Hyperglycaemia and ischaemia impair wound healing via Toll-like receptor 4 pathway activation *in vitro* and in an experimental murine model

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Introduction

Diabetes mellitus affects an estimated 451 million people, representing 8.5% of the adult population (1-3). It is not a single disease however, but represents a group of metabolic disorders characterised by chronic hyperglycaemia, of which type 2 (90%) and type 1 are the most common (2). Diabetes is associated with the development of micro- and macro-vascular complications which carry considerable morbidity and mortality. Macrovascular complications include accelerated atherosclerosis, manifest as coronary artery disease, cerebro-vascular disease and peripheral arterial disease (PAD). Microvascular complications include neuropathy, retinopathy and nephropathy (4).

Diabetic foot ulceration is a common, challenging and expensive complication. Diabetes confers a 15-20% lifetime risk of developing foot ulceration, leading to a 20 times greater risk of major amputation compared to non-diabetics (5, 6). The development of foot ulcers is multifactorial, involving infection, trauma, micro- and macro-vascular insufficiency, immunological dysfunction and neuropathy (7, 8). Despite current best medical and surgical intervention, 33% of these ulcers fail to heal (9).

Wound healing in diabetes is impaired with wounds failing to progress through the normal physiological phases of healing, often becoming stalled in the inflammatory phase (10). These

wounds are characterised by excessive inflammation, with significantly prolonged and sustained neutrophil and macrophage infiltration (11). In addition to the cellular components, the hyper-inflammatory environment also comprises a marked increase in the release of pro-inflammatory cytokines interleukin-6 (IL-6), tissue necrosis factor alpha (TNF-α), interleukin-1β (IL-1β) and matrix metalloprotease (MMP) production (10). This results in exaggerated destruction of the extracellular matrix, impairment in granulation tissue formation and dysfunction of other processes crucial to healing such as fibroblast migration and proliferation, and collagen synthesis (10).

Diabetes is now recognised as a systemic pro-inflammatory condition. There is compelling evidence the inflammatory process is mediated through pattern recognition receptors (PRRs) of the innate immune system (12). In particular, toll-like receptor 4 (TLR4) has been implicated in the systemic pathogenesis of diabetes and its complications (13). TLRs are key PRRs of the innate immune system, and are activated through recognition of exogenous microbial components termed pathogen associated molecular patterns (PAMPS) (14). Binding PAMPS leads to activation of downstream signalling pathways, ultimately resulting in the release of pro-inflammatory cytokines such as IL-6 and TNF-α (15). In addition to the PAMPs, TLRs are also activated by a variety of host derived endogenous ligands termed damage associated molecular patterns (DAMPs) (16). These are usually hidden from immune recognition, but are exposed by tissue damage alerting the innate immune system to injury (16). The resulting inflammatory response is a physiological mechanism for the recruitment of immune cells and stimulation of the normal process of wound healing and tissue repair (16).

This study investigates the effect of high glucose and hypoxic conditions on TLR4 activation

and signaling, using *in vitro* and *in vivo* models to replicate the 'real world' situation that exists in patients with foot ulceration as a manifestation of severe disease. These patients will have developed micro-vascular disease rendering local tissues ischaemic, macro-vascular disease in the form of atherosclerotic PAD or more likely combinations of both. It is therefore necessary to consider the hypoxic local tissue environment in addition to the hyperglycaemic conditions when attempting to simulate the diabetic-ischaemic wound environment found in chronic foot ulceration.

Materials and Methods

In vitro **experiments**

Cell culture:

Primary cultured human dermal fibroblasts were obtained from consenting non-diabetic patients undergoing major lower limb amputations from intact skin at the proximal margin. Skin samples were immediately placed in a culture medium of Dulbecco's modified Eagle's medium 4.5g/dL glucose (25mM), (DMEM, catalog number (cat. no.) 31966-021; Invitrogen, Paisley, UK), 10% fetal calf serum (GIBCOs, Paisley, UK), 100U/ml penicillin and 100g/ml streptomycin. The dermis and epidermis were separated using sharp dissection, and dermal cells seeded in a T75 flask (Falcon, Franklin Lakes, NJ, USA) with 25ml of culture medium and incubated in a humidified atmosphere of 21% O2 and 5% CO at 37°C. At passages 2 to 3, all cells were transferred to a low glucose media (5.5mM), and further cultured for at least two weeks.

Glucose dose treatments:

Low glucose (LG, 5.5mM) cultured dermal fibroblasts were seeded into 6 well plates (Falcon product code #353046) at a density of 80,000 cells per well. The media was changed daily and at 90% confluence a low serum media (0.1% FCS) was applied 24 hours before commencing the experiment. The cells were subsequently exposed to media containing glucose concentrations of 0mM to 25mM for 24 hours and included 14mM mannitol in low glucose as an osmotic control (17). Identical plates were simultaneously

placed in a hypoxic chamber (Modular Incubator Chamber, MIC-101; Billups-Rothenberg, Del Mar, CA, USA) utilising a gas mixture of 20% CO_2 and 80% N_2 for 8 hours (18).

Inhibitors:

The low glucose cultured fibroblasts were exposed to a very high glucose (VHG, 25mM) media for 24 hours utilising the above described protocol. Various inhibitors were added to the VHG treatment media. A selective TLR4 neutralising antibody at 1µg/ml (Anti-hTLR4 IgG, Invivogen) and inhibitor at 10µg/ml (LPS-RS, Invivogen).

Western Blots:

Protein lysates were prepared from the cultured fibroblasts following completion of the 24 hours of treatment exposure. Fibroblast monolayers were placed on ice and washed with PBS before addition of a 175µl volume of lysis buffer, containing RIPA buffer, protease and phosphatase inhibitors and 2-mercaptoethanol. The lysates were liberated using a cell scraper and agitation through a 23G needle and syringe. The lysates were placed into 1.5ml Eppendorff tubes and centrifuged at 10,000rpm for 4mins at 4°C.

Scratch migration assay:

Low glucose (LG, 5.5mM) cultured dermal fibroblasts were seeded into 24 well plates, cultured to 90% confluence and a standardised scratch wound inflicted (19). Following PBS wash, treatment media at glucose concentrations of 5.5mM, 25mM and 25mM with the inhibitors described above, were added in addition to 0.1% mM mitomycin c (Merck Millipore, UK) as a proliferation inhibitor. Duplicate plates were placed in a hypoxic chamber for 24 hours as above, and fibroblast migration was assessed via camera microscopy and percentage migration measured using a visual optical method (20).

ELISA:

IL-6 and TNF-α concentrations were measured from the supernatants of treated human dermal fibroblasts using ELISA (R&D systems, catalogue number DY206).

