INNATE IMMUNITY IN SYSTEMIC SCLEROSIS - ROLE OF TOLL-LIKE RECEPTORS, INTERFERON, AND THE POTENTIAL IMPACT OF VITAMIN D

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ABSTRACT

Systemic sclerosis (SSc) is an autoimmune disease in which vascular damage and immune activation leads to excessive accumulation of extracellular matrix in the skin and internal organs. Although the focus has been on adaptive immunity in SSc, recent data suggest that innate immunity is critically important. The innate immune system, the first-line barrier against pathogens, modulates mechanisms which activate adaptive immunity. Dysregulation of the innate immune system and toll-like receptors (TLRs) may link immune abnormalities and fibrosis in SSc. TLR signalling pathways might induce production of Type I interferon (IFN) and other cytokines, and represent one of the mechanisms that initiate and develop autoimmunity and subsequent fibrosis. Vitamin D displays many immunomodulatory effects on both innate and adaptive immune responses. Active vitamin D will produce signals via vitamin D receptor and influences TLR stimulation, IFN response, and antimicrobial peptide production. Vitamin D deficiency has been associated with many autoimmune disorders, and can influence clinical phenotype and immune disorders in SSc patients.

Keywords: Systemic sclerosis, innate immunity, toll-like receptor, interferon, vitamin D.

INTRODUCTION

Systemic sclerosis (SSc) is an autoimmune disease in which vascular injury and inflammation are followed by an excessive accumulation of extracellular matrix (ECM) in the skin and internal organs, leading to organ dysfunction and premature death. The cause of SSc is due to an interplay between genetic and environmental factors that lead to breach of immune tolerance.[1-4] Immune activation is common in SSc and, although the focus has been on adaptive immunity,[5,6] recent evidence suggests that innate immunity is critically important.[1-3]

AN OVERVIEW ON INNATE IMMUNE SYSTEM

The innate immune system constitutes the first-line barrier against pathogens that are present from birth, and protect the host between pathogen exposure and initiation of adaptive responses. Components of innate immune system include: physical barriers, enzymes, inflammation-related serum proteins (complement, C-reactive protein), antimicrobial peptides (AMPs: defensins, cathelicidins), pattern recognition receptors (PRRs: e.g. toll-like receptors [TLRs]), and cells (macrophages, mast cells, natural killer [NK] cells, neutrophils, dendritic cells [DCs]).[7]

The innate immune system recognises pathogens through PRRs, which are capable of distinguishing

Table 1: Toll-like receptor localisation and ligands.

PRRs	Localisation	Ligand
TLR1	Plasma membrane	Triacyl lipoprotein
TLR2	Plasma membrane	Lipoprotein
TLR3	Endolysosome	dsRNA
TLR4	Plasma membrane	lipopolysaccharide
TLR5	Plasma membrane	Flagellin
TLR6	Plasma membrane	Diacyl lipoprotein
TLR7/8	Endolysosome	ssRNA
TLR9	Endolysosome	CpG-DNA
TLR10	Endolysosome	Unknown

PRR: pattern recognition receptor; TLR: toll-like receptor; dsRNA: double-stranded RNA; ssRNA: single-stranded RNA; CpG: cytosine guanine dinucleotide.

between self-tissues and pathogens by recognising pathogen-associated molecular patterns (PAMPs) such as components of membrane bacteria, unmethylated microbial DNA, and double-stranded RNA of viral origin.[8,9] When PRRs on cell surface bind PAMPs, they initiate phagocytosis, release toxic oxidants, and in macrophages, pathogen-derived proteins are processed into peptides and presented to major histocompatibility complex (MHC) molecules to engage and instruct antigen-specific T lymphocytes.[8] PRRs can be circulating proteins and receptors.

