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Wounds that Heal and Wounds that Don't - The role of the IL-33/ST2 pathway in tissue repair and tumorigenesis.

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Abstract:

IL-33 is a member of the IL-1 family of cytokines. IL-33 is predominantly located within the nucleus of cells where it plays a role in gene regulation. Given the right combination of signals and cellular damage, stored IL-33 is released from the cell where it can interact with its receptor ST2, triggering danger-associated responses and act as a cellular “alarmin”. Whilst IL-33/ST2 signalling has been shown to induce potent pro-inflammatory responses that can be detrimental in certain disease states, a dichotomous, protective role of IL-33 in promoting wound healing has also emerged in multiple tissues types. This review will explore the current literature concerning this homeostatic role of IL-33/ST2 in tissue repair and also review its role in uncontrolled wound responses as seen in both fibrosis and tumorigenesis.

Keywords

Interleukin 33, ST2, tissue damage, fibrosis, tumorigenesis

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Introduction: IL-33/ST2 – an 'Alarmin' function in tissue damage

Wound healing, whether initiated by trauma, microbes or foreign materials, is a normal homeostatic process required for the resolution of tissue damage. It comprises multiple consecutive, overlapping phases including inflammation, epithelialization, angiogenesis and matrix deposition. A critical aspect of tissue repair is the deposition of key connective tissue proteins, however in situations of chronic inflammation, impaired tissue repair mechanisms, including continuous myofibroblast differentiation and dysregulated collagen deposition can lead to fibrosis and impaired organ function [1]. IL-1 family members cytokines (notably IL-1 and IL-18) are already implicated in the regulation of wound healing and tissue repair [2]. Herein we examine the role of a more recently described member of the IL-1 family, IL-33 and its receptor ST2L, in tissue repair highlighting its role in several of the mechanisms underpinning wound repair fibrosis and tumorigenesis.

Biological Properties of the IL-33/ST2 axis

IL-33

In 2005, IL-33 was identified as a member of the IL-1 family of cytokines [3]. The IL-33 N-terminus includes a nuclear localization signal and a homeodomain-like helix-turn helix DNA-binding domain as well as a chromatin-binding domain [4]. IL-33 mRNA is expressed in many organs - high levels of IL-33 mRNA are detectable in stomach, lung, spinal cord, brain and skin. It is expressed by a diverse range of cells with strongest expression observed in non-haematopoietic cells including endothelial cells, epithelial cells, keratinocytes, fibroblasts, fibrocytes and smooth muscle cells [5, 6]. Lower expression is reported in some activated leukocytes especially innate immune cells e.g. mast cells, macrophages and DCs. IL-33 can be induced by a variety of immune stimuli, for example pro-inflammatory TLR ligands, cytokines and immune complexes [7] [8]. Different IL-33 splice variants have been observed in human tissues. Moreover, IL-33 is generated as a precursor and as a full-length protein, the latter being bioactive, similar to IL-1α [9]. Unlike other IL-1 family members, IL-33 is deactivated by cleavage with caspase-3 or -7. Processing by these apoptotic caspases results in inactive fragments [10] [11] [12].

In terms of subcellular localisation, under homeostatic conditions, IL-33 is predominantly located in the nucleus of the cell [9]. Nuclear IL-33 regulates gene expression in numerous ways. Nuclear IL-33 binds to histones and regulates chromatin structure and by default, gene expression [4] [13]. IL-33 has also been reported to activate histone deacetylase-3 (HDAC3) activity, indicating a potential role for IL-33 in modulating epigenetic regulation [14]. Nuclear IL-33 has been reported to directly bind to NF-κB, suppressing NF-κB regulated gene expression [15]. However, upon cellular damage, such as necrosis, IL-33 is quickly released from the nucleus and exported from the cell leading to its definition as an "alarmin" and also to its categorisation as a danger-associated molecular pattern

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(DAMP). As IL-33 lacks a traditional signal sequence, it seems that cell death by either necrosis or necroptosis may be the predominant mechanisms by which IL-33 is liberated to the extracellular space [16]. This release of IL-33 from the cell facilitates its interaction with its cognate receptor ST2 and the subsequent initiation of signalling cascades. Localisation of IL-33 is tightly regulated. Recent studies utilising a murine transgenic model in which the nuclear localisation of IL-33 was abolished, demonstrate that such mice succumb to lethal inflammation characterised by eosinophil-dominated immune cell infiltration of multiple organs [17], highlighting the highly pro-inflammatory role of extracellular IL-33.

The IL-33 Receptor – ST2

The IL-33 receptor (ST2) was first described in advance of the detection of IL-33 hence the potentially confusing terminology. Suppression of tumourigenicity 2 (ST2), also known as T1, DER4 and FIT-1, was originally cloned as an oncogene-induced gene from murine fibroblasts. A second similar ST2 mRNA transcript was also detected and predicted to code for a receptor, now known as the transmembrane bound ST2L receptor [9]. The originally identified protein is now known to be a secreted soluble form or “decoy receptor” of ST2, termed sST2. Both of these proteins contain three identical Ig extracellular domains, although sST2 lacks the transmembrane sequence it contains an additional 9 amino acids at the C terminus. Both ST2 isoforms are members of the Ig superfamily and ST2L specifically belongs to the Toll/IL-1R (TIR) superfamily, as it shows ~29% homology to the IL-1R, and contains the three distinctive extracellular Ig domains and homology to the intracellular domain of IL-1R1 [18]. Furthermore, a third variant isoform, termed ST2V, has also been identified [19] (Figure1).

