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Review Article

Immune aging in diabetes and its implications in wound healing

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ABSTRACT

Immune systems have evolved to recognize and eliminate pathogens and damaged cells. In humans, it is estimated to recognize 10^9 epitopes and natural selection ensures that clonally expanded cells replace unstimulated cells and overall immune cell numbers remain stationary.

But, with age, it faces continuous repertoire restriction and concomitant accumulation of primed cells. Changes shaping the aging immune system have bitter consequences because, as inflammatory responses gain intensity and duration, tissue-damaging immunity and inflammatory disease arise.

During inflammation, the glycolytic flux cannot cope with increasing ATP demands, limiting the immune response's extent. In diabetes, higher glucose availability stretches the glycolytic limit, dysregulating proteostasis and increasing T-cell expansion. Long-term hyperglycemia exerts an accumulating effect, leading to higher inflammatory cytokine levels and increased cytotoxic mediator secretion upon infection, a phenomenon known as diabetic chronic inflammation.

Here we review the etiology of diabetic chronic inflammation and its consequences on wound healing.

1. Introduction

The immune system has evolved significantly from the rudimentary enzyme-based defense mechanisms we see in bacteria, to the structured and adaptive system we see in vertebrates. As pathogens have evolved to hide from the host's immunity, so did the immune system evolve to be able to recognize a plethora of antigenic motifs. This results in complex mechanisms that enable the immune system to adapt and memorize previous pathogen encounters. This continuous adaptation has led to the specialization of individual cells and tissues, which is coordinated to ensure that the potentially devastating effect of the immune response is not unleashed upon healthy tissue, before, during or after pathogen infection occurs.

However, the continuous adaptation of the immune system comes with a price. The recurrent exposure to foreign antigens leads to a state of chronic inflammatory that is characteristic of elder individuals and is now called inflammaging [1]. Recently it has been proposed that the major source of inflammatory stimuli is not exterior but comes from altered host cell proteins that are a consequence of damaged and/or dead cells and organelles, a phenomenon known as garb-aging [2].

Many theories on the mechanisms that drive inflammaging have been put forth. As with all aspects of aging, it is reasonable to expect that inflammaging also has many causes and their relative weight varies significantly between individuals, due to lifestyle, genetics and pathogen encounters.

Several reviews have discussed the similarities between inflammaging and the chronic inflammation observed in diabetic patients [3,4]. Some have even proposed that inflammaging could be a therapeutic target in diabetic patients [5]. Nonetheless, we believe that no other diabetic complication would benefit more from targeting inflammaging than chronic Diabetic Foot Ulcerations (DFU). This belief is based on the profound impact that chronic inflammation has on the exacerbation of the immune response and consequent healing dysregulation [6], hence the need for this literature review.

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In this review we argue that inflammaging is a physiological consequence of the evolution of the immune system towards a pathogen-specific response, that had to cope with many stress factors. These stress factors include increased longevity and consequent reduced thymic output, increased metabolic dysfunction, due to excessive caloric intake and alterations in skin/gut microbial ecology. In addition, in diabetic individuals, hyperglycemia and other factors increase the activation of various immune cells leading to inflammatory cytokine overexpression, as will be discussed in more detail below.

Ultimately, all these factors lead to an exacerbation of the chronic inflammation phenotype [7], causing most of the diabetes-associated complications, especially wound healing impairment, observed in some diabetic individuals.

We end this review by addressing possible therapeutic strategies targeted toward chronic inflammation, therefore substantially improving wound healing in chronic DFU patients.

1.1. Inflammaging

Inflammaging was first proposed by Franceschi and colleagues [1] in the year 2000, and was defined as a "global reduction in the capacity to cope with a variety of stressors and a concomitant progressive increase in the proinflammatory status". This theory proposes that continuous antigenic stress leads to a proinflammatory status which, upon reaching a threshold, hinders the immune response and promotes disability and disease.

This status is characterized by increased levels of circulating proinflammatory cytokines, specially Interleukin (IL)-6 and Tumor Necrosis Factor (TNF)-α [8] and the accumulation of effector ($CD28^{null}$) T-cells on both $CD4^+$ and $CD8^+$ subsets [9]. These cells are known to have a pathogenic potential in a variety of immune disorders, including autoimmune diseases, chronic inflammatory diseases and immune deficiency [10]. We will not discuss in detail the mechanisms and effects of inflammaging on immunosenescence, as they have recently been thoroughly reviewed [11]. Instead, we will focus on the causes of inflammaging and its relation with the chronic low-grade inflammation observed in diabetic patients.

Persistent viral infections, specially by Cytomegalovirus (CMV), are one of the driving forces behind inflammaging [12]. In fact, in old individuals, CMV seropositivity directly correlates with a decrease in naïve T-cells and an increase in memory and effector T-cell phenotypes, with concomitant increase in IL-6 levels [13], as well as an accumulation of terminally differentiated $NKG2C^+$ $CD57^+$ Natural Killer (NK) cells [14]. CMV infection also plays a role in the development of type 1 diabetes [15]. Furtheremore, a seroprevalence of anti-CMV IgG antibodies has been associated with Type 2 Diabetes Mellitus (T2DM) in middle-aged individuals [16]. Interestingly, high levels of the pro-inflammatory cytokines TNF-α, IL-1 and IL-6 are also observed in diabetic patients [17]. Furthermore, a decrease in naïve T-cells and an increase in memory and effector T-cell phenotypes has been associated with diabetes and is believed to cause wound healing impairment, observed in DFU patients [7].

Age-related changes in gut microbiome have also been described as a cause of inflammaging [18]. Among many other factors, age-related changes in dietary content have a significant impact on the gut microbiome [19], which modulates the host's immunity [20]. It is thus commonly accepted that poorer dietary content negatively impacts on morbidity and mortality, in old age [21]. On the other hand, changes in the gut microbiome are also associated with obesity [22] and T2DM [23]. In fact, recent evidence has even suggested that the gut microbiome can be a target for the treatment of T2DM [24].

