NAME; OGUNJOBI DAVID OLUWATOSIN
MATRIC. NO.; 16/MHS01/169
ANATOMY, 400L

HISTOPATHOLOGY ASSIGNMENT

CYTOKINE SIGNALLING AND ITS ROLE IN WOUND HEALING

Cytokine signaling is an important part of the human body regulation (John *et al.,* 2011). Most cytokines are cell-secreted proteins from glial cells in the nervous system and are necessary for intracellular signaling (John *et al.,* 2011). Most cytokines are local regulators that alert and activate lymphocytes. Some cytokine-signaling pathways involve hormones such as growth hormones and leptin, the hormone that controls fat storage (John *et al.,* 2011).

Cytokine biology is now recognized as a fundamental component of immunology and the actions of cytokines are understood to be essential mechanisms underlying host defense, immunoregulation, and autoimmunity (John *et al.,* 2011). Moreover, cytokines themselves and cytokine antagonists have become some of the most successful new drugs(John *et al.,* 2011) On a more basic level, the biochemistry of cytokine action has become a paradigm for understanding rapid, evolutionarily conserved membrane-to-nucleus signal transduction, offering remarkable opportunities for understanding how extracellular cues are sensed and translated into the control of gene expression. As we approach the 20th anniversary of the discovery of the Jak-STAT pathway, it is useful to reconsider the pivotal insights that led to these discoveries, to briefly comment on the present status of this field, and consider future challenges (John *et al.,* 2011). Members of the Type I/II cytokine receptor superfamily like erythropoietin, growth hormone, prolactin, and IFN were first purified more than 50 years ago. Colony stimulating factors began to be studied in the 1960s and ‘70s, and the discovery of the first lymphokines and interleukins followed quickly thereafter (John *et al.,* 2011). Thus, knowledge of the criticality of cytokines is by no means new. Less obvious was any notion that these factors were all structurally related and used common elements in mediating their biological actions. With molecular cloning and structural analysis, it began to become

clear that the 4-α helical family of cytokines comprises a rather large group of secreted factors with diverse functions Nonetheless, the question remained (and remains): how do these diverse factors exert their unique effects on cell behavior? During the late 1980s, the Darnell and Stark labs began to tackle this question by identifying rapidly inducible IFN-stimulated genes (ISGs). With the isolation of genomic clones of these inducible genes, it was appreciated that they shared promoter elements that were responsible for IFN-mediated induction. Two types of elements were identified: IFN-stimulated response elements (ISRE) and IFN-γ-activated sites (GAS elements) (John *et al.,* 2011)Jim Darnell, David Levy, Thomas Decker, and colleagues began to identify nuclear complexes induced by IFNs that bound to ISREs and soon recognized that the ISRE-bound complex ISGF3 comprised multiple subunits(John *et al.,* 2011). The cloning of these constituents led to the identification of the first two signal transducer and activator of transcription (STAT) proteins, STAT1 and STAT2. The third component of the complex was a member of the IFN response factor (IRF) family, IRF-9. Complexes bound to GAS (GAFs) also turned out to be STATs. Independent work from other labs, interested in prolactin and IL-6 signaling, identified similar complexes, the cloning of which also demonstrated the existence of new family members, STAT5 and STAT3, respectively (John *et al.,* 2011)

Many of the aforementioned discoveries represent groundbreaking work. However, the exciting finding we chose to highlight was the discovery that these new factors not only bound DNA, but they were also tyrosine phosphorylated, making it clear that this new transcription factor family might be directly linked to a signaling pathway. Schindler et al. analyzed the covalent modifications and trafficking of the constituents of the ISGF3 complex. Using metabolic labeling, they showed IFN-dependent tyrosine phosphorylation and translocation from the cytoplasm to the nucleus where, presumably, active transcription was induced. The figure in the paper depicting the findings described many of the features we now associate with STATs. (John *et al.,* 2011).

