Recent interdisciplinary studies provide insight into the next generation of Rheumatoid Arthritis treatments.

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Abstract:

Rheumatoid arthritis is a chronic inflammatory condition that can be treated in a variety of ways. Currently available options are discussed briefly but the main focus of this review is to provide some intuition into what will likely be the next generation of clinically available therapies. Potential medications to be discussed include the use of fusion proteins, matrix metalloproteinases (MMPs) and osteoprotegerin regulators, and binding immunoglobulin proteins (BiPs).

Introduction:

Rheumatoid arthritis is a chronic inflammatory disease that mainly targets the synovial membrane, cartilage and bone. Treatments for RA include the use of biologics and chemical disease modifying anti-rheumatic drugs (DMARDs). Prominent RA treatments involve the use of fusion proteins such as etanercept (Enbrel™), cytokine antibodies such as infliximab (Remicade™), adalimumab (Humira™), or methotrexate – an immunosuppressant that can be used in combination with biologics (Mcinnes, 2007; Pfaffen, 2010). Other treatments based around antibody are currently in development. Research into this often relies on collagin-induced arthritis (CIA), a method of mimicking the disease in mice, to evaluate the efficacy and performance of new therapies before clinical trials. The purpose of this review is to present some recent interdisciplinary studies into what are likely to become the next generation of RA treatments.

Molecular imagining techniques are also being used to facilitate early diagnosis, disease monitoring, and guidance of treatment strategy. Novel imaging modalities using molecular probes such as optical imaging (bioluminescence, fluorescence, and near-infrared, or NIR; 600 to 750 nm) or nuclear imaging (scintigraphy; positron emission tomography, or PET; and single-

photon emission computed tomography, or SPECT) are currently being improved for arthritis imaging. Some of the challenges to this include the high selectivity of molecular imaging probes and their resolution (Put, 2014). Despite this, a few of these studies to be discussed very much rely on molecular imaging techniques to evaluate their results.

Fusion Proteins and Antibodies

The purpose of a fusion protein is to target cytokines, or small cell signaling proteins, that are known to cause inflammation in order to block their effect. Examples of pro-inflammatory cytokines include tumor necrosis factor (TNF), IL-1-beta, and IL-6. Etanercept was among the first available for prescription. It works by specifically targeting TNF. It consists of two parts, the soluble portion of the p75-TNF receptor (TNFR) and the Fc fragment of human IgG1. Inhibitory or blocking antibodies, such as infliximab, adalimumab, and certolizumab pegol, are clinically available alternatives that achieve the same effect (Doll et al., 2013).

Another alternative currently in development involves the use anti-inflammatory cytokines. The most notable is Interleukin-10 (IL10). IL-10 is a 35-kd homomeric protein produced by a variety of immune cells. It has been shown to express a regulatory effect on tumor necrosis factor-alpha (TNF-alpha) and IL-1 (Walmsley, 1996). The use of this cytokine was met with limited success until 2013. The problem was that it was unable to specifically target affected areas. That changed when researchers developed the F8-IL10 fusion protein (also known as Dekavil™). Antibody fragment F8 binds to the extra-domain A (ED-A) of fibronectin, which is selectively expressed at sites of inflammation in RA (and in tumors) in both humans as well as animals. When combined with IL10 it was demonstrated to induce targeted delivery in the joints of CIA mouse models (Doll et al., 2013). Given the success of the Dekavil in murine models and Phase I clinical trials it has successfully moved on to multiple Phase II clinical trials (EudraCT

number 2013-005418-37, NCT02270632). It is even being tested alongside another TNF blocker in patients with ulcerative colitis (Murer & Neri, 2019; EudraCT number 2017-002108-28). While it is still in clinical trails, a patient study has confirmed that the fusion protein does show targeted delivery in patients with RA. One potential issue, however, is that unhealthy subjects display an increased uptake of the fusion protein in both the liver and the spleen when compared to healthy subjects. However, this has been currently mediated in subjects with the speed in which it will clear out of the body – less than 24 hours (Bruijnen et al., 2018).