In vivo **experiments**

Animal procedures

All procedures were conducted in accordance with UK home office regulations under Animals (Scientific Procedures) Act 1986 project license and with ARRIVE guidelines. Type 1 diabetes was induced in 8-week-old male C57BL-6 or TLR4 knock-out mice by intra-peritoneal (IP) streptozotocin (50mg/Kg doses daily for 5 consecutive days) (21). Diabetes was confirmed on tail capillary blood sampling at two weeks. A random blood glucose value of >16mmol/L was diagnostic; glycosuria was monitored with weekly urinalysis. Animals were kept with in individually ventilated cages and fed standard chow and water *ad libitum*.

In our novel model of diabetic-ischaemic ulceration, hindlimb ischaemia was induced four weeks post diabetes induction under sevofluorane anaesthesia by ligation of the external iliac artery (EIA) and ligation-excision of the superficial femoral artery (SFA) (22). A unilateral 4mm full thickness punch biopsy was then performed below the knee (23).

Animals were not formally randomised but were initially housed in individually ventilated cages. The cages were then randomly numbered and selection and separation for each treatment cohort was assigned based on sequential cage number. Animal numbers within each cohort was determined based on previous studies using the same model of hindlimb ischaemia in animals of different genetic backgrounds. It is estimated 4 animals in each group at each time point will enable an 80% power of detecting differences at a significance level of 5% (24).

Experimental protocol

Animals were sacrificed at days 3, 7 and 14 post surgery and hind limb wounds were photographed and excised en-mass. Surrounding skin was harvested and snap frozen.

Planimetry

Wound areas on days 0, 3, 7 and 14 were standardised and measured using image j digital planimetry software (https://imagej.nih.gov/ij/)

Statistics

Non-parametric data were expressed as median + ranges. Statistical analyses were performed using GraphPad Prism (GraphPad Software). Comparisons between two groups were performed using two-tailed Mann-Whitney test. Comparisons between multiple

independent groups were performed using the Kruskall-Wallis test. A p-value < 0.05 was considered statistically significant.

Ethical approval was granted for the collection of Human tissue samples, along with individual patient consent. All animal procedures were conducted in accordance with UK home office regulations under an Animals (Scientific Procedures) Act 1986 project license and within ARRIVE guidelines.

Results:
***In vitro* experiments:**

Increasing concentrations of glucose increased TLR4 protein expression in a dose response fashion (figure 1). Very high glucose conditions (VHG, 25mM) significantly increased TLR4 protein expression compared to physiological 'low glucose' (LG, 5.5mM) conditions (Figure 1B). The addition of 14.5mM mannitol to 5.5mM glucose media as an osmotic control had no significant effect on TLR4 protein expression. The increase in TLR4 protein expression in the VHG group reached significance over the LG group at 24 hours (data not shown). All further experiments were conducted for this duration.

Hypoxic conditions led to an increase in TLR4 protein expression (figure 2 **B**). This effect was significantly increased (p=0.017) in VHG concentrations and resulted in increased expression of MyD88 (figure 2 **C** p=0.014) and activation of NFkB (Figure 2 **A**). IL-6 release was significantly increased in the VHG-hypoxic conditions compared to LG-hypoxia groups (figure 2 **E**). VHG-hypoxic conditions significantly increased apoptosis (cleaved-caspase 3, figure 2 **D** p>0.0001) in dermal fibroblasts compared to LG and normoxic conditions.

To determine the effect of very high glucose and hypoxic conditions on TLR4 expression, activation and signalling, we utilised a specific TLR4 neutralising antibody and an TLR4 selective antagonist. In these hyperglycaemic-ischaemic conditions, TLR4 inhibition resulted in a significant reduction in apoptosis (figure 3 D), IL-6 release (figure 3 E), TLR4 protein expression (figure 3 B), and HMBG1 release (figure 3 C). Selective blocking of TLR4

mediated pathways in VHG-hypoxic conditions led to a significant reduction in IL-6 release, apoptosis and release of HMGB1, a potent TLR4 endogenous ligand. These experiments were conducted utilising three different fibroblast cell lines in triplicate (n=9) or four times repeated (n=12).

Very high glucose and hypoxic conditions significantly impairs fibroblast migration

The functional consequences of VHG and hypoxia on fibroblast migration were assessed by scratch migration assay. In normoxic conditions fibroblast migration was significantly improved in VHG media compared to LG (figure 4 C). The opposite effect was observed in hypoxic conditions (figure 4 F), where there was a significant impairment in fibroblast migration in VHG culture compared to LG.

Inhibition of TLR4 significantly improves fibroblast migration in very high glucose hypoxic conditions

The addition of a selective TLR4 neutralising antibody or antagonist to fibroblasts maintained in VHG-hypoxic environment ameliorates the effect of these simulated hyperglycaemic ischaemic conditions (figure 5 A and B).

***In vivo* experiments:**

Experimental diabetes and hind limb ischaemia impairs wound healing in a murine model of diabetic ulceration

Diabetic-ischaemic wounds (DM-I) in male wild-type C57BL6 mice demonstrated significantly impaired wound healing compared to diabetic non-ischaemic (DM-N), non-diabetic ischaemic (NDM-I) or non-diabetic, non-ischaemic wounds (NDM-N) at day 14 ($p < 0.05$) (Figure 6). There was no significant difference in healing rate between diabetes only and ischaemia only wounds, however at day 14, 50% of diabetic wounds were completely healed compared with 25% of ischaemic only wounds. Four animals were utilised at each time point in each of the four comparison groups (n=16).

TLR4 Knock-out accelerates wound healing in diabetic ischaemic mice

Diabetic-ischaemic wounds in TLR4 KO mice demonstrated significantly improved healing rates compared to those in WT mice at days 3, 7 and 14 post wounding (Figure 7). Two thirds (67%) of wounds were completely healed in TLR4 KO mice compared to 0% in WT mice by day 14. Endogenous knock out of TLR4 conferred a protective effect on wound healing in diabetic ischaemic animals, ameliorating the effect of pathological TLR4 mediated inflammation.

Discussion

This study has demonstrated that exposure to high glucose results in increased TLR4 expression, signaling, activation and function in human dermal fibroblasts. A similar effect has been observed in gingival fibroblasts (17), monocytes (25) and adipocytes (26). Data from studies of human tissue (27), and animal models (28) have consistently demonstrated the pro-inflammatory effect of diabetes and high glucose exposure, mediated via increased TLR expression and activation.

Other groups have shown high glucose increases TLR4 mediated inflammation, however the novel observation from this study is the effect of adding a second noxious variable, hypoxia. Our group has previously shown hypoxia induces TLRs 2, 6 and 4 expression and activation in skeletal muscle (29) however we have now demonstrated in dermal fibroblasts there is a significant increase in TLR4 expression and activation in hypoxia with concurrent exposure to high glucose. This inflammatory effect signals via TLR4 pathways, resulting in increased inflammation via downstream production of cytokines such as IL-6.

