Topical insulin application accelerates diabetic wound healing by promoting anti-inflammatory macrophage polarization

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Abstract

Besides regulating glucose levels, insulin has been reported to participate actively in many other functions such as modulating inflammatory reaction. In this study we investigated how insulin application topically would effort the diabetic wound healing process. We found that the excessive expression of insulin degrading enzyme in diabetic skin led to the insufficient insulin level in diabetic skin during the wound healing, which ultimately reduced the recovery rate of diabetic wounds. We confirmed the topical insulin application could reverse the impaired inflammation reaction in diabetic wound environment and promote diabetic wounds healing. Our study revealed that insulin promoted neutrophils apoptosis and following triggered macrophages phenotype polarization. Both in vivo and in vitro studies verified that insulin reestablished the damaged neutrophils phagocytosis function and promoted the process of phagocytosis induced apoptosis of neutrophils. Furthermore, we found that the insulin treatment also promoted macrophages efferocytosis of the apoptosed neutrophils and thus induced macrophages polarized from M1 to M2. In conclusion, our studies proved that the exogenous application of insulin could improve the diabetic wound healing via the normalization of inflammatory response.

Key words: diabetes; wound healing; insulin; macrophages; neutrophils

Abbreviations list: IDE (insulin degrading enzyme); M1 (pro-inflammatory macrophage phenotype); M2 (anti-inflammatory M2 like phenotype); IR (insulin receptor); IGF-1 (insulin like growth factor 1); IGFR (insulin like growth factor receptor);

Introduction

Diabetes is a group of metabolic diseases characterized by polydipsia, polyphagia polyuria, emaciation and weakness(American Diabetes, 2004), along with the long-term complications such as diabetic nephropathy, diabetic retinopathy and lower extremity ulcer. About 15%~25% diabetic patients would experience diabetic foot ulcer during their lifetime (Leone et al., 2012).

Wound healing is a complex physiological event. The body's reaction to tissue injury involves various cells, cytokines and growth factors. Usually, this process is divided into hemostasis and inflammatory phase, proliferative phase and remodeling phase(Gurtner et al., 2008). Hemostasis and inflammation is the first chapter of wound healing and the neutrophils were the first nucleated cells that infiltrated into the wounds after injury(Dovi et al., 2004). Once a wound was formed the damaged tissue would activate platelet and numerous cytokines collectively recruit neutrophils to wound site (Kolaczkowska and Kubes, 2013). The main function of neutrophils is to maintain the aseptic environment by destructing the invading microbes and to devitalize tissue through secreting antimicrobial substance and protease (Dovi et al., 2004, McCarty and Percival, 2013). A strong neutrophil response during an early stage after wounding benefits wound healing by controlling the infection and preparing wound beds. However, prolonged existence of neutrophils combined with massive proteases and ROS produced by neutrophils degrade the growth factors and extracellular matrix, displays harmful effect on healing (McCarty and Percival, 2013). Along with the healing progress, the pro-inflammation environment that induces neutrophils infiltration needs to be depleted and neutrophils infiltration needs be braked progressively, and inflammation resolution occurs gradually(Ortega-Gomez et al., 2013). Wound neutrophils proceed to apoptosis and macrophage phenotype polarization is the central step of inflammation resolution. The persistent presence of neutrophils or failure of resolution has been confirmed the cause of chronic inflammation (Headland and Norling, 2015, Ortega-Gomez et al., 2013). Normally, circulating neutrophils experiences constitutive apoptosis during 24 hours and be engulfed in liver, spleen and bone marrow (Li et al., 2011, Tofts et al., 2011, Pillay et al., 2010, McCracken and Allen, 2014). In inflammatory or injured site, neutrophils may increase their lifespan, and eventually occur apoptosis through the mediation of phagocytosis-induced, TNF-α-induced or Fas-ligand-induced signaling pathway (Fox et al., 2010, McCarty and Percival, 2013). The apoptosed neutrophils release numbers of substance, called "find me signal", attract macrophage migration and phagocytosis. Efferocytosis,

the process of macrophage engulf apoptosed neutrophils would induce macrophage phenotype transformation from M1 to M2. The M2 macrophages secrete a number of cytokines and growth factors such as IL-10, TGF-β, PDGF; VEGF promotes the resolution of inflammation and induce the wound transition into proliferative stage (Ortega-Gomez et al., 2013, Soehnlein and Lindbom, 2010, Ariel and Serhan, 2012).

Compared to the normal wound healing process, however; the functions of diabetic neutrophils, including adhesion to endothelium, chemotaxis, phagocytosis and bactericidal activity were all impaired (Alba-Loureiro et al., 2006). The inflammatory cells dysfunction in the wound environment is the main pathological feature of diabetic wound healing. Macrophages phenotype polarization impairment is the most significant and important impairment which is characterized by macrophages persisted in a pro-inflammatory phenotype (M1 phenotype). Macrophages were considered as the manipulator of the wound healing process as macrophages manipulate wound healing through phenotype polarization from M1 to pro-healing phenotype (M2 phenotype). Along with the phenotype transformation is the healing process that is switched from the inflammation phase to proliferation phase. Many researches intend to promote diabetic wound healing by targeting to promotion of the impaired inflammatory phase.

Our previous and others works have proved that insulin not only acts as glucose regulating hormone but also as a growth like factor and cytokine regulator that could accelerate healthy non-diabetic rodent wound healing (Chen et al., 2012, Zhang et al., 2007). Many researches revealed that insulin could regulate neutrophils and macrophages functions which were impaired in diabetic state. In recent years, some others reported that insulin application could also accelerate diabetic wound healing in type Ⅰ diabetic rodent models (Lima et al., 2012). Type Ⅰ diabetes is caused by the lack of insulin secretion not by insulin resistance. It has been proven by researchers that the extra insulin supplementary could enhance the wound healing by reverse the insufficient state of insulin. However; most diabetic non-healing wounds are originated from type Ⅱ diabetic wounds which are characterized by insulin resistance and metabolic disturbances. Many of these patients are still in a high insulinemia state. The effects of the insulin application during the healing process of type Ⅱ diabetic wounds were not well studied. Thus, in this article we thoroughly investigated the effects of a topical insulin treatment for type Ⅱ diabetic wound healing and tried to understand the mechanisms behind these effects.

First, we established a diabetic rat model that owns the major characteristics of type Ⅱ diabetes including insulin resistant and hyper-insulinemia state, et al (Yang et al., 2016). In order to compare how the serum insulin and directly-applied insulin affect in the wound healing process, we further proved that the

skin tissue of the type Ⅱ diabetic rats were as sensitive as liver tissues while applied by the insulin stimulation (Yu et al., 2017). However, we further found a deficiency of insulin in both uninjured diabetic skin tissue and diabetic wounds, as compared to the non-diabetic wounds, even the serum insulin level was relative higher. In chronic wounds, there are hyper proteolytic enzymes activities, such as matrix metalloproteinases (MMPs) and serine proteases been detected (Muller et al., 2008, Loffler et al., 2013). The excessive wound proteases degrade extracellular matrix, growth factors and/or their receptors; therefore, they could lead to deficient growth factors pool in the wounds area, which accordingly would induce an impaired/non-healing (Wlaschek et al., 1997, McCarty and Percival, 2013). In recent years, some researchers reported a hyper insulin degrading enzyme (IDE) activity in the diabetic wounds (Tang, 2016). To reveal whether the insulin deficiency in the wound were caused by hyper IDE activity, we investigated the IDE and insulin level in the diabetic rat model. We found that the hyper-activity of IDE could degrade insulin in local tissues and lead to an insulin deficient state in tissues. This finding gives an illustration about why the serum insulin level is still high whereas local wound may be short of insulin stimulation which is manifested as diminished response of the local tissues to insulin stimulation. To solve the shortage of insulin stimulation in the local wound, we studied the topical insulin application on the healing of diabetic wound and found that the topical insulin application expedited the wound healing process by promoting the macrophage phenotype polarization.

Results

A relatively lower insulin level of diabetic skin and diabetic wounds was detected due to the over-expressed IDE level in diabetic skin tissue.

To compare the expression of insulin in diabetic skin, we first investigated the insulin level of uninjured skin tissue in both the normal rat and the diabetic rats. A significant lower insulin level (Fig.1A-B) was detected in diabetic skin although the serum insulin level was higher in these diabetic rats according to our previous studies (Yang et al., 2016). Further investigation of the insulin level during the wound healing process also confirmed the insufficiency of insulin in diabetic wounds (Fig.1C-D). To reveal whether the insulin deficiency in the diabetic wound were caused by hyper IDE activity, we further analyzed the IDE levels by ELISA, and obtained the IDE distribution by IHC. There were significantly higher IDE levels (Fig.1E-F) and extensive IDE distribution (Fig.1G-H) found in diabetic skin and diabetic wounds comparing with the

normal skin and normal wound. To further verify whether high IDE level could impact wound healing, we use IDE inhibitor 6bK to the diabetic wound topically combined with intraperitoneal injection. We found that diabetic wound with IDE inhibitor application heal faster than the control diabetic group (Fig.1I-J). The observation that high IDE level induced insulin insufficient state in diabetic skins and IDE inhibitor could accelerate diabetic wound healing which set up the fundamental for the extra insulin supplement topically treatment in the diabetic skin.

