Osteoimmunology

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Osteoimmunology: The effect of autoimmunity on fracture healing and skeletal analysis

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ABSTRACT

Understanding factors that affect bone response to trauma is integral to forensic skeletal analysis. It is essential in forensic anthropology to identify if impaired fracture healing impacts assessment of post-traumatic time intervals and whether a correction factor is required. This paper presents a synthetic review of the intersection of the literature on the immune system, bone biology, and osteoimmunological research to present a novel model of interactions that may affect fracture healing under autoimmune conditions. Results suggest that autoimmunity likely impacts fracture healing, the pathogenesis however, is under researched, but likely multifactorial. With autoimmune diseases being relatively common, significant clinical history should be incorporated when assessing skeletal remains. Future research includes the true natural healing rate of bone; effect of autoimmunity on this rate; variation of healing with different autoimmune diseases; and if necessary, development of a correction factor on the natural healing rate to account for impairment in autoimmunity.

1. Introduction

Bone fractures are common injuries, with the average person experiencing at least two fractures in a lifetime [1]. With 5%–30% of people in 'Western' countries diagnosed with autoimmune diseases, the chance of these occurring together is inevitable [2–4]. Osteoimmunology is a relatively recent field of immunology that studies the reciprocal relationship between the skeletal and immune systems [5]. Over the past two decades, the study of osteoimmunology has become a conceptual framework for understanding both systems and developing novel therapies in many disciplines, from pharmacy to orthopaedics [6–9]. It is well documented that the immune system plays a role in diseases affecting bone resorption and formation, such as erosive arthropathies, osteoporosis, and rheumatoid arthritis [10,11]. In the process of bone fracture healing, inflammation is essential as it precedes bone formation and remodelling [12].

Forensic anthropology involves the analysis of human skeletal remains to obtain biological information about the decedent [13]. The biological profile includes ancestry, age, sex, and stature; however, assessing trauma and pathology can be influential in the identification process [14,15]. The application of estimating post-traumatic time interval of fractures is critical to understanding the chronology of events in abuse and torture cases. Estimating post-traumatic time interval is also significant in the identification of remains by matching to antemortem data [16,17].

This paper provides a review of the literature on the immune system and bone biology necessary for comprehension of osteoimmunological

Abbreviations: **APC**, antigen-presenting cell; **BCR**, B-cell receptor; **BMI**, body mass index; **BMU**, bone multicellular unit; **CCP**, cyclic citrullinated peptide; **CD4+**, cluster of differentiation (4) (regulator T cells); **CD8+**, cluster of differentiation (8) (cytotoxic T cells); **CT**, computed tomography; **DKK-1**, Dickkopf-related protein 1; **ELISA**, Enzyme-linked immunosorbent assay; **HIV**, human immunodeficiency virus; **I-L**, interleukin; **IgG**, immunoglobulin G; **INF-γ**, interferon gamma; **M-CSF**, macrophage colony-stimulating factor; **MRI**, magnetic resonance imaging; **mRNA**, messenger ribonucleic acid; **OA**, osteoarthritis; **OPG**, osteoprotegrin; **PTI**, post-traumatic time interval; **RA**, rheumatoid arthritis; **RANK**, receptor activator of nuclear factor kappa B; **RANKL**, receptor activator of nuclear factor kappa B ligand; **RF**, rheumatoid factor; **RNA**, ribonucleic acid; **SLE**, systemic lupus erythematosus; **SM**, smith-antibody (related to SLE); **T1D**, type 1 diabetes; **T-ALL**, T-cell acute lymphoblastic leukaemia; **TCR**, T-cell receptor; **Th**, T helper cell; **TNF-α**, tumour necrosis factor-alpha; **WNT**, wingless/integrated pathway.

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Glossary

Angiogenic factor A group of molecules that are key in the function of blood vessel formation
Antemortem Prior to death
Antigen Anything which elicits an immune response against it
Apoptosis Programmed cell death or 'suicide' normally functioning as a way for the body to remove unnecessary or abnormal cells
Bone morphogenic protein A group of proteins functioning to transform growth factors involved in bone and cartilage formation. They have significant roles in bone induction, maintenance, and repair
Cascade A series of reactions where the product of the first becomes the substrate for the next
Complex (protein) A group of 2 or more proteins or molecules that perform a new function together
Cytokine A type of protein made by both immune and non-immune cells that have a modulatory effect on the immune response
Eburnation Polished smooth articular surface of bone resulting from bone-on-bone contact
ELISA Enzyme-linked immunosorbent assay (ELISA) is an immunological assay used to measure antibodies, antigens, proteins, and glycoproteins in serum samples
Extra-articular Any region outside the joint space
Fine needle aspiration Type of biopsy procedure where a thin needle is inserted into the biopsy region and a sample is collected through the needle
Granuloma Benign small areas of inflammation
Growth factors Substances, usually hormones, the body produces to regulate cell division and survival
Hematoma Accumulation of clotted blood forming a solid mass. It is caused by a broken vessel due to surgery or trauma
Histological staining A series of staining processes to view tissue samples under a microscope
Homeostasis Term used to describe the balance of systems within the body needed for survival and correct function
Knock-out model An organism whose genome has been modified to remove or damage the sequence in examination, used to identify gene function by eliminating the expression and analysing the outcome
Lamellae Organised collagen fibres into layers of bone matrix
Lesion Refers to a bone lesion, which is an area of abnormal bone that has been changed or damaged from the standard morphology
Leukocyte Otherwise named white blood cells, these cells function as part of the immune system
Ligand A substance that forms a complex with a biomolecule to perform specific biological functions
Macroscopic Details able to be seen with the naked eye, not requiring a microscope
Non-specific Not directed to a particular function; instead, having a general effect
Osteoblastogenesis Formation of osteoblasts from osteoblast precursors
Osteoclastogenesis Formation of osteoclasts from osteoclast precursors
Osteon Primary structural unit of cortical bone
Pannus Inflamed, thick, and swollen synovial membrane in joints with rheumatoid arthritis
Pathogen An agent of diseases, e.g., virus, bacteria, fungi
Perimortem Occurring around time of death
Phagocyte An immune cell that surrounds, ingests, and kills foreign material and cell debris
Positive feedback loop: A process where the end products of a reaction continue to amplify further or enhance that change
Postmortem Occurring after death
Proteases Enzymes that break down proteins
Real-time PCR A sensitive method used to measure gene expression. Real-time, in comparison to conventional PCR, adds a reverse transcription step to allow for amplification
Receptors A molecule on the inside or on the surface of a cell that binds to a specific substance causing a particular response from the cell
Receptor desensitisation Decreased responsiveness due to repeated or chronic exposure to the agonist
RNA A nucleic molecule similar to DNA but contains ribose instead of deoxyribose and information transcribed from DNA
Self-antigen Any molecule from an organism that would induce an antibody response in another organism but to which the healthy immune system of the original organism is tolerant
Tolerance The prevention of an immune response against a particular antigen, usually referring to self-antigens
Transcription factor Proteins that bind to DNA and regulate gene expression by promoting transcription
Transgenic An altered genome by the induction or suppression of DNA sequences by artificial means
Wild-type The state (genotype or phenotype) predominated in a natural population. Wild-type is used in research to compare a test group against a control
Wnt An evolutionarily conserved group of signalling transduction pathways beginning with proteins passing signals into the cell through cell surface receptors

interactions, to provide a hypothetical model of the interactions between the two which may affect fracture healing in an individual with an autoimmune disease. Building on this model, the second aim of this paper addresses the practical application of fracture healing analysis in forensic anthropology and how new knowledge from osteoimmunological research may impact this analysis.

2. Autoimmunity

Comprehension of the immune system will contribute to the understanding of the osteoimmunological effects on fracture healing. This section introduces the function and branches of the immune system and discusses how dysregulation of these systems causes autoimmunity, concluding with rheumatoid arthritis as an example of an autoimmune disease.

2.1. General immunity

A pathogen must gain entry into the body (host) to invade, hijack, replicate and thrive. The immune system is a series of complex biological cascades that function to recognise and tolerate molecular markers to 'self' cells (antigens), to protect from and reject 'non-self' antigens creating immunity [18].

Three primary lines of defence are defined for resistance to infection, the first of which is the body's physical barriers, i.e., the skin and mucosal membranes (airways), and chemical barriers, such as the acidic environments (stomach) and bodily fluids (tears, saliva), which inhibit either the initial invasion or survival of the pathogen into the host

through antimicrobial effects [19,20]. If these barriers are breached, the pathogen encounters two further levels of defence, the innate and adaptive immunity, which are divided based on the speed and specificity of the response [21]. Innate, or non-specific immunity, is the first line of the host's cellular defense response through phagocytic cells that ingest and engulf particles and pathogens creating molecular cascades that allow for an immediate but non-specific defence to a particular pathogen [18]. In comparison, the adaptive immune response takes days to develop and is precise to a specific antigen using antigen-specific T and B lymphocytes [22]. These cells allow for the synthesis of antibodies and the development of 'memory'. Memory allows for a rapidly targeted defence response in cases of reinfection [21]. The innate and adaptive immune system responses are detailed below.

2.1.1. *Non-specific/innate*

The innate immune system comprises many non-specific defence mechanisms that act immediately after infection. The innate immune response is independent of specific antigens and will therefore respond to all pathogens each time there is infection or reinfection creating inflammation [23]. Inflammation is a biological response initiated by the innate immunity following the breach of physical barriers to a harmful stimulant such as tissue damage, a pathogen, or an irritant [24]. Inflammation functions to clear damaged cells, recruit cells, and promote blood vessel formation to initiate tissue repair [25]. See Fig. 1 for a summary of the innate immune response.

2.1.2. *Specific/adaptive*

Initiated by localised inflammation created by the innate immune system response the adaptive immune system begins to mobilise simultaneously. Unlike the innate immune system, the adaptive immune system uses antigen-specific mechanisms [21]. Adaptive immunity utilises lymphocytes which are a subset of leukocytes (white blood cells) that are the effector cells of the adaptive immune system [22]. The T and B lymphocytes contain antigen-specific receptors to recognise unique gene sequences on invading pathogens. Molecules recognised by receptors on lymphocytes (T and B cells) are termed antigens [27]. The process of developing these antigen-specific receptors requires random gene rearrangement and splicing of the antigen binding areas of the B cell (BCR) and T cell (TCR) receptors [27].