In clinical situations of chronic non-healing diabetic-ischaemic ulceration, it is possible to see the consequences of this dysregulated process. The release of pro-inflammatory cytokines via TLR4 activation, and the subsequent recruitment of the innate immune cellular response result in tissue damage and cellular apoptosis and the release of DAMPs such as HMGB1. These DAMPs act as potent ligands for TLR4 activation, resulting in the release of further pro-inflammatory cytokines, perpetuating a dysregulated positive feedback cycle of tissue damage and inflammation (16, 30). The resulting hostile wound environment is characterised by persistent and excessive inflammation, inhibiting normal progression of the healing process and a chronic, static wound results (16).

Dermal fibroblasts are essential cellular components of the normal process of skin wound healing through the production of extracellular matrix comprised of collagen, glycosaminosglycans, proteoglycans, fibronectin and elastin essential for the development of granulation tissue. In addition they undergo the crucial processes of proliferation, migration and differentiation into contractile myofibroblasts (31). Fibroblast migration in this study was improved by very high glucose conditions in normoxia, contrary to other studies in which high glucose impeded migration through overexpression of gap junction protein connexin 43 (Cx43) (32). However, while similar, the experimental protocols were not directly comparable to those presented in this study, utilising higher glucose doses and longer durations of exposure. It has previously been shown that hypoxia stimulates fibroblast migration, however cells harvested from diabetic animals failed to demonstrate this improved migratory property in hypoxia (33). We suggest the significant reduction in migration observed in our study by fibroblasts in high glucose and hypoxic conditions was due to the the pathological combination of both factors, resulting in a deleterious signalling change to the cell further study in this area is required.

Our murine model of diabetic-ischaemic ulceration demonstrated impairment in wound healing compared to controls at day 14, simulating chronic ulceration. We observed a significant improvement in wound healing at each time point in the diabetic-ischaemic TLR4 KO animals. The pathological effect of diabetes and ischaemia on wound healing was ameliorated by endogenous deletion of TLR4, we suggest by a reduction in the local and systemic hyper-inflammatory response observed in diabetic wounds. TLR4 and TLR2 knock out has previously been shown to have a protective effect on wound healing in diabetic animals (34, 35). Our data suggests this beneficial effect also persists when ischaemia is

applied, simulating the hyperglycaemic, locally ischaemic micro-environment of a diabetic foot wound.

This apparent synergistic effect between the innate inflammatory reaction to high glucose and hypoxia has significant clinical consequences. In addition to impaired wound healing, diabetic patients are observed to have poorer outcomes in other ischaemic pathologies such as acute myocardial infarction (36) and cerebro-vascular accident (37). This observation has led us to propose a two-hit hypothesis for the inflammatory basis of the clinical complications of diabetes. The process requires a subclinical 'priming' phase in diabetics of hyperglycaemia resulting in chronic low-grade inflammation and upregulated TLR expression. A second noxious stimulus such as infection, ischaemia or trauma, then triggers a significant TLR mediated response. TLR activation following injury is an important physiological mechanism (16), endogenous ligands such as HMBG1 released during tissue damage precipitate a TLR mediated inflammatory response, recruiting innate immune cells and stimulating tissue healing.

In diabetes, the normal physiological healing process is instead characterised by pathological hyper-inflammation, preventing the progression of the normal phases of wound healing. TLR4 antagonism therefore presents a novel therapeutic target in diabetic-ischaemic pathologies in both acute and chronic conditions.

The mechanism of how hyperglycaemia leads to TLR up-regulation remains uncertain. There is evidence high glucose induces TLR 2 and 4 expression through increased oxidative stress, via the activation of protein kinase C (PKC) and NADPH oxidase activity (25). There is also considerable overlap and convergence between TLRs 2 and 4, and receptors for the advanced glycosylated end-products (RAGE), which utilise common signalling pathways to

activate NFkB dependent gene expression (30). RAGEs are PRRs activated by a variety of ligands including advanced glycosylated end-products (AGE). These are protein, nucleic acid and fatty acid products produced non-enzymically in pro-oxidative environments, whose formation is accelerated by exposure to high glucose (30). Furthermore, HMGB1 is a highly potent endogenous ligand of TLRs 2 and 4 and RAGE (38). NFkB activation results in increased expression of TLR4 and TLR2, and it has been observed that TLR4 activation increases expression of TLR2 in endothelial cells (39). It is therefore possible that cross-talk between TLR2, TLR4 and RAGE and co-activation by ligands such as HMGB1 result in the over-expression of these pro-inflammatory receptors by high glucose. This principle requires further investigation and will form the basis of future work.

The authors acknowledge several limitations of this study. The use of primary cultured fibroblasts required the initial culture process to occur in high glucose conditions. While this was limited to the minimum period required, it is unclear how long phenotypic changes induced by hyperglycaemia persist. It is likely however this ultimately attenuated the glucose treatment response, and was accepted as an unavoidable limitation of this *in vitro* model. Non-diabetic donor patients were chosen for the same reason. The induction of diabetes through STZ treatment models type 1 diabetes in the *in vivo* model. Established mouse models of type 2 diabetes exist, but it was our aim to study hyperglycaemia rather than insulin resistance. Our later studies utilising TLR4 KO mice also required a simplified diabetes model, avoiding the complexity of double KO animals. The principle focus of this study has been TLR4, rather than downstream signaling pathways, as several TLR4 antagonist drugs have been developed and have even reached phase 3 clinical trials. The authors believed this increased the translational potential of this study.

Conclusion

Hypoxia stimulates up-regulation of TLR4 protein expression and this effect is exaggerated by hyperglycaemia. In TLR4 KO mice, there is a significant improvement in the healing of diabetic-ischaemic wounds compared to WT. We suggest that a synergistic effect between hypoxia and hyperglycaemia impairing wound healing exists, through TLR4-mediated inflammation.

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Increasing glucose concentration results in increased TLR4 protein expression in human dermal fibroblasts
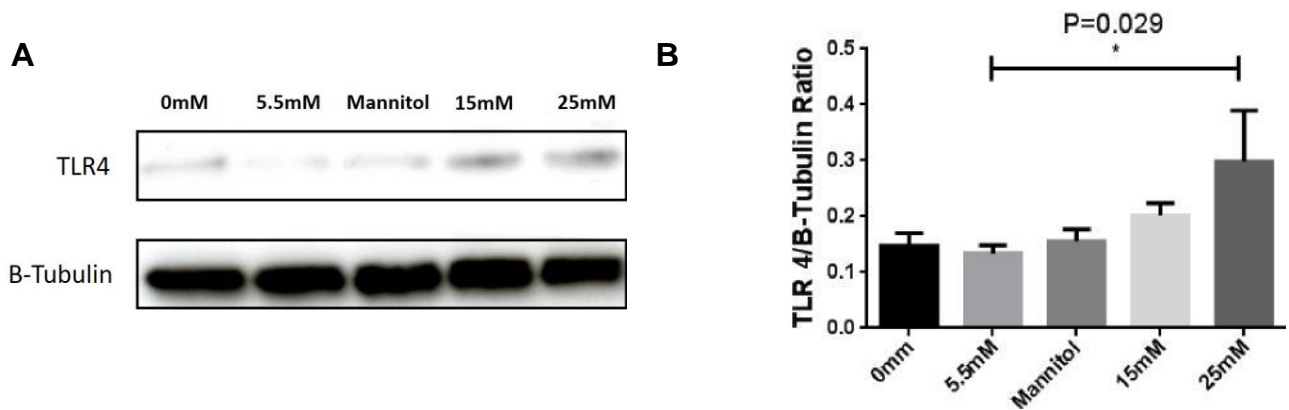
Figure 1: Glucose dose-response effect on TLR4 protein expression. Representative western blot analysis demonstrating the effect of increasing glucose concentration on TLR4 protein expression by fibroblasts. (**A**). Densitometric analysis of western blots (**B**). n=9, p=0.029, Mann-Whitney U test.

