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Mini review

Inflammatory bone destruction and osteoimmunology

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Objectives: The metabolism of hard tissue is influenced by the immune system. Research into the bone destruction associated with inflammatory diseases such as periodontal disease and rheumatoid arthritis has highlighted the importance of the interplay of the immune and skeletal systems. This interdisciplinary research field, called 'osteoimmunology', has become increasingly important for each system by itself as well as the biology linking them. The history and recent progress of this field are reviewed.

Material and methods: 'Osteoimmunology' was coined to describe the pioneering work on the T-cell regulation of osteoclastogenesis by the receptor activator of nuclear factor-κB ligand (RANKL) and interferon (IFN)-γ. Accumulating evidence suggests that the immune and skeletal systems share not only cytokines but also various signaling molecules, transcription factors and membrane receptors. The contribution of T cells to the pathogenesis of inflammatory bone destruction is discussed, and our recent findings are summarized to illustrate how the osteoimmunological network functions.

Results: RANKL is an osteoclastogenic cytokine that links bone and the immune system. Immunomodulatory cytokines such as IFNs also participate in the regulation of RANKL signaling and inflammatory bone loss. The transcription factor nuclear factor of activated T cells c1 (NFATc1) has been identified as a master switch regulator of osteoclastogenesis. In addition, immunoglobulin-like receptors are critically involved in bone homeostasis.

Conclusion: Bone turns out to be a dynamic tissue that is constantly renewed, where the immune system participates to a hitherto unexpected extent. This emerging field will be of great importance to a better understanding and treatment of diseases of the skeletal and immune systems, as well as to the fundamental biology underpinning both.

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The development and homeostasis of the vertebrate skeletal system depends on a dynamic balancing of the activities of bone-forming osteoblasts and bone-resorbing osteoclasts (1). This balance must be tightly controlled by various regulatory systems such as the endocrine system. Tipping this balance in favor of osteoclasts leads to pathological bone resorption, as seen in a variety of osteopenic diseases: autoimmune arthritis, periodontitis, postmenopausal osteoporosis, Paget's disease and bone tumors (2). Therefore, the regulatory mechanisms governing the osteoclast and osteoblast is critical for understanding the health and disease of the skeletal system.

It has long been known that the immune and skeletal systems have a variety of regulatory molecules, such as cytokines, in common. Furthermore, immune cells form in the bone marrow, interacting with bone cells. Therefore,

one can conceive that the physiology and pathology of one system may very well affect the other. It is worth noting that abnormal activation of the immune system leads to bone destruction in diseases like periodontitis and rheumatoid arthritis (3–5). More recently, animal models deficient in immunomodulatory molecules have been found to frequently develop an unexpected skeletal phenotype (6, 7). Thus, the crosstalk between the immune and skeletal systems and the interdisciplinary field called osteoimmunology has attracted much attention in recent years (8–11).

Identification of receptor activator of nuclear factor-κB ligand: a molecular bridge between two systems

Receptor activator of nuclear factor-κB ligand (RANKL) is a tumor necrosis factor (TNF) family cytokine essential for the induction of osteoclastogenesis (12, 13). RANKL was cloned as an activator of dendritic cells expressed by activated T cells, suggesting that this molecule is important in both the skeletal and immune systems (14, 15). The targeted disruption of RANKL results in defective formation of the lymph nodes and lymphocyte differentiation, as well as osteopetrosis, a sclerotic bone disease caused by impaired osteoclastic bone resorption (16). Thus, this molecule explicitly highlighted the close relationship between immune and bone systems (17).

We hypothesized that the bone loss in autoimmune arthritis is attributable to defective control of bone metabolism by the immune system, and revealed the molecular mechanism of T-cell-mediated regulation of osteoclast formation through signaling crosstalk between RANKL and interferon (IFN)-γ (5) (Fig. 1). In a com-