Human and murine ST2 genes contain two promoters, a distal and a proximal promoter. Both ST2L and sST2 isoforms can be transcribed from either promoter with promoter usage governed by the cell type. For example, while both the human leukaemic cell line, UT-7 and mast cells can transcribe ST2 isotypes using either the distal or proximal promoter, the distal promoter is predominantly used in this cell line for expression of both sST2 and ST2L [20]. Conversely, almost all transcription is initiated from the proximal promoter in fibroblasts [21]. Unlike other IL-1 family members, there is no known antagonistic ligand for ST2. High levels of sST2 and ST2L mRNA are expressed by the kidney, lung, placenta, and stomach. In humans, ST2L expression is higher in the spleen, heart, testis and colon than sST2, while the brain and liver express higher levels of sST2, than ST2L [22]. Many endothelial cells from lung, bronchus, coronary artery and umbilical cord express both ST2L and sST2 mRNA [23]. ST2L is strongly expressed on the surface of fibroblasts and hematopoietic cells such as T helper type 2 (Th2) lymphocytes, ILC2s and mast cells [24, 25]. Whilst not found constitutively expressed on Th1 cells and several other immune populations, some recent reports indicate that expression of ST2 can be induced in Th1 [26] and CD8+ T cells [27].

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IL-33/ST2 signalling

IL-33 has two mechanisms of action. Firstly, as a nuclear factor binding directly to chromatin in the nucleus, discussed previously, and secondly as a cytokine binding to ST2L. Biologically active full length IL-33 can be released in the extracellular space after cell damage or mechanical injury [10] whereas nuclear IL-33 is passively released upon cell death. [6]. Both of these mechanisms of cellular release facilitate the interaction of IL-33 with its receptor ST2. Upon binding of the extracellular IL-33 to ST2L; the receptor undergoes conformational change, which enables the recruitment of IL-1 Receptor Accessory Protein (IL-1RAcP). Heterodimerization of the two transmembrane molecules brings the two intracellular TIR domains together, and successive receptor adaptor proteins are recruited through protein-protein interactions. Once the IL-33/ST2L receptor complex is established the signal is transmitted by the MyD88-IRAK1/4-TRAF6 signalling pathway with resulting degradation of the inhibitory protein IκB and subsequent activation of the NFκB transcription factor [28]. MAP kinases, p38, JNK and ERK are also activated with activation of downstream transcription factors such as AP-1 (Figure 1). Activation of these transcription factors enables transcription of cytokines and chemokines in a cell-type restricted manner. For example activation of this pathway in Th2 cells leads to production of Th2 cytokines (i.e. IL-4, IL-5, IL-13) [3] whereas in epithelial cells, ST2 activation by IL-33 results predominantly in chemokine activation [29]. Multiple self-activated down-regulatory mechanisms of this pathway exist. Activation of ST2L by IL-33 results in activation of focal adhesion kinase (FAK) and glycogen synthase kinas 3β (GSK-3β) [30]. Once activated, GSK-3β binds to and phosphorylates ST2L resulting in the swift internalization of ST2L. Once internalized ST2L is polyubiquitinated by the E3 ubiquitin ligase FBXL19 and subsequently degraded by the proteasome [31]. As sST2 functions as a decoy receptor for IL-33 it negatively regulates ST2 signalling by sequestering IL-33 and has been shown to down regulate Th2 cell-mediated immunologic responses [32].

Epithelial barrier repair: A role for IL-33 in mucosal wound healing

Mammalian barrier surfaces including skin, lung and gastrointestinal tract are exposed to a plethora of potentially harmful agents deriving from external sources such as microbial or environmental as well as from intrinsic physical damage. The ability of these tissues to initiate effective repair processes is critical for their role in host defence. An absence of, or ineffective, wound healing and tissue repair processes can lead to chronic inflammatory states and the development of fibrotic disease. IL-33 is constitutively expressed by tissues involved in the maintenance of mechanical barriers, including keratinocytes, lung and gut epithelial cells, fibroblasts, fibrocytes and smooth muscle cells [33]. Emerging evidence is supportive of a critical role for the IL-33/ST2 axis in the initiation and maintenance of wound healing responses at these surfaces; a role that is somewhat divergent to its

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well-characterised function in promoting especially type II inflammatory responses. We will now consider the diverse wound repair properties of IL-33 across a range of tissues.

IL-33 in intestinal wound healing

The original report identifying IL-33 in 2005 described a putative role in epithelial repair, as IL-33 stimulation was shown to promote both epithelial proliferation and mucus production [33]. Subsequent human studies examining expression of both IL-33 and the different isoforms of ST2 showed that both IL-33 mRNA and protein were found to be up regulated in inflamed UC and CD [34], [35], while other reports describe IL-33 to be only up regulated in UC [36]. In particular, IL-33 was found to be increased in ulceration associated myofibroblasts in inflamed UC, although this was not observed in Crohns disease [37]. As the epithelium becomes damaged and mucosal lesions form in severe UC, IL-33 becomes increased in myofibroblasts indicating a potential involvement of IL-33 in wound healing in intestinal tissues[38].

