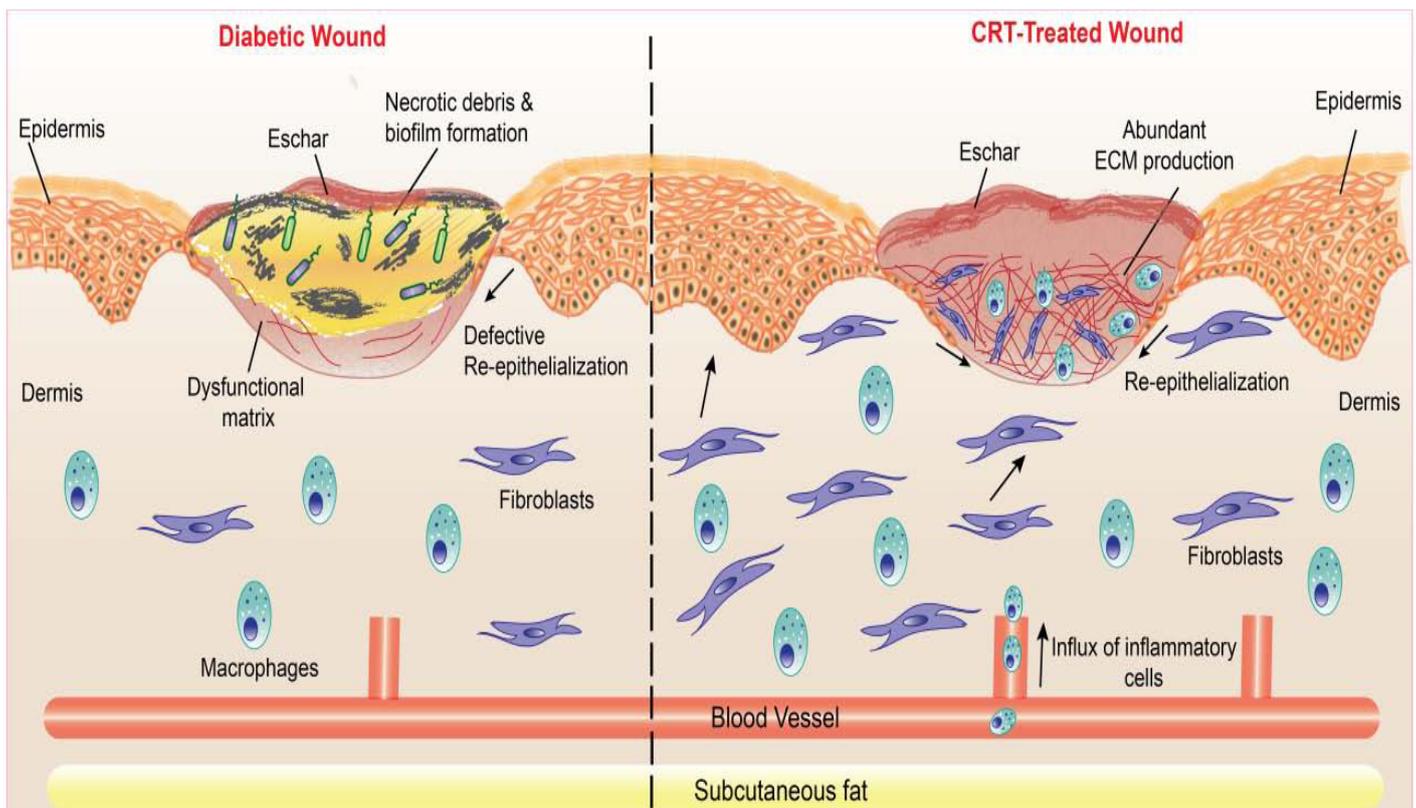


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## Review Article

# The Novel Biotherapeutic Calreticulin (CRT) Corrects Multiple Defects of Non-Healing Diabetic Wounds

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## Keywords

- Calreticulin (CRT)
- Diabetic foot ulcers (DFUs)
- Novel topical biotherapeutic
- Non-healing chronic diabetic wounds
- Diverse biological effects

