickening of the stratum corneum) and parakeratosis (presence of nuclei in the cornified layer). It is also manifest the elongation of rete ridges. The arrow shows the presence of inflammatory cells. Hematoxylin and eosin staining (Wagner EF *et al*, 2010).

In its classical presentation, that is psoriasis vulgaris, disease is characterized by well-circumscribed reddish and scaly papules; this is caused by the abnormal increase of the epidermis because of excessive keratinocytes (KCs) proliferation. The stratum corneum is strictly involved, characterized by retention of nuclei in cells, a phenomenon known as parakeratosis, and by hyperkeratosis, that is a thickness of the stratum itself. When psoriasis exacerbates, the histopathological features become more complex. The earliest changes can be not specific and affect generally the dermis, with a sparse superficial perivascular T-lymphocytic infiltrate. Then, other events follow. In the dermal papillae dilated and slightly tortuous blood vessels appear and a mild dermal edema become visible. Epidermis is affected by a minimal spongiosis and by a spreading of rare T lymphocytes

and/or neutrophils. At difference of intradermal T lymphocytes that are mostly CD4 positive, intraepidermal T lymphocytes are CD8 positive. At this stage, the papular early plaque is characterized by a slight epidermal hyperplasia, with neutrophils infiltration and a small parakeratosis. Lymphocytes, neutrophils, histiocytes form the inflammatory infiltrate, in which it is possible to recognize also extravasated red blood cells. The epidermal hyperplasia becomes manifest in the fully developed clinical plaques. Specifically the spinous and granular layers of the epidermis result thickened. A pallor characterizes the superficial layers of the epidermis, and spongiosis is minimal or absent. The hypercheratosis is well marked, and it is often composed by alternating orthokeratosis and horizontally confluent, vertically intermittent parakeratosis. Below parakeratosis zone, there is a situation of hypogranulosis. In most cases in the site of parakeratosis area rich of neutrophils, that are called the Munro's microabscesses, are identifiable. In the spinous layer this group of neutrophils, in truth less common, gets the name of spongiform pustules of Kogoj, formed by the migration of neutrophils from the papillary capillaries. A regular elongation of the rete ridges occurs; some rete ridges present typical bulbous enlargement of the tips, due to a widening of the deeper portion of the rete, or are fused to the adjacent one. Also dermal papillae are elongated and contain dilated and tortuous capillaries and fine fibrillary collagen. With a further worsening of the disease, KCs in the center of the pustules degenerate forming a cavity surrounding by thinned KCs. Neutrophils that migrate towards stratum corneum become pyknotic. A sparse superficial dermal perivascular lymphocytic infiltration is visible.

In psoriatic patients, the abnormal thickening of the epidermis can interest any or all layers of the epidermis itself. It was found that this hyperplasia is related to a down-regulation of markers of KCs differentiation, such as keratins K1 and K10, and an up-expression of the so-called hyperproliferation-associated keratins K6 and K16. Compared to normal skin, it was calculated that psoriatic skin has up to 27 times increase in mitotic activity, with a 12 fold decrease in the cell cycle time of basal and suprabasal KCs. The turnover time of the epidermis increases more than 5 fold: it is about 3-7 days in psoriasis in contrast to about 28-30 days in normal conditions. Angiogenesis in the dermal area is guided by angiogenic factors, first of all the vascular endothelial growth factor (VEGF), that is in fact expressed at

significantly high level in psoriatic plaques. Even more, the concentration of VEGF in psoriatic patients correlates with clinical disease severity. As previously described, neutrophils infiltrate in the intraepidermal level either in the stratum corneum (microabscesses of Munro), or in the spinous layer (pustules of Kogoj).

Considering the clinicopathologic correlation, the plaques elevation in psoriasis is principally due to the epidermal hyperplasia and the elongation of the rete ridges and to a lesser extent to dermal edema and inflammatory infiltrate. The fine silvery scale is related to the confluent parakeratosis. The erythematous appearance and the Auspitz sign are on the contrary the result of a combination of superficial dermal capillaries and overlying suprapapillary epidermal thinning. It is fair to remember that other forms of psoriasis can show different clinical appearances and some variations in the histopathological features.

During and after treatment, plaques show a progressive reduction in neutrophils presence in the stratum corneum, and a reduction in parakeratosis, with reformation of the granular zone. The epidermal hyperplasia resolves later. Even more, a mild superficial dermal fibrosis, with persistence of capillary dilatation and tortuosity in dermal papillae, can remain (Griffiths CE and Barker JN, 2007; Murphy M *et al*, 2007).

1.5. Psoriasis: an immune-mediated inflammatory disease

Psoriasis is a T cell mediated disorder. In response to triggering factors, multiple cell types and numerous cytokines are involved, leading to skin homeostasis disruption. The exact mechanisms that induce psoriasis are not yet fully clarified. However, it was showed that various insults can cause the release of the antimicrobial peptide LL37 (cathelicidin) by KCs. LL37 binds with pathogen-derived DNA or self-DNA released by stressed cells. The binding originates complexes that activate Toll-like receptor 9 (TLR9) on plasmacytoid dendritic cells (pDCs). The type I IFN is released; this cytokine along with TNF-α, IL-6 and IL-1β stimulates local myeloid DCs (mDCs) which in turn promote T cell-mediated inflammation (Mahil SK *et al*, 2016). Binding of LL37 to self-RNA induces the direct activation of mDCs via TLR7 and TLR8 (Ganguly D *et al*, 2009) and therefore provokes the expression of TNF-α and IL-6. Additionally, LL37 may directly stimulate circulating T cells, especially in case of moderate-to-severe psoriasis (Lande R *et al*, 2014). After migration in lymph nodes, mDCs

release cytokines, among which TNF-α, IL-23 and IL-12, promoting the differentiation of resident T cells population into Th1, Th17, and Th22 cells (Fig. 3).

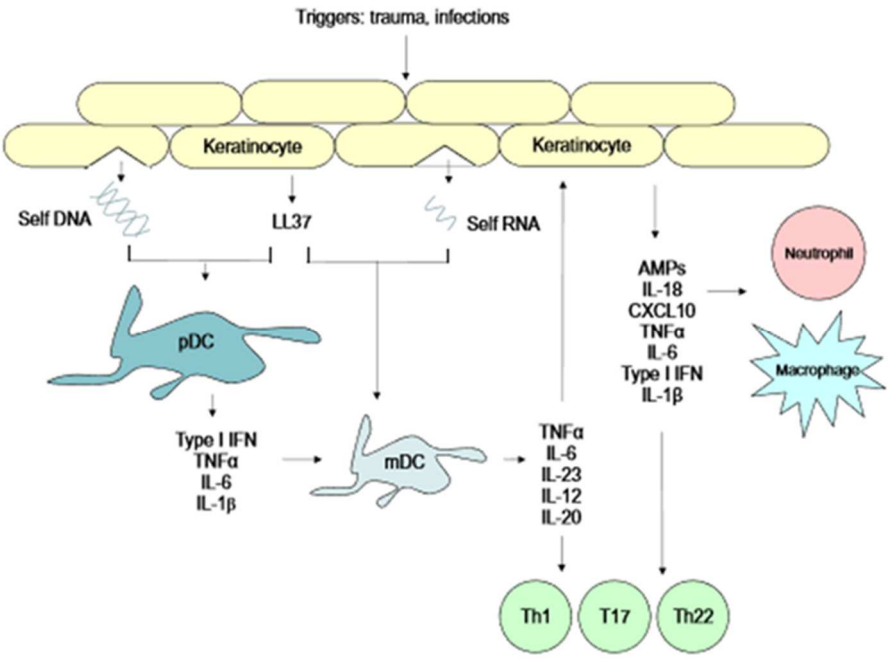

Figure 3. Schema of the initiation phase of psoriasis (Mahil SK *et al*, 2016).

Activated T cells move towards inflamed skin interacting with adhesion proteins on the endothelial cells of blood vessels. Here, they secrete molecules that activate KCs, which in turn release other cytokines and chemokines. In this way, the cutaneous inflammation is amplified. Therefore, it is clear that various immune cell types and cytokines are involved in psoriasis (Fig. 4). Between the cells, a primary role is assigned to DCs, KCs, T cells, neutrophils and macrophages.

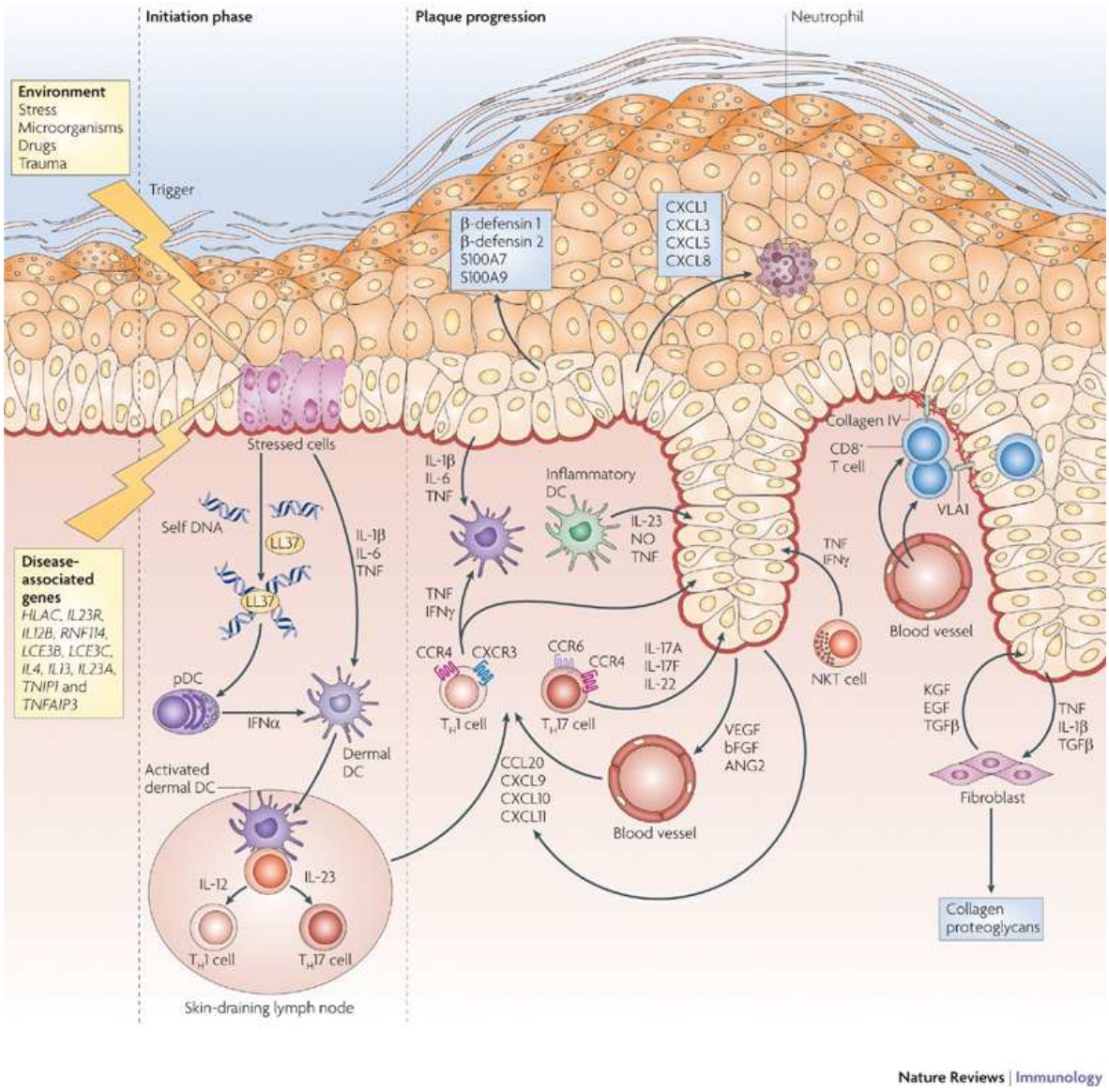

Figure 4. Psoriasis immunopathogenesis. After an initiation phase, activated by stressor factors sometimes favored by a genetic predisposition, numerous cells of inflammatory system and KCs release cytokines and chemokines. The cascade of events that occurs ultimately leads to the formation of psoriatic plaques (Nestle FO *et al*, 2009).

1.5.1. Principal immune cell types in psoriasis

Dendritic cells (DCs)

DCs connected to psoriasis pathogenesis are plasmacytoid DCs (pDCs) and myeloid DCs (mDCs), both localized in the dermis. The pDCs help to initiate the disease after their activation by LL37/DNA complexes. They represent a rich source of type I IFN; in particular,

pDCs induce an up-regulation of IL-17 and IL-23 by secretion of IFN-α (Acosta-Rodriguez EV *et al*, 2007). Different subsets of mDCs cells are recognized basing on the blood DC antigens (BDCA), such as BDCA-1^+ resident DCs and BDCA-1^- inflammatory DCs. The latter is present in greater amounts in the dermis of lesional psoriatic skin compared to non-lesional or normal skin. The mDCs with the marker α_X integrin CD11c are involved in the early stages of psoriasis.

Keratinocytes (KCs)

KCs form the first line of defense in the body against physical, chemical and microbial insults. KCs are fundamental in both the early stages of disease and the amplification of chronic inflammation. In fact, when there is a skin injury, resulting in cell death, KCs release antimicrobial peptides (AMPs), such as LL37, S100 and β-defensins. AMPs are key mediators of innate immune response. They modulate immune cells by promoting the up-regulations of pro-inflammatory cytokines and chemokines such as IL-6, IL-10, CXCL8, CXCL10, which in turn favour the recruitment of macrophages and neutrophils. KCs are also able to release IL-1 family cytokines themselves, including IL-1β and IL-18. In fact, KCs contain multi-proteins complexes, the inflammasomes, consisting of caspase 1, the adaptor protein ASC and a sensor protein. After stressors and pathogens identification, activated caspase-1 cuts pro-IL-1 and pro-IL-18 in their active forms, which are then released in the surrounding environment. Additionally, KCs are able to produce VEGF during the inflammatory state; KCs are therefore strictly involved also in the angiogenesis process (Mahil SK *et al*, 2016).

T cells

T cells are fundamental in psoriasis onset and progression, as demonstrated by the strong production of pro-inflammatory cytokines after their activation. At the beginning, studies on psoriatic skin lesions identified an increased expression of TNF-α and IFN-γ and therefore a pattern of cytokines corresponding to a type 1 profile (Uyemura K *et al*, 1993). More recently, a new type of T cells, that is Th17 cells, has been related to autoimmune disease in animal models (Bettelli E *et al*, 2007; Park H *et al*, 2005) and mixed populations of Th1 and Th17 cells were found in animals diseases previously classified as Th1-mediated

response (Weaver CT *et al*, 2007). Consequently, it was hypothesized that cytokines related to Th17 profile can be involved also in psoriasis. As expected, molecules such as IL-17 mRNA were found over-expressed in psoriatic lesions (Lowes MA *et al*, 2008). Psoriasis is now defined as a Th1/Th17 model of disease, linked to the crucial involvement of Th1 and Th17 cells. Some researchers suppose that Th1 cells are more relevant in the early stages of the disease, while Th17 cells maintain the inflammatory loop. In detail, it was proposed that Th1 cells produce IFN-γ. This molecule induces the secretion of IL-1 and IL-23 via the myeloid antigen presenting cells (APCs), so promoting the recruitment and expansion of IL-17 producing cells (IL-17$^+$ T cells). In parallel, IFN-γ stimulates APCs to produce CCL20 that supports the migration of IL-17$^+$ T cells. Even more, IFN-γ in synergy with IL-17 induces the production of β-defensin 2 (HBD-2), a chemotactic protein, by KCs (Fig. 5). Dysregulated KCs contribute to Th1 and Th17 cells differentiation through the release of IL-1β and IL-18 cytokines, respectively (Kryczek I *et al*, 2008).

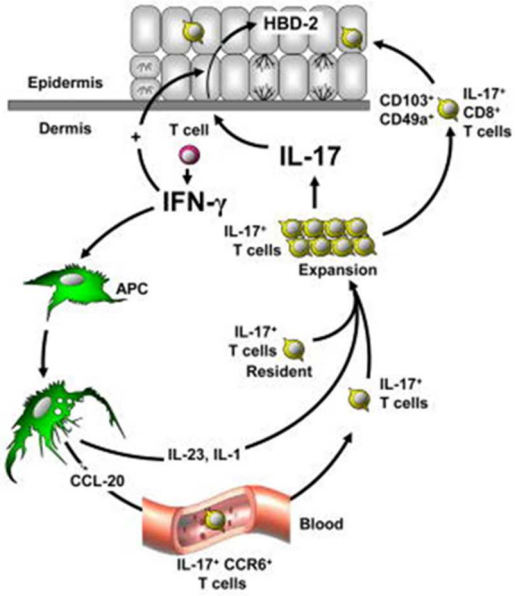

Figure 5. Schematic model of the hypothized interaction between Th1 cells and IL-17$^+$ T cells. Th1 and IL-17$^+$ T cells are thought to collaboratively work to promote psoriasis pathogenesis (Kryczek I *et al*, 2008).

Th17 cells establish an inflammatory loop with DCs and KCs. They promote the over-production of AMPs and inflammatory cytokines and can enhance the inflammatory

response, by secreting IL-17A, IL-17F and IL-9. IL-17 acts as an inflammatory gene expression regulator, able to altered KCs gene expression (Chiricozzi A *et al*, 2014). IL-17A is involved in a positive feedback loop with KCs: it stimulates the production of AMPs, cytokines and chemokines such as CCL20, CXCL1, CXCL3, CXCL8 by KCs, which in turn promote Th17 recruitment and so an over-production of IL-17 (Harper EG *et al*, 2009). IL-17F is able to increase the level of IL-6, highly expressed in lesional skin of psoriatic patients. IL-6 can stimulate KCs proliferation, thus contributing to epidermal hyperplasia; in addition, IL-6 is also able to induce Th17 differentiation. Therefore, a loop occurs: IL-17F induces IL-6 production, IL-6 in turn promotes the development of Th17 cells, enhancing inflammation of the lesional psoriatic skin (Fujishima S *et al*, 2010). A part of the already cited Th1 and Th17 cells, naïve Th cells can differentiate into other different subsets. More recently, two populations of $CD4^+$ T cells, Th9 and Th22, have been identified, probably strictly involved in psoriasis (Duhen T *et al*, 2009). Th9 cells produce IL-9, IL-10 and IL-21 and stimulate the production of IFN-γ, IL-13, IL-17 and TNF-α, thus enforcing the idea of a role in the skin inflammation onset and progression (Schlapbach C *et al*, 2014). Th22 cells secrete IL-22 and TNF-α but neither IL-17 nor IFN-γ. Another population of T cells greatly increased in psoriatic lesions are the dermal γδ T cells. They have a dendritic morphology but share many features with other IL-17^+ T cells, such as the constitutive expression of CCR6 and IL-23R. Under IL-23 stimulus and the presence of an endogenous IL-1β, secreted by KCs and infiltrating inflammatory cells, dermal γδ T cells produce large amounts of IL-17. Moreover, they constitutively express several chemokine receptors, including CCR1, CCR2, CCR4, CCR5, CXCR3, CXCR4, supporting the idea they are strongly involved in psoriasis pathogenesis (Cai Y *et al*, 2011). Several studies also demonstrated the importance of the entry of $CD8^+$ T cells in the epidermis; based on these researches, $CD8^+$ T cells are considered critical for the epidermal hyperproliferative response and inflammation in psoriasis (Kryczek I *et al*, 2008; Gudjonsson JE *et al*, 2004; Johnston A *et al*, 2004).

Neutrophils and macrophages

Neutrophils are activated by chemokines, such as CXCL1 and CXCL2, and by interleukins, in particular IL-8 and IL-18; once triggered, neutrophils release pro-inflammatory molecules

necessary for the recruitment and activation of T cells and for the proliferation and differentiation of KCs (Mahil SK *et al*, 2016).

Related to macrophages, a threefold increase of cells number in lesional skin was found. It was supposed that macrophages can participate to the pathogenic inflammation in psoriasis through the production of IL-23 and other inflammatory cytokines, and in this way contribute to KCs proliferation (Fuentes-Duculan J *et al*, 2010). However, the exact role of macrophages in psoriasis has to be better clarified (Zaba LC *et al*, 2007).

Natural killer cells (NK cells)

NK cells produce IFN-γ, that activates KCs. Moreover, NK cells are attracted by activated KCs following the secretion of CXCL10, CCL5 and to a lower extent CCL20 and CCL4 (Ottaviani C *et al*, 2006).

1.5.2. Principal cytokines in psoriasis

IFN-γ

IFN-γ is mainly produced by Th1 cells, DCs and NK cells. It has been confirmed that this cytokine is important in the early stage of the disease. In fact, IFN-γ induces the cross-phosphorylation of JAK2 and JAK3 leading to the activation of STAT3 and subsequently of STAT factors, that intervene in cell growth and gene expression regulation in psoriatic skin lesions. IFN-γ activates APCs such as DCs, promoting the release of cytokines IL-1 and IL-23, which induces Th17 and Th22 cell differentiation and activation. Moreover, IFN-γ can stimulate chemokines such as CXCL10 and CXCL11 from KCs (Deng Y *et al*, 2016).

TNF-α

TNF-α plays a crucial role in psoriasis. In cutaneous inflammation its production is induced by several cell types, such as macrophages, KCs, Th1 cells, Th17 cells, Th22 cells and BDCA-1$^-$ inflammatory DCs. TNF-α acts on APCs such as DCs, provoking the activation of T cells (Summers deLuca L and Gommerman JL, 2012) and stimulating the production of IL-23. Even more, TNF-α allows the recruitment on one side of mDCs and Th17 cells through the induction of CCL20 expression, and on the other of neutrophils through that of IL-8. The infiltration of T cells into the skin is promoted by TNF-α through the up-regulation of

intercellular adhesion molecule 1 (ICAM-1). TNF-α also favors the expression of C-reactive protein and of cytokines such as IL-6, that mediates T cell proliferation and KCs hyperproliferation (Mahil SK *et al*, 2016), and enhances the effects of other molecules such as IL-17 (Zaba LC *et al*, 2007).