The nature of the kinase responsible for such effects was still unknown but was presumed to be cytoplasmic in nature. Another equally striking feature of the STATs was the presence of an SH2 domain, a recognition motif for phosphotyrosine, which was further evidence of linkage to the action of tyrosine kinases. A key subsequent finding was that STATs bound cytokine receptors (John *et al.,* 2011). This put STATs in the position of being receptor-to-nucleus shuttles, directly connecting events from the extracellular milieu to de novo transcription. The importance of tyrosine phosphorylation as a mechanism of signal transduction became widely appreciated with the discovery of various oncogenes that were themselves tyrosine kinases (PTK) and the cloning of receptor tyrosine kinases like the insulin receptor and the epidermal growth factor receptor (John *et al.,* 2011). The race was on to identify other tyrosine kinases, and investigators used PCR-based approaches or low stringency screening to identify new members of this family. Out of such screens came tyrosine kinase 2 (Tyk2), Janus kinase (Jak)1 and Jak2, which were recognized to represent a new class of PTK but at this stage lacked a clearly assigned physiological function (John *et al.,* 2011)

In the meantime, George Stark and Sandra Pellegrini were engineering mutant cells that were defective in IFN-α/β and IFN-γ signaling (John *et al.,* 2011). This somatic cell mutagenesis approach yielded several classes of mutant lines, which were then used to identify a component that restores signaling. The approach led to an explosion of papers that established the criticality of various Jaks and STATs in signaling via different cytokines (John *et al.,* 2011). The study by Velazquez et al., was the first report showing that defective IFN-α signaling was complemented by a clone encoding a Jak, in this case Tyk2. The report linked the Jaks with a function for the first time – and it was an important one. Not only were Jaks involved in cytokine signaling, they were absolutely essential elements (John *et al.,* 2011). The approach of Pellegrini and colleagues involved the use of drug-sensitive cell lines mutagenized to select for insensitivity to IFN-α. Revertants were then isolated and genomic clones that conferred the IFN-sensitive phenotype were identified by construction of a cosmid library and transfection back into IFN-resistant cells. The cosmid responsible for phenotype conversion contained the gene coding for the tyrosine kinase Tyk2 which, at that point, had unknown function but exhibited homology to Jak1. This study provided unambiguous genetic evidence of the essential function of a Jak in IFN signaling (John *et al.,* 2011). The schematic model provided in the paper inserts Tyk2 as the receptor-associated proximal factor responsible for phosphorylation of ISGF3.

Other complementation studies quickly filled in the gaps, placing different Jaks and STATs with different cytokines (John *et al.,* 2011). Related studies showed that Jaks physically associated with cytokine receptors. The next phase in Jak/STAT biology was to assess whether data generated in a single mutant cell line had in vivo relevance. The answer was an unequivocal yes. Strikingly, in vivo evidence of the importance of the Jak/STAT pathway came from a human primary immunodeficiency, Jak3-SCID. Within two months, the same phenotype was revealed in Jak3-knockout mice, and a few months later the phenotype of STAT1-knockout mice was reported (John *et al.,* 2011). STAT2, STAT3, STAT4, STAT5a, STAT5b, and STAT6 knockouts were soon generated and the message was clear: the Jak/STAT pathway was fundamentally important for the development and differentiation of diverse cell types. Knocking out Jaks and STATs had profound effects on immune cells, host defense, and immunoregulation. Thus, it became clear very quickly that George Stark's mutant cell lines really did predict the essential functions of the Jaks in signaling by Type I/II cytokines. However, the impact of Jak-STAT signaling was far more extensive than just IFN signaling (John *et al.,* 2011)

The attempt to elucidate IFN-inducible genes was accelerated by microarray technology, which showed that hundreds of genes were induced by these cytokines; however, this technology did not allow one to discriminate direct vs. indirect actions of STATs (John *et al.,* 2011). Which of the genes were true STAT target genes? Newer technologies for mapping genome-wide transcription factor binding include ChIP-on-chip and ChIP-seq technology, and these technologies have quickly expanded our understanding of STAT action (John *et al.,* 2011). Coupling ChIP- seq data with expression data (either microarray or more recently RNA-seq data), now readily discriminates direct and indirect effects of STATs. Currently, genome-wide binding of all STATs has been profiled by ChIP–seq, and the original datasets are publicly available through the Gene Expression

Omnibus (GEO) repository (John *et al.,* 2011). Moreover, this technology permits one to examine the impact of STATs, not only on transcription but also on epigenetic changes in differentiating cells (John *et al.,* 2011)

During the last decade, more and more evidence for direct relevance of the Jak-STAT pathway in humans is emerging. We now know that gain-of-function JAK2 mutations result in the myelofibrosis spectrum of disorders, and many malignancies are associated with constitutive activation of the Jak-STAT pathway (John *et al.,* 2011). Loss-of-function STAT1 mutations are associated with impaired cellular responses to IFN-γ and susceptibility to viral and mycobacterial infections, but conversely, gain-of-function STAT1 mutations underlie a disorder termed chronic mucocutaneous candidiasis (John *et al.,* 2011). These STAT1 mutations result in enhanced IFN signaling and suppression of IL-17 production. Dominant-negative mutations of STAT3 in humans also have profound effects on Th17 cell generation. Such STAT3 mutations result in a disorder known as hyper-IgE syndrome (HIES; also known as Job's syndrome), a classic primary immunodeficiency (John *et al.,* 2011). Homozygous missense mutations of STAT5b are linked to a growth hormone insensitivity phenotype associated with autoimmunity and impaired Treg cell function (John *et al.,* 2011)

