IL-1β-primed Mesenchymal Stromal Cells improve epidermal substitute engraftment and wound healing via MMPs and TGF-β1


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IL-1β-primed Mesenchymal Stromal Cells improve epidermal substitute engraftment and wound healing via MMPs and TGF-β1

Running head of the title:

IL-1β-primed MSC favor wound healing

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ABSTRACT

Since the 1980s, deep and extensive skin wounds and burns are treated with autologous Split-Thickness Skin Grafts, or Cultured Epidermal Autografts (CEAs) when donor sites are limited. However, the clinical use of CEAs often remains unsatisfactory due to poor engraftment rates, altered wound healing and reduced skin functionality.

In the past few decades, Mesenchymal Stromal Cells (MSCs) have raised much attention due to their anti-inflammatory, pro-trophic and pro-remodeling capacities. More specifically, gingival MSCs have been shown to possess enhanced wound healing properties compared to other tissue sources. Growing pieces of evidence have also indicated that MSC priming could potentiate therapeutic effects in diverse in vitro and in vivo models of skin trauma.

In the present study, we found that, IL-1β-primed gingival MSCs (IL-MSCs) promoted cell migration, dermal-epidermal junction formation and inflammation reduction in vitro, as well as improved epidermal substitute engraftment in vivo. IL-MSCs had different secretory profiles from naive gingival MSCs (NV-MSCs), characterized by an overexpression of TGF-β and MMP pathway agonists. Eventually, MMP-1, MMP-9 and TGF-β1 appeared to be critically involved in IL-MSC mechanisms of action.
KEYWORDS

Mesenchymal stromal cells
Preconditioning / Priming
Wound healing
Severe burn
Skin graft

LIST OF ABBREVIATIONS

CM: Conditioned Medium
CEA: Cultured Epidermal Autograft
hPBES: human Plasma-based Epidermal Substitute
IL-CM: IL-1β primed MSC Conditioned Medium
NV-CM: naive MSC Conditioned Medium
COL-4: Collagen-4
CK-10: Cytokeratin-10
DEJ: Dermal-Epidermal Junction
ECM: Extracellular Matrix
IL-1β: Interleukin-1β
LAM-5: Laminin-5
LAM-γ2 : Laminin-5 gamma-2-chain
MSC: Mesenchymal Stromal Cell

IL-MSC: IL-1β-primed MSC

NV-MSC: naive MSC

NID-1: Nidogen-1

TNC: Tenascin-C
INTRODUCTION

Skin is essential to protect the body against infections and water loss. Upon injury, immune and skin cells trigger a cascade of events to orchestrate the wound repair. However, in case of full-thickness injuries or massive burns, this process is delayed. The current gold standard uses skin autografts, but is limited by donor site availability. Alternative therapies combine surgery and Cultured Epidermal Autografts (CEAs) or keratinocytes in spray (Chua et al., 2016, Ter Horst et al., 2018), but remain unsatisfactory due to persistent inflammation, poor skin engraftment and immature Dermal-Epidermal Junction (DEJ) (Auxenfans et al., 2015, Cirodde et al., 2011).

Mesenchymal Stromal Cells (MSCs) have become an attractive therapeutic option to improve tissue repair and treat severe skin disorders in clinical cases (Cerqueira et al., 2013, Gaur et al., 2015). Preclinical studies have highlighted their potential to reduce inflammation and promote reepithelialization and extracellular matrix (ECM) remodeling through paracrine mechanisms (Chen et al., 2016, Jackson et al., 2012). However, MSCs isolated from different tissue sources may not possess the same healing properties (Macrin et al., 2017). The gingiva is a tissue that heals fast with mild inflammation and minimal scar formation (Glim et al., 2013). Due to its location, it remains well-preserved from traumatic injuries, and easily accessible for cell harvest. From a clinical perspective, gingival tissues yield more proliferative (Li et al., 2018, Tomar et al., 2010) and clonogenic cells than the bone marrow (Fournier et al., 2010). At last, healing properties of gingival cells are seemingly superior to those of bone marrow MSCs to treat radiation burns (Linard et al., 2015).

MSCs are known to sense their environment and adapt accordingly (Kusuma et al., 2017). Using specific priming cues, such as cytokines, it is possible to guide MSC (Madrigal et al., 2014). However, to reach therapeutic outcomes, MSC priming must be carefully chosen depending on the disorder to treat (Magne et al., 2018). In the context of skin wound healing,
inflammatory primings have been reported to substantially improve MSC therapies (Heo et al., 2011).

Interleukin-1β (IL-1β) is released early after injury and can persist at high levels long after severe skin traumas, such as burns (Jeschke et al., 2008). In the gingival tissue, this cytokine is known to promote the secretion of matrix metalloproteinases (MMPs), chemokines and prostaglandins (Preshaw and Taylor, 2011), that contribute to the process of wound healing (Singer and Clark, 1999). Recent studies have shown that IL-1β-primed non-gingival MSCs could reduce inflammation (Fan et al., 2012, Song et al., 2017) and stimulate wound closure in vivo (Park et al., 2018). However, no study has yet addressed the effect of IL-1β-primed gingival MSCs (IL-MSCs) on skin wound healing and epidermal graft take after major skin injuries.

In the present study, we therefore investigated whether IL-MSCs were superior to naive MSCs (NV-MSCs) to improve wound healing and epidermal engraftment in vitro and in vivo. Our results demonstrated that IL-MSCs reduced inflammation, stimulated migration and DEJ formation and promoted epidermal engraftment, through the secretion of MMPs and TGF-β1. Taken together these results open up exciting avenues for future clinical applications.
RESULTS

**IL-1β-primed MSCs support wound healing and epidermal maturation in vitro**

MSCs isolated from gingival tissues were plastic adherent, able to differentiate into osteogenic, adipogenic and chondrogenic lineages, and expressed characteristic markers (Figure S1). We investigated the impact of naive and IL-1β-primed gingival MSCs (NV- and IL-MSCs) on skin wound healing *in vitro*. We first developed a wound closure assay in which we intoxicated keratinocytes with a cocktail of stress molecules known to be overexpressed during traumatic skin injuries (D'Arpa and Leung, 2017, Stanojcic et al., 2018) (see “Material & Method”). IL-MSCs strongly accelerated the wound closure of intoxicated keratinocytes (p < 0.05) and faster than NV-MSCs (p < 0.05, Figure 1a). Using an air/liquid differentiation assay, we then showed that IL-MSCs slightly increased epidermal thickness (p = 0.11), significantly supported basal layer organization (p < 0.05, Figure 1b), and promoted Cytokeratin-10 (CK-10, Figure 1c) expression compared to control or NV-MSCs in a human Plasma-Based Epidermal Substitute (hPBES, a CEA developed in our laboratory (Alexaline et al., 2015). Both NV- and IL-MSCs promoted the deposition of DEJ proteins including Tenascin-C (TNC), Laminin-5 (LAM-5) and Collagen-4 (COL-4) (Figure 1c). At last, NV-MSC and IL-MSCs drastically reduced the inflammatory response of a LPS-challenged monocytic THP1cell line, as depicted by a high increase of IL-1RA (p < 0.01, Figure 1d) and a strong decrease of TNF-α (p < 0.01, Figure 1e) in THP-1 culture supernatants. Interestingly, IL-MSCs were superior to NV-MSCs to decrease TNF-α secretions (p < 0.05, Figure 1e). Taken together, these results suggest that IL-MSCs are superior to NV-MSCs to promote wound closure, hPBES maturation and decrease of inflammation.

