Abstract

**Background:** Diabetic foot ulcers (DFU) are characterised by high levels of inflammatory mediators, resulting from sustained hyperglycaemic insult and the local microbial biofilm. The intralesional administration of epidermal growth factor (EGF) has emerged as an effective treatment that stimulates granulation and closure of DFU, reducing the risk of amputation. Within the wound, fibroblasts play key roles during the healing process, promoting granulation and contraction. The aim of the present study was to examine the anti-inflammatory effect of EGF in DFU-derived fibroblasts, challenged with lipopolysaccharide (LPS), under hyperglycaemic conditions, recreating in vitro what happens in a clinical scenario.

**Methods:** Healthy skin (HS) and DFU granulation tissue biopsies were used to isolate primary fibroblasts. The effect of LPS on cell proliferation was analysed. Transcriptional expression of toll-like receptor (TLR) pathway mediators (TLR4, TLR2, CD14, MYD88 and NFKB) and pro-inflammatory cytokines (TNF, IL-6 and IL-1B) were measured by semi-quantitative polymerase chain reaction (qPCR), in cells treated with appropriate concentrations of LPS, EGF and their combination. IL-6 protein concentration was quantified by ELISA.

**Results:** LPS stimulated proliferation of HS-derived fibroblasts, while inhibiting the proliferation of cells derived from DFU at the highest assayed concentration of 1 µg/mL. Regarding the TLR signalling pathway, LPS increased messenger RNA levels of mediators and pro-inflammatory genes, while EGF, alone or in the presence of LPS, downregulated them, except for IL-1B.

**Conclusion:** The results suggest that EGF might elicit an anti-inflammatory response in LPS-challenged fibroblasts, even in a hyperglycaemic milieu. Collectively, our findings contribute to explain newly observed effects of EGF in the clinical arena.

**Keywords**
Toll-like receptor, fibroblasts, diabetic foot ulcer, innate immunity, pro-inflammatory cytokines, lipopolysaccharide, Evidence Level 5: Evidence based on bench research.
Lay Summary

In this research article, we analyse the putative anti-inflammatory effect of epidermal growth factor (EGF) on fibroblast isolated from diabetic foot ulcer (DFU) granulation tissue. To induce the inflammatory response, the cells were treated with lipopolysaccharide (LPS), simulating the gram-negative bacterial infection that takes place in the wounds of diabetic patients. We studied the expression of genes involved in bacterial recognition receptors signalling pathway and those that code for different pro-inflammatory cytokines.

We obtained primary fibroblasts from biopsies of a neuropathic diabetic ulcer and from healthy skin, the former was used as the control. Cells were isolated and grown in high glucose Dulbecco’s Modified Eagle Medium (DMEM) culture medium, to simulate the hyperglycaemic insult. The effect of increasing concentrations of LPS on cell proliferation was analysed. Relative transcriptional expression of genes in the study was quantified by quantitative polymerase chain reaction (qPCR) in cells treated with LPS, EGF or a combination. Untreated cells served to normalise the expression.

In the present study, we demonstrated that EGF modulated the primary immune response by reducing the activation of pathogen-recognition receptors and common genes involved in these signalling pathways, even in hyperglycaemic conditions. This effect translated in a decreased expression of pro-inflammatory cytokines. These results contribute to explain our previous observations about the reduction of circulating levels of inflammatory cytokines after local administration of human recombinant EGF in DFU. Further molecular studies should be carried out to fully understand the biological mechanisms elicited by EGF in this clinical scenario.