IL-17

IL-17 is primary produced by IL-17$^+$ CD4$^+$ T cells (Th17 cells); however, also γδ T cells, IL-17$^+$ CD8$^+$ T cells, neutrophils and mast cells can secrete IL-17 (Cai Y *et al*, 2011; Kryczek I *et al*, 2008). IL-17 family includes at least six homodimeric cytokines (IL-17A-F), but IL-17A and IL-17F are the most involved in psoriasis. The active form of IL-17A is composed by either IL-17A homodimers or IL-17A/IL-17F heterodimers and it is over-expressed in both skin and blood of psoriatic patients (Gaffen SL, 2009). Its receptor (IL-17R) is especially expressed by KCs in psoriatic lesions. After the linkage, IL-17A stimulates these cells to produce AMPs, pro-inflammatory cytokines such as IL-1, IL-6, IL-19, IL-23 and chemokines for example IL-8; it is just through IL-8 that IL-17A promotes mobilization and activation of neutrophils. IL-17A also facilitates cutaneous recruitment of DCs and T cells through the increase production of CCL20 and ICAM-1 (Mahil SK *et al*, 2016). It is recently shown a synergy action with TNF-α, that induces KCs to produce pro-inflammatory cytokines; IL-17A seems to have a more relevant role than the other IL-17 members in this cooperation (Krueger JG *et al*, 2012).

IL-23

IL-12 was thought to drive the pathogenesis of psoriasis for more than a decade. However, new evidences focus no more on IL-12 but on IL-23. In fact, both IL-12 and IL-23 are composed by two subunits: IL-12p40, shared by both cytokines, and p35 or p19, respectively. In lesional skin, IL-12p40 and p19 were found at increased levels compared to healthy skin, while there is no difference for p35, demonstrating that IL-23 but not IL-12 is strongly involved in psoriasis (Lee E *et al*, 2004). IL-23 is released by DCs and macrophages, mediates the terminal differentiation and activation of Th17 cells and the up-regulation of TNF-α in macrophages. It also contributes to KCs hyperproliferation. The IL-23/IL-17 axis is fundamental in psoriasis. In fact, IL-23 binds to the IL-23 receptor (IL-23R) on Th17 cells. In

association with JAK2 and TYK2 it activates STAT3, causing the up-regulation of IL-17A. As previously described, IL-17A is engaged with IL-17R on KCs inducing the activation of NF-κB and so the transcription of pro-inflammatory cytokines (Fig. 6) (Mahil SK *et al*, 2016).

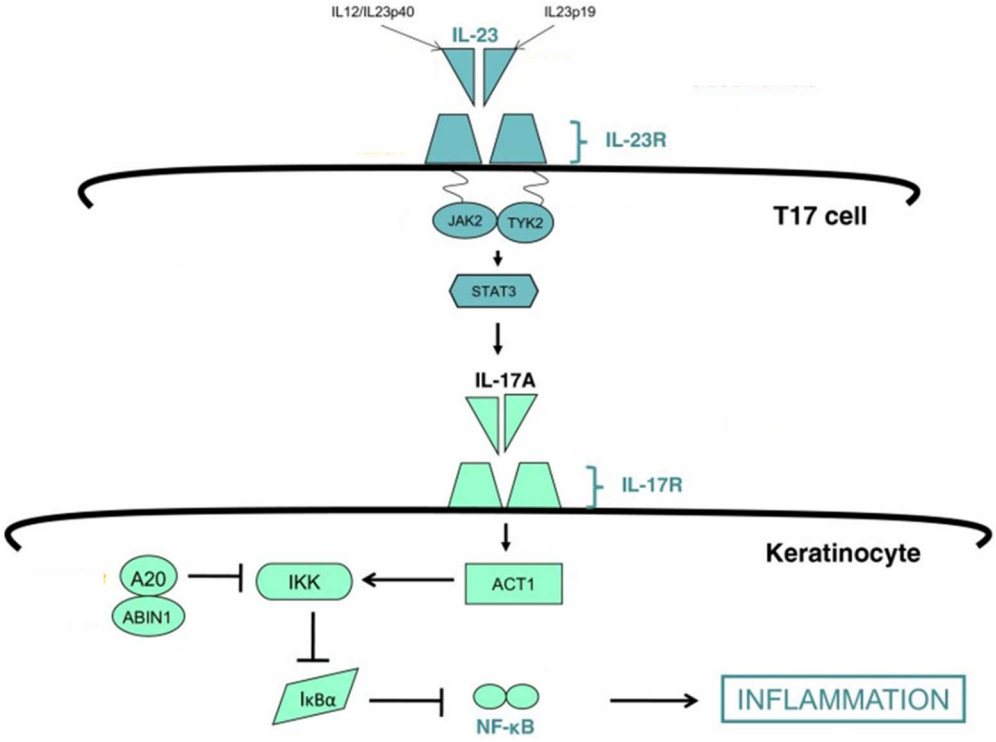

Figure 6. Representation of the IL-23/IL-17 axis, that provokes the start of an inflammatory process (Mahil SK *et al*, 2016).

IL-22

IL-22, produced by both Th17 and Th22 cells, can have a pro-inflammatory or tissue protective function, depending by different factors. IL-22 in synergy with IL-17 promotes a pro-inflammatory milieu and it is thought to provoke the typical hallmarks of psoriasis acting on KCs. In fact, KCs are inhibited to differentiate by the combination of these two cytokines, but increase their proliferation and mobility. This leads to parakeratosis, epidermal hyperplasia and elongation of the epidermal rete ridges (Deng Y *et al*, 2016). Even more, IL-22 activates IL-23 through STAT3 signaling, thus also indirectly stimulating KCs (Zheng Y *et al*, 2007). The involvement of IL-22 in psoriasis initiation and maintenance

has been enforced by different studies (Plank MW *et al*, 2017; MA HL *et al*, 2008; Boniface K *et al*, 2007).

1.6. The psoriatic march

As described in the previous paragraph (Paragraph 1.5.), psoriasis is an active source of various pro-inflammatory cytokines and chemokines. These molecules are over-expressed in lesional skin but are also detectable in circulation, causing systemic inflammation. Therefore, they are probably involved in developing comorbidities. Based on that, in the last ten years psoriasis, and in particular severe psoriasis, is no longer seen as a simple skin disease but it is considered as a chronic systemic inflammation. This concept is known as "psoriatic march". The theory is enforced by the presence of comorbidities, that is the prevalence of a higher than expected disorders in patients with psoriasis, mostly severe psoriasis. For example, psoriasis burden is often worsened by psoriatic arthritis, and approximately 15-30% of Caucasians with psoriasis develop psoriatic arthritis. Even more, psoriasis has a significant comorbidity with cardiovascular diseases and it is associated with myocardial infarction. Additionally, the connection of psoriasis with metabolic disorders such as obesity, glucose intolerance, hypertension, dyslipidemia is documented (Puig L *et al*, 2014; Takahashi H and Iizuka H, 2012; Henseler T and Christophers E, 1995). In a population of Italian patients, metabolic syndrome was reported to be more common in psoriatic patients than in patients with skin diseases other than psoriasis (30,1% vs 20,6%) after the age of 40 years (Gisondi P *et al*, 2007). Moreover, patients with severe psoriasis are more at risk of diabetes and obesity than patients with mild psoriasis. Psoriasis is frequently associated also with immune-mediated or autoimmune pathologies; among them, inflammatory bowel disease, multiple sclerosis, autoimmune hepatitis and Hashimoto's thyroiditis are the most represented (Furue M and Kadono T, 2017). It follows that both physical and psychological quality of life in psoriatic patients are impaired. In detail, based on the "psoriatic march" hypothesis, when the psoriasis specific onset occurs, due to genetic, environmental or other unknown factors, there are both innate and immune response, that lead to disease expression and progression. Pro-inflammatory cytokines are released into the circulation, producing a chronic low-grade of systemic inflammation. The state of chronic inflammation causes insulin resistance, followed by

endothelial cell dysfunction and cardiovascular disease. This cascade at the level of arteries, coronary or carotid can provoke myocardial infarction or stroke (Fig. 7) (Boehncke WH *et al*, 2011). Moreover, it can also cause obesity, hypertension, dyslipidemia, type 2 diabetes mellitus.

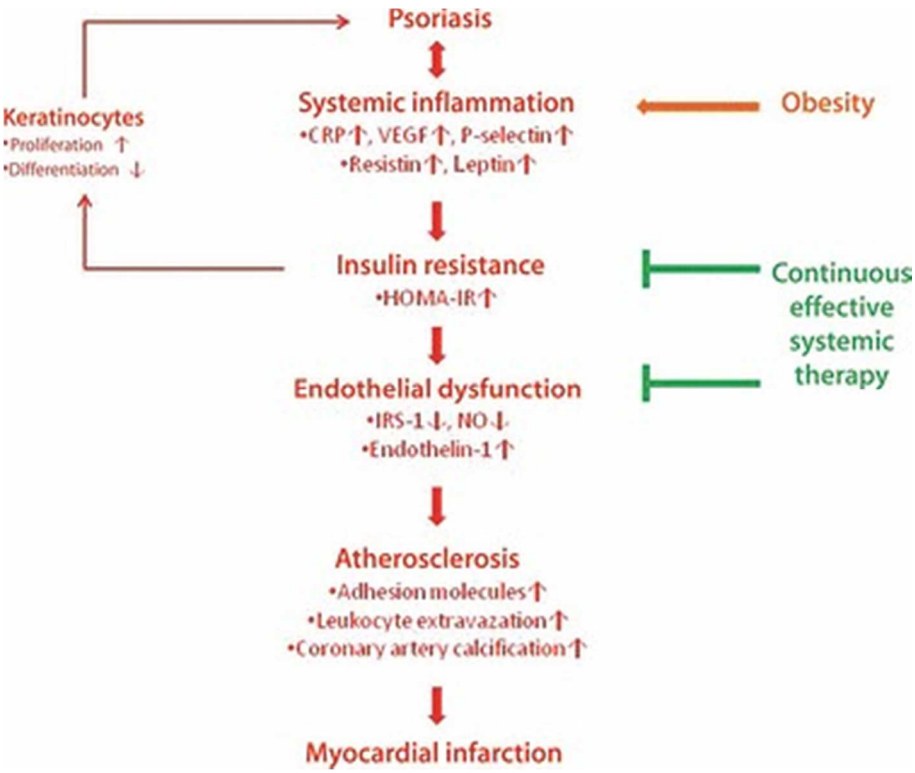

Figure 7. The "psoriatic march". Psoriasis as a condition of systemic inflammation can induce various comorbidities in psoriatic patients and lead to cascade up to myocardial infarction or stroke. In addition, complications such as insulin resistance can affect epidermal homeostasis through a blockade of KCs differentiation and an increase of proliferation, establishing a feedback that worsens psoriasis. Obesity is a risk factor for psoriasis. The "psoriatic march" can be stopped by the use of continuous systemic therapy (Boehncke WH *et al*, 2011).

In particular, pro-inflammatory cytokines, such as IL-6, adiponectin or other adipocytokines induce insulin resistance, and their effect is not limited to metabolically relevant cells. Two examples are the adipocytokines leptin, that acts as modulator of inflammatory and immune responses (Lago R *et al*, 2008), and chemerin, strictly associated with obesity and tryglicerides values, which has also a pro-inflammatory action since it is a chemotactic factor for pDCs (Gisondi P, 2013). Various studies demonstrated that insulin resistance

causes endothelial cell dysfunction (Jansson PA *et al*, 2003). An altered balance of vasodilating and vasoconstricting factors favors the atherogenic state (Hansson GK *et al*, 2005). Insulin resistance, visceral adiposity, hypertension, dyslipidemia, whose pathophysiologies are strictly related to the chronic state of inflammation, increase the risk of type 2 diabetes mellitus and cardiovascular disease development. Also obesity is strictly related to a systemic inflammation, that causes immune dysregulation (Wellen KE and Hotamisligil GS, 2005) and it is a risk factor for psoriasis, as confirmed by epidemiologic studies (Naldi L *et al*, 2005).

The "psoriatic march" concept is supported by various evaluations. Firstly, psoriasis patients with long-term continuous methotrexate treatment show a reduction in cardiovascular comorbidity. Even more, the use of the TNF-α inhibitor etanercept reduces C-reactive protein (CRP), a biomarker for cardiovascular risk, elevated in blood of psoriatic patients (Prodanovich S *et al*, 2005; Strober B *et al*, 2008). Also psoriatic patients with carotid arterial stiffness find a clear improvement by anti-TNF-α therapy (Pina T *et al*, 2016). Animal experiments proved that TNF-α plays a pivotal role in the connection of psoriasis with comorbidities onset, linking obesity, insulin resistance and inflammation. For example, a 2015 research demonstrated that the blockade of TNF-α by etanercept improved glucose tolerance in obese diabetic Zucker rats (Grauballe MB *et al*, 2015). In the same year, another study showed that repeated administration of etanercept could prevent exacerbation of cerebral damage caused by middle cerebral artery occlusion/reperfusion in both normal and diabetic rats, and it was mainly attributed to drug anti-inflammatory effects (Iwata N *et al*, 2015). Because of the complex context associated with psoriasis, when a psoriasis treatment is chosen, various parameters have to be taken into consideration, such as disease severity and presence of comorbidities. In the clinical practice the vitamin D dosage is also very important, being particularly poor in patients with psoriasis. In particular, topical vitamin D3 application is effective for the psoriasis treatment (Mattozzi C *et al*, 2016). However, some traditional systemic drugs can negatively interfere on comorbidities. For example, cyclosporine may worse hypertension, while methotrexate can be hepatotoxic. In this scenario, biological agents can be a valid therapeutic option. In turn, some conditions can alter the effectiveness of therapy. A case

is obesity, that can be considered as a risk factor for the development of the hepatotoxicity or renal toxicity induced by methotrexate and cyclosporine, respectively; even more, generally patients with a high body mass index (BMI) have a lower response to various treatments, including the biological ones (Clark L and Lebwohl M, 2008; Naldi L *et al*, 2008).

1.7. Assessment of injuries severity by quantitative indexes

Psoriasis is quantitatively evaluated on the basis of lesions severity and impact on lifestyle for the affected person.

The most common used indexes to classify the severity of psoriatic lesions are:

1. Psoriasis Area Severity Index (PASI). It is an evaluation index based on the characteristics of erythema, induration and desquamation and the extension area of affected regions;
2. Body Surface Area (BSA). BSA is a measure of the involved area in relation to the entire body surface;
3. Physician's Global Assessment (PGA). It is an overall assessment of injuries severity.

The impact of psoriasis on quality of life is evaluated by:

1. Dermatology Life Quality Index (DLQI). Developed in 1994, DLQI is a questionnaire of 10 questions, with five possibilities of answer (very much, a lot, a little, not at all, not relevant) (Finlay AY and Khan GK, 1994);
2. Skindex-29. It is created to measure quality of life in several populations and identify changes over time. It is composed by 30 questions.

1.7.1. The PASI score

PASI is the most used index to estimate the psoriasis severity. It was introduced in 1978 by the two Swedish doctors Fredriksson and Pettersson. More specifically, PASI evaluates:

- Spot extensions; a score (from 0 to 6) corresponding to the percentage of area involved in psoriatic lesions is assigned, according to the followed criteria:

0. 0%
1. < 10%
2. 10-29%
3. 30-49%
4. 50-69%
5. 70-89%
6. 90-100%

- Erythema; redness of skin is evaluated by comparing erythema with examples in photos. It is assigned a number from 0 to 4 (absent, mild, moderate, severe and very severe).
- Induration (Thickness); the degree of thickness is assessed using a scale from 0 to 4 (absent, mild, moderate, severe and very severe), by comparing the lesions with the examples in pictures.
- Desquamation (Scaling); also in this case a value from 0 to 4 (absent, mild, moderate, severe and very severe) is assigned visually comparing the degree of patients scaliness with examples shown in photos.

Body is divided in four parts, that are head and neck, upper limbs, trunk and lower limbs. Each region is considered in proportion to the total body surface, therefore a percentage is assigned (head and necks: 10%; trunk: 20%; upper limbs: 30%; lower limbs: 40%). For each region a value from 0 (no lesion) to 6 (> 90% lesions) is given. Finally, PASI index is calculated as

$$PASI = 0,1(R_h + T_h + S_h)A_h + 0,2(R_u + T_u + S_u)A_u + 0,3(R_t + T_t + S_t)A_t + 0,4(R_l + T_l + S_l)A_l$$

Where R is redness, T is thickness, S is scaling, h is the index representing head, u upper extremities, t trunk and l lower extremities. The assessment scale considers four levels: no psoriasis, mild psoriasis, moderate psoriasis and severe psoriasis.

PASI index is also used in clinical trials to evaluate the treatment response, and measure efficacy and outcomes. This is usually presented as a percentage response rate (PASI 50, PASI 75, PASI 90, PASI 100). For example, PASI 75 indicates a 75% or more reduction in the

patients PASI score from baseline after treatment. PASI 100 represents patients who received a complete resolution of psoriasis.

1.8. Treatment of psoriasis

Several treatments have been developed against psoriasis. Traditional therapies include vitamin D analogues, phototherapy and systemic treatments. The use of topical glucocorticoids and vitamin D analogues gives satisfactory results only on mild but not severe psoriasis. In the case of moderate to severe psoriasis, the approach is based on ultraviolet B light in combination with systemic drugs, of which the most common is methotrexate. It is estimated that more than 75% of patients receive a 50% reduction in disease severity by these treatments. However, cited therapies present adverse effects. Vitamin D analogues have a slow activation; continuous corticosteroids use can provoke loss of efficacy, cutaneous atrophy or appearance of pustular psoriasis. Lastly, methotrexate usage can induce adverse effects, including in the most serious cases liver fibrosis and cirrhosis. Some biological therapies are also now available. Among the others, based on their target it is possible to recognize TNF-α inhibitors (adalimumab, etanercept, certozulimab, golimumab, infliximab and onercept), IL-12/IL-23 inhibitor (ustekinumab), IL-17 inhibitor (secukinumab). A comparative study between methotrexate and etanercept use established that there is no statistical difference in PASI improvement; however, patients treated with the biological modulator revealed a higher remission suggesting etanercept is a better choice for long-term maintenance (Deng Y *et al*, 2016). Nevertheless, biological therapies are not without side effects. In fact, the blockade of one or more cytokines may impair the immune capacity against bacteria or infections; the response is also variable among the patients and biological modulators are more expensive than traditional therapy (Kalb RE *et al*, 2015; Bachmann F *et al*, 2010; Brown SL *et al*, 2002). A new intriguing therapeutic approach that is capturing researchers interest is based on the use of stem cells.

1.9. The stem cells

Stem cells are self-renewal not specialized cells with a differentiation potential. They are able to go through continuous divisions while maintaining the undifferentiated state.

Nevertheless, stem cells can enter into an asymmetric mitotic process, giving rise to two daughter cells: one maintains the same properties and phenotypic features of stem cell, while the other proceeds towards differentiation. The differentiation potential is the variety of different cell types that can be generated by an individual mitotic cell. It is important to note that degree of differentiation and potential are inversely related: when cell differentiation gradually proceeds, a progressive decrease in the differentiation potential is observed. Even more, stem cells can show various degrees of plasticity, depending on their origin. The stem cells plasticity refers to the ability to differentiate in other cell types than those of the tissue in which stem cells reside, under specific differentiating stimuli of the microenvironment.

1.9.1. Stem cell classification

Stem cells can be classified in different ways (Fig. 8). Based on their origins, they are subdivided in:

- Embryonic stem cells, derived from early stage embryos;
- Stem cells from extraembryonic tissues, such as from umbilical cord, amniotic fluid, amniotic membrane;
- Adult stem cells.

Based on their specific differentiation potential, stem cells are classified in:

- Totipotent stem cells, able to generate a complete embryo;
- Pluripotent stem cells, that can generate cells of the three germ layers;
- Multipotent stem cells, with the ability to generate only cells of the same germ layer;
- Unipotent stem cells, able to differentiate only in cells of the specific tissue from which they derive.

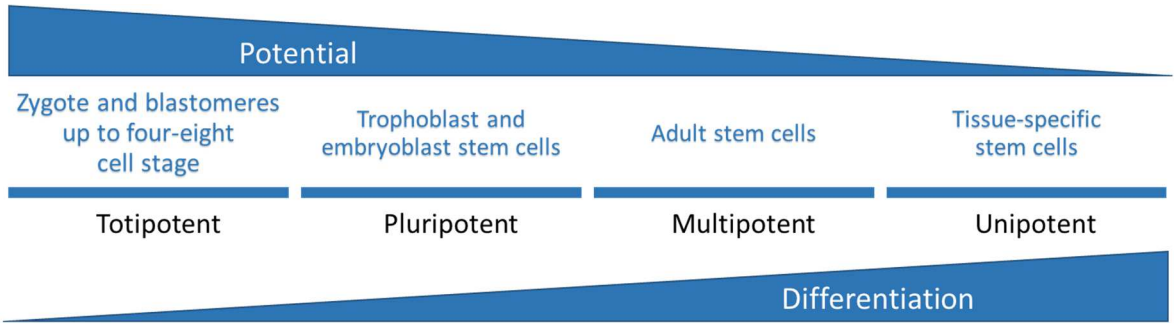

Figure 8. Schematic overview of stem cells classification. The differentiation potential gradually decreases for more specialized cells.

To these, two other types of stem cells can be identified:

- Induced pluripotent stem cells (iPS);
- Stem cells obtained from somatic nuclear cell transfer.

Totipotent and pluripotent stem cells

Totipotent stem cells are able to differentiate into the three primary germ cell layers of the embryo and into extra-embryonic tissues (placenta, chorion, amnios, yolk sac and umbilical cord). They can therefore give rise to a complete organism (Behr B *et al*, 2010). A fertilized egg, the zygote, is a totipotent stem cell. This ability is maintained up to the four-eight cell stage. After the morula stage, blastomeres undergo other mitotic divisions and blastocyst forms, characterized by a cavity. The outer cells of the blastocyst constitute the trophoblast while the inner blastomeres, connected to a trophoblast pole, form the inner cell mass or embryoblast. Cells of the embryoblast are considered pluripotent stem cells, because they have the capability to differentiate in all cell types but taken individually they can not give rise to the complete organism. Embryonic stem cells (ESCs) obtained from the inner cell mass are pluripotent stem cells. ESCs have been looked with enthusiasm by researchers owing to the proliferative capacity and the higher potential than other cells in culture. Once obtained, ESCs can be maintained in culture on a layer of embryonic fibroblasts or in the presence of the leukemia inhibitory factor (Kim HS *et al*, 2005); in absence of stimulus, these cells divide indefinitely without phenotypic alterations and maintain the undifferentiated state. Opportunely stimulated by specific growth factors in culture

medium, ESCs differentiate. For example, they have been successfully differentiated *in vitro* into neural precursors cells and neurons, smooth muscle cells, myocytes, cardiomyocytes, hepatocytes (Ikeda N *et al*, 2017; Shroff G *et al*, 2017; Wu Y *et al*, 2017; Zhang Q *et al*, 2011; Ozolek JA *et al*, 2010; Wu DC *et al*, 2007; Kawamura T *et al*, 2005; Singla DK and Sun B, 2005). ESCs are used in developmental biology field, for toxicity studies of drugs screening, and are paid into attention by the regenerative medicine. However, their use is compromised by various complications. First of all, there is an ethical issue because ESCs obtainment causes the death of the embryo that is developing. Even more, animal models that receive systemic administration of ESCs demonstrated the possibility of teratomas formation (Blum B and Benvenisty N, 2009). Another problem is linked to the histocompatibility system of the stem cells therapy recipient, that can induce rejection.