**IL-1β-primed MSCs support hPBES engraftment and wound healing in vivo**

We next compared the wound healing potential of NV- and IL-MSCs in a NOD/SCID mouse
model of full-thickness injury and hPBES grafting. Based on preliminary dose-effect studies, treated mice received 750,000 MSCs (Figure S2). Our results indicated that IL-MSCs strikingly increased the engraftment rate of hPBES compared to the control (p < 0.05, Figure 2a). While NV- and IL-MSCs did not improve the basal cell organization of grafted hPBES (p = ns), IL-MSCs significantly favored the epidermal thickness compared to control (p < 0.05) and tended to have a more prominent effect than NV-MSCs (p = 0.16, Figure 2b). NV- and IL-MSCs also improved the expression of epidermal differentiation marker CK-10, and DEJ proteins such as TNC, LAM-5 and COL-4 in grafted hPBES (Figure 2c). Lastly, we noted that IL-MSCs induced a shift towards a M2-polarization with a significant drop of iNOS⁺/CD206⁺ cell ratio compared to the control (p < 0.01), while NV-MSCs did not (p = ns, Figure 2c-d). To conclude, while NV- and IL-MSCs seemed to have similar effects on hPBES maturation and DEJ deposition, IL-MSCs were more efficient to support hPBES engraftment and thickening in vivo.

**MSC secretome is significantly modified by IL-1β priming**

As IL-MSCs appeared to possess superior repair properties in vitro and in vivo, we next sought to decipher their mechanisms of action. Therefore, we investigated how IL-1β priming could modify MSC secretome, since these cells mainly operate through paracrine mechanisms (Gneccchi et al., 2016). Mass spectrometry analysis of naive and IL-1β-primed MSC Conditioned Media (NV-CM and IL-CM respectively) revealed substantial differences in terms of protein content and expression level (Figure 3a, Table S1). We noted that the IL-1β priming induced the expression of 76 proteins uniquely found in IL-CM. Based on protein intensity scores, a higher expression of proteins related to migration, angiogenesis, remodeling, inflammation, SMAD, Integrin and Wnt pathways was found in IL-CM compared to NV-CM (Figure 3b). These data were supported by a protein interaction study using the online “String” data base (Figure S3a). To confirm these results, we quantified by
ELISA a selection of wound healing-related proteins and found that active MMP-1, active MMP-9, HGF, IGFBP-7, STC-1, TGF-β1 (Figure 3c), VEGF, FGF-2, FGF-7, IL-6, IL-1RA and SOD-2 (Figure S3b) were up-regulated in IL-CM compared to NV-CM (p < 0.05). Taken together, these findings reveal that MSCs secrete higher levels of wound healing-related factors after IL-1β priming.

**Beneficial effects of IL-1β-primed MSCs relies on distinct paracrine secretions**

Given the differences between NV- and IL-CM, we next sought to investigate their effect in vitro. Our findings revealed that IL-CM had a higher pro-migratory effect than NV-CM on intoxicated keratinocytes (p < 0.05, Figure 4a). IL-CM also tended to improve the expression level of several DEJ proteins, including Nidogen-1 (NID-1) (p = 0.094), TNC (p = ns) and Laminin-5 gamma-2 chain (LAM-γ2) (p = 0.094) compared to control (Figure 4b-d). Lastly, IL-CM presented stronger anti-inflammatory properties than NV-CM, as shown by the significant drop of TNF-α (p < 0.001, Figure 4e) and increase of IL-1RA (p < 0.001, Figure 4f) in LPS-challenged THP-1 supernatants. Importantly, THP-1 were the main producers of TNF-α and IL-1RA, as these factors were respectively absent (data not shown) or barely expressed in IL-CM (Figure S4a). Taken together, these data imply that IL-MSCs secrete distinctive factors that better regulate the skin wound healing than those derived from NV-MSCs.

**MMPs and TGF-β1 are key mediators involved in the mechanism of action of IL-1β-primed MSCs**

Rapid and scarless gingival repair is thought to rely on superior remodeling and anti-inflammatory properties of gingival cells (Leavitt et al., 2016, Mah et al., 2017). According to our secretome analysis (Figure 3b-c), we therefore focused on MMPs and SMAD signaling to investigate the mechanisms of action of IL-CM. We used Tigecycline, a broad-spectrum
inhibitor of MMPs (Pasternak and Aspenberg, 2009), SB431542, an inhibitor of the TGF-β receptor 1 (Inman et al., 2002), and human recombinant MMP-1, MMP-9, TGF-β1, HGF, IGFBP-7, STC-1 and QSOX-1 (used alone or combined in a cocktail, at the concentration they were found in IL-CM by ELISA). We showed that Tigecycline clearly prevented the IL-CM-induced migration of intoxicated keratinocytes (p < 0.01), while SB431542 did not (p = ns, Figure 5a). These results were confirmed using MMP-1 which significantly increased intoxicated keratinocytes migration (p < 0.01), while TGF-β1 or any other protein tested did not (p = ns, Figure 5b and S4b). Regarding DEJ protein expression, SB431542 abrogated the effect of IL-CM on NID-1 (p<0.05, Figure 5c) and tended to suppress the expression of TNC and LAM-γ2 (Figure 5d-e). Tigecycline abolished the effect of IL-CM on NID-1 only (p < 0.05, Figure 5c). When used alone in control experiments, both inhibitors did not induce DEJ protein expression drop, except for NID-1 with Tigecycline (p=0.086, Figure S5). We next observed that MMP-1, MMP-9 and TGF-β1 could possibly improve NID-1 (p = ns, Figure 5f and S4c) and TNC expression (p = ns, Figure 5g and S4d). However, none of the tested proteins increased LAM-γ2 expression (Figure 5h and S4e). At last, none of the tested inhibitors (Figure 5i), or tested proteins were able to block the effect of IL-CM on TNF-α production in LPS-challenged THP-1 supernatants (p = ns, Figure 5j and S4f). Conversely, both inhibitors abrogated the effect of IL-CM on IL-1RA (p < 0.05 and p < 0.01 respectively, Figure 5k). Recombinant TGF-β1 (p < 0.01), MMP-9 (p < 0.05), HGF (p < 0.01), IGFBP-7 (p < 0.05) and the protein cocktail (p < 0.01) significantly increased the expression of IL-1RA (Figure 5l and S4g). Taken together, these results suggest that IL-MSCs promote migration, DEJ protein deposition and reduction of inflammation through the synthesis of MMPs and TGF-β1.
DISCUSSION

In an attempt to improve the clinical management of full-thickness injuries and burns, we aimed to bring forward MSC therapy using a specific tissue source and an inflammatory priming. IL-1β is known to play important roles in oral mucosal wound healing (Graves et al., 2001), but is also a key pathological mediator in inflammatory oral diseases. In this study, the priming dose was below the levels observed in patients with gingivitis or periodontitis (Orozco et al., 2006). Therefore, we showed that IL-1MSCs promoted skin wound closure, DEJ protein deposition, reduction of inflammation and, epidermal engraftment through a paracrine mechanism involving MMPs and TGF-β1 signaling (Figure 6).