Murine models, however, have revealed complex roles for IL-33 and ST2 in intestinal disease [39]. Similar to the changes seen in humans, increased expression of IL-33 was observed in the colons of mice challenged by both the trinitobenzene sulphonic acid (TNBS) and the dextran sodium sulphate (DSS) models [40]. Whilst certain murine models implicate a pro-inflammatory, pathogenic role for the IL-33/ST2 signalling axis in intestinal disease, several studies have shown that this signalling axis can promote a homeostatic wound healing response in the intestinal epithelium. These divergent roles are dependent on whether the acute or chronic inflammatory state is investigated. Whilst mice deficient in ST2 demonstrate improved symptoms and reduced intestinal inflammation in the early stage of DSS colitis [41], a delay in the resolution of DSS dependent tissue damage in IL-33$^{-/-}$ mice was observed [42]. This dichotomous role of IL-33 in acute versus chronic IBD was also demonstrated using a different model, as whilst injection of IL-33 aggravated DSS-induced acute colitis [43], it alleviated DSS-induced chronic colitis [44-46]. Indeed, in the study of Duan *et al*, utilising the TNBS model of colitis and treatment with recombinant IL-33, it was noted that IL-33 promotes the induction of Type 2 II cytokines and upregulated expression of FoxP3^{+} cells. In addition, the protective effect of IL-33 was diminished after depletion of T-regulatory (Treg) cells [46], which are known to facilitate wound healing [47]. Further evidence supporting a role of IL-33 in promoting intestinal Treg function has been provided. The IL-33 receptor ST2 is preferentially expressed on colonic Tregs with IL-33 signalling leading to Treg accumulation and maintenance in inflamed intestinal tissues. Importantly, IL-23, a potent pro-inflammatory cytokine which contributes to the pathogenesis if IBD, inhibits the ability of IL-33 to activate intestinal Treg [48]. In contrast IL-33 has, also been shown to negatively regulate wound healing with both genetic ablation of ST2 and treatment with an ST2 blocking antibody shown to enhance wound healing. In this study, administration of IL-33 impaired epithelial barrier permeability both in vitro and in vivo [40].

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Whilst these lines of evidence, using the chronic model of DSS colitis, demonstrate a role for IL-33 in intestinal wound healing there is a relative paucity of mechanistic data associated with its role in epithelial repair and restoration. IL-33 has been shown to directly affect epithelial barrier function as stimulation of T84 monolayers increase transepithelial resistance in an ERK-dependant manner [49]. Hofmann *et al* demonstrated that IL-33-induced neutrophil influx during chronic intestinal inflammation was able to reduce the translocation of pathogenic bacteria across the damaged epithelium [45]. This reduction in bacterial load and concomitant reduction in inflammation may, therefore, indirectly facilitate epithelial repair. More recently, evidence that the role of IL-33 in intestinal epithelial repair may be linked to its effect on group 2 innate lymphoid cells (ILC2) has been demonstrated. In recent years it has become evident that ILC2s express high levels of ST2 and are critical cellular targets of IL-33 in consequence [50]. IL-33 stimulation of ILC2 causes induction of high levels of IL-4, IL-5, IL-9 and IL-13, GM-CSF and amphiregulin (AREG) expression, promoting eosinophil expansion, mast cell recruitment and macrophage polarisation. High levels of amphiregulin can aid in rebuilding the intestinal barrier by stimulating growth of new epithelial cells and creating a protective layer of mucus that repels future attacks [51]. In the DSS model of intestinal colitis/intestinal repair, stimulation with IL-33 induces and activates AREG-producing ILC2s, resulting in restoration of epithelial barrier function and maintenance of tissue homeostasis [51]. Given the dual roles of IL-33 in either promoting intestinal disease or in promoting intestinal wound healing, greater insight into the mechanistic basis underlying these is necessary to fully comprehend the function of IL-33/ST2 in intestinal health and disease.

IL-33 in cutaneous wound healing

In recent years, IL-33 has emerged as a key player in several dermatological diseases including psoriasis, atopic dermatitis and contact allergy [52, 53]. Recent attention has focussed on exploring the role of both IL-33 and ST2 in cutaneous wound healing. One of the first pieces of evidence in this field was shown by Sponheim *et al* who had previously identified recruitment of IL-33 positive fibroblasts and myofibroblasts to granulation tissue in the intestine [37]. In order to explore whether these may play a functional role in wound healing in organs other than the intestine, they investigated the role of these in a rat skin wound healing assay. Massive recruitment of cells with strong expression of IL-33 was observed in healing skin wounds in a rat model as early as 24h after wounding. These authors also observed activation of scattered tissue resident IL-33$^+$, PDGFRβ^+, αSMA$^+$ fibroblast-like cells at the site of wounding pointing to a role of these cells in mucosal healing and wound repair. Both mRNA and protein levels of IL-33 were also seen to be elevated in a murine model of cutaneous wound healing with the administration of exogenous IL-33 seen to accelerate wound healing and re-epithelialisation in the same model. The number of F480$^+$, CD206$^+$ cells was increased at the site of wounding in the IL-33 treated mice, suggesting that IL-33 promotes the

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development and/or recruitment of alternatively activated macrophages during wound healing [54]. These authors also utilised a murine model of *Staphylococcus aureus*-infected wound healing and demonstrated that exogenous administration of IL-33 inhibited MRSA-colonisation, whilst accelerating wound healing. It was noted that IL-33 administration promoted the proliferation of neutrophils and also of the chemokine receptor CXCR2, thereby facilitating neutrophil recruitment to the site of infection. In both these models an upregulation of both fibronectin and collagen IIIa was noted following IL-33 administration, indicating a role for IL-33 on matrix synthesis and re-epitheliatisation [54]. A direct link between ST2 and cutaneous wound healing has also been shown using standard skin wound healing models in ST2 knockout mice. Absence of ST2 results in impaired wound healing with reduced numbers of $Ly6c^{lo}$ $MHCII^{hi}$ pro-repair macrophages observed within the wounds. Examination of expression of the pan-endothelial marker CD31, as a marker of angiogenesis, revealed a nearly 50% decrease in angiogenesis in the absence of ST2 within the granulation tissue 5 days after wounding. Additionally, these studies highlight direct effects of IL-33 signaling on matrix synthesis, with reduced Col3 being apparent in $St2^{-/-}$ mice, whilst IL-33 treatment of wild type mice increased Col3 [54]. Although it seems from these reports that IL-33 signalling at the site of cutaneous damage may facilitate the transition of macrophages from a pro-inflammatory to a pro-repair phenotype thereby promoting dermal wound healing, the underlying mechanism for IL-33 in skin wound healing remains unclear. Given the interesting observation that IL-33 expression both varies at a basal level and is also differentially regulated between mouse and human keratinocytes [8], it is clear that more detailed studies need to be performed on human tissues and human cell lines to fully elucidate the role of IL-33 in cutaneous wound healing in humans.