Topical insulin supplement promoted diabetic wound healing, macrophage infiltration and resolution.

After confirming the insulin deficient state of the diabetic skin and wound, we next investigated whether an extra insulin application could accelerate the diabetic wound healing. We first examined insulin level in both diabetic and insulin application group. We found that insulin application significantly elevated wound insulin level in insulin application group (Fig.2A-B). We next set up our experiments, three groups were divided and compared with the out comings: the healthy normal rats as the standard group (Norm), the diabetic group (DM) without insulin treatment and the diabetic group with insulin treatment (DM+Ins). As one of the features to define the wound healing process, we recorded the healing time of Norm, DM and DM+Ins group respectively. We found that the insulin application efficiently accelerated diabetic wound healing, shown by a lower un-healed wound proportion (Fig.2C) and less healing time (Fig.2D) in DM+Ins group than the diabetic without insulin treatment group (DM group). Compared to the DM group, rapid re-epithelialization (Fig.2E-F) and extensive extracellular matrix deposition (Fig.2G) were found in DM+Ins group. Many researches revealed that macrophages are the orchestrator of wound healing which was realized by macrophage polarization from M1 to M2 (Mantovani et al., 2013). The process of re-epithelialization and extracellular matrix deposition are all intensively regulated by M2 macrophage. Thus, macrophage polarization from M1 to M2 and the resolution, that is, the further migration of M2 macrophage out to the wound, are the key steps of wound healing, marking the progress of healing from inflammatory phase to proliferation phase. We also observed the infiltration and resolution of macrophage on wound areas. We found significantly impaired macrophage infiltration and resolution in DM group wounds when compared with the Norm group wounds which are shown by less infiltration at early stage of healing and delayed resolution at late stage of healing. Insulin application ameliorated the abnormal macrophage infiltration and resolution of diabetic wounds (Fig.2H-I). In DM+Ins group wounds, on the 4th day after wounding, there were much more macrophages infiltration, while on the 12th day after wounding, these inflammatory cells

began to withdraw from the wounds area. Thus, based on the observation and comparison of wound healing time, un-healed wound proportion, rapid re-epithelialization and extensive extracellular matrix deposition and resolution, we confirmed that compared to DM group, the application of tropical insulin in diabetic wounds (DM+Ins group) had significantly promoted the wound healing process, which ultimately enhanced the infiltration and resolution of macrophages around the wound areas.

Insulin application regulated and promoted macrophage phenotype polarization from M1 to M2 in diabetic wound

It is well known that the macrophages are involved in manipulation of wound healing through their phenotype polarization. M2 macrophages progress the wounds towards the healing process, meanwhile M1 phenotype keeps the wound remaining in the chronic inflammation state (El Kebir and Filep, 2010, Mantovani et al., 2013). To investigate if the infiltration and resolution of macrophages were results of the macrophage polarization transformation, we next investigated the macrophages phenotype polarization after insulin application. We observed the extensive and persistent pro-inflammatory, M1(iNOS positive) phenotype of macrophage in the DM group wounds at day 9 and day 12 after wounds were created. Insulin application considerably alleviated M1 macrophage accumulation (Fig.3A-B). The number of M2 phenotypes marker (arginase 1) positive cells in the DM group were significantly lower than that of the Norm group at 9 and 12 days after wounding. M2 also became the major phenotypes of macrophage in wounds since day 9 after wounding in DM+Ins group which suggested that the topical application of insulin in the wounds promoted macrophage polarized to M2 phenotype (Fig.3C-D). To further confirm this observation, we subsequently detected pro-inflammatory and anti-inflammatory mediators/markers in the wounds. M1 macrophage related pro-inflammatory mediators/marker IL-1β and iNOS were significantly higher in the DM group wounds at day 9 and day 12 after wounding. Insulin application significantly decreased IL-1β and iNOS level, enhanced the expression of M2 related cytokine/marker, IL-10 and CD206 in the DM+Ins group wounds (Fig.3E-I). These results proved that insulin application simulated and promoted macrophage phenotype polarization from M1 to M2 and thus expedited the diabetic wound healing.

Insulin application promoted neutrophils infiltration and apoptosis

We have proven that the tropical insulin application could stimulate an improved macrophage reaction in diabetic wound. Since cytokine is the main substance secreted during hemostasis and inflammatory phase, we performed cytokines array analysis to comprehensively understand the influence of insulin application on macrophages response and inflammatory reaction (Fig.4A). We set the level of cytokines of the normal wound group in the first wounding day as a control/base number and the relative expression rates of other groups were laid out (Fig.4B). The relative expression rate is defined as the ratio of the expression level of cytokines of detected sample to the level of cytokines of the Norm group wounds in the first wounding day. The protein arrays further confirmed that the macrophage phenotype polarization from M1 to M2 was severely impaired in the DM group. The M1 phenotype related markers or related pro-inflammation cytokines such as CD86, IL-1β, CNTF and IL-6 were higher in the DM group wounds from the 9th day and even in the 12th day after wounding (Fig.4C). At the same time the expression of M2 related marker and related cytokine or growth factor such as IL-4, IL-10, PDGF and TIMP-1 were lower in diabetic group when compared with the Norm group wounds. Insulin application reversed these abnormalities as the DM+Ins group wounds showed decreased expression of M1 related cytokines and increased expression of M2 related markers (Fig.4C-D). These observations confirmed that insulin application promoted the macrophage phenotype polarizing from M1 to M2 which is impaired in diabetic wound environment. Besides, we found that the cytokines related to neutrophils chemotaxis, survive and apoptosis were also actively regulated by the insulin application. The chemokines functioning as neutrophils recruiting and activating such as Thymus Chemokine-1, CINC-1, CINC-2 and CINC-3, were relatively lower in the DM group wounds on the 1st day after wounding when compared with the Normal group wounds. Insulin application to DM+Ins group increased the expression of these chemokines to the levels that are very close to the Norm group. The expression of Thymus Chemokine-1, CINC-1, CINC-2 and CINC-3 were significant decreased on day 9 and day 12 after wounding in the Normal group wounds, whereas in diabetic wounds these chemokines were still in a relative high expression level. These chemokines expression pattern revealed that in diabetic wound the neutrophils infiltration and resolution were impaired and even in the late stage of inflammatory phase large amounts of neutrophils still exist in wounds. It is worth noting that consistent neutrophils infiltration is commonly seen in most chronic wounds. In insulin-treated wounds, the expression pattern of neutrophil chemokine was similarly to the normal wounds (Fig.4E), which suggested that topical insulin application

alleviates impaired neutrophils infiltration and resolution. The expression level of neutrophils survival and apoptosis related cytokines GM-CSF, Fas-ligand and TNF-α, and the neutrophil specific proteinase MMP-8 expression also confirmed this finding (Fig.4F). To further validate that insulin application improved neutrophils infiltration and resolution, we checked the neutrophils infiltration and resolution through H&E, IHC and TUNEL staining. H&E combined with IHC staining showed delayed neutrophils infiltration at the early stage and impaired resolutions at the late stage in the DM group wounds (Fig.4G-I). The TUNEL analysis was also applied to check the status of neutrophils apoptosis. Neutrophils apoptosis were detected using TUNEL and neutrophil marker CD66b double staining. Comparing with the Norm group wounds, fewer apoptotic neutrophils were found in DM group wounds at day 4 after wounding, insulin application significantly increased apoptotic neutrophil number in wound area of DM+Ins group (Fig.4J-K). Insulin application accelerating inflammatory cell infiltration and resolution in diabetic wounds suggested that topical insulin application reversed impaired diabetic inflammatory response.

Apoptosed neutrophils further positively regulated and promoted macrophage phenotype polarization from M1 to M2

To determine whether insulin could directly regulate inflammatory cells function, we detected the expression of insulin receptor (IR) and insulin like growth factor receptor (IGFR). These are main receptors that are involved in insulin signal pathway, in neutrophils and macrophages. We detected positive IR ad IGFR expression in both peritoneal neutrophils and macrophages (Fig.5A-B). Meanwhile, we also observed positive IR ad IGFR expression in wound neutrophils and macrophages (Fig.5C-D) which suggested that insulin could regulate inflammatory cell function in an IR or IGFR dependent manner.

Neutrophils were the first nuclear cells infiltrate into the wound after wounding. Recent researches reported that the apoptosed neutrophils could induce macrophages phenotype transition and this transition is regarded as the initiator of macrophage phenotype switching from M1 to M2(El Kebir & Filep, 2010; El Kebir & Filep, 2013; Wilgus, Roy, & McDaniel, 2013). Is the insulin application induced improvement of macrophages phenotype polarization correlated to the enhanced neutrophils apoptosis in diabetic wound environment? We next investigated the apoptosed neutrophils on macrophages phenotype transition. We isolated neutrophils and macrophages from rat peritoneal cavity. In an *in vitro* study, we used apoptosed neutrophils to stimulate M1 macrophages to check the apoptosed neutrophils on macrophages polarization. After efferocytosis of apoptosed neutrophils, macrophage initiated the phenotype polarization from M1 to

M2 phenotype. The higher expression of CD206 and IL-10 and lower expression of iNOS were detected by Western blot in the apoptotic group (Fig.5E-H). The immunofluorescence stain also confirmed this phenotype transformation of macrophages shown by down-regulated the expression of M1 related markers and up-regulated the expression of M2 related markers (Fig.5I-L). Validated by these results, we confirmed that the apoptosed neutrophils could promote macrophage phenotype polarization.