Multipotent stem cells

Multipotent stem cells show the capability to form cells of various tissues but belonging to the same germ layer. Mesenchymal stem cells (MSCs), which will be described in more detail later, are a classical example of multipotent stem cells.

Unipotent stem cells

Unipotent stem cells are able to differentiate in only one type of cells so they show the least potentiality among stem cells. They are responsible for the maintenance of a pool of stem cells in an adult individual tissue, to replace the lost cells. Some tissues, like skin, are continually replaced by new cells that differentiate starting from unipotent stem cells. It is now known that also other tissues, like heart and brain, present unipotent stem cells, going under a process of self-renewal and differentiation after an injury.

Induced pluripotent stem cells (iPS)

In 2006, Takahashi and Yamanaka published an article in which they defined a set of transcription factors that allow differentiated cells to acquire a pluripotent state (Takahashi K and Yamanaka S, 2006). Thus, adult cells can be reprogrammed inducing the expression of specific transcription factors. These cells, called induced pluripotent stem cells, are able to differentiate in various cell types, depending on stimuli in the microenvironment.

Stem cells obtained from somatic nuclear cell transfer

Nucleus of postnatal somatic cells can be transferred in unfertilized egg; mitotic divisions of the obtained cell can lead to the formation of a whole organism. The most famous experiment of somatic nuclear cell transfer was the sheep Dolly (Campbell KH *et al*, 1996). This procedure was successfully repeated also in other mammals such as mice, pigs, cats, dogs (Lee BC *et al*, 2005; Shin T *et al*, 2002; Polejaeva IA *et al*, 2000; Wakayama T *et al*, 1998). The ethical debate on the use of this method is still ongoing.

1.10. Stem cells of the epidermis

Stem cells are an attractive topic of research because of their involvement in skin development and their role in skin repair and regeneration. Doctor Withers in 1967 was the first to suppose that the epidermis is a heterogeneous compartment. In fact, when mouse epidermis was exposed to ionization or ultraviolet irradiation, only about 10% of cells in the epidermis could form new foci but about 60% of KCs were recycled (Whiters HR, 1967). The divided cells that regenerated the epidermis were recognized as stem cells, while the others that went towards differentiation were called transit amplifying cells (TA cells) (Potten CS, 1974). TA cells are defined as cells with limited self-renewal capacity (about three cycles of division) that go towards terminal differentiation in KCs. Studies continued and demonstrated as cells from epidermis used in burn people could form normal skin; even more, this population could be maintained in culture (Watt FM, 1998). The researchers Jones and Watt in 1993 made *in vitro* and *in vivo* experiments, in which they identified cells that formed large colonies and others that formed small colonies. The firsts were able to self-renewal and produce cells that went towards terminal differentiation. They were recognized as stem cells. The second group went to terminal differentiation in less than two weeks; they had the properties of the TA cells (Jones PH and Watt FM, 1993). Based on a variety of studies, a three tiers model of clonal organization of the epidermis is currently proposed. It is like a hierarchy of three orders. First of all, the Clonal Ectodermal Unit (CEU), produced by a progenitor from the ectoderm, composes the first tier. Many millions of cells make part to CEU, covering many centimeters. An epidermal stem cells (EpiSCs) unit (EpiSCU) follows. It is composed of hundreds to thousands cells and

spans from 0,2 to 3 mm. The EpiSCs divide giving rise to self-renewal cells that maintain stemness property and to TA cells that after few mitotic divisions differentiate. Thus, EpiSCs are responsible for the generation and the maintenance of adult epidermis and the relative appendices (hair follicles, sebaceous glands). In the end, a descendant of EpiSCU is involved in the formation of the Epidermal Proliferation Unit (EPU). This third tier spans 0,05 mm and is composed of about 20 cells with columnar shape. EPUs are considered the basic unit of epidermal organization (Fig. 9) (Strachan LR and Ghadially R, 2008).

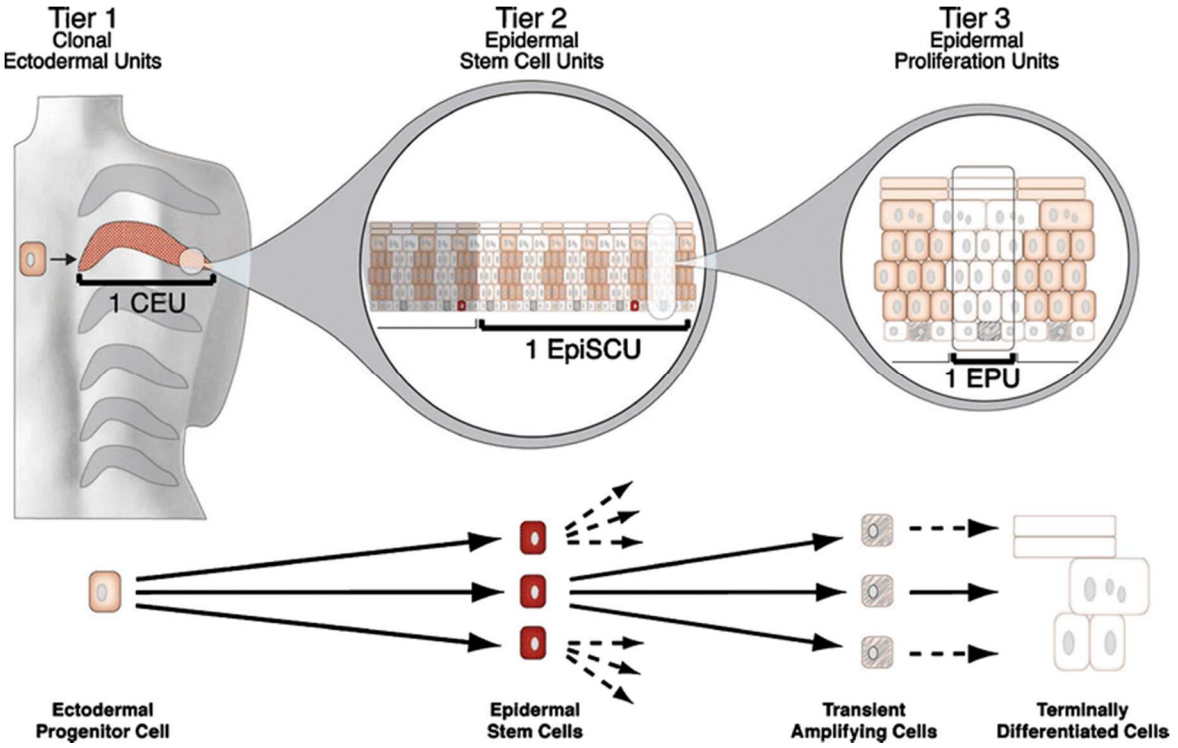

Figure 9. The three tiers model of the clonal organization of the epidermis. In the first order, ectodermal progenitors produce cutaneous cells that compose the Clonal Ectodermal Units (CEUs). In tier 2, epidermal stem cells, derived from the ectodermal progenitor cells, give rise to clonal units, called Epidermal Stem Cells (EpiSCs) Units (EpiSCUs). Third, probably transit amplifying cells derived from a descendant of EpiSCs produce the Epidermal Proliferation Units (EPUs). EPUs are considered the clonal units for the epidermal organization (Strachan LR and Ghadially R, 2008).

Therefore, many small clusters of cells form the base layer, well-organized at functional and spatial levels to guarantee the turnover of the stratum corneum. The cells produced by stem cells line migrate out of the basal layer; cells gradually change morphology

acquiring a columnar shape, in a stacked way, with a reduction in cell concentration moving closer to the surface. After an injury or a trauma that involves EPUs, cells that usually divided asymmetrically can go towards symmetrical division to repopulate the epidermis (Al-Barwari SE and Potten CS, 1976). Cells can also reorganize spatially to restore the spatial distribution of EPUs. However, while it is evident that the proliferation and organization of epidermis and the maintenance of a pool of stem cells are tightly regulated, the mechanisms have to be still understood in more detail. Even more, some researchers propose the presence of a greater number of distinct niches for skin stem cells: the epidermis, the outer part of hair follicle and the base of sebaceous glands (Fuchs E, 2008). Epidermis is constantly regenerated during the whole life of an individual by EpiSCs. The time necessary to replace epidermis with new KCs is the turnover time. It is determined by various cellular processes, such as cell density, cell proliferation, apoptosis and desquamation (Hou R *et al*, 2016). A healthy adult has an epidermis turnover time of about 30 to 79 days (Weinstein GD *et al*, 1984) After an injury, also cells of dermis contribute to the healing route (Blanpain C and Fuchs E, 2006). In psoriasis, this balanced set of mechanisms is dysregulated. It is calculated that even if stem cells cycle length does not show substantial variation, turnover time is shorter than normal of more than five times (Zhang H *et al*, 2015). It is natural to think that more EpiSCs or TA cells enter in cell cycle. Evidences confirm the involvement of both EpiSCs and TA cells in psoriasis onset. In fact, in the suprabasal layer of psoriatic patients skin two proteins, epidermal fatty acid-binding protein (FABP5), marker of TA cells, and nestin, supposed marker of EpiSCs, had been detected at higher level by immunostaining (Watarai A *et al*, 2013). Given that, psoriasis is characterized by hyperproliferation of KCs. Initially, EpiSCs and TA cells were considered the main responsible of psoriasis onset. However, it is now generally accepted that the microenvironment exerts a strong influence on stem cells proliferation and differentiation. The overexpansion of EpiSCs/TA cells that causes KCs hyperproliferation is probably induced by a first established inflammatory condition in the dermis, from which immune and growth factors are released (Jackson CJ *et al*, 2017; Jia HY *et al*, 2016; Rittié L, 2016; Nograles KE *et al*, 2008). Therefore, researches on psoriasis currently focus also on

environment surrounding epidermal stem cells and on the factors released from the dermis that reach and stimulate the layer above.

1.11. Mesenchymal stem cells: general aspects

Mesenchymal stem cells (MSCs) are self-renewal, multipotent stem cells. They can undergo asymmetric mitosis, giving rise to a cell that maintains the phenotypic characteristics of stem cells and a daughter cell committed to lineage-specific differentiation. Different sources of MSCs have been found. MSCs were isolated for the first time from bone-marrow by Friedenstein and colleagues in 1970 (Friedstein AJ *et al*, 1970). Other sources of MSCs were then identified, both from adult tissues, such as adipose tissue and skin (Ong WK and Sugii S, 2013; Orciani M and Di Primio R, 2013), and extra-embryonic tissues, including amniotic fluid, amniotic membrane and placenta (Soncini M *et al*, 2007). All MSCs isolated from different sources present common features. Based on that, the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy proposed three minimal criteria to the definition of human MSCs (Dominici M *et al*, 2006). First of all, cells have to be plastic adherent in standard culture conditions; they must have a specific immunophenotype characterized by the negativity for CD45, CD34, CD14 or CD11b, CD79α or CD19 and HLA-DR surface molecules and the expression of CD73, CD90, CD105. Third, cells must be able to differentiate *in vitro* in cells of the mesodermal lineages osteoblasts, adipocytes and chondroblasts. Even if minimal criteria are satisfied, it was debate if sources from which MSCs are isolated can affect their features and potentiality. In a study of 2014 and in the next analysis of 2016, Lazzarini and colleagues went beyond the simple evaluation of immunophenotype and differentiation potential. Researchers performed a molecular analysis based on mRNAs and miRNAs profiling of MSCs isolated from amniotic fluid, considered for their origin to have theoretically the greatest potentiality among MSCs, and skin, which contains MSCs that are believed to be the more committed and therefore less undifferentiated. Some relevant differences had been found, responsible of specific important cellular features (Lazzarini R *et al*, 2016; Lazzarini R *et al*, 2014). This and other similar researches confirm that actually MSCs are a heterogeneous group, whose features are strongly influenced by tissue origin. Nowadays MSCs are extensively studied and it is possible to have long-cultured MSCs in specific media, without abnormalities that

alter their features. On one part it is evaluated their role in the modulation of the microenvironment, on the other side MSCs are taken into consideration for cells replacement and tissue regeneration. In fact, as described below in detail, these cells can regulate the surrounding environment by releasing cytokines and factors, have immunomodulatory features and enhance angiogenesis. Even more, opportunely stimulated with factors in the media, they can differentiate into mature cells of mesodermal and non-mesodermal origin. For example, it was demonstrated their differentiation in vascular smooth muscle cells or cardiomyocytes (Lv Y *et al*, 2017; Zhang X *et al*, 2017).

Summarizing, the role of MSCs in the modulation of the microenvironment, their potential combined with the possibility to isolate them from numerous sources that not requires invasive procedures have made MSCs the subject of many studies in several areas. In particular, their involvement in the onset of various diseases or on the contrary their possible use in the field of regenerative medicine and stem cell transplant is deepening by even more increase number of researches.

1.11.1. The immune-modulatory effects of MSCs

Over the past 10 years, many researches focused on the effects of MSCs on immune system. It is now clear that MSCs are able to modulate both innate and adaptive responses. After an injury, specialized pathogen recognition receptors identify a pathogenic insult, and a signaling cascade brings to the recruitment of innate immune cells, such as neutrophils and macrophages. Probably, also MSCs are activated. It is debate if these cells migrate to the site of inflammation or exert their effect from a distance; however, various studies *in vitro* and *in vivo* demonstrated the MSCs migration (Liu H *et al*, 2012; Waterman RS *et al*, 2010; Sasaki M *et al*, 2008). The specific immunomodulation of MSCs is probably a multistage process (English K, 2013). First of all, MSCs sense the microenvironment and it is thinkable they migrate to the site of injury, where they are “licensed” or activated. This step can be induced directly by TLR stimulation or indirectly by activated macrophages. Depending on TLR signaling, MSCs become polarized towards either an anti- or pro-inflammatory phenotype, as clarified by Waterman and colleagues in 2010 (Waterman RS *et al*, 2010). TLR3 activation leads MSCs to produce indoleamine-2,3-dioxygenase (IDO),

prostaglandin E2 (PGE2), IL-4, IL-1RA, functioning with immunosuppressive effects. On the contrary, TLR4 activation of MSCs induces a pro-inflammatory phenotype with the secretion of IL-6, IL-8, TGF-β. In relation to the indirect process, the activated macrophages produce IFN-γ, TNF-α, IL-1β, that stimulate MSCs to modulate immune response (Ren G *et al*, 2008). In particular, IFN-γ, TNF-α, IL-1α or IL-1β stimulate the secretion of CXCL9 and CXCL10 by MSCs, while TNF receptor superfamily member 6 (FAS) induces the production of CCL2. CXCL9, CXCL10 and CCL2 chemoattract T lymphocytes; MSCs contact with effector T cells and produce NO or FAS/FASL to immunomodulate T cells and induce apoptosis (Akiyama K *et al*, 2012; Ren G *et al*, 2008). The hypothesis that MSCs can interact with macrophages and influence their polarization towards a M1 phenotype (pro-inflammatory) or M2 phenotype (anti-inflammatory) was confirmed by researches, thus MSCs have the ability to modulate monocyte function and alternatively activate macrophages (Cutler AJ *et al*, 2010; Kim J and Hematti P, 2009).

Briefly, depending on the stimulus received, MSCs are able to promote pathogen clearance or damp immune system (Fig. 10).

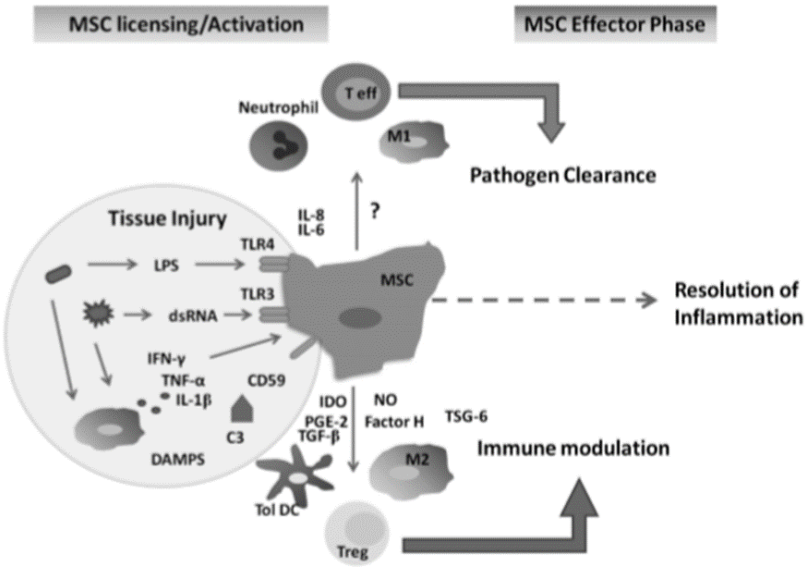

Figure 10. The participation of MSCs in inflammation. MSCs can be activated directly through TLR stimulation or indirectly by macrophages. Therefore, depending on the stimulus received, MSCs can promote pathogen clearance or regulate the immune modulation through the secretion of specific factors (English K, 2013).

To date, the known mechanisms through which MSCs promote pathogen clearance are the secretion of pro-inflammatory cytokines IL-6 and IL-8, the polarization of macrophages in the pro-inflammatory M1 phenotype, the anti-microbial activity and the enhanced neutrophils survival and activity. In alternative, MSCs deaden the immune response through the secretion of the immunosuppressive soluble factors such as IDO, PGE-2, TGF-β, NO, TSG-6 and complement factor H; even more, MSCs support the polarization of macrophage towards anti-inflammatory M2 phenotype, the induction of tolerogenic DCs and the generation of Treg (English K, 2013).

In conclusion, the researches made in the last 10 years enforce the concept that MSCs have sentinel functions allowing them to evaluate the microenvironment and promote or inhibit immune response. Because of local environment is fundamental for establish the MSCs activity, it is necessary to assess each particular environment to clarify the cascade of events and the specific mechanisms of MSCs immune modulation.

1.11.2. The interaction of MSCs with immune cells

Several studies pointed the attention on the interaction of MSCs with the cellular components of the innate and adaptive immune response, such as T lymphocytes, B lymphocytes, NK cells and DCs (Fig. 11).

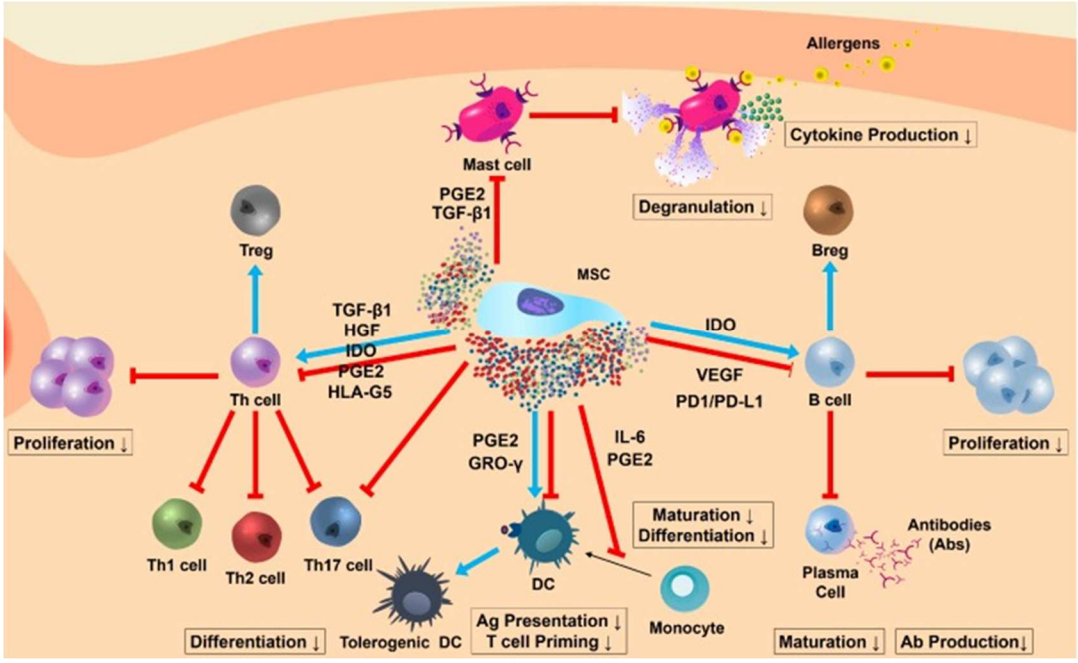

Figure 11. The mechanisms of MSCs immunomodulation towards principal cells of immune system. Red line: suppressive effect; blue line: stimulatory effect (Shin TH *et al*. 2017).

Concerning T lymphocytes, MSCs can interact with their activation, proliferation and differentiation. However, the exact role of MSCs in T lymphocytes activation is controversial: some researches observed a reduce stimulus to activation in presence of bone marrow-MSCs (Groh ME *et al*, 2005; Le Blanc K *et al*, 2004), while others evidenced no difference in activation in presence or not of MSCs (Ramasamy R *et al*, 2008). The effect of MSCs on the secretion of cytokines by activated T lymphocytes is also object of debate: MSCs can diminish (Ramasamy R *et al*, 2008) or increase (Castro-Manrreza ME *et al*, 2014) IFN-γ secretion by activated T lymphocytes. On the contrary, more homogeneity is related to the role of MSCs in T lymphocytes proliferation: several studies confirmed a capacity of MSCs to reduce it (Castro-Manrreza ME *et al*, 2014; Kronesteiner B *et al*, 2011; Ryan JM *et al*, 2007; Le Blank K *et al*, 2004). MSCs action takes place through secreted factors, between them TGF-β1, hepatocyte growth factor (HGF), IDO, PGE2, IL-10, HLA-G5, and contact dependent mechanisms by means of programmed death-ligand 1 (PD-L1) and HLA-G1. MSCs affect also the differentiation and function of inflammatory T lymphocytes populations, in particular in relation to their capacity to produce pro-inflammatory cytokines and in the induction of a Treg phenotype. In fact, in favorable conditions to differentiation of naïve T cells in Th1 or Th2, the presence of MSCs inhibits IFN-γ secretion by Th1 cells and increases IL-4 secretion by Th2 cells (Aggarwal S and Pittenger MF, 2005). Even more, MSCs was demonstrated to be able to inhibit the production of pro-inflammatory cytokines such as IL-17 and IL-22 preventing the differentiation of naïve $CD4^+$ lymphocytes in Th17. They also probably promote the differentiation towards Tregs by the secretion of IL-10 and the expression of the transcription factor Foxp3 (Ghannam S *et al*, 2010).