Circulating proteins include AMPs (e.g. defensins, cathelicidin LL-37), collectins, lectins, and pentraxins.[10] AMPs are important for skin and mucosal membrane protection and for killing phagocytosed organisms. Cathelicidins are released from neutrophils and epithelial cells, and exhibit antimicrobial activities. In both keratinocytes and macrophages, stimulation of CYP27B1, a member of cytochrome P450 superfamily of enzymes, converts vitamin D to its active form 1,25-dihydroxyvitamin D3 (1,25[OH]2D3), which induces the expression of the AMP cathelicidin LL-37.[11]

Receptors include TLRs, C-type lectin receptors, nucleotide-binding oligomerisation domain receptors (NOD-like receptors), and RIG-1-like receptors (RLRs).[10] Ten human TLRs (Table 1) have been identified as membrane proteins or expressed in endocytic vesicles. The ligands for these TLRs are represented by a wide variety of PAMPs, including microbial cell wall components, proteins, and nucleic acids. The final pathway for TLR signalling involves transcription factors that regulate a multitude of genes.[8,10] TLRs are expressed by many immune or non-immune cells such as fibroblasts, epithelial or endothelial cells, and can trigger the secretion of potent pro-inflammatory cytokines including TNF-α, interleukin-6 (IL-6), and pro-IL-1β. TLRs 3,4,7,8, and 9 can trigger production of Type 1 interferons (IFN).[12]

Cells of the innate immune system are phagocytes (neutrophils, monocytes, macrophages, DCs) and also other cells such as epithelial cells, mast cells, and platelets.[7] DCs migrate into lymphoid organs and peripheral sites, internalise microbial products, and molecules released from damaged tissue (called danger signals) present antigen to naïve T lymphocytes and induce their proliferation and activation.[13] PAMPs are potent inducers of DC production of IL-12 and IFN-α and β, and are key regulators of DC development and the Type 1 T helper (Th1) cell immune response. The effector T lymphocyte secretes IFN-γ that further primes DCs to produce great amounts of IL-12 (Figure 1).[14]

VITAMIN D AND INNATE IMMUNITY

Vitamin D receptors (VDRs) are present on antigen-presenting cells, NK cells, as well as B and T lymphocytes. Vitamin D displays many immunomodulatory effects on both innate and adaptive immune responses.[15-18] Vitamin D has been shown to have a plethora of actions on immune system cells. Active vitamin D negatively

regulates DC differentiation, maturation, and immunostimulatory capacity; decreased MHC-II expression and downregulation of co-stimulatory molecule expression (CD40, CD80, CD86) lead to suppression of antigen presentation to T cells.[19] Moreover, acting on DCs, vitamin D reduces other proinflammatory cytokines such as IL-6 and IL-17, reduces nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) activation,[20] impairs TNF, IL-1, and IFN-γ secretion,[21] and upregulates anti-inflammatory mediators such as IL-4 and IL-10,[15-18] inducing tolerogenic DCs. Vitamin D enhances monocyte phagocytosis but induces hyporesponsivness to PAMPs, and induces defensins and cathelicidins.[17]

