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REGULATION OF LANGERHANS CELL FUNCTIONS IN A HYPOXIC ENVIRONMENT

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Short title: Langerhans cell responses to hypoxia

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ABSTRACT

Langerhans cells (LCs) are a specialized dendritic cell subset that resides in the epidermis and mucosal epithelia and is critical for the orchestration of skin immunity. Recent evidence suggest LCs involvement in aberrant wound healing and the development of hypertrophic scars and chronic wounds, which are characterized by a hypoxic environment. Understanding LCs biology under hypoxia may, thus, lead to the identification of novel pathogenetic mechanisms of wound repair disorders and open new therapeutic opportunities to improve wound healing. In this study, we characterize a previously unrecognized role for hypoxia in significantly affecting the phenotype and functional properties of human monocyte-derived LCs, impairing their ability to stimulate naïve T cell responses, and identify the triggering receptor expressed on myeloid (TREM)-1, a member of the Ig immunoregulatory receptor family, as a new hypoxia-inducible gene in LCs and an activator of their proinflammatory and Th1-polarizing functions in a hypoxic environment. Furthermore, we provide the first evidence of TREM-1 expression *in vivo* in LCs infiltrating hypoxic areas of active hypertrophic scars and decubitous ulcers, pointing to a potential pathogenic role of this molecule in wound repair disorders

Keywords: Hypoxia, Langerhans cells, Immunoregulatory receptors, Wounds

Abbreviations: LCs, langerhans cells; TREM-1, triggering receptor expressed on myeloid cells; cDCs, classical dendritic cells; TGF-β1, transforming growth factor-β1; CLA, cutaneous lymphocyte-associated antigen; PAMP/DAMP, pathogen- or damage-associated molecular patterns; pO_2, partial oxygen pressure; HIF-1, hypoxia-inducible factor-1; AHS, active hypertrophic scars; U, decubitous ulcers; H-LCs, hypoxic LCs; OPN, osteopontin; MLR, mixed leukocyte reaction; lg+LCs/H-LCs, langerin+LCs/H-LCs; CM, central memory; qRT-PCR, quantitative real-time PCR; sTREM-1, soluble TREM-1; NS, normal skin.

INTRODUCTION

LCs are a specialized dendritic cell (DCs) subset that resides in the epidermal layer of the skin and the mucosal epithelia lining the cavities of respiratory, gastrointestinal, and urogenital systems. They represent the first line of defense against microbial pathogens that penetrate the epithelial barriers and play a key role in maintaining tolerance to self and harmless environmental antigens [1]. Although LCs share some features with classical (c)DCs, they are endowed with specific developmental, phenotypic, and functional properties. Unlike cDCs which originate from BM-derived progenitors, LCs renew under steady-state conditions from a pool of skin-resident myeloid precursors, whereas under pathologic conditions they are replenished by monocytes recruited from the bloodstream [1,2]. Furthermore, their development is strictly dependent on the presence of transforming growth factor (TGF)-β1 in addition to GM-CSF and IL-4, which are sufficient for cDCs differentiation [1,3,4]. LCs are characterized by a unique set of cell-surface molecules that are currently used as specific markers to distinguish them from other DCs subsets, namely: (i) Langerin (CD207), a type II C-type lectin receptor involved in microbial glycolipids uptake and internalization; (ii) E-cadherin, a homotypic adhesion molecule that anchors LC to neighbouring keratinocytes; (iii) cutaneous lymphocyte-associated antigen (CLA), a skin-homing antigen; and (iv) CD1a, a MHC class I-like molecule involved in microbial glycolipid antigens presentation to T cells. In addition, they express low/intermediate levels of MHC class II antigens and T cell costimulatory molecules, which are upregulated upon stimulation by pathogen/damage-associated molecular patterns (PAMP/DAMPs) or inflammatory cytokines released at sites of skin injury and infection [1,2]. LCs functional specialization is demonstrated by their higher efficiency at priming naive T cells and inducing cytotoxic high-avidity CD8+ T cells compared to dermal DCs which, in contrast, are more potent at initiating follicular Th cell responses and promoting naive B cells differentiation into plasma-cells and Ig class switching. Accordingly, LCs migrate to the T cell area of the draining lymph-nodes, whereas DCs mobilize near the B-cell follicles [2,5].

LC development and functions are intrinsically linked to the local microenvironment [6,7]. An important environmental factor to which LCs have to adapt in pathologic tissues is represented by hypoxia [8,9], a local decrease in partial oxygen pressure (pO_2) which affects cell phenotype, gene expression, and functions [10,11]; however the impact of hypoxia on LCs biology has not been explored. Hypoxia plays a prominent role in wound healing by regulating inflammatory cell influx and activation, fibroblast and keratinocyte migration, proliferation, and ECM protein production, angiogenesis, and tissue remodeling via induction of several transcription factors, in particular hypoxia-inducible factors -1 (HIF-1) [8,9], the master regulator of O_2 homeostasis [10], but successful wound repair ultimately requires restoration of normoxic conditions [8]. Chronic hypoxia or repeated ischemia-reperfusion injury, and consequent HIF-1 abnormal expression, lead to derailed wound healing by promoting exaggerated inflammatory responses, immune cell overactivation, augmented neovascularization, hyperproliferation of wound fibroblasts, and excessive matrix deposition, resulting in the formation of fibroproliferative scars and non-healing wounds [8,9]. LCs accumulation and/or functional abnormalities have been reported in the hypoxic areas of hypertrophic scars and ulcers, suggesting their potential contribution to aberrant wound healing [12,13]. Understanding how the hypoxic environment affects LCs responses may thus lead to the identification of new pathogenetic mechanisms of wound repair disorders and aid in the development of more specifically targeted therapeutic strategies.

Myeloid cells integrate stimulatory and inhibitory signals present in the microenvironment through a defined repertoire of cell surface immunoregulatory receptors [14], whose expression can be finely tuned by hypoxia [11]. Deregulated expression of these molecules can lead to aberrant cell responses and has been implicated in the pathogenesis of a number of chronic inflammatory diseases [5,14]. Hence, characterization of the effects of hypoxia on immunoregulatory receptor expression and functions in LCs will help unravel the regulation of their activity during pathologic wound healing.

In this study, we show for the first time that a hypoxic environment reflecting that occurring *in vivo* during wounding can functionally reprogram human monocyte-derived LCs and identify TREM-1, a member of the Ig-like immunoregulatory receptor family and a strong amplifier of inflammation [15-17], as a new regulator of LCs functions under hypoxic conditions with important implications for wound repair disorders.

MATERIALS AND METHODS

Blood and skin samples

Blood monocytes and naïve T cells were purified from platelet-apheresis of healthy donors obtained by the Blood Transfusion Center of the Gaslini Institute (Genova, Italy) according to the Gaslini's Ethics Committee-approved protocol, as described in Supplementary Materials and Methods. Skin biopsies were obtained from six patients with postburn active hypertrophic scars (AHT), three with decubitus ulcers (U) and four healthy individuals undergoing plastic surgery or scar correction procedures, according to a protocol approved by the CTO/Città della Salute e della Scienza Hospital Ethical Board (Torino, Italy) and in adherence with the Declaration of Helsinki Principles. Written informed consent was obtained from all subjects enrolled in the study.

Cytokines and antibodies

The list of reagents used in this study and their usage are detailed in Supplementary Materials and Methods.

Langerhans and T cells phenotypic and functional characterization.

Langerhans cell-like cells were generated from monocytes under normoxic (20% O_2) or hypoxic (1% O_2) conditions and cocultured with allogeneic naive T cells, as detailed in Supplementary Materials and Methods. Cell viability was assessed by trypan blue dye exclusion test and annexin V (AV)/propidium iodide (PI) staining. For phenotypic analyses, cells were incubated with fluorochrome-conjugated mAbs, and fluorescence was quantitated on a FACSCalibur flow-cytometer equipped with CellQuest softwar, as described [18]. TREM-1 mRNA expression in total RNA was determined by a 7500 Real Time PCR System, and TREM-1 cross-linking was carried out with agonist anti-TREM-1 mAb as detailed [18]. Cell-free supernatants were tested for cytokine/chemokine content by specific ELISA. Optical density was determined

using a Spectrafluor Plus plate reader from TECAN. Data were analyzed with the Graph Pad Prism 5 Software.

