IDO activation, inflammation and musculoskeletal disease

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Abstract
The IDO/kynurenine pathway is now established as a major regulator of immune system function. The initial enzyme, indoleamine 2,3-dioxygenase (IDO1) is induced by IFNγ, while tryptophan-2,3-dioxygenase (TDO) is induced by corticosteroids. The pathway is therefore positioned to mediate the effects of systemic inflammation or stress-induced steroids on tissue function and its expression increases with age. Disorders of the musculoskeletal system are a common feature of aging and many of these conditions are characterized by an inflammatory state. In inflammatory arthritis and related disorders, kynurenine protects against the development of experimental arthritis, while inhibition or deletion of IDO1 increases its severity. The long-term regulation of autoimmune disorders may be influenced by the epigenetic modulation of kynurenine pathway genes, with recent data suggesting that methylation of IDO may be involved. Osteoporosis is also associated with abnormalities of the kynurenine pathway, reflected in an inversion of the ratio between blood levels of the metabolites anthranilic acid and 3-hydroxy-anthranilic acid. This review discusses evidence to date on the role of the IDO/kynurenine pathway and the highly prevalent age-related disorders of osteoporosis and rheumatoid arthritis and identifies key areas that require further research.

1. Introduction
The kynurenine pathway (Fig. 1) has long been recognised as the endogenous synthetic route for the essential enzyme cofactor nicotinamide adenine dinucleotide (NAD), but its wider importance in cell biology was only recognised more recently. In the immune system, the key observation was that indoleamine-2,3-dioxygenase (IDO) was activated by interferon-γ [1], while in the same year it was discovered that compounds along the kynurenine pathway had unrelated, highly specific molecular targets and biological roles in the nervous system [2-5]. Appreciation of the importance of the kynurenine pathway in the immune system developed rapidly with the report of a major role for IDO in placental immunity and fetal protection [6,7]. In both these areas, immune and neural, it is now recognized that the kynurenine pathway is fundamentally involved in several central and peripheral disorders. In this review the initial emphasis is devoted to peripheral disorders such as arthritis, atherosclerosis and parasitic infection. A later section then analyses important links between peripheral and central inflammation. A later section then analyses important links between peripheral and central inflammation since the kynurenine pathway has been implicated in disorders such as Huntington's disease, schizophrenia and depression [8,9].
The interactions discussed are likely to be relevant to an understanding of physiology and disease pathogenesis, with the possibility that they may be common targets for potential therapeutic intervention in some central and peripheral disorders.

2. Rheumatoid arthritis
In rheumatoid arthritis (RA), localised joint and cartilage damage occurs, with immune cell infiltration and pannus formation resulting in a constriction of the synovial fluid volume. The leucocyte infiltration and activation results in increased production of cytokines which, especially when maintained chronically, are responsible for further tissue damage. Positive feedback results in the joint damage causing a greater inflammatory reaction and this in turn generates more bone and joint erosion.
Awareness of the relationship between arthritic damage and the kynurenine pathway arose from the discovery of the pathway in synovial fluid [10] and studies of IDO deletion or inhibition which exacerbated the severity of arthritic symptoms and tissue damage in a rodent model (collagen-induced arthritis) [11]. The symptoms were reduced by the direct administration of kynurenine to the test animals, confirming that this compound or its downstream catabolites were the predominant mediators of the arthritic symptoms and histopathology. The effects of IDO inhibition were accompanied by increased numbers of T lymphocytes releasing IFN-γ and IL-17, especially in the joints [11], implying that IDO normally functioned to suppress these cells. This would be consistent with the hypothesis that RA is associated with high levels of inflammatory thymocytes, including the Th1 and Th17 populations.