The advent of large-scale genome-wide association studies has also implicated cytokines, Jaks, and STATs in more common complex autoimmune diseases (John *et al.,* 2011). For example, polymorphisms of IL-23R, JAK2 and STAT3 are linked to susceptibility to inflammatory bowel disease and ankylosing spondylitis (John *et al.,* 2011). Similarly, a variant allele of STAT4 has been found to be associated with rheumatoid arthritis, systemic lupus erythematosus (SLE) Sjögren's syndrome, and inflammatory bowel disease (John *et al.,* 2011). SLE is associated with an "interferon-signature" and STAT4, like STAT1, is activated by type I IFNs (John *et al.,* 2011). Consistent with this idea, polymorphisms of TYK2 may also be associated with SLE (John *et al.,* 2011)

Finally, the clear genetic evidence of the essential functions of Jaks had equally clear implications for the development of a new class of immunosuppressive drugs (John *et al.,* 2011). The discovery that JAK2 mutations underlie myeloproliferative disorders provided a logical rationale for targeting this kinase in the clinical setting. Remarkable progress has been made in the generation of Jak inhibitors. Tofacitinib was the first clinically useful, highly selective and potent, oral Jak inhibitor and is now showing efficacy in rheumatoid arthritis, psoriasis, Sicca syndrome, and the prevention of renal transplant rejection. Ruxolitinib, a Jak1 and Jak2 inhibitor, has shown efficacy in myeloproliferative disease (John *et al.,* 2011). Many other Jak inhibitors are at different levels of development at this time in conclusion, we have endeavored to put the studies by Schindler et al. and Velazquez et al. in historical and biological context (John *et al.,* 2011). What arose from efforts to understand the rapid action of IFN on gene expression was the discovery of a new, linear biochemical pathway of membrane-to-nucleus signal transduction, which had implications for dozens of factors critical for human health and disease. Still, there is much to learn. Recent evidence points to roles of STATs in mitochondrial function and Jaks as histone modifiers (John *et al.,* 2011).

The ChIP-seq approach has vastly expanded our understanding of STAT action, but the challenge remains to decipher how STATs regulate transcription and control the epigenome on a biochemical and mechanistic level. In one sense, the pathway is remarkably simple and elegant, but do we really know all the elements? Hints from Drosophila suggest that there is more complexity (John *et al.,* 2011). In addition, STATs work in concert with other transcription factors, including NF-κB. This will all need to be sorted out (John *et al.,* 2011). The challenge, of course, is to really understand specificity in signaling; hopefully, genomic approaches will help elucidate this aspect of cytokine action as well (John *et al.,* 2011)

The Role of Chemokines in Wound Healing

Wound healing is a multistep process with four overlapping but distinct stages: hemostasis, inflammation, proliferation, and remodeling (Anisyah *et al.,* 2018). An alteration at any stage may lead to the development of chronic non-healing wounds or excessive scar formation. Impaired wound healing presents a significant health and economic burden to millions of individuals worldwide, with diabetes mellitus and aging being major risk factors (Anisyah *et al.,* 2018). Ongoing understanding of the mechanisms that underly wound healing is required for the development of new and improved therapies that increase repair. Chemokines are key regulators of the wound healing process. They are involved in the promotion and inhibition of angiogenesis and the recruitment of inflammatory cells, which release growth factors and cytokines to facilitate the wound healing process. Preclinical research studies in mice show that the administration of CCL2, CCL21, CXCL12, and a CXCR4 antagonist as well as broad-spectrum inhibition of the CC-chemokine class improve the wound healing process. The focus of this review is to highlight the contributions of chemokines during each stage of wound healing and to discuss the related molecular pathologies in complex and chronic non-healing wounds. We explore the therapeutic potential of targeting chemokines as a novel approach to overcome the debilitating effects of impaired wound healing (Anisyah *et al.,* 2018)

Chemokines are involved in all stages of wound healing but are most abundant and most varied during the inflammation and proliferation stages to promote angiogenesis (Anisyah *et al.,* 2018). They are, however, also present in the hemostasis and remodeling phase and work to inhibit the angiogenic process (Anisyah *et al.,* 2018)