Immunohistochemistry of skin biopsies

Immunohistochemical staining of frozen tissue specimens was performed by a three step immunoperoxidase technique, as detailed [19], using the Abs listed in the Supplementary Materials and Methods. Slides were counter-stained with Mayer's hematoxylin solution (DAKO) and examined under a DMLA Leica microscope. Microphotograph were taken with a digital camera (Leica DFC 425C), and images were acquired using Leica Application Suite software.

RESULTS

Hypoxia triggers phenotypic and secretory changes in monocytes-derived LCs

LCs development from monocytes recruited at sites of skin injury occurs in a hypoxic microenvironment [8]. To determine whether hypoxia affects LCs phenotypic features, surface expression of a set of LCs-specific Ag-presenting and T cell costimulatory molecules was assessed by flow cytometry in Langerhans cell-like cells generated from human monocytes cultured under normoxic (LCs) or hypoxic (H-LCs) conditions. As shown in Fig.1a, cells differentiated under normoxia displayed the typical LCs phenotype [2] characterized by high/intermediate expression of CD1a, Langerin, E-cadherin, CLA, HLA-DR, and CD86, and low expression of CD83, in line with previous reports [4]. The percentage of cells expressing Langerin, CD86, and CD83 was significantly decreased upon generation under hypoxia, although with some variability among individual donors. In contrast, hypoxia did not affect expression of CLA and HLA-DR, whereas it reduced that of CD1a and E-cadherin in a few donors (Fig.1a/b).

LCs orchestrate skin immune responses by secreting cytokines and chemokines in response to stimuli present in the tissue environment [5]. To determine hypoxia effects on LCs secretory profile, the release of several proinflammatory and Th1-priming cytokines/chemokines by LCs and H-LCs was assessed (Fig.1c). A 90% reduction in the amounts of secreted IL-12 was measured in the supernatants of H-LCs relative to LCs (from 1013±203 to 126±39 pg/ml), whereas osteopontin (OPN), TGF-β1, and CCL5 levels were increased by about 19-fold (from 59±10 to 1136±347 ng/ml), 3.7-fold (from 186±56 to 684±149 pg/ml), and 3-fold (from 4.7±1.7 to 14.3±4.4 pg/ml), respectively. Hypoxia also decreased the release of TNFα (from 14.5±9.2 to 3.3±2.2 pg/ml) and IL-8 (from 1950±887 to 1635±561 pg/ml), although not significantly.

These results indicate that hypoxia triggers major changes in H-LCs surface marker expression and cytokine/chemokine secretory profile.

Hypoxia impairs LCs capability to stimulate T cell responses

Experiments were then carried out to investigate whether the observed phenotypic and secretory changes affected H-LCs stimulatory activity on naive T cells in MLR. LCs and H-LCs capability to induce naive allogenic T cell proliferation was assessed by ^{3}H-thymidine incorporation (Fig.2a). In line with the decreased expression of costimulatory molecules, H-LCs were less efficient than LCs in triggering T cell proliferation. Similar results were obtained by performing MLR with purified langerin+ cells (lg+LCs vs lg+H-LCs), demonstrating that the differences in LCs and H-LCs stimulatory activity on T cell proliferation were not due to the different percentages of langerin+ cells present in the total cell populations (Supplementary Fig.1a). Accordingly, decreased expression of CD86 and CD83 costimulatory molecules was detectable in lg+H-LCs respect to lg+LCs, as revealed by double-staining experiments (Supplementary Fig.1b).

LCs and H-LCs ability to polarize T cell responses was then compared by analyzing the concentrations of various Th cytokines in coculture supernatants. T cells stimulated with LCs secreted large amounts of the Th1 cytokine, IFNγ (Fig.2b), indicating activation of a Th1-type response [20]. Coculture with H-LCs resulted in approximately 70% reduction of IFNγ secretion (from 784.5±218 to 212±33) (Fig.2b). No significant differences in the secreted levels of the Th2 cytokine, IL-10, and the Th17 cytokine, IL-22, were observed between T cells stimulated with LCs and H-LCs (Supplementary Fig.2a), whereas release of the Th2 cytokine, IL-4, and the Th17 cytokine, IL-17, was never detected (data not shown). These data suggest decreased Th1-priming ability of H-LCs.

Next, we compared LCs and H-LCs capacity to support allogenic naïve $CD4^{+}$/$CD8^{+}$ T cell differentiation [21] by four-color flow cytometric analysis with mAbs to CD4, CD8, CD27, and CD45RA (Fig.2c). A high proportion of central memory (CM) cells ($CD45RA^{-}$/$CD27^{+}$) was obtained upon naive T cell ($CD45RA^{+}$/$CD27^{+}$) coculture with LCs, representing a mean of 38% and 26% of the total $CD4^{+}$-and $CD8^{+}$-gated cell populations, respectively, in 5 different donors. A significantly lower percentage of CM cells was detectable upon coculture with H-LCs, accounting

for a mean of 21% and 10.6% of the total $CD4^+$ and $CD8^+$ cell subsets, respectively. Differentiation into effector memory (EM, EMRA) cells was not observed under these culture conditions.

Hypoxia was reported to have both proapoptotic and prosurvival consequences, depending on the cellular context[22-24]. To exclude the possibility that the decreased ability of H-LCs to promote T cell responses was due to an increased number of dying cells, we measured their viability by Annexin V-FITC/propidium iodide staining, both immediately after generation under hypoxia and following an additional 24 hr incubation period under normoxia in fresh medium deprived of differentiating cytokines to mimic MLR culture conditions (Fig.3). This time-point was chosen based on previous reports showing that naïve T cell activation by APCs occurs already within the first 20 hr of T/APC interaction [25,26]. Interestingly, H-LCs were more viable than LCs as indicated by the lower percentage of H-LCs undergoing both early and late apoptosis. Increased H-LCs, relative to LCs, viability was maintained when cells were exposed to normoxia for additional 24 hr (H/N-LCs *vs* N-LCs), suggesting a prosurvival effect of hypoxia on LCs, on line with previous evidence in other myeloid cells [23,24]. Accordingly, only minor, not significant changes in surface marker expression were observed after H-LCs reoxygenation (Table I).

Taken together, these results provide the first evidence that hypoxia specifically impairs H-LCs stimulatory activity on naive T cells..

TREM-1 is selectively expressed in LCs generated under hypoxic conditions

Hypoxia can tightly regulate monocytic-lineage cell responses in diseased tissue by differentially modulating the expression of immunoregulatory receptors. Among them, we recently identified TREM-1 as a common hypoxia molecular target in different monocytic cell populations [11]. We were interested in investigating whether TREM-1 was functionally relevant in LCs generated under hypoxic conditions. Initial experiments were performed to assess TREM-1 expression in H-LCs. As determined by quantitative real-time PCR (qRT-PCR) (Supplementary

Fig.3a), TREM-1 transcript levels were significantly and consistently higher in H-LCs than in LCs from all tested samples, paralleling those of CAXII assessed as an index of response to hypoxia [18], with the extent of induction ranging from 20- to 231-fold in different donors. TREM-1 surface expression was then measured by flow cytometry (Fig.4a). Fifty to 70% H-LCs expressed TREM-1, whereas no TREM-1$^+$ LCs were detectable in any of the donors examined, suggesting that expression of this molecule is restricted to cells generated under hypoxia. Similar TREM-1 expression profile was demonstrated by double-labeling experiments in langerin+ cells. (Supplementary Fig.3b). H-LCs reoxygenation by exposure to normoxic conditions for 24hr (H/N-LCs) resulted in decreased TREM-1 surface levels (Supplementary Fig.3c), suggesting that hypoxia stimulatory effects on TREM-1 expression were reversible. A parallel release of the soluble form of TREM-1 (sTREM-1), derived from the shedding of membrane-bound TREM-1 [17], was measured by ELISA in the supernatants of H-LCs but not of LCs, ranging from 100 to 202 pg/8x10^{5}cells/mL in 5 different donors (Supplementary Fig.3d), consistent with the expression pattern of the membrane-bound form.