The active form of vitamin D induces synthesis of cathelicidin AMP (hCAP, LL-37) in monocytes/macrophages, as well as other cells such as keratinocytes.[22] Cathelicidins have direct chemoattractant properties for neutrophils, monocytes, T cells, mast cells,[23,24] or can promote chemotaxis by inducing production
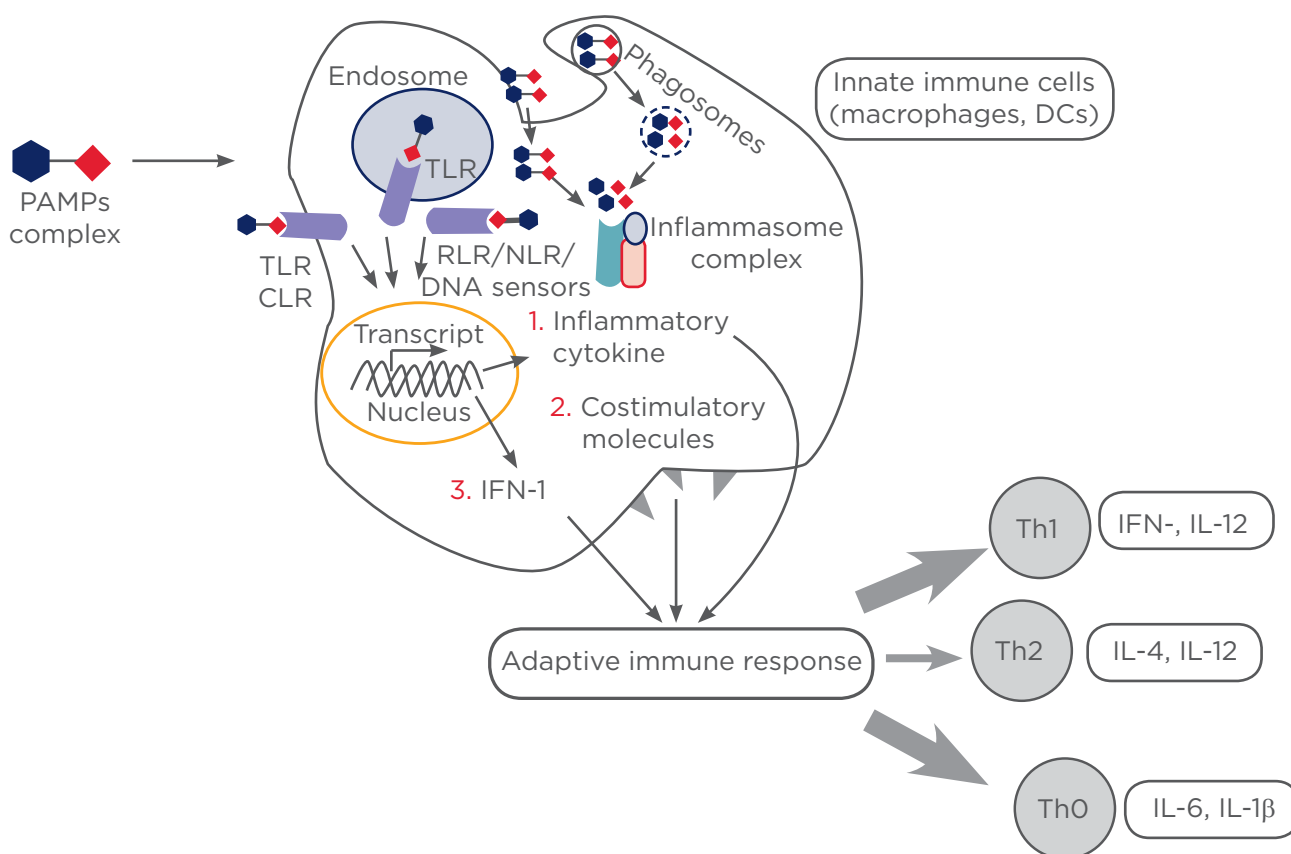

Figure 1: Link between innate and adaptive immune response.
Innate immune system responses are mediated by 'pattern recognition receptors' (PRRs). PRRs are capable of distinguishing between self-tissues and microbes by recognising highly conserved PAMPs. PRRs are either membrane and intracellular signal transducing (TLR, RLR, nucleotide-binding domain [NLRs], CLR) or secreted and circulating proteins (e.g. antimicrobial peptides, collectins, lectins). Activated TLRs initiate a signalling cascade that results in pro-inflammatory cytokines being produced along with chemokines and Type 1 IFNs. Innate immune cells internalise PAMPS binded to PRRs and this 'innate' step induces DC maturation, which is accompanied by upregulation of cytokine receptors, the major histocompatibility complex Class 2, and the co-stimulatory molecules CD80 and CD86. Once matured, DCs function as the most potent cells that present antigen to naïve T lymphocytes and induce their proliferation, activities that are central to the antigen-specific adaptive immune response. These effector T lymphocytes actively secrete IFN- that further primes the DCs to produce greater amounts of IL-12 in response to stimulation.
PAMPs: pathogen-associated molecular patterns; TLR: toll-like receptor; CLR: C-type lectin receptor; RLR: RIG-1 like receptor; NLR: NOD-like receptors; IFN: interferon; IL: interleukin; DC: dendritic cells; Th1: Type 1 T helper cell.