**Abstract**

A topical wound healing agent that could counter act the specific defects that prevent the healing of wounds sustained as a consequence of diabetes would be the first successful treatment for this serious unmet medical need. The application of calreticulin (CRT) to a porcine model of impaired wound healing surpassed, by far, Regranex®, the only current topical agent approved for the treatment of diabetic foot ulcers (DFUs). Whereas Regranex only affects dermal healing, CRT targets both epidermal resurfacing and dermal tissue regeneration. Moreover, CRT exerted an identical vulnerary effect in a diabetic mouse (db/db) wound healing model. In vitro studies confirmed the diverse and broad-reaching biological effects of CRT at the cellular level. Exogenous CRT promoted directed cellular migration of human keratinocytes, macrophages, and fibroblasts, stimulated proliferation of keratinocytes and fibroblasts, and induced fibroblasts to synthesize the extracellular matrix (ECM) proteins, collagen, fibronectin, and elastin. In addition, CRT induced  $\alpha 5$  integrin likely for migration on matrix. CRT also enhances the immune response. Importantly, as lack of recruitment of cells required for healing, acellular wounds, and a paucity of granulation tissue epitomizes chronic DFUs, CRT could be the first biotherapeutic to specifically attack the problems that characterize diabetic wounds, suffered by close to 4 million people in the United States and 30 million globally, with increased risk of amputation and death. This article presents proof of principle in vivo and extensive in vitro data to support a novel role for the heretofore intracellular ER chaperone protein, CRT, as a diversely functional biotherapeutic for DFUs and other chronic wounds.

**ABBREVIATIONS**

AMPs: Anti-Microbial Peptides; CRT: Calreticulin; DFUs: Diabetic Foot Ulcers; ECM: Extracellular Matrix; ER: Endoplasmic Reticulum; ERAD: Endoplasmic-Reticulum-Associated Protein Degradation; ICD: Immunogenic Cell Death; LRP1: Low-Density Lipoprotein Receptor-Related Protein 1; MEFs: Mouse Embryo Fibroblasts; PAD: Peripheral Arterial Occlusive Disease; PDGF: Platelet-Derived Growth Factor; PLC: Peptide Loading Complex; PS: Phosphatidyl Serine, SDF-1 $\alpha$ : Stromal Cell-Derived Factor 1-alpha; SOC: Standard of Care; TLR: Toll-like Receptor ; TSP1: Thrombospondin 1; UPR: Unfolded Protein Response ; VEGF: Vascular Endothelial Growth Factor

**INTRODUCTION****Demographics of chronic cutaneous diabetic wounds**

Failure to heal cutaneous wounds occurs in over 8 million

people in the US and 44 million worldwide (composite of pressure/decubitus ulcers, venous stasis ulcers, diabetic/neuropathic ulcers, and diabetic arteriole ischemic ulcers). Of the 29.1 million people in the US (in 2016) and 200 million worldwide diagnosed with diabetes mellitus (n.b., there were 2 million new cases diagnosed in the US in 2012), approximately 4.0 million and 30 million, respectively, have autonomic neuropathic diabetic foot ulcers (DFUs) (reported by NIDDK, National diabetes Information Clearing House [NDIC]). Type I diabetes (insulin-dependent diabetes [IDDM]) is caused by hyperglycemia due to destruction of pancreatic beta cells. This form of diabetes mellitus, which predominantly occurs in patients under 20 years, has doubled in the past 20 years (NIDDK-NDIC report). Type II diabetes (non-insulin-dependent diabetes mellitus [NIDDM]) is also associated with hyperglycemia but this type is due to insulin resistance. The alarming surge in obesity in both the US and globally has been a major cause of this form of diabetes. Type

I diabetes is most prevalent in non-hispanic whites and Type II mainly occurs in minority populations (African Americans, Hispanic/Latino Americans and American; NIDDK-NDIC). In addition to blindness, kidney disease, heart disease and stroke, it is notable that 60% of non-traumatic lower limb amputations, due to critical limb ischemia occur in the diabetic population [1-3]. Amputation is 15 times more common in diabetics followed by a 50% death rate. Etiologically, ulcers due to occluded arterioles causing insufficiency in arterial flow are a consequence of cardiovascular disease of Type I diabetics while neuro ischemic and neuropathic DFUs occur in both Type I and Type II diabetics. Approximately 20% of lower extremity ulcers in diabetics are due to peripheral arterial occlusive disease (PAD) and approximately 50% have primary diabetic neuropathy; 30% present with both conditions. Hyperglycemia resulting from type I and type II diabetics is a barrier to healing as cell walls become more rigid, impairing blood flow through small vessels at the wound surface leading to lack of oxygen (hypoxia) and nutrients to the wound. These ischemic wounds become gangrenous (i.e., irreversible damage) from continued necrosis of the skin and underlying structures, including osteomyelitis of the bone, and cannot heal without loss of at least part of the involved extremity [1,4].