Introduction

Diabetes mellitus (DM) is a worldwide pandemic disease. Type 2 DM is the most prevalent form of the disease and was recently acknowledged not as a single clinical condition, but importantly, as a group of metabolic disorders. Chronic hyperglycaemia is responsible for a wide range of downstream multi-organ complications.1 Among these, lower extremity ulceration, and its potential amputation, highly contributes to an increase of disability, morbidity and mortality in the diabetic population.2

Prolonged inflammation highly contributes to diabetic wound chronication.3 Ulcerated patients show high levels of local and circulating inflammatory mediators, such as tumour necrosis factor (TNF), interleukin (IL)-1β and IL-6, resulting from sustained hyperglycaemic insult, combined to a high susceptibility to wound infection and poor resolution of microbial colonisation.4 The resulting hostile environment promotes apoptosis in fibroblasts and vascular precursor cells and impairs some processes crucial to healing such as collagen synthesis and fibroblast migration and proliferation.5 In vitro models recreating ‘clinical hyperglycaemia’ have shown a disruption of normal fibroblasts physiology.5,6 Moreover, fibroblasts derived from DFU, grown in physiological levels of glucose and oxygen, exhibit a slow and declining proliferative response in vitro,7 mainly as a result of epigenetic modifications that perpetuate the in vivo ‘diabetic behavior’.8,9

Growth factors have been widely assayed in experimental models and clinical trials to promote wound healing.10,11 Specifically, intraleisional administration of epidermal growth factor (EGF) constitutes a highly effective therapeutic alternative to diabetic wound healing, reaching responsive cells while avoiding the deleterious effect of proteases and the biofilm on the wound’s surface.12,13 According to pharmacological surveillance studies, this infiltrative procedure is associated to a 16% absolute, 71% relative reduction of amputation risks and only 5% of re-ulcerations during a 12-month follow-up period.14,15 These results highlight the concept that EGF may potentially act as a senolytic agent for diabetic wounds, promoting the neoepidermal resilience and tolerance to physical and mechanical stress.16 Our group has recently demonstrated in small cohort of neuropathic patients that local EGF administration is related to a significant systemic recovery of oxidative stress and antioxidant reserve markers17 and to an attenuation of pro-inflammatory markers.18 All these molecular effects enable the resumption of a physiologic healing trajectory.
Toll-like receptors (TLRs) are pivotal innate immune receptors that recognise multiple pathogen and damage-associated molecular patterns, initiating downstream inflammatory responses necessary for pathogen clearance and tissue repair. Experimental data and human studies suggest that the expression and activation of TLRs are increased in a plethora of inflammatory disorders including DM and its complications.

To further investigate the contribution of dermal fibroblasts to innate immune response, in the present study we analysed the expression pattern of TLR signalling pathway mediators and inflammatory cytokines, in fibroblasts derived from granulation tissue of a neuropathic DFU in response to LPS stimulus, compared to normal skin fibroblasts. In addition, we evaluated the potential anti-inflammatory effect of EGF under hyperglycaemic conditions, simulating the DFU environment.

Materials and methods

Ethical issues

A fragment from DFU granulation tissue was collected from a 63-year-old male patient, with no chronic hypertension, with a seven-year history of type 2 DM and an open wound for 57 days. The patient was treated on a daily basis with 18 IU of insulin; at the time of sampling, he had a fasting glycaemic value of 5.5 mmol/L and HbA1c level of 7.1%. Healthy skin (HS) tissue was obtained from a 60-year-old woman, with no associated co-morbidities, after a cosmetic surgery procedure. Both tissue samples were acquired after obtaining informed consent from the donors, following the ethical guidelines of the 1975 Declaration of Helsinki.

Fibroblast isolation from human tissue explants

Granulation tissue from DFU was processed for culture as described previously. A 6-mm (diameter) cylinder biopsy was taken from a well-granulated area of the wound. Tissue fragments were dissected into small pieces and equally distributed in a 60-mm tissue culture plate, allowed to stay for 1 h at 37 °C in an atmosphere of 5% CO2 and 95% air without culture media, to guarantee adhesion to the plastic surface. Later, 3 mL of high glucose Dulbecco’s Modified Eagle Medium (DMEM) culture medium (4.5 g/L or 35 mM) (Gibco/Invitrogen, Breda, The Netherlands) supplemented with 10% fetal bovine serum (HyClone; Thermo Scientific, Etten-Leur, The Netherlands), 2 mM glutamine (Sigma-Aldrich, St Louis, MO, USA) and 100 U/mL penicillin/100 µg/mL streptomycin (Sigma-Aldrich, St Louis, MO, USA) were slowly added to the plate, to cover the fragments and avoid detachment. Culture medium was replaced every three days until fibroblasts sprouted from tissue fragments and reached confluency around 50%. The cells were then trypsinised and subcultured at a ratio of 7500 cells/cm². For further experiments, cells were maintained in supplemented high glucose DMEM culture medium. HS fibroblasts were previously isolated following the same protocol described above and were used as the control for all the studies. Early passages (<10) cells were used for subsequent experiments.