Dendritic cells (DCs) are an important site of IDO regulation which has proved highly relevant to understanding arthritis. CD80 and CD86, located on DCs, are ligated by Cytotoxic T-lymphocyte Antigen-4 (CTLA-4) located in the membrane of regulatory T cells. This interaction induces IDO leading to immune tolerance [12-14]. In patients with RA we have shown that there was aberrant methylation at the promoter of the CTLA-4 gene, resulting in the loss of IDO activation and reduced regulatory T cell function (Fig. 2)[15].
A number of studies have focussed on the role of kynurenine metabolites in RA. For example, Schroecksnadel et al measured kynurenine and tryptophan levels in serum of RA patients as a measure of IDO activity. It was found that tryptophan concentrations were reduced in patients compared to healthy controls with the decrease in tryptophan correlating with physician-graded disease activity [16]. However, a more recent study did not show significant differences in levels of tryptophan, kynurenine or the ratio of kynurenine to tryptophan between patient and control groups [17]. In another study it was shown that kynurenic acid, an antagonist of ionotropic glutamate receptors, was present in synovial fluid of 58 RA patients studied [18]. It was suggested that kynurenic acid could be playing a regulatory role in an attempt to control synoviocyte proliferation [18]. Surprisingly, however, Kang et al found evidence of downregulation of tryptophan-related metabolomic profile in RA synovial fluid [19]. It is of interest that both chondrocytes and synovial fibroblasts express IDO and this been suggested to account for the generally less inflammatory environment of the joint in osteoarthritis, compared to RA [20,21].
Measurement of the concentrations of kynurenines in RA before and after treatment with drugs revealed significantly decreased levels of tryptophan, 3-hydroxykynurenine and 3-hydroxyanthranilic acid and increased levels of kynurenine and xanthurenic acid before treatment. However, following six months of treatment with prednisolone or methotrexate there were no changes in the profiles of tryptophan metabolites despite significant therapeutic responses [22]. It was concluded that there was clear evidence of activation of the kynurenine pathway in RA, but the drug treatments studied did not alter this [22].
Collagen-induced arthritis (CIA) is an animal model of RA and to gain a better understanding of the role played by the IDO pathway in arthritis, levels of tryptophan and its catabolites were measured during the course of the disease [23]. The concentration of tryptophan in lymph nodes decreased during the development of arthritis and this was accompanied by an increase in kynurenine, indicating activation of IDO. Measurement of the downstream kynurenine metabolites in lymph nodes revealed an accumulation of anthranilic acid, and 3-hydroxyanthranilic acid during the resolution of arthritis suggesting that downstream kynurenine metabolites play a role in disease resolution [24,25].
Although many details of the above interactions remain to be clarified, it is clear that they are likely to make significant contributions to the development, progression and remission of RA. It may be that the relatively simple and expedient administration of kynurenine or one of its catabolites could be sufficient to help in the control of arthritis in human patients. Indeed compounds are being developed for other disorders, such as the KAT inhibitors for schizophrenia which indirectly promote the accumulation of endogenous kynurenine, and may prove to be useful in the arthritides. It is also likely that a more detailed appreciation of the molecular targets of individual kynurenine catabolites will be important [26]. For example, both kynurenine and kynurenic acid are agonists for the Aryl Hydrocarbon Receptors (AHR), which are now known to mediate some effects of the kynurenines in arthritis [27,28] raising the possibility that more selective agonists would be clinically useful.

3. Osteoporosis

A major problem of ageing is that of osteoporosis [29-31] and disorders of bone appear to develop as a normal part of the ageing process, dependent especially on hormonal status. Thus, the increased susceptibility of females following the menopause is difficult to avoid without intervention, whereas exposure to stress-induced glucocorticoids induces bone loss in both sexes. At the cellular level, osteoporosis results from an imbalance in the activity of bone resorbing osteoclasts and bone forming osteoblasts in favour of the former. An increased kynurenine/tryptophan ratio has been shown to be inversely correlated with bone mineral density and tryptophan levels have been shown to decrease significantly with increasing age, with an associated increase in kynurenine levels. Below we highlight some of the effects of the IDO pathway metabolites on osseous tissue.

3.1. Kynurenine

Treating animals with kynurenine by injection or in the diet promoted bone loss in parallel with increased levels of osteoclast markers such as Receptor Activator of Nuclear Factor k-B Ligand (RANKL). In

addition, suppression of bone marrow mesenchymal stem cell proliferation and differentiation to osteoblasts with reduced expression of markers including cathepsin K and alkaline phosphatase was observed (Fig. 3)[32,33]. Particularly notable was the finding that the characteristic increase in bone marrow adiposity known to occur in humans was also seen in the kynurenine-treated mice.