### **The difference between chronic and normal acute wound healing**

Temporally, normal wound healing involves four essential phases, coagulation and clot formation, the inflammatory phase (inflammatory cells migrate in wound and remove bacteria and debris, epidermal keratinocytes and fibroblasts migrate into the wound), the proliferative or tissue formation phase (keratinocytes resurface the wound, fibroblasts produce growth factors and extracellular matrix (ECM) proteins that provide granulation tissue to reconstruct the wound defect), and finally, the tissue remodeling and wound resolution phase, which occurs 10-14 days after injury and continues [1,5]. The events are stochastic involving numerous cell types and complex molecular events and biologic processes. At the cellular level, the most important functions and processes involved in normal wound healing are cell migration, proliferation, extracellular matrix production, angiogenesis, inflammatory cell functions, and wound contraction. The major cell types that accomplish these functions are keratinocytes, fibroblasts, endothelial cells, and immune cells, such as macrophages, which engulf bacteria and debris and release important cytokines. Chronic diabetic wounds are arrested in the inflammatory phase and are defined as lasting more than 8 weeks [1]. These wounds are characterized by persistent infection, including the formation of impenetrable biofilms secreted by bacteria [6], and a constant influx of inflammatory cells that release mediators of inflammation causing tissue destruction [5-7]. The presence of dead tissue prevents healing requiring frequent surgical debridement. Briefly, chronic non-healing diabetic wounds are defective in keratinocyte, fibroblast, and macrophage migration into the wounds, cell proliferation, ECM production, angiogenesis, appropriate cytokine release, clearance of dead tissue, cells and bacteria, and fibro myoblast differentiation, necessary for wound contraction [5,8-10]. Notably, the lack of fibroblast migration into wounds is a rate-limiting step in the formation of granulation tissue composed of ECM proteins that form the neodermis

[11]. In addition, the granulation tissue provides a substrate for keratinocytes to migrate over to resurface the wound. Diabetic wounds produce insufficient SDF-1 $\alpha$  for recruitment of mesenchymal stem cells [12] and endothelial progenitor cells [1,13] from the bone marrow; these cells are critical to the repair process. Therefore, an agent that could specifically affect these particular functions of wound repair would be highly valuable in attacking/solving the problem of non-healing diabetic wounds.

### **The intracellular chaperone protein calreticulin emerges with important extracellular functions that direct physiological and pathological processes including wound healing**

Calreticulin (CRT), a 46 kDa calcium-binding resident of the endoplasmic reticulum (ER), functions in directing proper folding (conformation) of proteins and homeostatic control of cytosolic and ER calcium levels [14,15]. The three domains that compose CRT are the globular N domain (1-80) of  $\beta$ -sheet structure, the extended flexible P domain (181-290), rich in prolines with low capacity, high affinity calcium binding, and the acidic C domain (291-400) with high capacity and low affinity for binding calcium. The C domain is a sensor for calcium and regulates cytoplasmic retro-translocation of CRT. CRT is targeted to the ER by the signal sequence and retained in the ER by KDEL at C-terminus. Among mammals, CRT has 96% conserved amino acid sequence homology except for its C-terminus. Together with other chaperones in the ER, CRT identifies mis-folded proteins banning them from the ER for ubiquitin-mediated destruction degradation (ERAD system) thereby protecting the cell from aberrant protein function. More recently, imbalance and/or insult in the ER has been shown to induce intracellular CRT as a stress response protein that is transiently involved in the unfolded protein response (UPR) with manifestation in a variety of pathological conditions including fibrotic diseases and Alzheimer's disease [Groenendyk et al 2016; [16, 17]. In addition, CRT has important functions in the innate and adaptive immune responses [18,19].

In the past decade, emerging evidence has exposed diverse roles for CRT localized outside the ER, on the surface of many cells, and in the extracellular space [15]. Importantly, CRT as a multi-compartmental protein has been shown to be important in many physiological and pathological processes [15,20,21]. Exogenous CRT mediates phagocytosis of apoptotic cells by macrophages and cancer cells by dendritic cells, and is involved in cell adhesion and migration. This implies that outside-in cell surface receptor signaling is involved in CRT-induced biological functions. However, although CRT does not signal directly from the cell surface, intracellular signaling through the low-density lipoprotein receptor-related protein 1 (LRP1) has been one of the only receptors shown to be involved in mediating certain functions of CRT [22-24]. For example both thrombospondin 1 (TSP1) mediated migration of fibroblasts and bovine aortic endothelial cells, and the phagocytosis of apoptotic cells by macrophages signal through LRP1 on fibroblasts and macrophages, respectively. Whereas extracellular signaling by CRT would require an intracellular route for CRT to exit the cell from the ER or cytoplasm, CRT does not have a secretory sequence and despite a large effort to solve this mystery, only one

publication suggests that CRT binds to phosphatidyl serine (PS) in a calcium-dependent manner and together CRT and PS are transported to the cell surface by oxidized amino phospholipid translocase [25]. Through the demonstration of extracellular biological activities of exogenous CRT, it has become evident that numerous physiological processes are driven by this protein. Among other physiological processes [15,17], CRT topically applied to experimental wound models is shown to have profound effects on wound healing by eliciting diverse cellular responses that drive the wound repair process [26-28]. CRT specifically corrects the deficiencies associated with poor healing of these wounds (affects more bioactivities required for wound healing than any other agent in its class). This remarkable discovery has the potential to shift past failures in the cure of non-healing diabetic wounds to efficacious healing.