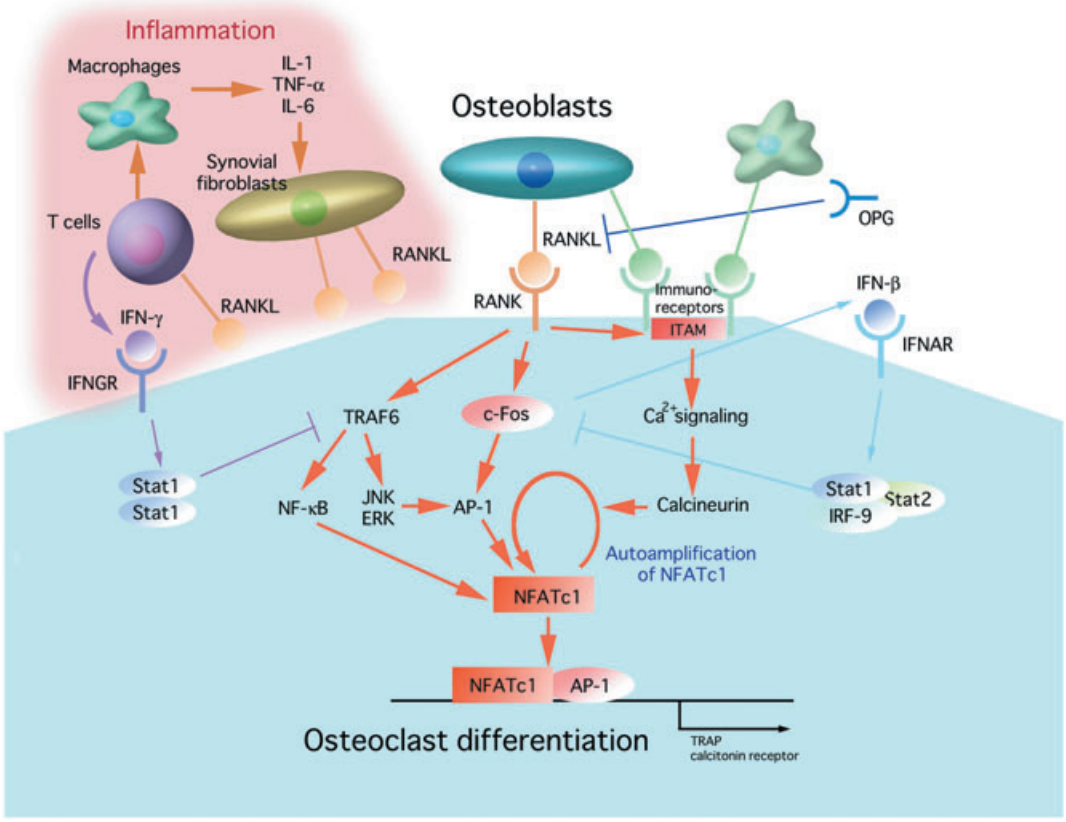

Fig. 1. Signaling crosstalk between immune and skeletal systems in osteoimmunology. Receptor activator of nuclear factor-κB ligand (RANKL) binds to its receptor, RANK, and activates essential signaling for osteoclastogenesis including TNF receptor-associated factor 6 (TRAF6), c-Fos and calcium pathways. The transcription factor nuclear factor of activated T cells c1 (NFATc1) integrates these pathways, acting as a master switch for osteoclast differentiation. Immunoglobulin-like receptors associated with immunoreceptor tyrosine-based activation motif (ITAM)-harboring adaptors are a novel type of essential receptor for osteoclastogenesis, acting as costimulatory molecules for RANKL. Under inflammatory conditions, the interferon-γ (IFN-γ) produced by activated T cells inhibits RANKL signaling by down-regulation of TRAF6 expression. In addition to osteoprotegerin (OPG), IFN-β, which is induced by RANKL, is an important physiological regulator of RANKL signaling, by inhibiting the expression of c-Fos. Immunomodulatory factors often influence RANKL expression in mesenchymal cells such as osteoblasts and modulate RANKL signaling through complex signaling crosstalk. This schematic provides a bird's-eye view of the osteoimmunological interaction described in this review. See the text for details. IL, interleukin; IFNGR, interferon-γ receptor; stat, signal transducers and activators of transcription; JNK, Jun N-terminal kinase; ERK, extracellular signal-related kinase; AP-1, activator protein-1; IRF, interferon regulatory factor.