Transcriptional activation by hypoxia is mediated primarily by HIF, a heterodimer of a constitutive HIF-1β subunit and an O_2-sensitive α-subunit (HIF-1α/2α), which binds to and transactivates the Hypoxia Responsive Element (HRE) present in the promoter of many hypoxia-inducible genes [10,27,28]. As shown in Supplementary Fig.4a, H-LCs, but not LCs, expressed high levels of HIF-1α protein, whereas HIF-2α was expressed constitutively and not modulated by hypoxia. Given the presence of a HRE sequence in the TREM-1 gene promoter [18], we investigated whether TREM-1 inducibility by hypoxia was mediated by HIF-1α by assessing the effects of echinomycin, a specific inhibitor of HIF-1α binding to HRE [29], on TREM-1 mRNA expression in H-LCs (Supplementary Fig.4b). Addition to the culture of increasing concentrations (0-5 nmol/L) of echinomycin decreased, in a dose-dependent fashion, mRNA expression of the known HIF-1 target gene, CAXII [30], assessed in parallel as an index of response to the drug, with

a 35% inhibition observed at a concentration of 2 nmol/L and a 70% reduction observed with 5 nmol/L of the drug. Treatment with echinomycin also resulted in the downregulation of TREM-1 mRNA levels, although to a lower extent than CAXII, with a 30% reduction achieved with 5 nmol/L of the drug (Supplementary Fig.4b), in line with previous findings in cDCs [31].

These data demonstrate that TREM-1 is inducible by hypoxia in LCs and that its expression is in part mediated by HIF-1α.

TREM-1 cross-linking stimulates H-LCs proinflammatory and Th1-polarizing activity

Crosslinking experiments were then carried out to investigate TREM-1 functions in H-LCs. As determined by flow cytometry (Fig.4b), CD86, CD83, and HLA-DR surface expression was significantly increased in response to TREM-1, relative to control IgG1, triggering, both in terms of percentage of positive cells and/or mean fluorescence intensity.

TREM-1 engagement also affected cytokine secretion. As shown in Figure 4c, a significant increase in the release of IL8 (from 2969±948 to 8742±1976 pg/ml), IL-12 (from 180±48 to 615±160 pg/ml), TNF-α (from 5.2±3.1 to 36.2±15.6 pg/ml), and CCL5 (from 20±7.9 to 81±25 pg/ml) was measured in response to TREM-1 cross-linking, whereas TGFβ1 (from 604±180 to 1006±216 pg/ml) and OPN (from 1295±394 to 1395±331 ng/ml) levels were only marginally affected.

The ability of TREM-1- and IgG-stimulated H-LCs to activate naïve allogeneic T cells in MLR was then compared. As shown in Fig.5a, T cell proliferation was significantly higher after culture with TREM-1- than IgG-triggered H-LCs and was associated with the production of significantly higher amounts of IFNγ (678±112 vs 284±80 pg/ml) (Fig.5b). In contrast, no significant differences were observed in the amounts of secreted IL-10 and IL-22 between T cells stimulated with TREM-1- and IgG-triggered H-LCs (Supplementary Fig.2b), whereas IL-4 and IL-17 secretion was not induced (data not shown). T cell differentiation into CM cells was significantly

increased upon naive $CD4^+$/$CD8^+$ T cell coculture with TREM-1-, compared to IgG-, stimulated H-LCs, accounting for a mean 37.5% vs 24% of the $CD4^+$ and 25% *vs* 14.5% of the $CD8^+$ population (Fig.5c).

Overall, these data demonstrate that TREM-1 activation promotes LCs proinflammatory and T cell stimulatory activities.

TREM-1 is expressed *in vivo* on H-LCs infiltrating AHS and U

Growing evidence suggest that epidermal LCs are implicated in the pathogenesis of fibroproliferative scars or chronic wounds [12,13], which are characterized by hypoxia [8,9]. The occurrence of TREM-1^+ LCs was evaluated in AHS and U. Biopsy specimens from AHS, U, and healthy skin (NS) were analyzed by immunohistochemistry with anti-TREM-1 Ab. LCs were identified based on DC-like morphology and langerin expression. HIF-1α immunostaining was carried out in parallel to confirm the presence of a hypoxic environment. Both upper epidermal layers and subpapillary dermis were examined (Fig.6a,b). Several $Langerin^+$ cells were regularly distributed within the epidermis of NS specimens (13.6 ± 1.7 cells/0.05 mm^2 of epidermal area). The number of $Langerin^+$ cells significantly increased in AHS (19.0 ± 3.4 cells/0.05 mm^2 of epidermal area) and U (20.0 ± 2.0 cells/0.05 mm^2 of epidermal area) specimens, suggesting LCs enrichment in diseased compared to healthy epidermis, in agreement with previous observations [12,13]. Strong positivity for HIF-1α was detectable in cells with DC morphology infiltrating AHS and U epidermal layers (20.0 ± 0.6 and 14.7 ± 0.5cells/0.05 mm^2 of epidermal area), confirming adaptation to the hypoxic environment, whereas NS showed significantly lower staining (2.0 ± 0.6 cells/0.05 mm^2 of epidermal area). Few scattered TREM-1^+ cells with DC morphology were present in NS epidermal specimens (3.0 ± 0.4 cells/0.05 mm^2 of epidermal area); the number of TREM-1^+ cells was significantly higher in AHS (21.5 ± 1.6 cells/0.05 mm^2 of epidermal area) and U (21.2 ± 1.0 cells/0.05 mm^2 of epidermal area) specimens. Langerin and TREM-1 costaining was confirmed by two-color immunofluorescence analysis in both AHT and U epidermal specimens (Fig.6c), with

TREM-1 expression detected in approximately 71% and 73% of langerin+ cells, respectively, demonstrating that TREM-1 expression is a common characteristic of LCs infiltrating different types of skin lesions. HIF-1α costaining was detected in TREM-1+ cells infiltrating the epidermis of both AHT and U (Fig.6c), indicating that TREM-1 is an *in vivo* marker of hypoxic LCs.

In contrast to epidermal sections, Langerin immunoreactivity was not observed in cells with DC-like morphology infiltrating the subpapillary dermis of normal and diseased skin (Fig.6a), which probably represented dermal interstitial DCs. However, several DC-like cells infiltrating U and, to a smaller extent, AHS dermal layers were positive for TREM-1 and HIF-1α, supporting our recent observations of TREM-1 expression in DCs infiltrating inflammatory tissues [18].

These findings provide the first evidence of the existence of a population of Langerin$^+$ cells expressing TREM-1 *in vivo* in the hypoxic epidermis of skin fibroproliferative lesions and chronic wounds.

.DISCUSSION

Wound healing is a tightly regulated multistage process involving a cascade of interactions among parenchimal and infiltrating cells, soluble mediators, ECM components, and microenvironmental factors. Alterations of these events may lead to wound healing disorders resulting in the formation of chronic wounds or abnormal scar [32-34]. Although chronic hypoxia [8,9] and prolonged inflammatory response [19,35,36] have been identified as the main determinants of aberrant wound healing, much remains to be elucidated regarding the molecular mechanisms underlying these processes, whose understanding may allow the identification of novel therapeutic targets. Results from this study provide novel mechanistic clues on the contribution of hypoxic LCs to the pathogenesis of hypertrophic scars and decubitous ulcers and identify TREM-1 as a potential new target for therapeutic intervention in these diseases.

.It is well recognized that hypoxia can differentially regulate the functions of innate and adaptive immune cells, boosting the former and inhibiting the latter [37]. We have previously reported that monocyte-derived cDCs generated under conditions of reduced oxygenation exhibited a Th1/Th17-polarized inflammatory phenotype, characterized by increased surface expression of T cell costimulatory and antigen-presenting molecules and enhanced production of proinflammatory Th1/Th17-priming cytokines [38]. Here we show that, as opposed to cDCs, monocyte-derived LCs differentiated under hypoxia displayed significantly lower expression of the CD86 and CD83 costimulatory molecules compared to the normoxic counterparts and produced significantly decreased amounts of the proinflammatory Th1-priming cytokine, IL-12 [20], as well as reduced levels of the proinflammatory/angiogenic mediators, TNFα and IL-8. These findings confirm and extend previous evidence that LCs are endowed with unique properties that distinguish them from cDCs [1,2,5]. Alterations of phenotypic and secretory features were paralleled by decreased H-LCs ability to stimulate allogenic naïve T cell functions, including proliferation, IFNγ production, and differentiation into CM cells, suggesting that LCs generated in a hypoxic environment are less efficient T cell activators than their normoxic counterparts. These findings identify a new regulatory

mechanism mediating hypoxia inhibitory effects on adaptive immune responses in addition to the direct impairment of T lymphocyte functions reported by other groups [39].