### CALRETICULIN ENHANCES THE RATE AND QUALITY OF WOUND HEALING IN PORCINE AND DIABETIC MOUSE EXPERIMENTAL WOUND MODELS

Measurable criteria most important to normal wound healing are rapid wound closure, wound resurfacing (re-epithelialization), and abundant granulation tissue to fill in the wound defect [7,27]. Topically applied CRT induced a faster rate of wound re-epithelialization and abundant granulation tissue/neodermal formation compared to Regranex® (platelet-derived growth factor [PDGF-BB] used as the positive control in a porcine cutaneous partial thickness (not through the dermis) wound healing model. This animal model was used because porcine cutaneous wound repair is most similar to humans [29]. Regranex is currently the only topical wound healing agent available, which is indicated only for DFUs and used off-label for other wounds [27]. Histologically, CRT-treated wounds showed evidence of a profound effect on both the epidermis and dermis whereas the Regranex-treated wounds had only dermal effects. Specifically, the CRT-treated wounds matured earlier being 100% re-epithelialized with a stratified epidermis whereas the Regranex-treated wounds barely showed one layer of keratinocytes by day 10 post-injury. High cellularity and collagen fibril deposition in the neodermis was evident in the Regranex and CRT-treated wounds but not the buffer-treated control. However, CRT-treated wounds contained more collagen fibrils and 3-fold more macrophages had migrated into the wounds compared to Regranex. The high cellularity of the dermis and full epidermal layering was explained by Ki67 immunostaining (for proliferation), which showed induction of proliferation of the regenerating basal and supra basal keratinocytes and fibroblasts of the dermis in the CRT-treated wounds. In addition, a statistically significant increase in wound tensile strength in wounded rats was observed at 21 days post-injury [27]. Whereas CRT stimulated proliferation of human microvascular endothelial cells, it did not increase microvessel density (angiogenesis) in porcine wounds.

Mice homozygous for the diabetes spontaneous mutation in the leptin receptor (*Lep<sup>rd/b</sup>*), a classic model for Type II diabetes have impaired/delayed wound healing. These mice become obese by 3-4 weeks of age, are hyperinsulinemic and hyperglycemic

by 4-8 weeks, and have peripheral neuropathy, and myocardial disease [30]. As both neurologic and vascular complications, and hyperglycemia are the basis for poor wound healing of both type I and type II diabetes, these mice are an appropriate experimental wound model for both forms of this disease and in addition, offer a longer window of time to test topical wound healing agents for efficacy. Topical CRT was applied to 6 mm full-thickness excisional wounds on the dorsum for four consecutive days post-wounding [26]. To better simulate human wound healing and allow morphometric analysis of re-epithelialization and granulation tissue formation, a splint was placed around the wound to prevent rapid contraction by the *panniculus carnosus* muscle layer located immediately below the dermis. This muscle layer is present in loose-skinned hairy animals but not pigs or humans. In these studies, VEGF and saline served as positive and negative control, respectively. Time points for histological analysis were 3, 4, 7, 10, 21 and 28 days post-wounding. A statistically significant reduction in time to wound closure, an increase in the rate of re-epithelialization, and an increase in granulation tissue formation was observed compared to the saline control. CRT-treated wounds contained granulation tissue at day 3 and 10 post-wounding whereas only fat was observed in the wound bed of the saline-treated wounds. Moreover, the granulation tissue (neodermis) was more cellular with a greater amount of collagen than the VEGF or saline treated wounds. However, as expected, the VEGF-treated wounds showed increased blood vessel density. As in the porcine study, cell proliferation was shown in the supra-basal and basal layers of the epidermis and the neodermis by uptake of BrUdR labeling. Interestingly, at 28 days post-wounding, the wounds were replenished with abundant black hair whereas the saline treated wounds showed a hairless scar. The histology of these wounds showed numerous hair follicles between the cuts in the *panniculus carnosus*. Epidermal appendages, such as hair follicles and sebaceous glands, do not regrow following injury through the dermis in any mammal. This is a remarkable finding implicating CRT as having the ability to regenerate tissue rather than classic cutaneous wound repair. Interestingly, further research in this regard indicates that CRT treatment induces hair regrowth through activation of the Wnt pathway and moreover, hair follicle neogenesis sprouting from sebaceous glands was observed as early as 7 days post-wounding under areas of hypertrophic epidermis and remaining eschar. In Wnt null mice, it was shown that hair follicle regeneration did not occur whereas with an intact Wnt pathway, mouse hair follicles regenerated in the wound bed originating from non-hair follicle epithelial stem cells that undergo an embryonic hair follicle development [31,32]. However, only scant white hair [in a black haired mouse] grew back and thus, CRT remarkably appears to affect melanocyte and melanin production. Whether CRT will have similar effects in humans remains a question to pursue. Importantly, that two mammals (pigs and mice) showed identical histological effects from topical application of CRT including increased cellularity and collagen deposition at exactly the same optimal concentration of CRT and treatment time, increases the likelihood that human healing would show similar beneficial effects. These animal studies implicated a significant role for CRT in cellular recruitment, proliferation, and ECM (ECM) induction to promote wound healing.