Chemokines in early and late phases of wound healing. Early wound healing, including clot formation, inflammation, and proliferation. Clot formation occurs to prevent the

loss of blood and platelets are activated and release, α-granules, which in turn release, CXCL4 as an early inhibitor of angiogenesis. Once the clot has fully formed other, chemokines such as CXCL8, CXCL1, and CXCL2 are released by α-granules to recruit inflammatory cells, including neutrophils and macrophages. Neutrophils are increased early in the healing process, then macrophages soon take over as the primary inflammatory cell (Anisyah *et al.,* 2018). Neutrophils and macrophages release chemokines such as CCL2, CCL3, and CCL5 into the wound to promote the recruitment of more inflammatory cells that release pro-angiogenic growth factors that increase neovessel formation in the wound (Anisyah *et al.,* 2018). Late wound healing is the remodeling stage. In this stage, the wound is fully healed and (1) a scar has formed. Type 3 collagen converts to type 1 collagen to promote scar formation and create a more stable wound seal. During the remodeling process, angiostatic chemokines (CXCL10, CXCL11) promote the regression of neovessels, as there is no longer a requirement for enhanced blood flow or the recruitment of immunological cells to the site. ↓: indicates decrease: indicates decrease.

Chemokines in Hemostasis Stage of Wound Healing

The hemostasis stage is an essential step that prevents further blood loss from the wound site. This involves immediate activation of the coagulation cascade to allow for clot formation (Anisyah *et al.,* 2018). During platelet activation, CXCL4 (also known as Platelet factor 4, PF4) is released from α-granules to inhibit angiogenesis. Other than being angiostatic, CXCL4 has been shown to inhibit hematopoiesis and collagenase activity (Anisyah *et al.,* 2018). CXCL4 inhibits angiogenesis through binding to vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF, also known as FGF-2), both potent promoters of angiogenesis, and through binding to its receptor CXCR3. When CXCL4 binds to VEGF and bFGF, it prevents binding to its receptors by disrupting cell surface heparin sulfates and is found to inhibit VEGF-induced proliferation of vascular endothelial cells (Anisyah *et al.,* 2018). Tubule formation, induced by VEGF, is found to be inhibited in the presence of CXCL4 in dermal human microvascular endothelial cells and in a human microvascular endothelial cell line. Similarly, the CXCL4 variant, CXCL4L1, inhibited tubule formation in the presence of VEGF and bFGF. CXCL4L1 was also more effective than CXCL4 in inhibiting bFGF-induced angiogenesis in rat corneas, and both forms inhibit bFGF-induced bovine aortic endothelial cell (BAOEC) proliferation and human umbilical vein endothelial cell (HUVEC) motility, with CXCL4L1 found to be 20 times more potent than CXCL4 (Anisyah *et al.,* 2018). Additionally, through binding to the receptor CXCR3, the CXCL4L1 variant was found to disrupt targeted cell migration towards CCL5 when compared to CXCL4 (Anisyah *et al.,* 2018). Interestingly, CCL5 and CXCL4 have been shown to have a heterophilic interaction that blocks CCL5-directed chemotaxis of monocytes on the

endothelium (Anisyah *et al.,* 2018).

Furthermore, CXCL4 and CXCL4L1 were found to be poorly chemotactic to T cells and monocytic THP-1 cells. Binding of CXCL4 to the CXCR3-B receptor variant was shown to upregulate human microvascular endothelial cell (HMEC-1) apoptosis [25] by activation of p38 and μ-calpain cleavage of integrins (Anisyah *et al.,* 2018). This is another endothelial cell function that causes inhibition of wound angiogenesis early post-wounding (Anisyah *et al.,* 2018). Whilst CXCL4 is the predominant chemokine released by platelet α-granules, CXCL1, CXCL4, CXCL5, CXCL7, CXCL8, CXCL12, CCL2, CCL3, and CCL5 are also released to a lesser extent (Anisyah *et al.,* 2018). This becomes important once the fibrin clot has formed, allowing the recruitment of inflammatory cells to initiate the next phase of wound healing (Anisyah *et al.,* 2018)

Chemokines in the Inflammatory Stage of Wound Healing

The inflammatory phase is characterized by an influx of inflammatory cells and an increase of pro-angiogenic molecules in the wound (Anisyah *et al.,* 2018). The main objective of chemokines in this phase is to recruit these inflammatory cells to remove dead cells, debris, and foreign bodies from the wound and to promote the release of pro -angiogenic molecules to facilitate the migration, proliferation, and differentiation of endothelial cells, endothelial progenitor cells (EPCs), and keratinocytes which eventually close the wound (Anisyah *et al.,* 2018)