Using a different approach, marked effects of raised kynurenine levels have been observed on bone strength [34]. In this study kynurenines were not administered directly, but the plasma concentrations of kynurenine and 3-hydroxykynurenine (3HK) were increased significantly in animals after nephrectomy. Although the mechanism was not clear, these changes were accompanied by increased expression of the aryl hydrocarbon receptor (AHR), one of few molecules thought to act as a receptor for kynurenine in the regulation of immune cell function. As animals aged there was a clear negative correlation between the increased levels of kynurenines and diminishing measures of bone strength and histological structure [34].

Specifically in relation to joint disorders, the induction of IDO in osteoclasts by the B7-CTLA-4 complex results in lowered extracellular tryptophan levels and the increased synthesis of kynurenine metabolites which together inhibit osteoclast generation and promote new bone formation. Hence the absence or dysfunction of the B7/CTLA-4 complex, or of IDO itself, results in osteopenia [35].

3.2. Kynurenine pathway metabolites

While there is no complete explanation for bone loss with ageing, reactive oxygen species are thought to play a key role and the kynurenine pathway is a source of redox active species. In particular, the two oxidized products of kynurenine – 3-HK and 3-HAA- are both redox active but with optimal activity at different levels of ambient redox status [36]. It is therefore of great interest that the levels of plasma 3HAA are substantially different in patients with newly diagnosed (and therefore as yet untreated) osteoporosis compared with control subjects. Indeed there is a remarkable switch between a high (>1) ratio of 3-hydroxyanthranilic acid (3HAA) to its reduced form anthranilic acid (AA), changing from approximately 10:1 in controls to 1:10 in the patients. This would be expected to have a highly significant effect on redox status in the patients. The potential relevance of this ratio is emphasised by its inversion to normal values after two years of treatment with standard anti-osteoporotic drugs such as etidronate and raloxifene [37].

The mechanism and pathological significance of these findings remain to be clarified, partly because the biochemical interface between anthranilate and 3HAA is still uncertain. There may be an enzymic route from anthranilic acid to 3HAA [38] which might be affected by the presence of inflammation which results in a change in the relative amounts of AA and 3HAA produced. Certainly the changes in ratio are not specific for osteoporosis, since many disorders, peripheral or central, in which inflammation is present, are associated with a similar shift in ratio [39,40]. The site of action of the two compounds remains unclear. 3HAA can inhibit Th1 cells proliferation and function with a resulting effect on the ratio of inflammatory IFN-γ-secreting Th1 cells relative to the anti-inflammatory IL-10 secreting Th2 cell population [41]. Any reduction in 3HAA synthesis is therefore expected to be associated with a pro-inflammatory environment such as observed in osteoporosis [42-44].

But what might be responsible for a lack of 3HAA? An enzymic hypofunction which converted less anthranilate to 3HAA could be involved, or a deficiency of kynurenine-3-monoxygenase (KMO) converting kynurenine to 3HK and then 3HAA might exist, allowing kynureninase to metabolise the increased kynurenine directly to anthranilate. In that case, however, the increased level of kynurenine should be converted by KAT into kynurenic acid but changes in kynurenate and anthranilate do not usually occur in parallel and may change in opposite directions. These questions apply not only to the understanding of their relevance in osteoporosis but also, as noted above, may be of more widespread relevance to inflammation control in many other disorders [39,40,45]. Some reports suggest that the AA:3HAA ratio may even change in parallel with the progression of disease symptoms in some conditions [46].

The role of these compounds has become of greater relevance with the results of the extensive Hordaland Health Study [47]. This group reported that anthranilic acid and 3HK exhibited a higher correlation with fracture rates than other measured components of the kynurenine pathway. There was no correlation overall with levels of 3HAA, but there was a positive correlation with bone density in females. Since there is evidence of gender differences in kynurenine pathway activity [45] understanding this factor would be of great value. Despite this anomaly, the strong links between AA, 3HK or 3HAA and bone density strongly support the conclusion that these compounds are of critical relevance in understanding bone formation and loss [37].