As many other investigators, we found that the IL-1β priming of MSCs led to major secretome changes in terms of growth factors, inflammatory mediators and ECM components (Figure 3 and S3) (Lee et al., 2010, Maffioli et al., 2017, Redondo-Castro et al., 2018). Up-regulated growth factors such as FGF-2, FGF-7, HGF and TGF-β1 may have helped promote the keratinocyte migration (Peplow and Chatterjee, 2013, Seeger and Paller, 2015), although we did not see a beneficial effect of individual factors (Figures 5b and S4b). As shown in our study, and in line with previous works (Benjamin and Khalil, 2012), MMP1 also clearly contributed to the promotion of keratinocyte migration (Figure 5b). Enhanced secretion of TGF-β1, MMP-9, HGF and IGFBP-7 following the IL-1β priming increased the release of IL-1RA by LPS-challenged THP-1 cells (Figure 5l and S4g), highlighting their anti-inflammatory role, as previously reported (de Araujo Farias et al., 2018). Our results investigating the mechanism of IL-CM on decreasing secretion of TNF-α also suggest that other paracrine factors might be involved, such as TSG-6 (Qi et al., 2014). The overexpression of TGF-β1 and MMPs in IL-CM was shown to have an overall positive impact on the production of DEJ proteins, although this effect was not always obvious in vitro (Figure 5f-h and S4c-e). Such inconsistencies are probably due to the dual role of MMPs that...
degrade DEJ proteins like COL-4 (Monaco et al., 2006) and activate DEJ-stimulating growth factors like TGF-β1 (Benjamin and Khalil, 2012). However, our results indicate that enhanced DEJ protein deposition and decreased inflammation in vivo might have contributed to the promotion of epidermal engraftment and maturation (Figure 2 and S2). Indeed, other studies have reported a better epidermal engraftment when DEJ proteins were preserved or added exogenously (Alexaline et al., 2019, Takeda et al., 1999). Recent studies have also shown that MSCs contribute to DEJ restoration through direct secretion of type VII collagen (Ganier et al., 2018) or exosomes containing both protein and mRNA (McBride et al., 2018). In our secretome analysis, increased expression levels of type IV and VII collagens, and nidogens were found in IL-CM, confirming previous results showing that IL-1β primed skin cells secrete basement membrane protein in higher amount (Furuyama et al., 2008, Matsushima et al., 1985, Mauviel et al., 1994, Vardar-Sengul et al., 2009). Therefore, the beneficial effect of IL-1β primed MSC must be due to a combination of direct DEJ protein deposition or an indirect stimulation of DEJ-producing resident cells, such as keratinocytes and fibroblasts.

In our study, we focused on MMPs and TGF-β signalings to unravel the mechanism of action of IL-MSCs. However, several other molecular pathways may be worth explore such as the MAPK, Akt, Integrin and Wnt/β-catenin (Longmate and Dipersio, 2014, Park et al., 2018). Importantly, previous works have shown that IL-1β could activate the Wnt/β-catenin pathway, resulting in more angiogenesis (Sun et al., 2016) and increased production of MMPs (Ge et al., 2009). This signaling pathway was also shown to promote cell migration in a model of burn wound through the release of Wnt4-carrying exosomes (Zhang et al., 2014, Zhang et al., 2015).

In conclusion, IL-1β-primed MSCs represent a promising therapeutic option for future cell-based therapy of full-thickness injuries, improving wound healing and epidermal substitute engraftment. Although our study focused on whole CM, we found that most of
them were composed of extracellular vesicles (data not shown). It is thus possible that the reported effects of IL-1β primed MSCs are accountable for the presence of extracellular vesicles carrying growth factors, cytokines or extracellular components. Therefore, the present study highlights the therapeutic benefit of using MSC secretory products to improve the treatment of severe skin traumas.

MATERIALS AND METHODS

Cell isolation, culture and characterization

After written informed patient consent, gingival MSCs, skin fibroblasts and keratinocytes were extracted from human donor biopsies (Supplemental Materials and Methods) and cultivated in medium, as described previously (Alexaline et al., 2015, Doucet et al., 2005). The human THP1 cell line (ATCC) was cultured in RPMI medium supplemented with 10% decomplemented FCS, 50 μM β-mercapto-ethanol (Sigma) and penicillin-steptomycin (100 U/mL, 100 μg/mL respectively, Gibco). Gingival MSCs were characterized by flow cytometry and differentiation assays (Supplemental Materials and Methods).

MSC Priming and Conditioned Medium (CM) preparation

Passage 4 MSCs were cultivated until 60% confluence, primed for 24h with 1 ng/ml human recombinant IL-1β (Peprotech) or left naive with no treatment. The priming dose was selected according to preliminary studies (data not shown). IL-MSCs or NV-MSCs were then washed three times in PBS and incubated in serum- and antibiotic-free medium for 48h. NV-CM and IL-CM were respectively derived from NV-MSC and IL-MSC supernatants and concentrated 40X using Amicon ultra centrifugal filter units (cutoffs 3K, Millipore) (Figure S6a). CM total protein amount was determined using the Bio-Rad Protein Assay kit. CM were analyzed using mass spectrometry and ELISA (Supplemental Materials and Methods).
**Wound closure Assay**

31,500 irradiated (60 grays of γ rays) passage 1 keratinocytes were seeded in each well of migration silicone inserts (Ibidi) with complete KSFM medium (Gibco). After insert removal, keratinocytes were washed with PBS and new medium was added along with an intoxication cocktail (1.31 mg/ml NaCl (Sigma), 0.23 mg/ml NaHCO\(_3\) (Sigma), 1 ng/mL IL-1β (Peprotech), 1 ng/mL IL-6 (Peprotech) and 10 ng/mL HMGB1 (Peprotech)), optimized in preliminary studies (data not shown). Keratinocytes were co-cultured with a pool of NV-MSCs or IL-MSCs from 7 donors at a 1:10 MSC-to-keratinocyte ratio in 0.4μm pore culture inserts (PET membrane, EMD-Millipore), a pool of NV-CM or IL-CM from 7 donors at 10μg/mL, SB431542 at 10μM (all from Calbiochem), Tigecycline at 50μM (Pfizer), or specific recombinant human factors including MMP-1 at 35.3 pg/ml, MMP-9 at 33.8 pg/ml, TGF-β1 at 5 ng/ml, STC-1 at 350 pg/ml, HGF at 50 pg/ml, IGFBP-7 at 5 ng/ml or QSOX-1 at 350 pg/ml (all from R&D) (Figure S6b). The cocktail includes TGF-β1, MMP-1, MMP-9, STC-1, HGF, QSOX-1 and IGFBP-7. Pictures of the entire gap were taken at 5 hours, and analyzed using the Image J software (1.47v). Wound closure percentage was calculated: 100 x \((A_{T0}-A_{T5H})/A_{T0}\), with \(A_{T0}\): Area of the gap at 0H, \(A_{T5H}\): Area of the gap at 5H.

**DEJ Formation Assay**

Passage 1 keratinocytes were seeded and cultured on a confluent irradiated fibroblast feeder layer (Supplemental Materials and Methods). At day 4 and day 6, culture medium was supplemented with a pool of NV-CM or IL-CM from 7 donors at 10μg/mL, inhibitors or specific recombinant human growth factors (see “Wound closure assay” section) (Figure S6c). At day 8, keratinocytes were washed with PBS and lysed for further analysis of LAM-γ2, NID-1 and TNC expression by Western Blot (Supplemental Materials and Methods).

**Air/liquid differentiation assay**
hPBES were prepared as described previously ((Alexaline et al., 2015), Supplemental Materials and Methods) and transferred in 0.4 µm pore 6-well culture inserts (PET membrane, EMD Millipore), previously seeded with a pool of 25,000 NV-MSCs or IL-MSCs from 7 donors, and were grown in keratinocyte culture medium at the air/liquid interface for 7 days, with medium change every 2 to 3 days (Figure S6d). At day 7, hPBES were fixed, embedded in paraffin and processed for Hematoxylin-Phloxin-Safranin (HPS) staining and CK-10, COL-4, LAM-5 and TNC immunostainings (Supplemental Materials and Methods). Basal epidermal organization was scored as described elsewhere (Figure S7).

**Inflammation Assay**

THP1 cells were seeded in 24-well plates at 170,000 cells/mL, exposed to 1 µg/mL LPS (Escherichia coli O55:B5; Sigma) and cultured with a pool of NV-MSCs or IL-MSCs from 7 donors at a 1:10 MSC-to-THP1 ratio, a pool of NV-CM or IL-CM from 7 donors at 10µg/mL, inhibitors or specific human recombinant growth factors (see “Wound closure assay” section) (Figure S6e). Supernatant of each condition was collected after 24h and assayed for TNF-α and IL-1RA levels by ELISA (DuoSet® Kits, R&D Systems).