The adaptive immune system can be subdivided into antibody-

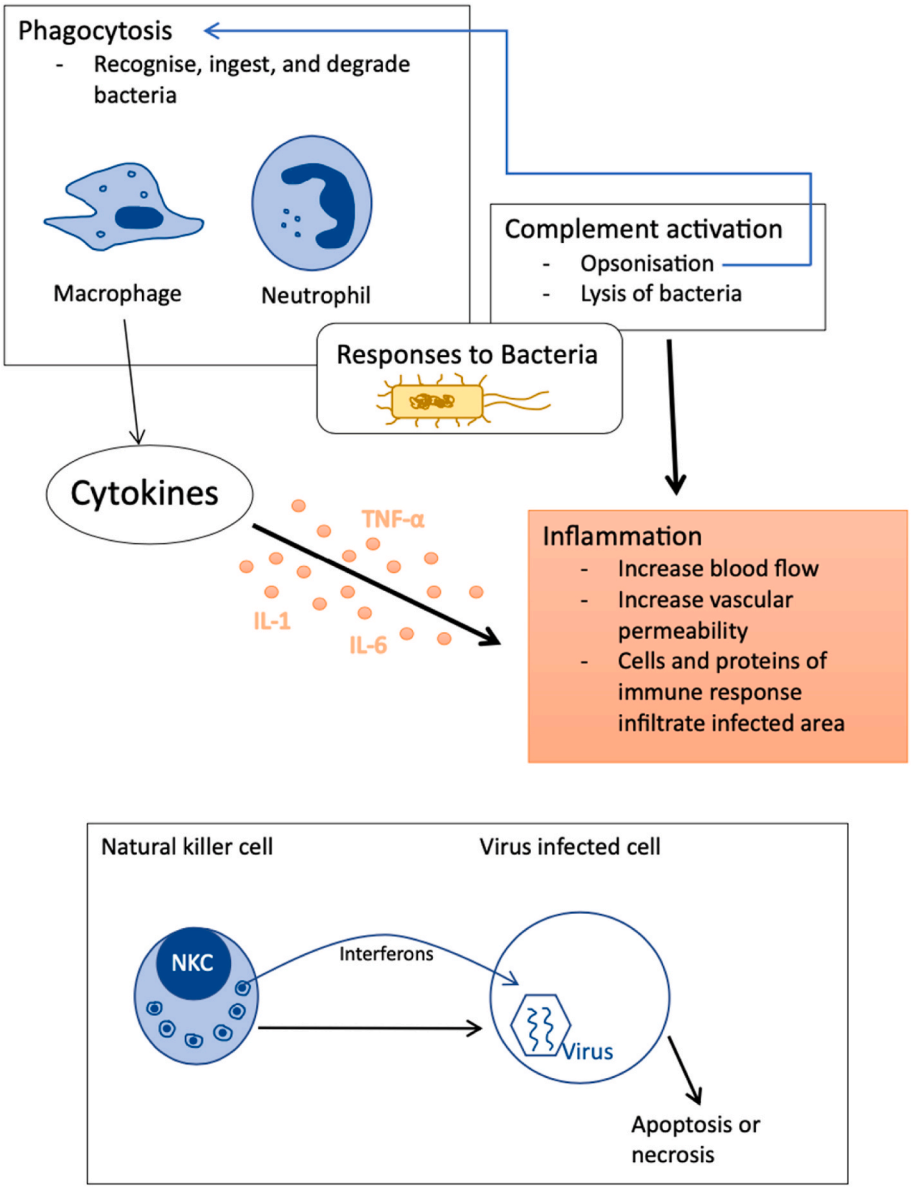

Fig. 1. Innate immune response overview. Image adapted from Keogan *et al.* ([26], p.10).

mediated humoral immunity and cell-mediated immunity [24]. These systems mutually function to recognise, destroy and create long-term immunity to the invading pathogen [23]. Survival and further differentiation of lymphocytes are dependent on the recognition of an antigen [24]. Fig. 2 summarises the humoral and cell mediated immune responses.

2.2. Pathogenesis of autoimmunity

The immune system displays immunological tolerance to healthy cells. This tolerance ensures that the immune cells do not mount a response against the body's own self-antigens [28]. To prevent this, self-antigens from all nucleated cells are displayed to leukocytes, and a specific co-stimulation allows for recognition and differentiation between self- and non-self-antigens [29]. However, breakdowns in these systems occur causing self-reactive leukocytes to be produced. Central tolerance is the process of deleting the T and B cells in the thymus and bone marrow, respectively, that have recombined T/BCR that are self-reactive [30]. Comparatively, peripheral tolerance is a secondary mechanism of immunological protection when a self-reactive T or B cell evades central tolerance, primarily maintained by regulatory T (Treg) cells in the lymph nodes and peripheral tissues [29].

Autoimmunity occurs when the tolerance to delete these self-reactive immune cells is lost alongside the ability to distinguish between these self-antigens and the invading pathogenic antigens [31]. This loss of tolerance and immune response to self-antigens is clinically termed 'autoimmunity.' Common autoimmune diseases include rheumatoid arthritis, type 1 diabetes (T1D), systemic lupus erythematosus (SLE), Graves' disease, and multiple sclerosis (MS) [32].

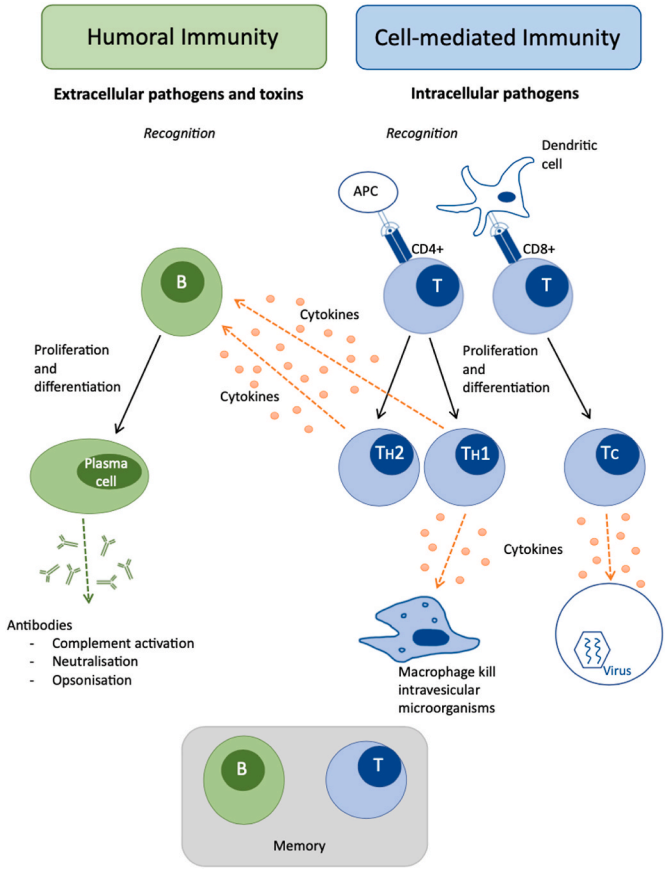

Fig. 2. Adaptive immune response overview showing branches of the humoral (left) and cell-mediated (right) responses. Figure shows interaction between the two, represented by where the peripheral tolerance mechanism of Th cell co-stimulates the B cells for antibody release (see dotted arrows from cell-mediated immunity to humoral immunity). Image adapted from Keogan *et al.* ([26], p.11).

The complexity of autoimmunity is still being researched [2], and no single cause has yet been identified [33]; however, there is a significant correlation between a genetic susceptibility initiating a loss of tolerance and dysregulation of the immune system. This provides the framework for pathological damage and a final environmental trigger such as a previous infection or ultra-violet light damage leading to the clinical appearance of an autoimmune disease [34–37]).

2.3. Inflammatory characteristics of rheumatoid arthritis

Rheumatoid arthritis (RA) is characterised by a dysregulation of the immune system. This causes a pathogenic effect on the skeletal system, highlighting a potential model for understanding the interactions between the skeletal and immune systems in the field of osteoimmunology [11].

RA is a chronic inflammatory autoimmune disorder that primarily affects the joints [38]. Although the precise pathogenesis is unascertained, RA is thought to have a genetic susceptibility in genes HLA-DR1 and HLA-DR4 followed by an environmental trigger such as a pathogen (bacteria or virus) or cigarette smoke [39]. These environmental changes cause modification of self-antigens in proteins such as collagen or vimentin through citrullination [40]. During citrullination, the amino acid arginine is converted to citrulline, and due to the susceptibility genes, immune cells respond to the altered proteins as foreign, representing the breakdown in tolerance [40]. $CD4^{+}$ Th cells are activated, which stimulates B cells to produce autoantibodies (anti-citrullinated protein (anti-CCP) and rheumatoid factor (RF)) [41,42]. T cells and macrophages secrete cytokines IL-1, IL-6, and TNF-α into the joint space to recruit more proinflammatory cells and stimulate synovial cells to proliferate, causing a pannus (thick swollen synovial membrane) [43]. Activated synovial cells of the pannus secrete proteases which damage cartilage and bone [43]. Inflammatory cytokines stimulate T cells to upregulate receptor activator of nuclear factor kappa-B ligand (RANKL) on their surface, which binds RANK on the surface of osteoclasts activating bone resorption [44,45]. The RANKL/RANK system will be further discussed in section 3.4.1. Within the joint synovium, autoantibodies bind to targets forming immune complexes. These complexes activate the complement system, which promotes joint inflammation and injury using an enzymatic cascade [46]. Chronic inflammation causes angiogenesis, encouraging more inflammatory cells to arrive [40]. Inflammatory cytokines travel through the blood, causing extra-articular problems throughout other organs. For example, IL-1 and IL-6 travelling to the brain, where they act as pyrogens that induce fever [41], promoting protein breakdown in skeletal muscle and forming rheumatoid nodules in the skin [47].