The study also noted that neopterin and the kynurenine:tryptophan ratio – a frequently used measure of IDO activity - were inversely correlated with bone density. Neopterin is widely regarded as a marker of

inflammation since it is generated by GTP cyclohydrolase activity induced by IFNγ, in parallel with this cytokine's activation of the kynurenine pathway [48]. Plasma levels of neopterin correlated well with fracture risk in the patient population (over 3000 individuals aged 71-74 years) strongly suggesting a role for inflammatory activity. Interestingly, there was no such correlation with C-reactive protein (CRP), a recognised acute phase reactant normally associated with the immediate response to inflammation. Similarly there was less of a correlation between fracture rate and the kynurenine:tryptophan ratio. This may be consistent with an ongoing, low level inflammation in bone dysfunction.
Finally, this Hordaland Study is one of few to see a link between xanthurenic acid, the primary product of 3HK cyclisation, and bone density in all patient subgroups [47]. The biological significance of this is unclear, although it does strengthen the view that kynurenine pathway activity is associated with the development or progression of osteoporosis.

3.3. Non-kynurenine tryptophan metabolites

Since the kynurenine pathway accounts for the metabolism of around 95% of plasma free tryptophan, any change in the activity of this pathway with inflammation is likely to have a disproportionate effect on other routes involving tryptophan or its non-kynurenine products. Thus, frequently overlooked is the fact that increased metabolism of tryptophan to kynurenines will reduce the availability of tryptophan for conversion to 5-HT (serotonin), tryptamine and melatonin. While these compounds have attracted much less attention, there are reports that increased levels of 5HT in blood platelets is associated with a loss of bone and increased evidence of osteoclast activity [49]. The concentration of tryptophan in erythrocytes has been shown to correlate with a wide range of measurements of bone density and histochemical parameters [50]. When rats were fed a tryptophan-free diet, there was reduced bone formation reflected in lowered blood levels of osteocalcin and IGF-1 [51] although it is difficult to exclude the impact of low tryptophan intake *per se* on general cell metabolism.
This is a serious clinical question since the increase in extracellular 5HT concentrations which are produced by psychotropic drugs such as the anti-depressant Selective Serotonin Reuptake Inhibitors (SSIRs) have been linked with losses of bone density. Certainly osteoblasts and osteoclasts express 5HT receptors, the 5HT synthesising enzyme tryptophan hydroxylase, and a tryptophan transporter, implying an ability to respond to the amine at appropriate concentrations. With the exception of citalopram, the SSRIs examined depressed the production of osteoclasts and inhibited the differentiation of osteoblasts, with the induction of apoptosis in both cell populations [52]. While of substantial practical, clinical interest, however, the true relevance of these observations is not entirely clear since 5HT itself had no effect on the osteocytes.
These concerns about the effect of kynurenine pathway activation and 5HT are relevant as there seems little doubt that 5HT does affect bone cell function. Certainly osteoclasts, exposed to RANKL, express tryptophan hydroxylase and synthesis tryptophan [53]. The amine is then presumably physiologically active since blocking the 1A or 2B 5HT receptors inhibited osteoclast formation. Entirely consistent with this result is the finding that inhibition of 5HT synthesis can prevent or reverse the signs of osteoporosis in ovariectomised mice [54], an action that could be of considerable practical application in human disorders involving low estrogen levels.
There may also be pathological significance in metabolites beyond 5HT such as melatonin (N-acetyl-5-methoxytryptamine) which has been quantified in relation to RA [55] and linked with increased osteoblast differentiation from mesenchymal stem cells [56]. The same report concluded that kynurenine levels inhibited stem cell proliferation and osteoblast differentiation, results which would be consistent with those of El Refaey et al [32]. It was also claimed that 3HK reduced the viability of osteoblasts but picolinic acid promoted bone formation. The latter result was also noted by Vidal et al. [57] but since 3HK is highly unstable and picolinic acid is present in very low concentrations *in vivo*, it is not certain whether these compounds contribute to the overall negative influence of kynurenine metabolites on bone status.

3.4 Kynurenine metabolism and sHLA-G

The question remains unresolved of whether osteoporosis should be regarded as a primarily inflammatory disorder or a condition which is facilitated, perhaps induced or triggered by a transient inflammatory stimulus [58]. Some role of inflammation would certainly be suggested by the association between increased levels of kynurenine or its catabolites and the progress and degree of bone dysfunction, since the first enzyme of the kynurenine pathway, IDO, is induced and activated by several inflammatory stimuli such as interferon-γ, LPS and TNFα. This activation of the kynurenine pathway has been demonstrated in human