Although the association between the gut microbiome and aging is, by far, the most extensively studied, evidence that other microbiomes also change with age and contribute to inflammaging is increasing. Aging-related changes in skin microbiome [25], bladder microbiome [26] and urinary tract microbiome [27] also have an impact in the immune system. Interestingly, changes in the urinary tract [27] and bladder [28] microbiome are also associated with T2DM. Recent studies have reported a significant reduction in diversity of the skin microbiome in diabetic patients [29]. The mechanism and the extent to which age or diabetes associated changes in these microbiomes have an impact on inflammaging is still elusive, but data from ongoing longitudinal studies will surely give new insights into this topic.

Oxidative stress and inflammaging are intimately related [30]. It is known that aging and diabetes share important features that include oxidative stress and low-grade inflammation. In aging, as in the early stages of diabetes there is a persistent accumulation of oxidative damage in biomolecules, caused by Reactive Oxygen Species (ROS) produced in all cells, especially by mitochondria [31]. Mitochondria are the main producers and also the main targets of ROS since it damages mitochondrial DNA (mtDNA), leading to a vicious cycle of increased ROS production. The consequence is the increase of mtDNA mutations that accelerate aging [32,33].

Cells have several antioxidant defense mechanisms that prevent ROS formation or neutralize ROS after being generated, including superoxide dismutase, catalase and glutathione reductase [34,35]. When the concentration of ROS overwhelms the capacity of these mechanisms, oxidative stress damages biomolecules, such as DNA, proteins, lipids and other cellular components [36]. So, a balance between ROS levels and antioxidants is needed for proper cell function [36]. Interestingly, it was shown that the increase in diet antioxidants can improve immune function and reduce oxidative stress, thus, the association between the redox state and immune function influences overall fitness [37]. Experimental evidence shows that low ROS levels extend lifespan in diverse organisms [38,39]. It has been reported that Endothelial Progenitor Cells (EPCs) of diabetic patients suffer oxidative stress-induced premature senescence [40,41]. The senescence of several tissues, including the endothelium, showed an upregulation of p53 in diabetic animal models and *in vitro* systems [42–44]. These data have shown treatment with antioxidants prevents endothelial senescence ameliorating endothelial dysfunction [42]. Also, diabetic patients treated with antioxidant compounds show better immune and endothelial function [45]. Moreover, an alternative to antioxidant administration is the promotion of the cellular antioxidant defense capacity to restore the redox status and prevent diabetes or aging-related damage [46]. As an example, Caloric Restriction (CR), which has a well-established antiaging action, diminishes oxidative stress and age-related diseases [47,48]. CR modulates several important inflammatory signaling pathways involved in aging and inflammation, such as mammalian Target Of Rapamycin (mTOR), Nuclear Factor (NF)-κB and Mitogen Activated Protein Kinases (MAPK) [49] and attenuates the age-related upregulation of IL-1β, IL-6, and TNF-α [47]. Moreover, sirtuins, a family of NAD^+-dependent deacetylases with epigenetic modulating activity, can prevent vascular senescence by increasing antioxidant defense [50]. Resveratrol and synthetic sirtuin activators mimic CR by conferring the attenuation of low-grade inflammation, especially in obesity and T2DM models [51–53]. Aging and diabetes lead to a reduced ability to preserve the cellular or system redox state resulting in a functional loss and an increase in oxidative stress which can lead to an increased production of proinflammatory cytokines that lead to a low grade chronic inflammatory condition, creating a vicious circle [54].

Even though the major driving force for inflammaging is probably external, recent evidence has suggested that cellular debris, organelles and other cellular components are a significant source of inflammaging [2]. Millions of cells die every day in our body and their contents can actively trigger an inflammatory response [55]. Some of these responses appear to be driven by stimulation of pattern recognition receptors [56] on dendritic cells or through various low molecular weight molecules that stimulate phagocytes. These have recently been named "find-me" signals [57].

Protein homeostasis, known as proteostasis, involves the activation of the Unfolded Protein Response (UPR), the Endoplasmic Reticulum-

Associated protein Degradation (ERAD)/Ubiquitin-Proteasome System (UPS) and different types of autophagy [58]. A direct link between aging and a decline in proteostasis has been established [58–61] and protein misfolding seems to be a major contributor to tissue functional decline during aging [62]. For instance, the high proteasome activity found in centenarians may be one of the reasons for their healthy ageing, because proteasome degrades small damaged proteins [63]. Moreover, autophagy degrades unwanted long-lived proteins, protein aggregates and damaged or functionally redundant organelles and parts of organelles and there are many studies relating increased autophagy and a long life [64]. Moreover, Meijer and Codogno suggest that insulin resistance is an adaptive response to increased autophagy that will prolong life [65]. In fact, loss of proteostasis with age leads to the accumulation of dysfunctional proteins and protein aggregates that are found in almost all tissues of aged organisms [66]. Furthermore, increasing evidence suggests that many age-related pathologies, such as neurodegenerative diseases [67,68], dementia [69] and osteoporosis [70], are associated with defects in proteostasis, as recently reviewed [71–73]. In addition, calorie restriction, which activates autophagy, provides protection against age-related diseases [65]. Interestingly, these pathologies and defects in proteostasis are associated with chronic inflammation [73–77].

Since the possible causes of inflammaging are also involved in the pathophysiology of diabetes, it is important to understand the relations between inflammaging and the chronic inflammation observed in diabetic patients.

1.2. Chronic inflammation in obesity and diabetes

Basal low-grade inflammation in obese and diabetic patients has multiple causes and multiple consequences (Fig. 1). In fact, immune cells, such as macrophages, dendritic cells and T cells usually infiltrate the adipose tissue of obese individuals and, as a result, adipose tissue secretes excess of free fatty acids and inflammatory cytokines.

The metaflammation theory, one of the most exciting new theories in the field of diabetes, put forth by Gokhan S Hotamisligil in 2006, links high nutrient intake and obesity with chronic inflammation [78]. In particular, chronic high-fat diet generates a low-grade inflammatory response, most notably through the Nucleotide-binding oligomerization domain, Leucine rich Repeat and Pyrin domain containing (NLRP)3 inflammasome [79], in tissues where free fatty acids reach the highest concentrations, such as the liver [80] and the adipose tissue [81]. More important, silencing NLRP3 can stop high fat diet induced chronic inflammation in animal models [82]. Moreover, damage to adipocytes and hepatocytes due to high fatty acid content leads to cell death that, in turn, is an additional source of inflammation [83]. There are many studies relating dysfunctional proteostasis and inflammation in different types of diseases [84]. Some studies demonstrated an increased activation of the UPR in diabetic and obese patients and that ER stress has a role in insulin resistance in obesity and T2DM [85].