3. Fracture healing

Comprehension of how fractures heal contributes to understanding the osteoimmunological effects of autoimmunity on the process. Healing and restoring function after bone damage is a detailed multi-stage process. Although these stages exhibit considerable overlap and act cooperatively, these will be discussed below in three major phases the inflammatory phase, repair phase, and remodelling phase.

3.1. Inflammatory phase

Immediately following a fracture, blood vessels in the bones Haversian canals rupture, forming a hematoma. Fibrinogen converted to fibrin (clotting) within the hematoma forms the initial scaffold for the fracture healing process [48]. The hematoma microenvironment, high in calcium and lactic acid, activates the peripheral blood-derived inflammatory cells to secrete proinflammatory cytokines [49,50]. This functions to further recruit inflammatory cells secreting a variety of

proinflammatory cytokines, including IL-1 from macrophages and antigen-presenting cells (APC), IL-6 from activated Th2 cells, APC and other somatic cells, and TNF-α from macrophages, mast cells and NK cells to initiate a local inflammatory response at the fracture site [51,52]. Neutrophils, monocytes, and macrophages infiltrate the area causing an acute inflammatory response [53,54]. After infiltration of macrophages, the immune response is shifted to adaptive immunity by the invasion of lymphocytes, proinflammatory cytokines, stimulated neutrophils and macrophages to clear debris [55].

Restoration of blood flow is essential to the healing process. Angiogenic factors are released due to disturbed vascularisation [12]. Endothelial cells from surrounding undamaged vessels migrate into the hematoma and are stimulated to form new blood vessels [56]. The tissue damage induces latent resident fibrocytes to differentiate into fibroblasts to secrete collagen fibres into the hematoma [57]. Cross communications between all cytokines, growth factors, and bone morphogenic proteins (BMPs) initiate osteogenesis and angiogenesis, creating a reparative granuloma, the template for callus formation [55,58,59].

The acute inflammatory period peaks around 24–48 h and remains until approximately seven days post-injury in adults [54,60]. Resolution of acute inflammation is the process where anti-inflammatory cytokines such as TGF- β, IL-4, IL-10, and IL-13 are secreted by macrophages, dendritic cells, and T cells [26]. These inhibit further macrophage activation, control excessive inflammation, promote B cell growth, and promote chronic fibrosis for soft tissue healing [59,61]. Cottrell and O'Connor [62] showed that treatment of anti-inflammatory drugs early after induction of a fracture showed marked impairment in the healing rate, signifying inflammation is a significantly important part of the initial stage of the fracture healing process.

3.2. Repair phase

Based on the anatomical location and the mechanical conditions of the fracture, either primary (direct) or secondary (indirect) bone healing will prevail [61]. Where stability is compromised, such as with an oblique fracture, secondary bone healing, or endochondral healing, through the formation of a callus is the dominant type of bone formation [54]. Secondary healing, occurring more frequently, involves the influx of immune cells [53,54] indicating the importance of immune cells in this process and, therefore, the close association with osteoimmunology.

The inflammatory and repair phases of adult fracture healing are marked by soft callus formation [63]. Following the formation of the granuloma and the secretion of collagen fibres from fibroblasts, chondrocytes differentiate from bone marrow progenitor cells and lay down a cartilage matrix throughout the granuloma [59,64]. Chondrocytes then undergo hypertrophic differentiation and mineralisation to form the soft fibrocartilaginous callus [64]. Resident osteoblasts and vascular networking infiltrate the soft callus, and simultaneously, hypertrophic chondrocytes apoptose. Proinflammatory cytokines (IL-1, IL-6) are absent during this period to allow for callus formation [65,66]. TNF-α is also low in the early repair phase allowing for chondrocyte and fibroblast action but increases in the late repair phase to facilitate chondrocyte apoptosis and cartilage resorption [66–68]. Glass *et al.* [68] used a murine model to assess the influence of TNF-α on fracture healing. Injection of TNF-α into the fracture site accelerated healing on day 14, seen by histological staining [68]. Comparing percentage callus mineralisation, results showed significantly greater mineralisation in the TNF-α injected mice, indicating improved healing with the addition of TNF-α at the repair phase of healing [68].

The cartilaginous soft callus begins to undergo endochondral ossification around days 11-28 in adults [54,69]. Within the fracture site, resident osteoblasts upregulate the production of RANKL on their surface, which binds to RANK on nearby monocytes. Binding stimulates the monocytes to fuse, forming a multinucleated cell, the osteoclast. RANKL allows for the maturation of the osteoclasts [45,70]. Lysosomal enzymes are secreted from osteoclasts, which digest the collagen protein in the organic matrix of the damaged bone [52]. Osteoclasts also secrete hydrochloric acid, which dissolves hydroxyapatite into calcium and phosphate ions which re-enter the bloodstream [71].

As with the inflammatory phase, the repair phase must be controlled to prevent damage and stimulate further healing. To control bone resorption, osteoblasts secrete osteoprotegerin (OPG), which binds RANKL and prevents it from activating the RANK receptor on osteoclasts, slowing resorption (see Fig. 3) [73,74]. The vascularisation within the cartilaginous callus allows for the continued migration of mesenchymal stem cells, facilitated by TNF-α, which promotes fracture repair by augmenting the recruitment and osteogenic differentiation of progenitor cells into osteoblasts [68,75]. Osteoblasts then secrete osteoid seam into the cartilaginous callus, forming the organic, elastic properties of the bony callus [52]. Calcium and phosphate deposition on the seam produce hydroxyapatite giving the callus mechanical strength [70].

3.3. Remodelling phase

In adults, the remodelling phase begins as early as day 18 post-trauma and can continue for months to years [54]. Once the bony callus bridges the fracture gap, remodelling is required to restore the pre-injury cellular and mechanical functions [76]. Remodelling is facilitated by a delicate balance between osteoblastic and osteoclastic activity, which is necessary for 'coupled remodelling' [76]. During coupled remodelling, woven bone formed in the bony callus is reorganised into trabecular bone lining the inner medullary cavity and is surrounded by cortical bone. Simultaneously, capillaries are formed, re-establishing vasculature to the new bone [77].

After restoring functional and structural integrity, normal bone homeostasis remodelling can continue. The basic multicellular unit (BMU) comprises osteoblasts, osteoclasts, and a capillary blood supply that maintains bone integrity [78]. Osteocytes are responsible for sensing stimuli, such as underperforming aged cells, and in response, activating the differentiation of osteoblasts and osteoclasts within the BMU.

Remodelling occurs cyclically for both bone fracture healing and normal bone homeostasis. It is divided into five overlapping steps: activation, resorption, reversal, formation, and termination of which, the primary function is to remove and replace damaged or aged bone [72,79,80]. Any disruption to the control and balance in the ratio of osteoblasts and osteoclasts may result in impairment [81].

3.4. RANK/RANKL/OPG signalling pathway

Understanding the molecular mechanisms of bone remodelling is fundamental to bone-related diseases and pathological skeletal conditions, including abnormal resorption of bone in conditions such as osteoperiostitis and rheumatoid arthritis. The RANKL/RANK pathway was first identified in 1990 and has since been the target of many drugs aiming to treat such conditions [82,83].

3.4.1. RANK/RANKL

The RANKL/RANK pathway mediates bone resorption [84]. Osteocytes are mature osteoblasts in the bone matrix and function to sense the bone microenvironment [85]. RANKL is a protein-ligand produced by osteoblasts and osteocytes during normal bone homeostasis upon recognition of a high concentration of M-CSF expressed by resident osteocytes [86]. The expression of M-CSF is increased in response to bone damage or stress factors such as cytokines (e.g., TNF-α) [87]. Based on an observation by Udagawa *et al.* [88] using an M-CSF knock-out mouse model which developed osteopetrosis due to a lack of mature osteoclasts, it was concluded that M-CSF along with RANKL is required for the differentiation of progenitor cells into osteoclasts. RANKL enhances osteoclastogenic bone resorption by binding its receptor RANK, expressed on the membrane of immature osteoclasts [73]. RANK

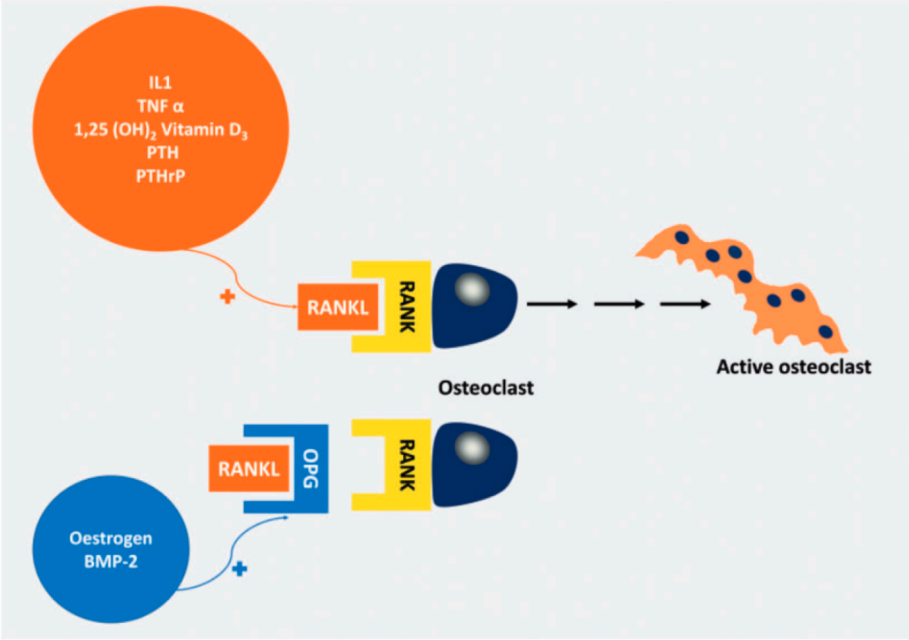

Fig. 3. Figure showing the factors influencing RANK/RANKL pathway activation and suppression by OPG. Image taken from Kenkre and Bassett ([72], p.315).

binding encourages further osteoclastic recruitment and stimulates osteoclastogenesis (osteoclast-mediated bone resorption) [89].

