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PhD thesis

Dextran-shelled oxygen-loaded nanodroplets promote *Enterococcus faecalis* killing and reduce macrophages-mediated inflammation

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SOMMARIO

Introduzione: Nei paesi sviluppati, le ulcere croniche rappresentano un problema sociale ed economico crescente, soprattutto negli anziani, a causa della mancanza di trattamenti efficaci e a basso costo. Nell'ambiente ipossico ed infiammatorio delle ulcere croniche i macrofagi perdono la capacità di transire dal fenotipo M1 pro-infiammatorio al fenotipo M2 antinfiammatorio. In aggiunta, i macrofagi contrastano le infezioni fagocitando ed eliminando i batteri attraverso diversi meccanismi, tra i quali la produzione di radicali dell'ossigeno e dell'azoto. *Eneterococcus faecalis* (*E. faecalis*) è un battere commensale Gram-positivo che colonizza il tratto gastrointestinale, ma anche capace di infettare le ferite e di sviluppare un'elevata resistenza agli antibiotici. Dal momento che nanogocce con guscio di destrano e cariche di ossigeno (OLNDs), in grado di rilasciare ossigeno lentamente nel tempo, si sono dimostrate in grado di contrastare gli effetti dell'ipossia su cellule endoteliali e monociti, lo scopo di questo lavoro è stato di studiare gli effetti delle OLNDs sul killing di *E. faecalis* mediato da macrofagi, e di valutare la modulazione dello stato infiammatorio, sia in normossia sia in ipossia.

Metodi: La citotossicità delle OLNDs e OFNDs (nangocce non ossigenate) è stata valutata con saggio MTT sia su macrofagi da midollo osseo di topo (BMDM) sia su monociti umani da paziente leucemico differenziati in macrofagi con la PMA (dTHP-1). La localizzazione citoplasmatica delle OLNDs coniugate con il FITC è stata confermata con la microscopia a fluorescenza. Gli effetti delle nanogocce su *E. faecalis* ATCC 29212 sono stati valutati calcolando la MIC e la MBC. La produzione dei radicali liberi dell'ossigeno e dell'azoto è stata valutata usando rispettivamente il sale fluorescente H_2DCFDA e il saggio di Griess. La fagocitosi e il killing di *E. faecalis* sono stati valutati contando il numero di macrofagi infetti su vetrini colorati con Giemsa o contando il numero di CFUs dopo il saggio di protezione dell'antibiotico e le successive 24 ore di trattamento. La produzione di citochine infiammatorie e di fattori angiogenici è stata valutata con saggio ELISA. Tutti gli esperimenti sono stati condotti in normossia e ipossia.

Risultati: Le nanogocce non sono risultate tossiche fino alla concentrazione del 10% v/v, e sono state internalizzate sia dai macrofagi murini sia umani.

Le nanogocce non hanno interferito con la crescita e la vitalità batterica. Le OLNDs hanno indotto i livelli di ROS e di NO nei BMDM, invece, i meccanismi di killing delle dTHP-1 non sono stati modificati. Dato l'incremento dei ROS e di NO, come atteso, il killing di *E. faecalis* da parte dei macrofagi murini è risultato essere migliorato dalle OLNDs e dalle OFNDs. Nessuna differenza è stata evidenziata tra nanogocce ossigenate e non ossigenate suggerendo che gli effetti erano probabilmente dovuti al guscio di destrano. Al contrario, il killing mediato dalle dTHP-1 non è stato modificato dalle nanogocce.
Nella seconda parte del lavoro, l'effetto delle nanogocce sui mediatori dell'infiammazione e dell'angiogenesi è stato valutato nei sovranatanti delle dTHP-1 infette con *E. faecalis*. Le OLNDs e le OFNDs hanno mostrato un'attività antinfiammatoria riducendo le citochine pro-infiammatorie IL-1β, IL-6 e TNFα in normossia e ipossia, di nuovo senza differenze tra i due carriers. Diversamente, come atteso, l'ipossia ha indotto il VEGF e solo le OLNDs hanno ripristinato un fenotipo simile alla normossia, confermando il rilascio di ossigeno.

Conclusioni: In questo lavoro abbiamo ulteriormente confermato il potenziale delle OLNDs in qualità di tools innovativi per le ferite croniche evidenziando il loro ruolo duale nel migliorare il killing batterico e nel ridurre la risposta infiammatoria mediata dai macrofagi.

ABSTRACT

Background: Chronic wounds represent nowadays an increasing social and economic burden in developed countries, especially in the elderly, due to the lack of effective and low-cost treatments. In the hypoxic and inflammatory milieu of non-healing ulcers, macrophages are unable to switch from the M1 pro-inflammatory to the M2 anti-inflammatory phenotype. Moreover, they counteract overlapping infections by phagocyting and killing bacteria with different mechanisms, among which the production of oxygen and nitrogen radicals. *Enterococcus faecalis* (*E. faecalis*) is a commensal Gram-positive bacterium, usually found in gastrointestinal tract, able to infect wounds and to develop high resistance to antibiotics. Since dextran-shelled oxygen-loaded nanodroplets (OLNDs), carriers able to release oxygen in a time-sustained manner, were proposed as tools able to counteract hypoxia in endothelial cells and monocytes, the aim of this work was to investigate their effects on macrophages-mediated killing of *E. faecalis*, and to evaluate their modulation of the inflammatory milieu, both in normoxia and hypoxia.

Methods: Cytotoxicity of OLNDs and OFNDs (oxygen-free nanodroplets) on murine bone marrow-derived macrophages (BMDM) and human leukemic monocytes differentiated into macrophages by PMA (dTHP-1) was evaluated by MTT assay. FITC-conjugated OLNDs internalization in cytoplasm was analyzed by fluorescent microscopy. Effects of nanodroplets on *E. faecalis* ATCC 29212, was evaluated by MIC and MBC. The production of oxygen and nitrogen radicals were evaluated using the fluorescent dye H_2DCFDA and the Griess assay, respectively. *E. faecalis* phagocytosis and killing were evaluated counting infected macrophages on Giemsa-stained slides or CFUs after the antibiotic protection assay and subsequent 24 hours of treatment. The production of inflammatory cytokines and angiogenic factors was evaluated by ELISA. All the experiments were performed in normoxia or hypoxia.

Results: Nanodroplets were not toxic up to the concentration of 10% v/v and were internalized by both murine and human macrophages. They did not interfere with bacterial growth and viability. ROS and NO levels were induced in BMDM in presence of OLNDs, while dTHP-1 killing mechanisms were not affected by nanodroplets. Given the increase in NO and ROS in the presence of NDs, as expected, *E. faecalis* killing by murine macrophages was

improved by OLNDs and OFNDs. No differences were observed between OLNDs and OFNDs indicating that the effects were probably due to the outer dextran shell of the NDs. On the contrary, dTHP-1-mediated killing was not affected by nanodroplets.
In the second part of the work, the effects of nanodroplets on inflammatory and angiogenic mediators was evaluated in *E. faecalis* infected dTHP-1. OLNDs and OFNDs exerted an anti-inflammatory activity by reducing the pro-inflammatory cytokines IL-1β, IL-6 and TNFα in normoxia and hypoxia. Again, no differences between OLNDs and OFNDs effects were observed. Differently VEGF, as expected, was induced by hypoxia and, only OLNDs were able to restore a normoxia-like phenotype reducing VEGF secretion and confirming the release of oxygen by OLNDs.

Conclusions: We further confirmed the potential of OLNDs as innovative tools for chronic wounds by deciphering their dual role in improving bacterial killing and reducing inflammatory response by macrophages.

INDEX

LIST OF SYMBOLS IN ALPHABETICAL ORDER

BMDM Bone Marrow-Derived Macrophages
CFUs Colonies Forming Units
CXCL8 C-X-C Motif Chemokine Ligand 8
DAMPs Damage-Associated Molecular Patterns
DAPI 4',6-diamidino-2-phenylindole
DFP Decafluoropentane
DFUs Diabetic Foot Ulcers
dTHP-1 PMA-differentiated THP-1
E. faecalis Enetorococcus faecalis
ECM Extracellular Matrix
EGF Epidermal Growth Factor
ELISA Enzyme Linked ImmunoSorbent Assay
FGF-2 Fibroblast Growth Factor-2
FITC fluorescein isothiocyanate
GSH Glutathione
H_2DCFDA 2'7'-dichlorofluorescein diacetate
H_2O_2 Hydrogen Peroxide
HIF Hypoxia Inducible Factor
hIFNγ human Interferon-gamma
IL-1β Interleukin 1 beta
IL-6 Interleukin 6
iNOS Inducible Nitric Oxide Synthase
LTA Lipoteichoic Acid
MBC Minimum Bactericidal Concentration
MH Mueller-Hinton
MIC Minimum Inhibitory Concentration

mIFNγ mouse Interferon-gamma

MMP-2 Matrix MetalloProteinases-2

MMP-9 Matrix MetalloProteinases-9

MOI Multiplicity Of Infection

MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NDs NanoDroplets

NOX2 NADPH Oxidase

O_2 Molecular oxygen

O_2^- Superoxide anion

OFNDs Oxygen-Free NanoDroplets

OH^- Hydroxyl radical

OLNDs Oxygen-Loaded NanoDroplets

$ONOO^-$ Peroxynitrite

PAMPs Pathogen-Associated Molecular Patterns

PI Phagocytic Index

PlGF Platelet Growth Factor

PMA Phorbol Myristate Acetate

ROS Reactive Oxygen Species

St. Dev. Standard Deviation

TGF-β Tissue Growth Factor-beta

TLR4 Toll-Like Receptor 4

TNFα Tumor Necrosis Factor alpha

VEGF Vascular Endothelial Growth Factor

1. INTRODUCTION

1.1 Wound healing process

1.1.1 Acute wound healing

The skin is a complex organ and it represents the first defence barrier against external insults. It is stratified in three different layers: an outer layer of epidermis, a middle layer of dermis, and the deepest layer of subcutaneous fat and connective tissue [1]. Integrity of the skin is essential to maintain its several and important functions among which the control of the body temperature, the prevention of fluid loss and the transmission of sensorial inputs. Moreover, it possesses a defensive role against infections and chemical or physical insults, also thanks to the presence of specialized cells of the immune system [2]. A cutaneous wound is defined as an interruption of the skin integrity and continuity with a loss of function of the underlying tissues, thus arising the necessity of a prompt restoration in order to re-establish the homeostasis [3]. All the mechanisms which take part to the restoration of the skin integrity are named as wound healing process.

Wound healing is a complex and highly coordinated process divided in four subsequent and overlapping phases: haemostasis, inflammation, proliferation and tissue remodelling [3, 4]. Haemostasis, with vasoconstriction and clot deposition, represents the immediate response to the skin injury. The main actors involved in haemostasis are the platelets, which undergo activation by adhering to the collagen of the exposed extracellular matrix (ECM). Activated platelets secrete soluble factors and adhesive molecules leading to self-aggregation and to the final fibrin clot stabilization, thus blocking fluid loss [5, 6]. The second phase of the healing process is inflammation, which partially overlaps the final steps of

haemostasis. About two days post injury, platelets degranulation, chemokines, complement factors and bacterial products attract neutrophil granulocytes, which represent the most abundant immune population in the bed of the wound until 4-5 days post injury. Neutrophils directly participate to the cleaning of the wound by phagocytizing necrotic tissue debris and bacteria. They also amplify inflammation by secreting pro-inflammatory cytokines and chemoattractant molecules which, together with the Damage-Associated Molecular Patterns (DAMPs) and the Pathogen-Associated Molecular Patterns (PAMPs), recruit circulating monocytes from the third day post injury. Monocytes rapidly develop into macrophages, which replace neutrophils in the defence of the host during the subsequent phases of healing. Macrophages are the key effectors of the promotion and subsequent resolution of inflammation, sustaining the transition to the third phase of healing [7, 8]. During the proliferation phase, which usually begins between the third and the tenth day after injury, endothelial cells, fibroblasts and keratinocytes cooperate in order to rebuild the damaged tissues. Under the strong VEGF stimulus, endothelial cells migrate to initiate angiogenesis, i.e. the birth of new vessels by capillary sprouting from pre-existing vessels [9]. Keratinocytes migrate from the edge of the wound to the forming granulation tissue in order to promote the re-epithelialization, while fibroblasts secrete collagen and fibronectin maintaining a balance between the synthesis and the degradation on the new ECM. At the end of the proliferation phase, the new deposed granulation tissue is ready to be remodelled in the scar [7]. Remodelling phase can last until one year post injury. During this phase the final scar reaches maturation by the replacement of the weak collagen type I with the stronger collagen type III, by the contraction of the wound's edges thanks to the action of the myofibroblasts, and by the regression of the dense capillary reticulum formed during angiogenesis [7, 8].

1.1.2 Risk factors for the development of chronic ulcers

A proper timeline of the events described in the 1.1.1 section is necessary to achieve a suitable healing. Nevertheless, many are the risk factors reported in literature which can interfere and finally lead to an impaired healing process. The different risk factors seem to be dependent from the type of ulcer taken into consideration, and most of them are well reviewed by Guo and Di Pietro [10]. They can generally be summarized as follow:

- **age**: improper healing increases with population aging, becoming more common in the elderly. This can be due to a general delay of the physiological responses in the healing process [10], to a major chance of concomitant chronic diseases, or to a general poor mobility [11-13];
- **sex**: men are more susceptible of non-healing wounds than women, probably due to their higher susceptibility to vascular and metabolic diseases [11]. Moreover, androgens seem to increase pro-inflammatory cytokines secretion, while oestrogens favour expression of genes involved in repair and regeneration [10, 14];
- **diabetes**: non-healing Diabetic Foot Ulcers (DFUs) are estimated to develop in 15% of diabetic population. Prolonged hypoxic environment and hyperglycaemia lead to an excessive oxidative stress, to the formation of advanced glycation end-products and to a reduced angiogenesis, thus impairing healing [10, 14, 15];
- **obesity**: a body mass index higher than 25 kg/m^2 or higher than 35 kg/m^2 seems to be correlated to higher risk of healing impairment [16], indeed obesity induces hypoperfusion and ischemia. Moreover, obesity often co-exists with other pathological conditions such as hypertension, diabetes, vascular diseases which all contribute to a strong delay in healing processes [10];

- **circulation abnormalities**: deep venous thrombosis, impaired microcirculation, venous abnormalities, edema, low blood pressure are all conditions reducing the possibility of a correct healing process [13, 16, 17];
- **immobility or reduced movement**: a lack of an adequate physical activity due to the sedentary behaviours or to the restricted mobility for health reasons, like people constricted in bed or wheelchair, represent a risk for impaired healing due to the prolonged pressures on the site of the wound [11, 13, 15, 16];
- **nutrition**: it is well-known that a correct intake of all the nutrients is essential for metabolism, thus malnutrition represent an obstacle to the correct healing process [10, 13, 15]. Moreover, a parenteral nutrition seems to be correlated to a worsen of wound healing [17];
- **smoking**: among the over 4000 substances identified in tobacco, some of them are detrimental for wound healing causing low oxygen supply, impaired white cells migration and decreased blood perfusion to damaged tissue [10];
- **stress**: psychological stress and depressive symptoms are correlated to a strongly reduced response of the immune system, which is deeply involved in the first phases of wound healing [10, 16];
- **first ulcer characteristics**: wound depth, area, localization and shape are all features directly correlated to the outcome of healing process [16].

The presence of one or more of these risk factors can finally lead to an inadequate and impaired healing process, determining the onset of a so-called chronic ulcer.

1.2 Chronic ulcers

1.2.1 Definition and classification

Even if the precise definition of "chronic wound" remains still debated due to the several interpretations, especially in clinical studies and depending on the type of the wound itself, it is generally accepted that chronic non-healing ulcers are "wounds that have not proceeded through an orderly and timely reparation to produce anatomic and functional integrity after 3 months" [18]. According to the classification of the Wound Healing Society, chronic wounds usually include venous ulcers, arterial ulcers, diabetic foot ulcers and pressure ulcers, which mainly differ for the underlying mechanisms of origin [19, 20]. Venous stasis ulcers are the consequence of a venous thrombosis or a reflux between the deep and the superficial venous system, due to non-functional valves. They represent more than a half of the lower extremity chronic wounds. Arterial ulcers usually derive from an insufficient supply of oxygen to skin, mainly caused by atherosclerosis or thromboembolic disease. DFUs, as already mentioned in 1.1.2 section, usually develop in more or less 15% of the diabetic population and are the result of the poor oxygenation and the hyperglycaemia. They represent the major risk for leg amputation. The last chronic wound type, i.e. pressure ulcers, are instead due to a prolonged pression, shear stress or friction, thus leading to an impaired blood supply and tissue low nutrition [20].