The endoplasmic reticulum (ER) is involved in calcium homeostasis, in the synthesis and folding of secreted and transmembrane proteins and in lipid biosynthesis. UPR, ERAD and autophagy are essential to maintain ER homeostasis [86,87]. Intracellular stress, including the accumulation of fatty acids or cholesterol, bacterial toxins and proinflammatory cytokines, can lead to the accumulation of misfolded or unfolded proteins inside the ER, known as ER stress. This can activate one to three branches of the UPR, starting with Inositol-Requiring Enzyme (IRE)1α, Protein Kinase R (PKR)-like Endoplasmic Reticulum Kinase (PERK) and Activating Transcription Factor (ATF)6, in order to preserve ER homeostasis [88–91]. Highly secretory cells, such as interferon-producing plasmacytoid dendritic cells, Paneth cells or B cells, are very dependent of the UPR for their correct differentiation and function [89,92]. On the other hand, an excessive or prolonged ER stress can lead to the activation of apoptotic pathways [88]. The presence of apoptotic bodies can induce inflammation [93].

Toxins derived from bacterial infection can enter the ER lumen and inhibit Glucose-Regulated Protein (GRP)78, the chaperone that inhibits the three initiators of UPR, or can increase anti-chaperone antibodies leading to UPR activation [94]. Furthermore, virus can also alter the UPR activating or inhibiting one of the UPR branches [89]. The UPR is part of the immune response, particularly the IRE1α branch, in many types of living organisms [92]. In mammals UPR, through IRE1α-TRAF2-Ikk, activates NF-kB leading to the proinflammatory TNF-α production [89]. The UPR branch initiated by PERK inhibits general protein translation and can also inhibit the translation of regulators of NF-kB, like nuclear factor of kappa light polypeptide gene enhancer in B-cells Inhibitor (IkB) [94]. NF-kB activation induced by ER stress promotes IL-1β secretion in various cell types, including macrophages and differentiated adipocytes [95]. However, low ER stress preconditioning has opposite effects attenuating TNF-α-induced NF-kB activation in endothelial cells [89]. Furthermore, UPR can activate proinflammatory kinases, such as c-Jun N-terminal kinases (JNK), through the IRE1α-TRAF2-ASK1 pathway [96]. Chaperones, known to decrease ER stress, reduced JNK activation and improved insulin sensitivity in mice [89]. Through PERK-ATF4-ATF3 and IRE1α-sXBP-1, UPR can also induce the transcription of cytokine genes [94,97]. For instance, in macrophages, the activation of IRE1α-sXBP-1 increases the

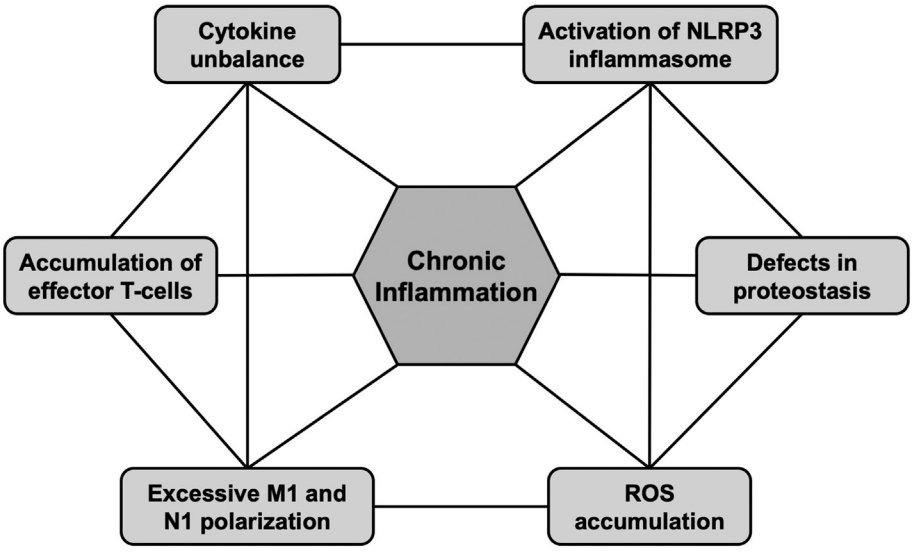

Fig. 1. Mechanisms of diabetes-associated chronic inflammation.
Chronic inflammation is at the center of DFU pathology and is caused by multiple factors that are both interconnected and interdependent. These factors have molecular cues (to the right of the figure) that have cellular consequences (to the left) and, altogether, define the chronic inflammation phenotype.

expression of proinflammatory factors, like IL-6 [98]. Moreover, ER stress can not only be due to, but also induce the accumulation of ROS [99]. An excessive accumulation of ROS can have proinflammatory effects, by inducing inflammasome activation [100] and promoting CASP-1-dependent IL-1β secretion [101]. Furthermore, ER stress can also activate the NLRP3 inflammasome in human macrophages triggering IL-1β secretion [102]. This positive feedback between infection inducing UPR activation and UPR inducing inflammation can lead to chronic diseases, like diabetes [103].

Insufficient or excessive nutrient supply leads to metabolic stress. A chronic excessive nutrient supply is involved in many metabolic diseases including obesity, insulin resistance and diabetes. Saturated fatty acids can incorporate in the ER membrane of pancreatic β-cells, and probably in other cell types and other membranes [104,105], This can lead to reduced sphingomyelin and cholesterol levels, that are needed to form ER membrane lipid rafts, essential to transport vesicles to the Golgi apparatus [106]. This leads to the accumulation of proteins inside the ER and consequently ER stress and the activation of the UPR [106,107]. On the contrary, unsaturated fatty acids can inhibit ER stress [108]. In macrophages, the increase of cholesterol in ER membranes depletes ER calcium stores leading to UPR activation and consequent activation of caspase-3 and the expression of the UPR-dependent proapoptotic CHOP, culminating in macrophage apoptosis [109]. High levels of circulating glucose activate the UPR, either directly or through the increase in ROS levels [110–112].