DCs are the most important antigen presenting cells in the body; MSCs can act on their recruitment, maturation and function. In particular, they can reduce the *in vitro* monocytes differentiation into DCs, but, interestingly, it is a reversible process, because the removal of stem cells allows the differentiation. Even more, MSCs favor the maintenance of DCs in an immature state by reducing the level of the maturation marker CD83 and of the costimulatory molecules CD80 and CD86 (Jiang XX *et al*, 2005). Several cytokines, important

for DCs maturation, are secreted at lower levels in presence of MSCs (Aggarwal S and Pittenger MF, 2005; Zhang W *et al*, 2004).

Researches on MSCs action were also conducted in relation to NK cells and B lymphocytes. IL-15 promotes proliferation, survival and function of NK cells, but MSCs can inhibit the proliferation induced by this cytokine (Sotiropoulou PA *et al*, 2006). Even more, activated NK cells by IL-2 secrete less IFN-γ in presence of MSCs (Aggarwal S and Pittenger MF, 2005). However, MSCs influence on NK cells is possible only if there is a direct contact between the two populations. About B lymphocytes, MSCs diminish B cell proliferation by cell cycle arrest in the G0/G1 phase, and can change the chemokines receptors expression (Corcione A *et al*, 2006). All these studies highlight that MSCs have immune-regulatory properties, exercised through direct cellular contact and/or secretion of soluble factors. As previously mentioned, the most known factors involved are TGF-β1, HGF, IDO, PGF2, Galectin-1, a series of membrane molecules such as PD-1/PD-L1 pathway, HLA-G1, Jagged-1, and the adhesion proteins intercellular adhesion molecule 1 (ICMA-1) and the vascular cell adhesion molecule 1 (VCAM-1). An inflammatory environment stimulates the MSCs immune-regulatory properties. T lymphocytes, macrophages, NK cells produce in turn pro-inflammatory cytokines such as IFN-γ, TNF-α, IL-1α, IL-1β, IL-17, thus there is a bidirectional interaction between MSCs and immune cells (Castro-Manrreza ME and Montesinos JJ, 2015). However, as it is previously described (Paragraph 1.11.1.), it was demonstrated that MSCs can be polarized to anti-inflammatory or pro-inflammatory phenotype: TLR3 in MSCs induces an anti-inflammatory phenotype through the activation of IL-10 and IL-12, while TLR4 supports the secretion of pro-inflammatory cytokines (Waterman RS *et al*, 2010; Tomchuck SL *et al*, 2008).

1.11.3. The pro-angiogenic capacity of MSCs

MSCs can promote angiogenesis by regulating the expression of VEGF. This process is stimulated by inflammatory cytokines, in particular IFN-γ and TNF-α (Liu Y *et al*, 2011; Crisostomo PR *et al*, 2008). However, it can not to be excluded that also other pro-inflammatory cytokines are involved. In 2008, Wang and colleagues evidenced that MSCs secrete VEGF after TGF-α stimulation through MEK- and PI3K- but not JNK- or ERK-

dependent mechanisms (Wang Y *et al*, 2008). In addition, an overexpression of stromal-derived factor 1 (SDF-1) by MSCs promotes angiogenesis (Tang J *et al*, 2009).

VEGF is an essential angiogenic molecule; however, its role is not limited to this. In fact, VEGF can regulate the synthesis of multiple angiogenic factors, such as angiogenin, IL-6, IL-8, TGF-β1, MCP-1 in an autocrine way (Liu Y *et al*, 2011; Niu J *et al*, 2008). VEGF can also chemoattract pDCs in psoriatic lesions, acting as an inflammation promoter (Suzuki T *et al*, 2014). Followed specific stimuli, VEGF augments IFN-γ and inhibits IL-10, promoting a Th1 phenotype; thus, this factor can increase T cell differentiation in a pro-inflammatory way (Mor F *et al*, 2004). All these activities explain the importance of defining the ways through which MSCs can regulate VEGF.

1.12. MSCs connection with psoriasis

Skin stroma contains a population of plastic adherent cells, with the same specific immunophenotype of MSCs, confirming the mesenchymal lineage, and the same differentiation potential (Vishnubalaji R *et al*, 2012). Cells with these features have been isolated also from injured skin such as that of patients affected by atopic dermatitis (AD) and psoriasis (Orciani M *et al*, 2017; Orciani M *et al*, 2011). Many new studies focus on MSCs influence on skin microenvironment, evaluate MSCs in the lesions and try to analyse the role that these cells can have in skin disease onset and/or progression.

1.12.1. MSCs in psoriatic lesions: state of art

Many researches studied the differentiated cells of psoriatic skin and focused on cytokines and growth factors over-expression that influence them and contribute to establish their altered state in the disease; however, until less than ten years ago, no data were available about MSCs isolated from skin of psoriatic patients. Orciani and colleagues in 2011 compared the psoriatic dermal MSCs with the normal dermal MSCs. Biopsies were made on the lesional skin, the perilesional skin and on a site 2 cm far from the nearest cutaneous lesion (called as non-lesional skin) of psoriatic patients, while only one biopsy was made on skin of healthy people as control. Also patients with AD, as a Th2 paradigm of skin disease, opposed to psoriasis, considered as a Th1/Th17 disease model, were included in the study. Researchers demonstrated that cells from psoriatic lesions hyper-proliferate compared to

the other cell groups; this is not surprising and confirms that the faster proliferation of differentiated cells in psoriasis is related to an increase proliferation of undifferentiated cells. Expressions of VEGF and inducible nitric oxide synthase (iNOS) were also evaluated, since proteins previously demonstrated over-expressed in lesions. Interestingly, the highest immunohistochemical expression of VEGF was found in perilesional-MSCs, in accordance with the fact that, during psoriasis development, one of the earliest detectable histological modifications is the formation of blood vessels within dermal papillae. On the contrary, iNOS was more expressed in lesional skin. Even more, MSCs from psoriatic lesions showed a lower antioxidant capacity (Orciani M *et al*, 2011). Therefore, MSCs interact with the microenvironment in lesional skin and produce angiogenic and pro-inflammatory mediators, significantly contributing to the pathophysiology of the disease.

TNF-α, as previously described strictly involved in establishing the inflammatory state in psoriasis (Paragraph 1.5.2.), was demonstrated to act also on MSCs and induce an increase of MSCs proliferation and human MSCs VEGF, fibroblast growth factor (FGF), HGF and insulin-like growth factor1 (IGF-1) production (Böcker W *et al*, 2008; Crisostomo PR *et al*, 2008). Because use of TNF-α inhibitors reduces KCs proliferation rate (Markham T *et al*, 2006) and ameliorates psoriasis symptoms, the specific effects of anti-TNF-α treatments on MSCs isolated from psoriatic lesions have been deepened. Campanati and colleagues in 2012 evaluated VEGF expression and production, nitric oxide (NO) production, iNOS expression and antioxidant response in MSCs isolated from lesional, perilesional and non-lesional skin of psoriatic patients before and after 12 weeks of treatment with TNF-α inhibitors adalimumab and etanercept. Both biological therapies were able to reduce expression and production of VEGF and NO in psoriatic-MSCs. Also the percentage of MSCs expressing iNOS was reduced. Anti-TNF-α treatments, with particular reference to adalimumab, were able to induce superoxide dismutase (SOD) activity helping to rebalance the pro-oxidant pressure and the oxidative damage in psoriasis. Therefore, the TNF-α inhibitors have an effect not only on the differentiated compartment related to psoriasis but also at level of MSCs (Campanati A *et al*, 2012).

In 2014, a new step forward was made to define MSCs profile in psoriasis. MSCs from psoriatic skin lesions have a greater expression of many of the genes encoding for Th1 and

Th17 cytokines, over-expressed also in differentiated cells in psoriasis, compared to healthy skin MSCs; no significant variation is present for the most part of the genes encoding for Th2 cytokines. This imbalance between Th1, Th17 and Th2 pathways in MSCs isolated from psoriatic people reflects the alterations found in differentiated psoriatic skin cells; the data seem indicate MSCs are psoriasis committed. However, not all the genes encoding for Th1 and Th17 cytokines known to be over-expressed in differentiated cells from psoriasis lesions change their expression also in MSCs (Campanati A *et al*, 2014). Different hypotheses have been made in this regard, trying to explain the differences: genes involved in the physiopathology of psoriasis can be regulate at different timing, or it is possible that the time required for *in vitro* culture reduced the minor differences.

Other researches confirmed the early involvement of MSCs in psoriasis. In fact, an altered expression in MSCs isolated from psoriatic patients compared to healthy subjects was also found for genes involved in the pro-angiogenic potential and immuno-regulatory effect. In particular, the angiogenesis related genes VEGF-A, angiopoietin, AMOT, EDIL3 are up-regulated in MSCs of psoriatic patients while it was noted a downregulation of platelet endothelial cell adhesion molecule-1 (PECAM1), facio-genital dysplasia-5 (FGD5) and prostaglandin-endoperoxide synthase-1 (PTGS1) (Niu X *et al*, 2016a; Niu X *et al*, 2016b; Hou R *et al*, 2014b). The new findings in support of the hypothesis that MSCs may promote angiogenesis and increase endothelial cells migration and proliferation make MSCs crucial to psoriasis onset and progression. Other studies followed to deepen the dysregulated expression of psoriatic-MSCs related genes (Niu X and Zhang K, 2016; Chang WJ *et al*, 2015). In addition, MSCs isolated from psoriatic skin patients have alterations at epigenetic level. They have a different genome-wide promoter methylation profile compared to MSCs derived from normal skin. The aberrant methylated state involves also many genes coding for proteins related to cell communication and migration, cellular response to stimulus and surface receptor signaling pathway. Some genes linked with epidermal proliferation, angiogenesis and inflammation with an alteration on the degree of methylation were demonstrated to be differently expressed in psoriatic patients compared to healthy people. Among them, the co-receptor for VEGF and pro-angiogenesis factor neuropilin 2 (NRP2) and the VEGF-induced negative feedback angiogenesis regulator vasohibin 1 (VASH1) are

hypomethylated and thus expressed at high level in psoriasis. The S100 family proteins, such as S100A7, S100A8 and S100A9, are differently methylated in psoriasis compared to healthy skin, and are proposed to have a role in KCs differentiation; also S100A10, which is related to migration of monocytes/macrophages, is hypomethylated and consequently over-expressed (Hou R *et al*, 2013). Further researches are necessary for a detailed clearance of this altered methylation state.

In conclusion, the connection between MSCs and inflammation is still object of several studies but researches already done confirm that MSCs of psoriatic people show a pro-inflammatory phenotype and promote local inflammation; even more, they stimulate expansions of blood vessels facilitating the angiogenesis process.

1.12.2. Bone marrow-MSCs in psoriatic patients

In psoriasis, the hematopoietic microenvironment of the bone marrow is also altered. MSCs isolated from bone marrow (BM-MSCs) of psoriatic patients present aberrant cytokines expression compared to that of healthy people, with an over-expression of stem cell factor (SCF), G-CSF, and IL-6, and a down-regulation of TNF-α, IL-1, IL-3, IL-8, leukemia inhibitory factor (LIF), HGF and platelet-derived growth factor (PDGF) (Zhang K *et al*, 2010). The datum that levels of secreted cytokines from BM-MSCs are not correlated to PASI suggests that the noted alterations are probably provoke by the inflammatory reaction of the body. In addition, most of the unbalanced cytokines regulate hematopoietic factors, thus are important for hematopoietic homeostasis. BM-MSCs from psoriatic patients show also an atypical proliferation activity and an increase of apoptosis (Hou R *et al*, 2014a). The data enforce the idea that another mechanism that drives psoriasis progression is an aberrant hematopoietic microenvironment.

Summarizing, according to the studies and their findings, MSCs are strictly involved in psoriasis. In particular, their influence is applied through three main mechanisms: the promotion of inflammatory cytokines secretion, the stimulation of angiogenesis and the decrease of the immunosuppressing and antioxidant activities.

2. SCOPE OF THE THESIS

MSCs reside in many adult tissues, and it is now well accepted that they can be involved in various pathologies. MSCs were found and successfully isolated from skin of psoriatic patients but their role in psoriasis pathophysiology needs more clarifications. Previous research of our group found an imbalance between cytokines of Th1/Th17 and Th2 pathways in MSCs of psoriatic patients, reflecting the alterations existing in differentiated skin cells. Based on obtained results, that suggest an early involvement of MSCs in disease onset, current research has the purpose to evaluate the effectiveness of biological therapies currently in use. In fact, if MSCs are strictly related to psoriasis, therapies that affect only differentiated cells are ineffective to arrive to the crux of the disease, on the contrary the staminal component must be stricken. Even more, present work focuses on two other aspects not yet known. On one hand, healthy MSCs exhibit immunomodulatory, tissue protective and repair-promoting properties; therefore, MSCs transplantation is considered a promising treatment strategy for various diseases, but many information for the purpose of their use is still lacking. On the other hand, few data are available about the influence of psoriatic immunodysregulated MSCs on KCs; the possibility that MSCs stimulate proliferation of differentiated skin cells and induce a pathological KCs profile has to be deepened. Briefly, to address these questions, MSCs were isolated from skin of psoriatic patients (PSO-MSCs) before and after treatment with TNF-α inhibitor adalimumab or etanercept and from healthy people (H-MSCs). Cells were characterized following minimal criteria of Dominici. Then, selected genes and secreted proteins related to inflammation were investigated on MSCs before and after treatment and compared to those of H-MSCs. To evaluate the effect of not inflamed MSCs on psoriasis, non-treated PSO-MSCs were indirectly co-cultured with H-MSCs. The proliferation of PSO-MSCs and the expression of 12 cytokines linked to Th1/Th17 and Th2 pathways in the secretome were evaluated before and after co-culture. In addition, the influence of PSO-MSCs on KCs was investigated by performing an indirect co-culture of PSO-MSCs with a KCs cell line, the HaCaT cells. In this case, individually or co-cultured HaCaT were counted; the same cytokines of Th1, Th2 and Th17 groups previously considered were analysed on the conditioned medium. The KCs cells cultured alone were used for comparison purpose.

3. MATERIALS AND METHODS

3.1. Patients recruitment and skin collection

15 patients affected by stable psoriasis (5 woman, 10 men; mean±standard deviation (SD) age 50,69±13,41 years) were enrolled in our study. All the patients had a moderate to severe plaque psoriasis (PASI - mean±SD: 14,23±2,87) (Table 1). Diagnosis of disease severity was made following the rule of 10: BSA > 10, PASI > 10, DLQI > 10. Two independent trained dermatologists calculated the Psoriasis Area and PASI indexes. The derived mean was used to classify the disease severity. No patient presented symptoms or signs related to psoriatic arthritis. Enrolled subjects were also not affected by guttate, erythrodermic, generalized pustular psoriasis or palmoplantar pustolosis, or by other inflammatory and immune-mediated diseases. All the patients were free from any systemic conventional treatments that could interfere with cutaneous expression of psoriatic lesions, such as PUVA therapy, methotrexate and cyclosporine for at least 12 weeks. All the people accepted to avoid sun or UVB and UVB-NB exposure for at least 4 weeks before the recruitment. Because of acitretin has a long tissue accumulation time, subjects who received this drug in the last 2 years were not included in the study. Moreover, individuals who were no naïve to biological treatments were excluded. Enrolled patients were randomly divided in two groups: 7 subjects were treated with etanercept 50 mg/bw for 12 weeks (Eta-treated); the other 8 received adalimumab, according to the recommended induction and maintenance system for plaque type psoriasis treatment (Ada-treated). In detail, in the last case, it was prescribed an initial dose of 80 mg/bw for the first week, and then 40 mg/bw for every other week. Disease severity was evaluated at baseline (T0) and after 12 weeks of TNF-α inhibitors treatment (T12), according to PASI and DLQI scores. After 12 weeks of TNF-α inhibitor administration, patients reached a PASI 75. All psoriatic patients received skin biopsy into lesional and perilesional skin with a sterile device of 5 mm in diameter (Gima, medical devices, s.r.l. Rome, Italy) after local anesthesia with lidocaine 2%. 15 people (7 woman, 8 men; mean±SD age 53,15±11,27 years) that not showed skin lesions, not apparently affected by other pathologies and who underwent surgical procedure for cutaneous benign lesions removal were also considered in our study. All the patients read and signed informed consent. This study was approved by Università

Politecnica delle Marche Ethical Committee and conducted in accordance with the declaration of Helsinki.

3.2. Isolation of mesenchymal stem cells

Skin samples were broken mechanically into small pieces and lacerated with scalpels. They were transferred into six-well plates with Mesenchymal Stem Cell Growth Medium (MSCGM, Lonza, Basel, Switzerland). Samples were incubated at 37°C and 5% CO_2. A phase-contrast microscopy (Leica DM IL; Leica Microsystems GmbH, Wetzlar, Germany) was used to evaluate cells morphology. Vitality and proliferation rate were assessed using an automatic cell counter (Invitrogen, Milan, Italy). The doubling time (DT) was calculated with an algorithm available online (http://www.doubling-time.com) as $DT=t\times lg2/(lgN_t-lgN_0)$, where N_0 is the number of inoculated cells, N_t is the number of harvested cells, t is the culture time expressed in hours.

3.3. Characterization of mesenchymal stem cells

5 random samples from psoriatic patients and the same number from the control group were characterized following the minimal criteria of Dominici for the isolation of human MSCs (Dominici M *et al*, 2006). All the samples were at third passage and at a confluence of about 80%. First of all, a cytofluorimetric analysis was performed to evaluate the immunophenotype of cells. The following surface markers were analysed:

- HLA-DR: distinctive of major histocompatibility complex, class II;
- CD14: marker for monocytes and macrophages;
- CD19: biomarker for normal and neoplastic B cells;
- CD34: marker for hematopoietic progenitor cells and endothelial cells;
- CD45: leukocyte common marker, expressed on almost all hematopoietic cells except for mature erythrocytes;
- CD73 and CD105: markers of mesenchymal and/or nerve cells, but not of ESCs;
- CD90: marker of mesenchymal stem cells;
- CD9: marker expressed in MSCs at significantly lower level than in fibroblasts, used to differentiate MSCs from fibroblasts.

In detail, 3 x 10^6 cells were washed with phosphate-buffered saline (PBS; Corning, New York, United States), centrifuged at 13000 rpm at 4°C and resuspended in RIA solution, made by bovine serum albumin (BSA) 0,2% in PBS. Cells were then divided in tubes and stained with fluorescein isothiocyanate-conjugated antibody (Becton Dickinson, Franklin Lakes, NJ, USA) against HLA-DR, CD14, CD19, CD34, CD45, CD73, CD90, CD105, CD9 for 30 minutes at 4°C. After two washes, cells were suspended in PBS and flow cytometry was performed with a Becton Dickinson FACScan instrument. Data acquisition was carried out with CellQUEST software (Becton Dickinson).

Then, the differentiation potential into osteogenic, adipogenic and chondrogenic lineages *in vitro* was evaluated. In particular, for the osteogenic and adipogenic differentiation, 5 x 10^4 cells were seeded in two-wells chamber slides. Cells were allowed to adhere for 24 hours; then, medium was replaced with STEMPRO® Osteogenesis and Adipogenesis Differentiation Kit (Gibco, Grand Island, NY, USA), respectively. For chondrogenesis differentiation, a pellet of 5 x 10^5 cells was prepared and maintained at 37°C in STEMPRO® Chondrogenesis Differentiation Kit (Gibco). Cells cultured in DMEM/F12 (Corning) with 10% FBS were used as negative control. All media were replaced twice a week.

The osteogenic differentiation was assessed by alkaline phosphatase (ALP) reaction after 7 days, and by Von Kossa staining after 14 days. In more detail, to evaluate levels of ALP in differentiated MSCs, cells were washed twice with PBS. A solution was prepared by adding 1 ml TRIS 1 M, 1 ml NaCl 1 M, 50 µl $MgCl_2$ 1 M, 8 ml deionized water, 66,6 µl NBT (nitroblue tetrazolium) stock solution, 33,3 µl BCIP (5-bromo-4-chloro-3-indolyl phosphate) stock solution and 10 µl of levamisole 100 mM; it was dispensed to cells at room temperature. Solution was left from 30 minutes to 24 hours, until it became darker, and cells washed with PBS-EDTA 20 mM. Chamber slides were dehydrated with subsequent steps in ethanol at increasing concentrations up to the absolute alcohol, then clarified with two xylene passages, mounted with balm and cover slide. For the Von Kossa staining, cells were washed twice and fixed in formalin 4% in PBS for 30 minutes. After two washes in deionized water, cells were covered with a silver nitrate solution at 5% in water and exposed to light for 1 hour. Cells were washed and a pyrogallic acid solution 1% was added for 3 minutes; finally, after two washes, cells were covered with sodium thiosulfate at 5% for 1 minutes,

then washed again. The chamber slides were dehydrated, clarified and mounted, as previously described.

Related to lipid vacuoles typical of adipocytes, they were highlighted by Oil Red staining after 14 days of culture. In particular, a Red Oil stock solution 0,5% (w/v) in isopropanol was diluted in deionized water in the ratio of 60% to 40%. After 10 minutes, solution was filtered in 0,22 µM pore membrane. Meanwhile, cells were washed twice with PBS and fixed in formalin 4% for 30 minutes. After washing, isopropanol at 60% in deionized water was added and cells incubated for 5 minutes. Then, cells were covered by the filtered Oil Red work solution and moved slowly on plate shaker for 30 minutes at room temperature in the dark. Cells were washed with water, nuclei counterstained with hematoxylin and immediately photographed.

Lastly, to verify the chondrogenic differentiation, after 21 days of maintenance in chondrogenic specific medium, the pellet was fixed in paraformaldehyde 4% for 24 hours; after that, it was conserved in phosphate buffer pH 7,4 for at least other 24 hours. Pellet was then subjected to steps with increasing ethanol concentrations (50% to 100%), two xylene passages for 1 hour each, finally included in paraffin and cut with microtome in 4 µm sections. Sections were left to dry for one night and stained with Safranin O. For the staining, firstly a stock solution of Safranin O 1% (w/v) in alcohol 95% was prepared, and diluted in deionized water 1:1 to have the work solution. The sections were treated with three changes in xylene for 10 minutes, and rehydrated with subsequent steps in alcohol at decreasing concentrations up to alcohol 70%. After wash with water, sections were stained with Safranin O work solution for 20 minutes. Lastly, sections were washed with alcohol 95% for 20 seconds, dehydrated in absolute alcohol, clarified in xylene and mounted.

3.4. HaCaT cells culture

HaCaT cells were purchased by AddexBio (San Diego, USA). Cells were cultured in DMEM/High glucose medium (Corning) with 10% FBS at 37°C, 5% CO_2.

3.5. Indirect co-culture conditions

Untreated PSO-MSCs at 4-6 passage were used for co-culture experiments with H-MSCs or HaCaT cells.