**Animal model of dorsal acute wound**

All experiments were approved by the Ethical Committee of “Paris-Sud n°26” in accordance with French regulations for animal experiments (#10045-2017052611235636v4). 8-week old NOD/SCID mice were premedicated with subcutaneous injection of 0.05 mg/kg Buprenorphine (Temgesic) and 0.04 mg/kg Atropine (Renaudin) and anesthetized 10 minutes later via intraperitoneal injection of 50 mg/kg Ketamine (Virbac) and 0.5 mg/kg Medetomidine (Domitor). Full-thickness excisional wounds of 1.5 x 1.5 cm² were created on the back of each animal. Both sides of Integra Dermal Regeneration Template Single Layers (Integra Lifesciences) were soaked with 50 µL of PBS 1X or a pool of 750,000 NV-MSCs or
IL-MSCs from 7 donors before being grafted on each animal wounds (Figure S6f). Grafted areas were covered with hPBES and protected by a silicon device (Interchim). Mice were left 14 days with the silicon protection before being sacrificed by sedation and overdose of anesthetics according to the French Institutional Animal Guidelines. Wounds were excised, fixed in formalin and embedded in paraffin. Samples were processed for HPS staining and Integrin-β1 (INT-β1), CD206, iNOS, CK-10, COL-4, COL-7, LAM-5 and TNC immunostainings (Supplemental Materials and Methods). hPBES organization and engraftment scores were obtained as described elsewhere (Figure S7).

**Statistics**

For all experiments, non-parametric Mann-Whitney and Kruskal-Wallis tests were used to determine statistical significance. When necessary, matched or repeated measures were taken into account using non-parametric Friedman test. All charts were plotted as mean ± sem on Prism 6 Graphpad software. Statistical analyses were conducted on R software (3.1.1v). Significance level (*) was set to p<0.05.

**DATA AVAILABILITY STATEMENT**

Datasets related to this article can be found at [http://dx.doi.org/10.17632/4r46w7rfsx.1], hosted at Mendeley Data, v1 (Magne, Brice; Dedier, Marianne; Nivet, Muriel; Coulomb, Bernard; Banzet, Sebastien; Lataillade, Jean-Jacques; Trouillas, Marina (2019), “Table S1. List of the up-regulated proteins found in the secretome of IL-1β primed MSC.”, Mendeley Data, v1

http://dx.doi.org/10.17632/4r46w7rfsx.1).
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Disclosure statement

The authors indicate no potential conflicts of interest.

CRediT statement

Conceptualization: BM, BC, MT; Data Curation: BM, MD, MT; Formal analysis: BM, MD, MT; Investigation: BM, MD, MN, MT; Methodology: BM, MT; Visualization: BM, MT; Writing-original draft: BM, MT; Funding acquisition: BC, SB, JJL; Supervision: SB, JJL; Project administration: MT; Validation: MT; Writing – review & editing: MT.
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FIGURE LEGENDS

Figure 1. IL-1β-primed MSCs improve wound closure, epidermal maturation and reduce inflammation in vitro. (a) Wound closure percentage at 5h of TOX, co-cultured in inserts with NV- or IL-MSCs (ratio to TOX, n=8). (b) Hematoxylin-Phloxin-Safranin stainings, epidermal layer number, basal organization score, and (c) IHC stainings of hPBES (CTRL) co-cultured with NV- or IL-MSCs (n=7). (d) Dosage of IL-1RA and (e) TNF-α in the supernatants of LPS-challenged THP-1 co-cultured for 24h with NV- or IL-MSCs (ratio to LPS, n=6). Scale bar = 50 (a), 100 (b) µm. Values are expressed as means± SEM. *p<0.05; **p<0.01; ns, not significant; CK-10, Cytokeratin-10; COL-4, Collagen-4; CTRL, control; hPBES, human Plasma-based Epidermal Substitute; IL, IL-1β; LAM-5, Laminin-5; MSC, mesenchymal stromal cells; NV, naive; TNC, Tenascin-C; TOX, intoxicated keratinocytes.

Figure 2. IL-1β-primed MSCs stimulate hPBES engraftment and epidermal maturation in vivo. (a) Reepithelialization scores are expressed as a percentage of mice present in each reepithelialization groups (bad: 0-20%, medium: 20-60%, good: 60-100%). (b) Hematoxylin-Phloxin-Safranin stainings, epidermal layer number, basal organization score, (c) IHC stainings and (d) marker quantifications of hPBES (CTRL) at 14 days after in vivo grafting and treatment with NV- or IL-MSCs (scale bar = 100 µm) (n=10 to 16). Values are expressed as means ± SEM. * p<0.05; ** p<0.01; ns, not significant; CK-10, Cytokeratin-10; COL-4, Collagen-4; CTRL, control; hPBES, human Plasma-based Epidermal Substitute; IL, IL-1β; LAM-5, Laminin-5; MSC, mesenchymal stromal cells; NV, naive; TNC, Tenascin-C.

Figure 3. Naive and IL-1β-primed MSCs possess distinct secretory profiles. (a) Protein repartition, and (b) intensity score analysis in NV- and IL-CM using mass spectrometry.
analysis (n=7 donors). (c) ELISA dosages of selected wound healing-related proteins in NV- and IL-CM used at 10µg/ml (n=7 donors). Values are expressed as means ± SEM. * p<0.05; ** p<0.01; ns, not significant; A.U., Arbitrary units; IL, IL-1β; MSC, mesenchymal stromal cells; NV, naive.

**Figure 4. IL-1β-primed MSC Conditioned Medium improves skin wound healing in vitro.** (a) Wound closure at 5h of TOX, cultured with NV- or IL-CM (scale bar = 500µm) (ratio to TOX, n=7). (b) Nidogen-1, (c) Tenascin-C and (d) Laminin-γ2 expression assessed by western blot at 8 days of keratinocyte and fibroblast co-cultures (CTRL) grown with NV- or IL-CM (ratio to control, n=7). (e) Dosage of TNF-α and (f) IL-1RA in the supernatants of LPS-challenged THP-1 cultured for 24h with NV- or IL-CM (ratio to LPS, n=10 to 16). Values are expressed as means ± SEM. * p<0.05; ** p<0.01; *** p<0.001; ns, not significant; CM, conditioned medium; CTRL, control; IL, IL-1β; LAM-γ2, Laminin-5 γ2 chain; NID-1, Nidogen-1; NV, naive; TNC, Tenascin-C; TOX, intoxicated keratinocytes.

**Figure 5. IL-1β-primed MSCs positively impact skin wound healing via TGF-β1 and MMPs in vitro.** (a-b) Wound closure at 5h of TOX (scale bar = 500µm) (ratio to TOX, n=7 to 8), (c-h) Nidogen-1, Laminin-γ2 and Tenascin-C protein expression assessed by western blot at 8 days in keratinocyte and fibroblast (CTRL) cultures (ratio to CTRL, n=6 to 7) and (i-l) dosage of TNF-α and IL-1RA in supernatants of LPS-challenged THP-1 cultured for 24h (ratio to LPS, n=5 to 19), grown or not with (a-c-d-e-i-k) SB431542- or Tigecycline-supplemented IL-CM, or (b-f-g-h-j-l) selected IL-CM-derived factors. Values are expressed as means± SEM. *p<0.05; **p<0.01; ***p<0.001; ns, not significant. CM, conditioned medium; CTRL, control; IL, IL-1β; LAM-γ2, Laminin-5 γ2 chain; NID-1, Nidogen-1; NV,
naive; TNC, Tenascin-C; TOX, intoxicated keratinocytes.