Fibroblast proliferation in LPS

DFU and HS fibroblasts (10,000 cells/cm²) were seeded in 96-well plates and incubated overnight for adhesion to plastic. Cells were treated with increasing concentrations of LPS (Sigma-Aldrich, St Louis, MO, USA) (0.005–1 µg/mL), as previously described. Cell proliferation was evaluated at one, four and seven days after the addition of LPS, by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) solution (Sigma-Aldrich, St Louis, MO, USA) reduction assay. A total of 20 µL of MTT solution (5 mg/mL) was added to each well, and cells were further incubated for an additional 4 h. The supernatant was removed, and the formazan deposits were dissolved with 200 µL/well of DMSO. The optical density was measured in a microplate reader (CLARIOstar®; BMG LABTECH, Ortenberg, Germany) at 450 nm.

Treatment of LPS and EGF

The effect of LPS, EGF and their combination was studied. Recombinant human EGF was obtained from the Center for Genetic Engineering and Biotechnology (Havana, Cuba). DFU and HS fibroblasts were seeded (10,000 cells/cm²) in six-well plates, in triplicate for each treatment, and allowed to attach for 24 h. Cells were then incubated for 48 h according to the following specifications: supplemented high glucose DMEM culture medium (control group); LPS 100 ng/mL (LPS group); EGF 75 µg/mL (EGF group); or LPS 100 ng/mL + EGF 75 µg/mL (LPS + EGF group). After incubation, culture supernatants were stored at −20 °C until further determination of inflammatory cytokines.
were preserved at −20 °C for cytokine quantification. Cells were rinsed twice with cold PBS 1X solution (Sigma-Aldrich, St Louis, MO, USA) to remove medium residues and collected for further purification of total RNA.

**Quantitative real-time polymerase chain reaction (qPCR)**

Total RNA was extracted from cell samples using the RNeasy® Plus Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s guidelines. A total of 500 ng of RNA was used for complementary DNA synthesis using the SuperScript® III First-Strand Synthesis SuperMix kit for qRT-PCR (Invitrogen, Waltham, MA, USA). Real-time PCR reactions were performed using Absolute qPCR SYBR Green Mix (Thermo Fisher Scientific, Waltham, MA, USA) in a total volume of 20 μL, according to the manufacturer’s protocol. A 400-μM mix of forward and reverse primers was used per reaction. The relative expression level of each messenger RNA (mRNA) of interest was normalised to the TATA binding protein (TBP) reference gene (mRNA) of interest was normalised to the expression level of each messenger RNA primers was used per reaction. The relative gene expression level was calculated using the formula: Ratio = \((E_{\text{target}})^{\Delta C_{\text{target}}(\text{control-sample})}/(E_{\text{ref}})^{\Delta C_{\text{ref}}(\text{control-sample})}\), as previously described.29 qPCR primer sequences are shown in Table 1.

**Enzyme-linked immunosorbent assay (ELISA)**

Cell supernatants collected from all experimental conditions were assayed to quantify levels of secreted IL-6 using a commercial kit (IL-6 Human ELISA Kit; Abcam, Cambridge, UK) according to the manufacturer’s instructions. Samples were diluted 1:200. The optical density was determined at 450 nm using a microplate reader (CLARIOstar®, BMG LABTECH).