Inducibility of chemoattractants for activated/memory T lymphocytes is a common feature of several monocytic lineage cell response to low pO_2, representing an important mechanism of regulation of T cell trafficking in pathologic tissues [28,38]. Results reported here extend to LCs this trend of response to hypoxia, demonstrating significantly increased release of CCL5 and OPN, which play a central role in activated/memory T cell chemotaxis [40,41], by H-LCs compared to LCs. Noteworthily, OPN overproduction has been reported to hinder wound healing and contribute to increased tissue scarring and inflammation-associated fibrosis [42]. Hence, the demonstration that H-LCs secreted large amounts of OPN (in the ng/μg range) is suggestive of an elevated capacity of LCs generated in a hypoxic environment to promote fibrosis. This hypothesis is further supported by the finding that H-LCs also released high levels of TGFβ1, which is another well known pro-fibrotic factor [43]. Based on these data, we speculate that H-LCs may contribute to skin fibrosis by overproducing OPN and TGFβ1.

LCs recognition of PAMP/DAMPs is the primary trigger for initiating skin immune responses and is mediated by a specific repertoire of membrane-bound and cytoplasmic pattern-recognition receptors [5]. The finding that the C-type lectin receptor, Langerin, is significantly downregulated in H-LCs respect to LCs and associated with decrease expression of the MHC class I-like molecule, CD1a, is consistent with the view that LCs generated in a hypoxic environment are endowed with decreased T cell stimulatory activity compared to their normoxic counterpart, given the critical role of these molecules in microbial glycolipid antigen uptake and presentation to T cells [44], further emphasizing the role of low pO_2 in skewing LCs-mediated adaptive immune responses.

An intriguing finding of this study is the demonstration that hypoxia strongly induces the expression of the TREM-1 proinflammatory receptor in LCs. This molecule was previously reported to be developmentally regulated in monocytic lineage cells, being selectively expressed on

neutrophils and a subset of monocytes/macrophages and completely down-regulated during their differentiation into DCs under normoxic conditions [18,31]. Our results support these findings, showing that TREM-1 is not expressed in monocyte-derived LCs generated under normoxia. Interestingly, however, we demonstrate TREM-1 inducibility upon LCs development under hypoxic conditions. TREM-1 mRNA was, in fact, consistently detected in H-LCs generated from different donors and paralleled by expression of the membrane-bound receptor and secretion of its soluble form. Interestingly, TREM-1 inducibility by hypoxia was reversible, because cell reoxygenation resulted in its downregulation, and appeared to involve, at least in part, the HIF/HRE system, in agreement with previous findings in cDCs [18,31]. TREM-1 engagement on H-LCs by an agonist Abs upregulated CD86 and CD83 costimulatory molecule expression and enhanced the production of proinflammatory and Th-1 priming cytokines, such as IL-8, TNFα, IL-12, and CCL5, associated with increased H-LCs ability to stimulate naïve T cell proliferation and differentiation and Th1 priming. These findings highlight the potential of TREM-1 to contribute to the functional reprogramming of LCs generated at hypoxic skin sites toward a Th-1 polarized inflammatory direction, emphasizing the relevance of this molecule not only as an amplifier of inflammation [15,16], but also as an inducer of adaptive immune responses.

TREM-1 expression was previously observed *in vivo* in macrophages and DCs recruited to some tumors and inflammatory tissues and reported to play a pathogenic role in these diseases [18,45-47]. Our data provide the first evidence of TREM-1 expression in LCs infiltrating AHS and U. AHS are a common complication of wound healing that follows deep or extensive cutaneous insults, such as burns and surgical incisions, and arise from exaggerated and persistent inflammation, hyperproliferation of wound fibroblasts and abnormal ECM protein accumulation [33,35]. U are non-healing, chronic wound frequently occurring in elderly and immobilized patients, characterized by local chronic inflammation, disruption of the collagen matrix, tissue damage, and cell death [34,36]. Both pathologies significantly affect patients quality of life, leading to severe complications and serious disabilities [33,34]. A significant enrichment of Langerin^{+} cells

was detected by immunohistochemistry and immunofluorescence in the epidermal layer of AHS and U, respect to NS, specimens, in agreement with previous evidence [12,13],. Noteworthily, Langerin^{+} cells infiltrating the epidermis of AHS and U, but not NS, specimens, coexpressed TREM-1, and TREM-1 expression was associated with positivity for HIF-1α confirming cell adaptation to the hypoxic environment. Taken together with *in vitro* data, these findings suggest that the hypoxic microenvironment is an important determinant of TREM-1 expression in LCs generated from monocytes recruited at sites of skin injury, pointing to a potential role of this molecule in the pathogenesis of fibroproliferative scars and chronic wounds.

Our results lead to new perspectives on the role of hypoxia in functionally regulating LCs responses during wounding. We hypothesize that, during physiologic wound healing, LCs differentiated from monocytes recruited to the wound site respond to local hypoxia by producing CCL5, OPN, and TGFβ1, which mediate activated/memory T cell as well as monocytes/macrophage recruitment and activation [40,41] and stimulate fibroblast proliferation/ECM secretion, neovascularization, and reepithelialization [42,43], thus contributing to both defense against invading pathogens and wound repair. This response is associated with decreased hypoxic LCs stimulatory activity on naïve T cells, which probably represents a negative feedback mechanism to prevent immune cell overactivation and collateral inflammatory tissue damage. It is conceivable that TREM-1 is induced in response to local hypoxia, but that its expression is rapidly reverted by restoration of normoxic conditions required for successful wound repair [8], which prevents TREM-1 activation. Conversely, conditions of chronic hypoxia or ischemic/reperfusion injury prolong survival of hypoxic LCs and their secretion of CCL5, OPN, and TGF-β1, initiating a cascade of events that ultimately leads to chronic inflammation and/or fibrosis, such as persistent monocyte and T cell accumulation/activation, extensive angiogenesis, excessive fibroblasts proliferation, and overabundant matrix deposition. In this inflammatory environment, sustained TREM-1 induction by chronic hypoxia is likely to contribute to the functional reprogramming of hypoxic LCs toward a Th-1 polarized proinflammatory direction,

which may be critical for the development of skin lesions. Upon recognition of specific PAMP/DAMP ligand(s) present in the wound environment or released in response to inflammation and/or tissue damage [17], TREM-1 will, in fact, promote IL-8, TNFα, and IL-12 production, driving further recruitment of monocytes/macrophages and neutrophils into the wound site, augmented neovascularization, and Th1 priming of naïve T cells, which in turn will stimulate macrophage polarization towards the M1 proinflammatory phenotype by producing IFNγ [20,40]. Interestingly, the same proinflammatory cytokines released by TREM-1-triggered LCs or other inflammatory cells, e.g. TNFα, as well as bacterial products present in the lesion [35,36], can upregulate TREM-1 expression [48], indicating the existence of both autocrine and paracrine positive feedback loops sustaining TREM-1 signaling. Based on these data, we hypothesize that targeting TREM-1 expression/activation with specific inhibitors [17] during pathologic wound healing may represent a potential novel therapeutic strategy to counteract the detrimental effects of H-LCs-induced inflammation and prevent or attenuate AHS and U formation.

In conclusion, these findings add novel and significant insights to our current understanding of LCs biology, leading to new perspective on the role of hypoxia in regulating their responses at sites of skin injury, and identify new potential pathogenetic mechanisms of chronic inflammation associated with wound repair disorders with important biological and therapeutic implications.