Hypoxia results in increased TLR4 expression and signalling and this effect is exaggerated in very high glucose conditions
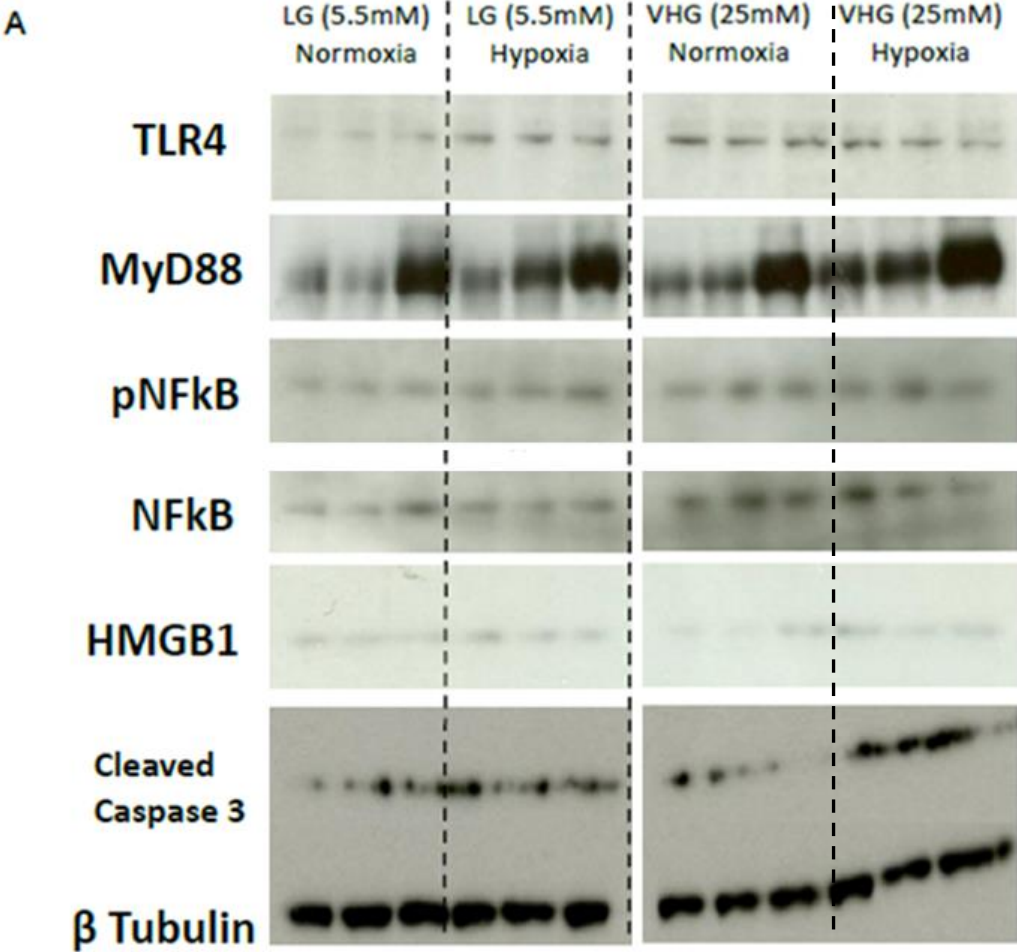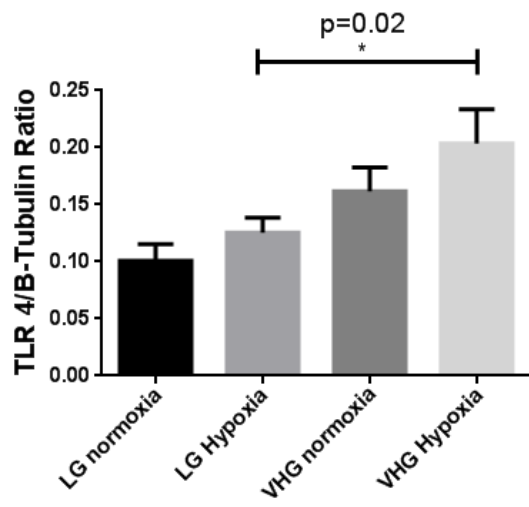
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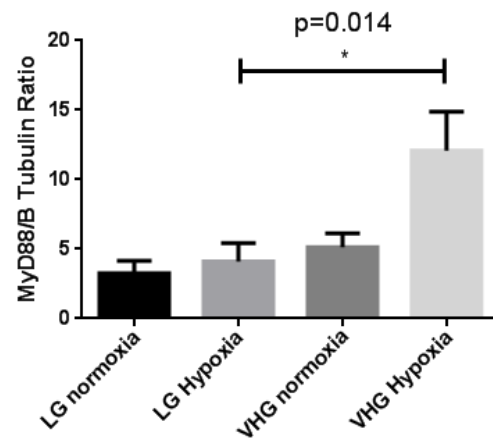
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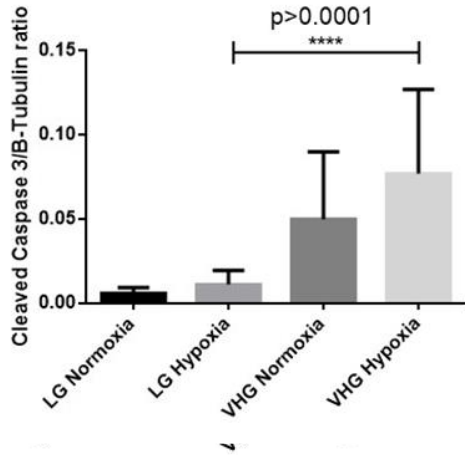
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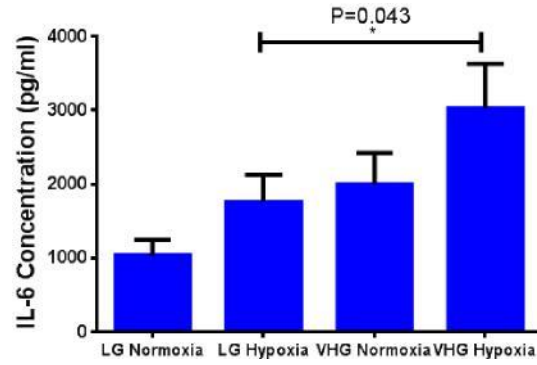
Figure 2: LG vs. VHG, Normoxia and Hypoxia. Representative Western Blots comparing the effect of LG (5.5mM) glucose and VHG (25mM) exposure in normoxia and hypoxia on expression of TLR4, cleaved caspase 3 (apoptosis), HMBG1 and MyD88 and NFkB activation (**A**). Densitometric analysis of TLR4 (**B**) MyD88 expression (**C**) and cleaved caspase 3 (**D**). (**E**) IL-6 concentration of cell supernatant samples. VHG conditions resulted in a greater release of IL-6 compared to LG. Hypoxia increased IL-6 release in both LG and VHG conditions. (n = 12, p = 0.043, Mann-Whitney U test).