**Figure 6. Summary of the main findings.** IL-MSCs mediate keratinocyte migration, differentiation, DEJ deposition and mitigate inflammation of THP1 cells through the release of MMP-1, MMP-9, TGF-β1, IGFBP-7 and HGF *in vitro*. They further improve skin wound healing and hPBES engraftment *in vivo*. CK-10, Cytokeratin-10; COL-4, Collagen-4; DEJ, dermal-epidermal junction; hPBES, human Plasma-based Epidermal Substitute; KC, keratinocyte; LAM-5, Laminin-5; MSC, mesenchymal stromal cells; NID-1, Nidogen-1; TNC, Tenascin-C.
IL-1β priming

MSC

MMP-1

TGF-β1

MMP-9

MMP-9

TGF-β1

HGF

IGFBP-7

THP-1

KC

MIGRATION

CK-10

NID-1

TNC

LAM-5

COL-4

IL-1RA

↑ IN VIVO WOUND HEALING & EPIDERMAL ENGRAFTMENT
MSC isolation and culture

Gingival biopsies of 7 healthy donors were cut and digested for 2.5 hours at 37 °C with 2.4 U/mL dispase II (Roche), 0.8 U/mL collagenase MTF (Roche), 100 µL of penicillan (Panpharma), 50 µg/mL gentamicin (Panpharma), 2.5 µg/mL amphotericin B (Bristol-Myers Squibb). Harvested cells were seeded at 5,000 cells/cm² and cultivated in MEM supplemented with 5% human platelet lysate (PL), 2 µL/mL heparin (Sanofi), 100 µL of penicillan, 50 µg/mL gentamicin and 2.5 µg/mL amphotericin B at 37 °C in a humid atmosphere under 5% CO2. After 2 days, cell medium was changed and amphotericin B was reduced to 1 µg/mL. Cells were trypsinized at 80% confluence using 0.05% trypsin/EDTA (Gibco) for 5 minutes at 37 °C and replated between 4,000 and 5,000 cells/cm² or frozen in a mixture of MEM, 10% DMSO (Sigma) and 9% human serum albumin (LFB Biomedicaments).

Keratinocyte isolation and culture

Human keratinocytes and dermal fibroblasts were obtained after informed consent from healthy donors undergoing breast reduction surgery. Skin pieces were digested overnight at 4°C in 1.8 U/µL dispase II and 0.0625% trypsin (Biochrom). After mechanical separation from the dermis, the epidermis was dissociated at 37°C in 0.05% trypsin/EDTA (Gibco) for 30 min. Keratinocytes were immediately frozen. Dermis was digested in 2.4 U/mL dispase II and 2.4 µg/mL collagenase II. Harvested dermal fibroblasts were plated at 4,000 cells/cm² and amplified in DMEM (Gibco) supplemented with 5% human PL, 10 µg/mL ciprofloxacin (Bayer Pharma) and 2 U/mL heparin. At 80% confluence, fibroblasts were frozen in liquid nitrogen. Keratinocytes were thawed and plated at 2,400 cells/cm² on a growth-arrested irradiated fibroblast feeder layer (60 Grays of γ-rays), seeded at 20,000 cells/cm² in a medium described elsewhere [Alexaline, 2015 #205]. Medium change was performed every two to three days. Before reaching 70% confluence, keratinocytes were trypsinized and used for wound closure assay, DEJ formation assay or hPBES preparation.

Human Plasma-Based Epidermal substitute (hPBES) preparation

hPBES were prepared as described previously [Alexaline, 2015 #205]. Briefly, a mix solution containing 39.8% of plasma (pool of fresh frozen human plasma), obtained from 10 donors, 4.66 mg/mL NaCl (Fresenius), 0.1mg/mL Calcium Chloride (Laboratoire Renaudin) and 0.39 mg/mL Exacyl (Sanofi) was poured on appropriate culture plates (0.3 mL/cm²²) and left to polymerize for minimum 3 h at 37°C. Growth-arrested fibroblasts and keratinocytes were then plated on this plasma matrix at 20,000 cells/cm² and 2,400 cells/cm² respectively. After 14 days of culture, epidermal substitutes were used for in vitro air/liquid differentiation assay or in vivo grafting.

Flow cytometry

MSCs were incubated with primary antibodies diluted in a PBS solution containing 2% w/v human serum albumin and 0.5% w/v human immunoglobulin G (LFB Biomedicaments) for 20 min at 4°C. Primary antibodies included anti-CD45-FITC, anti-CD90-FITC, anti-HLA-DR-FITC, anti-CD44-PE, anti-CD105-PE, anti-CD73-PE, anti-CD29-PE, anti-IgG1-FITC or PE (all from Beckman Coulter). After PBS washing, stained MSCs were analyzed using flow cytometry (Beckman Coulter Navios). Isotypic control was used as a negative control.

Differentiation Assays

For osteogenic differentiation assay, MSCs were seeded at 3,000 cells/cm² and cultured for 21 days in MEM supplemented with 10% FCS, 0.1µM dexamethasone, 0.05mM L-ascorbic acid-2-phosphate and 10mM -glycophosphate (all from Sigma), with medium change 2 to 3 times a week. Matrix mineralization was assessed using a 2% Alizarine Red (AR) solution (Sigma). It was further confirmed by a Von Kossa (VK) staining, incubating the cells for 15 min under UV-light in a 1% silver nitrate solution. For chondrogenic differentiation assay, MSCs were pelleted at 500g for 5min without brake and cultured for 21 days in DMEM high glucose (Gibco) supplemented with 10% FCS, 1 mM sodium pyruvate, 0.35 mM L-proline, 1X ITS, 0.17 mM ascorbic acid-2-phosphate, 0.1 µM dexamethasone, 10 ng/mL TGF-β3 (all from Sigma) and 5.3g/mL linoleic acid (Fluka). Pellets were embedded in paraffin and glycosaminoglycans were identified with Alcian Blue staining (VWR). For adipogenic differentiation assay, sub-confluent cultures of MSCs were exposed to three induction cycles with a medium composed of MEM supplemented with 10% FCS, 1 µM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine, 10 µM insulin, 200 µM indomethacin (all from Sigma). After 21 days, lipid droplets were stained using an Oil red O (ORO) solution (Cayman Chemical).

Mass spectrometry and secretome analysis

18 µg of NV-CM and IL-CM were pseudo-separated by 10% SDS-polyacrylamide gel electrophoresis. Proteins were reduced, alkylated, and digested in gel using a DigePro instrument (Intavics, Kiev) according to manufacturer’s instructions. Tryptic peptides were then dried in a vacuum centrifuge (Vacuum Concentrator, Thermoscientific) and analyzed by high performance liquid chromatography (HPLC) tandem mass spectrometry (LTQ-Orbitrap Velos, Thermoscientific). 2µL of peptides were injected in the system using a pre-concentration column (Acclaim PepMap C18, Thermoscientific), and separated by reversed phase chromatography using a C18 column (Acclaim PepMap nanoViper C18, Thermoscientific). Separation was achieved in a linear 45 min LC gradient from 4% to 55% acetonitrile in 0.1% formic acid (v/v) at a flow rate of 250 nL/min before direct electrospraying into the mass spectrometer. Raw MS file were processed with Proteome Discoverer (1.4v). The search included variable modifications for oxidation of methionine, peptide N-terminal acetylation and Carbamidomethylation of cysteine was set as a fixed modification. Peptides were matched using trypsin as a digestion enzyme and one missed cleavage site was allowed. The mass error for the precursor ions (full MS) was less than 10 ppm (errorppm = (m/zexperiment - m/zexact) x 106/ m/zexact). Mass error for ions from the MS/MS spectra was reported less than 1%, low if the FDR is greater than 5% and average (medium between 1 and 5 %). Peptide identifications were validated by determination of false positives by target decoy PSM validator. It is high if the false positive rate (FDR or false Discovery rate) is less than 0.6 Da. Peptides mass is searched between 350 Da and 7000 Da with time retention from 10 min to 60 min. Peptide identifications were validated by determination of false positives by target decoy PSM validator. It is high if the false positive rate (FDR or false Discovery rate) is less than 1%, low if the FDR is greater than 5% and average (medium between 1 and 5 %). Peptide identification Xcorr were calculated by the Xcorr data base (https://string-db.org/).