## STUDIES USING HUMAN CELLS IN TISSUE CULTURE EXPLAIN THE MECHANISMS INVOLVED IN THE WOUND HEALING EFFECTS OF CALRETI-CULIN

The increased rate of re-epithelialization (resurfacing) in the porcine and murine studies is due to both CRT's induction of keratinocyte migration, measured using human keratinocytes in the scratch plate assay and a chamber assay, showing concentration-dependent migration (chemotaxis), and stimulation of proliferation [27]. The ability of CRT to induce proliferation of keratinocytes in vitro supports the observation shown in vivo of Ki67 immunostaining of the proliferative supra basal and basal layers of the animal wounds leading to the full replenishing of the layers of the epidermis in CRT-treated wounds. In addition, CRT induces the matrix scaffold protein, fibronectin and  $\alpha 5$ ,  $\beta 1$  integrin in these cells. Therefore, to enable re-epithelialization, CRT might mediate migration by up-regulating the  $\alpha 5$  and  $\beta 1$  integrin for migration on a fibronectin substrate that keratinocytes deposit into the wound.

In vitro studies using human and mouse fibroblasts show that the robust granulation tissue found in both animal models is mechanistically due to the ability of CRT to induce fibroblast migration, proliferation of these cells, and a dose-dependent induction of ECM proteins, collagen type I, fibronectin, and elastin, the integrins,  $\alpha 5$  and  $\beta 1$ , and TGF- $\beta 3$  [26,27]. Again, the mechanism involved in CRT-induced fibroblast recruitment into the wound may in part be through up regulation of  $\alpha 5$  and  $\beta 1$  integrin for migration on fibronectin deposited in the granulation tissue. Another mechanism for CRT-mediated migration of fibroblasts was shown in the first studies that reported that CRT existed outside the ER as a cell surface protein but that cell surface CRT could not itself engage in receptor signaling for migration [24,33]. Instead, it was shown that CRT co-opts the matricellular protein, TSP1 and the LDL-receptor related protein 1 (LRP1), as the signaling receptor, to mediate concentration-dependent migration of endothelial cells and fibroblasts [24]. Interestingly, CRT co-immunoprecipitates with integrins during cell adhesion to substrate [34] and is co-localized with LRP1 in integrin-based adhesion complexes [15,35-37]. The specific physical binding of TSP1 and CRT [on the cell surface] occurs through amino acid residues 17-35 and 19-36, respectively [38]. It was further shown that intracellular CRT regulates collagen and fibronectin trafficking and processing to form the ECM as CRT null mouse embryo fibroblasts (MEFs) were defective in these functions [39]. The interaction of CRT with TSP1 is involved in induction of a fibrotic foreign body response [40] and interestingly, intracellular CRT was shown to be required for TGF- $\beta$  receptor mediated induction of collagen and fibronectin [41]. TGF- $\beta$  is a key mediator of organ fibrosis and tissue scarring [16,42]. Whereas CRT induces TGF- $\beta 1$  and TGF- $\beta 3$  isoforms by fibroblasts in vitro, post-healing scarring did not appear to occur in CRT-treated murine wounds. As shown by picrosirius red staining and polarized light microscopy, the wounds contained more organized collagen reflected by a yellow-green birefringence. This is likely related to induction of fibronectin and TGF- $\beta 3$  as both are involved in collagen organization [43,44]. Moreover, CRT also induced alpha-smooth muscle actin, indicating that

CRT might induce fibroblast differentiation into myofibroblasts necessary for wound contraction [7].