1.2.2 Acute vs. chronic wounds

The impaired mechanisms in chronic wounds are several and predominantly concern the inflammation and the proliferation phases of wound healing.
During the latter stages of haemostasis and the early stages of inflammation, endothelial cells increase their expression of ICAM (IntraCellular Adhesion Molecule) and VCAM (Vascular Cell Adhesion Molecules), contributing to the development of the subsequent excessive and prolonged inflammation with the recruitment of an abnormal number of neutrophils [21, 22]. Neutrophil granulocytes produce both high amounts of reactive oxygen species (ROS), detrimental for the cell populations involved in healing process, and proteases as collagenases and elastases which are responsible of the excessive degradation of either ECM or some essential growth factors like PDGF (Platelet-Derived Growth Factor) or TGF (Tissue Growth Factor) [21]. Among the collagenases, Matrix MetalloProteinases-2 (MMP-2) and Matrix MetalloProteinases-9 (MMP-9) are the ones mainly increased [23]. To their increase, also a decrease of their inhibitors further promotes ECM degradation [24]. To worsen the abnormal inflammatory response, the balance between M1 pro-inflammatory macrophages and M2 anti-inflammatory macrophages is impaired in favour of the first [22]. Nevertheless, despite the high number of phagocytic cells, diminished phagocytic and bactericidal activities are common in non-healing wounds [25]. Probably due to this decreased capability to clean ulcers from microorganisms, other typical features of chronic ulcers are bacterial colonization and infection, with a higher possibility to detect biofilm-forming communities compared to acute wounds [24, 26, 27]. Concomitantly to the disproportionate inflammation, proliferation and regeneration of new tissues are missing. Fibroblasts appear senescent, with a reduced response to growth factors due to the decreased number of surface receptors, and with a low mitogenic potential [24, 26]. Expression of genes coding for proteins

involved in the regulation of cellular cycle progression is impaired in keratinocytes, with overexpression of cyclins and downregulation of some checkpoint signals as p53, thus leading to an impaired proliferation [25]. In addition, their ability to migrate is defective, thus reducing re-epithelialization [26].

All these events take place in an environment lacking the correct oxygen and nutrients supply due to the impaired angiogenesis process, thus leading to a further damage of the surrounding tissues [24].

The main differences between a correct wound repair and a chronic ulcer are summarized in figure 1.

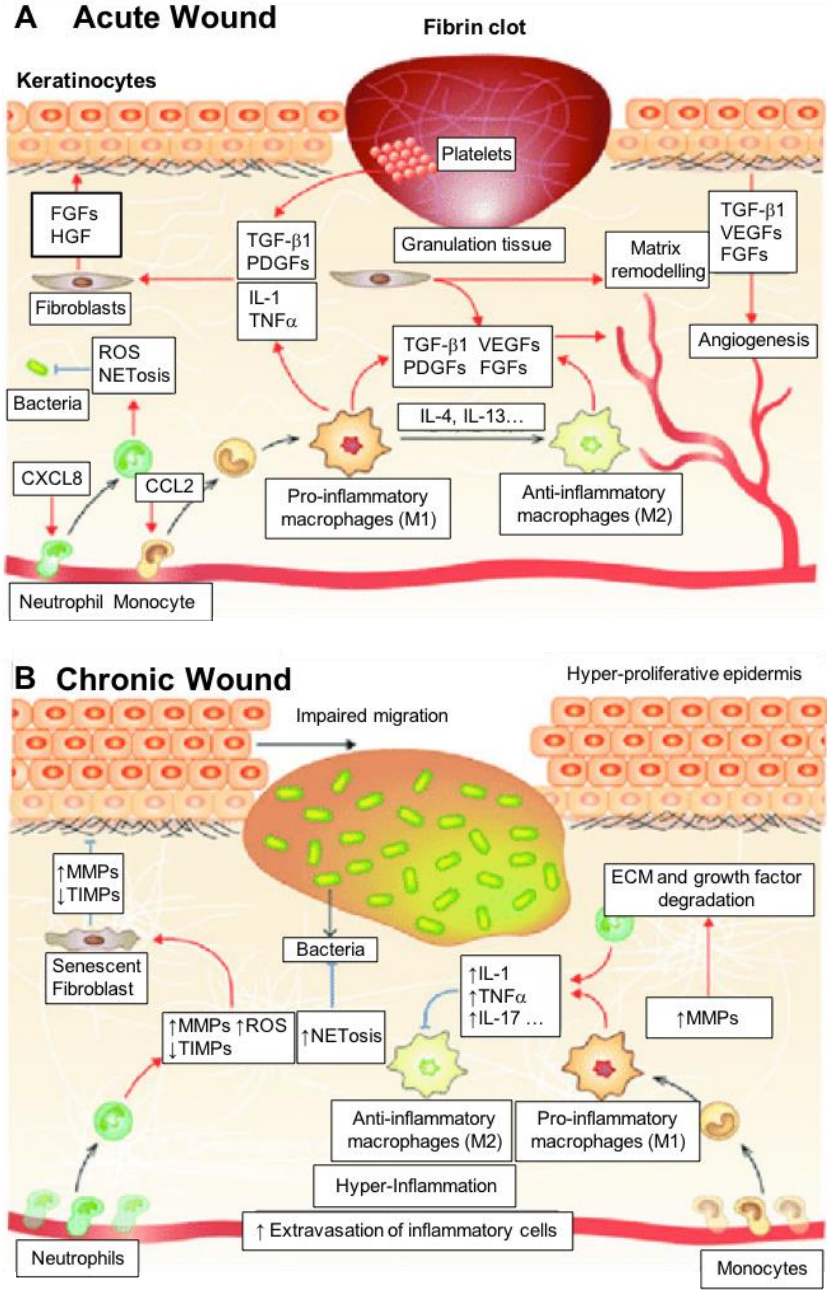

***Figure 1**. Overview of the impaired mechanisms of healing in chronic wounds (B) versus acute wounds (A). Modified version of Larouche et al. [28]*

1.2. Role of macrophages in wound healing and chronic wounds

1.2.1 Macrophages role in the inflammation phase of wound healing process

Macrophages are highly plastic cells, playing a pivotal role in wound healing process by orchestrating the inflammatory phase. Depending on the environmental stimuli, they are able to change their phenotype mediating tissue and cells destruction during the first stages of inflammation, but also promoting tissue repair in order to restore homeostasis during the latter stages of inflammation [29, 30].

At the site of the wound, macrophages derive from two different subsets: the tissue-resident macrophages which represent the first-line defence, and the macrophages differentiated from circulating monocytes [30]. When a skin injury occurs, tissue-resident macrophages are immediately activated by the binding of DAMPs (damage-associated molecular patterns), such as ATP (Adenosine Triphosphate) or HMGB-1 (High Mobility Box-1), and PAMPs (Pathogen-associated molecular patterns), like bacterial gram-negative LPS (lipopolysaccharide) or gram-positive LTA (Lipoteichoic Acid), to the PRRs (Pattern Recognition Receptors), among which the TLRs (Toll-Like Receptors), the NLRs (Nod-like Receptors) and the RIG-like (Retinoic acid-inducible gene 1) receptors [31, 32]. The same signals, taken together with the chemokine gradients, recruit the circulating monocytes which localize and enter the wound through the over-expressed adhesion molecules. Then, monocytes differentiate into macrophages in dermis. The first macrophages' set which develops in the site of the injury is the M1 phenotype, also called "classically activated" or pro-inflammatory phenotype. M1 macrophages are characterized by a switch of the glucose metabolism from the oxidative phosphorylation to glycolysis, which allows a prompt availability of energy and substrates for the pro-inflammatory functions [31]. Classically activated

macrophages have two main roles. At first, they sustain inflammation by producing typical inflammatory cytokines as IL-1β (interleukin-1beta), TNFα (tumour necrosis factor-alpha) and IL-6 (interleukin-6), and chemokines as CXCL8 (C-X-C Motif Chemokine Ligand-8). Always promoting inflammation, they support T-helper 1 activity through the secretion of IL-12 (interleukin-12) [29, 33]. Above this maintenance of the inflammatory environment, M1 macrophages exploit their phagocyting activity by cleaning the bed of the wound from debris deriving from necrotic tissues, and by engulfing microbes and apoptotic neutrophils [32, 34, 35]. The ingestion of apoptotic neutrophils, also known as efferocytosis, and the removal of DAMPs and PAMPs reduce inflammation, thus representing the crucial events for the beginning of the proliferative phase [36]. In the meanwhile, macrophages, passing through a continuum of changes with at least nine different subsets identified *in vivo*, switches to their M2 anti-inflammatory phenotype, also called “alternatively activated” phenotype. Differently from the M1 subset, they promote the regeneration of new tissues by secreting polyamines, growth factors and anti-inflammatory mediators such as TGFβ (Tissue Growth Factor-beta), IGF1 (Insulin-like Growth Factor-1) or IL-10, also useful for the recruitment of cells directly involved in tissue repair as fibroblasts, keratinocytes and endothelial cells [29, 30, 33].

1.2.2 Macrophages in chronic wounds

The typical feature of non-healing ulcers is the failure of the transition from the inflammatory phase to the repair stage due to the missing switch of macrophages from the pro-inflammatory M1 to the anti-inflammatory M2 phenotype [36]. It is indeed demonstrated that chronic wounds present a different cell composition compared to acute wounds, with a macrophage population composed of around 80% of M1 subset and only 20% of M2 subset [37]. This abundance of pro-inflammatory macrophages inevitably determines a reduced secretion of growth factors and an increased secretion of pro-inflammatory cytokines, chemokines and MMPs, thus furtherly sustaining inflammation. Moreover, the already compromised situation is worsened by the reduced capability of these macrophages to phagocyte apoptotic neutrophils, also inhibited by high levels of TNFα [37, 38].

1.2.3 Macrophages defence against bacterial infections

As already mentioned in 1.2.1 section, M1 polarized macrophages exploit their professional phagocytic activity by the engulfment of invading microorganisms, representing one of the first line of defence against pathogens. Phagocytosis starts when an antigen is recognised by phagocyting receptors as FcγR (Fc gamma receptor), CRs (Complement Receptors) or MRs (Mannose Receptors) [39]. Ingested bacteria are engulfed in a vesicle called phagosome which, in order to kill the microorganisms, proceeds to the fusion or fission with endosomes and lysosomes to form the so-called phagolysosome, characterized by an acid pH around 5.0 [39, 40]. Phagolysosomes are unhospitable; they reduce bacterial growth and promote bacterial degradation and killing through several mechanisms among which the production of reactive oxygen and nitrogen species. In response to phagocytic receptors binding, NOX2 (NADPH oxidase) is activated to form O_2^- (unstable superoxide anion), which

can directly contribute to bacterial killing. Moreover, O_2^-, under the acidic conditions of the phagolysosome, can dismutate in H_2O_2 (hydrogen peroxide), an oxygen radical able to damage proteins, DNA and lipids, thus destroying bacterial structures. In addition, superoxide anion can react with NO (Nitric Oxide) to form $ONOO^-$ (peroxynitrite), which again disrupt proteins and DNA. In the meanwhile, release of metals such as iron inside the phagolysosome induce the Fenton reaction, with the final formation of OH^- (hydroxyl radical), another ROS acting as explained above for O_2^-, H_2O_2 and $ONOO^-$ [41, 42]. NO is produced in the cytosol through the induction of the iNOS (inducible Nitric Oxide Synthase), also called NOS2 (Nitric Oxide Synthase 2). Cytosolic NOS2, strongly activated in M1 macrophages, is the catalyser of the reaction of L-arginine with O_2 (molecular oxygen) to form L-citrulline and NO. NO can directly affect bacterial enzymes, or it can diffuse in phagolysosomes to react with O_2^- to form $ONOO^-$ as previously described [41, 43].

Besides the production of ROS and NO, which are dependent on oxygen amounts, macrophages exert their antimicrobial activities by inducing antimicrobial proteins or peptides as cathepsins, cathelicidins, hydrolases and phospholypases, by producing mETs by NETosis, i.e. extracellular traps with host chromatin, and by the so-called “nutritional immunity”, i.e. the depletion of nutrients, like iron or manganese, for the pathogens [41]. The last killing mechanism is xenophagy, a well-conserved lysosomal degradation pathway induced by the binding of DAMPs and PAMPs to macrophages receptors [44].

The microbiocidal mechanisms actuated by macrophages to kill bacteria are summarized in figure 2.

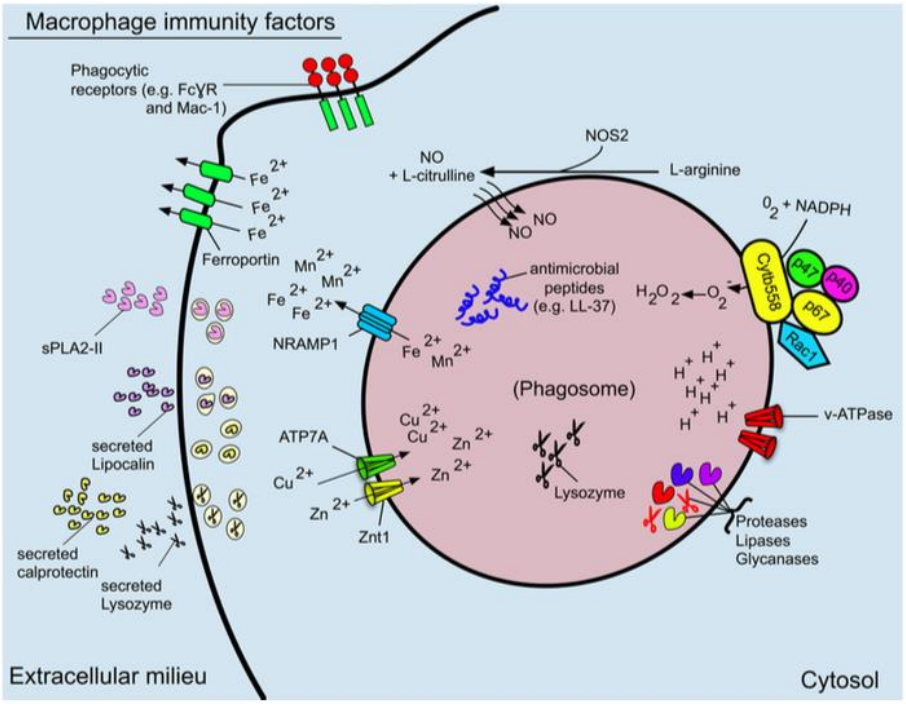

Figure 2. *Mechanisms of killing of macrophages against bacterial infections [41]*

1.2.4 Role of macrophages in angiogenesis

Besides the primary activities described before, macrophages also participate in the regulation of angiogenesis from the first sprouting of new and disordered vessels to the final maturation and re-organization [8, 45]. Regulation of endothelial cells proliferation and migration, and control and promotion of homeostasis are consequences of the secretion of various growth factors as VEGF-A (Vascular Endothelial Growth Factor-A), TGF-β (Tissue Growth Factor-beta), FGF-2 (Fibroblast Growth Factor-2), PlGF (Platelet Growth Factor) and EGF (Epidermal Growth Factor) [8, 46]. Among these, VEGF-A represent the most potent pro-angiogenic factor, constitutively expressed in macrophages, but increased especially in M1 phenotype [46, 47]. Nevertheless, it has been demonstrated that M2 anti-inflammatory phenotype has a higher angiogenic potential and that persistent inflammation inhibits angiogenesis, making the common M1 population found in chronic ulcers an obstacle to the birth of new vessels [8, 46].

1.3 Infections of chronic wounds

Another important cause of non-healing ulcer is infection, often presenting a polymicrobial population which leads to chronic inflammation [48]. Infections of skin ulcers represent an underestimated problem, indeed the sepsis and the multiple organ syndrome which can arise from a simple wound contamination are fatal for around 10000 people over 1 million of wounded patients [49, 50]. It is thus important to highlight the cut-off of 10^5 colonies forming units (CFUs) per gram of viable tissue below which it is considered a simple colonization with a low number of bacteria which do not affect wound healing, otherwise it is considered a potentially dangerous infection [51]. Both acute and chronic wounds are mainly colonized by endogenous and commensal bacteria which become pathogenic by entering the new site

of infection. Moreover, it is not unusual to find polymicrobial communities forming new ecosystems that worsen the situation due to the competition and the interactions among the different species [48].