mentary article in *Nature*, Arron and Choi coined the term 'osteoimmunology' to describe this new interdisciplinary field of bone biology and immunology (8). Choi and colleagues have greatly contributed to this field by their exhaustive researches on RANKL signaling (15, 18–21). Penninger and colleagues contributed to the development of osteoimmunology by generating a series of knockout mice and establishing the role of the RANKL/RANK system in the pathogenesis of bone-related diseases (16, 22–24). They published an extensive review article on the RANKL system (17). However, RANKL is not the only factor linking the immune and skeletal systems. The clinical effectiveness of anti-TNF-α therapy against bone destruction in rheumatoid arthritis patients also demonstrates the relationship between the immune and skeletal systems (25). In addition, targeted disruption of immunomodulatory molecules such as IFN-β or Stat1 results in an unexpected skeletal system phenotype (6, 7, 26, 27), further suggesting that osteoimmunology extends beyond RANKL and has a more profound significance (Fig. 1).

Receptor activator of nuclear factor-κB ligand and interferon-γ in arthritic bone destruction

The molecular mechanism by which abnormal immune responses damage bone has long been a mystery in autoimmune arthritis. Although there had already been circumstantial evidence that the osteoclasts generated from synoviocytes play a critical role (3), it was not until RANKL was cloned and found to be overexpressed in arthritic joints that researchers widely accepted that osteoclasts indeed do play a critical role in arthritic bone destruction (4, 28). Finally, recent studies have provided genetic evidence that RANKL as well as osteoclasts are central in the inflammatory destruction of bone (29, 30). However, in spite of the critical function of RANKL in the enhanced osteoclastogenesis in arthritis, it remains unclear whether synovial mesenchymal cells (synovial fibroblasts) or T cells are the major RANKL-expressing cells.

As T cell infiltration is a hallmark of rheumatoid synovium, we investigated the effect of activated T cells on osteoclast formation *in vitro*. However, the unexpected results revealed that activated T cells have a strong suppressive effect on osteoclastogenesis (5). It was hypothesized that the T cells might have a negative regulatory mechanism to counterbalance the action of RANKL, as abnormal bone resorption is not observed during normal T cell responses despite the expression of RANKL. Using mice lacking a receptor component for IFN-γ, we revealed that the IFN-γ produced by T cells strongly suppresses osteoclastogenesis by interfering with the RANKL signaling pathway (5). Thus, activated T cells not only positively regulate, but also negatively affect osteoclastogenesis. Rather, it is more appropriate to say that T cells are not osteoclastogenic under most physiological conditions. The question thus arises, how can anti-osteoclastogenic T cells have a positive effect on osteoclastogenesis under pathological conditions? T cells must possess a specific pathological mechanism to suppress IFN-γ-mediated inhibition of osteoclastogenesis, which is normally dominant in activated T cells. This is why abnormal bone resorption is observed only in pathological conditions such as arthritis but not in normal immune responses (8).

A possible mechanistic scheme of the bone destruction initiated by T cells in arthritis is summarized in Fig. 2. RANKL is abundantly expressed in synovial fibroblasts stimulated with inflammatory cytokines such as interleukin-1 (IL-1) or TNF-α in addition to RANKL in T cells. Interestingly, despite the significant T cell infiltration observed in arthritic joints, IFN-γ expression in these T cells is suppressed (31, 32). Therefore, the paucity of IFN-γ and the enhanced expression of RANKL may underlie the activation of osteoclastogenesis in arthritis. It is currently unknown why the T cells that have infiltrated into rheumatoid synovium have such 'frustrating features', i.e. an expression of surface markers for memory T cells, a low production