of chemokines.[25] Cathelicidins appear to be a link between the innate and adaptive immuneresponses by influencing DC activation and polarisation of T lymphocytes.[26,27] LL-37 upregulates the endocytic capacity of DCs and enhances the secretion of cytokines, leading to a Th1-driven immune response.[27]

On the other hand, it was shown that cathelicidins also exhibit anti-inflammatory properties, thus playing a significant role in balancing inflammation and maintaining homeostasis.[22] LL-37 can inhibit cellular immune responses triggered by IFN-γ, which is a key cytokine for polarisation of Th1 responses.[28] Cathelicidins alter the TLR-to-NF-κB pathway in the presence of exogenous inflammatory stimuli and selectively suppress specific proinflammatory cellular responses such as TNF-α, IL-1β, and NFκB.[25,29] The immunomodulatory effects of cathelicidin on macrophage TLR response may vary both on the exogenous/endogenous origin of peptide, upon the cell type and activation status, timing of exposure, and other immune mediators present.[22]

The intracellular TLRs are differentially regulated by vitamin D; TLR-2, 4, and 9 being downregulated, whereas TLR-3 was unaffected. This may have significant biological relevance and may lead us to speculate that the vitamin D deficiency observed in autoimmune disease may further potentiate autoreactivity to self-nucleic acids recognised by TLR.[30]

Actions of vitamin D of innate immunity have indirect effects on adaptive immunity suppressing effector T cell activation. Vitamin D also has direct actions on T cells: it decreases Th1 proliferation, promotes shift from Th1 to Th2, increases Th2 cell function, inhibits production of IL-2, IFN-γ, TNF-α, and IL-5, decreases levels of IL-2 in CD4+ T cells, enhances transforming growth factor beta (TGF-β)-1 and IL-4 transcripts, regulates Th17 cells and decreases IL-17 expression, and promotes regulatory T cell (Treg) development, differentiation, and functions.[17]

EVIDENCE FOR INNATE IMMUNE ACTIVATION IN SSC

Cells of the innate immune system are detected at the sites of tissue injury in SSc. Macrophages are present in the infiltrates of the dermis, especially in the perivascular region,[31] and their number is increased in both involved and uninvolved skin.[32]

DCs from SSc patients show an important TLR response especially in the early phase of the disease.[33,34] The increased production of Type I IFN and of other cytokines such as IL-6, TNF-α, and IL-10 is specifically found in response to TLR-2, TLR-3, and TLR-4 ligands in DCs.[35] The observations that circulating endogenous TLR-4 agonists are present in SSc patients,[36] combined with high circulating levels of inflammatory mediators often secreted by TLR stimulated DCs/macrophages (TNF-α, IL-6, and IL-12p70),[37,38] stress the potential role of TLRs in this condition.

Interplay between altered endothelial cells, immune cells, and their soluble mediators are responsible for the alteration of fibroblast functional phenotype and excessive accumulation of ECM.

The fibrosing phenotype of scleroderma is associated with the production of Th2 cytokines such as IL-4, IL-13, and profibrotic TGF-β.[4] Innate immune cells are responsible for the secretion of many cytokines mentioned above. IL-13 is produced by DCs and mast cells via TLR-2 activation; mast cells can be another source of TGF-β.[39]

Role of Different TLRs in SSc

Several studies suggested that TLRs can be important in disease initiation and progression, especially intracellular TLR-3, 7, 9, as well as plasma membrane TLR-2 and 4. Increased expression of Siglec-1, an IFN-regulated gene, in circulating SSc monocytes and tissue macrophages suggests that Type I IFN-mediated activation of monocytes occurs in SSc, possibly through TLR activation of IFN secretion. Stimulation with TLR-3, 7, or 9 agonists dramatically increased Siglec-1 expression on peripheral blood mononuclear cells.[40]