Infected chronic wounds present both aerobic and anaerobic bacteria, which cooperate: aerobic species provide growth factors to anaerobic ones; in the meanwhile, they consume oxygen, thus favouring the instauration of a hypoxic environment suitable for anaerobes and detrimental for host's immune response [48]. Different studies demonstrate a different composition of bacterial population in chronic ulcers. Gjodsbol and colleagues identified that bacteria coming from 46 patients with chronic venous leg ulcers presented in 93.5% of cases *Staphylococcus aureus*, 71.7% *Enterococcus faecalis*, 52.2% *Pseudomonas aeruginosa*, 45.7% coagulase-negative *Staphylococci*, 41.3% *Proteus* spp. and 39.1% anaerobic bacteria [52]. Anyway, it is commonly recognised that the two most common species infecting chronic wounds are *Staphylococcus aureus* and coagulase-negative *Staphylococci* [50, 53].

To further worsen the already complex situation, resistance to antibiotics induce an increase of bacterial specimens able to protect themselves by producing biofilm in chronic wounds: indeed, James and colleagues demonstrated that 60% of the bacterial species infecting chronic wounds were able to produce protective biofilm versus a 6% of bacteria infecting acute wounds [27].

1.3.1 *Enterococcus faecalis*

Enterococcus faecalis (*E. faecalis*) belongs to the Enterococcus genus. Enterococci are Gram-positive, facultatively anaerobic, lactic acid, catalase-negative (in absence of exogenous heme), oxidase-negative and non-spore forming cocci which usually grow in pair or in short chains [54, 55]. They are generally extracellular bacteria, but they are also able to survive and persist inside hosts' cells counteracting hosts' defence [56]. Until 1984 they were classified as streptococci. To date, Enterococci includes 37 species classified by 16S RNA sequencing and DNA-DNA hybridisation. Among the different species, *Enterococcus faecalis* and *Enterococcus faecium* represent the most important [54]. Enterococci are among the most resistant bacteria: they are mesophilic, i.e. they grow in a temperature range between 10°C and 45°C, but they can survive until 30 minutes at 60°C. Moreover, they grow in a pH range between 4.4 and 9.6 and in hyper salt broth with 6.5% NaCl [57]. Enterococci are ubiquitous bacteria, commensal in mammals' gastrointestinal tract, oral cavity and vagina, but also present in food, soil, plants or water. Nevertheless, they also represent some of the most dangerous and warrying opportunistic pathogens usually concerning hospital-acquired infections of urinary tract, wounds, blood and lungs [54, 58]. The main problem arising in the past decades is the easiness and the rapidity of emerging multi-drug resistant strains: indeed, Enterococci are classified among the top three bacteria resistant to antibiotics [57]. In addition, they are able to rapidly acquire virulence factors involved in different stages of infection from invasion, colonization to macrophages resistance and escape from immunity [58].

1.3.2 *Enterococcus faecalis* in chronic ulcers

Among all the Enterococci infections, up to 80% are caused by *Enterococcus faecalis*; of the remaining 20% the great part is caused by *Enterococcus faecium*, and only a small percentage by other species [57, 58]. *E. faecalis* is quite common in chronic ulcers, for example, it represents the third species of bacteria found in non-healing infected wounds derived from surgery [59]. When *E. faecalis* infects skin ulcers, it rapidly produces different virulence factors which lead to a chronicization of the wound due to a prolonged inflammation and to a strongly impaired tissue integrity, thus becoming difficult to eradicate [60].

1.4. Management of chronic wounds

1.4.1 Epidemiology of chronic wounds: a social and economic burden

Wounds, both acute and even more chronic, deeply affect people's lives and careers and have a strong impact in economy and healthcare systems, thus being now called "the silent epidemic" [61]. Epidemiology of chronic ulcers is not easy to decipher since data from poor and developing countries are often not available and data from developed countries are usually collected only from hospitalized patients. Moreover, the lack of well-established criteria to select chronic wounds leads to a high heterogeneity of studies. For all of these reasons, chronic ulcers burden is probably underestimated [62, 63]. In 2018 a meta-analysis of Martinengo and colleagues revealed a prevalence of 2.21 people per 1000 population with ulcers of various aetiologies, and a prevalence of 1.51 per 1000 population for leg ulcers [62]. In a review of Lindholm and Searle published in 2016 it was estimated that 3 to 4 people per 1000 in UK and Denmark suffered from a wound, with around 15% of ulcers unable to heal within 1 year. From these data it was extrapolated that

every 1 million of people, 3500 suffer from wounds, and 525 from ulcers which do not heal within 1 year. Moreover, it was registered a total of 6.5 patients suffering from chronic wounds in USA [61]. In general, it is estimated that a percentage between 1% and 2% of the population will suffer from a chronic wound during life; this prediction is intended to increase, especially in Western countries, due to the aging of population and the subsequent increasing of some of the risk factors already listed in 1.1.2 section [64].

What is certain is the social and economic burden. People suffering from nonhealing wounds have a reduced HRQoL (Health-Related Quality of Life). They suffer from specifically ulcer-related problems such as pain, odour and continuous exudates, but also from general conditions as depression, anxiety or sleep disturbances. Moreover, their social life is completely impaired due to the common social isolation and to the financial restriction, often related to the impossibility to maintain a job [65].

About the costs, it has been estimated that chronic wounds management cover 1%-3% of the healthcare expenditure in developed countries. Nevertheless, this data seems to be underestimated since, for example, in the years 2012-2013 Wales expenditure for chronic wounds was 5.5% of the total of the National Health System. In the USA it is reported a total cost of US $25 billion per year [66].

1.4.2 Current therapies for chronic wounds

TIME guidelines are a clinical tool defined in 2002 by a group of experts in the field of ulcers' management, where they identified the four key components for an optimal treatment. The acronym TIME, indeed, stays for **T**issue, **I**nfection, **M**oisture and **E**dges [67]. The first aim of the treatment is to clean the wound from necrotic and non-viable tissue, thus the **T**issue debridement. Tissue debridement can be obtained by autolytic debridement, mainly used in acute wounds where the non-totally compromised situation

allows the exploit of some hosts' enzymes as MMPs by keeping the ulcer wet, surgical removal with scalpel or scissors under anaesthesia, exogenous enzymes as collagenases, biologic debridement using medical grade maggots or sterile larvae, use of mechanical forces as "flushing" or ultrasound, or the less used chemical debridement due to the possible toxicity of chemical compounds [68, 69]. The second step is the prevention or the control of the excessive **I**nfections. At this scope, disinfectants and topical or systemic antimicrobials are proposed, even if local antibiotics can easily lead to sensitisation, while systemic antibiotics barely reach wounds due to the low vascularization, and even lower is their ability to penetrate biofilms. New antiseptic strategies as irrigations, negative pressure therapy, photodynamic therapy or cold plasma therapy are continuously proposed, but the difficulty to find the right balance between the antiseptic effect and the tolerance of living tissues makes them a challenging option [70]. The third aspect is the maintenance of the **M**oisture balance with the use of dry or wet dressings depending on the single wound characteristics. Finally, **E**dges are the most representative markers of the therapy outcome, by evaluating their progression. An adequate vascularization and the control of underlying pathologic conditions such as metabolic disorders are needed to promote edges progression. To do this, more classical or more innovative tools are used, described below [68, 69]. Thus, the current therapies used to treat chronic wounds can be summarize as follow:

- dressings: among the most commonly used tools, they must be accurately selected based on patients' necessities. Dressings can be loaded with drugs or antimicrobials; they are used to maintain the moisture balance, to treat infections, and to decrease the risk of frictions with dresses, bed or wheelchair. Besides their accessibility, comfort and low cost, the main problems are the lack of evidences of

their high-quality and their extremely vast market, making it difficult to select the best one [71];

- sterile gauzes: representing the standard of care, they are usually dry-to-wet tools able to absorb fluids and clean debris. A prolonged treatment with sterile gauzes can lead to an excessive drying of the skin. On the other side, an incorrect position covering also healthy skin can induce skin maceration and wound enlargement [71];
- advanced dressings: this category includes recently developed tools as alginates, hydrocolloids, hydrogels or foams with the aim to maintain a moisturizing environment, to reduce exudates and increase comfort. Also bioengineered and bioactive dressing releasing growth factors, antimicrobials or nucleic acids are part of this category, as well as skin substitutes. Despite their apparent efficacy from the little use done until now, they have high costs and very specific indications, making them difficult to use except for some niche cases [68, 69, 71];
- negative-pressure wound therapy (NPWT): a continuous or intermittent sub-atmospheric pressure is applied to the wound using tubes connected to a reticulated open-cell foam dressing. Despite evidences of improvements due to debridement and infections reduction, it is extremely uncomfortable [70, 71];
- hyperbaric oxygen therapy: used with the aim to revert the hypoxic environment of the ulcer and to facilitate anaerobic bacteria eradication, it consists in one or two expositions a day for 60 or 90 minutes to an oxygen pressure from 1.5 to 3 atmospheres. It is a highly selective treatment since it cannot be used with pneumothorax, restrictive airway diseases or concomitant chemotherapy, and it can cause several adverse effects including barotraumas, myopia and neurological oxygen toxicity. Moreover, it has a low compliance [72];

- stem cells: a promising, but still little studied, approach is the use of multipotent mesenchymal stem cells, able to transform in different population promoting inflammation resolution, angiogenesis and remodelling phases [68];
- specific approaches depending on the wound type.

In summary, despite a modest number of treatments available, the management of chronic wounds still remains challenging due to the extremely personalized approaches, to the high cost and to the low compliance.

1.5. Nanotechnologies

1.5.1 Nanotechnologies as new tools

In the recent years, nanotechnologies have emerged as new essential tools to improve diagnosis and treatments in several fields. Nanoparticles allow to overcome some criticism especially related to drug delivery as cell-specific targets, drugs' instability or inability to cross some membranes [73]. Nanoparticles can be classified into three major classes based on their structure: lipid, inorganic and polymeric nanoparticles [73].

Different nanoparticles have been proposed as new tools for treatment of several wounds' type, including acute and chronic wounds, such as the new technologies for burns [74], the nanomaterials for diabetic wound ulcers [75], or the nano-drug delivery systems for general improving of wound healing [76].

1.5.2 Dextran-shelled Oxygen-Loaded Nanodroplets

Among the different nanotechnologies proposed in the last decade, in 2015 Prato and colleagues described an innovative topical delivery system for the

time-sustained release of molecular oxygen in hypoxic cutaneous tissues: the so-called dextran-shelled oxygen-loaded nanodroplets (OLNDs), shown in figure 3. OLNDs are PBS-suspended nanocarriers made of an inner core of 2H,3H-decafluoropentane, able to establish bonds with molecular oxygen, thus improving stability of formulations without compromising the ability to release it, and an outer shell of dextran, a polysaccharide commonly used for topical formulations due to its biocompatibility with the skin. Dextran can be functionalized with drugs or with fluorescent dyes as FITC [77]. The ability of OLNDs to improve wound healing and related hypoxia has been already demonstrated *in vitro* in two cellular populations involved in the healing process: microvascular endothelial cells and monocytes. The ability of these cells to migrate and to secrete MMP-2 and MMP-9 and their inhibitors TIMP-1 (Tissue Inhibitor of Metalloproteinases-1) and TIMP-2 (Tissue Inhibitor of Metalloproteinases-2), involved in ECM remodelling, were perturbated by hypoxia. The treatment with OLNDs, but not with their counterpart not delivering oxygen OFNDs (Oxygen-Free Nanodroplets), restored a normoxia-like phenotype, thus counteracting hypoxia and making OLNDs eligible and promising tools for chronic wounds treatment [78, 79].

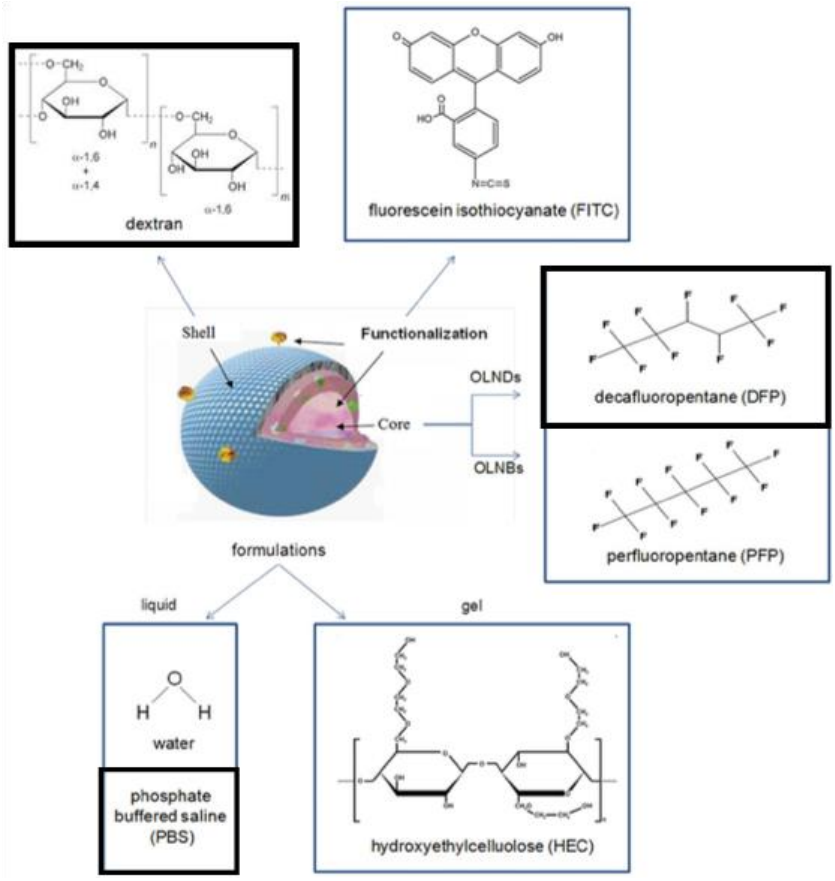

***Figure 3**. Dextran-shelled nanodroplets structure. Nanodroplets are nanocarriers made of an inner core of 2H,3H-decafluoropentane, an outer shell of dextran which can be functionalized with FITC, suspended in PBS. The components of the chosen formulation are highlighted by black squares. Modified version of Prato et al. [77]*

2. AIM OF THE STUDY

Chronic wounds, which represent an increasing emergency in Western countries, are constantly associated with low oxygen supply and persistent inflammation. Macrophages cover a pivotal role in all phases of wound healing by producing inflammatory and angiogenic mediators and controlling microbial infections. Oxygen loaded nanodroplets (OLNDs), carriers able to deliver oxygen to hypoxic tissues, has been proposed as possible innovative topical treatment able to counteract the effects of hypoxia on cells involved in wound healing such as endothelial cells, keratinocytes and monocytes. Since nothing was known about the possible role of nanodroplets on the activities of macrophages, the aim of this project was to investigate OLNDs effects on the two focal activities of macrophages in chronic wounds, i.e. bacterial killing and regulation of inflammation and angiogenesis.

In the first series of experiments the internalization of nanodroplets and their effect on viability of murine and human macrophages were investigated in normoxic and hypoxic conditions.

In the second part of the work we focused on bacterial killing, particularly by evaluating the effects of OLNDs on the production of reactive oxygen species (ROS) and nitric oxide (NO), the two main microbiocidal mechanisms directly dependent on oxygen levels, by murine and human macrophages in normoxia or hypoxia. Then, we set up an *in vitro* model of *Enterococcus faecalis* infection to verify if the effects of OLNDs on killing mechanisms were corroborated by a subsequent intracellular killing of ingested bacteria by macrophages.

In the third part of the work we investigated the ability of nanodroplets to modulate the inflammatory response of *E. faecalis* infected human macrophages in normoxia or hypoxia. The secretion of inflammatory cytokines and factors involved in angiogenesis was investigated.

3. MATERIALS AND METHODS

3.1 Nanodroplets formulations and characterization

Patented dextran-shelled Oxygen-Loaded NanoDroplets (OLNDs) and Oxygen-Free NanoDroplets (OFNDs) formulations were kindly provided by Dr. Adriano Troia from the Istituto Nazionale di Ricerca Metrologica, Turin, and Dr. Mauro Prato from the University of Turin.