Based on these results, we further studied whether promoting function of the insulin on macrophage phenotype transformation from M1 to M2 was realized by the promotion of neutrophils apoptosis. Neutrophils apoptosis during wound healing is mainly induced by phagocytosis (the process of phagocytosis induced apoptosis). We next investigate the effect of insulin on neutrophils phagocytosis function and the process of phagocytosis induced apoptosis. We found that the phagocytosis of diabetic derived neutrophils was significantly damaged. Fewest engulfed FITC-E-coli were observed in diabetic neutrophils. An obviously enhanced phagocytosis function was observed in diabetic neutrophils at the presence of insulin, which suggested that insulin promoted neutrophils phagocytosis function (Fig.5M). We next investigated the phagocytosis induced apoptosis of neutrophils after engulfing heat killed E-coli. After incubation, apoptosis of neutrophils was measured by analysis of caspase 3 expression. Significantly lower expression of cleaved caspase 3 was noticed in diabetic neutrophils. Insulin application promoted expression of cleaved caspase 3 and further apoptosis (Fig.5N-O). Macrophage efferocytosis, the process of macrophage phagocytosis of apoptotic neutrophil, induces macrophage phenotypes to switch from M1 to M2 phenotype (Ortega-Gomez et al., 2013). We then studied macrophages efferocytosis functions. We found significantly impaired diabetic peritoneal macrophages efferocytosis comparing with macrophages from normal rat peritoneal cavity. Insulin also significantly increased diabetic macrophages efferocytosis (Fig.5P).

All these results showed that insulin application could normalize the impaired functions of neutrophils and macrophages in diabetic wound, induce a balanced inflammation reaction and promote wound healing.

Discussion

Many researches have revealed that blood normalization by insulin could improve the neutrophils dysfunction of diabetes patients. Some researches attributed the recovery of neutrophil function to the blood glucose control whereas others discovered that the insulin promoted neutrophils function was independent of glucose normalization and attributed the improvement of neutrophil function to the direct effect/application of insulin (Lecube et al., 2011, Walrand et al., 2004). In this research we found a deficiency of insulin in both uninjured diabetic skin tissue and diabetic wounds, as compared to the non-diabetic wounds, even though the serum insulin level of diabetic group was relative higher than the non-diabetic group. In conjunction with the low insulin expression, high IDE expression was detected in diabetic skin and diabetic wounds. IDE is supposed to express in all tissues including the skin and it exists in many subcellular compartments and the extracellular milieu. In diabetic state many factors may contribute to the high expression of IDE, including high plasma glucose, high level of free fatty acids (FFA), and pro-inflammatory cytokines, such as IL-6 (Tang, 2016). In our study, we found a consistently high level of IDE level in diabetic wounds, and that IDE level in diabetic wound decrease along with the progress of healing process, which is similar to the change of pro-inflammatory cytokines level. As for insulin expression, in early stage of non-diabetic wound healing, the high level of insulin in wound area may come from plasma due to increased blood vessel permeability during inflammatory response. As healing achieved, vascular endothelial cells undergo apoptosis and wound blood vessel occlusion occur, and insulin level decrease correspondingly. In diabetic wound healing, however, a higher IDE level in early stage of healing degraded wound insulin and lead to a low insulin expression, meanwhile, delayed diabetic wound healing could induced a delayed and persistent insulin accumulation in wounds. Our result suggested that the excessive IDE activity lead to a lower insulin level in diabetic wounds; which provides the possibility of accelerating wound healing by topical insulin application.

The presence of IR and IGFR in neutrophils and macrophages propose the possibility of insulin regulate diabetic neutrophils and macrophages' function directly. We utilized various manners, including H&E and IHC staining, immunofluorescent staining and cytokine array analysis to investigate diabetic wound inflammatory response. All results conformably demonstrated the delayed but consistent infiltration of neutrophils in diabetic wounds, which is a typical feature of chronic inflammation (Pierce, 2001, Nunan et

al., 2014, Allen et al., 2007). *In vitro* study revealed an impaired phagocytosis function of neutrophils. Delightfully, the impaired neutrophils function was rectified by insulin application. Besides neutrophil, impaired macrophage function, specifically, impaired phenotype transition was also noticed in diabetic wounds. It has been known that apoptotic neutrophils are capable of inducing macrophage phenotype transition. To explore diabetic neutrophils apoptosis, we measured neutrophils apoptosis related mediators. As reported, TNF-α regulated neutrophils apoptosis in a bi-directions way, which means that TNF-α could promote the apoptosis of neutrophils in a high level and the survival of neutrophils in a moderate or low level. Protein array showed that the TNF-α level in the diabetic wound was persistent in a medium level, whereas the normal group and the insulin application group had an expression peak in the 4th day after wounding (Luo and Loison, 2008). Consist with the TNF-α expression peak in the normal group and insulin application group, the high rate of neutrophils apoptosis was found in both groups. The Fas-ligand and GM-CSF analysis also revealed that the neutrophils tend to survive in the diabetic wound environment (Martin et al., 2015). The prolonged survival of neutrophils may disrupt macrophage polarization for the lack of sufficient stimuli that trigger macrophage polarization(Mantovani et al., 2013). The macrophage phenotype polarization related cytokines also confirmed these discoveries. We found that the M1 related cytokines were relative higher whereas the M2 related cytokines were persistently lower in the diabetic group. Insulin supplement may normalize the impaired migration and phagocytosis function of neutrophils. Phagocytosis induced apoptosis was also confirmed by in vitro examination. By engulfing the apoptosed neutrophils, the macrophage initiates the phenotype polarization from M1 to M2 and promotes the resolution of inflammation of the wound (Poon et al., 2014). One flaw our study is that we did not exclude the possibility of insulin directly affect macrophage phenotype transformation. However, to the best of our knowledge, the apoptosis of neutrophils may be more crucial in triggering macrophage polarization during wound healing. In the supplementary figure we used BALB/c mice to observe the direct effect of neutrophils on macrophages polarization in wounded skin tissues. We found that neutrophils depletion could impair macrophages polarization from M1 to M2 during wound healing and this result was consistent with the other scientists' work (El Kebir & Filep, 2010; El Kebir & Filep, 2013; Wilgus, Roy, & McDaniel, 2013). In sum, our researches have revealed that the extra insulin application could normalize the wound insulin level locally, restore neutrophils and macrophage function in the wound, and ultimately promote diabetic wound healing.

In addition to the above conclusions, there are still some considerations need to be discussed. First of all, in diabetic state, there do exist some impairment of insulin signal pathway in neutrophils and

macrophages. However, many researches also reported that insulin could promote the function of neutrophils and macrophages in diabetes, such as insulin can promote the phagocytosis and killing of neutrophils, insulin can promote the expression of scavenger receptor in macrophages of diabetic rodents thus enhance their phagocytic function and so on (Walrand et al., 2004, Walrand et al., 2006, Alba-Loureiro et al., 2006). We argue that the damage of neutrophil and macrophage function in diabetes is caused by many mechanisms. Insulin level is one of them. Other mechanisms, such as high glucose level, AGEs accumulation and oxidative stress, can also lead to the damage of neutrophil and macrophage function (Berbudi et al., 2020, Baltzis et al., 2014, Frydrych et al., 2018, Forbes and Cooper, 2013). In this circumstance, the results in Fig. 5M and 5P could occur that showed reduced activity of neutrophils and macrophages isolated from diabetic rats comparing to those from normal rats which were treated with no insulin. The purpose of this research in focused on high level of insulin degrading enzyme in diabetic wound degrades insulin, which leads to the wound insulin insufficient state. Neutrophils and macrophages in the wound lack sufficient insulin to activate their functions and extra insulin supplement could improve the function of neutrophils and macrophages, and then promote diabetic wound healing. Secondly, we did not examine the exactly downstream signal pathway how insulin affect neutrophils and macrophages function. According to our previously and other researchers' work, insulin may regulate neutrophils activities through activation of PI3K-Akt, SPAK/JNK and p38MAPK signaling pathway when binding with IR or IGFR. Its downstream targets were related to the increased functions of neutrophils including chemotaxis, phagocytosis, and bactericidal capacities(Liu et al., 2018, Yano et al., 2012, Oldenborg and Sehlin, 1998).