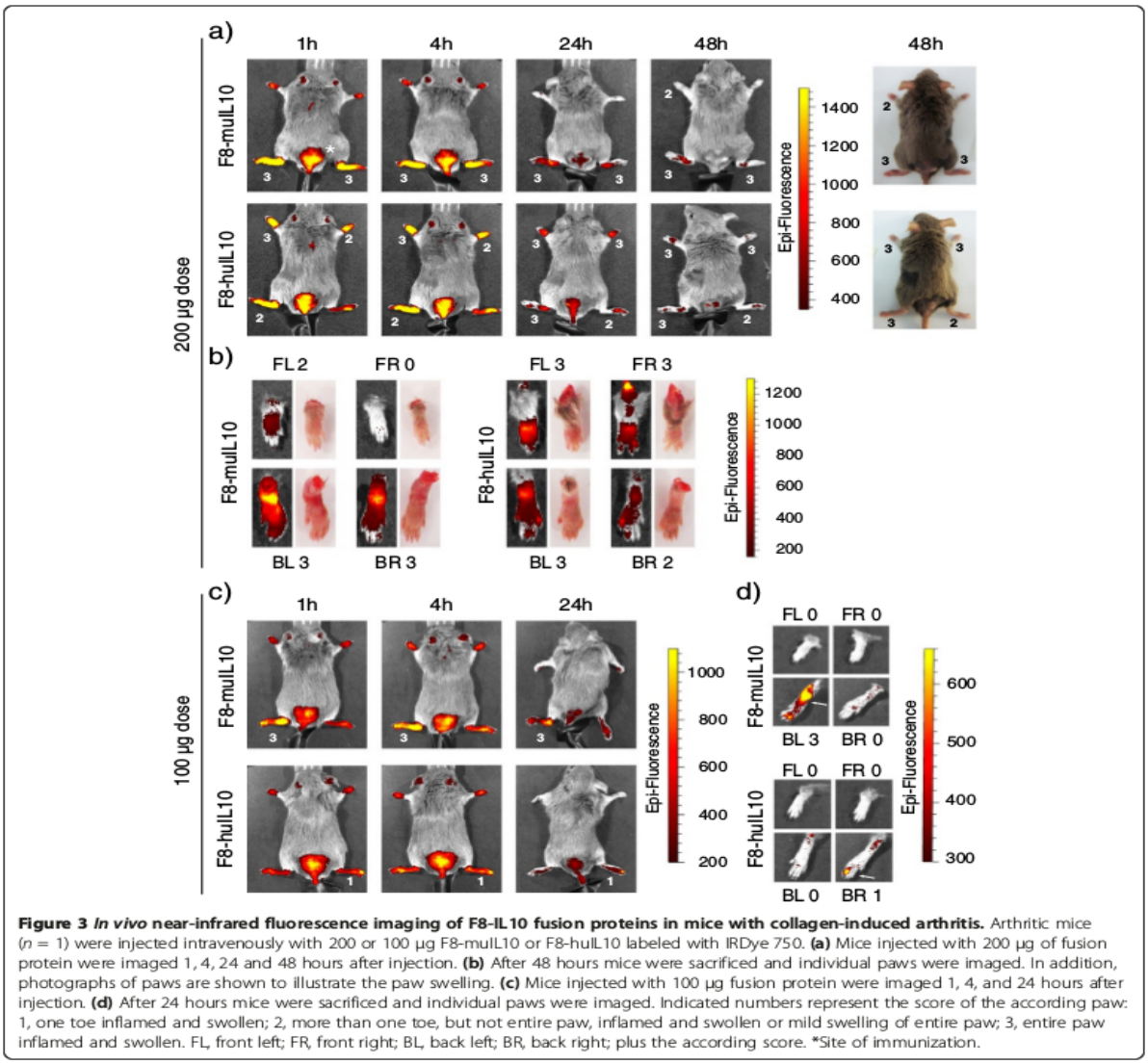


Figure 3 ***In vivo*** **near-infrared fluorescence imaging of F8-IL10 fusion proteins in mice with collagen-induced arthritis.** Arthritic mice (n = 1) were injected intravenously with 200 or 100 µg F8-mulL10 or F8-hulL10 labeled with IRDye 750. **(a)** Mice injected with 200 µg of fusion protein were imaged 1, 4, 24 and 48 hours after injection. **(b)** After 48 hours mice were sacrificed and individual paws were imaged. In addition, photographs of paws are shown to illustrate the paw swelling. **(c)** Mice injected with 100 µg fusion protein were imaged 1, 4, and 24 hours after injection. **(d)** After 24 hours mice were sacrificed and individual paws were imaged. Indicated numbers represent the score of the according paw: 1, one toe inflamed and swollen; 2, more than one toe, but not entire paw, inflamed and swollen or mild swelling of entire paw; 3, entire paw inflamed and swollen. FL, front left; FR, front right; BL, back left; BR, back right; plus the according score. *Site of immunization.