Usually, the chronic consumption of excessive lipids and excessive sugars is simultaneous. In fact, the modern diet with high consumption of refined vegetable oils and sugars, highly processed cereals, dairy products and a high ratio of omega-6 to omega-3 polyunsaturated fatty acids contributes to low-grade chronic inflammation [113]. Moreover, high levels of circulating saturated fatty acids and sugars are common in obesity and diabetes, leading to a synergistic effect named glucolipotoxicity [114]. In adipocytes and pancreatic β-cells, UPR activation is a mediator between glucolipotoxicity and inflammation [111]. In obesity, visceral adipocytes release fatty acids and inflammatory factors into the circulation leading to the accumulation of triglycerides in the liver, muscle and pancreas, impairing insulin and glucose homeostasis and contributing to chronic inflammation [115,116]. On the other hand, weight loss reduces insulin resistance, glucolipotoxicity, adipose tissue inflammation and the risk of diabetes, some times more effective than drug interventions, preserves β-cell function and increases beneficial adiponectin secretion [117].

UPR can induce the activation of autophagy through different pathways [118]. Autophagy is of major importance for the maintenance of cellular fitness through the degradation of unwanted proteins and organelles. It is also activated during nutrient deprivation, where it serves as a source of amino acids, free fatty acids for ATP production and other nutrients. On the other hand, amino acids and insulin inhibit autophagy initiation [119].

There is increasing evidence that activation of autophagy improves insulin resistance [120,121]. However, insulin resistance can also increase autophagy [122], because insulin is not activating its receptor to the same extent and consequently the activation of the pathway that leads to autophagy inhibition is decreased. In fact, defects in autophagy are associated with diabetes and obesity and can induce ER stress [123]. Moreover, pancreatic β-cells in T2DM patients have increased autophagic vacuoles and autophagosomes associated with cell death [124]. On the other hand, dysfunctional autophagy can induce insulin resistance [123,125,126].

Autophagy is also involved in antigenic peptide processing and promotes the development and survival of B and T cells [127,128], enhancing innate and adaptive immune responses. Defects in autophagic pathways lead to increased inflammation [129] and defects in ER stress induced autophagy are involved in chronic inflammation observed in diabetic patients [130].

Obesity and diabetes also have a large impact on specific populations of immune cells. Macrophage polarization is significantly altered by obesity and diabetes, favoring the M1 pro-inflammatory phenotype [131] that, through IL-6 and TNF-α secretion promotes low-grade chronic inflammation and insulin resistance [132]. This effect is partially due to the role of oxidized phospholipids, that regulate gene expression in adipose tissue macrophages, favoring M1 polarization [131]. Recent data puts microRNA (miR)-30 downregulation in the center of adipose tissue macrophage induced chronic inflammation [132]. Regardless of the driving force behind M1 polarization in obese and diabetic individuals, we know that the process is reversible because, at least in mice, dietary restriction leads to a decrease in macrophage adipose tissue infiltration and a consequent decrease in IL-6, TNF-α and IL-1β levels [133].

Neutrophils are also recruited to the adipose tissue in obese and diabetic individuals [134], where they differentiate to the pro-inflammatory N1 phenotype and contribute to chronic inflammation [135].

Diabetes and obesity have an enormous impact on T-cell differentiation and function, as recently and thoroughly reviewed [136]. Moreover, since T-cells express the insulin receptor upon stimulation [137] they are sensitive to differences in glucose concentrations, and hyperglycemia alone is able to activate NF-kB [138], impairing leukocyte activation [139] and migration [140]. We already know that Glucose transporter (Glut)1 is selectively essential for $CD4^+$ T-cell activation and effector function [141], but little else is known about how differences in glucose concentrations change the cytokine secretion profile of activated T-cells. Nonetheless, an accumulation of effector T-cells and consequent reduction in T-Cell Receptor (TCR) repertoire diversity has already been reported in diabetic patients [7].

Recently, new evidence has even suggested that B cells also play a role in chronic low-grade inflammation. B cells also infiltrate adipose tissues where they work as antigen presenting cells and contribute to insulin resistance and inflammation by secreting Interferon (IFN)-γ, IL-12 and TNF-α, as well as pathogenic antibodies [142]. Moreover, these effects seem to be, at least in part, regulated by leptin [143] and saturated fatty acids [144]. The same effect is also associated with aging [145].

It is clear that obesity and diabetes contribute to low-grade chronic inflammation, with impact on a variety of immune cells and processes. Accumulating evidence starts to suggest that the immunometabolism is itself altered by obesity and diabetes, with a strong impact in the immune response.

1.3. Immunometabolism

Immune cells switch between different metabolic pathways in response to different stimuli and in different conditions [146]. For example, M1 macrophages are avid glucose consumers and get most of their energy from glycolysis, whereas M2 macrophages prefer a steady state ATP generation program and favor the Tricarboxylic Acid (TCA) cycle and oxidative phosphorylation [147]. Moreover, PPAR-Gamma-Coactivator-1beta (PGC-1β), known for its central role in the transcriptional control of mitochondrial biogenesis and respiratory function [148], also decreases macrophage response to free fatty acids [149] and induces M2 polarization [150].

One of the hallmarks of immunometabolism has been the intrinsic relation between glycolytic metabolism and T-cell activation. In fact T-cell activation and differentiation relies so greatly on glycolysis that inhibiting this pathway completely blocks effector T-cell differentiation [151]. Moreover, accumulating evidence suggests that the extent of T-cell clonal expansion is controlled by the proficiency of the glycolytic metabolism [152] and thus glucose and oxygen availability is determinant. The result of this glycolytic burst is the production of lactate (Warburg effect) that regulates both cell migration and effector functions [153]. Despite the extensive glycolytic switch, mitochondrial function is still important for T-cells, both at the level of calcium and

ROS signaling and through the production of building blocks important for many anabolic processes that support cell proliferation and migration [154].