Materials and Methods

Reagents: Streptozocin（STZ）and thioglycolate were from Sigma-Aldrich（St. Louis, MO）, 60% high fat diet was from Research Diets Inc.（New Brunswick, NJ）, insulin was purchased from Lilly USA (LLC, Indianapolis), insulin for cell culture and TUNEL assay kit were obtained from Roche (Basel, Switzerland), DAPI (life technologies, Carlsbad, CA), FITC labeled E-coli and cell tracker green were purchased from Thermo fisher (Carlsbad, CA), RPMI 1640, DMEM and PBS was obtained from GIBCO (Carlsbad, CA), O.C.T. compound (Sakura Finetek, Torrance, CA), sandwich ELISA rat cytokine array G2 analysis was from

Ray Biotech (Norcross, GA), arginase was from cell signaling technology (Danvers, MA), chemiluminescence was from Millipore (Billerica, Massachusetts), Insulin degrading enzyme (IDE) inhibitor 6bK was from R&D Systems (Minnneapolis, MN) .

The following antibodies were obtained from various suppliers: The following antibodies were obtained from various suppliers: iNOS (life technologies, Carlsbad, CA, catalogue number: PA1-036); insulin receptor(catalogue number: ab5500), IGF-(catalogue number: ab36532), IGF receptor(catalogue number: ab182408) , IL-1β (catalogue number: ab9722),

IDE(catalogue number: ab32216), SOCS-1(catalogue number: ab9870)were from Abcam (Cambridge Science Park, Cambridge); insulin(catalogue number: 4590S), cleaved caspase-3(catalogue number: 9661S) and HRP labeled secondary antibody(catalogue number: 7074S, 7076S) (cell signaling technology, Danvers, MA); CD206 (catalogue number: sc-376232) and IL-10 (catalogue number: sc-365858) (Santa Cruz Biotechnology, Santa Cruz, CA); CD11b(ABclonal Biotech, Woburn, MA, catalogue number: A1581);CD68(Bio-Rad, Philadelphia, PA, catalogue number: MCA5709); donkey anti-rabbit alexa flour 488(catalogue number: R37118), donkey anti-mouse alexa flour 488(catalogue number: R37114), donkey anti-mouse alexa flour 594 (catalogue number: A-21203) were purchased from Thermo fisher (Carlsbad, CA).

Animal: 90 male Wistar rats, 6 weeks old, weighting 120~140 g were purchased from Shanghai Laboratory Animal Center in the Chinese Academy of Sciences and housed at the Animal Science Center of Shanghai JiaoTong University, School of Medicine (SJTUSM). The animals were maintained under a 12 hours light/dark cycle at 22°C. 60 rats were used to develop diabetes. The induction of diabetic was described previously(Yang et al., 2016). In brief, 60 rats were fed with 60% high fat diet (HFD) for 8 weeks. The rats were then fasted for 16 hours followed by multiple low dose intraperitoneal injections of STZ (10 mg/kg body weight (BW) dissolved in 0.1 mM citrate buffer) for four consecutive days. Rats were left to develop diabetes for 5 weeks. Random plasma glucose was measured and the glucose level >300 mg/L were considered as diabetic. The remained 30 rats fed with normal diet were set as normal control (Norm). The characteristics of this diabetic model have been described in our previously work. In general, a combination of HFD feeding for 8 weeks and four consecutive days of low dose STZ injections induced rats to develop type II diabetes with noticeable insulin resistance, persistent hyperglycemia, moderate degree of insulinemia, as well as high serum cholesterol and triglyceride levels. The animal procedures were performed in accordance with the rules of the Animal Care Committee of SJTUSM, and all experiments' protocols were

approved by the SJTUSM Institutional Animal Care and Use Committee.

Wounding procedure: One day before wounding, each 90 rats (30 healthy normal rats and 60 diabetic rats) was anaesthetized with a single intraperitoneal injection of thiopental sodium (40 mg/kg BW), the hairs on the back were shaved and thoroughly removed using Nair Hair remover lotion. On the day of wounding, the rats were anaesthetized with thiopental sodium and a dose of Tramadol. The animals were fixed on a hollow plank and the back was immersed in water at 80°C for 8 seconds to produce a 6*8 cm deep second degree burn wound covering almost 20% TBSA. 8 ml saline was intraperitoneally injected for resuscitation immediately after injury. The diabetic rats were then randomly divided into 2 groups randomly, the insulin group received 0.2 u insulin/2 ml saline through intrawound injection (DM+Ins) and the control group (DM) received 2 ml of saline injection every day. The healthy normal rats also received 2 ml saline every day after wounding until fully healed.

Measurement of wound closure: Rats were anaesthetized with thiopental sodium at day 1, 4, 12, 16 and 20 after the wounding. The wounds were photographed and drawn on transparent tracing paper. The wound sizes were analyzed using ImageJ software. The unhealed rate was calculated by comparing the unhealed wound area to the original wound area.

For the examination of IDE inhibition on diabetic wound healing: 3 healthy normal rats (Norm group) and 6 diabetic rats were allocated in this experiment. The diabetic rats were divided into 2 groups randomly (DM group and DM+IDE inhibitor group) after the preoperative preparation described as above. The animals' back was punctured to produce 6 wounds and the diameters were 1cm. IDE inhibitor 6bK（0.1mg/ml dissolved in PBS）was used by intraperitoneal injection (80mg/kg) for 3 consecutive days combined with 20ul daily wound topically application until wound healed. Wound were photographed and drawn as described above on day 1, 4, 9 and 12.

Histological observation: The wounds, including 5 mm adjacent normal skin, along with the subcutaneous fat tissue were harvested at day 1, 4, 9 and 12 after wounding. The tissue was fixed in 4% paraformaldehyde and embedded in paraffin. Sections with 6~7 μm thickness were stained with hematoxylin and eosin (H&E) for histological and morphometric observation and evaluation. Masson-trichrome staining was used for collagen deposition evaluation.

Immunohistochemistry and Immunofluorescence: The wounds were harvested and fixed as mentioned above and sections were then de-paraffinized, rehydrated, and washed in distilled water. The sections were

placed in 95~98℃ antigen retrieval citrate buffer in a container for 10~15 minutes. Endogenous peroxidase activity was blocked by placing the sections in 3% hydrogen peroxide in methanol for 10 minutes. Non-specific staining was blocked with normal goat serum, and the sections were incubated with anti-rat IDE, CD66b and CD68 overnight at 4°C. After washing, HRP-labeled secondary antibody was applied for 1 hour at room temperature then stained with diaminobenzidine, and counterstained with hematoxylin.

TUNEL procedure: The procedures were performed as the instruction of the Roche applied science. Dewax paraformaldehyde fixed tissue sections according to standard procedures. Place the slides in a plastic jar containing 200 ml 0.1 M citrate buffer, PH 6.0. Apply 750 W microwave irradiation for 1 minutes. Cooling rapidly by immediately adding 80 ml double distilled water. Transfer the sides into PBS. Immerse the slides for 30 minutes in Tris-HCl, 0.1 M PH 7.5, containing 3% BSA and 20% normal bovine serum. Rinse the slides twice with PBS. Add 50 µl of TUNEL reaction mixture on the section. Incubate for 60 mins at 37°C in a humidified atmosphere in the dark. Rinse slides three times in PBS for 5 minutes each and the incubate the neutrophils marker CD66b in the dark wet chamber overnight and then rinse slides three times in PBS for 5 minutes each. Add the deep-red conjugated second antibody for 1hour. Rinse the slide three times again with PBS. Mount the slides with prolong anti-fade reagent with DAPI and then evaluate the section under a fluorescence microscope.

Immunofluorescence: Tissues were fixed in 4% paraformaldehyde at 4℃ for 4 hours followed by grade dehydration in 30, 20 and 10% sucrose for 30 minutes. The specimens were then embedded in O.C.T. compound and stored in -80℃ until use. The tissues were sectioned in 5 µm and placed in poly-L-lysine pre-coated slides. The slides were washed three times in PBS and then blocked in 5% BSA for 30 minutes at room temperature. Then, the primary antibodies (iNOS and CD68 for detection of M1 macrophages, CD68 and arginase 1 for detection of M2 macrophages, IR and IGFR combined with CD68 and CD66b for detection of IR and IGFR expression in neutrophils and macrophages) were incubated overnight at 4℃ in wet chamber. After washing with PBS for three times the secondary antibodies conjugated with FITC or rhodamine were incubated in room temperature for 1hour in a darkened wet chamber. Finally, the sections were washed with PBS for three times and mounted with the Pro-Long anti-fade reagents with DAPI. Tissues were observed under a fluorescence microscope (ZESIS) at a magnification of 40X.

Isolation of neutrophils and macrophages:

Isolation of neutrophils and macrophages were performed as described(Swamydas et al., 2015). In brief, rats were injected intraperitoneally with 10 ml of 3% thioglycollate medium to induce sterile peritonitis.

Peritoneal exudate cells were harvested by intraperitonealy injection of 10 ml of cold PBS or DMEM at 4 hours and 3 days after thioglycollate injection for neutrophils and macrophages isolation respectively. The isolation and purification of neutrophils or macrophages were then performed according to followed procedures.

Neutrophils: The peritoneal exudate cells were centrifuged at 250 g for 10 minutes at 4℃ and then re-suspended in 10 ml of erythrocyte lysis and incubated for 10 minutes at 37°C. Neutrophils were collected by centrifuged cell suspension at 250 g for 10 minutes and re-suspended in RPMI 1640 for next analysis.