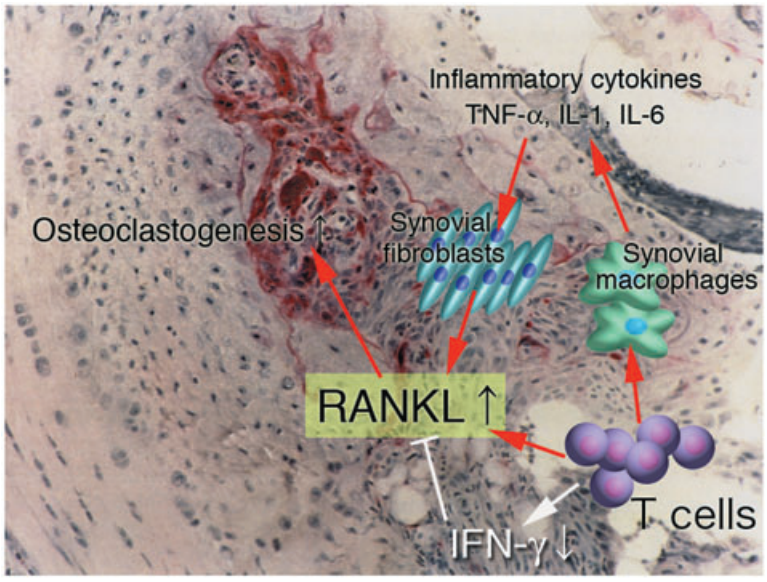

Fig. 2. Mechanism of arthritic bone destruction. Activated T cells have a positive effect on osteoclastogenesis in an indirect manner *in vivo*: they stimulate the macrophages to secrete proinflammatory cytokines such as tumor-necrosis factor-α (TNF-α) and interleukin-1 (IL-1), which strongly induce receptor activator of nuclear factor-κB ligand (RANKL) in synovial fibroblasts. In addition, T cells express RANKL themselves, but the contribution remains controversial. On the other hand, there is a very low level of interferon-γ (IFN-γ), which is a major T cell cytokine that inhibits osteoclastogenesis. This imbalance may be responsible for the aberrant activation of osteoclast formation in arthritis.

of IFN-γ or IL-2 and hyporesponsiveness to *in vitro* restimulation (32). We believe that synovial cells of mesenchymal origin are a major source of RANKL in arthritic joints, and that the RANKL expressed in T cells may have relatively limited contribution, as discussed below.

How do T cells contribute to osteoclastogenesis?

Bone destruction in rheumatoid arthritis is initially triggered by the activation and infiltration of T cells, which eventually enhances the expression of RANKL in synoviocytes as well as in T cells themselves. T cells may play an important role not only as an initial trigger but also as a constant stimulator of bone destruction, mainly by inducing inflammatory cytokines (e.g, TNF-α, IL-1) and RANKL in synovial fibroblasts. Although RANKL is expressed in T cells (24), T cells also produce inhibitors of RANKL, such as IFN-γ (5) and IL-4 (33). Thus, the direct effect of T cells on osteoclastogenesis depends on a dynamic balance among the cytokines they produce. It is tenable that even if RANKL is expressed on T cells, IFN-γ and other cytokines inhibit RANKL signaling, impeding T cells from participating in the direct positive control of osteoclastogenesis. The distribution of T cells in synovium is not always adjacent to bone destruction sites, where osteoclasts are abundant, suggesting that T cells mainly act on osteoclastogenesis in an indirect manner.

Are T cells absolutely required for osteoclastogenesis in rheumatoid arthritis? Lipopolysaccharide-induced bone destruction or collagen-induced arthritis can be induced in mice lacking T cells (34, 35). Therefore, T cell-mediated reactions are not essential for osteoclastogenesis, at least in some models of inflammatory bone destruction. Although the evidence for the considerable contribution of T cells to the exacerbation of bone destruction in rheumatoid arthritis must be admitted, the enhanced expression of RANKL in synoviocytes induced by synovial inflammation may still be part of the critical molecular basis for osteoclastogenesis in arthritis. The activation of T cells should be understood to be one of the causes initiating this inflammation, but the direct effect of RANKL expressed on T cells appears to have relatively low influence on osteoclastogenesis *in vivo*. Needless to say, further studies are necessary to determine exactly how T cells contribute to osteoclastogenesis in rheumatoid arthritis.

Although it is well documented that IFN-γ has a bone protective effect in antigen-specific autoimmune arthritis (36, 37), recent studies suggest that IFN-γ may have a causal role in the bone loss associated with estrogen deficiency. Pacifici and colleagues (38, 39) propose that IFN-γ activates antigen presentation through class II transactivator induction leading to the accumulation of a TNF-α-producing T cell population, but there is little evidence that estrogen deficiency results in the activation of antigen-specific immune reactions. It is an interesting hypothesis that T cell immunity is also involved in the pathogenesis of postmenopausal osteoporosis, but careful interpretation is still needed at this point because the antigens that activate T cells in estrogen deficiency are unknown, and the specific mechanism by which enhanced antigen presentation leads to the generation of bone-sparing T cell population is not well understood (40).