The inflammatory effects of high glucose hypoxic conditions are ameliorated by inhibition of TLR4
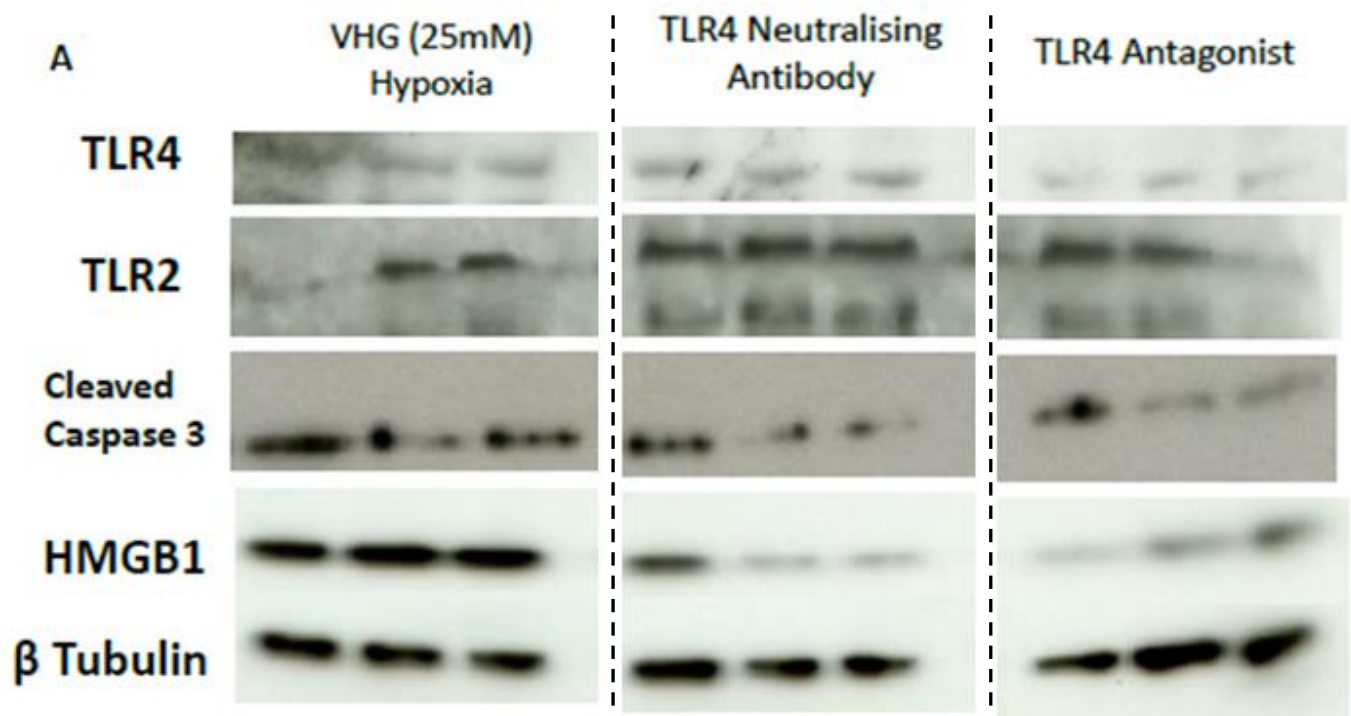
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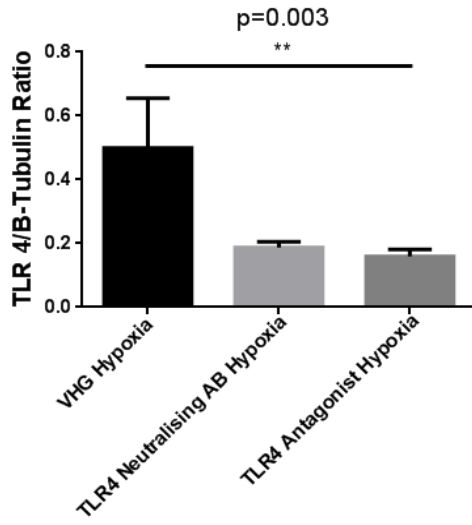
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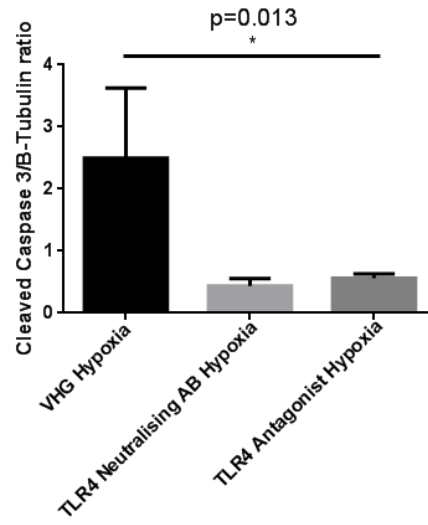
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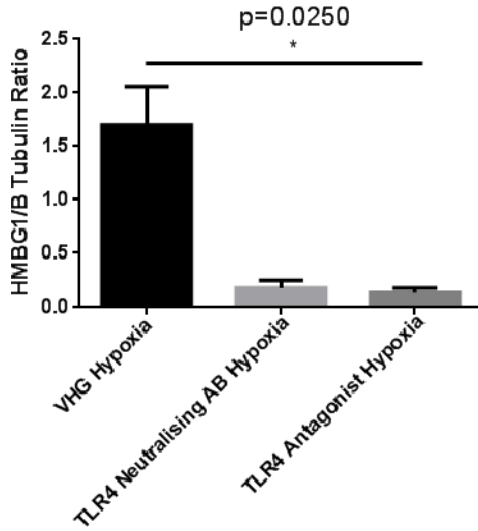
Figure 3: Inhibitors in VHG and hypoxia. Representative Western Blots comparing VHG vs. VHG + specific inhibitor treatment in hypoxic conditions on TLR4, TLR2, cleaved caspase 3 and HMBG1 (**A**). Densitometric analysis of TLR4 (**B**), cleaved caspase 3 (**C**) and HMBG1 (**D**). (**E**) IL-6 concentration of cell supernatant samples. (n= 12, Kruskal-Wallis test)