mesenchymal stem cells with a strong positive correlation between kynurenine pathway activity and osteoblast formation [57,59]. Correspondingly, inhibition of the kynurenine pathway using an inhibitor of IDO produced a reduction of osteoblastogenesis and genetic deletion of IDO resulted in the loss of bone structure and strength with fewer osteoblasts and a higher proportion of osteoclasts than wild-type animals.
We have noted previously a relationship between activation of the kynurenine pathway and expression of the soluble Human Lymphocyte Antigen-G (sHLA-G) in connection with the severity of Huntington's disease [60-63] where the plasma levels of sHLA-G correlated with the severity of symptoms. A relationship has also been described for this molecule in relation to the inhibition of lymphocyte proliferation [64]. Peripheral blood CD14+ cells produce sHLA-G [65] which appears to be responsible for the monocytic inhibition of T cell proliferation, an effect which was prevented by antibody-mediated inhibition of sHLA-G in culture supernatants.
sHLA-G shares with IDO the ability to regulate T cell activity, to the extent that it has been considered to be the dominant mediator of materno-fetal protection against effector T cell attack [66]. Indeed the suppression of effector T cells is blocked to a greater extent by antibodies to sHLA-G than by inhibition of IDO. sHLA-G also shares with IDO the property of being induced by IFNγ. Macrophages are polarized by sHLA-G to the M2 phenotype, which contribute to materno-fetal protection [67], (partly as a result of their expression of IDO). An additional result from Rizzo et al. (2008) [64] was that IL-10 promotes sHLA-G production, an intriguing observation since we have also noted an increased generation of this cytokine in parallel with kynurenine pathway activation in patients with cerebral trypanosomiasis [46,68]. Clearly the links between these molecules remains tenuous and speculative, but it may be important to understand the relationships between these three fundamentally relevant immune system regulators: IDO, sHLA-G and IL-10 in the future.

4. Steroid-induced osteoporosis

One of the more serious problems associated with the administration of corticosteroids to patients of any age, but especially the vulnerable older population, is the promotion of osteoporosis [69-71]. The loss of bone results from a combination of increased osteoclast activity and decreased osteoblast formation together with increased loss of osteoblasts by apoptosis [72]. A number of explanations have been attempted to develop a molecular explanation of these effects, such as a lowering of osteoprotegerin levels [73,74], inhibiting Insulin-like growth factor-1 (IGF-1) [74] or interference with Wnt signalling by steroid induction of the promoter element of DKK-1, a Wnt pathway inhibitor [75].
In the context of this review, however, it is pertinent to note that glucocorticoids are major inducers of tryptophan-2,3-dioxygenase (TDO). This enzyme has a lower affinity but higher substrate capacity than IDO. Therefore, whereas the focus of interest on kynurenines and the immune system has been on the latter enzyme and its potent induction by Toll-Like Receptor ligands leading to large but localised changes in tryptophan metabolism, the main interest in TDO has been in the regulation of high concentrations of tryptophan in the systemic circulation and extracellular fluids. Nonetheless, glucocorticoid secretion activates TDO as part of the homeostatic mechanisms to control tryptophan levels within physiological limits. Treating patients with glucocorticoids will, therefore, activate the kynurenine pathway with the increased generation of several of the metabolites discussed above and possessing the ability to modulate bone formation. From the above discussion, the overall effect is likely to be one of promoting bone destruction and suppressing bone formation. Such an effect of kynurenines may contribute significantly to the development of steroid-induced osteoporosis. While this concept does not seem to have been proposed it would imply that an inhibitor of TDO, several of which are in development for other clinical conditions [76-79], could be a useful adjunctive treatment to lower the probability of osteoporosis in these patients.

5. Summary and future directions

Inflammatory arthritis and osteoporosis appear to be disorders in which the IDO/kynurenine pathway may play a significant role in disease initiation or development, and as such this pathway represnts a potential avenue for drug discovery efforts.
It will be important to establish whether events occuring in the prenatal or neonatal periods or in early childhood predispose people to develop musculoskeletal disorders in later life. By analogy, inhibition or inactivation of the kynurenine pathway in the early stages of development has been shown to result in changes in brain morphology, neurochemistry, electrophysiological functionality and behaviour in the offspring which are detectable into adult life [80-82]. Changes in the adult brain are also seen in animals with a

genetic deletion of KMO [83-85]. The direct administration of kynurenine also has similar consequences [86-91]. The molecular basis of these effects of prenatal or neonatal increases in kynurenine pathway activity required further detailed investigation but probably involve the action of its metabolites such as quinolinic acid and kynurenic acid as agonist and antagonist respectively on glutamate receptors [2,3,5,92].