Mounting evidence suggests an important role for leptin in T-cell activation [155] and differentiation [156], through the up-regulation of Glut1 and concomitant increase in glucose uptake [155]. In fact, only effector T-cell Glut1 expression seems to be regulated by leptin and no effect was observed in naïve or regulatory T-cells [155]. Moreover, leptin signaling favors pro-inflammatory Th17 T-cell polarization [157] through a Hypoxia-Inducible Factor (HIF)-1α mediated pathway [156]. This is of particular importance since leptin levels are significantly higher in obese [158] and T2DM patients [159], thus explaining why effector T-cells accumulate in these individuals, contributing to the chronic low-grade inflammation phenotype [160] that is particularly more severe in the adipose tissue [161], where leptin is produced.

Moreover, molecules that mimic the biochemical and functional effects of caloric restriction, such as aspirin [162], 2-Deoxyglucose [163], fisetin [164] and others that have immunomodulatory effects, reduce chronic inflammation [165]. This is of particular interest to the field of diabetes because metformin, one of the most broadly used drugs for glycemic control, is also a caloric restriction mimetic [165] and has the ability to reduce chronic inflammation [166].

All recent evidence points in the direction of a strong and decisive impact of metabolism in T and B-cell priming and expansion and its important role in age and diabetes associated chronic inflammation.

1.4. B cells and aging

Tissues with regenerative capacity are able to continuously produce renewed cells throughout life, a process that is maintained due to the presence of tissue-specific stem cells. As described elsewhere [167,168], tissues with a regenerative capacity change with aging, but their regenerative capacity is not fully abolished. Thus, and as an example, despite accumulation of senescent characteristics in the aged skin, epidermal stem cells are maintained at normal levels throughout life [169]. Lymphocytes develop from Hematopoietic Stem Cells (HSC) that will posteriorly differentiate into early common lymphoid progenitors, that will differentiate either into T or B lymphocytes. In parallel, myeloid progenitors will generate myeloid leukocyte populations. Aleatory rearrangement of V(D)J segments in the gene that codes for the variable portion of immunoglobulins occurs during lymphocyte maturation and generates an almost infinite repertoire of antigen recognition on T and B lymphocytes. During adulthood, the size of the lymphocyte compartment is maintained by equal production and loss rates [170]. In adult mice, approximately 10^7 immature B cells are generated daily, however about only 3% of these survive positive and negative selection to form the pool of mature B cells in secondary lymphoid organs [170,171]. The rate of lymphopoiesis dramatically declines with aging because of cell-intrinsic and extrinsic factors that affect HSC, as well as early and late lymphoid progenitors. Several studies revealed that, with age, lymphoid differentiation diminishes in favor of myeloid differentiation, which affects directly the pool of naïve lymphocytes and clonal diversity, promoting higher frequencies of long-lived memory cells or innate-like lymphocyte populations. It was also proposed that DNA damage may alter lineage potential and responsiveness to trophic signals [172–175]. Importantly, it has been demonstrated that the composition of the stem cell compartment in aging is dominated by myeloid-biased HSCs [176,177], with down-regulation of genes associated with lymphoid lineage and up-regulation of genes directing myeloid lineage [173,178].

As a consequence of a reduced lymphopoiesis, the number and proliferative abilities of B-cell progenitors and immature B cells in the Bone-Marrow (BM) declines with age [171,179]. It is also described that, with age, there is decreased IL-7 production, and impaired V-DJ rearrangement [180,181]. Several studies show that when BM cells or purified HSCs from old mice were adoptively transferred into irradiated younger (adult) mice, they poorly reconstituted the peripheral B-cell compartment [173,182–184].

Despite the decline in lymphopoiesis, there are no significant changes in the absolute numbers of peripheral B and T-cells with age [185,186]. However, the cellular composition of the peripheral compartment is dramatically changed in old age. Perhaps the most significant change is the accumulation of long-lived memory cells and the decline in frequencies of naive cells. B cells in aged mice have a significantly longer life span than peripheral B cells in younger mice [187]. Indeed, analysis of B-lineage cells in old mice revealed a significant decrease in the number of naive B-2 cells (which are short-lived and primarily reside in the follicular compartment) and a significant increase in peripheral B cells expressing antigen-experienced long-lived phenotypes, including memory B cells, B-1 cells, and marginal zone B cells [185,188]. It is also interesting to notice that the onset of type I diabetes is also associated with an increase in the frequencies and total numbers of B-1 cells [189–192].

Until recently, it was thought that lymphocyte aging was an irreversible process. However, studies from Melamed and colleagues showed that this may not be the case, shedding some light into how inflammaging in chronic inflammatory conditions (such as diabetes) could be addressed or clinically ameliorated. The authors showed that the main cause for reduced lymphopoiesis and the consequent loss of clonal diverse naïve B cells are not cell intrinsic defects but, instead, a reduced number of HSCs with lymphocyte lineage potential. Keren et al. showed that aged mice had significantly lower number of HSCs that retained the potential to differentiate in early lymphoid progenitors; however, these lymphoid HSCs retained the same ability to proliferate and generate lymphocytes. Thus, when these lymphoid HSCs were transferred into irradiated aged recipients they were perfectly able to regenerate the pool of naïve and clonal diverse B cells [193,194]. Also, the same authors demonstrated that several rounds of peripheral lymphocyte depletion reverse the “aging” state of B cells in secondary lymphoid organs, originating a “young-like” repertoire of B-cells [194].

Thus, altogether, it seems that with aging, the demand for newly generated lymphocytes diminishes because the expansion of the memory cell pool is probably favored. In fact, it is plausible to assume that, with increased age, the number of neo antigens that our body is exposed to diminishes significantly. This, in turn, will favor the maintenance of antigen-experienced memory lymphocytes in detriment of recently-formed naïve B (and T) cells. Also, inflammatory signals or mitogen-induced polyclonal IgM seem to affect directly the pool of lymphocyte precursors in the BM [195,196], which can also explain why during chronic inflammatory conditions the clonal diversity of T and B-cells reduces dramatically. The ability to rejuvenate the peripheral compartment by depletion of B-cells is supported clinically in patients treated with rituximab (a monoclonal antibody directed towards the surface antigen CD20, that causes B-cell depletion). In these patients, the newly generated B-cells post-treatment, express a young phenotype and a diverse repertoire [197,198]. However, only replacing the B lineage is not enough to achieve complete restoration of a “young-like” immune response. Age-related defects are also developing in the T lineage and innate cells. Production of T-cells depends on further maturation and selection of progenitor cells in the thymus, which undergoes atrophy with age.