E

5.5mM

A

25mM

B

C
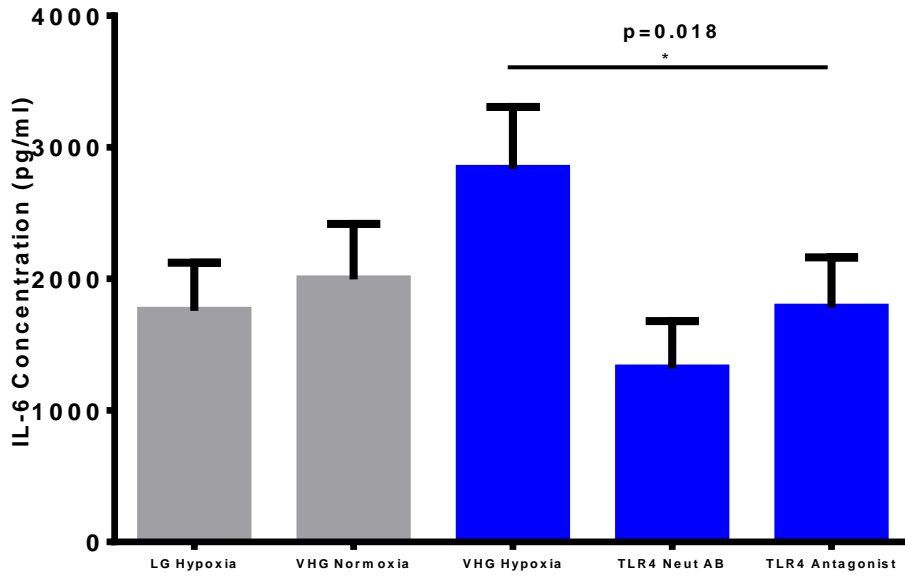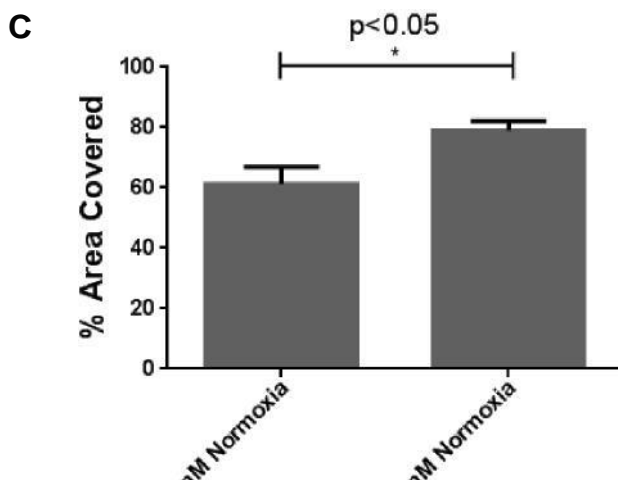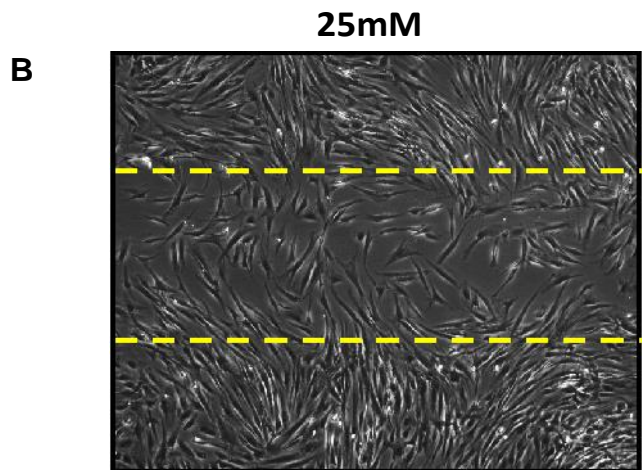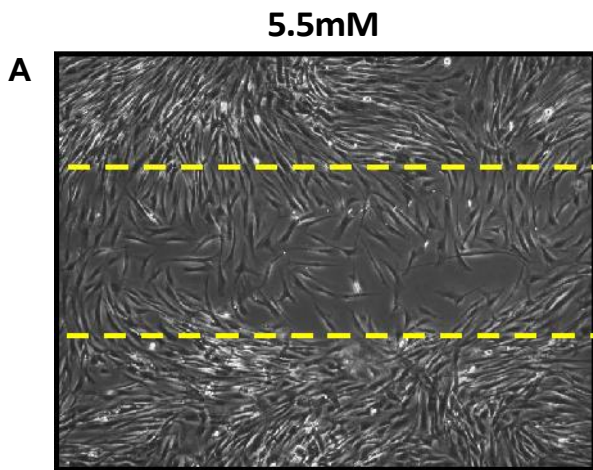
Figure 4: Scratch migration assay comparing (**A**) LG and (**B**) VHG in normoxic conditions. Fibroblast migration was significantly greater in VHG conditions (**C**). (n = 8, p<0.05, Mann-Whitney U test) (**D**) LG and (**E**) VHG migration in hypoxic conditions. Fibroblast migration was significantly impaired in VHG group compared to LG (**F**). (n =8, p<0.01, Mann-Whitney U test)
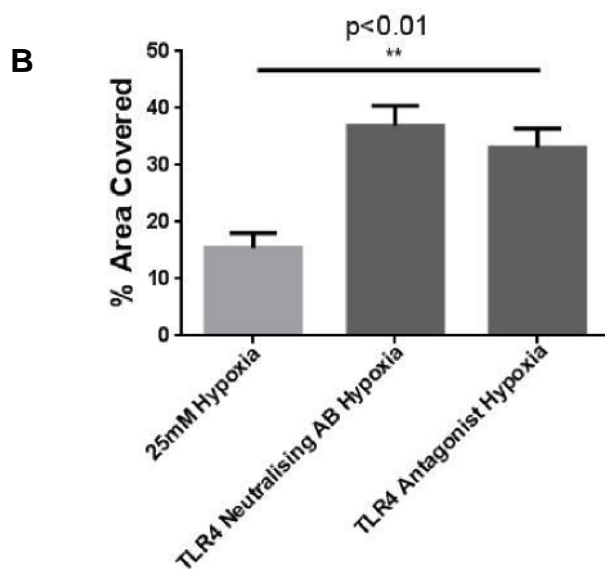
5.5mM