Macrophages: The harvested cells were cultured in the plates and left cells to adhere for 2 hours. The un-adherent cells were washed with PBS for 3 times and then cultured in DMEM with 10% FBS and PS for the further analysis.

Neutrophils phagocytosis analysis: neutrophils from both the diabetic rats and normal rats peritoneal were stimulated with FITC labeled E-coli for 1 hour. The culture medium was removed and washed. cells were subjected to the fluorescence microscope analysis. Neutrophils phagocytosis was observed when neutrophil uptake of FITC labeled E-coli and became FITC-positives. Phagocytosis function index was calculated as the ratio of the phagocytosis neutrophils to un-phagocytosis neutrophil.

Neutrophils phagocytosis induced apoptosis assay: both normal and diabetic neutrophils were subjected to the heat killed E coli stimulation, 18 hours after stimulation the cells were collected and lysed for apoptosis analyze.

Apoptotic neutrophils induce macrophage polarized from M1 to M2 phenotype: Neutrophils were collected from peritoneal and cultured for 24 hours. After confirming neutrophils were apoptosed. The apoptosed neutrophils were then collected and co-culture with macrophages. The latex beads were set as a negative control of apoptosed neutrophils.

Wound cytokine array analysis: At 1, 4, 9 and 12 days after injury, the wound tissue were harvested and stored in -80°C. The tissue was homogenized by pulverization in liquid nitrogen and transferred to tissue lysis buffer with a protease inhibitor cocktail followed by centrifugation at 12000 R.P.M for 15 minutes. The supernatant was removed and followed to the 34 cytokines preconfigured sandwich ELISA rat cytokine array G2 analysis. The procedures were accorded with the protocol offered by Ray Biotech (4). The analysis was performed using the manufacturer's recommended protocol, and the signals of Cy3 were imaged by Axon Gene Pix laser scanner (MDS analytical technologies, Sunnyvale, CA). Any ≥1.5-fold increase or ≤0.65-

fold decrease in signal intensity groups may be considered a measurable and significant difference in expression

Western blot: The tissues were homogenized by pulverization in liquid nitrogen and transferred to tissue lysis buffer with a protease inhibitor cocktail following centrifugation at 12000 R.P.M for 15 minutes. The supernatants were removed and stored at -80℃. An equal amount of protein per lane (50 μg) was separated by 5~12% SDS-PAGE and transferred to a polyvinylidene difluoride membrane. The membranes were blocked by 5% non-fat powdered milk in Tris-buffered saline with Tween-20 (TBST) and then incubated with anti-insulin IGF-1, iNOS, CD20, IL-1β, IR, IGFR, cleaved caspase-3 and IL-10 primary antibody in 5% non-fat milk in TBST overnight at 4℃. The membrane was then washed extensively with TBST, and then incubated with the secondary antibody for 1 hour at room temperature. Bands were visualized with enhanced chemiluminescence. Relative quantities of protein were determined using a densitometer and presented in comparison with β-actin expression.

Statistical Analysis: Data analysis was performed using Graph Pad Prism software (Graph Pad Software Inc.). T-tests were used to determine the significance of pair-wise differences between means, unpaired t-tests for comparison between two groups and one-way ANOVA (Dunnett's post hoc test) were used to determine significance between means of several groups. Data satisfying the assumptions of ANOVA were verified before performing the tests. The p-value less than 0.05 were considered statistically significant, and the p-value less than 0.01 were considered statistically highly significant. Data are shown as mean ± standard deviation (SD).

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Competing interests:

none declared.

Founding:

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Figures

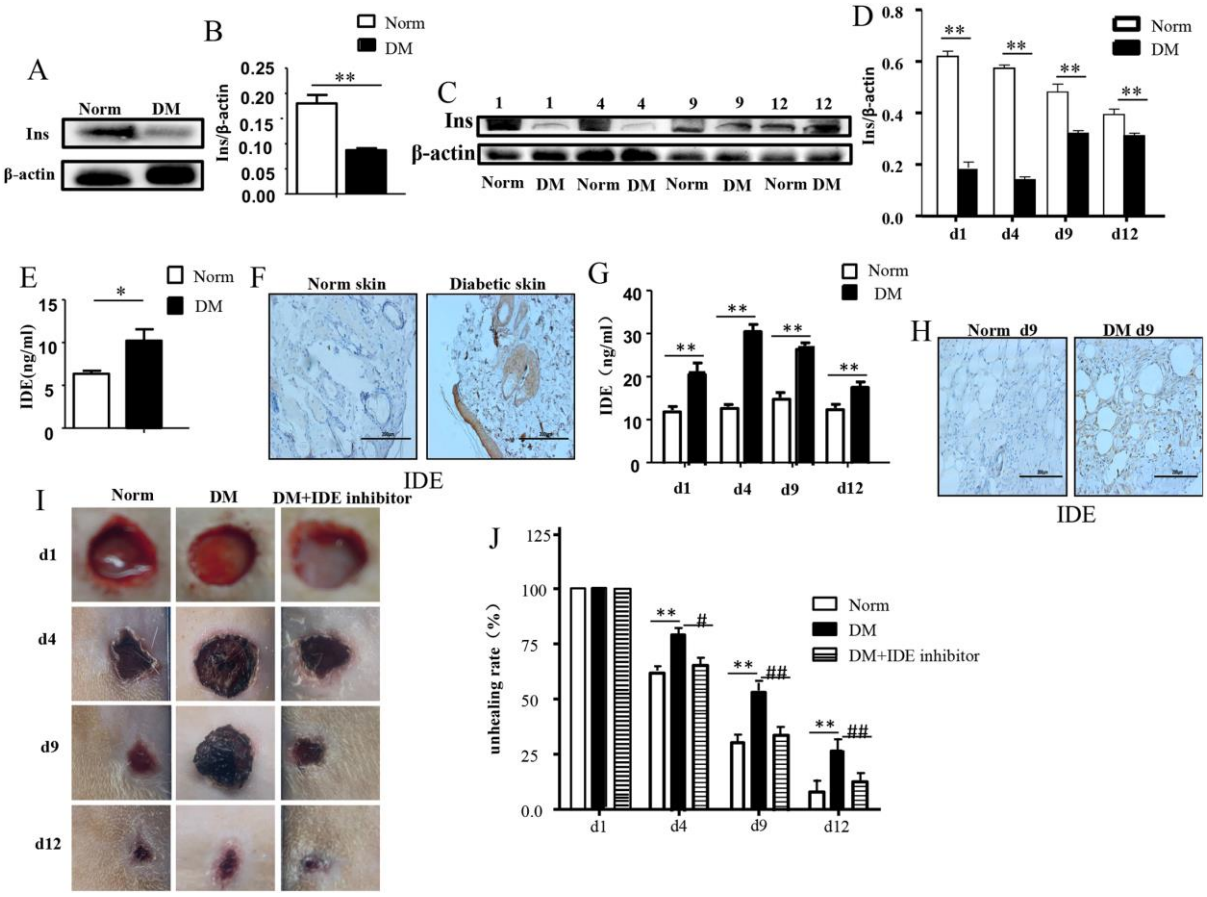

Figure 1 Diabetic skin and diabetic wounds showed a lower insulin level which possibly caused by over-expressed of IDE in diabetic skin tissue.

A-B. The insulin levels in the normal and diabetic rats' dermo were analyzed by western blot. Data are shown as the mean ± SD. *p <0.05, **$p<0.01$, n=5. **C-D.** Insulin levels in 1st, 4th, 9th and 12th day's wounds were also analyzed using western blot. Data are shown as the mean ± SD. *p <0.05, **$p<0.01$, n=5. **E.** To examine the IDE expression pattern in the diabetic state, the dorsal skins from diabetic and normal rats were harvested and homogenized, the supernatants were subject to ELISA examination. Data are shown as the mean ± SD. *p <0.05, **$p<0.01$, n=5. **F.** In situ examination of IDE expression in normal and diabetic skin were detected by immunohistochemistry. **G.** To further examine the IDE expression level during wound healing, the wounds were harvested and homogenized, the supernatant were subject to ELISA examination of the IDE expression in different time of wound healing. Data are shown as the mean ± SD. *p <0.05, **$p<0.01$, n=5. H. The in-situ examination of IDE expression in the wound were also examined by immunohistochemistry. **I-J.** IDE application on diabetic wound healing. Wounds size were record at day 1, 4, 9 and 12 days after wounding using transparent tracing paper. Unhealed rate of wounds was calculated by Image Pro Plus. Data are shown as the mean ± SD. *$^{/\#}p$ <0.05, **$^{/\#\#}p<0.01$, n=3. *means Norm VS DM, #means DM VS DM + IDE inhibitor.