Lessons from overuse – IL-33 and early tissue insult

Tendinopathy is a term used to describe a complex multi-faceted pathology most commonly associated with overuse injury of the tendon [55]. Tendons are sites of high mechanical stressing with the resident stromal cells (tenocytes) and the associated extracellular matrix undergoing microtrauma form persistent use [56]. Importantly type III collagen is produced in the initial phases of tendon damage [57] as a conserved mechanism to provide a rapid 'patch' to the area of damage. Type III is laid down in a haphazard fashion contributing to the irregular alignment seen microscopically as well as translating to inferior biomechanical strength in damaged tendon [58]. Increasing evidence has shown that inflammatory mechanisms and the innate immune system are activated within the tendon matrix microenvironment during tissue injury and dysregulated homeostasis [59] [60]. Based on previous observations that IL-33 may directly regulate tissue remodelling, this model system was utilised in human and rodent disease to explore the potential role of the IL-33/ST2 axis in early tissue insult and the possible interactions between inflammation and matrix remodelling. IL-33/ST2/IL-1RAcP message and protein expression was significantly increased in early human tendinopathy compared to both established tendinopathy and normal tendon suggesting that early ‘stressed’ tendon

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had an IL-33 alarmin phenotype which may account for the associated matrix changes in collagen I/III ratio associated with disease [61]. Utilising *in vitro* human tenocyte cultures the addition of exogenous IL-33 resulted in increased expression of type I but particularly type III collagen mRNA/protein in keeping with animal studies on cutaneous wound healing. IL-33-induced collagen expression was abrogated by ERK and NFκB inhibition. rhIL-33 also significantly elevated the production of IL-6, IL-8 and CCL-2, which was abrogated by NF-κB inhibition suggesting that IL-33 operates in tenocytes via its canonical IL-1R signaling pathway[61].

This work was expanded to include an *in vivo* patellar tendon injury model [62]to understand the molecular events implicating IL-33 in a tissue injury model. IL-33 mRNA and protein were elevated in early post tendon injury (days 1&3 of 21 day model) in WT mice. This was significantly reduced in injured $ST2^{-/-}$ mice, suggesting autocrine regulation. No significant changes in either IL-33 or ST2 transcript were found in WT mice at days 7 or 21 post injury, or for IL-33 expression in $ST2^{-/-}$ mice, suggesting that the impact of IL-33 expression is manifest early, in keeping with 'alarmin' type activity in tendon injury/repair. Analysis of collagen kinetics revealed significantly greater expression of collagen 3 at all time points post injury in WT mice compared with uninjured controls or injured $ST2^{-/-}$ mice. Importantly, injury of WT mice tendons resulted in a significant decrease in biomechanical strength at day 1 post injury compared with that of the $ST2^{-/-}$ mice. These data suggest altered collagen matrix synthesis in $ST2^{-/-}$ mice implicating IL-33/ST2 as an early modulator of collagen changes in tendon injury that has biomechanical significance. Taken together these studies demonstrate a key functional role for IL-33/ST2 in early injury induced matrix dysregulation and subsequent cytokine feedback mechanisms (Figure 2) that have an ultimate biomechanical and clinical effect[61].

Factors regulating the IL-33/ST2 axis continue to be elucidated. Caspases-3 and -7 inactivate IL-33 by cleaving its C-terminal IL-1 domain [63]. This inactivation event is believed to ensure specific roles of IL-33 in pathogenic situations such as parasitic infection, but not in non-inflammatory physiological situations, such as apoptosis. Invading pathogens mediate tissue damage leading to necrosis of barrier cells and this non- programmed cell death results in IL-33 release in the absence of inactivation by caspases-3 and -7. Others have found that the transcriptional regulators IRF4 and BATF cooperate at the *Il1rl1* locus to induce the expression of ST2 [64]. Additionally, proteases mainly secreted by neutrophils seem to play an active role. Calpain, cathepsin G, elastase and proteinase cleave pro-IL-33 and remove the N-terminal domain, leaving the mature form of IL-33 with a ten-fold higher affinity to its receptor [65] [33]. Emerging studies highlight miRNAs as key regulators of leukocyte function and the cytokine network while orchestrating proliferation and differentiation of stromal lineages that determine extracellular matrix composition [66]. Having established that IL-33 drives the differential regulation of collagen 1 and 3 in tenocytes, we postulated a mechanistic role for the miRNA network in this process. Previous studies have shown that the *miR-*