Doll, F., et al. (2013). Figure 3

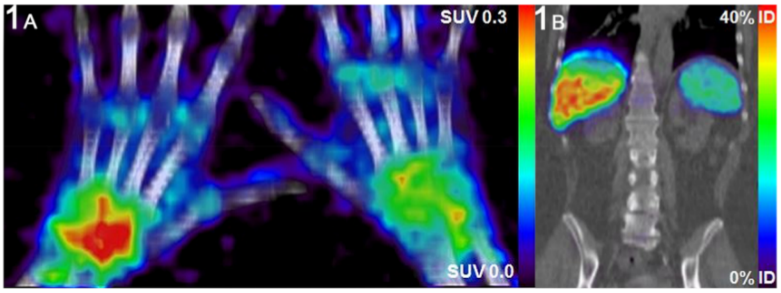


Figure 1. (A) Example of a [^{124}I]I–F8–IL10 PET-CT scan of the hands of RA patient 3 with clinically active disease. (B) [^{124}I]I–F8–IL10 PET scan of RA patient 1 showing clear tracer uptake in liver and spleen. %ID = percentage of the injected dose.

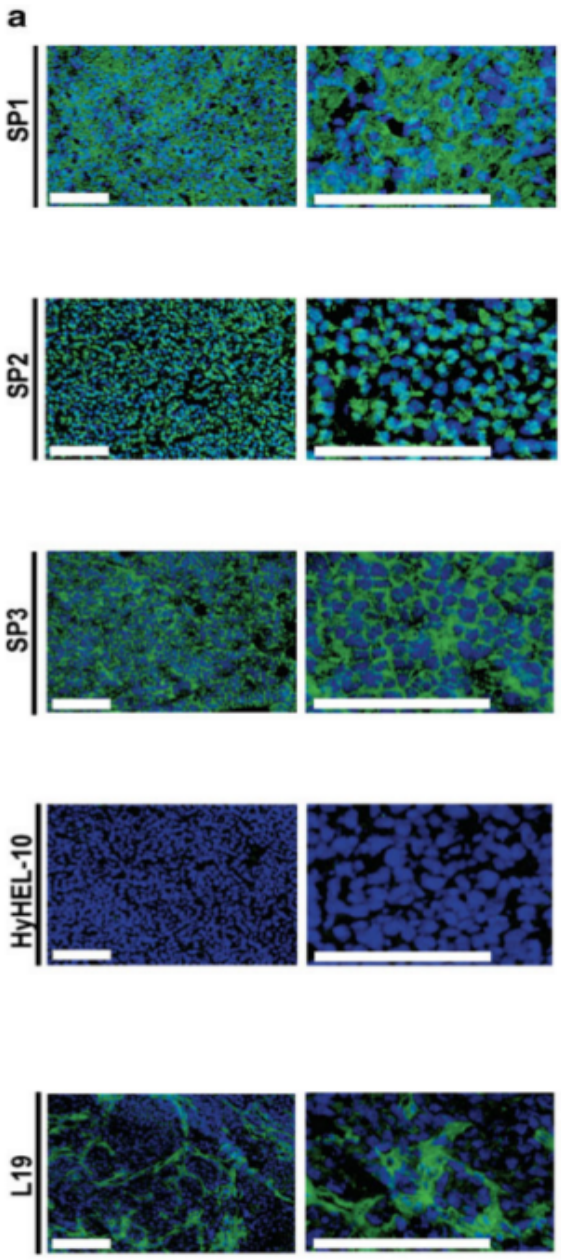
Bruijnen, S., et al. (2018) Figure 1

Micro-RNAs Targeting Matrix Metalloproteinases and Osteoprotegerin

Matrix metalloproteinases (MMPs) are a group of more than 20 zinc-containing extracellular proteinases that are capable of degrading multiple components of the extracellular matrix and are up-regulated in many diseases, thus they are potential inhibitory targets. Before 2010, researchers have unsuccessfully attempted to use radiolabeled MMP inhibitors for imaging tumors. One reason for this is possibly due to the limited specificity of these agents. Another could have also been related to their unsuitable properties relating to absorption, distribution, metabolism and excretion, also known as their pharmacokinetic properties. Afterwards, researchers instead investigated whether radiolabeled monoclonal antibodies – some of which are capable of targeted delivery – could be considered for the selective targeting and imaging of individual MMPs. Their method involved using mice bearing F9 teratocarcinoma (tumors) to study the biodistribution of the radiolabeled monoclonal antibodies SIP(SP1), SIP(SP2), and