The NMDA-sensitive subtype of glutamate receptors have a major influence on early brain formation and function. Since activity of the NMDA receptors in particular depends on the ratio of the agonist quinolinic acid to the antagonist kynurenic acid, a significant shift in structure and excitability is anticipated which may contribute to the emergence of diseases or increase susceptibility to them. Most work to date has been devoted to 'neurodevelopmental' disorders including schizophrenia [26], major depressive disorder [93,94] and suicidal tendencies [95]. However, less work has been performed to determine whether similar modifications to the kynurenine pathway during embryogenesis affect the musculoskeletal system. Since, as discussed above, there is ample evidence for a role of the kynurenine pathway in musculoskeletal disorders, it is probable that there will be some influence of early developmental manipulations on disease occurrence or severity.

Long-term influences on disease susceptibility in later life may also result from changes in nucleic acid structure in early life. Such 'epigenetic' changes may be induced by lifestyle factors such as exposure to stress, disease or dietary components which can modify the level of methylation and acetylation of nucleic acids with consequences for genetic transcription. Importantly, if epigenetic changes affect the germ-line cells of an individual, they may be inherited by the offspring and modify their susceptibility to external influences and disease. Changes in DNA methylation status have been observed in RA [96-100] where the extent of methylation may determine patient responsivity to the TNF-α inhibitor drug etanercept [101]. RA may also involve abnormalities in acetylation status [102], emphasised by the ability of a histone deacetylase inhibitor to inhibit symptoms in the collagen-induced model of arthritis [103]. Further work on the epigenetic regulation of the IDO/kynurenine pathway in musculoskeletal disease and whether those changes may then increase the susceptibility of the offspring to disease is required.

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Figure legends

Fig. 1. The kynurenine pathway for the oxidative metabolism of tryptophan. Tryptophan is oxidised by tryptophan-2,3-dioxygenase (TDO) in the liver, and indoleamine-2,3-dioxygenase (IDO) in other tissues to L-kynurenine. Several of the subsequent enzymes of the pathway, including kynureninase and kynurenine aminotransferase (KAT) are dependent on vitamin B6 for optimal activity, while KMO is partly dependent on riboflavine (vitamin B2). Figure created with BioRender.

Fig. 2. Under normal conditions, regulatory T cells express CTLA-4 which ligates CD80 and CD86 on myeloid APCs, resulting in activation of IDO. The resultant generation of kynurenine and downstream metabolites promotes the generation and activation of regulatory T cells, whilst inhibiting effector T cell responses. However, in patients with active RA, we have shown that there is a loss of regulatory T cell function, accompanied by reduced CTLA-4 expression due to increased methylation at the CTLA-4 promoter [15]. Figure created with BioRender.

Fig. 3. Cartoon which depicts the effect of Kynurenine on osteoclasts and osteoblasts. Figure created with BioRender.