The initial wave of inflammatory cells is recruited by CXCL8, CXCL1, and CXCL2 that are released by platelet α-granules (Anisyah *et al.,* 2018). CXCL8 is a potent neutrophil attractant, with neutrophils constituting nearly 50% of cells in the early stages of the wound (Anisyah *et al.,* 2018). In aged rats, the influx of neutrophils was dramatically reduced compared to young rats in the first 4 days post-wounding, causing delayed healing in older rats, indicating the importance of their early recruitment [27]. Neutrophils begin the phagocytosis of debris in the wound and release chemokines including CCL2, CCL3, and CCL5, which recruit macrophages to the wound (Anisyah *et al.,* 2018). Similarly, endothelial cells and keratinocytes already present in the wound border release chemokines to recruit macrophages to the wound site to further promote angiogenesis in the wound (Anisyah *et al.,* 2018). Macrophages quickly take over this process, targeting the dying cells, apoptotic neutrophils, and foreign bodies. The large influx of neutrophils and macrophages occurs quickly to prevent the risk of wound infection (Anisyah *et al.,* 2018).

After day 2–4 post-wounding, neutrophils are markedly reduced in the wound, leaving macrophages as the dominant inflammatory cells in the wound, persisting for approximately 14 days post-wounding (Anisyah *et al.,* 2018). Macrophages are important in the wound not only to keep the wound free from invading microorganisms but also to promote the repair of the wound. Macrophages release growth factors, cytokines, and chemokines such as VEGF, bFGF, platelet derived growth factor (PDGF),

tumor necrosis factor-α (TNF-α), and interferon-γ (IFN-γ) that stimulate angiogenesis. Additionally, macrophages release chemokines such as CCL2 and CCL5 to promote the migration of more macrophages to the wound (Anisyah *et al.,* 2018)

Chemokines found in the inflammatory phase are responsible for the recruitment of macrophages and the promotion of angiogenesis. CC-chemokines found in the first week after the initial wounding event include CCL1, CCL2, CCL3, CCL4, CCL5, and CCL7, which are all able to chemoattract macrophages, thereby suggesting a high content of macrophages in the wound (Anisyah *et al.,* 2018). CXC chemokines are also present in the wound, including CXCL1, CXCL2, CXCL5, CXCL7, CXCL8, and CXCL12, and are known to directly promote angiogenesis [13]. CXCL1, CXCL2, CXCL5, and CXCL12 are also released by macrophages. Angiogenesis is essential in this phase to facilitate the migration of cells into the wound and to meet the metabolic needs of the proliferating wound cells (Anisyah *et al.,* 2018).

Chemokines in the Proliferation Stage of Wound Healing

The third phase of wound healing occurs within the 3–10-day period after the initial wounding event. This phase is characterized by increased numbers of endothelial cells, keratinocytes, fibroblasts, and collagen in the wound area, which contribute to the formation of a temporary extracellular matrix (ECM) (Anisyah *et al.,* 2018). There is also a reduction in the number of inflammatory cells to promote the re-epithelialization process that closes the wound. In the first few days of the proliferation phase, many neovessels are present to support the rapid increase in cellular proliferation and migration in the wound. This is mediated through pro-angiogenic chemokines CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL7, and CXCL8 and their receptors CXCR1 and CXCR2 (Anisyah *et al.,* 2018). High expression levels of CXCL1 and CXCL8 have been associated with early wound neoangiogenesis in human wounds (Anisyah *et al.,* 2018). Additionally, CXCR2 knockout mice exhibit reduced neutrophil recruitment, reduced keratinocyte migration, reduced proliferation, and reduced neovascularization during re-epithelialization. Chemokines also play an indirect role in the proliferation stage, facilitating the recruitment of macrophages that secrete growth factors to promote angiogenesis. Chemokines CCL2 and CCL3 are highly expressed in wounds at this stage and coincide with an increased presence of macrophages (Anisyah *et al.,* 2018).