SIP(SP3). Two similar antibodies were used as a negative and positive control. Antibodies specific to MMP-3, the SIP(SP3) antibody and the positive control, did show a preference for the tumor sites. The rest were rapidly cleared from the bloodstream, from most normal tissue, and did not stick around in the tumor. The researchers hypothesized that the longer retention of SIP(SP3) and

the control in the blood could have been due to a non-covalent association with serum components. Thus, it was concluded that SIP(SP3) antibodies may serve as vehicles for the efficient and selective delivery of imaging agents or therapeutic molecules to wherever MMP-3 is strongly over-expressed, such as in the case of rheumatoid arthritis (Pfaffen et al., 2010).

Osteoprotegerin ligand (OPGL) or receptor activator of nuclear factor kB ligand (RANKL), is an essential factor for osteoclast differentiation from monocyte/macrophages (the role of osteoclasts will be mentioned later). Along with its receptor RANK they are both found in the synovial membrane of RA patients. Osteoprotegerin is a naturally occurring inhibitor of OPGL. A fine balance between OPG and RANK appear to be critical for the maintenance of bone architecture adjacent to joints. This balance seems to be disturbed in RA, which can be explained, at least in part, by the effects TNF-alpha , IL-1, and RANKL. Blockade of the RANKL/RANK pathway by systemic therapy with OPG leads to inhibition of erosions in TNF-alpha-triggered arthritis. This