Another cell type critical to the wound healing process is the macrophage. These cells were recruited into the CRT-treated porcine wounds as shown by the increase in vivo by immunostaining with MAC387 antibody [27]. In these same studies, in vitro, using a chamber migration assay, CRT dose-dependently induced monocyte and macrophage migration. CRT is claimed to be the "universal eat-me signal" on the surface of dead cells, which is required for their clearance by all phagocytes [22]. As another mechanism, CRT binds both the collagen-like domain and globular region of C1q and together with PS mediates phagocytosis of apoptotic cells [45]. Taken together, CRT not only recruits monocytes and macrophages from the circulation into the wound bed in both normal and steroid-impaired pigs, having delayed wound healing [27], but local activation of macrophages by the CRT present in wounds is ostensibly involved in the non-mechanical removal of debris or wound debridement essential for cutaneous healing. Broad effects on both the innate and adaptive immune response mechanisms are regulated by CRT (Reviewed in: [15, 19]). Briefly, under stress conditions including carcinogenesis, CRT translocates to the cell surface, usually in association with PS thereby providing a signal to phagocytes for engulfment of apoptotic cells [21,22,46]. In addition, intracellular CRT plays a significant role in MHC Class I assembly and is a component of the peptide loading complex (PLC) for cell surface antigen presentation important for cytotoxic T cells, NK cell recognition and the phagocytosis of cancer cells by dendritic cells (adaptive immune response) [47,48,19]. The cell surface exposure of CRT on cancer cells and phagocytosis by dendritic cells is important in immunogenic cell death (ICD) [49-51] and more recently, radiation-induced immunogenic modulation of tumor cells was shown to expose calreticulin which enhanced antigen processing resulting in heightened T-cell killing [52]. Moreover, CRT is released from activated neutrophils [53] and is a component of lytic granules of cytotoxic T cells [19]. Further to its immune function, CRT serves as a receptor on the surface of neutrophils that binds a synthetic anti-bacterial peptide, L5, shown to be an important chemotherapeutic agent against MRSA-infected mice through a G-coupled protein signaling mechanism that releases superoxide anion [54]. Notably, both the initial proinflammatory macrophages in the wound, M1, and the immune modulatory macrophages, M2 respond to CRT during wound repair [55,56]. Furthermore, CRT induces B cell activation in a Toll-like receptor (TLR)-4 dependent manner [57]. TLRs are bacterial sensing receptors on host cells and responsible for the initial innate immune response that commences the downstream stochastic events to combat invading organisms. The activation of macrophages by exogenous CRT is shown by membrane ruffling and pinocytosis of media via an LRP1-dependent mechanism [22] and by the release of nitric oxide (NO) [58]. CRT induces cytokine release including TNF- $\alpha$ , interferon- $\gamma$  and interleukins, that are essential for commencement of the innate immune response and the follow-on adaptive immune response [19,57], in part through the scavenger A receptor and also, by endocytosis [57]. In addition, both NF- $\kappa$ B and MAPK signaling pathways are involved in CRT-induced immune-related responses [59]. Furthermore, CRT has been shown to

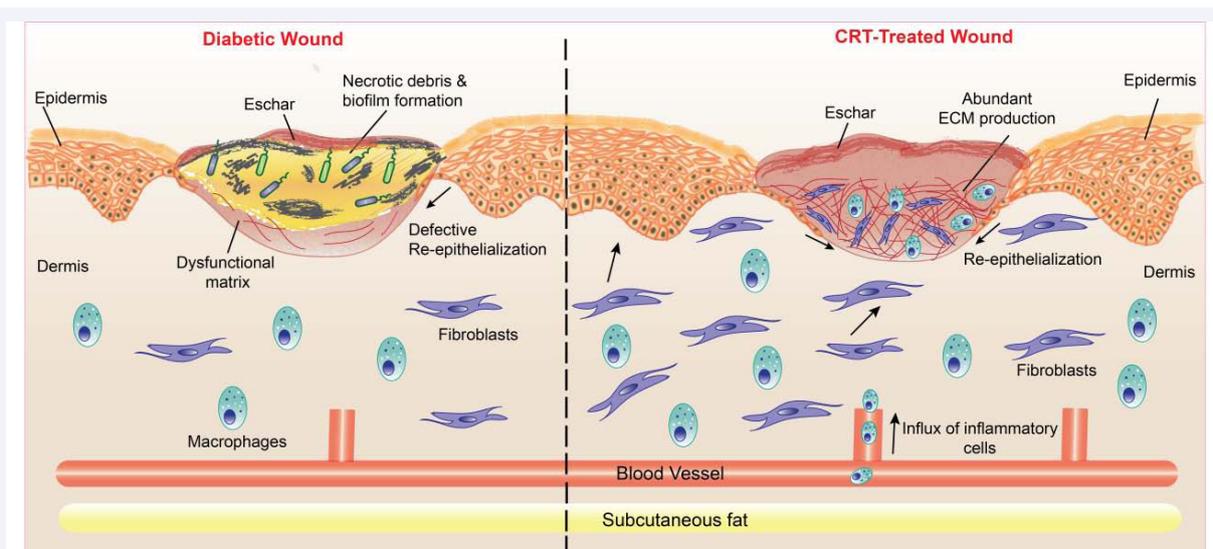
opsonize and induce the uptake of gram positive and negative bacteria, as shown in the fish, (*Branchiostoma Japonicum*) [60], the mitten crab (*EriachierSinensis*) [61], and the scallop (*PatinonpectenYessoensis*) [62] providing evidence for potential anti-microbial effects of CRT in human wound healing.