Western blots
Cells were lysed in a PBS solution containing 1% NP40, 0.1% of SDS, 0.5% of deoxycholic acid, protease and phosphatase inhibitor cocktails (all from Sigma). Supernatants were collected after centrifugation at 13,000 g for 15 min. Total protein content in cell lysates was evaluated with Bio-rad Protein Assay kit. 50 µg of protein samples were loaded on 10% SDS-polyacrylamide gels (Bio-Rad). After 45 min of migration, proteins were electro-transferred at 4°C for 2.5 hours on PVDF membrane (Immolohon-P Transfer Membrane, Millipore). Membranes were blocked at room temperature for 2 h in PBS 2% Tween 20 (Sigma) and 3% skimmed milk and incubated overnight at 4°C with primary antibodies (see table below). Membranes were then incubated for 45 min with horseradish peroxidase (HRP)-conjugated goat anti-rabbit, goat anti-mouse or donkey anti-goat immunoglobulin G (IgG) (Santa-Cruz). ECL substrate (Bio-Rad) was used to reveal antibody-binding sites. Signal intensity was detected with Chemidoc instrument and analyzed with Image Lab software. Then, signal intensity of protein of interest was normalized to signal intensity of β-actin.

<table>
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<tr>
<th>Antibody</th>
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**Histology, Immunohistochemistry (IHC), quantification and scoring**

Samples were washed in PBS, fixed in 4% formalin (LaboNord) and dehydrated with graded series of ethanol solutions prior to paraffin embedding (Thermo Scientific). Paraffin sections of 5 µm thickness were dried, deparaffinized, and stained with Hematoxylin-Phloxin-Safranin (HPS) (All from Dako). For IHC, paraffin sections of 5µm thickness were fixed on polylysine slides (Thermo Scientific). Sections were dried overnight at 37°C and deparaffinized. Antigen retrievals were performed in pH 6 solution for 20 min at 95°C, pH 9 solution for 20 min at 95°C, 0.1 mg/ml CaCl₂ solution containing 1 mg/ml pronase (Sigma) for 10 min at 37°C, or 1% trypsin (Gibco) for 30 min at 37°C. Endogenous peroxidases were blocked with 3% H₂O₂ (Dako, Denmark). Sections were incubated at room temperature for 30 minutes with primary antibodies (see table below). Detection was performed using LSAB™2 Kit (Dako) with Dako autostainer instrument.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Antigen retrieval</th>
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<td>CK10</td>
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<td>iNOS</td>
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<td>Integrin β1</td>
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<td>#4C8MS</td>
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M1-to-M2 ratio was calculated as the ratio of iNOS positive cell count to CD206 positive cell count. Basal layer organization and epidermal layer number were obtained from three observers. Basal organization was considered the best when nuclei reached an apical position, keratinocytes appeared cuboidal and no discontinuity in the basal layer was observed (Figure S2a). Human reepithelialization percentage was calculated as the ratio of human Integrin-β1 positive epidermal length to total wound length (Figure S2b).
<table>
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<th>Protein Name</th>
<th>Molecular function</th>
<th>Biological Process</th>
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<td>ATP hydrolysis coupled protein transport. phagosome acidification</td>
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<td>Appearance</td>
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<tr>
<td>Calumen</td>
<td>Calcium ion binding</td>
<td>Cell membrane protein</td>
<td>0.2207</td>
<td>Appearance</td>
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<tr>
<td>Macrophage-capping protein</td>
<td>Actin binding. Structural molecule activity</td>
<td>Macrophage function</td>
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<td>Appearance</td>
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<td>Centrosomal protein of 290 kDa</td>
<td>Protein binding</td>
<td>Protein transport</td>
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<td>Appearance</td>
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<tr>
<td>C-X-C motif chemokine-IIlerkur-8</td>
<td>Cytoxine activity</td>
<td>Inflammatory response</td>
<td>0.2207</td>
<td>Appearance</td>
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<tr>
<td>Endoplasm reticulum amminopeptidase 1</td>
<td>Metallopeptidase activity. Peptide and zinc ion binding</td>
<td></td>
<td>0.2207</td>
<td>Appearance</td>
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<td>ERB exoribonuclease 2</td>
<td>3'-5'-exodexoxyribonuclease activity</td>
<td></td>
<td>0.2207</td>
<td>Appearance</td>
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<td>Glutaredoxin-3</td>
<td>Metal ion binding</td>
<td></td>
<td>0.2207</td>
<td>Appearance</td>
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<tr>
<td>C-type natrio receptor 2</td>
<td>transmembrane signaling receptor activity</td>
<td>Remodeling ECM</td>
<td>0.2207</td>
<td>Appearance</td>
</tr>
<tr>
<td>Nidogen-1</td>
<td>ECM Structural component. ECM binding</td>
<td>ECM and basement membrane organization</td>
<td>0.2207</td>
<td>Appearance</td>
</tr>
<tr>
<td>Nidogen-2</td>
<td>ECM Structural component. ECM binding</td>
<td>ECM and basement membrane organization</td>
<td>0.2207</td>
<td>Appearance</td>
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</table>

**Table S1. List of the up-regulated proteins found in the secretome of IL-1β primed MSC.**
<table>
<thead>
<tr>
<th>Protein name</th>
<th>Binding activity</th>
<th>Functional description</th>
<th>Appearance</th>
<th>Fold Change</th>
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<tr>
<td>Perilipin-3</td>
<td>Cadherin binding</td>
<td>Vesicle-mediated transport</td>
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<td>Appearance</td>
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<tr>
<td>Actin-binding</td>
<td>Actin filament and calcium binding</td>
<td>Actin filament bundle assembly</td>
<td>0.2207</td>
<td>Appearance</td>
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<tr>
<td>Plasma protease C1 inhibitor</td>
<td>Serine-type endopeptidase inhibitor activity</td>
<td>Complement activation. Blood coagulation. (thromolysis</td>
<td>0.2207</td>
<td>Appearance</td>
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<td>Sphingomyelinase</td>
<td>Serine-independent protein</td>
<td>Endocytosis activity</td>
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<td>Appearance</td>
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<tr>
<td>Sushi-repeat-containing protein SRPX</td>
<td>Protein binding</td>
<td>Urokinase plasminogen-activator surface receptor. Angiogenesis. Cell migration and adhesion</td>
<td>0.2207</td>
<td>Appearance</td>
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<tr>
<td>Sushi von Willebrand factor type A</td>
<td>Actin filament and calcium binding</td>
<td>Actin filament bundle formation</td>
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<td>Appearance</td>
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<tr>
<td>Plastin-3</td>
<td>Actin filament and calcium binding</td>
<td>Actin filament bundle assembly</td>
<td>0.2207</td>
<td>Appearance</td>
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<td>Actin-related protein 2/3 complex subunit 4</td>
<td>Protein binding</td>
<td>Actin filament polymerization</td>
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<td>Mannan-binding lectin serine protease 1</td>
<td>Peptidase activity. Calcium-dependent protein and calcium binding</td>
<td>Complement activation</td>
<td>0.2449</td>
<td>15.9472</td>
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<td>Complement C4-B</td>
<td>Endopeptidase inhibitor activity.</td>
<td>Complement activation. Inflammatory response</td>
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<td>4.2798</td>
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<tr>
<td>Laminin subunit gamma-1</td>
<td>Protein binding</td>
<td>Cell adhesion. Migration. Hemidesmosome assembly</td>
<td>0.2492</td>
<td>4.1333</td>
</tr>
<tr>
<td>Thrombospondin-1</td>
<td>Protein binding</td>
<td>Fibronectin. Inflammation. Tissue remodeling</td>
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<td>2.9851</td>
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<td>Rab-GDP dissociation inhibitor beta</td>
<td>Complement component</td>
<td>Complement component C1q binding</td>
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<td>4.4105</td>
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<td>Apolipoprotein A-I</td>
<td>Apolipoprotein receptor binding</td>
<td>Cellular protein metabolic process</td>
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<td>Insulin-like growth factor-binding protein 2</td>
<td>Low-density lipoprotein particle binding</td>
<td>Immune response. Scavenging of lipid peroxidation products</td>
<td>0.3057</td>
<td>1.8392</td>
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<td>Collagen triple helix repeat-containing protein 1</td>
<td>Permanent binding. Water binding</td>
<td>Negative regulation of collagen matrix degradation</td>
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<td>15.0583</td>
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<td>Annexin A2</td>
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<td>Heat-shock response. Angiogenesis</td>
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<td>C-type lectin domain family 11 member A</td>
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<td>Primary response regulation</td>
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<td>Pentraxin-related protein PTX3</td>
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<td>Inflammatory response</td>
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<td>F-actin-binding protein subunit beta</td>
<td>Actin filament binding</td>
<td>Cytoskeleton organization. Endocytic vesicle-to-Golgi vesicle-mediated transport</td>
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<td>Complement component C1q binding</td>
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<td>Sulfhydryl oxidase 1</td>
<td>Flavin-linked sulfhydryl oxidase activity</td>
<td>Cellular protein metabolic process</td>
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<td>9.0444</td>
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<td>Troponin-related protein TTX1</td>
<td>Complement component C3q binding</td>
<td>Inflammatory response</td>
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<td>Cathepsin L1</td>
<td>Cathepsin-L endopeptidase activity</td>
<td>Collagen catabolic process</td>
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<td>Collagen alpha-1(V) chain</td>
<td>ECM structural component. Heparin binding</td>
<td>Collagen biosynthetic process and organization. Cell migration and adhesion</td>
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<td>Nucleotide diphosphate kinase</td>
<td>Cytochrome C oxidase activity</td>
<td>Differentiation. Endocytosis. Neurogenesis. Nucleotide metabolism</td>
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<td>Cathepsin Z</td>
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<td>Proteolysis. Endocytic vesicle-to-Golgi vesicle-mediated transport</td>
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</table>
ECM REMODELING