For the co-culture of PSO-MSCs with H-MSCs, 2,2 x 10^5 PSO-MSCs were seeded in 6-well plate; the same amount of H-MSCs were plated in 0,4 µm pore membrane inserts. All samples were maintained in DMEM/F12 10% FBS. After 24 hours of incubation to allow cells to adhere, medium in 6-well plate was replaced with fresh one; inserts were transferred in wells with plated PSO-MSCs in a total medium volume of 3 ml and co-cultured for 72 hours at 37°C. Conditioned medium was then collected as later described; cells were detached and counted, a cell pellet was made and stored at -80°C. PSO-MSCs cultured alone were used as mock for control purpose.

For the co-culture of PSO-MSCs with HaCaT, the same amount and condition were maintained except that HaCaT were plated in the 6-well plate and PSO-MSCs were seeded into inserts with 0,4 µm pore membrane. Control was represented by HaCaT cultured alone.

3.6. Conditioned medium preparation

Conditioned medium was collected from all PSO-MSCs at baseline (T0) and after 12 weeks of treatment with TNF-α inhibitors (T12), H-MSCs and HaCaT cultured alone and from co-cultured cells. A ratio of 7,3 x 10^4 cells/1 ml for each group was considered; individually or co-cultured cells were incubated at 37°C, 5% CO_2 with the culture medium DMEM/F12 10% FBS. After 72 hours of culture or co-culture conditions, medium was collected, centrifuged at 1000 rpm for 5 minutes and filtered in 0,22 µM pore membrane. Conditioned medium was stored at -80°C until use.

3.7. PCR Array

PSO-MSCs T0 and T12 were investigated for the expression of selected cytokines belonging to the Th1 (IL-6, IL-8, IL-12, IL-23A, IFN-γ, TNF-α), Th2 (CCL1, IL-2, IL-3, IL-4, IL-5, IL-13, TGF-β) and Th17 (CCL2, CCL20, CXCL2, CXCL5, IL-17A, IL-17C, IL-17F, IL-21, G-CSF) pathways by PCR Array. H-MSCs were used as control. Both MSCs at T0 and T12 and H-MSCs at 3rd passage were detached and a pellet of about 5 x 10^5 cells was made. Total RNA was isolated

by using PerfectPure™ RNA Cell & Tissue kit (5 PRIME, Hamburg, Germany). In detail, 200 µl of lysis solution was added to the frozen cell pellets, and cells vortexed vigorously for 2 minutes, then centrifuged at 13000 rpm for 1 minute. Each lysate was pipetted onto the purification column supplied by the kit, and centrifuged again. Solution contained residual of broken cells crossed the membrane in the column while RNA adhered to the resin. The column was washed by adding 400 µl of Wash 1 Solution. After centrifugation, the purification column was transferred to a new collection tube, and a DNase treatment was made by adding 50 µl of a DNase solution. Column was incubated for 15 minutes at room temperature, then washed twice with specific DNase wash solution. After 2 minutes of centrifugation, a new step of washing was made by using Wash 2 Solution. Lastly, the purification column was transferred to a new collection tube, 50 µl of elution solution was added and column centrifuged for 1 minutes. RNA was eluted into the collection tube, so purification column was discarded. RNA was quantified by using NanoDrop™ 2000 (ThermoFisher Scientific, Waltham, MA, USA). 1 µg of RNA of each sample was retro-transcribed using SABioscences RT^2 First Strand kit (Qiagen, ThermoFisher, Waltham, MA, USA). Therefore, a first step of genomic DNA elimination was made: buffer GE and water were added to RNA to a final volume of 10 µl. The mix was incubated at 42°C for 5 minutes, then placed on ice. A 10 µl solution of a reverse transcription mix prepared with a buffer, an internal control, a reverse transcriptase mix and RNasi free water was added. The samples were incubated again at 42°C for 15 minutes and the reaction stopped after 95°C for 5 minutes. Finally, 91 µl of RNasi free water was added to the obtained cDNA. Subsequently, Real-Time PCR (RT-PCR) was performed using a RT^2 Profiler Custom PCR Array (Qiagen). The RT^2 SYBR Green Mastermix was added to the cDNA synthesis reaction. 25 µl of the PCR components mix was dispensed in each well of the PCR Array plate using a 8-channel pipettor. Plate was incubated at 95°C for 10 minutes, then fluorescence data were collected by performing 40 cycles consisting of the following phases: a denaturation step at 95°C for 15 seconds, an annealing step at 62°C for 35 seconds, an extension part at 72°C for 30 seconds. At the end of the reaction, the internal controls GDC, detecting non-transcribed genomic DNA contamination, RTC, testing the efficiency of the reverse transcription reaction, and PPC, referring to the efficiency of the polymerase chain

reaction, were considered and reaction accepted if the controls fell in the established parameters. Both glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and the ribosomal protein large P0 (RPLP0) were used as housekeeping genes. RPLP0 gave the most consistent result and was therefore used for normalization analysis. Quantification of mRNA expression was calculated with the $2^{-\Delta\Delta Ct}$ method. The parameter threshold Cycle (Ct) is by definition the cycle number at which the first detectable increase in fluorescence above the threshold line was observed. ΔCt was calculated as Ct (gene of interest) − Ct (housekeeping gene) and ΔΔCt = ΔCt (PSO-MSCs T0 or T12) − ΔCt (H-MSCs) or ΔΔCt = ΔCt (PSO-MSCs T12) − ΔCt (PSO-MSCs T0). Controls were then considered as 1, and PSO-MSCs T0 or T12 accordingly calculated as X-fold. The values of the relative gene expression are reported as mean±SD over three independent experiments.

3.8. ELISA array analysis

Human cytokines belonging to Th1/Th17 and Th2 pathways secreted on the conditioned medium were studied by ELISA array with Multi-Analyte ELISArray Kit (Qiagen) following company instructions. In more detail, 50 µl of work solution assay buffer was added into each well of the ELISArray plate. The same volume of the appropriate sample dilution buffer was added into each well of Row A to set up the negative control; 50 µl of each experimental sample was dispensed in the respective rows and finally 50 µl of the antigen standard cocktail appropriately prepared and diluted was put into each well of row H to set up the positive control. Plate was incubated for 2 hours. Just before use, detection antibodies were diluted in the assay buffer and gently mix. Wells were washed three times and 100 µl of the diluted detection antibodies were added. Plate was incubated for 1 hour. After washing, avidin-HRP prepared in assay buffer was dispensed and plate one more time incubated for 30 minutes at room temperature in the dark. Lastly, after washing, a substrate solution was added to detect captured cytokines. Absorbances were read at 450 nm within 30 minutes of stopping the reaction, using a microtiter plate reader (Multiskan Go microplate reader, Thermo Scientific, Monza, Italy). Absorbances of samples cytokines were then compared to those of the standard antigens. Each sample was experimented three times. The determined concentrations are expressed in pg/ml.

3.9. VEGF quantification

PSO-MSCs treated with etanercept were also investigated to evaluate VEGF levels in the conditioned medium. Secretomes of lesional and perilesional PSO-MSCs at T0 and T12 were collected as previously described. VEGF was quantified using a commercial enzyme immunoassay kit (human VEGF TiterZyme® EIA kit; Assay Designs, Ann Arbor, MI, USA), following manufacturer instructions. Briefly, standards and samples were pipetted in duplicate into the wells; after 2 hours incubation, VEGF-conjugated antibody was added. Plate was incubate for 1 hour. After washing, a substrate solution was added to detect captured molecules. Absorbances were read at 450 nm using the microtiter plate reader. A standard curve was created with obtained values of the standards and samples concentrations accordingly calculated. Results are expressed as pg/ml.

3.10. Statistical analysis

Data were analysed using GraphPad Prism (version 5.0, El Camino REAL, San Diego, CA, USA) and QuickCalcs software package. All data were expressed as means±SD. The distribution of continuous variables was verified with Kolmogorov-Smirnov test. The significance of the variations regarding mRNA expression between samples was evaluated by performing a paired-sample *t*-test or nonparametric Wilcoxon signed ranks tests, depending on the normal distribution of the means tested by the Shapiro-Wilk test. For the analysis of VEGF quantification, the homogeneity of variance was established using Cochran's C test; post hoc comparison (Newmann-Keuls) was employed to discriminate between means of values and, when necessary, the nonparametric Mann-Whitney *U*-test was used. For all the analyses a *p*-value less than 0,05 was considered to be statistically significant.

4. RESULTS

All subjects included in the study (patients affected by psoriasis and healthy controls) were similar for age, sex and race. No statistically relevant difference was found in each cellular group among the donors. Therefore, results are reported as mean±SD for patients with psoriasis or for healthy subjects in each analysis.

4.1. Isolation and characterization of MSCs

For all the samples put in 6-wells plate obtained from psoriatic patients, at baseline or after treatment, and from healthy subjects the first cells were visible near to the explants after about 7 days of culture. All the samples showed the typical mesenchymal stem cells fibroblast-like morphology at phase contrast microscopy (Fig. 12).

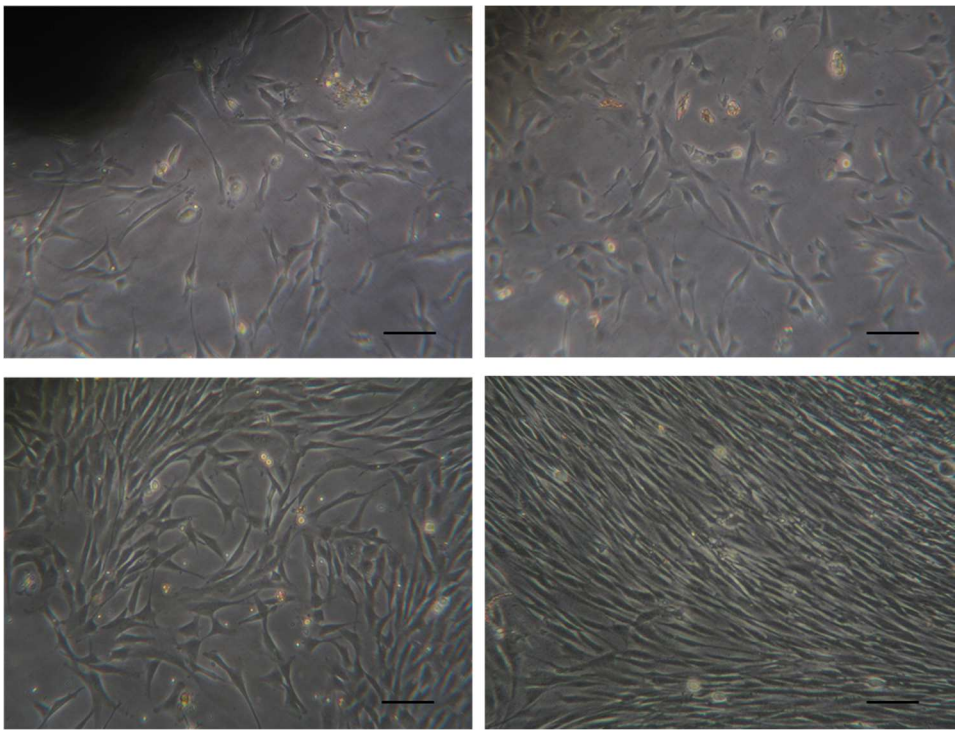

Figure 12. Morphology of cells isolated from skin biopsies at different time of culture by phase contrast microscopy. Cells isolated from healthy people and psoriatic patients showed the same fibroblast-like morphology. *Scale bar* 100 µm.

Cell growth was monitored from the first up to the twelfth passage and the DT was calculated. PSO-MSCs at baseline presented a higher proliferation rate than H-MSCs, with a DT equal to 12±2 hours for PSO-MSCs T0 and 19±2 hours for H-MSCs for the first 8 passages. Then, in both cases DT gradually increased up to passage 12 to about 36±1 hours for PSO-MSCs T0 and about 39±2 hours for H-MSCs. Cells after both adalimumab or etanercept treatments revealed a DT comparable to that of H-MSCs (Fig. 13).

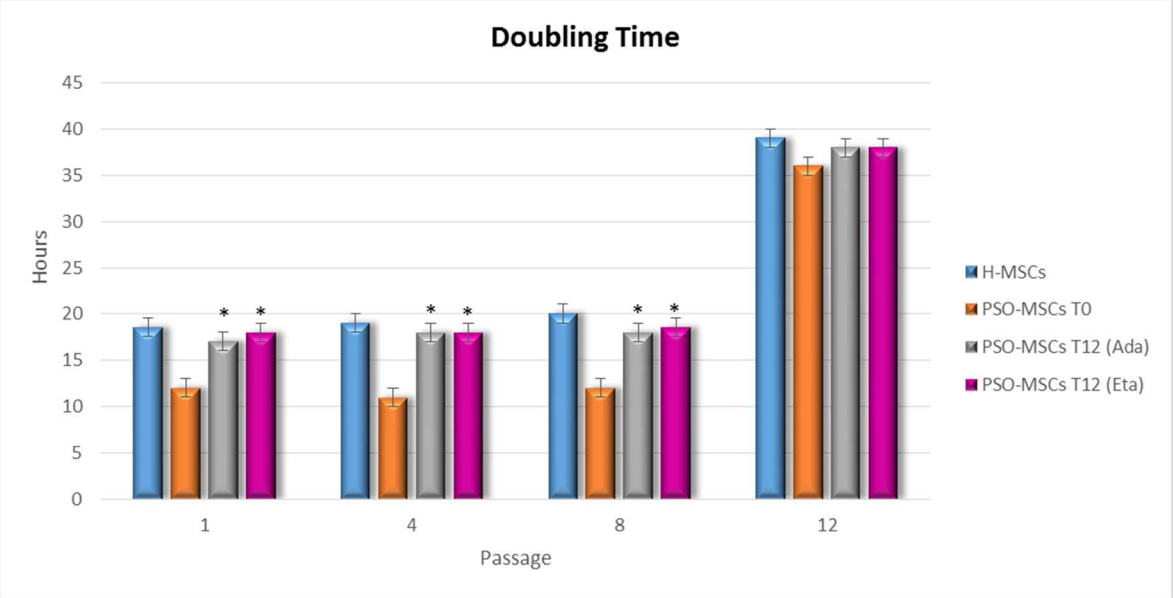

Figure 13. Doubling time (DT) analysis of the first to twelfth passage of control (H-MSCs) and PSO-MSCs before (PSO-MSCs T0) and after (PSO-MSCs T12) treatment with adalimumab (Ada) or etanercept (Eta). Data are showed as means±SD of three independent experiments.
*: *p*-value (PSO-MSCs T12 vs PSO-MSCs T0) <0,05.

Cells were characterized as established by the minimal criteria of Dominici; for this purpose, the immunophenotype and the differentiation potential were tested on 5 random samples of control group and 5 random samples of each PSO-MSCs group. In each group, samples analysed revealed the same expression profile and similar differentiation potential, with additionally no difference between H-MSCs and PSO-MSCs. The immunophenotype evaluated by the cytofluorimetric analysis revealed a strong positivity for the mesenchymal stem cells markers CD73, CD90 and CD105, and negativity for the other considered markers (HLA-DR, CD14, CD19, CD34, CD45) (Fig. 14).

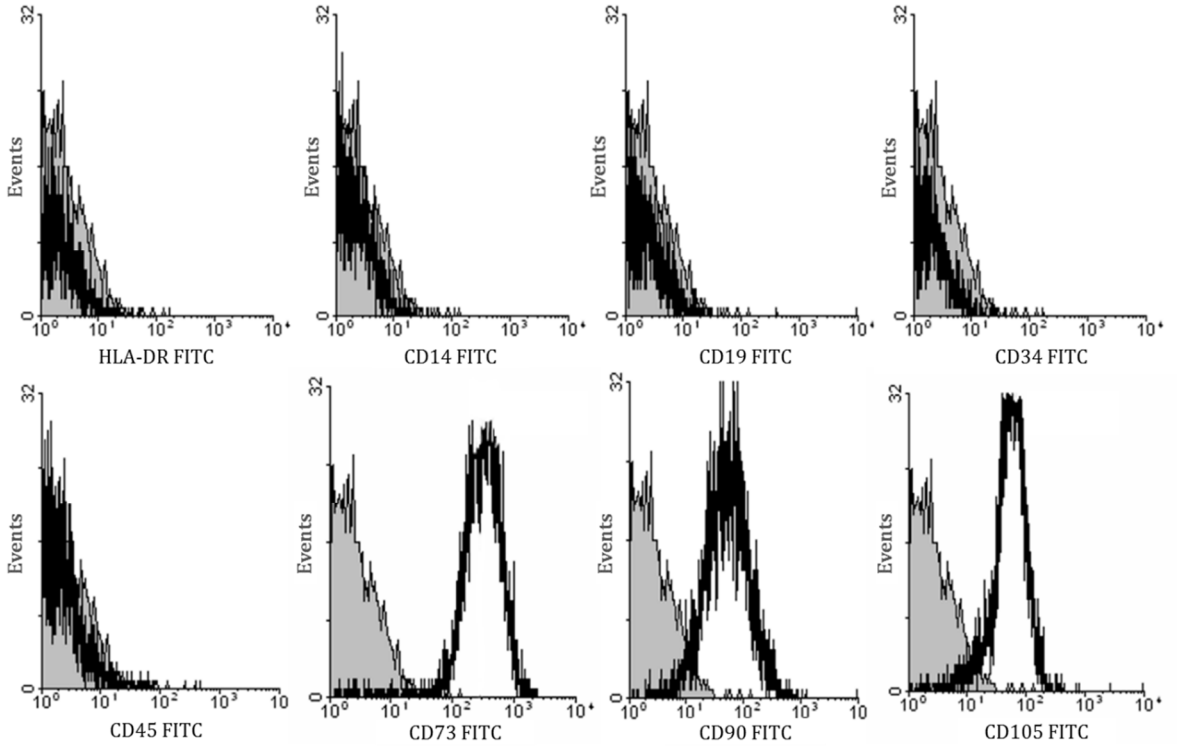

Figure 14. Immunophenotype of MSCs. The cytofluorimetric analysis was performed in 5 random samples of cells isolated from healthy subjects and 5 random samples from psoriatic patients. H-MSCs and PSO-MSCs revealed the same immunological profile and no difference was observed among samples. Solid grey fluorescence histograms are negative control (FITC-labelled IgG1 isotype control).

Because fibroblasts have the same profile for the cited markers, it was also tested CD9 to distinguish between MSCs and fibroblasts. Results revealed a weak positivity for the isolated cells compared to fibroblasts, confirming the mesenchymal-like immunophenotype.

Finally, the differentiation potential towards osteo-, chondro- and adipogenic lineages was tested. Focusing on the osteogenic differentiation, after 7 days of stimuli by using an osteogenic specific medium, reaction for alkaline phosphatase was made. In fact, ALP expression was noted to be high in bone, and it was hypothesized that this enzyme increases the local concentration of inorganic phosphate; therefore, ALP is important in the mineralization process, probably in the initial phase (Golub EE and Boesze-Battaglia K, 2007). Cells resulted positive to the ALP reaction (Fig. 15a). Cells maintained in osteogenic medium for 14 days were assessed using the Von Kossa staining. The Von Kossa staining

reveals the calcium deposits and, thus, the mineralization state of the extracellular matrix. Followed the protocol, calcium deposits appeared black and cells acquired a light yellow colouring, that confirmed the osteogenic differentiation (Fig. 15b). Cells that were cultured in adipogenic specific medium changed morphology and after 14 days of culture they showed a rounder and more cuboidal shape; therefore, cells were stained with Oil Red O. Oil Red O in solution is a lipophilic substance able to bind the neutral lipids contained in the fat vesicles of cells, highlighting them in red. The typical red vesicles were evidenced for cells tested (Fig. 15c). Lastly, cell pellets were maintained in specific medium to evaluate the chondrogenic differentiation; after 21 days, cells were included in paraffin and stained. The Safranin O staining revealed proteoglycan deposition, that confirmed the differentiation in the chondrogenic lineage (Fig. 15d).

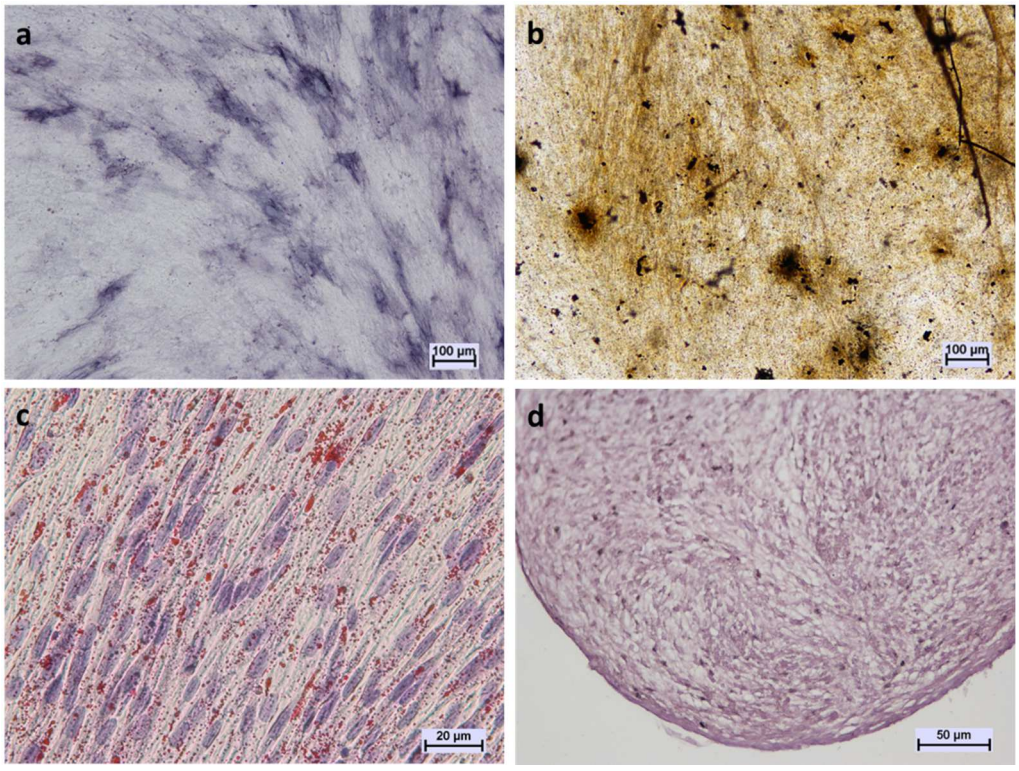

Figure 15. Representative images of differentiation potential of MSCs. Osteogenic differentiation was assessed by ALP reaction (a) and Von Kossa staining (b). Adipogenic differentiation was demonstrated by Oil Red O staining (c). Chondrogenic differentiation was confirmed by the Safranin O staining (d). Differentiation was assayed in 5 random samples of cells isolated from healthy subjects and 5 random samples from psoriatic patients. No difference was noted among different samples of H-MSCs or PSO-MSCs neither between H-MSCs versus PSO-MSCs.