CONFLICTS OF INTEREST

The authors state no conflict of interest

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REFERENCES

1. Romani N, Clausen BE, Stoitzner P (2010) Langerhans cells and more: langerin-expressing dendritic cell subsets in the skin. Immunol.Rev. 234:120-141.
2. Merad M, Ginhoux F, Collin M (2008) Origin, homeostasis and function of Langerhans cells and other langerin-expressing dendritic cells. Nat.Rev.Immunol. 8:935-947.
3. Borkowski TA, Letterio JJ, Farr AG, Udey MC (1996) A role for endogenous transforming growth factor beta 1 in Langerhans cell biology: the skin of transforming growth factor beta 1 null mice is devoid of epidermal Langerhans cells. J.Exp.Med. 184:2417-2422.
4. Geissmann F, Dieu-Nosjean MC, Dezutter C, Valladeau J, Kayal S, Leborgne M, Brousse N, Saeland S, Davoust J (2002) Accumulation of immature Langerhans cells in human lymph nodes draining chronically inflamed skin. J.Exp.Med. 196:417-430.
5. Ueno H, Klechevsky E, Morita R, Aspord C, Cao T, Matsui T, Di Pucchio T, Connolly J, Fay JW, Pascual V, Palucka AK, Banchereau J (2007) Dendritic cell subsets in health and disease. Immunol.Rev. 219:118-142.
6. Lin A, Schildknecht A, Nguyen LT, Ohashi PS (2010) Dendritic cells integrate signals from the tumor microenvironment to modulate immunity and tumor growth. Immunol.Lett. 127:77-84.
7. Musso T, Scutera S, Vermi W, Daniele R, Fornaro M, Castagnoli C, Alotto D, Ravanini M, Cambieri I, Salogni L, Elia AR, Giovarelli M, Facchetti F, Girolomoni G, Sozzani S (2008) Activin A induces Langerhans cell differentiation in vitro and in human skin explants. PLoS.One. 3:e3271.
8. Hong WX, Hu MS, Esquivel M, Liang GY, Rennert RC, McArdle A, Paik KJ, Duscher D, Gurtner GC, Lorenz HP, Longaker MT (2014) The Role of Hypoxia-Inducible Factor in Wound Healing. Adv.Wound.Care (New Rochelle.) 3:390-399.

9. Rezvani HR, Ali N, Nissen LJ, Harfouche G, de VH, Taieb A, Mazurier F (2011) HIF-1alpha in epidermis: oxygen sensing, cutaneous angiogenesis, cancer, and non-cancer disorders. J.Invest Dermatol. 131:1793-1805.
10. Semenza GL (2011) Oxygen sensing, homeostasis, and disease. N.Engl.J.Med. 365:537-547.
11. Bosco MC Varesio L (2014) Hypoxia and Gene Expression. In: Melillo G. (Ed) Hypoxia and Cancer. Biological Implications and Therapeutic Opportunities. Humana Press, pp 91-119.
12. Niessen FB, Schalkwijk J, Vos H, Timens W (2004) Hypertrophic scar formation is associated with an increased number of epidermal Langerhans cells. J.Pathol. 202:121-129.
13. Stojadinovic O, Yin N, Lehmann J, Pastar I, Kirsner RS, Tomic-Canic M (2013) Increased number of Langerhans cells in the epidermis of diabetic foot ulcers correlates with healing outcome. Immunol.Res. 57:222-228.
14. Colonna M, Nakajima H, Cella M (2000) A family of inhibitory and activating Ig-like receptors that modulate function of lymphoid and myeloid cells. Semin.Immunol. 12:121-127.
15. Ford JW and McVicar DW (2009) TREM and TREM-like receptors in inflammation and disease. Curr.Opin.Immunol. 21:38-46.
16. Colonna M and Facchetti F (2003) TREM-1 (triggering receptor expressed on myeloid cells): a new player in acute inflammatory responses. J.Infect.Dis. 187 Suppl 2:S397-S401.
17. Pelham CJ, Pandya AN, Agrawal DK (2014) Triggering receptor expressed on myeloid cells receptor family modulators: a patent review. Expert.Opin.Ther.Pat1-13.
18. Bosco MC, Pierobon D, Blengio F, Raggi F, Vanni C, Gattorno M, Eva A, Novelli F, Cappello P, Giovarelli M, Varesio L (2011) Hypoxia modulates the gene expression profile of immunoregulatory receptors in human mature dendritic cells: identification of TREM-1 as a novel hypoxic marker in vitro and in vivo. Blood 117:2625-2639.

19. Castagnoli C, Trombotto C, Ariotti S, Millesimo M, Ravarino D, Magliacani G, Ponzi AN, Stella M, Teich-Alasia S, Novelli F, Musso T (1999) Expression and role of IL-15 in post-burn hypertrophic scars. J.Invest Dermatol. 113:238-245.
20. Trinchieri G (2003) Interleukin-12 and the regulation of innate resistance and adaptive immunity. Nat.Rev.Immunol. 3:133-146.
21. Sallusto F, Geginat J, Lanzavecchia A (2004) Central memory and effector memory T cell subsets: function, generation, and maintenance. Annu.Rev.Immunol. 22:745-763.
22. Sun J, Zhang Y, Yang M, Zhang Y, Xie Q, Li Z, Dong Z, Yang Y, Deng B, Feng A, Hu W, Mao H, Qu X (2010) Hypoxia induces T-cell apoptosis by inhibiting chemokine C receptor 7 expression: the role of adenosine receptor A(2). Cell Mol.Immunol. 7:77-82.
23. Roiniotis J, Dinh H, Masendycz P, Turner A, Elsegood CL, Scholz GM, Hamilton JA (2009) Hypoxia prolongs monocyte/macrophage survival and enhanced glycolysis is associated with their maturation under aerobic conditions. J.Immunol. 182:7974-7981.
24. Walmsley SR, Print C, Farahi N, Peyssonnaux C, Johnson RS, Cramer T, Sobolewski A, Condliffe AM, Cowburn AS, Johnson N, Chilvers ER (2005) Hypoxia-induced neutrophil survival is mediated by HIF-1alpha-dependent NF-kappaB activity. J.Exp.Med. 201:105-115.
25. Iezzi G, Karjalainen K, Lanzavecchia A (1998) The duration of antigenic stimulation determines the fate of naive and effector T cells. Immunity. 8:89-95.
26. Celli S, Lemaitre F, Bousso P (2007) Real-time manipulation of T cell-dendritic cell interactions in vivo reveals the importance of prolonged contacts for CD4+ T cell activation. Immunity. 27:625-634.
27. Wenger RH, Stiehl DP, Camenisch G (2005) Integration of oxygen signaling at the consensus HRE. Sci.STKE. 2005:re12.

28. Bosco MC, Puppo M, Blengio F, Fraone T, Cappello P, Giovarelli M, Varesio L (2008) Monocytes and dendritic cells in a hypoxic environment: Spotlights on chemotaxis and migration. Immunobiology 213:733-749.

29. Kong D, Park EJ, Stephen AG, Calvani M, Cardellina JH, Monks A, Fisher RJ, Shoemaker RH, Melillo G (2005) Echinomycin, a small-molecule inhibitor of hypoxia-inducible factor-1 DNA-binding activity. Cancer Res. 65:9047-9055.

30. Kopecka J, Campia I, Jacobs A, Frei AP, Ghigo D, Wollscheid B, Riganti C (2015) Carbonic anhydrase XII is a new therapeutic target to overcome chemoresistance in cancer cells. Oncotarget. 6:6776-6793.

31. Pierobon D, Bosco MC, Blengio F, Raggi F, Eva A, Filippi M, Musso T, Novelli F, Cappello P, Varesio L, Giovarelli M (2013) Chronic hypoxia reprograms human immature dendritic cells by inducing a proinflammatory phenotype and TREM-1 expression . Eur.J.Immunol. 43:949-966.

32. Jumper N, Paus R, Bayat A (2015) Functional histopathology of keloid disease. Histol.Histopathol.11624.

33. Gauglitz GG, Korting HC, Pavicic T, Ruzicka T, Jeschke MG (2011) Hypertrophic scarring and keloids: pathomechanisms and current and emerging treatment strategies. Mol.Med. 17:113-125.

34. Mustoe TA, O'Shaughnessy K, Kloeters O (2006) Chronic wound pathogenesis and current treatment strategies: a unifying hypothesis. Plast.Reconstr.Surg. 117:35S-41S.

35. van der Veer WM, Bloemen MC, Ulrich MM, Molema G, van Zuijlen PP, Middelkoop E, Niessen FB (2009) Potential cellular and molecular causes of hypertrophic scar formation. Burns 35:15-29.