GO TERM | NUMBER | GO TERM | NUMBER
--- | --- | --- | ---
Clearance of damaged tissue involved in inflammatory response | GO:002247 | Wound healing | GO:1904597
Connective tissue replacement involved in inflammatory response | GO:002248 | Connective tissue remodeling | GO:0061048
Regulation of tissue remodeling | GO:0034103 | Regulation of blood vessel remodeling | GO:0060313
Positive regulation of tissue remodeling | GO:0034105 | Positive regulation of blood vessel remodeling | GO:0060313
Connective tissue development | GO:0061448 | Protein-containing complex remodeling | GO:0034367
Connective tissue remodeling | GO:007709 | TIMP family protein binding | GO:0098769
Regulation of connective tissue replacement involved in inflammatory response | GO:1904596 | Positive regulation of matrix metallopeptidase secretion | GO:1904466
Negative regulation of connective tissue replacement involved in inflammatory response | GO:1904597 | Negative regulation of matrix metallopeptidase secretion | GO:1904465
Positive regulation of connective tissue replacement involved in inflammatory response | GO:1904598 | Regulation of matrix metallopeptidase secretion | GO:1904464

PROLIFERATION

GO TERM | NUMBER | GO TERM | NUMBER
--- | --- | --- | ---
Cell proliferation | GO:0006939 | Positive regulation of epithelial cell proliferation | GO:0060876
Positive regulation of cell proliferation | GO:0006984 | Negative regulation of epithelial cell proliferation | GO:0060875
Mesenchymal cell proliferation | GO:0010465 | Regulation of stem cell proliferation | GO:0077091
Regulation of mesenchymal cell proliferation | GO:0010464 | Condensed mesenchymal cell proliferation | GO:0072157
Regulation of keratinocyte proliferation | GO:0010387 | Negative regulation of mesenchymal cell proliferation | GO:0072210
Regulation of keratinocyte proliferation | GO:0010385 | Mesenchymal stem cell proliferation | GO:0072168
Positive regulation of keratinocyte proliferation | GO:0010383 | Regulation of mesenchymal stem cell proliferation | GO:0072169
Regulation of cell proliferation | GO:0042127 | Negative regulation of mesenchymal stem cell proliferation | GO:1902461
Keratinocyte proliferation | GO:0042163 | Positive regulation of mesenchymal stem cell proliferation | GO:1902462
Tissue proliferation | GO:0046144 | Negative regulation of stem cell proliferation | GO:0072168
Regulation of fibroblast proliferation | GO:0046145 | Positive regulation of stem cell proliferation | GO:2000648
Positive regulation of fibroblast proliferation | GO:0046146 | Rho GTPase dissociation inhibitor activity | GO:0035094
Negative regulation of fibroblast proliferation | GO:0046147 | Mitotic cyclin-dependent protein kinase activity | GO:0035095
Epithelial cell proliferation | GO:0056763 | Phosphatidylinositol-3-phosphate biosynthetic process | GO:0039076
Regulation of epithelial cell proliferation | GO:0056768 | 1-phosphatidylinositol-3-phosphate biosynthetic process | GO:0039076

WOUND

GO TERM | NUMBER | GO TERM | NUMBER
--- | --- | --- | ---
Positive regulation of wound healing | GO:0003010 | Angiogenesis involved in wound healing | GO:0060353
Inflammatory response to wounding | GO:0000694 | Positive regulation of epithelial cell proliferation involved in wound healing | GO:0060548
Negative regulation of wound healing | GO:0001045 | Positive regulation of inflammatory response to wounding | GO:0061025
Regulation of wound healing | GO:0001046 | Negative regulation of inflammatory response to wounding | GO:0061026
Vascular wound healing | GO:0061042 | Regulation of inflammatory response to wounding | GO:0061026
Negative regulation of vascular wound healing | GO:0061043 | Wnt signaling pathway involved in wound healing, spreading of epithelial cells | GO:0072168
Negative regulation of vascular wound healing | GO:0061044 | Positive regulation of vascular wound healing | GO:0072168
Wound healing involved in inflammatory response | GO:0002246 | Wound healing | GO:0042060
Clearance of damaged tissue involved in inflammatory response | GO:0002247 | Positive regulation of connective tissue replacement involved in inflammatory response | GO:1904598
Connective tissue replacement involved in inflammatory response | GO:0002248 | Negative regulation of connective tissue replacement involved in inflammatory response | GO:1904597
Positive regulation of response to wounding | GO:1903036 | Regulation of connective tissue replacement involved in inflammatory response | GO:1904598
Regulation of response to wounding | GO:1903034 | Detection of wounding | GO:0041822
Negative regulation of response to wounding | GO:1903035 | Response to wounding | GO:0009611
Behavioral response to wounding | GO:0002210 | Wound healing, spreading of cells | GO:0044319
Regulation of wound healing, spreading of epidermal cells | GO:1903056 | Wnt signaling pathway involved in positive regulation of wound healing | GO:0072168
Negative regulation of wound healing, spreading of epidermal cells | GO:1903690 | Rho GTPase dissociation inhibitor activity | GO:0035094
Positive regulation of wound healing, spreading of epidermal cells | GO:1903691 | Wound healing, spreading of epidermal cells | GO:0035311