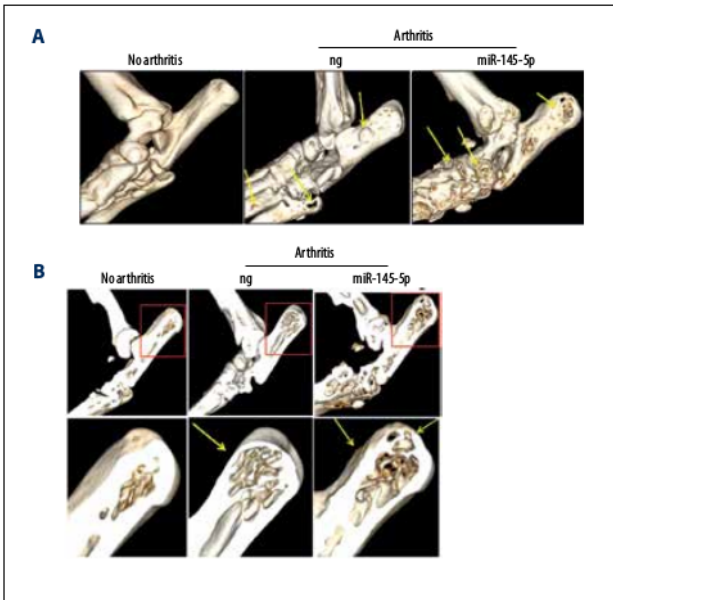


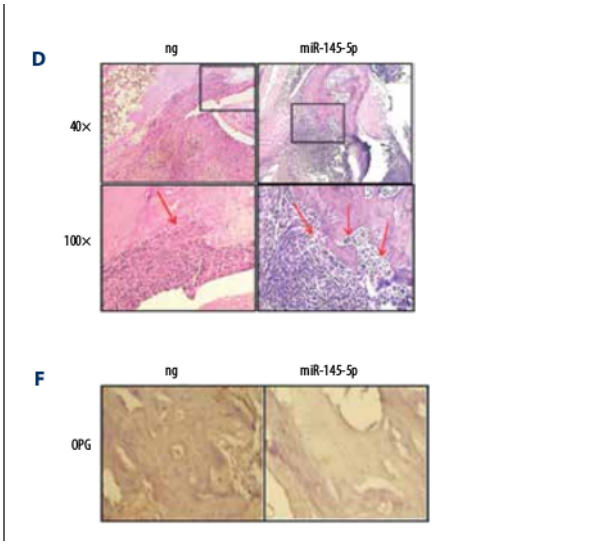
Pfaffen, S., et al. (2010). *Fig. 2 Immunofluorescence analysis and biodistribution studies after intravenous injection of antibodies in F9 tumour-bearing mice. a Immunofluorescence analysis was performed on tumour cryosections. MMP-1A, MMP-2, MMP-3 and extradomain B of fibronectin are stained in green, whereas cell nuclei were stained in blue using DAPI (scale bar 100 μm).*

approach may prove to be an effective targeted therapy for the prevention of bone damage in human RA (Redlich, et al., 2002).

A modern method of investigating the expression of MMPs and OPGs involve the use of micro-RNAs. These small non-coding RNAs undergo luciferase reporter assays to examine the predicted effects of on target mRNAs. Tissue samples are transfected with the experimental mi-RNA using a transfection reagent and their over-expression is analyzed. Afterwards studies on the inhibition of mi-RNAs are performed. In patients with RA it was found that miR-522 was up-regulated in blood samples. To study the effect of miR-522 researchers examined its over-expression in synovial fibroblasts (tissue samples) from RA patients and performed a series of *in vitrio* functional studies to elucidate the molecular mechanisms of miR-522-mediated biological behaviors of those tissue samples. What was discovered was that miR-522 over-expression significantly increases the levels of TNF-alpha and IL-1-beta, as well as the levels of MMP-1, MMP-3, and MMP-13. Inhibition of miR-522 significantly reduced the levels of the proinflammatory cytokines and the cartilage-destroying MMPs (Chen, et al., 2018). This study confirms that miR-522 has a role in the parthenogenesis of arthritis and reconfirms the possibility of targeting MMPs for therapeutic benefits.

Another investigation conducted within the same year sought to examine the over-expression of miR-145-5p in CIA mice by evaluating the levels of OPG. It was found that this micro-RNA, when over-expressed, downregulated OPG and exacerbated bone destruction in mice. Immunohistochemical (IHC) analysis also confirmed that when inhibited, OPG levels were higher. The mice were also healthier (Wan et al., 2018). This study reconfirms that when the balance of the RANKL/RANK/OPG system is broken, the risk of RA in patients can be increased.