D

25mM

E

F

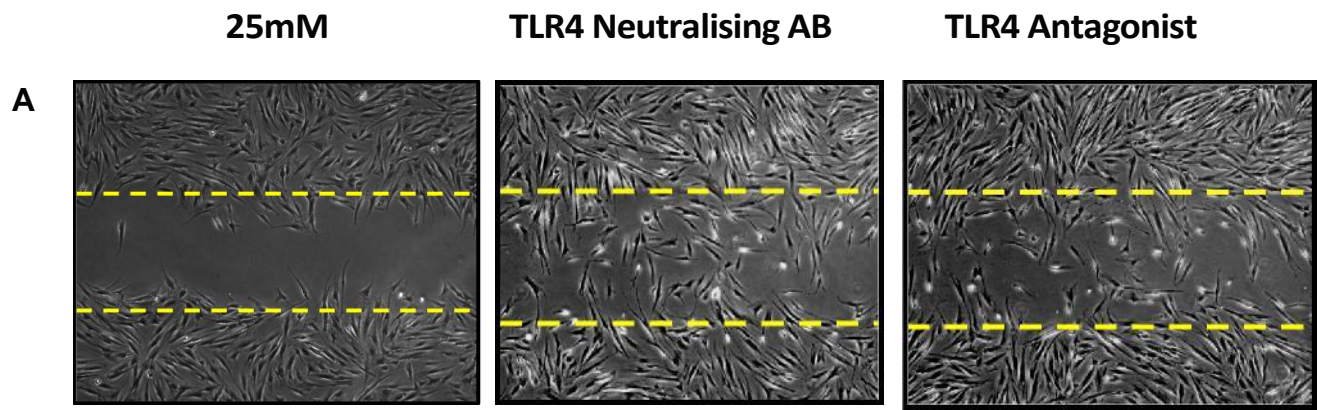**25mM**   **TLR4 Neutralising AB**   **TLR4 Antagonist**

A

B
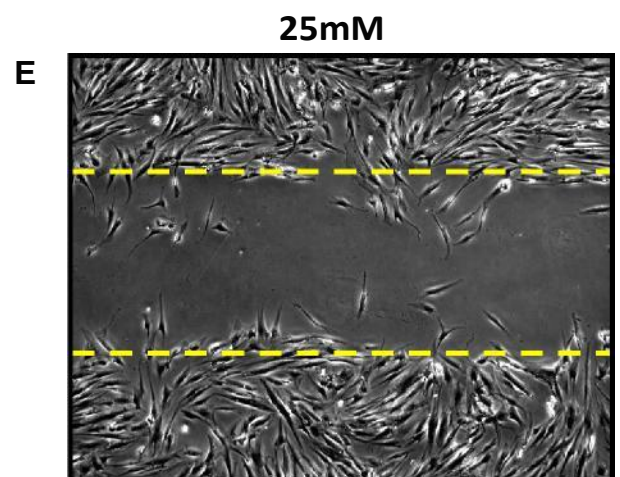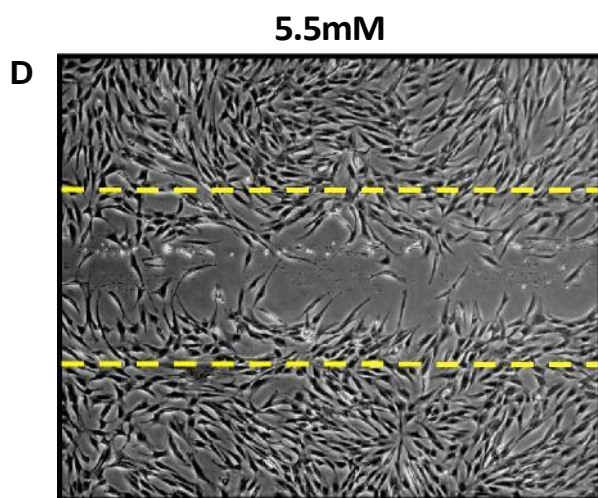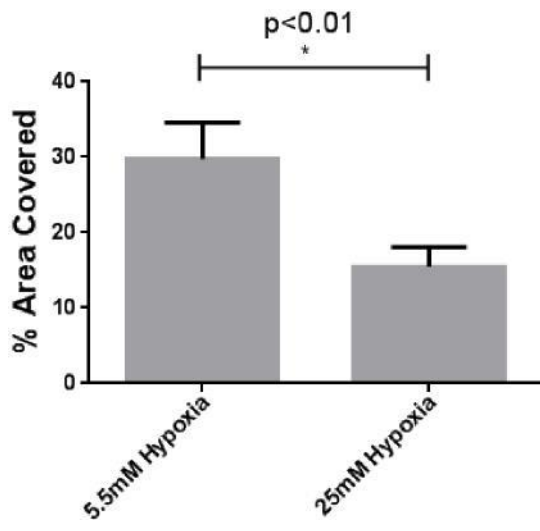
Figure 5: Scratch migration assay comparing fibroblast migration in VHG hypoxic conditions with the addition of a specific TLR4 neutralising antibody or antagonist (**A**). Fibroblast migration was significantly improved by the inhibition of TL4 in these conditions (**B**) (n=8, p<0.01, Krusal-Wallis test).