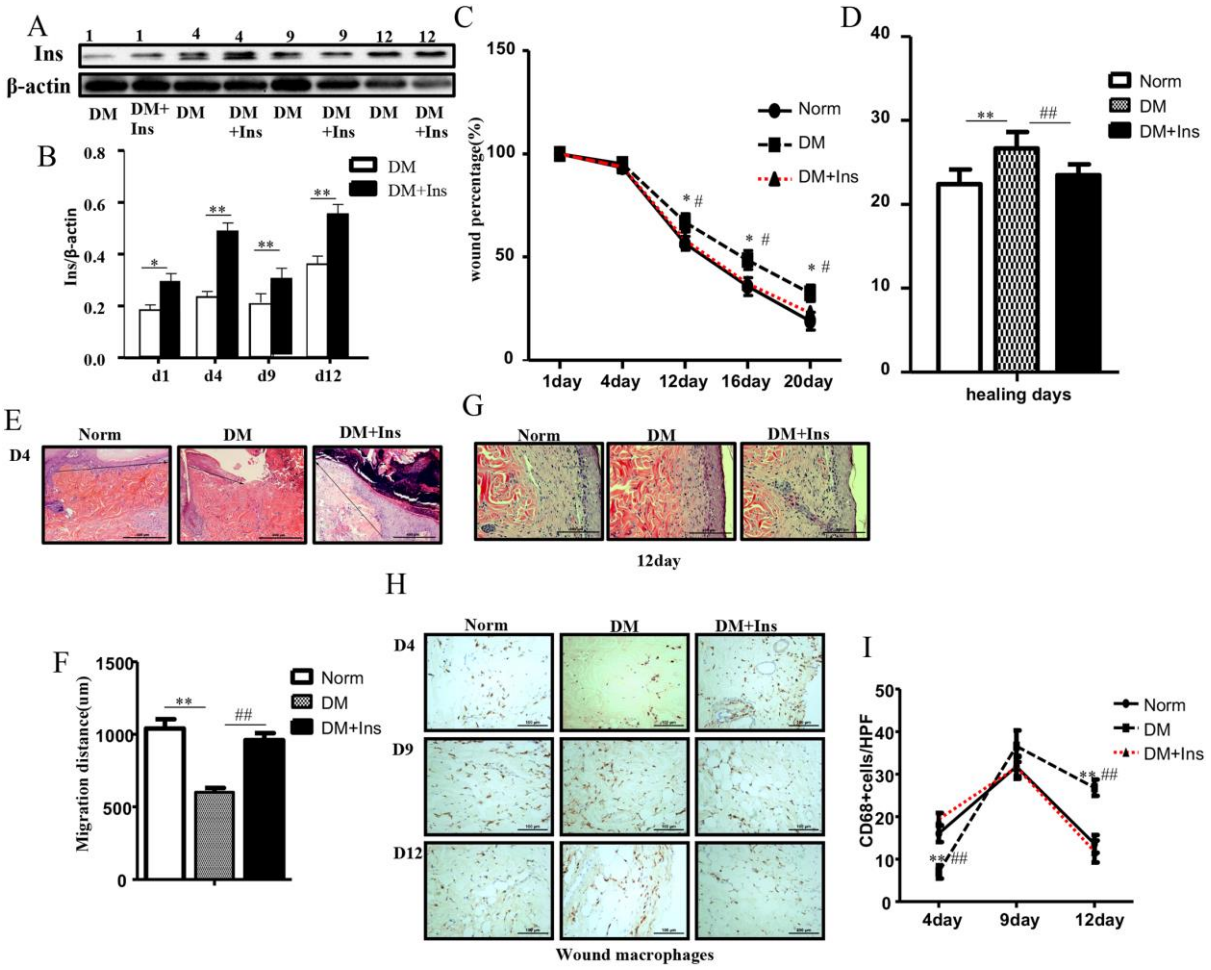

Figure 2. Topical insulin supplement promoted diabetic wound healing and macrophage infiltration and resolution.

A-B. Insulin topical application elevate wound topical insulin level in diabetic wound. Insulin levels in 1st, 4th, 9th and 12th day's wounds in DM and DM+Ins group were analyzed using western blot. Data are shown as the mean ± SD. **p* <0.05, ***p<0.01*, n=*5*. **C-D.** Wounds size were record at day 1, 4, 12, 16 and 20 days after wounding using transparent tracing paper. Unhealed rate of wounds was calculated by Image Pro Plus. Data are shown as the mean ± SD. */#*p* <0.05, **/##*p<0.01*, n=*5*. *means Norm VS DM, #means DM VS DM + Ins. **E.** Wound healing days were record and shown as the mean ± SD. **/##*p<0.01*, n=*5*. **F-G**. Wound re-epithelialization and extracellular matrix deposition in 4th and 12th day's wounds. Data are shown as the mean ± SD. **/##*p<0.01*, n=*5*. **H-I**. Macrophage infiltration and resolution were labeled by CD68 in 4th, 9th and 12th day's wounds using immunohistochemistry. The CD68 positive cell were calculated by Image Pro Plus in high power field (HPF) and Data are shown as the mean ± SD. **/##*p<0.01*, n=*5*.

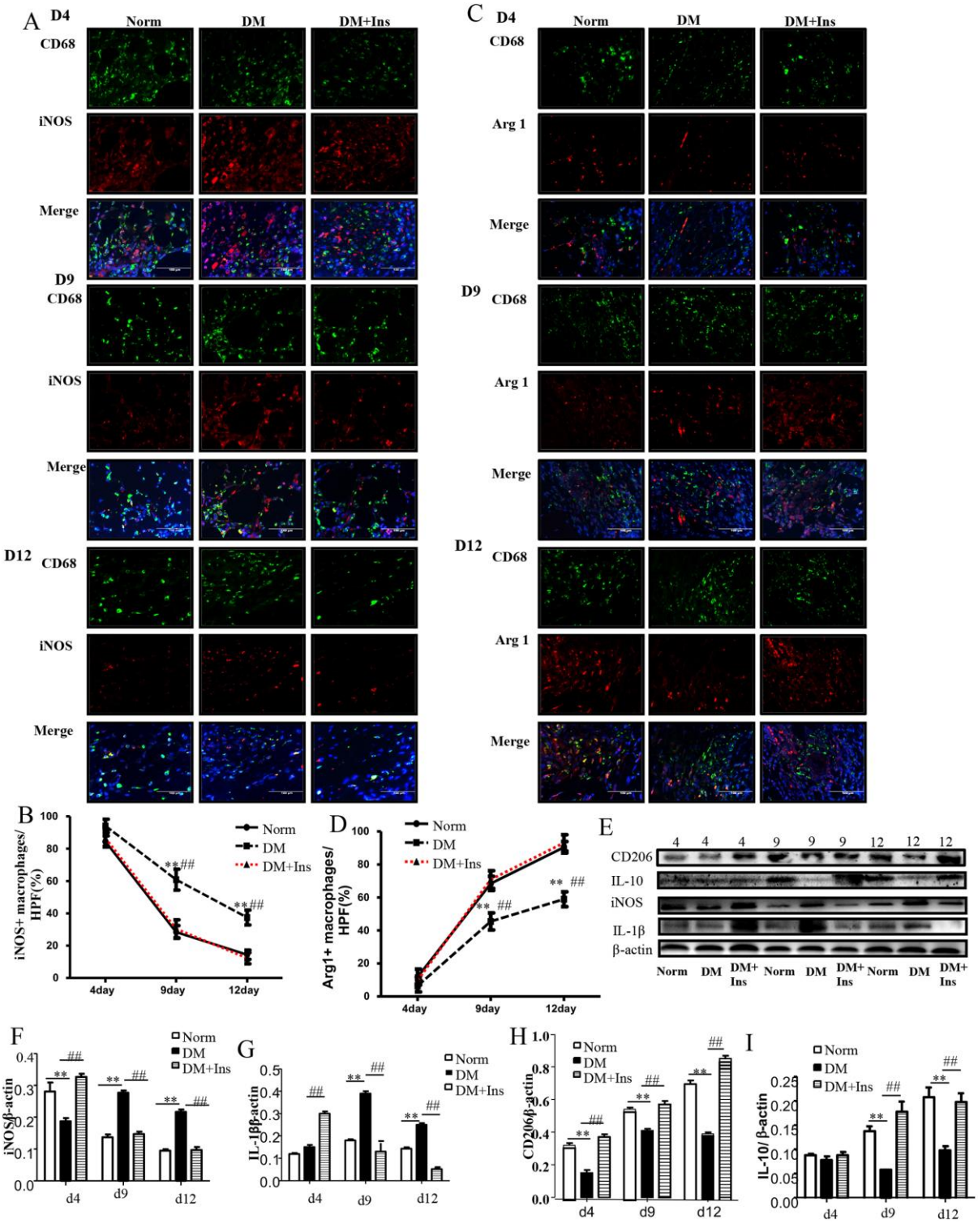

Figure 3. Insulin application promoted macrophage phenotype polarization in diabetic wound

A-B. M1 macrophage tendency were analyzed by immunofluorescence in 4th, 9th and 12th day's wounds. Fluorescent double staining was used in the examination, CD68 was used as a marker of total macrophage and iNOS was used as a marker of pro-inflammation marker, the double fluorescent positive cells were taken as the M1 macrophage. green: CD68; red: iNOS; blue: DAPI. The M1 macrophage were identified and

analyzed by Image Pro Plus and the percentage of M1 macrophage in different groups in relative wounding days were displayed in Figure D, Data are shown as the mean ± SD. $^{**/\#\#}p<0.01$, n=5. **C-D**. M2 macrophage tendency in 4th, 9th and 12th day's wounds. Fluorescent double staining was used in the examination, CD68 was used as a marker of total macrophage and arginase 1 was used as a marker of pro-healing marker, the double fluorescent positive cells were taken as the M2 macrophage. green: CD68; red: arginase1; blue: DAPI. The M2 macrophage were identified and analyzed by Image Pro Plus and the percentage of M2 macrophage in different groups in relative wounding days were displayed in Figure F, Data are shown as the mean ± SD. $^{**/\#\#}p<0.01$, n=5. **E-I**. iNOS and IL-1β were regarded as markers of M1 macrophage, whereas arginase 1 and CD206 were taken as markers of M2 macrophage. Western blot was used to exam the M1 and M2 macrophage related markers expression tendency in4th, 9th and 12th day's wounds. Photoshop was used in the quantification of immunoblots. Data are shown as the mean ± SD. $^{*/\#}p<0.05$, $^{**/\#\#}p<0.01$, n=5.