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29 family directly targets numerous extracellular matrix genes [65] and is implicated in the regulation of innate and adaptive immunity [66]. All members of the *miR-29* family were expressed in human tendon biopsies and explanted tenocytes with *miR-29a* showing the most altered expression in early tendinopathy biopsies. In tenocyte culture, IL-33 significantly reduced the expression of *miR-29a,* in an NF-κB*,* dependent manner [61]. *miR-29a* manipulation selectively regulated collagen 3 but not collagen 1 mRNA and protein expression in primary tenocytes. Moreover, *miR-29a* overexpression significantly decreased IL-33-induced collagen 3 mRNA and protein synthesis. In addition, *miR-29a* inhibition resulted in a significant increase in *COL 3A1* expression, indicating that *miR-29a* is not only actively regulating these transcripts in human tenocytes but its loss can be an important factor in the increase of type 3 collagen production observed in tendinopathy [61]. In contrast, *COL 1A1 and A2* transcript levels were relatively unchanged. Soluble ST2 message was significantly decreased by transfection with miR-29a mimic and increased by antagomir with a corresponding significant change in soluble ST2 protein confirming soluble ST2 as a target of miR-29a. Thus, IL-33-driven loss of *miR-29a* expression resulted in the simultaneous repression of collagen 3 and sST2, with a subsequent autoregulatory inhibition of IL-33 promoting the resolution of the immediate alarmin response [61]. The discovery of a single miRNA-dependent regulatory pathway in early tissue damage events highlights *miR-29a* replacement therapy as a promising therapeutic option for tendinopathy with implications for other human pathologies in which matrix dysregulation is implicated. The recent initiation of a Phase 1 clinical study of MRG-201, a synthetic microRNA mimic to microRNA-29b with possible extension to patients suffering from cutaneous scleroderma may therefore lead to future microRNA therapies in damage associated pathologies [67].

Given this role for IL-33 in tendons and the link between tendons and muscle it is interesting that a recent role for IL-33 in repair of skeletal muscle has also been shown. Normal repair of skeletal muscle requires expansion of local Treg cells. Using elegant studies in both aged and normal mice, Kuswanto and colleagues demonstrated that IL-33 regulates muscle Treg homeostasis in young mice and that a reduction of IL-33 in aged mice is associated with reduced skeletal muscle repair and regeneration. Administration of IL-33 in aged mice was associated with marked improvement in muscle Treg accumulation and muscle regeneration [68]. As defective muscle repair subsequent to injury and atrophy is a major health problem associated with aging populations, it is possible, therefore, that activation of the IL-33/ST2 axis is a potential therapeutic area in this field.

The mechanics of wound healing: a role for IL-33 in angiogenesis

Angiogenesis is critical to wound repair. Newly formed blood vessels participate in provisional granulation tissue formation and provide nutrition and oxygen to growing tissues. Angiogenesis, in response to tissue injury, is a dynamic process that is highly regulated by signals from both serum and the surrounding extracellular matrix (ECM) environment. Some cytokines implicated in inflammation

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have been shown to induce angiogenesis and increase vascular permeability and as such play a key role in regulating inflammatory angiogenesis. Given the role of IL-33 in promoting wound healing in response to both disease and trauma-induced tissue damage, it is therefore perhaps unsurprising that clear evidence has been presented to show that IL-33/ST2 can directly drive angiogenesis. IL-33 is strongly expressed in endothelial cells (EC), with IL-33 promoting proliferation, migration and morphological differentiation of EC [69]. In addition, IL-33 promotes angiogenesis, increases vascular permeability, and induces activation of endothelial cells toward an inflammatory phenotype through upregulation of IL-6, IL-8, monocyte chemoattractant protein-1, vascular cell adhesionmolecule-1, intercellular adhesion molecule-1, and endothelial selectin [70] [71, 72]. IL-33 has also been shown to increase the production of urokinase-type plasminogen activator (u-PA) in EC [73]. u-PA has been implicated in a variety of angiogenesis-dependent physiological as well as pathological events such as wound healing. Furthermore, IL-33 drives upregulation of Tissue Factor (TF) in ECs [74]. As TF is the primary trigger of coagulation, this further highlights the importance of IL-33 in the early stages of wound healing.

The findings above on the role of IL-33 in angiogenesis focus on the presence of extracellular IL-33 in tissues, and therefore on IL-33 functioning in its capacity as an alarmin. IL-33, however, is abundantly expressed in the nuclei of endothelial cells in most healthy human tissues with nuclear IL-33 observed to disappear rapidly from blood vessels on tissue injury and in lesions of acute inflammation [5]. In the context of wound-healing and wounding, it was noted that nuclear IL-33 was lost from all vessels close to the wound as early as 24 hours after injury with nuclear IL-33 remaining absent during wound-healing angiogenesis. Endothelial nuclear IL-33 expression was shown to be regulated by Notch signalling [75]. Thus, release of IL-33 appears to be a feature of activated endothelial cells, whereas nuclear IL-33 expression is related to a state of vascular quiescence with nuclear IL-33 potentially repressing angiogenesis and therefore supressing wound healing. The complexity of IL-33 biology is such that further examining the transcriptional repressor activity and other nuclear functions of IL-33 in endothelial cells is of high importance to fully understand the role of this protein in wound-healing angiogenesis.

Uncontrolled wound healing responses: The role of IL-33 in fibrosis

Chronic inflammation and uncontrolled wound repair mechanisms, including unrestrained myofibroblast differentiation, fibroblast activation and excessive collagen deposition can lead to fibrosis. The associated, often irreversible, impaired organ function is classified as fibrotic disease, a leading cause of human mortality and morbidity. Identifying key underlying mechanisms of fibrosis is of the utmost importance for the development of novel therapies to treat this wide-ranging disease. Recently, the IL-33/ST2 pathway has been implicated in a plethora of fibrotic diseases with beneficial

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effects being reported in some, whilst adverse effects have been noted in other systems, with these being recently reviewed in some detail [76] [77].