4.2. Evaluation of TNF-α inhibitors on PSO-MSCs

People affected by psoriasis were treated for 12 weeks with the anti-TNF-α adalimumab or etanercept. The effectiveness of the treatments in MSCs was evaluated, specially analysing the expression of selected cytokines dysregulated in psoriasis.

4.2.1. Clinical response to TNF-α inhibitors

All the patients included in the study responded well to anti-TNF-α treatments. After 12 weeks of usage of adalimumab or etanercept, all of them reached a 75% reduction in the PASI score (PASI 75). The mean baseline PASI value of 14,23±2,87 decreased after treatment to 2,68±1,5 for the adalimumab group (*p*-value<0,05) and 3,72±2,5 for the etanercept group (*p*-value<0,05). In both adalimumab and etanercept groups, the patients had a significant improvement of life quality. In fact, also mean DLQI score decreased from the baseline value of 23,45±4,2 to 6,3±2,1 in patients treated with adalimumab (*p*-value<0,05) and 5,6±3,7 for etanercept treated people (*p*-value<0,05).

4.2.2. Relative gene expression profiles of cytokines belonging to Th1, Th17 and Th2 pathways in PSO-MSCs T0 and PSO-MSCs T12

The levels of selected cytokines related to the Th1, Th17 and Th2 pathways expressed in PSO-MSCs T0 were firstly compared to those obtained from H-MSCs. In accordance with data previously found by our group and reported in literature (Campanati A *et al*, 2014), all the analysed cytokines related to the Th1 pathway were up-regulated in PSO-MSCs T0. In particular, IFN-γ, IL-6, IL-8 and IL-12 were considerably up-regulated more than two fold (*p*-value<0,001 for IFN-γ, IL-6, IL-12 and *p*-value<0,0001 for IL-8); TNF-α and IL-23A were more expressed in PSO-MSCs compared to control, but less than two fold. It was also noted a variated expression in most of the cytokines belonging to the Th17 pathway, with at least two-fold greater expression of CCL2, CCL20, CXCL2, CXCL5, IL-17C, IL-17F, IL-21, G-CSF, while no significant variation was observed for IL-17A. On the contrary, comparing cytokines of PSO-MSCs and H-MSCs related to the Th2 cluster, an up-regulated and a down-regulated group of genes were identified. The first included IL-13 and TGF-β; the latter comprised IL-2, IL-4 and IL-5, while CCL1 and IL-3 did not show significant variation. The comparison of relative gene expression between PSO-MSCs T0 and H-MSCs confirmed that

psoriasis is a Th1/Th17 model of disease. In fact, most of the cytokines related to the Th1/Th17 pathways were up-regulated in psoriatic patients at baseline.

Subsequently, PSO-MSCs T12 gene expression was compared to that of PSO-MSCs T0. At T12, all the analysed cytokines referred to Th1 and Th17 pathways were significantly down-regulated compared to PSO-MSCs T0. Among the genes encoding for the Th2 cytokines, treatment with TNF-α inhibitors was able to up-regulated genes encoding for IL-2, IL-4 and IL-5, and to reduce the expression of TGF-β and IL-13. Even more, comparing PSO-MSCs T12 to H-MSCs, TNF-α, involved in the Th1 cluster, and most of the cytokines part of the Th17 pathway (CCL2, IL-17F, IL-21 and G-CSF) were normalized, *id est* the gene expression of these cytokines in PSO-MSCs T12 was comparable to H-MSCs without any significant difference. The expressions of IL-5 and IL-13 were also normalized to the control (Table 2). The same results were obtained for both adalimumab and etanercept treatment.

4.2.3. Expression profiles of cytokines in the conditioned medium

Selected inflammatory cytokines of the Th1, Th17 and Th2 pathways were studied on the conditioned medium of all H-MSCs, PSO-MSCs T0 and PSO-MSCs T12. For each group, read absorbances were related to those of respective standards antigens and cytokines concentration was reported as pg/ml. Subsequently, levels of PSO-MSCs T0 cytokines were compared to cytokines concentration expressed by H-MSCs. The ELISA array demonstrated that all the investigated Th1 and Th17 cytokines (that are IL-6, IL-8, IL-12, IL-23A, IFN-γ, TNF-α, CCL2, CCL20, CXCL2, CXCL5, IL-17A, IL-17C, IL-17F, IL-21 and G-CSF) were significantly over-expressed by PSO-MSCs at baseline. These results confirmed RT-PCR analysis, with the only exception of IL-17A. Even more, there is a variation in the most of the Th2 cytokines, in fact IL-2, IL-4 and IL-5 were down-regulated at significant levels, while IL-13 and TGF-β resulted over-expressed in PSO-MSCs T0. Also in this case, the ELISA array results reflected the RT-PCR array outcome (Table 3, column T0 vs H-MSCs).

Then, cytokines released in the secretome by PSO-MSCs after treatment were compared to the values obtained by PSO-MSCs T0, to evaluate if TNF-α inhibitors are able to reduce the inflammatory state of cells. Results confirmed that all the cytokines of the Th1 and Th17 pathways were down-regulated after both adalimumab and etanercept treatment (Fig. 16a; Table 3, column T12 vs T0). Referring to the Th2 cytokines, it is interesting to note that

IL-2, IL-4 and IL-5 that were down-regulated at baseline resulted up-regulated after 12 weeks treatment, while the administration of TNF-α inhibitors caused a decreased expression of IL-13 and TGF-β, up-regulated in PSO-MSCs T0 (Fig. 16b; Table 3, column T12 vs T0). The ELISA and RT-PCR array analyses gave comparable results.

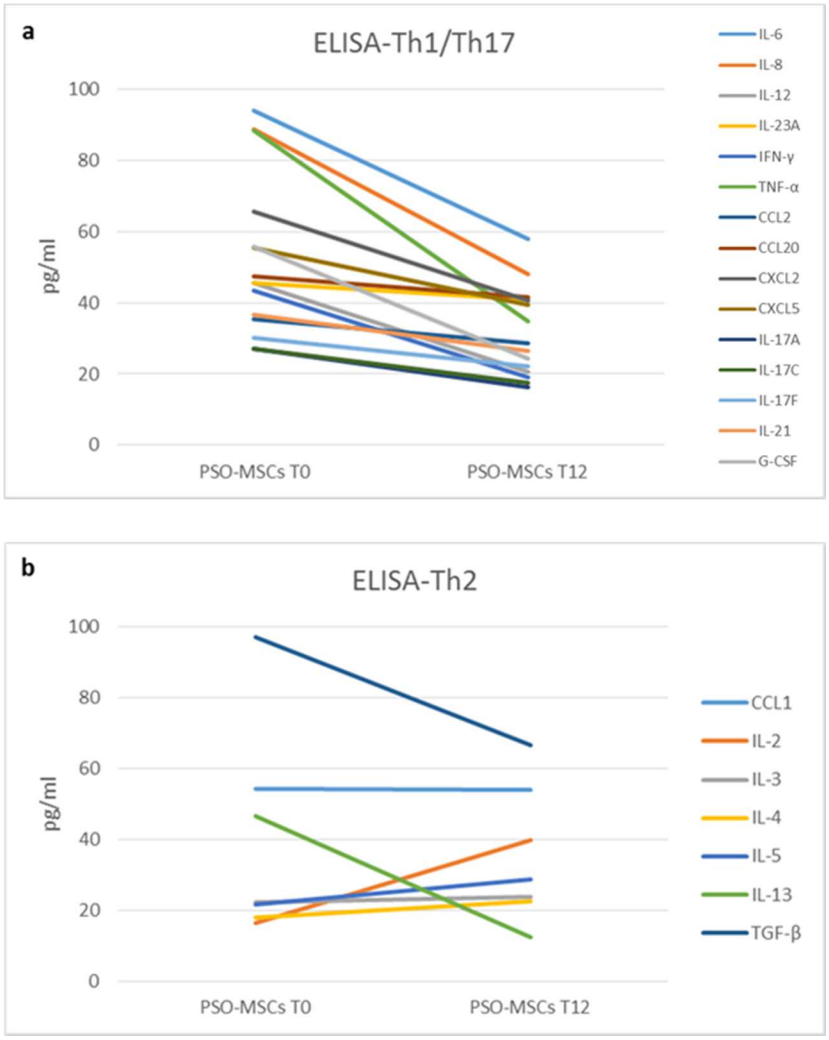

Figure 16. Cytokines expression variation of Th1/Th17 pathways (a) and Th2 pathway (b) in the conditioned medium of PSO-MSCs before (PSO-MSCs T0) and after treatment (PSO-MSCs T12). Cytokines were assessed by ELISA and expressed in pg/ml.

Furthermore, if PSO-MSCs T12 and H-MSCs were taken into consideration, the production of many proteins had been normalized after the administration of TNF-α inhibitors (Table

3, column T12 vs H-MSCs). These results were obtained regardless the type of administrated TNF-α inhibitor.

4.2.4. Quantification of VEGF after etanercept treatment on psoriatic patients

MSCs isolated from both lesional and perilesional skin of patients affected by psoriasis and treated with etanercept for 12 weeks were evaluated regarding the VEGF production. After treatment, it was noted a reduction in VEGF released in the conditioned medium, that reached significant level, in both lesional and perilesional MSCs compared with baseline. In detail, in lesional skin VEGF production was 256,42±3,07 pg/ml per 10^6 cells at baseline and it decreased to 27,66±2,03 pg/ml per 10^6 cells after treatment (*p*-value<0,05). In perilesional MSCs, from a starting value of 235,03±2,52 pg/ml per 10^6 cells at baseline, the VEGF concentration arrived to 41,65±4,72 pg/ml per 10^6 cells after etanercept usage for 12 weeks (*p*-value<0,05) (Fig. 17).

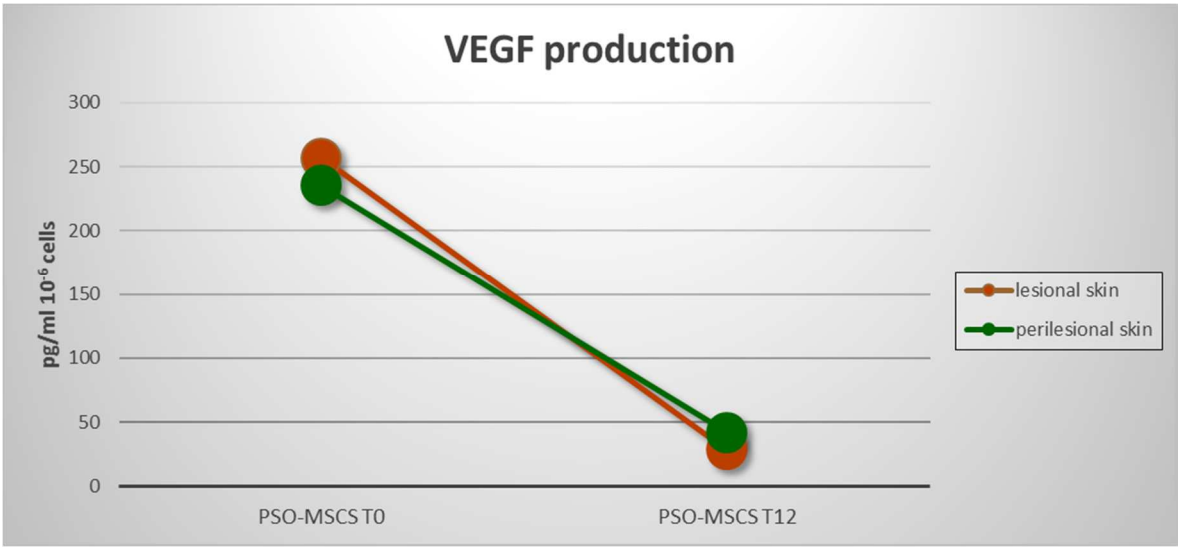

Figure 17. VEGF production before (PSO-MSCs T0) and after etanercept treatment (PSO-MSCs T12) in lesional and perilesional skin. Values are expressed in pg/ml per 10^6 cells. PSO-MSCs T0: MSCs derived from non-treated psoriatic patients; PSO-MSCs T12: MSCs derived from psoriatic patients after 12 weeks of treatment with etanercept. The reduction was statistically significant for both lesional and perilesional skin.

4.3. Influence of H-MSCs on PSO-MSCs

MSCs derived from healthy people were co-cultured with MSCs obtained from non-treated psoriatic patients to evaluate if H-MSCs, as cells with immunomodulatory properties and producers of a not-inflamed microenvironment, are able to positively influence PSO-MSCs and ameliorate their inflammatory state.

4.3.1. Impact of H-MSCs on PSO-MSCs proliferation

After 72 hours of co-culture, number of co-cultured or individually cultured PSO-MSCs was evaluated with an automatic cell counter. All the patients considered in the study gave consistent results, and for all of them, PSO-MSCs showed a reduction on cell proliferation of 30±0,20% after co-culture compared to mock (Fig. 18).

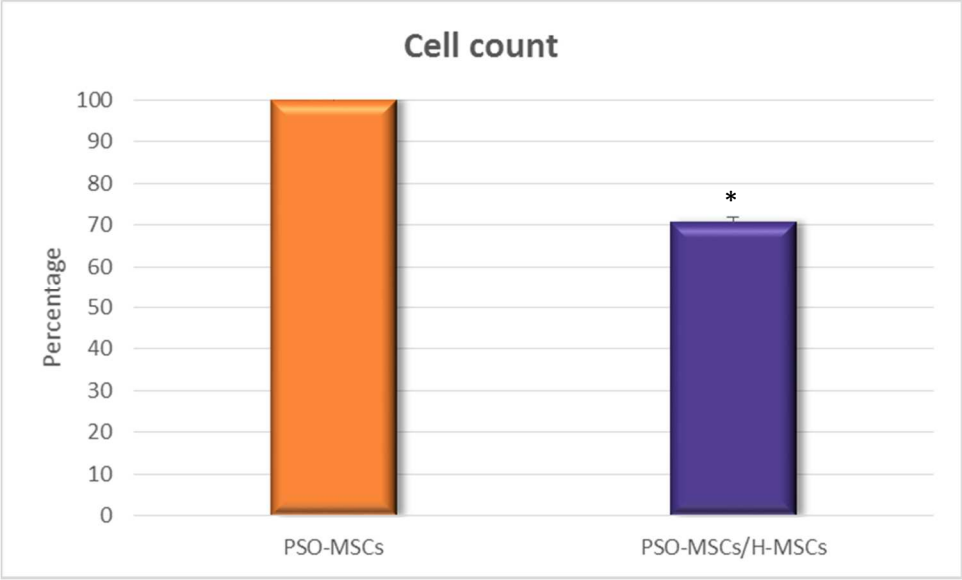

Figure 18. Reduction in cell number of MSCs derived from psoriatic patients after co-culture with MSCs obtained from healthy people. PSO-MSCs: individually cultured MSCs derived from non-treated psoriatic patients; PSO-MSCs/H-MSCs: co-cultured PSO-MSCs with MSCs derived from healthy people. Results are expressed in percentage as mean±SD for all the patients analysed. PSO-MSCs were used as control and referred as 100%; PSO-MSCs/H-MSCs number was accordingly calculated.
*: *p*-value<0,05.

4.3.2. Expression profile of cytokines belonging to Th1, Th17 and Th2 pathways

Expression of selected cytokines part of Th1, Th17 and Th2 pathways was evaluated on the conditioned medium of co-cultured and individually cultured PSO-MSCs by ELISA. As previously described (Paragraph 3.8.), the reading absorbances were calculated accordingly to the obtained absorbance values of respective standards antigens. Proteins concentration was reported as pg/ml. The expression of cytokines secreted by co-cultured cells was compared to that of PSO-MSCs individually cultured, used as mock. Co-culture induced a general decrease in the expression of cytokines. In detail, all the proteins belonging to the Th1 and Th17 pathways, over-expressed in PSO-MSCs compared to H-MSCs, were less expressed after co-culture. For all of them, a statistical significance was reached, with the only exception of IFN-γ. About cytokines of the Th2 pathway, IL-2 that was down-regulated in psoriatic patients resulted statistically more expressed after co-culture; on the contrary, all the other proteins (IL-4, IL-5, IL-10, IL-13, TGF-β1) were less expressed after co-culture condition (Fig. 19; Table 4).

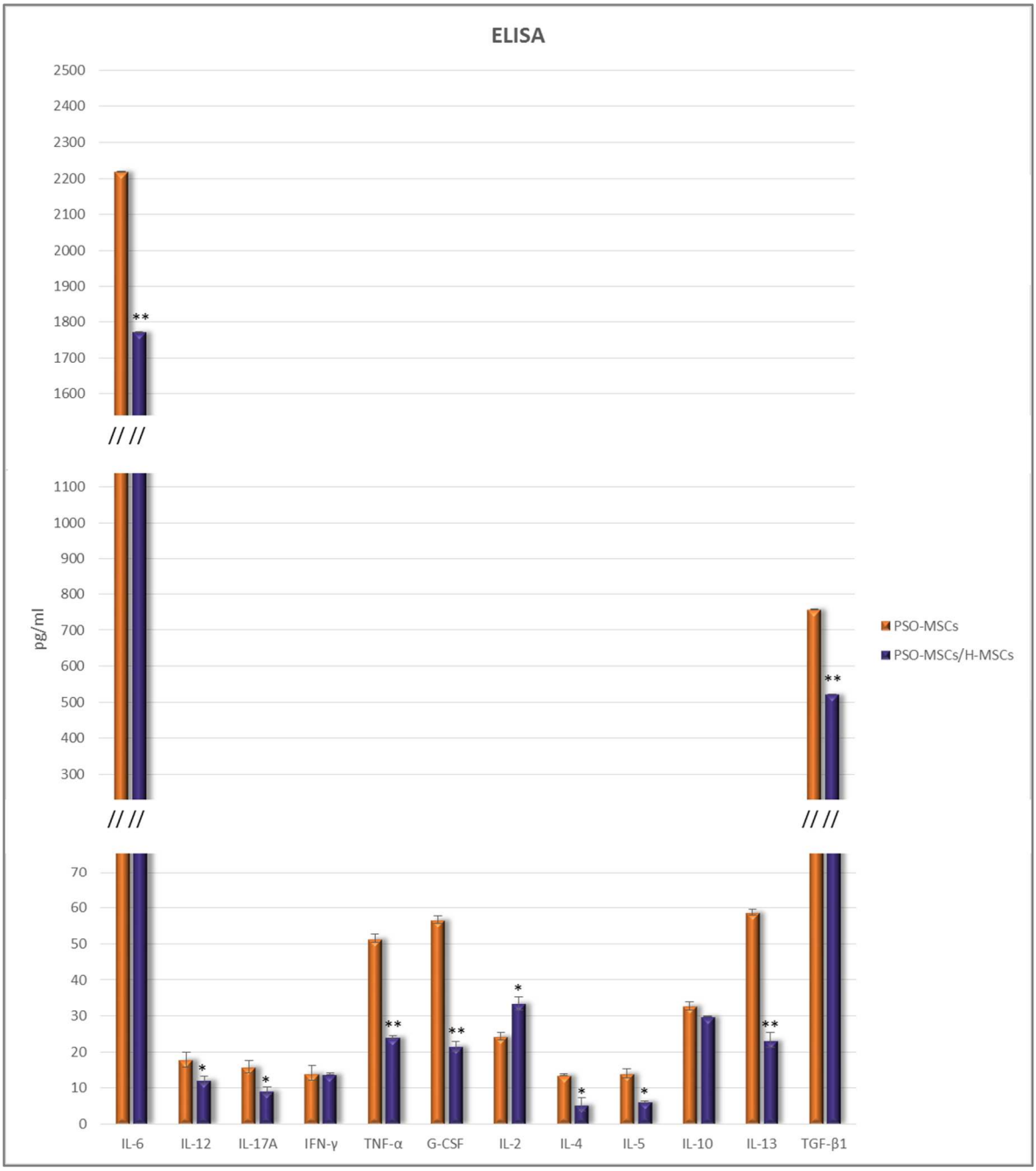

Figure 19. Secreted cytokines from individually and co-cultured PSO-MSCs with H-MSCs. PSO-MSCs: individually cultured MSCs derived from psoriatic patients; PSO-MSCs/H-MSCs: co-cultured PSO-MSCs with MSCs derived from healthy people. Results are expressed in pg/ml as mean±SD.
*: *p*-value<0,05; **: *p*-value<0,01.

4.4. Effect of PSO-MSCs on a human keratinocytes cell line

The abnormal cytokines secretion of non-treated PSO-MSCs was demonstrated. It was supposed that unhealthy cells may affect the proliferation of KCs and move them towards a disease phenotype. Therefore, in this study it was also investigated the influence that MSCs isolated from non-treated psoriatic patients can have on a KCs cell line isolated from adult human skin, the HaCaT cells.

4.4.1. Influence of PSO-MSCs on HaCaT proliferation

HaCaT cells were co-cultured with H-MSCs (HaCaT/H-MSCs) or PSO-MSCs (HaCaT/PSO-MSCs) for 72 hours. Then, co-cultured or individually cultured HaCaT were counted, and the latest used as mock. Number of control cells was set at 100% and the amount of co-cultured HaCaT with PSO-MSCs or H-MSCs accordingly calculated. HaCaT in co-culture with H-MSCs did not show reduction in cell proliferation (96,75% of co-cultured cells vs 100% of mock). In a similar way, the keratinocytes cell line in co-culture with PSO-MSCs demonstrated only a little decrease of proliferation, that did not reach statistical significance. In fact, in this case living cells were 91,15% compared to individually cultured HaCaT (Fig. 20).