3.4.2. *Osteoprotegerin*

Osteoprotegerin (OPG), first identified using a gene knock-out model by Simonet *et al.* [90] prior to the discovery of RANKL/RANK, is a decoy receptor for RANKL. Both transgenic mice overexpressing hepatic OPG and wild-type mice administered with OPG showed osteopetrosis. These results suggested that similarly to M-CSF, OPG blocked osteoclast differentiation from precursors. Therefore, it was concluded that increasing OPG in patients with osteoporosis, known to result from increased numbers and activity of osteoclasts, is likely to be a successful treatment [90]. Secreted by osteoblasts and osteocytes, it binds to RANKL, preventing its binding to RANK, thereby inhibiting and controlling bone resorption [73,89].

3.4.3. *Regulation and dysregulation*

Under normal homeostatic conditions, bone remodelling is controlled by a balance between osteoblasts and osteoclasts, mediated by the RANK/RANKL/OPG pathway [72]. However, as seen in Fig. 3, many mediators of the RANK/RANKL/OPG pathway exist. The complex regulation of the pathway causes it to be easily affected by pathologies. In inflamed joints, RANKL is also expressed by synovial cells and activated T cells [45,91,92]. The activated T cells also overexpress TNF-α. To study this effect on the RANK/RANKL pathway Li *et al.* [93] used overexpressing TNF-α-transgenic mice, and wild-type mice injected with TNF-α to investigate how TNF-α increased osteoclastogenesis in vivo. Results showed TNF-α mediated joint destruction in inflammatory joint diseases by systematically increasing the number of osteoclast precursors [93]. TNF-α promotes the fusion of precursors into osteoclasts and stimulates the expression of RANKL from osteoblasts, osteocytes, chondroblasts, synovial cells, and activated T cells [75,94]. Increased numbers of osteoclasts due to impacts on the regulation of the RANK/-RANKL pathway alter the fundamental balance between osteoblasts and osteoclasts required during coupled remodelling and the BMU in the repair and remodelling phases of fracture healing. Consequently, fracture healing is likely to be impaired.

4. Osteoimmunology

This section reviews the relatively new conceptual field of 'osteoimmunology,' that links the immune and skeletal systems and how changes in cytokines and T cells in autoimmunity may have implications on bone fragility and fracture healing.

4.1. *Osteoimmunology as a new conceptual framework*

Osteoimmunology combines interdisciplinary research from immunology and osteology to progress research into clinical pharmaceutical targets for conditions such as osteoporosis and RA and implications on the bone from treatments suppressing the immune system such as chemotherapy [40]. The initial discovery of the RANKL/RANK pathway was found to mediate osteoclastogenesis [86] and has been the focus of osteoimmunological development [95,96]. Within the field of osteoimmunology there has been significant research on the immunoregulatory factors in osteoclast differentiation, bone destruction, and cross-talks between autoimmune diseases such as RA [9,44,95,97,98]. Osteoimmunology has shown that the complex control mechanisms between bone and the immune system are intimately linked [8,9]. The interactions between immune and bone cells such as macrophages, T cells, osteoblasts, and osteoclasts are crucial to understanding the wider field of osteoimmunology and how a pathology in one system impacts the other [99]. Understanding how T cells are affected in autoimmunity is imperative to predicting the potential implications on fracture healing.

4.2. *Immune cells and cytokines involved in fracture healing*

The primary difference between endochondral ossification during skeletal development and endochondral healing is the initial inflammatory phase in healing. This phase allows for the recruitment of immune cells and the initiation of a local inflammatory response [12]. Secretion of proinflammatory cytokines from surveillant macrophages and antigen-presenting cells (APC) drives the inflammatory cascade [100]. Other proinflammatory cytokines such as IL-1 and IL-6 work synergistically with the primary inflammatory cytokine TNF-α to encourage inflammation, acute phase response, and B cell proliferation [51,75]. Activated Th1 cells generally act as further stimulators to

secrete IFN-γ, which continues to bind and activate MHC class I and II, promoting cell-mediated immunity [101]. As the fracture response progresses into the 'repair phase,' the activated T cells secrete anti-inflammatory cytokines IL-4, IL-10 and TGF-β to control excessive inflammation by suppressing the release of other cytokines and promoting cell-mediated immunity [51,102]. Cytokines act early in healing to promote activation and recruitment of immune cells, control over-inflammation, and mediate repair [60]. Without the fine balance in inflammatory and anti-inflammatory mediators throughout fracture healing, the process would be dysregulated, and callus formation would be impaired [12].

4.3. Autoimmunity and bone trauma

4.3.1. Autoimmunity and fracture risk

Bone fragility refers to bone that is more vulnerable to fractures [103]. Factors affecting bone fragility can be general or disease-specific. Fragility is usually due to a combination of factors [104]. For example, in RA, an initial genetic risk causes the serological factors, followed by systemic inflammation, which may be treated with glucocorticoids, all contributing in part to an increase in fragility [105]. The mechanisms explaining this increased fragility, particularly in systemic inflammation, have implicated the RANKL/RANK pathway as the cause of dysregulation. Briot *et al.* [105] analysed bone fragility within two autoimmune (RA and SLE) and three chronic inflammatory conditions. By measuring bone mineral density (BMD) it was concluded that inflammatory diseases show alterations in bone remodelling, which increase fragility [106].

Furthermore, using enzyme-linked immunosorbent assay (ELISA), serum concentration showed a higher ratio of RANKL to OPG, suggesting the implication of this system in systemic bone loss [105,107,108]. Significantly, fracture risk was doubled in RA compared to healthy age-matched controls [105]. These findings were first observed by Weiss *et al.* [109] in a case-control study of 53,108 patients with fractures, where it was found the risk of hip and vertebral fractures in rheumatic diseases and inflammatory diseases were significantly increased. Results of the review by Briot *et al.* [105] concluded that disruption of major pathways that affect normal bone remodelling, such as the RANK/RANKL/OPG pathway, are the likely determinants of bone loss and fragility [110,111].

Early observation of human immunodeficiency virus (HIV) patients showing osteoporosis and increased bone fragility characterised by an increase in fracture risk has raised interest in the commonalities between the immunosuppressive infection and a systemic over-activation of the immune system as seen in RA [112]. Although HIV is immunosuppressive, research has shown that HIV causes dysregulation of cytokines involved in fracture healing, particularly TNF-α, similar to autoimmunity, and appears to impair the blood supply of bone [113–116]. TNF-α has since been implicated as a likely cause for increased bone fragility in patients with HIV [117]. This raises the question of whether increased fragility seen in autoimmune patients may also be attributed to dysregulation of proinflammatory cytokines such as TNF-α.

4.3.2. Autoimmunity and fracture healing

4.3.2.1. Cytokines. Cytokines are essential to regular immune function by mediating cell signalling, recruitment, and function. TNF-α is a primary proinflammatory cytokine secreted by macrophages, mast cells, natural killer cells, T cells, B cells, stromal cells, and fibroblasts [51]. As discussed in sections 3 and 4.2, the inflammatory phase of fracture healing is essential for the recruitment of the cells and signals for successful restoration of function and mechanical strength of bone [12]. Proinflammatory cytokines (TNF-α, IFN-γ, and IL-1) are necessary for normal fracture healing [118]. High IL-10, an anti-inflammatory cytokine secreted by B cells to control levels of proinflammatory cytokines IFN-γ and TNF-α, causes a delay in early-stage fracture healing, representing the importance of an initial inflammation by these proinflammatory cytokines [95]. In vitro cell culture studies have shown that TNF-α and IL-1 activate human osteoclastogenesis directly onto osteoclast precursors, independent of the RANKL/RANK signalling pathway [94]. Osteoclastogenesis was not inhibited by OPG, indicating this was independent of the RANKL pathway [94]. This highlighted TNF-α′s role in the recruitment of mesenchymal stem cells for differentiation. Impaired fracture healing has been observed in the absence of TNF-α [119]. Using a TNF-α gene knock-out mouse model, Gerstenfeld *et al.* [75] showed that TNF-α has a critical modulatory role in endochondral bone formation. In the absence of TNF-α, chondrocyte differentiation and endochondral tissue resorption were delayed in the deficient mice compared with wild-type. Under normal fracture healing conditions, following recruitment, the mesenchymal progenitor cells are stimulated to differentiate into chondroblasts rather than osteoblasts by mechanical instability and chemical attractants [68,120]. The stability of the cartilaginous callus stimulates TNF-α to apoptose the chondrocytes within the scaffold to allow for the development of the bony callus [121].

The chronic state of inflammation in autoimmunity creates upregulation of proinflammatory cytokines. Increased cytokines contribute to clinically observed pathogenesis and tissue damage [122]. While the mechanisms are not fully understood, systemically increased levels of TNF-α have also been linked to impaired fracture healing, as seen in HIV [70,94,116]. It is suggested that as well as overstimulating the recruitment of osteoclast precursors, TNF-α activates osteoclastogenesis by promoting the activation of inflammatory cells such as T cells resulting in the secretion of RANKL, which results in activation of the RANKL/-RANK pathway. This leads to an imbalance between osteoblast and osteoclast activity required for fracture healing and remodelling [60,61,66,96].

A computer model created by Zhang *et al.* [66] predicted a 3.2-fold increase in TNF-α levels within the fracture callus in a diabetic (autoimmune) condition relative to non-diabetic control. The effect of this was slower healing, an impaired scaffold for new bone formation, therefore a smaller callus size, and diminished mechanical strength [123]. In a model of T1D, TNF-α′s interactions with bone homeostasis are likely to be synergistic with the RANKL/RANK pathway and in the absence of TNF-α, chondrogenic differentiation is delayed [75]. Results of the studies by Kayal *et al.* [123,124] support that TNF-α mediates chondrocyte apoptosis and controls osteoclast differentiation and recruitment for endochondral tissue remodelling. In vitro, treatments with anti-TNF-α antibodies have decreased osteoclast precursors and improved balance in the basic multicellular unit (BMU) [125]. Fig. 4 demonstrates the effects of TNF-α during fracture healing.

In summary, optimum levels of TNF-α are necessary for normal fracture healing [75,123–125]. Conversely, excessive levels of TNF-α, as in autoimmunity, or insufficient levels could significantly hinder the fracture healing process, particularly in the early stages of fracture healing [66].