1.5. T-cell priming and expansion

Since Burnet elucidated GOD (generation of diversity) and its central role in the adaptive immune response, immunologists have tried to estimate the size of the TCR repertoire and how it evolves with age. The minimal estimate of the Human naïve TCR repertoire is thought to be of the 10^8 magnitude at birth [199] and linearly declines after thymic involution, reaching a 10^6 magnitude in the 7th decade [200] and steeply declining thereafter [201]. This age-associated repertoire restriction generates holes that lead to impaired anti-pathogen [202] and

anti-cancer immunity [203]. Moreover, degenerate T-cell recognition of peptide-Major Histocompatibility Complex (MHC)-I complexes can further exacerbate the physiological importance of these repertoire holes [204].

Various studies have already demonstrated that some infections that cause a strong T-cell stimulation may cause a deleterious effect on TCR repertoire diversity and thus have a huge impact on the immune response to subsequent infections by different pathogens [205]. This effect is mainly due to the competition for resources (such as IL-2) that mediates the survival of naïve cells and limits the total T-cell count [206]. Other studies have demonstrated that regular pathogen encounters significantly reduce the diversity of the TCR repertoire in aged individuals [199].

Moreover, age-associated thymic involution [207] and hematopoietic progenitor cell decline [208] lead to a lower rate of naïve T-cell renewal, further contributing to the reduction in TCR repertoire diversity. Nevertheless, it has also been demonstrated that there is a fair amount of plasticity in the TCR repertoire, mainly due to cross-reactivity, that compensates for the reduction in TCR repertoire diversity [209], but this effect may be limited.

A reduction in TCR repertoire diversity and a decline in naïve T-cell numbers is also observed in diabetic patients [7], in much younger ages, possibly explaining the higher risk of infection [210] and cancer [211,212] in these individuals, especially those with longer disease durations and poorer glycemic control.

Besides the reduction in TCR diversity, aging is also associated with a reduction in naïve $CD8^+$ T-cell priming efficacy [213], that correlates with poor primary immune responsiveness [214]. This reduction in priming efficacy has already been reported in patients with persistent virus infections [215,216] and is associated with immune exhaustion.

In diabetic patients, excessive T-cell activation and expansion, driven by high glycemia and high leptin levels, leads to chronic inflammation and severe reduction in TCR diversity that is involved or even responsible for most of the diabetes-associated pathologies, such as chronic diabetic foot ulcerations (Fig. 2).

1.6. Impaired wound healing

DFUs are the most debilitating complications of diabetes and are a consequence of uncontrolled infection of foot wounds, often due to various diabetes-associated complications, such as neuropathy, impaired angiogenesis and chronic low-grade inflammation [17] (Fig. 3).

Peripheral neuropathy is present in nearly half of non-healing DFU cases [217] and neurological assessment is routinely used in the evaluation of DFU risk. Patients with reduced lower extremity pain sensation are prone to the development of chronic ulcers, because auto-diagnosis is hindered by the inability to detect wounds in the plantar foot regions, until these wounds become dangerously large.

Besides the decreased pain sensation, neuropathy also impairs neuro-immune interactions [218], either through decreased neuropeptide expression [219] or through a reduction in the cholinergic anti-inflammatory effect [220], both capable of modulating inflammation during wound healing. In fact, topical treatment of diabetic wounds with neuropeptides, such as substance P [221], neuropeptide Y [222] or neurotensin [223] reduce inflammation and improve wound healing.

Angiogenesis is also impaired in diabetic foot ulcers [224]. The reduced blood flow restricts macrophage and T-cell chemotaxis [225], impairing the immune response. It also reduces keratinocyte, fibroblast and endothelium progenitor cell migration to the wound [226], impairing tissue regeneration.

The reduced blood flow has a dramatic impact in the healing process, reducing oxygen availability and consequently ATP production [227]. In fact, hyperbaric oxygen therapy reliefs the need for lactic acid formation and improves wound healing [228], but it does not cure DFU [229]. This is partially because increased mitochondrial activity leads to oxidative stress, which can offset the healing effect in the long-term [230], but also because the lack of energy availability is not the only factor contributing to the wound healing impairment observed in DFU patients [231].

Long-term hyperglycemia leads to chronic inflammation [17] that, in turn, is involved in the etiology of all diabetic microvascular complications [232]. Despite the huge impact of long-term hyperglycemia in the etiology of diabetic complications, several large clinical trials [233,234] have shown that even though glucose-lowering treatments reduce the risk of cardiovascular disease, the risk of DFU still remains. This indicates that the mechanisms that impair wound healing are multifactorial [235].

ROS are essential for wound repair, however, excessive amounts of ROS have an adverse effect, due to lipid peroxidation, protein modification and DNA damage, all associated with impaired wound repair in chronic, non-healing wounds [236–240]. In addition to ROS-mediated persistent pro-inflammatory cytokine secretion and activation of matrix metalloproteases, excessive ROS can modify and/or degrade extracellular matrix proteins and cause impaired dermal fibroblast and keratinocyte function [241]. ROS are elevated in diabetic wounds [242] and the expression and activity of NADPH Oxidases (NOX), the main source of ROS in many cell types, are increased in hyperglycemic conditions [243]. Moreover, hyperglycemia activates protein kinase C in smooth muscle and endothelial cells which lead to the increased NOX activity [244]. Moreover, diabetic mouse wounds have increased expression and activity of H_2O_2-producing enzyme xanthine oxidase [245].