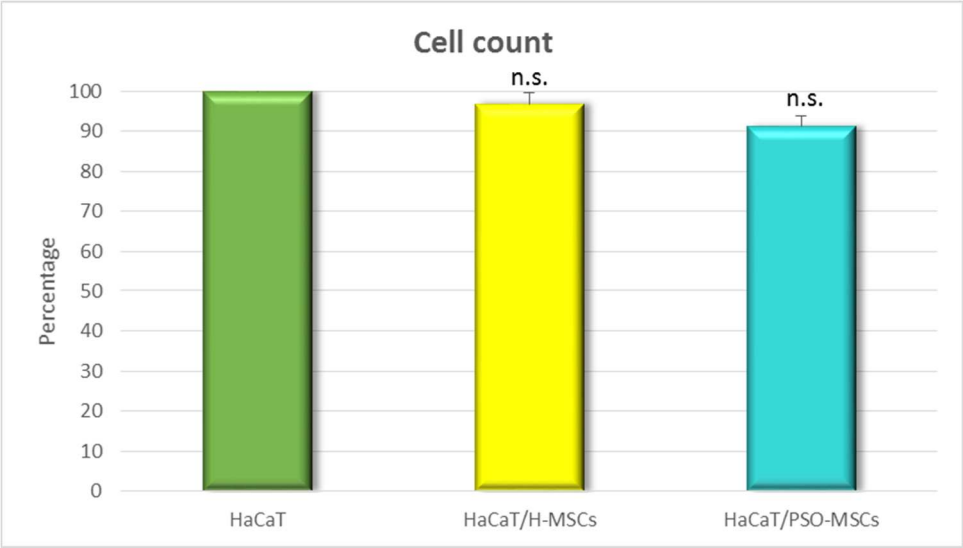

Figure 20. Cell count of individually or co-cultured HaCaT. HaCaT: individually cultured keratinocytes cell line; HaCaT/H-MSCs: co-cultured HaCaT with MSCs derived from healthy subjects; HaCaT/PSO-MSCs: co-cultured HaCaT with MSCs isolated from non-treated psoriatic patients.

Results are the average of three independent experiments and are expressed as percentage±SD. Number of individually cultured HaCaT was set as 100% and the number of co-cultured cells referred to that.
*: *p*-value<0,05; **: *p*-value<0,01; *** *p*-value<0,001; **** *p*-value<0,0001; n.s.: not significant.

4.4.2. Cytokines expression in the conditioned medium of individually or co-cultured HaCaT

The same groups of cytokines of Th1, Th17 and Th2 pathways previously considered, strictly related to psoriasis onset, were analysed by ELISA array on the conditioned medium of cultured and co-cultured HaCaT with H-MSCs or PSO-MSCs, respectively. For each cytokine, absorbance value was referred to that of the respective standard antigen, therefore the concentration was calculated and expressed as pg/ml. Statistical analyses were performed. The considered values are a mean of independent experiments made with all the patients included in the study. The concentration of cytokines produced by co-cultured cells was compared to that of cytokines released by only HaCaT, used as mock. Surprisingly, in both co-cultures, that is HaCaT/H-MSCs or HaCaT/PSO-MSCs, the expression of all the proteins was changed compared to mock. In detail, considering the Th1 and Th17 pathways, an up-regulated and a down-regulated group were recognizable. Specifically, IL-6, IFN-γ and G-CSF resulted up-regulated at significance level in co-cultures; on the contrary, the down-regulated group of cytokines included IL-12, and, with statistical significance, IL-17A and TNF-α. If Th2 pathway cytokines were analysed, also in this case both co-cultures gave almost the same results, and most of these cytokines resulted down-regulated (IL-2, IL-4, IL-5, IL-13). Only two proteins showed different expression between the two co-cultures compared to mock: in HaCaT/H-MSCs IL-10 resulted less expressed, although not with statistical significance, while TGF-β1 was increased; in HaCaT/PSO-MSCs IL-10 concentration was similar to value obtained by mock, on the contrary it was showed a reduction in TGF-β1 (Table 5).

5. DISCUSSION

5.1. The inflammatory state of PSO-MSCs

5.1.1. Psoriasis: a Th1/Th17 model of disease

Psoriasis is a chronic inflammatory skin disease. It is calculated to affect about 125 million people worldwide. Interestingly, African and American people have about half the rate of psoriasis compared to Caucasians. In psoriasis patients, skin cells multiply faster than normal, producing the typical thickened and scaling plaques. The disease is immune-mediated and it has been evidenced that Th1 cells are strictly related to the disease. In fact, in psoriasis plaques a large number of $CD4^+$ Th1 cells and elevated levels of IL-12, TNF-α and IFN-γ were found. Psoriasis was so firstly defined as a Th1 cell-mediated disorder (Cai Y *et al*, 2012). However, in 2005 a population of $CD4^+$ Th cells that produce Th17 was discovered, the so-called Th17 cells (Harrington LE *et al*, 2005; Park H *et al*, 2005). Even more, IL-23 was identified as a key cytokine for the maintenance of this cell population (Langrish CL *et al*, 2005). The theory that Th17 cells are involved in inflammatory and autoimmune diseases, included psoriasis, made its way. Actually, it was demonstrated that after the inflammatory stress signals from KCs trigger DCs, these last migrate into lymph nodes where naïve $CD4^+$ and $CD8^+$ are activated, and the differentiation into Th1 and Th17 cells, induced by IL-12 and IL-23 respectively, is promoted. After the triggering phase, KCs are stimulated, NK cells activated and the pro-inflammatory cytokines up-regulated, creating a system that propagates the inflammatory state itself. The cytokines released by Th1 and Th17 cells plus other keratinocytes-derived cytokines and chemokines stimulate KCs proliferation and open the way to the formation and perpetuation of psoriatic plaques on skin (Wagner E *et al*, 2010). The role of the Th17 cells in psoriasis is confirmed by their finding in psoriasis skin lesions and the increased levels of several cytokines belonging to this pathway, comparing to healthy subjects (Lewis BJ *et al*, 2013; Lowes MA *et al*, 2008; Chan JR *et al*, 2006). Also Th1 and Th22 cells are increased in psoriasis, but to a lesser extent (Kagami S *et al*, 2010).

Mesenchymal stem cells (MSCs) are self-renewing multipotent non-hematopoietic progenitor cells able to differentiate into tissue-specific cell type. MSCs were successfully

isolated from different sources, including skin. Only in the last 10 years researchers focused on MSCs isolated from skin of psoriatic patients, studying their immunological profile and the proteins secreted and trying to clarify their possible participation in the disease development. In fact, until that moment studies on psoriasis evaluated extensively the cytokines expression and the levels of factors related to the disease, such as iNOS and VEGF, only in differentiated cells from psoriatic skin, such as KCs, fibroblasts, dendritic antigen-presenting cells and leucocytes. Most recently, trying to clarify if the psoriatic process originates from undifferentiated cells of the skin, it was started to study the MSCs features in psoriasis. In the first study of 2011, researchers compared MSCs isolated from psoriatic skin with stem cells not only of healthy people but also of patients with atopic dermatitis. In fact, psoriasis and AD can be considered antithetical related to the Th1 and Th2 paradigm, presenting opposed differences in the disease microenvironment: the first is a Th1/Th17 model, in which it is prevalent the expression of chemokines known to attract Th1 and Th17 lymphocytes and neutrophils, while acute AD is considered a Th2 inflammatory example of disease because there is an increase in Th2 and eosinophil-attracting chemokines. The study confirmed that MSCs present typical features of psoriatic lesions, like the hyperproliferation, the expression of VEGF and iNOS and the reduction of total antioxidant capacity (Orciani M *et al*, 2011). Therefore, the microenvironment in the skin of psoriatic patients can stimulate the resident MSCs to produce angiogenic and pro-inflammatory mediators. This reduces the antioxidant ability of MSCs and consequently contributes to the development of the psoriatic lesions. Other researches followed; the genetic and epigenetic expression profiles, the cytokines production at both gene and protein levels were analysed. Step by step, new mosaic tails were added to clarify the MSCs features in psoriasis.

In particular, it was confirmed that MSCs in psoriatic skin share the typical Th1/Th17-Th2 imbalance of differentiated cells. In fact, most of the genes encoding for the Th1 and Th17 cytokines are over-expressed in MSCs of psoriatic patients compared to those of healthy people. Molecules such as IFN-γ, CXCL9, CXCL10, IL-6, IL-8, IL-17C, IL-17F, IL-17RA, IL-23A, CCL2 and CCL20 can promote the psoriasis process by stimulating the hyperplasia of

epidermal KCs, the angiogenesis, the activation and infiltration of T lymphocytes, DCs and neutrophils in the skin.

A predominant role in psoriasis development is held by TNF-α, expressed at higher levels by MSCs and differentiated cells in psoriatic lesions. In fact, it was reported that TNF-α stimulates MSCs proliferation and VEGF production *in vitro* and increases cytokines expression and migration of MSCs (Ke F *et al*, 2016; Böcker W *et al*, 2008; Crisostomo PR *et al*, 2008). This cytokine is able to promote the production of IL-17 and stimulate the differentiation of T cells toward a Th17 phenotype (Iwamoto S *et al*, 2007). Therefore, new therapeutic approaches to psoriasis make use of inhibitors of this cytokine (Brănişteanu DE *et al*, 2015; Lis K *et al*, 2014; De Simone C *et al*, 2013; Puig L *et al*, 2013).

5.1.2. The new biological therapies affecting TNF-α

TNF-α is synthesized as a transmembrane protein (tmTNF) and cleaved by a matrix metalloproteinase to soluble TNF (solTNF). Both forms result biologically active but with different functions. TmTNF plays a role in maintaining the physiological innate immune response to infections and provides tolerance to autoantigens. SolTNF drives the inflammatory response. Receptors of TNF (TNFR) are membrane glycoproteins. They can be either constitutively expressed (TNFR1, p55) or inducible (TNFR2, p75) and differ in expression, ligand affinity and signaling activation pathway. TNFR1 binds both solTNF and tmTNF, with a preference for the first one; expressed by most cell types, it is involved in inflammatory response beginning and apoptosis mediation. On the other hand, TNFR2 is preferentially activated by tmTNF; its expression is limited to specific cells such as endothelial cells, lymphocytes, cardiac myocytes, oligodendrocytes, microglia or astrocytes. TNFR2 is involved in the antiviral immune responses through the generation of cytotoxic T-lymphocytes (Grell M *et al*, 1998; Grell M, 1995). At the moment, five TNF-α inhibitors have been approved for the treatment of various autoimmune diseases: etanercept, infliximab, adalimumab, certolizumab and golimumab. Among them, etanercept, infliximab and adalimumab are generally used for the treatment of psoriasis while golimumab is used occasionally only for psoriatic arthritis. In the University Hospital from which samples for the researches here presented were collected, adalimumab and etanercept are the most used for psoriasis treatment; therefore the main features of these

two drugs will be presented. Adalimumab is a fully human bivalent monoclonal antibody; it presents a human-derived heavy and light chain variable regions and human IgG_1:κ constant regions, engineered through phage display technology and then produced in a Chinese hamster ovary mammalian cell line. It binds preferentially to TNF-α cytokine and blocks molecule interaction with both TNFR1 and TNFR2 receptors. Etanercept is a genetically engineered fusion protein. The Fc domain, consisting of human IgG1, is fused to a dimer of the extracellular ligand-binding domain of human TNFR2. It is able to bind both soluble and transmembrane forms of TNF-α with high affinity. Even more, it is the only clinically approved TNF-α biological therapy that can link the lymphotoxin α. However, compared to adalimumab, it was registered a lower complement-dependent cytotoxicity and apoptosis/cell cycle arrest through the transmembrane TNF-α linkage (Fig. 21) (Lis K *et al*, 2014; Mease PJ, 2007).

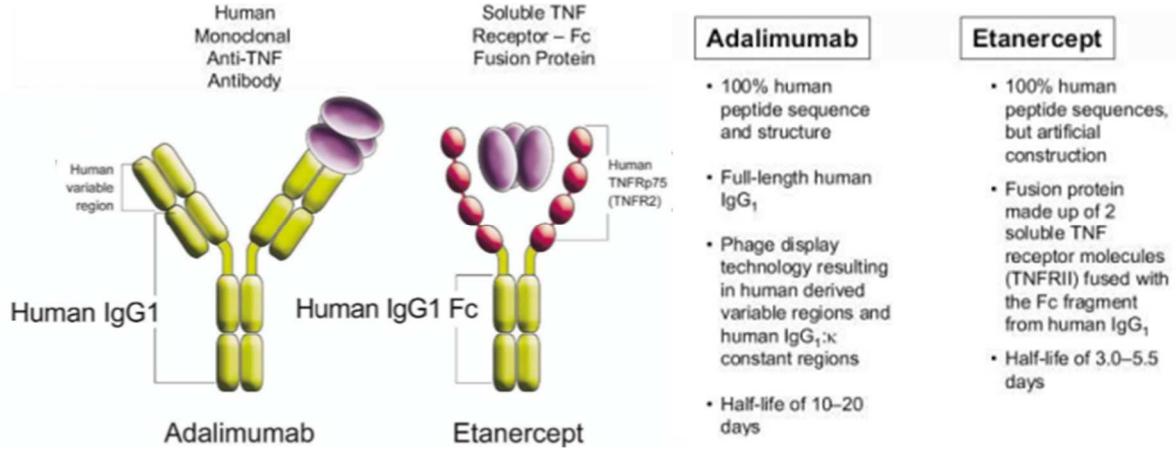

Figure 21. Adalimumab and etanercept structure (Mease PJ, 2007; Anderson PJ, 2005).

Both the drugs are approved for the treatment of moderate to severe psoriasis in adult patients contraindicated, intolerant or non responsive to other systemic therapies such as cyclosporine, methotrexate or PUVA.

Interestingly, the use of TNF-α inhibitors has been demonstrated to successfully reduce proliferation not only of differentiated cells in skin biopsies of psoriatic patients (Hendriks AG *et al*, 2014; Markham T *et al*, 2006) but also specifically of MSCs (Campanati A *et al*, 2012). Also the immunohistochemical expression and production of VEGF, the expression

of iNOS and the production of NO in MSCs from psoriatic patients show a reduction after the treatment (Campanati A *et al*, 2012). Therefore, TNF-α inhibitors help to rebalance the strong pro-oxidant pressure in psoriasis.

5.1.3. The effect of anti-TNF-α treatments on cytokines expression in PSO-MSCs

The present study is based on the state of art of MSCs and on the knowledge that both MSCs and differentiated cells isolated from psoriatic skin share the same cytokines expression profile. However, other aspects related to the effects of TNF-α inhibitors on resident MSCs of psoriatic patients need to be clarified. Here, it was firstly investigated if anti-TNF-α treatments, in particular adalimumab and etanercept, can be effective to ameliorate the imbalance of cytokines in MSCs. This research included patients treated for 12 weeks with one or the other drug. Cells were isolated from skin of psoriatic patients before (PSO-MSCs T0) and after therapy (PSO-MSCs T12). As control, cells were isolated with the same method from skin of healthy people (H-MSCs). Both psoriatic and healthy group cells showed the same fibroblast-like morphology. Cells were characterized assessing the correspondence to Dominici minimal criteria. The cytofluorimetric analysis and the evaluation of the differentiation potential into adipocytes, chondrocytes and osteocytes were similar in all the samples and in compliance with those of MSCs. Thus, both isolated PSO-MSCs and H-MSCs met the minimal criteria and fell in the definition of MSCs.

Clarified the features of isolated cells, the gene expression and the secretion of 22 cytokines involved in the Th1, Th2 and Th17 pathways were investigated by Real-Time PCR and ELISA array in MSCs, both before and after 12 weeks therapy with TNF-α inhibitors. First of all, the results confirmed that PSO-MSCs at baseline have an up-regulation of several genes encoding for Th1 (IL-6, IL-8, IL-12, IL-23A, IFN-γ, TNF-α) and Th17 cytokines (CCL2, CCL20, CXCL2, CXCL5, IL-17C, IL-17F, IL-21, G-CSF). Only gene expression of IL-17A was similar to that of control healthy cells. The ELISA array data were consistent with the PCR array profile, revealing the overexpression of all the analysed cytokines of these two pathways, included IL-17A. Therefore, data obtained from the isolated cells of psoriatic patients corroborate the literature and confirm the alteration of Th1/Th17 pathways in psoriatic MSCs. Furthermore, also the cytokines of Th2 pathway revealed a different trend in PSO-MSCs T0 compared to H-MSCs, with the down-regulation of IL-2, IL-4 and IL-5 genes

expression, whose secretions in the conditioned medium were correspondingly lower than H-MSCs, and the up-regulation of IL-13 and TGF-β, confirmed also by the data of ELISA array. The variations found for most of studied Th2 cytokines add new information to what is already known; probably the abnormal levels of Th2 cytokines in both PSO-MSCs gene and protein expressions contributes to alter the physiological state of cells. After the administration of adalimumab or etanercept for 12 weeks, the relative expression of all the genes encoding for the Th1 and Th17 cytokines was reduced. Similarly, a significant reduction in the corresponding secreted cytokines occurred; interestingly, some among them had been reported even to the physiological level (IL-6, IL-8, IL-12, IFN-γ, CCL2 and IL-17F). Anti-TNF-α inhibitors were also able to restore the atypical expressions of the Th2 cytokines both at gene and protein levels; even more, IL-5 and IL-13 were normalized to the control. No difference was reported between MSCs treated with adalimumab or with etanercept. This demonstrate that both drugs are able to reach the layer of undifferentiated cells and are successful treatments for reduce the altered inflammatory state of cells.

In addition, the effect of etanercept was deepened studying its influence on the VEGF secretion by PSO-MSCs. VEGF binds two receptors located on the endothelial cell membrane: VEGF receptor 1 (VEGFR-1) and VEGFR-2. When VEGF binds the receptors, particularly VEGFR-2, a signaling cascade starts, that stimulates the survival and proliferation of endothelial cells. This happens also in psoriasis where the interaction of VEGF with its receptors causes the formation of new blood vessels (Simonetti O *et al*, 2006). Synthesis and tissue expression of VEGF are regulated by TNF-α (Banno T *et al*, 2004). The angiogenic effect is amplified by endothelial cells, that release VEGF constitutively and in response to TNF-α. The TNF-α inhibitor adalimumab was already known to reduce the VEGF production in differentiated psoriatic cells, in both lesional and perilesional isolated MSCs, and in serum of psoriatic patients (Campanati A *et al*, 2013; Shimauchi T *et al*, 2013; Campanati A *et al*, 2012). Etanercept binds TNF-α with great affinity; the hypothesis that the interaction of this drug with TNF-α makes the protein biologically inactive and therefore induces a reduction of VEGF expression was confirmed on skin differentiated cells (Campanati A *et al*, 2009). In the present research, a 12 weeks etanercept treatment

resulted in a significant reduction in VEGF production compared with baseline in both lesional and perilesional MSCs. Subcutaneous administration of etanercept is therefore able to limit the angiogenic process that involves not only the differentiated cells but also the cutaneous undifferentiated layer. Summarising, biological TNF-α inhibitors can ameliorate psoriasis condition starting from the skin resident MSCs, cells probably early involved in the pathogenesis of psoriasis, modifying the cytokines milieu of MSCs, inhibiting angiogenesis and promoting a reversion of the MSCs from a psoriasis profile towards a more physiological state.

Inflammation and angiogenesis are considered key elements for the appearance of comorbidities in psoriasis; even more, it is thought that MSCs are involved in the early stage of the "psoriatic march". Treatments successfully able to reduce both inflammation and angiogenesis in MSCs may limit the occurrence of secondary events and slow down the continuation of the disease. Adalimumab and etanercept were both demonstrated to act on the inflammatory process of PSO-MSCs, lowering the over-expression of cytokines related to the Th1/Th17 pathways and bringing cytokines production to comparable values to those of healthy cells. In addition, both adalimumab and etanercept act on the reduction of VEGF production and therefore on the limitation of angiogenesis process. Having effect not only on differentiated cells, but also at the level of undifferentiated cells, the two drugs are extremely valuable for psoriasis treatment. However, not all the aspects related to the action of TNF-α inhibitors are clarified. Researchers suppose that adalimumab and etanercept can operate on MSCs also by other ways. In particular, one of the most accredited hypothesis believes that anti-TNF-α treatments act on the differentiation of MSCs. In fact, due to the microenvironment stimuli, resident MSCs of the skin may differentiate in endothelial cells and adipocytes. By reducing the altered state of these cells, it is possible that also the differentiation process is limited, thus slowing down the metabolic complications of psoriasis and therefore the so-called psoriatic march.

5.2. MSCs as therapeutic tool

5.2.1. The role of MSCs in cutaneous wound healing

A cutaneous wound induces the migration of inflammatory cells and stimulates an angiogenesis process followed by a granulation tissue formation, re-epithelialization and remodeling of the extracellular matrix. If this complex process is dysregulated, the wound healing fails. Many studies demonstrated that intravenous or intradermal administration of MSCs enhances cutaneous wound healing in both acute and chronic skin lesions (Wu Y *et al*, 2007; Li H *et al*, 2006). It was proposed that the acceleration of wound healing by MSCs is induced by two main processes: firstly, the MSCs paracrine communication established with cells in the wound, such as resident cells and infiltrating inflammatory cells, through the secretion of cytokines and growth factors; secondly, the differentiation into resident cells (Sorrell JM and Caplan AI, 2010). These mechanisms can enhance all the processes necessary for tissue repair and inhibit inflammation. MSCs in a wound area secrete growth factor and cytokines such as VEGF, PDGF, bFGF and angiopoietin-1, thus they probably promote angiogenesis. This process is also supported by the secretion of SDF-1, that induces survival of endothelial cells. By MSCs induction of macrophages polarization towards M2 group, that play a role in the progression of wound healing, there is an increase of M2 macrophages infiltration in wound (Zhang QZ *et al*, 2010). Even more, it has been reported that transplanted MSCs induce the recruitment of endogenous stem/progenitor cells. Conditioned medium from MSCs contains high levels of tissue inhibitor of metalloproteinases, that protect from the degradation induced by matrix metalloproteinases (MMP). MSCs also stimulate the synthesis of fibronectin, collagen, elastin by dermal fibroblasts and so contribute to the production and remodeling of the extracellular matrix (Motegi SI and Ishikawa O, 2017). The other mechanism through which MSCs contribute to tissue repair is linked to the capability to differentiate in cells of the mesenchymal lineage, or transdifferentiate in a site-specific way, in response to microenvironment stimuli. Consequently, MSCs can differentiate into cells of skin tissue, including fibroblasts, myofibroblasts, vascular endothelial cells, pericytes, KCs (Sasaki M *et al*, 2008). These mechanisms, together with the immune-regulatory properties of MSCs

previously described (Paragraph 1.11.1.), make MSCs an interesting tool for the treatment of cutaneous wound.