4.3.2.2. T cells. Recognition of an antigen (self or non-self) through binding of the TCR stimulates the proliferation of the T cell with the antigen-specific TCR, promoting a targeted (adaptive) immune response [127,128]. Chronic autoimmunity, therefore, significantly increases the repertoire of activated circulating auto-reactive T cells [129]. Upon activation of the TCR, the naïve $CD4^+$ T cell is stimulated to differentiate into different lineages of Th cells. Different subsets secrete varying types of cytokines that have other immune-modulatory functions [129].

The effect from Th cells on osteoclastogenesis depends on the dominant subset [44]. Activated Th1 and Th2 cells secrete IFN-γ and IL-4, inhibiting RANKL, functioning as a negative regulatory mechanism for osteoclastogenesis [130]. In contrast, Th17 cells stimulate osteoclastogenesis by producing IL-17, which promotes the secretion of

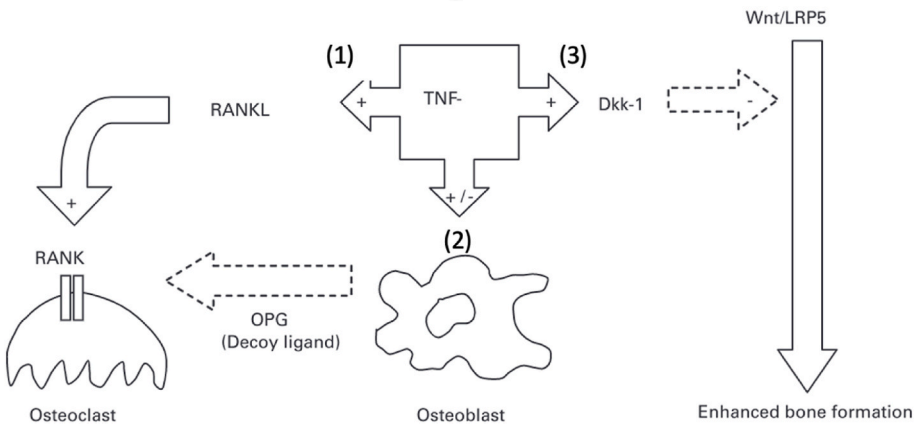

Fig. 4. Figure showing the effects of TNF-α in fracture healing. (1) Activates the release of RANKL, binding to RANK on osteoclasts, stimulating osteoclastogenesis, (2) depending on the phase of fracture healing, inhibits (early), or activates (later) osteoblasts to create new bone and secrete OPG, binding and inhibiting RANK on osteoclasts, (3) induces Dkk-1, a negative regulatory molecule of the Wnt pathway, inhibiting osteoblastogenesis [126]. Image adapted from Richardson *et al.* ([116], p.989).

RANKL from mesenchymal cells and further activates the differentiation of the Th17 subset [131]. Recently the Th17 subset has been the primary T lymphocyte implicated in autoimmune inflammation [132,133]. Research into the functions of Th17 and IL-17 on osteoclastogenesis has focused on RA due to its close connection to osteology and immunology. Increased osteoclast activity in RA is central to structural joint damage [40]. This increase in the bone breakdown is directly linked to bone fragility and fracture risk in RA [105]. It is thought that IL-17 induces the secretion of RANKL from the inflamed synovial fibroblasts within the joints, simultaneously stimulating synovial macrophages to secrete proinflammatory cytokines TNF-α and IL-1 [134]. These cytokines enhance osteoclastogenesis by further upregulating RANKL expression and activating osteoclast precursors [116] (Fig. 4). B cells releasing autoantibodies cause immune cells to secrete further cytokines and directly onto osteoclasts mediating bone resorption [10,42]. RA pathogenesis highlights the close inter-relationship between levels of cytokines and T cells (Fig. 5).

Building on the research from Arron and Choi [6], Takayanagi *et al.* [44] discovered that activated T cells, as well as activating, inhibited the RANKL-induced maturation and activation of osteoclasts by secreting another proinflammatory cytokine: interferon-gamma (IFN-γ). Knock-out mice for the gene encoding IFN-γ showed increased bone destruction, supporting that IFN-γ is essential for maintaining bone integrity through a T cell-mediated progress [7,44]. Furthermore, IFN-γ was found to block RANKL-induced osteoclast differentiation in vitro, likely by degrading TRAF6, a transcription factor in the molecular cascade initiating osteoclastogenesis [44]. Takayanagi *et al.* [44] concluded that IFN-γ prevents uncontrolled bone loss during inflammatory T cell responses.

Secreted from the activated Th1 subset, IFN-γ increases the antigen presentation function by upregulating MHC class II on APC and bone marrow cells, increasing the activation of T lymphocytes, which produce RANKL, TNF-α, and further IFN-γ [135]. Gao *et al.* [135] isolated macrophages and cultured them with RANKL and IFN-γ in a dose-dependent manner, showing that IFN-γ dose-dependently inhibited osteoclastogenesis from macrophages. To show that T cells were a major contributor to cytokines, T cells were purified and analysed using real-time PCR, with results showing that pre-treated APC with IFN-γ significantly increased the T cell expression of RANKL, TNF-α, and further IFN-γ compared to non-treated APCs [136,137]. These results suggested that IFN-γ inhibits osteoclastogenesis directly [7] but indirectly activates osteoclastogenesis. Gao *et al.* [135] stated the net result was bone resorption, concluding this was a complex system and likely dependent on multiple factors such as the specific conditions of the micro-environment and concentrations of other cytokines. Based on the current research, Fig. 6 hypothesises the mechanism of the net increase in osteoclastogenesis mediated by IFN-γ.

4.4. Impaired fracture healing: proposed hypotheses

Chronic inflammation in autoimmune disease has been shown herein to impact the balance of bone formation and bone resorption by

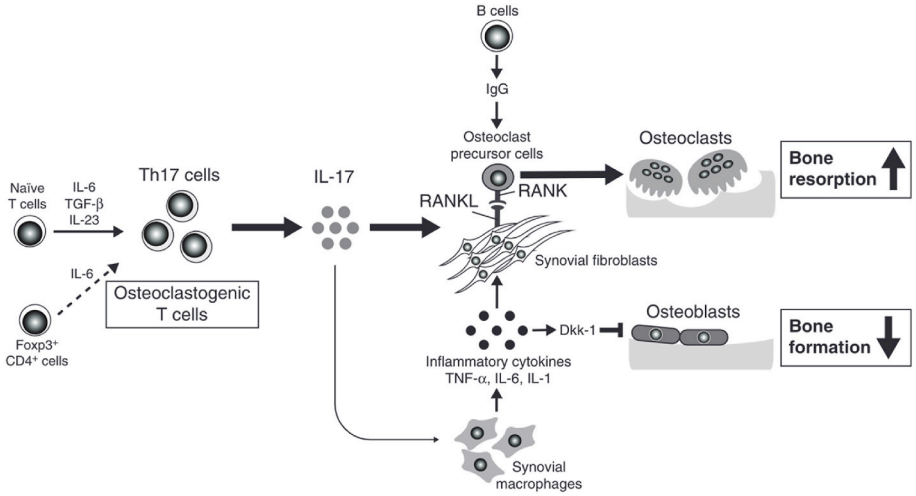

Fig. 5. Th17 mediated mechanism of bone destruction in RA. RANKL/RANK pathway mediated bone resorption. Inflammatory cytokines activate Dkk-1, inhibiting bone formation. Image taken from Okamoto and Takayanagi ([9], p.14).

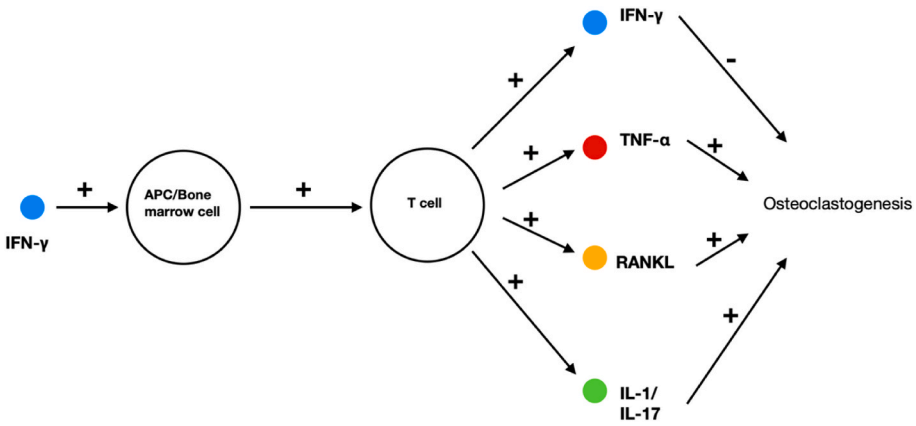

Fig. 6. Diagram showing the hypothetical mechanisms of osteoclastogenesis. Inhibitory IFN-γ was found to show a net increase in osteoclastogenesis. Hypothetically, one IFN-γ molecule stimulates a T cell to release one of each IFN-γ, TNF-α, RANKL, and further proinflammatory cytokines. This results in ~75% of the released molecules (TNF-α, RANKL, and IL-1/IL-17) activating osteoclastogenesis, and 25% inhibiting (IFN-γ). Diagram by Author.

osteoblasts and osteoclasts, with a tendency to increase osteoclastic resorption. This has implications for systemic bone fragility and fracture healing but how autoimmunity causes these implications has not been addressed in the literature. The following section proposes three likely pathways for how the immune systems alterations during autoimmunity likely affect fracture healing.