Negative feedback regulation of receptor activator of nuclear factor-κB ligand signaling by interferon-β

During the course of a genome-wide screening of the target genes induced by RANKL, multiple IFN-α/β-inducible genes in osteoclast precursor cells were detected. This led us to investigate the bone phenotype of mice deficient in an IFN-α/β receptor component, IFNAR1 (6). These mice spontaneously develop marked osteopenia (low bone mass) accompanied by enhanced osteoclastogenesis. We found that RANKL induces the *IFN*-β gene in osteoclast precursor cells and that IFN-β inhibits differentiation by interfering with the RANKL-induced expression of c-Fos, an essential transcription factor for osteoclastogenesis. Interestingly, unlike the case of induction by viruses, *IFN*-β gene induction by RANKL is not dependent on interferon regulatory factor (IRF)-3/IRF-7, but on c-Fos. Thus, a unique autoregulatory mechanism operates, wherein the RANKL-induced c-Fos induces its own inhibitor. A series of studies thus have placed both the IFN-α/β and -γ systems in the context of osteoimmunology, indicating that these cytokines are critical not only for immune responses but also for bone homeostasis under both physiological and pathological conditions (4, 27).

Nuclear factor of activated T cells c1: the master regulator of osteoclastogenesis

To gain insights into the mechanism underlying the RANKL-specific induction of the osteoclast differentiation program, we further pursued a genome-wide screening approach to identify the genes specifically induced by RANKL in bone marrow monocytes/macrophages (BMMs) (41). In this screening, we found that *NFATc1*, a member of the NFAT family of transcription factor genes (42), is the most strongly induced transcription factor gene following RANKL stimulation. The transcription factors of the NFAT family, originally discovered in the context of T cell activation (43), are also involved in the function and development of diverse cells in other biological systems, where they are under the control of a calcium-regulated phosphatase, calcineurin (44, 45).

RANKL also induces and activates NFATc1 through calcium signaling, and calcineurin inhibitors such as FK506 and cyclosporin A strongly inhibit osteoclastogenesis. Interestingly, the FK506-mediated inhibition of NFATc1 activity results in a defective induction of the mRNA of *NFATc1*, indicating that *NFATc1* induction is dependent on its own activity: NFATc1 autoamplifies its own gene, possibly by binding to its own promoter. We believe that this autoamplification of the *NFATc1* gene is the most specific event in osteoclast differentiation.

The necessary and sufficient role of the *NFATc1* gene in osteoclastogenesis

has been demonstrated by the observation that *NFATc1*$^{-/-}$ embryonic stem cells cannot differentiate into osteoclasts and that the ectopic expression of NFATc1 causes BMMs to undergo osteoclast differentiation in the absence of RANKL. Therefore, we propose that NFATc1 comprises the master switch for the terminal differentiation of osteoclasts (11, 41).

Nuclear factor of activated T cells c1 as a therapeutic target for inflammatory destruction of bone

Leflunomide is one of the disease-modifying antirheumatic drugs that have been shown to inhibit bone destruction in clinical trials. Leflunomide prevents the proliferation of activated lymphocytes by inhibiting a key enzyme of *de novo* pyrimidine synthesis, dihydroorotate dehydrogenase. However, it was unclear whether the drug suppresses bone destruction by acting directly on osteoclasts.

We evaluated the effect of leflunomide on RANKL-induced osteoclast differentiation and found that leflunomide inhibits osteoclast differentiation due to a blockade of *de novo* pyrimidine synthesis (35). Leflunomide also inhibits the RANKL-induced calcium signaling in osteoclast precursor cells, hence strongly inhibiting the induction of *NFATc1*. Importantly, a marked expression of NFATc1 in osteoclasts in rheumatoid joints was detected, revealing the clinical relevance of NFATc1 for the bone destruction that occurs in arthritis. Thus, the RANKL-dependent *NFATc1* induction pathway presents itself as an auspicious target for pharmacological intervention.