APC SIGNALING

GO TERM | NUMBER | GO TERM | NUMBER
--- | --- | --- | ---
Positive regulation of phosphatidylinositol 3-kinase signaling | GO:0040468 | Negative regulation of kinase activity | GO:0036731
Positive regulation of phosphatidylinositol 3-kinase signaling | GO:0040552 | Phosphatidylinositol 3-kinase signaling | GO:0041065
Positive regulation of phosphatidylinositol-3-phosphate 5-kinase activity | GO:0010319 | Protein kinase B binding | GO:0043422
Positive regulation of phosphatidylinositol-3,4,5-trisphosphate 5-kinase activity | GO:0010343 | Protein kinase B signaling | GO:0043491
Positive regulation of kinase activity | GO:0036784 | Positive regulation of protein kinase B signaling | GO:0036789
Negative regulation of phosphatidylinositol 3-kinase signaling | GO:0040567 | Negative regulation of protein kinase B signaling | GO:0036789
Negative regulation of phosphatidylinositol 3-kinase signaling | GO:0043553 | Phosphatidylinositol phosphate 5-kinase activity | GO:0036789
Negative regulation of 1-phosphatidylinositol-4-phosphate 5-kinase activity | GO:0010302 | Phosphatidylinositol phosphate kinase activity | GO:0036789
Negative regulation of 1-phosphatidylinositol-4-phosphate 5-kinase activity | GO:0010317 | Activation of protein kinase B activity | GO:0036789
Negative regulation of phosphatidylinositol-4,5-bisphosphate 5-kinase activity | GO:0010420 | Phosphatidylinositol 3-phosphate biosynthetic process | GO:0036902
Negative regulation of phosphatidylinositol-3,4,5-trisphosphate 5-kinase activity | GO:0010445 | 1-phosphatidylinositol-3-kinase activity | GO:0036789

SMAD SIGNALING

GO TERM | NUMBER | GO TERM | NUMBER
--- | --- | --- | ---
Positive regulation of pathway-restricted SMAD protein phosphorylation | GO:0010362 | Negative regulation of pathway-restricted SMAD protein phosphorylation | GO:0060394
Negative regulation of SMAD protein complex assembly | GO:0001091 | SMAD protein signal transduction | GO:0060395
Positive regulation of transforming growth factor beta receptor signaling pathway | GO:0030511 | Negative regulation of transforming growth factor beta signaling | GO:0072135
Negative regulation of transforming growth factor beta receptor signaling pathway | GO:0030512 | Negative regulation of transforming growth factor beta receptor signaling pathway | GO:0072136
Regulation of SMAD protein signal transduction | GO:0060390 | Negative regulation of transforming growth factor beta signaling pathway involved in primitive streak formation | GO:0072137
Positive regulation of SMAD protein signal transduction | GO:0060391 | Negative regulation of transforming growth factor beta activation | GO:0072138
Negative regulation of SMAD protein signal transduction | GO:0060392 | Positive regulation of transforming growth factor beta activation | GO:0072139
Regulation of pathway-restricted SMAD protein phosphorylation | GO:0060393 | Positive regulation of transforming growth factor beta translocation | GO:0072140

MAPK CASCADE

GO TERM | NUMBER | GO TERM | NUMBER
--- | --- | --- | ---
MAPK cascade | GO:000165 | Regulation of MAPK cascade | GO:0041048
Activation of MAPK activity involved in ommosensory signaling pathway | GO:000167 | Negative regulation of MAPK cascade | GO:0041049
Activation of MAPK activity involved in ommosensory signaling pathway | GO:000168 | Positive regulation of MAPK cascade | GO:0041100
Figure S1. Gingival MSC characterization
Representative pictures of (a) phase-contrast image of plastic-adherent gingival MSCs (scale bar = 200 μm) and of (b) osteogenic, adipogenic and chondrogenic inductions of gingival MSCs (scale bar = 500 μm). (c) Phenotype of gingival MSCs assessed by flow cytometry. AB, Alcian blue; AR, Alizarine Red; CTRL, control; MFI, Mean fluorescence intensity; MSC, mesenchymal stromal cells; ORO, Oil Red O; VK, Von Kossa.
Figure S2. Dose-effect study of IL-MSCs in vivo.

IL-MSCs were injected at different doses in NOD/SCID mice treated with Integra and hPBES after full-thickness excisional skin injury (n=6 in each experimental group). Aspect and histology of the skin were observed 14 days after treatment. HPS, Hematoxylin-Phloxin-Safran; COL-7, Collagen-7; CK-19, Cytokeratin-19.
Figure S3. Secretome analysis of naive and IL-1β-primed MSCs

(a) Protein network interaction analysis between selected IL-CM-derived factors and target proteins using the String online database (http://string-db.org/). Proteins in green are correlated with epithelial cell differentiation (GO:0030835), in yellow with cell migration (GO:006477), in red with ECM organization (GO:0030197) and in blue with immune response (GO:0006955). Links indicate different interaction types, including binding (blue), catalysis (violet), transcription regulation (yellow) and reaction (black).

(b) ELISA dosages of selected wound healing-related proteins present in NV- and IL-CM (n=7). * p<0.05; ns, not significant; CM, conditioned medium; IL, IL-1β; MSC, mesenchymal stromal cells; NV, naive.
Figure S4. Paracrine mechanisms of action of IL-1β-primed MSCs

(a) Dosage of IL-1RA in IL-CM, and in treated THP-1 culture supernatants (n=7 to 18).
(b) Wound closure of intoxicated keratinocytes (TOX) cultured with IL-CM-derived factors (ratio to TOX, n=3 to 7). (c, d, e) Nidogen-1, Tenascin-C and Laminin-5v2 protein expression at 8 days in keratinocyte and fibroblast co-cultures grown with IL-CM-derived factors (ratio to control, n=2 to 5). (f, g) Dosage of TNFα and IL-1αRA in the supernatants of LPS-challenged THP-1 cultured for 24h with IL-CM-derived factors (ratio to LPS control, n=3 to 10). Values are expressed as mean ± SEM. **p<0.01; ***p<0.001; ns, not significant. CM, conditioned medium; CTRL, control; IL, IL-1β; LAM, lamivudine; v2, vinyl; LAM, lamivudine; v2 chain; NID-1, Nidogen-1; NV, naive; TNC, Tenascin-C; TOX, intoxicated keratinocytes.
Figure S5. Inhibitors effect on migration, Inflammation and JDE model

(a) Wound closure of intoxicated keratinocytes (TOX) cultured with or without SBA431542- or Tegacycine and supplemented or not with IL-CM (ratio to TOX, n=5 to 7).
(b) Dosage of TNF-α and (c) of IL-1RA in the supernatants of LPS-challenged THP-1 cultured for 24h with or without SBA431542- or Tegacycine and supplemented or not with IL-CM (ratio to LPS control, n=3 to 19).
(d) Nidogen-1, (e) Tenascin-C and (f) Laminin-γ2 protein expression at 8 days in keratinocyte and fibroblast co-cultures grown with or without SBA431542- or Tegacycine and supplemented or not with IL-CM (ratio to control, n=6 to 7). Values are expressed as means ± SEM. ns, not significant. CM, conditioned medium; CTRL, control; IL, IL-1β; LAM-γ2, Laminin-5 γ2 chain; NID-1, Nidogen-1; NV, naive; TNC, Tenascin-C; TOX, intoxicated keratinocytes.
**Figure S6.** Experimental procedures and assays.

(a) MSC priming and CM preparation procedures. (b) Wound closure assay. (c) DEJ formation assay. (d) Air/liquid epidermal differentiation assay. (e) Inflammation assay. (f) In vivo model of dorsal acute wound and hPBES grafting. CM, Conditioned Medium; DEJ, dermo-epidermal junction; DF, dermal fibroblast; hPBES, human plasma-based epidermal substitute; KC, keratinocyte; MSC, mesenchymal stromal cells; PL, platelet lysate.
Figure S7. Histological quantifications and scores methods

(a) Method of epidermal basal layer scoring, made by three independent observers, on the basis of nucleus position, cell morphology and basal continuity (scale bar = 50 μm). (b) Method of quantification of hPBEs engraftment, based on human Integrin-β1 immunostaining (scale bar = 1500 μm). INT-β1, Integrin-β1; hPBEs, human plasma-based epidermal substitute.