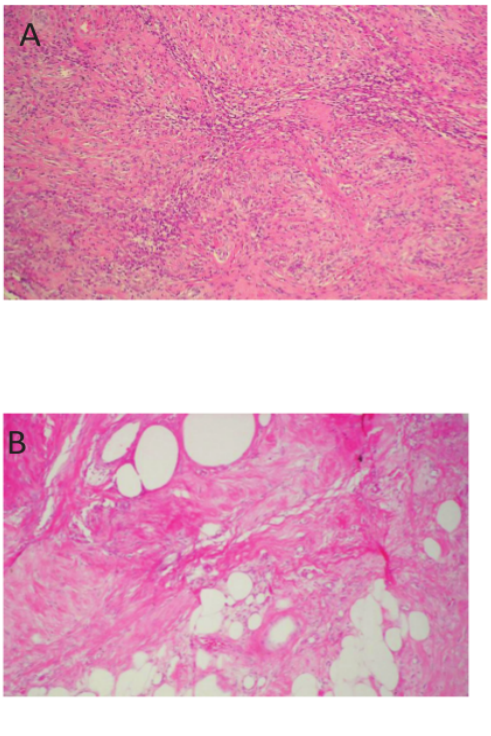


Chen, Y., et al. (2018). *Figure 4. miR-145-5p agomir aggravates bone destruction in CIA mice. (A, B) Comparison of bone erosion: micro-CT images of the ankles of non-CIA mice and of CIA mice treated with negative control (ng) or with miR-145-5p. (A) Bone surface; (B) Bone cross-section. Yellow arrows indicate bone erosion. Red boxed areas are shown the trabecular bone. (D) Hematoxylin and eosin staining of sections from the hind paws of the CIA mice (n=4, per group). Boxed areas in the top panels (original magnification×40) are shown at a higher magnification in the middle panels (original magnification×100). Red arrows indicate cartilage erosion. (F) IHC and quantification of OPG in CIA mice (n=4, per group). Bars=200 μm at 40×original magnification.*

Lentiviral Vector Delivery of Binding Immunoglobulin Protein

Binding immunoglobulin protein (BiP) is an anti-inflammatory protein found inside and outside of the endoplasmic reticulum. It displays potent immunomodulatory activity in both mice and humans on release from stressed cells (Shields et al., 2015). Studies before 2011 have shown that BiP downregulates both immune and inflammatory responses *in vitro*. On the other hand, using the CIA mouse model, BiP was shown to have long-lasting prophylactic and therapeutic action *in vivo*. These previous studies were confirmed during a xenogeneic study using RA synovial membrane transplants in severe combined immunodeficient (SCID) mice (Yoshida et al., 2011).

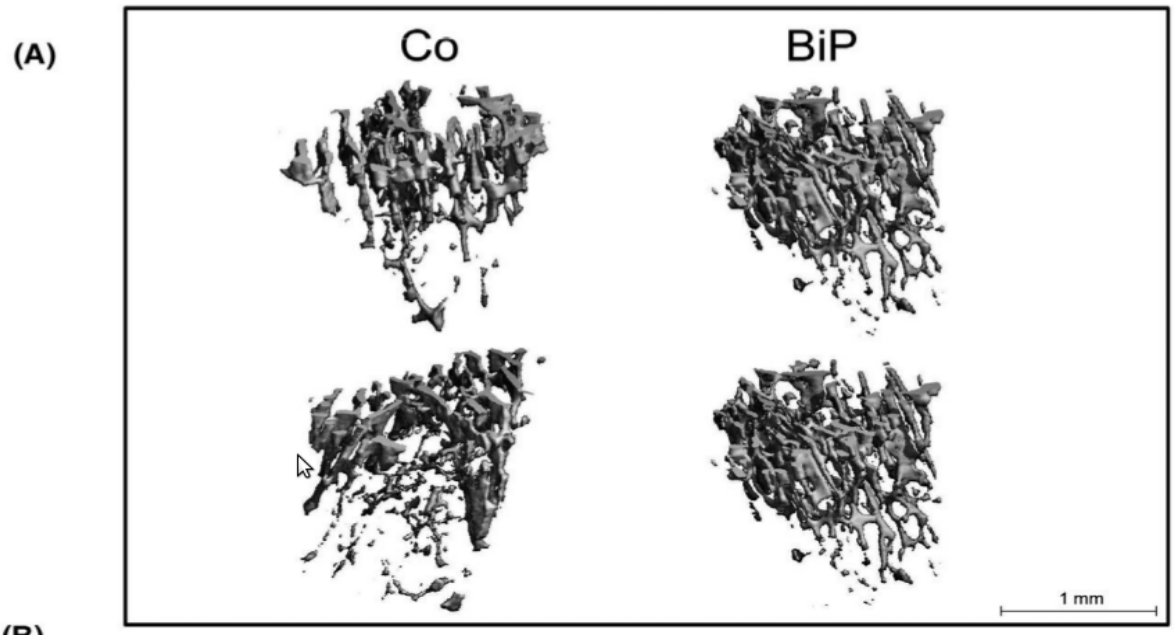
Following this discovery researchers examined whether lentiviral vector delivery could treat RA in mice models. Both a mouse version (mBiP) and a human version (rhuBiP) were examined and were even shown to have a similar immunological characteristics. Both a low dose and high dose study were conducted with results showing a positive impact on the clinical progression of CIA; the high dose study delayed the progression 5 days earlier than the low dose study. Specific cells that had been treated with the vector and co-cultured were shown to have significant increases in the levels of both IL-10 and IL-17 produced *ex vivo*. This strongly suggests that the vector alters the immunological parameters of the collagen-induced arthritis model and of lymphocytes derived from treated animals. Thus, BiP has the potential to work both on the inside and outside of inflammatory cells as it is being used to treat RA (Shields et al., 2015).