| | DAY 0 | DAY 3 | DAY 7 | DAY 14 |

A

DMI
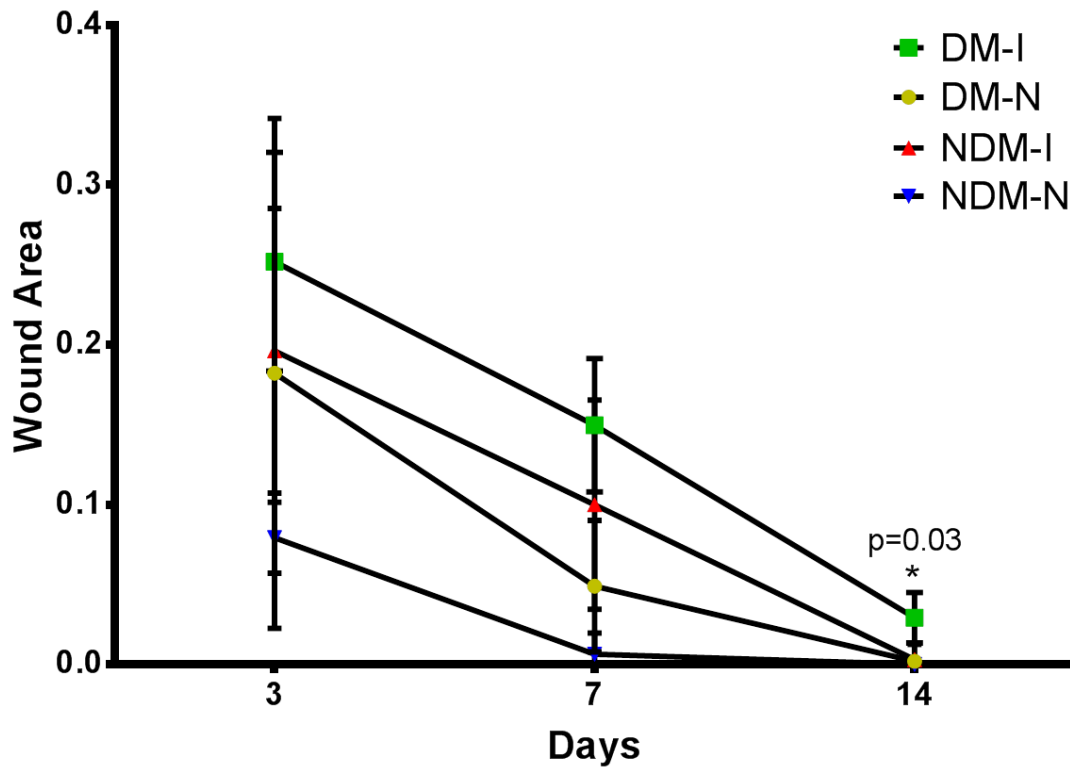
DMN

NDMI

B

NDMN

Figure 6: Wound healing in a murine model of diabetic ischaemic ulceration. Diabetic ischaemic (DM-I), diabetic non-ischaemic (DM-N), non-diabetic ischaemic (NDM-I) and non-diabetic non ischaemic (NDMN) wounds were photographed using digital photography. Representative images are shown (**A**). Wound area was measured using image J planimetry software. Wound areas at days 3, 7 and 14 were measured (**B**). Diabetes and hind limb ischaemia result in significant impairment of wound healing at day 14. (n=16, p=0.03, Kruskall Wallis test).
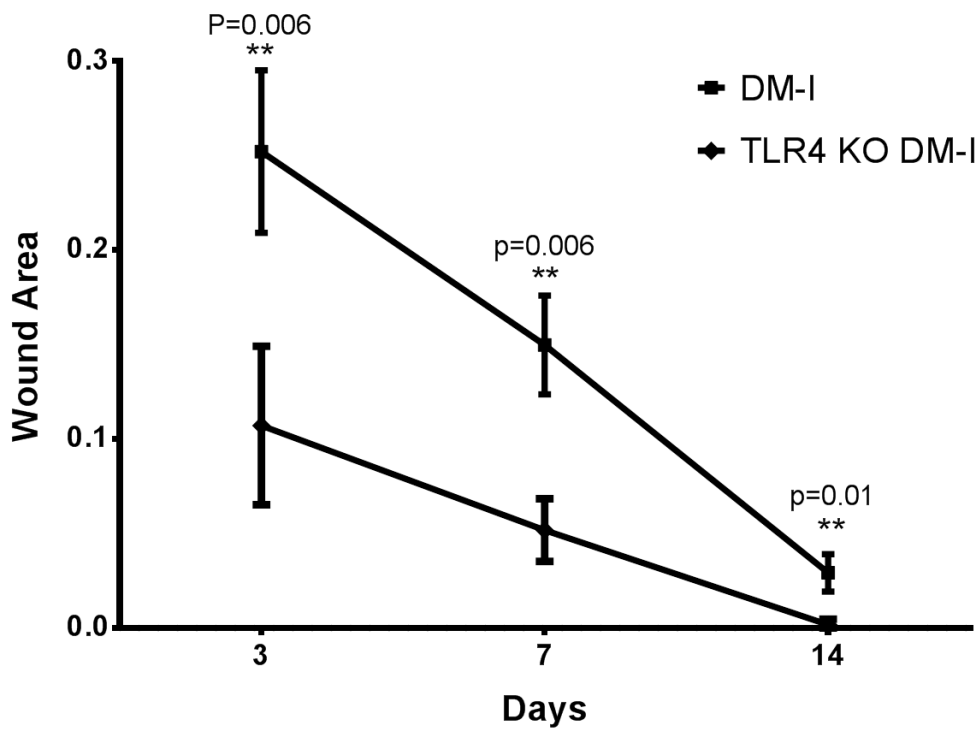
A
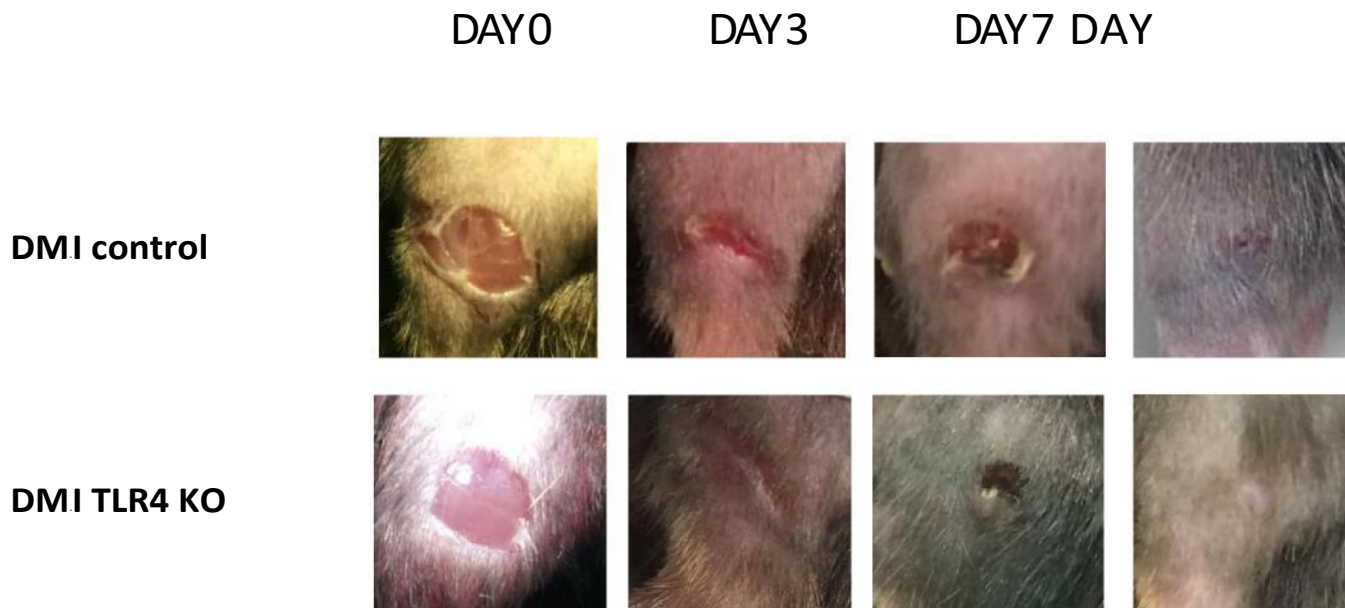
Figure 7: Wound healing in DM-I WT animals compared to DM-I TLR4 KO. Representative digital photographs shown (**A**). DM-I TLR4 KO animals demonstrate significantly improved wound healing at all time points compared to DM-I WT (**B**). (n=11, p<0.05, Kruskall Wallis test).