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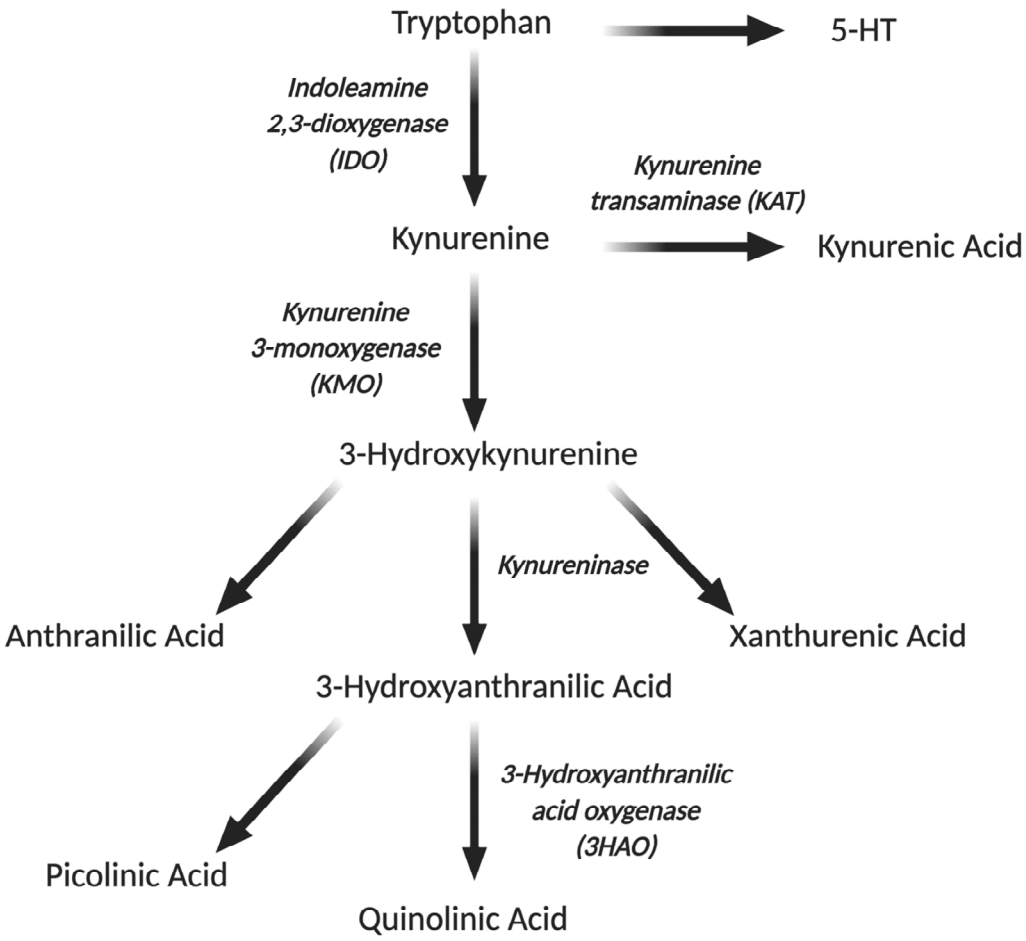
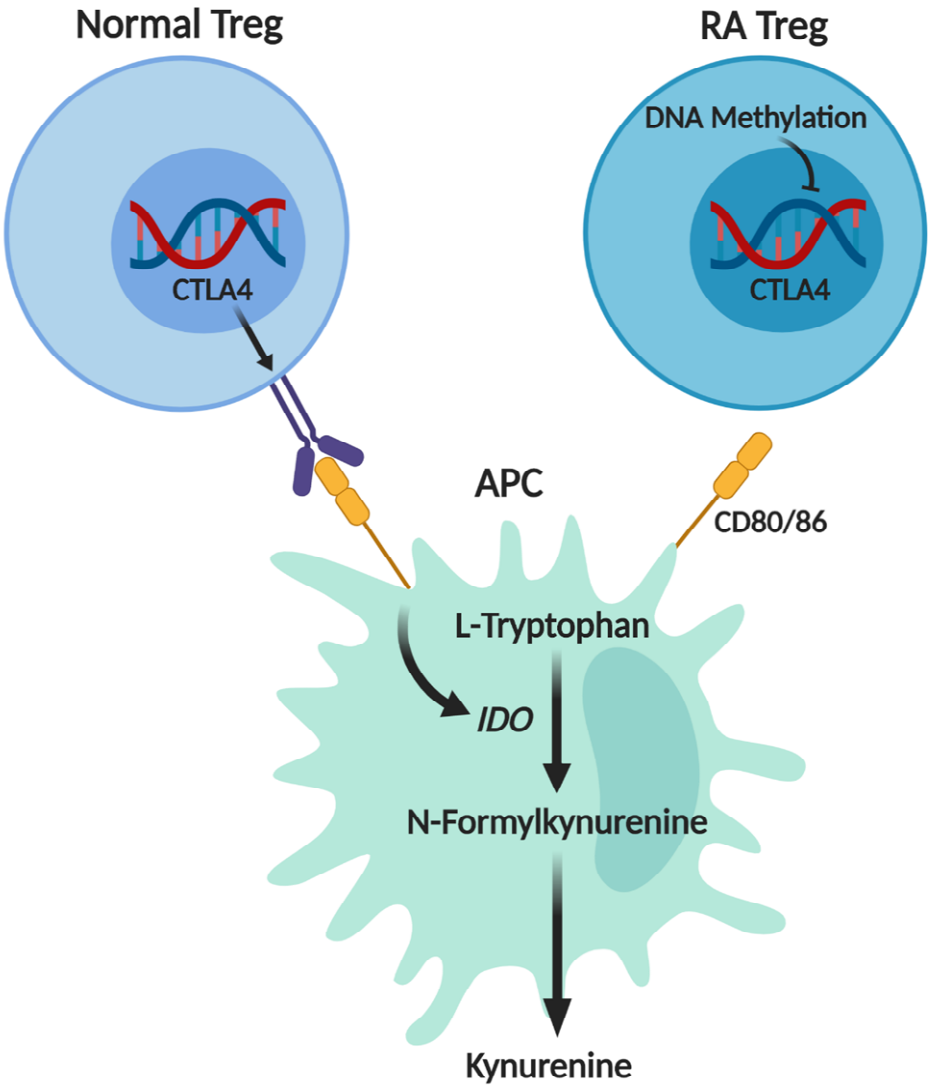
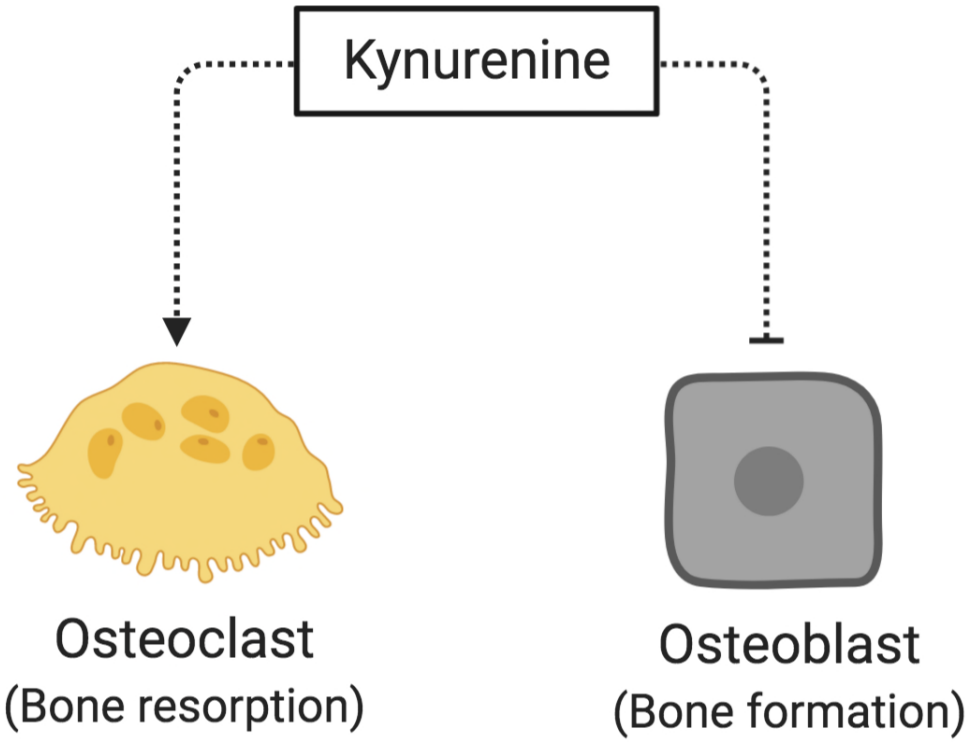

Tryptophan
5-HT
Indoleamine 2,3-dioxygenase (IDO)
Kynurenine transaminase (KAT)
Kynurenine
Kynurenic Acid
Kynurenine 3-monoxygenase (KMO)
3-Hydroxykynurenine
Kynureninase
Anthranilic Acid
Xanthurenic Acid
3-Hydroxyanthranilic Acid
3-Hydroxyanthranilic acid oxygenase (3HAO)
Picolinic Acid
Quinolinic Acid


Normal Treg
RA Treg
DNA Methylation
CTLA4
CTLA4
APC
CD80/86
L-Tryptophan
IDO
N-Formylkynurenine
Kynurenine


Kynurenine
Osteoclast
(Bone resorption)
Osteoblast
(Bone formation)