**Statistical analysis**

Statistical analyses were performed using Prism 6 software (GraphPad Software, Inc., La Jolla, CA, USA). All data are shown as mean ± SD. Comparisons were assessed among treatments for each individual cell line via regular two-way analysis of variance (ANOVA) combined with Sidak’s multiple comparisons test. A value of \(P<0.05\) was considered statistically significant.

**Results**

**Effect of LPS on fibroblast proliferation**

DFU and HS-derived fibroblasts were treated with increasing concentrations of LPS for seven days. After 24 h, there was no difference in proliferation among HS fibroblasts treated with each dose of LPS, compared to untreated cells. At day 4, LPS induced a dose-dependent increase in cell proliferation that was statistically significant for 0.05 μg/mL \((P<0.05)\) and from 0.1 to 1 μg/mL \((P<0.001)\). At day 7, all LPS concentrations significantly stimulated cell proliferation \((P<0.0001)\), compared to untreated cells. In general, there was a positive growth rate from day 1 to day 7, with an average slope of 0.10547 and a correlation index \((R^2)\) of 0.9873 for all treatments, as well as for non-treated cells (Figure 1).

DFU-derived fibroblasts responded completely differently to LPS. After 24 h, there were no differences in cell proliferation between LPS-treated and untreated cells. However, for the 1-μg/mL dose there was a slightly, although not significant, reduction. From day 1 to day 4, DFU fibroblasts almost duplicated cell growth in all experimental conditions, with an average slope of 0.4568 and a \(R^2\) of 0.9979. Nevertheless, although 1 μg/mL of LPS showed induction of proliferation, this was significantly lower compared to untreated cells \((P<0.01)\). From day 4 to day 7, untreated DFU fibroblasts and those grown in different LPS doses did not proliferate. Growing rate of cells in LPS 1 μg/mL remained significantly lower compared to untreated cells \((P<0.01)\) (Figure 1).

**EGF modulation of innate immunity mediators**

The effects of EGF, alone or in the presence of LPS, on the mRNA expression levels of innate immunity mediators were analysed in HS and DFU fibroblasts by real-time qPCR. Comparisons were carried out among treatments for each cell type independently. Statistically different expression levels for at least \(P<0.05\) are represented by different letters.

In HS fibroblasts (Figure 2), all the experimental groups exhibited lower levels of TLR4 compared to untreated cells. LPS induced a significant although discreet increase in TLR2, CD14 and MYD88. EGF, alone or in the presence of LPS, significantly reduced the expression of these genes, even at lower levels, compared to untreated cells, except for MYD88, where the
expression was slightly higher. Levels of nuclear factor kappa B (NFKB) among all groups were very similar, only faintly higher in LPS-treated cells. Regarding TNF and IL-6, LPS and EGF alone significantly induced their expression compared to untreated cells. Serendipitously, the LPS + EGF combination yielded the highest levels of expression for both genes. IL-1B showed the same expression pattern as TNF and IL-6, although there were no significant variations among the treatments.

In DFU fibroblasts (Figure 3), LPS induced a mild increase in TLR4 expression, compared to untreated cells. EGF, alone or in the presence of LPS, reduced the expression of this receptor. LPS doubled the basal expression of TLR2. On the contrary, EGF significantly reduced the expression of this gene near to or below the control level. LPS had no effect of CD14 expression but EGF, alone or in the presence of LPS, reduced its expression. For MYD88 and NFKB, the changes in expression level induced by LPS, although significant, were very slight. The rest of experimental conditions remained similar. Regarding TNF and IL-6, LPS significantly induced their expression compared to untreated cells. Cells treated with EGF exhibited lower expression levels than control cells. On the