### CALRETICULIN AS A NOVEL BIOTHERAPEUTIC THAT SPECIFICALLY TARGETS PROBLEMS ASSOCIATED WITH NON-HEALING DIABETIC WOUNDS

The consequences of diabetes in impaired chronic wound healing at the cellular level have been well-characterized [1,10,63-65]. As discussed herein, these particular defective functions including recruitment and proliferation of fibroblasts to form granulation tissue and the migration of monocytes from the vasculature and their local activation and differentiation into macrophages to combat infection were seemingly specifically ameliorated by CRT treatment in the murine diabetic and steroid-treated porcine wounds. Particularly, the strong induction of ECM proteins by CRT is important for reconstruction of the deep tissue defects of chronic diabetic wounds. The expression of fibronectin as a marker for granulation tissue formation is deficient in chronic wounds [5]. In vitro, fibroblasts isolated from adult diabetic (db/db) mice and the wild type background strain (C57/Bl6) and human dermal fibroblasts cultured in high glucose to simulate hyperglycemia and in normal glucose were compared for their responses to exogenous CRT-mediated migration and proliferation [66,67]. CRT induced proliferation in diabetic mouse and hyperglycemic human fibroblasts, albeit marginally [26]. However, the migratory response to CRT assessed by both chamber migration and scratch plate assays in these cells was strong but to lesser extent than the wild type fibroblasts. These results show that genetically diabetic mouse cells and human cells cultured in high glucose have impaired cell proliferation

and migration in vitro and that this proclivity can in part be ameliorated by CRT. However, in vivo, CRT has a more profound effect in the recruitment and proliferation cells in the diabetic mouse wounds.

The formation of biofilms by bacterial skin pathogen is a serious retardant to the healing of diabetic wounds [68]. Although bacterial infections can be treated by antibiotics with differing efficacies, many of these wound-related bacterial strains are becoming resistant to antibiotic treatments. These pathogens colonize open wounds and affect the healing process through interaction with host cells at the wound site. Bacterial toxins or their virulence factors not only adversely affect the ability of phagocytic cells to remove foreign materials, bacteria, and damaged tissue but, these toxins may affect other cell types present in the wound, such as endothelial cells and fibroblasts and convert their functions into a non-healing phenotype. The role for CRT in the innate and adaptive immune response, through induction of cytokines, and antimicrobial potential, as described above in lower taxonomic classification provides evidence for CRT to be effective in possibly preventing biofilm formation in diabetic wounds. Furthermore, keratinocytes make cathelicidin and other anti-microbial peptides (AMPs) that reduce bacterial skin pathogens [69] and increased expression of integrins and fibronectin by keratinocytes and fibroblasts interact with bacterial adhesions to increase ECM binding for enhanced bacterial uptake [70,71]. Therefore, as CRT both induces migration and proliferation of keratinocytes for rapid resurfacing of animal wounds, increased defense against bacteria might act through this mechanism as well. As shown in (Figure 1) and (Table I), CRT, as a multi-functional wound healing agent ameliorates the specific dysfunctional characteristics associated with the effects of prolonged high serum glucose on wound healing at the cellular level supporting the therapeutic



**Figure 1 Calreticulin (CRT) Ameliorates most Defects in Diabetic Wound Healing.** Diabetic wounds are characterized by lack of cell migration and proliferation, and a paucity of granulation tissue causing lack of normal wound closure. CRT enhances migration and proliferation of keratinocytes for wound resurfacing with full epidermal layering. CRT stimulates migration and proliferation of fibroblasts into the wound bed. Fibroblasts produce ECM proteins in response to CRT for reconstruction of the wound defect. CRT corrects the dysfunctional inflammatory response including defective phagocytosis contributing to bacterial infection (biofilms) and accumulation of necrotic debris by recruiting monocytes and macrophages into the wound and activating these cells enabling a functional inflammatory response including clearance of debris.