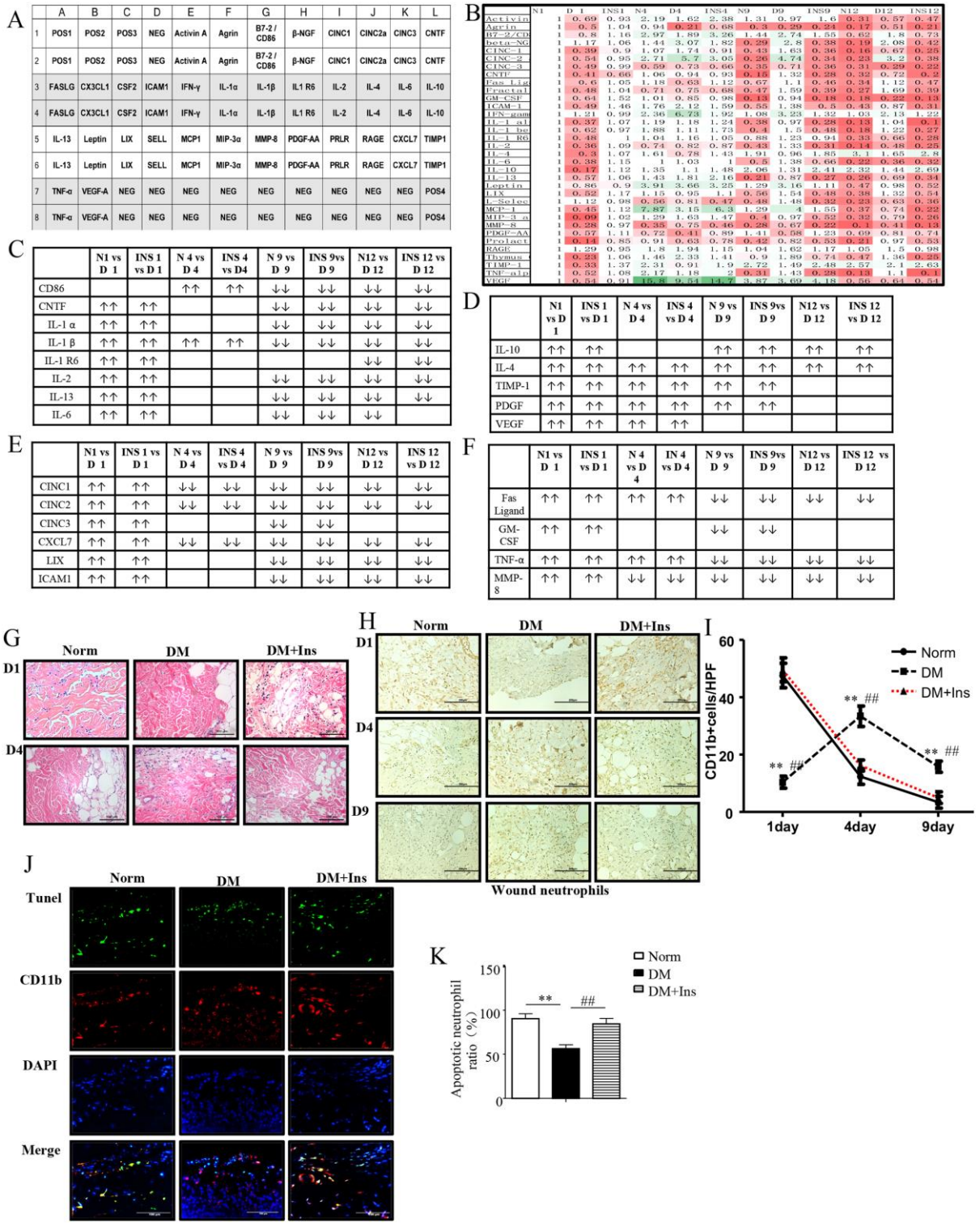

Figure 4 Insulin application promoted neutrophils infiltration and apoptosis

A. The layout of the cytokines array. **B**. The Fluoresce intensity of the in normal group day1 were set as a control and the relative expression intensity of other groups in different wounding days. **C.**M1 and pro-inflammation cytokine markers and cytokines expression tendency in 1st, 4th, 9th and 12th day's wounds were analyzed, listed and compared as normal group 1st day(N1) VS diabetic 1st day(D1); insulin

application group 1st day (INS1) vs diabetic 1st day(D1) and the like as N4 vs D4, INS4 vs D4; N9 vs D9, INS9 vs D9; N12 vs D12, INS12 vs D12. Data are shown as: ↑↑ means up regulated, the expression difference is significant; ↓↓ means down regulated, the expression difference is significant, n=3. **D**. M2 related cytokines and growth factors expression tendency in 1st, 4th, 9th and 12th day's wounds, the data were shown as described previously. **E**. The cytokines related to neutrophils attraction, migration and adhesion molecular in 1st, 4th, 9th and 12th day's wounds were analyzed, the data were shown as described previously. **F.** Neutrophils survive and apoptosis related cytokines expression tendency in 1st, 4th, 9th and 12th day's wounds, the data were shown as described previously. **G**. Neutrophils infiltration and resolution were labeled by CD66b in 1st, 4th and 9th day's wound using immunohistochemistry. The CD66b positive cell were calculated by Image Pro Plus in high power field and Data are shown as the mean ± SD. ***p<0.01*, n=*5* .**H-I.** The neutrophils apoptosis in the 4th day of wound were analyzed by TUNEL analysis combined with immunofluorescence. CD66b was used as a marker of neutrophils. green: TUNEL; red: CD66b; blue: DAPI. The apoptosis rates of neutrophils were calculated by Image Pro Plus. Data are shown as the mean ± SD. ***p<0.01*, n=5.