4.4.1. *The positive feedback loop of cytokines and T cells*

Due to self-reactivity in autoimmune disease, the increased levels of circulating cytokines and T cells promote chronic inflammation [97,138,139]. It has been shown that TNF-α at the correct concentration is imperative to fracture healing [66,75,125]. TNF-α, along with other proinflammatory cytokines, such as IL-1, work synergistically to 1) directly promote the recruitment and differentiation of mesenchymal stem cells into osteoclasts, 2) stimulate chondrocyte apoptosis to allow for bony callus formation, and 3) continue to stimulate inflammation by activating immune cells such as T cells secreting RANKL (Fig. 4) [66,68,124]. However, both over-activation and suppression result in impaired healing [66]. Increased cytokines create a positive feedback loop to increase T cells, further exacerbating cytokine levels [98]. The upregulation of circulating T cells, particularly Th17 in autoimmunity, continue to release proinflammatory cytokines and RANKL, enhancing the maturation and activity of osteoclasts [44,91,140]. Although T cells have a protective mechanism for over secretion of RANKL through upregulation of IFN-γ, this protective balance between IFN-γ and RANKL is lost in chronic inflammation [44]. Under inflammatory conditions, RANKL predominates, and IFN-γ loses its protective mechanism and instead has an indirect effect of further activating osteoclastogenesis (Fig. 6) [135].

Hypothesis 1. The positive feedback loop stimulating T cells to further increase cytokine levels increases osteoclastogenesis and affects fracture healing by altering the balance of the basic multicellular unit (BMU).

Whether increased osteoclastogenesis is stimulated via activation of the RANKL/RANK pathway or directly onto precursor cells, the increase in osteoclastic activity, and the inhibition of osteoblastic activity, through activation of DKK (Fig. 4) results in an imbalance in the BMU. The BMU relies on a carefully controlled balance of osteoclasts, osteoblasts, and capillary blood supply to maintain bone remodelling during fracture healing and normal homeostasis. Imbalance in the BMU causes an increase in porosity and decreases systemic bone mineral density [103,141–143]. These alterations increase fragility and bone fracture risk, and when occurring during autoimmunity, this is combined with further increased osteoclastogenesis and decreased osteoblastic activity. OPG secreted from osteoblasts is ineffective for inhibiting osteoclastogenesis via the RANKL independent pathway [94], and prolonged systemically high levels of proinflammatory cytokines maintain the production of RANKL from activated T cells, overwhelming the OPG suppression [142]. Premature removal of cartilage through chondrocyte apoptosis [124,126], lack of new bone formation [126], and local tissue inflammation [127] are likely to result in defective fracture healing, particularly in forming and maintaining the soft and bony callus [70].

4.4.2. *Alterations in blood supply*

Autoimmune diseases are associated with an increased risk of alterations in cardiovascular function, including hypertension and cardiovascular diseases [144]. These decrease the effectiveness of general vascular function and blood perforation, as arteries lose elasticity due to increased blood pressure [145]. As discussed in section 3, angiogenic factors are a significant part of fracture healing at the inflammatory and repair phases. Restoration of blood flow is essential for hematoma and granuloma formation [70]. Autoantibodies against angiogenic factors are upregulated in some autoimmune diseases (SLE and autoimmune pre-eclampsia), inhibiting the production of new blood vessels [146,147].

Hypothesis 2. Alterations in angiogenesis and blood flow would indirectly inhibit fracture healing by decreasing the ability of immune cells to infiltrate into the fracture hematoma in the early stage of the inflammatory phase.

Granuloma formation requires both angiogenesis and osteogenesis for the initial stabilisation of the fracture, forming the callus template [59]. Impairment of this could result in non-union or incorrect callus formation [148]. Furthermore, during the development of the cartilaginous callus, the outgrowth of new blood vessels could be impaired by autoantibodies to angiogenic factors. Impaired angiogenesis could encourage premature apoptosis of cells within the cartilaginous callus due to lack of blood supply and may result in instability, re-fracture, or non-union.

4.4.3. *Receptor desensitisation*

Receptor desensitisation of TNF-α was briefly mentioned by Richardson *et al.* [116] when discussing fracture healing impairment in HIV-positive patients. Receptor desensitisation is a decreased receptor responsiveness occurring when there is repeated or chronic exposure to an agonist [149]. The possibility of receptor desensitisation in autoimmunity hasn't been researched since; however, due to the chronic upregulation of TNF-α in autoimmunity, this idea warrants further exploration. TNF-α signals primarily through TNFR1, which is expressed on most cell types, and TNFR2, which is restricted to endothelial and immune cells [150,151].

Hypothesis 3. An increase in baseline TNF-α levels could lead to receptor desensitisation, preventing or decelerating the effectiveness of the cytokine during the inflammatory phase of healing. The result is likely similar to what Ono and Takayanagi [95] observed when high levels of anti-inflammatory cytokines impaired fracture healing by suppressing TNF-α.

The effect of autoimmunity on fracture healing is likely to be multifactorial. The immune system has its biggest effects early on in the inflammatory phase of fracture healing. Increasing porosity and decreasing systemic bone mineral density by the positive feedback loop of cytokines and T cells; decreasing the ability for immune cell infiltration and granuloma formation by alterations in blood supply; and TNF-a receptor desensitisation all create an increased initial risk of fracture, re-fracture, non-union and instability. These would impact forensic anthropological analysis of the post-traumatic time interval (PTI) since injury.

5. Implications for analysing skeletal trauma in forensic anthropology

This section considers the relevance of fracture healing rate and the impact of clinical conditions on the estimation of the post-traumatic time interval in the context of skeletal pathology and trauma analysis in forensic anthropology.

5.1. Fractures and forensic anthropology

In addition to the biological profile, a forensic anthropologist may also use their knowledge of pathology and biomechanics to identify diseases, congenital abnormalities, or past trauma present on the bone [152]. These can be uniquely identified and matched to personal antemortem records for identification or elimination [14].

5.1.1. Analysis of skeletal pathology

Correct analysis of bone pathology to identify autoimmune diseases requires extensive knowledge of bone biology and response to different pathological processes. Skeletal lesions are observed by response type (osteoblastic, osteoclastic or mixed), size, shape, and distribution [153]. Within each of these observations are subclassifications; some are disease-specific and may aid in a diagnosis. This analysis process is called 'differential diagnosis' [154]. However, because the bone is only able to respond with either an increase (osteoblastic lesion) or a decrease in bone production (osteoclastic lesion), differentiating between diseases presenting in bone can be difficult [155]. Autoimmunity, for example, except for RA, has not been shown to present distinctively in skeletal remains; as such, identifying autoimmunity with certainty is not possible without clinical history [156]. As a result, matching a patient who may have been significantly clinically affected by autoimmunity to a non-pathologically presenting set of skeletal remains can be challenging, and identification is likely to be done through other forensic identification means such as odontology or DNA [157].

Although having different pathogeneses, RA and osteoarthritis (OA) are both defined as arthropathies due to their pathological changes to joints [158]. While clinically, these are very different, with OA resulting from joint degeneration or injury and RA being a result of autoimmune disease, the bone response is very similar [159]. Differential diagnosis between RA and OA relies primarily upon the analysis of lesion distribution throughout the skeleton and presence or absence of eburnation which is pathognomonic of OA and absent in RA [159–162].

Skeletal fractures can manifest directly due to trauma or indirectly as comorbidities. Pathological fractures are often comorbid with metabolic imbalances (e.g. rickets), infection (e.g. tuberculosis), and genetic disorders (e.g. osteogenesis imperfecta) [163–166]. However, they are likely to heal through primary means due to the location and cause of these pathological fractures [59]. Due to this, the effect of autoimmunity on fractures will focus on those manifested through trauma and endochondral healing.

While disease pathology requires a complex knowledge of bone response, the analysis of trauma requires recognition and distinction between human variation, such as congenital abnormalities, for example, spinal curvatures in scoliosis [167], pathological fractures as discussed above, and direct trauma [15]. In the case of skeletal trauma, knowledge of the biomechanical response of force on bone is used to give details on how the trauma may have occurred [168]. Skeletal trauma is either identified as antemortem, occurring before death, or perimortem, occurring around the time of death [169]. Postmortem damage is classified as damage occurring after death, usually marked by a colour difference and lack of remodelling across the broken surface; however, it is dependent on the time since death and is typically only seen post-deposition [169]. Correct classification of the timing of the injury is pertinent to the analysis and imperative for forensic investigation in both living and deceased victims [170].

Due to bone healing being more relevant in fractures than in dislocations, these will be discussed below. Fractures are subclassified based on the force applied to the bone: tension (stretching), compression, torsion (twisting), flexion (bending), and shearing [168]. Most injuries are a combination of these types of fractures, and analysis can give information on the direction and method of the applied force [171].

5.1.2. Timing of injury and fracture healing

Fracture healing and its process is an integral part of skeletal trauma analysis in forensic anthropology [172]. The presence of healing (i.e., callus formation) at either a micro- or macroscopic level is used to indicate antemortem skeletal trauma [173,174]. Antemortem trauma is significant in the analysis of skeletal remains to eliminate the trauma from being part of the cause of death, to discern whether prosecution ensues, and to aid in identification when comparing ante- and post-mortem radiographs [175,176].

While the terms antemortem, perimortem, and postmortem are of general use, limitations arise when determining the post-traumatic interval. The post-traumatic interval (PTI) is the time frame between the trauma or fracture and the time of death [177]. Perimortem trauma is identified by the absence of healing whereas, antemortem lesions are typically classified as either 'healing' or 'healed' [178]. More precise information on the timing would aid forensic anthropologists in assessing the medical status and care at the time of death and may provide information on abuse and torture [174].

Microscopic evidence of healing can be analysed in the early stages of inflammation, within the first 48 hours showing reactivity around the lesion margins [174]. Under normal healing response, after 10–14 days, the fracture will show macroscopic signs of healing, such as the beginning of ossification of the cartilaginous callus [81,174,179]. Radiographs give macroscopic details of the fractures, which can be used to estimate the timing since the injury [180], making the analysis of antemortem trauma and the healing process also significant in the living.

De Boer *et al.* [174] explored a fracture dating system on human dry bones based on traditional forensic pathology methods. Features were assessed to see which were consistently detectable and applied to specific time frames. The study included inter-observer analysis and compiled a table of healing features that exhibited high agreement (Table 1). Although de Boer *et al.* [174] have demonstrated it is possible to estimate the PTI from the analysis of fracture healing, conclusions acknowledged that there must be adjustments for the time intervals of children (ages not specified), as it is well researched that they heal faster [181,182].