36. Jiang L, Dai Y, Cui F, Pan Y, Zhang H, Xiao J, Xiaobing FU (2014) Expression of cytokines, growth factors and apoptosis-related signal molecules in chronic pressure ulcer wounds healing. Spinal Cord. 52:145-151.

37. Sica A, Melillo G, Varesio L (2011) Hypoxia: a double-edged sword of immunity. J.Mol.Med.(Berl) 89:657-665.
38. Bosco MC and Varesio L (2012) Dendritic cell reprogramming by the hypoxic environment. Immunobiology 217:1241-1249.
39. Sitkovsky M and Lukashev D (2008) Regulation of immune cells by local-tissue oxygen tension: HIF1 alpha and adenosine receptors. Nat Rev Immunol. 5:712-721.
40. Baggiolini M and Loetscher P (2000) Chemokines in inflammation and immunity. Immunol Today 21:418-420.
41. Wang KX and Denhardt DT (2008) Osteopontin: role in immune regulation and stress responses. Cytokine Growth Factor Rev. 19:333-345.
42. Mori R, Shaw TJ, Martin P (2008) Molecular mechanisms linking wound inflammation and fibrosis: knockdown of osteopontin leads to rapid repair and reduced scarring. J.Exp.Med. 205:43-51.
43. Huang JS, Wang YH, Ling TY, Chuang SS, Johnson FE, Huang SS (2002) Synthetic TGF-beta antagonist accelerates wound healing and reduces scarring. FASEB J. 16:1269-1270.
44. Hunger RE, Sieling PA, Ochoa MT, Sugaya M, Burdick AE, Rea TH, Brennan PJ, Belisle JT, Blauvelt A, Porcelli SA, Modlin RL (2004) Langerhans cells utilize CD1a and langerin to efficiently present nonpeptide antigens to T cells. J.Clin.Invest 113:701-708.
45. Schenk M, Bouchon A, Seibold F, Mueller C (2007) TREM-1--expressing intestinal macrophages crucially amplify chronic inflammation in experimental colitis and inflammatory bowel diseases. J.Clin.Invest 117:3097-3106.
46. Ho CC, Liao WY, Wang CY, Lu YH, Huang HY, Chen HY, Chan WK, Chen HW, Yang PC (2008) TREM-1 expression in tumor-associated macrophages and clinical outcome in lung cancer. Am.J.Respir.Crit Care Med. 177:763-770.
47. Hyder LA, Gonzalez J, Harden JL, Johnson-Huang LM, Zaba LC, Pierson KC, Eungdamrong NJ, Lentini T, Gulati N, Fuentes-Duculan J, Suarez-Farinas M, Lowes MA

(2013) TREM-1 as a potential therapeutic target in psoriasis. J.Invest Dermatol. 133:1742-1751.

48. Bleharski JR, Kiessler V, Buonsanti C, Sieling PA, Stenger S, Colonna M, Modlin RL (2003) A role for triggering receptor expressed on myeloid cells-1 in host defense during the early-induced and adaptive phases of the immune response. J.Immunol. 170:3812-3818.

FIGURE LEGENDS

Fig. 1 Phenotypic and secretory features of monocyte-derived LCs and H-LCs. LCs and H-LCs were generated from human monocytes cultured with GM-CSF, IL-4, and TGF-β1 for 7 days plus TNFα for the last 48 hr, under 20% or 1% O_2, respectively. **(a,b)** Surface marker expression assessed by FACS. **(a)** Cells were stained with specific Abs (solid histograms) or isotype-matched controls (open histograms) and analyzed by FACS. Results from a representative of 5 tested donors are shown. Positive cell percentage is indicated. (**b)** Mean percentage of positive cells in LCs and H-LCs from 5 donors (*dots*). Horizontal lines represent mean values for each group. **(c)** Cytokine/chemokine content in supernatants from LCs and H-LCs assayed by ELISA after 24 hr culture with fresh medium. Results are expressed as pg or ng /8x10^5cells/mL and represent the mean ± SEM of five experiments. *p* values of H-LCs relative to LCs: *p<0.05;**p<0.01; n.s., not significant

Fig. 2 Inhibition by hypoxia of LCs stimulatory activity on naive T cells. Naive T cells purified from 5 donors were cocultured for 6 days with allogenic LCs or H-LCs. **(a)** [^{3}H]thymidine incorporation in T cells. Data are the mean ± SEM of five experiments. **(b)** IFN-γ concentrations in coculture supernatants. Results are expressed as pg/1x10^6cells/mL and are the mean±SEM of 5 experiments. **(c)** CD45RA and CD27 staining pattern of $CD4^+$ or $CD8^+$ T cells assessed by FACS. ***Left panels.*** Results from a representative donor. The percentage of single and double-positive T cells within the gated populations is reported. Upper right quadrants: $CD45RA^+/CD27^+$ cells (*naive cells*); upper left quadrants: $CD45RA^-/CD27^+$ cells (*Central Memory, CM*); lower left quadrants: $CD45RA^-/CD27^-$ cells (*Effector Memory, EM*); lower right quadrants: $CD45RA^+/CD27^-$ cells (*Effector Memory RA, EMRA*). ***Right panels.*** Mean percentage of $CD45RA^-/CD27^+$ cells in the CD4- and CD8-gated populations from 5 donors. *p* values of H-LCs relative to LCs: *$p \leq 0.05$; ** p≤0.01; ***$p \leq 0.001$

Fig. 3 Determination of LCs viability upon generation under normoxia and hypoxia. LCs and H-LCs were generated as described in the legend to Fig.1 and cultured for additional 24 hr under normoxic conditions in fresh medium w/o cytokines (N-LCs and H/N-LCs). Cells were stained with AV-FITC/PI and analyzed by FACS. One representative experiment of three performed is shown. The percentage of single and double-positive cells is indicated. Lower right quadrants: AV+/PI- *(early apoptotic):* upper right quadrants: AV+/PI+ *(late apoptotic);* upper left quadrants: AV-/PI+ *(necrotic)*; lower left quadrants: AV-/PI- *(healthy).*

Fig. 4 TREM-1 expression and activation in H-LCs. H-LCs were generated as described in Fig.1 **(a)** and cultured for additional 24 hr under hypoxic conditions in plates pre-coated with agonist anti-TREM-1 mAb or control IgG1 **(b,c).** Surface expression of **(a)** TREM-1 **(b)** HLA-DR, CD86, and CD83 assessed by FACS in LCs/H-LCs and TREM-1-/IgG-triggered H-LCs, respectively. Results are expressed as detailed in the legend of Fig.1a,b. ***Left panels.*** Positive cell percentage and mean (between brackets) in a representative of five different donors is indicated. ***Right panels.*** Mean percentage of positive cells in five different donors. **(c)** Cytokine/chemokine content in supernatants from TREM-1- and IgG-triggered H-LCs. Results are expressed as detailed in the legend of Fig.1C. *p* value: $*p \leq 0.05$; $**p \leq 0.01$; $***p < 0.001$; n.s., not significant

Fig. 5 T cell stimulatory activity of TREM-1-triggered H-LCs. Naïve T cells purified from 5 different donors were cocultured for 6 days with allogenic anti-TREM-1- or IgG1- triggered H-LCs **(a)** [^{3}H]thymidine incorporation in T cells. Data are the mean$\pm$SEM of five experiments. **(b)** IFN-γ concentrations in coculture supernatants. Results are expressed as pg/1x10^6/mL and are the mean$\pm$SEM of five different experiments. **(c)** CD45RA and CD27 staining pattern in $CD4^+$ or $CD8^+$ T cells, assessed as detailed in the legend of Fig.2c. *p* value of TREM-1- relative to IgG-triggered H-LCs: $*p \leq 0.05$; $**p \leq 0.01$

Fig. 6 TREM-1 expression in AHS and U. **(a)** Immunohistochemical images of biopsy specimens from healthy skin, hypertrophic scar, and decubitus ulcer stained with anti-Langerin, HIF-1α, and TREM-1 Abs. Positive cells are detectable in both upper epidermal and subpapillary dermal layers. Results are representative of at least three patients. **(b)** $Langerin^+$, $TREM\text{-}1^{+,}$ and $HIF\text{-}1a^+$ cell counts determined in an epidermal area of $0.05mm^2$ and reported as mean ± SEM. P values relative to healthy skin: $*p \leq 0.05$; $**p \leq 0.01$; $***p \leq 0.001$. **(c)** Representative two-color immunofluorescence of $Langerin^+$ (*red*)/$TREM\text{-}1^+$ (*green*) cells and of TREM-1 (*red*)/HIF-1α (*green*) in the epidermal layer of healthy skin, hypertrophic scar, and decubitus ulcer biopsies. Images are presented both as single and merged color stains

Table I. Surface marker expression in H/H-LCs and H/N-LCs[a]

Marker	H/H-LCs	H/N-LCs
CD1a	72±6	84±9
Langerin	17.7±2	20.8±3
E-Cadherin	60.8±1	66.3±1.2
CLA	85.3±6.2	93±4.5
HLA-DR	94.8±4	90.6±2.6
CD86	57.4±7.6	59±7.3
CD83	5.4±4	7±1

[a]LCs and H-LCs were generated as described in the legend of Fig.1 and reoxygeneted by exposure to normoxic conditions for 24 hr.