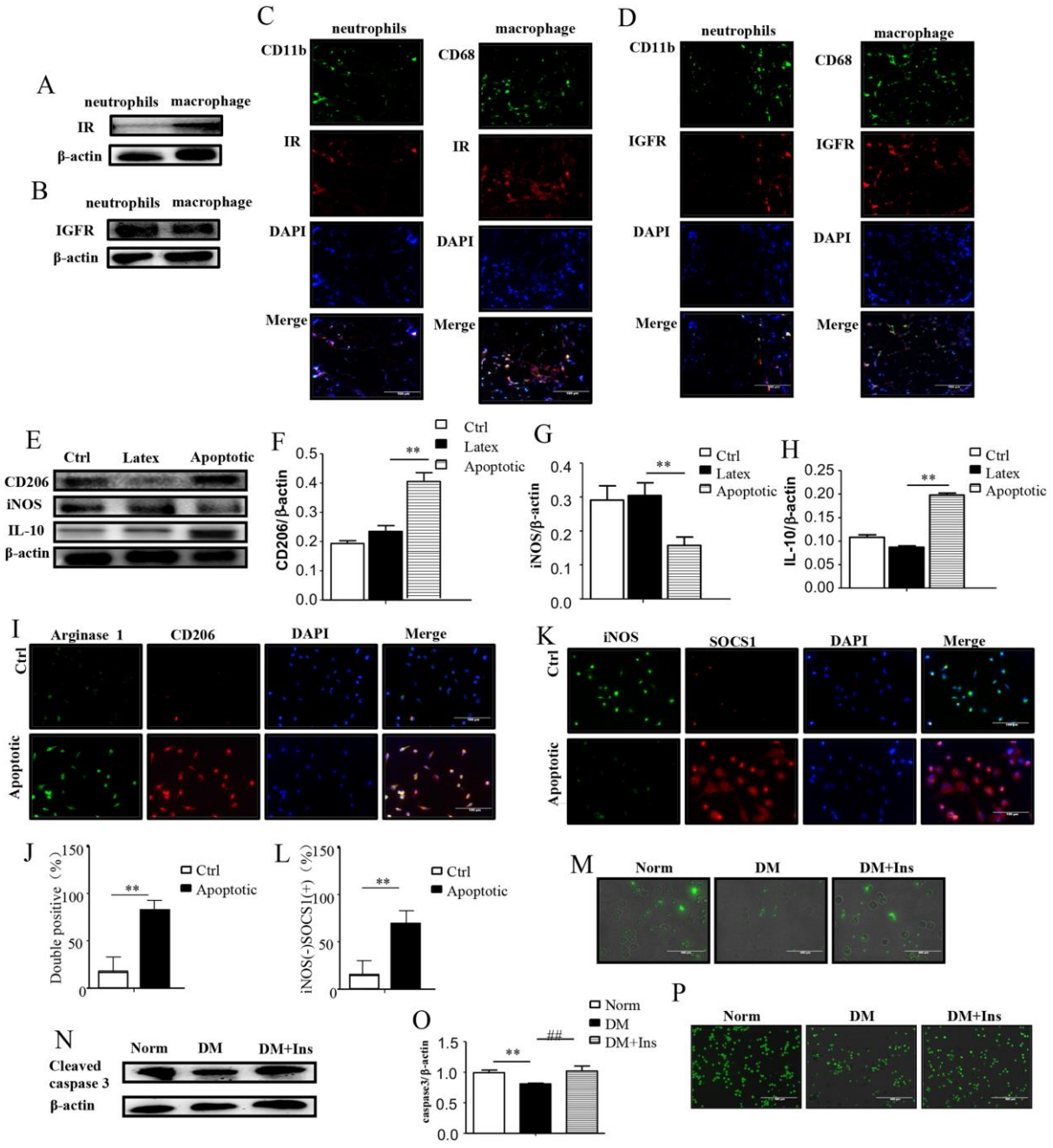

Figure 5 Apoptosed neutrophils promoted macrophage phenotype polarization from M1 to M2

A-B. Western blot analysis of IR and IGFR expression in neutrophils and macrophages. **C.** Fluorescent double staining was used in the examination of IR expression in wound neutrophils and macrophages. CD66b as a marker of neutrophils. CD68 as a marker of macrophages. red: IR, green: neutrophils marker or CD66b, macrophage marker CD68. **D.** Fluorescent double staining was also used examination of IGFR expression in wound neutrophils and macrophages. red: IGFR, green: neutrophils marker CD66b, macrophage marker CD68. **E-H.** 24 hours after apoptosed neutrophils treatment the macrophages were lysed and the expression of CD206, iNOS and IL-10 were analyzed by western blot and Photoshop was used in the quantification of immunoblots. Data are shown as the mean ± SD. ***p<0.01, n=3.* **I-L.** The apoptosed neutrophils were seeded as previously and 24 hours after challenge, the macrophages were fixed and

subjected to fluorescent double staining to analyze the macrophage phenotype transformation. Arginase 1, CD206 and SOCS1 was used as M2 macrophage marker and iNOS as M1 macrophage. Green: arginase 1(fig 4I), iNOS(Fig 5K); red: CD206(Fig 5I),SOCS1(Fig 5K). Data are shown as the mean ± SD. **p<0.01, n=3. **M.** Insulin improve the impaired diabetic neutrophils phagocytosis function. The neutrophils isolated from diabetic peritoneal were treated or untreated with 10^{-7}M of insulin for 1 hour then the FITC-labeled E-coli were added at a ratio of 10:1(E-coli: neutrophils), the neutrophils from the normal peritoneal were set as positive control, after 60min the cells were subject to the fluorescent microscope to analyze the phagocytosis function of neutrophils. **N-O.** After treatment or un-treatment for 10^{-7}M insulin treatment for 1 hour, the unlabeled E-coli were seeded at a ratio of 10:1 as previously. 18 hours later, the cells were lysed and subjected to the western blot analysis of the cleaved caspase 3 to evaluate the phagocytosis induced apoptosis and Photoshop was used in the quantification of immunoblots. Data are shown as the mean ± SD. ***p<0.01, n=3.* **P.** The neutrophils isolated from the peritoneal were labeled with cell tracker and cultured for 24 hours to induce apoptosis. After the neutrophils were apoptosed, the apoptosed neutrophils were seeded to the macrophages isolated from peritoneal of normal and diabetic rat at a ratio of 4:1(apoptosed neutrophils: macrophages). The diabetic macrophages were pre-treated or un-treated with 10^{-7}M insulin. 4 hours after the apoptosed neutrophils challenge the cells were washed with PBS twice and subjected to the fluorescent microscope to analyze the macrophage efferocytosis of apoptosed neutrophils.

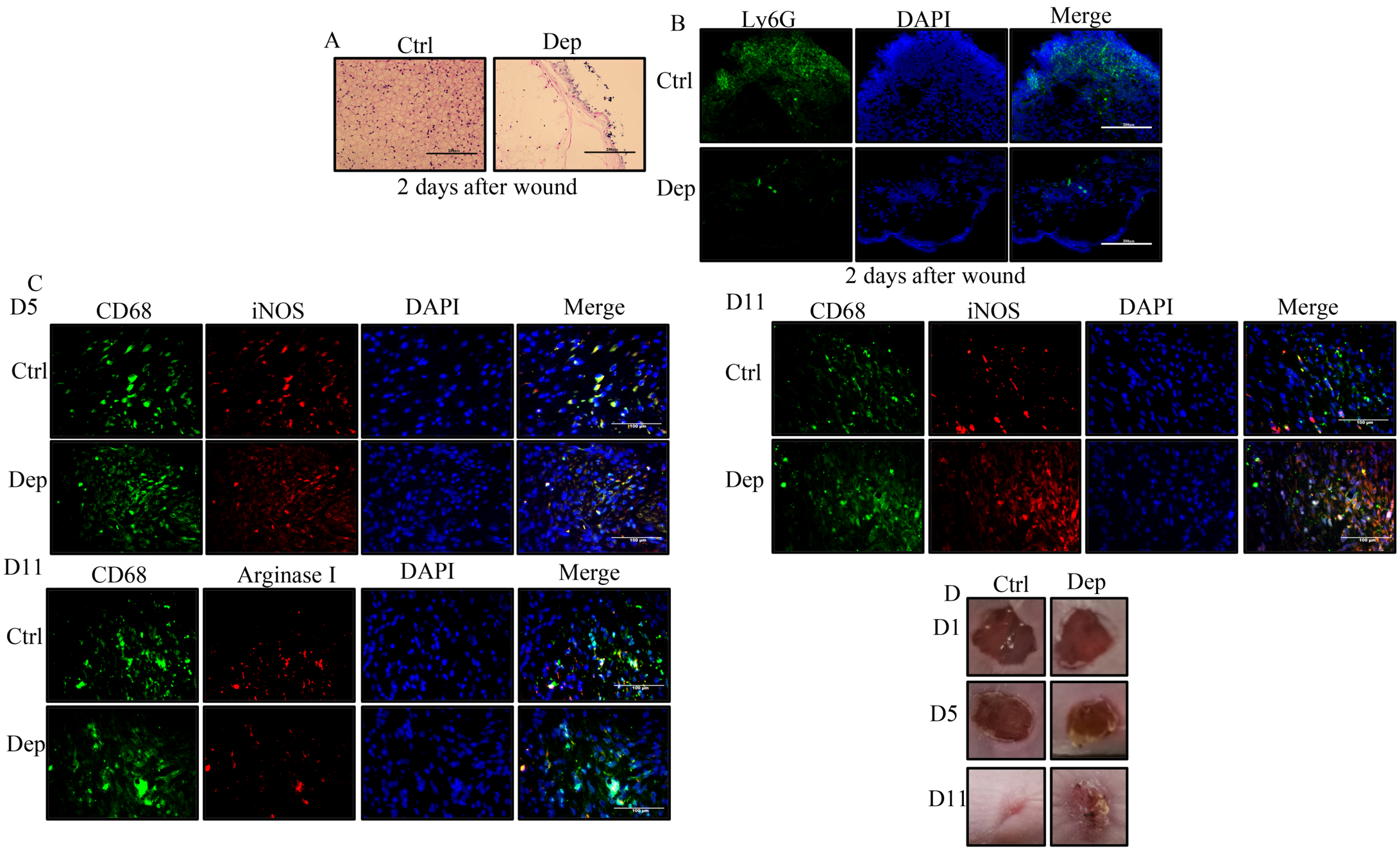

Fig. S1. **A-B.** Neutrophils were depleted by i.p. injection of 62.5 μg NIMP-R14 anti-Ly6G antibody in PBS on one day before wounding and 1st day of wounding. Control mice received 62.5 μg rat IgG2b. on the 2nd day of wounding, the wounds were harvested. H&E and Immunofluorescence were used to check the depletion efficiency of neutrophils. Both H&E and IF showed that anti-Ly6G antibody could deplete efficiently. **C.** IF were used to check the influence of neutrophils depletion on macrophages polarization during wound healing. On 5th and 11th day after wounding, wounds were collected and subjected to IF analysis. CD68 was used as a marker of total macrophage, iNOS was used as a marker of pro-inflammation marker and arginase 1 was used as a marker of pro-healing marker, the double fluorescent positive cells were taken as the M1 OR M2 macrophage. green: CD68; red: iNOS or arginase1; blue: DAPI. **D.** Neutrophils depletion on wound healing. Wounds size were record at day 1, 5 and 11 days after wounding.