Nanodroplets were obtained and characterized as described by Prato and colleagues in 2015 [77]. Briefly, 1.5 mL of decafluoropentane (DFP), 0.5 mL of a 2.5% solution of polyvinylpyrrolidone, 1.8 mL of a 1% w/v ethanol soy lecithin (Epikuron 200) and 0.3% w/v of palmitic acid were homogenized in 30 mL of Phosphate Buffer Saline (PBS) by an Ultra-Turrax SG215 homogenizer; only for OLNDs the solution was saturated with O_2. At the end, 1.5 mL of dextran conjugated or not with fluorescein isothiocyanate (FITC) was added drop by drop. As reported by Prato et al., nanodroplets suspensions in PBS were then characterized by transmitting electron microscopy and optical microscopy for their morphology, average diameters and shell thickness; dynamic light scattering was employed to determine size, particle size distribution and zeta potential; refractive index was determined by a polarizing microscope; viscosity and shell shear modulus were determined through Discovery HR1 Hybrid Rheometer; an oximeter was used to measure the oxygen content; stability was evaluated after up to 6 months [77]. Formulations were sterilized with UV-C exposure for 20 minutes.

3.2 Cell cultures

3.2.1 Macrophages cultures

THP-1, a human acute monocytic leukaemia cell line cultured for the first time from the blood of a boy in 1980 [80], were maintained in Roswell Park Memorial Institute Medium 1640 (RPMI 1640) supplemented with 2 mM L-Glutamine, 20 mM HEPES buffer pH 7.4, 50 μM β-mercaptoethanol, 10 μM sodium pyruvate and 10% of heat-inactivated Foetal Bovine Serum (FBS) (Euroclone). Differentiation into macrophages (dTHP-1) was obtained incubating cells with 10 ng/mL of Phorbol Myristate Acetate (PMA) (Sigma-Aldrich) for 72 hours at 37°C and 5% CO_2.

Wild type mouse C57Bl/6 immortalized Bone Marrow-Derived Macrophages (BMDM) were generated as described by Kalantari et al. [81] and kindly provided by Drs. Douglas Golenbock and Kate Fitzgerald, UMASS (MA, USA). BMDM were maintained in high glucose Dulbecco's Minimal Essential Medium (DMEM) (Euroclone) completed with 2 mM L-Glutamine, 20 mM HEPES buffer pH 7.4 and 10% of heat-inactivated FBS (Euroclone).

3.2.2 Bacterial cultures

The gram-positive *Enterococcus faecalis* (*E. faecalis*), purchased by American Type Culture Collection (ATCC 29212), was maintained on Mueller-Hinton (MH) agar (Liofilchem) petri dishes. The inoculum used to perform cell infections was prepared in cell culture media supplemented with 2.5% of FBS by transferring some Colonies Forming Units (CFUs) till a concentration of 10^8 bacteria/mL measured by reading turbidity with a BIORAD SmartSpec Plus spectrophotometer at a wavelength of 600 nm. To better quantify alive bacteria in the inoculum, titration with 1:10 serial dilutions in physiologic solution was performed on MH agar petri dishes, and CFUs

were counted after 24 hours of incubation at 37°C. In the meanwhile, the inoculum was maintained on ice at 4°C until the use for the experiments.

3.3 Incubation conditions

All the experiments were performed both in normoxia, with an atmosphere composition of 20% O_2, 5% CO_2 and 75% N_2, and hypoxia. Hypoxia was obtained in a modular incubation chamber (StemCells Technologies) insufflated with a microaerophilic gas mixture made of 1% O_2, 5% CO_2 and 94% N_2 for 4 minutes at a rate flow of 10 L/min.

3.4 Evaluation of nanodroplets toxicity

3.4.1 Viability assay on macrophages

Non-toxic doses of OLNDs and OFNDs on cells were established performing the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. THP-1 were seeded $5*10^4$ cells/well in 96-wells flat bottom tissue culture plates (Corning®) and differentiated for 72 hours with 10 ng/mL of PMA at 37°C and 5% CO_2. Treatments with 5%, 10% and 20% v/v of OLNDs and OFNDs in serum-free medium were tested for 24 hours both in normoxia and hypoxia. BMDM were seeded 10^5 cell/well in 96-wells flat bottom tissue culture plates (Corning®) for 24 hours of adhesion at 37°C and 5% CO_2. Treatments with 2.5%, 5% and 10% v/v of both NDs were performed in serum-free medium for 24 hours in normoxia and hypoxia. At the end of the incubation of both the cell lines, 10% v/v of a solution 5 mg/mL of MTT (Sigma-Aldrich) in PBS was added in normoxia at 37°C. After 3 hours of incubation, supernatants were discarded and 100 μL of a solution made of 40% N,N-dimethylformamide (Sigma-Aldrich) and 20% sodium dodecylsulfate (Sigma-Aldrich) in PBS were added to dissolve formazan crystals. After 24 hours at room temperature plates were read with a Synergy

4 plate reader spectrophotometer (BioTek®) at a wavelength of 550 nm, and a reference wavelength of 650 nm.

3.4.2 Evaluation of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration on *Enterococcus faecalis*

MIC and MBC were evaluated following the method described by Mazzaccaro et al., as follow [82].

One single colony of *E. faecalis* was grown in a T25 cell culture flask with 5 mL of MH broth (Liofilchem) with a gentle shaking for 24 hours at 37°C. Concentration of bacteria was measured reading turbidity with a BIORAD SmartSpec Plus spectrophotometer at a wavelength of 600 nm. Serial 1:2 dilutions in MH broth of OLNDs and OFNDs, from 50% to 0.7% v/v, were done in 96-wells round bottom plates (Euroclone). Vancomycin, used as positive control, was serially diluted 1:2 in MH broth from 32 μg/mL to 0.5 μg/mL. Suddenly, $5*10^4$ bacteria were added to each well. After 24 hours of incubation in normoxia and hypoxia plates were shaken to resuspend bacterial pellet and MIC, i.e. the lowest concentration with no bacterial growth, was determined reading turbidity in each well with a Synergy 4 plate reader spectrophotometer (BioTek®) at a wavelength of 600 nm. 100 μL of bacterial suspension were taken from the wells which did not present bacterial growth and plated in MH agar petri dishes. After 24 hours of incubation at 37°C the MBC was established as the lower concentration of NDs without any CFUs grown on MH agar.

3.5 OLNDs internalization by macrophages

THP-1 were seeded $5*10^4$ cells/well in 16-wells LabTek chamber slides (Nunc) and differentiated into macrophages with 10 ng/mL of PMA for 72 hours at 37°C and 5% CO_2. For BMDM seeding, 16-wells LabTek chamber

slides (Nunc) were pre-treated for 1 hour with 50 μL of 0.1 mg/mL of Poly-L-Lysine (Sigma-Aldrich) in PBS in order to facilitate cells adherence, then BMDM were seeded $2.5*10^4$ cells/well and incubated for 24 hours at 37°C and 5% CO_2. Both dTHP-1 and BMDM were treated with 10% v/v of FITC-labelled dextran OLNDs for 24 hours in normoxia and hypoxia, with a contemporaneous activation of BMDM with 25 U/mL of mIFNγ. At the end of incubation wells were washed with DPBS, and 100 μL of 10 μg/mL 4',6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich) were added for 2 hours for nuclei staining. Images acquisition was done with an exposition of 200 ms for nuclei staining and 800 ms for FITC-labelled OLNDs using a Nikon Eclipse Ti-S fluorescence microscope with 400x magnification. Nuclei were detected with an excitation wavelength of 330-380 nm, a dichromatic mirror of 400 nm and a barrier of 420 nm. OLNDs were detected with an excitation wavelength of 465-495 nm, a dichromatic mirror of 505 nm and a barrier of 515-555 nm.

3.6 Infection of macrophages and antibiotic protection assay

THP-1 were seeded $5*10^5$ cells/well in 24-wells tissue culture plates (Corning®) with 10 ng/mL of PMA to obtain differentiation during the 72 hours of incubation at 37°C and 5% CO_2. A modified version of the antibiotic protection assay [83] was performed as explained below. After bacterial titration as previously described (3.2.2), *E. faecalis* was added at a Multiplicity Of Infection (MOI) of 10. Plates were centrifuged to induce a contact between bacteria and macrophages; then, plates were incubated in a humified incubator at 37°C and 5% CO_2. After 2 hours of phagocytosis, wells were washed four times with DPBS to remove free bacteria, and 40 μg/mL of vancomycin (Sigma-Aldrich) was added for 2 hours at 37°C in order to kill non-phagocytized bacteria. Four DPBS washings were performed to clean environment from the death non-phagocytized bacteria, and cells were

treated with or without 10 ng/mL of human interferon-γ (hIFNγ) and 10% v/v OLNDs or OFNDs in serum-free medium for 24 hours in normoxia or hypoxia. During these 24 hours, 4 μg/mL vancomycin was added in order to avoid the growth of sporadic residual alive bacteria outside macrophages. At the end of the incubation supernatants were collected to quantify the production of inflammatory factors, while cells were used to evaluate killing.

Differently from dTHP-1, BMDM were seeded $6*10^6$ cells in each T25 tissue culture flask (Corning®) previously treated with 0.1 mg/mL Poly-L-Lysine (Sigma-Aldrich) in order to strengthen macrophages adherence. After infection with *E. faecalis* at a MOI of 10 and subsequent treatment with 40 μg/mL vancomycin, infected BMDM were scraped, counted with a 1:10 dilution in Trypan Blue with a Neubauer chamber, and plated $5*10^5$ cells/wells in 24-wells tissue culture plates (Corning®). Cells were activated by adding 25 U/mL of mIFNγ and treated as previously described with OLNDs or OFNDs and with vancomycin in serum-free medium for 24 hours, in normoxia and hypoxia. At the end of the incubation, cells were collected to evaluate killing.

3.7 Evaluation of hypoxia and nanodroplets effects on killing mechanisms

3.7.1 Measurement of Reactive Oxygen Species (ROS) production by macrophages

THP-1 were seeded $5*10^4$ cells/well in 96-wells black tissue culture plates (Corning®) with flat and clear bottom and differentiated with 10 ng/mL PMA for 72 hours at 37°C and 5% CO_2. At the end of the incubation, dTHP-1 were primed or not with 10 ng/mL of hIFNγ for 2 hours. BMDM were seeded $2*10^5$

cells/well in the same black plates pre-treated for 1 hour with 0.1 mg/mL Poly-L-Lysine (Sigma-Aldrich) to improve adherence. After 24 hours of incubation, BMDM were primed or not with 25 U/mL of mIFNγ for 2 hours at 37°C and 5% CO_2.

To detect ROS production, macrophages were incubated for 15 minutes at 37°C with 10 μM of the fluorescent 2'7'-dichlorofluorescein diacetate (H_2DCFDA) (Sigma-Aldrich) diluted in DPBS, a dye able to enter cells and to bind oxygen radicals. After DPBS washings, cells were treated with 2.5%, 5% and 10% v/v of OLNDs or OFNDs in Hanks' Balanced Salt Solution (HBSS) without red phenol, and 0.5 mM of hydrogen peroxide (H_2O_2) (Sigma- Aldrich) was used as positive control. Fluorescence was detected using the Synergy 4 plate reader (BioTek®) after 30, 60 and 120 minutes of incubation at 37°C in normoxia or hypoxia. Fluorescence was read with an excitation wavelength of 485/20 nm and an emission wavelength of 528/20 nm.

3.7.2 Measurement of nitric oxide (NO) as nitrites released in the supernatants

BMDM were seeded in 96-wells flat bottom tissue culture plates (Corning®) at 10^5 cells/wells and incubated for 24 hours at 37°C and 5% CO_2. Then, macrophages were activated with 25 U/mL of mIFNγ and simultaneously treated for 24 hours with 10% OLNDs or OFNDs both in normoxia and hypoxia. Treatment with 10 ng/mL of lipopolysaccharide (LPS) (Sigma-Aldrich) was used as positive control. At the end of the incubation supernatants were collected, and nitrites levels were evaluated following the widely used Griess method [84] as described below. 50 μL of supernatants or of a 7-point standard curve were added to the same volume of Griess

reagent in a 96-wells clear plate (Euroclone). Griess regent was a mixture 1:1 of the reagent A made of 1% w/v sulphanilamide (Sigma-Aldrich) in water, and the reagent B made of 0.1% w/v naphthylethylenediamine dihydrochloride (Sigma-Aldrich) and 2.5% w/v phosphoric acid (Sigma-Aldrich) in water. After 10 minutes of incubation in the dark, the developed colorimetric reaction was quantified by measuring absorbance with the Synergy 4 plate reader spectrophotometer (BioTek®) at a wavelength of 550 nm and 620 nm as the reference wavelength. Nitrites concentrations were calculated interpolating the optical density of the samples in a standard calibration curve made of seven serial 1:2 dilutions starting from the concentration of 100 μM of sodium nitrite ($NaNO_2$) in BMDM medium.

3.8 Evaluation of E. faecalis infection and killing by macrophages

At the end of the treatments described in 3.6 section, infected cells were collected. dTHP-1 were detached from plates incubating cells with 250 μL/well of EDTA-Trypsin (Euroclone) for 5 minutes at 37°C. Detachment reaction was stopped by adding 25 μL/well of heat-inactivated FBS, and cells were then washed with DPBS. BMDM were detached by scraping wells in DPBS.

Both macrophages were then counted in a Neubauer chamber with a dilution 1:10 in Trypan blue. $1.5*10^5$ cells for each condition were diluted in 150 μL of DPBS, and 100 μL of the suspension were cytocentrifuge using a Cytospin 3 (Shandon) to obtain a cells spot on a slide. Then, after cells fixation with methanol, slides were coloured with a 1:10 dilution of Giemsa stain (Sigma-Aldrich) in Weise's buffer (Sigma-Aldrich) for 15 minutes. Counts were performed with an optical microscope (Nikon Eclipse E200) with the oil immersion lens 1000x.

The percentage of infected macrophages was calculated as follow:

% infected macrophages = (n° of infected macrophages / total n° of macrophages counted) * 100

For each condition the percentage of infected macrophages was evaluated on a total of 300 cells counted from different fields.

Then, phagocytic index (PI) was calculated following Chen and colleagues method [85] with the formula:

PI = (n° of total bacteria / n° of infected macrophages) * (n° of infected macrophages / total n° of macrophages counted) *100

Which means:

PI = Average n° of bacteria per infected macrophages * percentage of infected macrophages

The percent of killing was calculated in control and treated cells after 24 hours with respect of PI after 2 hours of phagocytosis, when PI was taken as 100%:

% of killing = 100 – ((PI at 24h/PI at 2h of phagocytosis) * 100

To confirm data obtained by counts on Giemsa-stained slides, at the end of the assay described in 3.6 section, a CFU-based counting method was used. Macrophages were harvested, counted and $1.5*10^5$ cells for each condition were lysed with a solution of 1% Triton-X-100 (Sigma-Aldrich) in water in order to obtain internalized bacteria. Bacteria were then washed two times with DPBS to discharge residual Triton, and three 1:10 serial dilutions in physiological saline solution were plated on MH agar petri dishes. After 24 hours of incubation at 37°C the number of CFUs was counted. Results are expressed as Log_{10} CFUs.

3.9 Quantification of cytokines, chemokines and growth factors in the supernatants

At the end of the antibiotic protection assay described in the 3.6 section, sandwich Enzyme Linked ImmunoSorbent Assay (ELISA) was performed on supernatants collected from dTHP-1. The levels of pro-inflammatory Tumor Necrosis Factor alpha (TNFα), Interleukin 1 beta (IL-1β), Interleukin 6 (IL-6) and C-X-C Motif Chemokine Ligand 8 (CXCL8), and the levels of the Vascular Endothelial Growth Factor (VEGF) were measured using the DuoSet ELISA Kit (R&D Systems) following the manufacturer's instruction. Briefly, 96-wells flat bottom high binding plates (Greiner Bio-One) were coated overnight with the capture antibody. After blocking the unspecific binding sites with bovine serum albumin, 100 μL of samples or of a 7-points standard curve were left for 2 hours at room temperature. Then, the secondary detection antibody bound to biotin was added, followed by the addition of the streptavidin conjugated to horseradish peroxidase. To develop the colorimetric reaction 3,3',5,5'-Tetramethylbenzidine was added. At the end, reaction was stopped with 2N sulphuric acid. The plate was then read with the Synergy 4 plate reader spectrophotometer (BioTek®) at a wavelength of 450 nm and a reference wavelength of 540 nm. Concentrations were obtained by interpolating the optical densities of samples in the equation of a seven 1:2 serial dilutions standard curve.