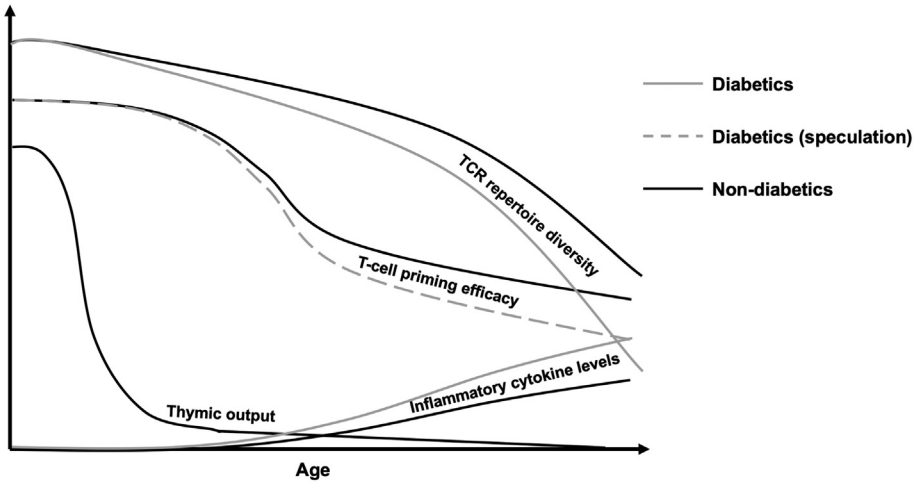

Fig. 2. T-cell activation in diabetic conditions.
T-cell expansion is limited by the extent of the glycolytic burst. In diabetic patients, due to chronic hyperglycemia, T-cells expand to greater numbers, reducing the naïve compartment and, consequently, the TCR repertoire diversity. Accumulating effector T-cells increase inflammatory cytokine levels and loss of the naïve pool is not compensated by an increase in thymic output, leading to a probable reduction in T-cell priming efficacy, as seen in elder individuals.

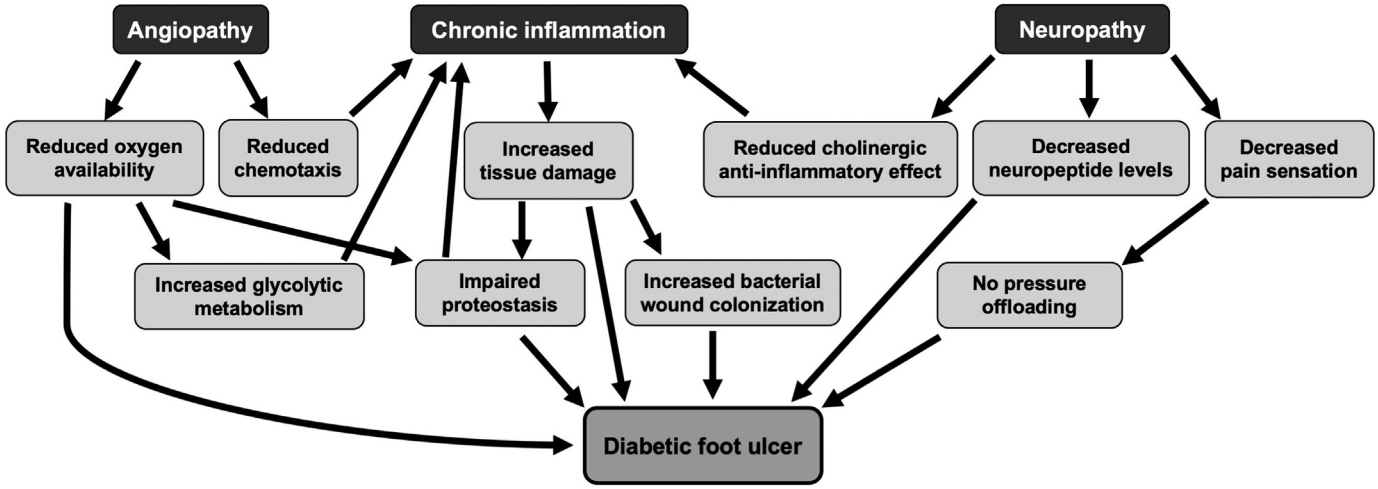

Fig. 3. Causes of diabetic wound healing impairment.
The diabetic foot ulcer pathology has multiple causes that can roughly be divided in three categories (angiopathy, chronic inflammation and neuropathy). All these underlying conditions trigger multiple interconnected effects that lead to an impaired immune response, a poorer tissue remodeling and, consequently, an increased and persistent bacterial wound colonization.

The mitochondrial electron transport chain is one of the main important sources of ROS in diabetes [244]. Hyperglycemia causes an increase in the production of mitochondrial superoxide [244,246], promoting the activation of the polyol, protein kinase C, and hexosamine pathways [244,246] and ultimately depleting antioxidant resources, and promoting further ROS and advanced glycation end products production.

The deleterious effects of ROS on cell function are also related to the reduction in antioxidant defenses. Superoxide Dysmutase (SOD), catalase, and glutathione peroxidase activity were reduced in the blood of diabetic patients [247]. In addition, the expression of Nuclear factor erythroid 2-Related Factor 2 (Nrf2), a regulator of antioxidant defense gene expression, is impaired in diabetes [248]. Oxidative stress decreases Nrf2 activity and is associated with reduced catalase, NADPH dehydrogenase Quinone 1 (NQO1), glutathione reductase, and glutathione S-transferase expression [249]. Moreover, high glucose levels decrease nuclear translocation of Nrf2 in dermal fibroblasts and the expression of MnSOD and NQO1 is reduced [250].

Some researchers propose that impaired wound healing in DFU is due to a concomitant excess of pro-inflammatory cytokines, like TNF-α, and a deficit of anti-inflammatory and healing associated cytokines, like IL-10 and TGF-β [251]. This cytokine unbalance explains the excessive M1 macrophage polarization [131] and the accumulation of effector $CD8^+$ T-cells in the wound site that, in turn, are responsible for the excessive granzyme secretion that leads to tissue destruction [252]. Moreover, under diabetic conditions, excessive TNF-α levels prime neutrophils for NETosis [253], even under sterile inflammation [254], also promoting tissue damage. Nonetheless, despite the obvious contribution of the cytokine unbalance to the impaired wound healing, this theory fails to explain why only some DFUs surprisingly become chronic and inflammation is not resolved, when all DFU patients have high pro-inflammatory cytokine levels [252].