Observing the healing rate is particularly important in forensic anthropology when analysing the skeletal remains of infants and children in abuse cases [183,184]. Abuse is often identified by the presence of multiple fractures at different healing stages, with healing often disrupted by repeated incidents [185]. However, the fast-healing rate of

Table 1
Figure showing consistently (those with high inter-observer agreement) detected healing features in human dry bone. Adapted from de Boer *et al.* ([174]; p.101).

Healing feature	Time interval
• Frayed bone lamellae at the lesion margins	Before 48 h
• First Howship's lacunae at the lesion margins	After 4–7 days
• Smoothening of the lesions margins	After 4–7 days
• Start of periosteal callus formation, distant from the lesion margins, separable from the cortex.	After 7 days
• Endosteal callus formation clearly visible	After 10–12 days
• Osteoporotic appearance of the cortex	After 12 days
• Start of the transition of primary woven bone into secondary lamellar bone	After 14 days
• Cortical cutting and closing cones orientated towards the lesion	After 12–21 days
• Clearly visible periosteally situated callus	After 15 days
• Endosteal callus becomes indistinguishable from the cancellous bone in the marrow cavity	After 17 days
• Periosteal callus becomes firmly attached (inseparable) to the cortex	After 6 weeks
Features specific for fractures	
• Union by bridging of the cortical bone discontinuity o By primary woven bone o By secondary lamellar bone	After 21–28 days
• Smoothening of the callus outline	After 2–3 months
• After adequate immobilisation: quiescent appearance indicating subsided healing.	After 1–2 years

children and infants and poor preservation of skeletal material make PTI estimation difficult in both the living and deceased [176,186]. This increased healing is thought to be due to a more extensive fracture hematoma and thicker subperiosteum, contributing to rapid callus formation in children [183]. The growth environment within children's skeletal systems provides an osteogenic environment which is then already present at the time of fracture, decreasing the time needed for cellular recruitment [187]. Further detail on the healing of subadult fractures is beyond the scope of this review. However, this highlights the possibility that other factors influence healing significantly enough to warrant adjustment.

5.2. Implications of autoimmunity on the analysis of fractures in forensic anthropology

5.2.1. Implication of healing rate

Implications of antemortem analysis include local or systemic factors impacting the healing rate. Local factors include the location of the fracture, the type of bone impaired, and the mobility at the fracture site [169]. These, along with the degree of damage measured by the separation of bone ends, may also interfere with the revascularisation of the fracture site and, therefore, impair fracture healing rates [188]. Systemic factors include those which indirectly influence the healing process by affecting the body's overall health. Comorbidities such as diabetes mellitus [189], anaemia [190], malnutrition [191], vascular diseases [188], hypothyroidism [192] and infection [193] are among some of the pathologies that have been shown to directly impact on the fracture healing process by impairing blood flow, cell recruitment, inflammation, and cell health. Prescription drugs to treat these comorbidities, such as non-steroidal anti-inflammatories, corticosteroids, and statins, as well as recreational non-prescription drugs such as alcohol and smoking [194], have also been implicated as inhibitors of the fracture healing process [195].

6. Discussion

6.1. Current research: implication of autoimmunity on the rate of healing

The complexity of autoimmune diseases means there are still many unanswered questions despite decades of research, and disease interactions are still to be discovered. Research into how bone and the immune system interact has been central to research into RA pathogenesis [161], as well as decreased bone mineral density and increased fracture risk studies in type 1 diabetes [196]. In addition, contemporary research is increasingly focussing on whether other autoimmune diseases have skeletal implications that may not present with the same severity [61,105]. This review postulated how autoimmune disease may affect the bone fracture healing rate and the implications on skeletal analysis in forensic anthropology.

Cells involved in fracture healing have been studied extensively in animal models, particularly murine models. Knock-out gene or transgenic mouse models, antiserums, and viral vector overexpression are all ways of studying small mammals' biological responses to predict how these changes occur in humans [197–199]. Mouse models are the most commonly used to study human fracture healing and autoimmune diseases due to their low cost and availability for specific genotypes and phenotypes, allowing researchers to study cells with specific characteristics [198,200]. However, murine bones lack Haversian systems, likely affecting the pathophysiological response to bone fracture healing [201]. Due to biological differences such as these, research obtained from animal models has occasionally failed to translate into clinical studies, limiting the potential to represent the complexity of human disease modelling [202]. Nevertheless, animal models, including mice, are likely good models for cellular interaction during fracture healing. Gross morphological analysis is best researched through human radiographic studies and magnetic resonance imaging (MRI) to account for structural and mechanical variations between animals and humans and are preferred due to the lack of ionising radiation [69]. Furthermore, penetrating ionising radiation from x-ray for non-diagnostic or treatment purposes does not comply with the current mainstream medical ethics due to possible long-term health implications [203].

Early case reports showed delayed union and increased healing time in diabetic patients compared with matched controls [204,205]. As autoimmunity becomes more researched in the 21st century, analysis has shifted to investigating the cause of these observed impairments [206]. To research the rate of fracture healing in autoimmunity, models have been used to simulate the situation of a fracture in an autoimmune patient and observe the biological response. These models allow for direct comparison of a healthy fracture model with an autoimmune model. Research obtained from this could then be applied to analysing existing fractures in autoimmune patients. Li *et al.* [93] used a mouse model of systemic inflammation by injecting TNF-α into circulation to simulate an autoimmune condition. Cell assays were used to quantify and characterise osteoclast-precursors in the spleen and bone marrow and a TNF-α antagonist to observe if this conferred protection. Results found that increased levels of TNF-α increased osteoclast precursors compared to wild-type, an action protected by a TNF-α antagonist [93]. These results suggested that osteoclast activity would increase in systemic inflammation, as in autoimmunity, which likely caused the decreased bone mineral density and impaired fracture healing observed in early clinical cases. This founding study showed the possibility of a clinically quantifiable test to evaluate a patient's potential for erosive diseases such as RA and the efficacy of anti-TNF therapy [93]. However, as this is a simulation of autoimmunity using TNF-α, it cannot be directly linked to autoimmunity pathogenesis; this prompted the development of a diabetic model for research.

Using genetic research methods to investigate if osteoclastic activity and TNF-α proteins are also increased in a diabetes model, Kayal *et al.* [123] and Kayal *et al.* [124] analysed premature chondrocyte apoptosis and cartilage resorption specifically in a diabetic mouse model of

fracture healing. The initial study analysed pro-apoptotic RNA and TNF-α proteins within the fracture callus at 12, 16, and 22 days post-trauma to determine if diabetes caused premature chondrocyte apoptosis through TNF-α mediated upregulation of pro-apoptotic genes [123]. Day 16 and 22 fracture callus showed reduced levels of cartilage compared to controls. Biomedical genetic research using animal analogues has shown to be very effective when researching human clinical studies, particularly with genetic research [207–209]; for example, the development of insulin for people with diabetes [210]. However, as mentioned, murine bone structure varies significantly from that of humans. Therefore, histomorphometric analysis of fracture healing in Kayal *et al.* [124] is not directly translatable to human fracture healing. Nonetheless, it provides a preliminary observation and possible mechanism of impairment in humans. Future development of this research could use fine needle aspirations of the fracture callus in human patients as a clinical study to analyse the same features. Fine needle aspiration is already commonly used for bone and tissue biopsy as it is cost-effective and minimally-invasive [211]. A meta-analysis of diagnostic literature by Chambers *et al.* [212] supported the expansion of fine needle aspiration as a diagnostic tool for bone and soft tissue lesions. Research of this type would also aid in understanding a murine model's validity when researching fracture healing.

Currently, studies on autoimmunity's effect on fracture healing are primarily limited to animal models of systemic inflammation and diabetes [124,205,213]. This is likely due to the complexity in attempting to simulate a specific autoimmune condition in an animal analogue and ethical issues in simulating fractures in humans with specific autoimmune diseases. The pathogenesis of most autoimmune diseases is still not fully understood, making simulation difficult. Type 1 diabetes is a well-researched and understood autoimmune disease [156]. Although similar impairments are seen in the systemic inflammation models as with the diabetic models, applying these results to autoimmunity must be done with caution. The lack of research into skeletal morphological impairments in autoimmune states proves it is not well understood. Gene studies have shown that there is an impairment in the fracture healing process, likely mediated by systemic inflammation [123,124]; however, the effect of this in the analysis of dry skeletal remains is yet to be determined.

6.2. Current research: known time frames of fracture healing

There is a large amount of research spanning many disciplines on the histology and radiology of the fracture healing process [185,214–216]. As this research is primarily from a medical and clinical standpoint, the literature tends to focus on aspects that delay the natural healing process, e.g., malnutrition, vascular disease, and hormone imbalances [188,189,191,192]. Despite the importance of the topic in forensic anthropology, there is a lack of standardised definitions of fracture healing times in the analysis of dry skeletal remains leads to highly subjective fracture age estimations dependent upon observer experience and understanding of histological and radiographic features of healing bone [176]. Timelines of fracture healing are challenging to research due to controlled scientific studies on human subjects being unethical, therefore limitations in fracture ageing research are significant. While confounding variables such as age, sex and fracture location have been investigated, further studies such as the current review are required to explore more confounding variables to refine present knowledge [16]. Finding a robust set of fracture specimens with date-of-injury and contextual information is necessary as small datasets are likely to confound results.

Research around the gross and radiological appearance of fractures at different stages of healing and time correlations has not been well researched, particularly in adult populations with most of the research centred on paediatric research [16,180]. However, Maat [217] was the first to publish a compilation of 13 research articles published prior to the year 2000 on the timing of the natural healing process as observed in skeletal remains. However, these articles' accuracy or testing methods are not included within Maat [217]. Presented as a case study from the Serbian-Albanian hostilities in Kosovo (1998–1999), Maat [217] analysed a possible case of torture by the intentional infliction of bone fractures prior to execution. Through histology and gross anatomical analysis looking at bone tissue unrest, activity, and remodelling, the study concluded the presence of antemortem fractures inflicted approximately 2–3 weeks before death. While a limitation of this study is that the true timing of the antemortem fractures cannot be known, the research aimed to predict and substantiate, by scientific means, the minimum period of time that must have passed since the injuries were inflicted. In this case, the presence of multiple healing fractures, at different stages, distributed throughout the body, and the recovery context were significant enough to prove torture before death. However, a significant limitation is the lack of clinical medical history on any of the deceased. In addition, underlying malnutrition or other diseases may have significantly impacted the outcome analysis. However, these are likely to have delayed healing of the fractures, and Maat [217] aimed to establish the 'minimum period of time' before death that these fractures occurred. As a result, despite the impact of clinical history on healing, the conclusion of 2–3 weeks before death is likely to be the same; however, the possible impact was not acknowledged in the publication. Maat [217] did acknowledge that these timings are likely to be used in the future as forensic standards, and "timings should be substantially reduced" ([217]; p. 245) when analysing subadult skeletal remains.