Figure 1.

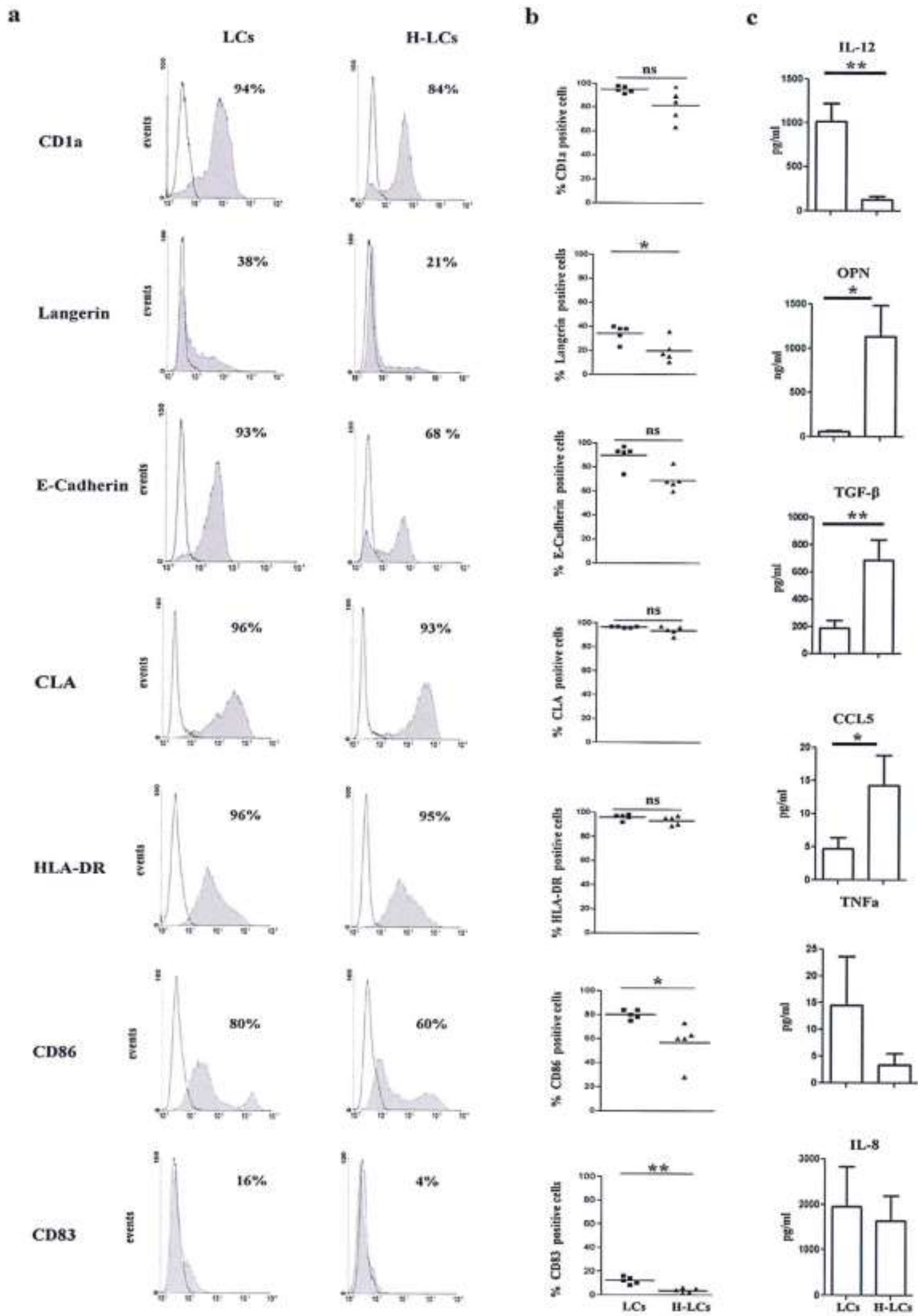


Figure 2

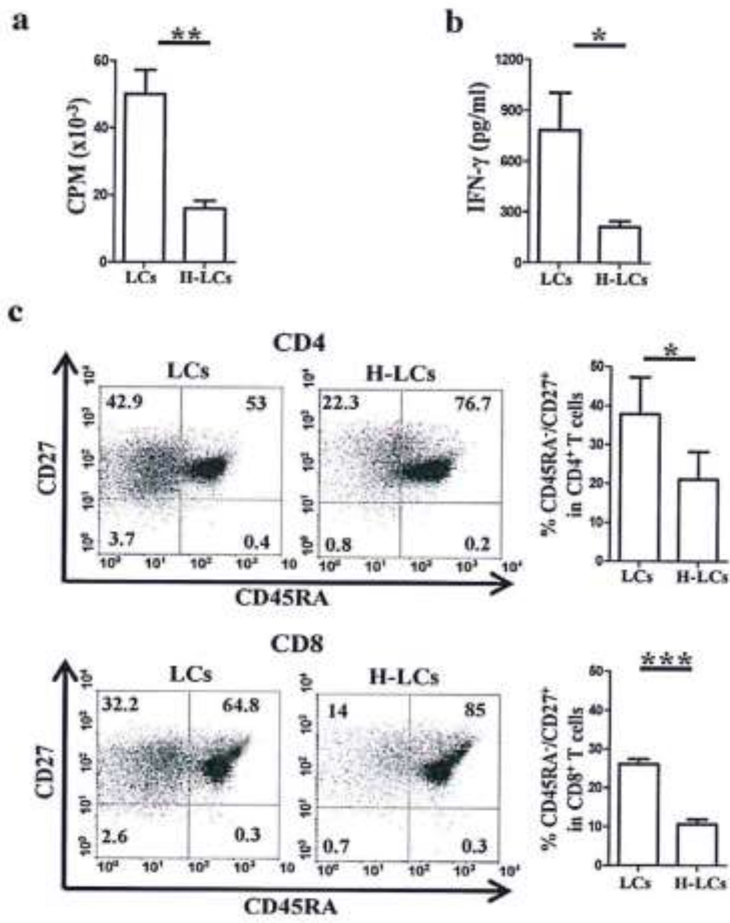
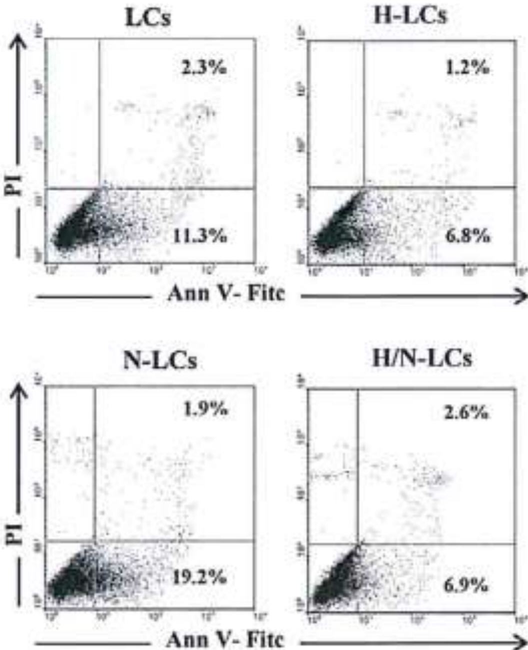


Figure 3.