Unpublished data from ongoing longitudinal studies by our group suggests that loss of TCR repertoire diversity and accumulation of effector T-cells, together with loss of naïve priming efficacy is the tipping effect that leads to immune exhaustion and the inability to successfully mount *de novo* immune responses that can resolve pathogen infections and heal diabetic wounds.

1.7. Vaccination as a strategy to slow immune aging

The rising prevalence and severity of infectious diseases among the elderly population has a significant negative impact on their quality of life and increases their dependency on healthcare systems. Attempts to improve vaccination efficacy among elderly populations has had only limited success [255], indicating that new innovative approaches are necessary to enhance immune competence and avoid cellular senescence in aging. A major limitation for these attempts is the composition of the different cellular compartments in aging, which can have poor responsiveness [256] and a limited repertoire [257,258].

Age-related defects are not limited to lymphocytes. Although the number of neutrophils remains unaltered with age, neutrophils from elderly subjects are characterized by a reduced capability to migrate towards a chemotactic signal. Of interest, this reduced chemotactic capacity could also lead to a diminished egress of neutrophils from inflamed tissue, thereby contributing to higher local inflammation, as observed in aged mice after burn-associated lung injury [259,260]. Moreover, it has also been described that recognition of Pathogen-Associated Molecular Patterns (PAMP) by endogenous Pattern Recognition Receptors (PRR), such as Toll-Like Receptors (TLR), induces increased IL-10 production in the elderly compared with younger adults, increasing their susceptibility to bacterial infections [261–265]. Interestingly, this ability of elder individuals to produce higher levels of IL-10 upon microbial/mitogenic stimulus is shared by neonates and may be due to the increased numbers of B-1 or marginal-zone B lymphocyte sub-populations [261,266,267].

Thus, new strategies for developing vaccines would be extremely important to tackle the increased susceptibility of elderly individuals to infections, specifically those caused by fast-growing extracellular bacteria. On the other hand, the stimulus with neo antigens such as those offered by peptide-based epitope targeted vaccines [268–270], would also increase the demand for newly generated B and T cells favoring new lymphopoiesis, which would most likely increase the levels of lymphoid HSCs and slow immune aging. In this regard, vaccination schedule in the elderly (or diabetic) should be rethought, increasing the number of immunizations for each vaccine – 2 to 3 doses, as it is done in the early years of life – in order to potentiate the proliferation of lymphoid HSCs and the output of recently lymphocyte emigrants from BM and thymus. Another issue to be considered is the type of adjuvant used. For many years, aluminum hydroxide (Alum) has been used with great success. Nevertheless, due to its ability to activate the NALP3 inflammasome and induce a very strong inflammatory response mediated by the IL-1 family of cytokines, it is not the best fit to use in the elderly or in individuals with an already excessive inflammatory condition [271]. Lipid emulsions, analogs of Lipopolysaccharide (LPS) or even agonists of TLR are already being used, inducing very strong

immunization responses without the exacerbated peak of inflammation seen with Alum [271–273].

Also, bacterial infections, namely those caused by multi-drug resistant strains of *Staphylococcus aureus*, are known to be responsible for serious complications in diabatic patients [260,267]. Again, a successful new vaccination approach would be the best-case scenario to avoid complications caused by infection, which would decrease the degree of inflammation and, in turn, would also reduce the tendency of a skewed lymphocyte repertoire.

2. Conclusion

Increasing evidence suggests that immune aging is potentiated by dysmetabolism, resulting in chronic inflammation and loss of immune fitness, with an enormous impact in age-related pathologies [274]. Chronic inflammation can lead to immune exhaustion, as observed in HIV infection [275], autoimmune disorders [276], cancer [277] or simply with old age [278].

Age-related decline in immune fitness is unavoidable because, until our last breath, we are constantly adapting to continuing pathogen infections and, by doing so, accumulating various immune effector cells. As we get better at fighting recurrent infections, we also increase collateral tissue damage and gradually reduce our ability to mount *de novo* immune responses. Due to the gradual loss of homeostatic fitness mechanisms, collateral tissue damage becomes increasingly problematic, creating the perfect habitat for the colonization of new microorganisms.

In diabetic patients, due to chronic dysmetabolism, the magnitude of the immune response is potentiated, leading to accelerated immune aging. Increased energy and metabolite availability are not the sole culprits of diabetes-associated chronic inflammation. Metabolic hormones, especially leptin, also play an important role in immune dysregulation, making them ideal targets for chronic inflammation-associated diabetes complications, such as retinopathy, nephropathy, neuropathy and impaired wound healing.

Impaired wound healing may be a consequence of the accelerated immune aging observed in diabetic patients. Based on this theory and as previously proposed [7], the restriction in TCR repertoire diversity and the accumulation of effector T-cells, both associated with inflammaging, can be used as biomarkers for chronic DFUs.

Thymic involution eventually abolishes the output of naive T-cells in aging, and any attempt to reverse T-cell aging should include the restoration of functional thymic tissue. Several hormones, sex steroids, growth factors, and cytokines have been shown to inhibit or partially restore thymic tissue [279]. The plasticity of the thymic tissue also has been shown in a recent study where the cortical thymic epithelial cell compartment regenerated-itself after conditional ablation and restored T-cell development [279–281].

Also, immunomodulatory therapies that can decrease non pathogen-specific tissue immunity, as previously reported in lung infection [277], may prove more useful in DFU care, than any currently used treatment.

As a final remark, despite not being the focus of this review, we would like to reflect on the fact that most of the current knowledge considers inflammaging and age- and diabetes-associated decline in the immune response to be gender-independent. This assumption is not based on empirical knowledge but on the lack of data on gender-associated differences in immune responses. Moreover, increasing evidence suggests that there are significant gender differences regarding prevalence and incidence of the most important age-related diseases [282]. Moreover, diabetes-associated complications, such as impaired wound healing, differently affect man and women [283] and male gender is considered one of the risk factors for DFU [284].

Conflicts of interest

The authors declare that there are no conflicts of interest.

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