Following Maat [217], de Boer *et al.* [174] expanded on this research to include both human dry bone fractures and amputations in various stages of healing. The study aimed to establish the extent to which histomorphological features can indicate specific time intervals. This study compiled fracture healing timings studied by Maat [217] and Barber [218] to observe 22 fracture specimens and nine amputation specimens. Unlike Maat [217], the sample used for de Boer *et al.* [174] included a wide demographic spread (South Africa, Netherlands, and Norway) and three intentionally sawn and broken control specimens. Interobserver studies were conducted using a questionnaire. Researchers were analysing the presence or absence of features on histological and gross anatomical specimens against standard terminology from the compilation of research (see Table 1) [174]. To prevent bias, samples were anonymised by random numbering.

While de Boer *et al.* [174] is a good modern rendition of the timings of fracture healing, the accuracy is again unknown as the results are based on interobserver agreement and consistency of traits, appearance timings of which are compared against the compiled research. Due to this, there may be carry-over errors across the literature, as the true accuracy has not been researched. Similar to Maat [217], de Boer *et al.* [174] did not have clinical history on the samples. This is a more significant limitation in this study as it is not a case study where 'minimum' time was sufficient. Here, the PTI is likely to be underestimated by all observers, creating a systemic observer error and false security in the results.

Isaac *et al.* [176] have recently addressed the major gap in the research on healing rates of skeletal injuries. Isaac *et al.* [176] created the Repository of Antemortem Injury Response (REPAIR) archived by the National Archive of Criminal Justice Data to counter fracture research's practical and ethical limitations. REPAIR is an online database (https://repair.orainc.com) accessible to forensic practitioners of cases of skeletal injuries with known PTI, histology, radiography, and gross photography. Currently, the database comprises 187 cranial fracture samples from 86 individuals obtained during postmortem examination ageing between infant (0–3 years) and adult (16+ years). If known, case information includes decedent demographics, date, time of death, medical conditions, and medications.

The most significant limitation of REPAIR is the presence of only cranial fractures within the database. Cranial fractures do not heal with soft or bony callus formation because there are no weight-bearing or supportive demands [177]. Expansion of the database to include

postcranial elements has been proposed [176]. The database allows for the effects of age and other biological and medical variables to be analysed. As the database grows, there may be opportunities to assess the differences between those samples with autoimmunity, as recorded in their medical history, and those samples without.

The topic of natural fracture healing rates lacks original research and is made up primarily of review-based methods, drawing on data from multiple publications rather than primary analysis-based research. Due to the published research in the specific fracture healing rates being limited, it is highlighted that this field requires more research. In addition, many studies have shown there is an impairment in the fracture healing rate, however, have not applied this impairment back into the time frames and how they would be affected [93,112,123,124]. New research is beginning to address the possibility of other confounding variables such as age, sex and location of the fracture however "further studies are needed to explore more confounding variables to refine the present outcomes ([16]; p.1.)"

6.3. *Future advances*

While this review has shown an impairment during fracture healing under autoimmune conditions, it is not understood how or if autoimmunity shows a clinically significant effect on fracture healing rate. For this reason, it is important to research this. Within the field of forensic anthropology, it is imperative to assess if fractures are antemortem or perimortem, and the practice is also developing to attempt to provide PTI for healing antemortem fractures [169,174,180]. This is important for the corroboration or contradiction of victims or suspect statements in domestic abuse or torture cases and asylum seekers claiming abuse however, also in deceased victims [16,219]. In a forensic setting, the clinical history of the deceased is often not known prior to identification. However, if the clinical history could be obtained following the identification, a correction (if necessary) could be used to provide a more accurate and precise estimation of the fracture PTI based on the presence or absence of autoimmunity. Therefore, a comprehensive understanding of the implications in chronic clinical conditions such as autoimmunity is imperative to conducting accurate and precise skeletal analysis in forensic anthropology.

In order to fill the research gap, future studies should aim to answer the following research questions:

1) What is the true natural healing rate of bone?
2) What is the effect of autoimmunity on the rate of fracture healing?
3) Is the impact on fracture healing rates the same across different autoimmune diseases (i.e., RA, SLE, MS, and Graves' disease)?

Animal and cell studies have shown the impairment observed during fracture healing in autoimmune models is both at the cellular and molecular level [66,124,220], but limitations arise when using animal models for human physiological and anatomical changes [221]. To address questions 1–3 specifically to human physiology and anatomy, databases such as REPAIR are recommended for future research and are plausible from both a practical and ethical standpoint. When histological and gross morphological information is collected from postmortem examinations and clinical history is obtained, the data will contain the true PTI of the fracture. These can be used to accurately establish the natural healing rate, allowing the creation of a standard, based on original observation. The same can be done by sorting the data by autoimmune disease to assess the healing rate under autoimmune conditions and observing if there is variation in the rate among different types of autoimmune diseases.

4) If found to be necessary, what is the correction factor required to be used on the natural healing rate to account for the impairment seen in autoimmunity?

Based on the data collected from the natural and autoimmune healing rates, if "healing" was able to be translated into a numerical value for quantitative analysis (e.g., certain observations account for a percentage of overall healing, similar to assigning 'phase' using descriptions by Brooks and Suchey, [222] for age estimation) then "percentage healed" could be plotted over "time (likely in weeks)" on a graph. Fracture healing is non-linear, rather progressing in a sigmoidal manner [223–226].

Hypothesising that the plotted data collected from the database would produce a sigmoidal model, a line of best fit could be fitted using non-linear regression, as seen in Fig. 7. Transforming the data using the Lineweaver-Burk formulation would allow the sigmoidal graph to be transformed into a linear model, where linear regression analysis could be used to compare the gradients between natural and autoimmune healing rates to confirm or reject the null hypothesis. If a significant result is found, a correction factor could be determined to analyse the healing rate in an autoimmune individual [227].

The model would aim to show if there is a significant difference between the two healing states. If the null hypothesis was rejected and there was a significant difference between the two healing rates, PTI could not be reliably estimated unless clinical history is known. On the contrary, this model would be a useful resource when the clinical history of the remains is known. Furthermore, by analysing the remains for 'percentage healed', the error rates of the model would provide a time frame, the PTI (see Fig. 7*).

7. Conclusion

Although attempting to measure and create standards for the many influences on healing rate may seem an overwhelming prospect, it is important to understand how broad concepts that may govern many of these may affect fracture healing rates. For example, understanding the impact of chronic systemic inflammation on healing will provide information on how diabetes mellitus and other autoimmune diseases, infections, and anti-inflammatory medications may influence the healing rate [61,105]. In addition, accurate PTI dating may aid in the identification process, particularly in mass disaster situations, where identifying features such as the presence of antemortem fractures that can be linked back to medical records may be imperative for identification.

This paper aimed to synthesise the literature on the immune system, bone biology, and osteoimmunology to assess interactions that may affect fracture healing in an individual with an autoimmune disease. A model with three hypotheses was presented that are likely part of a multifactorial impact of autoimmunity on fracture healing: 1) the positive feedback loop of cytokines and T cells; 2) alterations in blood

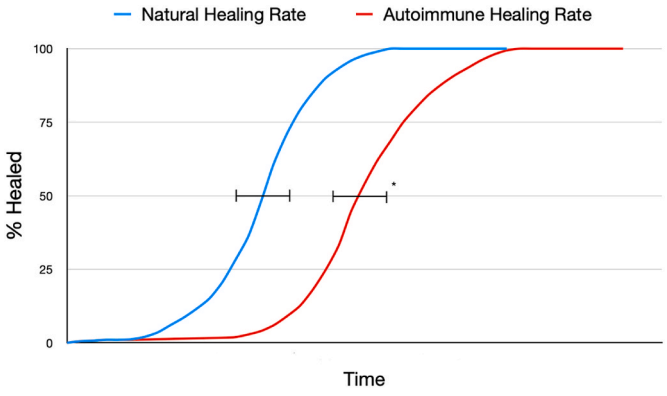

Fig. 7. Proposed graph for investigating the relationship between natural and autoimmune fracture healing rates. * Represent an example of the error bars that would show the 95% confidence interval at 50% observed healing. These would correspond to a time range (PTI) Image by Author.

supply; and 3) receptor desensitisation. Building on the first, the second aim addressed and discussed the practical application of fracture healing in forensic anthropology and how the new information regarding fracture healing under autoimmune conditions is likely to impact this. It was found that research into determining the natural healing rate is dated and that there is no correction for clinical conditions when estimating post-traumatic time-interval from fractures. However, our review of the interaction between the immune and skeletal systems from an osteoimmunological perspective has shown the fracture healing rate is likely impaired in patients with autoimmune diseases. While autoimmunity may not always be a presenting condition in skeletal remains, the impact of how these non-presenting conditions affect skeletal trauma such as fractures is not well researched and easily overlooked. With forensic anthropological methods developing to estimate the post-traumatic time interval more accurately, it is vital to understand how clinical conditions may impact this analysis. This review has shown that direction for future advances in research should aim to analyse the extent autoimmunity has on fracture healing to determine if a correction factor is required in the analysis of skeletal remains when clinical history can be obtained.

CRediT authorship contribution statement

Stephie R. Lončar: Conceptualization, Methodology, Investigation, Writing – original draft, Writing – review & editing, Visualization. **Siân E. Halcrow:** Verification, Writing – review & editing, Funding acquisition. **Diana Swales:** Verification, Supervision, Writing – review & editing.

Declaration of competing interest

The authors have no competing interests to declare.

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