Pro-fibrotic roles for IL-33 and ST2 have been reported in the liver, GI, lung, skin and kidney [76]. IL-33 is elevated in patients with idiopathic pulmonary fibrosis (IPF) as well as in the bleomycin-induced murine model of lung injury and fibrosis. This was observed to be predominantly full-length intra-nuclear IL-33 (flIL-33). Combined flIL-33 and bleomycin exerted a synergistic effect on pulmonary fibrosis. Interestingly, this effect was still observed in ST2 knockout mice, implying that this response may be mediated by nuclear located flIL-33 effecting gene expression [78]. In contrast Li *et al* demonstrated that both ST2 deficiency and administration of a blocking IL-33 antibody was able to attenuate bleomycin-induced pulmonary fibrosis [79]. Furthermore, intranasal administration of lentiviral expressing soluble ST2 significantly attenuated pulmonary fibrotic change and improved survival rate [80]. A variety of studies have revealed that IL-33 overexpression is implicated in the development of cutaneous fibrotic diseases, such as cutaneous fibrosis [81], psoriasis [82], and progressive systemic sclerosis [83]. To model chronic IL-33 release caused by sustained tissue damage, repeated administration of rhIL-33 revealed that it induces ST2- dependent cutaneous fibrosis and inflammation. Moreover it was noted that IL-13 is a critical downstream mediator of IL-33-induced cutaneous fibrosis requiring eosinophils and RAG-dependent lymphocytes [81]. A direct correlation between both IL-33 and ST2 levels has been observed in both mouse and human liver fibrosis correlating closely with collagen expression, again highlighting a role for IL-33/ST2 in regulation of matrix proteins [84]. IL-33 has been shown to directly promote hepatic fibrosis, at least in part, through its ability to recruit and activate liver-resident ILC2s [85]. IL-33 has also been shown to be elevated in biliary atresia patient serum and in the livers and bile ducts of mice with experimental biliary atresia. Injury to the biliary epithelium triggers inflammation and can result in both intra- and extra- hepatic fibrosis. Administration of IL-33 markedly increased cholangiocyte proliferation and promoted sustained cell growth, resulting in dramatic and rapid enlargement of extrahepatic bile ducts. This increased proliferation was mediated by an increase in the ILC2 population [86].

Extensive research has detailed an increase in expression of sST2 as early as 1 day after myocardial infarction (MI) and with this increase correlating with the ongoing process of cardiac inflammation, and fibrosis. Indeed, levels of sST2 are a good predictor of clinical outcome following MI, with high levels correlating with a poor prediction and lower levels correlating with a more favorable prognosis [87]. As sST2 is a negative regulator of IL-33 signaling, it is not surprising therefore, that a critical role of IL-33 in regulating cardiac myocyte activities and a protective role for IL-33 in cardiac fibrotic diseases have been suggested. It was demonstrated that IL-33 inhibits cardiomyocyte apoptosis both *in vitro* and *in vivo* and that IL-33 improved cardiac contractile function after ischemia/reperfusion myocardial injury in rats [88]. In addition, *in vivo* administration of IL-33 significantly decreased

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cardiac interstitial fibrosis in wild type mice that had undergone transaortic constriction surgery to increase cardiovascular load [89]. Cardiac fibroblasts, themselves, express ST2 and respond to IL-33 by inducing pro-inflammatory cytokines and chemokines IL-6 and MCP-1[90]. A similar protective function for IL-33 and ST2 has also been observed in atherosclerosis, a fibrosing disease of the arteries. Indeed, it is proposed that low levels of IL-33 may predispose to the development of atherosclerotic plaques [91]. Clearly, therefore this important signaling pathway exerts notably divergent effects in varying types of fibrosis and as such further work is required to fully understand these roles before the therapeutic potential of this pathway can be fully exploited.

"Wounds that do not heal" – Understanding the role of IL-33 in tumorigenesis

Wound healing and cancer progression have striking similarities. In 1986, Harold Dvorak suggested that "tumours are wounds that do not heal" [92]. His observations were that the mechanisms of wound healing and the formation of tumour stroma had similar connective tissue components, including fibroblasts, blood and lymphatic vessels, inflammatory cells, and extracellular matrix. In contrast to healing wounds, chronicity of the inflammatory phase results in uncontrolled cell proliferation, invasion, and metastasis. As outlined above, the IL-33/ST2 pathway participates in many of these processes, demonstrating clear direct effects on angiogenesis, production of matrix components, on fibrosis that can lead to tumour formation, and on modulation of immune populations which can therefore affect the tumor microenvironment. Unsurprisingly, therefore, links between the IL-33/ST2 signalling axis and tumorigenesis have recently been identified. In a parallel manner to the divergent roles of IL-33/ST2 reported in many of the processes associated with wound healing, both pro- and anti-tumorigenic roles have been reported for IL-33 and ST2 in cancer.