During the re-epithelialization phase, basal keratinocytes express CXCL10 and CXCL11, suggesting an important role of these chemokines (Anisyah *et al.,* 2018). This is highlighted in CXCR3 knockout mice, the receptor for CXCL10 and CXCL11, in which wound re-epithelization and basement membrane regeneration is delayed in both partial and full thickness wounds (Anisyah *et al.,* 2018). These mice also had reduced epidermal maturation compared to wild-type wounds. Conversely, keratinocyte

migration, wound closure, and granulation tissue were significantly increased in the presence of fibrin functionalized with fibronectin and CXCL11 in 10-day full thickness mouse wounds (Anisyah *et al.,* 2018). Additionally, CCL27 has been reported to promote the migration of bone-marrow-derived keratinocyte stem cells in full thickness wounds, resulting in faster wound healing (Anisyah *et al.,* 2018).

CXCL12 promotes the recruitment of bone-marrow-derived stem cells which differentiate into endothelial cells and fibroblasts to form the granulation tissue (Anisyah *et al.,* 2018). The granulation tissue fills the wound from the base up to form a new ECM ready for the deposition of collagen. While this occurs, keratinocytes and endothelial cells at the wound edge proliferate and migrate to close the wound surface (Anisyah *et al.,* 2018). This is stimulated by the release of TGF-β from M2 macrophages. Additionally, TGF-β increases endothelial, fibroblast, and keratinocyte cell proliferation and migration (Anisyah *et al.,* 2018). TGF-β is also important for collagen formation, remodeling of the extracellular matrix, and the initiation of granulation tissue formation that stimulates the contraction of fibroblasts, which is important for wound closure (Anisyah *et al.,* 2018).

Chemokines in the Remodeling Stage of Wound Healing

The remodeling phase is the longest phase occurring for several months or years after wounding. The processes of angiogenesis and proliferation cease, excess cells either leave the wound or undergo apoptosis, and neovessels undergo regression, leaving mostly collagen and ECM proteins in the wound. During this process, the ECM is broken down by matrix metalloproteinases (MMPs) and metalloproteinase tissue inhibitors (TIMPs), allowing for neovessel regression and the deposition of type I collagen (Anisyah *et al.,* 2018). The type III collagen deposited during the proliferation phase is degraded and replaced with stronger, thicker, more permanent type I collagen forming the final scar (Anisyah *et al.,* 2018). In vitro CCL2 was found to promote expression of MMP-1 and TIMP-1 in human dermal fibroblasts, indicating a profibrotic and collagen degradative role (Anisyah *et al.,* 2018). Additionally, CCL3, CCL4, and CCL5 upregulated the secretion of MMP-9 by lymphocytes in vitro, and CCL2 increased the secretion of MMP-12 from macrophages in vitro, suggesting a role for these CC-chemokines in ECM degradation (Anisyah *et al.,* 2018).

Chemokines are also involved in promoting the regression of neovessels, formed in the previous phases. Whilst this process is not fully understood, it is thought to be due to the expression of two angiostatic chemokines, CXCL10 and CXCL11. These chemokines bind to their receptor CXCR3, which is the same anti-angiogenic receptor that binds to CXCL4 in the early hemostasis phase. Binding of CXCL10 to CXCR3 prevents VEGF-induced endothelial cell tubulogenesis (Anisyah *et al.,* 2018). Similarly, activation of μ-calpain by CXCL10 cleaves β3 integrin, leading to the dissociation of endothelial cells and death (Anisyah *et al.,* 2018). Furthermore, knockout of the CXCR3 receptor in mice was found to produce wounds with a weakened healed dermis caused

by insufficient remodeling and reorganization of collagen in the wound (Anisyah *et al.,* 2018)

Chemokines in complex wounds

Wounds are not only simple open cuts or lacerations but also occur in several other forms and by various methods, including surgical incisions, combat, burns, and skin grafts. The type of wound and environment can also determine the presence of chemokines and rate of wound healing.

1. Combat Wounds

One type of complex wound is the combat wound or blast-related wound. Wounds such as these are characterized by large injury zones affecting soft tissue, bone, and muscle and largely result in amputation. The healing of combat wounds is highly dependent on the regulation of the inflammatory response, whereby prolonged inflammation promotes prolonged wound healing times (Anisyah *et al.,* 2018). Combat wounds are also prone to bacterial infection, which may lengthen the healing process even further. Wounds which have been sutured closed may also experience wound rupture or dehiscence. Studies have discovered the presence of several chemokines which may play a role in the prolongation of combat wound healing (Anisyah *et al.,* 2018)

2. Burns

The wound healing phase in response to burn injury is similar to that of a normal wound, consisting of inflammation, proliferation, and remodeling. However, in burns, there is a higher degree of inflammation, resulting in increased capillary permeability, persistent vasodilation, and edema (Anisyah *et al.,* 2018). Cellularly, neutrophils and macrophages are responsible for removal of necrotic debris, removal of toxins, and prevention of infection of the burn (Anisyah *et al.,* 2018)