3.10 Statistical analysis

All the experiments, unless differently stated, were performed at least three times. Data about cells viability and cytokines, chemokines and growth factors secretion in *E. faecalis* infected vs. uninfected cells in normoxia vs. hypoxia were analysed by two-way ANOVA with all vs. all comparisons, and followed by Sidak's test. Results obtained from ELISA on treated infected cells in normoxia and hypoxia were analysed by two-way ANOVA comparing treatments vs. control, followed by Sidak's test. Kinetics of ROS production were analysed by two-way AOVA comparing all the conditions to the control, performing Dunnett's post hoc test, while results about nitrites secretion were analysed by ordinary one-way ANOVA, comparing all the conditions among them and followed by Tukey's test. Data about killing were analysed by two-way ANOVA, with comparisons all vs. all, followed by Tukey's post hoc test. All the statistical analyses were performed using the software GraphPad Prism 9.

4. RESULTS

4.1 Characterization of nanodroplets

Before use, each batch of either OLNDs or OFNDs was characterized for size (average diameter), polydispersity index and zeta potential (Table 1).

***Table 1**. Characterization of OLNDs and OFNDs formulations.*

Formulation	Average diameter (nm)	Polydispersity index	Zeta potential (mV)
OLNDs	600 ± 90	0,13	-27 ± 1
OFNDs	350 ± 80	0,11	-27 ± 1

Average diameters and zeta potentials are presented as means ± standard deviations, polydispersity indexes as means of different batches tested.

As can be seen in Table 1, the different preparation of nanodroplets are quite homogenous as polydispersity index and Z potential, whereas they differ in size being OLNDs almost double in diameter than OFNDs. All sizes were in the nanometer range, with average diameters of 600 nm for OLNDs and 350 nm for OFNDs. The larger size of OLNDs can be related to the different solubility of oxygen in DFP. Indeed, the presence of oxygen in the core of the NDs can change the interfacial layer structure, modify the surface tension, and lead to different hydrophobicity [86, 87]. Despite this difference in the average diameter, the presence of only one peak in the size distribution graph already shown by Prato and colleagues confirms the homogeneity of both the samples tested [77].

4.2 Nanodroplets toxicity on macrophages and bacteria

The effect of OLNDs and OFNDs on dTHP-1 viability was assessed by MTT assay after 24 hours of treatment with 5%, 10% and 20% v/v of NDs in normoxia or hypoxia. As shown in figure 4 OFNDs and OLNDs were not toxic on dTHP-1 both in normoxia and hypoxia. Only the treatment with 20% v/v OFNDs in normoxia significantly reduced dTHP-1 viability to 65.99% ± 8.06% compared to control (figure 4). Compared to normoxia, hypoxia did not modify cells viability in any of the conditions tested.

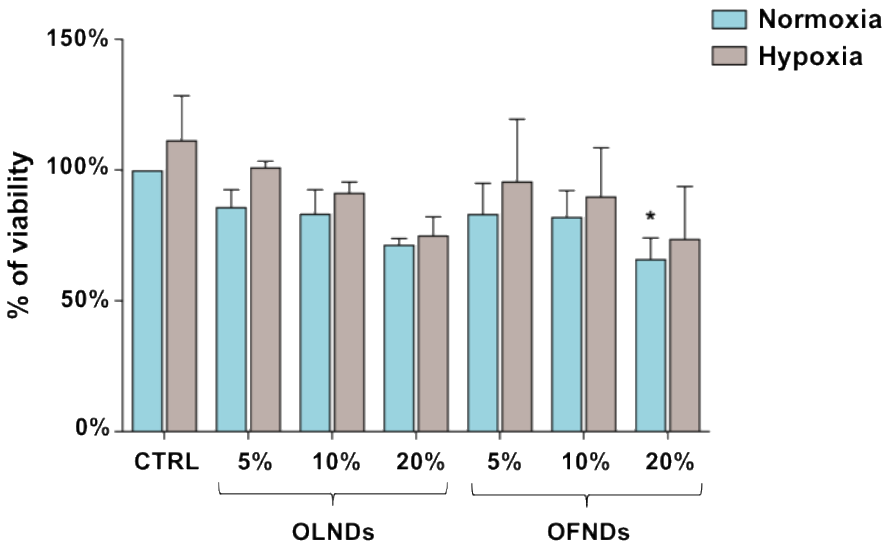

***Figure 4**. Effects of NDs and hypoxia on d-THP1 viability. Viability was evaluated by MTT assay after 24 hours of treatment in normoxia or hypoxia. Data are the means ± standard deviations of at least three independent experiments. Statistical analysis: two-way ANOVA, multiple comparisons all vs. all, followed by Sidak's test. *p<0.05*

Nanodroplets toxicity on BMDM was checked starting from the highest non-toxic concentration evaluated on human macrophages, i.e. 10% v/v. None of the concentrations tested (2.5%, 5%, 10% v/v) significantly reduced BMDM viability both in normoxia and hypoxia (figure 5).

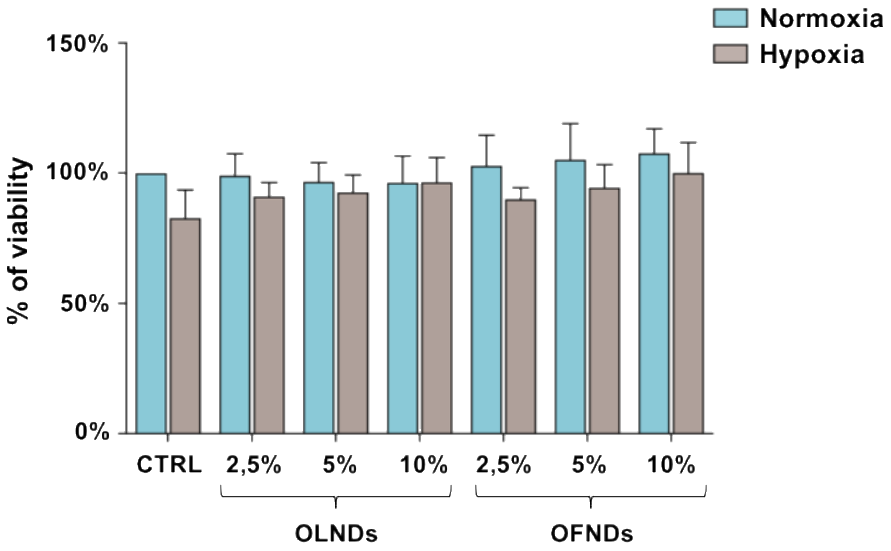

***Figure 5**. Effects of NDs and hypoxia on BMDM viability. Viability was evaluated by MTT assay after 24 hours of treatment in normoxia or hypoxia. Data are the means ± standard deviations of at least three independent experiments. Statistical analysis: two-way ANOVA, multiple comparisons all vs. all, followed by Sidak's test.*

The potential toxicity of OLNDs or OFNDs was evaluated also against *E. faecalis* measuring MIC and MBC. Bacteria were exposed to serial dilutions of NDs starting from the concentration of 50% v/v and vancomycin was used as control drug. MIC and MBC of NDs resulted higher than the highest concentration of NDs (50% v/v) tested, confirming an absence of a direct toxicity of the carriers on *E. faecalis* growth and viability at all the concentrations tested (table 2). On the contrary vancomycin MIC and MBC were 1 µg/mL and 32 µg/mL both in normoxia and hypoxia, confirming that

E. faecalis was sensitive to vancomycin and the sensitivity was not influenced by the oxygen concentration.

***Table 2**. MIC and MBC on E. faecalis after 24 hours of treatments with OLNDs or OFNDs in normoxia or hypoxia.*

	Normoxia		**Hypoxia**	
Treatments	**MIC**	**MBC**	**MIC**	**MBC**
OLNDs	NT	NT	NT	NT
OFNDs	NT	NT	NT	NT
Vancomycin	1 µg/mL	32 µg/mL	1 µg/mL	32 µg/mL

Vancomycin was used as positive control. NT = Not Toxic.

Since 10% v/v was the highest non-toxic concentration of NDs either in normoxia or hypoxia on human and murine macrophages, and it was not toxic against *E. faecalis*, it was chosen as the dose to perform all the subsequent experiments.

4.3 OLNDs internalization and localization in dTHP-1 and BMDM

A confirmation that OLNDs were internalized by macrophages was obtained using FITC-conjugated OLNDs and acquiring images by fluorescent microscopy after 24 hours of treatment with 10% v/v of in normoxia and hypoxia.

As shown in figures 6 and 7, where nuclei stained with DAPI appear in blue, the green FITC-conjugated OLNDs were localized in the cytoplasm both in normoxia (figures 6A – 6F and figures 7A – 7F) and hypoxia (figures 6G – 6N and figures 7G – 7N) in dTHP-1 (figure 6) and BMDM (figure 7).

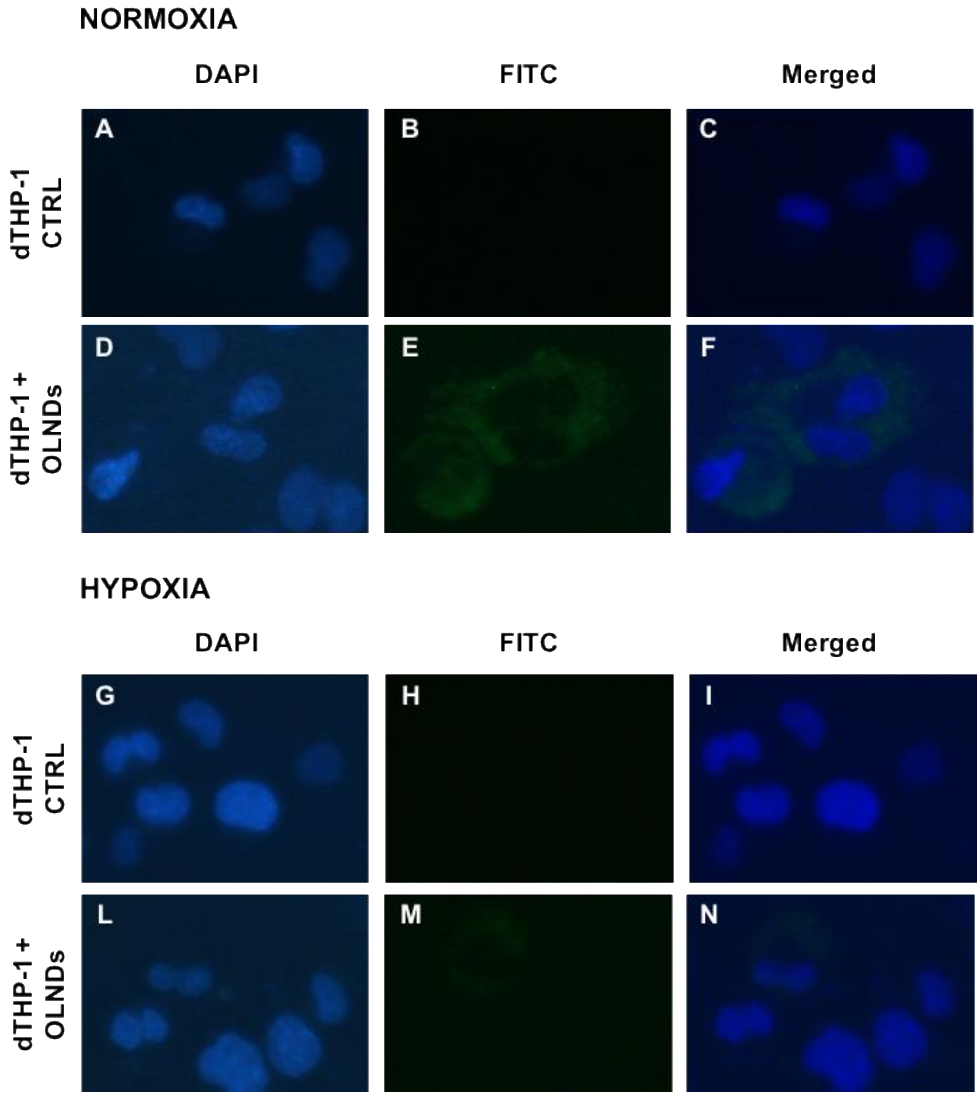

***Figure 6**. OLNDs internalization by human dTHP-1. Macrophages were treated with FITC-labelled OLNDs (panels D-F and L-N) or left untreated (panels A-C and G-I) for 24 hours in normoxia (panels A-F) or hypoxia (panels G-N). After nuclei staining with DAPI, images were acquired at 400x magnification with a fluorescent microscope. Left panels: nuclei stained with DAPI; central panels: FITC-labelled OLNDs; right panels: merged images.*

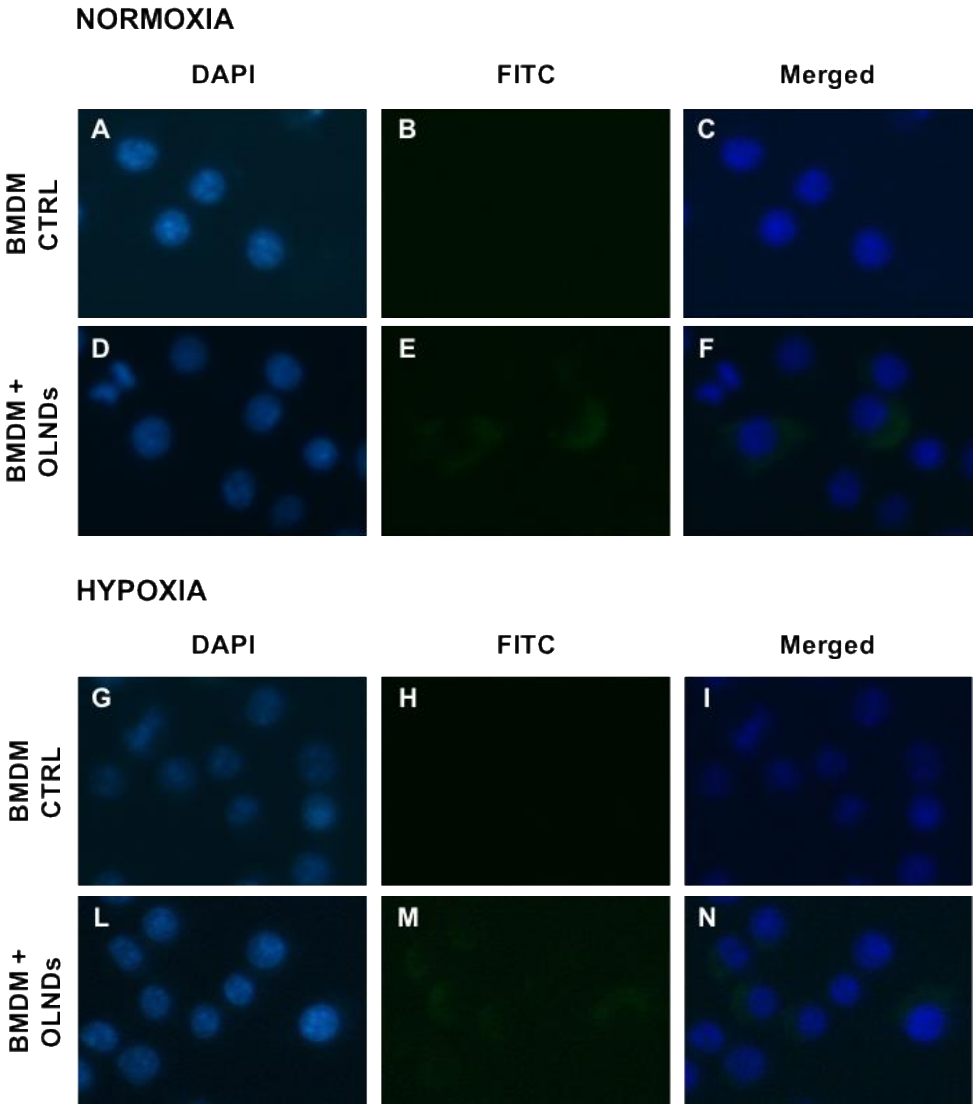

***Figure 7**. OLNDs internalization by murine BMDM. Macrophages were treated with FITC-labelled OLNDs (panels D-F and L-N) or left untreated (panels A-C and G-I) for 24 hours in normoxia (panels A-F) or hypoxia (panels G-N). After nuclei staining with DAPI, images were acquired at 400x magnification with a fluorescent microscope. Left panels: nuclei stained with DAPI; central panels: FITC-labelled OLNDs; right panels: merged images.*

4.4 Effects of hypoxia and nanodroplets on macrophages killing mechanisms

4.4.1 Regulation of reactive oxygen species production by hypoxia and nanodroplets

Since macrophage killing activity depends on the production of toxic radicals, in a first set of experiments, the kinetic of ROS production in the presence of OLNDs or OFNDs was evaluated after 30, 60 and 120 minutes of treatment in normoxia or hypoxia using the fluorescent probe H_2DCFDA. In BMDM, both OLNDs and OFNDs induced a strong increase of ROS production in normoxia, even at the lowest concentration tested (2.5% v/v) and rapidly after stimulation (30 minutes) (figures 8A and 8C). ROS levels were dependent on NDs concentrations at all time points tested (figures 8). In hypoxia, however, the effects of OLNDs became significant only after 60 minutes at the doses of 5% and 10% v/v and after 120 minutes at all the doses tested (figure 8B and 8D). OFNDs displayed the same effect of OLNDs both in normoxia and hypoxia (figures 8C and 8D). Hydrogen peroxide (H_2O_2), used as positive control, induced significant levels of ROS starting from 30 minutes in normoxia and 60 minutes in hypoxia.