Initially the link between IL-33/ST2 and cancer was identified in breast cancer. Early studies utilising $ST2^{-/-}$ mice demonstrated that ST2 deletion inhibited breast cancer progression and increased the intra-tumoral accumulation of both innate (NK cell) and acquired immunity (CD8+ T-cells) and Th1/Th17 cytokines, indicating that a lack of IL-33 signalling through ST2L promotes a Th1 response 21484786. In addition, suppressing sST2 reduced ErbB2-induced cell motility in breast cancer cells. Furthermore, breast cancer patients with metastatic disease showed increased levels of circulating sST2 compared to patients with primary tumours [93]. Further studies in breast cancer also showed significantly higher levels of both IL-33 and sST2 in the serum of patients with ER positive breast cancer relative to healthy controls [94]. In a subsequent study, administration of IL-33 to breast cancer-bearing mice accelerates tumour growth and increased metastasis. The proposed mechanism responsible for the enhanced tumour growth was the increase in the number of infiltrating immunosuppressive immune cells and innate lymphoid cells, providing further evidence of the role of IL-33 in driving carcinogenesis [95]. Consistent with a role for IL-33 and ST2 in promoting tumour metastasis and invasion, inhibition of IL-33 and ST2 in glioma cells resulted in reduced tumour

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growth and colony formation *in vitro*, and reduced tumour size *in vivo* [96]. In head and neck squamous cell carcinoma (HNSCC), it has been shown that administration of IL-33 promoted cancer cell migration and invasion through induction of epithelial-mesenchymal transition [97]. Recently, it has been suggested that IL-33 can promote gastric cancer cell invasion and migration, which was suggested to be mediated by activation of ERK1/2 [98]. In this study, $ST2^{-/-}$ mice showed attenuated IL-33-mediated invasion and migration of cancer cells. In squamous cell carcinoma of the tongue, IL-33 and ST2 were shown to be expressed in cancerous cells in 100% of cases examined and cases with higher protein expression of IL-33 and ST2 showed poor overall survival [99]. More recently elegant work by Akimoto et al, investigating the role of IL-33 and ST2 in lung demonstrated that IL-33 enhanced the cell death of ST2L-positive low-metastatic cells, but not of ST2L-negative high-metastatic cells. These authors concluded that IL-33 enhances lung cancer progression by selecting for more malignant cells in the tumour microenvironment [100]. Finally several recent reports have reported a pro-tumorigenic role for IL-33 in colon cancer [101, 102] [103, 104]. These studies reported an increase in IL-33 in colorectal cancer as compared to adjacent normal tissue and healthy volunteers, with IL-33 having a protective anti-tumourigenic effect in colorectal cancer. Inhibition of IL-33 in colon cancer cells resulted in reduced tumour growth, migration and colony formation in vitro, and smaller tumours in vivo [102]. IL-33 was also shown to activate tumour stroma and promote polyposis in APC(Min/+) mice [103].

Other studies, however, have shown divergent anti-tumorigenic effects of IL-33 and ST2 in cancer. Levels of IL-33 have been reported to be reduced in the plasma of non-small cell lung cancer patients relative to controls [105], and levels of IL-33 have also been shown to negatively correlate with tumour stage in multiple myeloma patients [106]. Interestingly, IL-33 has been observed to be increased in response to viral infection and to be important for the eradication of a viral insult, as it can differentiate CTLs into anti-viral $CD8^+$ T cells [107]. IL-33 has also been shown to synergize with IL-12 to promote $CD8^+$ T cell effector function [108]. In line with the ability of IL-33 to promote a $CD8^+$ T cell response and the fact that $CD8^+$ T cells mediate a vital role in the defence against cancer, over-expression of IL-33 potently inhibited tumour growth and metastasis in both B16 melanoma and 4T1 breast cancer models with both CD8+ T cell and NK cell numbers seen to be increased [109]. Similarly, transgenic expression of IL-33 reduced tumour metastasis in a Lewis lung carcinoma and B16 melanoma model. Both the number and the cytotoxicity of $CD8^+$ T cells and NK cells were increased in response to IL-33 expression [110]. In contrast to the above finding detailed concerning a pro-tumorigenic role for IL-33 in CRC, we recently demonstrated that the IL-33/ST2 axis plays an anti-tumorigenic role in colon cancer as ST2L expression is decreased in human colon cancer tissue as compared to adjacent non-tumour tissue, with lower ST2L expression correlating with poorer patient prognosis. Consistent with this, knockdown of ST2 in murine colon cancer cells, resulted in enhanced

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tumour growth ($p<0.05$) in vivo with reduced CD8+ T cell infiltration observed in the ST2L knockdown tumours as compared to the control tumours [29].

It appears therefore, that the role of both IL-33 and ST2 in tumorigeneisis may be defined by the tumour type and source of the tumour. However, the models utilised above often differ in terms of reducing expression of these proteins either within the tumour or within the tumour microenvironment. These differing approaches appear to be in part responsible for the seeming divergent roles of these proteins in tumorigenesis.

Conclusions:

In certain tissue microenvironments the IL-33/ST2 axis appears to have a role in the immediate tissue response to injury in keeping with its 'alarmin' function while additionally regulating matrix homeostasis via immune cell crosstalk. It is equally clear, however, that divergent roles in wound healing, angiogenesis, fibrosis and tumorigenesis have also been ascribed to both IL-33 and ST2. Thus while manipulation of the IL-33/ST2 pathway represents a promising new therapeutic strategy for targeting damage associated tissue repair further mechanistic elucidation of its biology is required. Whilst some of these divergent roles can potentially be explained as a tissue/cell type specific function, it seems likely that a greater understanding of the biology and regulation of IL-33, in particular dissecting the conflicting roles of intracellular versus extracellular IL-33 may shed much needed light on the biological functions of the IL-33/ST2 axis and its role in wound healing, fibrosis and cancer.

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Figure Legends

Figure 1: The IL-33/ST2 signalling pathway.