Chemokines are essential to orchestrate this inflammatory response. In mice, serum CCL2, CXCL1, CCL3, and CCL11 are found to be significantly higher at day 1 post-burn injury compared to unwounded controls (Anisyah *et al.,* 2018). This reflects a significant increase in monocytes at day 3 post-burn injury, suggesting an early acute macrophage inflammatory response (Anisyah *et al.,* 2018). Interestingly, neutrophils are found to be strikingly increased in burn wounds at day 7, which may be related to the elevated CXCL1 (neutrophil chemoattractant) at day 1(Anisyah *et al.,* 2018)

3. Skin Grafts

Skin grafts may be required after injuries such as burns, large open wounds, bed sores, skin infections, or skin cancer surgery. There are two main types of skin grafts: split

thickness and full thickness grafts. In a split thickness graft, the epidermis and some of the dermis are replaced, and this type of graft is usually used to cover larger areas. (Anisyah *et al.,* 2018). However, in a full thickness graft, the whole epidermis and dermis is replaced. These grafts tend to cover smaller areas and closely match the recipient skin as they cover visible parts of the body, for example, the face or arms (Anisyah *et al.,* 2018)

The immunological response to skin grafts is controlled by the use of immunosuppressants to prevent the rejection of the graft. This is particularly pertinent in an allograft, when the donor and recipient are not the same person. Following a skin graft, there is a high infiltration of neutrophils and macrophages. As new connections are established, dendritic cells from the donor tissue migrate through lymphatic vessels to the lymph nodes (Anisyah *et al.,* 2018)

Cytokines in chronic non-healing wounds

Non-healing or chronic wounds occur when normal healing is impaired due to either a metabolic disease such as diabetes or through age-related decline in repair and regeneration. These wounds are characterized by prolonged inflammation and inadequate or weak wound closure. With an increasing ageing population and the elevated incidence of diabetes worldwide, there is a significant population that develops foot ulcers, resulting in a significant health impact to the individual and that may lead to lower-limb amputation, as very few treatments have proven therapeutic efficacy. Chronic wounds typically have an increased chemokine milieu.

1. Diabetic Wounds

Diabetes mellitus is a metabolic disease characterized by high blood glucose levels, sustained inflammation, and endothelial dysfunction. These characteristics contribute to a prolonged wound healing process. When the wound does eventually heal, the wound area is weak and prone to reinjury (Anisyah *et al.,* 2018). Several key chemokines have been studied in diabetic wounds, including CCL2, CXCL2, and CXCL12. These chemokines have been targeted for their role in sustained inflammation, formation of granulation tissue, and effects on re-epithelialization (Anisyah *et al.,* 2018)

2. Ageing Wounds

The aged population suffers from non-healing wounds such as chronic venous leg ulcers, pressure ulcers, and diabetic foot ulcers. Although the elderly can heal most wounds, the wound healing process is often delayed compared to wounds in younger individuals (Anisyah *et al.,* 2018). There is evidence showing that there are age-related

alterations in angiogenesis, keratinocyte proliferation delayed synthesis of new ECM, reduced macrophage function, reduced neutrophil infiltration, and reduction in VEGF, bFGF, and TGF-β (Anisyah *et al.,* 2018)

In aged wounds, following the initial wounding event, there is a markedly reduced inflammatory infiltrate compared to young wounds. In wounds from young mice, neutrophils are found to peak at day 3, however older mice do not experience this same peak despite the significant increase of CXCL1 at day 1 in older mice compared to younger mice (Anisyah *et al.,* 2018)

The therapeutic targeting of chemokines to improves wound healing

Due to their extensive involvement in the wound healing process of both "normal" and complex wounds, chemokines have been considered as potential therapeutic targets to improve wound healing (Anisyah *et al.,* 2018). Several studies have either targeted key individual chemokines and chemokine receptors or performed broad-spectrum chemokine inhibition approaches. To date, studies have reported varied levels of success. Further studies are still required to determine the best strategy for manipulating chemokines to improve wound healing (Anisyah *et al.,* 2018)

1. Single Chemokine Targeting

There are several key chemokines which have been targeted to improve wound healing (Anisyah *et al.,* 2018). These include CXCL8, CCL2, CCL5, and CXCL12. Studies inhibiting single CC-chemokines have shown varied effectiveness. CCL2 knockout mice exhibit delayed re-epithelialization and reduced angiogenesis early in the wound repair process, whilst CCL3 knockout mice follow a normal wound healing pattern when compared to wild-type litter mate mice (Anisyah *et al.,* 2018). Recent studies have reported that there is an improvement in healing with a single administration of CCL2 onto diabetic wounds (Anisyah *et al.,* 2018). CCR1 knockout mouse models display no alteration in wound healing, while intraperitoneal infusion of the CCL5 antagonist Met-CCL5 was shown to improve liver fibrosis in mice and accelerate regression of fibrosis (Anisyah *et al.,* 2018).