In general, an overall slight decrease of ROS production was observed in hypoxia compared to the same conditions in normoxia (figure 8B and 8D).

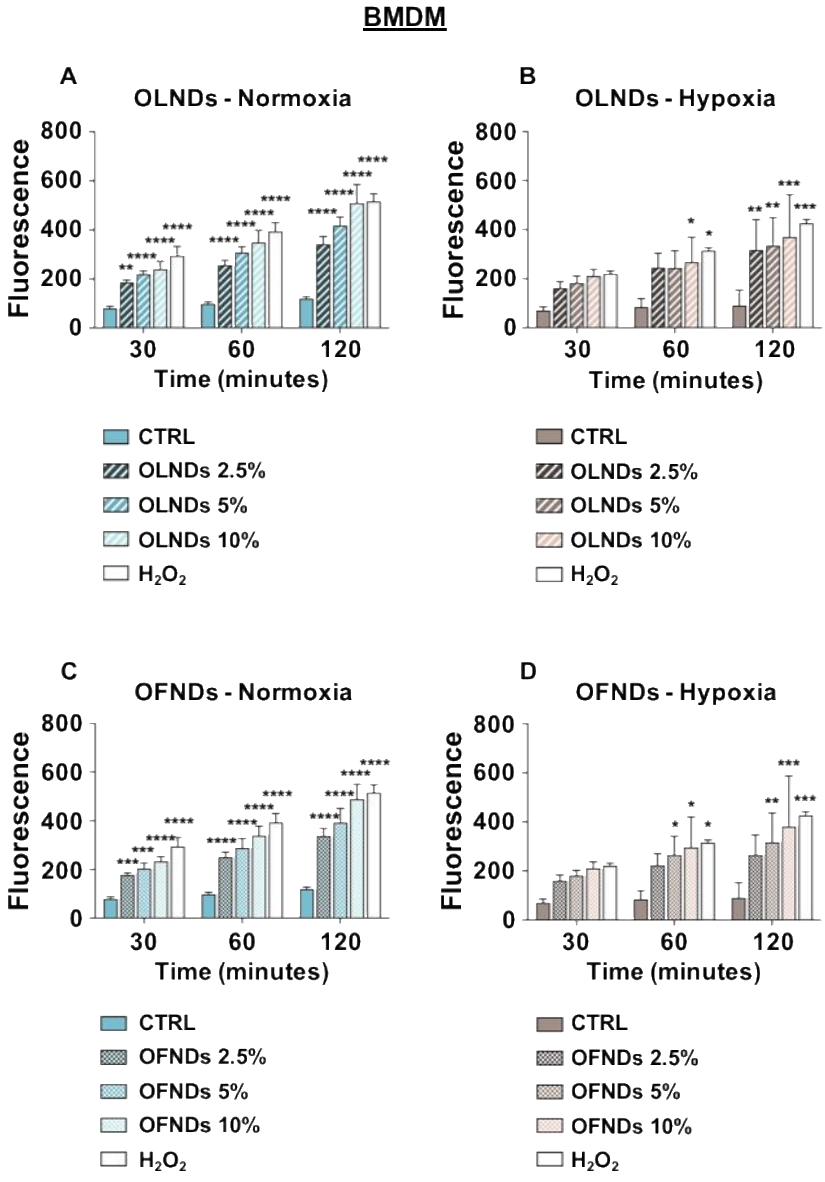

Figure 8. *Reactive Oxygen Species production detected using the fluorescent probe H_2DCFDA, by non-primed BMDM after 30, 60 and 120 minutes of treatment with 2.5%, 5% and 10% v/v of OLNDs (panels A and B) or OFNDs (panels C and D), in normoxia (panels A and C) or hypoxia (panels B and D). H_2O_2 was used as positive control. Data are the means ± standard deviations of at least three independent experiments. Statistical analysis: two-way ANOVA, comparisons of treated cells vs. control (untreated cells) at each time point, followed by Dunnett's test. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.*

The production of ROS by BMDM was evaluated also after macrophages priming with mIFNγ. ROS levels did not change compared to non-primed BMDM, except for a slight gain in hypoxia at 30 minutes of incubation where the increase of ROS induced by the highest concentration of NDs (10% v/v) or H_2O_2 became statistically significant (figures 9B and 9D). On the other side, in normoxia at 30 minutes only 10% v/v OLNDs and H_2O_2 maintained a statistically significant effect compared to untreated cells (figures 9A and 9C).

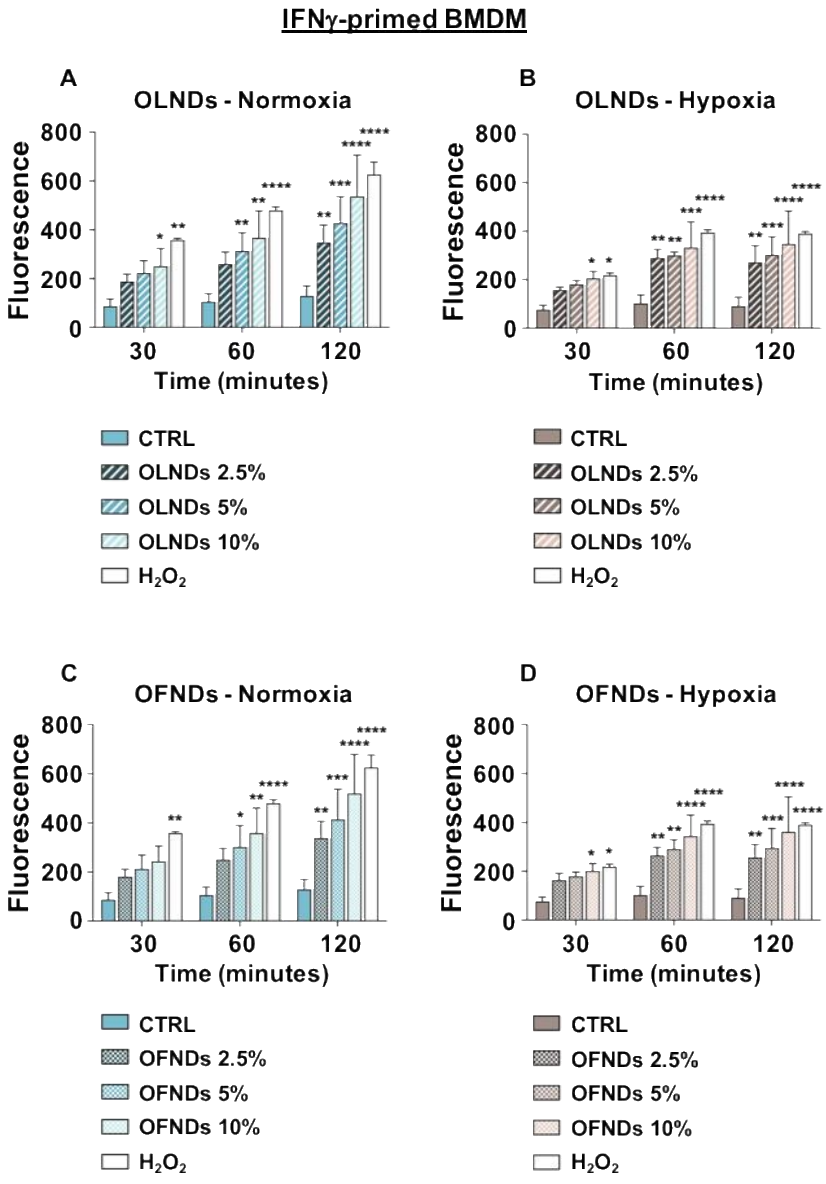

Figure 9. *Reactive Oxygen Species production detected using the fluorescent probe H_2DCFDA, by mIFNγ-primed BMDM after 30, 60 and 120 minutes of treatment with 2.5%, 5% and 10% v/v of OLNDs (panels A and B) or OFNDs (panels C and D), in normoxia (panels A and C) or hypoxia (panels B and D). H_2O_2 was used as positive control. Data are the means ± standard deviations of at least three independent experiments. Statistical analysis: two-way ANOVA, comparisons of treated cells vs. control (untreated cells) at each time point, followed by Dunnett's test. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.*

When human dTHP-1 cells were used, neither OLNDs nor OFNDs induced significant levels of ROS both in normoxia and hypoxia at all the time points tested, compared to untreated cells. This was at variance of what observed with H_2O_2 which significantly induced ROS production starting from 60 minutes after treatment (figure 10).

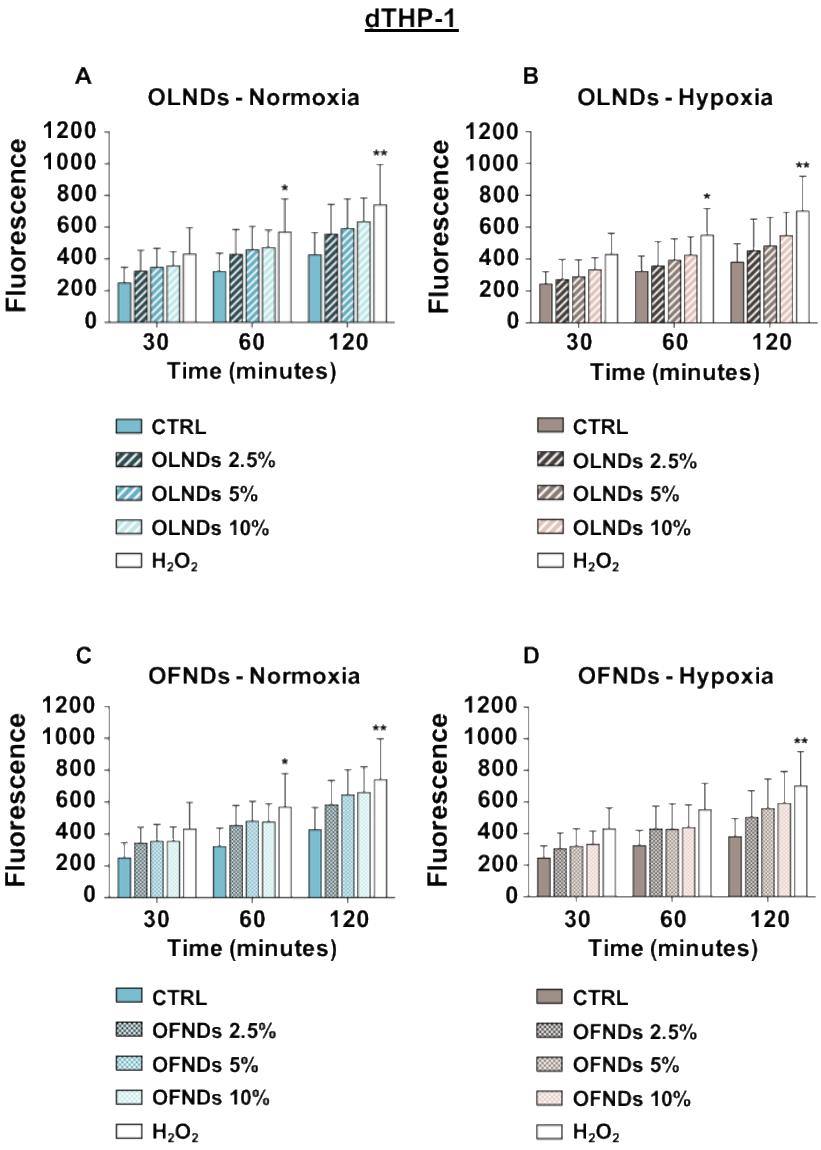

***Figure 10**. Reactive Oxygen Species production detected using the fluorescent probe H_2DCFDA, by non-primed dTHP-1 after 30, 60 and 120 minutes of treatment with 2.5%, 5% and 10% v/v of OLNDs (panels A and B) or OFNDs (panels C and D), in normoxia (panels A and C) or hypoxia (panels B and D). H_2O_2 was used as positive control. Data are the means ± standard deviations of at least three independent experiments. Statistical analysis: two-way ANOVA, comparisons of treated cells vs. control (untreated cells) at each time point, followed by Dunnett's test. *p<0.05; **p<0.01.*

Priming of dTHP-1 with hIFNγ did not induce any change in the production of ROS (figure 11). It has to be considered however, that baseline ROS production by dTHP1 cells appeared to be constantly higher than BMDM at all the time point tested, thus masking the stimulation induced by OLNDs or OFNDs.

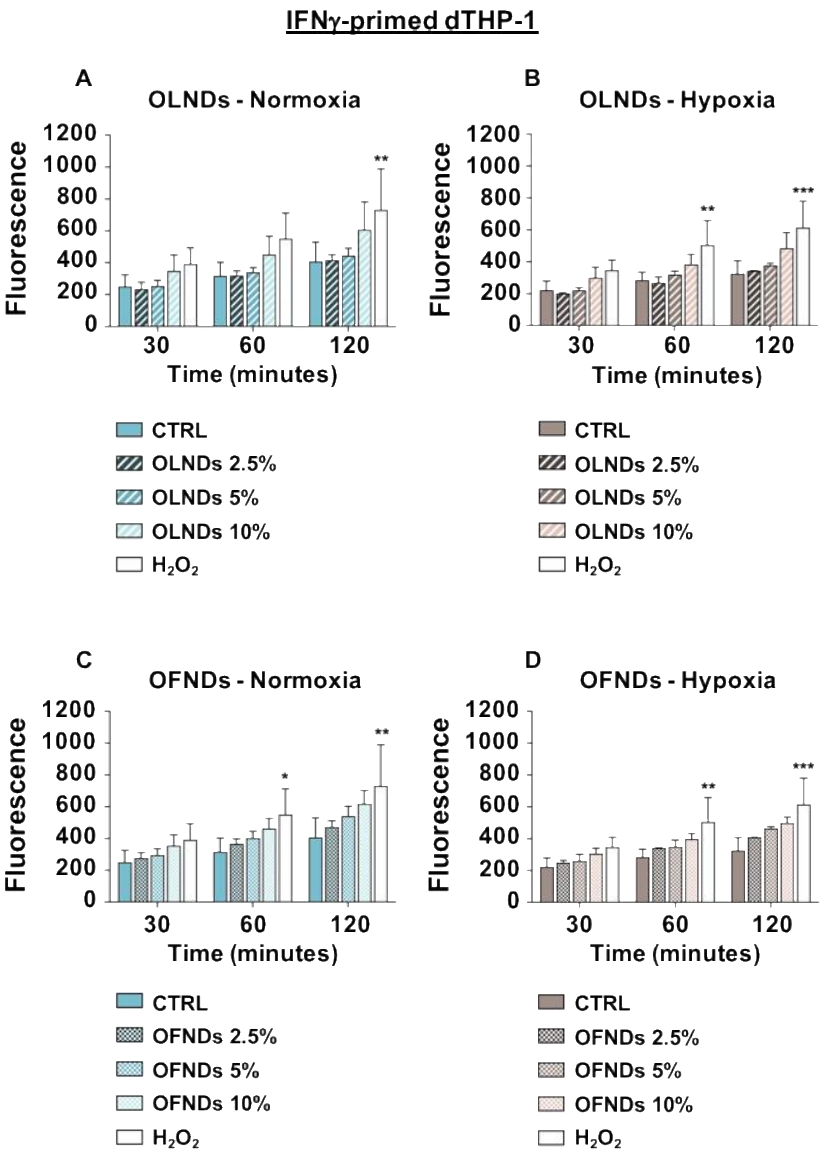

Figure 11. *Reactive Oxygen Species production detected using the fluorescent probe H_2DCFDA, by hIFNγ-primed dTHP-1 after 30, 60 and 120 minutes of treatment with 2.5%, 5% and 10% v/v of OLNDs (panels A and B) or OFNDs (panels C and D), in normoxia (panels A and C) or hypoxia (panels B and D). H_2O_2 was used as positive control. Data are the means ± standard deviations of at least three independent experiments. Statistical analysis: two-way ANOVA, comparisons of treated cells vs. control (untreated cells) at each time point, followed by Dunnett's test. *p<0.05; **p<0.01; ***p<0.001.*

4.4.2 Hypoxia and nanodroplets effects on nitric oxide secretion by murine macrophages

Together with ROS, the reactive nitrogen radicals, namely NO, may contribute to the toxic activity of macrophages, thus NO production was evaluated by Griess assay after 24 hours of treatment with nanodroplets in normoxia or hypoxia. NO production was observed only in the IFNγ-primed BMDMs, but not in the absence of IFNγ-priming (data not shown).