In vitro stimulation of DC subsets from patients with early SSc with TLR-2, 3, and 4 ligands produced increased secretion of IL-6 and TNF-α by plasmaytoid DCs (pDCs) compared with controls.[33] A rare polymorphism in the TLR-2 gene (Pro631His) is highly associated with the diffuse form of SSc, high levels of antitopoisomerase-I antibodies, and pulmonary hypertension.[41] SSc monocytes carrying this polymorphism also secreted higher levels of proinflammatory and profibrotic cytokines.[41]

Van Lieshout et al.[42] demonstrated that the level of the chemokine ligand CCL18, which was a T cell chemoattractant and profibrotic factor, is higher in the serum from SSc patients compared with

healthy controls. CCL18 and IL-10 were secreted by CD14+ circulating monocytes and DCs after TLR-4 stimulation. Monocytes derived from SSc patients with interstitial lung disease have an enhanced pro-fibrotic phenotype and could differentiate into fibrocytes and secrete higher collagen after exposure to a TLR-4 agonist.[43] A recent study[44] demonstrated that TLR-4 was overexpressed in the skin and lung tissue from patients with SSc.

Farina et al.[45] showed that TLR-3 ligand (dsRNA) strongly upregulated ET-1 mRNA expression in dermal fibroblasts isolated from SSc patients, whereas selective blocking of TLR-3 with bafilomycin attenuated ET-1 expression. Agarwal et al.[46] also demonstrated that dermal fibroblasts from patients with SSc had an increased expression of TLR-3 in response to Type I IFN, resulting in an enhanced secretion of IL-6 and monocyte chemotactic protein 1 (MCP-1). The increased IL-6 secretion could contribute to dermal fibrosis through increases in fibroblast survival and proliferation, ECM deposition, and myofibroblast differentiation.

In nephrogenic systemic fibrosis, gadolinium-based contrasting agents bind TLR-4 and 7 in differentiated macrophages and induce activation of NF-κβ and expression of profibrotic IL-4, IL-6, and TGF-β.[47] HSP70 (heat shock protein 70) and HMGB-1 (high mobility group box 1) are PAMPs - danger signals released from damaged cells that can bind TLRs and induce gene expression of proinflammatory mediators.[48,49] Serum HSP70 has been demonstrated to be elevated in SSc compared with controls and associated with pulmonary fibrosis, skin sclerosis, renal vascular damage, oxidative stress, and inflammation.[48] HMGB-1 is elevated in tissue and serum from SSc patients and correlates with the skin score.[49]

Interaction between TLR and Type I IFN in SSc

Type I IFNs-α/β are a family of cytokines induced rapidly by viral and bacterial infections, and are well recognised for their crucial role in innate defence. Moreover, IFNs-α/β enhance immune responses through the stimulation of DCs, demonstrating that IFN-α/β serves as a signal linking innate and adaptive immunity. In the last years a lot of research focused on the role of Type I IFN in the pathogenesis and severity of SSc. TLR activation stimulates production of IFNs and other cytokines, and IFNs regulate the behaviour of key cells involved in the development of SSc, including DCs, T cells, and dermal fibroblasts.[50]

In SSc, approximately half of patients have an increased expression of IFN-stimulated genes (ISGs), termed the IFN signature, and pDCs were the main source of IFN production upon TLR-7 or 9 activation.[51,52] Probably the strongest evidence implicating IFN in the disease pathogenesis is detection of IFN and ISGs in affected tissues, especially skin.[53-55]

Kim et al.[56] showed that autoantibody subsets in SSc sera differentially induce IFN-α: anti-topoisomerase 1 induced significantly higher levels of IFN-α as compared with anticentromere or antinucleolar antibodies. IFN inducing activity was significantly higher in patients with diffuse SSc than in those with limited forms, and correlated with lung fibrosis, digital ulcers, pulmonary hypertension, or cardiac involvement.[51] Interferon regulatory factors (IRFs) coordinate the expression of Type I IFNs and IFN–inducible genes, and several polymorphisms have been associated with susceptibility for development of SSc.[53,57]