**Table 1:** Calreticulin corrects multiple defects of non-healing diabetic wounds.

DEFECTS IN DIABETIC WOUNDS	EFFECT OF CRT ON DIABETIC WOUNDS
Decreased Re-epithelialization	Increased Re-epithelialization
Delayed wound closure	Accelerates wound closure
Impaired cell proliferation	Increases proliferation of keratinocytes and fibroblasts
Decreased cell migration	Induces directed migration of keratinocytes, fibroblasts, monocytes and macrophages Induces $\alpha 5$ and $\beta 1$ integrins for cell migration
Impaired fibromyoblast differentiation	Induces alpha smooth muscle actin required for fibromyoblast differentiation and wound closure
Poor granulation tissue formation/ paucity of extracellular matrix	Increased cellular recruitment, cell proliferation and stimulation of extracellular matrix production (Type I Collagen, Fibronectin, Elastin and TGF $\beta$ -3)
Dysfunctional inflammatory response, poor removal of necrotic cells and biofilm formation	Mediates phagocytosis of dead cells by macrophages and neutrophils (tissue debridement) Activation of macrophages and cytokine release
Decreased angiogenesis	Induces proliferation of micro vascular endothelial cells

topical application of CRT to compensate for, and therefore, treat impaired healing of diabetic wounds. Intracellular CRT is dynamically expressed during wound repair and it is known that this stress response protein is increased during cell injury. Without a well-proven exit route of CRT from the cell, CRT might have evolved to heal wounds following cell injury and release from dead cells. Ostensibly, topical application of CRT provides an additional amount of protein for the observed enhanced porcine and murine wound healing.

## DISCUSSION AND CONCLUSION

The failure of efficacious and cost-effective pharmacological treatments for chronic diabetic wounds has caused impaired diabetic wound healing to remain a serious unmet medical need and economic burden (\$174 billion direct and indirect costs [NIDDK-NDIC]). The uniqueness of CRT's broad-reaching diverse effects on the wound healing process has not been shown by any other topical wound healing agent. For example, Regranex® (becaplermin; PDGF-BB dimer), [72] the only topical agent approved for DFUs and used off-label to treat other chronic wounds, only targets the dermis whereas, as described above, CRT affects both the epidermis and dermis. In the porcine wound healing experiments described herein, Regranex was the benchmark positive control. The CRT-treated wounds showed a stratified epidermis and highly cellular neodermis containing abundant collagen fibrils compared to Regranex-treated wounds that barely showed one microscopic layer of keratinocytes and, the neodermis was less cellular with less collagen [27]. Other remarkable effects that were not obtained with Regranex include induction of proliferation of the regenerating basal and supra basal keratinocytes and fibroblasts, induction of

TGF- $\beta 3$ , and recruitment of many more macrophages. These cells engage in growth factor release and engulfment of dead cells and bacteria [27]. Furthermore, CRT induces hair follicles neogenesis in diabetic mice and the neodermis contained organized collagen fibrils in the diabetic murine wound healing studies [26]. Moreover, Regranex is not cost-effective and has been a disappointment for treatment of DFU as it is only slightly better than standard of care (SOC) [73,74]. Notably, Regranex has incurred a black box label as it is associated with a 5-fold risk for all types of cancers [75]. However, it is still being used since it is the only topical agent approved for DFUs. CRT is not a growth factor, normally circulates in the plasma (2-6 ng/ml) and is thus, unlikely to be immunogenic. SOC for DFUs includes wound debridement, infection management, and off-loading of the ulcer [76]. Add-on therapies are hyperbaric oxygen therapy, use of advanced wound care products (e.g., Regranex), and negative-pressure wound therapy [77]. However, efficacy and cost-effectiveness of these add-on treatments have not been shown.

Novel topical agents for chronic wounds including DFUs, venous stasis ulcers and pressure ulcers, and others have failed, generally in Phase II and Phase III clinical trials. For example, Aclerastide (DSC127; Derma Sciences; angiotensin analog) and HO/03/03 (HealOR; protein kinase C moderator) clinical trials are two topical agents that were developed for DFUs [78] that have failed. These agents might have failed because unlike CRT, both of these agents affect only one enzyme or factor. Currently, Granexin, a connexin 43 peptide mimetic (First String Research) and Galnobax, an aldolase inhibitor (NovaLead) have completed Phase II clinical trials. Other forms of topical therapy are cell devices such as Apligraf® and Dermagraft® that have been shown to be effective in impaired wound healing. However, these cell impregnated scaffolds are difficult to store (-80°C), prepare (37°C incubator), apply, and are very expensive. In summary, because of the compelling evidence for the varied effects of CRT on both the cells and functions necessary for wound healing, the application of CRT to wounds is expected to be superior to currently available products indicated for DFUs and chronic wounds, in general.

## CONFLICT OF INTEREST

LIG is an equity owner in CalRegen, Inc. UMP has no financial interest or other conflicts of interest to disclose.

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