2. Broad Spectrum Chemokine Targeting

Several studies that have focused on targeting a single chemokine have been ineffective. Due to some redundancy in the chemokine system, a broad-spectrum approach to chemokine inhibition may have the potential to be more effective for the improvement of wound healing (Anisyah *et al.,* 2018). Two broad-spectrum chemokine inhibitors have thus far been tested in wound healing models. There is the CC-chemokine class inhibitor "35K" and NR58-3.14, which inhibits a number of both CC and

CXC chemokines (Anisyah *et al.,* 2018)

35K is produced by the Vaccinia virus and uniquely inhibits the entire CC-chemokine class (Anisyah *et al.,* 2018). Topical application of 35K protein to subcutaneous wounds placed on the sub flanks of mice was found to enhance wound closure and neovascularization early post-wounding (Anisyah *et al.,* 2018). 35K also increased TGF-β, a pro-angiogenic and pro-repair cytokine, and the expression of the inflammatory transcription factor NF-κB. During the later remodeling phase of wound healing, 35K reduced the deposition of collagen, suggesting a reduction in scar formation. Taken together, broad-spectrum CC-chemokine inhibition with 35K may therefore present as a therapeutic strategy to enhance the healing of chronic wounds and reduce scar formation (Anisyah *et al.,* 2018)

Conclusion

In conclusion, chemokines play key roles at each stage of the wound healing process to help coordinate the interaction of cells within the wound. Whilst chemokines do play an important beneficial role early post-wounding, a prolonged inflammatory response that is exacerbated by chemokines can lead to the formation of chronic non-healing wounds. This suggests that approaches to chemokine inhibition in which the stage of wound healing is considered may present as a possible therapeutic strategy to improve wound healing (Anisyah *et al.,* 2018).

WHEN IS WOUND HEALING REFERRED TO AS 'IMPAIRED'? AND WHY?

In wounds where oxygenation is not restored, healing is impaired(Guo S and DiPietro L.A 2010) Temporary hypoxia after injury triggers wound healing, but prolonged or chronic hypoxia delays wound healing (Guo S and DiPietro L.A 2010)In acute wounds, hypoxia serves as a signal that stimulates many aspects of the wound-healing process (Guo S and DiPietro L.A 2010)

Because there is inadequate oxygen supply (Guo S and DiPietro L.A 2010)

EXAMINE THE ROLE OF OXIDATIVE STRESS IN THE

DEVELOPMENT AND PROGRESSION OF IMPAIRED WOUND HEALING.

1. Oxidative stress was a condition which was the imbalance of prooxidant and antioxidants, abnormally high levels of free radicals and/or the decline of antioxidant defense mechanisms (Yang et al., 2007). Excessive oxidative stress could lead to damage of tissue, which played an important role in the development of many kinds of diseases.

2. Free radical relatively increased during oxidative stress. Normally free radical was necessary for defense of organism and there was a balance between its produce and scavenge(Yang et al., 2007)

   Oxidative stress was closely associated with reactive oxygen species. Reactive oxygen species could play an important role in physiology in some extent, also it led to damage of tissue or cells when organism could not defend excessive reactive oxygen species (Yang et al., 2007)

3. Excessive reactive oxygen species and its degradation product generated during the healing of cutaneous wound. Oxidation increased in acute and chronic wound. After wound oxidative stress generates, antioxidation increased in chronic wound, which indirectly reflected the increasing of oxidative stress and compensation and defense of tissue to oxidative stress (Yang et al., 2007)

4. The generation of oxidative stress in wound maybe closely relate to inflammatory reaction. In the inflammatory stage of wound healing, oxidative stress induced the damage of tissue because of the imbalance of prooxidant and antioxidant. Conclusion: Oxidative stress should be considered in the inflammatory processes of wound healing and treatment of chronic wound (Yang et al., 2007). The treatment of antioxidation is a good strategy. If it is used in wound healing in time, it can be good to wound healing (Yang et al., 2007)

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