As shown in figure 12, in normoxia mIFNγ-primed BMDM produced significant NO levels compared to the control in the presence of OLNDs, but not, or much less, in the presence of OFNDs, at all the concentrations tested. These data confirm the release of oxygen by OLNDs since it has been shown that NO production is dependent on oxygen concentration. The positive control LPS strongly induced NO secretion (figure 12).

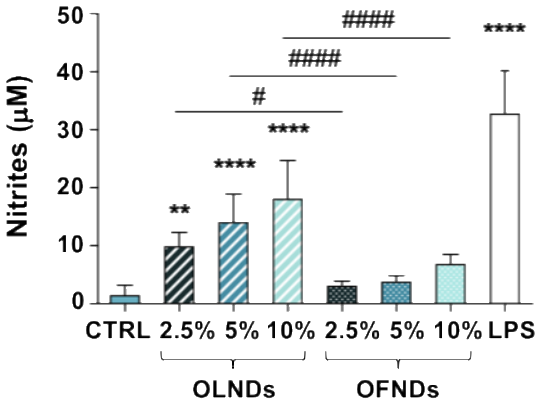

***Figure 12**. NO secretion by mIFNγ-primed BMDM, evaluated by Griess assay, after 24 hours of treatment with 2.5%, 5% and 10% v/v of OLNDs or OFNDs in normoxia. LPS was used as positive control. Data are the means ± standard deviations of at least three independent experiments. Statistical analysis: ordinary one-way ANOVA, comparisons all vs. all, followed by Tukey's test. Asterisks represent the comparisons vs. CTRL (untreated cells): **p<0.01; ****p<0.0001. Hashtags represent the comparisons between the same concentrations of OLNDs and OFNDs: #p<0.05; ####p<0.0001.*

In the hypoxic environment, the NO secretion was completely abolished with all the treatments, except for the very low concentration of 0.62 μM obtained in LPS-stimulated and mIFNγ-primed BMDM (data not shown).
In our model, dTHP-1 cells did not produce detectable levels of NO under any of the conditions tested. It is known, indeed, that even with IFNγ stimulation human macrophages do not produce detectable NO *in vitro* [34].

Since OLNDs induced ROS and NO, the main microbiocidal molecules produced by macrophages, the killing of *E. faecalis* by dTHP-1 and BMDM treated with NDs was evaluated.

4.5 Effects of hypoxia and nanodroplets on E. faecalis killing by macrophages

Killing of *E. faecalis* by IFNγ stimulated BMDM and dTHP-1 cells was evaluated after 24 hours in control cells and in cells treated with 10% v/v of OLNDs and OFNDs in normoxia or hypoxia by counting the percentage of infection and the number of intracellular bacteria in Giemsa-stained slides.
As shown in figure 13, after 24 hours of incubation the percentage of killing by control BMDM was near to zero both in normoxia and hypoxia. Differently, both OLNDs and OFNDs induced bacterial killing in normoxia (percentage of killing OLNDs: 44.2% ± 11.2%; OFNDs: 41.4% ± 16.9%) and hypoxia (percentage of killing OLNDs: 43.1% ± 18.4%; OFNDs: 37.7% ± 23.3%), with no differences between the two carriers (figure 13).
In dTHP-1 bacterial killing was not observed at any of the conditions tested. Indeed, the percentages of killing were all negative, thus indicating the proliferation of bacteria inside cells (figure 13).

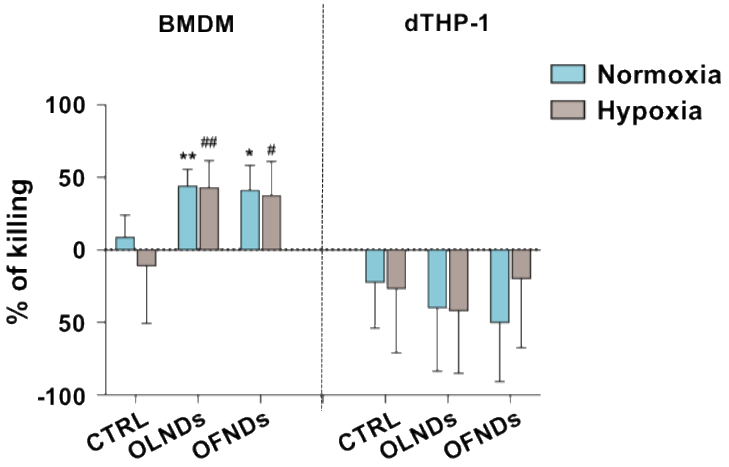

***Figure 13**. Killing of E. faecalis by mIFNγ-activated BMDM and dTHP-1 after 24 hours of incubation with 10% v/v OLNDs and OFNDs in normoxia or hypoxia. The percentage of killing was evaluated by counting the number of infected macrophages and the number of intracellular bacteria on Giemsa-stained slides. Data are the means ± standard deviations of at least three independent experiments. Statistical analysis: two-way ANOVA, comparisons all vs. all, followed by Tukey's test. Asterisks represent comparisons vs. CTRL in normoxia; *p<0.05; **p<0.01. Hashtags represent comparisons vs. CTRL in hypoxia; #p<0.05; ##p<0.01.*

The means ± St. Dev. of the percentage of infection and of the average number of bacteria inside each infected macrophage used to calculate the percentage of killing are listed in the Appendix A.

To further demonstrate the microbicidal activity of NDs on treated macrophages, a CFUs-based counting method for quantitating the number of intracellular live bacteria was performed by lysing macrophages and counting the CFUs after 2 hours of phagocytosis and 24 hours of treatment both in normoxia and hypoxia. As shown in table 3, in BMDM the Log_{10} CFUs in control cells after 2 hours of phagocytosis was 4.36, becoming 4.64 after 24 hours of incubation. The treatment with OLNDs and OFNDs decreased Log_{10} CFUs to 4.10 and 3.80, respectively in normoxia, and from 4.36 to 3.24

and 3.66 in hypoxia. As expected, in dTHP-1 cells none of the treatments increased bacterial killing (table 3).

***Table 3**. Log$_{10}$ CFUs of E. faecalis after treatment with OLNDs or OFNDs by IFNγ-activated BMDM and dTHP-1 in normoxia or hypoxia.*

	Log$_{10}$ CFUs			
Treatments	**BMDM**		**dTHP-1**	
	Normoxia	**Hypoxia**	**Normoxia**	**Hypoxia**
CTRL 2h	4.36	-	6.84	-
CTRL 24h	4.64	4.36	6.74	6.65
OLNDs 24h	4.10	3.24	6.82	6.76
OFNDs 24h	3.80	3.66	6.80	6.80

Data represent Log$_{10}$ CFUs of a representative experiment.

4.6 Modulation by NDs of the inflammatory environment induced by E. faecalis infected macrophages

In addition to bacterial killing, the microenvironment generated by infected macrophages treated with NDs was examined.

In a first series of experiments, the production of primary inflammatory cytokines, namely TNFα, IL-1β, IL-6 was evaluated. Subsequently, since OLNDs may play a peculiar role in the repair process, the production of pro-angiogenic factors CXCL8 and VEGF was also analysed.

The cytokines were measured by ELISA in the culture supernatants of *E. faecalis* infected dTHP-1 cells after 24 hours of treatment with 10% v/v of OLNDs or OFNDs, in both normoxia and hypoxia.

4.6.1 Effects of *E. faecalis* infection and hypoxia on pro-inflammatory cytokines

E. faecalis infection significantly increased the secretion of all the pro-inflammatory cytokines (IL-1β, IL-6, TNFα) both in normoxia and hypoxia compared to control (figure 14). On the contrary, hypoxia did not affect the levels of any of the cytokines tested either in uninfected or infected dTHP-1 (figure 14).

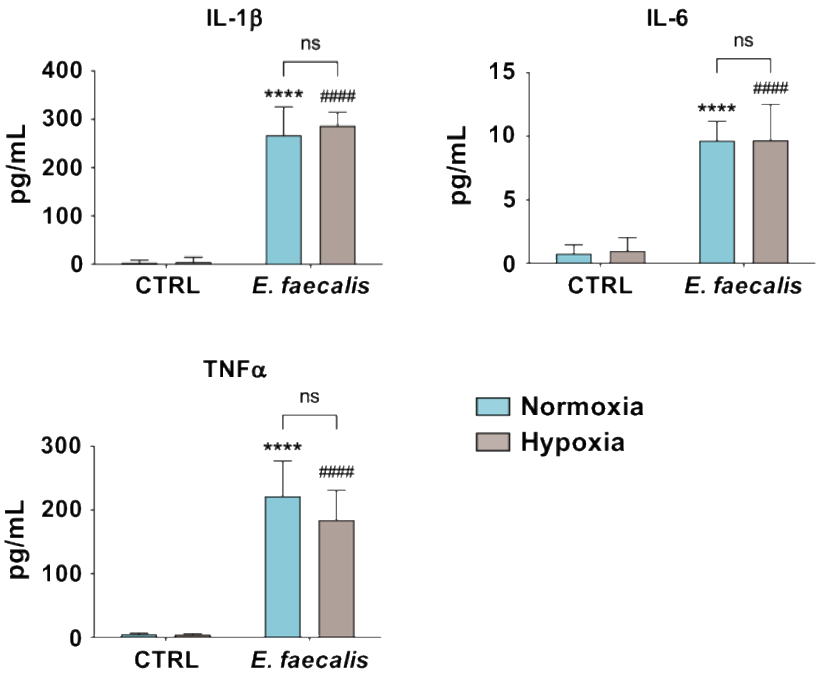

***Figure 14**. Effects of E. faecalis infection and hypoxia on the secretion of the pro-inflammatory cytokines IL-1β, IL-6 and TNFα by dTHP-1 were evaluated by ELISA after 24 hours of treatment in normoxia or hypoxia. Data are the means ± standard deviations of at least three independent experiments. Statistical analysis: two-way ANOVA, multiple comparisons all vs. all, followed by Sidak's test. Asterisks represent comparisons vs. CTRL in normoxia ****p<0.0001. Hashtags represent comparisons vs. CTRL in hypoxia ####p<0.0001. ns = non-significative.*

4.6.2 Effects of nanodroplets on pro-inflammatory cytokines secretion by *E. faecalis* infected dTHP-1

Both OLNDs and OFNDs significantly reduced IL-6 and TNFα secretion in normoxia and hypoxia, while IL-1β was significantly reduced only in hypoxia (figure 15). Since the effect was observed with both nanodroplets, these data suggested an anti-inflammatory property due to the dextran structure and not to the release of oxygen.

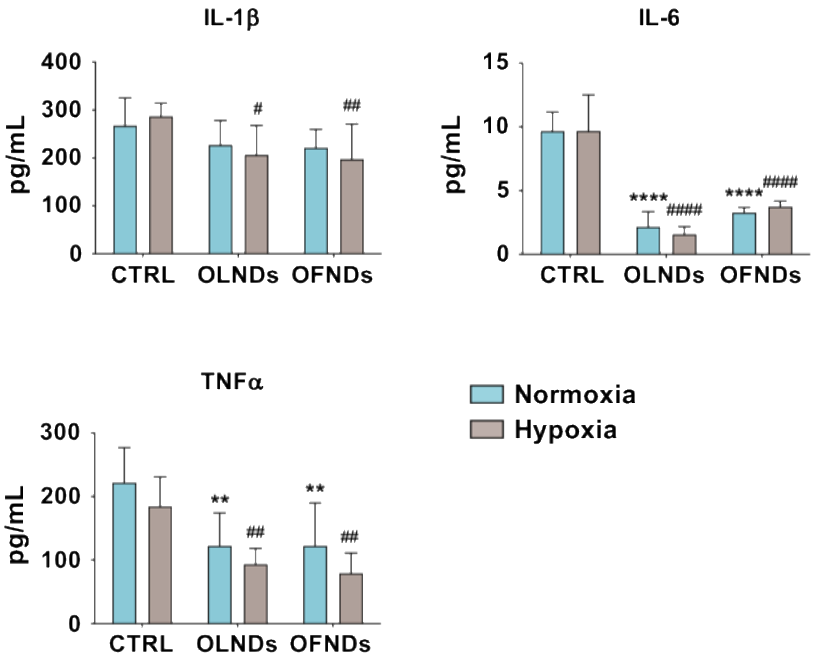

***Figure 15**. Effects of nanodroplets on the secretion of the pro-inflammatory cytokines IL-1β, IL-6 and TNFα by dTHP-1 were evaluated by ELISA after 24 hours of treatment in normoxia or hypoxia. Data are the means ± standard deviations of at least three independent experiments. Statistical analysis: two-way ANOVA, multiple comparisons treated vs. CTRL, followed by Sidak's test. Asterisks represent comparisons vs. CTRL in normoxia **p<0.01; ****p<0.0001. Hashtags represent comparisons vs. CTRL in hypoxia ##p<0.01; ####p<0.0001.*

4.6.3 Effects of *E. faecalis* infection and hypoxia on pro-angiogenic factors

Infection by *E. faecalis* significantly increased the secretion of CXCL8 both in normoxia and hypoxia compared to control, while hypoxia did not affect CXCL8 production. Differently VEGF, which is known to be regulated by oxygen levels [88], was significantly induced only with *E. faecalis* infection in hypoxic environment, probably as the result of the sum of the two stimuli (figure 16).

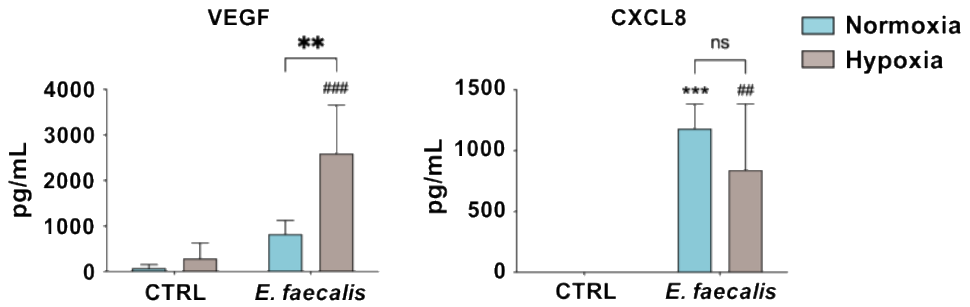

***Figure 16**. Effects of E. faecalis infection and hypoxia on the secretion of the pro-angiogenic factors VEGF and CXCL8 by dTHP-1 were evaluated by ELISA after 24 hours of treatment in normoxia or hypoxia. Data are the means ± standard deviations of at least three independent experiments. Statistical analysis: two-way ANOVA, multiple comparisons all vs. all, followed by Sidak's test. Asterisks represent comparisons vs. CTRL in normoxia; **p<0.01; ***p<0.001. Hashtags represent comparisons vs. CTRL in hypoxia ##p<0.01; ###p<0.001. ns = non-significative.*

4.6.4 Effects of nanodroplets on pro-angiogenic factors secretion by *E. faecalis* infected dTHP-1

The increase of VEGF in infected dTHP-1 by hypoxia was counteracted only by the treatment with OLNDs, able to bring the levels of VEGF back to the ones of control in normoxic environment, confirming the release of oxygen by OLNDs. Again, none of the conditions tested affected CXCL8 levels (figure 17).