In humans three splice variants of ST2 exist: ST2L, sST2 and ST2V. sST2 is a soluble protein with no transmembrane sequence, it is excreted extracellularly and binds to IL-33. sST2 is thought to act as a decoy receptor sequestering IL-33 away from the transmembrane bound receptor ST2L. ST2V is a membrane-bound receptor that contains a hydrophobic tail. It contains two Ig domains and is expressed in the gut, the function of ST2V has not been fully elucidated. IL-33 binds to the ST2L receptor and once IL-1RAcP is recruited, the TIR domain of IL-1RAcP interacts with the ST2L TIR domain. The heterodimeric complex acts as a scaffold for the recruitment of MyD88, IRAK-1 and IRAK4. This results in the phosphorylation of IκB which activates the transcription factor NF-*κ*B. AP-1 has also been shown to be activated through activation of the MAPK signalling pathway. This activates a pro-inflammatory response via the induction of cytokines and chemokines.

Figure 2

The role of the IL-33/ST2 axis in the immediate tissue response to injury – lessons from tendon

Schematic depicting the IL-33/ST2 signaling in tendon disease, representing tissue microtrauma. Tissue injury/stress results in the release of IL-33 which acts to both promote immune cell recruitment /enhanced cytokine production (IL-6, IL-8, CCL-2) and matrix remodeling via the ST2/IL-1RaCP complex and subsequent repression of microRNA29a. The IL-33-driven loss of *miR-29a* expression results in the simultaneous repression of collagen 3 and sST2, with a subsequent autoregulatory inhibition of IL-33 promoting the resolution of the immediate alarmin response. The tissue microenvironment subsequently displays an inflammatory phenotype compared to the homeostatic tendon. The resultant crosstalk between IL-33/ST2 and matrix proteins determines resolution versus pro damage pathways reflecting in clinical disease.

Figures 1 and 2

Figure 1

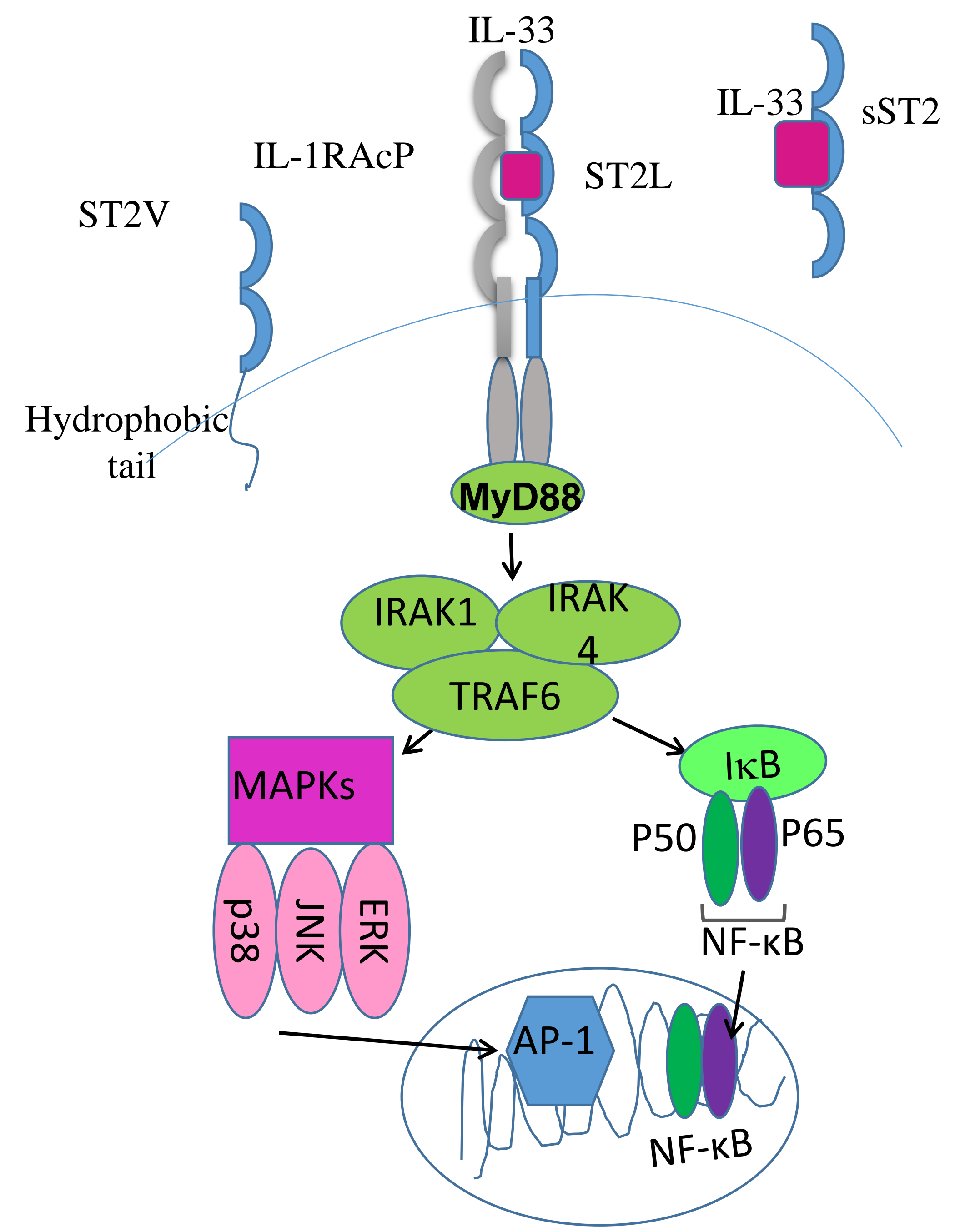

Figure 2