| Gene name | Gene symbol | Accession number | Forward primer | Reverse primer |
|-----------|-------------|-----------------|----------------|---------------|
| Toll-like receptor 4, transcript variant 1 | TLR4 | NM_138554.4 | GTGGAGCTGTACCGCCTTCTCA | CCAGCAGGGCTTTTCTGAGTCG |
| Toll-like receptor 2, transcript variant 1 | TLR2 | NM_001318787.1 | ACTGGACTTCTCCCATTTCCGTC | GCCACTCCAGGTAGGTCTTGTG |
| CD14 molecule, transcript variant 1 | CD14 | NM_000591.3 | TGGGCTGGAACAGGTGCCTAAA | AGGATTCCCCGTCAGTCAG |
| MYD88, innate immune signal transduction adaptor, transcript variant 1 | MYD88 | NM_001172567.2 | GCTGCTCTCAACATGCGA | TGTCGCACGTTCAAGAAC |
| Nuclear factor kappa B subunit 1, transcript variant 1 | NFKB | NM_003998.3 | CAGATGGCCCATACCTTCAAAT | CGAAAGCAAAATCCTCTCTTG |
| Tumour necrosis factor | TNF | NM_000594.3 | TGAGGCCAAGCCCTGATGAGT | CCCAGACTGGCAAGGAAGTCGAG |
| Interleukin 6, transcript variant 1 | IL-6 | NM_000600.4 | AGCCAGAGCTGTCGATGATGTG | CTTGCAGCAGGCTGGCATTT |
| Interleukin 1 beta | IL-1B | NM_000576.2 | GCTGGAATTTGAGTGGCTGGCATT | ATATCCGGGCTCGTTGTT |
| TATA binding protein | TBP | NM_003194 | GCCATAAGGCATGAGAG | AACAACAGCTGCCACCTTA |
other hand, EGF lessened the effect of LPS, as TNF and IL-6 showed lower expression levels. Finally, LPS increased IL-1B expression and, as for HS fibroblasts, the LPS + EGF combination synergistically induced IL-1B in a very remarkable way.

We later verified whether the results observed for gene expression translated into the protein environment. In this regard, levels of IL-6 were quantified in culture supernatants from all the experimental conditions (Figure 4). For HF, there was a correlation between gene expression and protein concentration, except for cells treated with EGF, where the protein level was similar to untreated cells, while gene expression was comparable to LPS-treated cells. For DFU-derived fibroblasts, there was also a direct correlation but with the difference that gene expression in LPS-EGF experimental condition was similar to that in control cells; however, protein level was significantly higher in LPS-EGF compared to untreated cells supernatant.

**Discussion**

Inflammation plays a key role during the wound-healing process. Immediately after a skin injury, there is a release of pro-inflammatory cytokines that constitute potent chemoattractant signals to recruit immunocompetent cells to the wound, which are responsible to clear both pathogens and cellular debris.\(^{30}\) In addition to chemokines derived from platelets, non-specific factors with danger and pathogen-associated molecular patterns are released from host dying cells and microbial agents. After being recognised by TLRs, these factors stimulate recruitment, proliferation and activation of local macrophages and other immune cells, as well as fibroblasts, mesenchymal stromal cells and various epithelial cells that promote tissue repair.\(^{31,32}\) This innate immune response in the skin serves not only to eliminate infections after injury, but also to maintain homeostasis and functional integrity, and may be active in restoring structure to damaged tissues.\(^{33}\)

In DM, sustained hyperglycaemia impairs almost all the events involved in the healing process. In particular, the inflammatory reaction is prolonged and exacerbated\(^{3}\) and does not necessarily imply a local physiological control of bacteria.\(^{34}\) Diabetic wounds host an abnormal bacterial biofilm that is able to rapidly regenerate and cover a group of genotypically distinct bacteria that symbiotically produce a polymicrobial community difficult to diagnose and eradicate.\(^{35}\) Among contaminants, gram-negative bacteria are well represented and contribute to inflammation by releasing endotoxins to the wound bed.\(^{36}\) LPS is the main component of endotoxin, so taking this into account, in the present study we used purified LPS from *Escherichia coli* to partially simulate an in vitro model of infection. The main objectives were to study the capacity of DFU-derived fibroblasts to respond to LPS stimulus and mainly to elucidate the potentiality of EGF as an anti-inflammatory agent in this particular context.