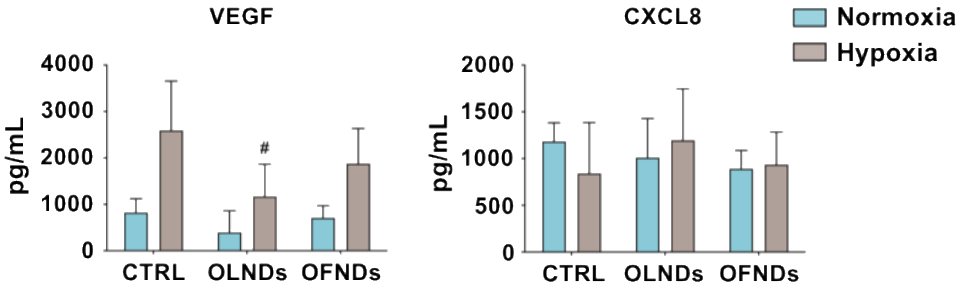

***Figure 17**. Effects of nanodroplets on the secretion of the pro-angiogenic factors VEGF and CXCL8 by dTHP-1 were evaluated by ELISA after 24 hours of treatment in normoxia or hypoxia. Data are the means ± standard deviations of at least three independent experiments. Statistical analysis: two-way ANOVA, multiple comparisons treated vs. CTRL, followed by Sidak's test. Hashtags represent comparisons vs. CTRL in hypoxia $^{\#}p<0.05$.*

5. DISCUSSION

Chronic ulcers are defined as “wounds that have not proceeded through an orderly and timely reparation to produce anatomic and functional integrity after 3 months” [18], indeed, they are characterized by a prolonged and excessive inflammation, a lack of oxygen and nutrients supply, a reduced cell proliferation and the overlap of bacterial infections [32, 89]. In non-healing wounds macrophages are not able to switch from a M1 pro-inflammatory phenotype to a M2 anti-inflammatory phenotype, thus sustaining and worsening inflammation [22]. Moreover, they are professional phagocytic cells involved in the defence of the host from bacteria, among which *Enterococcus faecalis*, a commensal Gram-positive, facultatively anaerobic and catalase-negative bacteria usually resident in mammals’ gastrointestinal tract, which is found to be one of the most common and antibiotic-resistant bacterial species detected in chronic wounds, especially in hospitalized patients [59, 90, 91].

A gold standard therapy for chronic wounds is still missing, thus making chronic wounds an increasing emergency for the healthcare system and a social and economic burden, especially in developed countries and in the elderly [66, 69]. In the last decade different nanotechnologies have been proposed in several fields, including wound therapy [76, 92] and delivery of oxygen to hypoxic tissues, as it happens in cancer [93, 94]. Among these, oxygen-loaded nanodroplets (OLNDs) with an outer structure made of dextran, a polysaccharide demonstrated to be highly biocompatible with cells and tissues [95, 96], were proposed in 2015 as innovative carriers delivering oxygen in a time-sustained manner, and able to revert the effects of hypoxia on endothelial cells and monocytes, two cellular populations involved in wound healing [77-79].

In this work we give additional proofs of the possible use of these topical OLNDs as innovative tools in the field of chronic wounds by proving their safety also on murine and human macrophages. Moreover, we provide evidences of their efficacy in improving killing of *E. faecalis* through the secretion of ROS and NO by *in vitro* murine macrophages. To conclude, we also demonstrate their anti-inflammatory effects on an *in vitro* model of human macrophages.

Since it is largely known that oxygen can be both beneficial or detrimental for cells and tissues depending on its levels ad doses [97], it has been important, at first, to define the non-toxic concentrations of OLNDs against cells used in this study. OLNDs and OFNDs were not toxic up to concentration of 10% v/v, both in a normoxic and in a hypoxic environment. Too high levels of oxygen, indeed, can induce an excessive oxidative stress by increasing oxygen radicals, nitrogen radicals and oxidizing agents, which can bind to cells structures as lipids, protein and DNA causing damages [98-100]. Moreover, the subsequent infection of macrophages with *E. faecalis* and the evaluation of the effects of nanodroplets on its killing, made it indispensable to test the toxicity of nanodroplets also on the bacterial strain. *E. faecalis* is a facultative anaerobic bacterium able to adapt to a broad range of conditions and to different oxygen levels [90, 101]. We proved the absence of a direct effect of OLNDs or OFNDs on *E. faecalis* viability and growth in normoxia and hypoxia. Localization of nanodroplets in both dTHP-1 and BMDM cytosol was observed by fluorescent microscopy, confirming the nanodroplets intake already proved by Basilico and colleagues in a human endothelial cell line, and by Gulino and collaborators in primary monocytes [78, 79].

Considered the cytoplasmic localisation of OLNDs, we investigated whether their oxygen release inside cells could improve two of the main microbiocidal mechanisms directly dependent from oxygen levels, i.e. the production of reactive oxygen species as OH^-, H_2O_2, O_2^- from the activation of NOX2, and

the production of nitrogen radicals as $ONOO^-$ and NO both from NOX2 and NOS2 activation [41, 43, 102]. In BMDM both OLNDs and OFNDs induced ROS production starting from 30 minutes after treatment, thus immediately inducing the respiratory burst. Due to the dependence of oxidative stress from the availability of oxygen, hypoxia can modulate ROS production by increasing it as demonstrated by Kim and colleagues on THP-1 after a long-term hypoxia [103], or decreasing oxidative stress response as shown by Sgarbi et al. on fibroblasts after a short-term hypoxia [104, 105]. In our *in vitro* model of murine macrophages, hypoxia slightly reduced ROS levels, but not in a significant way, confirming again that the effect can be dependent on the conditions of incubation, the concomitant treatments, and the targets of HIF (Hypoxia-Inducible Factor) transcriptional factor [106]. If both nanodroplets were able to strengthen the production of oxygen radicals in the murine model, in the human dTHP-1 they exerted just a slight non-significant increase, thus suggesting a lower sensitivity to the treatment. It is also interesting to notice that the basal levels of ROS were much higher in dTHP-1 compared to BMDM, thus probably reducing the affection by nanodroplets. The resulting different response of murine and human models to the same treatment can be justified considering that murine and human immune systems and metabolism do not always overlap in their responses [107, 108]. Differential gene expression depending from TLR4 (Toll-Like Receptor 4) pathway, usually activated by LPS, has been evidenced between primary human and mouse macrophages [109], as well as a different regulation of gene expression after polarization with M1 or M2 stimuli of murine BMDM and dTHP-1 [110]. Murine and human macrophages also differ both in terms of reactive oxygen species production after the treatment with the same oxidative agent [111], and in terms of antioxidant defence against radicals as glutathione (GSH) levels. Indeed, murine tumour cells are reported to have lower levels of GSH compared to human tumour

cells [112]. Taken together, it is not surprising that in our models human and mouse macrophages resulted in a different activation by NDs, explaining the reduced ROS production of dTHP-1 compared to murine BMDM.

Although IFNγ is an inducer of oxidative stress response in several cell types [113, 114] and an *in vitro* stimulation for the polarization of macrophages versus a pro-inflammatory M1 phenotype, thus enforcing the response against microorganisms [115], production of ROS in our models did not substantially differ with IFNγ priming both on murine and human macrophages. The missed ROS induction by IFNγ in our models could be explained with the low dose of IFNγ used, chosen because able to activate the NO pathway, and with the absence of a co-stimuli. Indeed, literature data report that a low concentration of IFNγ of 10 ng/mL is enough to induce ROS in a different *in vitro* model of mouse macrophages, the RAW 264.7 cells [116]. Differently, a higher concentration of IFNγ of 200 U/mL [117], the concomitant treatment with another pro-inflammatory stimuli as LPS [118] or a higher concentration of sugars in the culture medium [115] are necessary to determine an increase of ROS production by BMDM. The ability of IFNγ to induce ROS production in the presence of a co-stimuli as LPS is also assessed on dTHP-1, as described both by Lewis et al. and Koo and colleagues [119, 120]. Moreover, it has been described that low doses of IFNγ have anti-inflammatory effects in an *in vivo* asthma model and suppress T cells trafficking *in vitro* and *in vivo*, further supporting the different effect of IFNγ based on the dose and the model [121].

The second killing mechanism taken into consideration was NO production. Furtherly confirming the different behaviours of murine and human models, NO production was observed just on IFNγ-activated BMDM since the stimulation with IFNγ, which is essential to activate the inducible nitric synthase [122], is not sufficient in human cells to obtain detectable levels of

NO [34, 123]. In normoxia NO was induced in a dose-response manner only by OLNDs, and not by OFNDs, thus confirming both the dependence of NO release from environmental oxygen levels [124] and the ability of OLNDs to improve killing mechanisms. On the other side, NO secretion was completely abrogated in hypoxia with all the treatments, suggesting that the oxygen released by OLNDs inside cells is not sufficient to counteract the effects of hypoxia. The depletion of NO in hypoxia was somehow expected. Indeed, Daniliuc and colleagues demonstrated how hypoxia causes a different intracellular localization of the enzyme iNOS with a missed interaction with the cytoskeletal α-actinin 4 in the murine RAW 264.7 macrophages cell line, compared to normoxia. This displacement of the enzyme in an environment with a low oxygen tension was hypothesized to be cause of the absence of the production of NO in hypoxia [125].

Proved the ability of OLNDs to increase the production of microbiocidal molecules without affecting macrophages' viability, we evaluated if this improvement was corroborated by a consequential increase in *E. faecalis* killing. As mentioned above, INFγ priming is essential to promote NO production by macrophages [122] and to induce the M1 phenotype more prone to kill microorganisms [115], therefore *E. faecalis* killing was investigated by INFγ-activated macrophages. As expected from the data collected on microbiocidal mechanisms, *E. faecalis* killing mediated by murine macrophages was improved both by OLNDs and OFNDs in normoxia and hypoxia. To the induction of NO exclusively by OLNDs in normoxia did not follow a major improvement of killing in the same conditions, probably suggesting a prevalence of ROS-mediated killing to the detriment of NO-mediated microbiocidal mechanism. On the contrary, *E. faecalis* internalized by human dTHP-1 was able to survive, and no nanodroplets-mediated effect was shown, confirming the failure of NDs to induce ROS and NO by this cell type. The ability of *E. faecalis* to survive inside phagocytic cells was already

reported by Zou and colleagues infecting RAW 264.7 murine macrophages with different *E. faecalis* strains [126], by Baldassarri and collaborators in peritoneal rat macrophages [127, 128] and by Sabatino et al., who observed not only the survival of three different *E. faecalis* strains isolated from environment, but also their ability to replicate inside human blood-derived macrophages [129]. This behaviour can be explained by the ability of *E. faecalis* to protect itself from oxidative stress as reported by Szemes and colleagues [55].

In addition to bacterial killing and related mechanisms, the microenvironment generated by infected macrophages in the presence of NDs was examined measuring the secretion of inflammatory cytokines and mediators involved in the angiogenic process by *E. faecalis* infected dTHP-1. IL-1β, IL-6 and TNFα are the three primary pro-inflammatory cytokines known to be deeply involved in the inflammation phase of chronic wounds [130]. In our model, the infection of dTHP-1 with *E. faecalis* induced the secretion of the three inflammatory mediators with no differences between normoxic and hypoxic cytokines' levels. This data is in accordance with literature since it is well-known that infection of macrophages with different microorganisms activate the inflammatory response, thus leading to an increase of the primary pro-inflammatory cytokines [131, 132]. As already mentioned about the role of hypoxia on the modulation of the microbiocidal ROS production, also inflammatory cytokines seem to be regulated by low oxygen tension in different manners depending on the model chosen and the experimental conditions [133]. Supporting this evidence, it has been demonstrated both an induction of inflammatory mediators by hypoxia, as summarised in Riboldi et al. review [134], but also a decrease of TNFα and IL-1β, as shown by Ke and colleagues on LPS and IFNγ-activated THP-1 [135]. Treatment with nanodroplets significantly reduced the secretion of IL-6 and TNFα both in normoxia and hypoxia, and the levels of IL-1β only in hypoxia. The difference

in the regulation of inflammatory cytokines' secretion by nanodroplets can be explained with the different pathways involved in their production. IL-1β, indeed, is the only one of the three cytokines to be dependent on the inflammasome activation [136]. This hypothesis has been already supported in literature, for example by Hirano and colleagues who evidenced a different modulation of IL-1β and IL-6 in murine macrophages [137]. The reduction of IL-1β, IL-6 and TNFα by OLNDs and OFNDs suggests a potential anti-inflammatory effect of nanodroplets but, since no differences were evidenced between the carriers with and without oxygen, the anti-inflammatory action is not oxygen-dependent, but it is probably due to the structure itself of the nanoparticles. To our knowledge, no literature data are available about a possible anti-inflammatory effect of the dextran polysaccharide. On the contrary, dextran salts are well established inducers of a murine model of colitis [138]. Thus, we hypothesized that this peculiar formulation of dextran with the other nanoparticles' components can induce an anti-inflammatory effect of which mechanisms still remain unclear.

Besides being the main actors of the inflammatory response, macrophages also play a pivotal role in regulating angiogenesis by secreting growth factors as VEGF or chemokines as CXCL8 [9, 139]. VEGF regulation is strongly dependent on oxygen levels, indeed, its secretion is demonstrated to be increased by hypoxia [9, 88], as confirmed also in our *in vitro* infected dTHP-1. Furtherly confirming the dependence of VEGF secretion from oxygen levels, we demonstrated that treating hypoxic infected dTHP-1 with OLNDs, but not with OFNDs, reduced VEGF to concentrations similar to the normoxic ones, thus suggesting a restoration of a normoxia-like behaviour in macrophages. *E. faecalis* infection of dTHP-1 induced CXCL8 secretion in normoxia and hypoxia, confirming the increased levels of this chemokines already reported to be a consequence of bacterial infection in macrophages

[131]. Neither hypoxia nor the treatments with nanodroplets affected CXCL8 secretion in any manner.

6. CONCLUSIONS

Chronic wounds represent an increasing emergency in Western countries due to the lack of effectiveness, low-cost and comfortable treatments. In the field of nanotechnologies, topical suspension of OLNDs has been proposed as innovative tools able to deliver oxygen in hypoxic tissues and to counteract hypoxia effects on the regulation of ECM deposition and angiogenesis, in endothelial cells and circulating monocytes [77-79]. Starting from these promising data, our idea was to further investigate the contribution of OLNDs as a possible device for chronic ulcers by studying their effects on macrophages, the protagonists of the inflammatory response, and *E. faecalis* infection, both in a normoxic and in a hypoxic environment.

In the first part of our work we demonstrated the ability of the nanodroplets to increase the secretion of the microbiocidal radical species by murine macrophages without affecting cells viability, thus promoting *Enterococcus faecalis* killing both in normoxia and hypoxia. Since bacterial infections represent a tricky hurdle of chronic wounds, an improvement of the infection eradication not directly dependent on the use of the antibiotics, which are not always suitable due to the bacterial resistance, could represent a great advantage.

In the second part of the project, our aim was to verify the modulation of the inflammatory status by OLNDs. Our data suggested an anti-inflammatory activity of both the nanodroplets by reducing the inflammatory cytokines IL-1β, IL-6 and TNFα, and a capability exclusive of OLNDs to promote a restoration of a normoxic-like phenotype under hypoxia. Since prolonged and excessive inflammation is a central and critical point of chronic ulcers, the promotion of a more anti-inflammatory M2 phenotype could be beneficial.

Despite the low evidences of an effective advantage of OLNDs versus OFNDs, these data allow us to encourage once again a more deeply

investigation on these interesting and innovative oxygen-carriers since they represent a double-edged sword by simultaneously improving bacterial killing and promoting a reduction of the inflammatory response by macrophages.

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8. APPENDIX A

Table 4 reports the percentages of infected macrophages counted on 300 macrophages on Giemsa-stained slides as:
((n° of infected macrophages / total n° of macrophages) * 100).

Table 4. *Percentage of infected macrophages.*

	BMDM		**dTHP-1**	
	Normoxia	**Hypoxia**	**Normoxia**	**Hypoxia**
CTRL 2h	35.3 ± 18.7		40.7 ± 9.3	
CTRL 24h	34.6 ± 28.3	39.4 ± 21.6	45.0 ± 4.7	44.5 ± 6.0
OLNDs	26.2 ± 13.6	25.0 ± 14.0	43.7 ± 7.5	45.7 ± 3.3
OFNDs	26.3 ± 15.1	27.4 ± 17.2	45.7 ± 7.1	41.0 ± 2.4

Data are the means ± standard deviations of at least three independent experiments.

Table 5 reports the average number of bacteria inside each infected macrophage, counted on Giemsa-stained slides.

Table 5. *Average number of bacteria inside each infected macrophage.*

	BMDM		**dTHP-1**	
	Normoxia	**Hypoxia**	**Normoxia**	**Hypoxia**
CTRL 2h	5.8 ± 1.8		5.2 ± 0.5	
CTRL 24h	5.4 ± 0.9	5.8 ± 0.7	5.7 ± 1.6	5.9 ± 1.6
OLNDs	4.4 ± 0.6	4.7 ± 0.6	6.7 ± 1.8	6.4 ± 1.1
OFNDs	4.6 ± 1.1	4.9 ± 1.0	6.9 ± 1.9	5.9 ± 1.4

Data are means ± standard deviations of at least three independent experiments.

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