The development of SSc was reported in patients undergoing IFN treatment.[58] A randomised placebo-controlled trial evaluating effects of subcutaneous IFN-α injection on the severity of skin involvement in patients with early SSc showed that treatment with IFN-α resulted in worsening lung function and a smaller degree of improvement in skin thickening scores.[59]

A combined score of the plasma IFN-inducible chemokines, IFN-inducible protein 10, and IFN-inducible T cell chemoattractant highly correlated with the IFN gene expression signature in SSc patients in the Genetics versus Environment in Scleroderma Outcome Study.[60] The chemokine score correlated positively with Medsger Severity Index for muscle, skin, and lung involvement, as well as creatine kinase levels in SSc. There was also a negative correlation with forced vital capacity and diffusing capacity for carbon monoxide. The fact that the IFN chemokine score did not show a consistent trend of change in time suggested that the IFN signature was a stable marker for the more severe subtype of disease rather than a time-dependent immune dysregulation that improved after the initial phase of SSc.[60]

VITAMIN D IN SSC

The impact of vitamin D on innate immune activation in SSc is not fully understood. Vitamin D could be beneficial by its capacity to inhibit maturation

of DCs, to decrease important inflammatory cytokines such as IL-1, 6, TNF-α, and IFN-γ, to induce hyporesponsiveness to PAMPs, and to decrease TLR-2, 4, and 9 expression that indirectly inhibits T cell activation. Some of the effects of vitamin D could have the potency to worsen SSc, such as Treg cell activation and cathelicidins. Vitamin D activates Tregs, which have tolerogenic properties but can also increase TGF-β production - a key fibrotic cytokine.[15-18] On the other hand, *in vitro* studies showed that impaired VDR signalling with reduced expression of VDR, and decreased levels of its ligand, may thus contribute to hyperactive TGF-β signalling and aberrant fibroblast activation in SSc.[61] Little is known about the role of cathelicidins in the pathogenesis of SSc; whether their chemoattractant properties and Th1 driven immune response would lead to worsening of the disease remains to be studied.

Yet, vitamin D deficiency has been documented in a high proportion of SSc patients (about 80%),[62] but low vitamin D levels were universal and independent of geographic origin or vitamin D supplementation.[63,64] Vitamin D deficiency in SSc is potentially related to several factors: dermal fibrous thickening with reduced synthesis of provitamin-D3, gastrointestinal involvement, and malabsorption. Moreover, patients with SSc experience impairment in physical functioning, and are prone to a sedentary lifestyle and diminished sunlight exposure.[66-68] Vitamin D is still an undiscovered field in SSc; data reported so far are not homogeneous. Different studies correlated levels of vitamin D with higher levels of parathyroid hormone,[68] higher incidence of acro-osteolysis and calcinosis,[68] systolic pulmonary artery pressure,[65,67] inflammatory syndrome,[62,65,67] Rodnan score,[65,69] activity and severity score,[65,67] low bone mass density,[63,68] disease duration, and pulmonary fibrosis.[62,65,67]

Despite these data, evidence linking vitamin D supplementation with reduced disease activity is still lacking. Nevertheless, given the immunomodulatory properties of vitamin D, we advocate vitamin D supplementation to SSc patients in order to raise vitamin D serum concentrations to desired levels, achieving also as a secondary outcome a potentially mitigating effect of an overactive autoimmune response in such patients.

CONCLUSION

Although great progress has been made in recent years in elucidating the pathogenesis of SSc, the exact molecular mechanisms are still unclear and it still remains a challenging disease to treat. Components of innate immunity-like cells, AMPs, and PRRs, serve to link innate and adaptive immunity. TLR intracellular signalling pathways might be one of the mechanisms that initiate and drive autoimmunity and subsequent fibrosis. Activation of the immune system results in IFN sensitive gene transcription. TLRs may represent the link between immune activation and tissue fibrosis; several TLR agonists are under investigation. Vitamin D modulates key elements of innate immunity like TLRs, IFNs, cathelicidins, DC function, and T cell activation. Whether vitamin D could have a role in the complex pathogenesis of SSc still remains unclear but low levels of vitamin D may represent a marker of aggressive disease.

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