Calcium signaling and immunoreceptor tyrosine-based activation motif-harboring adaptors

Despite the revelation of the importance of the calcium-NFAT pathway, it remained unclear how RANKL activates calcium signals leading to the induction of *NFATc1*, because RANK belongs to the TNF receptor family that is not directly related to calcium signaling. Therefore, we became interested in a report by Kaifu *et al*. that *in vitro* osteoclast differentiation is severely inhibited in mice deficient in DAP12, a membrane adaptor molecule containing immunoreceptor tyrosine-based activation motif (ITAM) that activates calcium signaling in immune cells (46). In spite of the *in vitro* blockade of osteoclast differentiation, DAP12-deficient (*DAP12*$^{-/-}$) mice exhibit only mild osteopetrosis and possess a normal number of osteoclasts in bone tissue, suggesting that DAP12-mediated signals play a critical role in the RANKL/macrophage colony-stimulating factor (M-CSF)-induced culture system but other molecules can help overcome DAP12 deficiency *in vivo*. Consistent with a normal number of osteoclasts *in vivo*, we observed that *DAP12*$^{-/-}$ BMMs undergo osteoclast differentiation when the BMMs are cocultured with osteoblasts. This indicates that osteoblasts stimulate the signal that compensates for the loss of DAP12.

We hypothesized that the compensating molecule is Fc receptor common γ subunit (FcRγ), and generated mice deficient in both molecules (*DAP12*$^{-/-}$*FcRγ*$^{-/-}$ mice). *DAP12*$^{-/-}$*FcRγ*$^{-/-}$ mice exhibit severe osteopetrosis due to a defect in the differentiation of osteoclasts (47). Another group also independently generated the same double knockout mice and reported a similar phenotype (48). The retroviral transfer of *DAP12* into *DAP12*$^{-/-}$*FcRγ*$^{-/-}$ cells, but not the *DAP12* mutant deficient in ITAM, efficiently rescued osteoclast differentiation, suggesting that the ITAM signal is indispensable for RANKL-induced osteoclastogenesis. In addition, it was found that calcium signaling and *NFATc1* induction was impaired in *DAP12*$^{-/-}$*FcRγ*$^{-/-}$ cells. This indicates that the ITAM signal is critical for calcium signaling in the osteoclast lineage as well as in lymphocytes.

Immunoglobulin-like receptors and costimulatory signals for osteoclastogenesis

FcRγ or DAP12 associates with several specific immunoreceptors for cell activation in myeloid lineage cells and natural killer (NK) cells (49, 50). Among the multiple candidate immunoreceptors, we identified the pairing of paired immunoglobulin-like receptor (PIR)-A and osteoclast-associated receptor (OSCAR) with FcRγ, and that of triggering receptor expressed by myeloid cells (TREM)-2 and signal-regulatory protein (SIRP)β1 with DAP12 in osteoclast lineage cells. The triggering of either receptor by cross-slinking with an antibody accelerated RANKL-induced osteoclast differentiation, indicating the activating role of these immunoglobulin-like receptors in osteoclastogenesis. However, in the absence of RANKL, the stimulation of these receptors alone was not able to induce osteoclast differentiation, suggesting that these receptor-mediated signals act cooperatively with RANKL but cannot substitute for the signal. Therefore, we propose that such immunoreceptor-ITAM signaling should be labeled 'costimulatory signals' for RANKL, as their function is analogous to that of costimulatory signals for the activation of immune cells such as T cells. It is now clear that RANKL and M-CSF are not sufficient for osteoclastogenesis. Immunoreceptors need to be stimulated to activate the requisite ITAM-dependent costimulatory signals (47).

Conclusion

The discovery of the RANKL–RANK system has brought about rapid progress in the understanding of the regulatory mechanisms of osteoclast differentiation exerted by the immune system. However, osteoclast differentiation is not only regulated by RANKL and its decoy receptor osteoprotegerin. For example, RANKL induces its own inhibitor IFN-β and autoregulates RANKL signaling. The identification of NFATc1 as the master transcription factor for osteoclastogenesis led us to realize the specific importance of calcium–calcineurin signaling in osteoclast differentiation in addition to the growing awareness of the intimacy between the skeletal and immune systems. Similar to immune cell activation, osteoclast differentiation is

delicately regulated by multiple receptors in addition to RANK and the M-CSF receptor. Thus, emerging findings in the field of osteoimmunology suggest that the field is reaching an advanced stage, in which novel therapeutic strategies for both skeletal and immune disorders are coming to light even as this article goes to press.

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