Yoshida, K., et al. (2011) *Figure 1 Binding immunoglobulin protein reduces gross pathology of synovial membrane transplants. Pieces of human synovial tissue from patients with rheumatoid arthritis were transplanted into severe combined immunodeficient mice. After successful engraftment, binding immunoglobulin protein (BiP) (10 μg/animal) or human serum albumin (HSA) (10 μg/animal) were administered intravenously, and 12 days later the tissue was removed for immunohistological examination. Representative figures of haematoxylin-stained tissue removed from (a) HSA-treated mice or (b) BiP-treated mice*

These studies mentioned earlier demonstrate that BiP is a cytokine inhibitor but there exists yet another cell target that has not been mentioned. Osteoclasts (mentioned previously) are a type of cell that breaks down bone tissue. Normally this would be ideal for maintaining blood calcium. The problem with these cells is that when TNF is up-regulated certain macrophages become differentiated into osteoclasts, causing bone destruction. An investigation conducted in 2019 demonstrates that BiP can inhibit osteoclast differentiation

alongside TNF-induced arthritis (Zaiss et al., 2019). This is remarkable since current biologics do not directly act on osteoclast precursors.



Zaiss, M. et al. (2019). *Figure 2. Binding immunoglobulin protein (BiP) protects from tumor necrosis factor (TNF) mediated systemic bone loss. Structural parameters of trabecular bone from the tibiae of 10 week old BiP- or phosphate-buffered saline–treated human TNF transgenic (hTNFtg) mice. (A) Representative micro-computed tomography reconstruction of proximal tibiae from hTNFtg mice treated for 5 weeks in the absence or presence of 10 μg BiP showing increased trabecular bone mass in BiP-treated animals.*

Discussion

Further research into F8-IL10 seems to have lagged behind or has yet to reach the public domain. An incomplete study involving 5 patients reported a high tolerability of F8-IL10 used in combination with Methotrexate (Mauro et al., 2012). In 2018 a present translational study was performed to study the uptake of a radiolabeled form of F8-IL10. The results demonstrate a targeting of the protein in the arthritic joints and a rapid clearance from the bloodstream, but also a high uptake in the liver and spleen. Overall, this study demonstrates the value of *in vivo* biodistribution and PET-CT-guided imaging in development of new and potential anti-rheumatic drugs. The study only used 3 RA patients so further research is needed to demonstrate whether this specific fusion protein is clinically feasible (Bruijnen et al., 2018).

There is no publicly available research on the use of SP(SP3) antibodies and their use in delivering any sort of anti-rheumatic treatment. Whether this is because the antibody is not as functional as initially demonstrated has yet to be determined. Assuming is a lack of interest is the reason, someone with insight could model a fusion protein based on etanercept using this antibody in place of the Fc fragment.

There are several more limitations that need to be addressed. The mechanism by which miR-145 regulates OPG/RANK/RANKL pathway and RA bone erosion is also not clear. Therefore, further investigations are required to fully understand the mechanism of miR-522 and its target genes in the pathogenesis of RA. Even though BiP has a similar effect to IL-10, the relationship between the two remains unclear. However, one study mentioned earlier provides evidence that BiP stimulates IL-10 (Zaiss et al., 2019). BiP gene therapy for rheumatic disease is also still in its infancy. Several other potential new therapies have not been reported here due to the insufficient amount of information that has been published about them.

Conclusion

There exist several interdisciplinary studies that provide insight into the next generation of RA treatments. New fusion proteins will enhance targeted delivery of anti-inflammatory cytokines. Micro-RNA transfection and lentiviral vector deliveries – in other words, gene therapy – have the potential to mediate RA pathogenesis as further research into their mechanisms continue. BiP therapies are capable of addressing the destruction of bone that accompanies RA over the long term. On top of all that, modern molecular imaging techniques allow for novel methods of study.

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