5.2.2. Preclinical studies of MSCs therapy in psoriasis

To date, few studies focused on the efficacy of MSCs as a therapeutic instrument against psoriasis. In 2016 Sah and colleagues treated mice affected by imiquimod (IMQ)-induced psoriasis-like skin inflammation with subcutaneous infusion of MSCs isolated from human umbilical cord blood and demonstrated that this therapy suppressed the Th1, Th2 and Th17 differentiation and up-regulated Treg population. Also level of reactive oxygen species (ROS) and the immune cell infiltration into skin lesions were decreased, thus limiting the inflammatory state. In the same study it was also tested the effect of the strong antioxidant enzyme superoxide dismutase 3 (SOD3) in combination with MSCs. SOD3-transduced MSCs inhibited the severity of mice disease to a greater extent compared with MSCs alone (Sah SK *et al*, 2016). At the moment, according to the NIH ClinicalTrials.gov database, only one clinical trial is underway (NCT02491658). It is based on the use of umbilical cord-MSCs (UC-MSCs) for the treatment of patients with moderate-to-severe psoriasis vulgaris. The results are encouraging: treated patients remained free from psoriasis relapse for four-five years (Chen H *et al*, 2016). Also MSCs from adipose tissue are under study for the treatment of psoriasis arthritis and psoriasis vulgaris. In a case report, two injections of autologous adipose tissue-MSCs (AT-MSCs) in a psoriatic arthritis patient, unresponsive to standard treatments, decreased PASI. The psoriatic vulgaris patient, previously dependent on methotrexate, showed a decrease in PASI after three infusion: interestingly, this clinical improvement was maintained for 292 days without drug use (De Jesus MM *et al*, 2016). All these studies have been conducted on a strict number of cases, so more researches have to be done; however, these data are encouraging and once again suggest the possibility of MSCs use in psoriasis.

5.2.3. The *in vitro* immune-modulatory impact of H-MSCs on PSO-MSCs

Based on the data previously reported, referred to the anti-inflammatory action of MSCs, their role in ameliorate skin disease and the encouraging preclinical studies, present research evaluated the influence of MSCs isolated from skin of healthy people on inflamed

PSO-MSCs, in particular with reference to the inflammatory state modulation. MSCs isolated from healthy people and MSCs obtained from non-treated psoriatic patients were indirectly co-cultured for 72 hours. Then, PSO-MSCs proliferation and the level of cytokines in the conditioned medium were analysed. Usually, PSO-MSCs have a proliferation rate higher than normal cells; however, after co-culture, psoriatic cells number was reduced of about 30%. No difference was noted between patients with moderate or severe psoriatic plaques. Therefore, it is plausible to hypothesize that H-MSCs secrete factors that slow down the proliferation rate. Focusing on cytokines levels, PSO-MSCs cultured alone showed altered production of almost all the considered molecules. Cytokines related to the Th1 and Th17 pathways resulted up-regulated, confirming as psoriasis is a Th1/Th17 model of disease. 72 hours of co-culture made interesting variations in cytokines production. In fact, all the over-expressed molecules of the Th1 and Th17 groups were down-regulated in the conditioned medium produced by co-cultured cells. All of them, with the only exception of IFN-γ, reached statistical level. Also the secretion of cytokines of Th2 pathway changed before and after co-culture in PSO-MSCs: all the molecules resulted less expressed after co-culture; only IL-2, down-regulated comparing to H-MSCs, was more expressed after co-culture. If the levels of PSO-MSCs inflammatory cytokines after co-culture with H-MSCs are compared to those observed after the 12 weeks treatments with anti-TNF-α drugs interesting correspondences are found. In fact, H-MSCs and TNF-α inhibitors gave similar results: all the cytokines of the Th1 and Th17 pathways that were over-expressed by PSO-MSCs at baseline resulted down-regulated. Even more, level of IL-2 increased, while IL-13 was less expressed after co-culture or treatment. Additionally, both co-culture conditions and 12 weeks treatments were able to reduce the higher proliferation rate of PSO-MSCs. These data enforce the concept that skin MSCs have immunomodulatory properties that are strictly linked to the local environment. MSCs isolated from an inflammatory situation such as psoriasis lesions do not have immune-suppressive features, but on the contrary are characterized by altered secretome that reflects the pathology. MSCs isolated from healthy people reside in a not-inflamed environment, and present immune-suppressive properties. Even more, in the current research, it was demonstrated that H-MSCs are able to positively influence the altered state of psoriasis *in vitro*, acting directly at stem level. The effect is

comparable to the use of two of the most employed treatments for psoriasis, adalimumab and etanercept, for 12 weeks. More researches have to be done, to better evaluate the impact that H-MSCs have on psoriasis. In particular, PSO-MSCs are characterized by altered levels of iNOS and VEGF, that are directly implicated in determining both skin lesions and systemic involvement, and by an oxidative damage, in which ROS actively promote the secretion of inflammatory Th1 cytokines. Therefore, it is interesting to assess the effect of H-MSCs in PSO-MSCs also related to VEGF production and expression, to iNOS expression and to the antioxidant response. Even more, another open question is if H-MSCs and TNF-α treatments can work synergistically, or in other words, if administered together there is a better or faster response of PSO-MSCs than one cure alone. A positive answer to these queries makes under active consideration how and how long carry out the cure with H-MSCs. At this regard, the preclinical studies already made (Paragraph 5.2.2.) can help solve the latest doubts. Anyway, results are encouraging, even because MSCs isolated from healthy subjects were demonstrated to act at the level of stem cells. The use of stem cells for psoriasis treatment gets closer and closer.

5.3. The influence of PSO-MSCs on the keratinocytes cell line HaCaT

5.3.1. The background art of PSO-MSCs impact on KCs proliferation and apoptosis

Epidermal acanthosis is one of the pathological features founded in psoriasis; it is associated with an abnormal proliferation and altered apoptosis of KCs. Normally, epidermal homeostasis is maintained by the loss of KCs adhesion followed by terminal differentiation to corneocytes, apoptosis and therefore desquamation. Proliferation and differentiation of KCs are regulated by growth factors and cytokines, among them epidermal growth factor (EGF), IGF-1, TGF-α, IL-1, IL-6, TNF-α. The apoptosis of KCs is induced by the loss of cell-cell contact, caused by cross-linking of the Fas (CD95) molecule or by UV radiation (Rückert R *et al*, 2000). In psoriasis, the chronic inflammation leads to a reduction in apoptosis rate and KCs proliferate more and are aberrantly resistant to apoptosis (Wrone-Smith T *et al*, 1997; Wrone-Smith T *et al*, 1995). PSO-MSCs reflect the unbalanced cytokines production typical of psoriasis and produce pro-inflammatory mediators, releasing them in the surrounding microenvironment (Campanati A *et al*, 2014;

Orciani M *et al*, 2011). Liu RF and colleagues in 2015 hypothesized that MSCs isolated from skin lesions may influence KCs proliferation and apoptosis in psoriasis. Therefore, researchers isolated MSCs from patients affected by psoriasis vulgaris and from healthy people; they co-cultured them with a spontaneously immortalized human KCs cell line (HaCaT) for 72 hours. Cell cycle progression and apoptosis of HaCaT cells were assessed by a real-time dynamic cell proliferation test and by Annexin V-FITC cell apoptosis analysis kit, respectively. Results evidenced an increase in HaCaT cells proliferation in co-culture both with PSO-MSCs and H-MSCs compared to human KCs cultured alone; however, PSO-MSCs showed a stronger effect on stimulate proliferation than H-MSCs. Maximum proliferation was reached between 32 and 60 hours of cultivation, then count gradually decreased for all 3 groups (HaCaT individually cultured, HaCaT in co-culture with PSO-MSCs or H-MSCs). Co-cultured HaCaT with MSCs exhibited also an increase in the proportion of cells in S phase of cell cycle. In addition, even if both H-MSCs and PSO-MSCs could induce apoptosis of human KCs, PSO-MSCs had a reduced ability to stimulate this process than H-MSCs (Liu RF *et al*, 2015). Therefore, MSCs isolated from psoriatic patients lesions seem to promote KCs proliferation and decreased induction of apoptosis more potently than MSCs isolated from healthy people. To our knowledge, this is the only study that analysed the effect of PSO-MSCs on human KCs.

5.3.2. Cytokines expression variation in the HaCaT/MSCs co-culture

In the work here presented, the study of MSCs influence on a human KCs cell line was extended to the effect of the cytokines released in the surrounding environment. For this purpose, HaCaT cells were co-cultured with PSO-MSCs or H-MSCs for 72 hours. Conditioned medium was then collected and cells count. Co-culture conditions for 72 hours did not significantly affect cell proliferation, that was about 97% for HaCaT co-cultured with H-MSCs compared to HaCaT alone (referred as 100%) and about 91% for KCs co-cultured with PSO-MSCs. Also if apparently these data did not correlate to those of previous research on the matter, it is important to remember that in Liu and colleagues work maximum proliferation was reached after 32 hours for HaCaT cultured alone and after 60 hours for co-cultured cells, then in both cases proliferation decreases. In present study, cell count was performed after 72 hours. It means that also if it is possible that co-culture with MSCs

promotes KCs proliferation, this influence is not long-lasting and KCs return to their physiological proliferation rate. Furthermore, HaCaT are a cell line expressing involucrin, filaggrin, keratin 10 (Lemaître G *et al*, 2004; Boukamp P *et al*, 1988); these molecules are produced by cells in the exterior part of the stratum spinosum and in the stratum granulosum. It is probable that MSCs are able to influence proliferation of cells in the stratum basale and in the nearest part of stratum spinosum but not of KCs in the outer layers of the epidermis. The conditioned medium of individually cultured and co-cultured HaCaT was then analysed, to evaluate the influence of inflamed MSCs on KCs, using a multi-analyte ELISA array that detects 12 key cytokines of the Th1, Th2 and Th17 pathways. Co-culture with PSO-MSCs or H-MSCs induced variation in the expression of cytokines of all the considered pathways. A part for IL-10, whose level of expression did not change in co-culture with PSO-MSCs while had a little, not significant, reduction in co-culture with H-MSCs, and TGF-β1, that decreased in PSO-MSCs co-culture, and increased in H-MSCs co-culture, all the other analysed cytokines showed the same trend in both co-cultures. In particular, referring to Th1/Th17 groups of cytokines, IL-6, IFN-γ and G-CSF increased after co-culture; on the contrary, IL-12, TNF-α and IL-17A decreased. About proteins of Th2 pathways, except for the already mentioned IL-10 and TGF-β1, all the others molecules showed a down-expression after co-culture with both PSO-MSCs and H-MSCs. PSO-MSCs because of their imbalance in cytokines expression were expected to induce a pro-inflammatory state towards a psoriatic profile. However, most of the cytokines show variations in lineage with what happens in psoriatic disease (IL-6, IFN-γ, G-CSF, IL-2, IL-4 and IL-5), but others had an opposite behaviour (IL-12, IL-17A, TNF-α, IL-13). In particular, it was surprising that some cytokines of Th1 and Th17 pathways (IL-17A, IL-12, TNF-α) were less secreted in the conditioned medium after co-culture with PSO-MSCs. IL-17A is usually expressed at high levels in site of inflammation in psoriasis and other autoimmune diseases, where it has probably a role in promoting the autoimmune pathology. However, a recent study shows that another member of IL-17 family, that is IL-17C, is abundant in lesional skin. The over-expression of IL-17C in mouse KCs promotes a psoriasiform skin inflammation leading to the development of a psoriasis-similar skin phenotype (Johnston A *et al*, 2013). Additionally, IL-17A is most produced by innate immune cells such as NK

cells, mast cells, neutrophils, γδ T cells, and Th17 ($CD4^+$ $IL\text{-}17^+$) and Tc17 ($CD8^+$ $IL\text{-}17^+$) cells of the acquired immune system; instead, IL-17C is mainly expressed by KCs in lesional skin (Baliwag J *et al*, 2015). Therefore, the expression of IL-17C has to be included in the study. In a similar way, it is important to also evaluate the expression of IL-23 in the secretome after MSCs co-culture. In fact, as mentioned above (Paragraph 1.5.2.), IL-12 and IL-23 share the same p40 subunit, but in addition IL-12 has a p35 subunit and IL-23 has a unique p19 subunit. Even if both proteins have been identified as key inflammatory cytokines involved in psoriasis, studies on the subunits expression gave to IL-23 a more important role than IL-12 in the pathogenesis of psoriasis (Lee E *et al*, 2004). IL-23 is produced from both KCs and DCs (Piskin G *et al*, 2006; Lee E *et al*, 2004). Since IL-23 is an IFN-γ inducing molecule, an IL-23 increase may explain the higher level of IFN-γ in co-culture compared to KCs cultured alone. Referring to TNF-α expression, it is produced by most activated T cells and APC, but TNF-α alone does not provoke significant responses from cultured KCs (Baliwag J *et al*, 2015). Therefore, it is stimulating to extend the analysis to other cytokines and assess if there are some triggering factors secreted by MSCs that may influence KCs towards a psoriasis phenotype. In addition, further aspects need an in-depth examination. It has to be clarified why MSCs isolated from inflamed and not-inflamed sources in co-culture with KCs influence in the same way the conditioned medium, giving the same results in terms of cytokines expression variation. Even more, starting from Liu and colleagues research, the present work used HaCaT cell line. However, some studies pointed the attention on the differences between HaCaT cells and normal human KCs. For example, it was demonstrated that Th cell cytokines, such as IL-4, IFN-γ, IL-17A and IL-22, differently affect the transcriptional profiles of cornified envelope-associated genes, such as filaggrin or loricrin, in HaCaT compared to normal KCs (Seo MD *et al*, 2012). In addition, HaCaT cell line has features of stratum granulosum cells; it is important to consider also cells of stratum basale and spinosum, nearest the MSCs stratum, where cytokines released by MSCs are expected to be at higher levels. These evaluations suggest that HaCaT may not be a perfect model to study Th cell cytokine-mediated changes.

In conclusion, the knowing of MSCs features in psoriatic lesions and the understanding of their relationship with cells typical of the skin and with mobile cells that migrate following injuries help to clarify the features of psoriasis. A better knowledge of this disease, also at staminal level, is a solid starting point to develop new and efficacy therapeutic approaches, especially for those patients unresponsive to therapies currently in use. Additionally, on the other front an intriguing turning point in the study of MSCs is their use in therapy for pathologies that show an immune dysregulation, taking advantage of MSCs immune-regulatory properties.

6. TABLES

Table 1. Demographic and clinical features of subjects included in the study.

	Psoriatic patients	Healthy subjects	*p*-value
Number	15	15	n.s.
Sex (woman/men)	5/10	7/8	n.s.
Age (years)	50,69±13,41	53,15±11,27	/
PASI (mean±SD)	14,23±2,87	n.a.	/
Years of psoriasis	10,56±5,79	n.a.	/

n.s.: not significant; n.a.: not applicable.

Table 2. Gene expression of cytokines of Th1/Th17 and Th2 pathways.

Pathway	*Cytokines*	H-MSCs	T0	T12	T0 vs H-MSCs	T12 vs T0	T12 vs H-MSCs
Th1	*IL-6*	1	2,78±1,07	1,14±0,52	***	*	*
	IL-8	1	4,33±1,27	2,41±1,58	****	**	**
	IL-12	1	3,55±1,56	1,13±0,40	***	*	*
	IL-23A	1	1,53±0,80	0,14±0,06	*	*	*
	IFN-γ	1	10,79±1,89	1,45±0,30	***	*	*
	TNF-α	1	1,78±1,14	1,01±0,86	**	*	n.s.
Th17	*CCL2*	1	3,17±1,58	0,27±0,20	***	**	n.s.
	CCL20	1	6,06±5,12	1,62±1,08	*	*	*
	CXCL2	1	5,01±2,17	3,06±2,21	****	**	**
	CXCL5	1	10,72±5,27	2,22±2,74	****	*	*
	IL-17A	1	0,89±0,78	0,14±0,07	n.s.	**	*
	IL-17C	1	2,57±1,72	0,89±0,63	*	*	*
	IL-17F	1	2,52±1,41	0,90±0,16	**	*	n.s.
	IL-21	1	21,16±3,23	2,12±0,48	***	***	n.s.
	G-CSF	1	3,69±0,48	1,35±0,20	***	**	n.s.
Th2	*CCL1*	1	1,40±1,09	0,80±0,48	n.s.	n.s.	n.s.
	IL-2	1	0,27±0,08	0,59±0,12	**	****	**
	IL-3	1	1,02±0,27	1,06±0,23	n.s.	n.s.	n.s.
	IL-4	1	0,42±0,12	0,77±0,25	****	*	***
	IL-5	1	0,44±0,14	1,08±0,45	*	****	n.s.
	IL-13	1	1,88±0,85	1,16±0,49	**	*	n.s.
	TGF-β	1	1,53±0,36	1,24±0,30	****	*	*

H-MSCs: MSCs derived from healthy subjects, used as control; T0: MSCs derived from non-treated psoriatic patients; T12: MSCs derived from psoriatic patients after 12 weeks of treatment.
H-MSCs are considered as 1; PSO-MSCs T0 and PSO-MSCs T12 are accordingly calculated as X-fold.
All the reported values are expressed as mean±SD.
*: *p*-value<0,05; **: *p*-value<0,01; *** *p*-value<0,001; **** *p*-value<0,0001; n.s.: not significant.

Table 3. Levels on the conditioned medium of the Th1/Th17 and Th2 pathways cytokines.

Pathway	*Cytokines*	H-MSCs (pg/ml)	T0 (pg/ml)	T12 (pg/ml)	T0 vs H-MSCs	T12 vs T0	T12 vs H-MSCs
Th1	*IL-6*	53,71±4,96	93,88±1,34	58,01±1,00	***	**	n.s.
	IL-8	49,04±1,4	88,88±5,83	48,01±1,00	***	***	n.s.
	IL-12	13,16±0,89	45,48±0,50	20,67±0,48	***	***	n.s.
	IL-23A	38,16±2,7	45,48±0,50	41,01±2,70	***	*	*
	IFN-γ	15,75±0,75	43,40±2,70	18,93±1,30	***	***	n.s.
	TNF-α	27,38±0,26	88,39±3,02	34,88±0,69	****	***	*
Th17	*CCL2*	27,36±1,97	35,41±0,88	28,68±3,58	***	**	n.s.
	CCL20	37,81±1,89	47,32±2,8	41,48±1,30	***	*	*
	CXCL2	23,16±0,39	65,56±0,39	40,67±0,48	****	**	**
	CXCL5	27,83±1,64	55,56±0,41	39,29±1,51	***	*	*
	IL-17A	12,48±0,20	27,02±1,11	16,15±0,05	****	**	*
	IL-17C	14,20±1,10	27,20±1,67	17,49±0,65	***	*	*
	IL-17F	22,67±0,57	30,27±1,48	22,22±2,06	**	*	n.s.
	IL-21	15,36±0,32	36,71±1,22	26,42±0,45	***	**	**
	G-CSF	16,56±0,30	55,84±1,01	24,40±0,23	****	**	*
Th2	*CCL1*	52,82±7,40	54,26±6,55	54,01±7,25	n.s.	n.s.	n.s.
	IL-2	50,51±0,25	16,41±0,44	39,80±1,80	***	**	**
	IL-3	22,78±2,56	22,13±0,95	23,68±2,64	n.s.	n.s.	n.s.
	IL-4	45,70±0,68	18,04±0,25	22,64±0,59	***	**	***
	IL-5	30,81±1,87	21,66±0,87	28,88±0,14	****	**	n.s.
	IL-13	15,51±1,93	46,52±2,05	12,50±1,50	****	***	n.s.
	TGF-β	59,15±0,36	97,26±2,78	66,67±3,08	***	***	*

H-MSCs: MSCs derived from healthy people, used as control; T0: MSCs derived from non-treated patients affected by psoriasis; T12: MSCs derived from psoriatic patients after 12 weeks of treatment with TNF-α inhibitors.

All the values are reported in pg/ml and expressed as mean±SD.

*: *p*-value<0,05; **: *p*-value<0,01; *** *p*-value<0,001; **** *p*-value<0,0001; n.s.: not significant.

Table 4. Cytokines expression variation after co-culture of PSO-MSCs with H-MSCs.

	Cytokines	H-MSCs (pg/ml)	PSO-MSCs (pg/ml)	PSO-MSCs/ H-MSCs (pg/ml)	PSO-MSCs/ H-MSCs vs PSO-MSCs
Th1/Th17	*IL-6*	1830,13±0,18	2218,34±0,91	1771,10±0,41	**
	IL-12	15,43±2,18	17,91±2,11	12,21±0,95	*
	IL-17A	12,43±0,23	15,96±1,68	9,30±1,00	*
	IFN-γ	9,99±2,31	14,16±2,01	13,91±0,27	n.s.
	TNF-α	33,47±2,01	51,53±1,15	24,06±0,48	**
	G-CSF	40,60±0,92	56,76±0,97	21,53±1,32	**
Th2	*IL-2*	28,42±1,52	24,49±1,02	33,48±1,90	*
	IL-4	13,14±0,97	13,73±0,13	5,42±1,87	*
	IL-5	11,10±1,15	14,07±1,21	6,19±0,23	*
	IL-10	31,19±0,46	32,78±1,05	29,89±0,17	n.s.
	IL-13	35,96±0,89	58,83±0,76	23,31±2,05	**
	TGF-β1	479,18±0,27	756,91±0,54	522,13±0,94	**

H-MSCs: MSCs derived from healthy people; PSO-MSCs: MSCs derived from non-treated psoriatic patients; PSO-MSCs/H-MSCs: PSO-MSCs co-cultured with H-MSCs.
All the values are reported in pg/ml and expressed as mean±SD.
*: *p*-value<0,05; **: *p*-value<0,01; n.s.: not significant.

Table 5. Levels of evaluated cytokines in the co-culture of HaCaT with MSCs.

	Cytokines	HaCaT (pg/ml)	HaCaT/ H-MSCs (pg/ml)	HaCaT/ PSO-MSCs (pg/ml)	HaCaT/ H-MSCs vs HaCaT	HaCaT/ PSO-MSCs vs HaCaT
Th1/Th17	*IL-6*	1666,90 ±0,17	13171,63 ±1,54	13266,90 ±0,15	***	***
	IL-12	8,31±0,66	6,73±0,55	6,93±0,75	n.s.	n.s.
	IL-17A	41,71±0,75	8,63±0,49	8,27±0,96	**	**
	IFN-γ	5,88±0,94	11,19±1,47	7,63±0,61	*	*
	TNF-α	35,09±1,91	26,14±1,87	21,69±0,70	*	*
	G-CSF	66,12±2,37	1255,61 ±2,68	940,02 ±3,32	***	***
Th2	*IL-2*	55,31±0,22	34,71±2,63	27,26±1,53	*	*
	IL-4	8,46±1,14	5,59±0,54	7,53±0,86	*	n.s.
	IL-5	11,28±1,64	7,70±1,15	5,79±0,38	*	*
	IL-10	22,53±0,32	18,86±2,80	22,83±0,97	n.s.	n.s.
	IL-13	250,10 ±0,43	19,61±1,37	32,59±2,82	**	**
	TGF-β1	694,15 ±1,59	736,90 ±2,06	366,04 ±0,72	*	**

HaCaT: individually cultured KCs cell line; HaCaT/H-MSCs: HaCaT co-cultured with MSCs derived from healthy subjects; HaCaT/PSO-MSCs: HaCaT co-cultured with PSO-MSCs.
All the values are reported in pg/ml and expressed as mean±SD.
*: *p*-value<0,05; **: *p*-value<0,01; *** *p*-value<0,001; n.s.: not significant.

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