The result concerning the stimulatory effect of LPS on fibroblast cell growth coincided with previous findings obtained by other groups.\(^{27,37,38}\) In our model, LPS increased the growth rate in healthy fibroblasts in a dose-dependent way, even at the highest concentration.
tested. Over the next seven days, cells remained in a logarithmic phase without reaching a plateau, which, for untreated cells, agreed with the previous results from our group.\textsuperscript{7} However, for DFU-derived fibroblasts, the higher dose of 1 μg/mL significantly inhibited cellular proliferation compared to untreated cells, as previously described for other types of fibroblasts.\textsuperscript{27} In addition, the logarithmic growth for DFU-derived cells continued only during the first four days in culture. From this day forward, the cells reached a non-dividing plateau stage that lasted until day 7. This fact might contribute to explain the \textit{in vivo} limited dividing capacity of fibroblasts from granulation tissue of DFU in the presence of sustained infection. We selected the 100μg/mL LPS dose for further experiments based on our results and previous findings obtained specifically for

\textbf{Figure 2.} Relative gene expression of innate immune related genes in HF. Relative gene expression was measured as the fold change of target genes normalized to TATA binding protein reference mRNA and compared to control untreated cells. Data are presented as mean ± SD. Statistical analyses were performed using an ordinary one-way ANOVA followed by Sidak’s multiple comparisons test. Different letters indicate significant differences for at least \( P < 0.05 \). Experimental groups: 48 h exposure to LPS (100 ng/mL), EGF (75 μg/mL) or the combination (LPS + EGF), control: untreated cells. CD14, cluster of differentiation 14; EGF, epidermal growth factor; HF, healthy fibroblasts; IL, interleukin; LPS, lipopolysaccharide; MYD88, innate immune signal transduction adaptor; NKB, nuclear factor kappa B; TLR, toll-like receptor; TNF, tumour necrosis factor.
Regarding EGF, we assayed the dose of 75 µg/mL as it has been widely used in clinical trials\textsuperscript{12,13} as well as in daily practice, proving to be effective in promoting granulation and wound closure.\textsuperscript{40}

TLR4 and TLR2 were expressed in cultured human skin fibroblasts of both origins, in agreement with previous results.\textsuperscript{41} But, although LPS is the main ligand for TLR4,\textsuperscript{42} it did not stimulate the transcriptional expression of this receptor in either cell line. This might be related to the chemical structure of LPS we used during the experiment. The recognition and response to LPS by TLR4 strongly depend on the chemical modification of the lipid A structure. According to previous studies, the number and carbon chain length of acyl groups attached to the phosphorylated glucosamine backbone are critical for

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**Figure 3.** Relative gene expression of innate immune related genes in DFU-derived fibroblasts. Relative gene expression was measured as the fold change of target genes normalized to TATA binding protein reference mRNA and compared to control untreated cells. Data are presented as mean ± SD. Statistical analyses were performed using an ordinary one-way ANOVA followed by Sidak’s multiple comparisons test. Different letters indicate significant differences for at least $P < 0.05$. Experimental groups: 48 h exposure to LPS (100 ng/mL), EGF (75 µg/mL) or the combination (LPS + EGF), control: untreated cells. CD14, cluster of differentiation 14; DFU, diabetic foot ulcer; EGF, epidermal growth factor; IL, interleukin; LPS, lipopolysaccharide; MYD88, innate immune signal transduction adaptor; NKB, nuclear factor kappa B; TLR, toll-like receptor; TNF, tumour necrosis factor.
TLR4 activation, and that alteration of these factors can reduce the magnitude of the activation.\textsuperscript{43} For further experiments, different sources of LPS should be used, to clearly induce TLR4 expression.