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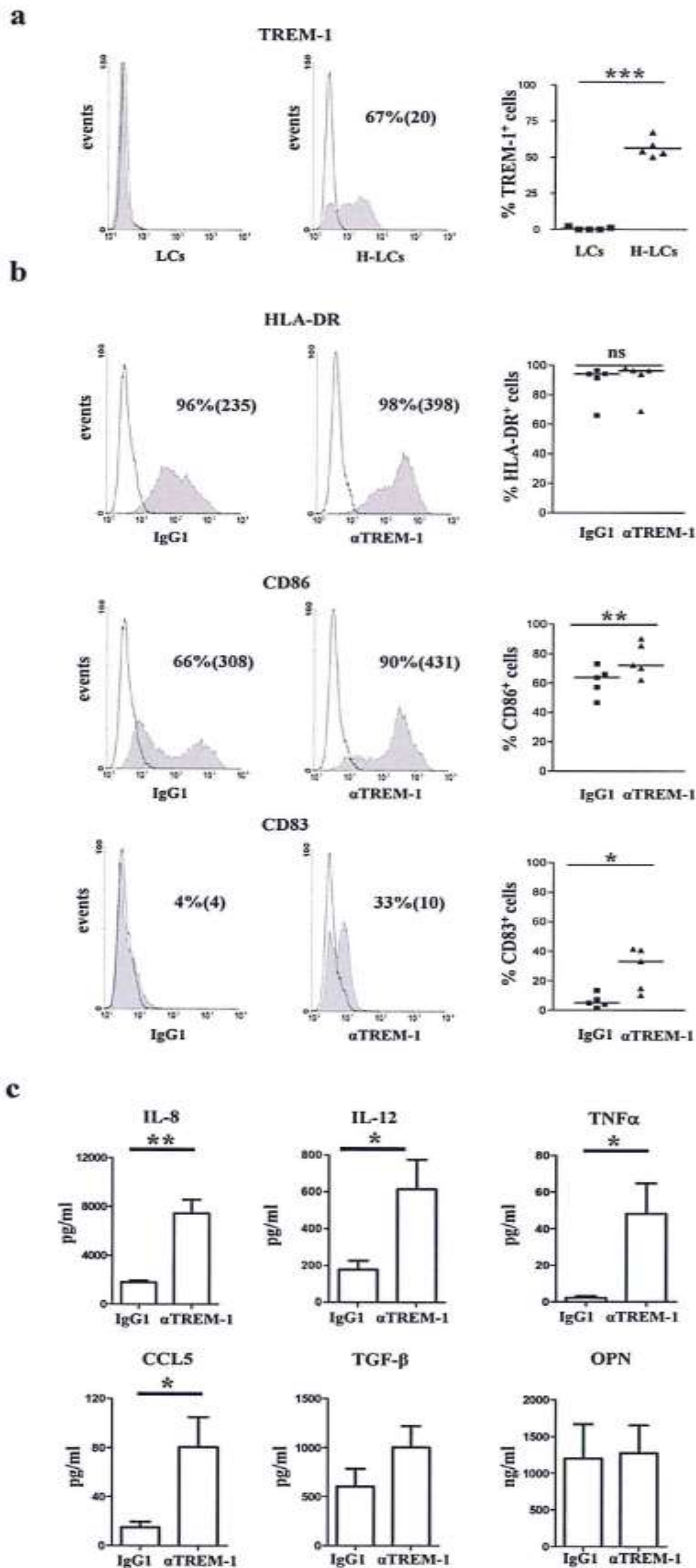

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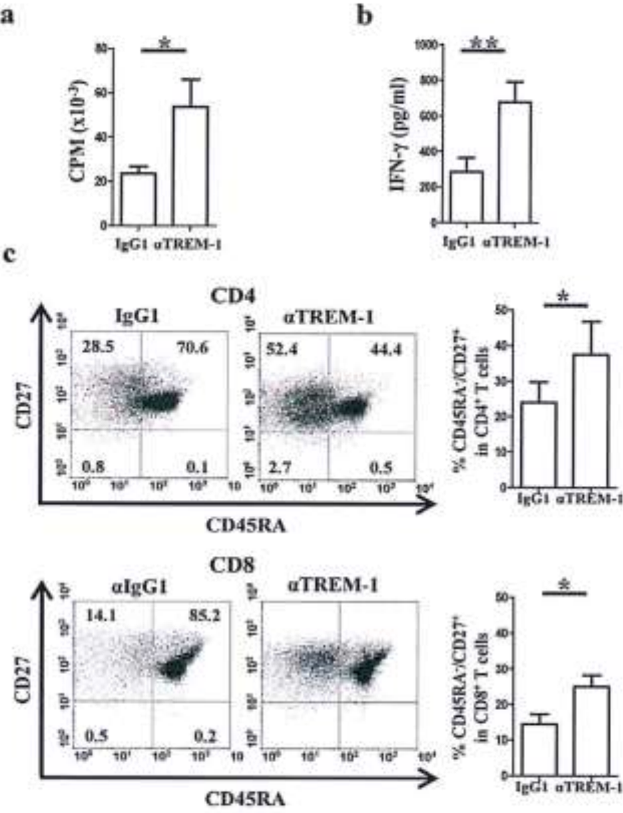
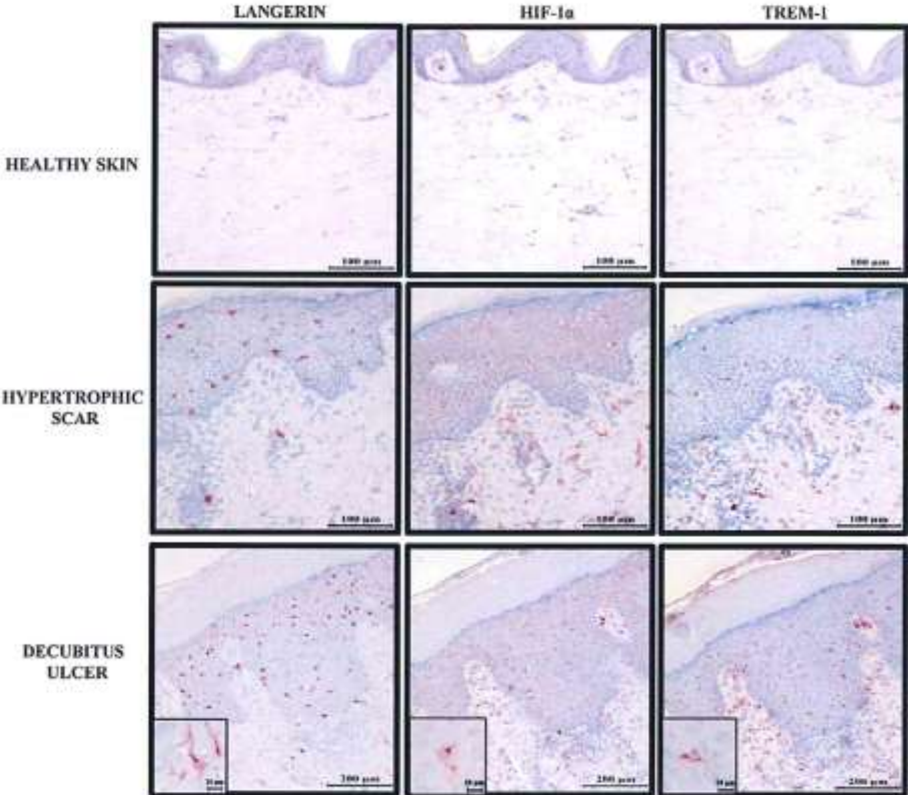
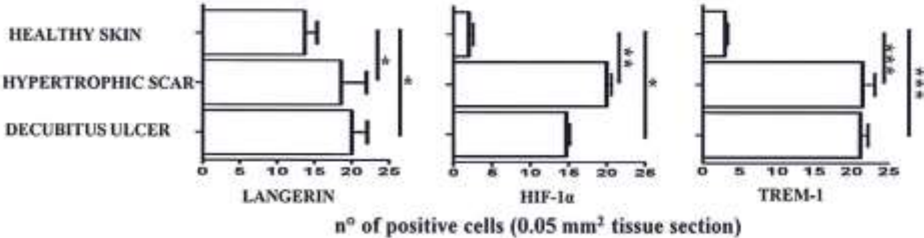


Figure 6.

a



b



c