TLR4 inhibition exerted by EGF, alone or in combination with LPS, is consistent with previous results in intestinal mucosa and in IEC-6 cells in which downregulation of TLR4 was associated to stimulation of EGFR signalling and downregulation of the downstream target GSK3\textbeta.\textsuperscript{44} Growth factor receptor/ligand interaction initiates a signalling cascade that affects GSK3\textbeta, which negatively affects NF\kappaB activation\textsuperscript{45} thus decreasing NF\kappaB-dependent pro-inflammatory cytokine production.\textsuperscript{46}

TLR2 has also been implicated in the response to LPS.\textsuperscript{47} The ability of human TLR2 to be activated by LPS and the inhibition of LPS-induced cytokine activation by an anti-human TLR2 blocking antibody suggests that there may be some factors in common between TLR4 and TLR2 specificities.\textsuperscript{48,49} In our study, LPS was shown to highly increase TLR2 transcriptional expression in UPD-derived fibroblasts and slightly, but yet significantly, increase in healthy cells. As for TLR4, EGF, alone or in presence of LPS, diminished TLR2 expression. This is in agreement with previous results showing that the expression of TLR2 was downregulated by recombinant human EGF in \textit{Propionibacterium acnes}-treated normal human epidermal keratinocytes, inhibiting pro-inflammatory cytokines through the suppression of NF\kappaB.\textsuperscript{50}

CD14 may play a critical role in TLR signalling by binding many lipid-containing molecules, including LPS,\textsuperscript{51} and promoting the internalisation of the TLR4-MD2-LPS complex into endosomes.\textsuperscript{52} According to our results, the effect of EGF in decreasing the transcriptional expression of CD14 could contribute to limit the biological effect of LPS by reducing its presentation to TLR. MYD88 is also essential for the inflammatory responses mediated by TLR family members.\textsuperscript{53} It has been demonstrated that the transcript level of MYD88 is significantly elevated in the peripheral blood of diabetic patients compared to controls.\textsuperscript{54} In our experiment, the downregulating effect observed for EGF also might contribute to diminish the signal transduction from TLRs.

It is well established that TLR stimulation, except TLR3, results in the activation of nuclear transcription factor NF\kappaB.\textsuperscript{55} In diabetic animals, activation of NF\kappaB is increased compared to non-diabetic controls\textsuperscript{24,26} suggesting that, in diabetes, the stimulation of this signalling pathway is associated to hyperinflammation and to a delay of the wound-healing process. The increase of NF\kappaB is also related to an excessive expression of matrix metalloproteases production, leading to extracellular matrix degradation and impaired formation of granulation tissue.\textsuperscript{3} As expected, in our experimental system, LPS treatment increased the transcriptional expression of the NF\kappaB gene. The addition of EGF restored its levels to those of untreated cells, which could be contributing to further regulate the expression of inflammatory cytokines driven by NF\kappaB.
DFU are characterised by elevated levels of pro-inflammatory cytokines as TNF, IL-1β and IL-6. This pro-inflammatory phenotype reduces the ability of dermal fibroblasts to proliferate, migrate and synthesize collagen and promotes their apoptosis, impairing wound closure. In our experimental conditions, LPS significantly increased transcriptional expression of the three cytokines in DFU-derived fibroblasts. EGF, alone or in the presence of LPS, significantly reduced levels of TNF and IL-6 genes, and IL-6 protein in culture medium, contributing to reduce the pro-inflammatory environment. Serendipitously, our results revealed that the addition of EGF exacerbated the stimulatory effect of LPS on IL-1β expression. This could be in agreement with previous findings in which EGF induced the expression of IL-1β through transcriptional activation of the gene. Specifically, in this study, EGF increased the binding of NFκB to the NFκB binding site in IL-1B promoter through the activation of the Akt/NFκB pathway.

Taken together, our in vitro findings helped to elucidate the active role of dermal fibroblasts in the innate immune response against pathogen insult. Our study showed that fibroblasts, particularly DFU-derived ones, not only act as sentinel cells, but also respond to therapeutic stimuli such as EGF, by decreasing the aberrant activation of the TLR signalling pathways and, consequently, the levels of inflammatory mediators. These results contribute to explain our previous in vivo observations about the reduction of circulating levels of inflammatory cytokines after local administration of human recombinant EGF in DFU, which stimulate granulation and wound closure in diabetic patients.

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